Effects of Ketamine and Related New Psychoactive Substances in Rat Urinary Bladder

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Abstract

Introduction: In 2007, Shahani et al. first described the clinical entity of ketamine-induced cystitis (KC) — a condition seen in recreational users of the drug, characterised by the development of various lower urinary tract (LUT) symptoms indicative of bladder overactivity. Histopathologically, individuals with KC typically present with thinning or denudation of the urothelium and inflammatory cell infiltration, and in severe cases, complete or partial cystectomy are the only recourse. Despite being investigated for more than 15 years, the aetiology surrounding KC remains incompletely understood, and there is currently no known treatment. The development of KC has also been reported in individuals using therapeutic ketamine, and so, when considering ketamine's expanding clinical application, uncovering its mechanism of action in KC is becoming increasingly important. The aim of this thesis was to provide further insight into this phenomenon, with the ultimate hope of developing treatments and/or prevention of KC. Also explored were the effects of ketamine-related new psychoactive substances (NPS), which, despite a lack of information regarding their pharmacological effects or potential toxicity, are generally regarded as safer alternatives to ketamine.

Methods: For functional assessment, rat bladder strips were suspended in organ baths containing oxygenated Krebs-Henseleit solution and maintained at 37°C. The effects of acute (20-minute) exposure to ketamine and ketamine-related compounds (up to 3mM) were investigated on contractility evoked by carbachol (CCH), high potassium, and electrical field stimulation (EFS). Longer-term (3-day) exposure to ketamine was examined by contracting tissues with either CCH or high potassium, incubating for 3 days in tissue culture media containing 3mM ketamine, and reassessing the contractile response. For histological and immunohistochemical assessment, rat bladder was dissected in half longitudinally and cultured at 37°C with ketamine or related compounds (up to 3mM). Bladders were then fixed in 10% formalin, processed, and embedded in paraffin wax. Tissue sections were stained with haematoxylin and eosin for visualisation of nuclei and cytoplasm, or stained with antibody for visualisation of various proteins of interest.

Results: Ketamine and all ketamine-related compounds tested inhibited rat bladder strip contractility following acute exposure. In contrast, 3-day ketamine exposure enhanced bladder contractility to CCH, but not high potassium, which is a novel finding. Ketamine and nifedipine (an L-type calcium channel antagonist) concentration-dependently attenuated EFS-induced contractility potentiated by Bay K8644 (an L-type calcium channel agonist), and Bay K8644 concentration-dependently enhanced EFS-evoked contractility inhibited by ketamine or nifedipine. Histological examination revealed a loss of mucosal cells following 3 days culture with ketamine, (S)-ketamine, norketamine, or ketamine-related NPS (3mM). Three-day exposure to ketamine and (S)-ketamine also induced a loss of urothelial umbrella cells, which has not previously been reported in *in vitro* rat bladder.

Conclusion: The evidence presented here suggests that acute inhibition of rat bladder contractility by ketamine and related compounds is mediated through L-type calcium channel blockade. The mechanism behind increased rat bladder strip contractility to CCH following 3-day ketamine exposure remains elusive; however, it seems likely that this effect is not L-type calcium channel-mediated, and may be due to loss of a diffusible inhibitory factor released from the urothelium following muscarinic receptor activation. It is suggested that, acutely, direct contact of ketamine and its metabolites with the bladder reduces the magnitude of bladder contractility, leading to bladder relaxation or poor bladder efficiency, ultimately resulting in an increased contact time of ketamine and its metabolites with the urothelium. Over time, urothelial damage (which can at least be partly explained by loss of umbrella cells) and reduced expression of tight junction-associated proteins, presumably induced by ketamine and/or norketamine, leads to defective barrier function of the urothelium. Increased urothelial permeability can lead to leakage of irritative agents and urinary potassium to the underlying tissue layers, which could cause an inflammatory response and also directly depolarise nerves and smooth muscle, potentially contributing to the LUT symptoms of KC. Overall, the mechanisms mediating KC appear complex, and are likely the result of the interplay of various ketamine-related effects. The novel *ex vivo* tissue culture assay described here shows clear functional and histopathological effects of ketamine, and so could form the basis of a functional or histopathological screening assay that is sensitive to ketamine toxicity. The evidence presented in this thesis provides further avenues to explore in the hope of fully understanding the aetiology of KC.

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Abbreviations

ACH	Acetylcholine
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
CCB	Calcium channel blocker
CCH	Carbachol
CCR	Carbachol concentration-response
DAB	3,3’-diaminobenzidine
DHNK	Dehydronorketamine
DMEM	Dulbecco’s modified Eagle’s medium
DPD	Diphenidine
DXM	Dextromethorphan
EFS	Electrical field stimulation
FBS	Fetal bovine serum
GAG	Glycosaminoglycan
GC-MS	Gas chromatography mass spectrometry
H&E	Haematoxylin and eosin
IHC	Immunohistochemistry
IP_3	Inositol triphosphate
KC	Ketamine-induced cystitis
KCl	Potassium chloride
Krt7	Cytokeratin-7
LUT	Lower urinary tract
MLC	Myosin light chain
mTOR	Mechanistic target of rapamycin
MXE	Methoxetamine
MXP	Methoxphenidine
NK	Norketamine

NMDA	N-methyl D-aspartate
NMDAR	N-methyl D-aspartate receptor
NPS	New psychoactive substances
PBS	Phosphate buffered saline
PCP	Phencyclidine
PDGFRα	Platelet-derived growth factor receptor alpha
PLC	Phopholipase C
PSA	Psychoactive Substances Act
RA	Retinoic acid
ROCK	Rho-kinase
SR	Sarcoplasmic reticulum
TrkB	Tyrosine kinase receptor B
VOCC	Voltage-operated calcium channel

Chapter 1: Introduction

1.1 BACKGROUND AND RATIONALE

1.1.1 Ketamine

Ketamine is a dissociative anaesthetic that was first synthesized in 1962 by Calvin Stevens. It is a derivative of phencyclidine (PCP) and was developed with the intent of replacing PCP as a more favourable anaesthetic. As such, the safety profile of ketamine is generally considered to be excellent (Dolansky, Shah, Mosdossy & Rieder, 2008), and, in comparison to PCP, the incidence and duration of its hallucinogenic effects are greatly reduced (Duperon & Jedrychowski, 1983; Mion & Villevieille, 2013). Ketamine has been used clinically since 1970, and to this day is still used as a short-acting, potent anaesthetic in both human and veterinary medicine due to its rapid onset and swift recovery time. More recently, the clinical application of ketamine has expanded to include its use in a number of different chronic pain settings such as neuropathic pain (Israel et al., 2021) and post-surgical pain (McNicol, Schumann & Haroutounian, 2014); and as a fast-acting antidepressant that is able to show efficacy in patients with treatment resistant-depression (Serafini, Howland, Rovedi, Girardi & Amore, 2014).

1.1.2 Ketamine-Induced Cystitis

Due partly to its dissociative and hallucinogenic properties, ketamine is also used globally as a recreational drug. Its recreational use dates back to as early as 1965 (Stevenson, 2005), and more recently ketamine's popularity has increased, particularly amongst young party-goers and those in the Far East (Chu et al., 2007; Wood et al., 2011; Lee, Jiang & Kuo, 2013; Tam, Kwok, Lo, Lam & Lee, 2018). Ketamine is most commonly obtained as a powder and either snorted or smoked (Morgan & Curran, 2012), with the majority of users taking from 0.25g to over 1g of ketamine in a typical session (Winstock, Mitcheson, Gillatt & Cottrell, 2012). This increased recreational use of ketamine likely facilitated the discovery of a link between the long-term use of ketamine and the development of the clinical entity known as ketamine-induced cystitis (KC) or ketamine-associated ulcerative cystitis (Shahani, Streutker, Dickson & Stewart, 2007).

Clinically, patients with KC present with an array of lower urinary tract (LUT) symptoms, including debilitating urinary frequency, urgency, urge incontinence, dysuria (painful urination), and haematuria (Shahani et al., 2007; Chu et al., 2007; Chu et al., 2008; Mak et al.,

2011). Urodynamic evaluation of KC patients has revealed detrusor overactivity, decreased bladder compliance, decreased bladder capacity, hydronephrosis, and secondary vesicoureteral reflux (flow of urine into the kidneys caused by bladder or urethral obstruction), typically in the absence of an infection (Chu et al., 2008; Mak et al., 2011; Tsai & Kuo, 2015). Additionally, cystoscopic inspection often shows epithelial inflammation, ulceration, neovascularisation, and widespread loss of urothelium (Wood et al., 2011; Tsai & Kuo, 2015; Kidger et al., 2016), as well as inflammation and fibrosis of the detrusor muscle (Chu et al., 2008; Tsai & Kuo, 2015).

1.1.3 Prevalence of Ketamine-Induced Cystitis

It is reported that approximately 25-50% of ketamine recreational users experience bladder dysfunction, and the prevalence and severity of the associated symptoms appear to be positively correlated with the dose and frequency of ketamine use (Muetzelfeldt et al., 2008; Mak et al., 2011; Winstock et al., 2012). Mak et al. (2011) reported that individuals using ketamine three or more times a week over a two-year period displayed measurable bladder dysfunction, but that some users reported urinary symptoms as soon as one month after starting ketamine use. Any report of ketamine-induced bladder dysfunction is complicated by the fact that ketamine is often taken recreationally with other club drugs or alcohol (Kalsi, Wood & Dargan, 2011; Sassano-Higgins, Baron, Juarez, Esmaili & Gold, 2016). Moreover, many studies detailing KC amongst drug users are lacking information regarding dosage, frequency, and polysubstance use.

It is important to note that KC is not a phenomenon exclusive to recreational users of the drug. Lower urinary tract symptoms indicative of KC have been reported in individuals taking lower doses of ketamine (200mg – 1g/day; 8mg/kg/day in children) for therapeutic reasons (Gregoire, MacLellan & Finley, 2008; Shahzad, Svec, Al-Koussayer, Harris & Fulford, 2012). One case study reported that a woman taking ketamine (50mg four times per day) for abdominal pain developed frequency, dysuria, and haematuria, with biopsies revealing moderate inflammatory cell infiltration and areas of mucosal denudation (Storr & Quibell, 2009). However, three weeks after cessation of ketamine use, these LUT symptoms were resolved. Moreover, although rare, there have been some reports of ketamine-induced bladder dysfunction in patients undergoing ketamine treatment for depression. Feifel,

Dadiomov & Lee (2020) conducted a survey among providers of parenteral ketamine for depression which revealed three instances (0.06% of 6630 patients) of bladder dysfunction. Additionally, a literature search uncovered one instance (16.6% of 6 patients) of symptomatic cystitis (Diamond et al., 2014) and one instance (10% of 10 patients) of an increased urge to urinate (George et al., 2017) among individuals who had received six or more repeated parenteral ketamine treatments for depression treatment (0.5mg/kg/40min; Feifel et al., 2020). There are no reports of KC following a single anaesthetic dose of ketamine.

1.1.4 Treatment of Ketamine-Induced Cystitis

There is currently no definitive disease modifying or symptomatic treatment for KC, and conventional treatments such as antibiotic, anticholinergic, and non-steroidal anti-inflammatory medications are unable to offer a significant and durable effect (Mak et al., 2011; Meng et al., 2011; Tsai & Kuo, 2015). Intravesical administration of hyaluronic acid is reported to have some short-term effectiveness in relieving LUT symptoms in recreational ketamine users (Meng et al., 2015). Moreover, there is one case report of a complete reversal of the urinary symptoms and image morphology, including regeneration of the urothelium, in a KC patient following hyaluronic acid treatment (Ou, Liu, Cha, Wu & Tsao, 2018), however further studies are necessary to determine its long-term efficacy (Jhang, Hsu & Kuo, 2015). Cessation of ketamine use is reported to improve the urological symptoms in some patients; however, this is not true for all individuals (Storr & Quibell, 2009; Mak et al., 2011; Winstock et al., 2012). In extreme cases, the LUT symptoms and extent of bladder damage are so severe that patients have been treated with a partial or full cystectomy (Shahani et al., 2007; Shahzad et al., 2012). Bladder augmentation to increase bladder capacity has also been employed to alleviate the urinary symptoms associated with KC, however this comes with the risk of complications, including persistent LUT symptoms and impaired renal function (Ng et al., 2013; Hung, Hsieh, Chen & Lin, 2019; Tan, Zhu, Zheng, Zheng, Kang & Tang, 2021).

The existence of KC has been known since 2007, however the aetiology surrounding this phenomenon is still incompletely understood. With the medicinal use of ketamine recently expanding to the chronic treatment of depression, and with both its present role in chronic pain management and use as a recreational drug, the long-term effects of ketamine are becoming increasingly important to consider. Current research is aiming to uncover

ketamine's mechanism of action in the bladder, with the hope of developing treatments and prevention of KC.

1.2 KETAMINE PHARMACOLOGY

1.2.1 Ketamine ADME

1.2.1.1 Absorption

Aside from intravenous infusion, ketamine can be administered through several different routes, including intramuscular, nasal, oral, rectal, subcutaneous, and sublingual (Das, 2020). Intramuscular administration, which is used in children and emergency situations where the patient is uncooperative, has a bioavailability of 93% (Zanos et al., 2018). Ketamine's bioavailability for intranasal, rectal, oral, and sublingual administration is 8-50%, 11-25%, 16-29%, and 24-30%, respectively (Mion & Villevielle, 2013; Zanos et al., 2018; Das, 2020). The limited bioavailability of orally administered ketamine is due to extensive first pass metabolism (Zanos et al., 2018).

Intravenous ketamine results in a rapid onset of action that occurs within seconds (Sassano-Higgins, Baron, Juarez, Esmaili & Gold, 2016; Das, 2020). Ketamine administered intranasally, which is a common route among recreational users, is associated with a fairly rapid onset of action of approximately 15-30 minutes (Sassano-Higgins et al., 2016; Das, 2020).

1.2.1.2 Distribution

Ketamine is a highly lipophilic molecule and has a short distribution half-life of approximately 7-11 minutes, as it is rapidly distributed into highly perfused tissues, including the brain (Sassano-Higgins et al., 2016; Das, 2020). The rapid distribution is reflected in its low plasma protein binding which is around 10-47% (Mion & Villevieille, 2013; Das, 2020), and results in a large steady state volume of distribution (V_d=3-5l/kg; Zanos et al., 2018).

1.2.1.3 Metabolism

Ketamine is a racemic mixture comprised of equal parts (R)-ketamine and (S)-ketamine. (S)-ketamine is typically reported to have a higher clearance than (R)-ketamine (Ihmsen, Geisslinger & Schüttler, 2001; Kamp et al., 2020), and it is generally accepted that metabolism

of (S)-ketamine is favoured over (R)-ketamine, supposedly due to the higher affinity of (S)-ketamine for the CYP3A4 enzyme (Kamp et al., 2020).

Ketamine undergoes extensive metabolism in the liver via nitrogen demethylation (Zanos et al., 2018). It is mostly metabolised to norketamine (80%; Figure 1.1) — an active metabolite that is reported to have a potency of around 30% of that of ketamine (Lin & Lua 2005, Tsai et al., 2009; Mion & Villevieille, 2013). Norketamine (NK) is then further metabolised into dehydronorketamine (DHNK; Figure 1.1) and hydroxynorketamines (Zanos et al., 2018). The metabolites of ketamine are conjugated to water soluble glucuronide derivatives and roughly 90% are excreted in the urine (Chu et al., 2008).

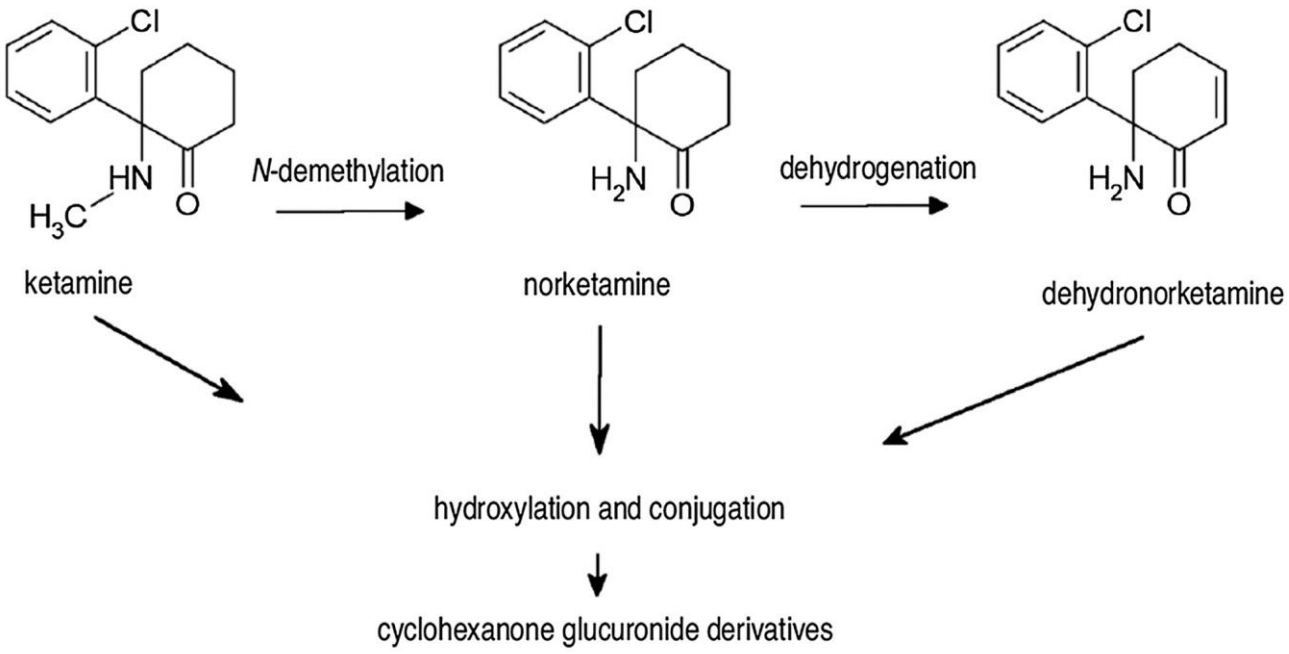

Figure 1.1. Chemical structure of ketamine and its major metabolites. Ketamine is metabolised to norketamine via *N*-demethylation, and norketamine is dehydrogenated to form dehydronorketamine. Ultimately, the metabolites of ketamine are conjugated to glucuronide derivatives.

1.2.1.4 Excretion

Ketamine, NK, and DHNK can be detected in the urine for up to 5, 6, and 10 days, respectively, following a small oral dose (50mg) of ketamine (Parkin et al., 2008), and this was extended to up to 11 and 14 days for ketamine and NK, respectively, in hospitalised children who had received ketamine as an anaesthetic (Adamowicz & Kala, 2005).

There is limited data regarding the concentrations of ketamine and its metabolites present in the urine. Of the data that is available, important information such as the dosage, route of administration, and the time interval between ketamine use and urine assessment is often absent. Regardless, the high upper-limit concentrations of ketamine and its metabolites in the urine (Table 1.1) raises the possibility that ketamine, NK, and DHNK may accumulate in the urine and contribute to the bladder damage and inflammation observed in KC through direct contact with the bladder (Shahani et al., 2007). Indeed, there is evidence to suggest that ketamine has a direct toxic effect on the urothelium (Baker et al., 2016), and more recently, NK has been reported to induce apoptosis in a human urothelial cell line, with greater cytotoxicity (0.01-0.5mM) than ketamine (0.1-3mM; Lin et al., 2022). The *in vitro* effects of DHNK on the bladder, however, have not yet been investigated. Therefore, further research into the potential involvement of NK and DHNK in KC would be of benefit.

Table 1.1. The reported concentration ranges of ketamine and its major metabolites present in the urine of ketamine users, or following a single administration of ketamine.

REFERENCE & DRUG USAGE DETAILS	**DRUG**		
	Ketamine	**Norketamine**	**Dehydronorketamine**
Moore et al. (2001) ***(Suspected drug users - no usage details)***	6-7744ng/mL 25nM-33µM	7-7986ng/mL 31.3nM-35.7µM	37-23,239ng/mL 167nM-105µM
Cheng et al. (2008) ***(Suspected drug users - no usage details)***	21-5620ng/mL 94nM-24µM	66-9031ng/mL 295nM-40µM	
Wang et al. (2005) ***(No usage details)***	114-2925ng/mL 480nM-12.3µM	453-9805ng/mL 2µM-44µM	307-33,715ng/mL 1.4µM-152µM
Parkin et al. (2008) ***(Following single oral dose of 50mg ketamine)***	up to ~3µM	up to ~7.6µM	
Chen et al. (2007) ***(Following single intramuscular dose of 10mg ketamine)***	5.4-131ng/mL 22.7nM-551nM	12.5-74.1ng/mL 55.8nM-331nM	22.8-278.9ng/mL 102nM-1.25µM

1.2.2 Ketamine Metabolite Pharmacology

Norketamine is detected in the blood approximately 2-3 minutes following intravenous administration of ketamine (Mion & Villevieille, 2013), and 15 minutes following oral administration of ketamine (Kubota et al., 2013). The maximum serum concentration of NK was reported to be almost ten times that of ketamine (250.2 ± 28.7ng/mL and 29.9 ± 5.3ng/mL, respectively), and its half-life was reported to be nearly five times longer than ketamine (5.3 ± 1.1hr and 1.1 ± 0.5hr, respectively) in healthy individuals following a single oral administration of a liquid formulation of ketamine (50mg; Kubota et al., 2013).

There is little information regarding the pharmacokinetic behaviour of DHNK in humans. Dehydronorketamine is reportedly a major circulating metabolite, and significant concentrations of the metabolite are present in 24-hour plasma samples of patients following a single 40-minute infusion of a sub-anaesthetic dose of ketamine (Zhao et al., 2012). In the same study, wide inter-patient variations of ketamine metabolite plasma concentrations were reported, with DHNK being the predominant metabolite in four of nine patients, and NK in three of nine patients (Zhao et al., 2012).

1.2.3 Receptor Pharmacology

1.2.3.1 N-Methyl D-Aspartate Receptor

Ketamine's most notable pharmacological properties, including the induction of anaesthesia, are generally attributed to its interaction with the N-methyl D-aspartate (NMDA) receptor, whereby it acts as a non-competitive antagonist (Bergman, 1999; Sleigh, Harvey, Voss & Denny, 2014). The NMDA receptor (NMDAR) is an ionotropic glutamate receptor, which is vital to, amongst other things, central nervous system development, and the processes underlying memory, learning, and neuroplasticity (Blanke & Van Dongen, 2009; Sanz-Clemente, Nicoll & Roche, 2013).

Activation of the NMDAR requires the simultaneous binding of glutamate and co-agonists glycine or serine (Figure 1.2; Mion & Villevieille, 2013). At resting membrane potential, however, the NMDAR channel pore is blocked by extracellular magnesium (Wilcock & Twycross, 2011), and so activation also requires a depolarisation sufficient to dislodge magnesium from its binding site (Blanke & VanDongen, 2009; Mion & Villevieille, 2013).

Subsequent calcium influx through the channel increases intracellular calcium levels, enabling activation of calcium-specific signalling cascades that can modify synaptic efficacy and neuronal morphology (Blanke & VanDongen, 2009; Sanz-Clemente et al., 2013).

As a non-competitive NMDAR antagonist (Anis, Berry, Burton & Lodge, 1983; Martin & Lodge, 1985), ketamine acts by binding to the PCP site of the NMDAR, which is only accessible when the channels are in their open, activated state (Wilcock & Twycross, 2011; Zorumski, Izumi & Mennerick, 2016). The binding of ketamine to this site reduces the mean channel opening time, leading to a reduction in excitatory neurotransmission (Britt & McCance-Katz, 2005). Ketamine may also decrease the frequency of channel opening by binding to a second site located in the hydrophobic domain of the NMDAR (Figure 1.2; Orser et al., 1997; Mion & Villevieille, 2013).

1.2.3.2 Other Ketamine Receptor Pharmacology

Although ketamine is most notably an NMDAR ligand, it is reported to interact with many other receptors in the central and peripheral nervous system. This reported binding promiscuity, along with its complex mechanisms of action, have led to ketamine being dubbed "the nightmare of the pharmacologist" (Persson, 2013), and has likely contributed to the difficulty in elucidating ketamine's mechanism of action in certain disease states.

Ketamine has been reported to interact with mu, delta, and sigma opioid receptors, and inhibit nitric oxide synthase, which contributes to its analgesic properties (Table 1.2; Zanos et al., 2018). It is also reported to act as a muscarinic receptor antagonist, and interact with sodium, potassium, and L-type calcium channels (Table 1.2; Mion & Villevieille, 2013). There is also evidence that ketamine acts as a partial agonist at D_2 receptors, inhibits dopamine and serotonin reuptake (Table 1.2; Kapur & Seeman, 2002; Yeung et al., 2009), and increases dopamine efflux in the rat nucleus accumbens (Hancock & Stamford, 1999).

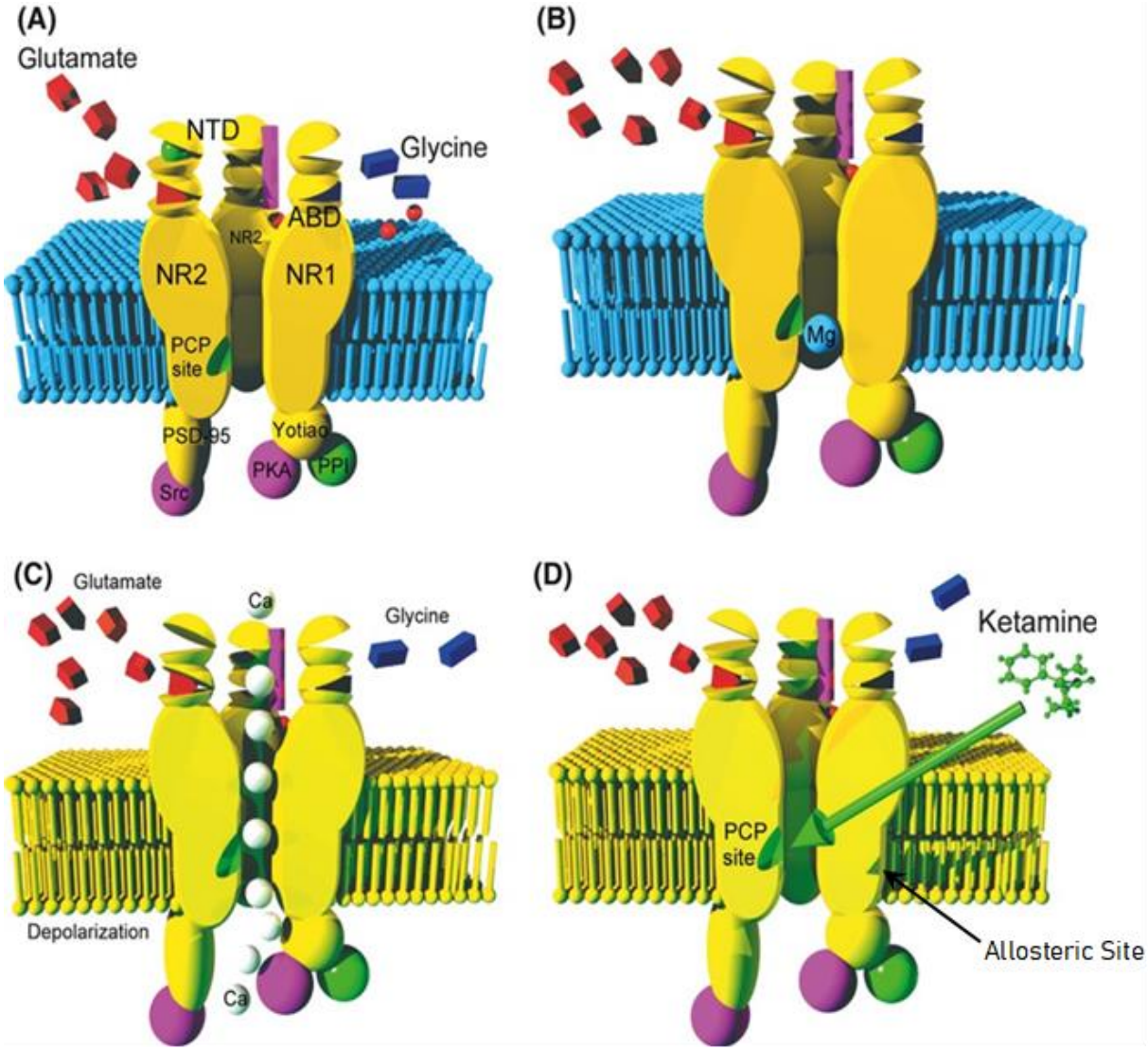

Figure 1.2. N-methyl-D-aspartate receptor pharmacology. **(A)** The binding site for glutamate is located in the ABD domain of the NR2 subunit, and the binding site for co-agonist glycine is located in the ABD domain of the NR1 subunit. **(B)** When the membrane is not depolarised, even when the co-agonists occupy the ABD binding sites, that channel is blocked by Mg^{2+}. **(C)** Removal the Mg^{2+} block requires both membrane depolarisation and occupancy of the ABD sites by the co-agonists, which subsequently allows influx of calcium. **(D)** Ketamine inactivates the NMDA receptor by binding to the PCP site, which partially covers the magnesium binding site. A second binding site for ketamine is thought to be located in the hydrophobic domain. (Adapted from Mion & Villevieille, 2013).

Table 1.2. Reported action and potency of ketamine and ketamine-related compounds discussed herein at various receptors/targets.

Receptor/ Target	Drug	Action	Potency (μM)	Method	Tissue/ System	Species	Reference
NMDAR	Ketamine	Antagonist	IC_{50}=0.43 ± 0.10	Whole-cell patch-clamp recordings	Hippocampus	Rat	Parsons et al. (1996)
NMDAR			IC_{50}=0.92 ± 0.21	Whole-cell patch-clamp recordings	Striatum	Rat	Parsons et al. (1996)
NMDAR			IC_{50}=5.4 ± 0.6	Autoradiographic binding – [^{3}H]MK-801	Frontal cortex	Rat	Porter & Greenamyre (1995)
NMDAR			IC_{50}=6.7 ± 0.8	Autoradiographic binding – [^{3}H]MK-801	Hippocampus	Rat	Porter & Greenamyre (1995)
NMDAR			IC_{50}=5.0 ± 0.6	Autoradiographic binding – [^{3}H]MK-801	Striatum	Rat	Porter & Greenamyre (1995)
NMDAR			IC_{50}=7.97	FLIPR calcium influx assay	HEK293 Cells	Human	Gilling et al. (2009)
NMDAR			IC_{50}=0.71 ± 0.03	Whole-cell patch-clamp recordings – holding potential at -70mV		Human	Gilling et al. (2009)
NMDAR			IC_{50}=6.05 ± 0.66	Whole-cell patch-clamp recordings – holding potential at 0mV	HEK293 cells transfected with GluN1/2A receptors	Human	Gilling et al. (2009)
NMDAR	(S)-Ketamine	Antagonist	IC_{50}=1.6-1.9	RBA – [^{3}H]MK-801 binding	Hippocampus	Human	Oye et al. (1992)
NMDAR			IC_{50}=1.5-1.8	RBA – [^{3}H]MK-801 binding	Frontal Cortex	Human	Oye et al. (1992)
NMDAR			IC_{50}=1.6-2.1	RBA – [^{3}H]MK-801 binding	Occibital cortex	Human	Oye et al. (1992)
NMDAR			IC_{50}=0.8	Whole-cell patch clamp recordings	Hippocampus	Rat	Zeilhofer et al. (1992)

NMDAR			IC_{50}=0.9 ± 1.4	NMDA- (µM) evoked currents	Cortex	Rat	Ebert et al. (1997)
NMDAR	Nor-ketamine	Antagonist	IC_{50}=2.00	Whole-cell recordings	Hippocampal neuron culture	Rat	Emnett et al. (2016)
M1	Ketamine	Not reported	K_i=45	Radiobinding assay	Human receptor expressed in CHO cells	Human	Hirota et al. (2002)
M1		Antagonist	IC_{50}=0.7	Two-microelectrode recording	Rat receptor expressed in *Xenopus* oocytes	Rat	Durieux (1995)
M2	Ketamine	Not reported	K_i=294	Radiobinding assay	Human receptor expressed in CHO cells	Human	Hirota et al. (2002)
M3	Ketamine	Not reported	K_i=246	Radiobinding assay	Human receptor expressed in CHO cells	Human	Hirota et al. (2002)
D_2	Ketamine	Partial agonist	EC_{50}=0.9 ± 0.4	Radiobinding assay	Human D_2R expressed in CHO cells	Human	Kapur & Seeman (2002)
DAT	Ketamine	Uptake inhibitor	K_i=62.9 ± 2.3	[^{3}H]dopamine uptake	Rat transporter expressed in HEK293 cells	Rat	Nishimura et al. (1998)
SERT	Ketamine	Uptake inhibitor	K_i=161.7 ± 28.3	[^{3}H]serotonin	Rat transporter expressed in HEK293 cells	Rat	Nishimura et al. (1998)
***µ* opioid receptor**	Ketamine	Agonist	K_i=42.1	RBA - [^{3}H]DPN	Human receptor expressed in CHO cells	Human	Hirota et al. (1999)

μ opioid receptor	(S)-Ketamine	Agonist	K_i=28.6	RBA - [^{3}H]DPN	Human receptor expressed in CHO cells	Human	Hirota et al. (1999)
δ opioid receptor	Ketamine	Agonist	K_i=272	[^{3}H]DPN	Human receptor expressed in CHO cells	Human	Hirota et al. (1999)
δ opioid receptor	(S)-Ketamine	Agonist	K_i=205	[^{3}H]DPN	Human receptor expressed in CHO cells	Human	Hirota et al. (1999)
Estrogen receptor alpha	Ketamine	Not Reported	K_D=0.34 ± 0.01	Radiobinding assay		Human	Ho et al., (2018)
L-type calcium channel	Ketamine	Antagonist	IC_{50}= 1000	Whole cell recording	Tracheal smooth muscle	Pig	Yamakage et al. (1995)
L-type calcium channel			IC_{50}=9.2	Whole cell recording	Atrial myocytes	Bulfrog	Hatakey-ama et al. (2001)

1.2.4 Ketamine Therapeutic efficacy

1.2.4.1 Anaesthesia & Analgesia

When administered at a dose of approximately 0.5-4.5mg/kg intravenously or 4-13mg/kg intramuscularly, ketamine induces anaesthesia (Pai & Heining, 2007; Rosenbaum, Gupta, Patel & Palacios, 2022), which is thought to be primarily mediated by blockade of NMDA channels (Pai & Heining, 2007). In mice, ketamine-induced anaesthesia was shown to be dose-dependently antagonised by NMDA, and NMDAR antagonists were reported to dose-dependently potentiate anaesthesia produced by low doses of ketamine (Irifune, Shimizu, Nomoto & Fukuda, 1992). Moreover, (S)-ketamine has a higher affinity to the NMDAR binding site than the R(-) isomer, and was shown to have an anaesthetic potency around 3-4 times greater than (R)-ketamine in human volunteers (White et al., 1985).

Other molecular targets of ketamine have also been reported to be involved in the induction of anaesthesia, however. Muscimol, a γ-aminobutyric acid type A ($GABA_A$) receptor agonist, was able to potentiate ketamine-induced anaesthesia in a dose-dependent manner, while bicuculline, a $GABA_A$ receptor antagonist, dose-dependently inhibited ketamine-induced anaesthesia (Irifune et al., 2000). Thus, ketamine is speculated to act as a $GABA_A$ receptor agonist, and ketamine-induced anaesthesia is thought to be, at least partly, mediated by this property. A role for hyperpolarisation-activated channels (HCN1) in ketamine-induced anaesthesia was also reported following the discovery that the potency of ketamine to provoke a loss-of-righting reflex was strongly reduced in HCN1 knockout mice (Chen, Shu & Bayliss, 2009).

At subanaesthetic doses of approximately 0.2-0.75mg/kg intravenously or 2-4mg/kg intramuscularly, ketamine produces analgesic effects which have been reported to alleviate both acute and chronic pain (Pai & Heining, 2007).

The first clinical trial of ketamine was carried out in 1963, where its anaesthetic and analgesic effects were reported (Domino, Chodoff & Corssen, 1965). The potential of ketamine as an analgesic at subanaesthetic doses emerged from reports that its parent compound, PCP, displayed analgesic properties distinct from its sedative ones (Persson, 2013). Reports of a role for peripheral NMDA receptors in pain signalling encouraged experiments investigating the effects of peripheral administration of ketamine (Sawynok, 2014). Indeed, in rodents, ketamine was shown to produce antinociception in various models (Davidson & Carlton, 1998; Oatway, Reid & Sawynok, 2003), and was reported to reduce primary hyperalgesia and the development of secondary hyperalgesia in humans following thermal injury (Warncke, Jorum & Stubhaug, 1997).

Its analgesic properties are thought to be due to NMDAR inhibition and activation of descending inhibitory monoaminergic pain pathways (Hirota & Lambert, 2011). Ketamine is also reported to interact with mu, kappa, and delta opioid receptors (Panzer, Moitra & Sladen, 2009; Williams et al., 2018), which is thought to contribute to its analgesic effects. Indeed, Finck & Ngai (1982) demonstrated that ketamine was able to bind opioid receptors in rat brain homogenate and displace the opioid 3H-etorphine in regional areas of the mouse brain. It appears to show a preference to mu rather than delta opioid receptors, as *in vitro* radioligand binding studies showed that ketamine was four-times more effective at inhibiting the binding

of 3H-dihydromorphine (a highly selective mu opioid agonist) than 3H-[D-Ala2, D-Leu5] enkephalin (a delta opioid agonist; Smith et al., 1987). Ketamine is reported to activate mu and kappa opioid receptors (Bonaventura et al., 2021), and opioid receptor antagonists have been shown to inhibit ketamine-induced analgesia in rats (Fidecka, 1987), and humans (Williams et al., 2018).

Activation of opioid receptors allows coupling of $G_{i/o}$ proteins to the C terminus of the receptor, where it dissociates into G_{α} and $G_{\beta\gamma}$ (Stein, 2016). The G_{α} subunit inhibits adenylyl cyclase, which reduces cyclic AMP production, and leads to activation of downstream signalling pathways (Kudla & Przewlocki, 2021); whereas the $G_{\beta\gamma}$ subunit inhibits voltage-gated calcium channels and opens rectifying potassium channels, reducing the excitability of neurons (Stein, 2016; Kudla & Przewlocki, 2021). Together, these effects are thought to mediate analgesia. The role of ketamine activation of opioids in analgesia has been debated, however, as in some experimental settings naloxone, an opioid receptor antagonist, was not able to inhibit ketamine-induced analgesia (Mikkelsen et al., 1999; Sleigh, Harvey, Voss & Denny, 2014).

Several animal studies have reported that NK also possesses antinociceptive properties, with an estimated potency of one-fifth to one-third compared to ketamine (Peltoniemi, Hagelberg, Olkkola & Saari, 2016). The analgesic effects of NK in humans, however, are largely unknown (Peltoniemi et al., 2016).

1.2.4.2 Antidepressant

The discovery of ketamine's antidepressant effects in humans were first detailed in the 1970s; however, this was not followed up due the illicit nature of the drug (Wei, Chang & Hashimoto, 2020). A study in 2000 demonstrated that ketamine (0.5mg/kg intravenously) significantly improved depressive symptoms in subjects suffering with major depression (Berman et al., 2000). Since then, many studies have investigated the antidepressant effects of both ketamine (Murrough et al., 2013; Fond et al., 2014; McGirr et al., 2015) and esketamine ((S)-ketamine; Fedgchin et al., 2019; Popova at al., 2019), and their efficacy in patients with treatment-resistant depression is well established. Consequently, in 2019, (S)-ketamine was approved for treating resistant major depressive disorder in both the United States and Europe (Wei, Chang & Hashimoto, 2022). Despite being investigated for more than 20 years,

the precise cellular and molecular mechanisms underlying ketamine's antidepressant effects are still not known.

It is generally acknowledged that blockade of NMDARs located on GABAergic inhibitory interneurons accounts for the rapid antidepressant effects of ketamine (Yang, Yang, Luo & Hashimoto, 2019; Wei et al., 2020). This blockade causes disinhibition of pyramidal cells (an excitatory neuron) in the prefrontal cortex (Homayoun & Moghaddam, 2007), which leads to a burst of glutamatergic transmission, and subsequent activation of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor (Yang et al., 2019; Wei et al., 2020). The ability of AMPA receptor antagonists to inhibit the antidepressant effects of ketamine in rodents suggests that activation of the AMPA receptor is required for ketamine's antidepressant effects (Maeng et al., 2008).

Other non-ketamine NMDAR antagonists, however, do not show robust ketamine-like antidepressant effects in patients. Further, (R)-ketamine has greater antidepressant effects when compared to (S)-ketamine in a rodent model of depression, even though (S)-ketamine is a more potent NMDAR antagonist (Yang et al., 2019). This suggests the potential involvement of other pathways and/or molecular targets that contribute to the antidepressant effects of ketamine. For example, reports that naltrexone, an opioid receptor antagonist, was able to significantly reduce ketamine's antidepressant effect in ketamine-responsive treatment-resistant depression patients suggests a role for opioid receptor activation (Williams et al., 2018). The mechanistic target of rapamycin (mTOR), and the brain-derived neurotrophic factor (BDNF) tyrosine kinase receptor B (TrkB) pathways, are also postulated to contribute to ketamine's antidepressant properties.

Brain-derived neurotropic factor is a growth factor that is significantly decreased in serum levels of patients with major depressive disorder (Schmidt & Duman, 2010). Antidepressant treatments can reverse this effect, and systemic or intra-hippocampal administration of BDNF is shown to have antidepressant-like effects in rodent models (Hoshaw, Malberg & Lucki, 2005; Schmidt & Duman, 2010). Activation of TrkB, the high affinity receptor of BDNF, is required for the antidepressant effects of BDNF, and ketamine both increases the activation of this receptor, and increases overall BDNF protein levels (Autry et al., 2011). Moreover, BDNF was shown to be critical for the antidepressant effects of ketamine, as it failed to exert

any antidepressant action in mice with *Bdnf* knockdown (Autry et al., 2011; Zanos & Gould, 2018).

The mechanistic target of rapamycin is a serine/threonine kinase that regulates, amongst other things, neurogenesis, dendritic outgrowth, protein translation initiation, and protein synthesis (Zanos & Gould, 2018), and has been implicated in the antidepressant effects of several antidepressant drugs (Park et al., 2014). Indeed, low levels of mTOR activity have been reported in patients with depression and are associated with increased depressive symptoms (Cardona-Acosta & Bolaños-Guzmán, 2023). Activation of TrkB by BDNF causes downstream signalling cascades that can lead to activation of mTOR complex 1, driving protein translation (Zanos & Gould, 2018). Ketamine is reported to induce mTOR activation within thirty minutes of administration in the prefrontal cortex and hippocampus of rodents (Zanos & Gould, 2018), and pre-treatment with the selective mTOR inhibitor rapamycin prevents the antidepressant effects of ketamine in rats (Li et al., 2010).

Given its role in the processing of reward and reinforcement, the mesolimbic dopaminergic system is an appropriate target for the treatment of depression, and ketamine's antidepressive effects are reported to be, at least partly, mediated by molecular and synaptic adaptations of areas within mesolimbic brain regions (Cardona-Acosta & Bolaños-Guzmán, 2023).

A role for NK in the antidepressant effects of ketamine has also been suggested. Yang et al. (2018) reported that (S)-NK and (R)-NK displayed antidepressant effects in inflammation and chronic social defeat stress models in mice, and that (S)-NK was the more potent of the two enantiomers. The antidepressant effects of (S)-NK in mice are thought to be, at least partly, mediated by BDNF-TrkB and mTOR signalling, as TrkB and mTOR inhibitors were able to block its antidepressant effects (Yang et al., 2018).

1.3 RECREATIONAL USE OF KETAMINE

1.3.1 Legal Status of Ketamine

Ketamine is currently a controlled substance in many countries including the UK, the US, India, Australia, Canada, and Hong Kong. The Misuse of Drugs Act was passed in the UK in 1971. It implements the UK's obligations under the Single Convention on Narcotic Drugs (1961) and

the Convention on Psychotropic Substances (1971), with the aim of preventing the misuse of controlled drugs by prohibiting the possession, supply, manufacture, import, or export of controlled drugs, except where permitted by regulations or possession of a licence. The Misuse of Drugs Act 1971 divides controlled drugs into the classes A, B, or C, broadly based on their relative harms, with higher classification drugs carrying stricter penalties for possession, supply, and importation.

In the UK, ketamine is a class B schedule 2 drug. Other class B drugs include oral amphetamines, cannabis, codeine, and barbiturates. Schedule 2 drugs are only available as prescription medicines and so can be legally possessed and supplied by pharmacists and doctors. Regulations also require record keeping and put forward rules relating to the storage requirements of the drugs. Ketamine carries a penalty of up to 5 years in prison and/or an unlimited fine for possession, and up to 14 years and/or an unlimited fine for supply or production (UK Government, n.d.). Despite this, ketamine use is still on the rise, and can be unlawfully obtained via the internet, dark web, or the street. It is reportedly easy to obtain, and relatively inexpensive, at around £20-30 per gram (Mason, Cottrell, Corrigan, Gillatt & Mitchelmore, 2010; Advisory Council on the Misuse of Drugs, 2013; DrugWise, 2016).

1.3.2 Demographics

Ketamine abuse is becoming an increasing problem worldwide. Despite widespread prohibition the recreational use of ketamine has been on the rise since 1997, with the latest data in the UK indicating that prevalence of use among adults is currently the highest on record (Focal Point, 2019). The number of ketamine users is particularly of concern in Asia, where large quantities of the drug are manufactured (Wood et al., 2011); and in Hong Kong, where it is the second most abused psychotropic drug in the region (Tam et al., 2018).

Ketamine use is reportedly most common in males and those who attend night clubs (Advisory Council on the Misuse of Drugs, 2013). In the UK, recreational ketamine use is most common in young people, with individuals aged 16-24 being four times more likely to use the drug than those aged 16-59 (Focal Point, 2019). In the same report, 2.8% of 16–24-year-olds stated that they had used ketamine in the last year.

A study investigating individuals attending electronic dance music parties in New York revealed that 14.6% had reported using ketamine in the past year. However, after testing hair

samples for detection of ketamine, the estimation of ketamine use was up to 2.8 times higher than self-reported use, suggesting that ketamine use is extensively underreported in some areas (Palamar, Salomone, Rutherford & Keyes, 2021).

1.3.3 Ketamine Use Disorders

Ketamine's popularity as a drug of abuse can be explained by its reported side effects, which include auditory and visual hallucinations, out-of-body experiences, novel body sensations, and euphoric rush (Shahani et al., 2007; Mason et al., 2010). A study by Dillon, Copeland & Jansen (2003) revealed that the vast majority (80%) of ketamine users considered these psychological effects to be 'positive effects'.

There appears to be a widely held belief amongst ketamine users that the drug has a wide margin of safety and is relatively non-addictive (Chu et al., 2008). However, it is estimated that 25-50% of ketamine users develop urological symptoms that are indicative of KC (Chu et al., 2008; Wood et al., 2011). Ketamine has also been shown to increase the release of dopamine in the nucleus accumbens of rats using *in vivo* microdialysis (Witkin et al., 2016), and increased dopamine release in this area of the brain has been associated with addiction liability (Cadoni & Di Chiara, 2007). Moreover, although not numerous, the development of ketamine addiction has been previously reported (Bonnet, 2015).

1.3.4 Dose Range and Route of Administration

A UK based study found that 96% of ketamine users ingest the drug via insufflation (n=7,700; Advisory Council on the Misuse of Drugs, 2013), which may be due to the rapid onset of effects when administered this way. The reported recreational dose of ketamine varies between individuals. A survey of 1,285 individuals who had used ketamine in the previous year revealed that, during a single session, 35% use between 0.25 and 0.5g; 34% typically use more than 1g; and 5.2% regularly take ≥3g (Winstock et al., 2012). Moreover, in reference to the maximum amount of ketamine taken, 24.6% reported using ≥3g in a session, and 6.6% had used ≥5g. In the same study, the majority of users were classified as low frequency users having used the drug 1-4 days/month, with 15.6% using 5-8 days/month, and 13.4% taking ketamine ≥9 days/month.

The dose range for ketamine administered for therapeutic reasons varies and is dependent on its intended use. When taken orally for analgesia, initial doses are typically 2-25mg to be taken three to four times a day, and can increase to 40-60mg four times a day (Visser & Schug, 2006). The upper limits of continuous subcutaneous injection (600mg/24h) are similar doses to those reported in recreational users of ketamine (Visser & Schug, 2006). It is, however, important to consider the difference in the route of administration between therapeutic and recreational use of ketamine, as insufflation, the primary route of administration amongst recreational ketamine users, results in a much lower bioavailability compared to intravenous or intramuscular injection.

1.3.5 Ketamine Toxicity

Ketamine can cause a variety of cardiovascular, neurological, psychiatric, urogenital, and abdominal symptoms (Othurhu, Vashisht, Claus & Cohen, 2019). In recreational users, acute ketamine toxicity is associated with neuro-behavioural abnormalities, including hallucinations, anxiety, agitation, and psychosis, with such effects also putting the user at an increased risk of physical harm or trauma (Kalsi, Wood & Dargan, 2011). Features of chronic ketamine use include neuropsychiatric disorders, and gastrointestinal toxicity, including abnormal liver function and abdominal pain (Kalsi et al., 2011). Abdominal pain associated with regular ketamine use is a known phenomenon amongst recreational users and is often referred to as “k cramps” (Wang, Kivovich & Gordon, 2017). The aetiology of these cramps is not known; however, as both ketamine and NK are reported to be partly excreted by the biliary system (Ireland & Livingston, 1980), it is possible that they have a direct toxic effect on the biliary tree epithelium, leading to abdominal pain (Wang et al., 2017). Aside from the well-established LUT symptoms associated with long-term ketamine use, toxicity to the upper urinary tract has also been reported. Chu et al. (2008) reported that 51% of recreational ketamine patients who presented with urinary symptoms had developed hydronephrosis (the swelling of one or both kidneys due to a build-up of urine). Additionally, Chu et al. (2008) reported of one patient with severely impaired kidney function, and ketamine-induced kidney damage has been reported in both humans (Lee et al., 2015), rats (Jang et al., 2017), and mice (Yeung, Rudd, Lam, Mak & Yew, 2009).

1.3.6 Polysubstance Abuse

Potential polysubstance use is important to consider in individuals who abuse ketamine, as this could contribute to the bladder dysfunction and LUT damage associated with chronic ketamine use. A study conducted in Hong Kong by Mak et al. (2011) reported that 80% of ketamine users surveyed took multiple drugs, and two-thirds reported consuming alcohol and ketamine at the same time. Therefore, as many ketamine users undertake polysubstance abuse, drug interactions make it difficult to ascertain the individual effects of ketamine on the LUT (Mak et al., 2011). There is, however, evidence to suggest that ketamine alone can induce the described phenomena of cystitis and the numerous associated LUT symptoms. The first case study to identify KC reported ulcerative cystitis in nine ketamine users who had not taken any other illicit drugs (Shahani et al., 2007), and a number of *in vivo* animal studies have reported the development of KC following administrations of ketamine alone. Additionally, samples of illegally distributed ketamine have been shown to contain no other agent that is likely to contribute to bladder dysfunction (Chu et al., 2007).

1.4 NEW PSYCHOACTIVE SUBSTANCES

New psychoactive substances (NPS) are substances that have been produced to mimic the effects of illicit drugs. As NPS are analogues, they are often able to circumvent legal prohibition, and as such are sometimes referred to as “legal highs”. Unlike traditional illicit drugs, NPS are typically sold online, where they are often marketed as “research chemicals”, “plant food”, or “bath salts” (Liechti, 2015).

Despite growing clinical and public concern, the prevalence of NPS in the recreational drug scene has been on the increase since the turn of the 21st century (Wood & Dargan, 2012). Many newly emerging NPS are produced by modifying the molecular structures of previously controlled compounds, and importantly, even a small molecular difference can result in a large difference in terms of biological activity and pharmacokinetic parameters (Zawilska & Andrzejczak, 2015). Alarmingly, as NPS are typically novel or untested, there is often very little information regarding their pharmacological effects or the potential toxicity associated with these substances following acute or chronic use (Wood & Dargan, 2012; Baumeister, Tojo & Tracy, 2015). Further, inter- and intra-product concentration variability, the presence of multiple psychoactive substances in a single product, no information of active ingredients,

and different compounds being advertised under the same name all represent risk factors for users (Zawilska & Andrzejczak, 2015). These issues can lead to severe adverse effects, especially when considering that little is known about NPS metabolism or their interaction with other medicines (Zawilska & Andrzejczak, 2015). Public awareness of NPS and their potential associated risks is lacking, highlighting the need for drugs education and intervention by policy makers, particularly in schools and universities (Delligianni, Corkery, Schifano & Lione, 2017).

Some governments attempt to resolve this problem by banning the newly identified substances as soon as possible, but due to the rapid nature of the NPS market and the fast production of new agents, any drug that is made illegal can be quickly replaced by new legal alternatives (Baumeister et al., 2015). Indeed, between 2005 and 2014, the European Union reported the emergence of over 300 NPS, with around one new substance identified every week (Liechti, 2015); and between 2020 and 2022, 139 NPS were reported for the first time to the European Monitoring Centre for Drugs and Drug Addiction (Figure 1.3; European Monitoring Centre for Drugs and Drug Addiction, 2023).

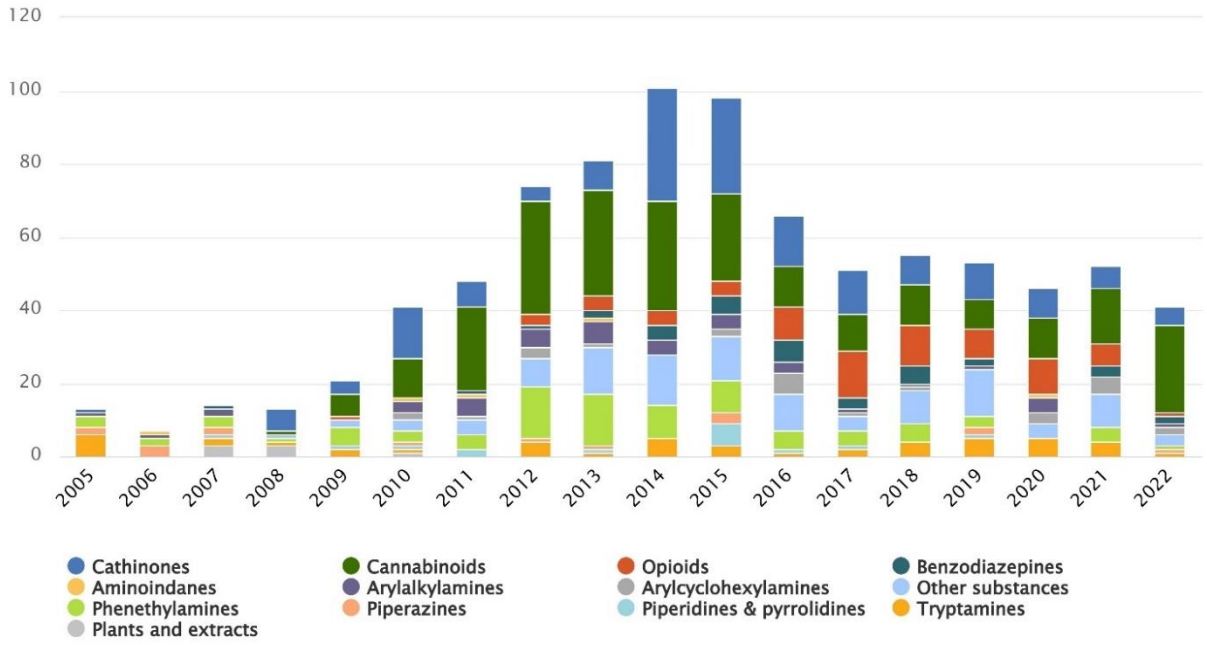

Figure 1.3. Annual number of new psychoactive substances reported for the first time to the EU Early Warning System, by category, for the period of 2005-2022. (Adapted from The European Monitoring Centre for Drugs and Drug Addiction, 2023).

In 2016, the UK government introduced the Psychoactive Substances Act (PSA), making it an offence to, amongst other things, produce, supply, or import/export psychoactive substances. The act came into force on 26th May and was intended to battle the growing problem of NPS and decrease their use amongst the general population. Despite these new laws, however, legal high websites still exist in the UK by selling substances that they claim adhere to the act, and the sale and use of many NPS is still legal in other parts of the world. Interestingly, a survey undertaken in the UK one year after the PSA reported that overall awareness of NPS and their risks has not increased, and that the legislation has had no impact on overall NPS use amongst those surveyed (Deligianni, Daniel, Corkery, Schifano & Lione, 2019). A review of the PSA by the Home Office in 2018, however, suggests that there has been a substantial reduction in NPS use amongst the overall adult population since the introduction of the PSA, and that sales of NPS in shops and online had been fundamentally eliminated (Home Office, 2018). Further, they reported that the PSA has caused the price of NPS to rise and their availability to fall, and that users have switched to street vendors and the dark web to source them (Home Office, 2018; Deligianni et al., 2019). Despite the act, however, emergence of new NPS has not ceased, and the use of NPS among vulnerable groups in some areas remains unaffected (Home Office, 2018).

1.4.1 Categories of New Psychoactive Substances

New psychoactive substances are generally broken down into four broad categories: synthetic stimulants, synthetic cannabinoids, synthetic hallucinogens, and synthetic depressants (Tracy, Wood & Baumeister, 2017). Synthetic hallucinogens are further broken down into hallucinogens and dissociatives (Figure 1.4), with ketamine falling under the latter subcategory (Shafi, Berry, Sumnall, Wood & Tracy, 2020).

Dissociatives are made up of two main classes: arylcyclohexylamines (to which ketamine and methoxetamine belong), and diarylethylamines (to which diphenidine and methoxphenidine belong; Figure 1.4), and these drugs modify perceptions of sight and sound and create an emotional state of detachment from reality (Shafi et al., 2020).

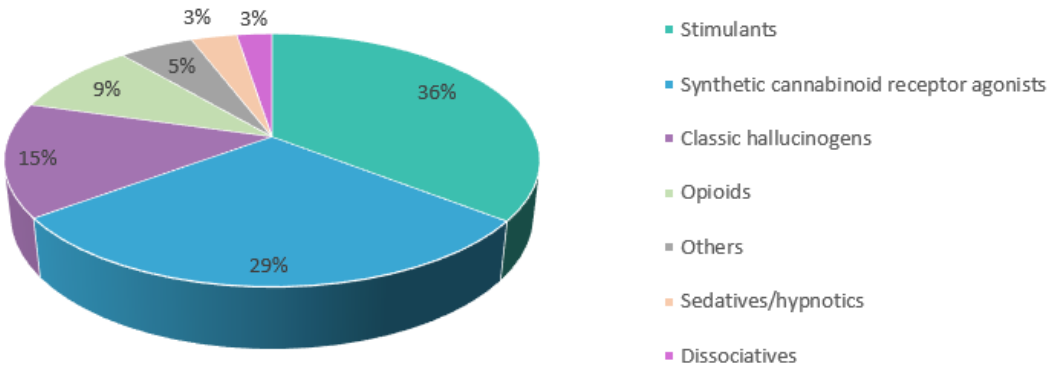

Figure 1.4. Proportion of new psychoactive substances, by psychoactive effect group, as of December 2020 (Adapted from The European Monitoring Centre for Drugs and Drug Addiction, 2023).

1.4.2 Methoxetamine

Methoxetamine (MXE) is an NPS that first appeared on the market in 2010 (Kjellgren & Jonsson, 2013; Craig & Loeffler, 2014). As MXE is an analogue of ketamine, it produces similar dissociative and hallucinogenic effects, leading to its use as a recreational drug (Lawn, Borschmann, Cottrell & Winstock, 2016). In the UK, a study on the prevalence of MXE use among polydrug users revealed that, of the 7,700 respondents, 326 (4.2%) had used MXE in the past 12 months (Winstock, Lawn, Deluca & Borschmann, 2016). Methoxetamine was designed to have greater potency than ketamine so that lower doses would produce similar effects (Horsley et al., 2016). In theory, this should limit the accumulation of urotoxic metabolites, and so MXE was extensively advertised online as a "bladder friendly" alternative to ketamine, despite no pharmacological data to corroborate this claim (Zawilska, 2014; Horsley et al., 2016; Lawn et al., 2016). Interestingly, a recent study investigating the inhibitory activity of MXE on the NMDA receptor in HEK293 cells (a cell line derived from human embryonic kidney cells), revealed that ketamine is more potent than MXE (IC_{50} of 0.351µM and 0.524µM, respectively; Botanas et al., 2021).

1.4.2.1 Pharmacology of Mexthoxetamine

Structurally, like ketamine, MXE is classed as an arylcyclohexylamine, but it has two distinct structural differences (Figure 1.5). The 2-chloro group on the phenyl ring of ketamine is replaced with a 3-methoxy group in MXE, which is purported to reduce the analgesic and anaesthetic properties compared to ketamine (Coppola & Mondola, 2012). Additionally, the

N-methyl group on the amine of ketamine has been replaced by an *N*-ethyl group, which is thought to increase MXE's potency and duration of action compared to ketamine (Coppola & Mondola, 2012).

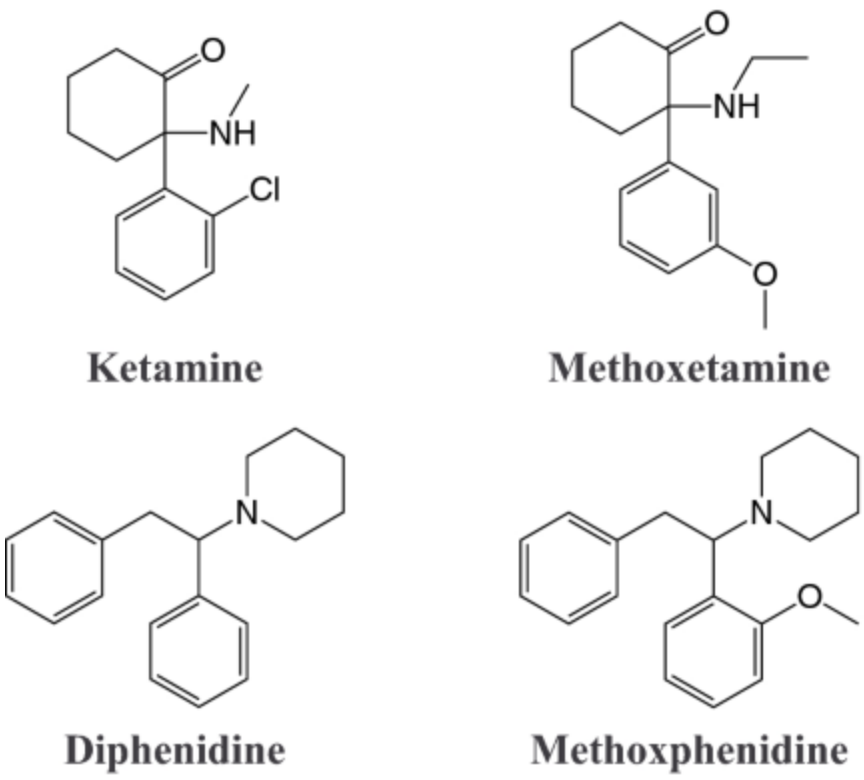

Figure 1.5. Molecular composition of ketamine and the ketamine-like new psychoactive substances methoxetmine, diphenidine, and methoxphenidine (adapted from Luethi & Liechti, 2020).

The mechanism of action of MXE is thought to be similar to that of ketamine, including non-competitive NMDAR antagonism and dopamine reuptake inhibition (Corazza et al., 2012; Horsley et al., 2016). Methoxetamine has been shown to inhibit human dopamine transporter, serotonin transporter, and norepinephrine transporter in HEK293 cells (Hondebrink et al., 2017), and Roth et al. (2013) also reported an affinity of MXE for the serotonin transporter.

Due to the pharmacodynamic similarities between MXE and ketamine, MXE was hypothesized to produce rapid antidepressant effects (Coppola & Mondola, 2012), and subsequent studies have supported its therapeutic potential as an antidepressant (Botanas et al., 2017; Botanas

et al., 2021). Methoxetamine is also reported to have analgesic properties following a report of self-medicated MXE to alleviate chronic foot pain (Maskell, Bailey & Rose, 2016), and the ability of MXE to exhibit analgesic effects in rats (Zanda et al., 2017).

1.4.2.2 Legal Status of Methoxetamine

In April 2012, the UK government used a temporary class drug order for the first time to ban MXE, and, following an assessment by the Advisory Council on the Misuse of Drugs, the drug was made permanently illegal in February in 2013. Methoxetamine is still legal in many other countries around the world, however (Lawn et al., 2016).

1.4.2.3 Methoxetamine Toxicity

In recent years, there has been a significant growth in reported cases of fatal and non-fatal intoxications related to MXE use (Botanas et al., 2019). Symptoms of acute MXE-associated toxicity include neuro-behavioural abnormalities, such as agitation, anxiety, hallucinations, and aggression (Botanas et al., 2019); and reversible cerebellar features, including ataxia and nystagmus (involuntary rhythmic motion of the eyes; Shields et al., 2012).

A study in 2014 looked at the effects of daily MXE injections (30mg/kg) in mice (Dargan, Tang, Liang, Wood & Yew, 2014). They concluded that, after 3 months, MXE administration was associated with significant bladder and renal toxicity, and that these observations were similar to other animal model studies investigating the effects of chronic ketamine administration. Similarly, MXE was shown to induce increased micturition frequency, urothelial damage, and inflammatory cell infiltration in the bladders of female Sprague-Dawley rats following daily intraperitoneal injection of 30mg/kg MXE for 4 or 12 weeks (Wang et al., 2017).

The chronic use of MXE has also been linked to bladder toxicity in humans. Lawn et al. (2016) reported that, in a group of MXE users, 23% had experienced urinary symptoms that were associated with MXE use. However, all respondents had also used ketamine at least once in their lifetime, and so ketamine use could not be ruled out as a potential cause of the urinary dysfunction.

1.4.3 Diphenidine and Methoxphenidine

Following the ban of MXE in early 2013, several legal ketamine alternatives emerged on the market, including methoxphenidine (MXP; also referred to as methoxydiphenidine or 2-MeO-Diphenidine) and diphenidine (DPD). As such, MXP and DPD are reported to display similar dissociative and hallucinogenic effects to ketamine and MXE (Van Hout & Hearne, 2015; Beharry & Gibbons, 2016).

1.4.3.1 Legal Status of Diphenidine and Methoxphenidine

Since 2016, the production, supply, or importation of DPD and MXE is prohibited in the UK under the PSA. Methoxphenidine is also a controlled substance in Canada, China, Germany, Italy, and Sweden (World Health Organisation, 2020a). The legal status of diphenidine, on the other hand, is reportedly in a legal grey area, with the drug being easily obtainable from online shops (World Health Organisation, 2020b).

1.4.3.2 Diphenidine and Methoxphenidine Toxicity

Importantly, as MXP and DPD are still relatively new drugs, there is limited information regarding their pharmacology and acute or long-term health consequences (Van Hout & Hearne, 2015; Wallach et al., 2016). A case study of hospital patients with suspected or admitted NPS intake reported that acute DPD or MXP toxicity is associated with hypertension, tachycardia, anxiety, and an altered mental status including confusion, disorientation, dissociation, and hallucinations (Helander, Beck & Bäckberg, 2015). It was noted, however, that polysubstance use was documented in 87% of patients, making it difficult to assess the individual effects of DPD or MXP. The acute and long-term effects of these compounds on the bladder are yet to be investigated.

1.4.3.3 Pharmacology of Diphenidine and Methoxphenidine

Wallach et al. (2016) carried out radioligand binding studies in rat brain homogenate and showed that both MXP and DPD are relatively selective NMDAR antagonists. They also reported that DPD and MXP displayed moderate activity at dopamine and norepinephrine reuptake inhibition in HEK293 cells, and that both have moderate affinities for sigma-1 and sigma-2 receptors (Wallach et al., 2016). Another study, however, reported that, in rat striatal

sections, DPD, but not MXP, can bind the dopamine transporter and in turn increase dopamine efflux (Sahai, Davidson, Dutta & Opacka-Juffry, 2018).

1.4.4 Concentration of Methoxetamine, Diphenidine, and Methoxphenidine in the Urine

It has been postulated that the bladder damage often observed in KC patients is due to a direct toxic effect of ketamine on the bladder. Ketamine and its metabolites reportedly accumulate in the bladder, and relatively high concentrations of these compounds have been observed in the urine of recreational users (Moore, Sklerov, Levine & Jacobs, 2001; Wang et al., 2005; Cheng et al., 2008).

Although limited, there is some information available regarding the concentration of MXE, DPD, and MXP in the urine of recreational NPS users (Table 1.3). In the case of a 29-year-old male who died following MXE intoxication, a urine concentration of 85µg/mL (343µM) was observed (Adamowicz & Zuba, 2014). Additionally, an MXE urine concentration of 660ng/mL (2.7µM) was reported in a 28-year-old male following acute poisoning via nasal MXE (Lukasik-Glebocka, Sommerfeld, Tezyk, Zielinska-Psuja & Druzdz, 2013).

A DPD urine concentration of 631ng/mL (2.37µM) was reported in a 30-year-old male with acute intoxication following DPD intake (Gerace, Bovetto, Corcia, Vincenti & Salomone, 2017). Diphenidine and MXP have been reported at urine concentrations ranging 8-19,000ng/mL (30nM-72µM) and 3-8,367ng/mL (10nM-28.3µM), respectively, in Swedish emergency room patients with suspected NPS intake (Helander et al., 2015). Additionally, an MXP urine concentration of 1,066µg/L (3.6µM) was reported in a male who died from stab wounds following a chemsex party (Gonclaves, Castaing, Titier & Dumestre-Toulet, 2021).

Due to the wide range and high upper-limit concentrations of MXE, DPD, and MXP reported in the urine of users, it is possible that, like ketamine, these NPS are able to accumulate in the urine and have a direct toxic effect on the bladder. The direct effects of MXE, MXP, and DPD on the bladder have not been investigated, however.

Table 1.3. The reported concentration ranges of methoxetamine, diphenidine, and methoxphenidine present in the urine of recreational new psychoactive substance users.

REFERENCE & DRUG USAGE DETAILS	DRUG		
	Methoxetamine	**Diphenidine**	**Methoxphenidine**
Adamowicz & Zuba (2014) ***(MXE intoxication fatality)***	85µg/mL 343µM	-	-
Lukasik et al. (2013) ***(Suspected drug users - no usage details)***	660ng/mL 2.7µM	-	-
Gerace et al. (2017) ***(Acute intoxication following DPD intake)***	-	631ng/mL 2.37µM	-
Helander et al. (2015) ***(Swedish emergency room patients with suspected NPS intake)***	-	8-19,000ng/mL 30nM-72µM	3-8,367ng/mL 10nM-28.3µM
Gonclaves et al. (2021) ***(Methoxetamine use in forensic chemsex case)***	-	-	1,066µg/L 3.61µM

1.5 BLADDER MORPHOLOGY AND FUNCTION

The LUT is comprised of the bladder and urethra, and is responsible for the storage and controlled release of urine (Figure 1.6; Chapple & MacDiarmid, 2000). Disruption of the storage function of the bladder can lead to bladder dysfunction and LUT symptoms (Andersson & Arner, 2004).

1.5.1 Histology of the Bladder

Histologically, the bladder consists of three distinct layers: an outer layer of connective tissue; a middle layer of smooth muscle cells which make up the detrusor muscle; and an inner mucosal layer that is comprised of the urothelium, basement membrane, and lamina propria (Chapple & MacDiarmid, 2000; Andersson & McCloskey, 2014).

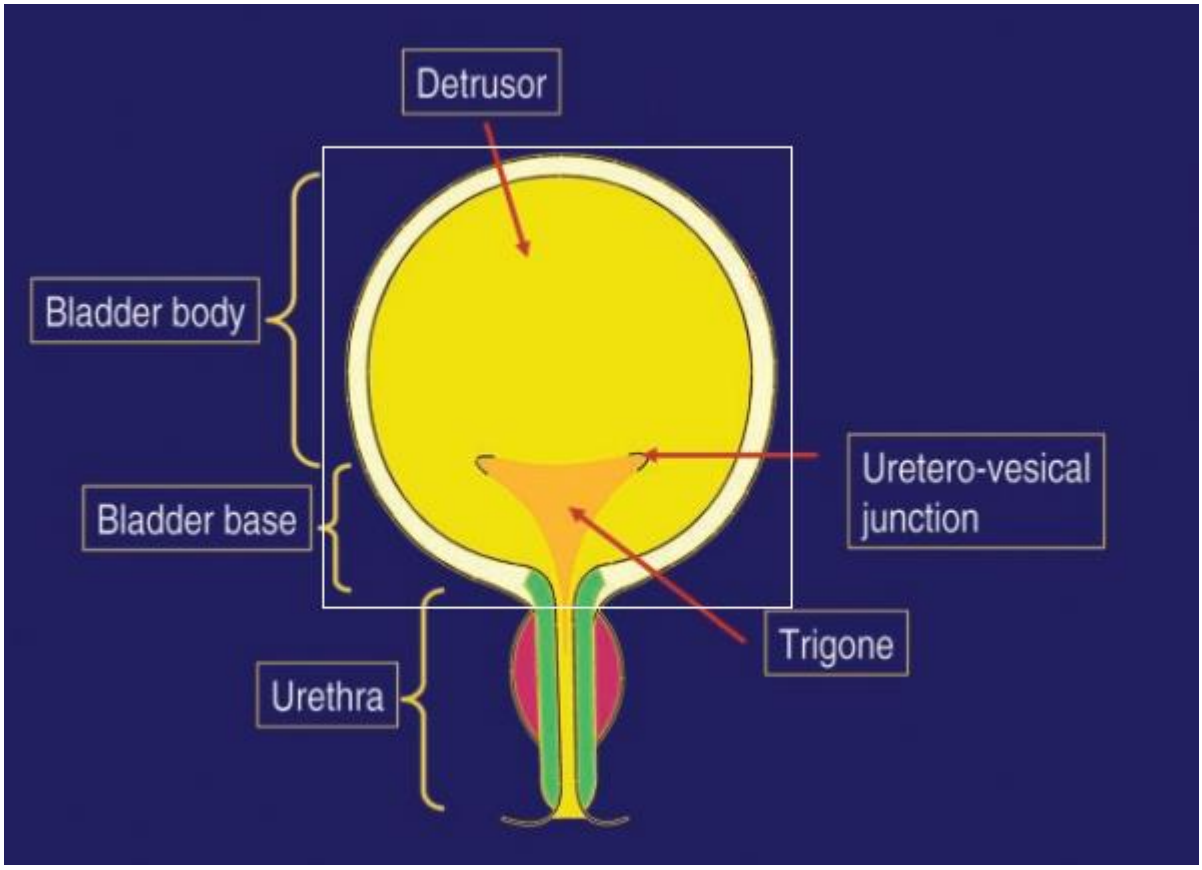

Figure 1.6. Morphology of the bladder. The outlined area indicates the section of the bladder used in this study. (Diagram adapted from Andersson & Arner, 2004).

1.5.1.1 The Urothelium

The urothelium lines the lumen of the urinary bladder and plays an important role in protecting the underlying tissue layers from toxic components in the urine. It is a stratified epithelium consisting of three distinct cell layers: the basal cell layer; the intermediate cell layer; and a superficial layer, comprising a single layer of large hexagonal cells known as umbrella cells (Dalghi, Montalbetti, Carattino & Apodaca, 2020).

The umbrella cells constitute the cell layer that is most important for maintaining the barrier function of the urothelium (Arrighi, 2015), and several of its properties contribute to this function. They are interconnected by high resistance tight junctions, and the majority of their apical surface is covered by crystalline proteins called uroplakins which assemble into hexagonal plaques (Birder & de Groat, 2007). The apical surface of the urothelium is also covered by a glycosaminoglycan (GAG) layer which is a thick mucus layer comprising of

glycoproteins and proteoglycans (Klingler, 2016). Disruption to any of these properties can compromise the barrier function of the urothelium and lead to increased permeability.

Although previously believed to act solely as a passive barrier, more recent evidence suggests that the urothelium is an adaptive structure that can detect and respond to various physiological and chemical stimuli, and release various signalling molecules such as acetylcholine, adenosine triphosphate (ATP), and nitric oxide (Birder & de Groat, 2007; Dalghi et al., 2020).

1.5.1.2 Lamina Propria

The lamina propria separates the urothelium and the detrusor muscle. It is made up of an extracellular matrix containing many different cell types, including fibroblasts, adipocytes, interstitial cells, various immune cells, and afferent and efferent nerve endings (Andersson & McCloskey, 2014; Bolla, Odeluga & Jetti, 2021). Although incompletely understood, the roles of the lamina propria are thought to be numerous. Such roles include determining bladder compliance, relaying signals to the central nervous system, the production of factors which affect both the urothelium and detrusor muscle, and acting as a mediator of information from the urothelium to the underlying tissue layers (Andersson & McCloskey, 2014).

1.5.2 Histology of the Rat Urinary Bladder

The function and histological profile of the human urinary bladder is similar to that of the rodent urinary bladder (Figure 1.7), with the main differences being the size and intra-abdominal position (Chamorro, Engberg & Fossum, 2020). Histologically, like humans, the rat urinary bladder is made up of three distinct layers: an innermost mucosal layer, a middle layer of smooth muscle, and an outer layer of connective tissue. However, the rodent urothelium is typically three cell layers thick, whereas, in larger mammals, including humans and monkeys, the urothelium usually has more than three layers, occasionally up to ten, due to the intermediate cells consisting of multiple layers (Jost, Gosling & Dixon, 1989).

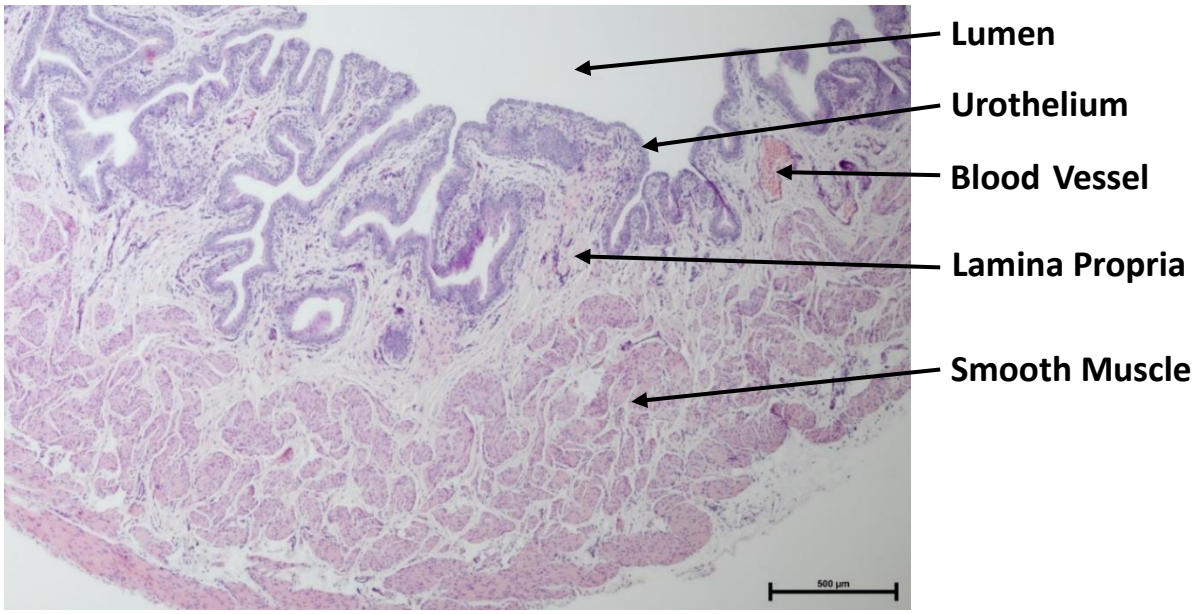

Figure 1.7. Haematoxylin and eosin-stained section of rat urinary bladder.

1.5.3 Micturition

Micturition is the process of emptying urine from the urinary bladder. In humans, micturition through detrusor contraction is generally attributed to the release of acetylcholine (Andersson & Arner, 2004), however, a small component (likely equating to less than 3%) of this response is thought to be attributed to ATP (Burnstock, 2014; Andersson, 2015). In rats, the micturition response is also thought to be primarily mediated through muscarinic receptor activation by acetylcholine, but the extent of the contribution of ATP is thought to be greater in rats than humans (Sultana, Berger, Cox, Kelly & Lehmann, 2021). In one study, intra-arterial administration of atropine close to the bladder in conscious rats decreased micturition pressure by 54% (Igawa, Mattiasson & Andersson, 1993).

1.5.4 *In Vitro* Bladder Contractility

The *in vitro* bladder is able to contract in response to various different stimuli. Carbachol (CCH), a cholinergic agonist, has long been known to produce contractions in *in vitro* bladder preparations, and this is largely attributed to activation of M_3 muscarinic receptors (Schneider, Hein & Michel, 2003). Also well documented is the contraction of rat bladder strips in response to ATP, which is thought to be mediated through P2X receptors (Wibberley, Chen, Hu, Hieble & Westfall, 2003; Meng et al., 2011).

Electrical field stimulation (EFS) has been employed to contract bladder strips *in vitro*, and in human bladder tissue such contractions are thought to be solely mediated by acetylcholine release, as atropine entirely inhibits these EFS-induced responses (Bayliss, Wu, Newgreen, Mundy & Fry, 1999). In bladder strips from most other mammals, *in vitro* EFS responses are thought to be mediated by the release of both acetylcholine and ATP (Foster & Ross, 2004; McCarthy et al., 2019). However, studies in guinea pigs and rats have shown a portion of the EFS-evoked response that is resistant to both atropine and ATP desensitization, suggesting a potential role for other unknown transmitters/modulatory pathways (Patra & Westfall, 1994; Wibberley et al., 2003).

Many studies have also utilized high potassium solutions to contract bladder strips (Jezior et al., 2001; Meng et al., 2011). Potassium chloride causes bladder contractility *in vitro* through membrane depolarisation, which subsequently leads to calcium influx through voltage-operated calcium channels (Jezior et al., 2001; Wibberley et al., 2003).

1.5.5 Receptor Expression in the Bladder

There is limited information regarding the receptor targets of ketamine in the bladder. The presence of the NMDAR in both human and rat bladder has been suggested by Gonzalez-Cadavid, Ryndin, Vernet, Magee & Rajfer (2000). They were able to bind an NMDAR ligand to membrane preparations of these tissues but were not able to determine where they are localised within the bladder. However, Baker, Shabir, Georgopoulos & Southgate (2016) reported the absence of GRIN (genes that encode NMDAR subunits) transcripts in the urothelium.

There is also evidence supporting the presence of dopamine D_1 and D_2 receptors in the bladder (Escaf, Cavallotti, Ricci, Vega & Amenta, 1994), and ketamine is reported to act as a partial agonist at the D_2 receptor in rat striatal tissue (Kapur & Seeman, 2002). Therefore, a potential role for the dopaminergic system in the pathogenesis of KC seems plausible, especially when considering that individuals with Parkinson's disease commonly present with LUT symptoms similar to those observed in KC (Mitra et al., 2015). In rat bladder strips, dopamine is reported to have no effect on contractility when administered alone, but inhibited responses to both non-adrenergic non-cholinergic nerve stimulation and high potassium, likely, in part, through D_2 receptor activation (Lot, 1993). Therefore, although not

directly demonstrated in bladder, the potential ability of ketamine to increase dopamine signalling in the bladder through dopamine reuptake inhibition, increased dopamine efflux (Hancock & Stamford, 1999), and/or direct partial agonism of D_2 receptors by ketamine would likely lead to bladder contractile inhibition. Further investigation of the potential role of dopamine signalling in KC may be of interest.

Sigma-1 receptors are reportedly present in both human and mouse urothelium (Gonzalez et al., 2015). Ketamine has been shown to bind to sigma-1 receptors in rat liver homogenate (Robson, Elliot, Seminerio & Matsumoto, 2012), and sigma-1 receptor agonists have been shown to inhibit rat bladder contractility to various stimuli (Levin, Whitbeck, Sourial, Tadrous & Millington, 2006). Further, radioligand binding assays have demonstrated that ketamine can directly bind to estrogen receptor alpha (Ho et al., 2018), and estrogen receptors are present in human bladder (Teng, Wang, Jarrard & Bjorling, 2008). However, the effect of ketamine or its ability to bind these receptors in bladder has not been reported.

1.6 KETAMINE-INDUCED CYSTITIS IN HUMANS

The first documented report of KC was in 2007, where Shahani et al. (2007) described nine patients who were daily recreational ketamine users and presented with dysuria, haematuria, urgency, and increased micturition frequency. Patient urine cultures were sterile, indicating the absence of infection, and tomography revealed a thickened bladder wall. Cystoscopically, all had severe ulcerative cystitis, and biopsies in four patients uncovered urothelial denudation and inflammation.

Since the initial publication in 2007, the characteristic features of KC detailed by Shahani et al. (2007) have remained consistent in subsequent reports. Chu et al. (2008) reported similar LUT symptoms of frequency, urgency, dysuria, haematuria, and urge incontinence in 59 ketamine users referred to urology units in Hong Kong, and similar LUT symptoms have been observed in many other case reports and studies of KC (Colebunders & Erps, 2008; Tsai et al., 2009; Winstock et al., 2012). Bladder wall thickening (Mason et al., 2010; Huang, Wang, Shen, Lin & Chang, 2014; Jhang, Birder, Chancellor & Kuo, 2016) and a thinning or denuded urothelium (Shahani et al., 2007; Chu et al., 2008; Tsai et al., 2009; Baker et al., 2013; Jhang et al., 2015; Lin et al., 2015) are also often reported in KC patients. Further, inflammatory cell infiltration within the bladder tissue is often observed, with eosinophils and mast cells being

the most commonly reported (Chu et al., 2008; Tsai et al., 2009; Chen, Lee, Chen & Lin, 2011; García-Larrosa et al., 2012; Jhang et al., 2015).

Although the mechanisms underlying KC are still not known, unique insight was provided by Kidger et al. (2016), who reported on a patient undergoing cystectomy for incessant pain related to ketamine abuse. The patient was discovered to have a rare urachal cyst near the bladder dome, and it was determined that, phenotypically, the epithelium lining the urachus was similar to that of the urothelium, and thus could be classified as such. Interestingly, Kidger et al. (2016) reported widespread loss of bladder urothelium, but no apparent abnormalities of the urothelium within the urachus. Moreover, it was noted that the only multi-layered areas of bladder urothelium remaining were those located in von Brunn's nests (invaginations of the urothelium into the lamina propria). Therefore, it was concluded that the loss of urothelium in this case of KC arose from direct ketamine or metabolite exposure, and not through systemic factors, as the only areas of intact urothelium observed were those that would not have come into direct contact with ketamine (urachal urothelium) or were partially protected by their physical location in the lamina propria (von Brunn's nests urothelium).

1.6.1 *In Vitro Studies*

The direct effects of ketamine on human urothelium were investigated by Baker et al. (2016). Human ureteric organ cultures after 72-hour exposure to ketamine (3mM) showed thinning of the urothelium and signs of apoptosis. It was postulated that ketamine has a direct toxic effect on the bladder urothelium, resulting in apoptosis and widespread loss of the urothelium, disrupting the barrier function.

Similarly, ketamine (0.1-8mM) was shown to induce cytotoxicity *in vitro* in human urothelial cells in a concentration- and time-dependent manner (Shen et al., 2015). The barrier function of these cells was also compromised, with an increase in permeability reported following ketamine exposure.

A study investigating the effects of ketamine on a human tissue-engineered three-dimensional urothelium reported concentration-dependent damage of the urothelium (particularly in the intermediate layers) by ketamine (0.5-10mM; Bureau et al., 2015). The integrity of the urothelium was also disrupted following ketamine exposure. It was noted that

the effects observed are likely a result of a direct toxic effect of ketamine, and as the tissue model is lacking a vascular or immune system, the observed effects were not due to immunologic or vascular factors as has been previously suggested.

Gonzalez-CaDavid et al. (2000) investigated the effects of ketamine on human bladder strips *in vitro*. They reported that, following contraction mediated by the muscarinic agonist bethanechol, the tissue was able to completely relax with increasing concentrations of ketamine (0.05-0.5mM), and these same concentrations of ketamine were also able to completely relax bethanechol-induced contractions in rat bladder. Dextromethorphan (DXM; an NMDAR antagonist) displayed similar effects (0.04-0.2mM), and pre-treatment with 0.1mM DXM was also able to block the contractile response to bethanechol.

1.7 KETAMINE AND NEW PSYCHOACTIVE SUBSTANCE-ASSOCIATED BLADDER DYSFUNCTION IN ANIMAL MODELS

Following reports of ketamine-associated bladder dysfunction in the late 2000s, many animal models of KC have been utilized in an attempt to elucidate ketamine's mechanism of action in the bladder. Additionally, although sparse, there have been some reports of the *in vivo* effects of methoxetamine on the rodent bladder.

1.7.1 Ketamine *In Vitro* Studies

Ceran, Pampal, Goktas, Pampal and Olmez (2010) looked at the effects of the anaesthetics ketamine, midazolam, and propofol on the urinary rat bladder. They reported a significant decrease in CCH-induced detrusor muscle contractility following pre-treatment with ketamine (≥1mM), however, under the same conditions, EFS-evoked contractility was unaffected.

Similarly, ketamine was shown to induce complete relaxation in rat bladder strips contracted with bethanechol (Gonzalez-CaDavid et al., 2000), and was also reported to inhibit contractility mediated by EFS. Interestingly, DXM and MK-801, two other NMDAR antagonists, were also able to relax precontracted bladder strips, and simultaneous addition of MK-801 and bethanechol eliminated the contractile response. This finding is similar to the observation that DXM concentration-dependently inhibited rat and mouse bladder strip contractility evoked by EFS (Levin, Whitbeck, Sourial, Tadrous & Millington, 2006).

Ketamine (365µM and 1.8mM) was also shown to concentration-dependently inhibit mouse bladder smooth muscle strip contractility evoked by EFS, CCH, α,β-meATP, and 50mM potassium chloride (KCl; Chen et al., 2020). These functional effects of ketamine were thought to be due to Cav1.2 (an L-type calcium channel isoform) antagonism, as both nifedipine (a Cav1.2 specific antagonist) and ketamine concentration-dependently inhibited EFS-evoked contractility potentiated by Bay K8644 (a Cav1.2 agonist), and inhibition of mouse bladder smooth muscle contractility by ketamine or nifedipine was able to be reversed by subsequent exposure to Bay K8644 (Chen et al., 2020). Moreover, Bay K8644 induced Ca^{2+} influx and intracellular Ca^{2+} signalling in primary cultured mouse bladder smooth muscle cells, and this was shown to be blocked by ketamine and nifedipine.

1.7.2 Ketamine *In Vivo Studies*

A study undertaken by Meng et al. (2011) looked at the effects of daily ketamine injections (100mg/kg) in female mice. After four weeks, ketamine-dosed mice displayed lower baseline detrusor pressure and high bladder compliance when compared to the saline treated controls, indicative of bladder underactivity. After 8 weeks of ketamine treatment, however, mice displayed symptoms that are characteristic of long-term ketamine use in humans; that is, an increase in micturition frequency and a reduction in bladder capacity. Meng et al. (2011) reported that this observed bladder overactivity was associated with an increased sensitivity of bladder strips to ATP, and upregulation of the P2X1 receptor. These subsequent *in vitro* organ bath studies were carried out on urothelial denuded detrusor muscle strips, however, removing any potential involvement of the urothelium on the bladder contractile response in this model of ketamine toxicity.

Several studies have investigated the functional changes in rat bladder following daily administration of ketamine. Juan et al. (2015) reported that rats receiving daily ketamine (25mg/kg) developed bladder hyperactivity and decreased bladder capacity after 28 days. This is similar to the findings presented by Chuang et al. (2013), whereby rats dosed with daily ketamine (25mg/kg) developed bladder hyperactivity, with the number of non-voiding contractions significantly increased.

Chen et al. (2020) reported that NR1 (an essential NMDAR subunit) knockout mice displayed normal voiding function compared to wildtype mice, and that bladder smooth muscle

contractility in response to EFS, CCH, α,β-meATP, and KCl was normal. Further, EFS-evoked contractile force in these mice were still concentration-dependently inhibited by ketamine, suggesting that the NMDAR is not necessary for normal bladder smooth muscle contractility, and is not likely to be a mediator of KC.

Histologically, rat models of KC typically show urothelial damage following ketamine treatment. Chuang et al. (2013) observed sloughing of mucosal cells and a denuded or thinning urothelial layer after 14 and 28 days of ketamine treatment. Similarly, Juan et al. (2015) reported that after 28 days, ketamine treated rats had a denuded urothelium.

Wang et al. (2017) looked at the effects of daily ketamine (30mg/kg) in rats for 4 or 12 weeks. Cystometry revealed decreased bladder capacity and compliance, and the number of non-voiding contractions and micturition frequency was increased in drug-treated rats at both time points. Histologically, the urothelial barrier was damaged and decreased amounts of GAG were observed in the urothelium. Additionally, increased levels of inflammatory cell infiltration and fibrosis in the bladders of ketamine rats were observed.

Gu et al. (2014) investigated the effects of a daily low dose (5mg/kg) or high dose of ketamine (50mg/kg) in rats for 16 weeks. Ketamine treated rats displayed an increased urinary frequency compared to the saline controls and a thicker, more compact urothelium. This is contrary to the numerous histological findings reporting urothelial thinning or denudation in animal models of KC. Despite this observation, immunohistochemistry and western blot analysis showed a decreased expression of the tight junction protein zonula occludens-1 in the urothelium, suggesting a defective barrier function. Surprisingly, the expression of another tight junction protein, occludin, was increased following western blot analysis. However, immunohistochemical analysis showed that the increased expression of occludin was primarily localised to endothelial cells in the submucosal layer, suggesting the development of angiogenesis and hypervascularity. The findings therefore appear to be in line with the defective barrier function of the urothelium in animal models of KC.

1.7.3 New Psychoactive Substance Studies

The effects of daily intraperitoneal injections of MXE (30mg/kg) in rats was reported by Wang et al. (2017). Micturition frequency was increased in a time-dependent manner in rats following MXE treatment for 4 and 12 weeks. Wang et al. (2017) noted that, histologically,

MXE-treated rats displayed areas of mucosal denudation and had mild tissue congestion (localised increase in blood). Moreover, reduced alcian blue staining was observed in MXE treated rats compared to controls, suggesting decreased levels of GAG in the urothelium.

Bladder toxicity associated with MXE was also noted in mice following three months of MXE administration (Dargan et al., 2014). A greater density of mononuclear cells was observed in the lamina propria and submucosa of MXE treated mice, and an increase of sirius-red collagen was noted in the submucosa and muscle layers, indicating fibrosis in these areas.

The acute or chronic effects of DPD and MXP on the bladder have not yet been investigated. Moreover, the acute effects of these ketamine-related NPS on bladder contractility has not been reported. Further research into this area is needed to determine their effects on the bladder and their potential safety profile in recreational users.

1.7.4 Translatability of Rat Models of Ketamine Cystitis

Rats are more commonly used for studying KC in rodents, and the functional and histological changes observed in the bladder in KC rat models closely mimic those observed in KC patients (as reviewed in section 1.6), suggesting translatability of these models. As discussed, *in vivo* rat models of KC typically report bladder hyperactivity, with an increased micturition frequency, and decreased bladder capacity and compliance (Meng et al., 2011; Juan et al., 2015; Wang et al., 2017), which is similar to the KC phenotype in humans. Moreover, histopathologically, both rat models and human cases of KC commonly report thinning or denudation of the urothelium (Shahani et al., 2007; Chuang et al., 2013; Juan et al., 2015), immune cell infiltration (Wei et al., 2013; Wang et al., 2017), decreased expression of the tight junction-associated protein ZO-1 and cellular adhesion protein E-cadherin (Gu et al., 2014; Tsai, Birder & Kuo, 2016; Wang et al., 2017), and an increase in urothelial apoptosis (Liu et al., 2015; Tsai et al., 2016).

The reported *in vitro* effects of ketamine on both human and rat bladder are also similar. In bladder strips from both species, ketamine has been shown to inhibit contractions evoked by various stimuli, and also concentration-dependently relax bladder tissue contracted with bethanechol (Gonzalez-CaDavid et al., 2000; Ceran et al., 2010). Additionally, cytotoxicity of ketamine on urothelial cells has been reported in both humans (Bureau et al., 2015; Shen et al., 2015; Baker et al., 2016), and in rats (Chapter 6 & 7).

There are limitations to rat models of KC that are of importance here. Histologically, the urothelium of rats is typically three cell layers thick. In humans, however, the urothelium usually consists of more cell layers, occasionally up to ten (Jost et al., 1989), which may complicate comparisons between the effects of ketamine on the urothelium of rats and humans. The relative contribution of acetylcholine and ATP in the micturition response also differs between humans and rats. Micturition in humans is generally attributed to release of acetylcholine, with ATP only responsible for a small portion of this response (Burnstock, 2014; Andersson, 2015). In rats, however, the contribution of ATP is thought to be much greater, approaching that of acetylcholine (Igawa et al., 1993, Sultana et al., 2021).

Taken together, although there are known limitations, rat models present many of the major hallmarks of KC, and as such appear to be an appropriate species to investigate the symptoms and bladder damage observed in KC.

1.8 AIM OF THESIS

The aim of this thesis is to examine the mechanism of action of ketamine in the bladder and to determine how long-term use can lead to bladder damage and dysfunction. This could help to provide a basis for the development of prevention or treatment of patients with KC, which is especially important when considering the recent discovery and licencing of the antidepressant properties of ketamine.

The direct *in vitro* effects of ketamine on rat bladder histopathology have not previously been reported in the literature. As such, a further aim of this thesis is to address the direct effects of ketamine on the urothelium and on the expression of various proteins of interest in the pathogenesis of KC. Additionally, although the acute *in vitro* effects of ketamine on bladder contractility have been investigated, the effects of longer-term exposure have not. Therefore, the direct functional effects of three-day ketamine exposure in rat bladder strips are presented.

Another major goal of this thesis is to assess the direct functional and histopathological effects of NK and DHNK on the bladder, as the potential role of these metabolites in the development of KC is often overlooked in the literature, despite the relatively large concentrations reported in the urine of ketamine users (Moore et al., 2001). The *in vitro* effects of various ketamine-related NPS on bladder function and histology are also

considered, as little pharmacological information is available with regards to both their short- and long-term effects on the bladder.

A brief outline of the chapters and their objectives is as follows:

- Chapter 3: Study of the acute effects of ketamine and related compounds on rat bladder strip contractility using an *in vitro* organ bath assay. The functional effects of the ketamine metabolites norketamine and dehydronorketamine, as well as the ketamine-related new psychoactive substances methoxetamine, diphenidine, and methoxphenidine on rat bladder strips are examined, which has not been previously reported.
- Chapter 4: Exploring the mechanism of action behind acute inhibition of rat bladder contractility following ketamine or ketamine-related drug treatment, and assessing the functional effects of longer-term ketamine incubation on rat bladder strips. Specifically, a tissue culture assay was developed to determine the effects of 3-day ketamine incubation on rat bladder strip contractility, which has not previously been reported.
- Chapter 5: Assessing the histological effects of ketamine and ketamine-related compounds in rat urinary bladder. A novel *ex vivo* tissue culture assay was developed to determine the direct effects of ketamine, norketamine, dehydronorketamine, methoxetamine, diphenidine, and methoxetamine on the rat bladder mucosa, which has not been previously reported.
- Chapter 6: Exploring the mechanisms behind the direct toxic effects of ketamine and ketamine-related compounds on the bladder. Immunohistochemistry was employed to determine the expression levels of various target proteins of interest following incubation with ketamine, its metabolites, and related new psychoactive substances, many of which have not been reported.

Chapter 2: Methods

2.1 MATERIALS

Table 2.1. List of materials used herein. * Denotes controlled substances that were purchased under UK Home Office Licence.

Chemical / Compound	Supplier	Product Number
(S)-Ketamine	Sigma	K1884
ABC Kit	Vector Laboratories	PK-4001
Animal Free Blocker	Vector Laboratories	SP-5035
Anti-CACNA1C Antibody	Invitrogen	PA5-77297
Anti-Cytokeratin 7 Antibody	Abcam	ab154334
Anti-Myosin 11 Antibody	Abcam	ab53219
Anti-Uroplakin III Antibody	Abcam	ab231576
Anti-ZO-1 Antibody	Abcam	ab214228
Atropine	Sigma	A0257
Bay K8644	Tocris	1544
Calcium Chloride Dihydrate	Fisher Scientific	C/1500/60
Carbachol	Sigma	C4382
D(+)-Glucose	Fisher Scientific	G/0500/61
DAB Kit	Vector Laboratories	Sk-4100
Dehydronorketamine	LGC Standards	LGCFOR0144.07
Dextromethorphan	Sigma	D-2531
Dimethyl Sulfoxide (DMSO)	Fisher Scientific	D/4121/PB08
Diphenidine*	Researchchemist.co.uk	N/A
DPX Mounting Medium	Fisher Scientific	D/5319/05
Dulbecco's Modified Eagle Medium (DMEM)	Sigma	D6429
Eosin Y	Acros Organics	152881000
Ethanol	Fisher Scientific	E/0665DF/15
Fetal Bovine Serum (FBS)	Fisher Scientific	10500-064
Formaldehyde	Fisher Scientific	10532955
Haematoxylin	Sigma	HHS32
Hydrochloric Acid	Fisher Scientific	H/1200/PB17
Hydrogen Peroxide	Sigma	31642
Industrial Methylated Spirits	Fisher Scientific	M/4450/17
Isradipine	Tocris	2004
(±)-Ketamine Hydrochloride	Sigma	K2753
M30 CytoDEATH	Sigma	12140322001
Magnesium Sulfate Heptahydrate	Fisher Scientific	M/1050/53
Methoxetamine*	LGC Standards	LGCFOR1275.65
Methoxphenidine*	Researchchemist.co.uk	N/A
Nifedipine	Tocris	1075
Norketamine	Tocris	1970
Panexin	Pan Biotech	P04-96950
Penicillin/Streptomycin	Sigma	P0781
Potassium Chloride (KCl)	Fisher Scientific	P/4280/53

Potassium Dihydrogen Orphophosphate (KH2PO4)	Fisher Scientific	P/4800/53
Retinoic Acid	Sigma	R2625
RPMI 1640	Sigma	R8758
Sodium Chloride (NaCl)	Fisher Scientific	S/3160/65
Sodium Hydrogen Carbonate	Fisher Scientific	S/4200/63
Tris	Fisher Scientific	T/3710/60
Trisodium Citrate Dihydrate	Sigma	S1804
Triton X-100	Sigma	T3783
Tween 20	Sigma	P1379
U73122	Tocris	1268
Xylene	Fisher Scientific	X/0250/17
Y-27632	Tocris	1254

2.2. TISSUE PREPARATION

Male Wistar rats (Charles River, UK) weighing 300-550g were used. Their wellbeing was ensured in accordance with the Animals (Scientific Procedures) Act 1986, under establishment license number PEL7003708. Rats were housed in cages (Techniplast standard rat housing; 2-4 rats per cage) under a 12-hour light/dark cycle and kept at a temperature of approximately 22°C (Biological Services Unit, University of Hertfordshire). All rats were fed a normal diet (Labdiet) and had free access to water.

Euthanization was carried out via CO_2 exposure followed by cervical dislocation (approved Schedule 1 methodology). Immediately following euthanization, the abdominal cavity was opened, and the bladder was removed through incisions behind and below the bladder base. Tissues were immediately transferred to fresh Krebs-Henseleit buffer (NaCl 118.3 mM, KCl 4.4 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 1.2 mM, $NaHCO_3$ 25.0 mM, Glucose 11.1 mM, $CaCl_2$ 2.5 mM) equilibrated with 95% O_2 5% CO_2.

2.2.1 Organ Bath Bladder Preparation

The bladder was transferred to a cork board covered in absorbent tissue. Krebs buffer was then applied (and reapplied as necessary) to the tissue to ensure that the bladder did not dry out. A scalpel was used to dissect away any undesired connective tissue, including the suspensory ligament, making sure not to cut into the bladder itself. The bladder was then oriented ventral side upwards and cut in half longitudinally. The two pieces of tissue produced were then pinned down at the very edge of their corners, and again cut in half resulting in

two dorsal and two ventral bladder strips. Coloured pins were used to enable identification of dorsal and ventral strips.

Cotton thread was tied into a loop, which was then tightened onto one end of one bladder strip and subsequently tied into a knot. A second, small loop was then made and knotted at the same end of the bladder strip. A second piece of thread was tied and knotted to the other end of the bladder strip in a similar manner, whilst leaving a sufficiently long piece of thread. The looped side of the tissue was then attached to a hook rod to suspend the tissue in the organ bath. The long piece of thread was attached to an isometric force transducer, which converts tissue contractility into grams of force using the software Iworx Labscribe © (Version 1.817).

For all experiments, dorsal bladder strips were used where possible, as there is some evidence to suggest that they exhibit less variability in response to EFS and are more sensitive to CCH compared to ventral strips (Longhurst, Leggett & Briscoe, 1995b). These differences, however, are reported to be so small that they will likely have no influence on data interpretation (Longhurst et al., 1995b). Regardless, experiments requiring two bladder strips always used strips from the same side of the bladder to account for any potential variability.

2.2.2 Preparation of 3-Day Ketamine Cultured and Denuded Bladder Tissues

Whole bladder was placed on a tissue covered cork board that had been saturated with Krebs. Any connective tissue was carefully removed with a scalpel and discarded. The bladder was then oriented ventral side up and cut into two equal halves via a longitudinal incision down the centre of the ventral wall with a scalpel. The two resultant strips therefore consist of both dorsal and ventral portions of the bladder. Each strip was then tied with cotton thread as previously described and suspended in organ baths containing 15mL Krebs-Henseleit buffer.

2.2.3 Organ Bath Ileum Preparation

The ileum was transferred to a dissection tray filled with a sufficient supply of Krebs solution. Tissue sections of approximately 1.5cm in length were dissected from the middle portion of the ileum and placed in a new dissection tray containing fresh Krebs buffer. Mesentery was carefully removed from the tissue with scissors.

Using a needle, cotton thread was threaded at one end of the tissue, through the lumen and out through the wall of the ileum. A small loop was then made and knotted at the end, with the excess thread being cut away. The same method was repeated to tie the opposite end of the ileum; however, a long piece of string was left emanating from the end of the loop. Tissues were then suspended in organ baths using a hook rod which attached to the small loop, and a transducer which attached to the long piece of thread.

2.3 ORGAN BATH SETUP

Organ baths were maintained at 37°C using thermocirculators (Lab Companion CW3-20 Heating Bath Circulator). Baths were filled with 15mL Krebs-Henseleit buffer equilibrated with 95% O_2 5% CO_2 and allowed to warm to the desired temperature prior to tissue setup.

Isometric force transducers (iWorx FT-104) were used, enabling the change in tension of the tissue (contraction/relaxation) to be converted into grams of force using the software iWorx Labscribe © (Version 1.817). The transducers were connected to the computer via an iWorx IX-214 data recorder. A sampling speed of 50 per second was used for all organ bath experiments. Transducers were calibrated before every individual experiment using a 10-gram weight. Tissues were then attached to the transducers and set to an initial resting tension of 1 gram. The bladder strips were then left to equilibrate for at least thirty minutes before the addition of any drug.

2.4 FUNCTIONAL ORGAN BATH EXPERIMENTS (CHAPTER 3)

2.4.1 The Carbachol Concentration-Response in Rat Urinary Bladder

After a resting tension of approximately 1-gram had been maintained by the bladder strips for thirty minutes, a single concentration of 10µM CCH was added to the organ bath to assess the tissue's viability. Viable tissues were then washed three times with fresh Krebs buffer and left for twenty minutes before performing a concentration-response to CCH (10nM, 30nM, 100nM, 300nM, 1µM, 3µM, 10µM, 30µM, 100µM, 300µM). After applying the final concentration of CCH, or once the tissue had achieved a maximum response, strips were washed three times and left for twenty-five minutes before continuing with the next concentration response. This process was continued until a sufficient number of concentration responses had been carried out.

2.4.2 Effect of Ketamine and Related Compounds on Carbachol Induced Contractions in Rat Urinary Bladder

Matching bladder strips (two dorsal or two ventral) were mounted in organ baths containing Krebs-Henseleit solution as previously described. After testing the viability with 10μM CCH, tissues were washed three times in Krebs-Henseleit buffer and left to equilibrate for twenty-five minutes. A CCH concentration-response (CCR) was then performed on each strip as described previously. After washing, strips were left for five minutes, and the lowest concentration of the drug to be tested was added to the non-control organ bath and left to incubate for twenty minutes before carrying out the next CCR (see Table 2.2). Strips were then washed three times and left for five minutes before adding the next concentration of drug. After twenty minutes incubation, the next CCR was carried out. This procedure was repeated until all concentrations of the drug had been tested.

Table 2.2. List of drugs and the concentrations tested in functional organ bath studies on rat urinary bladder strips contracted with carbachol. All compounds were incubated with bladder strips at the concentrations listed for 20 minutes before a carbachol concentration-response was carried out to determine whether bladder contractility was affected.

Drug	Concentrations Tested
(±)-Ketamine	10nM, 100nM, 1μM, 10μM, 100μM, 300μM, 1mM, 3mM
Norketamine	100nM, 1μM, 10μM, 100μM, 300μM, 600μM, 1mM
Dehydronorketamine	100nM, 1μM, 10μM, 100μM, 300μM, 600μM
(S)-ketamine	10nM, 100nM, 1μM, 10μM, 100μM, 300μM, 1mM
Dextromethorphan	3μM, 10μM, 30μM, 100μM
Methoxetamine	1nM, 10nM, 100nM, 1μM, 10μM, 100μM, 300μM
Methoxphenidine	1nM, 10nM, 100nM, 1μM, 10μM, 100μM, 300μM
Diphenidine	1nM, 10nM, 100nM, 1μM, 10μM, 100μM, 300μM
MK-801	10nM, 100nM, 1μM, 10μM, 100μM, 300μM

The second strip acted as either a time-matched control (ketamine, (S)-ketamine norketamine, dehydronorketamine), or as a vehicle control (methoxetamine, methoxphenidine, diphenidine, MK-801). In the case of time-matched control strips, all subsequent CCRs were carried out twenty-five minutes after washout from the previous concentration-response. This was repeated concurrently with the drug treated strip until all

concentrations had been tested. Vehicle control-treated strips were incubated with the equivalent percentage of dimethyl sulfoxide (DMSO) as present in the drug-treated strips. The lowest desired concentration of DMSO was added five minutes after washout of the initial CCR and left for twenty minutes before carrying out the next CCR. This procedure was repeated until all concentrations of DMSO had been tested.

After all concentrations of drug or vehicle control had been tested, strips were washed three times and left for twenty-five minutes, at which point a single concentration of CCH (10µM) was added to the organ bath to ensure that the bladder strips had retained their contractile ability and that the drug/DMSO had been successfully washed off.

2.4.3 Simultaneous Effect of Ketamine, Norketamine, and Dehydronorketamine on Carbachol Induced Contractions in Rat Urinary Bladder

Matching bladder strips were setup in organ baths as previously described and left to equilibrate for thirty minutes. Their viability was then tested with 10µM CCH, at which point the strips were washed and left for twenty-five minutes. After carrying out a control CCR, tissues were washed and left for five minutes. Ketamine (30µM), norketamine (30µM), and dehydronorketamine (100µM) were then added to one of the baths and left to incubate for twenty minutes before carrying out a second CCR.

2.4.4 Effect of Ketamine and Related Compounds on High Potassium-Induced Contractions in Rat Urinary Bladder

Matching tissue strips were mounted in organ baths and their viability was tested as previously described. Bladder strips were then washed three times and left to equilibrate for twenty-five minutes. At this point, the organ baths were drained of Krebs, and 15mL of a modified high potassium Krebs-Henseleit solution (KCl 126.8 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 1.2 mM, $NaHCO_3$ 25.0 mM, Glucose 11.1 mM, $CaCl_2$ 2.5 mM) was immediately added to the organ bath. After a maximum contractile response had been observed, the tissue was washed three times in Krebs-Henseleit solution and left for twenty-five minutes. The bath was then drained again of Krebs, and 15mL of the high potassium Krebs was added to the bath. This was continued until a consistent contractile response was established.

After observing at least three consistent, consecutive responses to high potassium Krebs (a response within a 5% difference to the previous), tissue was washed three times and left for five minutes before adding the lowest concentration of drug to be tested (see Table 2.3). After twenty minutes, the organ bath was drained of Krebs and a solution consisting of 15mL high potassium Krebs and the drug to be tested (with a concentration equivalent to that exposed to the tissue in the organ bath) was added. Following the response, the tissue was washed three times and left for five minutes before adding the next concentration of the drug. After twenty minutes, tissue strips were again contracted with a mixture of 15mL high potassium Krebs, and a final concentration of drug equivalent to that which was in the organ bath. This procedure was repeated until all concentrations of drug had been tested.

Table 2.3. List of drugs and the concentrations tested in functional organ bath studies on rat urinary bladder strips contracted with high potassium Krebs. All compounds were incubated with bladder strips at the concentrations listed for 20 minutes before being contracted with high potassium to determine whether bladder contractility was affected.

Drug	**Concentrations Tested**
Ketamine	1nM, 10nM, 100nM, 1µM, 10µM, 100µM, 1mM
Norketamine	1nM, 10nM, 100nM, 1µM, 10µM, 100µM, 300 µM, 1mM
Dehydronorketamine	1nM, 10nM, 100nM, 1µM, 10µM, 100µM, 300 µM, 1mM
Methoxetamine	1nM, 10nM, 100nM, 1µM, 10µM, 100µM, 300 µM
Methoxphenidine	1nM, 10nM, 100nM, 1µM, 10µM, 100µM, 300 µM
Diphenidine	1nM, 10nM, 100nM, 1µM, 10µM, 100µM, 300 µM

The second strip acted as a time-matched control. After the initial CCH viability response and washout, strips were left to equilibrate for twenty-five minutes, and then contracted with high potassium Krebs every twenty-five minutes until a consistent contractile response had been established. Control strips were then incubated with distilled water at a volume equivalent to that used in the matched, drug-treated bladder strip. After twenty minutes, a mixture of 15mL high potassium Krebs and the same volume of distilled water as previously described was applied to the tissue. This procedure was then repeated concurrently with the drug-treated tissue until all concentrations of drug had been tested.

For the drugs dissolved/diluted in DMSO (methoxetamine, methoxphenidine, diphenidine), the same procedure as above was followed; however, distilled water was replaced with the equivalent percentage of DMSO as present in the matched, drug-treated strip.

After all concentrations of drug or vehicle control had been tested, bladder strips were washed three times and left for twenty-five minutes. The baths were then drained of Krebs and high potassium Krebs was again added to assess the contractility of the strips. If bladder contractility had not been seen to return to baseline levels, this was repeated a second time.

2.4.5 Effect of Ketamine on Rat Urinary Bladder Contractions Evoked by Electrical Field Stimulation

Bladder strips were dissected and tied as previously described. Each strip was then attached to an electrode so that the tissue was positioned longitudinally between the two platinum wire electrodes. Electrodes were then secured in organ baths containing 15mL Krebs solution, and tissues were left to equilibrate to a resting tension of approximately 1-gram for thirty minutes. Each electrode was attached to a stimulator (Harvard Apparatus 6002 Stimulator) and a cycle timer (Harvard Apparatus Variable Cycle Timer). A viability test was carried out using 10µM CCH to assess the contractility of the tissue, at which point the bladder strips were washed three times and left for twenty-five minutes.

A voltage response was undertaken (20, 30, 40, 50, 60, 70, 80, 90V) using a frequency of 20Hz and a pulse width of 0.1ms. Cycle timers were setup so that bladder strips would be stimulated for 10 seconds every minute. After all voltages had been tested, or a maximal response had been achieved, tissues were washed three times and left for twenty minutes. A submaximal voltage was selected from the previous response, and a frequency response was carried out (0.25, 0.5, 1, 2, 4, 8, 20 and 40Hz) using the selected voltage and a pulse width of 0.1ms. After all frequencies had been tested, or a maximal response was observed, tissues were again washed three times in fresh Krebs buffer and left for twenty minutes.

The submaximal voltages and frequencies previously obtained, as well as a pulse width of 0.1ms, were set as the parameters on the stimulator. Strips were then stimulated with 10 second pulse trains every minute, until a consistent contractile response (within 10% of the previous contractile response) had been established for at least 5 pulses. At this point, ketamine was added to the non-control organ bath immediately following the next

stimulation. The pulse cycle was carried out until either the contractility of the ketamine treated bladder strip became indistinguishable from the baseline, or until it elicited a consistent contractile response for at least five minutes. This protocol was followed for ketamine concentrations of 100µM, 300µM and 1mM.

The second bladder strip acted as a time-matched control, following the same procedure as described but instead replacing ketamine with a vehicle control of distilled water, with a volume equal to that of the ketamine solution added to the non-control bath.

2.4.6 Gas Chromatography Mass Spectrometry

Solutions of diphenidine (DPD) and methoxphenidine (MXP) were dissolved in DMSO at a concentration of approximately 1mg/mL. These solutions were then transferred to vials suitable for gas chromatography mass spectrometry (GC-MS) analysis, at a volume of 1mL each. DMSO was also used for GC-MS analysis to act as a control.

Gas chromatography was performed using a Varian 450 equipped with an autosampler. Helium was used as a carrier gas at a flow rate of 1 mL/min. The injector port was heated to 275°C. An initial column temperature of 60°C was held for two minutes and then increased to 300°C at a rate of 15°C/min and held for 5 minutes.

Mass spectrometry was performed with a Varian 240 mass spectrometer. The trap, manifold, and transfer line setpoint for the ion trap detector were set at 150, 80 and 250°C, respectively. After a 5-minute solvent delay, samples were subjected to electron ionization. The scan range was 40-1000m/z.

2.4.7 Effect of Ketamine on Rat Ileum Contractility Evoked by Carbachol

Two sections of ileum were mounted in organ baths as previously described and set to a 1-gram resting tension. After allowing the tissue to equilibrate for thirty minutes, a single concentration of 3µM CCH was applied to both tissues to assess their viability. Both tissues were then washed three times and left for twenty-five minutes. A single application of 3µM CCH was then applied to both tissues, and after observing a maximal response, tissues were washed. This process was repeated until a consistent contractile response (a response within a 10% difference to the previous) had been observed over three consecutive responses. At this point, both sections of ileum were washed three times and left for five minutes before

applying the lowest concentration of ketamine (10µM) to one tissue, and the equivalent volume of distilled water to the control tissue. Each treatment was left to incubate for twenty minutes. Carbachol (3µM) was again added to each organ bath and washed off after a maximal response had been observed. The next concentration of ketamine was added to the ketamine-treated tissue five minutes after the previous washout. This protocol was continued for all concentrations of ketamine to be tested (10µM, 100µM, 300µM, 1mM).

2.5 MECHANISTIC ORGAN BATH EXPERIMENTS (CHAPTER 4)

2.5.1 Effect of Isradipine, U73122, and Y27632 on Carbachol-Induced Bladder Contractility

Rat bladder was dissected into four equal strips, and matching strips were suspended in organ baths and set to 1-gram resting tension as previously described. After assessing tissue viability using 10µM CCH, strips were washed and left for twenty-five minutes before carrying out a CCR on both tissues. Tissues were then washed and, after five minutes, either isradipine (1µM), U73122 (10µM), or Y27632 (10µM) was added to both organ baths and left to incubate for twenty minutes. The contractility of the tissue strips was then assessed by carrying out a CCR.

2.5.2 Effect of Ketamine on Isradipine or U73122 Resistant Bladder Contractility

In strips treated with isradipine or U73122, the tissues were washed three times and then left for five minutes. At this point, 1µM isradipine or 10 µM U73122 was added to the control bath and both 1µM isradipine or 10 µM U73122 and 1mM ketamine were added to the ketamine-treated bath. After twenty minutes, a CCR was carried out and the contractility of the strips was assessed.

2.5.3 Effect of Ketamine on Bladder Contractility Resistant to both Isradipine and U73122 or Isradipine and Y27632

As described above, bladder strips were suspended in organ baths and contracted with 10µM CCH to confirm their viability, at which point a CCR was carried out to determine a control level of contractility. Isradipine (1µM) was then added to both baths and, after twenty minutes, a CCR was carried out on both tissues. After washing, tissues were left for five minutes before adding either isradipine (1µM) and U73122 (10µM), or isradipine (1µM) and

Y27632 (10µM). After twenty minutes incubation, a CCR was carried out and tissues were again washed three times and left for five minutes. The same combination of isradipine and either U73122 or Y27632 were then added to the organ baths, with 1mM ketamine added to one bath, and a vehicle control of distilled water being added to the other organ bath. After twenty minutes, a CCR was then carried out on both bladder strips.

2.5.4 Effects of Ketamine, Nifedipine, and Bay K8644 on Rat Bladder Contractility Evoked by Electrical Field Stimulation

2.5.4.1 Tissue Setup

Two matched bladder strips were attached to electrodes with the tissue positioned between the two platinum wire electrodes. The electrode was secured in the organ bath by clamping the plastic body of the electrode, and the thread attached to the top of the tissue was tied to the force transducer. The electrodes were connected to a stimulator (Harvard Apparatus 6002 Stimulator) and a cycle timer (Harvard Apparatus Variable Cycle Timer). After setting the tissues to a resting tension of approximately 1-gram, the strips were left for thirty minutes. A viability test was then undertaken by adding 10µM CCH to each bath. Tissues were then washed three times and left for twenty-five minutes. A frequency response curve (1, 2, 5, 10, 20, and 50Hz) was carried out on both tissue strips, using a voltage of 50V and a pulse width of 0.1ms, to determine a baseline response. Cycle timers were programmed to stimulate the bladder for 3 seconds every 2 minutes. After the final frequency of the response curve had been tested, the tissues were washed three times in fresh Krebs buffer and left for twenty-five minutes.

2.5.4.2 Bay K8644

A second frequency response curve in the absence of drug was carried out (control response), and after washing three times, tissues were left for five minutes. At this point, the lowest concentration of Bay K8644 to be tested (1nM) was added to one organ bath, while the equivalent concentration of DMSO (0.0001%) was added to the other bath and acted as a vehicle control. After twenty minutes incubation, a frequency response was undertaken, after which the tissues were washed three times and left for five minutes before adding the next concentration of Bay K8644. This procedure was repeated for all subsequent concentrations

of Bay K8644 to be tested (10nM, 50nM, 200nM), and their respective matched DMSO concentrations (0.001%, 0.005%, 0.02%).

2.5.4.3 Effect of Ketamine and Nifedipine on Bay K8644 Potentiated Contractile Responses

Following the baseline frequency response, Bay K8644 (200nM) was added to both organ baths and, after twenty minutes, another frequency response was carried out on both tissues. After washing, both bladder strips were left for five minutes before again adding Bay K8644 (200nM) to both tissues. The first concentration of ketamine (30µM) or nifedipine (1µM) to be tested was then added to one of the organ baths, while the equivalent volume of distilled water (30µL; vehicle control for ketamine experiments) or equivalent concentration of DMSO (0.01%; vehicle control for nifedipine experiments) was added to the control organ bath. After twenty minutes, a frequency response was again carried out and the strips were washed and left to rest for five minutes. Bay K8644 (200nM) was then added to both baths, and the next concentration of ketamine or nifedipine to be tested was added to the organ bath, with an equivalent volume of distilled water or equivalent concentration of DMSO added to the control bath. This procedure was undertaken for all subsequent concentrations of ketamine (300µM and 1mM), nifedipine (10 and 100µM), or DMSO (0.1 and 1%) to be tested.

2.5.4.4 Effect of Bay K8644 on Bladder Contractile Responses Attenuated by Ketamine or Nifedipine

After the baseline frequency response had been carried out, tissues were left for 5 minutes and either ketamine (300µM) or nifedipine (1µM) were added to both organ baths and allowed to incubate for twenty minutes before carrying out another frequency response. Both strips were again washed three times and left for five minutes. At this point, a combination of either ketamine (300µM) and Bay K8644 (10nM), or nifedipine (1µM) and Bay K8644 (10nM) were added to the first organ bath. The second organ bath acted as a time-matched vehicle control and was exposed to either ketamine (300µM) or nifedipine (1µM), and an equivalent concentration of DMSO (0.001%) as was present in the solution of Bay K8644 added to the first bath. After 20 minutes, a frequency response curve was carried out, and both strips were washed and left for five minutes. This procedure was continued for all further concentrations of Bay K8644 (50nM, 200nM, 1µM) and DMSO (0.005%, 0.02%, 0.1%) to be

tested. Tissues were then washed three times and left for twenty-five minutes before carrying out a final frequency response without the presence of any drug.

2.5.5 Effect of Urothelial Denudation on Carbachol and High Potassium-Induced Contractions in Rat Urinary Bladder Strips

Whole bladder was dissected into two equal strips as previously described. Strips were suspended in organ baths and set to an initial resting tension of 1 gram. After thirty minutes, 10µM CCH was added to each bath to test tissue viability.

2.5.5.1 Carbachol-Induced Contractility

The tissues were washed and left for twenty-five minutes before carrying out a CCR. After washing, the tissues were removed from tension and placed on an elevated, Krebs-soaked cork board with the thread still intact. The strip to be denuded was oriented with the urothelium facing upwards and was gently swabbed with a Krebs-soaked cotton bud ten times lengthways. The strip was then swabbed ten times in the opposite direction, and this process was repeated once for a total of forty swabs. The control tissue was removed from the organ bath and placed on a cork board saturated with Krebs solution for the same duration as the denuded strip but was not mechanically handled any further. Both strips were then returned to their respective organ baths and set to 1-gram resting tension. After twenty-five minutes, a second CCR was carried out on both tissues.

2.5.5.2 High Potassium Krebs-Henseleit Solution-Induced Contractility

After the initial viability, both tissues were washed and left for twenty-five minutes, at which point, the organ baths were drained of Krebs and 15mL of a high potassium Krebs-Henseleit solution was added to each bath. After observing a maximal response, both tissues were washed and left for twenty-five minutes. This process was repeated until a consistent contractile response was observed over three consecutive responses, as explained earlier. After washing, the tissues were then removed from the organ baths and one strip was mechanically denuded with a cotton bud as previously described. Both tissues were then resuspended in their respective organ baths, set to 1-gram resting tension, and left for twenty-five minutes. Each organ bath was then drained of Krebs and replaced with 15mL of the high potassium alternative.

2.5.5.3 Electrical Field Stimulation-Induced Contractility

Following the viability test, tissues were washed and left for twenty-five minutes. Strips were then contracted with increasing frequencies (1, 2, 5, 10, 20 & 50 Hz), at a voltage of 50V, and a pulse width of 0.1ms (Chen et al., 2020). Tissues were then washed, and one strip was denuded. Both tissues were then resuspended as previously described, and, after twenty-five minutes, a frequency response was undertaken on both tissues.

2.5.6 Effect of 72-Hour Ketamine Incubation on Carbachol Contracted Rat Urinary Bladder Strips

2.5.6.1 Tissue Culture Media

To assess the effects of longer-term exposure of bladder strips to ketamine and other compounds of interest, the strips were maintained in tissue culture media for 72 hours. The tissue culture media was comprised of a 50:50 mix of Dulbecco's Modified Eagle Medium (DMEM) and RPMI 1640, supplemented with 10% fetal bovine serum, and 1% penicillin/streptomycin. This composition is based on that used by Baker, Shabir, Georgopoulos & Southgate (2016) to culture human ureteric tissue.

2.5.6.2 Tissue Preparation

Whole bladder was placed onto a tissue-covered cork board that had been saturated with Krebs solution. After removing any connective tissue, the bladder was oriented ventral side up, and cut into two equal halves via a longitudinal incision down the centre of the ventral wall with a scalpel. The two resultant strips were tied with cotton thread and suspended in organ baths as previously described.

2.5.6.3 Pre-Incubation Assessment of Contractility

Bladder strips were set to an initial resting tension of 1 gram and left to equilibrate for thirty minutes before testing tissue viability with 10µM CCH. Both tissues were then left for twenty-five minutes before carrying out a CCR. At this point, the tissues were washed three times and carefully removed from the organ bath, ensuring each strip had both threads still attached at either end.

2.5.6.4 Tissue Culture

After assessing pre-incubation contractility, tissues were transferred to separate bijous containing tissue culture media and placed in a biological safety cabinet that had been cleaned with 70% industrial methylated spirit to ensure a sterile work environment. Both bladder strips were transferred into new, separate bijous containing 1mL fresh tissue culture media and left for 30 seconds, and this process was repeated one additional time. Finally, the two tissue strips were placed into new, separate bijous containing different mixtures depending on the intended treatment. The ketamine-incubated tissue was placed in a bijou containing 2.91mL tissue culture media and 90µL of 100mM ketamine to achieve a final ketamine concentration of 3mM. The control-incubated tissue was transferred to a bijou containing 2.91mL tissue culture media and 90µL distilled water. Care was made to ensure that both tissues were fully submerged in the tissue culture mixture. Lids were loosely placed on each bijou to ensure adequate air supply, and they were then placed in an incubator set to 37°C and supplied with 95% oxygen 5% carbon dioxide.

2.5.6.5 Post-Incubation Assessment of Contractility

After 72 hours of incubation, both tissues were removed and resuspended in organ baths containing Krebs solution as previously described. After thirty minutes, a 10µM concentration of CCH was added to each bath to assess tissue viability, and each strip was then washed three times with fresh Krebs. After twenty-five minutes, a CCR was carried out on both bladder strips.

2.5.7 Effect of 72 Hour Isradipine Incubation on Carbachol Contracted Rat Urinary Bladder Strips

The effects of 72-hour exposure of isradipine (1µM) on rat urinary bladder strips was also investigated. As described above, whole bladder was dissected in half and a pre-incubation assessment of tissue contractility by means of a CCR was carried out. Both tissue strips were then transferred to a biological safety cabinet and rinsed twice in 1mL fresh tissue culture media. One strip was then placed in a bijou containing 2.97mL fresh tissue culture media and 30µL of isradipine (100µM) to achieve a final concentration of 1µM. The other strip acted as a time-matched vehicle control and so was placed in a bijou containing 2.97mL tissue culture media and 30µL of 10% DMSO to achieve a DMSO concentration of 0.1%. After incubation at

37°C for 72 hours, both strips were resuspended in organ baths and their contractility was reassessed by carrying out a CCR.

2.5.8 Effect of 3 Day Incubated Ketamine Solution on Rat Urinary Bladder Contractility Evoked by Carbachol

In a sterile bijou, a ketamine solution was made up by dissolving 12.3mg of ketamine in 450µL of tissue culture media to achieve a ketamine concentration of 100mM. This was then transferred to an incubator and maintained at 37°C for 3 days. The lid of the bijou was loosened prior to incubation to ensure the solution was supplied with oxygen and carbon dioxide (95% and 5%, respectively).

After 3 days, the solution was removed from the incubator. Fresh bladder strips were suspended in organ baths containing 15mL Krebs-Henseleit as previously described. Following a viability test using 10µM CCH, tissues were washed and left for twenty-five minutes. A CCR was then carried out, after which the tissue was washed and left for five minutes. The 450µL solution of ketamine and tissue culture media was then added to the organ bath to achieve a final bath concentration of 3mM. This was left for twenty minutes before carrying out a second CCR.

2.6 DATA MEASUREMENT FOR FUNCTIONAL ORGAN BATH EXPERIMENTS (CHAPTER 3 & 4)

Tissue contractile responses were recorded using Labscribe, which measures generated tension in grams of response and displays this raw data as a trace. To record a contractile response, one cursor was positioned on the baseline (a stable section of the trace just before addition of a drug or initiation of a stimulus), and the second cursor was placed at the peak of the drug- or stimulus-induced response. Labscribe then displays the net tension (drug or stimulus-induced response minus baseline tension), and these values were recorded in Microsoft Excel. For CCR curves, the baseline measurement was kept in the same place, and only the second cursor was repositioned to record the peak of each successive CCH concentration (Figure 2.1).

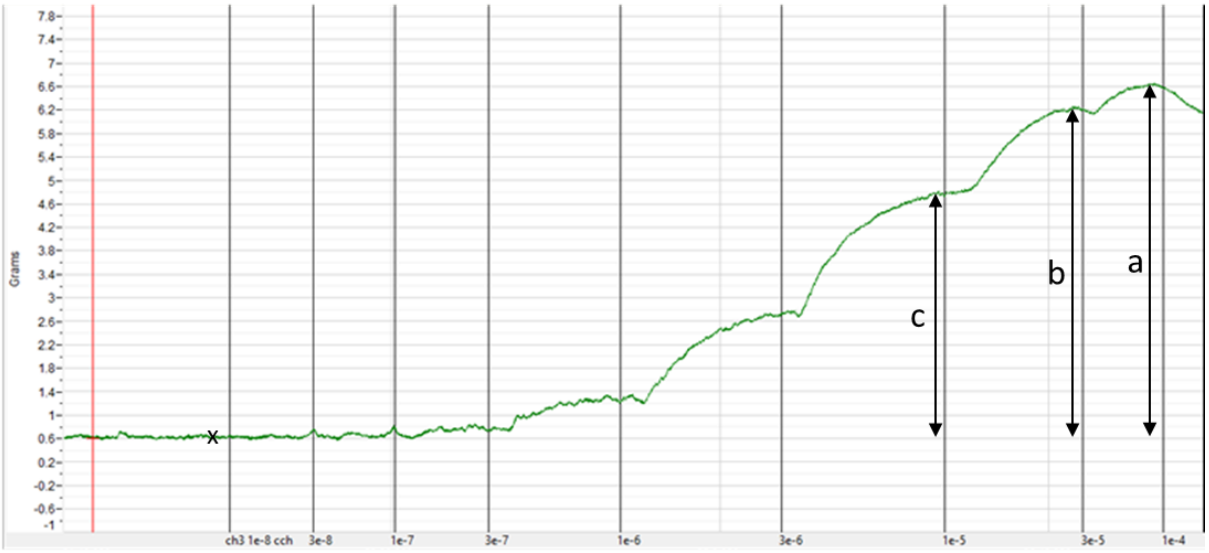

Figure 2.1. Quantification and normalisation of carbachol concentration-response data in rat bladder strips. To calculate the tension generated by each carbachol concentration, cursor 1 was placed on the baseline (represented by 'x'), and the cursor 2 was positioned at the peak of each response (the top of the arrow for a, b, c, etc.). The net contractile response for any given carbachol concentration was calculated by subtracting the baseline value (cursor 1) from the peak response (cursor 2). Data was then normalised by dividing the peak of each response (for example, a, b, or c), by the maximum control response (denoted in this example by 'a'), and multiplying by 100 (e.g. (c/a)*100 would calculate the percentage of the maximum control response for response c).

2.6.1 Carbachol Concentration-Response Experiments

For CCR experiments, raw data (grams of response) were normalised in excel by converting to a percentage of the maximum control response. The maximum control response (the greatest tension generated in the initial control concentration-response) for each bladder strip was determined, and then all CCH responses generated by that strip were individually divided by the maximum control response and multiplied by 100 (Figure 2.1).

2.6.2 High Potassium, Single Concentration Carbachol, and Electrical Field Stimulation Experiments

For high potassium or single concentration CCH experiments, bladder or ileum strips were contracted with either high potassium Krebs-Henseleit solution or 3µM CCH every 25 minutes until a consistent response was observed for at least three separate contractions (see section 2.4.4). The last of these three responses was taken to be the baseline response. A further high potassium- or CCH-induced response was carried out 25 minutes after the baseline response, in the absence of any other drug, and this was referred to as the control response. In each tissue, the control response and all additional potassium- or CCH-induced responses were

divided by the baseline response and multiplied by 100 to convert the data into a percentage of the baseline response.

The EFS frequency responses presented in Chapter 4 were normalised to a percentage of the control response. A control frequency response curve in the absence of any drug was initially carried out. Further EFS responses (in the presence of drug) were then normalised as a percentage of the control response at the respective frequency (Figure 2.2).

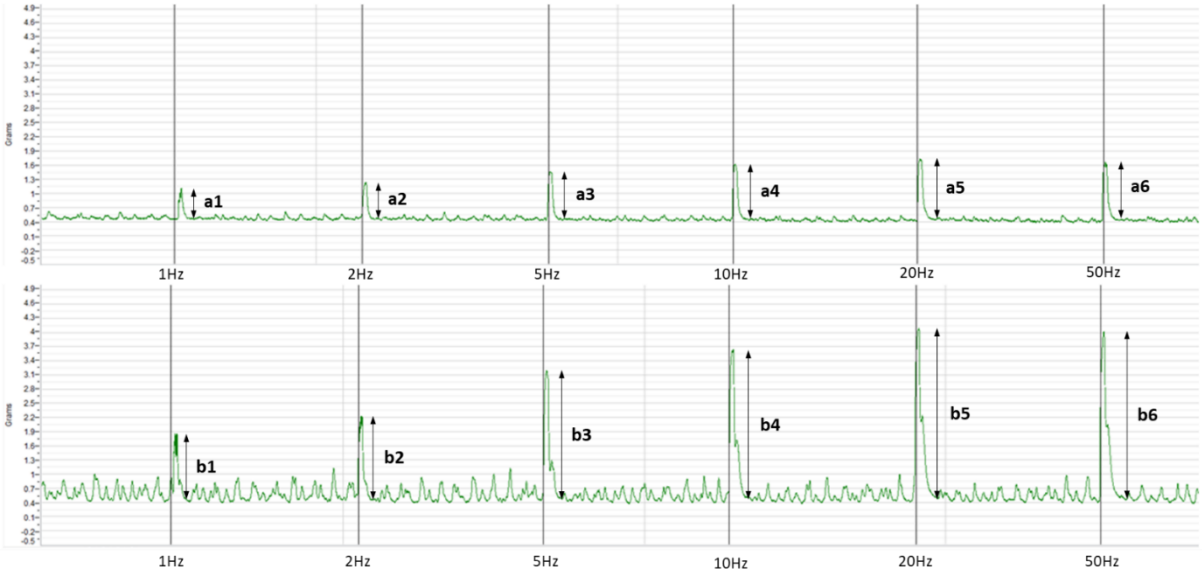

Figure 2.2. Normalisation of electrical field stimulation frequency responses as presented in Chapter 4. An initial control frequency response was carried out (top panel). All subsequent frequency responses were normalised to a percentage of the control response at the respective frequency. For example, for a frequency response in the presence of drug (bottom panel), the response at 1Hz was normalised by diving the response 'b1' by the control response 'a1' and multiplying by 100. For 2Hz, this would be: (b2/a2)*100. This representative frequency response trace shows potentiation of electrical field stimulation responses by Bay K8644 (bottom panel).

The EFS experiments presented in Chapter 3 were normalised to a percentage change from the baseline. A baseline value was determined for each tissue by calculating the average contractile amplitude of the five EFS-induced responses prior to addition of ketamine. The baseline was calculated in this way as contractile responses to EFS tend to be quite variable, and this method prevented the possibility of any extreme values just prior to addition of a drug from over- or under-representing the baseline. Each subsequent EFS response was then divided by the baseline response and multiplied by 100.

2.7 RAT BLADDER HISTOLOGY AND MUCOSAL CELL COUNT (CHAPTER 5)

2.7.1 Tissue Preparation

Whole rat bladder was excised from the animal as previously stated and placed into a sterile bijou containing tissue culture media. The bladder was transferred to a cork board covered in absorbent tissue and soaked in tissue culture media. Using a sterile scalpel, any connective tissue was carefully dissected away from the bladder, and the bladder was then oriented ventral side up. An incision was then made down the centre of the ventral wall so that the whole bladder was dissected into two equal halves. Each half was then placed into separate sterile bijous containing fresh tissue culture media.

In a sterile biological safety cabinet, the tissues were transferred into two separate bijous containing 1mL tissue culture media and gently swirled within the vial for approximately 10 seconds, and this was repeated once more. One bladder strip was then transferred into a final bijou containing tissue culture media and the chemical compound of interest. Compounds were dissolved beforehand and added to the tissue culture media ensuring that the final volume of the mixture equalled 3mL and the desired final concentration of the drug was achieved. The second bladder strip acted as a time-matched vehicle control and was incubated in a 3mL mixture containing tissue culture media and an equal volume of the solvent used to dissolve the drug for the drug-treated tissue. With the lids loosened, both bijous were then transferred to an incubator that was maintained at 37°C and supplied with a mixture of 95% oxygen 5% carbon dioxide and were kept there for the desired duration (between 1 and 7 days).

2.7.2 Tissue Processing

After both tissues had been incubated for the appropriate amount of time, they were removed from the incubator and transferred to separate Falcon tubes containing 10mL of 10% formalin solution. As a rule, the volume of formalin to be used was approximately 20x that of the tissue size and so was adjusted where appropriate. Tissues were left in the fixative at room temperature for at least 24 hours, and then, using forceps, were carefully transferred into separate glass jars containing 30mL of 70% industrial methylated spirit (IMS) and left for 1 hour. Both bladder strips were then transferred into new separate glass jars containing 90% IMS, and 1 hour later were transferred into 100% IMS and left for a further 1 hour. Tissues

were then transferred into separate vials of 30mL xylene for an hour each, and this stage was repeated two more times in fresh xylene for a total of 3 hours treatment time. Both tissues were then placed in a large metal pan containing molten paraffin wax that was maintained at 58°C using a hot plate, with care taken to ensure that the temperature did not exceed 70°C. After 2 hours, both bladder strips were transferred to separate metal base moulds containing fresh molten paraffin wax maintained at 58°C. A summary of this process is displayed in Figure 2.3.

After 2 hours, one metal base mould was removed from the hot plate. Fine tip forceps were used to carefully orientate the bladder strip on its side, or in a suitable position so that all layers of the bladder would be visible when sectioning. With the forceps still holding the tissue in place, the bottom of the base mould was gently placed on ice until the very bottom layer of wax began to set. At this point, a labelled cassette was placed on the top of the base mould, and molten wax was poured onto the cassette to the top of the rim. The base mould was then immediately placed on ice, where it was left until the wax had fully set. The same process was carried out on the remaining bladder strip.

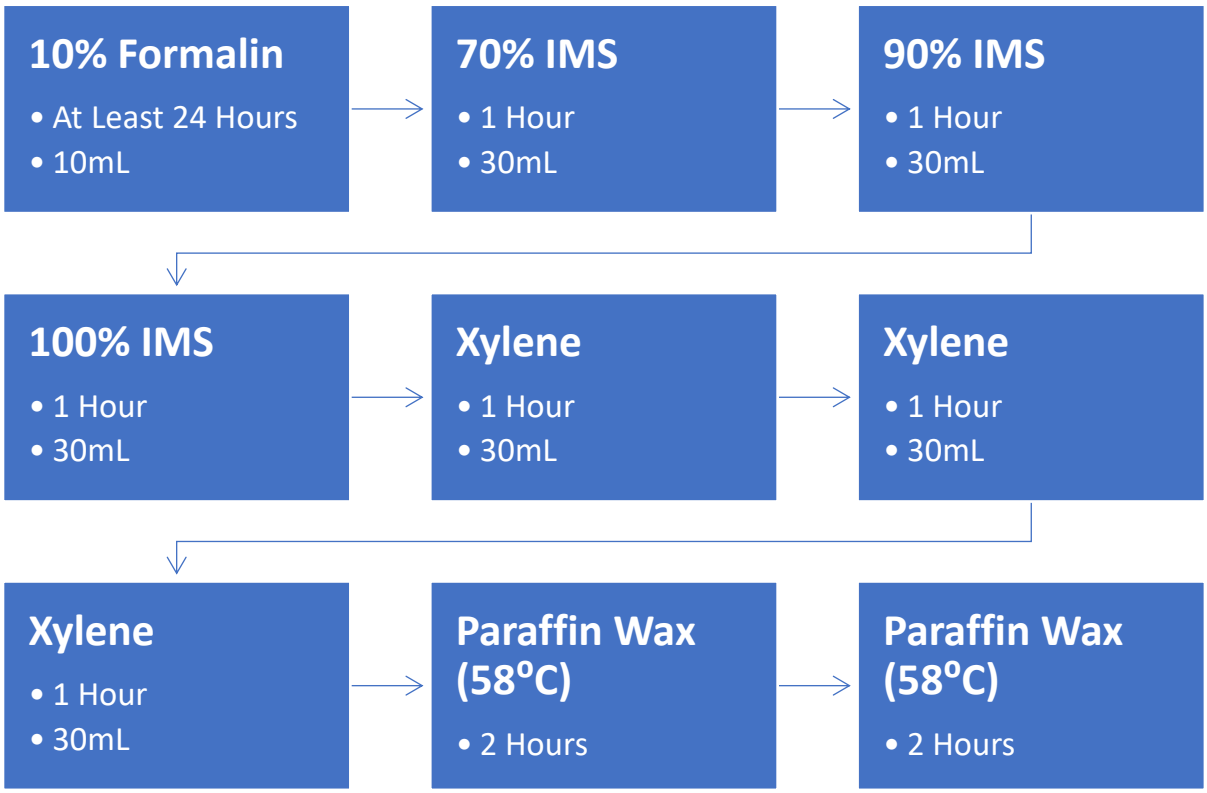

Figure 2.3. The procedure by which uncultured tissues, or cultured tissues post-incubation, were processed prior to staining. All stages were undertaken at room temperature, except for treatment in molten paraffin wax which took place at 58°C.

Bladder sections were cut at 5µm thickness from the paraffin block using a microtome (Shandon AS325 or Leica HistoCore BIOCUT R). After cutting, sections were placed into a water bath containing distilled water maintained at 45°C for approximately 1 minute, ensuring no air bubbles were present on the section. Each section was transferred onto a labelled Polysine Adhesion Slide (Thermo Scientific™) and were lightly tapped to remove any excess water. Slides were transferred to a slide rack so that they were oriented sideways and were air-dried overnight.

2.7.3 Haematoxylin and Eosin Staining

Once the sections were dry, deparaffinization was carried out via two separate xylene treatments of 2 minutes each. They were then rehydrated in a series of graded alcohols starting with two stages of 100% ethanol for 2 minutes each, followed by one stage of 70% ethanol for 2 minutes. Sections were then placed in distilled water for two minutes, at which point they were transferred to a staining rack. Haematoxylin was dispensed onto each slide making sure that each tissue section was completely covered by the solution. The haematoxylin was then washed off after 4 minutes with tap water. Each section was then de-stained by quickly dipping into acid alcohol (70% ethanol, 1% hydrochloric acid). Individual slides were immediately rinsed under tap water for approximately 10 seconds following the acid alcohol treatment and were placed back on the slide rack. Once all slides had been de-stained, they were continually rinsed in tap water for a further 5 minutes. Any excess water was then removed from the slides, and eosin was dispensed onto the sections and left for 2 minutes. The slides were again washed in tap water until all excess eosin had been removed and were left to air-dry. A summary of the staining process is displayed in Figure 2.4.

After drying, slides were treated with two stages of 100% ethanol for 2 minutes each, and two stages of xylene for 2.5 minutes and 3 minutes, respectively. Coverslips were mounted to the slides using DPX Mountant (Fisher Scientific) and left overnight in a fume cupboard. After the DPX Mountant had set, the slides were ready for visualisation.

Figure 2.4. A summary of the haematoxylin and eosin staining procedure for 5µm paraffin embedded tissue sections. All stages were undertaken at room temperature.

2.7.4 Haematoxylin and Eosin Cell Count

2.7.4.1 Capturing Images for Quantification

Bladder sections were visualised using an inverted light microscope (Nikon Eclipse TE2000-U) at x40 magnification to locate the mucosa. The slides were oriented so that the tissue was positioned longitudinally within the field of view, and a magnification of x200 was used to isolate the mucosa. The slides were moved on the mechanical stage to visualise the section of mucosa to the extreme left of the tissue, and an image was captured in .tiff format. The slide was then moved to the right, and the next section of the mucosa was captured, ensuring no overlap with the previous image. This was continued until the entirety of the mucosal layer had been captured, or until six unique images had been taken.

2.7.4.2 Quantification of Cell Count

Images of the mucosa at x200 magnification were opened in ImageJ (version 1.51k; https://imagej.nih.gov/ij/). The software was then calibrated for scale to enable measurements in µm. This was achieved by using the straight-line tool to measure the exact length of the scale bar and selecting “Set Scale” from the “Analyze” toolbar (Figure 2.5). The known distance was entered depending on the magnification of the image and scale bar being used, and the “Global” box was ticked to enable the scale to be set for all images.

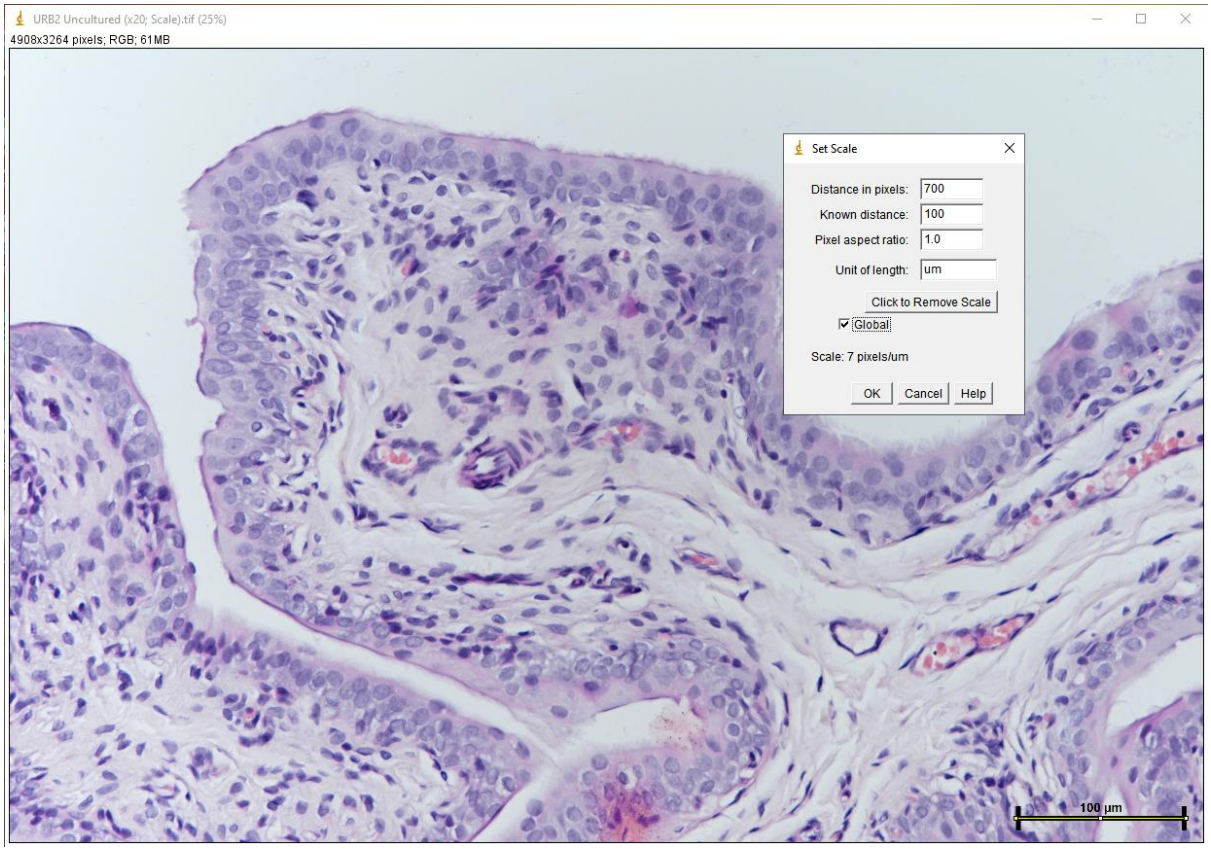

Figure 2.5. Setting the scale on ImageJ to enable all measurements taken to be a true reflection of the distance/size in µm. The straight-line tool is used to measure the length of the scale bar, and this known distance is entered in the 'set scale' dialogue box. The global box is ticked to apply this scale to all future images analysed.

The following steps were then undertaken to process the images for quantification (Figure 2.6):

1. Process → Subtract Background.

   A rolling ball radius of 50 pixels was used, and the 'Light background' option was selected.
2. Image → Type → 8-bit
3. Image → Adjust → Threshold

   'B&W' was selected in the rightmost dropdown menu. The lower and upper limits were set to 0 and 231, respectively. Occasionally the upper limit was adjusted slightly for images where the outline of the urothelium was overly visible which is commonly seen in the uncultured samples. Determining a suitable upper threshold limit was achieved by viewing the original image and adjusting the limit until as many nuclei as possible are accounted for in the processed image.

4. Process → Binary → Fill Holes
5. Process → Binary → Convert to Mask
6. Process → Binary → Watershed

Once the image had been processed, a rectangle of width=60 and height=40 (µm) was created using the rectangle tool. The dimensions were set by navigating to 'Selection', and then 'Specify' under the 'Edit' toolbar. The rectangle was then positioned to the leftmost portion of the mucosa, and the number of cells present within the area was measured by selecting the 'Analyze Particles' option under the 'Analyze' toolbar and setting the pixel (µm) size to 1.8-30 and the circularity to 0.25-1. Two more measurements were taken by placing the rectangle in the middle of the mucosa and the rightmost section of the mucosa, with the two remaining measurements being taken in the intermediates between the three previous measurements (see figure 2.8). The number of cells present in each of the five separate 2,400µm sections were recorded for each unique image of a particular treatment, and these values were averaged to obtain the mean number of cells per 2,400µm for each tissue.

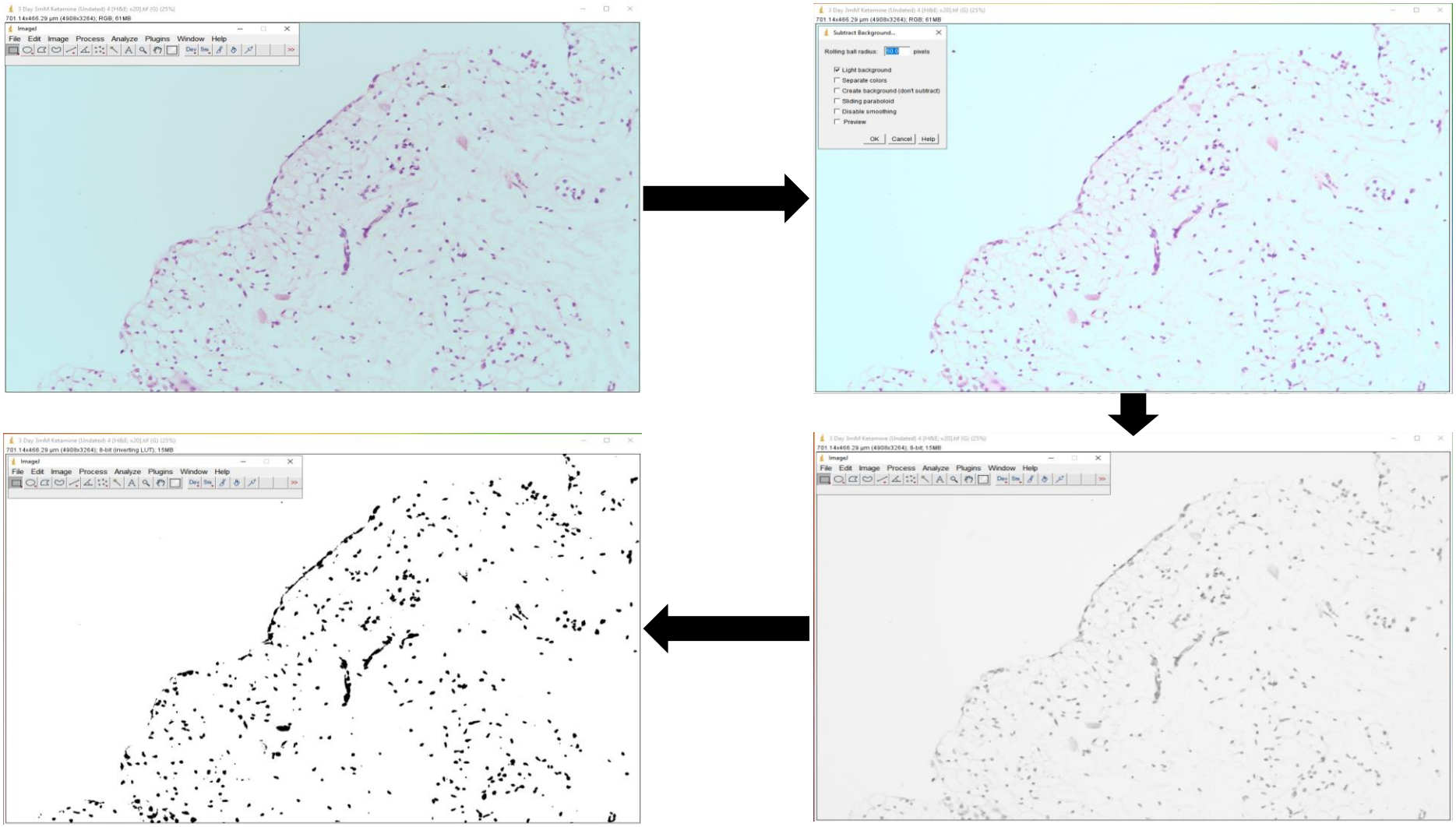

Figure 2.6. Processing haematoxylin and eosin-stained bladder images for quantification in ImageJ. The first image (top left) shows the original image. The second (top right) shows the image after subtracting the background (step 1). The third (bottom right) shows the result of changing the image type to 8-bit (step 2). The fourth image (bottom left) shows the result of adjusting the image threshold, filling any resultant holes in the remaining particles, converting to mask, and applying watershed to segment any particles believed to multiple particles grouped together steps 3-6).

2.8 RAT BLADDER IMMUNOHISTOCHEMISTRY (CHAPTER 6)

2.8.1 Tissue Processing

Tissues were processed and embedded in paraffin wax as previously described. Tissue sections of 5µM thickness were cut using a microtome and were transferred to a water bath containing distilled water maintained at 45°C. Each section was left for approximately 1 minute before being transferred onto a labelled Polysine Adhesion Slide. All slides were then oriented sideways using a slide holder and left to air-dry overnight.

2.8.2 Immunohistochemistry Protocol

After drying, slides were placed in an oven maintained at 60°C for 40 minutes and were then treated in the following conditions at room temperature:

- Xylene – 10 minutes
- 100% ethanol – 10 minutes
- 97% ethanol – 10 minutes
- 70% ethanol – 10 minutes

The slides were then dipped in distilled water and left in phosphate buffered saline (PBS) for 10 minutes. Next, slides were transferred to a plastic container filled with antigen retrieval solution (2.94g trisodium citrate dihydrate /L distilled water, adjusted to pH 6, with 0.5mL Tween 20), making sure that the tissue sections were fully submerged. This container was then placed into a decloaking chamber (Biocare Medical Decloaking Chamber DC2012) containing 500mL of distilled water and was gradually heated to a maximum temperature of 110°C, which was then maintained for 30 minutes. After heating, the internal temperature of the decloaking chamber was left to cool to 50°C, at which point the slide container was put on ice to speed the cooling process. Once the antigen retrieval solution had reached room temperature, the slides were dipped in tap water and then placed in a container of PBS for 5 minutes. Excess moisture was removed from the slides by dabbing with tissue paper, and a PAP pen was then used to draw a circle around the tissue samples. At this point, the slides were transferred to a humid chamber filled with distilled water and a few drops of a 0.3% hydrogen peroxide solution were pipetted onto each tissue section within the area created by the PAP pen. After 30 minutes, the excess hydrogen peroxide was removed, and the tissues

were washed using a few drops of PBS. The excess PBS was then removed from the tissue and animal free blocker was pipetted onto the tissue section and left for 30 minutes. Excess animal free blocker was then removed from the slides, and the antibody solution was pipetted onto the tissues. The humid chamber was then stored at 2°C with the lid on for the desired amount of time (Table 2.4).

Table 2.4. Dilution factors, incubation temperatures, and incubation times for the different primary antibodies used for immunohistochemical analysis of rat bladder sections.

Antibody	Dilution	Incubation Temperature	Incubation Time
Uroplakin III	1:100	2°C	16 hours
Cytokeratin 7	1:200	2°C	16 hours
Zonula Occludens-1	1:200	2°C	16 hours
Myosin-11	1:100	2°C	16 hours
CACNA1C	1:300	2°C	16 hours
M30 CytoDEATH	1:10	2°C	40 hours

After incubating for the desired duration, excess antibody was removed from the tissues and the slides were placed in PBS for 10 minutes. The slides were then transferred into fresh PBS, at which point the secondary antibody and avidin/biotin solutions were made up (Vector Laboratories ABC kit, PK-4001). Excess PBS was removed from the slides, and they were placed back into the humid chamber before pipetting liberal amounts of the secondary antibody solution onto the tissues. The slides were left for 1 hour and regularly checked to ensure they did not dry out. The secondary antibody solution was then washed off by placing them in fresh PBS for 5 minutes, after which any excess PBS was removed. The avidin/biotin solution was then pipetted onto the tissues and left for 45 minutes, and slides were then placed in Tris buffer (0.05M, pH 7.4) for 5 minutes. During this time, a 3,3'-diaminobenzidine (DAB) solution was made up (Vector Laboratories DAB Kit, SK-4100). One slide was then removed from the Tris buffer and any excess moisture was removed. The DAB solution was added to the tissue and, whilst timing, the slide was visualised under a stereo microscope (Zeiss Stemi 2000) to observe any colour change. The time taken to produce a colour change was recorded, and excess DAB solution was removed from the tissue. The slide was then

placed in Tris buffer to stop the DAB reaction. The rest of the slides were then treated with the DAB solution for the same amount of time and placed in Tris buffer.

The slides were then treated in the following conditions at room temperature:

- Distilled water – 10 minutes
- 70% ethanol – 10 minutes
- 97% ethanol – 10 minutes
- 100% ethanol – 10 minutes
- Xylene – 10 minutes

The slides were individually removed from the xylene and coverslips were mounted to the slides using DPX Mountant. They were then left in a fume cupboard overnight to dry.

2.8.3 Quantification of Immunohistochemistry Staining

Images of the immunostained tissues were taken on an inverted light microscope (Nikon Eclipse TE2000-U) at x40 magnification to get an overview of the staining of all bladder layers and to locate the layer or section of interest. Further images of the tissues were taken at x200 magnification to isolate the portion of the tissue to be quantified. Images were taken until the entire area of interest was captured, or until six unique pictures had been taken, whilst making sure that there was no overlap between successive images.

Each image was individually opened on ImageJ, where they were initially changed into 8-bit format by navigating to the “Image” tab and then changing the “Type”. The images were then inverted by navigating to the “Edit Tab” and selecting “Invert” (Figure 2.7). The software was calibrated for scale and the rectangle selection was set to an area of 60x40µm as previously described. The rectangle selection tool was then used to calculate the mean grey value of both the image background (the portion of the image with no tissue present) and the area of interest (the immunostained section of the tissue; Figure 2.7). The background of each image was calculated by positioning the rectangle selection onto a section of the background and pressing ‘m’ on the keyboard. Using this method, as many measurements were taken as possible, while ensuring that the sections did not overlap. An average of these grey value measurements was taken, and this value was considered the mean background of its respective image.

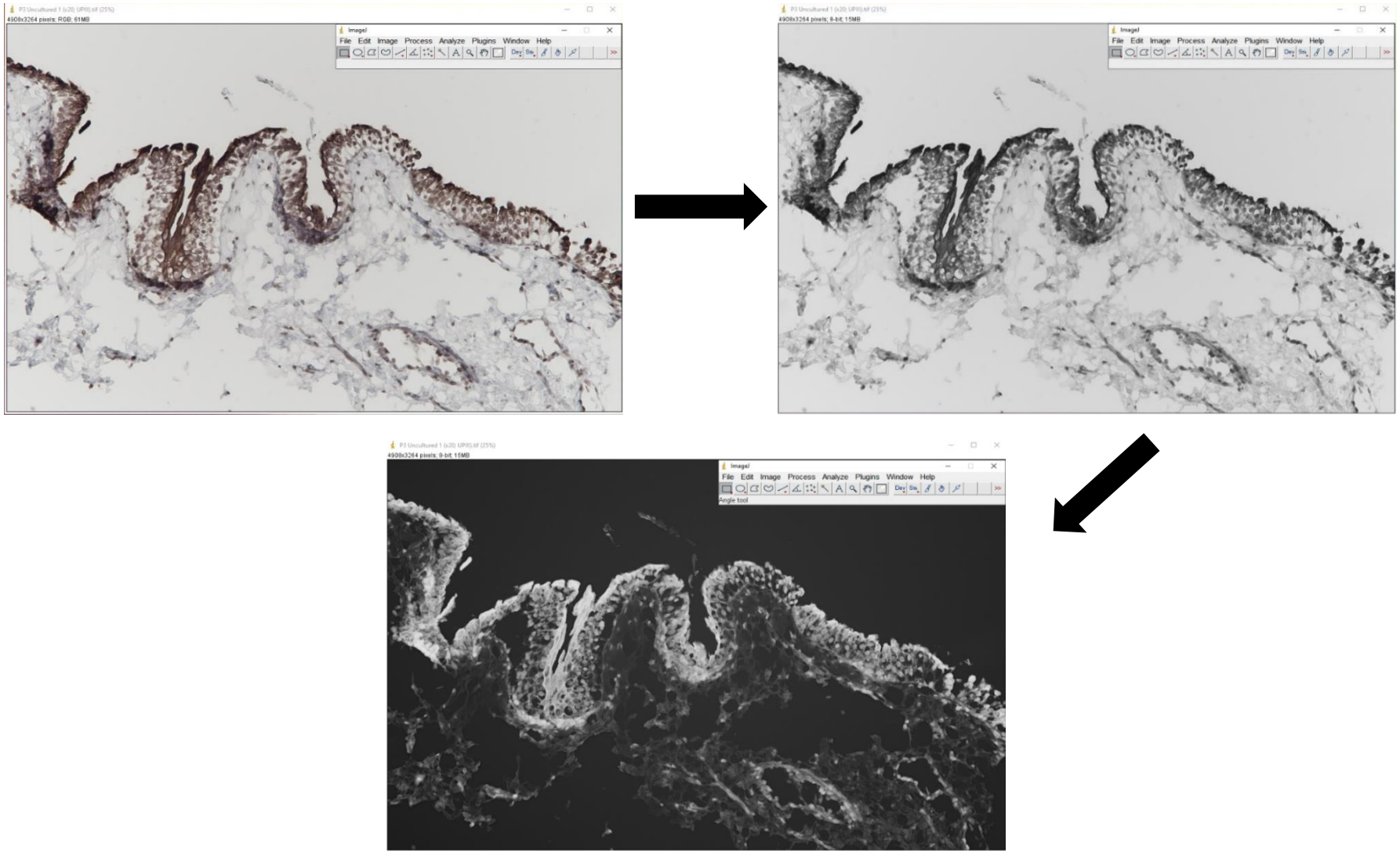

Figure 2.7. Processing immunostained bladder images for quantification in ImageJ. The first image (top left) shows the original image. The second (top right) shows the image after changing to 8-bit. The third (bottom middle) shows the image after inverting. The top third of each image where no tissue is present is considered the image background. The immunostained section of the tissue can be clearly seen in white in the bottom image.

Grey value measurements of the area of interest were then taken. This was achieved by taking five separate measurements, as previously described. Briefly, the rectangle selection was oriented along the area of interest, and a measurement was taken to the extreme right and left of the image, the middle of the image, and in the two intermediaries (Figure 2.8). The mean grey value of all five selections was then subtracted from the mean background, and the resulting value was referred to as the mean optical density for the respective image.

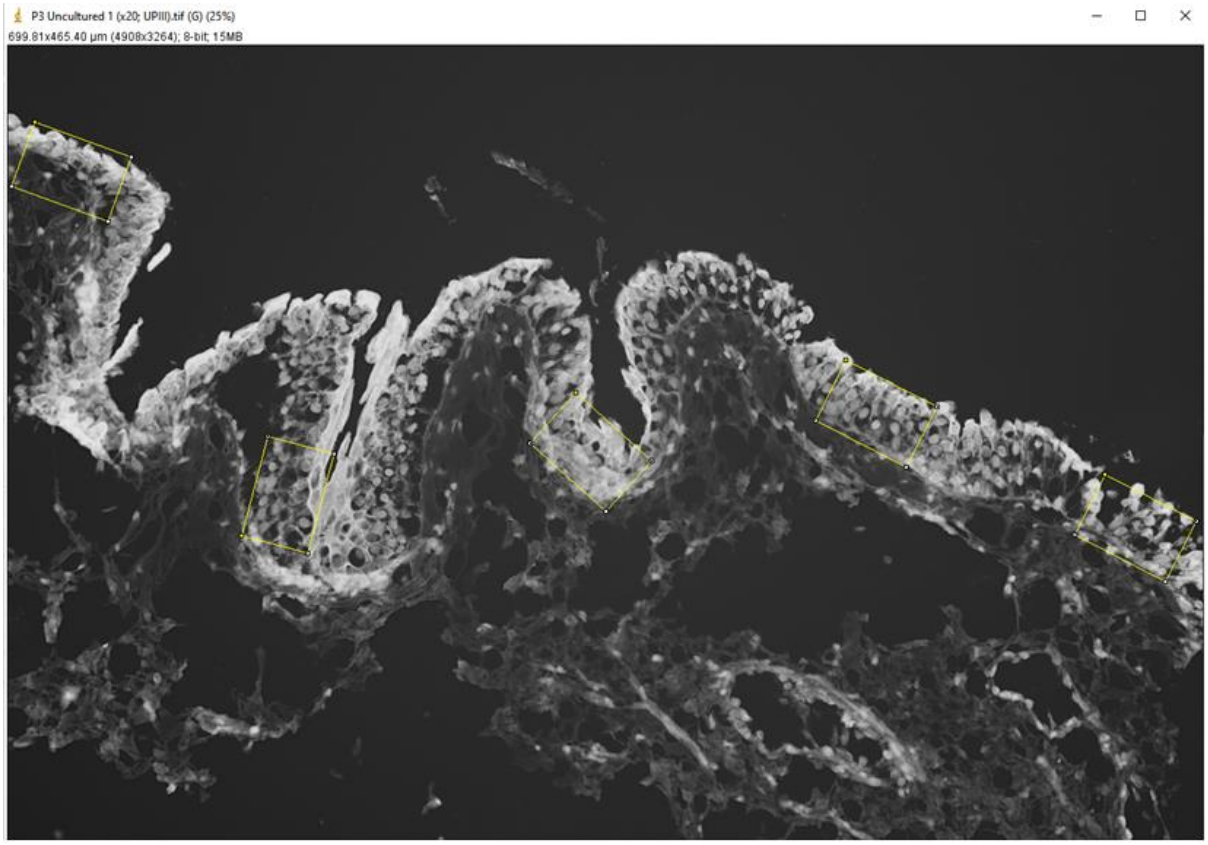

Figure 2.8. Example of the five measurements used to calculate optical density of immunostained rat bladder tissues. Measurements were taken using a 60x40µm rectangle selection positioned to the extreme right, extreme left, and middle of the image, and the two intermediaries.

2.9 STATISTICAL ANALYSIS

The number of animals used in any given experiment is referred to as *n*, and for statistical analysis to be carried out each experiment required at least *n*=3.

Statistical analysis was performed using Graphpad Prism 8. A two-way ANOVA was used when comparing agonist concentration response data in the presence and absence of a second drug. One-way ANOVA with a Dunnett post-test was performed when comparing more than

two groups with a single independent variable (e.g. high potassium or single concentration CCH experiments). A Dunnett post-test was performed when comparing two or more drug-treated groups to the control, and a Bonferroni post-test was used when comparing all tested groups to one-another. A paired t-test was used for comparison of two matched data groups (e.g. when comparing the cell count of matched tissue strips in response to different treatments). An unpaired t-test was used to compare cell count data between two different (unmatched) tissues.

In general, mean contractile responses of a particular group were compared to the mean control response. However, when investigating the effects of drugs dissolved in DMSO (MXE, MXP, DPD, or MK-801) on the CCR, CCH-induced contractility was compared to the vehicle control response with an equivalent percentage of DMSO at the relevant CCH concentration. This is because DMSO was shown to significantly affect contractility to CCH, therefore drug effects were compared back to the DMSO control to account for the effect of DMSO alone.

Statistical significance was taken at $p<0.05$, with extra levels of significance being defined at $P<0.01$, $p<0.001$, and $p<0.0001$.

Chapter 3: Effects of Ketamine and Related Compounds on Rat Bladder Contractility *in vitro*

3.1 INTRODUCTION

The two main functions of the urinary bladder are the storage and efficient release of urine; disturbances in these processes can lead to urinary dysfunction (Chapple & MacDiarmid, 2000; Andersson & Arner, 2004). In humans, the contractile response of the detrusor muscle, which leads to bladder emptying, is thought to be mediated predominantly, if not entirely, by muscarinic receptor activation via acetylcholine (Andersson & Arner, 2004; Schneider, Fetscher, Krege & Michel, 2004; Abrams & Andersson, 2007).

As previously discussed, the long-term use of ketamine can lead to ketamine-induced cystitis (KC), which is typically associated with an array of lower urinary tract (LUT) symptoms indicative of an overactive bladder. Perhaps paradoxically, however, is has been reported that high concentrations of ketamine significantly decrease bethanechol-induced contractility of human bladder (Cadavid, Ryndin, Vernet, Magee & Rajfer, 2000), and carbachol-induced contractility of rat bladder strips (Ceran et al., 2010) *in vitro*. Further, dextromethorphan (DXM) and MK-801 (which, like ketamine, are NMDA receptor antagonists), have been shown to inhibit contractions in isolated rat and mouse bladder strips (Levin et al., 2006), and bethanechol-induced contractions in rat bladder (Cadavid et al., 2000), respectively.

These direct, acute effects of ketamine on the bladder have not been widely discussed in literature investigating the mechanisms behind KC. Further, there is little or no information regarding the effects of ketamine metabolites norketamine (NK) and dehydronorketamine (DHNK), or other ketamine-related compounds on the bladder. There is evidence, however, to suggest that both NK and DHNK are present in the urine of ketamine users at concentrations similar to or higher than that of ketamine (Moore et al., 2001). It was therefore hypothesized that ketamine-related NMDA receptor antagonists, and the ketamine metabolites NK and DHNK will decrease rat bladder contractility *in vitro*.

The direct acute effects of ketamine analogue methoxetamine (MXE), and its alternatives methoxphenidine (MXP) and diphenidine (DPD) are also yet to be investigated in rat bladder. Despite a lack of pharmacological investigation, there are unsupported claims that MXE is a ‘bladder safe’ alternative to ketamine (Zawilska & Andrzejczak, 2015; Lawn, Cottrell & Winstock, 2016). Methoxetamine has been shown to cause dysfunction of rat bladder *in vivo*, displaying pathological features comparable to that of KC (Wang et al., 2017). Therefore, it

was hypothesized that acute exposure to MXE, MXP, or DPD will cause inhibition of rat bladder strip contractility.

Organ bath studies can be employed to investigate muscle contractility in specific tissues *ex vivo*. In particular, the assay allows the introduction of various exogenous compounds to assess what impact, if any, they have on tissue contractility.

As such, the aim of this chapter is to investigate the direct functional effects of ketamine and other related compounds on rat bladder contractility. The objectives are as follows:

- To assess the direct, acute effects of ketamine on rat bladder contractility *in vitro* and to determine whether the observed results are in line with current published data.
- To assess the direct, acute effects of the ketamine metabolites norketamine and dehydronorketmine, and the S(+) enantiomer (S)-ketamine on rat bladder contractility *in vitro*.
- To assess the direct, acute effects of the ketamine-related new psychoactive substances methoxetamine, methoxphenidine, and diphenidine on rat bladder contractility *in vitro*.
- To assess the direct, acute effects of the N-methyl D-aspartate receptor antagonists MK-801 and dextromethorphan on rat bladder contractility *in vitro.*
- To investigate the direct, acute effects of ketamine on rat ileum contractility *in vitro.*

3.2 METHODS

In short, rat urinary bladder strips or ileum sections were suspended in organ baths and attached to force transducers to enable measurement of isometric tension. Control responses of tissues were assessed by contracting with either increasing concentrations (bladder strips) or a single concentration (ileum sections) of carbachol (CCH); a single concentration of high potassium Krebs-Henseleit solution (KCl 126.8 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 1.2 mM, $NaHCO_3$ 25.0 mM, Glucose 11.1 mM, $CaCl_2$ 2.5 mM); or electrical field stimulation (EFS). Tissues were then incubated with ketamine or ketamine-related compounds at various concentrations for 20 minutes before recontracting with the aforementioned stimuli.

For full methodology, please refer to sections 2.2-2.4.

3.3 RESULTS

3.3.1 Carbachol Cumulative Concentration-Response

Bladder strips were contracted with increasing concentrations of CCH (10nM-100µM), and the resultant CCR produced a sigmoidal curve (Figure 3.1B). An example of a CCR trace as seen in Labscribe is shown in Figure 3.1A.

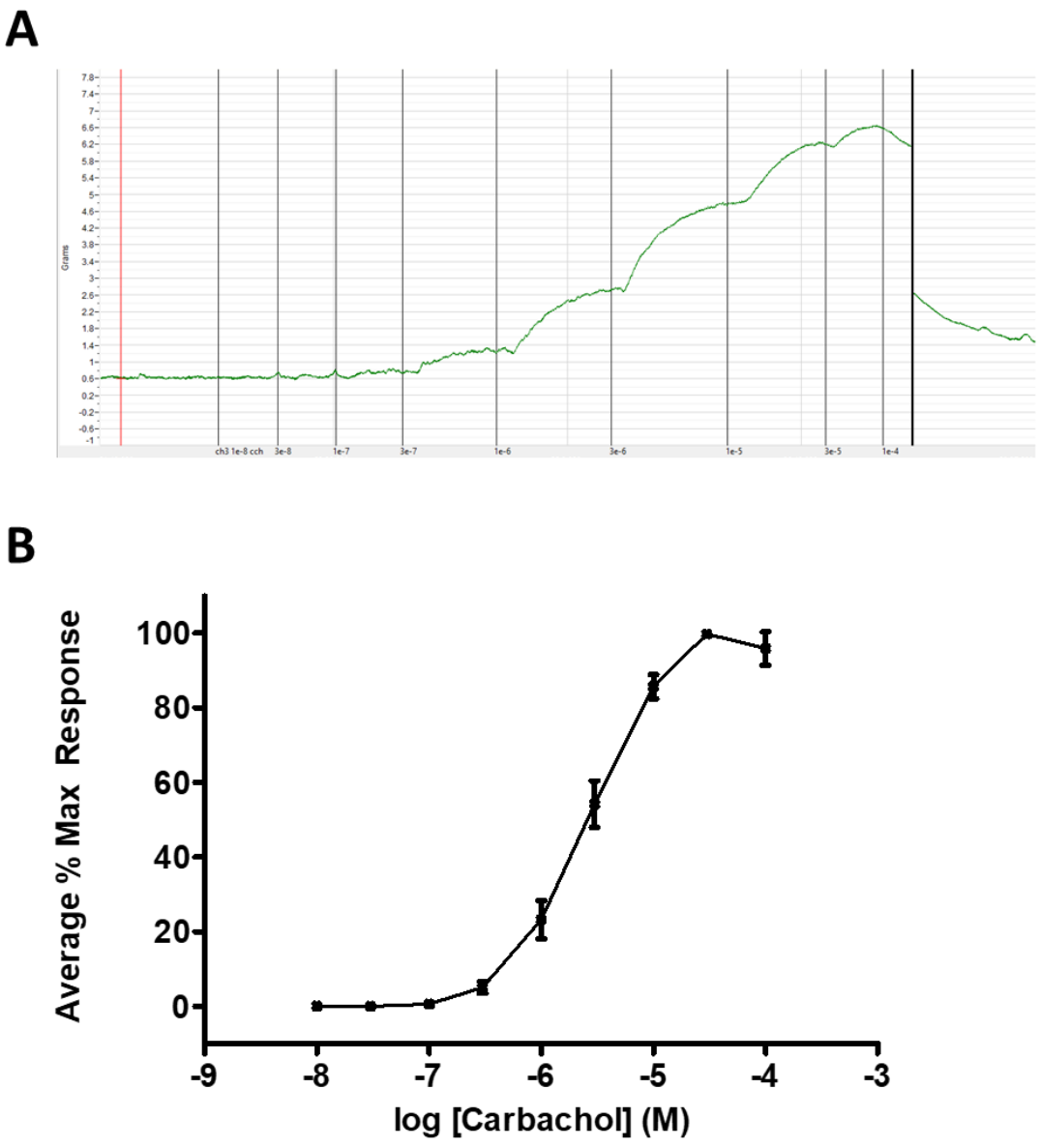

Figure 3.1. Effect of carbachol on rat urinary bladder strip contractility. **A:** Representative Labscribe trace of a carbachol cumulative concentration-response in rat bladder represented by grams of response (Y-axis). Each black line marks each carbachol addition to the bath, with the concentration (M) listed along the X-axis. **B:** Effect of carbachol on rat bladder strips expressed as a mean percentage of the maximum contractile response ± SEM. Carbachol concentrations 10nM-30µM represent *n*=4 and 100µM represents *n*=3.

3.3.2 The Contractile Response of Rat Urinary Bladder Strips to Carbachol Does Not Change Over Time

To ensure that any change observed in the contractile response of bladder strips after incubating with a compound is an effect of this treatment, it was first necessary to establish whether the CCR changes over time. Six successive CCRs were carried out on rat bladder strips, with a 25-minute interval between each concentration-response to match the interval used in subsequent experiments. The contractile response of bladder strips to CCH did not change over time (P>0.05; Figure 3.2A), and the EC50 and Emax of these responses was also unaltered over the duration tested (P>0.05; Figure 3.2B&C).

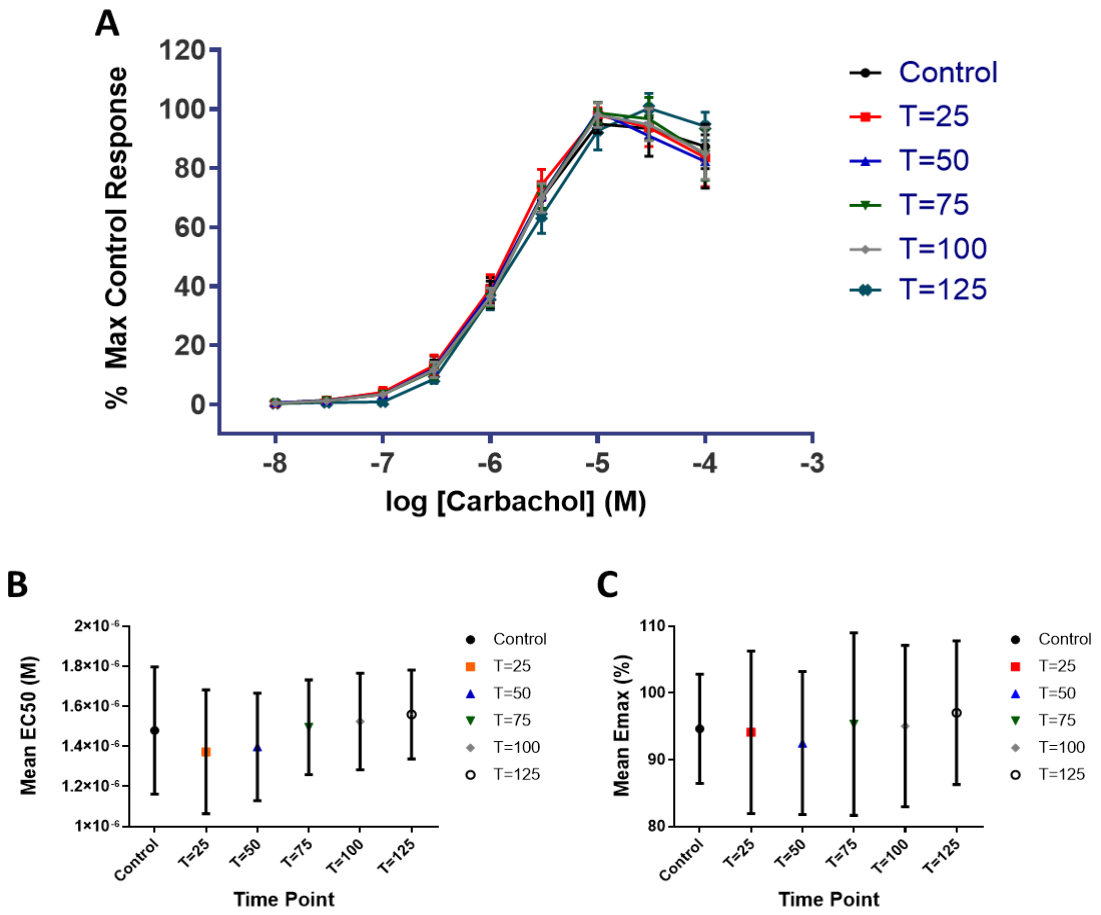

Figure 3.2. The effect of time on the cumulative concentration-response to carbachol in rat bladder strips. **A:** Effect of 25-minutes intervals between carbachol concentration-responses in rat bladder strips. Data is expressed as the mean percentage of the maximal contractile response of the control response ± SEM. **B:** Effect of time on EC50 of carbachol concentration-responses in rat bladder strips. **C:** Effect of time on Emax of carbachol concentration-responses in rat bladder strips. | Each consecutive response represents an elapsed time of twenty-five minutes from the end of the previous concentration-response. Control represents a time of 0 minutes, T=25 represents 25 minutes after the control response, T=50 represent 50 minutes after the control response, etc. Data represents *n*=5. Analysed using a two-way ANOVA and a Dunnett's post-test **(A);** analysed using a one-way ANOVA and a Dunnett's post-test **(B&C)**. No significant difference was observed between any of the time points tested.

3.3.3 Ketamine Decreases Carbachol-Induced Contractions in Rat Urinary Bladder Strips

Concentrations of ketamine ≥300µM significantly reduced the contractile response of rat bladder strips to CCH (Figure 3.3). Twenty-minute exposure to 300µM ketamine attenuated the response of bladder strips at CCH concentrations 1, 3, and 10µM ($P<0.0001$), and 30µM ($P<0.05$) compared to the control. Bladder strips incubated with 1mM ketamine had a significantly reduced contractile response to CCH concentrations of 1, 3, 10, 30, and 100µM after twenty minutes, compared to the control ($P<0.001$).

All other concentrations of ketamine tested had no significant effect on the contractile response of bladder strips to CCH.

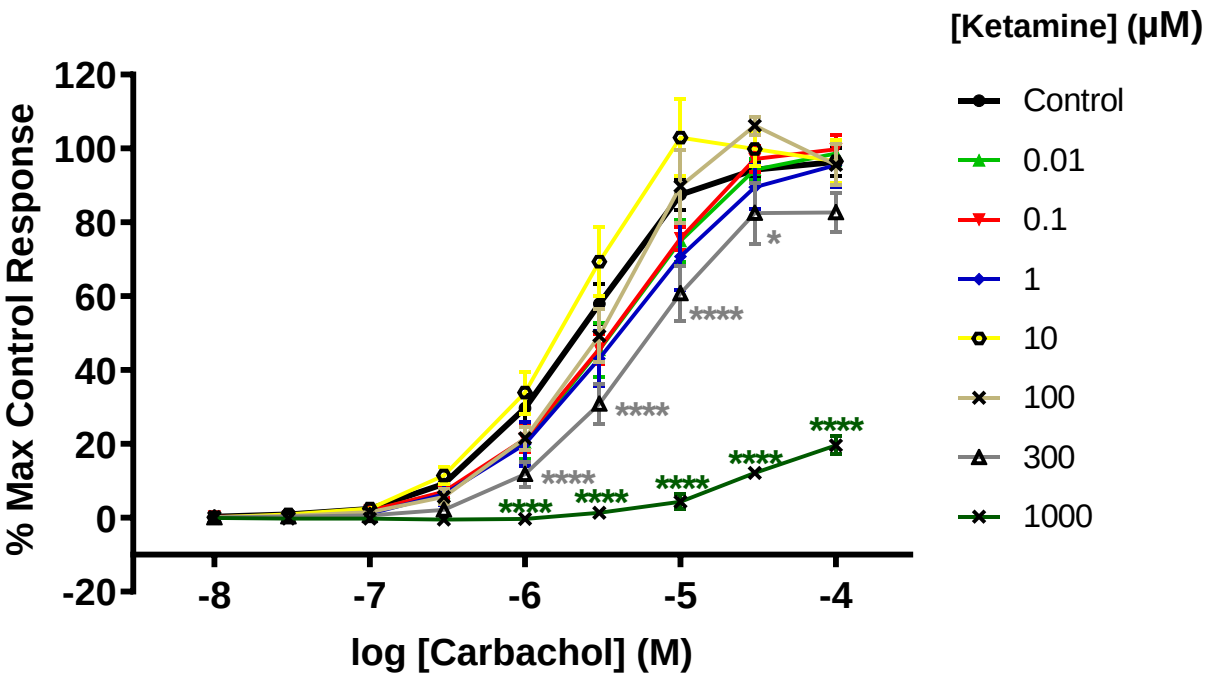

Figure 3.3. Effect of ketamine on the cumulative carbachol concentration-response in rat urinary bladder strips after 20-minute incubation. Data is expressed as a mean percentage of the maximum control response ± SEM. Data represents: control *n*=7; 0.01, 0.1, 1 & 10µM ketamine *n*=4; 100, 300, & 1000µM ketamine *n*=3. Data analysed using a two-way ANOVA and a Dunnett's post-test. *P<0.05, ****P<0.0001 significantly different from control at the respective carbachol concentration.

3.3.4 Norketamine Decreases Carbachol-Induced Contractions in Rat Urinary Bladder Strips

Concentrations of NK ≥300µM significantly reduced the contractile response of rat bladder strips to CCH in a concentration-dependent manner (Figure 3.4). Bladder strips exposed to 300µM NK saw a decrease in contractility evoked by CCH concentrations of 1 and 3µM ($P<0.001$), 10µM ($P<0.01$), and 100µM ($P<0.05$). Application of 600µM NK reduced bladder

contractility at CCH concentrations of 300nM (P<0.05) and 1, 3, 10, 30, and 100µM (P<0.0001). Similarly, strips exposed to 1mM NK saw a decrease in contractility at CCH concentrations of 300nM (P<0.05) and 1, 3, 10, 30, and 100µM (P<0.001).

All other concentrations of NK tested had no effect on the contractile response of bladder strips to CCH.

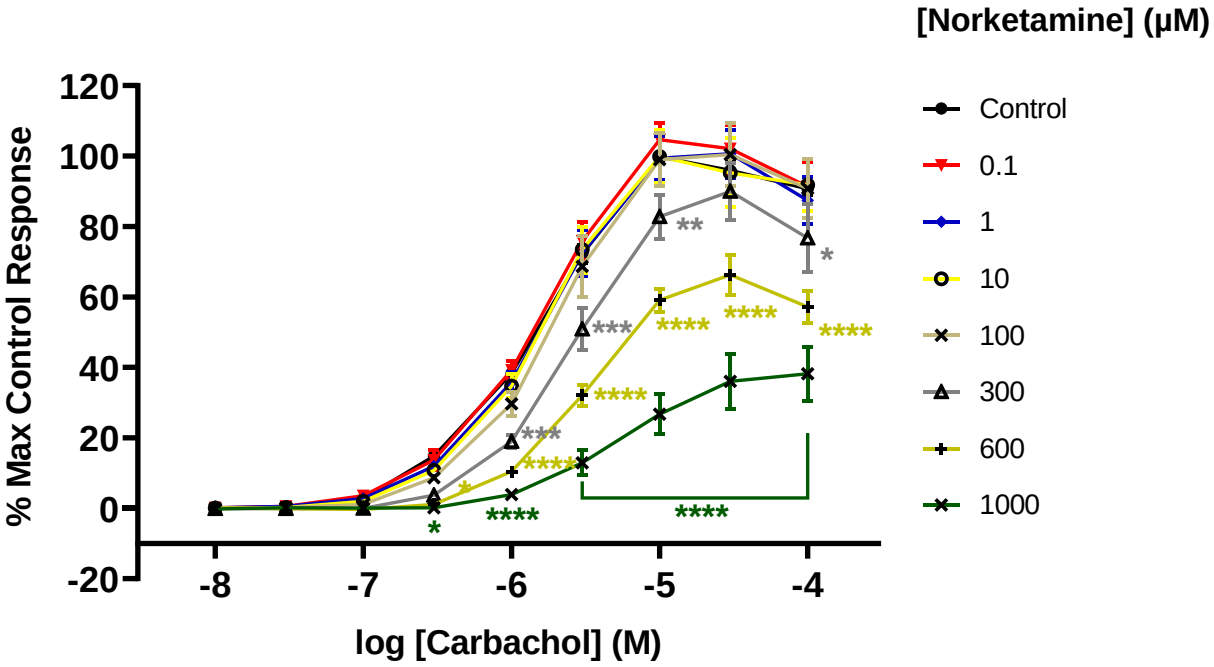

Figure 3.4. Effect of norketamine on the cumulative carbachol concentration-response in rat urinary bladder strips after 20-minute incubation. Data is expressed as a mean percentage of the maximum control response ± SEM. Each data point represents *n=4.* Data analysed using a two-way ANOVA and a Dunnett's post-test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 significantly different from control at the respective carbachol concentration.

3.3.5 Dehydronorketamine Decreases Carbachol-Induced Contractions in Rat Urinary Bladder Strips

Dehydronorketamine (≥300µM) significantly attenuated CCH-evoked contractile responses in rat bladder strips following 20 minutes exposure (Figure 3.5). Bladder strips exposed to 300µM DHNK depressed contractile responses induced by 100 (P<0.01) and 300µM (P<0.05) CCH. Additionally, 600µM DHNK decreased bladder contractility evoked by 1µM (P<0.05), and 3, 10, 30, 100, and 300µM CCH (P<0.0001).

All other concentrations of DHNK tested had no effect on the contractile response of bladder strips to CCH.

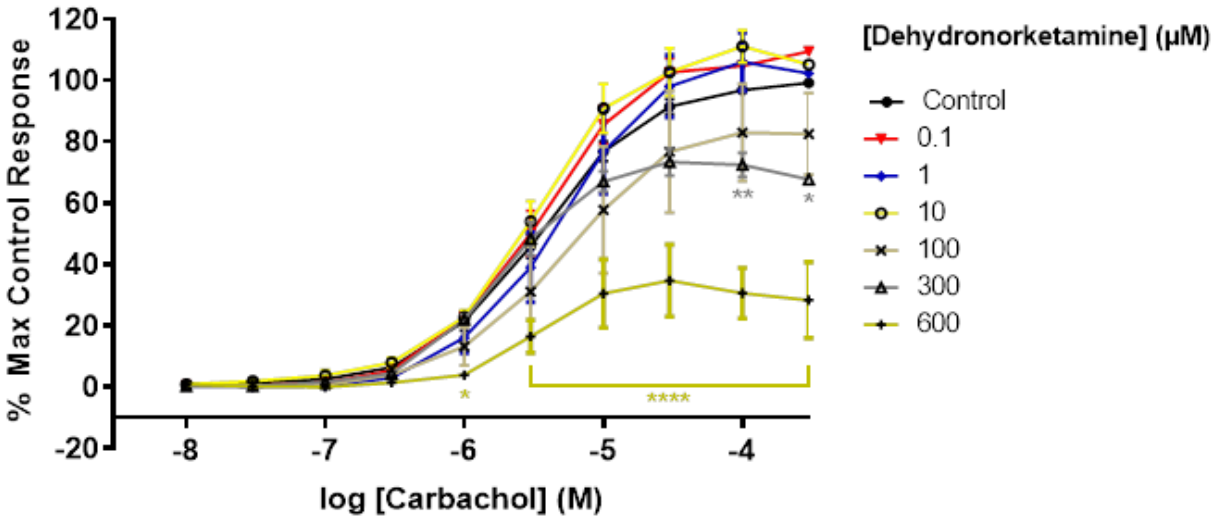

Figure 3.5. Effect of dehydronorketamine on the cumulative carbachol concentration-response in rat urinary bladder strips after 20-minute incubation. Data is expressed as a mean percentage of the maximum control response ± SEM. Data represents *n=4.* Data analysed using a two-way ANOVA and a Dunnett's post-test. *P<0.05, **P<0.01, ****P<0.0001 significantly different from control at the respective carbachol concentration.

3.3.6 Ketamine, Norketamine, and Dehydronorketamine Have an Additive Effect at Non-Inhibitive Concentrations That Can Reduce Rat Bladder Strip Contractility to Carbachol

Concentrations of ketamine, NK, and DHNK that do not affect the contractility of rat bladder strips to CCH alone (30μM, 30μM, and 100μM respectively) can reduce the contractile response of strips to CCH when applied together (Figure 3.6). Bladder strips exposed simultaneously to all three compounds displayed a decrease in their contractile response at CCH concentrations of 1 and 3μM (P<0.05) compared to the control response. Further, the mean EC50 of strips treated with ketamine and its metabolites was significantly higher than the control (Table 3.1), however the Emax was unchanged.

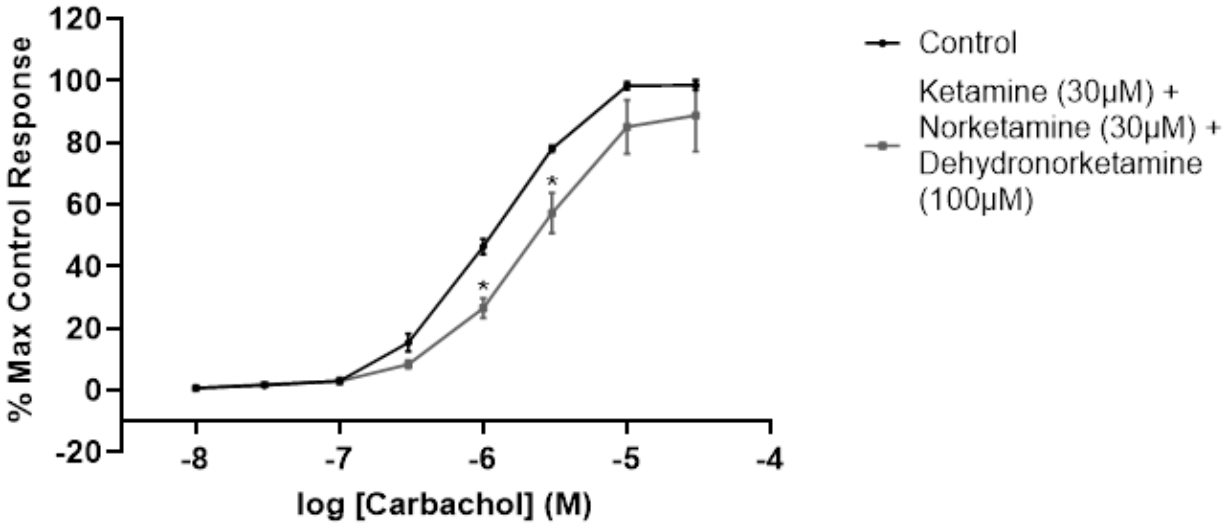

Figure 3.6. Simultaneous effect of ketamine (30µM), norketamine (30µM), and dehydronorketamine (100µM) on the cumulative carbachol concentration-response in rat urinary bladder strips after 20-minute incubation. Data is expressed as a mean percentage of the maximum control response ± SEM. Each data point represents *n=3.* Data analysed using a two-way ANOVA and a Bonferroni post-test. *P<0.05 significantly different from control at the respective carbachol concentration.

Table 3.1. EC50 and Emax of rat bladder strips contracted with carbachol (10nm-100µM) in the presence or absence of ketamine (30µM), norketamine (30µM), and dehydronorketamine (100µM) preincubated simultaneously for 20 minutes. Data are expressed as mean EC50 or Emax ± SEM and were calculated using non-linear regression. Data represents *n*=3. Data analysed using a paired two-tailed t-test. **P<0.01 significantly different from control.

	EC50 (µM)	**Emax (%)**
Control	**1.15 ± 0.06µM**	**101.45 ± 1.09**
Ketamine + Norketamine + Dehydronorketamine	**2.05 ± 0.11µM****	**92.61 ± 11.62**

3.3.7 (S)-Ketamine Decreases Carbachol-Induced Contractions in Rat Urinary Bladder Strips

Concentrations of (S)-ketamine ≥100µM significantly reduced the contractile response of rat bladder strips to CCH in a concentration-dependent manner (Figure 3.7). Bladder strips incubated with 100µM (S)-ketamine displayed a reduced contractile response at CCH concentrations of 3µM (P<0.001) and 10µM (P<0.01), and exposure to 300µM (S)-ketamine reduced bladder strip contractility to CCH concentrations of 1, 3, 10, 30 and 100µM (P<0.001). Exposure to 1mM (S)-ketamine practically abolished the response of bladder strips to CCH, with a significantly reduced contractile response at CCH concentrations of 1, 3, 10, 30 and

100µM (P<0.001). All other concentrations of (S)-ketamine tested had no effect on the contractile response of bladder strips to CCH.

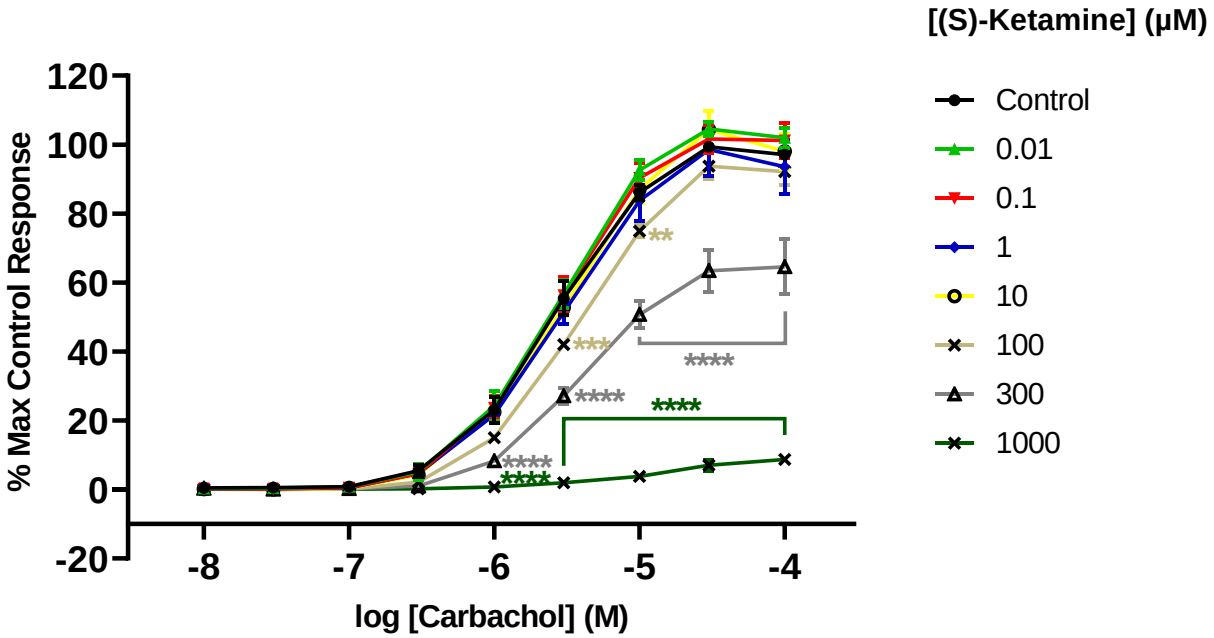

Figure 3.7. Effect of (S)-Ketamine on the cumulative carbachol concentration-response in rat urinary bladder strips after 20-minute incubation. Data is expressed as a mean percentage of the maximum control response ± SEM. Each data point represents *n=3.* Data analysed using a two-way ANOVA and a Dunnett's post-test. **P<0.01, ***P<0.001, ****P<0.0001 significantly different from control at the respective carbachol concentration.

Table 3.2. EC50 and Emax of rat bladder strips contracted with carbachol (10nm-100µM) in the absence of drug (control) or following 20 minutes exposure to ketamine, norketamine, dehydronorketamine, or (S)-ketamine (300µM). Data are expressed as mean EC50 or Emax ± SEM, and were calculated using non-linear regression. Data represents *n=3* (ketamine, (S)-ketamine); *n=4* (norketamine, dehydronorketamine). Data analysed using a one-way ANOVA and a Bonferroni post-test. *P<0.05, ****P<0.0001 significantly different from control; ## P<0.01, ###P<0.001, ####P<0.0001 significantly different from (S)-ketamine.

	EC50 (µM)	Emax (%)
Control	**2.75 ± 0.30**	**99.14 ± 0.82**
Ketamine (300µM)	**8.10 ± 2.63** (*) (##)	**86.82 ± 6.06** (*) (##)
Norketamine (300µM)	**3.64 ± 1.03** (####)	**88.72 ± 10.69** (##)
Dehydronorketamine (300µM)	**5.12 ± 1.09** (###)	**72.46 ± 3.41** (****)
(S)-Ketamine (300µM)	**17.39 ± 5.57** (****)	**64.93 ± 7.22** (****)

3.3.8 Dextromethorphan Decreases Carbachol-Induced Contractions in Rat Urinary Bladder Strips

Concentrations of DXM ≥10µM significantly reduced the contractile response of bladder strips to CCH in a concentration-dependent manner (Figure 3.8). Bladder strips incubated with 10µM DXM displayed a reduced contractile response at CCH concentrations of 10µM ($P<0.01$), 30, and 100µM ($P<0.05$). Exposure to 30µM DXM reduced bladder strip contractility at CCH concentrations of 1, 3, 10, 30 and 100µM ($P<0.0001$), and strips exposed to 100µM DXM displayed a reduced contractility to CCH at concentrations of 300nM ($P<0.05$), and 1, 3, 10, 30 and 100µM ($P<0.001$).

Exposure to 3µM DXM did not affect the contractile response of bladder strips to CCH.

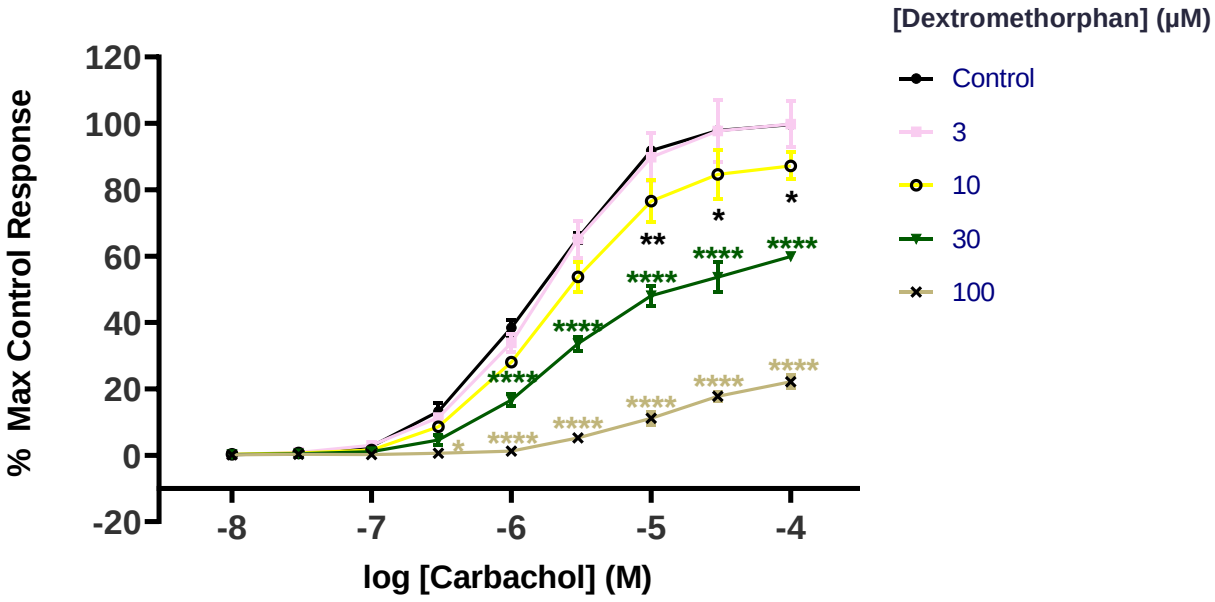

Figure 3.8. Effect of dextromethorphan on the cumulative carbachol concentration-response in rat urinary bladder strips after 20-minute incubation. Data is expressed as a mean percentage of the maximum control response ± SEM. Each data point represents *n=3.* Data analysed using a two-way ANOVA and a Dunnett's post-test. *P<0.05, **P<0.01, ****P<0.0001 significantly different from control at the respective carbachol concentration.

3.3.9 Gas Chromatography-Mass Spectrometry Analysis of Diphenidine and Methoxphenidine

To confirm the identity of the samples, both DPD and MXP were dissolved in dimethyl sulfoxide (DMSO) and the resultant solutions were analysed using gas chromatography-mass spectrometry (GC-MS). A control analysis of DMSO was also carried out to account for it when interpreting the GC-MS traces.

The gas chromatography chromatogram for DMSO displays a major peak at a retention time of 5.27 minutes (Figure 3.9). At this retention time the mass spectrum shows a major peak at 79 m/z, and two smaller peaks at 94 and 78 m/z.

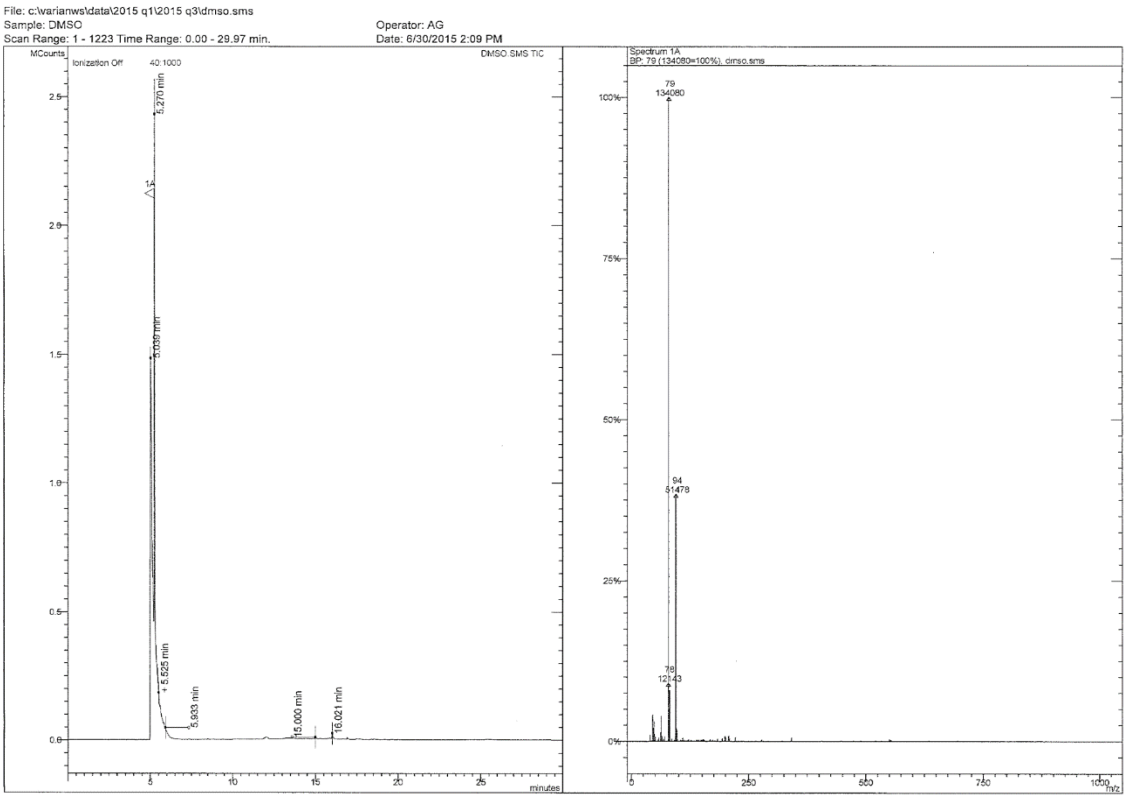

Figure 3.9. Gas chromatography-mass spectrometry trace of DMSO. M counts = number of counts in millions; m/z = mass to charge ratio.

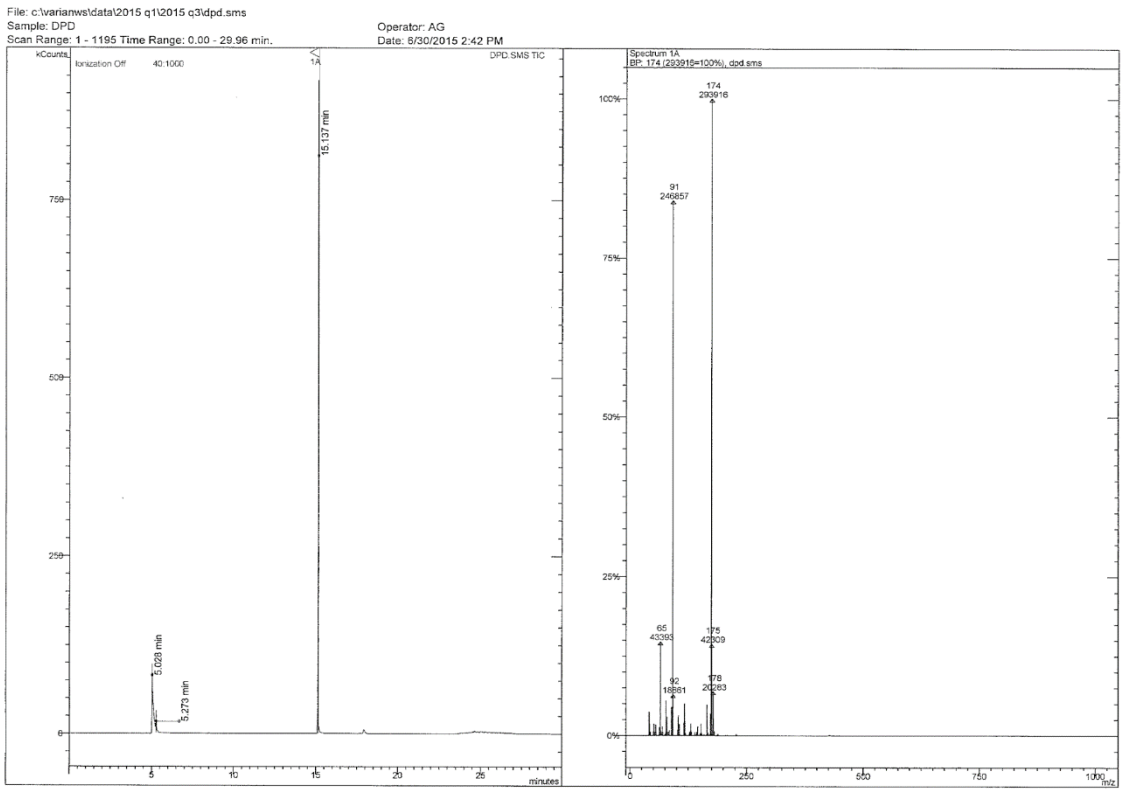

Figure 3.10. Gas chromatography-mass spectrometry trace of diphenidine. K counts = number of counts in thousands; m/z = mass to charge ratio.

The chromatogram for DPD shows a major peak at a retention time of 15.137 minutes, and a smaller peak at 5.028 minutes (Figure 3.10). At a retention time of 15.137 minutes, the mass spectrum shows two major peaks at 174 and 91 m/z, and four smaller peaks at 65, 175, 178, and 92m/z.

The chromatogram for MXP displays a major peak at a retention time of 16.006 minutes, and a smaller peak at 5.028 minutes (Figure 3.11). At a retention time of 16.006 minutes, the mass spectrum shows two major peaks at 204 and 91m/z, with two smaller peaks at 205 and 121m/z.

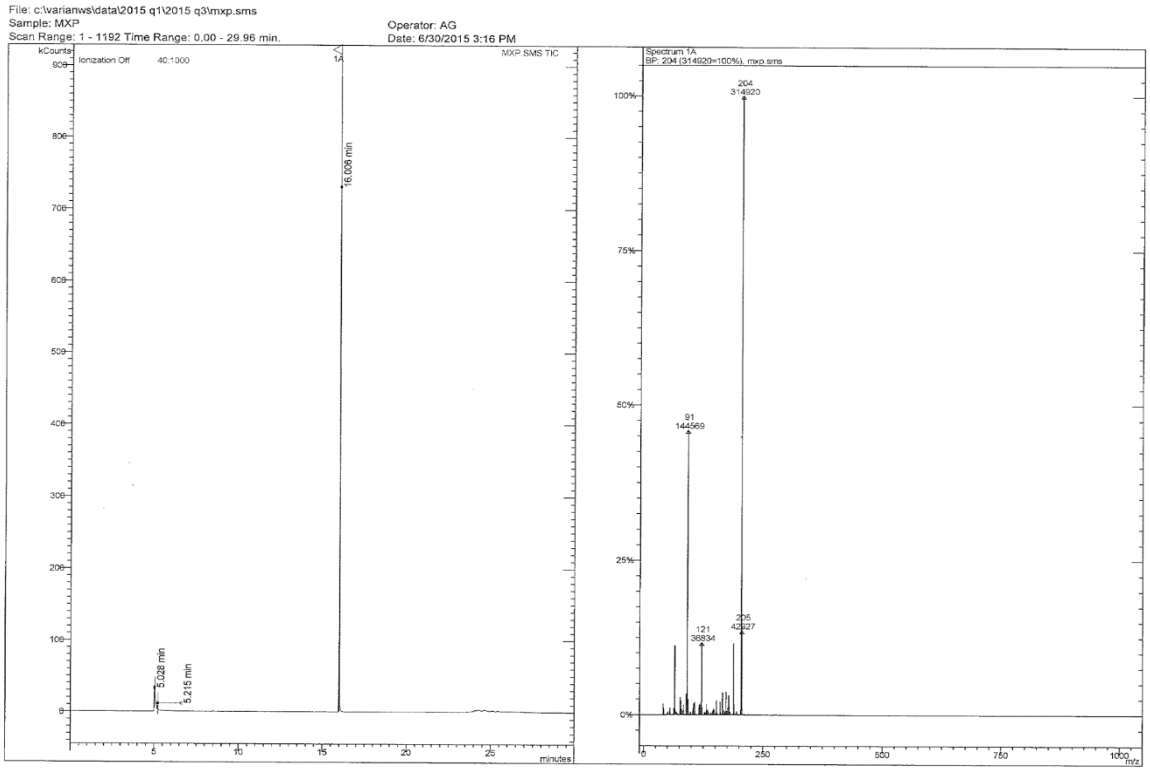

Figure 3.11. Gas chromatography-mass spectrometry trace of methoxphenidine. K counts = number of counts in thousands; m/z = mass to charge ratio.

3.3.10 Dimethyl Sulfoxide Decreases Carbachol-Induced Contractions in Rat Urinary Bladder Strips

As the NPS tested here are dissolved in DMSO, it was necessary to determine whether DMSO alone has any effect on the contractile response of bladder strips to CCH. Therefore, bladder strips were incubated with varying percentages of DMSO, matching the amount present in the different concentrations of NPS to be tested.

Statistical analysis of bladder contractility in the presence of DSMO revealed a significant treatment factor variation (Figure 3.12A), and therefore the effects of MXE, DPD, and MXP were compared back to the DMSO control curve at their respective concentration to account for this effect.

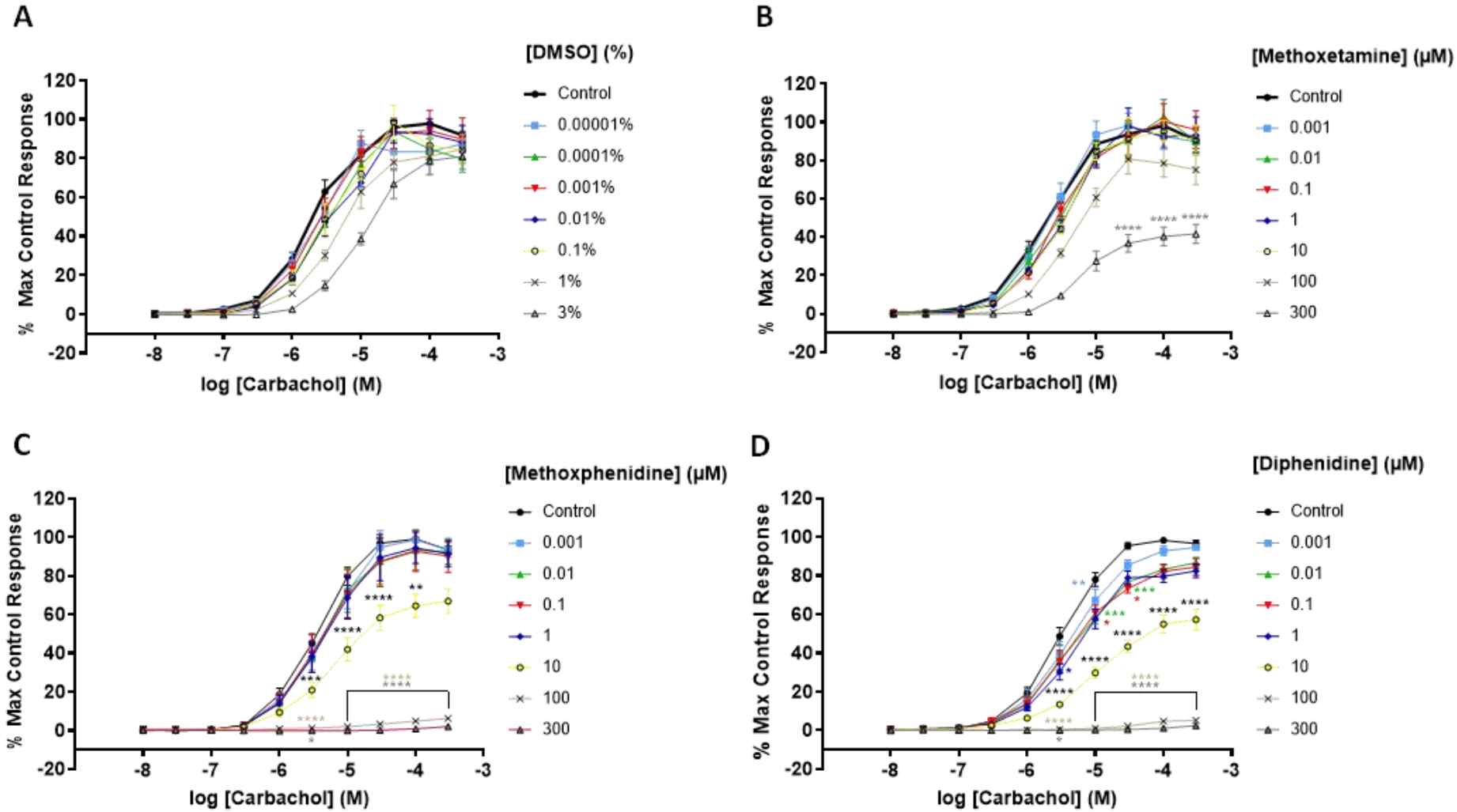

Figure 3.12. Effect of various drugs on the cumulative carbachol concentration-response in rat urinary bladder strips after 20-minute incubations. **A:** Effect of dimethyl sulfoxide. **B:** Effect of methoxetamine. **C:** Effect of methoxphenidine. **D:** Effect of diphenidine. Data expressed as a mean percentage of the maximum control response ± SEM. Each data point represents *n=4.* Data analysed using a two-way ANOVA **(A-D)** and a Bonferroni post-test **(B-D)**. Analysis revealed a significant treatment factor (DMSO) variation **(A)**; *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 significantly different from respective DMSO control at the respective carbachol concentration **(B, C, D)**.

3.3.11 Methoxetamine Decreases Carbachol-Induced Contractions in Rat Urinary Bladder Strips

Bladder strips incubated with 300µM MXE displayed a reduced contractile response at CCH concentrations of 30, 100 and 300 µM ($P<0.001$) compared to vehicle-control tissue strips incubated with 3% DMSO (Figure 3.12B).

All other concentrations of MXE tested did not alter bladder contractility to CCH when compared to their respective DMSO controls (Figure 3.12B).

3.3.12 Methoxphenidine Decreases Carbachol-Induced Contractions in Rat Urinary Bladder Strips

Concentrations of MXP ≥10µM reduced the contractile response of bladder strips to CCH in a concentration-dependent manner (Figure 3.12C). Strips exposed to 10µM MXP displayed reduced contractility at CCH concentrations of 3µM ($P<0.001$), 10 and 30µM ($P<0.0001$), and 100µM ($P<0.01$) compared to vehicle-control tissue strips incubated with 0.1% DMSO.

The contractile response of bladder strips to CCH was practically abolished when treated with ≥100µM MXP. Application of 100µM MXP significantly reduced the contractile response at CCH concentrations of 3, 10, 30, 100 and 300µM ($P<0.0001$) compared to tissues treated with 1% DMSO. Similarly, exposure to 300µM MXP significantly decreased contractility elicited by CCH concentrations of 3µM ($P<0.05$), and 10, 30, 100 and 300µM ($P<0.0001$) compared to tissues treated with 3% DMSO.

3.3.13 Diphenidine Decreases Carbachol-Induced Contractions in Rat Urinary Bladder

Concentrations of DPD ≥1nM attenuated the contractile response of rat bladder strips to CCH in a concentration-dependent manner (Figure 3.12D). Bladder strips exposed to 1nM DPD displayed a reduced contractile response to 10µM CCH ($P<0.01$) compared to strips treated with 0.00001% DMSO, and strips incubated with 10nM DPD displayed a reduced contractile response to CCH concentrations of 10 and 30µM ($P<0.001$) compared to strips exposed to 0.0001% DMSO. Exposure to 100nM DPD reduced contractility induced by CCH concentrations of 10 and 30µM ($P<0.05$) when compared to strips incubated with 0.001% DMSO. Application of 1µM DPD reduced bladder contractility to 3µM CCH ($P<0.05$) compared to the 0.01% DMSO control, and 10µM DPD attenuated the contractile response of strips

evoked by CCH concentrations of 3, 10, 30, 100 and 300µM (P<0.0001) compared to the 0.1% DMSO control.

The contractile response of bladder strips was practically abolished when exposed to DPD concentrations ≥100µM. Strips incubated with 100µM DPD displayed a significantly reduced contractile response to CCH concentrations of 3, 10, 30, 100 and 300µM (P<0.0001) when compared to tissue exposed to 1% DMSO. Similarly, strips exposed to 300µM DPD produced a significantly reduced contractile response to CCH concentrations of 3µM (P<0.05), 10, 30, 100 and 300µM (P<0.0001) compared to the 3% DMSO control.

3.3.14 MK-801 Decreases Carbachol-Induced Contractions in Rat Urinary Bladder

Concentrations of MK-801 ≥100µM reduced the contractile response of bladder strips to CCH (Figure 3.13). Strips incubated with 100µM MK-801 displayed a reduced contractile response at CCH concentrations of 10, 30, 100 and 300µM (P<0.0001) compared to the 0.1% DMSO control. Exposure to 300µM MK-801 practically abolished the contractile response of bladder strips to CCH, with significantly reduced responses at CCH concentrations of 3µM (P<0.05), 10, 30, 100 and 300µM (P<0.0001) when compared to the 0.3% DMSO control.

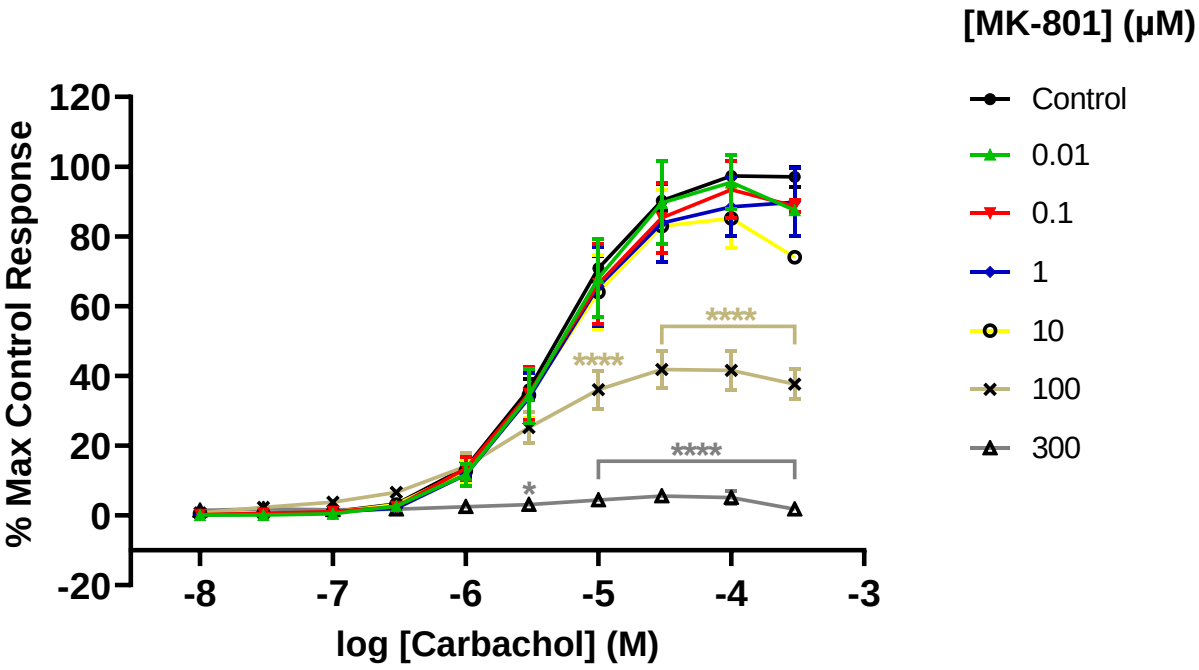

Figure 3.13. Effect of MK-801 on the cumulative carbachol concentration-response in rat urinary bladder strips after 20-minute incubation. Data expressed as a mean percentage of the maximum control response ± SEM. Each data point represents *n=4.* Data analysed using a two-way ANOVA and a Bonferroni post-test. *P<0.05, ****P<0.0001 significantly different from respective DMSO control at the respective carbachol concentration.

3.3.15 The Contractile Response of Rat Bladder Strips to High Potassium Krebs Solution Does Not Change Over Time

To determine whether any change in the contractile response of bladder strips to high potassium after drug incubation was a drug effect, it was necessary to establish whether bladder contractility evoked by high potassium changes over time. Tissue strips were therefore contracted with high potassium Krebs solution every twenty-five minutes (the same time interval used in drug treated bladder strips). The contractile response of rat bladder strips to high potassium Krebs did not change over time (Figure 3.14).

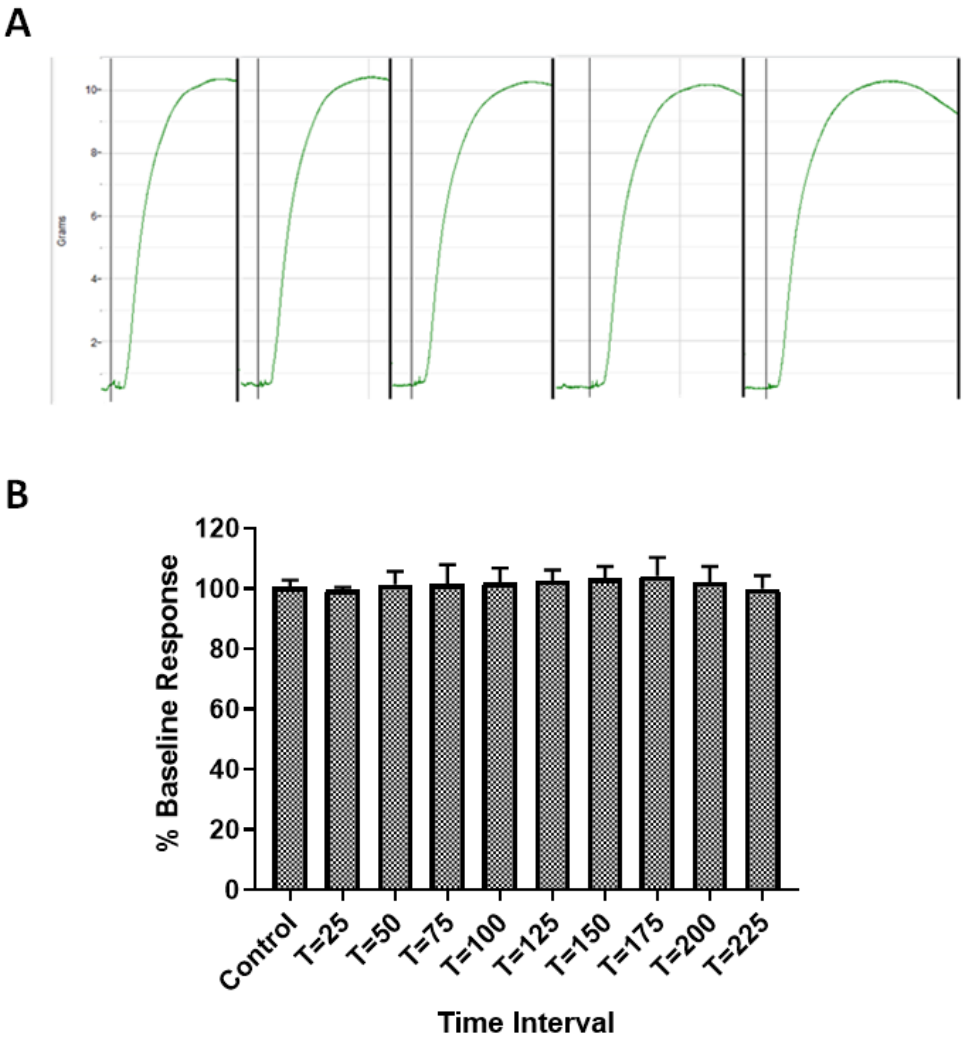

Figure 3.14. Effect of time on rat urinary bladder strip contractility evoked by high potassium Krebs-Henseleit solution. **A:** Representative Labscribe trace of rat bladder strip contractility evoked by high potassium Krebs-Henseleit solution over time. Y-axis represents grams of response. **B:** Effect of rat bladder strip contractility to high potassium Krebs-Henseleit solution over time. Each time interval represents twenty-five minutes (i.e. T=25 represents twenty-five minutes elapsed from control, T=50 represents fifty minutes elapsed from control, etc.). Data are expressed as a mean percentage change from the baseline response ± SEM. Each data point represents *n=7*. Data analysed using a one-way ANOVA and a Dunnett's post-test. No significant difference in contractility was observed over time.

3.3.16 Ketamine Decreases Rat Bladder Strip Contractility Evoked by High Potassium Krebs Solution

Bladder strips incubated with ≥100µM ketamine displayed a reduced contractile response to high potassium stimulation. Exposure to 100µM ketamine attenuated the response of bladder strips to high potassium compared to the control (P<0.001; Figure 3.15). Moreover, exposure to 1mM ketamine practically abolished the contractile response of tissue strips to high potassium Krebs (P<0.001; Figure 3.15). Tissue contractility was able to return to control levels after 2 washouts (recovery; Figure 3.15).

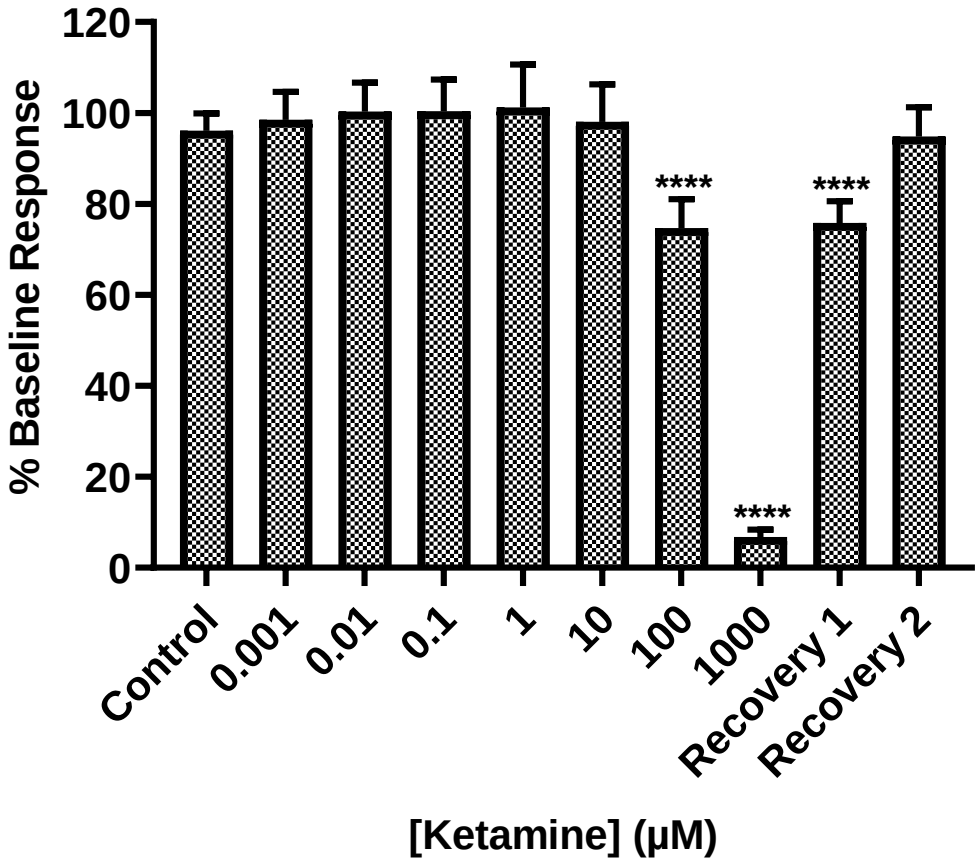

Figure 3.15. Effect of ketamine on rat bladder strip contractility evoked by high potassium Krebs solution after 20-minute incubation. Data are expressed as a mean percentage change from the baseline response ± SEM. Each data point represents *n=4*. Data analysed using a one-way ANOVA and a Dunnett's post-test. ****P<0.0001 significantly different from the control response.

3.3.17 Norketamine Decreases Rat Bladder Strip Contractility Evoked by High Potassium Krebs Solution

Concentrations of NK ≥100µM depressed bladder strip contractile responses to high potassium Krebs solution in a concentration-dependent manner, and the contractile response of the tissue was able to return to control levels after washout (P<0.0001; Figure 3.16).

All other concentrations of NK tested had no effect on the contractile response of bladder strips to high potassium Krebs.

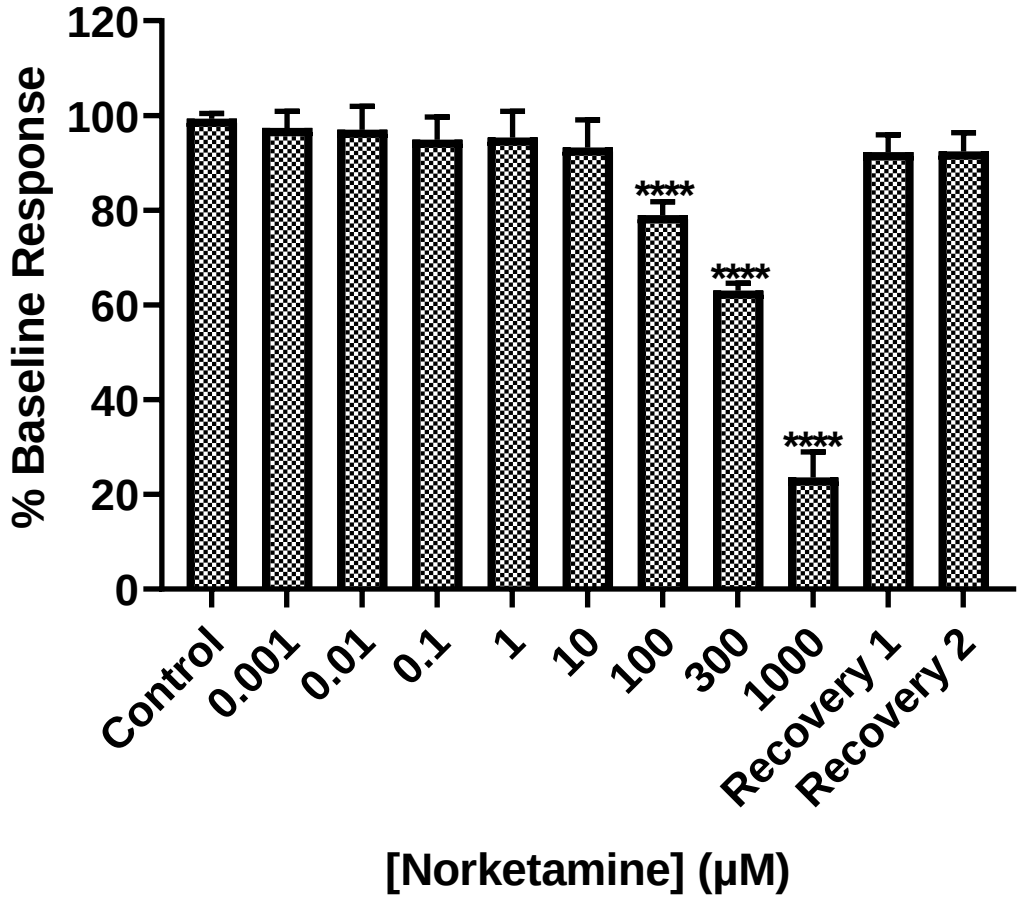

Figure 3.16. Effect of norketamine on rat bladder strip contractility evoked by high potassium Krebs solution after 20-minute incubation. Data are expressed as a mean percentage change from the baseline response ± SEM. Each data point represents *n=3*. Data analysed using a one-way ANOVA and a Dunnett post-test. ****P<0.0001 significantly different from the control response.

3.3.18 Dehydronorketamine Decreases Rat Bladder Strip Contractility Evoked by High Potassium Krebs Solution

Exposure to 1mM DHNK reduced the contractile response of bladder strips to high potassium ($P<0.0001$; Figure 3.17). After two washout periods, this response was still significantly depressed from the control response ($P<0.0001$).

All other concentrations of DHNK tested had no effect on the contractility of rat bladder strips to high potassium Krebs.

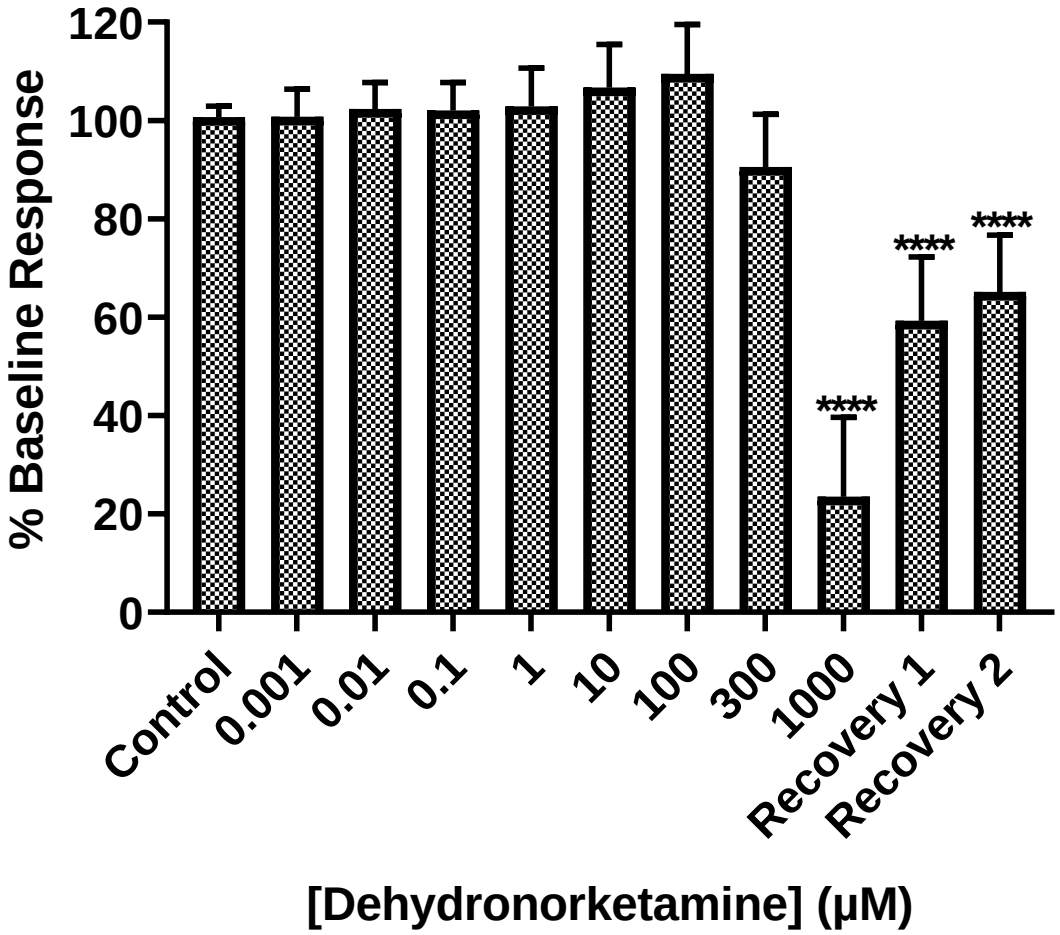

Figure 3.17. Effect of dehydronorketamine on rat bladders strip contractility evoked by high potassium Krebs solution after 20-minute incubation. Data are expressed as a mean percentage change from the baseline response ± SEM. Each data point represents *n=3*. Data analysed using a one-way ANOVA and a Dunnett post-test. ****P<0.0001 significantly different from the control response.

3.3.19 Dimethyl Sulfoxide Does Not Affect Rat Bladder Strip Contractility Evoked by High Potassium Krebs Solution

To establish whether DMSO alone affects bladder strip contractility induced by high potassium Krebs solution, strips were incubated with varying percentages of DMSO to match the percentage of DMSO present in NPS treated tissues (Figure 3.18A).

All concentrations of DMSO tested had no effect on bladder strip contractility evoked by high potassium.

3.3.20 Methoxetamine Decreases Rat Bladder Strip Contractility Evoked by High Potassium Krebs Solution

Bladder strips incubated with 100μM ($P<0.05$) and 300μM ($P<0.0001$) MXE had a reduced contractile response to high potassium Krebs solution (Figure 3.18B). This contractile response was able to recover back to control levels after a washout.

All other concentrations of MXE tested had no effect on the contractile response of bladder strips to high potassium Krebs.

3.3.21 Methoxphenidine and Diphenidine Decrease Rat Bladder Strip Contractility Evoked by High Potassium Krebs Solution

Concentrations of MXP or DPD ≥10μM reduced the contractility of rat bladder strips evoked by high potassium ($P<0.001$) in a concentration-dependent manner (Figure 3.18C&D). Subsequent recovery periods display a trend towards tissue contractility returning to control levels, however full recovery of the tissue was not able to be obtained after three washouts.

All other concentrations of MXP and DPD tested had no effect on the contractile response of bladder strips to high potassium Krebs.

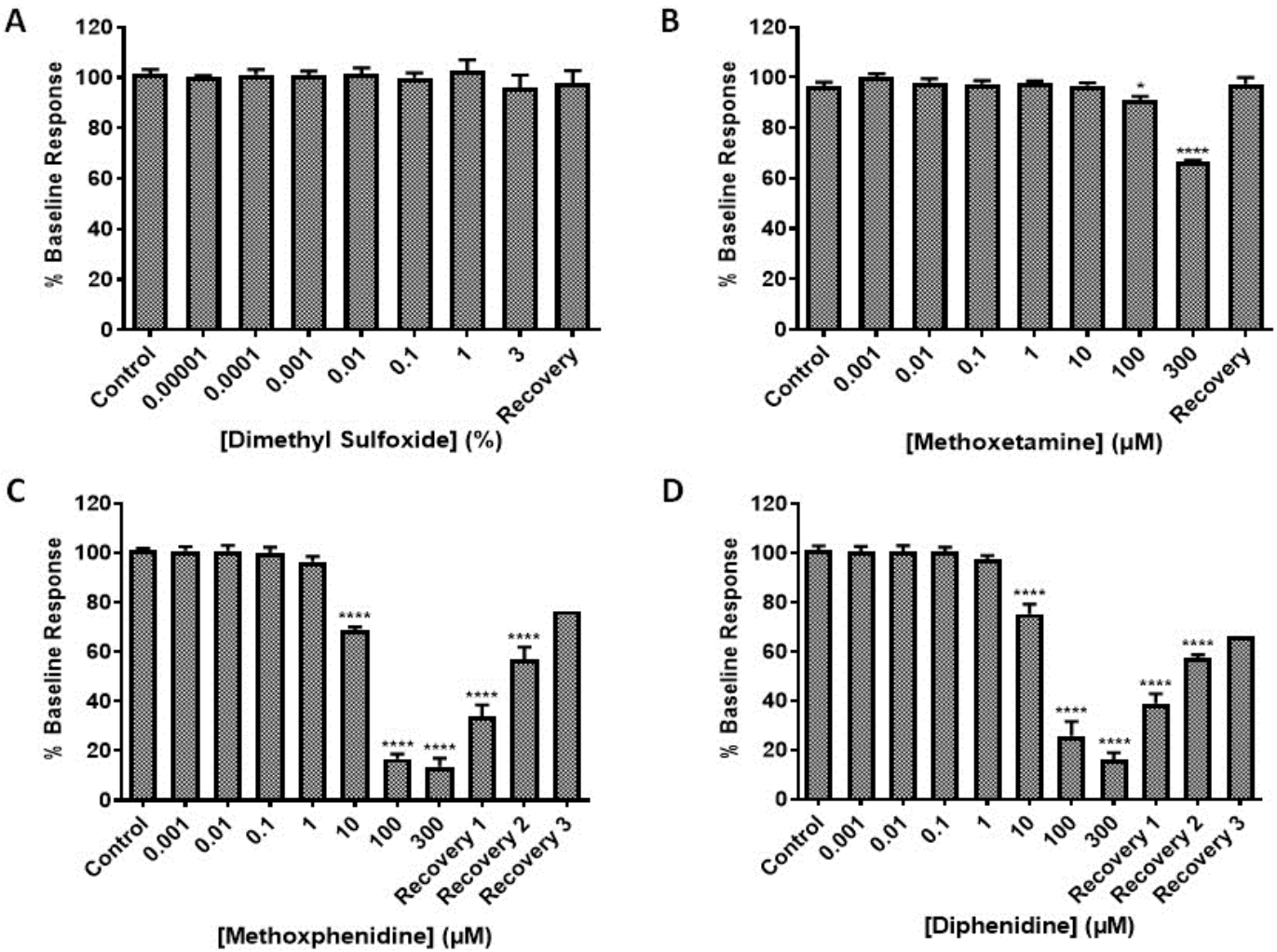

Figure 3.18. Effect of DMSO and NPS on rat bladder strip contractility evoked by high potassium after 20-minute incubation. **A:** Effect of dimethyl sulfoxide. **B:** Effect of methoxetamine. **C:** Effect of methoxphenidine. **D:** Effect of diphenidine. Data are expressed as a mean percentage change from the baseline response ± SEM. Data analysed using a one-way ANOVA and a Dunnett post-test. * P<0.05, ****P<0.0001 significantly different from the control response. Each data point represents *n=3* (**A & B**); *n=4*, except Recovery 1 & 2, where *n=3,* and recovery 3, where *n=1* (**C & D**).

3.3.22 Ketamine Reduced Rat Urinary Bladder Strip Contractility Evoked by Electrical Field Stimulation

Bladder strips stimulated by EFS contracted in a voltage- and frequency- dependent manner (Figure 3.19), and were subsequently contracted using a submaximal frequency and voltage.

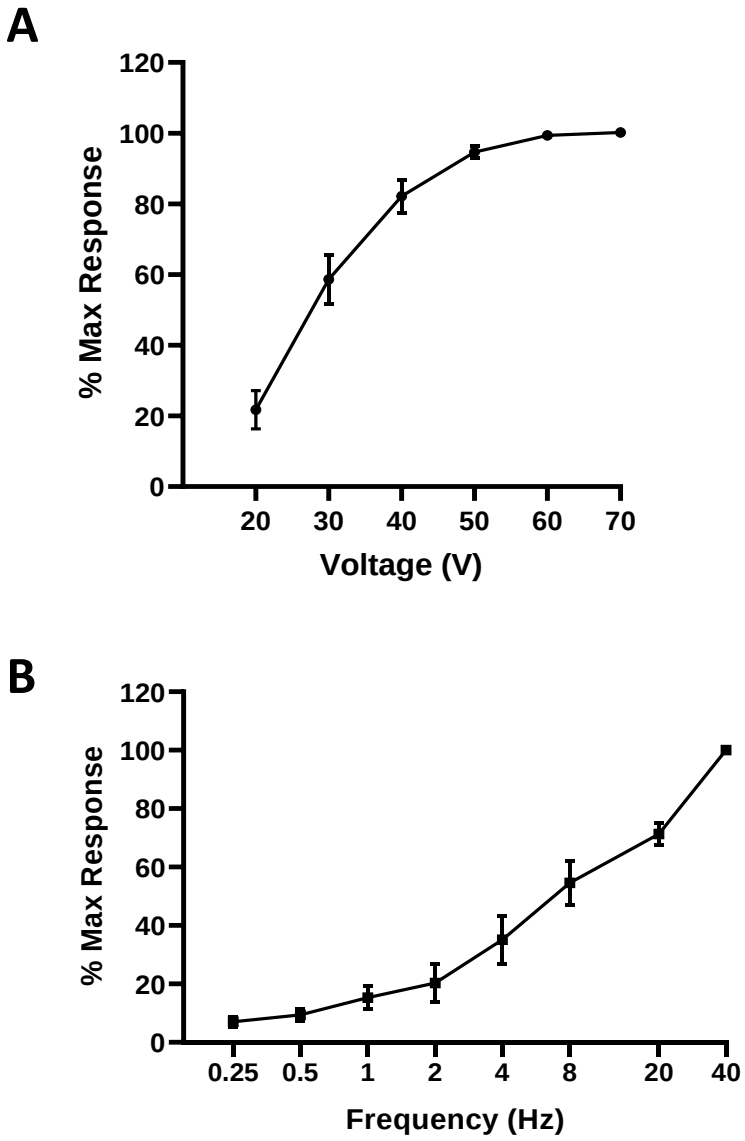

Figure 3.19. Effect of electrical field stimulation on rat bladder strip contractility. Voltage **(A)** and frequency **(B)** responses were undertaken on rat bladder strips to determine submaximal parameters for subsequent electrical field stimulation experiments. Data expressed as a mean percentage of the maximum contractile response ± SEM. Data represents *n=4*.

Bladder strips incubated with 300µM ketamine displayed a reduced contractile response to EFS after three minutes compared to time-matched tissues ($P<0.01$; Figure 3.20B), and inhibition of the contractile response was observed for the entire duration tested ($P<0.001$). Exposure to 1mM ketamine decreased the contractile response of strips to EFS after one-minute compared to time-matched strips (Figure 3.20C; $P<0.05$), and this inhibitory effect was observed for the entire duration tested ($P<0.001$). After nine minutes of incubation with 1mM ketamine, the response of bladder strips to CCH was practically abolished (2.6 ± 1.52%).

There was no change in contractility observed in bladder strips exposed to 100µM ketamine ($P>0.05$; Figure 3.20A).

3.3.23 Ketamine Decreases Carbachol-Induced Contractions in Rat Ileum

Rat ileum was contracted with increasing concentrations of CCH (1nM-30µM), and the resultant CCR produced a sigmoidal curve (Figure 3.21A), with an EC50 of 0.29 ± 0.09µM. A submaximal concentration of 3µM CCH was selected to investigate the effects of ketamine on CCH-induced contractility in rat ileum.

Sections of ileum treated with ≥300µM ketamine produced a significantly reduced response to 3µM CCH compared to control-treated sections (Figure 3.21C). After 20 minutes incubation with 300µM ketamine, the response of rat ileum to CCH was decreased compared to the control response ($P<0.0001$). Exposure to 1mM ketamine practically abolished the response of ileum sections to 3µM CCH ($P<0.0001$) compared to the control. Exposure to 10µM ketamine tested had no effect on the contractile response of ileum to CCH (Figure 3.21C).

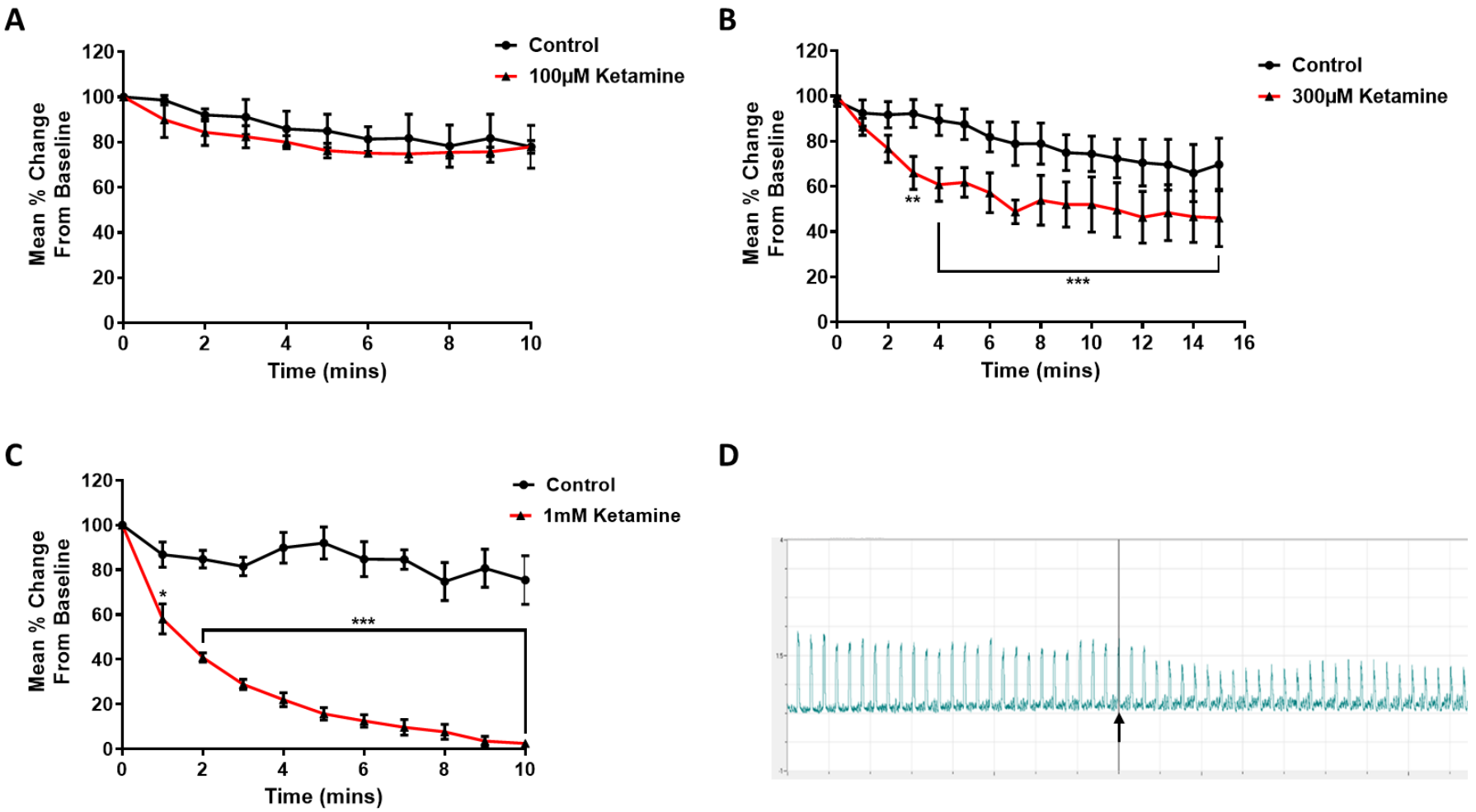

Figure 3.20. Effect of ketamine on rat bladder strips contractility evoked by electrical field stimulation. Bladder strips are exposed to ketamine at time=0. **A:** 100µM ketamine. **B:** 300µM ketamine. **C:** 1mM ketamine. Data are expressed as mean percentage change from the baseline response ± SEM. Each data point represents *n=3*. Data analysed using a two-way ANOVA and a Bonferroni post-test. *P<0.05, **P<0.01, ***P<0.001 significantly different from control at respective time point. **D:** Representative Labscribe trace showing the effect of 1µM atropine on bladder strip contractility evoked by electrical field stimulation. Addition of atropine denoted by the arrow.

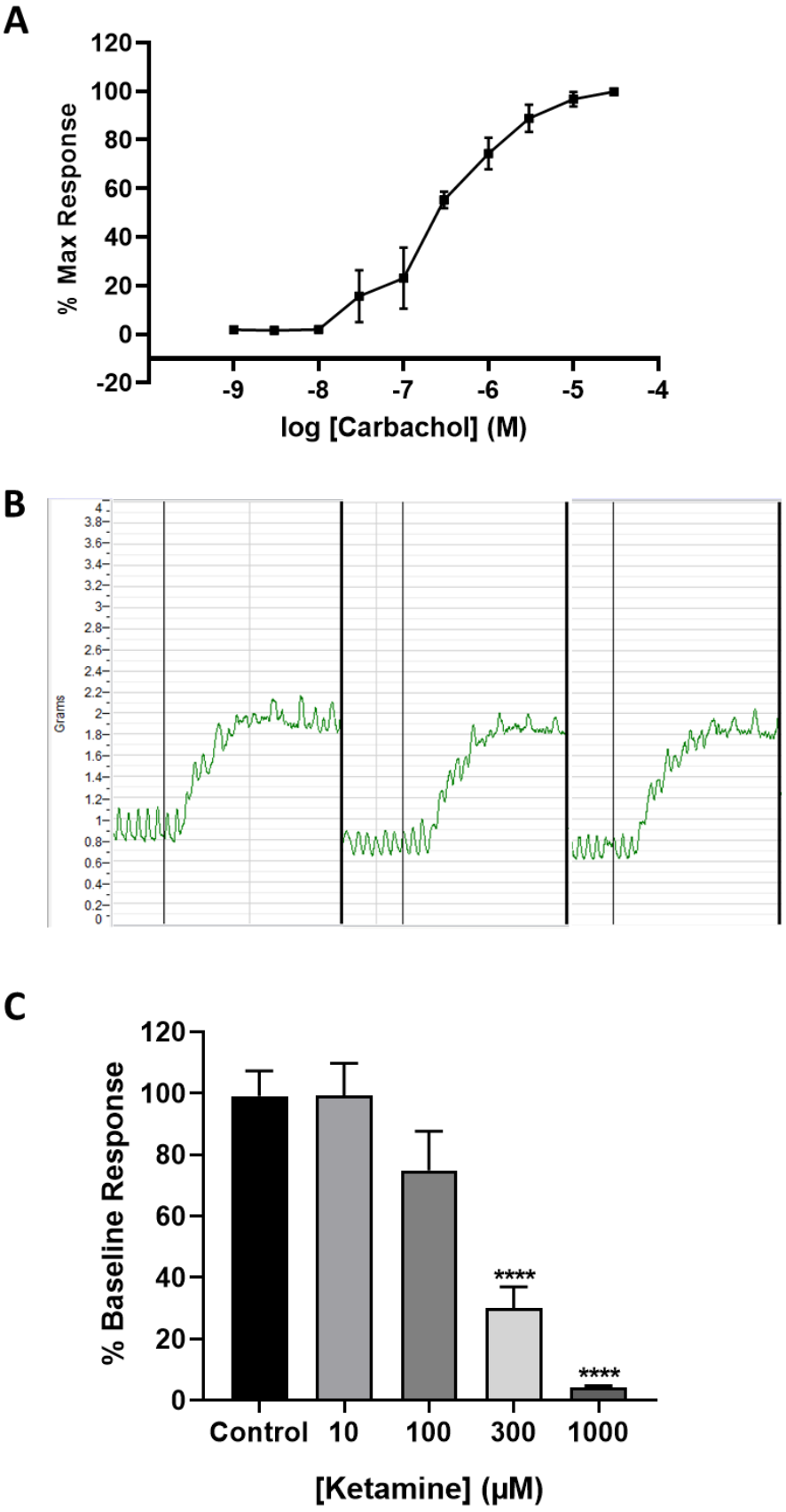

Figure 3.21. Effect of carbachol on rat ileum contractility with or without preincubation with ketamine. **A:** Carbachol concentration-response in rat ileum. **B:** Representative Labscribe trace of rat ileum contractility induced by carbachol (3µM). A consistent response for 3 consecutive carbachol-induced contractions was required before preincubating with ketamine **C:** Effect of 20-minute preincubation of ketamine on rat ileum contractility evoked by 3µM carbachol. Data are expressed as a mean percentage of the baseline response ± SEM. Each data points represents *n=3*. Data analysed using a one-way ANOVA and a Dunnett post-test **(C)**. **** P<0.0001 significantly different from the control response **(C)**.

3.4 DISCUSSION

3.4.1 Effect of Ketamine and Related Compounds on Carbachol-Induced Contractions in Rat Urinary Bladder Strips

Carbachol contracted rat urinary bladder strips in a concentration-dependent manner (Figure 3.1). The EC50 of the CCH response (1.48 ± 0.32μM) is similar to some other reports in rats (1.3 ± 0.3μM, Braverman & Ruggieri, 1999; and 1.26 ± 0.21μM, Braverman, Luthin & Ruggieri, 1998). Additionally, the response of tissue strips to CCH was unaltered by repeated administrations of CCH (Figure 3.2A), and the EC50 and Emax of these responses was shown to not change over time (Figure 3.2B&C). As such, any change in contractility observed after applying ketamine would not be a result of deterioration of the contractile capacity of the tissue over time or repeated applications of CCH, and could therefore be attributed to the effects of the drug.

3.4.1.1 Ketamine

After twenty minutes of exposure, ketamine concentrations ≥300μM inhibited rat bladder contractility evoked by CCH (Figure 3.3). These findings are similar to those observed by Ceran et al. (2010), who reported that 1mM ketamine, but not 10 or 100μM, significantly decreased CCH-evoked contractions in rat bladder strips.

In both human and rat urinary bladder, CCH-induced contractions occur via M3 muscarinic receptors (Schneider, Fetscher, et al., 2004; Schneider, Hein & Michel, 2004). Indeed, the micturition contraction *in vivo* and the neurogenic response *in vitro* in normal human detrusor muscle has been attributed primarily, if not entirely, to muscarinic receptor stimulation, as blockade of muscarinic receptors with atropine essentially abolishes these responses (Kinder & Mundy, 1985; Luheshi & Zar, 1990; Andersson & Arner, 2004; Chess-Williams, Chapple, Yamanishi, Yasuda & Sellers, 2008). Urinary bladder emptying in rats is also thought to be mediated primarily through acetylcholine receptor activation (Wang, Luthin & Ruggieri, 1995; Andersson & Arner, 2004), however a non-adrenergic non-cholinergic component also contributes to the micturition contraction (Igawa et al., 1993; Longhurst, Leggett & Briscoe, 1995).

There is some evidence to suggest that ketamine can directly interact with muscarinic receptors. Schneider and de Lores Arniaz (2013) reported that in rat cerebellar and

hippocampal membranes, binding of the muscarinic antagonist quinuclidinyl benzilate was reduced after a single administration of ketamine. Additionally, ketamine was able to concentration-dependently decrease quinuclidinyl benzilate binding in rat cerebellar and hippocampal membranes *in vitro* at concentrations ≥100μM, and so a direct interaction of ketamine at muscarinic receptors was suggested. Ketamine has also been shown to interact with recombinant human M1, M2, and M3 muscarinic receptors expressed in Chinese hamster ovary cells (Hirota, Hashimoto & Lambert, 2002). It is therefore possible that the inhibitory effects of ketamine on CCH-induced contractions seen here are a result of ketamine interaction at muscarinic receptors, however such interaction has not been reported in rat or human bladder. Nevertheless, as ketamine was able to inhibit contractility elicited by high potassium (Figure 3.15) — which bypasses muscarinic receptor activation entirely — this possibility seems unlikely.

It is widely recognised that the key signal required to induce smooth muscle contraction is an increase in the concentration of intracellular calcium ($[Ca^{2+}]_i$). Much evidence indicates that M3-mediated contraction of the bladder is largely dependent on influx of extracellular calcium through L-type calcium channels, and rho-kinase activation (Figure 3.22; Wibberley, Chen, Hu, Hieble & Westfall 2003; Takahashi et al., 2004; Hedge, 2006). This is because, in various mammalian species, CCH-induced bladder contractions are significantly inhibited by L-type calcium channel blockers (Schneider, Fetscher, et al., 2004; Scheneider, Hein, et al., 2004; Badawi et al., 2006; Frei, Hofmann & Wegener, 2009), rho-kinase inhibitors (Wibberley et al, 2003; Fleichman, Schneider, Fetscher & Michel, 2004; Takahashi et al., 2004), removal of extracellular calcium (Uchida et al., 1994; Wegener et al., 2004), or in $Ca_v1.2$ (subunit of L-type calcium channels) deficient animals (Wegener et al, 2004; Frei et al., 2009). Interestingly, CCH-induced contractions were largely unaffected by U73122 (a phospholipase C inhibitor) in rats (Schneider, Fetscher, et al., 2004), humans (Schneider, Hein, et al., 2004), and mice (Wegener et al., 2004); however, it has been demonstrated that PLC/IP_3 signalling is possible in the absence of L-type calcium channel activity (Frei et al., 2009). Moreover, while the importance of calcium release from intracellular stores has been debated, it appears likely that it at least plays a small role in CCH-evoked contractility in both human and rat bladders (Schneider, Fetscher, et al., 2004; Schneider, Hein, et al., 2004; Wuest et al., 2007).

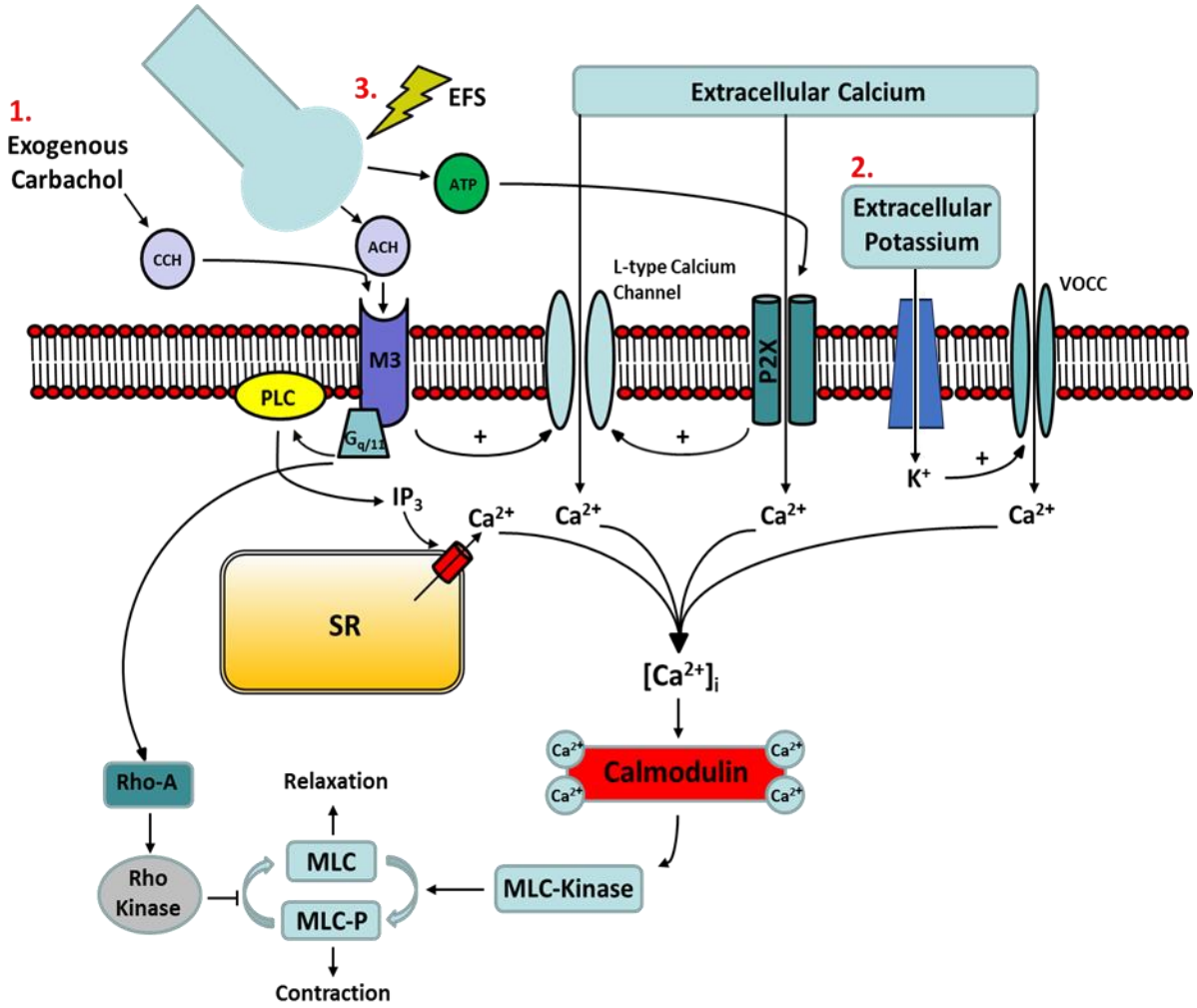

Figure 3.22. Schematic representation of the methods used here to contract rat bladder strips *in vitro*. An increase in intracellular calcium concentration $[Ca^{2+}]_i$ causes contraction by binding calmodulin which in turn leads to phosphorylation of MLC by MLC-kinase. **1**. Exogenous carbachol added to the organ bath binds and activates M_3 muscarinic receptors. This leads to an increase in intracellular calcium by influx of extracellular calcium through L-type calcium channels and through mobilization of intracellular calcium from the SR. Rho kinase activation also prevents dephosphorylation of MLC. **2**. Following application of a high potassium Krebs solution, potassium enters the cell via potassium leak channels. The increased intracellular potassium concentration causes membrane depolarisation, leading to influx of extracellular calcium through VOCCs. **3**. Electrical field stimulation leads to nerve mediated release of endogenous transmitters ATP and acetylcholine. Acetylcholine binds M_3 muscarinic receptors and leads to an increase in intracellular calcium as previously described. ATP binds and activates P2X receptors which allows influx of extracellular calcium directly through the channel and also through L-type calcium channels. ACH: acetylcholine; CCH: carbachol; EFS: electrical field stimulation; MLC: myosin light chain; PLC: phospholipase C; SR: sarcoplasmic reticulum; VOCC: voltage operated calcium channel.

Therefore, another possibility is that ketamine inhibits CCH-induced contractions in rat urinary bladder strips through inhibition of L-type calcium channel current. Indeed, ketamine has been shown to concentration-dependently inhibit Ca^{2+} influx through L-type calcium channels in bullfrog arterial cells (concentrations of 10µM-1mM; Hatakeyama, Yamazaki, Shibuya, Yamamura, & Momose, 2001), canine tracheal smooth muscle (Hanazaki, Jones &

Warner, 2000), and in smooth muscle from rabbit portal vein (concentration 100µM-1mM; Yamazaki, Ito, Kuze, Shibuya & Momose, 1992). Additionally, Hirota, Zsigmond, Matsuki and Rabito (1995) reported that ketamine has a relaxant effect in guinea-pig intestinal smooth muscle, and that this is brought about by a reduction of calcium influx through L-type calcium channels. Therefore, given ketamine's ability to inhibit calcium influx in a variety of different tissues, and the importance of Ca^{2+} influx through L-type calcium channels in rat bladder contractility, the possibility that ketamine inhibits rat bladder contractility through blockade of L-type calcium channels was considered and is further explored in Chapter 4. Indeed, ketamine has recently been reported to inhibit Bay K8644-induced influx of calcium and intracellular calcium signalling in primary cultured mouse and human bladder smooth muscle cells (Chen et al., 2020).

3.4.1.2 Norketamine and Dehydronorketamine

The ketamine metabolites NK and DHNK both inhibited CCH-evoked contractility in rat urinary bladder strips (Figure 3.4 & 3.5; ≥300µM), and this is believed to be the first study to show such inhibitory effects.

There is evidence to suggest that both NK and DHNK are present in the urine of ketamine users at concentrations similar to or higher than that of ketamine (Moore et al., 2001). Additionally, after daily intraperitoneal injections of ketamine (100mg/kg) for twenty weeks, the concentrations of NK and DHNK in the urine of both male and female mice were markedly higher than ketamine (Shen et al., 2016). Therefore, the direct effects of ketamine on the bladder may be mediated, or in part mediated, by the relatively high concentrations of NK and DHNK in the urine. Despite this, few studies have considered the potential involvement of these compounds in KC.

As both NK and DHNK possess the ability to inhibit contractions in rat bladder strips, and both are detected in the urine of ketamine users, the possibility of an additive effect between ketamine and its metabolites was explored. Ketamine (30µM), NK (30µM), and DHNK (100µM), when incubated together, were able to decrease the CCH-evoked contractile response of rat bladders strips (Figure 3.6), at concentrations that would not alter contractility if administered alone (Figure 3.3-3.5). As these concentrations are within the range reported in the urine of recreational ketamine users (Moore et al., 2001), this result indicates that

ketamine and its metabolites may cause direct inhibition of bladder contractility in recreational users.

3.4.1.3 (S)-Ketamine

(S)-ketamine was able to concentration-dependently inhibit rat bladder contractility induced by CCH at concentrations ≥100μM (Figure 3.7). Despite an extensive literature search, no other such studies have been reported, and so this is the first study to investigate the direct effects of (S)-ketamine on rat bladder contractility.

Racemic ketamine consists of two optical enantiomers: (S)- and (R)-ketamine. The affinity of (S)-ketamine for the NMDA receptor is reported to be approximately four-fold greater than (R)-ketamine, and approximately two-fold greater than racemic ketamine (Zanos et al., 2018). Here, 100μM (S)-ketamine, but not 100μM ketamine, was able to decrease rat bladder strip contractility evoked by CCH, and (S)-ketamine was a more potent inhibitor of rat bladder contractility at 300μM compared to ketamine (Table 3.2). Therefore, a potential role for the NMDA receptor in rat bladder contractility should be considered.

(S)-ketamine has also been shown to relax bovine middle cerebral arteries following preconstriction with either U46619 (a thromboxane A_2 mimetic that binds specific G-protein coupled receptors leading to an increase in $[Ca^{2+}]_i$) or potassium (Kamel et al., 2008). Kamel et al. (2008) also reported that (S)-ketamine inhibited $^{45}Ca^{2+}$ uptake through both receptor- and potential-operated channels in cerebrovascular smooth muscle. Therefore, when also considering ketamine's ability to inhibit Ca^{2+} influx through L-type calcium channels, it is plausible to hypothesize that (S)-ketamine may reduce rat bladder contractility evoked by CCH through inhibition of Ca^{2+} influx through calcium channels.

3.4.1.4 Dextromethorphan

After twenty minutes of exposure, DXM concentrations ≥10μM concentration-dependently reduced rat bladder contractility evoked by CCH (Figure 3.8). This is similar to the findings of Levin et al. (2006), who reported that DXM concentration-dependently inhibited both rat and mouse bladder contractility induced by CCH. However, Levin et al. (2006) reported that 3μM DXM was able to inhibit CCH-induced contractions, whereas here the same concentration had no effect.

Like ketamine and (S)-ketamine, DXM acts as a non-competitive antagonist at the NMDA receptor, and there is some evidence to suggest that it can relax smooth muscle via a decrease in calcium influx through calcium channels. Kamel et al., (2008) demonstrated that DXM is able to relax preconstricted bovine cerebral arteries, and block $^{45}Ca^{2+}$ uptake induced by potassium or U46619. Additionally, DXM has been reported to decrease high potassium induced $^{45}Ca^{2+}$ uptake in cultured neural (PC12) cells and brain synaptosomes (Carpenter et al., 1988). It is therefore possible that DXM could exert similar effects here - acting as a calcium antagonist by inhibiting Ca^{2+} influx through calcium channels, leading to a decrease in bladder contractility.

3.4.1.5 MK-801

Concentrations of MK-801 ≥100µM were able to inhibit rat bladder contractions evoked by CCH compared to the respective DMSO control (Figure 3.13). This is similar to the findings reported by Gonzalez-CaDavid et al. (2000), who showed that 500µM MK-801, when applied simultaneously with bethanechol (a muscarinic receptor agonist), eliminated the contractile response in rat bladder strips. Additionally, in strips precontracted with bethanechol, MK-801 was able to accelerate relaxation at concentrations ≥30µM.

MK-801 is a potent NMDA receptor antagonist and is speculated to act as an L-type calcium channel blocker in rat hippocampus as it increased recovery rate of neural function in rat hippocampal slices to a similar degree to other L-type calcium channel blockers, whereas other NMDAR antagonists had little effect (Schurr, Payne & Rigor, 1996).

3.4.2 Effect of Methoxetamine and Related New Psychoactive Substances on Carbachol-Induced Contractions in Rat Urinary Bladder Strips

Methoxetamine is a ketamine analogue that shares several of its properties, including NMDA receptor antagonism (Corazza et al., 2012), and was advertised as a 'bladder friendly' alternative despite no pharmacological evidence to support this claim (Zawilska & Andrzejczak, 2015; Lawn et al., 2016). Methoxphenidine and DPD are NPS that emerged as alternatives to MXE, and they are also reported to be relatively selective NMDA receptor antagonists (Wallach et al., 2016). As little is known regarding their effects on the bladder, the direct, acute effects of these NPS were investigated in rat bladder.

3.4.2.1 Gas Chromatography Mass Spectrometry Analysis of Diphenidine and Mexthoxphenidine

Samples of DPD and MXP were analysed using GC-MS to confirm their identity (Figure 3.10 & 3.11). The chromatogram for both DPD and MXP displayed one peak (when accounting for the DMSO control) which is a strong indication of purity. At the relevant retention times, the mass spectrum for both DPD and MXP displayed a major peak at 91 m/z, and also 174 and 204 m/z, respectively. These results are in line with reported mass spectrum data from both DPD samples and reference standards, which displayed a base peak at 174 m/z and a smaller peak at 91 m/z (Wurita, Hasegawa, Minakata, Watanabe & Suzuki, 2014), and MXP sample data, which displayed peaks at 204 m/z and 91 m/z (Stachel, Jacobsen-Bauer & Skopp, 2015). The mass spectrum data presented here and reported in the literature is accounted for by the suspected fragmentation of the compounds (Figure 3.23), and thus the identity of both DPD and MXP were confirmed with a reasonable degree of certainty.

3.4.2.2 Methoxetamine

As all the NPS tested here were dissolved in DMSO, the effect of DMSO alone on rat bladder contractility was considered. Statistical analysis of rat bladder contractility in the presence of DSMO revealed a significant treatment factor variation (Figure 3.12A), and so any effects exhibited by the NPS were compared back to the DMSO vehicle-control data.

Rat bladder contractility to CCH was inhibited by 300µM MXE (Figure 3.12B). Like ketamine and many of the other compounds tested here, MXE is an NMDA receptor antagonist (Table 3.3). It has also been shown to induce a small but significant inhibition in $[Ca^{2+}]_i$ in human SH-SY5Y cells (a neuronal cell line) evoked by both potassium and acetylcholine (Honderbrink et al., 2017). In the same study however, MXE had no effect on potassium-induced increases in $[Ca^{2+}]_i$, and actually increased glutamate evoked $[Ca^{2+}]_i$ in rat primary cortical cells.

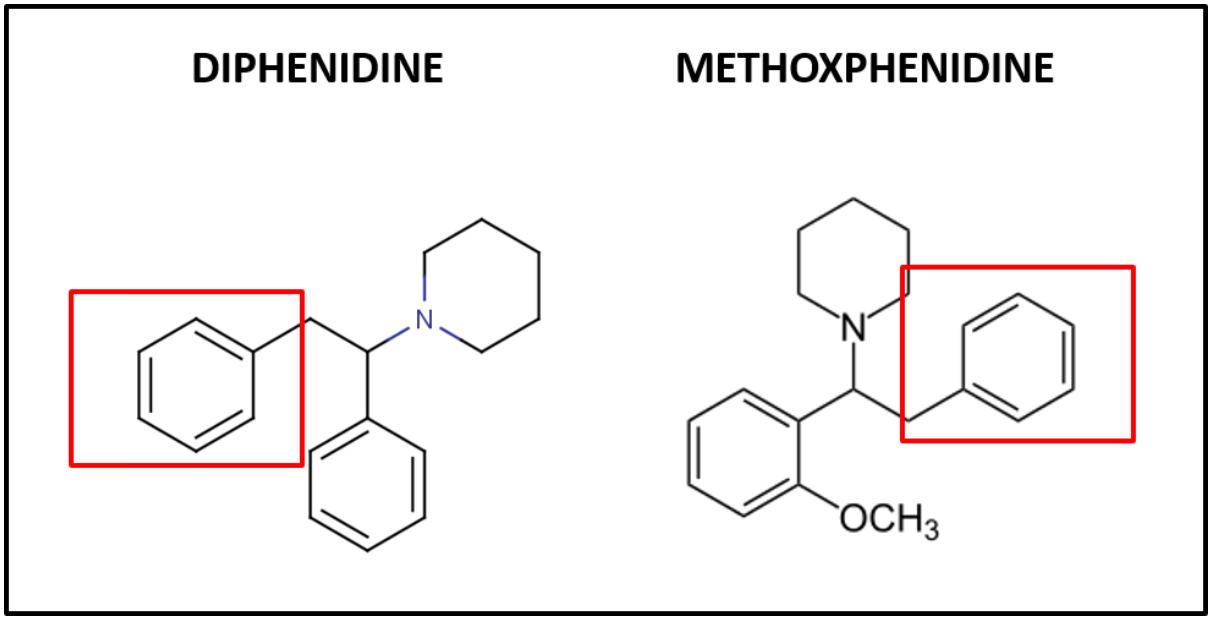

Figure 3.23. Molecular structure of diphenidine and methoxphenidine. Area highlighted in red indicates the suspected fragmentation during mass spectrometry. Molecular mass for the fragment of the molecule outlined in red (C_7H_7) is ~91. The other resulting fragment for diphenidine ($C_{12}H_{16}N$) has a molecular mass of ~174, and for mexthoxphenidine ($C_{13}H_{18}NO$) has a molecular mass of ~204.

3.4.2.3 Methoxphenidine and Diphenidine

Tissue strips exposed to MXP (≥10µM) or DPD (≥1nM) displayed a decreased contractile response to CCH (Figure 3.12C-D).

As with many NPS, information regarding their pharmacology is limited. However, both MXP and DPD are reported to be NMDA receptor antagonists (Table 3.3), as determined by radioligand binding in rat forebrain (Wallach et al., 2016).

The fact that all of the compounds used here are either NMDA receptor antagonists, or display affinity to the NMDA receptor in radioligand binding assays (Ebert et al., 1997; Shimoyama et al., 1999; Werling et al., 2007; Roth et al., 2013; Sałat et al., 2015; Wallach et al., 2016), and that they all were able to inhibit CCH-induced contractility in rat bladder suggests a potential role for the NMDA receptor in this phenomenon.

NMDA receptor activity has been reported in some peripheral tissues (Liu et al., 2015), including the cardiovascular system (McGee & Abdel-Rahman, 2012). Gonzalez-Cadavid et al. (2000) reported that the NMDA receptor subunits required to form a potentially active NMDA receptor (1, 2A and 2B) are present in both human and rat bladder. Moreover, they were able to bind an NMDA ligand to membrane preparations from these tissues. In humans, it is

unlikely that these subunits are located within the urothelium, as RT-PCR of NMDA receptor transcripts found no expression of GRIN isoforms in normal human urothelial cells (Baker et al., 2016). It is possible that the NMDA receptor reported in bladder is restricted to nerve terminals (Gonzalez-CaDavid et al., 2000).

As various NMDA receptor antagonists have been shown to inhibit rat bladder contractility, the ability of NMDA agonists to potentially contract bladder strips was investigated by CaDavid et al. (2000). Glutamate and NMDA (0.1-10mM) did not induce contractions in rat or human bladder strips that were fully relaxed, and only produced weak contractions in strips that were precontracted with bethanechol and slowly relaxed with ketamine (Gonzalez-CaDavid et al., 2000), suggesting that contractile inhibition of bladder by ketamine and the ketamine-related compounds tested here is not, or at least is not primarily mediated by NMDAR antagonism.

Radioligand binding data shows that the high-affinity NMDA antagonist MK-801 has the highest affinity for the NMDA receptor out of all the compounds investigated here (Table 3.3). However, 10µM MK-801 had no effect on CCH-induced contractility in rat bladder strips (Figure 3.13), whereas 10µM MXP or DPD displayed a significant reduction in the contractile response (Figure 3.12C&D). Additionally, DXM, which is a relatively low affinity NMDA receptor antagonist (Table 3.3; Taylor et al., 2016), was able to decrease rat bladder contractility evoked by CCH at concentrations of 10, 30, and 100µM (Figure 3.8), whereas ketamine at these concentrations had no effect (Figure 3.3). Moreover, DHNK is reported to show weak or no ability to displace [^{3}H]MK-801 binding to NMDA receptors (Moaddel et al., 2013; Zanos et al., 2018), but was able to cause inhibition of rat bladder contractility induced by CCH comparable to ketamine (Figure 3.3 & 3.5). Therefore, as some of the lower-affinity NMDA receptor antagonists tested here are able to more potently inhibit rat bladder contractility than other higher-affinity NMDA receptor antagonists, it is plausible to suggest that the mechanism of contractile inhibition observed is not mediated by NMDA receptors, or at least that NMDAR-mediated inhibition is not the main mechanism by which this occurs. It should be noted, however, that the binding affinity data cited are for centrally located NMDA receptors, so how this relates to peripherally located NMDA receptors is not known. Nevertheless, Gonzalez-CaDavid et al., (2000) demonstrated that CGP 39653 (a high affinity

antagonist to the NMDA receptor) was able to bind NMDA receptors in both human and rat bladder membrane fractions.

Table 3.3. Radioligand binding data of ketamine and the related compounds investigated here at the N-methyl-D-aspartate receptor. All data are from [^{3}H]-MK-801 binding displacement assays in rat.

Compound	**K_i ± SEM (µM)**	**Tissue/System**	**Reference**
Ketamine	0.3239 ± 0.0192	Forebrain	Wallach et al. (2016)
	0.53 ± 0.078	Brain homogenate	Roth et al. (2013)
	0.119 ± 0.01	Brain homogenate	Ebert et al. (1997)
	0.659	Cortex homogenate	Sałat et al. (2015)
Norketamine	3.6 ± 0.49	Brain homogenate	Ebert et al. (1997)
	0.97 ± 0.1	Cortex homogenate	Sałat et al. (2015)
Dehydronorketamine	3.21 ± 0.3	Cortex homogenate	Sałat et al. (2015)
(S)-Ketamine	0.30 ± 0.013	Brain homogenate	Ebert et al. (1997)
Dextromethorphan	2.21 ± 0.8	Hippocampus	Werling et al. (2007)
MK-801	0.0019 ± 0.0010	Brain homogenate	Ebert et al. (1997)
Methoxetamine	0.259	Brain homogenate	Roth et al. (2013)
Diphenidine	0.0182 ± 0.0022	Forebrain	Wallach et al. (2016)
Methoxphenidine	0.036 ± 0.0037	Forebrain	Wallach et al. (2016)

Ketamine and the NPS tested here are also reported to display dopamine reuptake inhibition (Corazza et al., 2012; Wallach et al., 2016), although one report suggests that DPD, but not MXP, increases dopamine efflux in rat nucleus accumbens, likely through dopamine transporter inhibition (Davidson et al., 2014). There is evidence to suggest that dopamine D_1 and D_2 receptors are present in the bladder (Escaf, Cavallotti, Ricci, Vega & Amenta, 1994; El-Mas, Elmallah, Omar & Sharabi, 1999), and dopamine has been shown to relax cholinergic contractile responses in cat stomach fundus (Lefebvre, 1992), and reduce responses to non-adrenergic non-cholinergic nerve stimulation and potassium evoked contractions in rat bladder (Lot et al., 1993). The presence of the dopamine transporter in the bladder has not

been reported however, and so whether the binding of these compounds to the dopamine transporter is relevant in modulating bladder contractility is questionable.

3.4.3 Effect of Ketamine and Related Compounds on Rat Bladder Contractility Evoked by High Potassium

3.4.3.1 Ketamine

The contractile response of rat bladder strips was not affected by repeated administration of high potassium Krebs over the typical time course of the experiment (Figure 3.14). Ketamine (≥100μM) inhibited the contractile response of rat bladder strips induced by high potassium Krebs solution, and the contractions were able to return to control levels after washout (Figure 3.15).

Interestingly, 100μM ketamine was able to inhibit contractility induced by potassium but not CCH. This could potentially be explained by considering the additional intracellular signalling pathways that are activated following M_3 muscarinic receptor activation. High potassium solutions are thought to elicit rat urinary bladder contraction by causing membrane depolarisation, which subsequently leads to influx of calcium through voltage-operated calcium channels (VOCCs; Figure 3.22). Indeed, experiments which utilize calcium free buffers or VOCC blockers indicate that potassium-induced contractions in bladder are dependent upon extracellular calcium in humans (Visser & van Mastrigt, 2000), rabbits (Jezior et al., 2001), and guinea pigs (Lowe & Noronha-Blob, 1991). Therefore, as previously discussed (see section 3.4.1.1), ketamine may inhibit rat bladder strip contractility by inhibiting L-type calcium channel current, as potassium-induced contractility is more sensitive to ketamine. Carbachol induces contractility not only through influx of calcium through L-type calcium channels, but also by increasing calcium sensitivity of the bladder smooth muscle (Wibberley et al., 2003; Takahashi et al., 2004). As such, although CCH-mediated contractions in bladder are largely dependent upon extracellular calcium influx through L-type calcium channels, they are not entirely dependent upon it. In mouse bladder, PLC/IP_3 mediated contractility evoked by CCH is still possible in the absence of L-type calcium channel activity (Frei et al., 2009). Additionally, potassium-induced contractions in rabbit bladder are abolished in the presence of verapamil (a VOCC blocker) and calcium free solution, whereas the response to bethanechol was reduced, but not abolished when subjected to the same conditions (Jezior

et al., 2001). This may explain why CCH-evoked contractility is less sensitive to ketamine than potassium, as some degree of contractility independent of extracellular calcium influx is possible. Also consistent with this idea is the finding that Y-27632 (a specific inhibitor of Rho-kinase) was able to inhibit rat bladder contractility evoked by CCH, but not potassium (Wibberley et al., 2003). Rho-kinase (ROCK) plays an important role in calcium sensitisation by inhibiting smooth muscle myosin phosphatase and thus inhibiting dephosphorylation of myosin light chain (Somlyo & Somlya, 2000), and in mice devoid of L-type calcium channel activity, CCH is still able to elicit modest contractions that are largely dependent on ROCK activity (Wegner et al., 2004). As such, the difference in ketamine sensitivity between CCH and potassium mediated contractions could be partially explained by involvement of PLC/IP_3 and/or Rho-kinase activation following muscarinic M_3 receptor activation.

3.4.3.2 Norketamine and Dehydronorketamine

Norketamine (≥100μM) decreased the contractile response of rat bladder strips to high potassium Krebs solution, and contractility was able to recover to control levels after washout (Figure 3.16). This is similar to the effect of ketamine as 100μM NK was able to inhibit rat bladder contractility induced by potassium but not CCH. As such, NK may also inhibit rat bladder contractility through L-type calcium channel current blockade.

Dehydronorketamine was only able to inhibit potassium-induced contractions in rat bladder at a concentration of 1mM (Figure 3.17). This inhibition was unable to be reversed back to control levels after two washout periods. The fact that 300μM DHNK was able to depress bladder contractility evoked by CCH, but not high potassium suggests that this effect may in part be mediated through a non-L-type calcium channel mechanism.

3.4.3.3 Methoxetamine, Methoxphenidine and Diphenidine

Methoxetamine (≥100μM), MXP (≥10μM), and DPD (≥10μM) were all able to inhibit potassium-induced contractions in rat urinary bladder strips (Figure 3.18B-D). The inhibitory effect of MXE was able to be recovered after washout, and, although there was a trend towards recovery to control contractility in MXP and DPD treated strips, full recovery was not able to be demonstrated here. The fact that all NPS were able to decrease contractility induced by high potassium Krebs suggests they act by inhibition of calcium influx and/or inhibition of calcium-dependent signalling pathway(s).

As the NPS tested here are dissolved in DMSO, and DMSO displays an ability to inhibit CCH- but not potassium-induced contractions in rat bladder, it is difficult to make direct comparisons regarding the potency and efficacy. However, the finding that low concentrations of DPD (1nm-1µM) can inhibit CCH- but not potassium-induced contractions may suggest a selective receptor site for DPD that can reduce bladder contractility that is not dependent upon extracellular calcium influx.

3.4.4 Effects of Ketamine on Rat Bladder Strip Contractility Evoked by Electrical Field Stimulation

Ketamine concentrations of 300µM and 1mM significantly attenuated responses to EFS after just two minutes and one minute, respectively (Figure 3.20 B&C). These findings are in opposition with Ceran et al. (2010), who reported that ketamine concentrations of 10µM – 1mM did not significantly alter the contractile response of rat bladder strips evoked by EFS. This discrepancy may be due to the different EFS parameters employed. Here, a pulse width of 0.1ms was used, whereas Ceran et al. (2010) used a pulse width of 5ms, and higher pulse widths have an increased potential to directly stimulate the muscle (Williamson, Okpako & Evans, 1996).

In rat bladder, the contractile response to EFS is reportedly mediated by the release of both adenosine triphosphate (ATP) and acetylcholine (Figure 3.22), though a role for other transmitters cannot be ruled out (Brading & Williams, 1990; Wibberley et al., 2003). Blockade of the cholinergic and purinergic response evoked by EFS in mice does not completely abolish bladder contractility, and the remaining response can be further decreased by tetrodotoxin (Yu, Sun, Robson & Hill, 2013).

In rats, the phasic portion of the response to EFS is thought to be primarily mediated by ATP, and the tonic portion by acetylcholine (Wibberley et al., 2003). Addition of 1µM atropine, a concentration commonly used for maximal cholinergic block in rat bladder strips (Rouget et al., 2014; Stenqvist, Carlsson, Winder & Aronsson, 2020), produced EFS-induced contractility with an amplitude approximately 60% of the response without atropine (Figure 3.20D).

Adenosine triphosphate is reported to induce contractions in rat bladder through activation of purinergic $P2X_1$ receptors (Aronsson, Andersson, Ericsson & Gigilo, 2010). $P2X_1$ receptor activation reportedly allows influx of calcium directly through the receptor, and also through

L-type calcium channels (Figure 3.22; Schneider, Hopp & Isenberg, 1991; Wibberley et al., 2003). However, nifedipine (an L-type calcium channel blocker) has been shown to nearly abolish rat bladder contractions evoked by beta,gamma-MeATP (Bo & Burnstock, 1990), and more or less wipe out responses to EFS in strips pretreated with atropine (Bo & Burnstock, 1990; Zar, Iravani & Luheshi, 1990). Thus, the extent to which calcium influx through $P2X_1$ receptors plays a role in EFS-induced contractility in rat bladder is questionable, though it does seem likely that in isolated rat bladder, the purinergic component is mediated predominantly, if not entirely, through ATP-mediated influx of calcium through nifedipine-sensitive channels (Bo & Burnstock, 1990; Schneider et al., 1991).

Therefore, the ability of ketamine to reduce the maximum contractile response to EFS is in line with the possibility that ketamine works by inhibiting calcium influx through L-type calcium channels. Indeed, the rapid onset of inhibition displayed by ketamine suggests a target site that is readily accessible. Although there is evidence to suggest that ketamine can directly interact with muscarinic receptors, this seems unlikely to be the cause of the inhibition seen here, as approximately 60% of EFS induced contractility still remains after treatment with atropine (Figure 3.20D), whereas ketamine nearly abolishes these contractions. Reports suggesting that the atropine-resistant portion of the EFS response is mediated by VOCCs might therefore explain why ketamine inhibits such a significant portion of the response to EFS in rat bladder strips. Experiments investigating the effects of ketamine on rat bladder contractility induced by ATP or the atropine-resistant portion of the EFS response would provide further insight into this possibility.

3.4.5 Effects of Ketamine on Rat Ileum Contractility Evoked by Carbachol

Ketamine (≥300µM) was able to inhibit the contractile response of rat ileum to 3µM CCH (Figure 3.21C). This is similar to the effect of ketamine on bladder contractility which was also inhibited by ≥300µM ketamine. Hirota et al., (1995) also reported the ability of ketamine to inhibit contractile responses in guinea-pig intestinal smooth muscle.

The fact that ketamine can inhibit contractility in a variety of tissue types and in a variety of different species suggests that it is acting upon a receptor and/or interfering with a signalling pathway that is common within different tissues and within different species, and not

something that is unique to the bladder. This is consistent with the idea that ketamine inhibits contractility though L-type calcium channel blockade.

3.4.6 Clinical Relevance

3.4.6.1 Concentration of Ketamine and Metabolites in the Urine

The clinical relevance of the higher concentrations of ketamine used here is questionable. Several studies have reported the concentrations of ketamine and its metabolites in the urine in the context of clinical ketamine usage (Table 1.1), however, many of these studies are based upon small doses of ketamine (≥50mg) that are much lower than reported recreational doses (Chen, Lee, Cheng & Wu, 2007; Parkin et al., 2008). Indeed, Winstock et al., (2012) reported that 34% of ketamine users take >1g in a typical session, and 54% have used >1g at least once.

There is little information regarding ketamine concentrations present in the urine of recreational users of the drug. Moore, Sklerov, Levine & Jacobs (2001) reported urinary concentrations of ketamine (25nM-33μM), NK (31nM-36μM), and DHNK (167nM-105μM) after urine analysis of 33 ketamine users. Additionally, Cheng, Lee, Liu & Chien, (2008) reported ketamine and NK concentrations of 94nM-24μM and 295nM-40μM, respectively, in the urine from 14 suspected drug users.

Therefore, the concentrations of ketamine, NK, and DHNK used here that were able to inhibit rat bladder contractility to CCH (≥300μM), are greater than those reported in ketamine users. However, in both reports, important information such as the dosage, route of administration, polysubstance use, and the time interval between ketamine use and urine assessment for individuals is not known. It has been suggested that a urinary ketamine concentration in excess of 1mM is possible in users taking high doses (≥1g) of ketamine, when considering a typical voiding rate of 6x300mL per day and that approximately 85% of the drug will be eliminated via the urine within 24 hours (Baker et al., 2016).

Nevertheless, concentrations of ketamine, NK, and DHNK that are within the ranges reported in the urine of ketamine users were able to inhibit CCH-induced contractions in rat bladder when incubated together after twenty minutes (Figure 3.6). Indeed, studies investigating concentrations of ketamine in the urine show that all three compounds can be present in the

urine simultaneously (Moore et al., 2001; Wang, Shih & Cheng, 2005; Chen et al., 2007), and this can occur for at least four hours after ketamine was administered (10mg intramuscularly; Chen et al., 2007). Therefore, the finding that lower concentrations of ketamine, NK, and DHNK have an additive effect that can inhibit rat bladder contractility is novel, and these concentrations are within the ranges of ketamine and its metabolites observed within the urine of ketamine users.

3.4.6.2 Concentrations of New Psychoactive Substance in the Urine

Information regarding the concentrations of MXE, MXP, and DPD in the urine of users is limited. Łukasik-Głebocka, Sommerfeld, Tezyk, Zielińska-Psuja and Druzdz (2013) observed an MXE concentration of 2.7µM in the urine of a 29-year-old male with nasal MXE acute poisoning. Additionally, in a reported case of an MXE-related fatality, an MXE concentration of 343µM was measured in the urine of a 29-year-old male (Adamowicz & Zuba, 2014). In the urine of rats after a subcutaneous injection of 40mg/kg MXE, MXE was present at concentrations ranging from 12-24µM, and was found at much greater concentrations than its metabolites over a twenty-four hour period (Horsley et al., 2016). Here, a concentration of 300µM MXE was able to decrease rat bladder strip contractions to CCH.

In a study of suspected NPS users in a Swedish emergency room, Helander, Beck and Bäckberg (2015) reported a concentration of DPD in the urine ranging from 30nM-72µM (mean 8.3µM), and a urine concentration of 2.4µM was reported in a 30-year-old male following a nonfatal intoxication related to DPD use (Gerace, Bovetto, Di Corcia, Vincenti & Salomone, 2017). These reported concentrations of DPD are well within the range of concentrations used here that were able to decrease CCH- and potassium-induced contractility. Helander et al. (2015) also reported a urine concentration of MXP in suspected NPS users ranging from 10nM-28.3µM, and these concentrations are also within the ranges used here to attenuate bladder strip contractility evoked by both CCH and high potassium. Therefore, this indicates that recreational DPD or MXP may cause a decrease in bladder contractility in users.

3.4.6.3 Organ Bath Experiments

In the *in vitro* organ bath studies undertaken here, the bladder is dissected into strips, and so when incubated with a drug in the organ bath, the drug is exposed to multiple tissue layers. However, *in vivo*, ketamine-induced bladder dysfunction is thought to possibly arise from the

direct contact of ketamine and its metabolites with the urothelium. As one of the main roles of the urothelium is to maintain a very low passive permeability (Lewis, 2000), it is questionable whether ketamine is able to act on multiple tissue layers *in vivo*. However, ketamine has been shown to increase the permeability of human urothelial cells *in vitro* within 24 hours, in a dose- and time-dependent manner (Shen et al., 2015). Experiments which compare the contractility of urothelium denuded bladder strips to intact strips in the presence of ketamine may be of interest.

3.4.6 Future Work and Conclusions

The findings that acute exposure to ketamine or DXM can decrease rat bladder contractility are in agreement with those reported by Ceran et al., (2010), and Levin et al., (2006), respectively. The observation that similar concentrations of ketamine and DXM can also relax human bladder strips precontracted with bethanechol (Gonzalez-CaDavid et al., 2000) suggests that these findings may be clinically relevant in ketamine/DXM users, or individuals who abuse any of the related NPS investigated here. Also presented here are the novel findings that acute exposure to (S)-ketamine, NK, DHNK, MXE, MXP, or DPD can inhibit contractions of rat bladder strips.

The ability of ketamine and related compounds to inhibit rat bladder contractility is interesting. The long-term use of ketamine is associated with the development of various LUT symptoms that are indicative of bladder overactivity, and so how or if the observed acute inhibition of bladder contractility relates to long-term bladder overactivity, or indeed if this is relevant *in vivo*, is uncertain. However, Meng et al. (2011) reported that at 4 weeks, female mice receiving daily injections of ketamine (100mg/kg) displayed higher compliance and lower baseline detrusor pressure, suggesting that short-term ketamine use may induce detrusor relaxation. Additionally, recent research appears to suggest that KC results from the direct effects of ketamine on the urothelium. Baker et al. (2016) reported that exposure of urothelium to ketamine leads to apoptosis, and histological assessment of human ureteric tissues revealed thinning of the urothelium after 72 hours of exposure to 3mM ketamine. Indeed, many *in vivo* studies have reported urothelial denudation or thinning after long-term ketamine use (Shahani et al., 2007; Chuang et al., 2013; Kidger et al., 2015; Duan et al., 2017; Lee et al., 2017; Wei, Wu, Li & Shan, 2020). Therefore, one potential link between the reported opposing short-term and long-term effects of ketamine exposure in the bladder is

that acute ketamine use initially causes bladder underactivity. This would allow ketamine and its metabolites to accumulate in the urine and would also increase the contact time between these compounds and the urothelium, exacerbating the damage. Indeed, ketamine has been shown to induce cytotoxicity in human urothelial cell lines in a dose- and time-dependent manner (Shen et al., 2015).

The findings here report the effects of acute ketamine and NPS exposure, whereas KC is associated with long-term use of ketamine. Therefore, the development of a more chronic model of ketamine exposure would be of interest. This idea is explored in Chapter 5, where an organ culture protocol was developed to allow incubation of rat bladder strips with ketamine, its metabolites, or related NPS for up to 72 hours to determine whether these compounds have direct toxic effects upon rat urothelium.

Finally, the possibility presented here that ketamine and related compounds may inhibit rat bladder contractility through inhibition of L-type calcium channel current could be explored through further *in vitro* organ bath investigation. This is explored in Chapter 4 by utilising compounds that can manipulate calcium channel activity and the intracellular signalling pathways activated following M3 receptor activation in an attempt to elucidate the mechanism of action behind ketamine-induced inhibition of bladder contractility.

Chapter 4: Exploring the Mechanism(s) Behind Ketamine-Induced Inhibition of Bladder Contractility

4.1 INTRODUCTION

The long-term use of ketamine has been linked to the development of cystitis and bladder overactivity. However, as previously reported in Chapter 3, high concentrations of ketamine can decrease rat bladder contractility evoked by various stimuli *in vitro*. The onset of inhibition is rapid (electrical field stimulation-induced contractions were significantly attenuated after just one minute exposure to 1mM ketamine) and reversible as bladder strip contractility was able to return to baseline levels after washout. Contractile inhibition of rat bladder strips was also observed after acute exposure to all ketamine-related compounds tested, including ketamine metabolites, the ketamine analogue methoxetamine and related new psychoactive substances, and various NMDA receptor antagonists. The mechanisms underlying bladder underactivity following exposure to these compounds are not known.

The reported binding promiscuity of ketamine makes identifying the receptor(s) and/or signalling pathway(s) responsible for this phenomenon tricky, especially when considering the relatively high concentrations of ketamine that are required to inhibit stimuli-induced contractility of the detrusor. Although the functional data presented in Chapter 3 does not specifically address the molecular mechanisms behind the observed decrease in bladder contractility, some insight may be gained by considering two things: the different intracellular signalling pathways that are activated in response to the various stimuli used to contract bladder strips; and the reported receptor interactions that are shared by ketamine and the ketamine-related compounds used.

As ketamine and the ketamine-related compounds used in Chapter 3 were able to decrease bladder contractility evoked by all the different stimuli tested, it is plausible to suggest that they are acting upon a common contractile pathway target, and one that is downstream of muscarinic and purinergic signalling. In this case, that is the influx of calcium through voltage-operated calcium channels. Additionally, as all compounds tested in Chapter 3 were able to decrease rat bladder strip contractility after acute exposure, it is valuable to consider any receptor interactions or relevant reported *in vitro* effects that these compounds have in common. As previously described, all the compounds tested are reported to have affinity for the NMDA receptor, and many of the compounds tested are reported to inhibit the influx of calcium through L-type calcium channels. The rapid onset of action of ketamine presented in Chapter 3 also suggests a readily accessible receptor target. Therefore, it was hypothesized

that ketamine decreases rat bladder contractility through inhibition of L-type calcium channel current.

Various exogenous compounds can be introduced into the organ bath assay, and this can be utilized to manipulate specific signalling pathways that may be of interest. Here, the antagonists isradipine, nifedipine, U73122, and Y27632, and the calcium channel agonist Bay K8644, have been used to directly inhibit/enhance various signalling pathways involved in the carbachol-induced contractile response (Figure 4.1; Schneider, Fetscher, Krege & Michel, 2004; Morelli et al., 2007; Chen et al., 2020). Isradipine and nifedipine inhibit bladder contractility through inhibition of L-type calcium channel current. U73122 is an inhibitor of phospholipase C (PLC) which can prevent the inositol triphosphate- (IP_3) mediated mobilization of intracellular calcium from the sarcoplasmic reticulum, and Y27632 inhibits Rho-associated kinases (ROCK) from preventing the dephosphorylation of myosin light chain (MLC). Finally, Bay K8644 increases bladder contractility by increasing the mean open time of L-type calcium channels (Kokubun & Reuter, 1984; January, Riddle & Salata, 1988), producing a positive inotropic effect.

The ability of ketamine and various ketamine-related compounds to decrease rat bladder strip contractility after acute (20-minute) exposure contrasts the detrusor overactivity associated with chronic ketamine use. Therefore, a secondary objective for this chapter was to develop an assay in which bladder strips could be exposed to ketamine for longer periods (up to 3 days; see Baker et al., 2016) to determine what effect, if any, this has on rat bladder contractility. In human bladder tissue, 3mM ketamine can cause thinning of the urothelium after 72 hours exposure (Baker et al., 2016), and a compromised urothelium can cause symptoms including urinary frequency (Birder & de Groat, 2007). Therefore, it was hypothesized that exposure to 3mM ketamine for 72 hours will lead to an increase in rat bladder contractility *in vitro*. Moreover, it has been reported that urothelium-denuded bladder preparations display enhanced contractility to carbachol (CCH) in several species (Hawthorn, Chapple, Cock, & Chess-Williams, 2000; Chaiyaprasithi, Mang, Kilbinger & Hohenfellner, 2003; Koşan et al., 2005). Therefore, the effects of urothelial denudation on rat bladder strip contractility will be investigated.

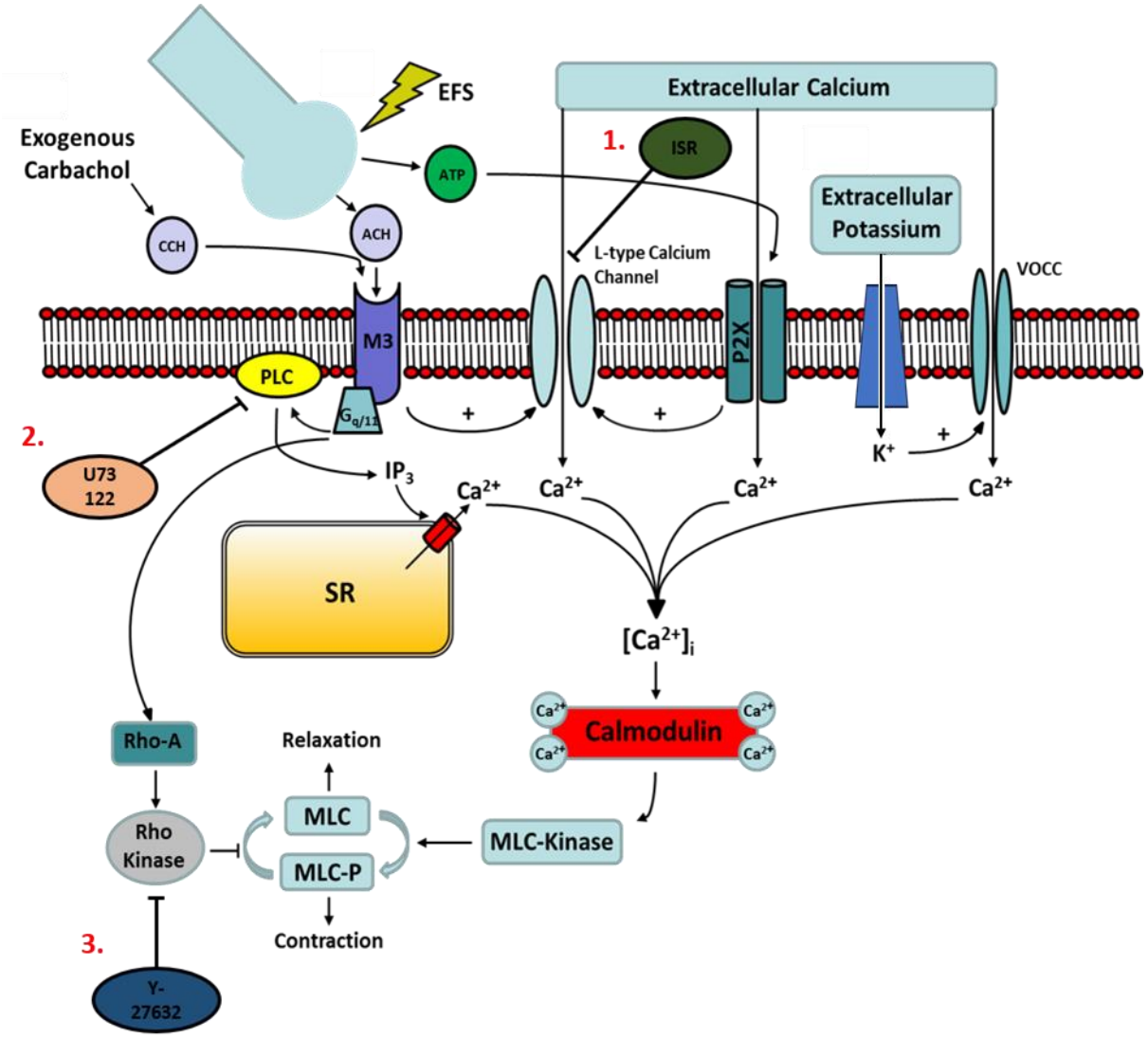

Figure 4.1. Schematic representation of the antagonists used here to manipulate the contractile signalling pathway of rat bladder strips *in vitro*. **1.** Isradipine (ISR) is an L-type calcium channel antagonists that inhibits influx of calcium via these channels. **2.** U73122 is an inhibitor of phospholipase C (PLC) and inhibits the production of inositol triphosphate (IP_3), thus inhibiting IP_3 mediated calcium release from the sarcoplasmic reticulum (SR) leading to a decrease in free cytosolic calcium. **3.** Y-27632 inhibits Rho kinase from preventing the dephosphorylation of myosin light chain (MLC), leading to muscle relaxation.

Overall, the aim of this chapter is to investigate the mechanism(s) behind inhibition of bladder contractility following acute ketamine exposure, and to determine the functional effects of longer-term ketamine exposure. The objectives are as follows:

- To assess whether ketamine can inhibit carbachol-induced contractility that is resistant to isradipine, U73122, or Y27632, or a combination of these compounds.
- To assess the effects of nifedipine and ketamine on bladder contractile responses potentiated by Bay K8644.

- To assess the effects of Bay K8644 on bladder strip contractility inhibited by nifedipine and ketamine.
- To investigate whether denudation of rat bladder urothelium effects bladder strip contractility.
- To investigate the functional effects of 3mM ketamine after 72 hours exposure in rat bladder strips.
- To investigate the functional effects of isradipine after 72 hours exposure in rat bladder strips.

4.2 METHODS

4.2.1 Effect of Ketamine, Isradipine, U73122, and Y27632 on Carbachol-Induced Bladder Contractility

Rat urinary bladder strips were suspended in organ baths and attached to force transducers to allow measurement of isometric tension, and a control CCH concentration-response was carried out. Bladder strips were then incubated for 20 minutes with either isradipine (10μM), U73122 (1μM), Y27632 (1μM), or a combination of these compounds, before reassessing the contractile response to CCH (see section 2.5.1). Tissues were then incubated for 30 minutes with ketamine (1mM) and either isradipine, U73122, Y27632, or a combination of these compounds, and a CCH concentration-response was again carried out (see section 2.5.2 & 2.5.3).

4.2.2 Effects of Ketamine, Nifedipine, and Bay K8644 on Rat Bladder Contractility Evoked by Electrical Field Stimulation

Matched bladder strips were attached to electrodes and suspended in organ baths as previously described (see section 2.5.5), and a frequency response (1, 2, 5, 10, 20, and 50Hz) was carried out using a voltage of 50V and a pulse width of 0.1ms (Chen et al., 2020).

Bladder contractile responses to electrical field stimulation (EFS) were potentiated with Bay K8644 (200nM), and then the effect of ketamine (0.03-1mM) or nifedipine (1-100μM) on these potentiated EFS frequency responses were tested. Conversely, EFS-induced contractile

responses of bladder strips were attenuated by ketamine (300μM) or nifedipine (10μM), and the effects of Bay K8644 (0.01-1μM) on these responses was examined (see section 2.5.4).

4.2.3 Effect of Urothelial Denudation on Rat Bladder Strip Contractility

Half bladder strips were suspended in organ baths and contracted with either high potassium Krebs solution, increasing concentrations of CCH, or EFS. One tissue strip was then mechanically denuded using a cotton bud, and the contractility of both tissues was then reassessed (see section 2.5.5).

4.2.4 Effect of Three-Day Ketamine or Isradipine Exposure on Rat Bladder Strip Contractility

Whole rat bladders were dissected longitudinally into two equal halves and contracted with a high potassium Krebs-Henseleit buffer or with increasing concentrations of CCH. One half bladder strip was then incubated for three days in tissue culture media containing either ketamine (3mM), isradipine (1μM), or ketamine (3mM) and Bay K8644 (200nM). The second strip was incubated for three days in tissue culture media and an appropriate vehicle control. Both tissues were then resuspended in organ baths and their contractility was assessed with high potassium or CCH (see section 2.5.6 & 2.5.7).

4.3 RESULTS

4.3.1 Isradipine, U73122, and Y-27632 Decrease Rat Bladder Strip Contractility to Carbachol

Rat bladder strips incubated with 1μM isradipine displayed a reduced contractile response to CCH (≥1μM) compared to untreated control strips ($P<0.0001$; Figure 4.2A). Twenty-minute exposure to U73122 (10μM) also reduced the contractile force of bladder strips evoked by CCH concentrations of 1μM ($P<0.05$) and 3, 10, 30, 100, and 300μM ($P<0.0001$) compared to the control (Figure 4.2A). Similarly, Y-27632 decreased bladder strip contractility to CCH (≥1μM) compared to the control ($P<0.0001$; Figure 4.2A).

Bladder strips incubated with both isradipine (1μM) and U73122 (10μM) saw a decrease in contractility evoked by CCH concentrations of 1μM ($P<0.01$), 3, 100, and 300μM ($P<0.001$) and 10 and 30 μM ($P<0.0001$) compared to isradipine (1μM) alone (Figure 4.2B). Likewise, strips simultaneously exposed to isradipine (1μM) and Y-27632 (10μM) displayed reduced

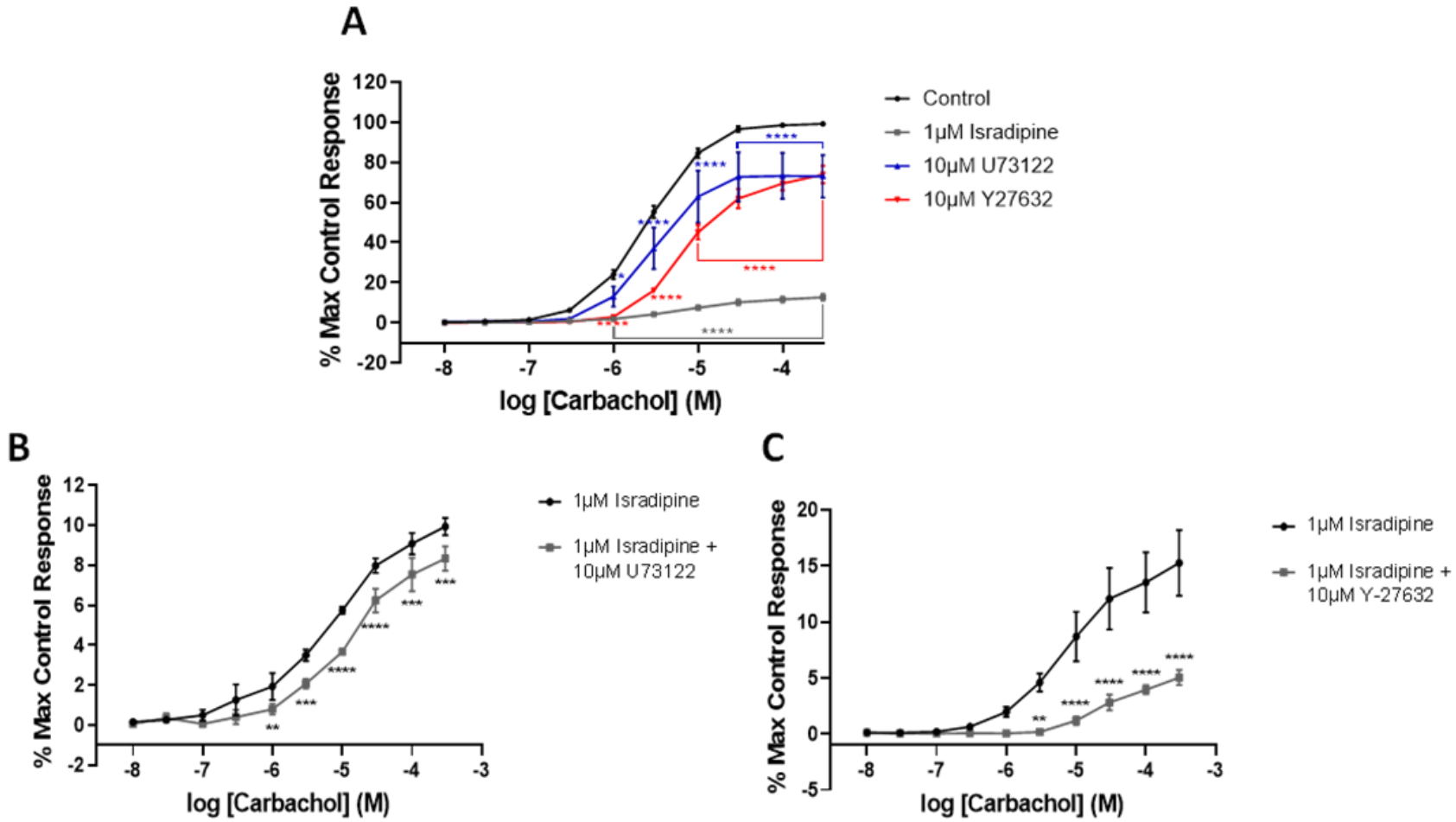

Figure 4.2. Effects of various drugs on the carbachol concentration-response curve in rat urinary bladder. **A:** Effect of isradipine, U73122, and Y27632 vs untreated strips. Each data point represents *n*=10 control; *n*=6 isradipine; *n*=3 U73122; *n*=4 Y-27632. **B:** Isradipine vs isradipine & U73122 (*n*=3). **C:** Isradipine vs isradipine & Y-27632 (*n*=3). Data is expressed as a mean percentage of the maximum control response ± SEM. Data analysed using two-way ANOVA and a Dunnett **(A)**, or Bonferroni **(B,C)** post-test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 significantly different from the untreated control strip **(A)** or the 1µM isradipine treated strip **(B, C)** at the respective carbachol concentration.

contractility to CCH concentrations of 3μM ($P<0.01$) and 10, 30, 100, and 300μM ($P<0.0001$) compared to strips incubated with isradipine (1μM) alone (Figure 4.2C).

4.3.2 Ketamine Inhibits Isradipine, U73122, and Y-27632-Resistant Rat Bladder Strip Contractility

Twenty-minute exposure to isradipine (1μM) and ketamine (1mM) together inhibited CCH-induced contractility to a greater degree than isradipine (1μM) or ketamine (1mM) exposure alone (Figure 4.3A). Decreased contractility by simultaneous incubation of ketamine and isradipine was observed at CCH concentrations of 3μM ($P<0.01$) and 10, 30, 100, and 300μM ($P<0.001$) when compared to isradipine alone, and CCH concentrations of 100 and 300μM ($P<0.0001$) when compared to ketamine alone.

Bladder strips incubated with both U73122 (10μM) and ketamine (1mM) displayed a reduction in CCH-induced contractility compared to U73122 or ketamine alone (Figure 4.3B). Simultaneous exposure to U73122 and ketamine decreased bladder strip contractility at CCH concentrations of 3μM ($P<0.01$), and 10, 30, 100, and 300μM ($P<0.0001$) when compared to U73122 alone, and CCH concentrations of 100μM ($P<0.01$) and 300μM ($P<0.001$) when compared to ketamine alone. Additionally, ketamine decreased rat bladder contractility to a higher degree than U73122 at CCH concentrations ≥3μM ($P<0.0001$).

Ketamine was also able to inhibit isradipine and U73122-resistant contractility in rat bladder strips (Figure 4.3C). Strips incubated with isradipine (1μM), U73122 (10μM), and ketamine (1mM) produced significantly reduced contractile responses to CCH concentrations of 3μM ($P<0.001$), and 10, 30, 100, and 300μM ($P<0.0001$) when compared to the effects of isradipine (1μM) and U73122 (10μM).

Similarly, ketamine inhibited the isradipine and Y-27632-resistant portion of the CCH contractile response in bladder strips (Figure 4.3D). Strips exposed to isradipine (1μM), Y-27632 (10μM), and ketamine (1mM) displayed a decreased contractile response at CCH concentrations of 10μM ($P<0.01$), and 30, 100, and 300μM ($P<0.0001$), when compared to the effects of isradipine (1μM) and Y-27632 (10μM).

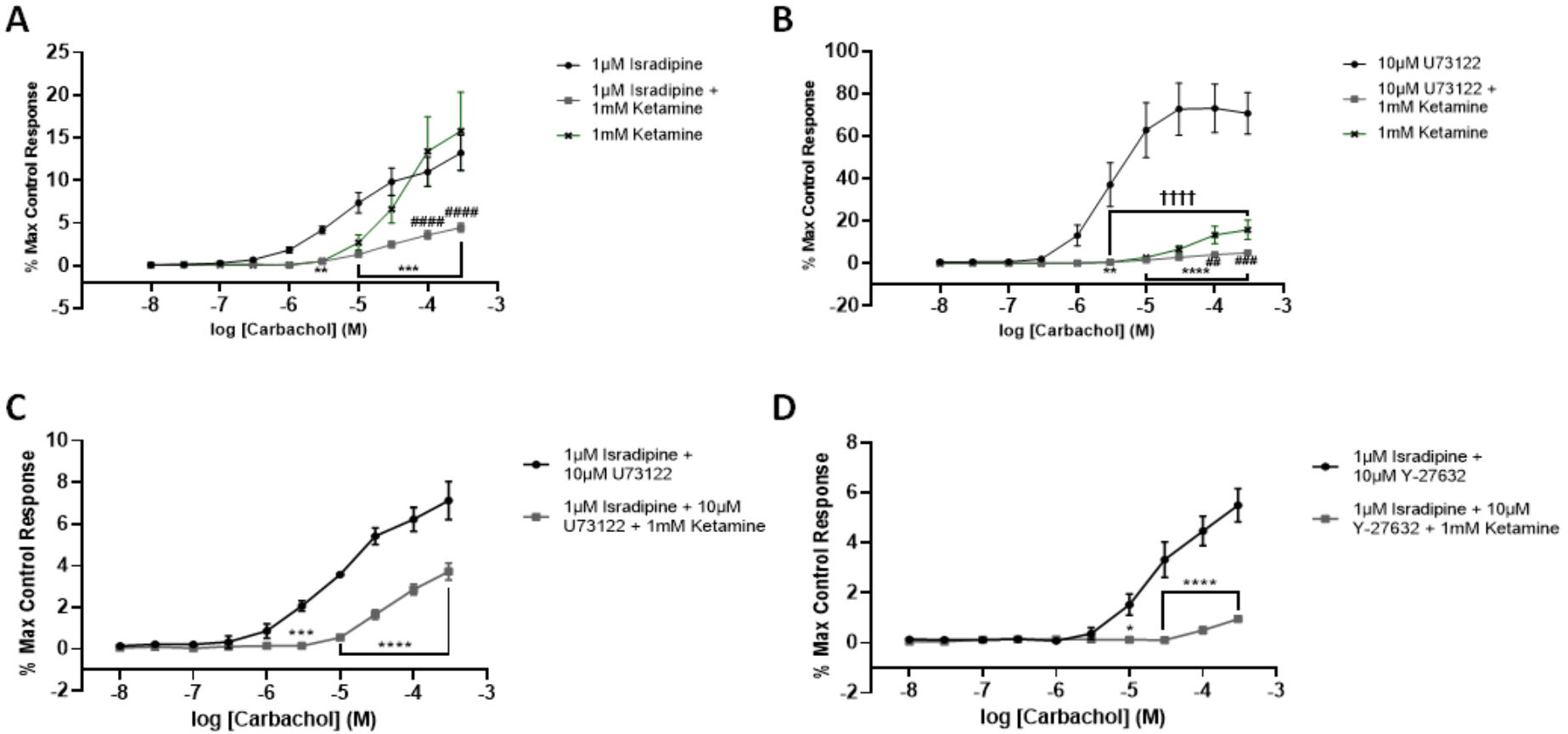

Figure 4.3. Effect of 1mM ketamine on isradipine, U73122, and Y-27632-resistant carbachol-induced contractions in rat urinary bladder strips. **A:** Effect of 1mM ketamine on carbachol contractility resistant to 1µM isradipine. **B:** Effect of 1mM ketamine on carbachol contractility resistant to U73122. **C:** Effect of 1mM ketamine on carbachol contractility resistant to 1µM isradipine & 10µM U73122. **D:** Effect of 1mM ketamine on carbachol contractility resistant to 1µM isradipine and 10µM Y-27632. Data are expressed as mean percentage change from the maximum control response ± SEM. Each data point represents at least *n*=3. Data analysed using a two-way ANOVA and a Dunnett **(A,B)**, or Bonferroni **(C.D)** post-test. **(A)****P<0.01, ***P<0.001 isradipine & ketamine treated significantly different from isradipine treated, ####P<0.0001 isradipine & ketamine treated significantly different from ketamine treated. **(B)****P<0.01, ****P<0.0001 U73122 & ketamine treated significantly different from U73122 treated, ##P<0.01, ###P<0.001 U73122 & ketamine treated significantly different from ketamine treated, ††††P<0.0001 ketamine treated significantly different from U73122 treated. **(C)*****P<0.001, ****P<0.0001 isradipine & U73122 & ketamine treated significantly different from isradipine & U73122 treated. **(D)** **P<0.01, ****P<0.0001 isradipine & Y-27632 & ketamine treated significantly different from isradipine & Y-27632 treated.

4.3.3 Ketamine Inhibits Bay K8644-Potentiated Rat Bladder Strip Contractility

Bay K8644 concentration-dependently and significantly enhanced rat bladder strip contractility induced by EFS (Figure 4.4A). A vehicle control using the same concentration of DMSO as present in the respective Bay K8644 concentration was carried out in parallel in matching half bladder strips (Figure 4.5A), and DMSO was shown to affect bladder strip contractility evoked by EFS. Bay K8644 (10nM) enhanced bladder contractility at 5Hz only ($P<0.01$), whereas 50nM Bay K8644 potentiated responses elicited by 1-5Hz, and 200nM potentiated EFS responses from 1-10Hz (at least $P<0.05$).

Bay K8644- (200nM) potentiated bladder contractile responses were then tested in the presence of ketamine. Ketamine inhibited Bay K8644-potentiated rat bladder strip contractility at concentrations ≥300µM (Figure 4.4B). A control strip was run in parallel with Bay K8644. The contractility of bladder strips was unaffected by repeated administration of Bay K8644 over the time course of the experiment (Figure 4.5B).

Similarly, nifedipine (≥1µM) was also able to concentration-dependently inhibit Bay K8644-(200nM) potentiated bladder contractility to EFS at all frequencies tested (Figure 4.4C; at least $P<0.01$). Repeated administration of Bay K8644 and DMSO over the time course of the experiment did not affect the contractility of the vehicle control-treated bladder strips (Figure 4.5C).

4.3.4 Bay K8644 Reverses Inhibition of Rat Bladder Strip Contractility by Ketamine

Prior inhibition of rat bladder strip contractility by ketamine (300µM) was concentration-dependently reversed by Bay K8644 (Figure 4.4D). A matched control strip was run in parallel and incubated with ketamine (300µM) and the respective concentration of DMSO for each Bay K8644 concentration tested, and it was shown that this treatment did affect the contractility of bladder strips to EFS (Figure 4.5D). Concentrations of Bay K8644 ≥50nM significantly increased the EFS-induced contractile response in rat bladder strips compared to the matched control strip in the presence (Figure 4.4D) and absence of ketamine (300µM) (Figure 4.4A), with the exception of Bay K8644 (10nM) which significantly increased EFS-induced contractile responses in rat bladder strips at 5Hz only in the absence of ketamine (300µM).

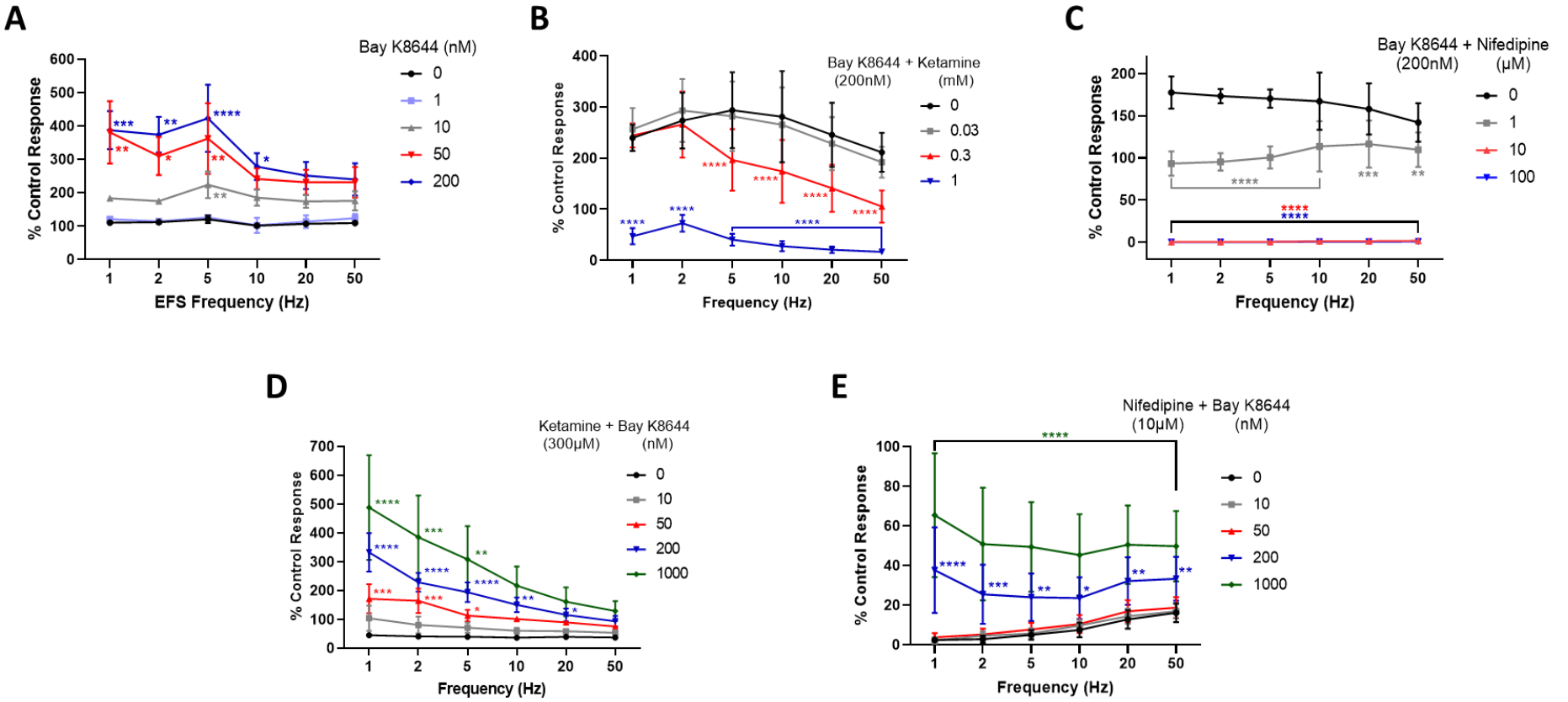

Figure 4.4. Effects of various calcium channel agonists/antagonists on rat bladder strip contractility induced by EFS after 20-minute incubations. **A.** Effect of various concentrations of Bay K8644 on the frequency response contraction curve in rat bladder strips. **B.** Effect of increasing concentrations of ketamine on EFS induced rat bladder contractility potentiated by Bay K8644. **C.** Effect of increasing concentrations of nifedipine on EFS induced rat bladder contractility potentiated by Bay K8644. **D.** Effect of increasing concentrations of Bay K8644 on EFS induced rat bladder contractility attenuated by ketamine. **D.** Effect of increasing concentrations of Bay K8644 on EFS induced rat bladder contractility attenuated by nifedipine. Data is expressed at the mean percentage of the control untreated EFS response at the respective frequency ± SEM. Each data point represents at least *n*=4. Data analysed using a two-way ANOVA and a Bonferroni post-test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 significantly different from the DMSO control response **(A & D)**, Bay K8644 treated response **(B & C)**, or nifedipine treated response **(E)** at the respective frequency.

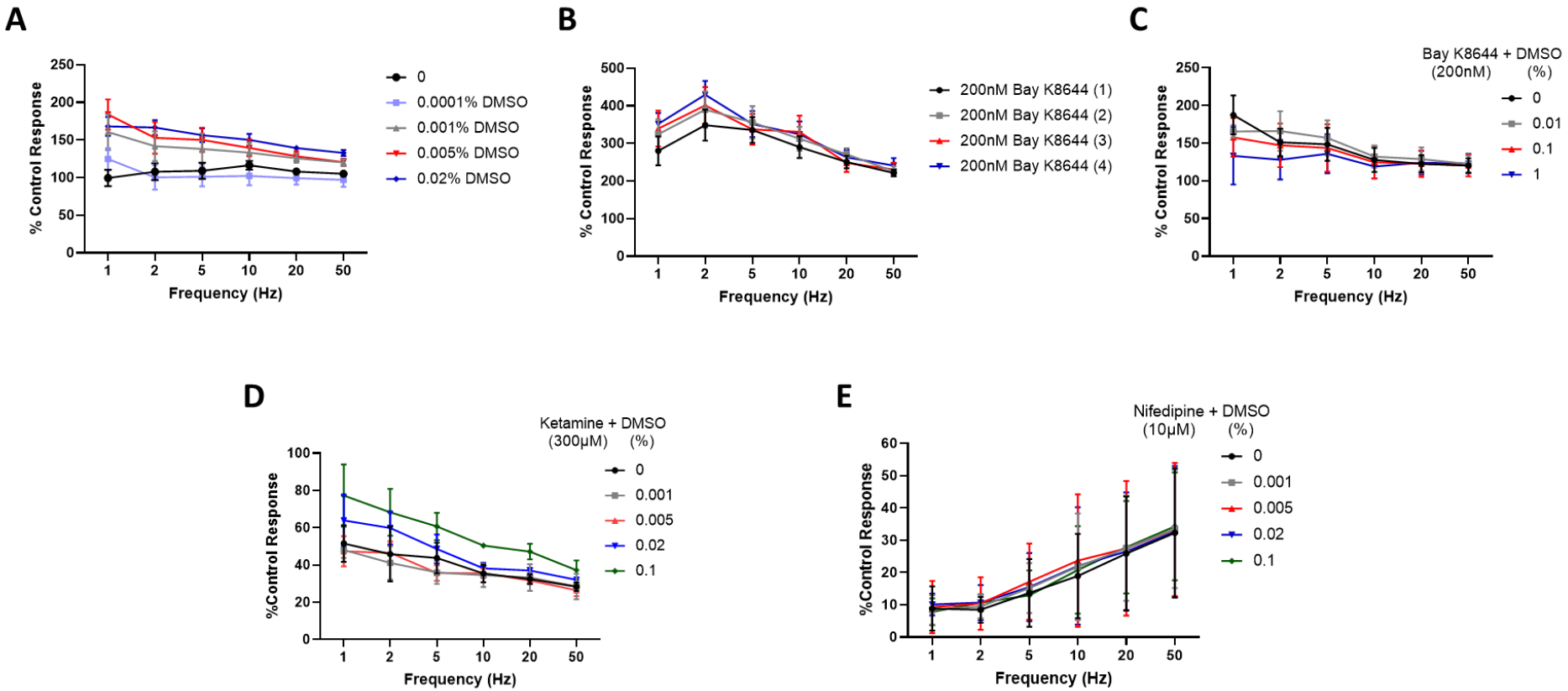

Figure 4.5. Effect of DMSO or repeated applications of Bay K8644, ketamine, or nifedipine on rat bladder strip contractility induced by EFS after 20-minute incubations. **A.** Effect of various concentrations of DMSO on the frequency response curve in rat urinary bladder strips. **B.** Effect of repeated treatment of rat bladder strips with Bay K8644 at 25-minute intervals. **C.** Effect of various concentrations of DMSO on rat bladder strip contractility to EFS in the presence of Bay K8644. **D.** Effect of various concentrations of DMSO on rat bladder contractility to EFS in the presence of ketamine **E.** Effect of various concentrations of DMSO on rat bladder strip contractility to EFS in the presence of nifedipine. Data is expressed as the mean percentage of the control untreated EFS response at the respective frequency ± SEM. Each data point represents at least *n*=3. Data analysed using a two-way ANOVA. **B, C, & E:** Treatment was not shown to significantly affect contractility (P>0.05; B, C, & E). **A & D:** Treatment was shown to significantly affect contractility (P<0.05).

Bladder strip contractility inhibited by nifedipine (10μM) was enhanced by Bay K8644 only at concentrations ≥200nM (Figure 4.4E). A matched vehicle control-treated tissue strip was also run in parallel with nifedipine (10μM) and equivalent concentrations of DMSO for each respective Bay K8644 concentration tested (Figure 4.5E). These treatments were shown to have no effect on rat bladder strip contractility evoked by EFS.

4.3.5 Urothelial Denudation Does Not Affect Rat Bladder Strip Contractility

Half rat bladder strips were contracted with CCH and then the urothelium was mechanically removed. The absence of the urothelium was noted via histological evaluation (Figure 4.6). Mechanically denuded tissue strips did not display any change in their CCH-evoked contractility compared to intact strip responses (Figure 4.7A; $P>0.05$), and the calculated EC50 values for intact (4.52 ± 0.82μM) and denuded (11.2 ± 4.3μM) strips were not significantly different ($P>0.05$). Mechanical denudation of the urothelium also had no effect on KCl-induced contractility when compared to intact strip responses (Figure 4.7B; $P>0.05$). Similarly, urothelium-denuded bladder strips stimulated via EFS produced similar contractile responses to intact bladder strips at all tested frequencies (Figure 4.7C).

4.3.6 Three-Day Ketamine Exposure Increases Rat Bladder Strip Contractility to Carbachol, But Not to High Potassium

Both 3-day control-incubated rat bladder strips and 3-day ketamine- (3mM) incubated strips displayed a reduction in contractility evoked by CCH when compared to their pre-incubation responses (Figure 4.8A&B). The CCH-induced contractile response of 3-day ketamine-treated strips, however, was significantly greater than the response of 3-day incubated control tissues (Figure 4.8C). Additionally, strips exposed to ketamine (3mM) for 20-minutes displayed a marked reduction in contractility induced by CCH compared to strips incubated with ketamine (3mM) for 3-days (Figure 4.8D).

To determine whether culturing bladder strips with ketamine for 3 days metabolised the drug or altered its potency, ketamine solution incubated for 3 days was tested on fresh bladder tissue. Bladder strips exposed to the 3-day-incubated ketamine solution for 20 minutes displayed a similar contractile response to CCH compared to bladder tissue exposed to freshly prepared ketamine (Figure 4.9).

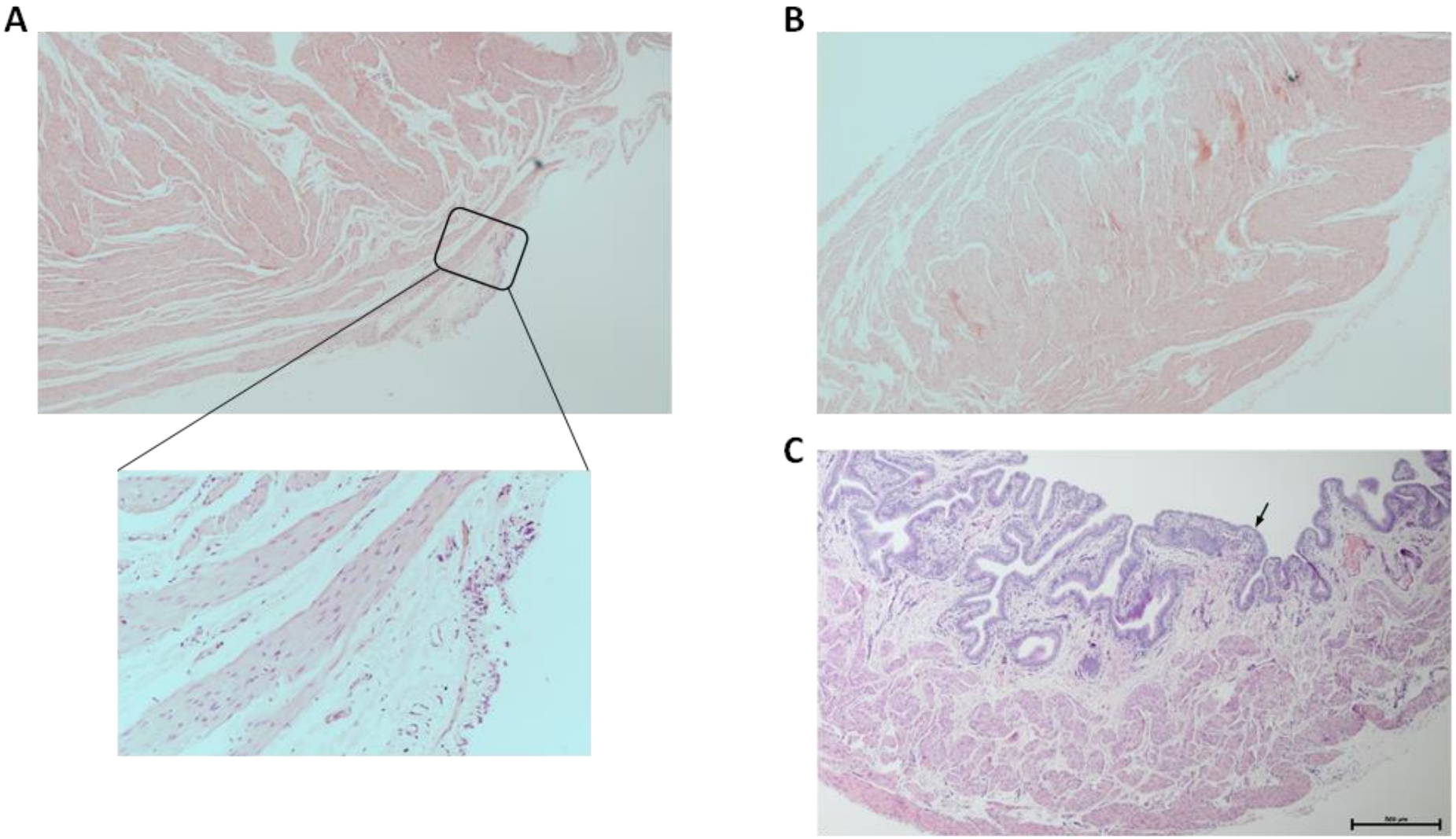

Figure 4.6. Effect of urothelial denudation on the histological composition of rat urinary bladder. Both denuded (**A, B**) and intact (**C**) strips were embedded in paraffin wax, sectioned, and stained with haematoxylin and eosin. Mechanically denuded strips showed a marked loss of urothelium (**A**) or a complete absence of urothelium (**B**) compared to control tissues (**C**). Arrow indicates the location of the urothelium **(C)**. Magnification x40 (**A-C**), and x200 (zoomed in **A**).

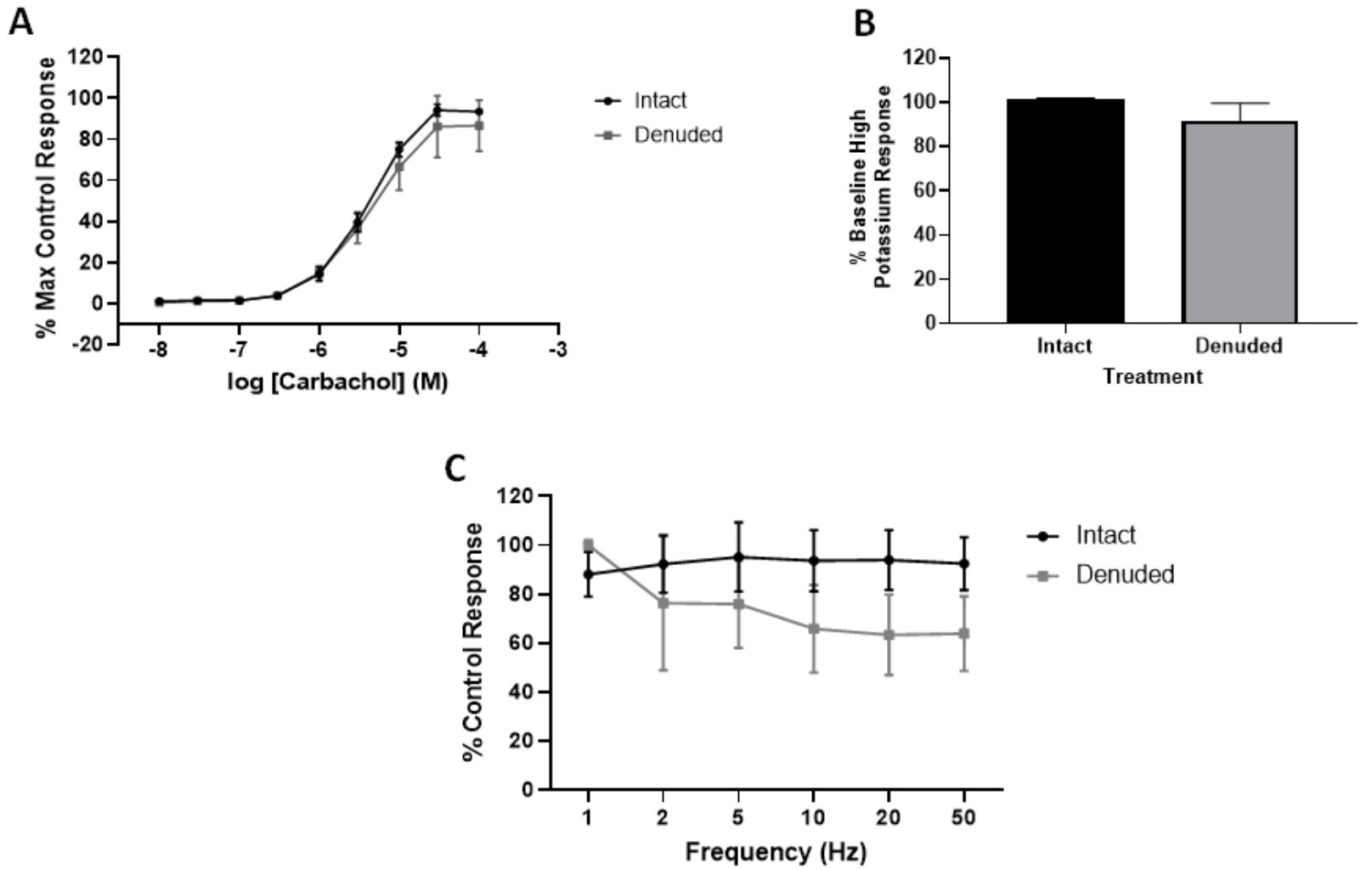

Figure 4.7. Effect of mechanical denudation of the bladder urothelium on rat bladder contractility. **A.** Effect of denudation on carbachol induced contractility. **B.** Effect of denudation on contractility evoked by high potassium. **C.** Effect of denudation on contractility evoked by electrical field stimulation. Data is expressed as a mean percentage of the: max control response (**A**); the initial baseline response (**B**); the control response at the respective frequency, ± SEM. Each data point represents *n=6* (**A**); *n=5* (**B**); and *n=3* (**C**). Data analysed using a two-way ANOVA and a Bonferroni post-test (**A, C**); or a paired t-test (**B**). Denudation was not shown to significantly affect contractility (P>0.05).

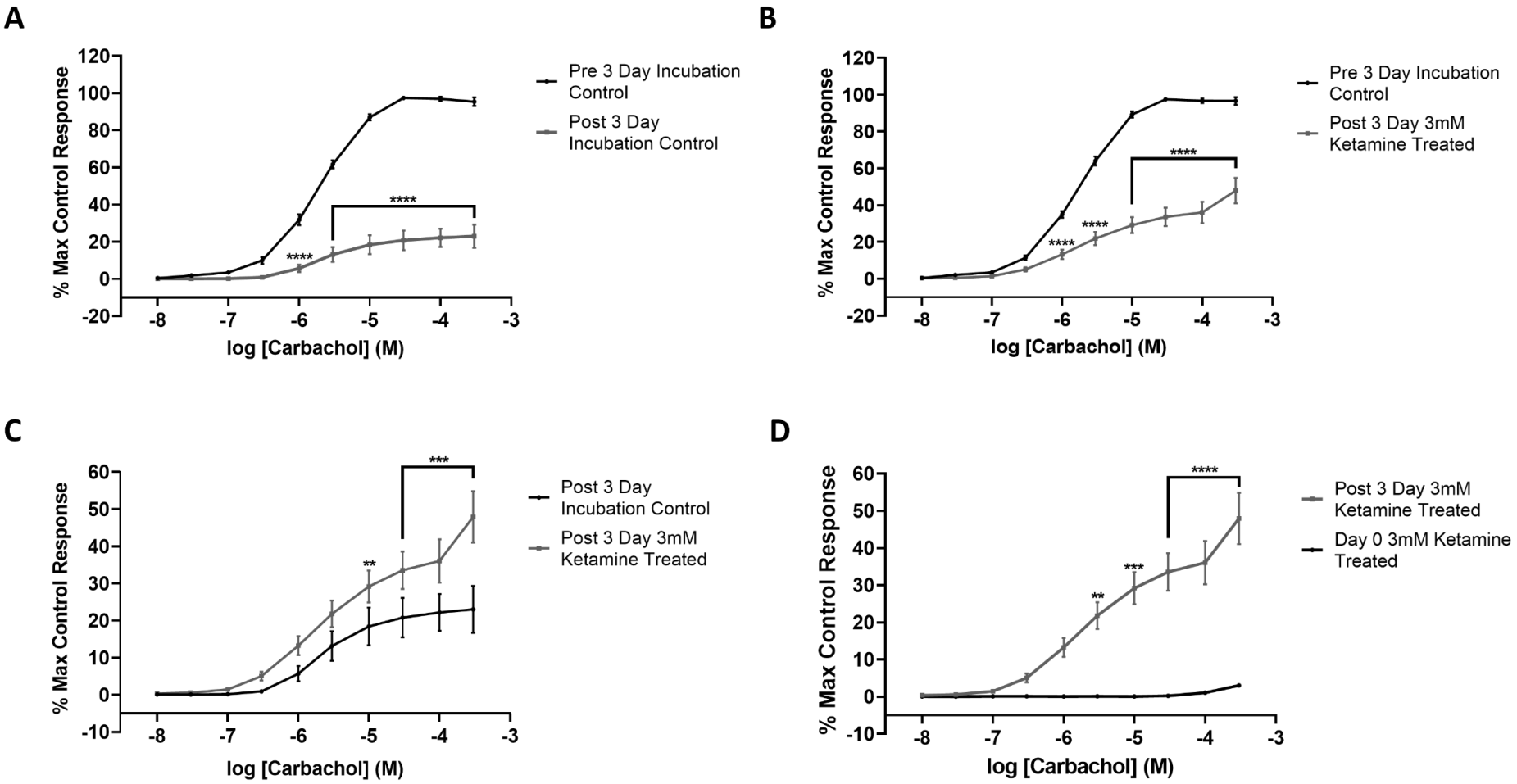

Figure 4.8. Effect of 3-day ketamine exposure on the carbachol concentration-response in rat urinary bladder strips. **A:** 3-day control incubation vs matched pre-incubation carbachol concentration-response. **B:** 3-day ketamine incubation vs matched pre-incubation carbachol concentration-response. **C:** 3-day control incubated vs 3-day ketamine incubated bladder strips. **D:** 3-day ketamine incubated bladder strips vs uncultured bladder strips exposed to 3mM ketamine for 20 minutes. Data expressed as a mean percentage of the maximum control response ± SEM. Each data point represents *n=14.* Data analysed using a two-way ANOVA and a Bonferroni post-test. **P<0.01, ***P<0.001, ****P<0.0001 significantly different from the: matched pre-incubation control at the respective carbachol concentration (**A & B**); the 3-day incubation control strip at the respective carbachol concentration (**C**); acute 3mM ketamine treated strip at the respective carbachol concentration.

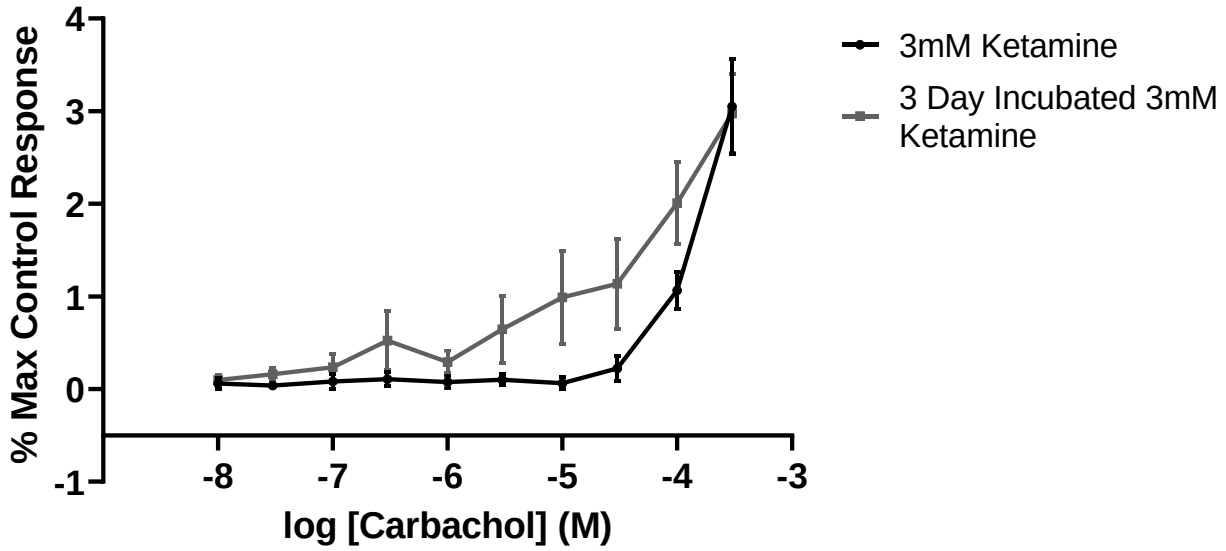

Figure 4.9. Effect of freshly prepared ketamine vs 3-day incubated ketamine on the carbachol concentration-response in rat urinary bladder following 20 minutes exposure. Data expressed as a mean percentage of the maximum control response ± SEM. Each data point represents *n=4.* Data analysed using a two-way ANOVA and a Bonferroni post-test. No significant difference was observed between the two treatments (P>0.05).

Bladder strips exposed to ketamine for 3-days displayed a similar contractile response to 3-day control incubated strips when stimulated with a high potassium Krebs-Henseleit solution (Figure 4.10).

The pre- and post-incubation weights of the bladder tissues were recorded to see if ketamine-treated strips had any significant change in their mass. Both control and ketamine-treated tissues displayed a similar degree of weight loss compared to the pre-incubation tissue weights (Figure 4.11).

4.3.7 Three-Day Isradipine Exposure Does Not Affect Rat Bladder Strip Contractility

Both 3-day vehicle control-treated rat bladder strips and 3-day isradipine-incubated strips had a reduced contractile response to CCH compared to their pre-incubation responses (Figure 4.12A&B). Bladder strips exposed to isradipine (1µM) for 3 days had a contractile response to CCH that was similar to 3-day vehicle control treated strips (Figure 4.12C).

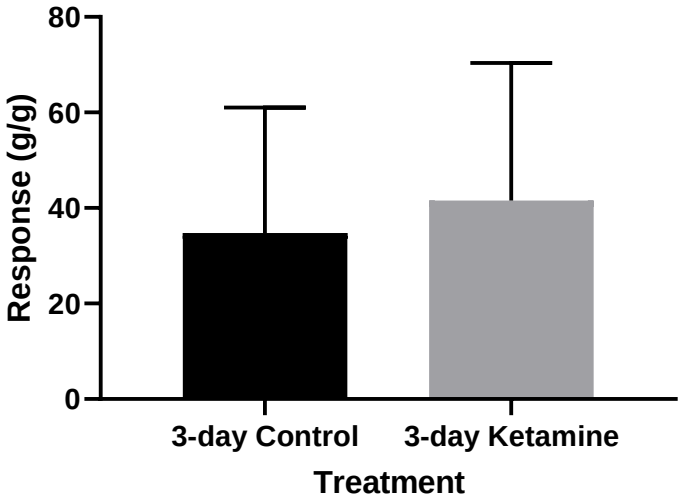

Figure 4.10. Effect of 3-day control vs 3-day ketamine incubations on rat bladder strip contractility evoked by high potassium. Data expressed as grams of tension per gram of tissue weight ± SEM. Each data point represents *n=7*. Data analysed using a paired two-tailed t-test. No significant difference was observed between the two treatments (P>0.05).

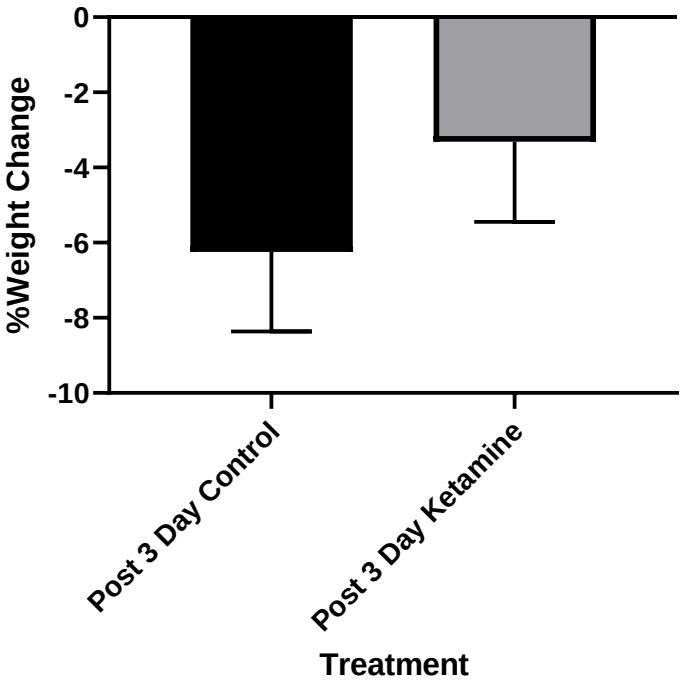

Figure 4.11. Effect of 3-day control vs 3-day ketamine cultured rat bladder strips on tissue strip weight. Data is expressed as a mean percentage change from the respective pre-incubation tissue weight ± SEM. Each data point represents *n=7.* Data analysed using a paired two-tailed t-test. No significant difference was observed between the two treatments.

4.3.8 Enhancement of Rat Bladder Contractility by Three-Day Ketamine Exposure is Not Affected by Bay K8644

Rat bladder strips incubated for 3 days with either ketamine (3mM) or both ketamine (3mM) and Bay K8644 (200nM) displayed a reduction in contractility to CCH when compared to the pre-incubation response (Figure 4.13A&B). There was no difference observed in the CCH-evoked contractile response of 3-day ketamine treated strips and 3-day ketamine and Bay K8644 treated strips (Figure 4.13C; $P>0.05$).

4.3.9 Acute Exposure to Ketamine Inhibits Contractility of Three-Day Control and Ketamine Incubated Rat Bladder Strips

To assess whether the acute inhibitory effects of ketamine on rat bladder contractility were retained in incubated strips, 3-day incubated bladder strips were exposed to ketamine for 20 minutes. Exposure to 1mM ketamine for 20 minutes reduced the CCH-induced contractile response of both 3-day control- and 3-day ketamine- (3mM) incubated bladder strips (Figure 4.14A&B, respectively).

4.3.10 Spontaneous Activity of Three-Day Ketamine Incubated Bladder Strips

Pronounced spontaneous contractile activity was noted in some three-day ketamine incubated bladder strips and occurred prior to the addition of CCH to the organ bath (Figure 4.15). Such activity was not observed in any three-day control-incubated tissues.

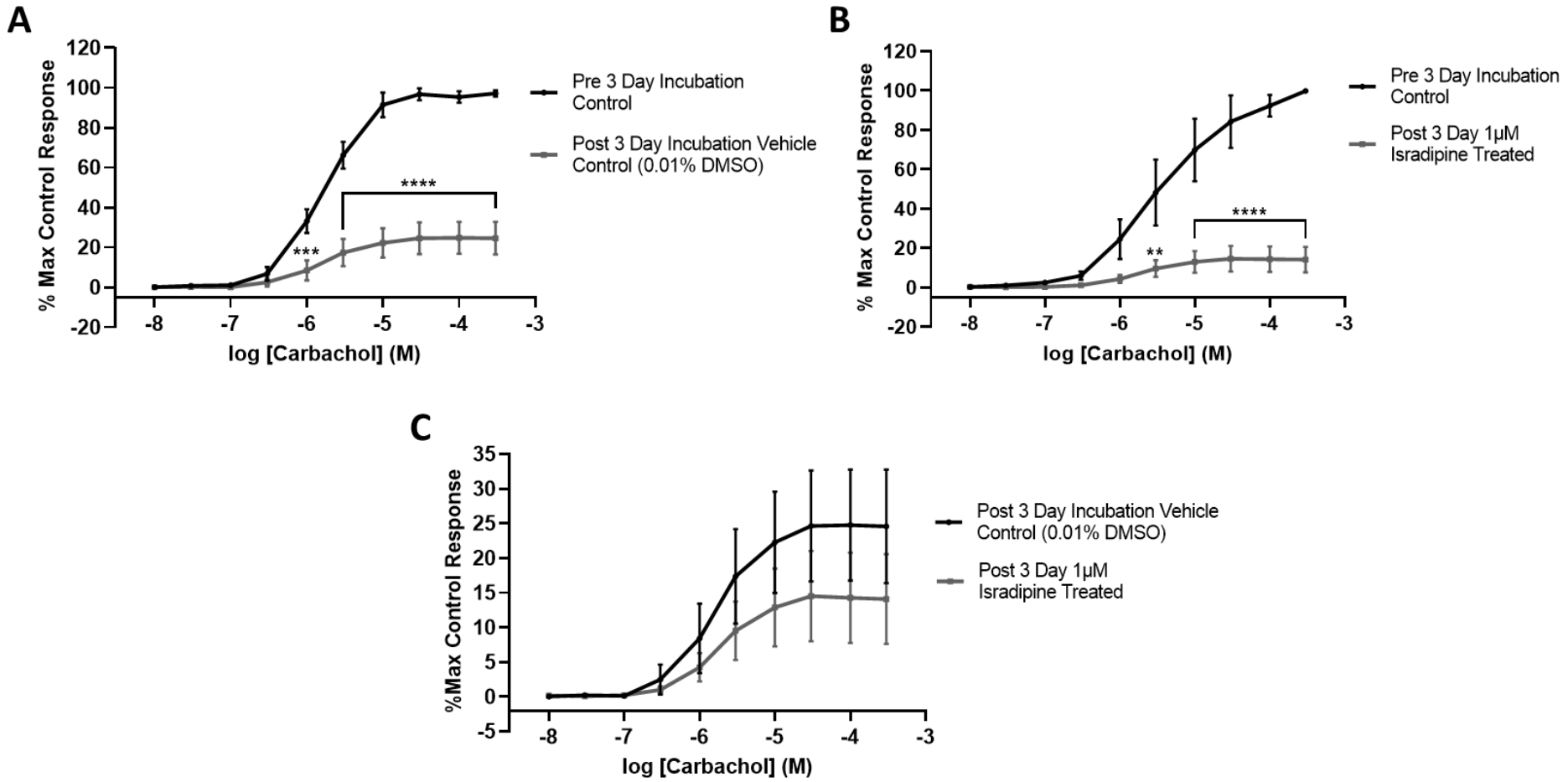

Figure 4.12. Effect of 3-day isradipine (1µM) exposure on the carbachol concentration-response in rat urinary bladder strips. **A:** 3-day DMSO (0.01%) vehicle control incubation vs matched pre-incubation carbachol concentration-response. **B:** 3-day 1µM isradipine incubation vs matched pre-incubation carbachol concentration-response. **C:** 3-day 1µM isradipine incubated vs 3-day DMSO (0.01%) incubated bladder strips. Data expressed as a mean percentage of the maximum control response ± SEM. Each data point represents *n=3.* Data analysed using a two-way ANOVA and a Bonferroni post-test. **P<0.01, ***P<0.001, ****P<0.0001 significantly different from the matched pre-incubation control at the respective carbachol concentration (**A & B**). No significant difference was observed between strips following 3-days exposure to DMSO or isradipine (P>0.05; **C**).

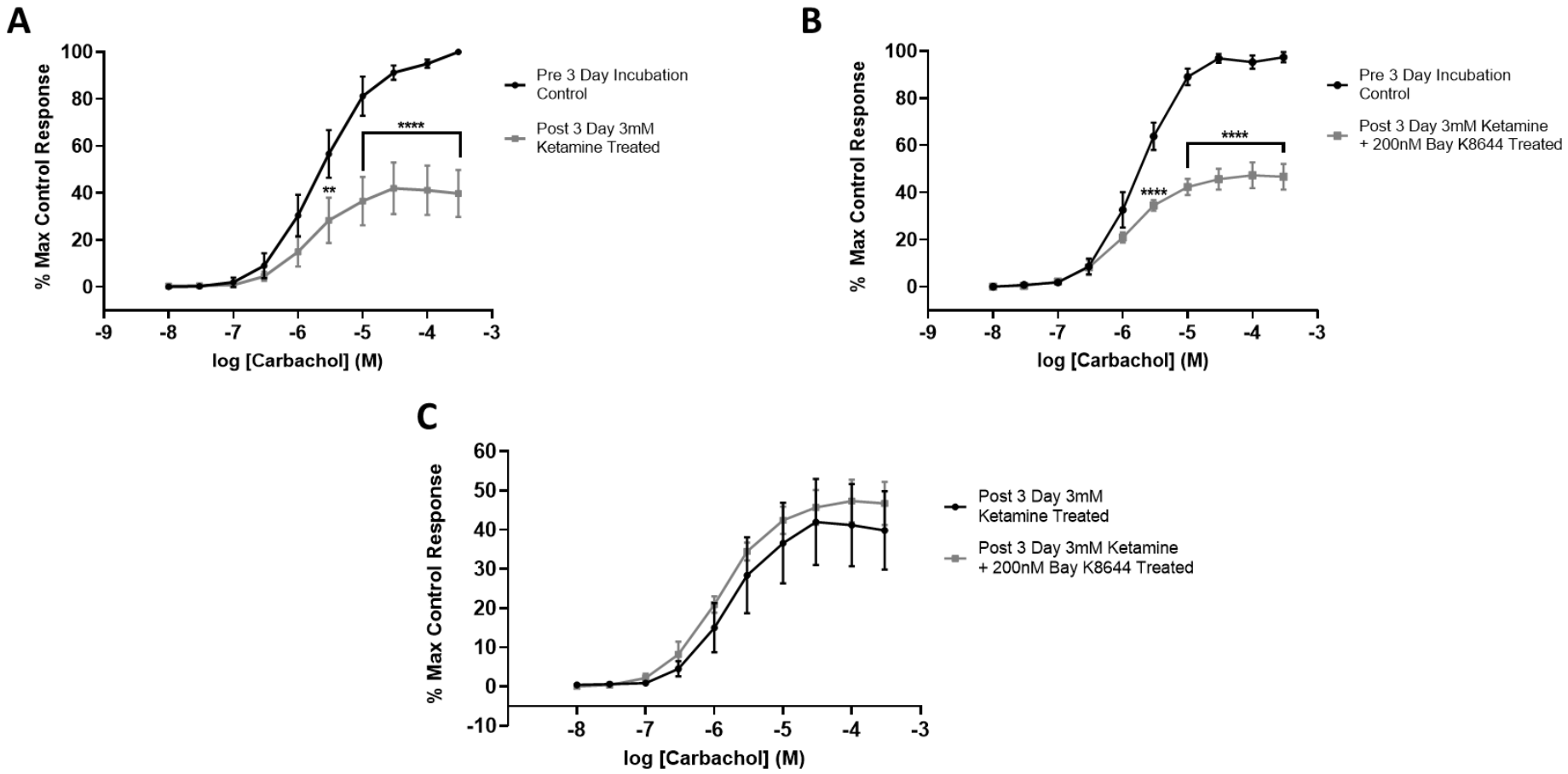

Figure 4.13. Effect of 3-day exposure to ketamine and Bay K8644 on rat bladder strip contractility. **A:** 3-day ketamine (3mM) incubated vs matched pre-incubation carbachol concentration-response. **B:** 3-day ketamine (3mM) and Bay K8644 (200nM) incubated vs pre-incubation carbachol concentration-response. **C:** Carbachol evoked contractility of 3-day ketamine incubated (3mM) bladder strips vs 3-day ketamine (3mM) and Bay K8644 (200nm) incubated bladder strips. Data expressed as a mean percentage of the maximum control response ± SEM. Each data point represents *n=3.* Data analysed using a two-way ANOVA and a Bonferroni post-test. **P<0.01, ****P<0.0001 significantly different from the matched pre-incubation control at the respective carbachol concentration (**A & B**). No significant difference was observed between strips following 3-days exposure to ketamine and ketamine + Bay K8644 (P>0.05; **C**).

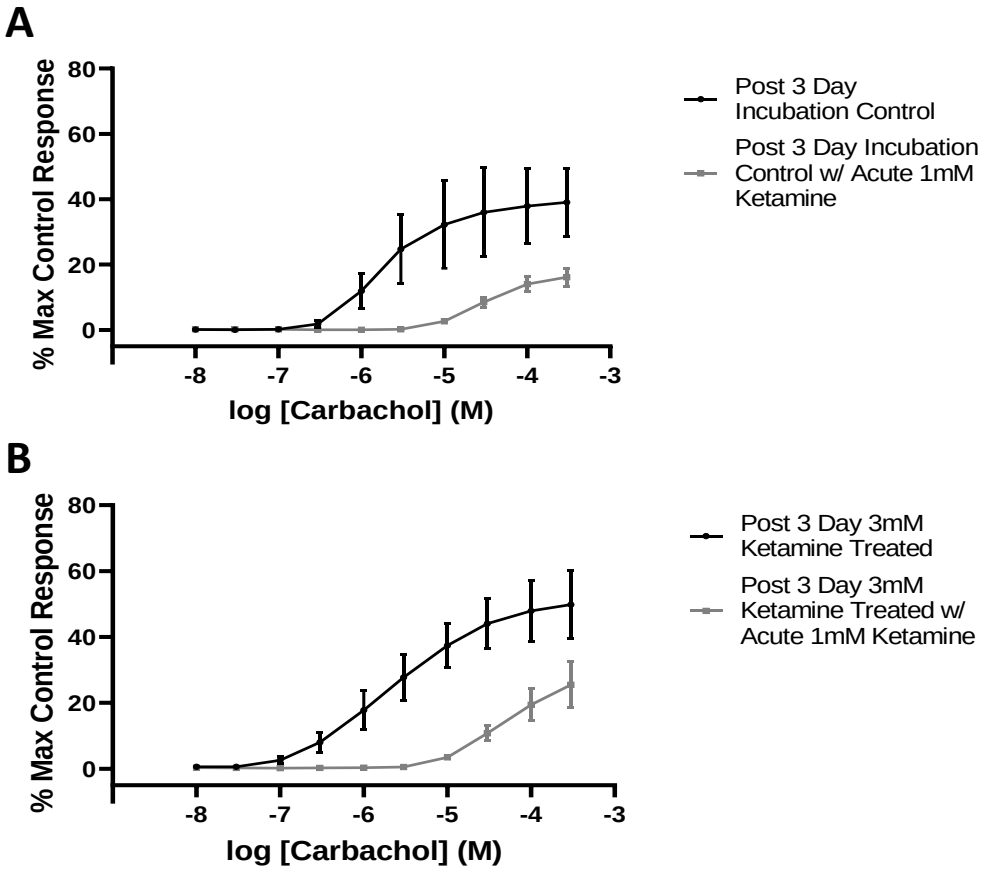

Figure 4.14. Effect of acute (20 minutes) ketamine (1mM) exposure on 3-day incubated rat bladder strip contractility. **A:** Effect of acute ketamine exposure on the contractile response of 3-day control incubated bladder strips. **B:** Effect of acute ketamine exposure on the contractile response of 3-day 3mM ketamine incubated bladder strips. Data expressed as a mean percentage of the respective maximum pre-incubation response ± SEM**.** Each data point represents *n=4.* Analysis via a two-way ANOVA revealed a significant treatment (ketamine) factor effect **(A&B)**.

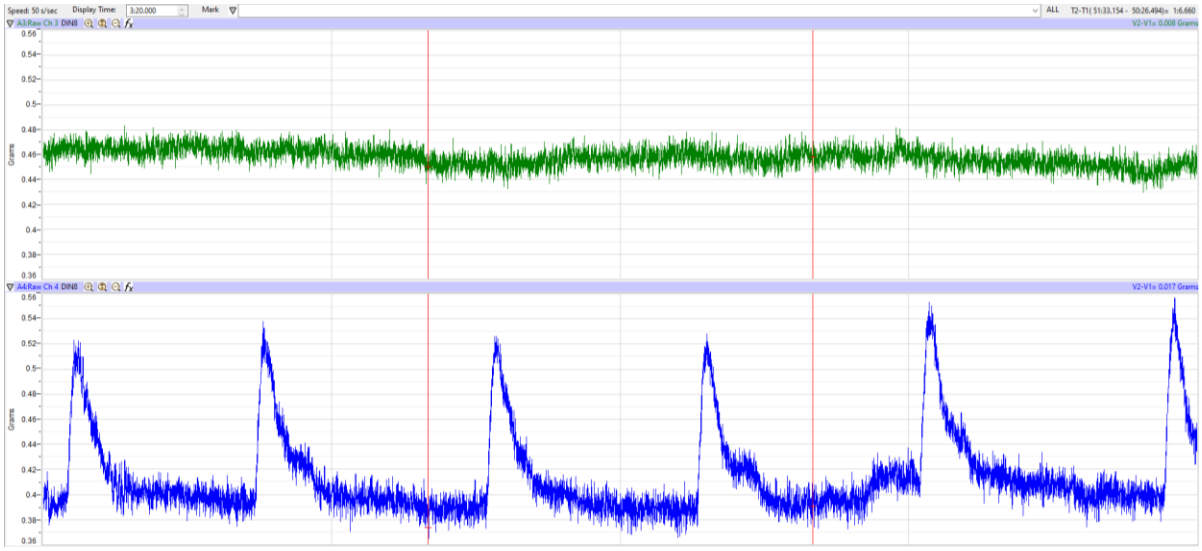

Figure 4.15. Representative trace of spontaneous contractile activity seen in bladder strips following exposure to ketamine for 3 days. Top trace (red) represents spontaneous activity of a 3-day vehicle control treated rat bladder strip. Bottom trace (blue) represents spontaneous activity of a 3-day 3mM ketamine treated rat bladder strip. Y-axis displays grams of tension. X-axis represents time (200 seconds shown).

4.4 DISCUSSION

4.4.1 Effect of Isradipine, U73122, and Y-27632 on Carbachol-Induced Rat Bladder Contractility

4.4.1.1 Isradipine

Isradipine (1μM) significantly inhibited the contractile response of rat bladder strips to CCH (≥1μM; Figure 4.2A). The ability of isradipine to attenuate detrusor contractility has been previously reported in rat (Morelli et al., 2007) and mouse bladder (Wegener et al., 2004), and this effect is attributed to $Ca_v1.2$ L-type calcium channel blockade by isradipine (Wegener et al., 2004; Figure 4.1). This is supported by the observation that detrusor contractility induced by CCH or high potassium was 10-fold lower in mice deficient in the smooth muscle $Ca_v1.2$ calcium channel (SMACKO) compared to corresponding control mice (Wegener et al., 2004). Moreover, isradipine inhibited mouse bladder contractility evoked by high potassium or CCH in control mice but had no effect on detrusor muscle from SMACKO mice (Wegener et al., 2004).

4.4.1.2 U73122

U73122 (10μM) significantly inhibited the contractile response of rat bladder strips to CCH (≥1μM; Figure 4.2A). U73122 is an inhibitor of PLC, which prevents IP_3-mediated release of calcium from the sarcoplasmic reticulum (SR; Figure 4.1). In contrast to this finding, Schneider, Hein, and Michel (2004) reported that, although U73122 (10 μM) reversed CCH-induced inositol phosphate formation, it did not significantly affect CCH-induced contractility in rat bladder strips (1-10μM). Despite not being statistically significant, however, U73122 (3-10μM) treated bladder strips produced a maximum response that was approximately 75% of the control contractile response (Schneider et al., 2004), which is similar to the effect reported here (73.03 ±10.63%).

4.4.1.3 Y-27632

Y-27632 (10μM) significantly inhibited the contractile response of rat bladder strips to CCH (≥1μM; Figure 4.2A). Y-27632 is an inhibitor of ROCK (Figure 4.1) and so prevents ROCK from inhibiting the dephosphorylation of MLC, leading to relaxation. The ability of Y-27632 to attenuate CCH-evoked contractions in rat bladder strips was reported by Wibberley, Chen,

Hu, Hieble and Westfall (2003). They observed a decrease in rat bladder contractility evoked by the G-protein coupled receptor agonists CCH and neurokinin A following pre-treatment with Y-27632 (10µM). Potassium chloride-evoked contractility, however, was unaffected, suggesting that ROCK mediated Ca^{2+}-sensitisation is dependent on G-protein activation.

Simultaneous incubation of isradipine and U73122, or isradipine and Y-27632, produced greater inhibition of rat bladder strip contractility to CCH than isradipine alone (Figure 4.2B&C), presumably as each compound affects a different mechanism of the contractile pathway (Figure 4.1), resulting in additive effects.

4.4.2 Effect of Ketamine on Isradipine, U73122, and Y-27632-Resistant Rat Bladder Contractility

4.4.2.1 Isradipine

Ketamine (1mM) significantly attenuated the isradipine- (1µM) resistant portion of the CCH response in rat bladder strips (Figure 4.3A). Further, strips exposed to both ketamine and isradipine displayed a depressed contractile response compared to either drug incubated alone (Figure 4.3A), suggesting a possible additive effect. These results do not necessarily suggest, however, that ketamine and isradipine are acting through a different mechanism. Initial experiments to determine the concentration of isradipine required for maximum inhibition in this system were not carried out, and so it is possible that the concentration of isradipine used here is not sufficient for maximum inhibition. Therefore, ketamine's ability to inhibit the isradipine-resistant portion of the CCH response could be a result of further enhancement of L-type calcium channel blockade. Ketamine (1mM) and isradipine (1µM) were shown to inhibit CCH-induced rat bladder strip contractility to a similar degree (Figure 4.3A); however, this does not necessarily suggest that they are working through the same mechanism.

4.4.2.2 U73122

Ketamine (1mM) significantly attenuated the portion of the CCH concentration-response resistant to U73122 (10µM), and likewise, U73122 was able to further inhibit ketamine-resistant CCH contractility (Figure 4.3B). Ketamine also inhibited the remainder of the CCH response following inhibition by simultaneous incubation with isradipine and U73122 (Figure

4.3C). This suggests that ketamine does not act through blockade of PLC-mediated intracellular calcium release, or at least that this is not the entire mechanism of ketamine's action. This is supported by the observation that U73122, despite being a specific blocker of PLC (Smith et al., 1990), is only responsible for ~25% inhibition of CCH-mediated contractility (Figure 4.2A).

4.4.2.3 Y-27632

Ketamine (1mM) decreased CCH contractility resistant to isradipine and Y-27632 (Figure 4.3D). This suggests that ketamine does also not act through, or at least does not entirely act through, ROCK inhibition.

Ketamine was previously shown to inhibit rat bladder contractility evoked by high potassium (Chapter 3), whereas Y-27632 is reported to have no effect on these contractions in rat bladder (Wibberley et al., 2003). Contractions induced by KCl bypass G-protein coupled M_3 receptor activation of ROCK (Figure 4.1), which is likely why Y-27632 does not affect bladder contractility to high potassium and suggests that ketamine is not an inhibitor of ROCK. Further evidence of this comes from the observation that ketamine inhibited KCl contractility more potently than CCH-induced contractility (Chapter 3). Potassium-induced contractions in bladder have been shown to be dependent on extracellular Ca^{2+} (Wibberley et al., 2003), whereas bethanechol contractility was reduced, but not abolished, in the absence of extracellular Ca^{2+} (Jezior et al., 2001), and small CCH-induced contractile responses that were largely dependent on ROCK activity were possible in mice devoid of L-type calcium channel activity (Wegner et al., 2004). Thus, as CCH contractility is less sensitive than KCl contractility to ketamine, ketamine may be acting as an L-type calcium channel antagonist, with the ketamine-resistant portion of the CCH response being largely dependent on ROCK activation.

4.4.3 Effect of Ketamine, Nifedipine, and Bay K8644 on Rat Bladder Strip Contractility Evoked by Electrical Field Stimulation

4.4.3.1 Bay K8644

Bay K8644 (≥10nM) significantly potentiated the contractile response of rat bladder strips to EFS (Figure 4.4A). The ability of Bay K8644 to potentiate bladder contractility has also been reported in mouse bladder (Chen et al., 2020).

4.4.3.2 Effect of Ketamine and Nifedipine on Bay K8644 Potentiated Contractility

Rat bladder contractility potentiated by Bay K8644 (200nM) was inhibited by both ketamine (≥300μM) and nifedipine (≥1μM; Figure 4.4B&C). Ketamine and nifedipine have recently been reported to inhibit Bay K8644-enhanced bladder contractility in mouse bladder strips (Chen et al., 2020), demonstrating the reproducibility and validity of the data presented here.

Nifedipine is a known $Ca_V1.2$ antagonist, and so its ability to inhibit contractility potentiated by Bay K8644 is presumably mediated via blockade of L-type calcium channels. The fact that ketamine was also able to reverse Bay K8644 potentiated contractility suggests L-type calcium channel antagonism.

4.4.3.3 Effect of Bay K8644 on Rat Bladder Contractility Inhibited by Ketamine and Nifedipine

Ketamine (300μM) and nifedipine (10μM) inhibited EFS-induced contractility in rat bladder strips which was reversed by Bay K8644 (≥50nM and ≥200nM, respectively; Figure 4.4D&E). Bay K8644 has also been reported to restore mouse bladder contractility that had been attenuated by ketamine or nifedipine (Chen et al., 2020).

Taken together, the results (Figure 4.4B&D) indicate mutual antagonism of ketamine and Bay K8644 and suggests that inhibition of rat bladder contractility by ketamine occurs via L-type calcium channel blockade.

4.4.4 Effect of Mechanical Urothelial Denudation on Rat Bladder Contractility

Mechanical removal of the bladder urothelium did not have any effect on rat bladder contractility evoked by CCH (Figure 4.7A). Previous studies, however, have shown that urothelium-free bladder preparations display an increased contractility in response to CCH in pigs (Hawthorn, Chapple, Cock, & Chess-Williams, 2000), humans (Chaiyaprasithi, Mang, Kilbinger & Hohenfellner, 2003) and rats (Koşan et al., 2005). This apparent ability of the urothelium to depress bladder contractility does not appear to be due to the constitutive release of inhibitory agents, as removal of the urothelium did not alter the contractile response of the bladder to KCl in rats (Figure 4.7B; Koşan et al., 2005), humans (Chaiyaprasithi et al., 2003), or pigs (Hawthorn et al., 2003). Moreover, it is unlikely that removal of the urothelium is significantly aiding the ability of CCH to diffuse into the underlying muscle. The

evidence for this is twofold: firstly, reports detailing the inhibitory nature of the urothelium typically use bladder strips and so multiple layers of the bladder are exposed to any agents added to the organ bath; secondly, the presence of a second bladder strip with an intact urothelium can reverse the increased contractile response to CCH in denuded strips (Hawthorn et al, 2000; Koşan et al., 2005). Therefore, the presence of a diffusible inhibitory factor that is derived from the urothelium seems plausible. As urothelial denudation only seems to increase bladder contractility to CCH and in some instances EFS (Chaiyaprasithi et al., 2003; Koşan et al., 2005), but not to other contractile mediators such as KCl (Hawthorn et al., 2000; Chaiyaprasithi et al., 2003; Koşan et al., 2005) and neurokinin A (Chaiyaprasithi et al., 2003), it seems likely that the release of the purported inhibitory factor from the urothelium is dependent on muscarinic receptor activation.

Another consideration is that urothelial denudation reduces inhibitory regulation of the bladder detrusor mediated by a subpopulation of interstitial cells. In the detrusor and suburothelial layers of the bladder, the majority of interstitial cells express platelet derived growth factor receptor α (PDGFRα; Koh, Lee, Ward & Sanders, 2018). In response to an increase in intracellular calcium, PDGFRα$^+$ interstitial cells generate outward current and become hyperpolarised via activation of SK3 potassium channels, and this inhibitory influence is thought to be passed onto smooth muscle cells via electrical coupling (Koh et al., 2018). However, PDGFRα$^+$ interstitial cells were absent in mouse urothelium (Koh et al., 2012), and their presence in the urothelium of other species has not been reported. Moreover, as the urothelium does not connect to smooth muscle cells directly, loss of PDGFRα$^+$ interstitial cells in the urothelium appears unlikely to be the mechanism of increased bladder contractility following urothelial denudation. A potential loss of suburothelial PDGFRα$^+$ interstitial cells following denudation could possibly lead to increased detrusor activity, but loss of these cells would not be resolved by the introduction of a second intact bladder strip and therefore cannot explain subsequent restoration of normal bladder strip contractility as reported in the literature.

The finding here that removal of the urothelium does not affect the contractile response of rat bladder to KCl (Figure 4.7B) is in agreement with previous reports. There was also no change in contractility observed when denuded rat bladder strips were stimulated via EFS (Figure 4.7C). In human bladder, contractions to EFS at 10Hz and 30Hz were significantly

increased in denuded bladder strips when compared to intact strips (Chaiyaprasithi et al., 2003), which is consistent with the idea that urothelial inhibitory factor release is mediated by muscarinic receptor activation, as acetylcholine release is reportedly greater at higher EFS frequencies (Koşan et al., 2005). In rat bladder, it was reported that removal of the urothelium increased contractile responses to EFS; however, these results were not statistically significant (Koşan et al., 2005).

While most reports observe an increase in CCH-induced bladder contractility following urothelial denudation, one study saw that denudation caused a decreased response in rat bladder strips to CCH, EFS, and ATP (Munoz, Gantitano, Smith, Boone & Somogyi, 2010). One possible explanation for these conflicting results is the method used to remove the urothelium. Munoz et al. (2010) used a cotton bud and a gentle sweeping motion on the urothelial surface, which was reportedly confirmed through histological assessment to remove the urothelium whilst leaving the suburothelium intact. Other reports which demonstrated increased bladder contractility to CCH following denudation either used sharp surgical dissection to remove the urothelium, or did not specify the method of removal. It is therefore possible that surgical removal removes both the urothelial and suburothelial layers, and that it is the suburothelium that is the source of the diffusible inhibitory factor, while the urothelium actually exerts an excitatory effect on the underlying muscle (Munoz et al., 2010).

Regardless of the different techniques employed in the literature to remove the urothelium, many reports share a lack of clarity and detail when describing the protocol used. This makes it hard to determine whether similar denudation protocols have been used amongst reports and what tissue layers these techniques are likely to remove, especially when histological confirmation is typically absent. As such, it makes these reports difficult to replicate and could explain the observed discrepancies in bladder contractility. Here, histological examination shows that the denuded bladder strips used are almost completely void of the entire mucosal layer (Figure 4.6). It therefore seems likely that the denudation protocol used was too harsh, and reducing the number of longitudinal swabs or the amount of pressure used may help to preserve the suburothelial layers.

4.4.5 Effect of Longer-Term Ketamine Exposure on Rat Bladder Strip Contractility

The acute effects of ketamine on rat bladder strip contractility were explored in Chapter 3, however, the development of KC is associated with long-term ketamine use. Therefore, a novel assay to investigate the effects of longer-term ketamine exposure on rat bladder tissue function was developed.

4.4.5.1 Increased Bladder Contractility Following 3-day Ketamine Exposure

Both ketamine-treated and vehicle control-treated bladder strips showed a markedly reduced contractile response to CCH after being incubated for three days (E_{max}: 47.9 ± 6.9 and 23.0 ± 6.3, respectively; Figure 4.8A&B). Interestingly, however, 3-day ketamine-treated strips displayed an increase in CCH-evoked contractility when compared to the 3-day incubated control strips (Figure 4.8C), and the increase in bladder contractility observed appears to be consistent with the effects of long-term ketamine use in humans. This is a novel finding and is in stark contrast to the effects of acute ketamine exposure, whereby 20-minute incubations inhibit bladder contractility induced by CCH (Chapter 3).

How and if the increase in bladder contractility seen here relates to the lower urinary tract (LUT) symptoms and bladder overactivity often associated with long-term ketamine use in humans is not clear. Bladder overactivity is defined as the presence of urinary urgency and is typically accompanied by frequent urination and nocturia (Peters, Schmidt & Michel, 2007). The aetiology underlying the overactive bladder, however, is not completely understood.

Disruption of the storage function of the bladder may lead to LUT symptoms such as urgency and frequency (Andersson & Arner, 2004). In several species, including humans, phasic increases in detrusor pressure can be observed during the storage phase (Drake et al., 2017), and these contractions, which are unrelated to intentional voiding, are believed to underly the biological basis of urgency (Peters et al., 2007). It is therefore possible that the increased detrusor contractility seen here after 3-day ketamine exposure contributes to bladder overactivity by modulating these normal non-voiding contractions, and enhancement of non-voiding contractions could theoretically result in increased bladder sensation, leading to urinary urgency (Biallosterski, van Koeveringe, van Kerrebroeck, Gillespie & de Wachter, 2011).

The mechanism(s) behind the increased bladder contractility following 3-day ketamine exposure are unclear. The ability of ketamine to increase bladder contractility after longer-term exposure, although seemingly consistent with the clinical model of KC, is somewhat surprising when considering that, at the same concentration (3mM), ketamine drastically attenuated the contractile response of bladder strips to CCH after 20 minutes (Figure 4.8D), presumably through L-type calcium channel blockade.

One mechanism proposed in the pathogenesis of KC is dysfunction of the urothelial barrier (Xie et al., 2021), and indeed, denudation of the urothelium is the most common pathological finding of KC (Jhang et al., 2015). Ketamine has been shown to induce apoptosis in human urothelial cells, leading to a widespread loss of urothelium after 3-day exposure (Baker et al., 2016). It seems unlikely here that the functional changes observed are a result of changes in the permeability or barrier function of the urothelium, as the use of bladder strips would likely allow ketamine and the contractile mediators used access to multiple tissue layers regardless of urothelial permeability. As discussed previously, mechanical denudation of the urothelium in bladder strips of different species has generally been shown to increase contractility to CCH (Chaiyaprasithi et al., 2003; Hawthorn et al., 2003; Koşan et al., 2005), and this is thought to be due to the presence of a diffusible inhibitory factor that is released from the urothelium (or suburothelium) following muscarinic receptor activation. This would also potentially explain why the KCl contractile response was unaffected by 3-day ketamine exposure (Figure 4.10). The histological effects of 3-day ketamine exposure on rat bladder, however, have not been reported in the literature, and are explored in Chapter 5 and 6. In Chapter 5, it is reported that three-day exposure to ketamine (3mM) resulted in a significant loss of urothelial cells, whereas urothelial cell count was unaffected in strips incubated with ketamine (3mM) for 20 minutes. Therefore, a loss of urothelium after longer-term (3-day), but not acute (20 minute) ketamine exposure could explain the contrasting functional effects of ketamine exposure duration.

The possibility that the functional changes seen after 3-day ketamine exposure were due to ketamine being metabolised during the incubation period was considered by incubating ketamine for 3-days and testing its effect on bladder contractility. The 3-day incubated ketamine solution affected bladder contractility to the same degree as freshly prepared ketamine (Figure 4.9), and so this possibility seems unlikely.

Multiple animal models of KC have reported an increase in bladder weight in ketamine-treated animals (Liu et al., 2015; Lee et al., 2017), and so it was considered that the increase in contractility seen was due to a higher degree of tissue degeneration in the control tissue compared to ketamine-treated tissue. After 3-day incubations, both the control and ketamine-treated group displayed a similar relative change to their pre-incubation weights (Figure 4.11). Moreover, there is evidence to suggest that ketamine inhibits bladder smooth muscle cell proliferation and can even cause cell death at concentrations similar to those used here (≥1.82mM; Chen et al., 2020); and in Chapter 6 it was reported that SMMHC (a marker of differentiated smooth muscle cells) expression was similar in control and 3-day ketamine-(3mM) incubated rat bladder. Therefore, a higher degree of muscle retention or a decrease in tissue degeneration in the ketamine group does not explain the increase in contractility seen here.

4.4.5.2 Increased Bladder Contractility Following Acute Ketamine Exposure

As discussed previously, evidence reported both here and in various other studies suggest that acute ketamine exposure inhibits bladder contractility through antagonism of the $Ca_V1.2$ L-type calcium channel (Chen et al., 2020). Due to the critical role of $Ca_V1.2$ in bladder smooth muscle and the excellent inhibitory effects of calcium channel blockers (CCBs) such as nifedipine, it was the expectation of clinical urologists that CCBs would be an effective drug for the treatment of bladder dysfunction (Yu, 2022). However, an initial trial for the treatment of urgency and/or urge incontinence with nifedipine revealed that the drug did not affect bladder pressure or bladder capacity, and it was ultimately concluded that nifedipine lacked effectiveness to treat these symptoms (Forman, Andersson, Henriksson, Rud & Ulmsten, 1978). Since then, numerous CCBs have been investigated for their effectiveness in treating bladder overactivity, mostly without therapeutic success (Yu, 2022). In contrast to this, associations between CCB use and bladder dysfunction have emerged more recently in clinical studies. A study looking at Japanese male hypertensive patients, for example, reported an increase in frequency, urgency, and nocturia in patients taking CCBs compared to those taking other medications (Ito, Taga, Tsuchiyama, Akino & Yokoyama, 2013).

Interestingly, Chen et al. (2020) reported that intravesical infusion of ketamine or nifedipine significantly decreased peak bladder pressure while increasing voiding frequency in mice. This is in stark contrast to the long-held belief that inhibition of bladder contractility through

calcium channel blockade would lead to an increased bladder capacity and a reduction in voiding frequency (Yu, 2022). Moreover, instillation of Bay K8644 into the bladder lumen was able to correct these abnormalities, suggesting that $Ca_V1.2$ antagonism by ketamine is a major mediator of KC (Chen et al., 2020). Therefore, although contrary to current urologic dogma, the apparent ability of ketamine and nifedipine to increase micturition frequency is reflected clinically in some studies looking at the effects of CCB use.

One potential explanation for this counter-intuitive idea is proposed by Yu (2022) who suggests that inhibition of bladder smooth muscle by nifedipine reduces voiding efficiency which, in turn, increases residual volume. Indeed, an increase in residual volume was reported in patients taking nifedipine for treatment of overactive bladder (Forman et al., 1978). A higher volume of residual urine would then reduce the space available for urine generated by the kidneys, leading to an increased micturition frequency. These effects reported by Chen et al. (2020) were observed almost immediately after infusion of ketamine or nifedipine; however, there is no report of bladder dysfunction following a single dose of ketamine. Therefore, how this relates clinically to KC, which is associated with long-term ketamine use, is not known. It would be of interest to see if any urodynamic parameters indicative of overactive bladder could be observed in patients following a single dose of nifedipine or ketamine.

4.4.6 Effect of Three-Day Isradipine Exposure on Rat Bladder Strip Contractility

To determine whether $Ca_V1.2$ antagonism could explain the increase in bladder strip contractility observed following 3-day ketamine exposure, strips were incubated with isradipine for three days. Both vehicle control and isradipine-treated strips displayed a marked decrease in contractility to CCH following the 3-day incubation period compared to their respective pre-incubation controls (Figure 4.12A&B). Three-day isradipine-treated strips, however, displayed a similar contractile response to CCH when compared to 3-day vehicle control-treated strips (Figure 4.12C). These results suggest that the increase in bladder contractility following 3-day ketamine exposure is unlikely explained by $Ca_V1.2$ antagonism.

4.4.7 Effect of Three-Day Ketamine and Bay K8644 on Rat Bladder Strip Contractility

To further investigate whether $Ca_V1.2$ modulates rat bladder contractility following 3-day ketamine exposure, strips were incubated with either ketamine or ketamine and Bay K8644. Strips incubated with both ketamine and Bay K8644 had a similar contractile response to those incubated with ketamine alone following the 3-day incubation period (Figure 4.13C). This provides further evidence that the functional effects seen after 3-day ketamine incubations are not mediated through $Ca_V1.2$.

4.4.8 Effect of Ketamine on Rat Bladder Contractility Potentiated by Three-Day Ketamine Exposure

The observation that 3-day ketamine exposure can potentiate the contractile response of rat bladder strips to CCH is a surprising one, and directly opposes the acute (20 minute) effects of ketamine reported here. It is important to note that the "longer-term" effects of ketamine reported here are occurring in the absence of ketamine, as strips are removed from the tissue culture media containing ketamine and washed in Krebs-Henseleit solution prior to contracting with CCH, and as reported in Chapter 3, ketamine's effects are reversed after wash-out. As such, the effects of acute exposure to ketamine (1mM) on the potentiated contractile response of bladder strips following 3-day ketamine incubation was investigated.

Exposure to 1mM ketamine for 20-minutes decreased the contractile response of both 3-day ketamine-incubated and vehicle control-incubated rat bladder strips (Figure 4.14). This suggests that some degree of $Ca_V1.2$ functionality is retained in bladder strips following 3-day ketamine and 3-day control incubations, and that the reduction in CCH contractility of 3-day-incubated strips after acute ketamine exposure is due to $Ca_V1.2$ antagonism by ketamine. Further, we can conclude that 3-day ketamine exposure induces some type of change in the bladder tissue that persists, at least in the short term, that is able to increase CCH-induced contractility in the absence of ketamine.

4.4.9 Effect of Ketamine on Spontaneous Contractile Activity

Pronounced spontaneous contractile activity was observed in some 3-day ketamine-treated tissues, but not in control-incubated tissues (Figure 4.15). These contractions may therefore reflect changes seen in KC and could theoretically lead to enhancement of non-voiding

contractions and/or smooth muscle cell excitability, contributing to urinary urgency (Biallosterski et al., 2011). The source of this spontaneous activity, however, is not known.

As reported in Chapter 5, three-day exposure to ketamine leads to loss of mucosal cells, and this may contribute to the spontaneous activity seen here. The urothelium is thought to be the source of a diffusible inhibitory factor that is released following muscarinic receptor activation, thus modulating smooth muscle excitability (Chaiyaprasithi et al., 2003; Hawthorn et al., 2003; Koşan et al., 2005). Loss of urothelial cells by ketamine may therefore inhibit the release of this inhibitory factor, enhancing excitability of the detrusor and thereby leading to the generation of spontaneous contractility. Evidence for this possibility is provided by a study conducted by Meng, Young & Brading (2008), who looked at spontaneous activity of mouse bladder strips where the urothelium was either kept intact or removed. It was noted that the frequency of spontaneous action potentials in mouse detrusor smooth muscle cells were significantly increased in urothelium-denuded strips compared to intact tissues, providing evidence for the release of an inhibitory factor by the urothelium.

Another possibility is that ketamine exposure leads to loss of PDGFRα^{+} interstitial cells in the suburothelium. In response to increased intracellular calcium, these cells activate SK3 potassium channels which generates an outward current and hyperpolarisation, and this inhibitory influence is thought to suppress bladder contractility via direct electric coupling to smooth muscle cells (Koh et al., 2018). As such, loss of these cells would presumably reduce the inhibitory influence they have on bladder smooth muscle which may give rise to spontaneous contractile activity. Indeed, blocking SK3 channels or inhibiting pathways that enhance intracellular calcium levels in PDGFRα^{+} interstitial cells leads to a cystometric patten of bladder overactivity (Koh et al., 2018). The ability of ketamine to induce loss of PDGFRα^{+} interstitial cells in bladder tissue, however, has not been reported.

Increased contractile frequency was observed in mouse bladder after infusion of ketamine, and this effect is thought to be due to Cav1.2 antagonism (Chen et al., 2020). It is unlikely, however, that the effects seen here are mediated via the same mechanism, as the enhanced contractility reported here is observed in the absence of ketamine, and subsequent addition of ketamine to the organ bath inhibits bladder contraction. Nevertheless, to what extent spontaneous contractility generated in isolated bladder strips reflects those observed in the

intact bladder is not known. Further investigation into the origin of these spontaneous contractions would be of interest.

4.4.10 Future Work and Conclusions

The findings presented here suggest that acute exposure to ketamine decreases rat bladder strip contractility via L-type calcium channel blockade. This is in agreement with Chen et al. (2020), who concluded that ketamine also acts as an L-type calcium channel antagonist in mouse bladder to reduce smooth muscle contractility.

Also presented here is the novel finding that 3-day ketamine (3mM) exposure potentiates the contractile response of rat bladder strips to CCH. This observation appears to be more consistent with the long-term effects of ketamine in humans. However, the bladder dysfunction seen in KC is associated with long-term ketamine use and urinary symptoms typically take months to years to present (Mak et al., 2011). Therefore, how or if this finding relates to the clinical presentation of KC is not known. However, in the assay developed here, bladder strips are constantly exposed to high concentrations of ketamine (3mM). Though such concentrations of ketamine in the urine are theoretically possible (Baker et al., 2016), micturition would ensure that any build-up of ketamine (or its metabolites) in the urine would be periodically eliminated, limiting the contact time of ketamine to the urothelium. As such, the bladder dysfunction seen in KC could be due to multiple episodes of direct damage to the urothelium by ketamine, which over time, results in a disrupted urothelium. Indeed, ketamine (3mM) has been shown to have a direct toxic effect on human urothelial cells (Bureau et al., 2015; Baker et al., 2016), and, at lower concentrations (0.5mM), ketamine was reported to increase the doubling time of urothelial cells (Bureau et al., 2015). It is therefore possible that, in some instances, ketamine use exposes the urothelium of users to high concentrations of ketamine that are directly toxic to the urothelial cells, or to sub-toxic concentrations that can impede urothelial regeneration. As the bladder tissue used here is exposed continuously to toxic concentrations of ketamine, the functional effects seen after 3-day ketamine exposure may be the result of a sped-up version of this process that would not occur clinically.

The report that acute intravesical infusion of ketamine in mice can simultaneously decrease peak pressure and increase voiding frequency is interesting. In theory, blockade of $Ca_V1.2$ by ketamine reduces detrusor contractility, which effectively limits the efficiency of bladder

emptying during micturition. This would lead to an increase in residual volume and reduce the amount of space available for urine generated by the kidneys, increasing urinary frequency. Indeed, as reported in Chapter 3, 20-minute ketamine incubations decreased bladder contractility to a range of different stimuli, and this explanation would provide a link between the apparent opposing effects of detrusor contractile inhibition and bladder overactivity. However, both of these observations were reported following acute ketamine exposure, whereas KC is associated with long-term ketamine use, and so the link between the increased voiding frequency seen in animal studies following acute ketamine instillation and the bladder dysfunction observed in KC patients or animal models of KC is unclear.

It seems unlikely that these acute effects of ketamine are related to the 3-day ketamine effects reported here, as they appear to be mediated through different mechanisms. The acute voiding dysfunction reported in mice by Chen et al. (2020) are $Ca_V1.2$-dependent, as antagonism by ketamine or nifedipine induces urinary frequency which is reversed by the $Ca_V1.2$ agonist Bay K8644. In the 3-day ketamine experiments reported here, however, simultaneous incubation of Bay K8644 and ketamine produces a contractile response to CCH that is similar to bladder strips incubated with ketamine alone. Further, 3-day incubations of bladder strips with isradipine did not potentiate contractility, suggesting that the effects seen after 3-day ketamine exposure are not mediated by $Ca_V1.2$ antagonism.

As previously discussed, disruption of the urothelium has been proposed as one of the major mechanisms in the pathogenesis of KC (Xie et al., 2021). Moreover, several *in vitro* bladder studies in different species have reported the presence of a diffusible inhibitory factor that is likely derived from the urothelium or suburothelium (Hawthorn et al., 2000; Chaiyaprasithi et al., 2003; Koşan et al., 2005; Munoz et al., 2010). Therefore, histological evaluation of rat bladder strips will be investigated in the next chapter to assess whether 3-day ketamine exposure leads to loss of urothelial cells, and if so, this could provide an explanation for the increase in contractility following longer-term ketamine exposure seen here.

Chapter 5: Histological Effects of Ketamine and Related Compounds on Rat Bladder Mucosa

5.1 INTRODUCTION

In Chapter 3, it was detailed that acute (<20 minutes) exposure to ketamine leads to inhibition of *in vitro* rat bladder strip contractility. This is in stark contrast to the ability of ketamine (3mM) to potentiate the contractility of rat bladder strips to carbachol after 72 hours incubation, as reported in Chapter 4.

Bladder overactivity is a well-established clinical presentation of ketamine-induced cystitis (Shahani, Streutker, Dickson & Stewart, 2007; Chu et al., 2008; Tsai et al., 2009; Mak et al., 2011), and similar observations have also been reported in animal studies of ketamine-induced cystitis (KC). For example, an increased voiding frequency and decreased bladder capacity was reported in mice receiving a daily intraperitoneal injection of 100mg/kg ketamine for 8 weeks (Meng et al., 2011). Similarly, in a study by Gu et al. (2014), rats that received either a low (5mg/kg) or high (50mg/kg) daily dose of ketamine displayed an increased urinary frequency compared to the normal saline group after 16 weeks.

In both humans and rats, the bladder is made up of 3 main layers: the mucosa, the detrusor muscle, and the adventitia/serosa. The mucosa encompasses the urothelium, lamina propria, and basement membrane, with the urothelium being the superficial-most cell layer. Histopathological examination of patients with KC often reveals a thinning or denuded urothelium (Shahani et al, 2007; Baker et al., 2013; Lin et al., 2014; Jhang, Hsu & Kuo, 2015), and disruption of the urothelial barrier is often postulated to be a potential cause of KC (Jhang et al., 2015; Castellani et al., 2020). It has been suggested that ketamine and/or its metabolites have a direct toxic effect on the urothelium leading to an impaired barrier function, which subsequently allows leakage of urinary potassium or other noxious substances to the underlying layers of the bladder, leading to cystitis and overactive bladder (Birder & de Groat, 2007; Jhang et al., 2015).

More recently, evidence has emerged to suggest that, like ketamine, methoxetamine (MXE) can also induce bladder overactivity in rats. Wang et al. (2017) reported that rats injected intraperitoneally with either 30mg/kg MXE or ketamine displayed increased micturition frequency bladder dysfunction after 4 weeks. Urothelial thinning was also observed in both ketamine and MXE treated rats, suggesting that these substances are toxic to urothelial cells.

A direct toxic effect of ketamine on human urothelial cells has been reported by Baker, Shabir, Georgopoulos & Southgate (2016). It was demonstrated that ketamine induces apoptosis in urothelial cells, and human ureteric tissues maintained in organ culture showed urothelial thinning after 72 hours exposure to 3mM ketamine.

The previous chapters have focused primarily on the acute effects of ketamine, however the bladder overactivity seen in patients with KC is associated with long-term ketamine use. Therefore, the aim of this chapter is to develop a longer-term model of ketamine exposure in which rat bladder can be maintained and exposed to ketamine and related compounds over a longer period of time to determine any resultant histological/histopathological effects. It was hypothesized that ketamine and MXE would have a direct toxic effect upon rat urothelium, leading to a loss of cells in the bladder mucosa.

The objectives for this chapter are as follows:

- To develop and optimise an organ culture protocol that allows rat bladder tissue to be incubated with ketamine and related compounds over a period of days.
- To determine whether ketamine or its metabolites cause a loss of cells in the bladder mucosa following direct exposure.
- To determine whether MXE or its alternatives cause a loss of cells in the bladder mucosa following direct exposure.
- To determine whether the NMDA receptor antagonists MK-801 or dextromethorphan cause a loss of cells in the bladder mucosa following direct exposure.

5.2 METHODS

Whole rat bladders were dissected longitudinally into two equal halves. One half was incubated for up to three days in tissue culture media containing either ketamine or ketamine-related compounds, while the other half acted as a matched-control and was incubated for the same duration in tissue culture media and an appropriate vehicle control. Tissues were then fixed in formalin, dehydrated through graded alcohols, cleared in xylene, and embedded in paraffin wax.

Tissue sections were stained with haematoxylin and eosin (H&E) and visualised using microscopy. Captured images were processed on ImageJ to isolate the haematoxylin-stained nuclei in the mucosal layer, and a cell count was conducted to determine what effect, if any, ketamine or its related compounds have on mucosal cell count.

For full methodology, please refer to section 2.7.

5.3 RESULTS

5.3.1 Histological Structure of Uncultured Rat Urinary Bladder

Half rat bladders were immediately placed in 10% formalin following dissection and, after processing, were stained with H&E for visualisation of their histological structure (Figure 5.1). The various layers of the bladder were easily distinguishable, with strong blue staining on the apical surface of the innermost layer suggesting the presence of nuclear cells, and therefore the presence of an intact urothelium (Figure 5.2).

5.3.2 The Different Concentrations of Fetal Bovine Serum Tested Do Not Have an Effect on Mean Mucosal Cell Count in Rat Urinary Bladder Strips After 3 Days Culture

Before incubating bladder tissue with ketamine or ketamine-related compounds, it was first necessary to optimise the tissue culture protocol (adapted from Baker et al., 2016). Half bladder strips were maintained in 3mL Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 (50:50 mix), supplemented with 1% penicillin/streptomycin and either 5% or 10% fetal bovine serum (FBS) to determine whether a higher concentration of FBS would help minimise degeneration of the bladder tissue. This was assessed by quantifying the number of mucosal cells present on the apical surface of the bladder. Post incubation, tissues were fixed and embedded in paraffin before staining with H&E. There was no statistically significant difference in the mean mucosal cell count between tissues supplemented with 5% or 10% FBS (Figure 5.3A, $P>0.05$).

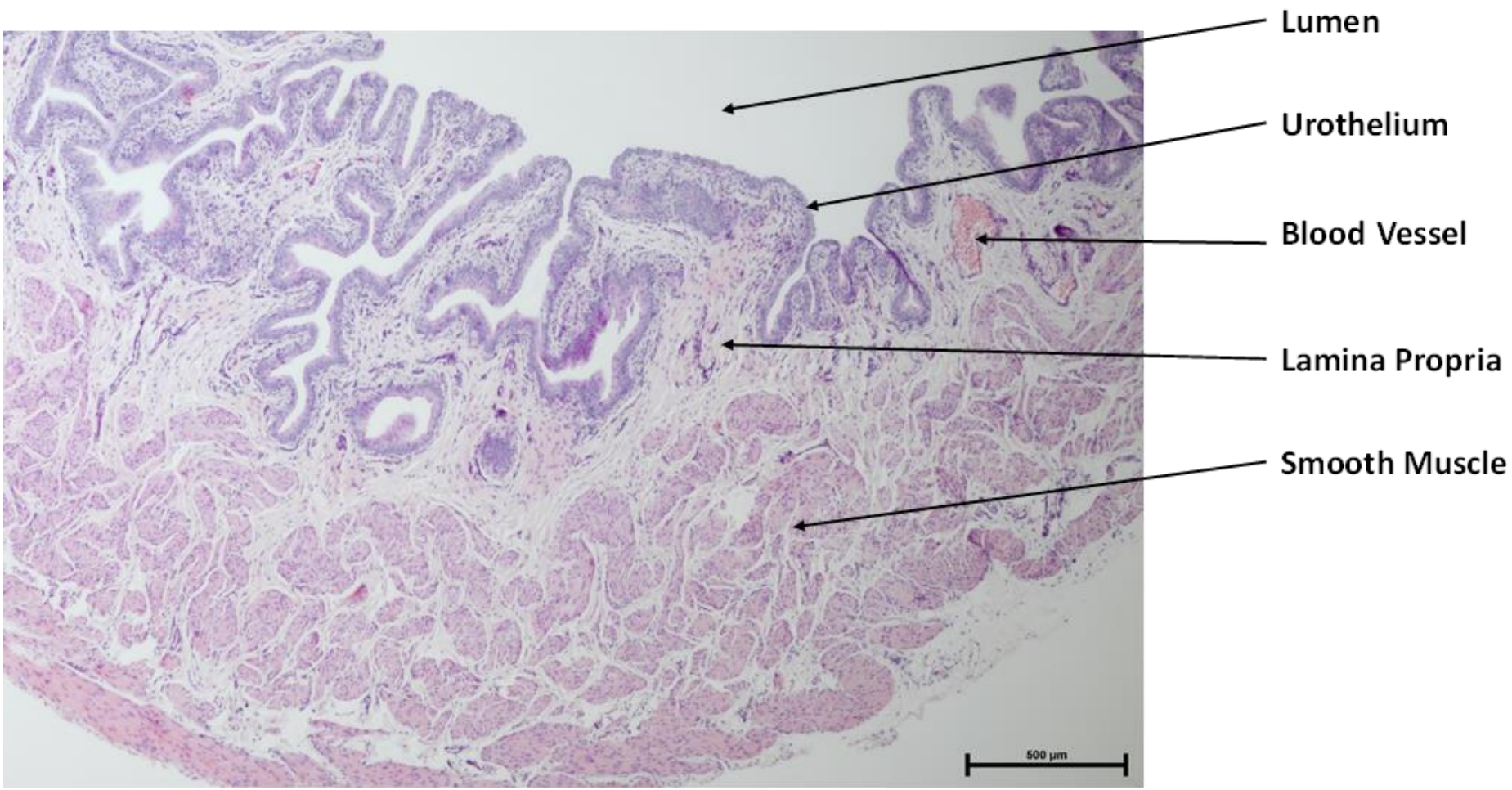

Figure 5.1. Histological structure of an uncultured rat urinary bladder stained with haematoxylin and eosin (x40 magnification). The urothelium is the superficial-most cell layer and so will come in direct contact with ketamine, or its metabolites, present in the urine.

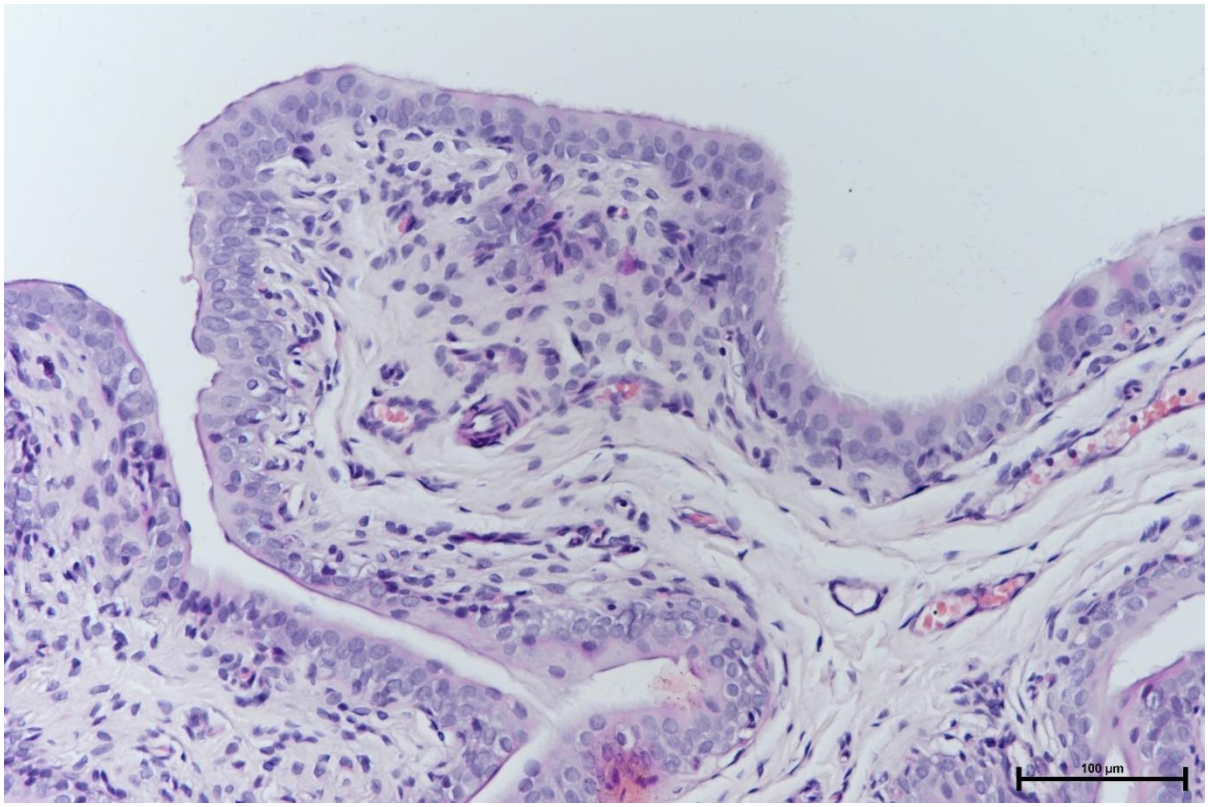

Figure 5.2. The urothelium and underlying lamina propria visualised in uncultured rat urinary bladder stained with haematoxylin and eosin (x200 magnification).

5.3.3 The Different Tissue Culture Medias Tested Did Not Affect Mean Mucosal Cell Count in Rat Urinary Bladder Strips After 3 Days Culture

Two different culture medias were tested to determine which was optimal for preservation of the tissue samples over the course of the incubation period. One half bladder strip was incubated for 3 days in a mixture of DMEM and RPMI 1640 (50:50 mix), while the other was maintained in Panexin (PAN-Biotech) culture media. All medias were also supplemented with 10% FBS and 1% penicillin/streptomycin. A mucosal cell count post incubation revealed no significant difference in mean cell count between the two culture medias (Figure 5.3B, $P>0.05$).

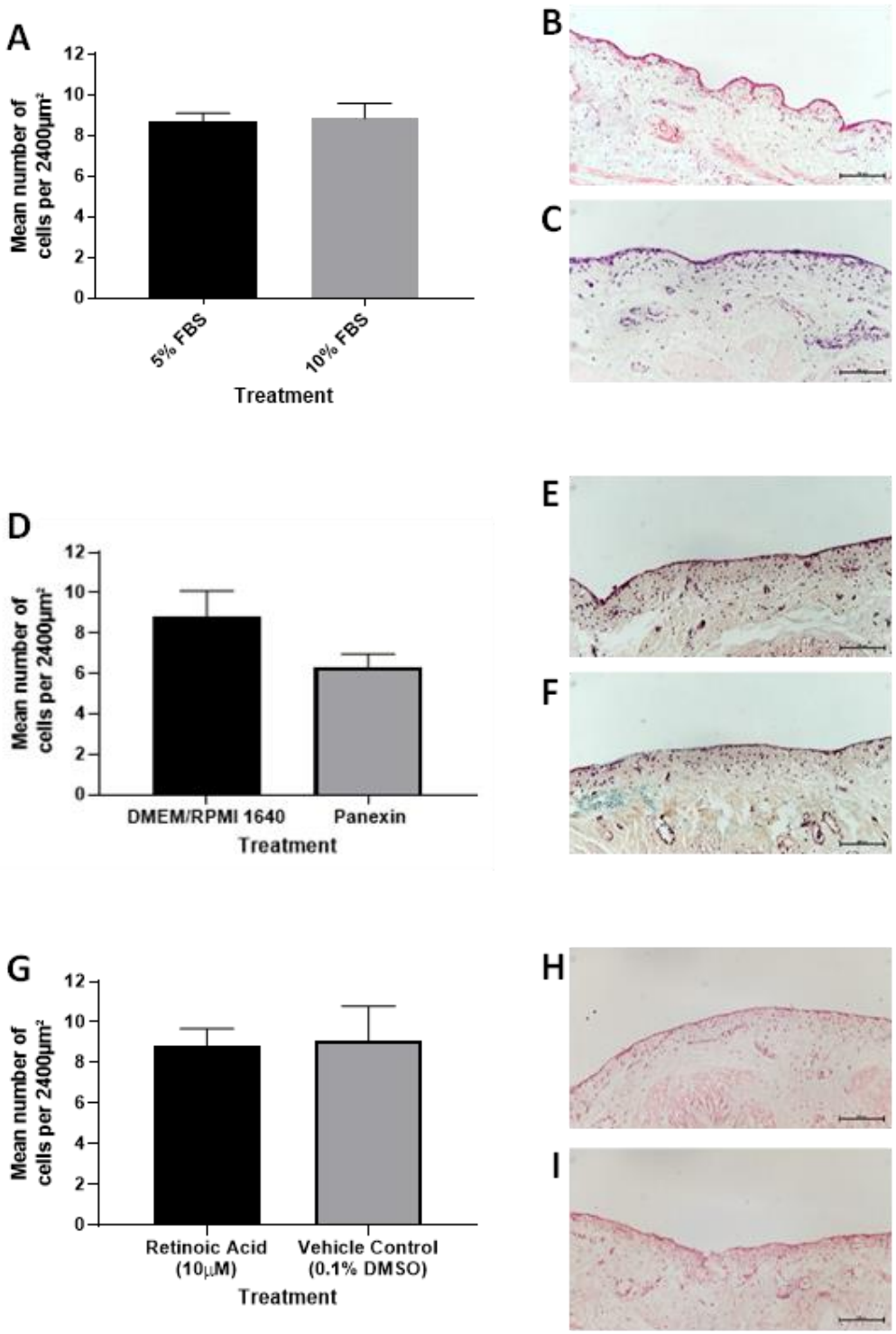

Figure 5.3. Effect of different medias and media supplements on mean mucosal cell count in rat bladder strips incubated for 3 days. Post incubation, tissues were processed, embedded in paraffin wax, and 5µM sections were stained with haematoxylin and eosin. **A,B,C:** Effect of 5% **(B)** and 10% **(C)** fetal bovine serum (in DMEM/RPMI 1640 media) on mean mucosal cell count **(C)**. **D,E,F:** Effect of DMEM/RPMI 1640 media **(E)** and Panexin media **(F)** (supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin) on mean mucosal cell count **(D)**. **G,H,I:** Effect of 10µM retinoic acid **(H)** or 0.1% DMSO vehicle control **(I)** (in DMEM/RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin) on mean mucosal cell count **(G)**. Mean mucosal cell counts were quantified by counting the number of stained nuclei on the apical surface of the mucosa in a 2400µm area. Data is expressed as mean number of cells per 2400µm ±SEM. Each data point represents *n*=3. Data were analysed using a two-tailed, paired t-test. P>0.05, not significantly different from the paired treated strip. For representative images, magnification is x200 and scale bar represents 100µm.

5.3.4 Retinoic Acid Does Not Affect Mean Mucosal Cell Count in Rat Urinary Bladder Strips After 3 Days Culture

Retinoic acid (RA) is important in the maintenance of the urothelium, and urothelial regeneration following injury (Gandhi et al., 2013). Therefore, RA (10µM) was used to supplement the tissue culture media (DMEM/RPMI 1640, with 10% FBS and 1% penicillin/streptomycin) to determine whether it would help preserve the tissue during incubation. Bladder strips incubated for 3 days with media supplemented with retinoic acid were quantified as having a mean mucosal cell count that was similar to the matched vehicle control (0.1% DMSO; Figure 5.3C, $P>0.05$).

When considering the effects of the different culture medias and supplements on mucosal cell count, all further experiments were undertaken using DMEM/RPMI 1640, supplemented with 10% FBS and 1% penicillin/streptomycin.

5.3.5 Increased Culture Duration Leads to a Reduction in Mean Mucosal Cell Count in Rat Urinary Bladder Strips After Three Days

The effect of culture duration was investigated to assess its effect upon mucosal cell count. Half bladder tissues were maintained in DMEM/RPMI 1640 (50:50 mix) supplemented with 10% FBS and 1% penicillin/streptomycin for 1-10 days. All culture durations tested (1, 3, 5, and 10 days) had a significantly lower mean mucosal cell count compared to the uncultured control tissues ($P<0.0001$; Figure 5.4). A loss of intensity in urothelial staining was noted in cultured tissues compared to the uncultured control, with intensity also appearing to decrease as incubation duration increased (Figure 5.5 & 5.6). Sloughing (detachment of cells from the underlying tissue layer) of the urothelium was also noted in incubated tissues after 1 and 3 days (Figure 5.5 & 5.6).

Tissues incubated for 5 or 10 days had a reduced number of mucosal cells compared to both 1-day ($P<0.05$ and $P<0.01$, respectively; Figure 5.4), and 3-day ($P<0.01$ and $P<0.001$, respectively) incubated tissues; however, there was no significant difference in mucosal cell count between bladder strips cultured for 1 or 3 days. Therefore, as the aim was to develop a longer-term model of ketamine exposure, 3 days was selected as the standard incubation period for future tissue culture experiments.

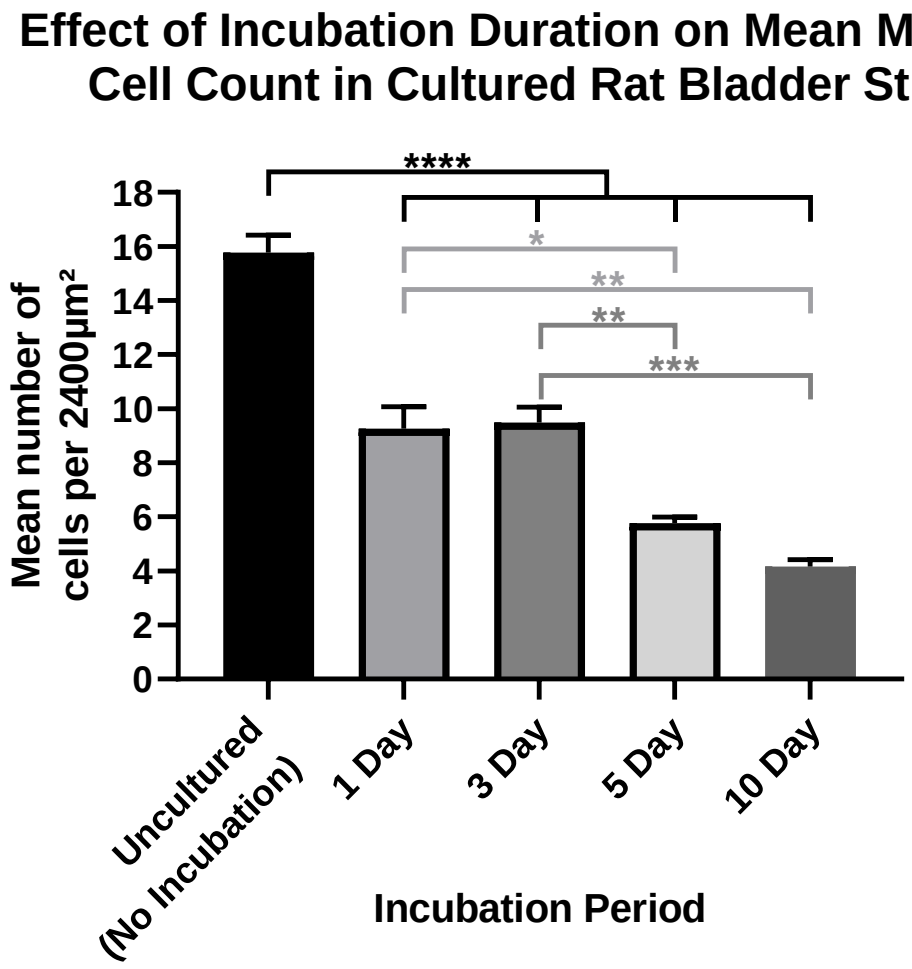

Figure 5.4. Effect of incubation duration on mean mucosal cell count in rat bladder strips. Strips were maintained in DMEM/RPMI 1640 (50:50 mix) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and incubated for 1-10 days. Post incubation, tissues were processed, embedded in paraffin wax, and 5µM sections were stained with haematoxylin and eosin. Mean mucosal cell counts were quantified by counting the number of stained nuclei on the apical surface of the mucosa in an area of 2400µm. Data is expressed as mean number of cells per 2400µm ±SEM. Uncultured group represents *n*=6; 3 day represents *n*=8; all other culture durations represent *n*=3. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 significantly different from the specified incubation period. Data were analysed using a one-way ANOVA with a Bonferroni post-test.

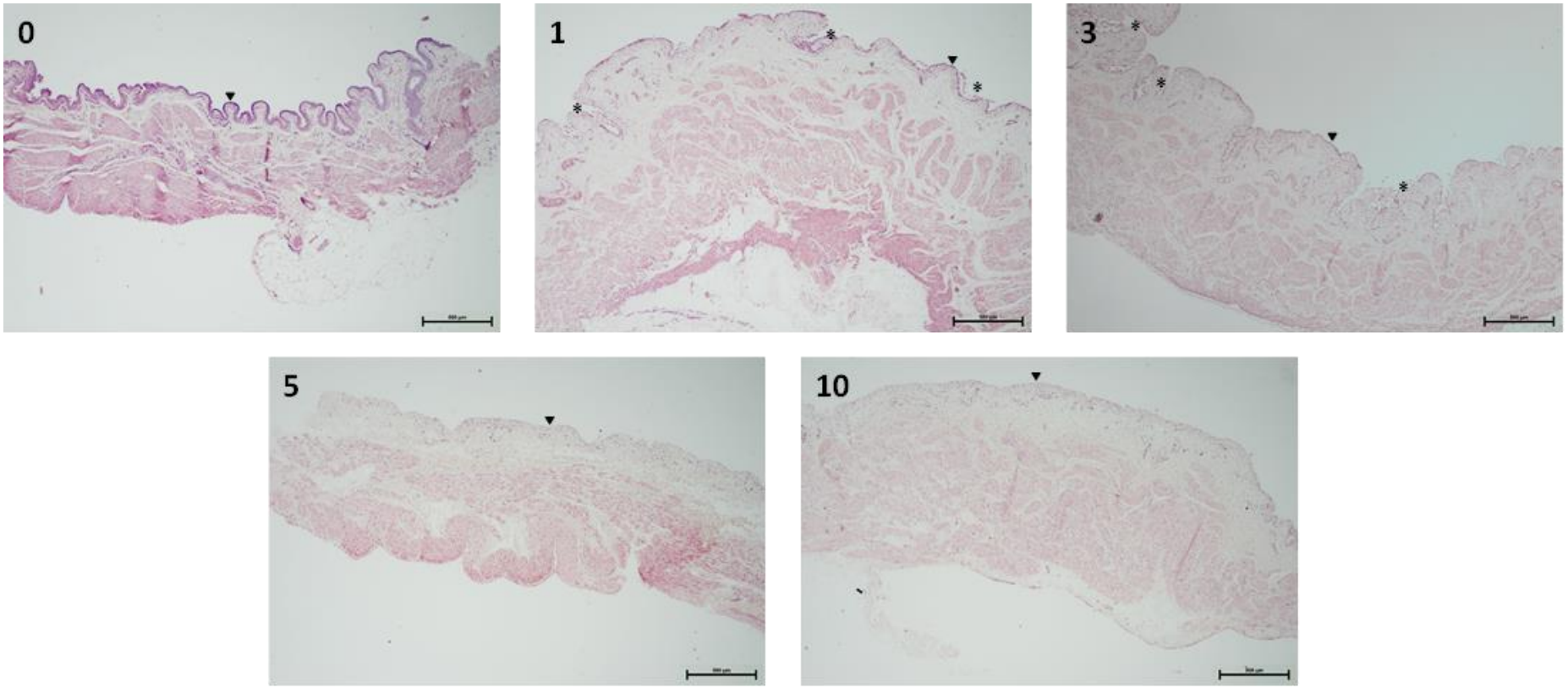

Figure 5.5. Histological assessment of representative rat bladder sections following 0-10 days incubation. There is a marked observable loss of intensity in urothelial staining after incubating (1, 3, 5, and 10 days) compared to the uncultured control (0). Arrows indicate the supposed location of the urothelium. Asterisks indicate sloughing of the urothelium. Magnification x40; scale bar represents 500µm. Bladders stained with haematoxylin and eosin.

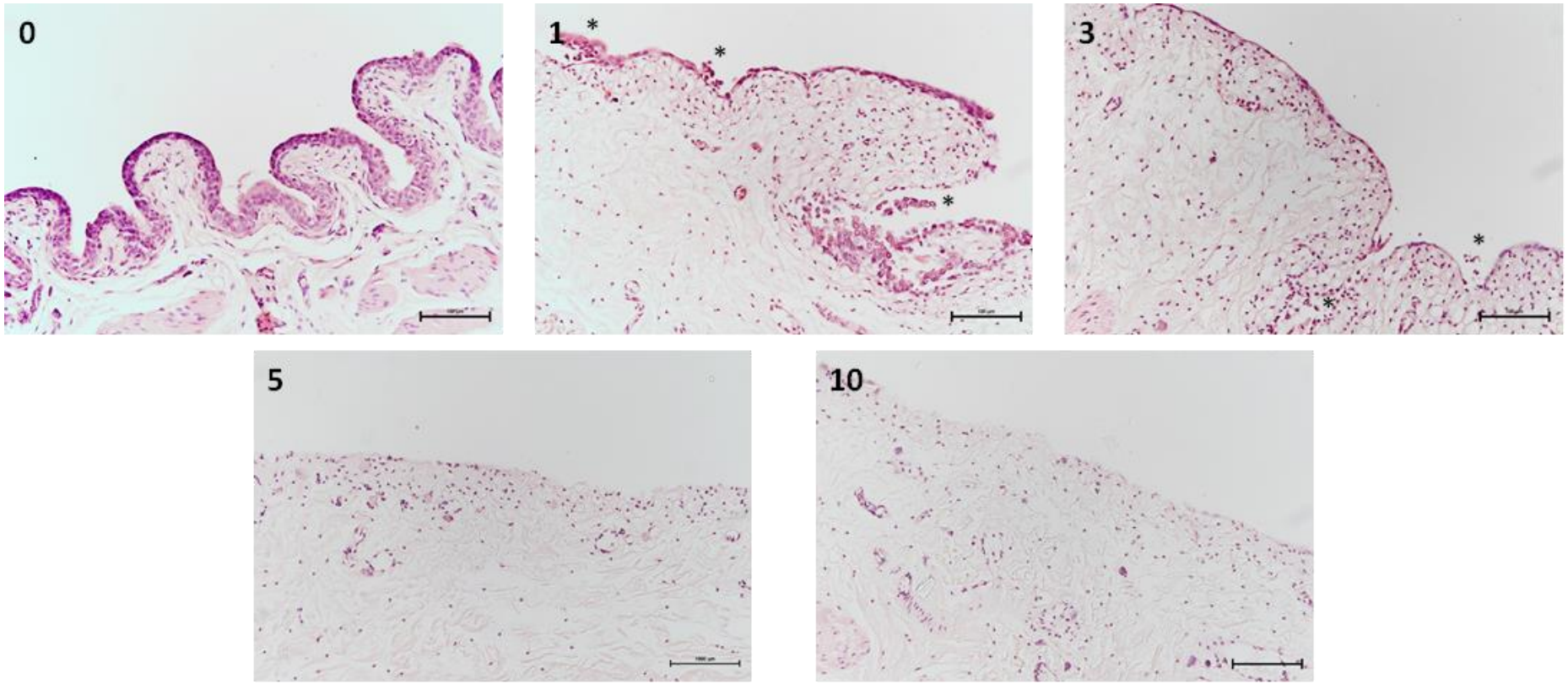

Figure 5.6. Histological assessment of representative rat bladder sections following 0-10 days incubation. There is a marked observable loss of intensity in urothelial staining after incubating (1, 3, 5, and 10 days) compared to the uncultured control (0). Asterisks indicate sloughing of the urothelium. Magnification x200; scale bar represents 100µm. Bladders stained with haematoxylin and eosin.

5.3.6 Ketamine Reduces Mean Mucosal Cell Count in Cultured Urinary Rat Bladder

Bladder strips exposed to 3mM ketamine for 3 days had a significantly reduced mean mucosal cell count compared to untreated control-cultured tissues (P<0.01; Figure 5.7), and there was an observable loss of apical staining intensity in ketamine-treated tissues, suggesting a decreased nuclear cell presence (Figure 5.8). Evidence of karyorrhexis (fragmentation of the nucleus) was also noted in both 3-day control and 3-day ketamine-treated tissues (Figure 5.8). Strips that were exposed to 1mM ketamine for 3 days (Figure 5.7), or 3mM ketamine for 1-day (Figure 5.9), had a similar number of apical mucosal cells compared to control-treated tissues, suggesting that the ketamine effect is concentration- and time-dependent.

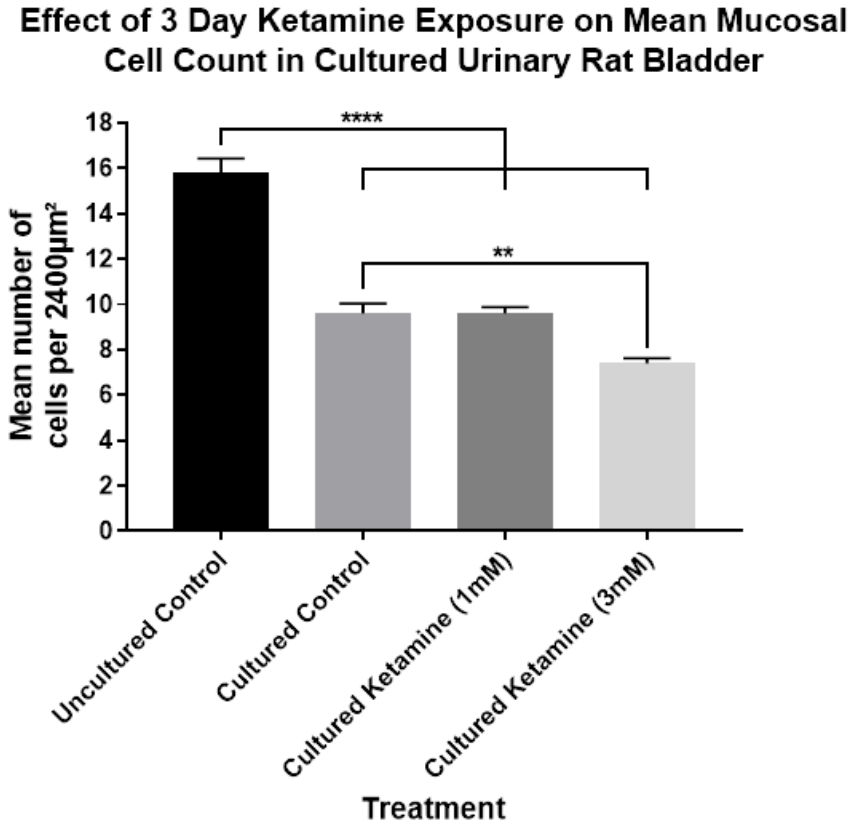

Figure 5.7. Effect of 3-day ketamine exposure on mean apical mucosal cell count in cultured rat bladder strips. Matched half bladder strips were incubated for 3 days in tissue culture media alone, or tissue culture media and 1 or 3mM ketamine. Post incubation, strips were processed, embedded in paraffin wax and stained with haematoxylin and eosin. Mean mucosal cell counts were quantified by counting the number of stained nuclei on the apical surface of the mucosa within an area of 2400µm. Data is expressed as mean number of cells per 2400µm ±SEM. Uncultured group represents *n*=6; cultured control *n*=11; 1mM ketamine *n*=3; 3mM ketamine *n*=8. **P<0.01; **** P<0.0001 significantly different from the specified treatment. Data were analysed using a one-way ANOVA with a Bonferroni post-test.

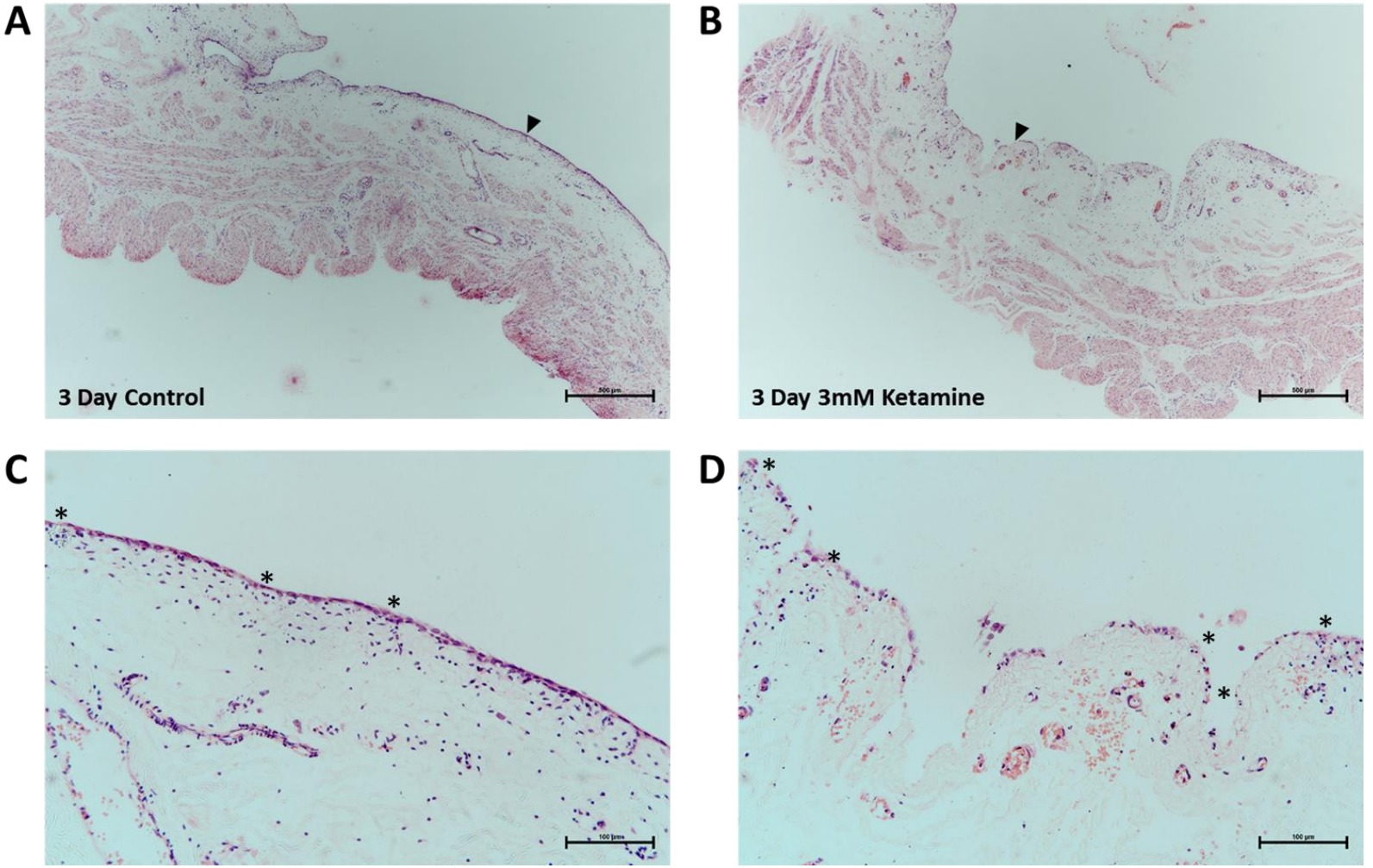

Figure 5.8 Haematoxylin and eosin-stained representative half rat bladder strips (matched) incubated for 3 days with tissue culture media (**A&C**), or tissue culture media and 3mM ketamine (**B&D**). Reduced intensity of apical mucosal layer staining was generally observed in the strips exposed to ketamine. Magnification x40, scale bar represents 500µm (**A&B**), and magnification x200, scale bar represents 100µm (**C&D**). Arrows indicate the zoomed in portion of the mucosa displayed below each treatment. Asterisks indicate evidence of karyorrhexis.

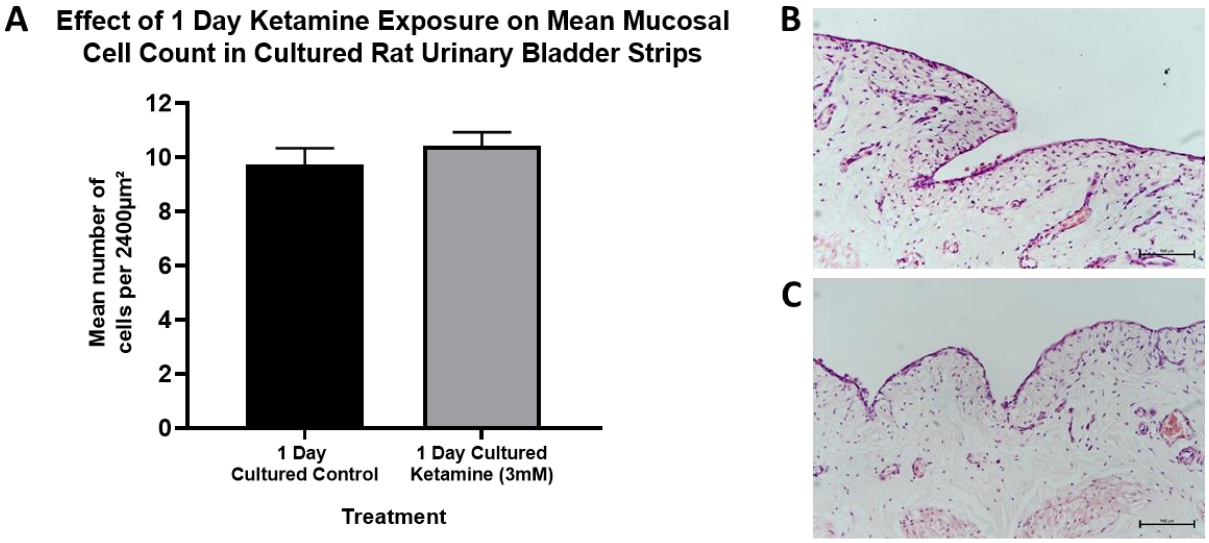

Figure 5.9 Effect of 1-day 3mM ketamine exposure on mean apical mucosal cell count in cultured rat bladder strips. Matched half bladder strips were incubated for 1 day in tissue culture media alone, or tissue culture media and 3mM ketamine. Post incubation, strips were processed, embedded in paraffin wax and stained with haematoxylin and eosin. **A:** Graphical representation of mean mucosal cell count. Mean mucosal cell counts were quantified by counting the number of stained nuclei on the apical surface of the mucosa within an area of 2400µm. Data is expressed as mean number of cells per 2400µm ±SEM. Uncultured group and cultured control represents *n*=3. Data were analysed using a paired two-tailed t-test. No significant difference was observed in the mean mucosal cell count of the treatments tested (P>0.05). **B,C:** Representative images of haematoxylin and eosin staining in 1-day cultured control **(B)**, and 1-day cultured 3mM ketamine **(C)** matched half rat bladder sections. Magnification x200; scale bar represents 100µm.

5.3.7 Norketamine, But Not Dehydronorketamine, Reduces Mean Mucosal Cell Count in Cultured Rat Urinary Bladder

Exposure to 3mM norketamine (NK) for 3 days led to a significant decrease in the number of apical mucosal cells compared to control-cultured tissues (P<0.05; Figure 5.10). A reduced staining intensity on the apical surface of the mucosa was generally observed in the NK-treated tissues (Figure 5.11).

Rat bladder strips incubated with 3mM dehydronorketamine (DHNK) for 3 days had a similar number of apical mucosal cells when compared to control-cultured tissues (Figure 5.12).

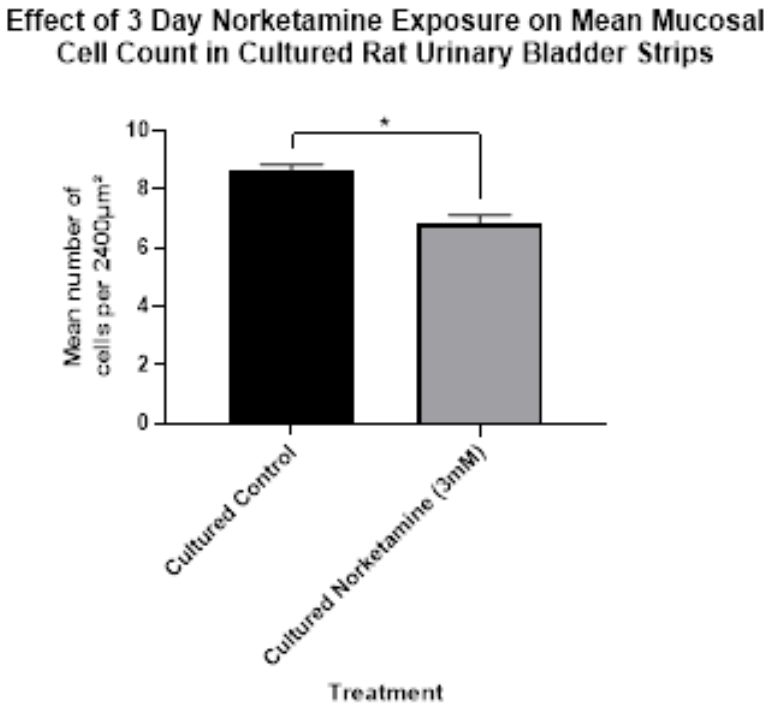

Figure 5.10. Effect of 3-day 3mM norketamine exposure on mean apical mucosal cell count in cultured rat bladder strips. Matched half bladder strips were incubated for 3 days in tissue culture media alone, or tissue culture media containing 3mM norketamine. Post incubation, strips were processed, embedded in paraffin wax and stained with haematoxylin and eosin. Mean mucosal cell counts were quantified by counting the number of stained nuclei on the apical surface of the mucosa within an area of 2400µm. Data is expressed as mean number of cells per 2400µm ±SEM. Data represents *n*=4. *P<0.05, significantly different from the cultured control. Data were analysed using a paired two-tailed t-test.

5.3.8 (S)-Ketamine Reduces Mean Mucosal Cell Count in Cultured Urinary Rat Bladder

Rat bladder strips exposed to 3mM (S)-ketamine for 3 days had a significantly reduced mean mucosal cell count when compared to matched control-incubated strips (P<0.01; Figure 5.13). Reduced apical staining intensity was generally observed in (S)-ketamine-treated tissues, suggesting a potential loss of urothelial cells (Figure 5.13).

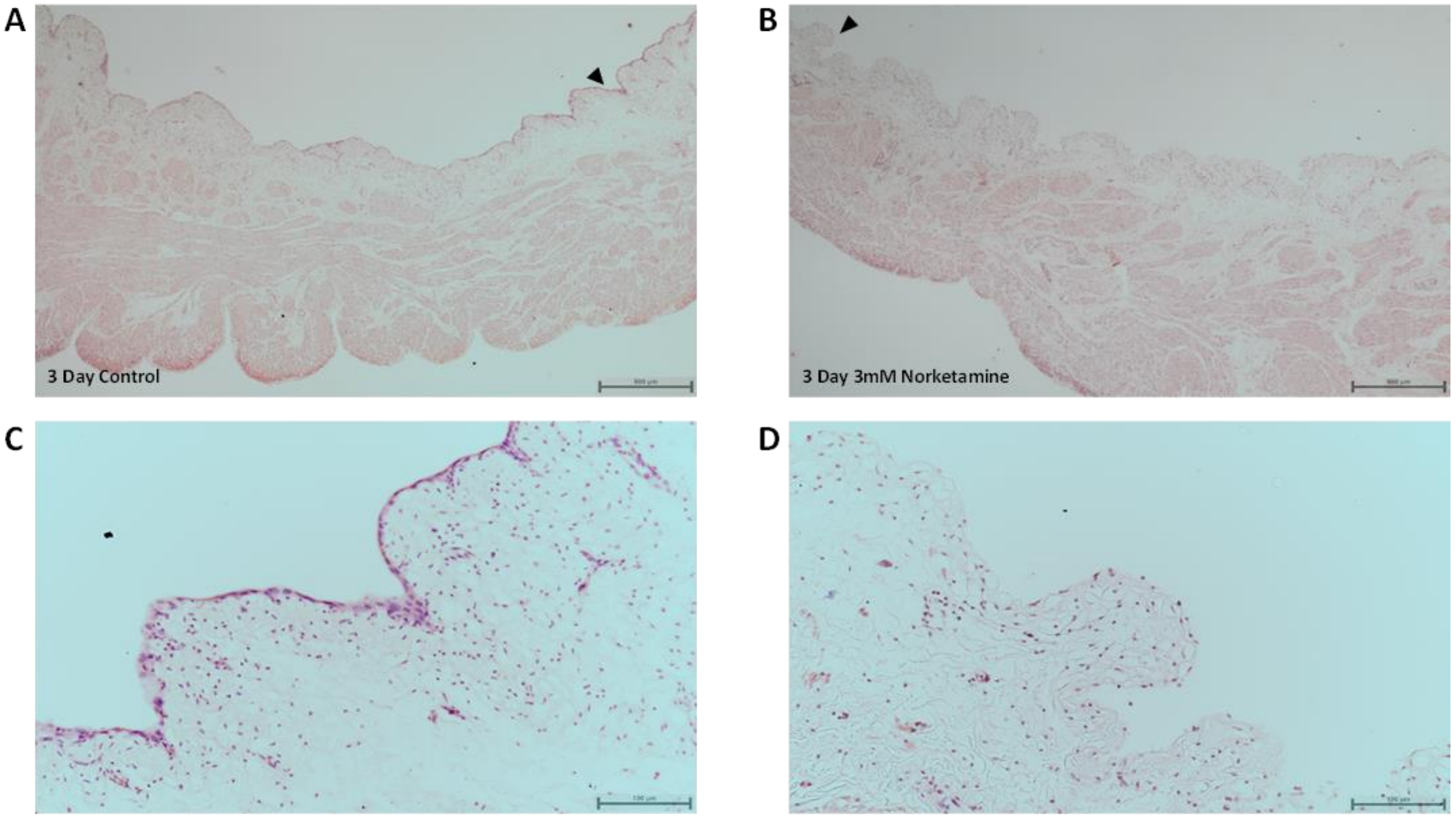

Figure 5.11. Haematoxylin and eosin-stained representative half rat bladder strips (matched) incubated for 3 days with tissue culture media (**A&C**), or tissue culture media and 3mM norketamine (**B&D**). Reduced intensity of apical mucosal layer staining was generally observed in the strips exposed to norketamine. Magnification x40 (**A&B**), and x200 (**C&D**). Arrows indicate the zoomed in portion of the mucosa displayed below each treatment.

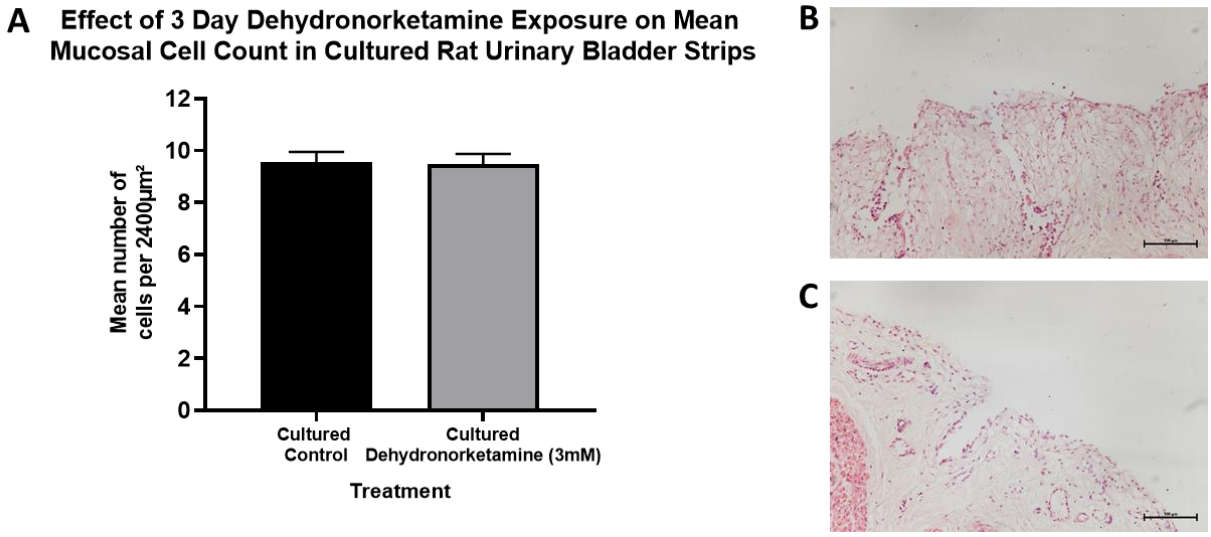

Figure 5.12. Effect of 3-day 3mM dehydronorketamine exposure on mean apical mucosal cell count in cultured rat bladder strips. Matched half bladder strips were incubated for 3 days in tissue culture media alone, or tissue culture media containing 3mM dehydronorketamine. Post incubation, strips were processed, embedded in paraffin wax and stained with haematoxylin and eosin. **A:** Graphical representation of mean mucosal cell count. Mean mucosal cell counts were quantified by counting the number of stained nuclei on the apical surface of the mucosa within an area of 2400µm. Data is expressed as mean number of cells per 2400µm ±SEM. Data represents *n*=11 3-day cultured control; *n*=3 3-day dehydronorketamine. Data were analysed using an unpaired two-tailed t-test. No significant difference was observed in the mean mucosal cell count of the treatments tested (P>0.05). **B,C:** Representative images of haematoxylin and eosin staining in 3-day cultured control **(B)**, and 3-day cultured 3mM dehydronorketamine **(C)** half rat bladder sections. Magnification x200; scale bar represents 100µm.

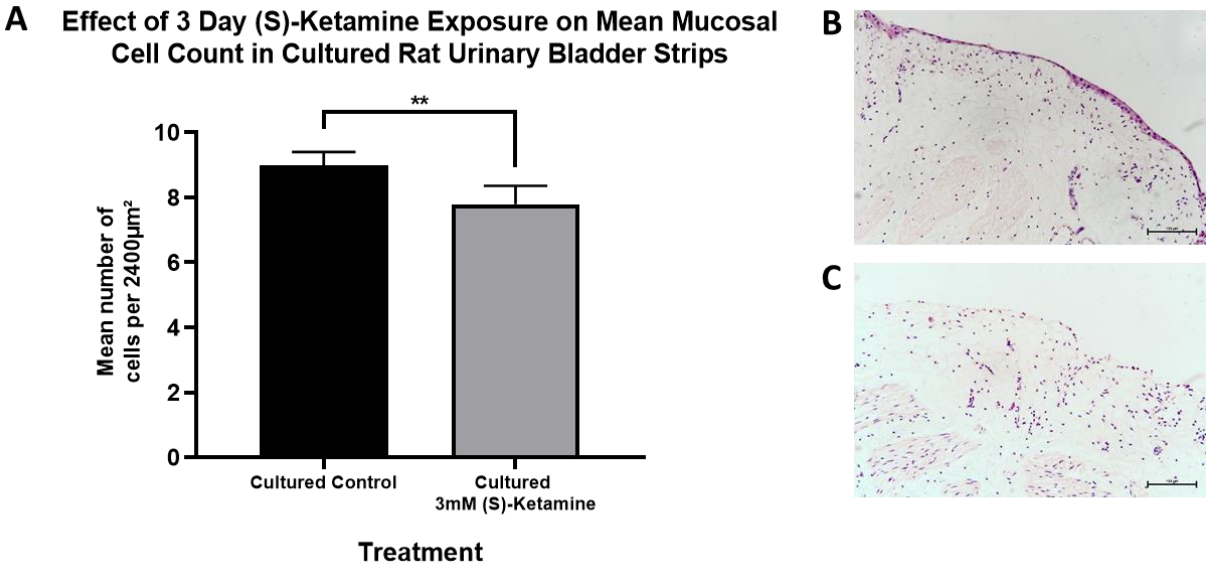

Figure 5.13. Effect of 3-day 3mM (S)-ketamine exposure on mean apical mucosal cell count in cultured rat bladder strips. Matched half bladder strips were incubated for 3 days in tissue culture media alone, or tissue culture media and 3mM (S)-ketamine. Post incubation, strips were processed, embedded in paraffin wax, and stained with haematoxylin and eosin. **A:** Graphical representation of mean mucosal cell count. Mean mucosal cell counts were quantified by counting the number of stained nuclei on the apical surface of the mucosa within an area of 2400µm. Data is expressed as mean number of cells per 2400µm ±SEM. Data represents *n*=4. **P<0.01, significantly different from the cultured control. Data were analysed using a paired two-tailed t-test. **B,C:** Representative images of haematoxylin and eosin staining in 3-day cultured control **(B)**, and 3-day cultured 3mM (S)-ketamine **(C)** matched half rat bladder sections. Magnification x200; scale bar represents 100µm.

5.3.9 Mean Mucosal Cell Count Was Unaffected in Rat Urinary Bladder Strips Exposed Simultaneously to Lower Concentrations of Ketamine, Norketamine, and Dehydronorketamine

Simultaneous exposure to ketamine (30µM), NK (30µM), and DHNK (100 µM) for 3 days did not lead to a loss of apical mucosal cells in rat bladder strips compared to control-cultured tissues (P>0.05; Figure 5.14).

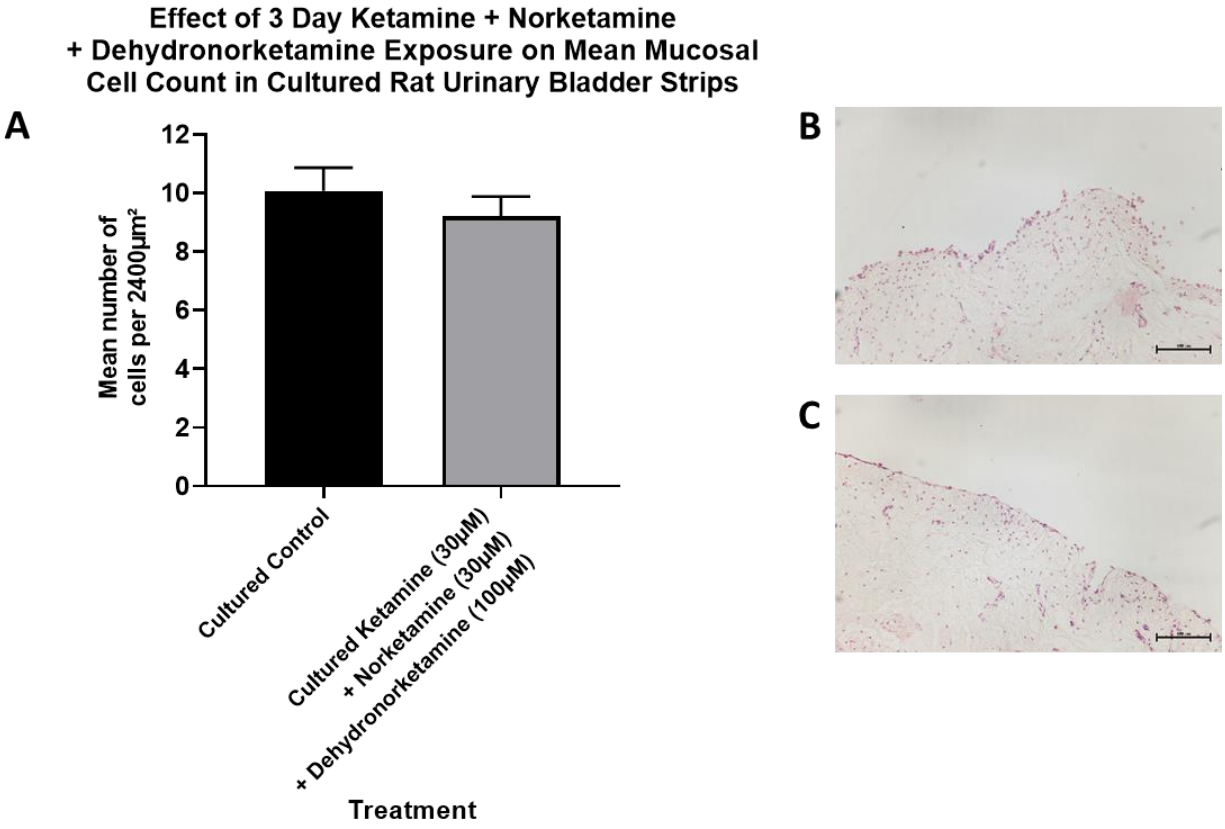

Figure 5.14. Effect of 3-day 30µM ketamine + 30µM norketamine + 100 µM dehydronorketamine exposure on mean apical mucosal cell count in cultured rat bladder strips. Matched half bladder strips were incubated for 3 days in tissue culture media alone, or tissue culture media containing ketamine, norketamine, and dehydronorketamine. Post incubation, strips were processed, embedded in paraffin wax and stained with haematoxylin and eosin. **A:** Graphical representation of mean mucosal cell count. Mean mucosal cell counts were quantified by counting the number of stained nuclei on the apical surface of the mucosa within an area of 2400µm. Data is expressed as mean number of cells per 2400µm ±SEM. Data represents *n*=3. Data were analysed using a paired two-tailed t-test. No significant difference was observed in the mean mucosal cell count of the treatments tested (P>0.05). **B,C:** Representative images of haematoxylin and eosin staining in 3-day cultured control **(B)**, and 3-day cultured 30µM ketamine + 30µM norketamine + 100µM dehydronorketamine **(C)** matched half rat bladder sections. Magnification x200; scale bar represents 100µm.

5.3.10 Methoxetamine, Methoxphenidine, and Diphenidine Reduce Mean Mucosal Cell Count in Cultured Rat Urinary Bladder

The ketamine-related new psychoactive substances (NPS) methoxetamine (MXE), methoxphenidine (MXP), and diphenidine (DPD) were investigated to see what effect, if any, they had upon mucosal cell count in rat bladder strips. After 3 days exposure, all NPS (3mM) tested significantly reduced the number of cells in the apical portion of the mucosa when compared to the matched vehicle control-cultured tissues (P<0.05; Figure 5.15A-C).

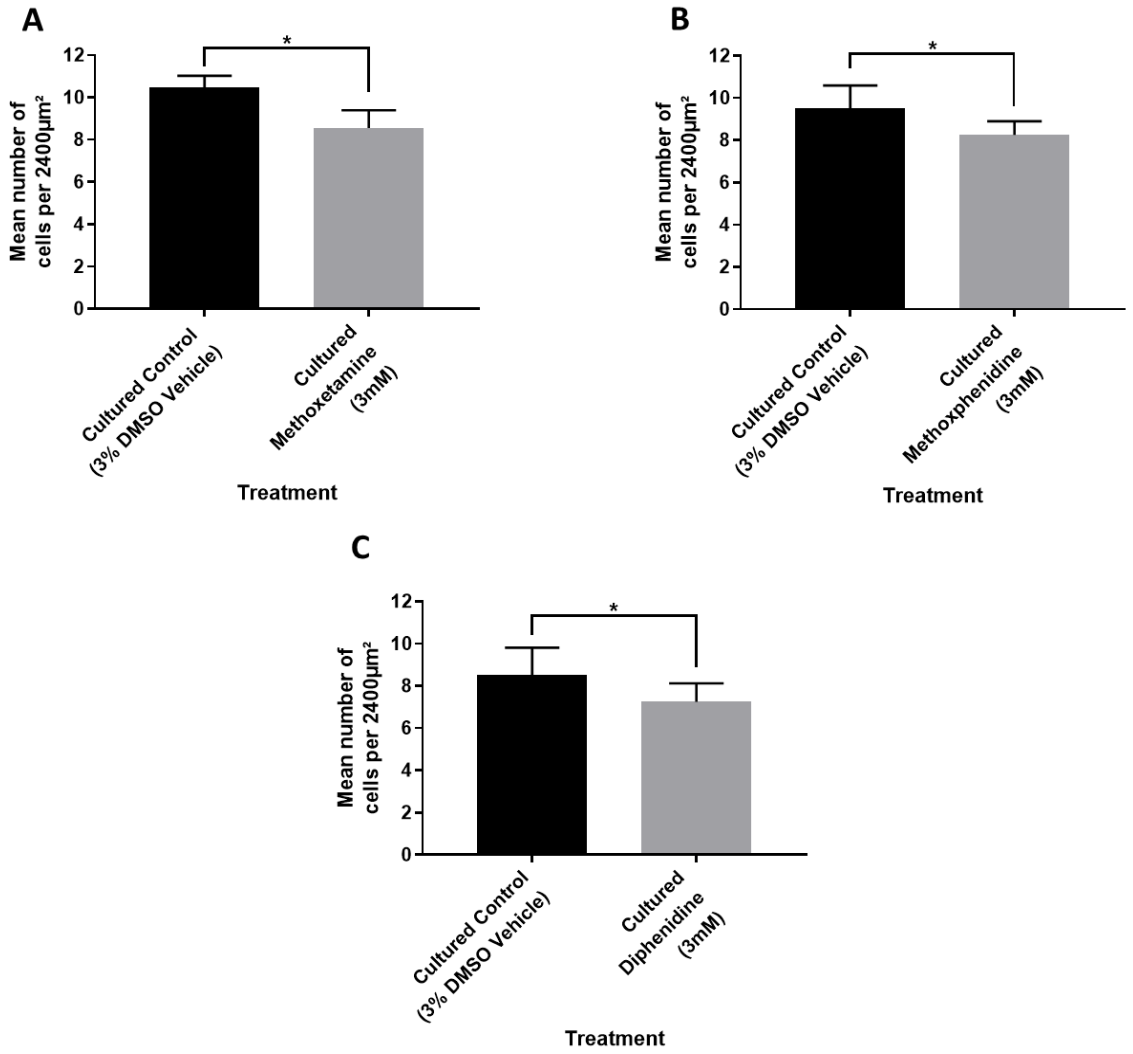

Figure 5.15. Effect of 3-day methoxetamine, methoxphenidine, or diphenidine exposure on mean apical mucosal cell count in cultured rat bladder strips. Matched half bladder strips were incubated for 3 days in tissue culture media containing 3mM of the specified new psychoactive substance, or tissue culture media and a vehicle control of 3% dimethyl sulfoxide. Post incubation, strips were processed, embedded in paraffin wax, and stained with haematoxylin and eosin. Mean mucosal cell counts were quantified by counting the number of stained nuclei on the apical surface of the mucosa within an area of 2400µm. Data is expressed as mean number of cells per 2400µm ±SEM. Data represents *n*=5 methoxetamine; *n*=4 methoxphenidine; *n*=6 diphenidine. *P<0.05, significantly different from the cultured vehicle control. Data were analysed using a two-tailed paired t-test.

A decrease in staining intensity of the apical mucosa was also generally observed in tissues treated for 3 days with MXE, MXP, and DPD, compared to the matched vehicle control tissues (Figure 5.16 & 5.17).

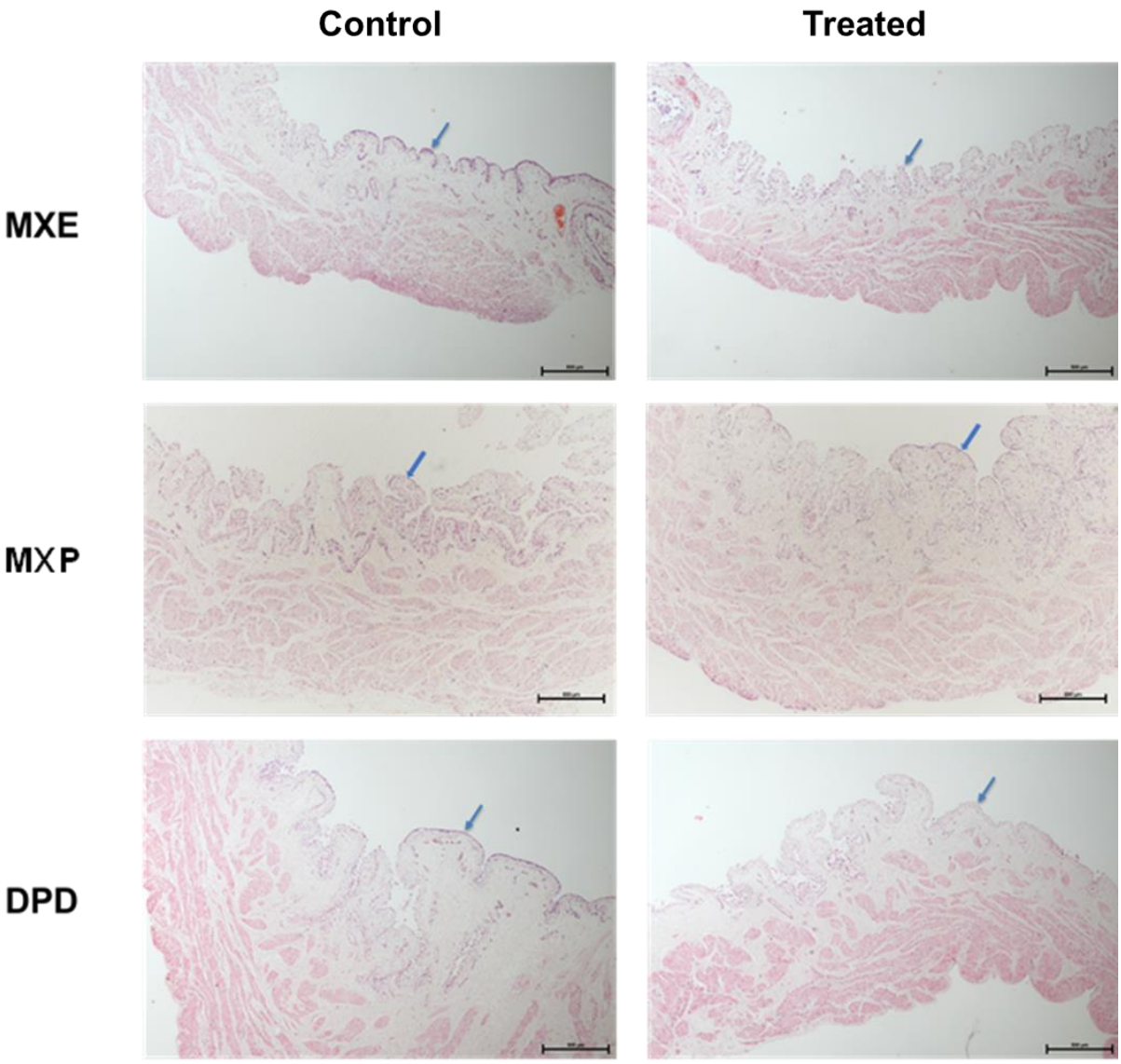

Figure 5.16. Haematoxylin and eosin-stained representative rat bladder strips following 3 days incubation with either ketamine (3mM), or ketamine-related new psychoactive substances (3mM). Matched half bladder strips were incubated for 3 days in tissue culture media, and either distilled water (ketamine), or with 3% dimethyl sulfoxide (MXE, MXP, DPD) and acted as a vehicle control. Decreased intensity of the apical mucosal layer was generally observed in ketamine and new psychoactive substance treated tissues compared to the vehicle control tissues. Arrows indicate the location of the urothelium. Magnification x40; scale bar represents 500µm.

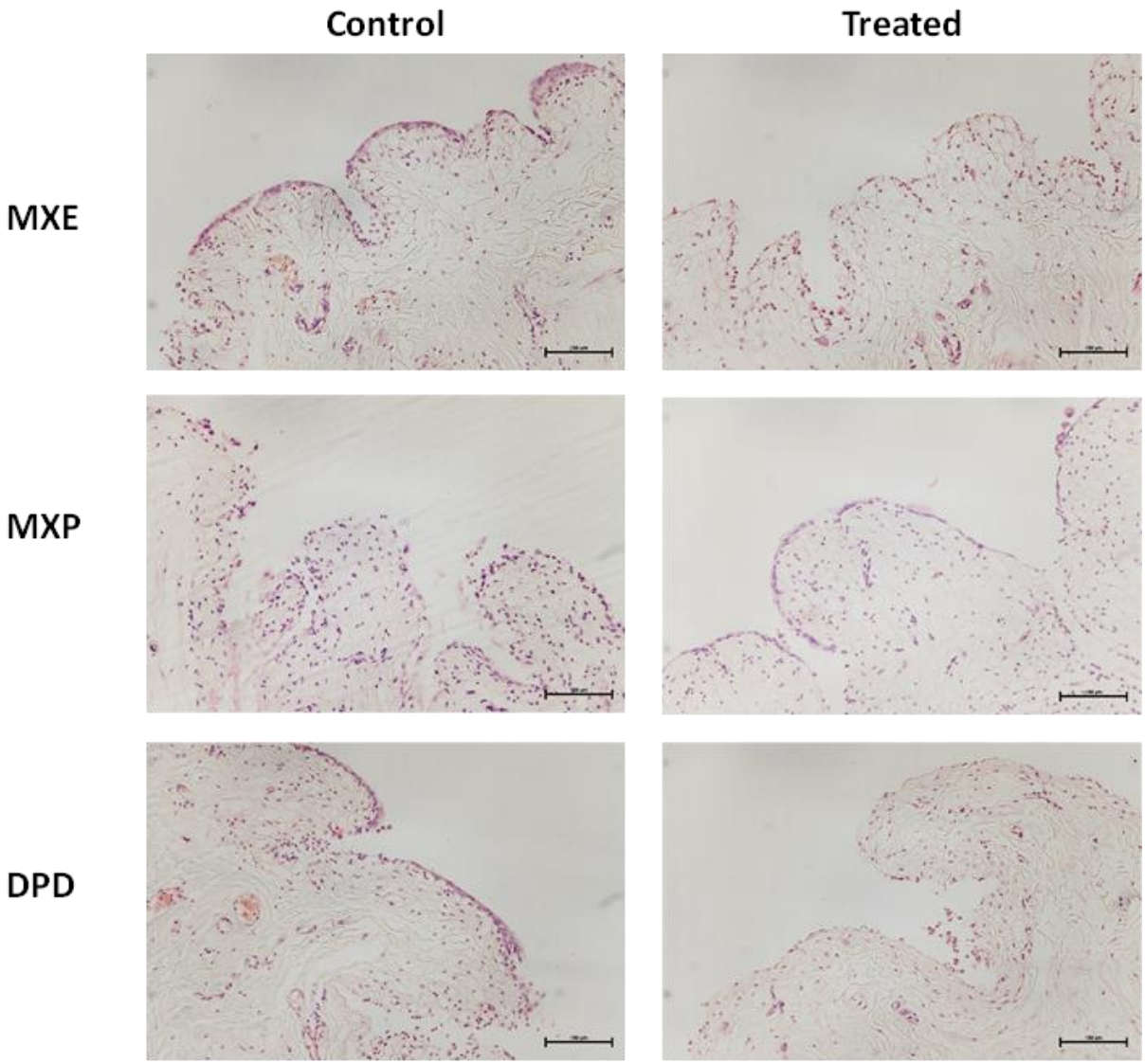

Figure 5.17. Haematoxylin and eosin-stained representative rat bladder strips following 3 days incubation with the ketamine-related new psychoactive substances methoxetamine, methoxphenidine, or diphenidine (3mM). Control matched half bladder strips were incubated for 3 days in tissue culture media and 3% dimethyl sulfoxide. Decreased intensity of the apical mucosal layer was generally observed in new psychoactive substance-treated tissues compared to the vehicle control tissues. Magnification x200; scale bar represents 100µm.

5.3.11. MK-801 and Nifedipine Did Not Affect Mean Mucosal Cell Count in Cultured Rat Urinary Bladder

Bladder strips cultured with 3mM MK-801 did not alter mean mucosal cell count when compared to matched control tissue strips (P=0.089; Figure 5.18). Similarly, exposure to 10µM nifedipine for 3 days also had no effect on mean mucosal cell count in rat bladder strips when compared to their matched vehicle cultured counterparts (P>0.05; Figure 5.19).

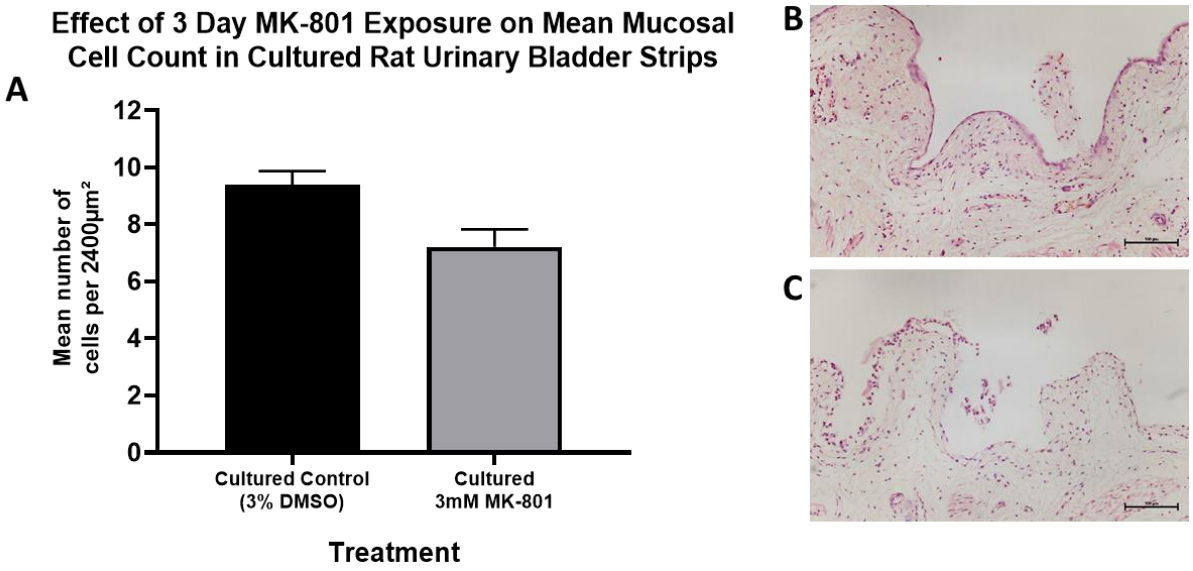

Figure 5.18. Effect of 3-day 3mM MK-801 exposure on mean apical mucosal cell count in cultured rat bladder strips. Matched half bladder strips were incubated for 3 days in tissue culture media and 3% dimethyl sulfoxide, or tissue culture media containing 3mM MK-801. Post incubation, strips were processed, embedded in paraffin wax, and stained with haematoxylin and eosin. **A:** Graphical representation of mean mucosal cell count. Mean mucosal cell counts were quantified by counting the number of stained nuclei on the apical surface of the mucosa within an area of 2400µm. Data is expressed as mean number of cells per 2400µm ±SEM. Data represents *n*=4. Data were analysed using a paired two-tailed t-test. No significant difference was observed in the mean mucosal cell count of the treatments tested (P=0.089). **B,C:** Representative images of haematoxylin and eosin staining in 3-day cultured vehicle control **(B)**, and 3-day cultured 3mM MK-801 **(C)** matched half rat bladder sections. Magnification x200; scale bar represents 100µm.

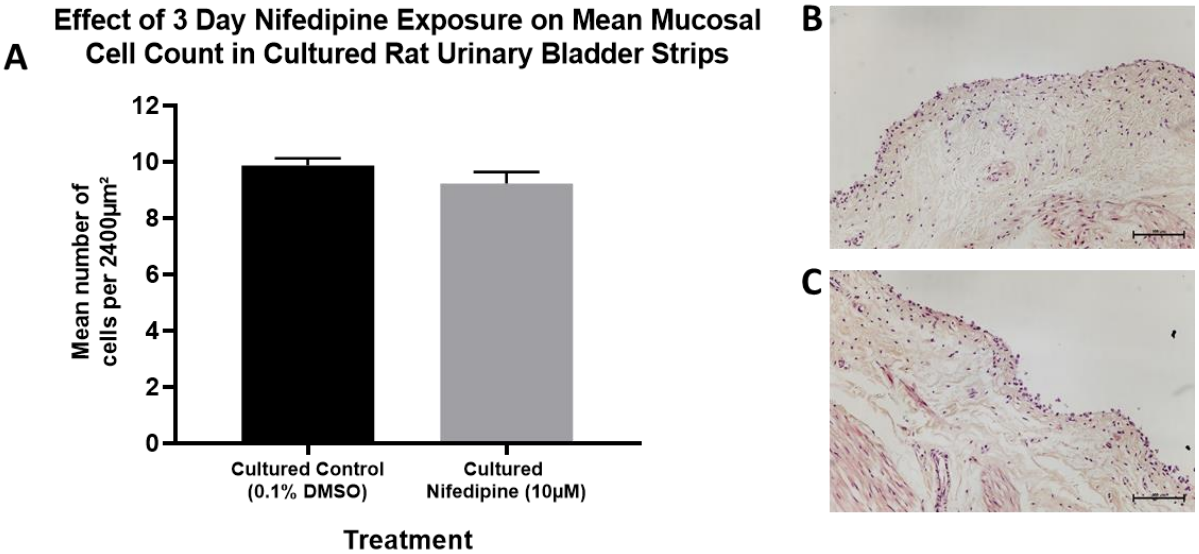

Figure 5.19. Effect of 3-day 10µM nifedipine exposure on mean apical mucosal cell count in cultured rat bladder strips. Matched half bladder strips were incubated for 3 days in tissue culture and 0.1% dimethyl sulfoxide, or tissue culture media containing 10µM nifedipine. Post incubation, strips were processed, embedded in paraffin wax, and stained with haematoxylin and eosin. **A:** Graphical representation of mean mucosal cell count. Mean mucosal cell counts were quantified by counting the number of stained nuclei on the apical surface of the mucosa within an area of 2400µm. Data is expressed as mean number of cells per 2400µm ±SEM. Data represents *n*=3. Data were analysed using a paired two-tailed t-test. No significant difference was observed in the mean mucosal cell count of the treatments tested (P>0.05). **B,C:** Representative images of haematoxylin and eosin staining in 3-day cultured vehicle control **(B)**, and 3-day cultured 10µM nifedipine **(C)** matched half rat bladder sections. Magnification x200; scale bar represents 100µm.

5.3.12 Bay K8644 Could Not Rescue Loss of Mucosal Cells in Ketamine Cultured Rat Urinary Bladder Strips

Rat bladder strips incubated with 3mM ketamine and 200nM Bay K8644 had a similar number of apical mucosal cells when compared to matched tissue strips incubated with ketamine (3mM) alone (P>0.05; Figure 5.20).

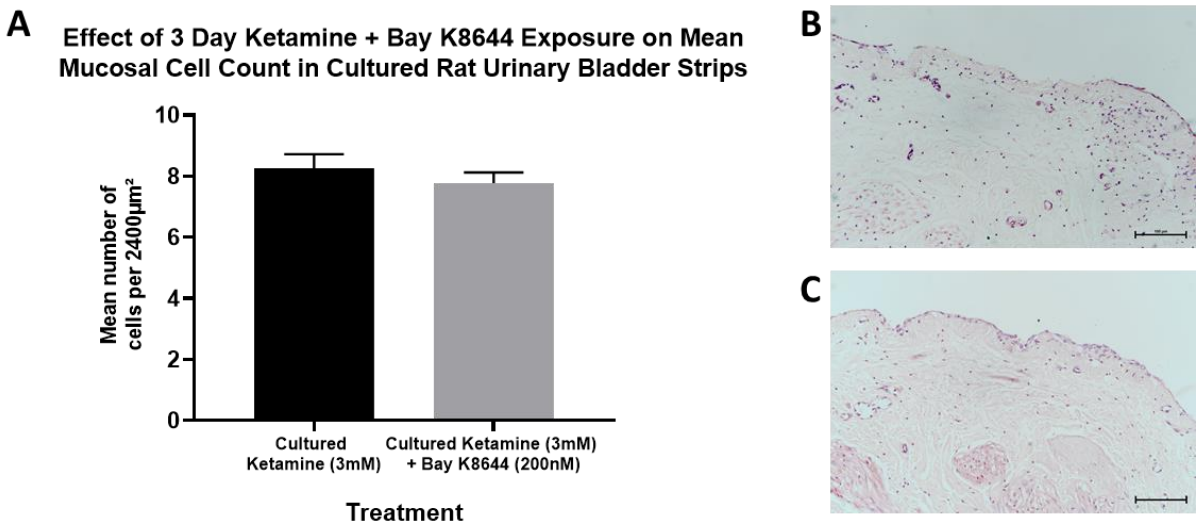

Figure 5.20. Effect 3-day 3mM ketamine and 200nM Bay K8644 exposure on mean apical mucosal cell count in cultured rat bladder strips. Matched half bladder strips were incubated for 3 days in tissue culture media containing 3mM ketamine, or tissue culture media containing 3mM ketamine and 200nM Bay K8644. Post incubation, strips were processed, embedded in paraffin wax and stained with haematoxylin and eosin. **A:** Graphical representation of mean mucosal cell count. Mean mucosal cell counts were quantified by counting the number of stained nuclei on the apical surface of the mucosa within an area of 2400µm. Data is expressed as mean number of cells per 2400µm ±SEM. Data represents *n*=3. Data were analysed using a paired two-tailed t-test. No significant difference was observed in the mean mucosal cell count of the treatments tested (P>0.05). **B,C:** Representative images of haematoxylin and eosin staining in 3-day cultured 3mM ketamine **(B)**, and 3-day cultured 3mM ketamine + 200nM Bay K8644 **(C)** matched half rat bladder sections. Magnification x200; scale bar represents 100µm.

5.4 DISCUSSION

5.4.1 Optimisation of Organ Culture Protocol

A novel tissue culture protocol was developed to investigate the longer-term direct effects of ketamine on rat urinary bladder. A composition of tissue culture media as described by Baker et al. (2016) was initially utilized; however, as there is limited information regarding the culture of rat bladder strips, experiments investigating different media compositions were

undertaken in an attempt to optimize the protocol and minimize any degenerative effects on the histological composition of the tissue.

5.4.1.1 Effect of Fetal Bovine Serum

The concentration of FBS (5% or 10%) present in the media did not affect the mean mucosal cell count after 3 days of culture (Figure 5.3A). Although not statistically significant, an FBS concentration of 10% was used for future experiments as the mean cell count with this concentration was slightly greater than that of tissues cultured with 5% FBS. The percentages of FBS used here are in line with the majority of *in vitro* bladder smooth muscle cell and urothelial cell culture experiments reported in the literature, which typically use between 2.5 and 10% (Zupančič, Poljšak & Kreft, 2017).

5.4.1.2 Tissue Culture Media

Mucosal cell count was not significantly different in bladder strips maintained in DMEM/RPMI 1640 when compared to Panexin (Figure 5.3B). For future experiments, DMEM/RPMI 1640 was used as the culture media as, although not statistically significant, bladder strips cultured with DMEM/RPMI 1640 had a higher mean mucosal cell count compared to those cultured in Panexin (8.82 ±1.26 and 6.28 ±0.69 cells per 2400µm, respectively; P=0.16).

A tissue culture media composed of DMEM/RPMI 1640 (50:50 mix) was successfully used by Baker et al. (2016) to culture human ureteric tissue; however, why this specific media composition was used was not discussed. There is very little information regarding the *ex vivo* culture of bladder tissue in the literature. Reports detailing the culture of urothelial cells and bladder smooth muscle cells utilize a wide range of different culture medias and compositions (Roby, Olsen & Nagatomi, 2008; Zupančič et al., 2017), with keratinocyte serum free media and DMEM being commonly used for the culture of urothelial cells. Keratinocyte serum free media was used by Baker et al. (2006) to culture human urothelial cells but not to culture human ureteric tissue, and so whether this media would be more effective than DMEM/RPMI 1640 in preserving the urothelium in the tissue culture assay presented here is unknown. Further work to determine whether different media compositions would help to minimise mucosal cell loss may be of benefit.

5.4.1.3 Retinoic Acid

The effect of RA on mean mucosal cell count in cultured rat bladder strips was investigated. Retinoic acid is important in the steady state maintenance of the adult urothelium, and RA signalling is required in the intermediate cells for regeneration of the urothelium after injury (Gandhi et al., 2013). Moreover, RA can increase urothelial cell expression of heparin-binding epidermal growth factor (Harris, Cheng & Liebert, 2008), which is a critical mediator of urothelial regeneration (Daher et al., 2003). Increased urinary levels of antiproliferative factor have been reported in ketamine-treated rats (Gu et al., 2014), and in patients with interstitial cystitis, which can inhibit production of heparin-binding epidermal growth factor from urothelial cells (Harris et al., 2009) and therefore potentially inhibit regeneration of the urothelium. However, in the *ex vivo* bladder culture protocol investigated here, RA (10µM) did not affect the mean mucosal cell count of bladder strips when used as a supplement to the culture media (Figure 5.3C).

5.4.1.4 Culture Duration

The effect of culture duration was also considered in the development of the tissue culture protocol. All culture durations tested resulted in a marked loss of apical mucosal cells compared to the uncultured control (Figure 5.4). The urothelium was present in uncultured rat bladder strips, identified by blue staining of the apical portion of the mucosa and an abundance of closely packed, large cells (Figure 5.5 & 5.6). The continuous urothelium observed in uncultured tissues was markedly thinner and patchy in rat bladder strips cultured for 1 or 3 days (Figure 5.5 & 5.6), and almost entirely absent in tissues cultured for 5 or 10 days, suggesting a time-dependent loss of urothelial cells (Figure 5.5 & 5.6). Sloughing of urothelium was noted in some 1- and 3-day cultured tissues (Figure 5.5), indicating that detachment of urothelial cells from underlying layers is at least partially responsible for the loss of these cells following culture. This is the first report of culture duration effects on rat bladder tissue. Mucosal sloughing has been reported in rats receiving daily ketamine (25mg/kg) injections for 14 days (Chuang et al., 2013); however, whether the mechanism of urothelial sloughing in *in vivo* treated animals is the same as that observed here is not known.

As bladders cultured for 5 or more days had a significantly reduced mean mucosal cell count compared to 1- or 3-day cultured tissues, but there was no difference in cell count between

1 and 3 days, the longer culture duration period of 3 days was implemented for future experiments to ensure minimal degeneration of cultured tissues, therefore optimising the length and sensitivity of the bioassay to determine longer-term drug effects.

5.4.2 Effect of Ketamine on Mean Mucosal Cell Count in Cultured Rat Urinary Bladder

Bladder strips exposed to 3mM ketamine for 3 days had a reduced number of cells in the apical mucosa compared to matched cultured controls (Figure 5.7). This effect appears to be concentration- and time-dependent, as 1mM ketamine exposure for 3 days and 3mM ketamine exposure for 1-day did not reduce mean cell count (Figures 5.7 & 5.9, respectively).

A loss of staining intensity on the apical surface of the mucosa was also noted in 3-day 3mM ketamine treated tissues, with observable thinning or denudation of the urothelium in multiple areas compared to control tissues (Figure 5.8). These findings are similar to those reported by Baker et al. (2016), where, although not quantified, 3mM ketamine exposure led to observable thinning of the urothelium in human ureteric organ cultures after 3 days. Baker et al. (2016) reported signs of apoptosis, including karyorrhexis (fragmentation of the nucleus) in H&E-stained tissues, which could explain the reduced mucosal cell count in ketamine-treated tissues seen here. Importantly, this shows similar histopathological effects of ketamine on both rat and human urothelium, suggesting that this model of direct toxicity by ketamine has translational value.

Loss of urothelium has also been reported in rats following daily intraperitoneal injections of ketamine (30mg/kg). After 4 weeks of treatment, H&E staining revealed areas of ulceration and degeneration, as well as a decreased thickness of the urothelium, in the bladders of albino rats (El-Azab, El-Mahalaway & Mostafa, 2020). Similarly, loss of urothelium was also observed in the bladders of rats following daily intraperitoneal injection of ketamine (30mg/kg) for 4 and 12 weeks (Wang et al., 2017). The findings reported here and in other *in vitro* reports of ketamine toxicity to urothelial cells therefore appear translatable *in vivo*. Hence, the novel *in vitro* assay presented here may provide the basis for a useful preclinical toxicity screen with higher throughput (3 days), and a reduced number of animals (n=3 per group), compared to lower throughput (>4 weeks) *in vivo* studies (n=6-10 per group), which is beneficial in drug discovery and in line with the 3Rs principals (replacement, reduction, and refinement).

5.4.2.1 (S)-Ketamine

Like ketamine, (S)-ketamine also reduced the mean mucosal cell count in rat bladder strips following 3 days exposure (Figure 5.13). This is believed to be the first report to describe direct toxic effects of (S)-ketamine on rat bladder mucosa.

Given the recent licensing of (S)-ketamine as a treatment for treatment-resistant depression, its direct effects on the bladder are important to consider, but have not been reported in the literature. Trials of (S)-ketamine in patients typically show little to no incidence of urinary dysfunction, however, one long-term observational study reported adverse bladder effects in 137 (19%) individuals (The United States Food and Drug Administration, 2019). Further research into potential bladder toxicity by (S)-ketamine would be of benefit to help determine its safety profile for clinical use. The direct effects of (S)-ketamine on the bladder are further explored in Chapter 6.

5.4.3 Effect of Norketamine and Dehydronorketamine on Mean Mucosal Cell Count in Cultured Rat Urinary Bladder

Exposure to 3mM NK reduced the mean mucosal cell count in cultured rat bladder strips after 3 days (Figure 5.10). Histological examination shows observable loss of urothelium in NK-treated tissues compared to the control, characterised by decreased staining intensity (suggesting a lack of nuclear staining), and loss of large cells within the apical mucosa (Figure 5.11). Norketamine toxicity has been reported *in vitro* in cultured normal human urothelial cells at concentrations of 0.5-6mM (Shahzad, 2011). Shahzad (2011) showed that NK was toxic to normal human urothelial cells in a concentration-dependent manner, and that the degree of toxicity was equal to that of ketamine. Similarly, another study reported that NK (0.1-0.3mM) induced apoptosis in human urothelial cells; however, it was reported that NK was more cytotoxic than ketamine (Lin et al., 2022).

Bladder strips exposed to 3mM DHNK for 3 days had a similar number of mucosal cells when compared to cultured control tissues (Figure 5.12). No studies have investigated the direct toxic effects of DHNK in rat bladder, and so the finding here suggests that DHNK is not cytotoxic to bladder mucosal cells.

The direct toxic effects of both ketamine and NK on urothelium raises the possibility that the two may have an additive effect that leads to the thinning or denudation of the urothelium as reported in KC patients. Simultaneous exposure of rat bladder to ketamine (30µM), NK (30µM), and DHNK (100µM) — concentrations which have been reported in the urine of recreational ketamine users (Moore et al., 2001) — did not, however, affect the mean mucosal cell count (Figure 5.14).

5.4.4 Effect of Ketamine-Related New Psychoactive Substances on Mean Mucosal Cell Count in Cultured Rat Urinary Bladder

5.4.4.1 Methoxetamine

Rat bladder tissue incubated with 3mM MXE for 3 days displayed a reduced number of apical mucosal cells compared to the vehicle-treated controls (Figure 5.15A). Histological examination revealed a decrease in apical staining intensity and sloughing of the urothelium in some samples after exposure to MXE (Figure 5.16 & 5.17). In line with these findings, urothelial thinning and mucosal degeneration were noted in the bladders of rats receiving daily intraperitoneal injections of MXE (30mg/kg) for 4 or 12 weeks (Wang et al., 2017). Further, mice treated daily with MXE (30mg/kg) displayed a higher density of mononuclear cell infiltration in both the submucosa and lamina propria compared to saline controls (Dargan, Tang, Liang, Wood & Yew, 2014), which are similar to the reported effects of KC in humans (Chu et al., 2008). Taken together, these findings and those presented here suggest a direct toxic effect of MXE on urothelial cells, refuting the claim that MXE is a 'bladder friendly' alternative to ketamine. Additionally, the MXE effect seen here further supports the application of this novel bioassay for preclinical toxicological screening of ketamine-related NPS in place of lower throughput *in vivo* screening.

5.4.4.2 Methoxphenidine and Diphenidine

Exposure to either MXP or DPD (3mM) reduced the mean mucosal cell count in cultured rat urinary bladder (Figure 5.15B&C). Reduced apical staining and sloughing of the urothelium was also noted in drug-treated strips compared to the vehicle controls (Figure 5.16 & 5.17). This is the first report to show loss of mucosal cells following direct exposure to MXP or DPD. Given the apparent translatability of the tissue culture assay used here to reported *in vivo*

data, it is plausible to suggest that MXP and DPD may also have direct toxic effects on bladder urothelium *in vivo.*

5.4.5 Effect of MK-801 on Mean Mucosal Cell Count in Cultured Rat Urinary Bladder

There was no difference in the number of mucosal cells in rat bladder strips cultured with MK-801 compared to control cultured tissues (Figure 5.18; P=0.089), and this result is consistent with the finding that MK-801 (≤100µM) was not cytotoxic to human urothelial cells (Baker et al., 2016).

5.4.6 Histopathological Changes Following Ketamine Exposure

As well as thinning or denudation of the urothelium, histological examination of the bladders of KC patients often reveals eosinophil and mast cell infiltration in the suburothelium (Shahani et al., 2007; Jhang et al., 2015). Similarly, in ketamine-treated animal models, immune cell infiltration in the suburothelial tissue has also been reported in rats (Lee et al., 2019; El-Azab et al., 2020), with increased mast cell infiltration observed in both rats (Wang et al., 2017) and mice (Li et al., 2005). Toluidine blue is a stain that has been used to assess the degree mast cell infiltration in animal models of KC (Wang et al., 2017), however this has not been investigated here as the *ex vivo* assay does not model downstream inflammatory mechanisms.

The microvasculature of the bladder is often altered in KC patients, with histological and cystoscopic studies revealing an increased vascular density and distribution (Xie et al., 2021). This formation of new blood vessels (angiogenesis) has also been reported in mice following daily ketamine treatment (100mg/kg for 20 weeks) and was associated with an upregulation of two angiogenesis-related genes (Shen et al., 2016). There are conflicting reports regarding the effects of ketamine on angiogenesis in rats, however, with one study reporting a decrease in angiogenic markers following ketamine treatment (30mg/kg/day for 12 weeks; Lu et al., 2021), and another indicating the development of angiogenesis and hypervascularity following daily ketamine injections (50mg/kg) for 16 weeks (Gu et al., 2014). This discrepancy could potentially be explained by the different concentrations of ketamine used and the length of the study.

Angiogenesis is thought to be intrinsic to chronic inflammation and many inflammatory cells are able to release numerous angiogenic factors, including vascular endothelial growth factor, and platelet derived growth factor (Xie et al., 2021). In the case of KC, immune cell infiltration therefore likely plays a role in the development of angiogenesis.

While the immune response observed in KC patients has been linked to its pathogenesis, it appears unlikely that it plays a major role in the thinning or denudation of the urothelium seen here and in other reports. It was demonstrated using a human tissue engineered model of KC that direct exposure to ketamine induced apoptosis of urothelial cells and affected the structure of the urothelium in a concentration-dependent manner (Bureau et al., 2015). Interestingly, these effects were observed in the absence of a vascular or immune system – things that the tissue model lacked. This suggests that the urothelial changes often reported in KC patient and animal models are unlikely to be a result of immunologic or vascular factors, but rather are observed as a result of direct exposure of ketamine with the urothelium (Bureau et al., 2015).

Evidence of apoptosis following ketamine exposure has been demonstrated both *in vitro* and *in vivo* in human and animal models. Haematoxylin and eosin staining of human urothelial cells exposed to ketamine showed urothelial thinning with evidence of pyknotic nuclei (condensed nuclei) and karyorrhexis (Baker et al., 2016). Similarly, urothelial degeneration with evidence of karyolysis (chromatin dissolution) and vacuolisation was observed in rats following 4 weeks of daily ketamine injections (El-Azab et al., 2020). Such findings indicate a direct toxic effect of ketamine on urothelial cells. Evidence of karyorrhexis was observed in both 3-day control and ketamine-treated tissues (Figure 5.8), and so apoptosis as a potential explanation for loss of mucosal cells is explored in Chapter 6.

5.4.7 Mechanism of Mucosal Cell Toxicity

Although not directly explored here, some insight may be gained regarding the potential mechanisms behind the apparent loss of mucosal cells following exposure to ketamine or other related compounds. As explored in Chapter 3, ketamine and the ketamine-related compounds tested here are all reported to act as NMDAR antagonists (Table 5.1), and there is evidence to suggest that many are also antagonists of L-type calcium channels (Table 5.2).

Table 5.1. Radioligand binding data of ketamine and the related compounds investigated here. All data are from [^{3}H]-MK-801 binding displacement assays in rat.

Compound	K_i ± SEM (µM)	Tissue/System	Reference
Ketamine	0.3239 ± 0.0192	Forebrain	Wallach et al. (2016)
	0.53 ± 0.078	Brain homogenate	Roth et al. (2013)
	0.119 ± 0.01	Brain homogenate	Ebert et al. (1997)
	0.659	Cortex homogenate	Sałat et al. (2015)
Norketamine	3.6 ± 0.49	Brain homogenate	Ebert et al. (1997)
	0.97 ± 0.1	Cortex homogenate	Sałat et al. (2015)
Dehydronorketamine	3.21 ± 0.3	Cortex homogenate	Sałat et al. (2015)
(S)-Ketamine	0.30 ± 0.013	Brain homogenate	Ebert et al. (1997)
Dextromethorphan	2.21 ± 0.8	Hippocampus	Werling et al. (2007)
MK-801	0.0019 ± 0.0010	Brain homogenate	Ebert et al. (1997)
Methoxetamine	0.259	Brain homogenate	Roth et al. (2013)
Diphenidine	0.0182 ± 0.0022	Forebrain	Wallach et al. (2016)
Methoxphenidine	0.036 ± 0.0037	Forebrain	Wallach et al. (2016)

5.4.7.1 N-Methyl D-Aspartate Receptor Antagonism

Given that MK-801, the most potent NMDAR antagonist tested here (Table 5.1), did not affect the mucosal cell count, but other, lower affinity compounds such as ketamine and NK did, it seems unlikely that the bladder toxicity seen here is mediated through NMDAR antagonism. Further, MK-801 (≤100µM) did not trigger any toxicity in normal human urothelial cells, and pre-treatment with D-serine and NMDA was unable to block ketamine-induced toxicity (Baker et al., 2016). Expression of NMDAR subunits were also not reported in freshly isolated human urothelium (Baker et al., 2016), so it is questionable whether ketamine present in the urine would come into contact with bladder NMDAR *in vivo* given the barrier function of the urothelium. However, in the tissue culture assay presented here, the bladder was dissected in half allowing ketamine present in the culture media to come into direct contact with

multiple layers of the bladder. Therefore, it is possible that ketamine is able to interact with bladder NMDAR in this instance. However, taken together, it seems unlikely that ketamine-(and related compound) induced toxicity is mediated through NMDAR antagonism.

5.4.7.2 L-type Calcium Channel Antagonism

The acute inhibitory effects of ketamine and related compounds on rat bladder contractility observed in Chapter 3 are postulated to be a result of L-type calcium channel antagonism. There is evidence that many of the compounds tested here can inhibit Ca^{2+} influx through L-type channels in multiple different tissues and species (Table 5.2).

Table 5.2. Evidence of calcium channel antagonism by ketamine or the ketamine-related compounds investigated here.

Compound	Evidence	Tissue/System	Reference
Ketamine	Caused concentration-dependent ↓ in force and $[Ca^{2+}]$ evoked by acetylcholine	Canine tracheal smooth muscle	Pabelick et al. (1997)
	Concentration-dependently ↓ L-type calcium channel current	Bullfrog atrial myocytes	Hatakeyama et al. (2001)
	↓ ileum contractility which could be restored by Bay K8644	Guinea pig ileum	Hirota et al. (1996)
	Caused ↓ of peak L-type calcium channel current (1mM ketamine)	Pig tracheal smooth muscle	Yamakage et al. (1995)
	Concentration-dependent ↓ of peak L-type voltage-dependent Ca^{2+} current (0.1-1mM ketamine)	Rabbit portal vein smooth muscle	Yamazaki et al. (1995)
	↓ calcium influx and calcium signalling induced by Bay K8644, KCl, carbachol, and ATP	Cultured mouse and human bladder smooth muscle cells	Chen et al. (2020)
(S)-Ketamine	↓ in Ca^{2+} uptake evoked by potassium or U46619	Bovine cerebral arteries	Kamel et al. (2008)
Dextromethorphan	↓ in Ca^{2+} uptake evoked by potassium or U46619	Bovine cerebral arteries	Kamel et al. (2008)
MK-801	Speculative	Rat hippocampus	Schurr et al. (1995)
Methoxetamine	↓$[Ca^{2+}]$ in human neuronal cell line evoked by acetylcholine or potassium; but ↑$[Ca^{2+}]$ in rat primary cortical cells evoked by glutamate	Human neuronal culture	Honderbrink et al. (2017)

Ketamine and the Cav1.2 antagonist nifedipine were reported to inhibit proliferation of human bladder smooth muscle cells and induce cell death at high concentrations (Chen et al., 2020). Moreover, the ability of ketamine and nifedipine to reduce the number of Ki67-positive cells (a marker of proliferating cells) could be reversed by treatment with the L-type calcium channel agonist Bay K8644 (Chen et al., 2020). The bladder toxicity observed is thought to be mediated through inhibition of Cav1.2 signalling, which can inhibit upregulation of the immediate early genes c-fos and c-jun which are important for cell survival, proliferation, and differentiation (Chen et al., 2020). Therefore, the idea that ketamine-induced toxicity of mucosal cells is mediated through L-type calcium channel antagonism was explored, and the effects of 3-day ketamine (3mM) culture on smooth muscle are reported in Chapter 6.

Nifedipine (10μM) did not induce a loss of mucosal cells in rat bladder strips after 3 days (Figure 5.19). Further, mucosal cell loss induced by ketamine could not be reversed by co-incubating with Bay K8644 (Figure 5.20). Therefore, it seems unlikely that loss of mucosal cells following 3-day exposure to ketamine and ketamine-related compounds can be explained by L-type calcium channel antagonism. MK-801-induced bladder toxicity was also not shown here, and while it is speculated to be an L-type antagonist in rat hippocampus (Schurr, Payne & Rigor, 1996), no experimental data was found that has confirmed antagonistic action of L-type channels.

5.4.7.3 Prolonged Elevation of Cytosolic Calcium

As described previously, thinning of the urothelium and evidence of urothelial cell apoptosis was observed in human ureteric organ cultures following three-day ketamine exposure (Baker et al., 2016). It was reported that the ketamine-induced cytotoxicity was associated with prolonged elevation of cytosolic calcium which ultimately led to mitochondrial depolarisation, release of cytochrome c, and apoptosis via caspase activation. Ketamine is proposed to induce the release of ATP from urothelial cells (via an unknown mechanism), which binds P2Y receptors on their cell surface and causes IP_3-mediated release of calcium from the endoplasmic reticulum. Concentrations of ketamine ≤1mM were shown to be cytostatic and induced a calcium transient that returned to baseline after approximately 90 seconds, whereas >1mM ketamine was cytotoxic to urothelial cells and elicited a larger increase in cytosolic calcium that remained unresolved after ten minutes. The source of calcium that

delivers this prolonged elevation following >1mM ketamine exposure, however, was not discovered.

The observation here that 3mM, but not 1mM, ketamine led to a loss of mucosal cells is in agreement with the findings presented by Baker et al. (2006) and suggests that, although 1mM ketamine is not directly toxic to urothelial cells, it may still participate in the pathogenesis of KC by preventing regeneration of the urothelium.

5.4.8 Clinical Relevance

5.4.8.1 Concentration of Ketamine in the Urine

The clinical relevance of the high concentrations of ketamine used here is discussed in section 3.4.6.1. A ketamine concentration of 3mM was shown to significantly reduce mean mucosal cell count in rat bladder strips (Figure 5.7) and is higher than the concentration of ketamine required to induce functional changes in Chapter 3 (≥300µM).

As mentioned previously, a urine concentration of ketamine in excess of 1mM is theoretically possible in individuals taking high doses (≥1g) of ketamine (Baker et al., 2016). One ketamine use survey reported that 34% of individuals regularly take >1g, and 5.2% regularly take ≥3g in a typical session (Winstock et al., 2012). Moreover, regarding the maximum amount of ketamine used in a single session, 24.6% had taken ≥3g, and 6.6% had taken ≥5g. Therefore, it is not uncommon for ketamine users to take well in excess of 1g at a time. In the same study, it was reported that most individuals were classified as low frequency ketamine users, taking the drug 1-4 days per month. However, 29% of users were said to take ketamine more than once per week, and a survey amongst ketamine users in Taiwan reported that the vast majority (92.9%; n=106) took ketamine several times per day (Li et al., 2019). Given that ketamine can be detected in the urine for days to weeks, it is possible that in higher frequency users, ketamine concentrations in the urine could accumulate. Therefore, in individuals frequently taking large amounts of ketamine, a urine concentration of 3mM is theoretically possible. Studies investigating the concentrations of ketamine in the urine of recreational users lack details regarding the amount and frequency of use, and also the interval between the time of the urine sample and last drug use. As the elimination half-life of ketamine is 2-3 hours (Mion & Villevieille, 2013), peak ketamine concentrations in the urine may occur within hours after taking the drug. Therefore, reports detailing urine ketamine concentrations in

users shortly after taking the drug would help ascertain whether the concentrations of ketamine used here are clinically relevant.

5.4.9 Limitations

The initial intent of this chapter was to assess the direct effects of ketamine and related compounds on the urothelium. The urothelium of uncultured rat bladder tissue is easily identifiable through the blue apical staining and the presence of stratified, somewhat larger cells (Figure 5.1 & 5.2). Upon culturing the tissue however, these characteristic features can be degraded or entirely lost. It is unclear whether under these pathological conditions the loss of these identifying features necessarily equates to the specific loss of urothelium, especially when considering that H&E staining does not definitively distinguish between cell types. Using immunohistochemistry to stain for specific urothelial cell markers would help to address this issue.

As the cell counts presented here were not performed blind, the potential for bias arises. This issue was somewhat offset, however, by the automation of the cell count through the use of ImageJ. Additionally, the placement of three of the five 2400µm boxes (within which the cell counts took place) were consistent throughout all images, as they were positioned to the extreme left, extreme right, and middle of the urothelium. Regardless, quantification of bladder toxicity through use of a blind cell count would have been preferable.

There is a potential for the cell count of some tissues to be inflated due to the use of the ImageJ 'watershed' tool. In some images, stained nuclei can be closely grouped and appear to be touching or overlapping. As all images are converted to binary, overlapping of multiple cells would normally be counted as a single cell. The watershed tool attempts to identify such occurrences and draws a one-pixel line between the cells to separate them so that they are not misidentified as a single cell. In some instances, however, the tool will divide up a single cell meaning that it will be incorrectly counted as multiple cells. However, as all images undergo the same process prior to the cell count the impact this may have when comparing cell counts between tissues is minimized.

5.4.10 Future Work and Conclusions

Loss of urothelial barrier function is commonly postulated to be one of the major mechanisms behind KC and its associated urological symptoms. The tissue culture protocol developed here suggests that exposure to ketamine, NK and ketamine-related NPS (MXE, MXP, DPD) leads to a loss of mucosal cells in the rat bladder. Although the mechanism of action behind the loss of mucosal cells has not been identified, it seems likely that it is not mediated through the NMDAR as exposure to the most potent NMDA antagonist tested here (MK-801) did not alter the mucosal cell count compared to control-cultured tissues. Exposure to nifedipine also did not affect the mucosal cell count, indicating that L-type calcium channel antagonism does not explain loss of mucosal cells following exposure to ketamine, NK, or related NPS. The fact that Bay K8644 could not reverse loss of mucosal cells induced by ketamine further strengthens this idea.

Although H&E staining is a very useful tool for investigating the structure of the bladder following ketamine exposure, it does not allow for identification of specific cell types. Utilizing immunohistochemistry techniques will allow for staining of specific proteins of interest. For example, uroplakin III (a specific marker of urothelial umbrella cells) could be utilised to determine whether the loss of bladder mucosal cells by direct exposure to ketamine equates to a loss of umbrella cells. Further, an antibody to cleaved cytokeratin 18 has been previously employed as a marker of apoptosis in human ureteric organ cultures following ketamine exposure (Baker et al., 2016), and could therefore be used here to assess the degree of apoptosis in ketamine-treated tissues, potentially providing insight into why we see a widespread loss of mucosal cells following ketamine exposure. Staining for proteins that play a role in the integrity of the urothelial layer, such as zonula occludens-1, would also be of benefit, and provide insight into whether the barrier function is altered and how.

Chapter 6: Immunohistochemical Effects of Ketamine and Related Compounds in Rat Bladder

6.1 INTRODUCTION

In Chapter 5, histological evaluation via haematoxylin and eosin (H&E) staining revealed a loss of mucosal cells in rat bladder strips following exposure to ketamine and other ketamine-related compounds. Indeed, a previous report showed urothelial thinning in human ureteric tissue following ketamine exposure (Baker, Shabir, Georgopoulos & Southgate, 2016), and rats injected with either ketamine or methoxetamine (MXE) over a four-week period were shown to display thinning of the urothelium (Wang et al., 2017).

In this context, H&E staining is a useful tool for investigating the overall morphological and cellular structure of the bladder, and identifying any pathological changes associated with ketamine exposure. It is limited, however, in that it does not definitively distinguish between specific cell types. Immunohistochemistry (IHC) is a technique which allows for the staining of specific proteins of interest, and this has helped provide insight into the phenomenon of ketamine-induced cystitis (KC).

Disruption of the urothelium by ketamine is commonly cited as a potential mechanism for KC, and immunohistochemical examination of the bladders of ketamine-treated animals has revealed altered expression and/or distribution of several tight junction-associated proteins. Lee et al. (2017) looked at the effects of 4 weeks of daily ketamine injections (30mg/kg) in rats and investigated the levels of two such proteins: zonula occludens-1 (ZO-1) and claudin-4. Ketamine-treated rats displayed a reduced expression of both ZO-1 and claudin-4, and in a similar study, a reduced expression of these proteins was reported in ketamine-treated rats (25mg/kg) after just 14 days (Liu et al., 2015). Decreased expression of ZO-1 and E-cadherin (a glycoprotein involved in intercellular adhesion) has also been reported in the bladders of KC patients (Tsai, Birder & Kuo, 2016), and abnormal distribution of ZO-1 in the bladder was observed in mice following daily ketamine injections for four weeks (Duan et al., 2017).

The apical urothelial layer, which is known as the umbrella cell layer, plays an important role in the barrier function of the urothelium. Uroplakin III is a protein that is an umbrella cell-specific marker, and so investigating the effects of ketamine on UPIII antibody immunoreactivity would determine whether ketamine exposure leads to loss of umbrella cells. Further, immunohistochemical staining of cytokeratin 7 (Krt7), a protein expressed in all layers of the urothelium, would reveal whether ketamine has a direct toxic effect on urothelial

cells in general. Such investigations have not yet been reported but would help to determine whether the loss of mucosal cells following ketamine exposure observed in Chapter 5 could be explained by loss of urothelial cells.

Immunohistochemical evaluation of human ureteric organ cultures following exposure to ketamine for three days showed immunoreactivity with a cleaved cytokeratin 18 antibody (M30) in cells sloughing from the urothelium (Baker et al., 2016), suggesting the importance of apoptosis in the thinning or denudation of the urothelium that is commonly observed in KC.

In some animal models of KC, ketamine also induced loss of smooth muscle (Tan et al., 2011), and high concentrations of ketamine have been reported to lead to cell death of human bladder smooth muscle cells (Chen et al., 2020). Therefore, immunohistochemical staining of smooth muscle myosin heavy chain 11 (myosin-11), a protein expressed in smooth muscle cells, would be of interest to determine whether ketamine exposure leads to a loss of smooth muscle cells in rat bladder.

$Ca_v1.2$ is also another interesting target for immunohistochemical evaluation. This protein was identified in Chapters 3 & 4 as a likely receptor target of ketamine and has also been purported to be an important target in the pathogenesis of KC (Chen et al., 2020). Thus, the effects of ketamine exposure on $Ca_v1.2$ expression were explored.

The aim of this chapter is to utilize immunohistochemical techniques to evaluate the expression of several proteins of interest in rat bladder strips following exposure to ketamine and other ketamine-related compounds. It is hypothesized that exposure to ketamine and its metabolites will lead to a reduced expression of tight junction-associated proteins and UPIII, and an increased expression of cleaved cytokeratin 18 in rat bladder mucosa.

The objectives for this chapter are as follows:

- To determine the effects of ketamine, its metabolites, (S)-ketamine, methoxetamine, MK-801, and nifedipine on the umbrella cell layer via immunohistochemical staining of the umbrella cell specific protein uroplakin III.

- To investigate the effects of ketamine, (S)-ketamine, norketamine, methoxetamine, and diphenidine on the urothelium through immunohistochemical staining of cytokeratin 7.
- To assess the degree of apoptosis in ketamine-treated bladder tissues via immunolabelling of cleaved cytokeratin 18.
- To determine the effect of ketamine on the expression of the tight junction-associated protein zonula occludens-1 using immunohistochemistry.
- To assess whether ketamine induces loss of smooth muscle in rat bladder through immunohistochemical staining of smooth muscle myosin heavy chain 11.
- To determine whether ketamine exposure affects the expression of $Ca_V1.2$ through immunohistochemical staining with anti-CACNA1C antibody.

6.2 METHODS

Whole rat bladders were dissected in half via a longitudinal incision through the centre of the ventral wall. One strip was incubated for up to three days in tissue culture media and ketamine (up to 3mM) or a related compound of interest, while the other strip acted as a time-matched vehicle control.

After the desired incubation period, tissues were fixed in formalin, dehydrated through graded alcohols, cleared in xylene, and embedded in paraffin wax. Tissue sections were incubated with primary antibody and visualised using a diaminobenzidine substrate reaction.

Immunostained tissue sections were visualised using light microscopy, and images were captured at x200 magnification along the urothelium (for UPIII, Krt7, M30, ZO-1, and $Ca_V1.2$), or the smooth muscle layer (for myosin-11 and $Ca_V1.2$). The images were then processed in ImageJ and the mean optical density was calculated for each image.

For full methodology, pleases refer to section 2.8.

6.3 RESULTS

6.3.1 Bladder Strips Exposed to 1mM or 3mM Ketamine for Twenty Minutes Displayed Uroplakin III Expression Levels Similar to Control Bladder Tissues

As acute (twenty-minute) exposure to ketamine induced functional changes in rat bladder contractility (Chapter 3), the ability of acute ketamine exposure to alter UPIII expression was investigated. The mean optical density of UPIII staining in bladder tissues exposed to 1mM or 3mM ketamine for twenty minutes was similar to that of uncultured tissues (P>0.05; Figure 6.1). Additionally, there was no difference in the mean optical density of UPIII staining between tissues cultured with 1mM or 3mM ketamine following twenty minutes exposure (P>0.05; Figure 6.1).

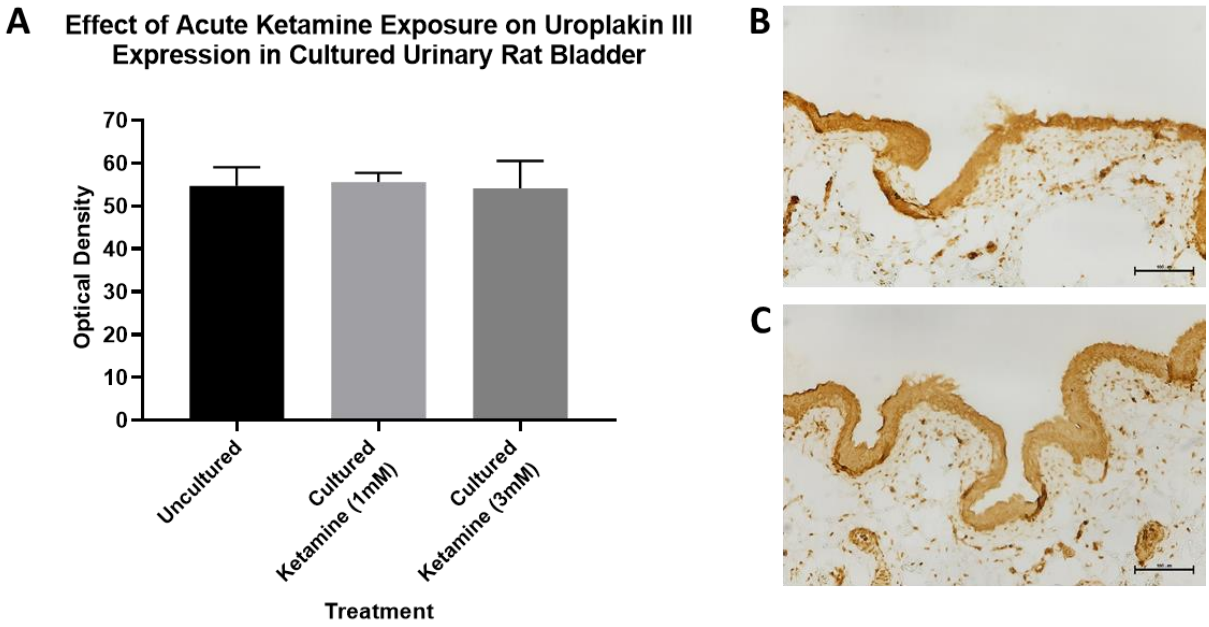

Figure 6.1. Effect of twenty-minute ketamine exposure on uroplakin III expression in rat bladder strips. Matched half bladder strips were incubated for twenty minutes in oxygenated Krebs-Henseleit solution containing 1mM or 3mM ketamine. Post incubation, strips were processed, embedded in paraffin wax, and visualised using immunoperoxidase labelling to UPIII antibody. **A:** Graphical representation of UPIII staining intensity. Expression of UPIII was quantified by calculating the mean optical density of staining per tissue section. Data is expressed as mean optical density ±SEM. Uncultured group represents *n=6*; cultured ketamine (1mM) *n=3;* cultured ketamine (3mM) *n=4*. Data were analysed using a one-way ANOVA with a Bonferroni post-test (uncultured vs cultured ketamine), or a two-tailed paired t-test (1mM cultured ketamine vs 3mM cultured ketamine). No significant difference was observed in the mean optical density of the treatments tested (P>0.05). **B,C:** Representative images of immunoreactivity with UPIII antibody in cultured 1mM ketamine **(B)**, and cultured 3mM ketamine **(C)** rat bladder sections. Magnification x200; scale bar represents 100µm.

6.3.2 Cultured Bladder Strips Express Less Uroplakin III Than Uncultured Bladder Tissues

To establish the effects of 3-day ketamine exposure in rat bladder, tissues were incubated for 3 days in culture media. Uncultured rat bladder tissues were also taken and examined to obtain a control value of UPIII expression to enable comparison between uncultured and cultured tissues. A markedly decreased expression of UPIII was observed in cultured control tissues compared to uncultured tissues (P<0.001; Figure 6.2).

6.3.3 Ketamine Decreased Uroplakin III Expression in Cultured Urinary Rat Bladder

Bladder strips exposed to 3mM ketamine for 3 days had a reduced expression of UPIII compared to the 3-day cultured vehicle control tissue strips (P<0.01; Figure 6.2), and also compared to uncultured control tissues (P<0.0001; Figure 6.2). An observable loss of UPIII staining was noted in the urothelium of ketamine treated tissues compared to matched cultured control and to uncultured tissues (Figure 6.3).

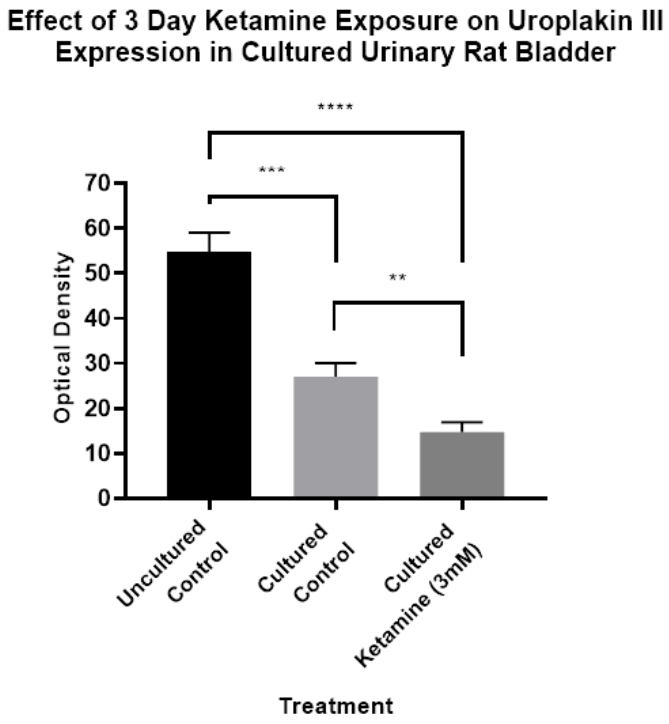

Figure 6.2. Effect of 3-day ketamine exposure on mean optical density of uroplakin III in rat bladder strips. Matched half bladder strips were incubated for 3 days in tissue culture media alone, or tissue culture media containing 3mM ketamine. Post incubation, strips were processed, embedded in paraffin wax, and visualised using immunoperoxidase labelling to UPIII antibody. Expression of UPIII was quantified by calculating the mean optical density of staining per tissue section. Data is expressed as mean optical density ±SEM. Uncultured group represents *n=6*; cultured control and cultured ketamine *n=5*. **P<0.01; ***P<0.001; ****P<0.0001 significantly different from the specified treatment. Data were analysed using a one-way ANOVA with a Bonferroni post-test (uncultured vs cultured control/cultured ketamine), or a two-tailed paired t-test (cultured control vs cultured ketamine).

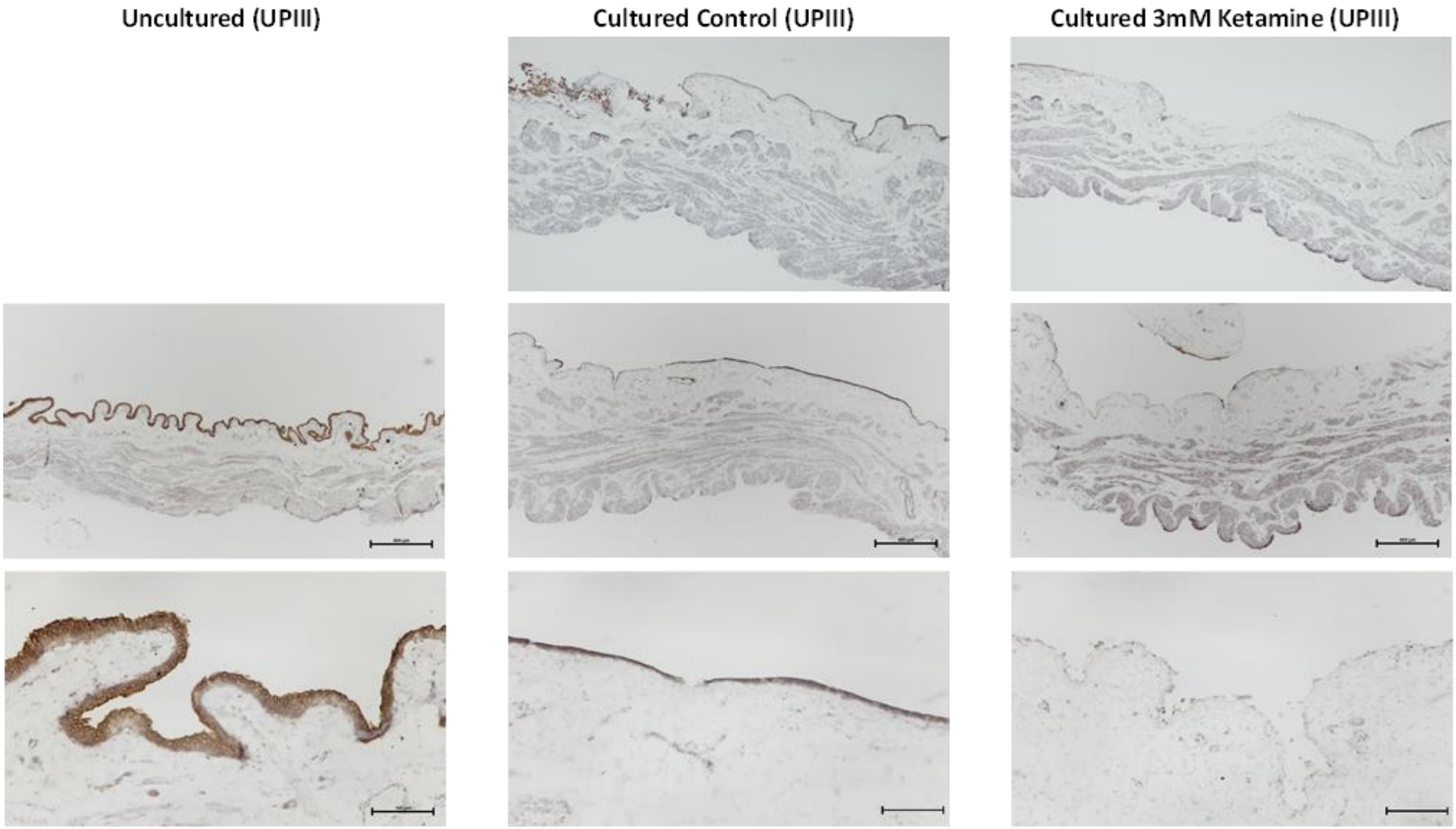

Figure 6.3. Immunohistochemical assessment of representative rat bladder sections following no treatment (uncultured), or 3 days culture with or without ketamine. Uncultured or post-cultured half bladder strips were processed, embedded in paraffin wax, and visualised using immunoperoxidase labelling to UPIII antibody. An observable loss of uroplakin III staining was seen in cultured tissues compared to uncultured, and decreased uroplakin III staining was also seen in 3-day 3mM ketamine treated tissues compared to 3-day cultured control tissues. Magnification x40, scale bar represents 500µm (top two rows); magnification x200, scale bar represents 100µm (bottom row).

6.3.4 (S)-Ketamine Decreased Uroplakin III expression in Cultured Urinary Rat Bladder

Exposure to 3mM (S)-ketamine for 3 days significantly reduced UPIII expression in rat bladder strips compared to 3-day cultured control tissues (P<0.05; Figure 6.4A). An observable loss of UPIII staining was noted in the urothelium of (S)-ketamine treated bladder strips compared to cultured control tissues (Figure 6.4B&C).

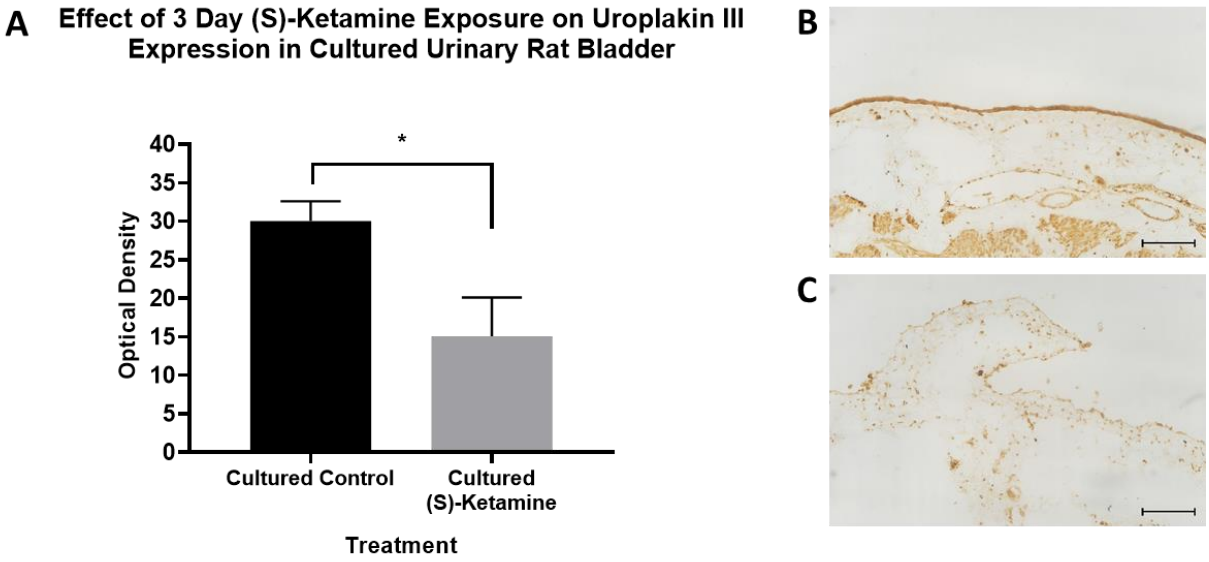

Figure 6.4. Effect of 3-day (S)-ketamine exposure on uroplakin III expression in rat bladder strips. Matched half bladder strips were incubated for 3 days in tissue culture media alone, or tissue culture media containing 3mM (S)-ketamine. Post incubation, strips were processed, embedded in paraffin wax, and visualised using immunoperoxidase labelling to UPIII antibody. **A:** Graphical representation of UPIII staining intensity. Expression of UPIII was quantified by calculating the mean optical density of staining per tissue section. Data is expressed as mean optical density ±SEM. Cultured control group represents *n=9*; cultured (S)-ketamine *n=3*. *P<0.05 significantly different from the cultured control. Data were analysed using an unpaired two-tailed t-test. **B,C:** Representative images of immunoreactivity with UPIII antibody in cultured control **(B)**, and cultured 3mM (S)-ketamine **(C)** rat bladder sections. Magnification x200; scale bar represents 100µm.

6.3.5 The Ketamine Metabolites Norketamine and Dehydronorketamine Do Not Affect Uroplakin III Expression in Cultured Urinary Rat Bladder

Bladder strips incubated with 3mM norketamine (NK) for 3 days displayed a similar mean optical density of UPIII staining compared to their matched cultured control tissues (P>0.05; Figure 6.5A). Similarly, dehydronorketamine (DHNK) also had no effect on the mean optical density of UPIII staining when compared to cultured control tissues (P>0.05; Figure 6.5D).

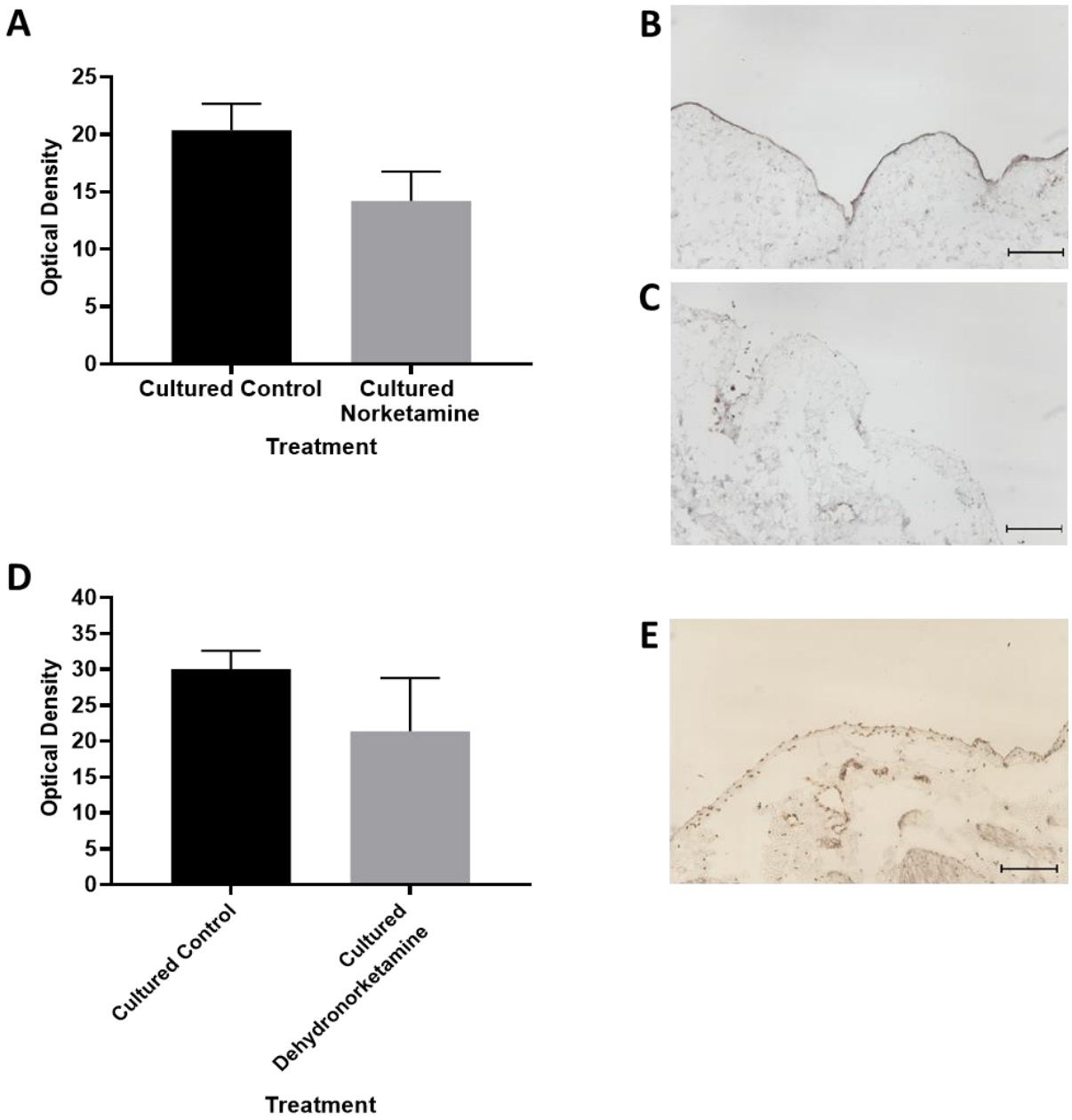

Figure 6.5. Effect of 3-day norketamine **(A,C)** or dehydronorketamine **(D,E)** exposure on uroplakin III expression in rat bladder strips. Matched half bladder strips were incubated for 3 days in tissue culture media alone, or tissue culture media containing 3mM norketamine or dehydronorketamine. Post incubation, strips were processed, embedded in paraffin wax, and visualised using immunoperoxidase labelling to UPIII antibody. **A&D:** Graphical representation of UPIII staining intensity. Expression of UPIII was quantified by calculating the mean optical density of staining per tissue section. Data is expressed as mean optical density ±SEM. Cultured control and cultured norketamine group represents *n=3* **(A)**; Cultured control group represents *n=9*, cultured dehydronorketamine group represents *n=3* **(D)**. Data were analysed using a paired two-tailed t-test **(A)**, or an unpaired two-tailed t-test **(D)**. No significant difference was observed in the mean optical density of the treatments tested (P>0.05). **B,C,E:** Representative images of immunoreactivity with UPIII antibody in cultured control **(B)**, cultured 3mM norketamine **(C)**, and cultured 3mM dehydronorketamine **(E)** rat bladder sections. Magnification x200; scale bar represents 100µm.

6.3.6 Bladder Strips Exposed to a Combination of Ketamine, Norketamine, and Dehydronorketamine Had a Similar Uroplakin III Expression to Control Tissues

As both ketamine and its major metabolites NK and DHNK have been detected in the urine of ketamine users, all three compounds were tested simultaneously to determine whether they had an effect on UPIII expression. Exposure to ketamine (100µM), NK (100µM), and DHNK (300µM) did not significantly alter the mean optical density of UPIII staining compared to control bladder strips (P>0.05; Figure 6.6).

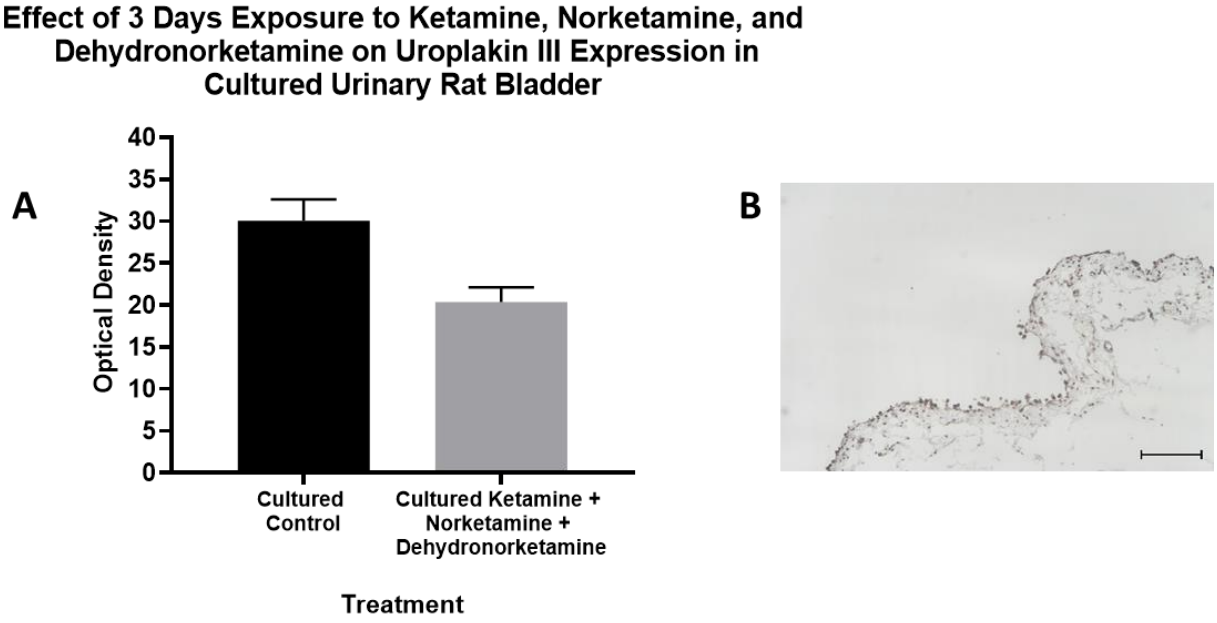

Figure 6.6. Effect of 3-day ketamine, norketamine, and dehydronorketamine exposure on uroplakin III expression in rat bladder strips. Matched half bladder strips were incubated for 3 days in tissue culture media alone, or tissue culture media containing 100µM ketamine, 100µM norketamine, and 300µM dehydronorketamine. Post incubation, strips were processed, embedded in paraffin wax, and visualised using immunoperoxidase labelling to UPIII antibody. **A:** Graphical representation of UPIII staining intensity. Expression of UPIII was quantified by calculating the mean optical density of staining per tissue section. Data is expressed as mean optical density ±SEM. Cultured control group represents *n=9*; Cultured ketamine + norketamine + dehydronorketamine *n=3*. Data were analysed using an unpaired two-tailed t-test. No significant difference was observed in the mean optical density of the treatments tested (P>0.05). **B:** Representative image of immunoreactivity with UPIII antibody in rat bladder sections following simultaneous exposure to ketamine (100µM), norketamine (100µM), and dehydronorketamine (300µM) for 3 days. Magnification x200; scale bar represents 100µm.

6.3.7 Methoxetamine Does Not Affect Uroplakin III Expression in Cultured Rat Urianry Bladder

Bladder strips cultured with MXE for 3 days displayed a similar optical density of UPIII staining to the cultured vehicle control tissues (P>0.05; Figure 6.7).

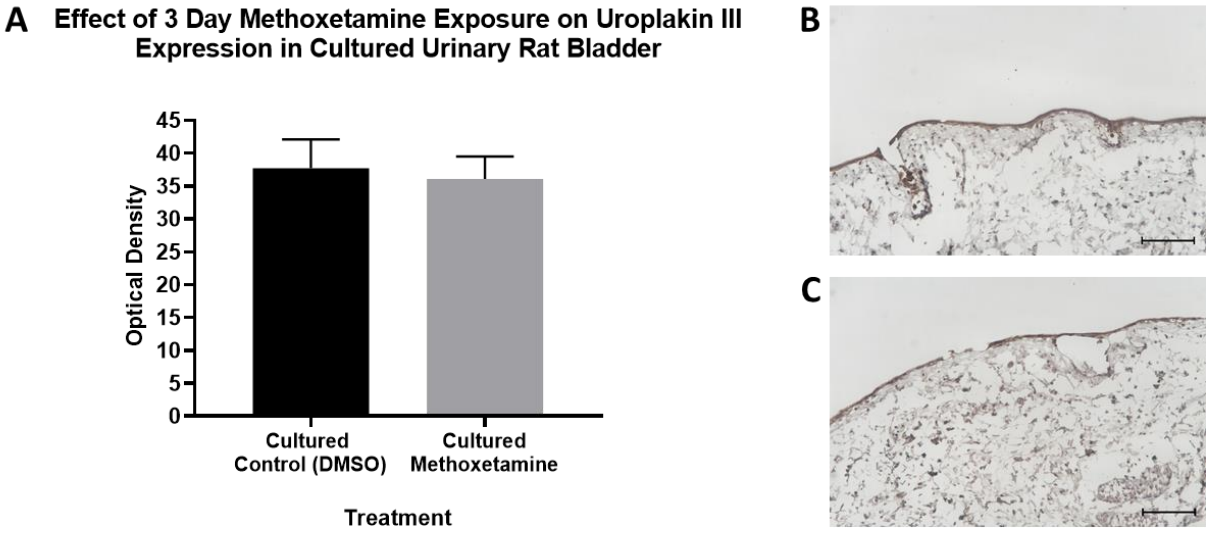

Figure 6.7. Effect of 3-day methoxetamine exposure on uroplakin III expression in rat bladder strips. Matched half bladder strips were incubated for 3 days in tissue culture media and 3% dimethyl sulfoxide (cultured control), or tissue culture media containing 3mM methoxetamine. Post incubation, strips were processed, embedded in paraffin wax, and visualised using immunoperoxidase labelling to UPIII antibody. **A:** Graphical representation of UPIII staining intensity. Expression of UPIII was quantified by calculating the mean optical density of staining per tissue section. Data is expressed as mean optical density ±SEM. Cultured control (DMSO) and cultured methoxetamine group represents *n=5*. Data were analysed using a paired two-tailed t-test. No significant difference was observed in the mean optical density of the treatments tested (P>0.05). **B,C:** Representative images of immunoreactivity with UPIII antibody in cultured control **(B)**, and cultured 3mM methoxetamine **(C)** rat bladder sections. Magnification x200; scale bar represents 100µm.

6.3.8 (+)-MK-801 Does Not Affect Uroplakin III Expression in Cultured Rat Urinary Bladder

Exposure to 3mM (+)-MK-801 for 3 days did not significantly alter the mean optical density of UPIII staining in bladder tissues compared to the cultured vehicle control (P=0.093; Figure 6.8A). The effects of (-)-MK-801 on UPIII expression were also tested, however the sample size was not sufficient for statistical analysis (Figure 6.8D).

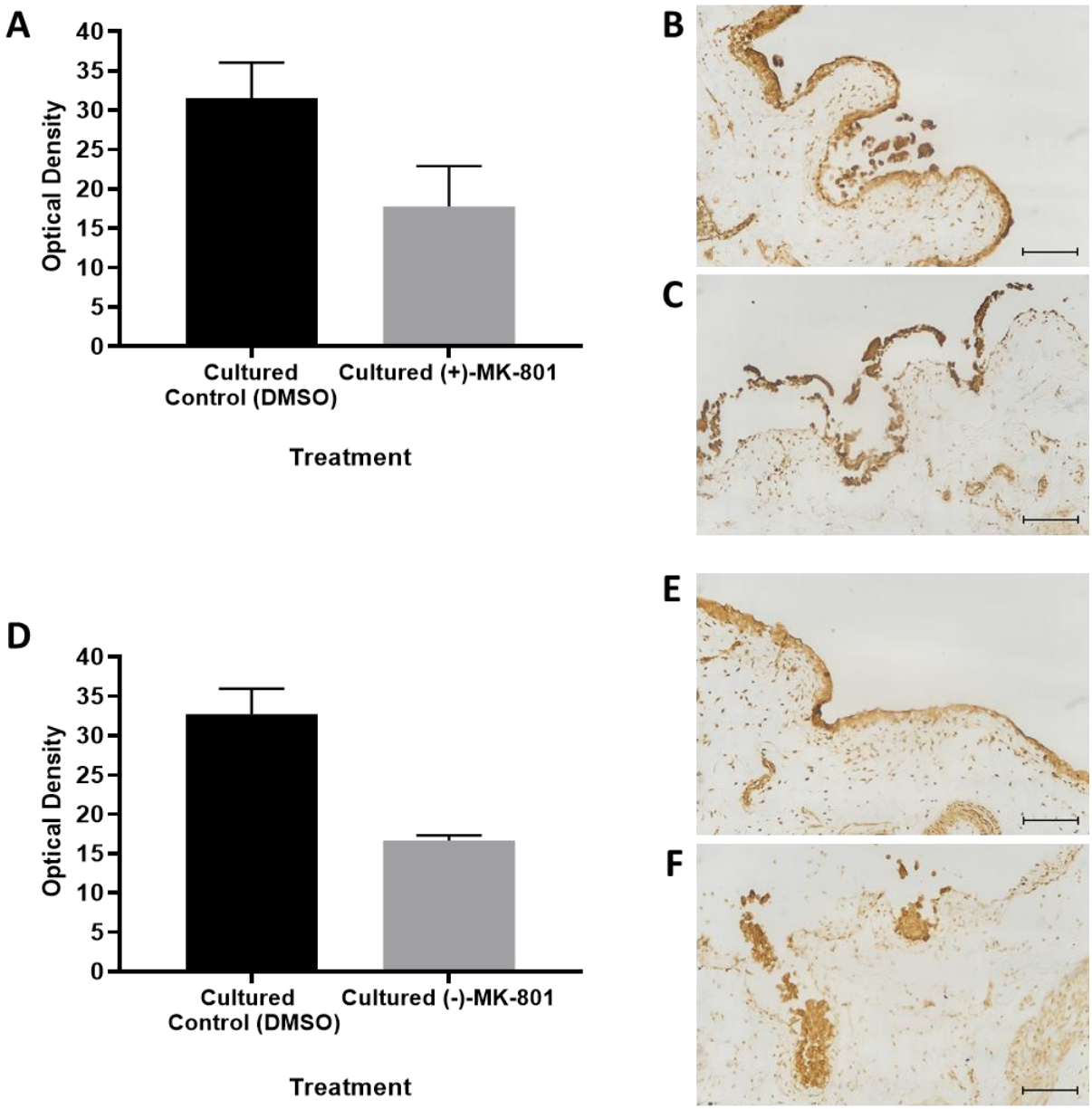

Figure 6.8. Effect of 3-day (+)-MK-801 **(A,C)** or (-)-MK-801 **(D,F)** exposure on uroplakin III expression in rat bladder strips. Matched half bladder strips were incubated for 3 days in tissue culture media and 3% dimethyl sulfoxide (cultured control), or tissue culture media containing 3mM (+)-MK-801 or (-)-MK-801. Post incubation, strips were processed, embedded in paraffin wax, and visualised using immunoperoxidase labelling to UPIII antibody. **A,D:** Graphical representation of UPIII staining intensity. Expression of UPIII was quantified by calculating the mean optical density of staining per tissue section. Data is expressed as mean optical density ±SEM. Cultured control (DMSO) and cultured (+)-MK-801 group represents *n=4* **(A)**; Cultured control (DMSO) group represents *n=6*, cultured (-)-MK-801 group represents *n=2* **(D)**. Data were analysed using a paired two-tailed t-test **(A)**. No significant difference was observed in the mean optical density of the treatments tested ($P>0.05$; **A**), or the sample size was not sufficient for statistical analysis **(D)**. **B,C,E,F:** Representative images of immunoreactivity with UPIII antibody in cultured control **(B)**, and cultured 3mM (+)-MK-801 **(C)** rat bladder sections; and cultured control **(E)**, and cultured 3mM (-)-MK-801 **(F)** rat bladder sections. Magnification x200; scale bar represents 100µm.

6.3.9 Nifedipine Does Not Affect Uroplakin III Expression in Cultured Rat Urinary Bladder

Bladder strips incubated with 10µM nifedipine for 3 days displayed a similar mean optical density of UPIII staining when compared to the vehicle control (DMSO) treated tissues (P>0.05; Figure 6.9).

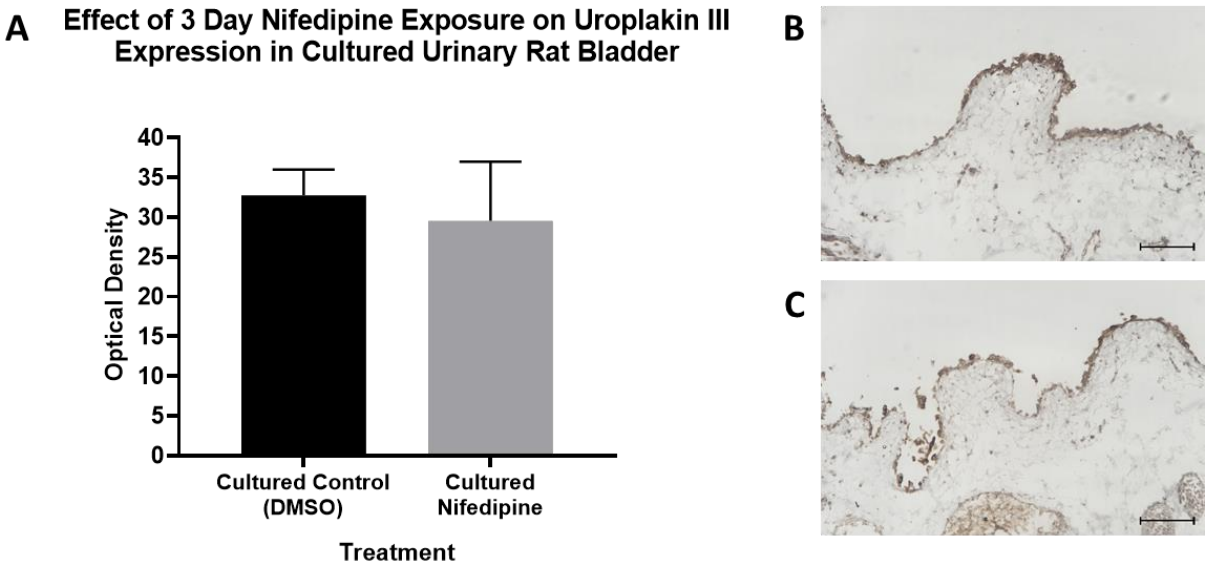

Figure 6.9. Effect of 3-day nifedipine exposure on uroplakin III expression in rat bladder strips. Matched half bladder strips were incubated for 3 days in tissue culture media and 0.1% dimethyl sulfoxide (cultured control), or tissue culture media containing 10µM nifedipine. Post incubation, strips were processed, embedded in paraffin wax, and visualised using immunoperoxidase labelling to UPIII antibody. **A:** Graphical representation of UPIII staining intensity. Expression of UPIII was quantified by calculating the mean optical density of staining per tissue section. Data is expressed as mean optical density ±SEM. Cultured control (DMSO) group represents *n=6*; cultured nifedipine *n=3*. Data were analysed using an unpaired two-tailed t-test. No significant difference was observed in the mean optical density of the treatments tested (P>0.05). **B,C:** Representative images of immunoreactivity with UPIII antibody in cultured control **(B)**, and cultured 10µM nifedipine **(C)** rat bladder sections. Magnification x200; scale bar represents 100µm.

6.3.10 Cultured Bladder Strips Express Less Cytokeratin-7 Than Uncultured Bladder Tissues After 3 Days

Uncultured bladder strips were stained for Krt7 alongside cultured control and treated tissues. Uncultured tissues displayed a greater mean optical density of Krt7 staining than 3-day cultured control and 3-day cultured ketamine bladder tissues (P<0.05 and P<0.01, respectively; Figure 6.10).

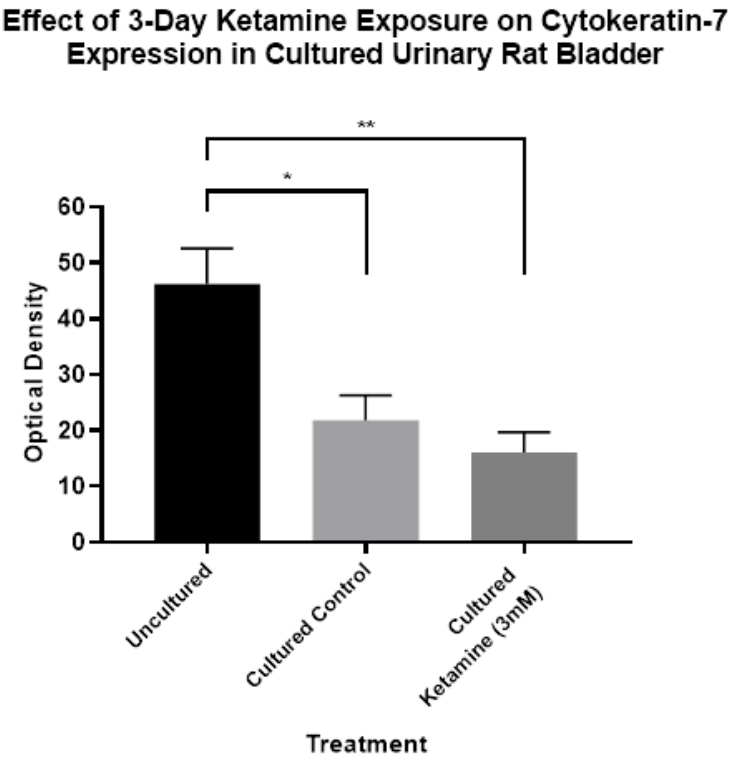

Figure 6.10. Effect of 3-day ketamine exposure on cytokeratin-7 expression in rat bladder strips. Matched half bladder strips were incubated for 3 days in tissue culture media alone, or tissue culture media containing 3mM ketamine. Post incubation, strips were processed, embedded in paraffin wax, and visualised using immunoperoxidase labelling to Krt7 antibody. Expression of Krt7 was quantified by calculating the mean optical density of staining per tissue section. Data is expressed as mean optical density ±SEM. Uncultured group represents *n=3*; cultured control and cultured ketamine *n=4*. *P<0.05; **P<0.01 significantly different from the specified treatment. Data were analysed using a one-way ANOVA with a Bonferroni post-test (uncultured vs cultured control/cultured ketamine), or a two-tailed paired t-test (cultured control vs cultured ketamine).

6.3.11 Ketamine Does Not Affect Cytokeratin-7 Expression in Cultured Rat Urinary Bladder

Bladder strips incubated with 3mM ketamine for 3 days were observed to have a mean optical density of Krt7 staining similar to 3-day cultured control bladder strips (P>0.05; Figure 6.10). There was an observable loss of Krt7 staining in cultured strips compared to uncultured strips, and a decreased continuity of Krt7 staining in 3-day ketamine-treated strips compared to cultured control tissues (Figure 6.11).

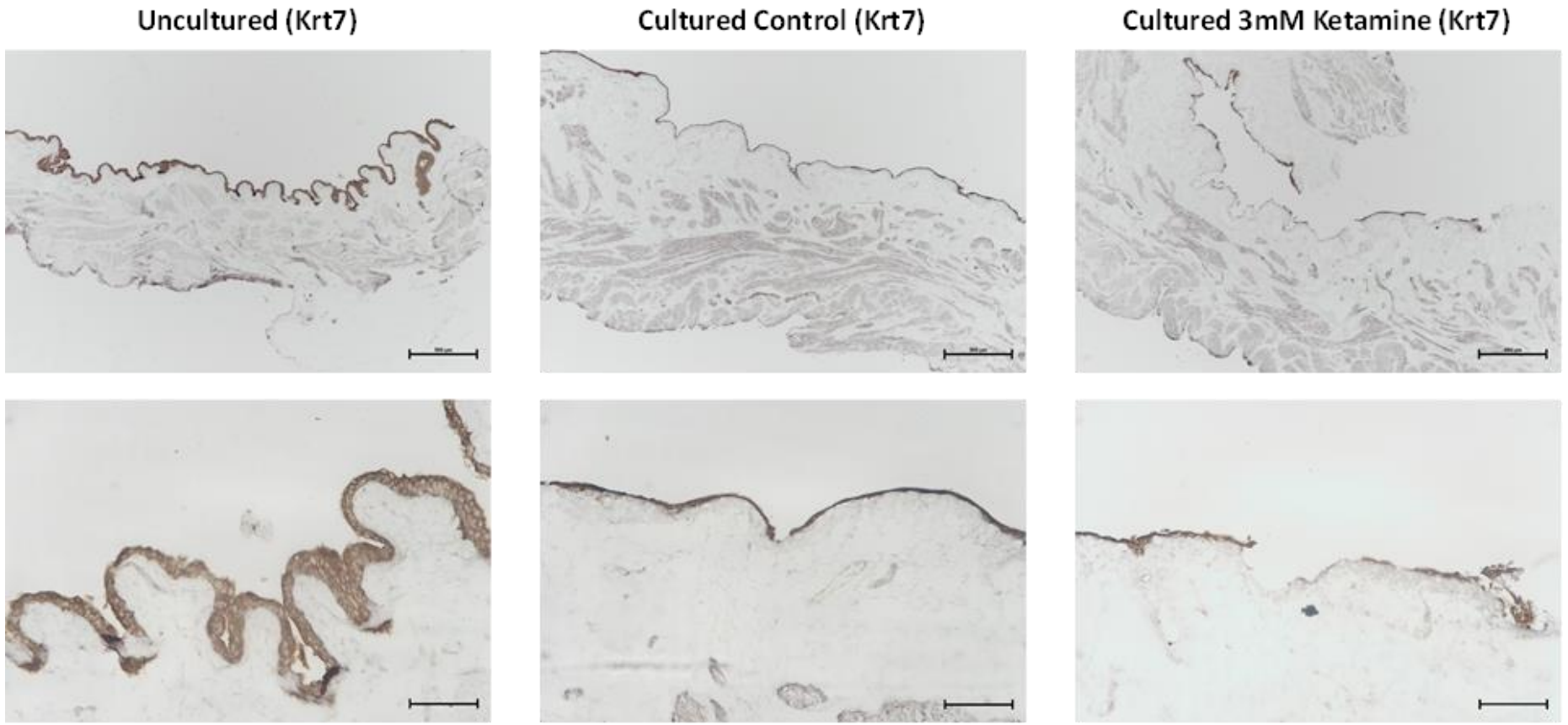

Figure 6.11. Immunohistochemical assessment of representative rat bladder sections following no treatment (uncultured), or 3 days culture with or without 3mM ketamine. Uncultured or post-cultured half bladder strips were processed, embedded in paraffin wax, and visualised using immuoperoxidase labelling to Krt7 antibody. An observable loss of cytokeratin 7 staining was seen in cultured tissues compared to uncultured tissues. Disrupted continuity of cytokeratin 7 staining was also observed in some ketamine-treated tissue. Magnification x40, scale bar represents 500µm (top row); magnification x200, scale bar represents 100µm (bottom row).

6.3.12 (S)-Ketamine Does Not Affect Cytokeratin-7 Expression in Cultured Rat Urinary Bladder

Exposure to 3mM (S)-ketamine for 3 days produced a mean optical density of Krt7 staining in rat bladder strips that was similar to cultured control bladder strips (P>0.05; Figure 6.12).

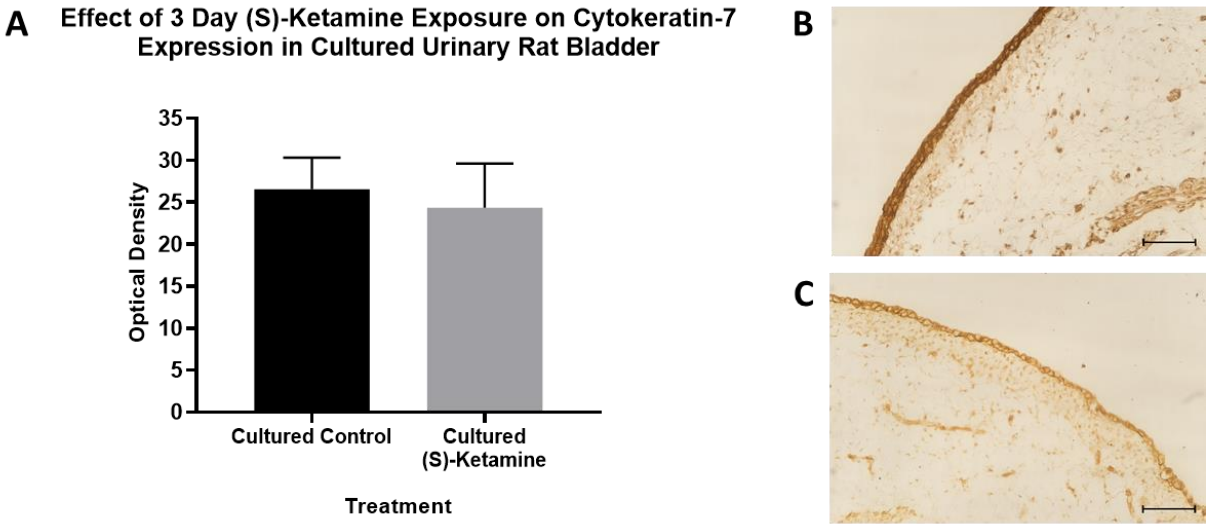

Figure 6.12. Effect of 3-day (S)-ketamine exposure on cytokeratin-7 expression in rat bladder strips. Matched half bladder strips were incubated for 3 days in tissue culture media alone, or tissue culture media containing 3mM (S)-ketamine. Post incubation, strips were processed, embedded in paraffin wax, and visualised using immunoperoxidase labelling to Krt7 antibody. **A:** Graphical representation of Krt7 staining intensity. Expression of Krt7 was quantified by calculating the mean optical density of staining per tissue section. Data is expressed as mean optical density ±SEM. Cultured control group represents *n=8*; cultured (S)-ketamine *n=3*. *P<0.05; **P<0.01 significantly different from the specified treatment. Data were analysed using a two-tailed unpaired t-test. No significant difference was observed in the mean optical density of the treatments tested (P>0.05). **B,C:** Representative images of immunoreactivity with Krt7 antibody in cultured control **(B)**, and cultured 3mM (S)-ketamine **(C)** rat bladder sections. Magnification x200; scale bar represents 100µm.

6.3.13 Norketamine Does Not Affect Cytokeratin-7 Expression in Cultured Rat Urinary Bladder

No significant difference was observed in Krt7 expression between 3-day 3mM NK-treated tissues and 3-day vehicle control-treated tissues (P>0.05; Figure 6.13A). The effects of DHNK on Krt7 expression were investigated, however the sample size was not sufficient for statistical analysis (Figure 6.13D).

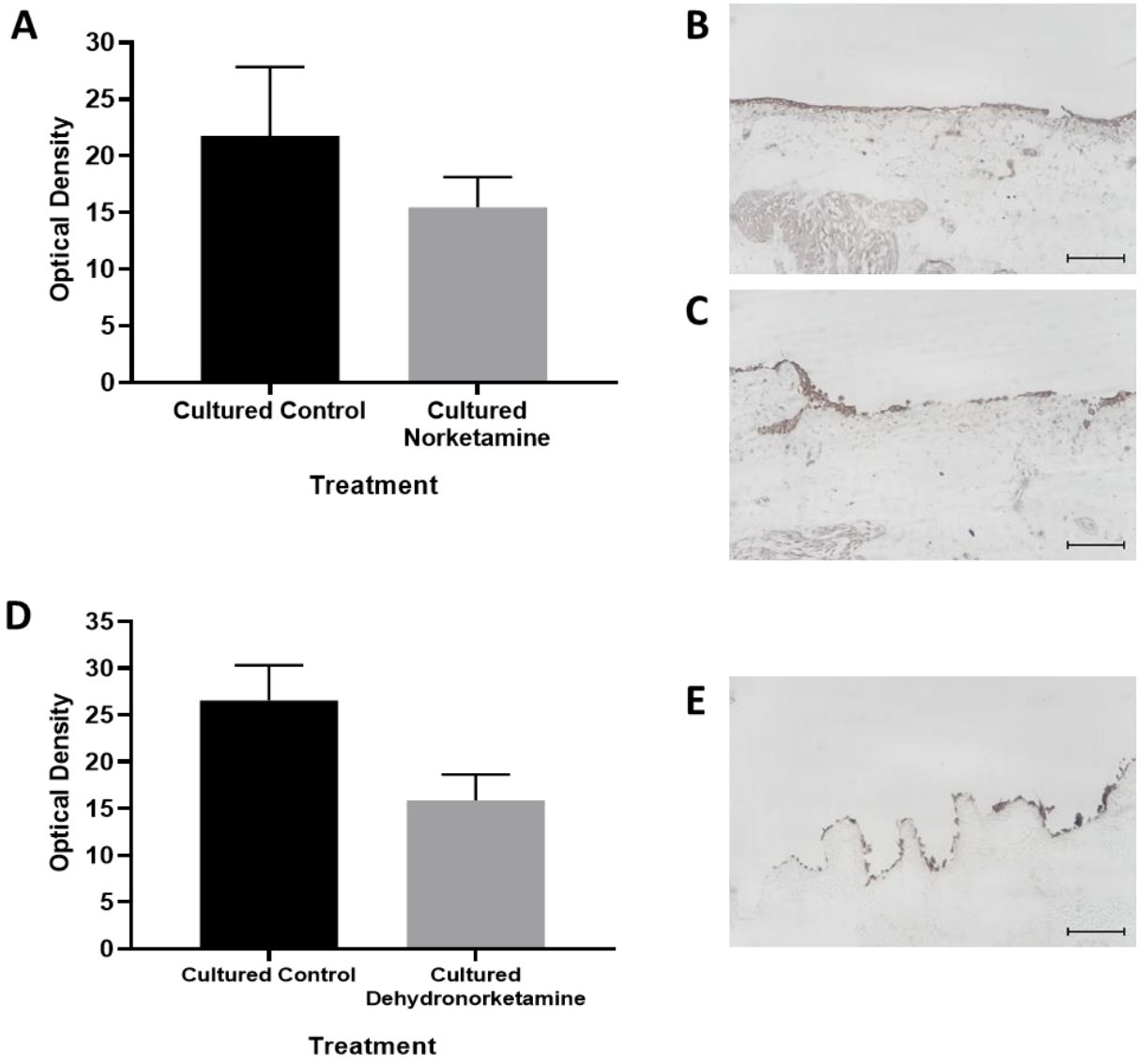

Figure 6.13. Effect of 3-day norketamine **(A,C)** or dehydronorketamine **(D,E)** exposure on cytokeratin-7 expression in rat bladder strips. Matched half bladder strips were incubated for 3 days in tissue culture media alone, or tissue culture media containing 3mM norketamine or dehydronorketamine. Post incubation, strips were processed, embedded in paraffin wax, and visualised using immunoperoxidase labelling to Krt7 antibody. **A,D:** Graphical representation of Krt7 staining intensity. Expression of Krt7 was quantified by calculating the mean optical density of staining per tissue section. Data is expressed as mean optical density ±SEM. Cultured control and cultured norketamine group represents *n=4* **(A)**; Cultured control group represents *n=8*, cultured dehydronorketamine group represents *n=2* **(D)**. Data were analysed using a paired two-tailed t-test (**A**). No significant difference was observed in the mean optical density of the treatments tested (P>0.05; **A**), or the sample size was not sufficient for statistical analysis **(D)**. **B,C,E:** Representative images of immunoreactivity with Krt7 antibody in cultured control **(B)**, cultured 3mM norketamine **(C)**, and cultured 3mM dehydronorketamine **(E)** rat bladder sections. Magnification x200; scale bar represents 100µm.

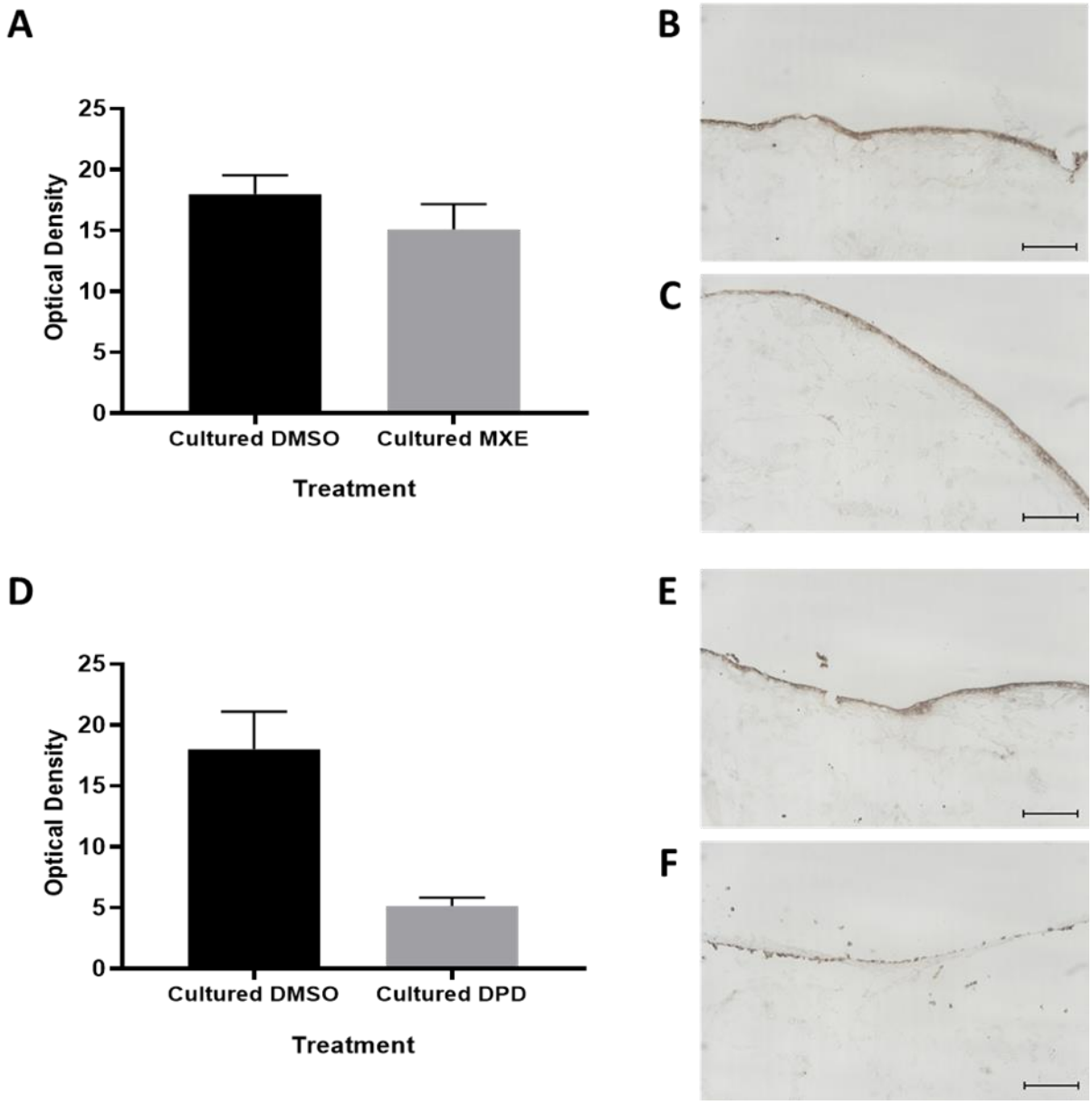

Figure 6.14. Effect of 3-day methoxetamine **(A,C)** or diphenidine **(D,F)** exposure on cytokeratin-7 expression in rat bladder strips. Matched half bladder strips were incubated for 3 days in tissue culture media and 3% dimethyl sulfoxide (cultured control), or tissue culture media containing 3mM methoxetamine or diphenidine. Post incubation, strips were processed, embedded in paraffin wax, and visualised using immunoperoxidase labelling to Krt7 antibody. **A,D:** Graphical representation of Krt7 staining intensity. Expression of Krt7 was quantified by calculating the mean optical density of staining per tissue section. Data is expressed as mean optical density ±SEM. Cultured DMSO group represents *n=4*, cultured methoxetamine group represents *n=3* **(A)**; Cultured DMSO group represents *n=4*, cultured diphenidine group represents *n=2* **(D)**. Data were analysed using an unpaired two-tailed t-test **(A)**. No significant difference was observed in the mean optical density of the treatments tested (P>0.05; **A**), or the sample size was not sufficient for statistical analysis **(D)**. **B,C,E,F:** Representative images of immunoreactivity with Krt7 antibody in cultured control **(B)** and cultured 3mM methoxetamine **(C)**; or cultured control **(E)**, and cultured 3mM diphenidine **(F)** rat bladder sections. Magnification x200; scale bar represents 100µm.

6.3.14 Methoxetamine Does Not Affect Cytokeratin-7 Expression in Cultured Rat Urinary Bladder

Exposure to 3mM MXE for 3 days did not significantly alter the mean optical density of Krt7 staining in rat bladder strips compared to 3-day cultured control tissues ($P>0.05$; Figure 6.14A). The effects of diphenidine (DPD) on Krt7 expression were also tested, however the sample size was not sufficient for statistical analysis (Figure 6.14D).

6.3.15 Effect of Ketamine on Expression of Cleaved Cytokeratin 18

The expression of cleaved cytokeratin 18 was assessed in both 3-day ketamine- and 3-day control-treated tissues to determine whether ketamine exposure increases apoptosis in rat bladder strips. Immunoreactivity to cleaved cytokeratin 18 appeared to be increased in 3-day ketamine treated bladder (Figure 6.15); however, an insufficient number of samples were successfully stained, and so statistical analysis was not possible.

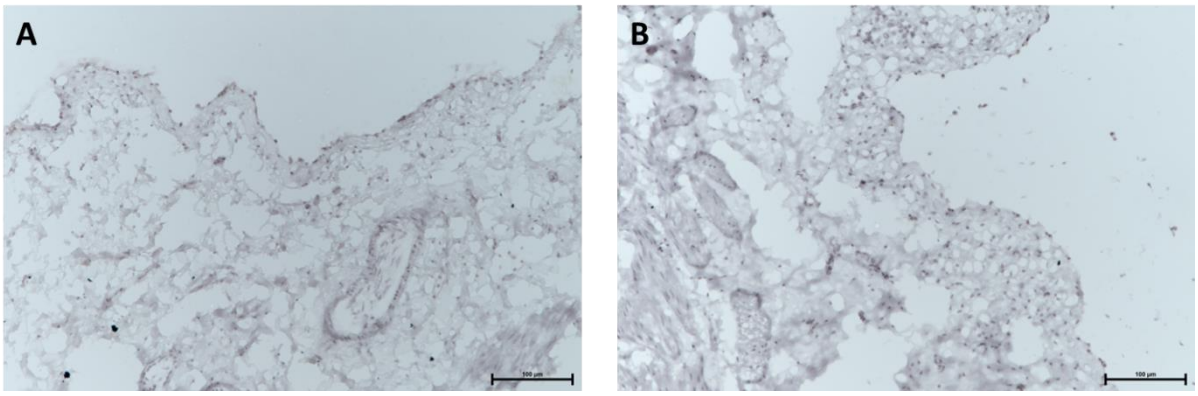

Figure 6.15. Immunostained rat bladder sections for cleaved cytokeratin 18 following 3 days incubation in tissue culture media alone **(A)**, or 3 days incubation in tissue culture media and 3mM ketamine **(B)**. The ketamine-treated tissue (B) appears to display an increased number of immunostained cells compared to the cultured control tissue (B). Magnification x200; scale bar represents 100µm.

6.3.16 Cultured Bladder Strips Express Less Zonula Occludens-1 Than Uncultured Bladder Tissues After 3 Days

Bladder strips that were cultured for 3 days with either ketamine (3mM) or vehicle control expressed less urothelial ZO-1 than uncultured bladder strips ($P<0.01$; Figure 6.16). No difference in mean optical density of ZO-1 staining was observed between cultured control and cultured ketamine tissues ($P>0.05$; Figure 6.16). In uncultured tissues, ZO-1 expression was primarily localised to the urothelial layer, whereas in cultured control or cultured ketamine tissues, ZO-1 appeared to be primarily expressed in the suburothelial layers, or was absent (Figure 6.17).

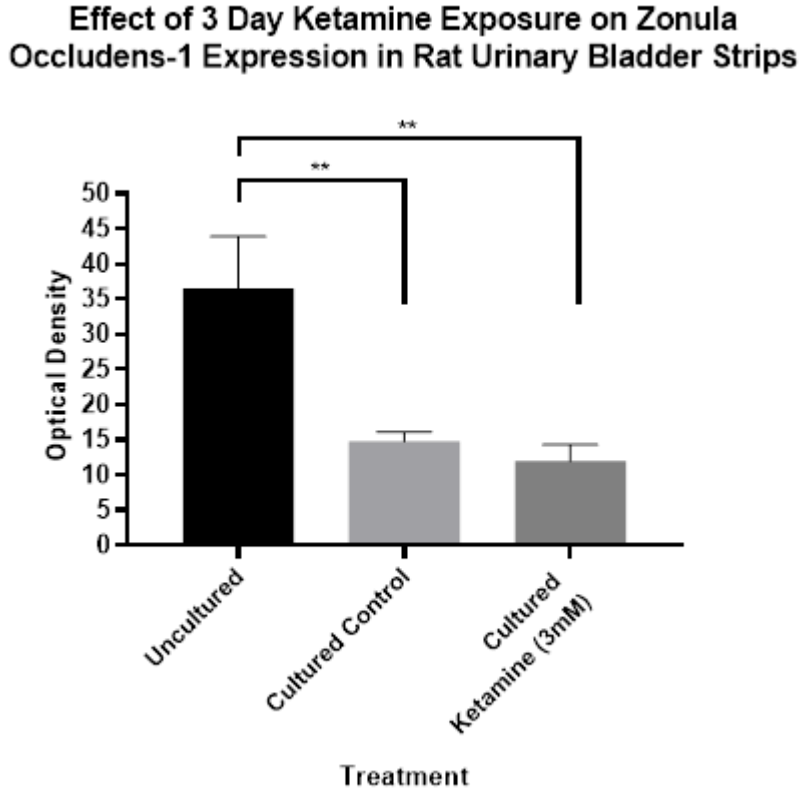

Figure 6.16. Effect of 3-day ketamine exposure on zonula occludens-1 expression in rat bladder strips. Matched half bladder strips were incubated for 3 days in tissue culture media alone, or tissue culture media containing 3mM ketamine. Post incubation, strips were processed, embedded in paraffin wax, and visualised using immunoperoxidase labelling to ZO-1 antibody. Expression of ZO-1 was quantified by calculating the mean optical density of staining per tissue section. Data is expressed as mean optical density ±SEM. Uncultured group represents *n=3*; cultured control *n=4*; cultured ketamine *n=5*. **P<0.01 significantly different from the specified treatment. Data were analysed using a one-way ANOVA with a Bonferroni post-test (uncultured vs cultured control/cultured ketamine), or a two-tailed paired t-test (cultured control vs cultured ketamine).

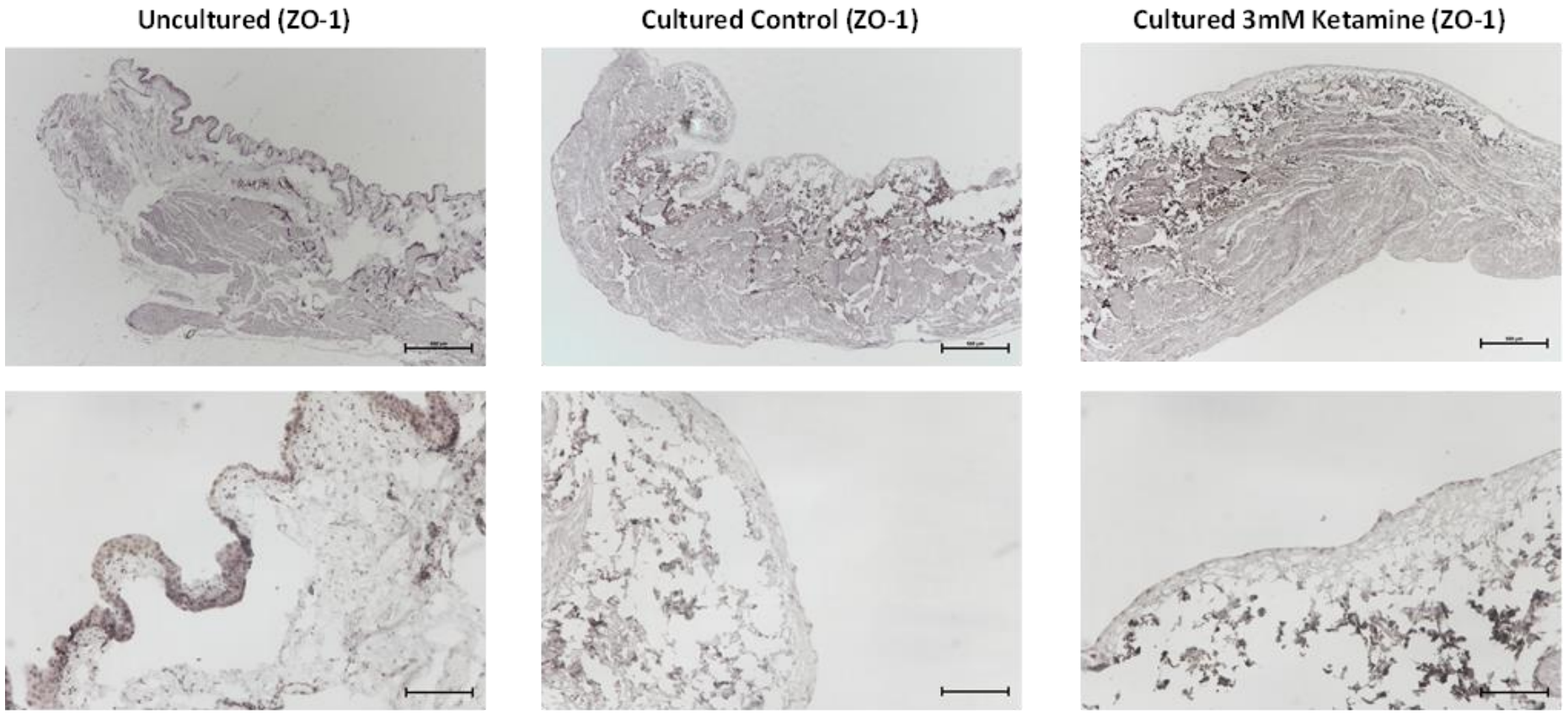

Figure 6.17. Immunohistochemical assessment of representative rat bladder sections following no treatment (uncultured), or 3 days culture with or without 3mM ketamine. Uncultured or post-cultured half bladder strips were processed, embedded in paraffin wax, and visualised using immunoperoxidase labelling to ZO-1 antibody. Zonula occludens-1 staining appeared to be primarily localised in the urothelium of uncultured tissues. In cultured tissues, zonula occludens-1 was primarily localised in suburothelia layers or was absent. Magnification x40, scale bar represents 500µm (top row); magnification x200, scale bar represents 100µm (bottom row).

6.3.17 Ketamine Does Not Affect Myosin-11 Expression in Cultured Rat Urinary Bladder

Bladder strips exposed to 3mM ketamine for 3 days expressed similar levels of myosin-11 to 3-day control bladder strips (P>0.05; Figure 6.18). There was also no change in myosin-11 expression in cultured bladder strips when compared to uncultured strips (P>0.05; Figure 6.18).

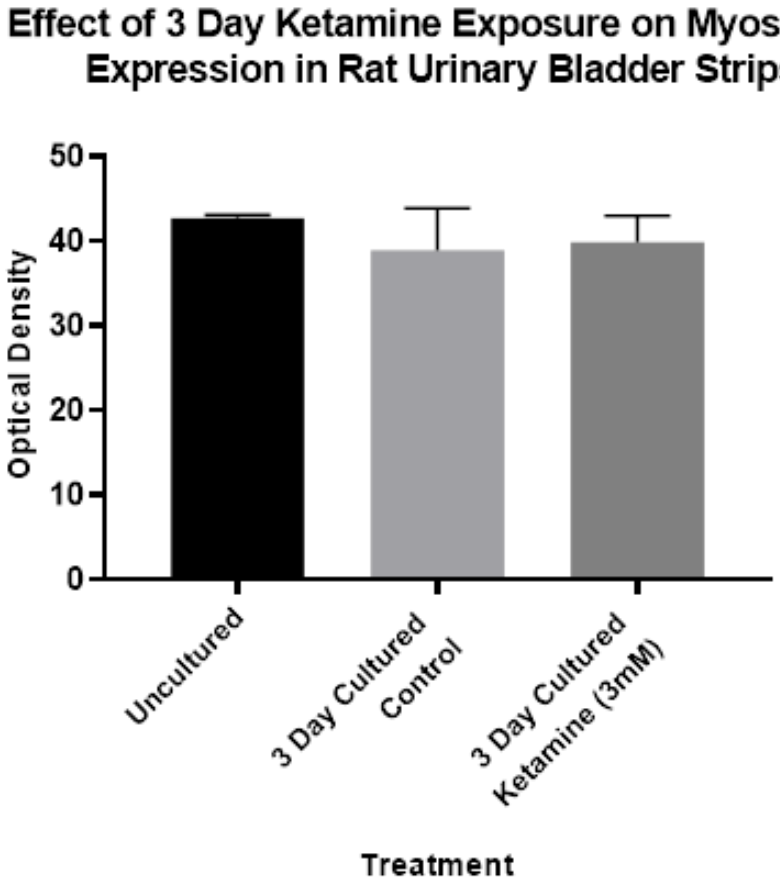

Figure 6.18. Effect of 3-day ketamine exposure on myosin-11 expression in rat bladder strips. Matched half bladder strips were incubated for 3 days in tissue culture media alone, or tissue culture media containing 3mM ketamine. Post incubation, strips were processed, embedded in paraffin wax, and visualised using immunoperoxidase labelling to SMMHC antibody. Expression of myosin-11 was quantified by calculating the mean optical density of staining per tissue section. Data is expressed as mean optical density ±SEM. Uncultured, cultured control, and cultured ketamine group represents *n=3*. Data were analysed using a one-way ANOVA with a Bonferroni post-test (uncultured vs cultured control/cultured ketamine), or a two-tailed paired t-test (cultured control vs cultured ketamine). No significant difference was observed in the mean optical density of the treatments tested (P>0.05).

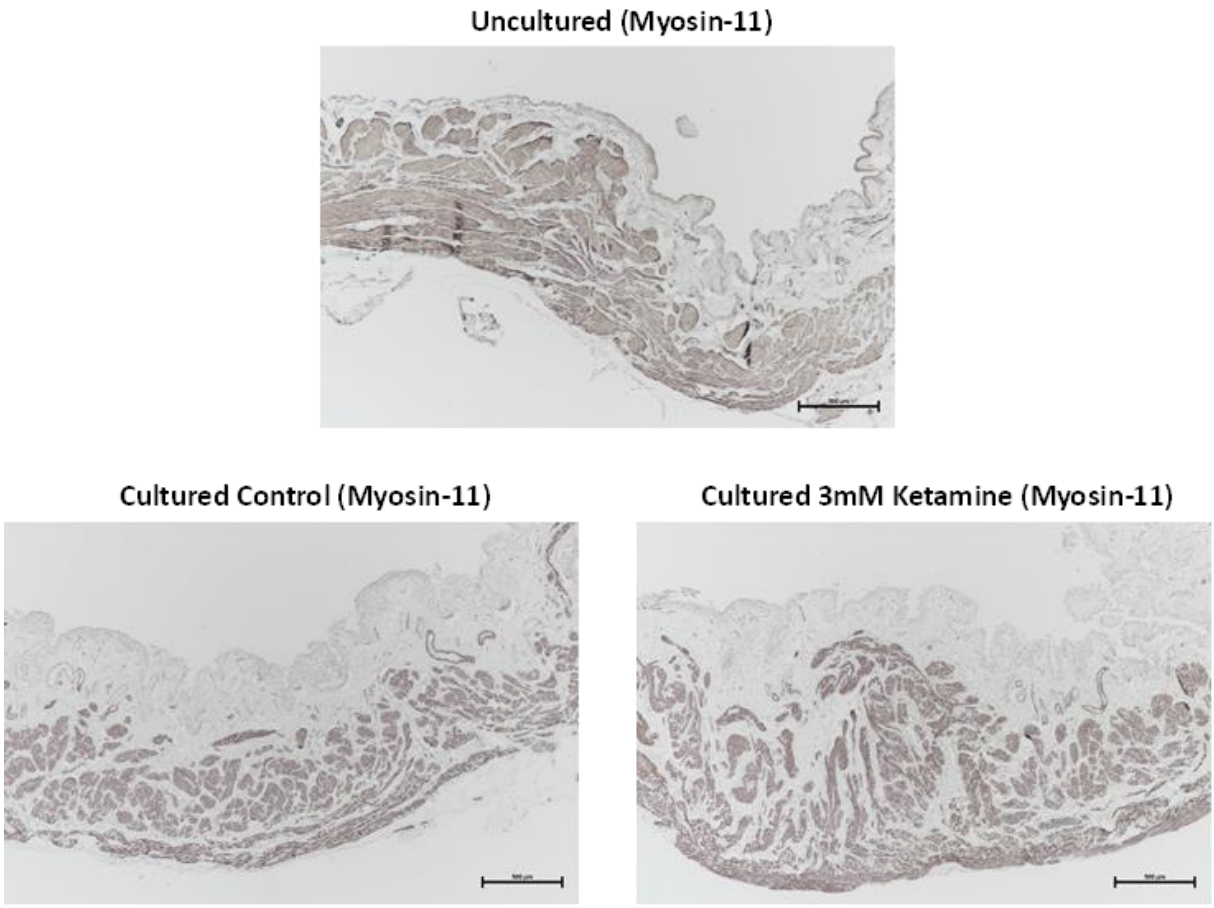

Figure 6.19. Immunohistochemical assessment of representative rat bladder sections following no treatment (uncultured), or 3 days culture with or without 3mM ketamine. Uncultured or post-cultured half bladder strips were processed, embedded in paraffin wax, and visualised using immunoperoxidase labelling to SMMHC antibody. There was no observable change in myosin-11 staining between any of the treatments. Magnification x40; scale bar represents 500µm.

6.3.18 Ketamine Does Not Affect CaV1.2 Expression in Cultured Rat Urinary Bladder

Expression of $CaV_{1.2}$ was unaffected by 3-day exposure to ketamine (3mM) in both the urothelium and muscle layers when compared to cultured control bladders ($P>0.05$; Figure 6.21). Cultured tissues also had a similar mean optical density of $CaV_{1.2}$ staining to uncultured bladder strips ($P>0.05$; Figure 6.21).

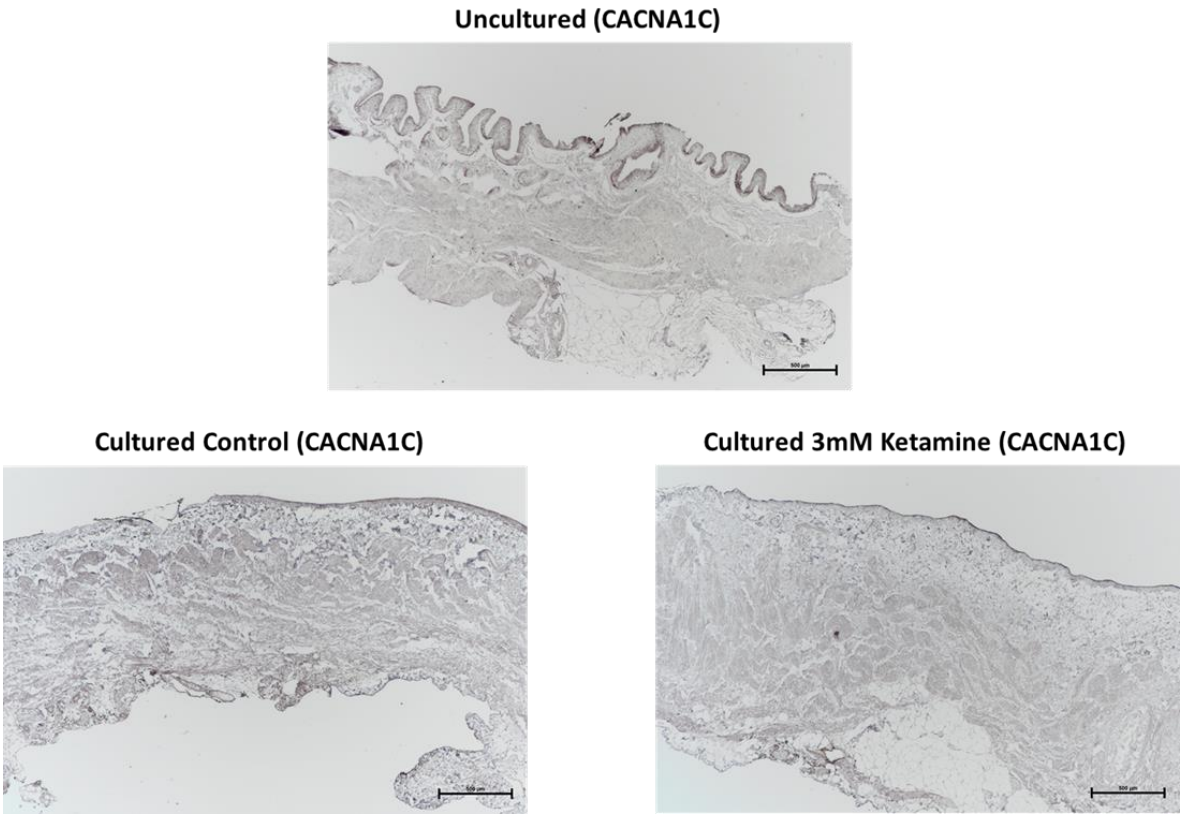

Figure 6.20. Immunohistochemical assessment of representative rat bladder sections following no treatment (uncultured), or 3 days culture with or without 3mM ketamine. Uncultured or post-cultured half bladder strips were processed, embedded in paraffin wax, and visualised using immunoperoxidase labelling to CACNA1C antibody. There was no observable change in $Ca_V1.2$ staining between any of the treatments. Magnification x40; scale bar represents 500µm.

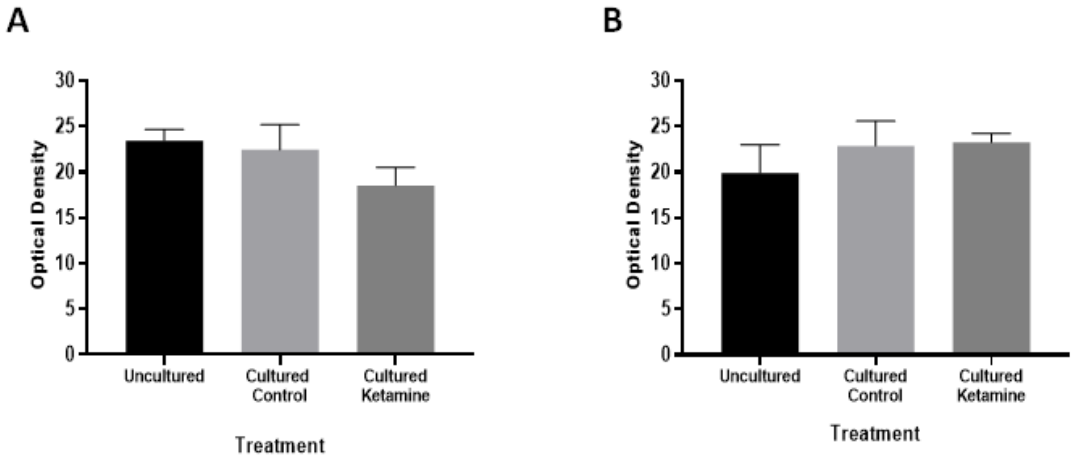

Figure 6.21. Effect of 3-day ketamine exposure on $CaV_{1.2}$ expression in rat bladder mucosa (**A**) or smooth muscle (**B**). Matched half bladder strips were incubated for 3 days in tissue culture media alone, or tissue culture media containing 3mM ketamine. Post incubation, strips were processed, embedded in paraffin wax, and visualised using immunoperoxidase labelling to CACNA1C antibody. Expression of $CaV_{1.2}$ was quantified by calculating the mean optical density of staining per tissue section. Data is expressed as mean optical density ±SEM. Uncultured group represents *n=3*, cultured control and cultured ketamine represents *n=4* (**A**); uncultured group represents *n=3*, cultured control *n=5*, and cultured ketamine *n=4* (**B**). Data were analysed using a one-way ANOVA with a Bonferroni post-test (uncultured vs cultured control/cultured ketamine), or a two-tailed paired t-test (cultured control vs cultured ketamine). No significant difference was observed in the mean optical density of the treatments tested (P>0.05).

6.4 DISCUSSION

6.4.1 Time Effects of Ketamine on Uroplakin III Expression in Cultured Rat Urinary Bladder

6.4.1.1 Short Term (20-Minute) Ketamine Exposure

Rat bladder strips exposed to 1 or 3mM ketamine for twenty minutes displayed similar expression levels of UPIII to uncultured control strips (Figure 6.1). This suggests that the decrease in bladder contractility observed after acute ketamine exposure (Chapter 3) cannot be explained by a loss of urothelial umbrella cells.

6.4.1.2 Three-Day Ketamine Exposure

Bladder strips exposed to 3mM ketamine for 3 days displayed a reduction in UPIII expression compared to cultured control bladders (Figure 6.2). Loss of urothelial cells as a result of direct exposure to ketamine *in vitro* has been previously reported here (Chapter 5), and in the literature (Baker et al., 2016). Moreover, thinning or denudation of the urothelium is a commonly reported histopathological finding in both patients with KC (Shahani et al, 2007; Baker et al., 2013; Lin et al., 2014; Jhang, Hsu & Kuo, 2015) and in animal models of KC (Lee et al., 2017; Wang et al., 2017). The reduction of UPIII expression following ketamine exposure seen here would therefore suggest that the thinning of the urothelium observed after direct exposure to ketamine (3mM), or in *in vivo* studies is at least partially explained by a loss of umbrella cells from the urothelium.

This finding appears to be in line with several animal studies that reported reduced UPIII expression following daily ketamine injections. Western blot analysis revealed that rats administered with ketamine (30mg/kg) daily for 4 weeks had a significantly reduced expression of UPIII compared to the saline control group, and this effect was able to be partially reversed following a 2-week recovery period (Lee et al., 2017). Uroplakin III protein levels were also markedly decreased in rats following 8 weeks of daily ketamine (35mg/kg) injections, as determined by western blot analysis (Chueh et al., 2022). Immunohistochemical evaluation of UPIII expression also revealed absent or disrupted staining of UPIII at the apical surface of the urothelium following 4 weeks ketamine injection (25mg/kg) in rats (Huang et al., 2022).

Loss of UPIII in knockout mice is reported to increase the permeability of the urothelium (Hu et al., 2000), and impaired barrier function of the urothelium is thought to be a potential cause of KC (Jhang et al., 2015; Castellani et al., 2020). In animal models of KC, treatment with hyaluronic acid or platelet-rich plasma has been shown to rescue decreased expression levels of UPIII and ameliorate bladder overactivity (Lee et al., 2017; Chueh et al., 2022). Loss of UPIII alone, however, is unlikely to induce the bladder overactivity seen in such models. In mice, loss of UPIII was reported to induce changes in certain cytometric parameters indicative of bladder overactivity — namely, an increase in non-voiding contractions and a decrease in threshold pressure — without increasing micturition frequency (Aboushwareb et al., 2009). Additionally, the direct effect of ketamine on the urothelium has been shown to alter the expression of many urothelium-associated proteins, and so it seems unlikely that decreased UPIII expression alone, if at all, is responsible for the bladder dysfunction observed in KC.

6.4.2 Effect of Ketamine-Related Compounds on Uroplakin III Expression in Cultured Rat Urinary Bladder

6.4.2.1 (S)-Ketamine

Rat bladder strips exposed to 3mM (S)-ketamine for 3 days also displayed a reduced expression of UPIII when compared to control bladder strips (Figure 6.4). This is thought to be the first report to show a direct toxic effect of (S)-ketamine on the urothelium and suggests that the loss of mucosal cells following 3 days exposure to (S)-ketamine (Chapter 5) can at least be partially explained by loss of umbrella cells. The average mean optical density of UPIII staining in (S)-ketamine-treated bladder strips was similar to ketamine-treated strips (15.07±5.06 and 14.71±2.24, respectively).

There is currently no published data on the direct effects of (S)-ketamine on the urothelium, presumably because it is racemic ketamine that is typically taken by recreational users. However, interest into the effects of (S)-ketamine has grown in recent years due to its potential use as an antidepressant, and so data regarding the safety of the drug through clinical trials is available. Generally, trials of (S)-ketamine show little to no incidence of urinary dysfunction in patients; however, in one long-term observational study 137 (19%) individuals reported adverse bladder effects (The United States Food and Drug Administration, 2019). The report concluded, however, that it was difficult to determine whether (S)-ketamine

results in LUT dysfunction without a comparison arm. Moreover, the dose of (S)-ketamine used in these studies was ≤84mg, which is a relatively small amount compared to the large amounts of ketamine typically taken by recreational users, or those used in animal studies.

6.4.2.2 Norketamine and Dehydronorketamine

Exposure to 3mM NK or DHNK had no effect on the UPIII expression of rat bladder strips (Figure 6.5). A combination of ketamine, NK, and DHNK, at lower concentrations (100µM ketamine and NK; 300µM DHNK) that are within the range reported in the urine of recreational users (Moore et al., 2001), also did not affect UPIII expression (Figure 6.6).

Norketamine, a major metabolite of ketamine, has been detected at high concentrations in the urine of ketamine abuse patients (Shahani et al., 2007); and urine levels of NK were reported to be similar to those of ketamine in humans (Moore et al., 2001), and higher than ketamine urine levels in rats (Liu et al., 2015). Therefore, as relatively high levels of NK in the urine will be exposed to the urothelium, NK is postulated to contribute to the LUT toxicity associated with KC.

Indeed, one recent study demonstrated that NK induced apoptosis and reduced cell viability in human urinary bladder epithelial-derived RT4 cells and was reported to be more cytotoxic than ketamine (Lin et al., 2022). When also considering that 3 days of exposure to 3mM NK reduced the mean number of mucosal cells in bladder strips (Chapter 5), the observation here that 3-day NK (3mM) exposure did not affect UPIII expression is somewhat surprising.

Dehydronorketamine is also reported to be present in the urine of ketamine users at concentrations higher than ketamine or NK (Moore et al., 2001), and so there is potential for DHNK to accumulate in the urine and have a direct toxic effect on the urothelium. However, DHNK had no effect on UPIII expression here, and there are also no available reports on the effects of DHNK on the urothelium.

6.4.2.3 Methoxetamine

Methoxetamine exposure did not affect UPIII expression in rat bladder strips (Figure 6.7). This result was somewhat surprising as 3-day MXE exposure was previously shown to decrease the mean mucosal cell count in rat bladder strips (Chapter 5). Additionally, rats administered MXE (30mg/kg) for 4 or 12 weeks displayed damaged urothelium with areas of interrupted

continuity (Wang et al., 2017). This result suggests that loss of mucosal cells or damage to the urothelium induced by MXE is potentially mediated by a different mechanism to ketamine and may not be due to loss of umbrella cells.

6.4.2.4 MK-801

Exposure to (+)-MK-801 did not affect UPIII expression in rat bladder strips (Figure 6.8A). Similarly, MK-801 also had no effect on mean mucosal cell count in rat bladder strips following 3 days incubation (Chapter 5). MK-801 (≤100μM) was also reported to not trigger any toxicity following exposure to normal human urothelial cells, and ketamine-induced urothelial toxicity in these cells was unaffected by pre-treatment with D-serine and NMDA (Baker et al., 2016). Together, these results suggest that ketamine's mechanism of urothelial toxicity is independent of the NMDA receptor, especially when considering that MK-801 is a much more potent NMDA receptor antagonist than ketamine.

Bladder strips exposed to (-)-MK-801 for 3 days displayed a trend towards a reduced expression of UPIII compared to control incubated strips, however the sample size was insufficient for statistical analysis (Figure 6.8D). Further investigation into the effects of MK-801 and other selective NMDAR antagonists in this assay would help to confirm or deny the suggestion that loss of urothelial cells following ketamine exposure is not NMDAR-mediated.

6.4.2.5 Nifedipine

There is some evidence to suggest that ketamine and nifedipine can inhibit bladder smooth muscle cell proliferation and even lead to cell death at high concentrations and that these effects are mediated by $Ca_V1.2$ (Chen et al., 2020). As nifedipine (10μM) reportedly decreased the number of Ki67- (a marker of cell proliferation) positive cells in human bladder smooth muscle cell cultures, bladder strips were incubated with 10μM nifedipine to determine whether $Ca_V1.2$ antagonism could lead to urothelial damage. Rat bladder strips incubated with 10μM nifedipine for 3 days displayed similar levels of UPIII expression to vehicle control incubated strips (Figure 6.9). Therefore, it seems unlikely that the urothelial toxicity displayed by ketamine is mediated by $Ca_V1.2$ antagonism.

6.4.3 Effect of Ketamine and Ketamine- Related Compounds on Cytokeratin 7 Expression in Rat Urinary Bladder Strips

6.4.3.1 Ketamine

Rat bladder strips exposed to 3mM ketamine for 3 days expressed similar levels of Krt7 to 3-day cultured control tissues (Figure 6.10 & 6.11). This is thought to be the first time that the effects of ketamine on Krt7 expression in rat bladder have been investigated.

Cytokeratin 7 is a filament protein that is expressed in all urothelial cell layers in both humans and rodents (Khandelwal, Abraham, & Apodaca, 2009; Dalghi, Montalbetti, Carattino & Apodaca, 2020). The result that 3-day 3mM ketamine exposure does not affect Krt7 expression when considering that the same treatment reduced UPIII expression and reduced mean mucosal cell count is a surprising one. This seems to suggest that umbrella cells are the primary urothelial cell type being lost as a result of 3-day ketamine exposure, as UPIII expression is significantly reduced (Figure 6.2), but Krt7 expression is similar, presumably due to expression of Krt7 by intermediate and/or basal urothelial cells "masking" a loss of umbrella cells. A disproportionate loss of umbrella cells compared to other urothelial cell types is also reasonable to suggest as umbrella cells are the apical-most cell type expressed in the urothelium and so would come into direct contact with ketamine in this assay.

6.4.3.2 (S)-Ketamine

Exposure to 3mM (S)-ketamine for 3 days did not alter the expression of Krt7 in rat bladder strips (Figure 6.12). This also seems to suggest that umbrella cells are the primary urothelial cell type being lost to (S)-ketamine exposure, because UPIII levels were significantly reduced under the same conditions (Figure 6.4).

6.4.3.3 Norketamine and Dehydronorketamine

No change in Krt7 expression was observed in rat bladder strips following exposure to 3mM NK or DHNK for 3 days (Figure 6.13). Both metabolites also did not alter UPIII expression under the same conditions; however NK, but not DHNK, reduced the mean mucosal cell count in rat bladder strips (Chapter 5). These results therefore suggest that 3mM DHNK does not have a cytotoxic effect on rat urothelial cells *in vitro*. This does not necessarily mean that DHNK does not contribute to the urothelial damage seen in KC patients and animal models, however, as

non-cytotoxic concentrations of ketamine have been shown to inhibit cell division of urothelial cells (Baker et al., 2016).

Given the seemingly conflicting results of 3-day NK treated strips, the ability of NK to induce a loss of urothelial cells in this particular assay is inconclusive.

6.4.3.4 Methoxetamine and Diphenidine

The sample size for 3-day 3mM DPD-treated tissues was insufficient for statistical analysis; however, a trend towards reduction of Krt7 expression by DPD was noted (Figure 6.14D). Bladder strips exposed to 3mM MXE for 3 days had no change in Krt7 expression (Figure 6.14A). Methoxetamine also had no effect on UPIII expression under the same conditions; however, both MXE and DPD induced a loss of mucosal cells in rat bladder strips (Chapter 5), and loss of urothelium has been reported in MXE-treated rats (Wang et al., 2017). As such, the ability of MXE or DPD to induce a loss of urothelial cells in this particular assay is inconclusive.

The discrepancy between the histological effects of MXE reported in Chapter 5 and those reported here could in part be explained by the limitations of this dataset. Loss of tissues during the IHC protocol resulted in a small sample size for MXE-treated bladder strips (*n=3*), and the inability to compare these tissues to their matched controls. Moreover, differences in the methodology used to quantify staining in Chapter 5 (cell count) and here (optical density) could partly explain these seemingly conflicting results.

6.4.3 Effect of Ketamine on Cleaved Cytokeratin 18 Expression in Rat Urinary Bladder Strips

The expression of cleaved cytokeratin 18 in ketamine-treated tissues was investigated to see if loss of umbrella cells following 3 days exposure to ketamine could be explained by an increase in apoptosis. There appeared to be an increased number of immunostained cells in the urothelium of 3-day cultured ketamine strips compared to 3-day cultured control strips (Figure 6.15); however, this was unable to be verified as the number of samples was insufficient for statistical analysis.

Human urothelial cell apoptosis induced by ketamine (Baker et al., 2016; Lin et al., 2022) and NK (Lin et al., 2022) *in vitro* has been reported in the literature. Moreover, evidence of increased apoptosis in the mucosa of KC patients (Lee, Jiang & Kuo, 2013; Tsai et al., 2016)

and in the bladders of rats administered daily ketamine for 14 or 28 days (Liu et al., 2015) has been reported. It therefore seems likely that ketamine exposure induces urothelial cell apoptosis, contributing to the development of KC. Optimising the IHC protocol used here to enable statistical analysis and improve the staining of cleaved cytokeratin 18 would help determine whether ketamine, NK, and related compounds induce apoptosis in *in vitro* rat bladder strips.

6.4.4 Effect of Ketamine on Zonula Occludens-1 Expression in Rat Urinary Bladder Strips

No change in ZO-1 expression was observed in rat bladder mucosa following 3 days exposure to 3mM ketamine when compared to cultured control strips (Figure 6.16). Cultured control and cultured ketamine bladder strips did, however, express significantly less urothelial ZO-1 than uncultured rat bladder strips (Figure 6.16).

Zonula occludens-1 is thought to be an essential component of the barrier function of the urothelium by maintaining the high resistance paracellular pathway and mediating signal transduction (Gu et al., 2014). Disruption of the urothelium's barrier function may result in leakage of urine components to the underlying tissue layers resulting in irritation and LUT symptoms typically associated with KC (Duan et al., 2017). Indeed, decreased levels of ZO-1 have been observed in patients with interstitial cystitis and bladder pain syndrome (Slobodov et al., 2004; Liu, Shie, Chen, Wang & Kuo, 2012), and in the bladder mucosa of patients with KC (Tsai et al., 2016).

In rats, ZO-1 is typically localised in the tight junctions of umbrella cells (Acharya et al., 2004), so it was therefore expected that a loss of umbrella cells following exposure to ketamine (Figure 6.2) would in turn lead to reduced expression of ZO-1. Indeed, ZO-1 expression was reduced in rats receiving daily ketamine injections (30mg/kg) for 4 weeks (Lee et al., 2017), and rats receiving either low-dose (5mg/kg) or high-dose (50mg/kg) ketamine daily for 16 weeks displayed a decreased protein expression of ZO-1 (Gu et al., 2014). In a study of KC in mice, ZO-1 in the ketamine-treated group (100mg/kg daily for 4, 8, and 12 weeks) was either absent or located in the cytoplasm and not organised into tight junction structures, whereas ZO-1 in control mice was present in the tight junctions of umbrella cells (Duan et al., 2017). A similar localisation of ZO-1 was seen here in some samples from cultured control and cultured

ketamine bladder strips, whereas ZO-1 was confined to the urothelial layer in uncultured bladders (Figure 6.17).

One potential explanation for animal models of KC reporting a loss of ZO-1 expression but no significant difference being observed here is differences in the comparative controls. In *in vivo* animal models, control animals are typically administered saline, and so normal bladder function and signalling is maintained. In the *ex vivo* tissue culture assay investigated here, however, bladders are removed from the rat, and so do not receive the normal endogenous signals that are necessary for cell survival, growth, and proliferation. Indeed, the act of culturing bladder strips leads to a loss of mucosal cells (Chapter 5), and a significant reduction in UPIII, Krt7, and ZO-1 expression compared to uncultured bladder strips. Therefore, as damage to the urothelium occurs in cultured control bladder strips, this may partially mask some of the effects of ketamine. When considering the evidence from multiple animal models of KC and reports of KC patients, it seems likely that ketamine exposure does cause decreased expression of ZO-1 in humans, rats, and mice.

6.6.5 Effect of Ketamine on Myosin-11 Expression in Rat Urinary Bladder Strips

Histological evaluation in some patients with KC has revealed smooth muscle degeneration (Lin et al., 2015), and ketamine has been shown to inhibit cell proliferation and induce cell death at high concentrations (≥1.82mM) in primary cultured human bladder smooth muscle cells (Chen et al., 2020). The effects of ketamine on the expression of myosin-11, a specific marker of differentiated smooth muscle cells, was therefore examined. No change in myosin-11 expression was observed following ketamine exposure when compared to both 3-day cultured control tissues and uncultured tissues (Figure 6.18). Therefore, in cultured rat urinary bladder strips, 3-day exposure to 3mM ketamine does not lead to a loss of smooth muscle cells.

6.6.6 Effect of Ketamine on CaV1.2 Expression in Cultured Rat Urinary Bladder

Evidence from the functional studies reported here indicate that ketamine is a $Ca_V1.2$ antagonist and this function likely mediates the inhibition of smooth muscle contractility seen following acute ketamine exposure (Chapters 3 & 4). Recently, it has also been reported that ketamine and other L-type calcium channel blockers (CCBs) such as nifedipine can inhibit the magnitude of bladder smooth muscle contractility but at the same time also induce bladder

frequency and bladder spasm after instillation into mouse bladder (Chen et al., 2020). This is consistent with studies that revealed an increase in frequency, urgency, and nocturia in patients taking CCBs (Elhebir, Hughes & Hilmi, 2013; Ito, Taga, Tsuchiyama, Akino & Yokoyama, 2013). Therefore, the effects of ketamine on the expression of $Ca_V1.2$ were investigated. Exposure to 3mM ketamine for 3 days had no effect on the expression of $Ca_V1.2$ in either the mucosal or smooth muscle layers in rat urinary bladder (Figure 6.21), suggesting that the increased contractility of rat bladder strips to carbachol following 3-day 3mM ketamine exposure likely cannot be explained by a change in $Ca_V1.2$ expression.

There are some limitations to this dataset, however. No reports were found detailing the immunohistochemical expression of $Ca_V1.2$ in human or rat bladder, and no negative antigen controls were used, making it difficult to ascertain the degree of non-specific staining in this instance (Figure 6.20).

6.6.7 Limitations

6.6.7.1 Immunohistochemistry Protocol

One of the major difficulties faced during the IHC protocol was partial or complete loss of tissue samples from the slides. During the initial trials of the IHC protocol, roughly one third of all slides would experience some degree of tissue loss, typically following the antigen retrieval stage or the proceeding treatment stages. As such, steps to remedy this issue and optimise the tissue culture protocol were taken. To ensure that excess water was not trapped underneath the paraffin wax which would impede adhesion of the sample to the slides, slides were left to dry on their sides overnight and also heated to 60°C in an oven prior to commencing the IHC protocol. These steps did seem to improve adhesion of the samples to the slides and improve the number of samples that experienced loss of tissue, but did not completely rectify the problem. Superfrost slides were also trialled in place of Polysine slides; however, this did not seem to affect the number of samples that experienced loss of tissue. For future experiments, reducing the temperature at which antigen retrieval was performed and/or the reducing the duration of this process could further help to reduce loss of samples during the IHC protocol.

The issue with loss of tissue from slides, although improved, was not able to be completely remedied, which resulted in several issues. Tissue sections from particular treatment groups

would have to be tested in duplicate or retested to ensure that a viable sample was obtained, thus limiting the overall number of samples that could be tested. This resulted in a smaller number of samples that were able to be analysed per treatment compared to the number of samples analysed with H&E staining, making direct comparison difficult. Additionally, some treatments were unable to be analysed statistically due to an insufficient sample size, or lacked the number of matched controls necessary to enable comparison via a paired t-test. To overcome this issue, it may have been beneficial to focus on a smaller, select number of treatments to ensure sufficient sample sizes were obtained and to enable robust statistical analysis.

Another limitation was the lack of some antigen controls during the IHC procedure. Positive controls constitute a sample from a tissue that is known to express the protein of interest, and that, if positive, validates that the procedure is working even if other samples return a negative result. A positive control was used for all antibodies tested apart from the M30 antibody. As immunoreactivity with cleaved cytokeratin-18 was minimal or lacking in the samples tested, it would have been invaluable to have a positive control as this would determine whether the antibody was working as expected.

Negative controls are also used by taking a sample from a tissue that does not express the protein of interest, and this helps to determine if there is any non-specific staining or any false positive results (e.g. using ileum and staining for immunoreactivity to UPIII). Such negative controls were not used making it difficult to determine the extent of non-specific staining. 'No primary antibody control' sections were conducted for all treatments, however, and displayed no staining, indicating that all staining observed in primary antibody-incubated tissues results from antigen detection by the primary antibody.

6.6.7.2 Tissue Culture Protocol

Both H&E staining and IHC examination of tissue samples revealed degradation of the urothelium in cultured control tissues, thus reducing the sensitivity of the assay. Optimisation steps, such as testing different culture medias and different supplements, were taken in an attempt to conserve the integrity of the bladder strips as much as possible; however, these steps were not as effective as hoped. Minimising urothelial degeneration during the tissue

culture stage would help when comparing treated bladder strips to their matched controls and determining the effects that ketamine or related compounds have on the urothelium.

6.6.7.3 Low Sample Sizes

As discussed, loss of tissue during the immunohistochemistry procedure resulted in a small sample size for some treatment groups. Lower sample sizes can lead to type I errors, whereby a study falsely concludes that there is a significant effect when there is none; or type II errors, where a study concludes that there is not a significant effect when there is. This issue can be addressed by increasing sample sizes, as larger sample sizes are more likely to produce a result that is reflective of the population. Too large a sample size here, however, could lead to unnecessary use of animal tissue, and would not be in line with the 3Rs principals. The use of power analysis, to determine the smallest sample size necessary to detect an effect, would have been of benefit here.

6.6.8 Future Work and Conclusions

Thinning or denudation of the urothelium is one of the most common histopathological findings in both patients with KC and in animal models of KC. Ketamine has also been reported to induce apoptosis in human urothelial cell cultures (Lin et al., 2022), induce thinning of the urothelium in human ureteric organ cultures (Baker et al., 2016), and reduce mucosal cell count in rat bladder organ cultures (Chapter 5), suggesting a direct toxic effect of ketamine on the urothelium. The finding that ketamine exposure reduces the expression of UPIII therefore suggests that thinning of the urothelium by ketamine can be explained, at least in part, by a direct toxic effect of ketamine on urothelial umbrella cells. Loss of umbrella cells is reported to impair the barrier function of the urothelium (Hu et al., 2000; Xie et al., 2021), and this is thought to be a potential cause of KC (Jhang et al., 2015; Castellani et al., 2020).

The mechanism(s) behind loss of umbrella cells following ketamine exposure remain elusive. It seems unlikely that NMDAR antagonism is responsible as MK-801, a more potent antagonist of NMDAR than ketamine, did not decrease UPIII expression. Additionally, it is unlikely that the toxic effects of ketamine on urothelial umbrella cells are mediated by $Ca_V1.2$ antagonism, as nifedipine had no effect on UPIII expression.

The ability of ketamine (≥300μM) to decrease bladder contractility *in vitro* after 20 minutes of exposure was reported in Chapter 3. Here, it was demonstrated that UPIII expression was unchanged after 20-minute ketamine (1 & 3mM) incubations when compared to uncultured bladder strips, confirming that these functional changes brought about by ketamine are not due to loss of umbrella cells. Enhanced bladder contractility to carbachol was observed in rat bladder strips exposed to ketamine (3mM) for 3 days (Chapter 4). As loss of urothelial cells is reported under these same conditions, it is possible that this functional change is caused by disruption of the urothelium.

The potential contribution of ketamine metabolites in KC has been proposed, in part due to their relatively high concentration in the urine of recreational users. The findings presented here suggest that NK and DHNK have no effect on the expression of UPIII or Krt7. Recent studies, however, have reported that NK significantly reduced cell viability and induced apoptosis in human urothelial cells, and was more cytotoxic than ketamine (Lin et al., 2022). It is therefore possible that NK contributes to the direct toxic effects of ketamine on the urothelium. The degree of apoptosis in rat bladder following ketamine or NK exposure was unable to be assessed from the IHC data obtained here; however, optimisation of the IHC protocol and the inclusion of negative controls could help to resolve this issue. Alternatively, a different assay, such as the TUNEL assay, could be employed instead.

The effects of ketamine and related compounds on glycosaminoglycan (GAG) expression would be of interest as GAG deficiency is a common histopathological finding in patients with KC (Xie et al., 2021), and lower GAG staining intensities were observed in a rat model of KC (Wang et al., 2017). Glycosaminoglycans cover the apical surface of the urothelium and contribute to its barrier function (Xie et al., 2021). The direct effects of ketamine or related compounds on the GAG layer have not been investigated, and may provide further insight into the pathogenesis of KC.

Further optimisation of both the IHC and tissue culture protocol would be beneficial for any future IHC experiments utilising the methodology presented here. Supplementation of the tissue culture media with hyaluronic acid or platelet-rich plasma could be considered to potentially reduce degeneration of the urothelium, as they have been shown to rescue decreased expression of UPIII in animal models of KC (Lee et al., 2017; Chueh et al., 2022). Additionally, optimisation of the IHC protocol to reduce loss of tissue samples would be of

benefit by enabling increased sample sizes and comparison of drug-treated tissues to their matched controls, thereby addressing some of the limitations of the data as discussed here.

The *ex vivo* tissue culture assay presented here shows clear effects of ketamine and (S)-ketamine on UPIII expression in rat bladder, and so could form the basis of a histopathological screening assay that is sensitive to ketamine toxicity.

Chapter 7: Overall Discussion

In 2007, Shahani et al. (2007) first described the clinical entity of ketamine-induced cystitis (KC) — a condition seen in recreational users of the drug, characterised by the development of various lower urinary tract (LUT) symptoms indicative of bladder overactivity. Urothelial thinning or denudation and inflammatory cell infiltration are common histopathological features of KC, and in some cases the damage to the bladder can become so severe that partial or complete cystectomy is the only recourse. To date, the aetiology of KC is not completely understood.

In recent years, the clinical application of ketamine has expanded to include its use in a number of chronic pain settings and as a fast-acting antidepressant. As KC is typically associated with the long-term use of ketamine, its long-term safety profile in such applications is important to consider. Indeed, LUT symptoms indicative of KC have been reported in individuals taking low doses of medicinal ketamine (Gregoire et al., 2008; Storr & Quibell, 2009; Shahzad et al., 2012; Schifano, Chiappini, Castiglione, Salonia & Schifano, 2021), making it even more pertinent to uncover ketamine's mechanism of action in KC. To help determine the mechanism of action, LUT symptoms associated with KC have been characterised preclinically in rodents, demonstrating their importance to model human bladder function despite recognised species differences in micturition.

7.1 Acute Effects of Ketamine on Rat Bladder Contractility

Ketamine is detected in the urine of users for up to 14 days even after a single dose (Moore et al., 2001; Castellani et al., 2020); therefore, the direct functional effects of ketamine on the bladder were examined. Ketamine decreased carbachol-induced contractility of rat urinary bladder strips after 20 minutes exposure, and this finding is in agreement with previous reports in both rat (Ceran et al., 2010), and human (Gonzalez-Cadavid, Ryndin, Vernet, Magee & Rajfer, 2000) bladder. Ketamine also facilitated a decrease in bladder contractility evoked by high potassium Krebs-Henseleit solution and electrical field stimulation (EFS). The ability of ketamine to inhibit bladder contractility to various stimuli that work through slightly different mechanisms suggest that it is acting on a common contractile pathway target that is downstream of muscarinic receptor signalling. Ketamine was also shown here to inhibit contractility of rat ileum, implicating a molecular target that is common to multiple tissues and not one that is specific to bladder. Therefore, it is suggested that ketamine may inhibit rat bladder strip contractility through inhibition of L-type calcium channel current, and

indeed, ketamine has been reported to concentration-dependently inhibit Ca^{2+} influx through L-type calcium channels in multiple species and tissue types (Yamazaki et al., 1992; Hirota et al., 1995; Hanazaki et al., 2001; Hatakeyama et al., 2001; Chen et al., 2020).

Norketamine (NK) and dehydronorketamine (DHNK), the major metabolites of ketamine, are also present in the urine of users at concentrations similar to or higher than that of ketamine (Moore et al., 2001); however, their effects on bladder contractility have not been reported. Both compounds were able to decrease rat bladder contractility evoked by either carbachol (CCH) or high potassium Krebs-Henseleit solution. Moreover, when incubated together, ketamine, NK, and DHNK were able to reduce rat bladder strip contractility to CCH at concentrations that would not affect contractility when administered alone. This suggests a possible additive effect between ketamine and its metabolites, and that NK and DHNK should also be considered when examining the functional effects of ketamine in KC.

The effects of various other ketamine-related compounds on rat bladder contractility were also examined. Dextromethorphan and MK-801 attenuated rat bladder contractility to CCH, and the ketamine-related new psychoactive substances (NPS) methoxetamine (MXE), methoxphenidine, and diphenidine all decreased rat bladder contractility to both CCH and high potassium Krebs-Henseleit solution, which has not previously been reported. Interestingly, many of these compounds are also reported to act as L-type calcium channel antagonists.

7.2 Mechanisms Underpinning Rat Bladder Strip Contractility Following Acute Exposure to Ketamine

Given the recognized receptor-binding promiscuity of ketamine, multiple potential targets in the contractile signalling pathway could be implicated. The presence of the N-methyl-D-aspartate receptor (NMDAR) has been reported in both human and rat bladder (Gonzalez-CaDavid et al., 2000), and all compounds tested here are reported to be NMDAR antagonists. Therefore, NMDAR antagonism was explored as a potential mechanism behind ketamine-induced inhibition of rat bladder contractility. The affinity of each compound to the NMDAR, however, did not correlate with their potency. Ketamine has also been reported to interact with M3 muscarinic receptors (Hirota et al., 2002); however, as contractions induced by high potassium were more sensitive to ketamine than CCH-evoked contractions, M3 receptor

antagonism by ketamine is unlikely as high potassium-mediated contractility bypasses muscarinic receptor activation. Ketamine also decreased CCH-induced contractility resistant to U73122 and Y-27632, suggesting that it does not act through, or at least does not entirely act through, blockade of phospholipase C-mediated calcium release or through Rho-kinase (ROCK) inhibition, respectively. Further, Y-27632 is reported to have no effect on potassium induced contractility in rat bladder strips, which further suggests that ketamine does not act through ROCK inhibition (Wibberley et al., 2003).

L-type calcium channel blockade by ketamine was further explored as a potential mechanism for acute inhibition of rat bladder contractility. Ketamine and the L-type calcium channel blocker nifedipine concentration-dependently inhibited EFS-induced rat bladder contractility that had been potentiated by the L-type calcium channel activator Bay K8644. Moreover, Bay K8644 was able to reverse contractile inhibition induced by ketamine or nifedipine. Taken together, these results indicate mutual antagonism of ketamine and Bay K8644, suggesting that acute inhibition of bladder contractility by ketamine is mediated by L-type calcium channel blockade.

7.3 Three-Day Effects of Ketamine on Rat Bladder Contractility

Rat bladder strips incubated for 3 days with ketamine displayed an increased contractility to CCH, which is a novel finding and is in stark contrast with the acute (20-minute) functional effects reported here. Contractions induced by high potassium Krebs-Henseleit solution, however, were unaltered following 3-day exposure to ketamine.

7.4 Mechanisms Behind Ketamine-Induced Potentiation of Rat Bladder Contractility Following Three Days Exposure

Given that the acute functional effects of ketamine appear to be mediated by L-type calcium channel antagonism, L-type calcium channels were explored as a potential source of the longer-term functional effects. Incubation for 3 days with the L-type calcium channel blocker isradipine had no effect on rat bladder contractility evoked by CCH compared to 3-day vehicle-treated control tissues. Further, incubating tissues with both ketamine and Bay K8644 did not reverse the increased contractility to CCH. Taken together, these results do not implicate L-type calcium channel antagonism as the mechanism underlying increased bladder contractility following 3-day ketamine exposure.

Interestingly, acute (20 minutes) exposure to ketamine was able to reduce the enhanced contractility of 3-day ketamine-incubated bladder strips. This appears to suggest that, within 3 days, ketamine is inducing some type of change (e.g. a microstructural change) in the bladder that leads to an increased contractile response to CCH, and that this increased response is not dependent on the physical presence of ketamine.

7.5 Effect of Three-Day Ketamine Exposure on Rat Bladder Mucosa

Thinning or denudation of the urothelium is a commonly reported histopathological feature of individuals with KC (Shahani et al., 2007; Chu et al., 2008; Tsai et al., 2009; Baker et al., 2013; Jhang et al., 2015; Lin et al., 2015), and in rat models of KC (Chuang et al., 2013; Juan et al., 2015; Wang et al., 2017). Moreover, ketamine has been reported to induce apoptosis in human urothelial cell cultures (Lin et al., 2022), and induce urothelial thinning in human ureteric organ cultures (Baker et al., 2016). Therefore, rat bladder strips were incubated with ketamine for 3 days to examine its direct histopathological effects. Ketamine concentration- and time-dependently reduced the mean mucosal cell count in rat bladder strips. This effect is unlikely to be mediated by NMDAR, as MK-801 had no effect on the mucosal cell count. Furthermore, 3-day exposure to nifedipine did not affect the number of mucosal cells, and incubating tissues with both ketamine and Bay K8644 did not reverse the effects on mucosal cell count when compared to ketamine alone. These data indicate that L-type calcium channel antagonism by ketamine cannot explain the reduction in mucosal cell count of rat bladder strips following 3-day ketamine exposure.

Data presented by Baker et al. (2016) suggests that toxicity of urothelial cells by ketamine is associated with a prolonged elevation of cytosolic calcium. At ketamine concentrations ≤1mM, these calcium transients are resolved after an average of 90 seconds and are largely dependent on activation of purinergic receptors brought about by ketamine-induced release of ATP. At higher ketamine concentrations (>1mM), elevation of cytosolic calcium remained unresolved after 10 minutes and the primary source of this calcium remains elusive. Further investigation into this novel mechanism of ketamine-induced cytotoxicity of urothelial cells would be of interest.

Norketamine, but not DHNK, reduced the number of mucosal cells in rat bladder strips. Few studies have examined the potential role of these active metabolites in KC, however this

finding suggests that NK may directly contribute to the urothelial damage seen in KC, and future research should also focus on the contribution of the metabolite, as well as the potential additive effects of the metabolite in combination with ketamine.

All the ketamine-related NPS tested here were shown to decrease mucosal cell count in rat bladder strips following 3 days exposure. There is little information regarding the effects of these compounds on the bladder; however, one study reported that histological examination of rats receiving daily MXE for 4 or 12 weeks revealed damaged urothelium with areas of disrupted continuity (Wang et al., 2017). Together, this suggests that MXE is likely not a "bladder friendly" alternative to ketamine as previously speculated. Use of these NPS may lead to bladder damage similar to that seen in KC, and so increased awareness would help to warn NPS users of the potential risks.

7.6 Immunohistochemical Effects of Three-Day Ketamine Exposure

To further examine the loss of mucosal cells following ketamine exposure, immunohistochemistry was employed to investigate the expression of specific proteins of interest. Decreased expression of uroplakin III (UPIII) was observed in rat bladder strips after incubating for 3 days with ketamine, suggesting that thinning of the urothelium can at least be partially explained by loss of umbrella cells induced by ketamine exposure.

Similar to this finding, decreased UPIII expression has been reported in several *in vivo* animal models of KC. In rats, western blot analysis revealed a reduced expression of UPIII after daily ketamine administration for 4 weeks (Lee et al., 2017), or 8 weeks (Chueh et al., 2022). Additionally, immunohistochemical evaluation of rat bladders following daily ketamine injections for 4 weeks revealed absent or disrupted UPIII staining (Huang et al., 2022).

In the *ex vivo* tissue culture experiments presented here, unlike in *in vivo* studies, ketamine is exposed to all tissue layers. No change in smooth muscle myosin heavy chain 11 expression was observed following 3-day ketamine exposure, suggesting that ketamine selectively damages urothelial cells and not smooth muscle cells in this assay. Although no quantifiable data was obtained here from cleaved cytokeratin 18 antibody immunoreactivity, several human studies have shown increased urothelial cell apoptosis following ketamine exposure in both *in vivo* and *in vitro* settings (Lee, Jiang & Kuo, 2013; Baker et al., 2016; Lin et al., 2022).

As such, the loss of umbrella cells reported here could potentially be explained by increased urothelial cell apoptosis.

7.7 Mechanisms Behind Ketamine-Induced Cystitis

The diverse biological effects and recognised receptor-binding promiscuity of ketamine has almost certainly complicated efforts to uncover its mechanism of action in KC. Since 2007, numerous mechanisms have been proposed, and it seems increasingly likely that this phenomenon is the result of the interplay of numerous ketamine-induced effects.

7.7.1 L-type Calcium Channel-Mediated Inhibition of Bladder Contractility

The finding that acute ketamine exposure causes inhibition of bladder smooth muscle contractility, although previously reported, seems rather counter-intuitive given that the LUT symptoms associated with KC indicate overactivity of the bladder. It was suggested here that ketamine may initially cause bladder relaxation or inhibit contractility of the bladder, thus increasing the contact time of ketamine and its metabolites with the urothelium, exacerbating urothelial damage, and ultimately leading to bladder overactivity. As models of KC do not tend to report the acute effects of ketamine, there is little to no data indicating whether ketamine may initially display these effects *in vivo*, although one study reported lower baseline detrusor pressure and increased bladder compliance in female mice after four weeks of daily ketamine administration (Meng et al., 2011).

Recently, however, is has been hypothesized that Cav1.2 antagonists, including ketamine, induce bladder overactivity by reducing bladder voiding efficiency which in turn leads to increased residual volume (Yu, 2022). This increase in residual volume would reduce the amount of available space for urine generated by the kidneys, and as such would cause an increase in voiding frequency. Indeed, increased residual volume has been reported in individuals taking nifedipine, and instillation of ketamine or nifedipine into the bladder of mice during cystometrogram reduced peak pressure while simultaneously increasing voiding frequency (Chen et al., 2020). If indeed ketamine is inducing increased residual volume in users, this would also increase the exposure time of the urothelium to ketamine and its metabolites present in the residual volume.

Given the reported role of Cav1.2 in ketamine-induced bladder dysfunction, Cav1.2 agonists such as Bay K8644 have been highlighted as potential treatments for LUT symptoms. It appears likely, however, that KC is the result of multiple mechanisms that are not all necessarily Cav1.2-mediated. Moreover, ketamine- and nifedipine-induced voiding dysfunction is seen almost immediately upon instillation into the bladder, whereas KC is associated with long-term ketamine use. It is unknown whether the acute Cav1.2-mediated effects of ketamine are related to the long-term effects of ketamine in KC. The acute Cav1.2-mediated effects of nifedipine appear to persist in long-term users, as a similar urodynamic profile of increased residual volume and increased frequency was reported in individuals being treated with the drug (Forman, Andersson, Henriksson, Rud & Ulmsten, 1978). Reports detailing the effects of long-term ketamine use on residual volume are sparse; however, one study in rats reported a decreased residual volume and increased micturition frequency after daily ketamine injections (Chung et al., 2022). Moreover, the longer-term effects of ketamine reported here — that is, increased CCH contractility and loss of urothelial cells following 3 days exposure — were not observed in tissues incubated under the same conditions with nifedipine, suggesting that the long-term effects of ketamine and nifedipine may be mediated through different mechanisms.

7.7.2 Increased Bladder Contractility Following Three-Day Ketamine Exposure

The mechanism behind increased bladder strip contractility to CCH after 3-day ketamine exposure remains elusive, however this effect was shown to not be Cav1.2-mediated. Incubating bladder strips for 3 days with compounds that interact with other ketamine-associated targets would help to elucidate this effect.

One potential explanation for the increase in bladder contractility is urothelial damage induced by ketamine. Ketamine was shown here to reduce mucosal cell count which could be at least partially explained by loss of urothelial cells, and urothelial denudation is typically associated with an increase in bladder contractility to CCH (Hawthorn et al., 2000; Chaiyaprasithi et al., 2003; Koşan et al., 2005). The mechanism for this increased bladder contractility is thought to be mediated by a diffusible inhibitory factor that is released by the urothelium, and so removal of the urothelium prevents this inhibitory effect. This is also reported to be dependent on M3 receptor activation, as other contractile mediators such as KCl did not cause increased detrusor contractility in denuded strips. This could therefore

explain why 3-day ketamine cultured bladder strips did not show and increase in contractility when stimulated by KCl. This possibility could be confirmed by introducing an intact bladder strip into the organ bath of a 3-day ketamine incubated strip to see if this reverses the enhanced contractile response to CCH.

7.8 Translational Value of Rat Models of Ketamine Cystitis

The novel *ex vivo* rat model presented here could serve as a useful tool for further investigation into the direct effects of ketamine, its metabolites, and related NPS on bladder histopathology and function. Rat models of KC do appear to have translational value given the similar LUT symptoms observed in both *in vivo* rat models and human cases of KC; the similar histopathological findings observed in the bladder from both KC rat models and KC patients; the reported cytotoxicity of ketamine to both rat and human urothelial cells *in* vitro; and the similar acute *in vitro* functional effects of ketamine in both human and rat bladder. While the higher concentrations of ketamine (3mM) used here to model urothelial damage are theoretically possible, it is very unlikely that such concentrations would be sustained for three days *in vivo*. However, the effects observed here do appear predictive of reported *in vivo* effects, and so this model may represent a 'sped-up' version of the urothelial damage seen in KC.

One limitation of this *ex vivo* assay is its inability to model downstream inflammatory mechanisms that are associated with chronic LUT symptoms, as there is no infiltration of inflammatory cells. Chronic inflammation in KC is thought to result in bladder fibrosis (Wang et al., 2017), and may also lead to direct activation and sensitisation of afferent nerves which potentially contributes to the LUT symptoms (Xie et al., 2021).

7.9 Ketamine Use in Depression

Given the increasing use of ketamine as an antidepressant, its potential effects on the bladder are important to consider. The prevalence of KC appears to be positively correlated with the amount and frequency of use (Mak et al., 2011; Winstock et al., 2012), and, although treatment plans for ketamine patients vary (Andrade, 2017; Andrade, 2019), the dosage and frequency of use is generally less than those reported in recreational users of the drug (Winstock et al., 2012). Perhaps not surprisingly then, instances of bladder dysfunction in patients undergoing ketamine treatment for depression are rare compared to the estimated

25-50% of recreational users that develop KC (Muetzelfeldt et al., 2008; Mak et al., 2011; Winstock et al., 2012; Feifel et al., 2020). Moreover, there is the potential for bladder dysfunction to be misdiagnosed as KC due to the coincidental ketamine treatment and its similar clinical and histopathological presentation to other conditions such as interstitial or bacterial cystitis (Tsai & Kuo, 2015).

Overall, considering the low reported incidence of bladder dysfunction in patients taking therapeutic ketamine, and the relatively high doses of ketamine necessary here and in the literature to induce functional and/or histopathological changes in the bladder, the risk of developing KC as a result of ketamine treatment for depression appears low. However, studies investigating its longer-term therapeutic use remain an unmet need.

7.10 Summary Statement

The aetiology surrounding KC remains incompletely understood; however, uncovering ketamine's mechanism of action is becoming increasingly important given its expanding clinical application. The aim of this thesis was to provide further insight into this phenomenon, with the ultimate hope of developing treatments and/or prevention of KC. Based on the findings presented here and those published in the literature, it seems likely that multiple ketamine-induced effects contribute to the development of KC.

The direct contact of ketamine and its metabolites with the bladder appears to be crucial. Acutely, ketamine reduces the magnitude of bladder contractility which either leads to increased micturition frequency due to poor bladder efficiency and increased residual volume, or a reduction in urinary frequency due to reduced bladder contractility. In either case, the result is the same — an increased contact time of ketamine and its metabolites with the urothelium. This increased contact time will exacerbate the damage to the urothelium, as evidenced by reports of increased urothelial cell cytotoxicity with increasing exposure durations to ketamine or NK across species.

Over time, *in vivo*, this toxic effect on the urothelium will result in impairment of its barrier function. Although no change in ZO-1 expression following ketamine exposure was observed here relative to control-incubated tissues, expression levels of ZO-1 and other key components involved in urothelial barrier function, such as glycosaminoglycan, are reportedly reduced in KC. Increased urothelial permeability can lead to leakage of irritative agents and

urinary potassium to the underlying tissue layers which could cause an inflammatory response; and also directly depolarise nerves and smooth muscle, potentially contributing to the LUT symptoms of KC. Chronic inflammation by persistent use of ketamine can progress to fibrosis of the bladder, which can cause decreased bladder elasticity and compliance.

The contribution of the increased bladder contractility following 3-day ketamine incubation in KC is not known; however, it is potentially the result of the loss of a diffusible inhibitory factor that is released by the urothelium following M3 receptor activation. In KC, urothelial denudation mediated by ketamine- and/or NK-induced toxicity could therefore increase bladder contractility and contribute to the characteristic LUT symptoms.

Importantly, the concentrations of ketamine tested here that inhibited bladder contractility (≥300μM) are clinically relevant. In users taking 1g of ketamine, a urine concentration in excess of 1mM is theoretically possible, and more than a third of recreational users reported that they use more than 1g in a typical session (Winstock, Mitcheson, Gillatt & Cottrell, 2012).

In recreational users of the drug, cessation of use appears to be the most effective treatment. Given the findings presented here, drugs that help to regenerate the urothelium and restore its barrier function may also be effective in treating ketamine-induced bladder dysfunction. Even the simple preventative measure of drinking more water may help by increasing urinary frequency and/or diluting the concentrations of ketamine and its metabolites in the urine, limiting their toxic effects on the bladder.

The evidence presented here provides further avenues to explore in the hope of fully understanding the aetiology of KC.

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