Understanding the Role of BMP Signalling in the Formation of Neural Circuits in the chick embryo

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2 Abbreviations

SCI – Spinal Cord Injury

PNS – Peripheral Nervous System

CNS – Central Nervous System

BMP – Bone Morphogenetic Protein

INs – Excitatory Interneurons

ES – Embryonic Stem Cells

NSPCs – Neural Stem/Progenitor Cells

iPSCs – Induced Pluripotent Stem Cells

DRG – Dorsal Root Ganglia

NT – Neural Tube

NCCs – Neural Crest Cells

Shh – Sonic Hedgehog

MN – Motor Neurons

STC – Spinocerebellar Tract

DREZ – Dorsal Root Entry Zone

DMN – Dorsomorphin

ISL – Isoliquirtigenin

RP – Roof Plate

Abstract

Regenerative cell-based therapies for spinal cord injuries (SCI) will benefit from a deeper understanding of proprioceptive neural circuit formation in the developing embryo. The function of sensory interneurons, such as dI3, and the Dorsal Root Entry Zone (DREZ), which are components of proprioceptive neural circuits, are well characterised, but the developmental processes and molecular environment needed for their formation are not well understood. We investigated the Bone Morphogenic Pathway (BMP) which is important for the development of dorsal interneurons. However, its involvement in the birth of dI3 neurons and the formation of the DREZ is not well understood. In this study, we used the chick embryo model to investigate the effect of dorsomorphin (DMN) which is a BMP pathway inhibitor, and isoliquiritigenin (ISL) which is a BMP pathway activator on dI3 interneuron generation and DREZ anatomy. Our objective was to inject these small molecule drugs sub-blastodermally (10µl for DMN, 10µl for ISL) at defined stages (16, 18) of chick embryo development and use immunohistochemistry to investigate dorsal interneuron and DREZ formation. We found that DMN broadens and dampens levels of Smad1/5/9, a downstream effector of BMP signalling within a specific time window before the birth of dI3 interneurons in the dorsal neural tube which correlates with significant increases in the number of dI3 neurons that are generated at the expense of dI1 and dI2 neurons and ventrally positioned motor neurons. ISL has no significant impact on the dI3 neuron population but does significantly increase the number of dI1 interneurons as well as the number of dorsal rootlets innervating the DREZ. These observations suggest that both the level and the duration of BMP signalling are important for dI3 interneuron generation and provide new insight into the role of BMP signalling in DREZ formation. Future work will involve the investigation of other downstream signalling markers to confirm disruption of the BMP signalling by DMN and ISL, as well as the investigation of BMP receptor and BMP inhibitor expression.

1 Introduction

Spinal cord injury (SCI) has a catastrophic impact on the physical and socioeconomic status of those who are injured, as SCI causes a disruption in nerve connections between the peripheral nervous system (PNS) and central nervous system (CNS). This connection is important as the PNS links the CNS with the rest of the body enabling communication of sensory and motor information and thus sensation and movement. Depending on the severity of the injury, different therapeutic approaches are being used and developed for SCI. Many of them involve stimulation of dI3 sensory interneurons which play a crucial role in movement coordination and integration with sensory feedback (Kozlova, 2008; Mahrous et al., 2023; Poopalasundaram et al., 2023; Xu et al., 2023; Zheng & Tuszynski, 2023) One approach is stem cell therapy, however for this strategy to be successful knowledge of how to generate and amplify the neural cells of interest is required (Huang et al.; 2021). It is currently unclear how dI3 interneurons are generated *in vivo*, however, there is strong evidence that the process is dependent on the Bone Morphogenetic Protein (BMP) signalling pathway (Duval et al., 2019; Laliberte et al., 2022; Andrews et al., 2017). Moreover, there is limited information regarding the impact of the timing of BMP signalling on the birth of dI3 interneurons.

This project aimed to investigate the temporal role of BMP signalling in the birth of dI3 dorsal interneurons using the developing chick embryo as a model. This study also explored the impact of BMP signalling on the integrity of the dorsal root entry 'gateway'. This "gateway" serves as a zone for sensory axon innervation into the CNS, preventing neuronal migration out of the CNS and this develops in the same region of the dorsal neural tube as dI3.

1.1 Spinal Cord Injury and Repair Strategies

SCI is a neurological condition resulting in paralysis and impaired sensational and autonomic dysfunction. SCI leads to severe damage of white matter, and often loss of motor function below the injury site (Xue et al., 2021; Zipser et al., 2022). SCI can be either complete, where there is no nerve communication below the injury site, or incomplete (National Institute of Neurological Disorders and

Stroke, n.d.). Complete SCI usually occurs in young adults and the elderly (Alito et al. 2021). This can be related to two major causes of complete SCI: motor vehicle accidents and falls (Zárate-Kalfópulos et al; 2016). The annual incidence varies across the region and countries, but it approximately ranges from 10.4 to 83 cases per million per year (Wengel et al., 2020). Incomplete SCI interrupts the flow of motor and sensory information between the spinal cord and brain. The most common form of this injury leads to walking and motor difficulties.

There are two phases of SCI, necrotic death of tissue surrounding the injury followed by the formation of a glial scar, as reactive astrocytes migrate to the site to contain the wound. Both the injury itself and glial scar can damage or remove neural connections and neural circuits and thus a major therapeutic goal is to restore lost neural circuits and connectivity around and across the injury site. Restoring the ability to walk is a primary concern for individuals with SCI and is frequently addressed through occupational therapy, physical therapy e.g. muscle strengthening, and positioning (McHugh et.al., 2020) as well as functional electrical stimulation (Shen et al., 2022; Xue et al., 2021). These physical therapies, often involve stimulation of dI3 sensory interneurons, as these interneurons are shown to be necessary for recovery of locomotion (Bui et al., 2013; Kozlova, 2008; D. C. Lu et al., 2015). This specific class of interneurons is recognised for processing sensory information from skin mechanosensitive receptors (Bui et al. 2015). dI3 neurons are important for grasp circuits due to their role in mediating sensory information from mechanosensitive receptors through low threshold afferents (Bui et al. 2015; Wilson & Sweeney; 2023). Unfortunately, such physical therapies are not 100% effective and do not always allow injured patients to achieve full recovery (Yang et al., 2020). As an alternative, transplantation of neural stem cell populations into patients with SCI has been extensively investigated, including the use of glial astrocytes and oligodendrocytes (Davies et al. 2008) and excitatory interneurons (INs) (Asboth et al. 2018). Recent work has focused on stem cell therapies, i.e. transplanting neural stem cells into patients with SCI to enhance neural circuit repair and reactivation. Research has shown that the transplantation of neural stem cells into SCI lesions contributes to the generation of neurons that extend axons and make connections with healthy neural

circuits even across long distances (Wilcox et al. 2014). Neural cells/neurons for explant into patients can be derived from several sources. One example is embryonic stem (ES) cells. ES cells are derived from pluripotent blastocyst embryos and can differentiate into any cell in the body including neural precursors specific neurons or glial cells. ES cells are obtained from preimplantation or blastocyst-stage embryos. They can be also sourced from the parthenogenic activation of eggs or created by somatic cell nuclear transfer (Mothe & Tator, 2012). When transplanted they can form teratomas, tumour-like cell formations, therefore ES cells must be predifferentiated prior to transplantation (Prokhorova et al.; 2009). In experiments performed on rats, this therapy shows partial motor recovery and allows axon regeneration (Huang et al. 2021; Mothe & Tator, 2012). The advantage of this therapy means ES cells can be propagated in vitro indefinitely; however, there are difficulties in the generation of cell lines without genetic abnormalities. ES therapy has its ethical issues due to the possibility of tumorigenesis caused by incomplete differentiation (Mothe & Tator, 2012). Neural stem/progenitor cells (NSPCs) are also candidates for use in stem cell therapies for SCI. NSPCs derived from the subventricular region of the ventricles, hippocampus, and the ependymal region of the central canal of the spinal cord, are pluripotent cells. These cells have the potential to differentiate into specific neuronal or glial cells, contributing to remyelination and providing nutritional support (Huang et al. 2021). Xu et al. 2023 used early embryonic NSPCs isolated and cultured on a collagen scaffold to construct spinal cord–like tissues, which then were transplanted into rats and rhesus monkeys that suffered SCI. The experiments showed that NSCPs could differentiate into mature cells with specific neuron identities when transplanted into the monkeys. This method contributed to the recovery of damaged neuronal circuits and promoted hindlimb motor recovery (Gazdic et al., 2018; Mothe & Tator, 2012; Xu et al., 2023). Induced Pluripotent Stem Cells (iPSCs), similar to NSPCs, are generated from a patient's cells e.g. hair follicle adult stem cells. They overcome the ethical issues associated with ES cells, for example, hair follicle stem cells are easily accessible and can be reprogrammed, re-differentiated into neural cells, and transplanted (Xia et al. 2023). The strategy of stem cell therapy

however requires knowledge of the developmental mechanisms which drive both the patterning and birth of specific neuroepithelial progenitors and specific neurons and glial cells in the embryo/foetus.

1.2 Development of the Central Nervous System and Peripheral Nervous System

The central nervous system (CNS) consists of neurons and glia. Axonal projections connect neurons in the spinal cord via ascending and descending tracts (Fig.1). The role of the CNS includes receiving, processing, and responding to information. The peripheral nervous system (PNS) is crucial for connecting the CNS to the tissues and organs in the periphery of the body and for sending information from different areas of the body of an animal into the CNS (Nowakowski & Hayes, 1999). The PNS is composed of two types of nerve fibres: afferent and sensory, which transmit information from the periphery to the CNS, and efferent and motor, which carry signals from the CNS to muscles (Catala & Kubis, 2013). The cell bodies of peripheral sensory neurons reside within dorsal root ganglia (DRG; Fig.1C) and send sensory axons to both the CNS and peripheral tissues, these neurons are special in that they possess two axon projections (bi-polar) (Shwarz et al. 2009). The cell bodies of motor neurons reside within the CNS and send their single axon out via the ventral root directly to the tissue/organ or via neuron connections (neuron relay) within the sympathetic ganglia. The connection between PNS and CNS is important for proprioception, which is how the CNS perceives and regulates movement and distinguishes the location of body parts e.g. limbs without any visual cues. (Han et al., 2016). Proprioception is necessary for motor control as well as effective performance during task execution (Hiller S. & Immink M. & Thewlis D; 2015). The connection between CNS and PNS is also crucial for the function of ascending and descending pathways (Figure 1B). Ascending tracts carry information, such as pain, up to the spinal cord and to the brain, where the information is processed. Then, descending pathways carry motor information which enables movement (Canedo, 1997; Lemon, 2008).

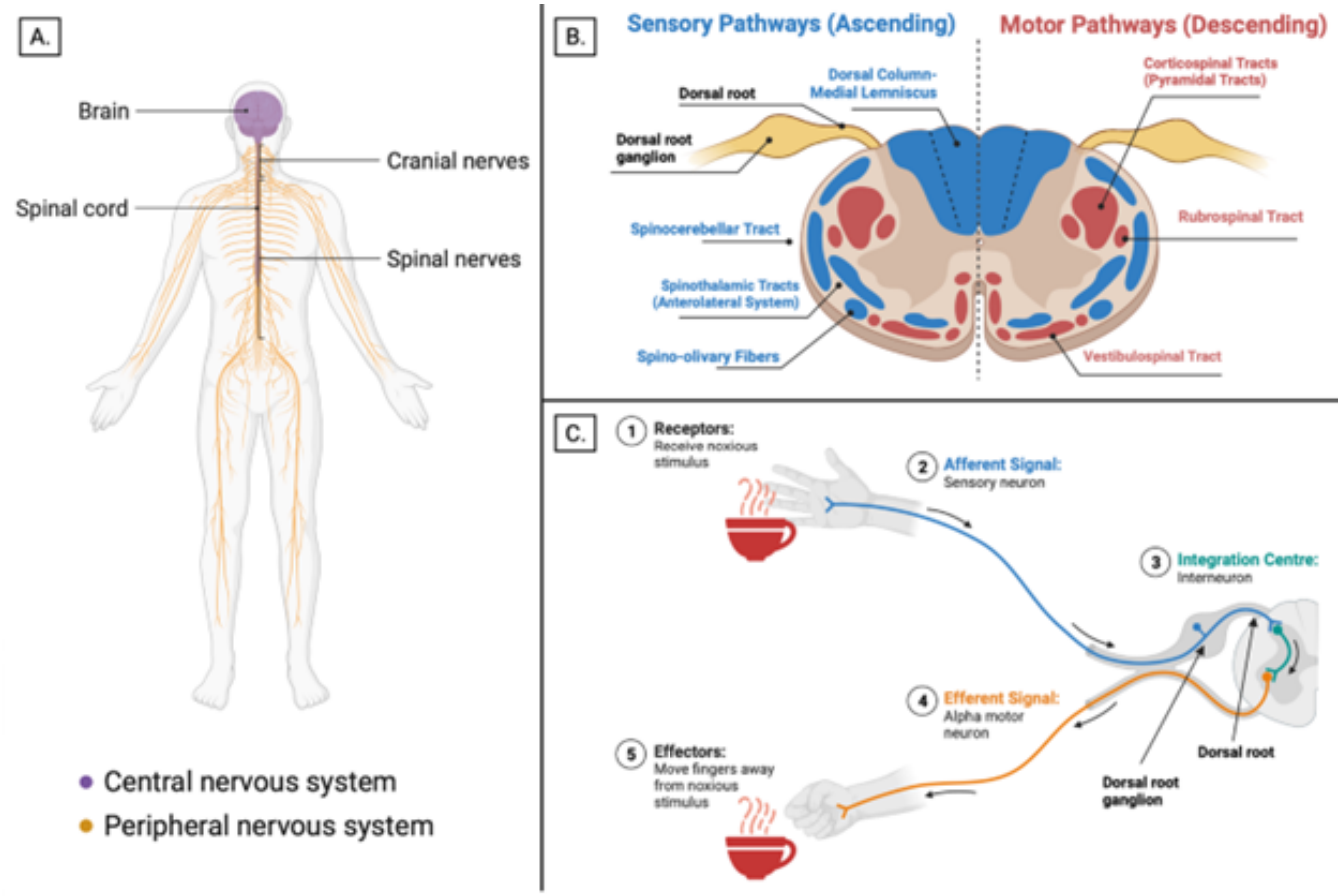


Figure 1.*Schematic representation of central and peripheral nervous system.*

A. Schematic representation of central and peripheral nervous system. B. Schematic presentation of section through spinal cord, showing anatomy of ascending sensory pathways in blue and descending pathways in red and their location within the spinal cord. C. Schematic representation of reflex nerve pathway and association of sensory, interneuron and motor neurons and their pathways Figure created by author using Biorender.com and based on (Bui et al., 2013; Canedo, 1997; Catala & Kubis, 2013; Han et al., 2016; Lemon, 2008).

1.3 Neural Tube Formation

The CNS and PNS are derived from the neural tube (NT) which develops at early embryonic stages and is derived from the dorsal ectoderm. NT development starts with the induction of the neural plate, which appears within the dorsal ectoderm at the dorsal midline of the developing body (Murray L. Barr & John A. Kiernan, 1983). Firstly, the neural plate undergoes primary neurulation, where neural folds elevate using a midline neural groove hinge point. Subsequently, dorsolateral hinge points form which enable further neural plate folding on the dorsal side which drives the fusion of the neural

folds and eventual neural tube closure (Lowery & Sive, 2004; van der Spuy et al., 2023). Neural tube closure proceeds posteriorly as the anterior-posterior axis of the embryo trunk is generated (Figure 2).

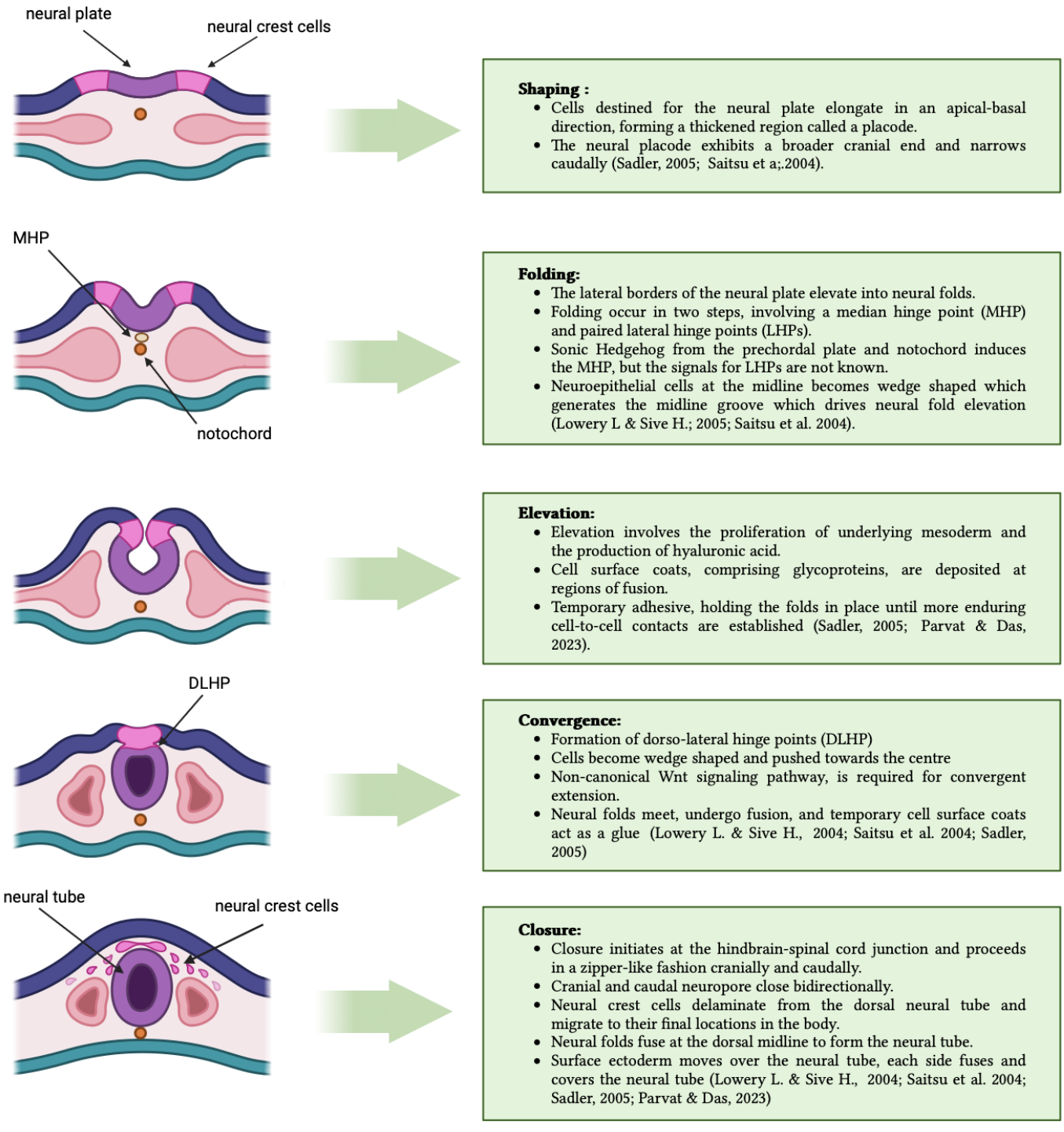


Figure 2. *Schematic presentation of neural tube formation.*

Primary neurulation, divided into five steps of development. Each step is described in the figure. Figure created by author in biorender.com based on Gilbert SF, 2000; Lowery & Sive, 2004; Parvat Kuwar Chhetri & Joe M Das, 2023; Sadler, 2005; Saitsu et al., 2004.

The posterior neural tube develops through a secondary neurulation process (Gilbert SF, 2000) which involves the transformation of mesenchymal cells in the tailbud into neuroepithelial cells which gain apical-basal polarity, organise into a rosette formation of cells, and finally generate a central luminal cavity.

Along the dorsal NT, neural crest cells (NCCs) are generated following closure. Neuroepithelial cells undergo epithelial to mesenchymal transition, then they migrate away from the NT and contribute to the PNS and other tissues. For example, cranial neural crest cells give rise to cranial sensory ganglia and sense organs. In the trunk, the NCCs give rise to the neurons of the dorsal root ganglia and sympathetic chain ganglia (Butler & Bronner, 2015; Maden, 2002).

As the neural tube matures into the spinal cord it develops ventricular, mantle, and marginal layers, with progenitor stem cells in the ventricular zone, neurons in the mantle layer, and their axon tracts in the marginal layer. Different classes of neurons are born at distinct positions along the dorsal-ventral axis of the spinal cord, leading to the formation of groups of neurons with specific functions. This dorsal-ventral patterning of neurons has been shown to require morphogen signals arising from both the most dorsal (surface ectoderm/roof plate) and most ventral (notochord/floor plate) aspects of the neural tube. Ventral neural progenitors respond to Sonic Hedgehog (Shh) signalling from the underlying notochord and the floor plate of the NT, giving rise to motor neurons and ventral interneurons. Dorsal neural progenitors are influenced by the BMP and Wnt signalling from the roof plate of the NT and adjacent surface ectoderm (BMP) and paraxial mesoderm (Wnt), leading to the formation of dorsal interneurons. Different classes of spinal cord neurons, including motor neurons, are born at specific segmental levels along the rostral-caudal axis, controlled by the Hox gene code. The formation of spinal circuits is tightly regulated, ensuring the proper wiring and function of the nervous system (Butler & Bronner, 2015; Hegarty et al., 2013; Murray L. Barr & John A. Kiernan, 1983).

1.4 Neural Tube Patterning

Morphogens such as Sonic Hedgehog (Shh), Wnts and bone morphogenic proteins (BMPs) are versatile proteins taking a crucial part in CNS development. They function in a gradient-dependent manner along specific body axes. Morphogen gradients form as early as when the neural plate is formed and throughout the formation and moulding of the neural tube (Le Dréau, 2022a; Stone & Rosenthal, 2000). Dorso-ventral patterning of the neural tube and the generation and differentiation of dorsal interneurons require multiple morphogens signalling pathways.

1.4.1 The BMP pathway

As outlined in Table 1, BMPs are members of TGFβ family (Kubiczkova et al. 2010). The BMP pathway plays a significant role in the patterning of dorsal progenitors and is pivotal for the formation of dorsal classes of sensory interneurons, especially dI1, dI2, and dI3 (Andrews et al., 2017a). As presented in Figure 3, BMP signalling initiates with the dimerization of two distinct BMP cell surface receptors, type I (ALK1, ALK2, ALK3, and ALK6) and type II (BMPRII, ActRIIa, and ActRIIb) (Table 1). BMP binding connects these receptors in the plasma membrane and induces their dimerization (Antebi et al.; 2017). Type II receptors contain an integral Serine/Threonine kinase within their intracellular domains. Dimerization leads to the phosphorylation by the type II S/T kinase of the intracellular domain of the Type 1 receptor. This activated receptor dimer then triggers cell signalling resulting in phosphorylation of the BMP effectors Smad1, Smad5, and Smad8. Subsequently, the complex of phosphorylated pSmad,5,8, together with Smad4, is translocated to the nucleus (Yu et al., 2008). pSmad1,5,8-Smad4 bind to CG-rich regions in the genome and thus activates changes in gene transcription (Antebi et al.; 2017). CG-rich regions often mark sites of gene transcription (Fainsod et al., 1997; Hegarty et al., 2013; Liem et al., 1995). BMP-Smad1/5/9 signalling is extensively involved in the patterning of the dorsal spinal cord. Its source is at the roof plate (RP), located above dorsal part of the NT (Chizhikov & Millen; 2004) and BMP ligand spreads in a gradient-dependent manner in the dorsal neural tube (Andrews et al. 2017). The signalling participates in patterning on the neural tube

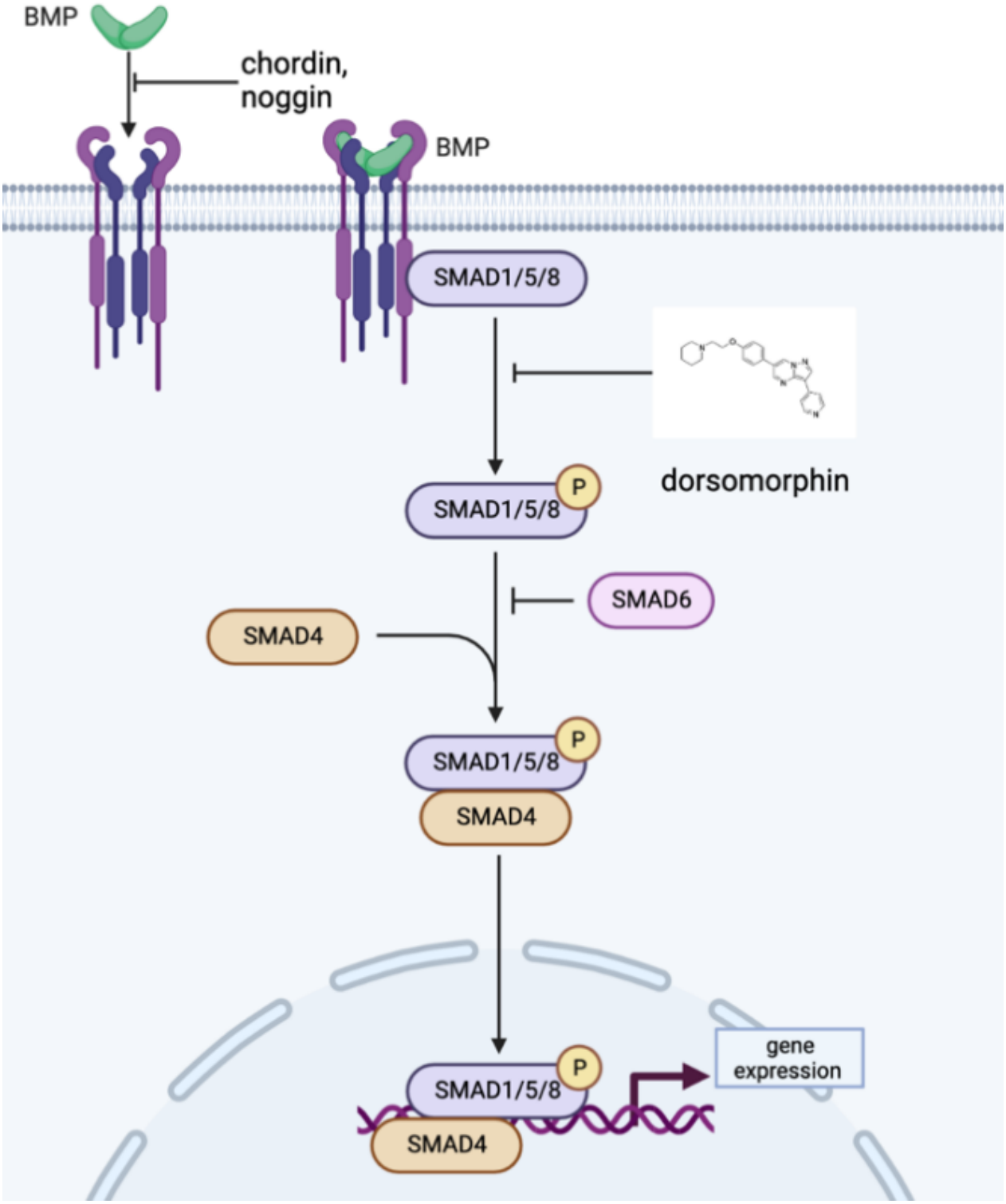


Figure 3. *The BMP signalling pathway*

BMP canonical signalling in Smad-dependent manner. BMP initiates the signal cascade by inducing dimerization of BMP receptors which induces a phosphorylation signalling cascade leading to activation of Smad effectors which translocate to the nucleus. Chordin, noggin are BMP antagonists. Dorsomorphin inhibits Smad phosphorylation by binding to type I receptors. Illustration created by author with Biorender.com.

from early embryonic stages. The signalling participates in patterning on the neural tube from early embryonic stages. BMP signalling acts upon dorsal progenitor domains such as those which express the transcription factors Olig3 and Lbx1 to promote birth of dorsal interneurons (Le Dreau, 2022; Le Dreau & Marti 2012). In experimental settings, it was demonstrated that BMPs induce the generation of dorsal interneurons: dI1, dI2, dI3 (Tozer et al., 2013a). It was found that, loss or reduction of BMP activity within dorsal progenitor domains could lead to decreases in dorsal interneuron populations (Le Dreau & Marti, 2012). Additionally, experiments performed on mice with hyperactivation of BMP revealed an increase in dorsal interneuron populations (Timmer et al., 2002, Le Dreau & Martin, 2012).

Different BMP proteins have divergent functions in embryo development, dorso – ventral patterning and the development of dorsal interneurons (Table 1). In chick, BMP4 and BMP7 are the only BMPs expressed in the dorsal spinal cord before neurogenesis. In loss-of-function studies performed in chick embryos, it was demonstrated that BMP 4 knock down, at early neurogenesis, resulted in reduction of dI1 population (Le Dreau et al., 2012). Additionally, it was found that the birth of dI3 neurons alone is induced by BMP4 as well as BMP7 (Andrews et al., 2017). Studies suggest that the timing of expression of BMP4 and BMP7, and the level and distribution of the receptors they bind to (Table 1), is crucial for the specification of dorsal Interneurons (Alvarez-Medina et al., 2008a; Andrews et al., 2017b; Selleck et al., 1998; Tozer et al., 2013b).

1.4.2 Developmental signalling pathways which inhibit BMP signalling.

Noggin is a glycoprotein secreted by notochord and encoded by the NOG gene (Nog for mouse and chick). The structure of noggin reassembles bilateral molecular symmetry like BMPs, forming a dimer. Noggin undergoes dimerization through its core body, featuring two pairs of β-strands that extend along with an N-terminal segment of approximately 20 amino acids known as the "clip" segment. This clip segment winds around the BMP ligand, effectively obstructing the surfaces of the growth factor and preventing its binding to both BMP type I and type II receptors (Zhang et al., 2008; Karunaraj et al. 2022). Noggin plays a crucial role in the dorso-ventral patterning of the NT by antagonising the

actions of BMP4 and BMP7, effectively Noggin neutralises BMP signalling (Krause et al., 2011; Groppe et al., 2002; Hirsinger et al., 1997), however a balance between BMP4 signalling and Noggin

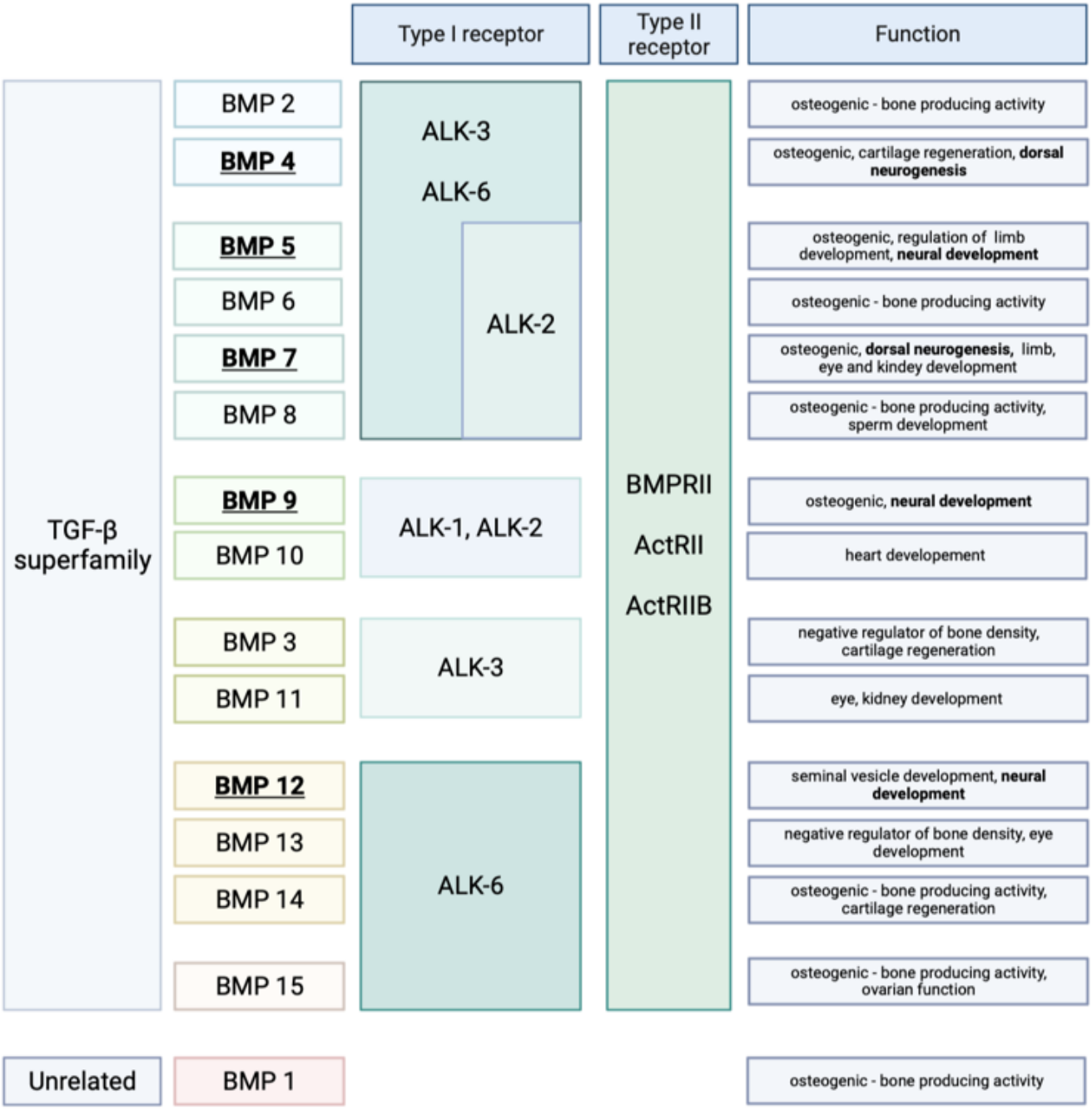


Table 1. *TFG-β BMP family members and their functions. Table presents which BMP receptors each protein binds to. Underlining and bold font presents protein involved in neural development. Table created by author in biorender.com based on Katagiri & Watabe, 2016; Sanchez-Duffhues et al., 2020; Wang et al., 2014.*

antagonism of BMP4 signalling is needed for healthy neural development e.g. control of neural crest cell emigration (Sela-Donenfeld & Kalcheim, 1999).

1.4.3 Dorso-ventral patterning interactions between BMP signalling and SHH/Wnt pathways

Wnts are a family of secreted palymitoylated glycoproteins proteins related to Drosophila wingless protein and take part in cell-to-cell interaction regulation during embryogenesis. Wnt expression in the dorsal neural tube is induced by BMP protein (Burstyn-Cohen et al., 2004). Wnt protein binds to the receptor of the Frizzled family on the cell surface, then a signal is transduced through the canonical β-catenin pathway, which then enters the nucleus to form a complex with specific transcription factors to activate Wnt target genes (Figure 4) (Alvarez-Medina et al., 2008b; Le Dréau & Martí, 2012). Wnts are components of roof–plate signalling (Ulloa & Martí, 2009). In studies with mice lacking specific Wnts i.e. Wnt1, Wnt3a (Chesnutt et al.; 2004) it was found that generation of dI1 and dI2 classes of interneurons was reduced; the loss was compensated by expansion of dI3 neuron population (Muroyama et al., 2002).

On the ventral side, initially from the notochord and then from the floor plate of the NT, Shh is released (Figure 6). Shh ectopic expression is crucial for the patterning of ventral neuroepithelial progenitors and the subsequent generation and differentiation of ventral interneurons and motor neuron (MN) cells (Litingtung & Chiang, 2000). Shh signalling involves two transmembrane proteins: Patched (Ptch) and Smoothened (Smo). Where Shh ligand is lacking, Smo activity is inhibited by Ptch. Shh pathway starts with Shh binding to Ptch which releases inhibition of Smo, which leads to activation of intracellular signalling (Figure 5) (Ulloa & Martí, 2009). Absence of Shh signalling stops development of ventral interneurons and reduces cranial motor axons (Litingtung & Chiang; 2000). In vertebrates, the tight regulation of the dorso-ventral BMP signalling gradient is crucial for proper tissue growth and differentiation (Le Dréau & Martí; 2012). Dorso-ventral morphogen gradients provide the positional information that define boundaries of progenitor domains (Bier & Robertis, 2015). Based on the French flag model devised by Lewis Wolpert (Sharpe; 2019), morphogen activity areas are

determined by threshold concentration (Vetter & Iber, 2022). Figure 5 presents a schematic diagram of the proposed morphogen gradients of BMP/Wnt and Shh, signalling which result in establishment of dorso-ventral axes of the neural tube and subsequent dorso-ventral patterning (Vetter & Iber, 2022; Fuentealba et al., 2007). Shh signalling, acts upon progenitor domains p3, pMN, p2 and p1 leading to generation of ventral interneurons and motor neurons (MN) whilst BMP and Wnt signalling derives the dorsal classes of interneurons (Kutejova, Briscoe & Kicheva; 2009).

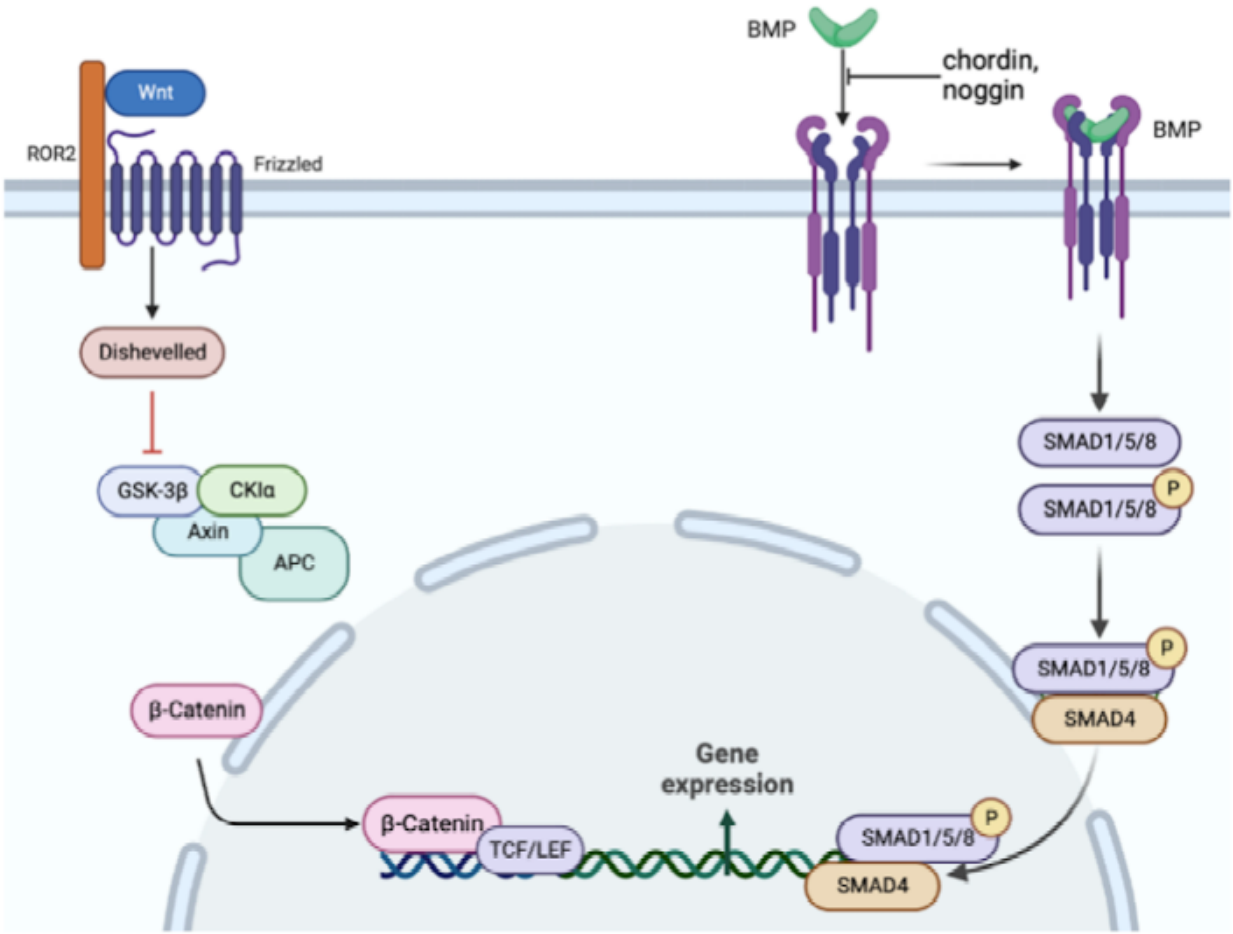


Figure 4. *The WNT/BMP ß-catenin pathway.*

WNT protein binds Frizzled receptor on the surface of the cell membrane, then a signal is converted through the canonical B-catenin pathway, and, enters the nucleus to form a complex with transcription factors (T-cell factors; TCF) to activate Wnt target genes., BMP-Smad 1,5,8 is acting upstream to WNT- β- catenin. Chordin, noggin are BMP antagonists. Figure created by author using Biorender.com.

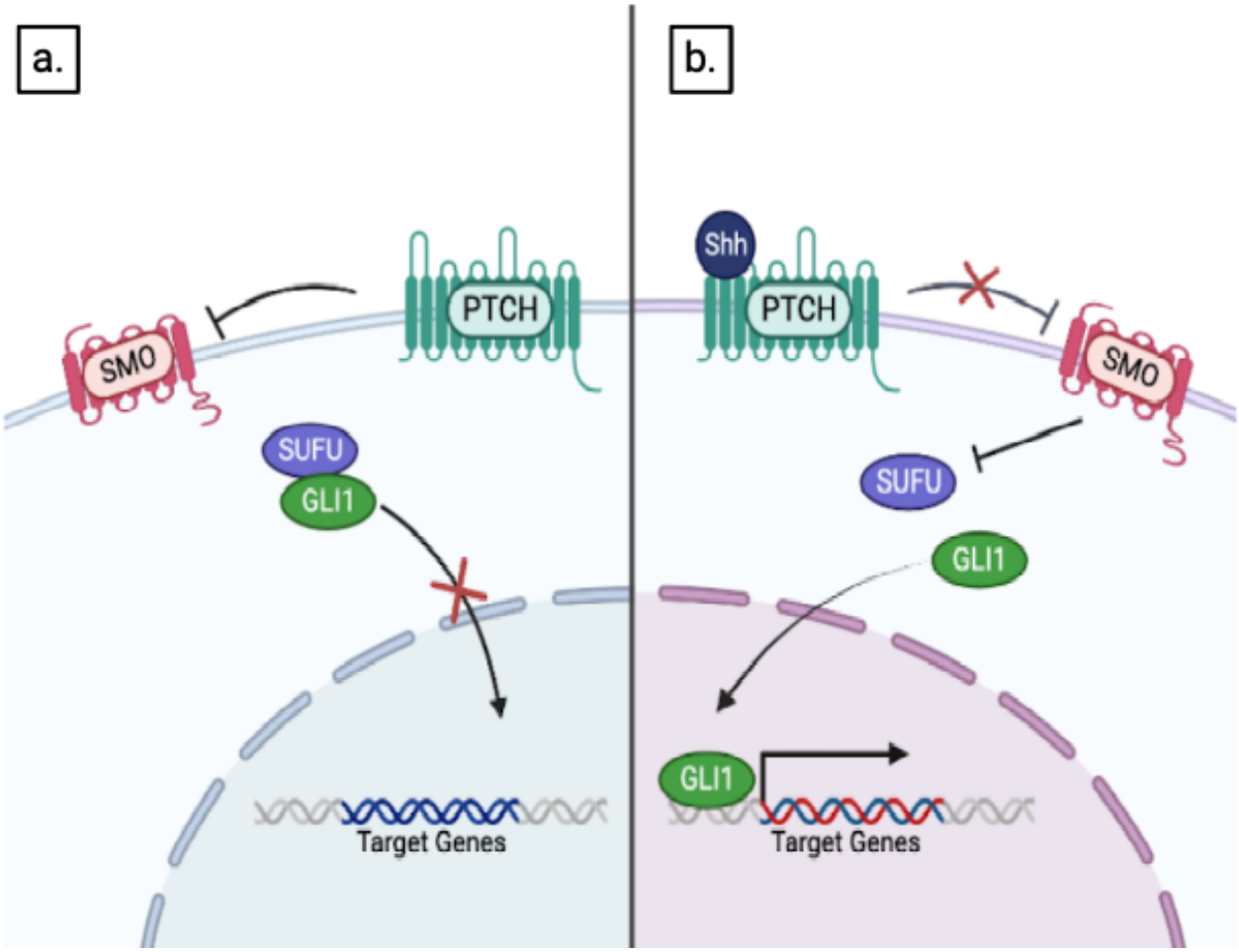


Figure 5. *Shh signalling.*

A. shows inactive Shh signalling, where Smoothened (SMO) receptor is inhibited by Patched (PTCH). Transcription factor GLI1 binds to protein kinase SUFU. B. Shh binds to PTCH which causes activation of SMO. This causes release of GLI1 and leads to its dislocation to nucleus which induces transcription of target genes. Figure created by author using Biorender.com.

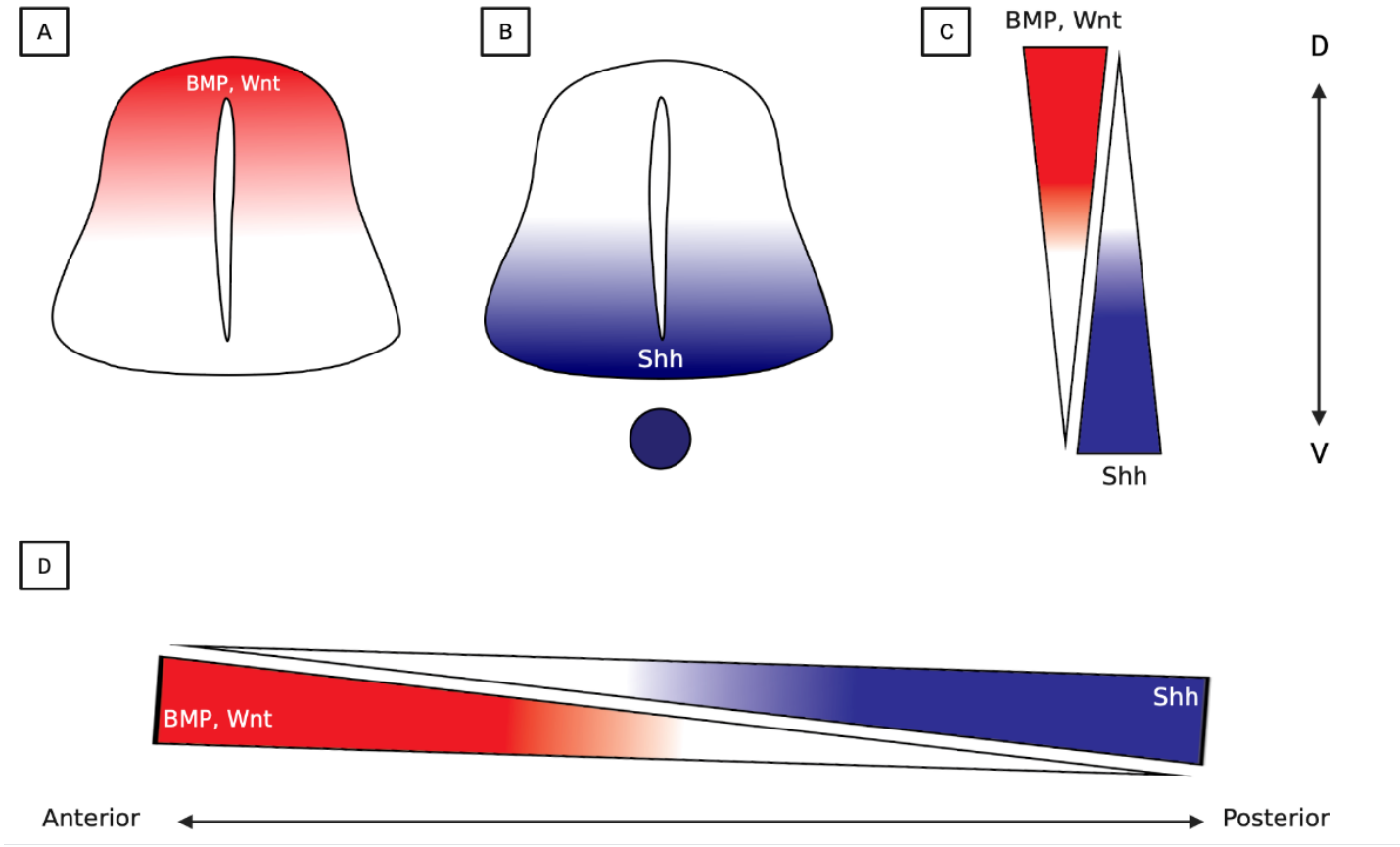


***Figure 6**. Morphogen gradients within the neural tube*

BMPs and Wnts are produced within the most dorsal part of the NT and the rood plate (A, C), while in contrast Shh signalling comes from the most ventral side of the NT and goes up towards the middle (B, C). Additionally, BMP/Wnt signalling in observed in the anterior side and Shh in the posterior side (D). Illustration made by author using biorender.com and based on Alvarez-Medina et al., 2008; Liem et al., 1995; Litingtung & Chiang, 2000

1.4.4 Development of dorsal interneurons

There are eight canonical classes of dorsal neurons in the spinal cord: dI1-6, dILA, dILB. Classes dI1-dI3 are known as class A, remaining are referred as class B. The most dorsal progenitors pd1-pd3 are born between days E9.5 and 10.5. Class B cells are usually born between E11 and E13 (stage 12-15 in chick (George et al. 2011)) (Lu et al., 2015). Throughout the neural tube, several classes of dorsal progenitors give rise to different neuronal sub-types (Figure 6). Dorsal interneurons dI1-dI4 are generated by Pax7-positive progenitor cells. The dorsal-most progenitor domain (pd) called pd1 expresses the transcription factor Math1/Atoh1 and gives rise to two subclasses of glutamatergic neurons, dI1A and dI1B (Lai et al. 2011). These subclasses are characterised by their expression of

Lim-HD transcription factors (TF) and their functional contributions to the spinocerebellar tract (SCT) (Lu & Niu & Alaynick; 2015). dI1A neurons are defined by high expression levels of Lhx2 TF and low levels of Lhx9 TF, while dI1B neurons express Lhx9. The dI1A and B interneurons migrate to the deep dorsal horn and intermediate grey regions of the spinal cord, where they receive proprioceptive input and form commissural projections of the dorsal and ventral SCTs (Buckley et al., 2020; Lu et al., 2015; Osorio et al., 2009).

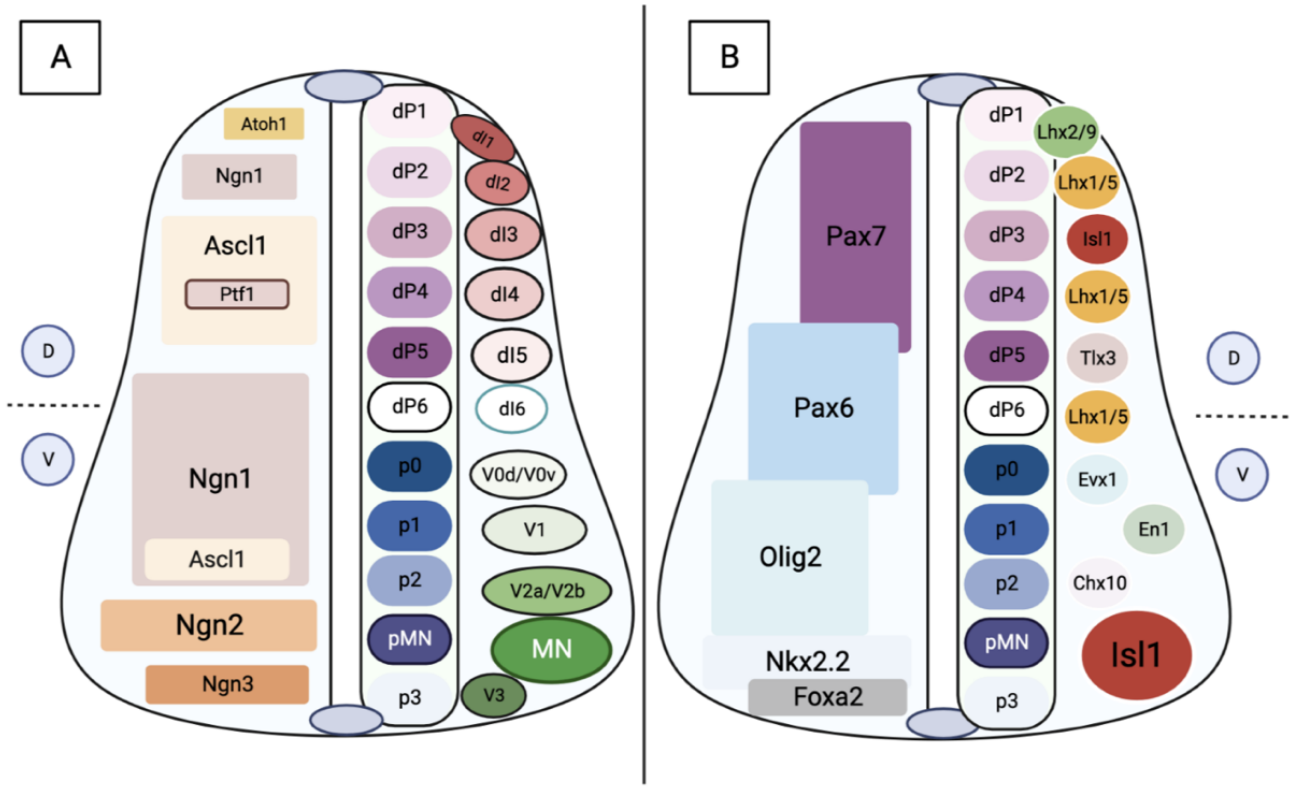


Figure 7. *Dorsal-ventral (D/V) patterning of developing spinal cord of chick embryo.*

Schematic representation of transversal section of chick neural tube. Left hand side of the neural tube presents the expression profile of helix-loop-helix factors (Atoh1, Ngn1, Ascl1, Ptf1, Ngn1, Ngn2, Ngn3) which take part in shaping and neurogenesis during development. Right hand side presents subdivision of the neural tube into 11 domains (dP1, dP2, dP3, dP4, dP5, dP6, p0, p1, p2, pMN, p3) of neural progenitors, and its differentiating progeny (dI1, dI2, dI3, dI4, dI5, dI6, V)d/V0v, V1, V2a/V2b, MN, V3). Left hand side in (B) represents expression of ventral and dorsal progenitors' proteins (Pax7, Pax6, Olig2, Nkx2.2, Foxa2) patterning the neural tube. Right hand side shows subdivision of the neural tube into 11 domains of neural progenitors and subpopulations of differentiating neurons generated during the primary neurogenetic wave (Le Dreau, Martil 2012). Illustration created by author using Biorender.com.

dI2 interneurons are a type of ascending relay interneurons that project contralaterally (Avraham et al.; 2009). They migrate to the intermediate spinal cord and ventral horn. It is suggested that these

interneurons transmit sensory information through the spinothalamic tract to the thalamus based on their location. These interneurons originate from progenitor cells expressing the bHLH transcription factors Ngn1 and Ngn2 (Ding et al., 2012). Post-mitotically, they express LIM-HD transcription factors Lhx1, Lhx5 (Lu & Niu & Alaynick; 2015).

dI3 interneurons express several sub-types such as Tlx3, Ascl1 and Islet-1. Isl1 expression is specific for dI3 neurons (Helms and Johnson, 2003; Xu et al., 2008). dI3 neurons are located between sensory and motor (locomotor) circuits and play an important role in integrating sensory input into useful locomotor output (Anderson et al., 2022; Laliberte et al., 2022; Zavvarian et al., 2020). dI3 neurons are classified as excitatory interneurons that relay sensory information (Xu et al., 2008; Avraham et al., 2010). Their axons create two long bundles (longitudinal fascicules) on the same side of the neural tube (Anderson et al., 2022). The developmental biology of dI3 neurons is not completely clear. Their birth is known to be promoted by BMP and Wnt signalling from the roof plate (Su at al.; 2010). The overexpression of constitutively active BMP type 1 receptors (Bmpr1) through *in-ovo* electroporation in chick embryos was observed to increase the size of domain containing dI1 – dI3 progenitors and the number of dI1- dI3 interneurons, at the expense of other dI classes (Timmer & Chesnutt & Niswander; 2005). Intriguingly, in the receptor knockout mice, dI3 neurons continued to be generated, suggesting the potential operation of other signals influencing dI3 subtype identities. Activin signalling, known to independently promote dI3 interneuron differentiation without relying on BMPs, raises the possibility that both BMPs and activin signalling contribute to the specification of dI3 neurons *in vivo* (Timmer & Chesnutt & Niswander; 2005). This notion aligns with the finding that, in mice, knockdown of Smad4, capable of mediating both BMP4 and activin signalling through short interfering RNA (siRNA), leads to a reduction in dI3 neuronal subtypes (Zhuang & Sockanathan; 2006).

Additionally, it was found that Wnt1−/−; Wnt3a−/− double homozygous mutant embryos exhibit reduced populations of cells expressing LH2 (a marker of dI1 neurons) whereas Islet1 (a marker of dI3 neurons) increased at the dorsal margin of the neural tube. (Muroyama et al. 2002). This

indicates a substantial reduction of dI1 and dI2 neurons, accompanied by a compensatory increase in dI3 neurons. These findings highlight the necessity of Wnt proteins for proper generation of dI1 and dI2 interneurons (Burrill et al. 1997; Liem et al. 1997). The loss of dI1 and dI2 interneurons was observed only in Wnt1−/−; Wnt3a−/− double mutants and not in Wnt1−/− or Wnt3a−/− single mutants, suggesting that both Wnt1 and Wnt3a together are required to maintain normal dI1 and dI2 interneuron populations. Examination of progenitor populations in the ventricular zone revealed that Math1+ and Ngn1+ cells in the dorsal-most region was significantly reduced, while Mash1+ cells expanded dorsally, aligning with the changes observed in dI neuron populations. Thus, the loss of Wnt1/Wnt3a signalling appears to affect the progenitor neuroepithelial cells responsible for generating dI1 and dI2 interneurons. (Muroyama et al. 2002). Considering BMP involvement, the expression of *Bmp4, Bmp6, Bmp7, Gdf7*, and *Noggin* in the roof plate of $Wnt1^{-/-}$; $Wnt3a^{-/-}$ embryos was comparable to wild-type embryos. Moreover, *Msx1* and *Msx2* expression, which responds to BMP activity, was unchanged (Lee et al. 1998). Thus, the reduction in dI1/dI2 neurons and the compensatory increase in dI3 neurons appear to occur without alterations in the BMP signalling system, implying a direct regulatory role for Wnt proteins in dI1/dI2 interneuron determination (Muroyama et al. 2002).

1.5 Formation of the dorsal root and dorsal root entry zone (DREZ)

Connectivity between the PNS and CNS requires PNS sensory axons to breach the outer surface of the neural tube/spinal cord (Sternini, 1997). The cell bodies of PNS sensory neurons are located in the dorsal root ganglia (DRG) which sit alongside the neural tube/spinal cord in a segmented manner (Figure 8). Sensory axons enter the CNS via the dorsal root (Bradbury et al., 2000) and dorsal root entry zone (DREZ).

The dorsal root is a cluster of neurons that carry signals to the CNS from the PNS (Gebreyohanes & Ahmed & Choi; 2021). Neural crest cells (NCC) are precursors for the DRG, sympathetic ganglia and Schwann cells (Kalcheim & Teillet; 1989). NCCs follow specific migration pathways influenced by the metameric organisation of the somite (Weston 1963).

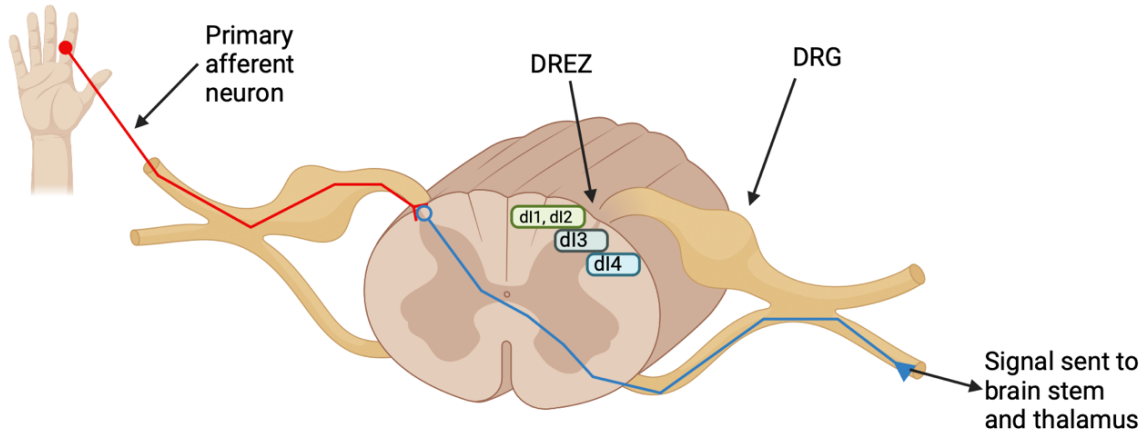


Figure 8. *Schematic cross-section of spinal cord.*

Schematic cross-section of spinal cord, presenting position of dorsal root entry zone, dorsal root ganglia and location of dI1, dI2, dI3 and dI4 classes of interneurons. Figure created by author in Biorender.com.

It was found that, DRG form opposite the cranial half of each somite. Neural crest cells originating from the caudal half undergo redistribution along the craniocaudal axis, localising opposite cranial somitic segments, thereby participating in the formation of two consecutive ganglia (Teillet et al., 1987).

The DREZ contains multiple cell types and undergoes a maturation over time (Golding & Cohen; 1997). Understanding its cellular diversity is crucial to grasp how the connection between the peripheral nervous system (PNS) and central nervous system (CNS) is established and stabilised, creating distinct environments (Chilton, 2006; Giger, Hollis, & Tuszynski, 2010). Importantly, in zebrafish, chick, mouse neural tube it is reported that an outer membrane called the glial limitans is formed by astrocytic end-feet cells which form a continuous sheath around the neural tube/spinal cord but at the site of the DREZ these end-feet are associated with a series of ‘holes’ in the glial limitans that allow axons to pass through into the CNS (Middleton & Perez-Sanchez & Dawes; 2022; Golding & Bird & McMahon, 1999).

1.6 Formation of neural circuits within the dorsal neural tube

1.6.1 Innervation of sensory axons via the DREZ

The peripheral axon of the sensory dorsal root neuron extends towards its target in the periphery e.g. skin (Wang & Julien & Sagasti; 2013). The other axon of the bi-polar sensory neuron enters the CNS via the dorsal root and DREZ and splits into two afferent growth cones which extend in opposite directions along the axis of the neural tube (Middleton & Perez-Sanchez & Dawes; 2022). Primary sensory axons display distinct branching patterns. Typically, Aβ fiber axons bifurcate into ascending and descending arms, giving rise to collaterals at regular intervals along several segments before penetrating deep into the grey matter of the spinal cord (Abraira & Ginty, 2013; Lucas-Osma et al., 2018). This branching pattern is illustrated in Figure 8. In contrast, axons related to Aδ and C fibers do not bifurcate at the Dorsal Root Entry Zone (DREZ) but instead turn unidirectionally before promptly projecting towards the grey matter of the spinal cord. (Zylka, Rice & Anderson, 2005).

Interestingly, axon branching also determines how axons regenerate after a lesion, according to the position of the lesion with respect to a branch point (Lorenzana et al., 2015). This splitting of the innervating sensory axon serves two crucial purposes. Firstly, it enables sensory information to reach higher-order relay neurons in the spinal cord or brain stem through its upward projections. Additionally, after the waiting period in the longitudinal sensory axon tracts they innervate into the dorsal horn and connect with motor neurons and interneurons. These branches from cutaneous and synapses on motor afferents generating reflex circuits (Gibson & Ma; 2011).

1.6.2 Neural circuit formation

Neurons do not function in isolation: they are organised into circuits that process and integrate complex information leading to a specific outcome. The arrangement of neural circuits depends on their function (Lu & Wang & Nose; 2009). Neural circuits are typically defined by synaptic connections formed within a network of dendrites, axon terminals whose activity is regulated by glial cells (Purves D, 2001). Neural circuits are initially generated during CNS development, but their final

function is defined through experience. The formation of these initial neural circuits (Blankenship & Feller, 2010) involves a combination of genetic regulations, and adaptation in response to environmental factors (Imai & Sakano, 2011). Neural circuit development is dependent on the type of neurons within these circuits. Two major types of neurons: GABAergic and glutamatergic send excitatory and inhibitory signals to their target neurons (Lu & Wang & Nose; 2009). Establishment of excitatory and inhibitory synapses is based on neurons progressing through four developmental processes (Sanes and Yamagata 2009). These stages include cell fate determination, where progenitor cells differentiate into GABAergic and glutamatergic neurons (Guillemot 2007; Schuurmans and Guillemot, 2002) and neuronal migration to appropriate regions (Marin and Rubenstein, 2003). Axons of these neurons are guided to their targets, recognise them, and synapses are formed (Terauchi & Umemori; 2012).

1.6.3 Formation of dI3-specific proprioceptive neural circuits

dI3 neurons play an important role in integrating sensory input for useful locomotor output (Anderson et al., 2022; Laliberte et al., 2022; Zavvarian et al., 2020). dI3 neurons are classified as excitatory interneurons that relay sensory information (Xu et al., 2008; Avraham et al., 2010). Their axons create two long bundles on the same side of the neural tube (Anderson et al., 2022). Initially, dI3 neuron axon outgrowth might also be influenced by signals from the roof plate (i.e. BMP and Wnt signalling) either making them move away or towards certain areas. dI3 neurons are subdivided to be dorsally and ventrally projecting neurons (Avraham et al.;2010). The dI3 axons projecting dorsally alter their course from the transverse to the longitudinal plane upon encountering the axons of dorsal root ganglion (DRG) neurons at the dorsal root entry zone (DREZ). Simultaneously, the dI3 axons projecting ventrally shift their trajectory plane from ventral to lateral when they encounter the axons of motor neurons (Bui et al.; 2013; Avraham et al.;2010).

1.7 Modelling dI3 interneuron patterning and birth *in vivo*

Studies which have investigated the contribution of BMPs to the generation of dorsal interneurons through mice, chick, and zebrafish (Yu et al. 2008; Andrews et al. 2017; Liem et al. 1995; Nguyen et al. 2000) have proven less informative due to issues such as redundancy and early lethal phenotypes. Recent alternative approaches have provided additional insights into the involvement of BMPs in the development of dorsal interneurons *in vivo*. dI3 neurons arise from a dorsal progenitor domain that expresses the bHLH transcription factor Ascl1 (Figure 7) (Goulding et al.;2002). In mice studies, where *Bmp 7* was knocked out, dI3 neurons were still generated (Chesnutt et al.; 2004). This suggests that other BMP-like signals, such as activin might promote dI3 neuron birth in mice (Wine-Lee et al.; 2004). In studies interfering with Smad4 via gene knockdown in mice, a reduction in dI3 neuron numbers was observed. As Smad4 is a downstream signalling component common to many if not all BMP/TGFβ ligands (Goulding et al.;2002; Chesnutt et al.; 2004; Zhuang & Sockanathan; 2006) this could suggest that both BMP and activin signalling are crucial for dI3 development. Additionally, in mouse embryonic stem cell culture work, it was found that increase of BMP4 leads to increased number of dI3 neurons (Andrews et al., 2017)

1.7.1 Chick as a model organism

The avian (chick) model is considered an excellent model organism for studying embryonic development in higher vertebrates. Chick eggs are readily available, do not require killing of the mother and are inexpensive to purchase. Avian (chick) embryos allow for flexible experimental design as they can be incubated to any desired developmental stage which can be identified with high precision for a given batch of incubated eggs. Additionally, due to rapid development, use of chick embryo allows for timely data collection and their development *in ovo* lends itself to experimental manipulation (Stern, 2005). Avian models can be used in many types of research and for developing new methods of experiments. This involves gain and loss of function experiments, promoter analysis, stem cells and transgenesis as well as precise gene manipulation (Darnell & Schoenwolf, n.d.). A recent novel

strategy to disrupt cell signalling involves direct injection *in ovo* of small molecule inhibitors into the sub-blastoderm of chick embryos at specific developmental stages, then allowing the embryo to develop further for 24hours in presence of the drug (Poopalasundaram & Richardson & Graham, 2023).

1.7.2 Use of the small molecule BMP inhibitor, Dorsomorphin

Dorsomorphin (DMN) is small-molecule inhibitor of BMP signalling (Cai et al. 2013). The molecule is a selective inhibitor of the BMP I receptors ALK2, ALK3, ALK6 (Boergermann et al., 2010a). Dorsomorphin, in dose dependent manner, blocks BMP-mediated Smad 1/5/8 phosphorylation, therefore, blocks transcription of the target genes (Madhu et al., 2016; Yu et al., 2008a). DMN achieves the inhibitory effect on BMP signalling by binding to the ATP binding site in the kinase domain in Type I BMP receptor – specifically Alk1, Alk2, Alk3 and Alk6 (Figure 3; Table 1). (Andrews et al., 2017; Boergermann et al., 2010b). In zebrafish studies, it was found that DMN causes dorsalisation of the embryo by inhibiting the BMP pathway. Moreover, it was found that in already ventralised zebrafish embryos, addition of DMN would diminish ventralization effect by causing dorsalisation of the embryo (Yu et al., 2008b).

1.7.3 Isoliquirtigenin

Isoliquirtigenin is a small molecule found in the roots of various liquorice plants (Peng et al., 2015). Isoliquirtigenin is reported to be a BMP stimulator (Virjens et al., 2013), however specific cellular targets and its mechanism of action remains unclear. The drug showed good potency with an EC50 of 10mM and efficacy 80% in experiments on cell lines and zebrafish (Vrijens et al., 2013). In the studies on Id1 and Id2 proteins involved in differentiation of keratinocytes, it was found that compound activates Smad 1,5 phosphorylation, and triggers BMP signalling by phosphorylation and expression of its downstream receptors (Riege et al., 2023; Vrijens et al., 2013). Through experimentation in zebrafish, Isoliquirtigenin has been named ‘ventromorphin’ (Virjens et al., 2013). In zebrafish, isoliquirtigenin induced ventralization defects in zebrafish embryos are identified as a

decrease in brain size and, at higher concentrations, loss of eyes, abnormal accumulation of cells caudal to the anus, and loss of the ventral tail fin. Increasing concentrations of this compound exacerbated the phenotype, while treatment with DMSO alone had no effect on embryonic development (Wang et al.; 2023; Genthe et al. 2017).

1.8 Aims and objectives of the study.

BMPs are involved in sensory interneurons development but its role in dI3 interneuron development is less well understood than for dI1 and dI2 interneurons. dI3 neurons are born in an area of the NT that also gives rise to the DREZ. Previous independent studies in the Formstone lab (C.J. Arnell, A. Drabik, unpublished) had revealed a 'burst' of pSmad1/5/8 in the area destined to generate dI3 interneurons around stage 17-stage 18, raising the hypothesis that sustained BMP signalling at this time was required for dI3 interneuron birth. The primary aim of this project therefore was to manipulate BMP signalling over these specific stages of chick embryo development *in vivo* via sub-blastoderm injection of small molecule drugs and to investigate their impact on the position and integrity of the DREZ and the overall position and effects on number of rootlets and number of dI3 interneurons. Secondary aims were to find an 'injection window' for DMN and ISL, by injecting drugs sub-blastodermally at stages 16 and 18 of chick embryo and section them at FLB and HLB level. This is because in injected embryos, FLB stage 18 is equivalent to HLB stage 16, and embryos injected at stage injected at stage FLB 16, their HLB would be equivalent to stage 14. Changes in number of dI3 neurons were analysed against other classes of dorsal interneurons: dI1, dI2, dI4 and dI6 to determine if the increase/ loss of dI3 interneurons was at the cost of other dorsal neuron classes.

The objectives were to:

A) Independently inject the commonly used BMP inhibitor, dorsomorphin (DMN; 10μM), and a less well documented BMP activator, isoliquirtigenin (ISL; 10μM) into intact chick embryos at stage 16 and 18 of development.

B) Allow embryos to develop for further for 24hours, to fix and freeze embryos and generate frozen sections of DMN injected embryos at the level of the FLB and HLB to find the specific drug injection window that impacts the development of dorsal interneurons.

C) Perform immunohistochemistry on frozen sections of fixed injected chick embryos that had been allowed to develop for a further 24 hours post-injection using a pSmad 1,5,9 antibody, that stains pSmad 1/5/8 as a proxy for BMP signalling and antibodies raised against dorsal interneuron-specific TFs (Lhx2 for sI1, Lhx5 for dI2, dI4, dI6) and neurofilament (NFM), to label axonal projections around the DREZ.

2 Materials and Methods

2.1 Hen Eggs

Fertilised chicken eggs (Henry Stewart & Co; Medeggs, UK) were incubated at 37°C until the appropriate stage (HH) (Hamburger & Hamilton, 1992), which was determined by cracking an egg and observation of embryo morphology, counting of somite number and comparison to photographs described in Hamburger & Hamilton (1992). The Table below indicates the time required, in our hands, for development of chick embryos to stages 16-22, based on Hamburger & Hamilton (1992).

Table 2. Approximate incubation time, based on Hamburger and Hamilton (1992).

Stage of development	Approximate incubation time at 37°C (hrs)
16	54
18	60
20	80
22	96

2.2 Sub-blastoderm injection of chick embryos

Chick embryos were incubated on the side until stage 16 to 18 depending on the experiment. Eggs were sprayed with 70% ethanol (v/v) and air dried. 5ml of albumin was removed from each egg to allow embryos to sink. A window was then cut on top of the egg and 50µl of each diluted drug or diluted DMSO (D2438, SigmaAldrich) vehicle was injected underneath the embryo within the yolk as described by Poopalasundaram et al, 2023. Dorsomorphin (P5499, SigmaAldrich) was used at a final concentration of 10µM, isoliquirtigenin concentration (I3766, SigmaAldrich) was 10µM following dilution in PBS. Same volume of DMSO as drug was diluted in PBS.

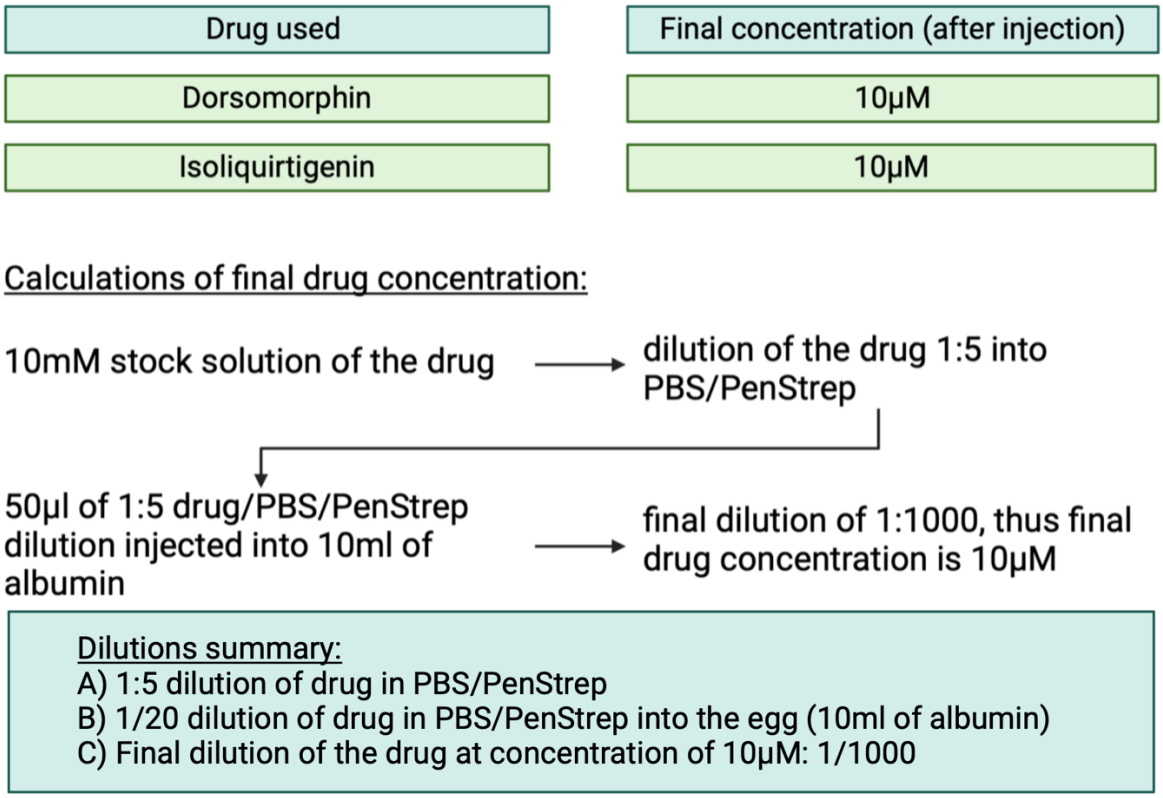


Figure 9. *Schematic representation of calculation of final concentration of drugs injected.*

Calculation based on methodology provided by Professor Anthony Graham, Kings Collage London. Illustration created by author in Biorender.com.

Sub-blastodermal injection allowed the maintaining drug concentration and enabled lower doses of the drug to be used due to the drug being contained within the yolk (Poopalasundaram et al., 2023). 500 µl of 1X Penicillin-Streptomycin (15070063, ThermoFisher) in sterile phosphate buffered saline (PBS) was applied on top on the chick embryo to prevent bacterial growth. Eggs were sealed with sellotape and incubated for another 24 hours to achieve the appropriate stage for analysis (Figure 10) (Poopalasundaram et al., 2023). Embryos were dissected in PBS and subsequently fixed in 4% (v/v) formaldehyde (FA) in PBS overnight at 4ºC.

2.3 Preparation of frozen sections

Fixed embryos were washed in 1x PBS and then incubated at 4ºC in 30% sucrose/1 xPBS until the embryos had completely sunk. Prior to embedding the anterior of the embryo from the top of the forelimb upwards was removed by cutting across the embryo trunk with a knife. The remaining forelimb-tail was then mounted upright with the forelimb to the top in a foil mould filled with OCT

(VWR, 361603E). The foil mould was then incubated on dry ice until the OCT was frozen through. Embryos mounted in OCT were stored at -80°C.

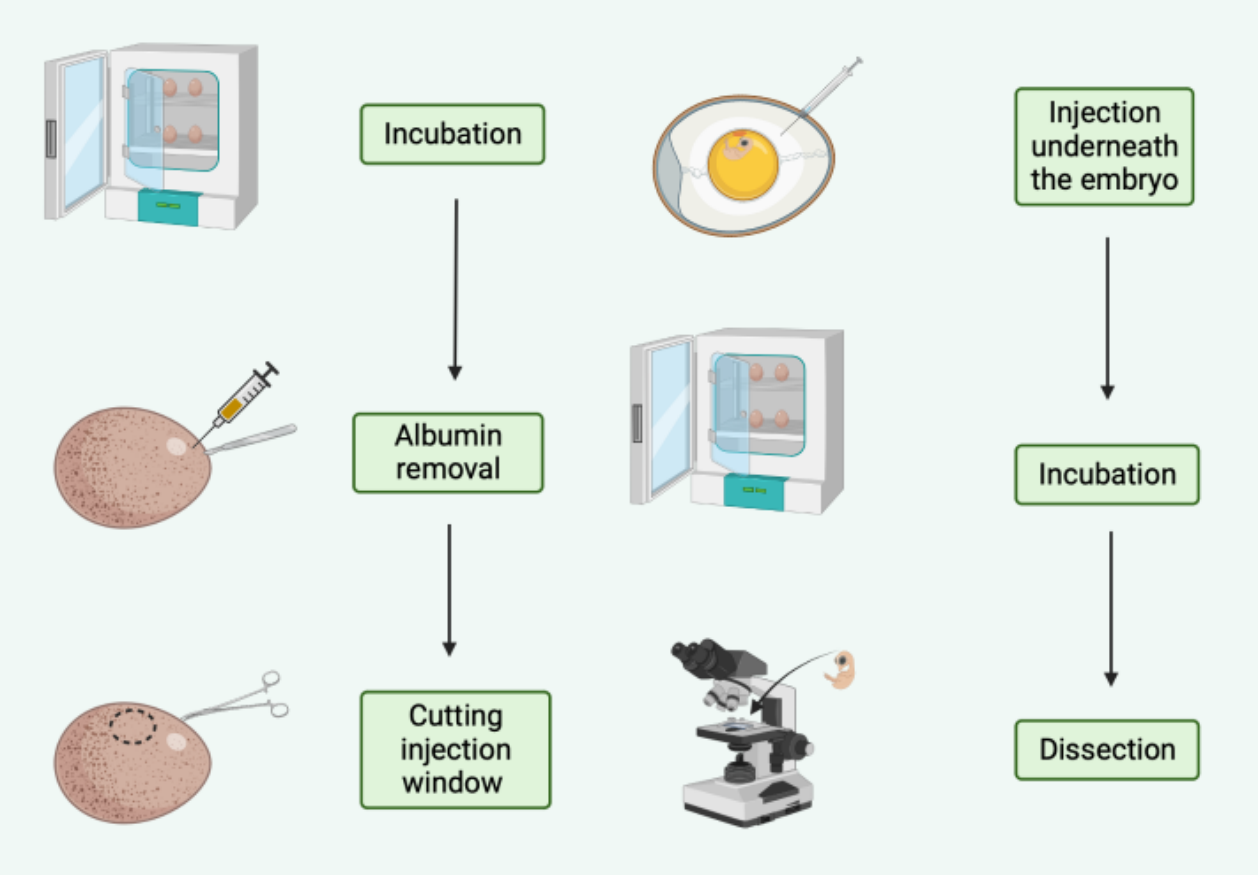


Figure 10. *Schematic representation of sub-blastema drug injection underneath the chick embryo.*

Frozen sections (30µm) were sequentially taken at the forelimb and subsequently the hindlimb level using a Leica CM1860 cryostat. Sections were captured on Super frost plus slides (VWR, 631-9483), air dried and a DAKO pen (S200230-2, Agilent) was used to define the area on the slide for antibody incubation (Figure 11)

2.4 Immunochemistry

Air-dried frozen sections were rehydrated in 1x PBS and then blocked in 12% BSA, 1xPBS, 0.1% Triton-X 100 (BSA block) for at least one hour at room temperature. Primary antibodies were

diluted in BSA block (Table 3), centrifuged briefly at 13,000g and incubated with sections overnight at 4°C.

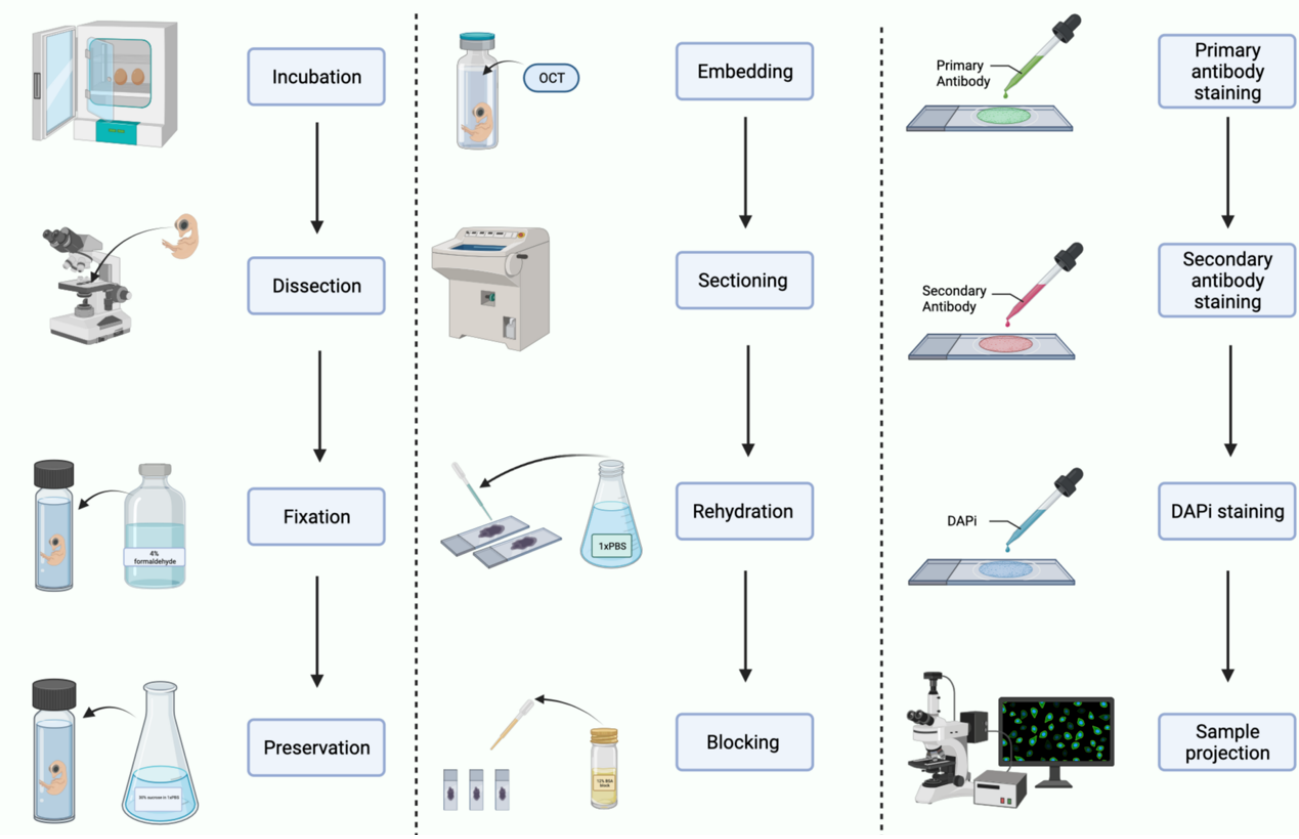


Figure 11. *Schematic representation of chick embryo dissection and generation of frozen sections.*

Sections were then washed three times in 1xPBS/0.1% Triton X-100 for at least 5 minutes followed by incubation with fluorescently labelled secondary antibody (Table 3) for at least 1 hour at room temperature. Sections were then washed three times in 1xPBS/0.1% Triton X-100 for at least 5 minutes, DAPI (1:5000, 1mg/ml stock, ThermoFisher, 62248) was included in the final wash to fluorescently label nuclei. Sections were then mounted in glycerol under a coverslip. The next day immunostained sections were analysed using an Apotome 2 (ZEISS) and photographs taken of individual neural tubes, for both control and experimental embryos, at 100X magnification. Negative controls (data not shown) were sections stained with secondary antibodies only.

2.5 Image analysis and measurements

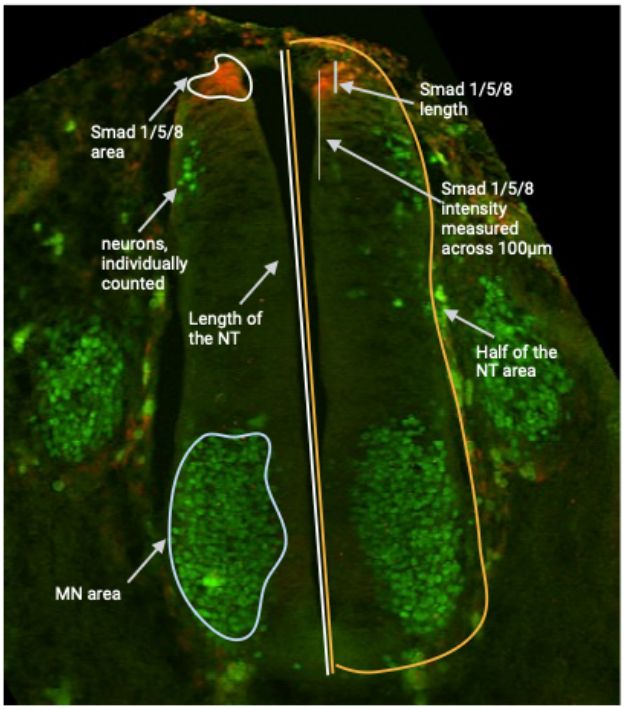
Levels of Smad 1,5,9 staining was examined. Smad 1/5/9 antibody was used as a marker for Smad 1/5/8. Software used for image analysis was ZEN PRO, following settings we used: The area of Smad, NFM and Islet-1 staining was measured using measuring tools (as line, area measurements) of ZEN PRO software, and dI3 neurons were counted manually. For Lhx5 and Lhx2 staining dI1 and dI2, dI4, dI6 neurons were counted manually. For example, Smad staining area, was measured as a ratio of Smad against the area of one side of the NT whereas the extent of Smad staining was measured along a line of 100μm length along the Smad-expressing domain (Figure 12). All measurements were based on n=5 embryos, to provide reliable data.

Table 3. Primary and Secondary Antibodies

Antibody		**Protein stained by antibody**	**Dilution in BSA block**
NFM Monoclonal Antibody (RMO-270) – Invitrogen # 13-0700	Primary	neurofilaments	1:800
ISLET – 1 (gift from Dr Ivo Lieberman, Kings College London, UK) Lipovsek et al, 2017	Primary	Islet-1 (dI3, MN neurons)	1:1000
LHX2 Monoclonal Antibody – (DSHB Hybridoma Product PCRP-LHX2-1C11)	Primary	Lhx2 (dI1 neurons)	1:1000
LHX5 Monoclonal Antibody – (DSHB Hybridoma Product PCRP-LHX5-1B7)	Primary	Lhx5 (dI2, dI4, dI6 neurons)	1:1000
Phospho-SMAD1/SMAD5/SMAD9 (Ser463, Ser465) Polyclonal Antibody – Invitrogen # PA5-104523	Primary	Marker for Smad 1/5/8	1:500
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 594 – Invitrogen # A-11012	Secondary		1:1000

Goat anti-Mouse IgG (H+L), Recombinant Secondary Antibody, Alexa Fluor™ 488 – Invitrogen # A28175	Secondary		1:1000

For all data, a statistical student t-test for independent samples was performed, significance level was set to 5% ($P<0.05$). Data was analysed and represented in graphical form using Graphpad Prism. Photographs of IHC were cropped and annotated in Biorender.com.



Biological replicates, Smad area:

$$\frac{\left(\text{Smad area left}/\text{NT area left} + \left(\text{Smad area right}/\text{NT area right}\right)\right.}{2}$$

Biological replicates, Smad length:

$$\frac{\left(\text{Smad length left}/\text{NT length} + \left(\text{Smad length right}/\text{NT length}\right)\right.}{2}$$

Biological replicates, number of neurons:

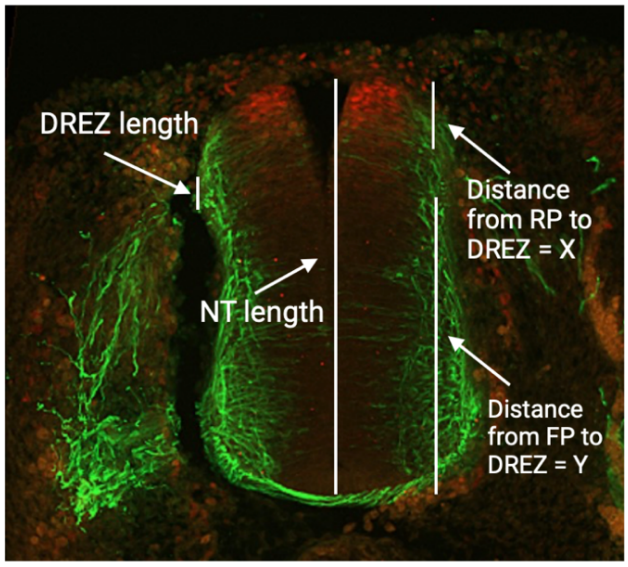
$$\frac{\left(\text{Number of neurons left}/\text{NT length}\right) + \left(\text{Number of neurons right}/\text{NT length}\right)}{2}$$

Biological replicates, MN area:

$$\frac{\left(\text{MN area left}/\text{NT area left} + \left(\text{MN area right}/\text{NT area right}\right)\right.}{2}$$

Figure 12. *Schematic representation of measurements taken.*

Area of the neural tube (NT), area and length of Smad staining and area of motor neurons (MN) was measured for both sides of the NT. Firstly, technical replicates for each side of the NT were calculated, summated, and divided by two, to generate a value for the biological replicate, n=5. Unpaired student t-test for statistical analysis was performed. Figure created by author in Biorender.com.



Biological replicates, DREZ length:

$$\frac{(\text{DREZ length left}/\text{NT length}) + (\text{DREZ length right}/\text{NT length})}{2}$$

Biological replicates, distance from RP to DREZ:

$$\frac{(\text{X left}/\text{NT length}) + (\text{X right}/\text{NT length})}{2}$$

Biological replicates, distance from FP to DREZ:

$$\frac{(\text{Y left}/\text{NT length}) + (\text{Y right}/\text{NT length})}{2}$$

Figure 13. *Schematic representation of measurements taken – DREZ*

Length of DREZ, distance between roof plate (RP) and DREZ and distance between floor plate (FP) and DREZ was measured on both sides. Firstly, technical replicates for each side of the NT were calculated, summated, and divided by two, to generate a value for the biological replicate, n=5. Unpaired student t-test for statistical analysis was performed. Figure created by author in Biorender.com.

3 Results

In ovo sub-blastoderm injection of small molecule inhibitors (Poopalasundaram et al., 2023) allows control of the developmental stage for intervening in BMP signalling in the chick embryo. A pilot study had been performed previously as part of an undergraduate project, supervised by Dr Caroline Formstone, to test the effect of DMN on BMP signalling and dI3 neuron number and distribution. DMSO vehicle control and DMN were injected at stage 16 and embryos allowed to develop further until stage 20, at which point they were checked for gross abnormalities, fixed, and processed for frozen sections which were taken at the level of the forelimb bud (FLB). Immunohistochemistry (IHC) was performed to investigate BMP signalling using a Smad1,5,9 antibody and dI3 neurons using an Islet-1 antibody. At the start of this research project the resultant data were analysed and were used to direct subsequent experimental analysis.

3.1 Dorsomorphin injections at stage 16 *in ovo* increase breadth of Smad 1/5/9 staining but decrease its intensity at the level of the forelimb bud (FLB)

Data analysed from the pilot study is shown in Fig.14A-E. The area of Smad staining, length, and intensity were measured and calculated against neural tube (NT) area and length (see Methods, Figure 12 for the explanation of how measurements were made) for n=5 DMSO control and DMN injected embryos. It was found that for FLB of DMN-injected embryos there is a significant increase in Smad staining area and length compared to vehicle control (Fig.14C, D), however, there was a significant decrease in intensity of Smad staining (Fig.14 E) suggesting that Smad1/5/9 expression is broader but dampened in the presence of DMN at the level of the FLB from stage 16. This trend can be seen in IHC photos of frozen sections for FLB (Fig. 14 A, B). Smad1/5/9 in DMSO control is much 'tighter' and in the very dorsal part of the neural tube, just below the roof plate. However, for DMN-injected embryos, Smad 1/5/9 can be seen as much broader. Its intensity is higher in the very dorsal part of the neural tube, then it dissipates. Frozen sections were then taken at the level of the hindlimb bud (HLB), from the same set of injected embryos, immunostained, and analysed as for the FLB. Data

was not significantly different, however (Fig.14) the IHC photos of the frozen HLB embryos, show Smad1,5,9 in the very dorsal part of the neural tube. This can be seen for both, DMSO control and DMN-injected embryos.

An intensity profile of Smad 1/5/9, for FLB stage 16 DMN and DMSO injected embryos across 100µm on the neural tube as well as the standardised (400µm) length of the neural tube was generated and is presented in Fig.15. This demonstrates the broader but dampened staining of Smad1/5/9 across DMN injected samples. For DMSO control, it can be seen, that Smad 1/5/9 staining has its maximum intensity around 20µm from the RP, then diminishes. In contrast, for DMN injected embryos, Smad 1/5/9 intensity is apparent around 20µm from the RP and reaches its 'highest' intensity level around 30µm, however, the intensity is much less intense than for DMSO control. Across 100µm length, it can be noticed, that for DMN samples, Smad 1/5/9 dissipates, around 70-80µm, while for DMN the 'faded' levels for Smad 1/5/9 remain at a similar level across the 100µm length measured. Measurements were made on n=5 embryos. Intensity measurements across both 100µm and the full length of the neural tube were taken for both the left and right side of the neural tube and average intensity was taken. The average of intensity measurements was calculated for all five embryos. Data was uploaded to Graphpad Prism to create heat maps.

3.2 Dorsomorphin injection at stage 16 *in ovo* increased number of dI3 neurons but had no impact on DREZ size and position at the level of the FLB when analysed at stage 20.

Islet-1 staining neurons were counted manually, then normalised against the length of the NT for each embryo section analysed (Fig. 12, methods). Then MN area was measured and divided by the area of the NT (Fig. 12); n=5. Presented results for FLB showed that injection of DMN at stage 16 led to a significant increase in the number of dI3 neurons and a decrease in MN area (Fig. 16). These trends were not observed for HLB. This may suggest that there is a correlation between the timing of BMP signalling and the birth of dI3 neurons. DREZ forms in the same region of the dorsal neural tube as the dI3 neurons are generated. The observed increase in the number of dI3 neurons raised the

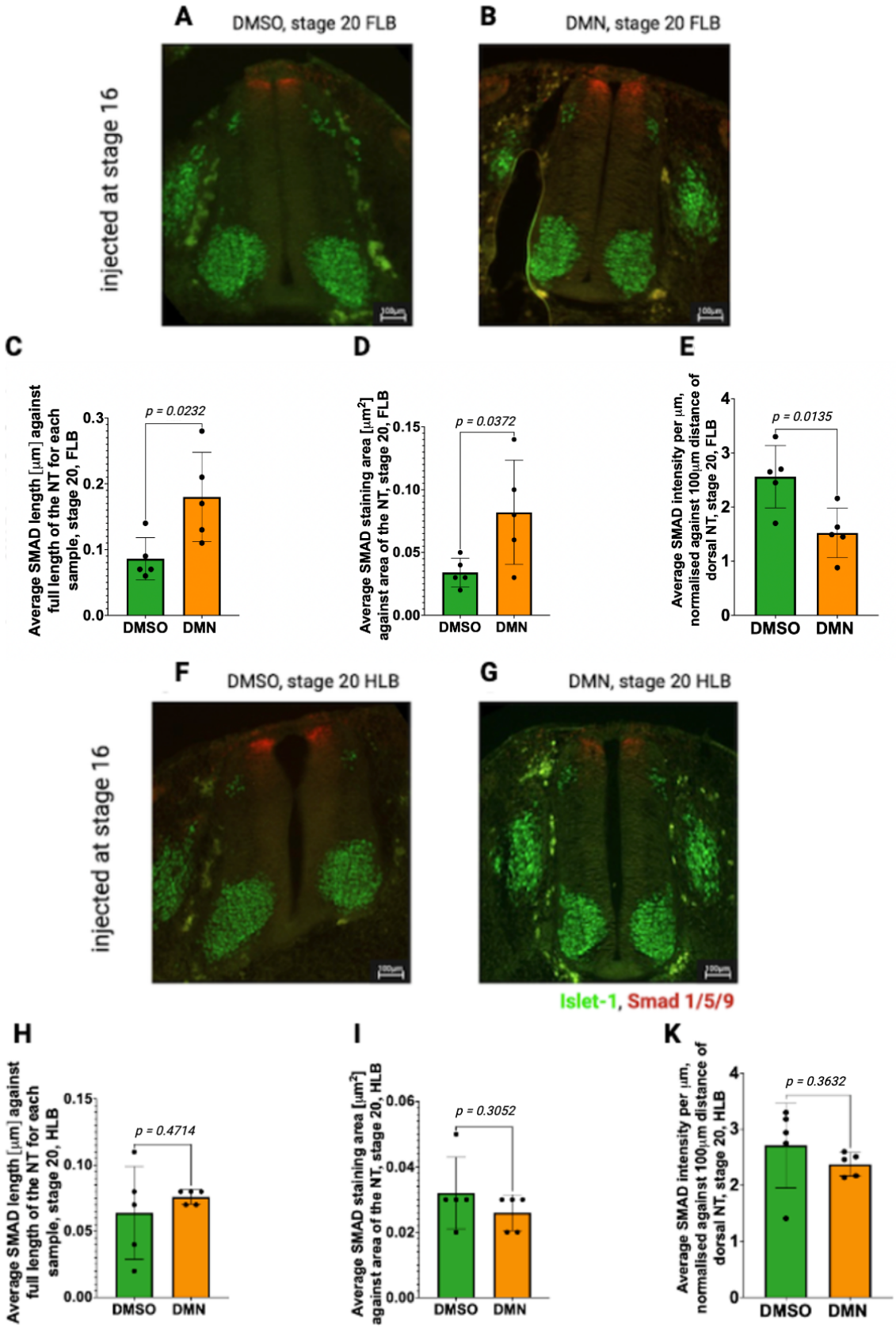


Figure 14. *IHC analysis of chick embryo neural tube, stage 20, Smad 1/5/9.*

(A, B, F, G) Representative photographs from control DMSO (A, F) and DMN (B, G) injections at stage 16 (n=5 embryos), scale bars are shown. (C-E, H-J) Histograms of Smad 1/5/9 staining area, length, and intensity for FLB (C-E) and HLB (H-J) embryos (n=5 embryos). Statistical analysis was performed using an unpaired t-test, $p<0.05$.

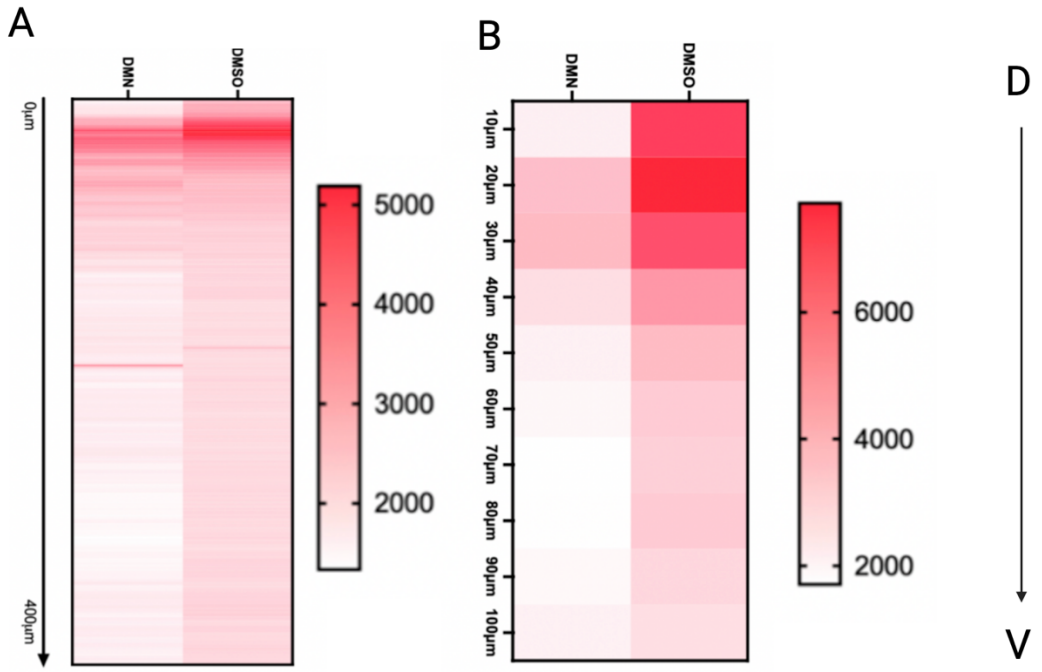


Figure 15. *Heat maps presenting Smad 1/5/9 intensity along 100 µm of the neural tube, stage 20, DMN.*

Standardised length – 400 µm of the neural tube (B). Measurements were taken using ZEN ZEISS software, and presented results are an average on n=5 embryos. Graphs generated in GraphPad PRISM. Presented results show changes in intensity of Smad 1/5/9 alongside DV axis for DMSO and DMN injected embryos.

question of whether DREZ position and structure might also be affected by DMN treatment (Purves et al. 2001) Embryos sections were stained with NFM antibody and analysed (n= 5 embryos). Measurements of DREZ size, the distance between the roof plate (RP) and DREZ, and the distance between the floor plate (FP) were taken (Fig. 13, methods) and an unpaired student t-test was performed to analyse results. DMN treatment had no significant impact on the size and position of the DREZ. This could be related to timing, as the DREZ develops slightly earlier than dI3 neurons, at stage 16 (Formstone lab, unpublished).

3.3 Dorsomorphin injection at stage 16 *in ovo* decreased number of dI1 and dI2 neurons at the level of the stage 20 FLB.

The next step was to investigate, if, in stage 20 FLB embryos, the increased number of dI3 neurons is at the expense of other classes of neurons. Therefore, embryo sections were stained with Lhx2 marking for dI1 neurons and Lhx5 antibody marking for dI2, dI4 and dI6 neurons. Each class of neurons was counted manually and then the number was divided by the length of the NT (Fig. 12).

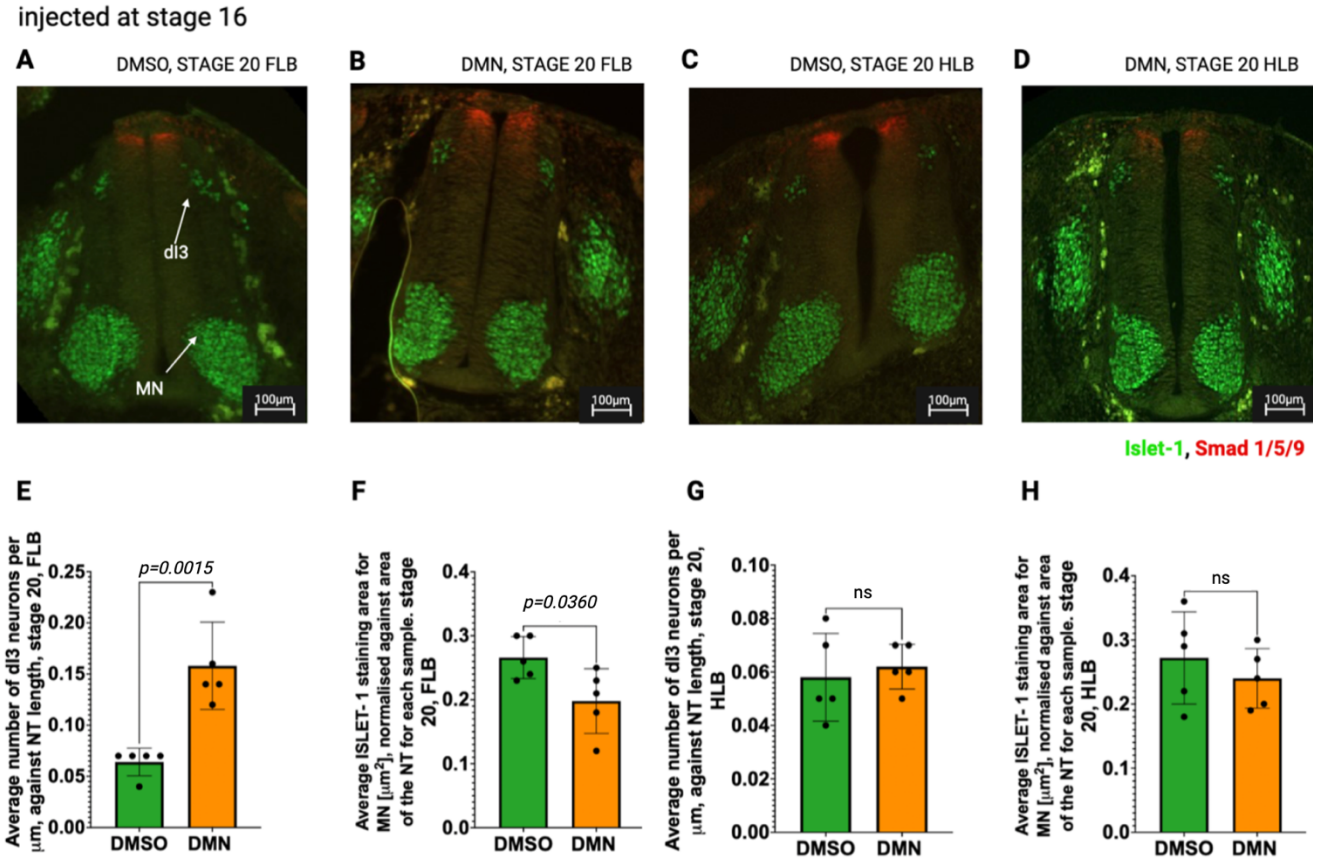


Figure 16. *IHC analysis of chick embryo neural tube, stage 20, Islet-1*

Representative photographs from control DMSO (A, C) and DMN (B, D) injections at stage 16 (n=5 embryos), scale bars are shown. (E, F) Histograms of Islet-1 staining for dI3 and MN area FLB and HLB (G, H) embryos (n=5 embryos). Statistical analysis was performed using an unpaired t-test., p values are shown, ns – nonsignificant, p<0.05.

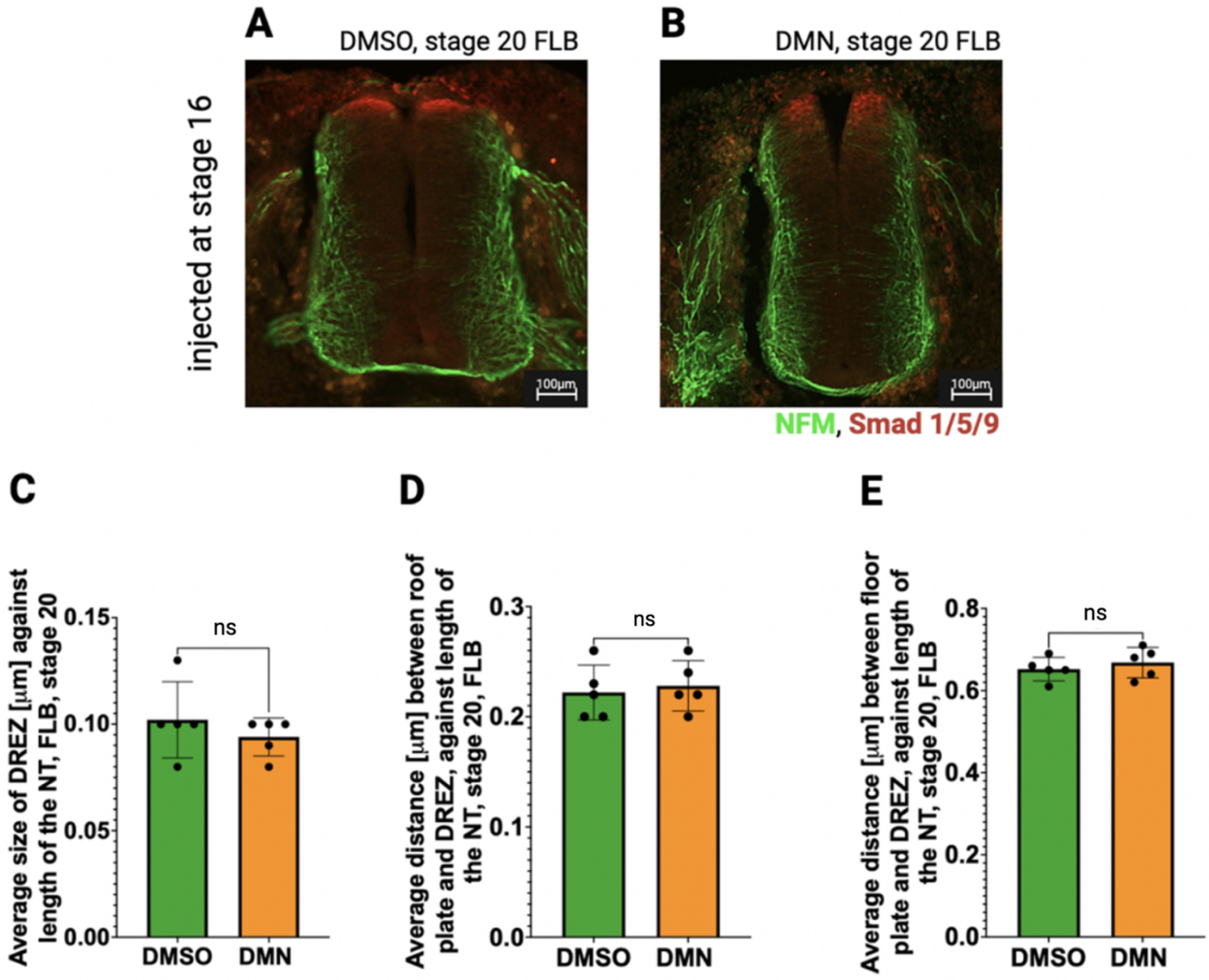


Figure 17. *IHC analysis of chick embryo neural tube, stage 20, NFM.*

(A, B) Representative photographs from control DMSO (A) and DMN (B) injections at stage 16 (n=5 embryos), scale bars are shown. (C) Histogram of DREZ length (n=5 embryos). (D) Histogram presenting distance between RP and start of DREZ (n= 5 embryos). (E) Histogram presenting distance between FP and start of DREZ (n= 5 embryos). Statistical analysis was performed using an unpaired t-test, ns – nonsignificant, $p<0.05$.

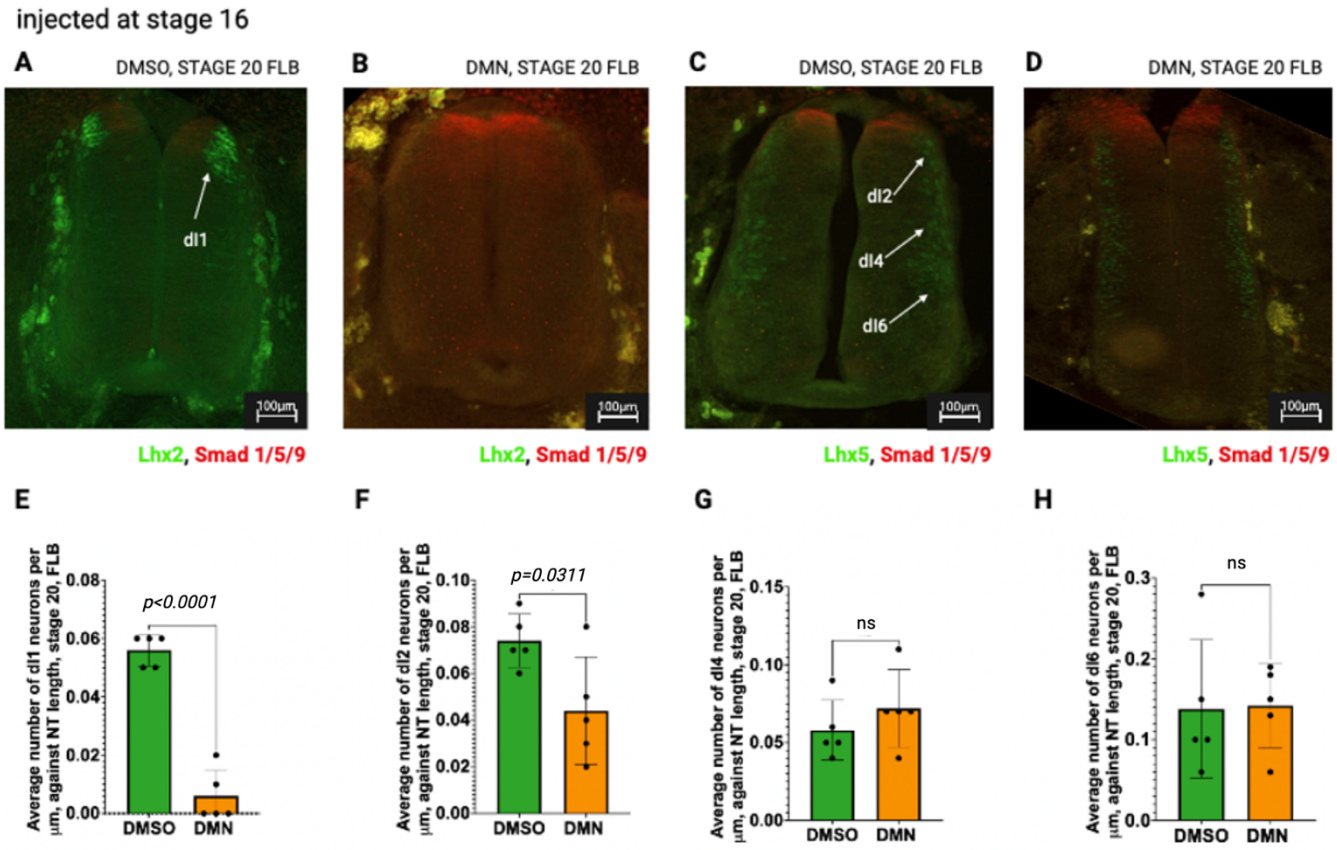


Figure 18. *IHC analysis of chick embryo neural tube, stage 20, Lhx2, Lhx5*

Representative photographs of DMSO (A, C) and DMN (B, D) injected at stage 16 (n=5embryos) scale bars are shown. (E) histogram representing Lhx2 staining for dI1 neurons FLB. (F, G, H) histograms representing Lhx5 staining for dI2, dI4 and dI6 neurons, respectively (n = 5 embryos). Statistical analysis was performed using an unpaired t-test., p values are shown, ns – nonsignificant , p<0.05.

Obtained results showed a significant decrease in the number of dI1 and dI2 classes of neurons (Fig. 18 A, B, C, D, E, F), but no statistical significance in number of dI4 and dI6 classes of neurons (Fig. 18, C, D, G, H). This data suggests that an increase in dI3 neurons could be at the expense of dI1 and dI2 classes of neurons.

3.4 Dorsomorphin injection at stage 18 *in ovo* increased area of Smad 1/5/9 when analysed at stage 22 in the HLB.

Observed decreases in Smad intensity at the level of the FLB but not the HLB in stage 16 DMN injected embryos led to the hypothesis that the timing of BMP/Smad signalling might be important for the birth of dI3 neurons. To test this hypothesis, chick embryos were injected individually with DMSO

or DMN at stage 18 and allowed to develop further until stage 22 at which point they were checked for gross abnormalities, fixed, and processed for frozen sections which were taken at the level of the forelimb bud (FLB) and hind limb bud (HLB). IHC was performed to investigate BMP signalling using a Smad1,5,9 antibody and dI3 neurons using an Islet-1 antibody. Then, the area, length, and intensity of Smad 1/5/9 were measured and analysed (Fig. 12).

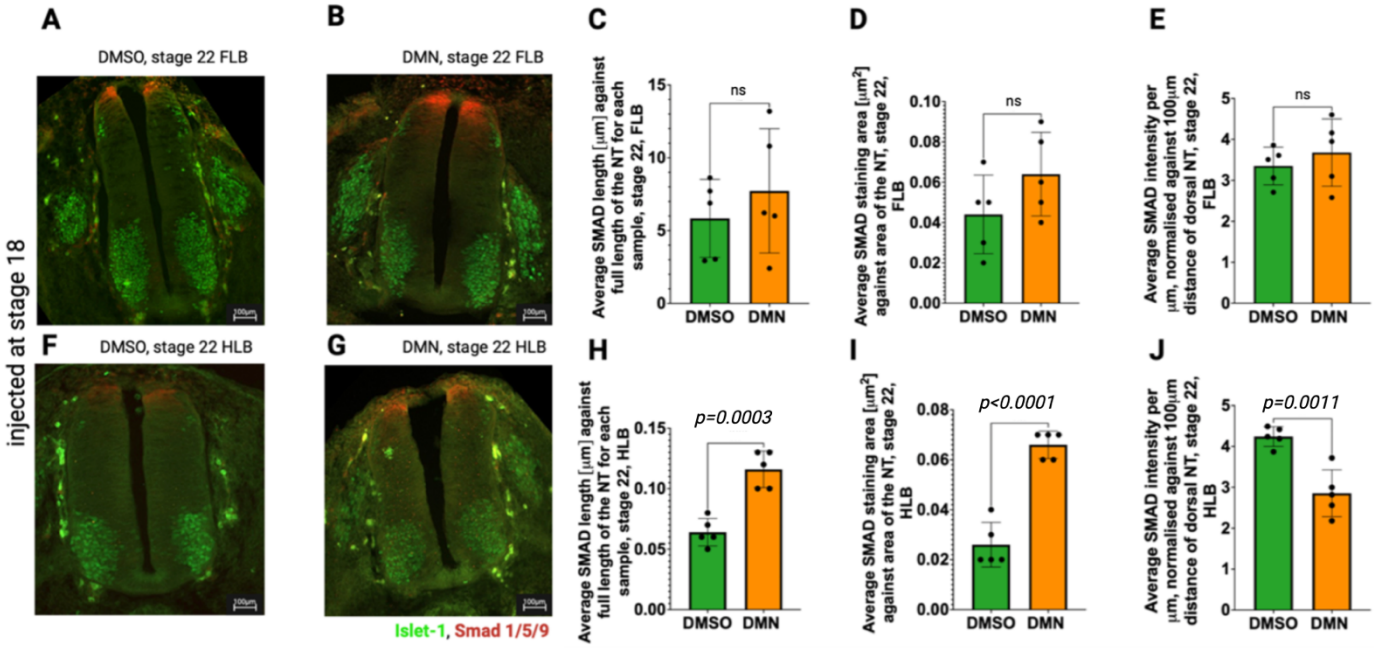


***Figure 19**. IHC analysis of chick embryo neural tube, stage 22, Smad 1/5/9*

Representative photographs from control DMSO (A, F) and DMN (B, G) injections at stage 18 (n=5 embryos), scale bars are shown. (C-E, H-J) Histograms of Smad 1/5/9 staining area, length, and intensity for FLB (C-E) and HLB (H-J) embryos (n=5 embryos). Statistical analysis was performed using an unpaired t-test., p values are shown, ns – non-significant, p<0.05.

Presented results showed that at the FLB in DMN injected embryos there is a modest but not significant increase in the length of the Smad 1/5/9 domain and its surface area (Fig. 19A, B, C, D), and the intensity of the Smad 1/5/9 was not impacted (Fig. 19A, B, E). Interestingly, significant differences were observed at the HLB level in DMN-injected embryos compared to vehicle controls. There was a significant increase in Smad 1/5/9 length and area (Fig. 19F, G, H, I) and a significant decrease in Smad 1/5/9 intensity (Fig. 19F, G, J). Overall, changes in staining pattern for Smad1/5/9 in stage 22 HLB DMN injected embryos were similar to stage 20 FLB DMN injected embryos. HLB

is younger than FLB, we predict that stage 22 HLB is approximately equivalent in age to stage 20 FLB. The intensity of Smad 1/5/9, for FLB and HLB stage 18 DMN and DMSO injected embryos was measured across 100µm on the neural tube as well as a standardised (400µm) length of the neural tube. Results presented highlight differences in the profile of Smad1/5/9 intensity for DMN injected samples at FLB and HLB levels (Fig. 20).

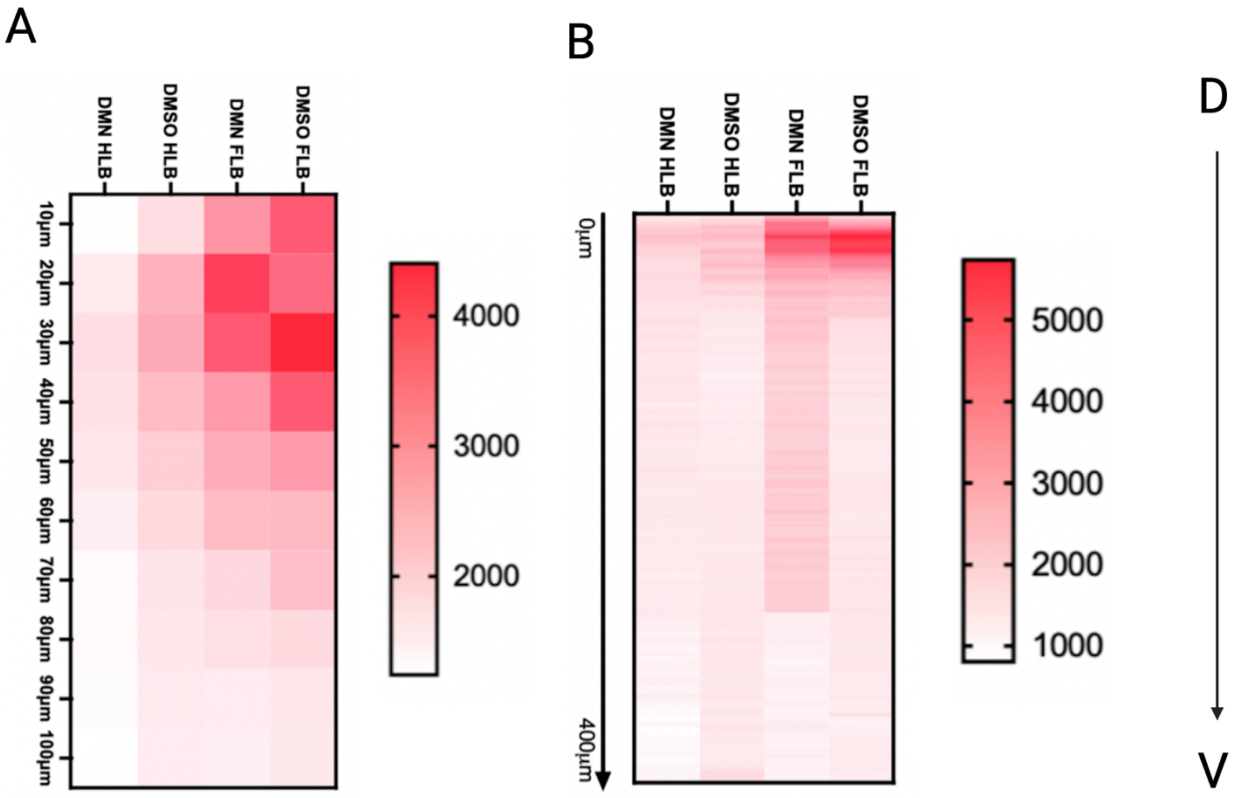


Figure 20. *Heat maps presenting Smad 1/5/9 intensity along 100 µm of the neural tube, stage 22, DMN. (A) and standardised length - 400µm of the neural tube (B). Injections of the drugs were done at stage 18 and embryos were allowed to develop until stage 22. Measurements were taken using ZEN ZEISS software, and presented results are an average on n=5 embryos. Graphs generated in PRISM GraphPad. Presented results show changes in intensity of Smad 1/5/9 alongside DV axis for DMSO and DMN injected embryos.*

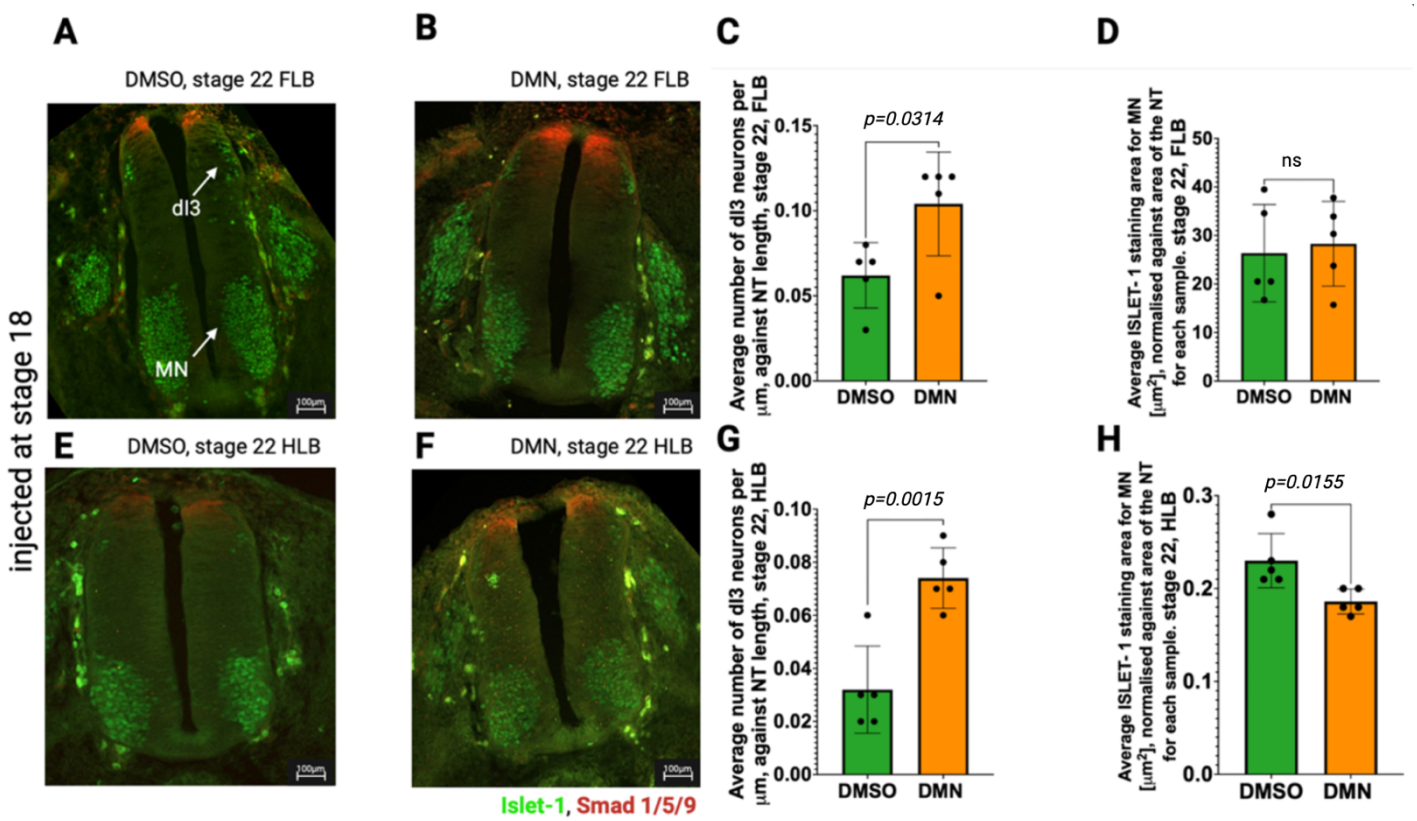


Figure 21. *IHC analysis of chick embryo neural tube, stage 22, Islet-1.*

Representative photographs from control DMSO (A, E) and DMN (B, F) injections at stage 18 (n=5 embryos), scale bars are shown. (C, D) Histograms of Islet-1 staining for dI3 and MN area FLB and HLB (G, H) embryos (n=5 embryos). Statistical analysis was performed using an unpaired t-test., p values are shown, ns – nonsignificant, p<0.05.

3.5 Dorsomorphin injection at stage 18 *in ovo* significantly increased number of dI3 neurons at level of the stage 22 HLB but had no impact on DREZ size and position.

Based on Smad 1/5/9 results, Islet-1 staining for dI3, and MN neurons was analysed. The next step was the analysis of DREZ size and position in stage 18 DMN injected embryos for both FLB and HLB levels. The presented data shows a significant increase in the number of dI3 neurons at both FLB and HLB levels (Fig. 21A, B, C, E, F, G). MN area was however unaffected at FLB levels (Fig.21A, B, D) but MN area was significantly smaller in the HLB part of the embryo (Fig. 21E, F, H). Injection of the DMN at stage 18 did not affect DREZ size or position at either FLB or HLB levels (Figure 22).

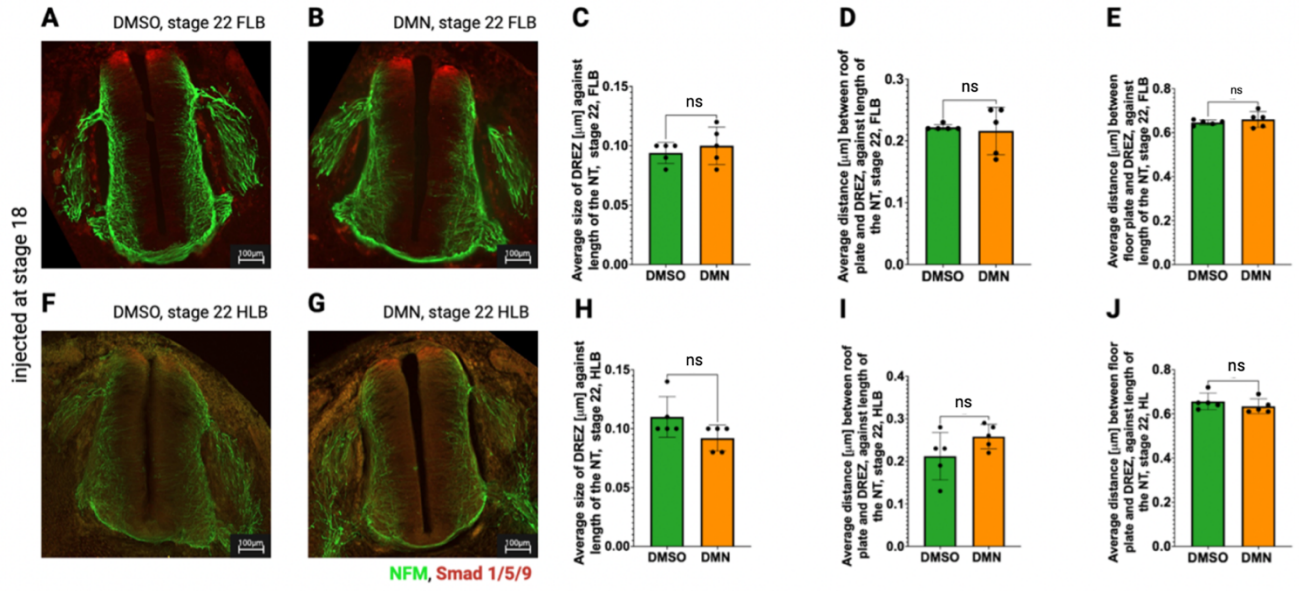


Figure 22. *IHC analysis of chick embryo neural tube, stage 22, NFM.*

Representative photographs from control DMSO (A, F) and DMN (B, G) injections at stage 18 (n=5 embryos), scale bars are shown. (C, H) Histogram of DREZ length (n=5 embryos) for FLB and HLB respectively. (D, I) Histogram presenting distance between RP and start of DREZ (n= 5 embryos). (E, J) Histogram presenting distance between FP and start of DREZ (n= 5 embryos). Statistical analysis was performed using an unpaired t-test., ns – nonsignificant, p<0.05.

3.6 Dorsomorphin injection at stage 18 *in ovo* decreased number of dI1 and dI2 neurons at stage 22 FLB and HLB levels.

The next step was to investigate, if, in stage 22 FLB, and HLB levels, the increase of the dI3 number is again at the expense of other classes of neurons. Therefore, embryo sections were stained with Lhx2 marking for dI1 neurons and Lhx5 antibody marking for dI2, dI4 and dI6 neurons. Each class of neurons was counted manually and then the number was divided by the length of the NT (Fig. 12).

Obtained results showed a significant decrease at both FLB and HLB levels in the number of dI1 and dI2 neurons (Fig.23, A, B, E, F, I, J, M, N). There was no significant difference between DMN and DMSO injected embryos for dI4 and dI6 neurons however, at both FLB and HLB levels (Fig. 23, C, D, G, H, K, L, O, P).

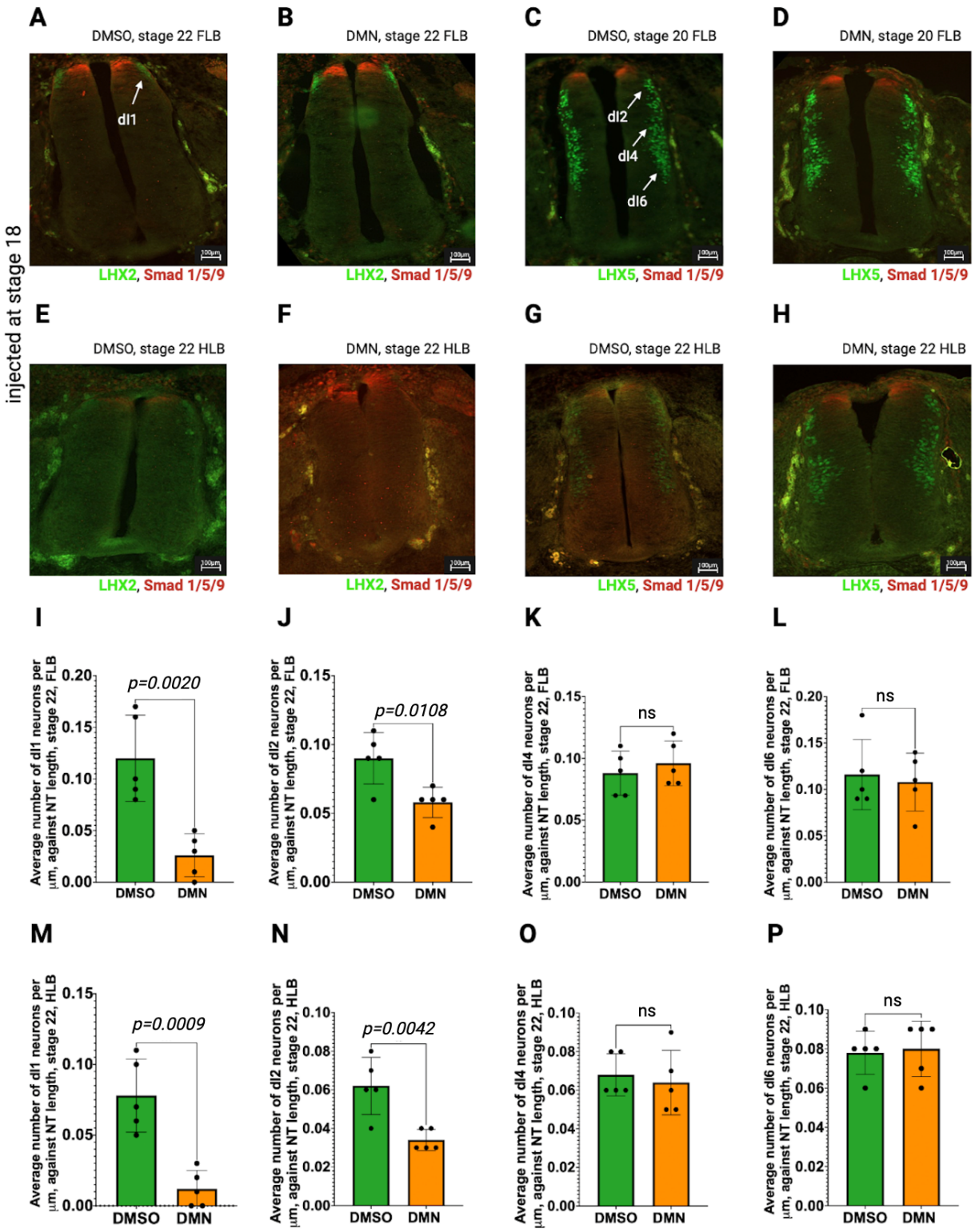


Figure 23. *IHC analysis of chick embryo neural tube, stage 22, Lhx2, Lhx5*

Representative photographs of DMSO (A, C, E, G) and DMN (B, D, F, G) injected at stage 18 (n=5embryos) scale bars are shown. (I, M) histograms representing Lhx2 staining for dI1 neurons FLB, HLB respectively. (J, K, L, N, O, P) histograms representing Lhx5 staining for FLB, HLB dI2, dI4 and dI6 neurons, respectively (n = 5 embryos). Statistical analysis was performed using an unpaired t-test., p values are shown, ns – nonsignificant, $p<0.05$.

3.7 Isoliquirtigenin injection at stage 16 *in ovo* led to smaller chick embryos by stage 20.

Based on the data obtained from DMN injections, an 'time window' for its impact on Smad1/5/9 staining and dorsal interneuron birth at FLB level was determined as stage 16 to stage 18. Isoliquirtigenin (ISL) is proposed to have the opposite effect of DMN on zebrafish dorso-ventral axis development (Alvarado et al. 2016; Riege et al., 2023; Genthe et al., 2017; Vrijens et al., 2013), To test its effect in our avian model for BMP signalling ISL was injected at stage 16 and chick embryos were allowed to develop further until stage 20 at which point they were checked for gross abnormalities, fixed, and processed for frozen sections which were taken at the level of the FLB.

Our first observation was that ISL-injected embryos were grossly smaller in size than vehicle controls (data not shown), which is represented here by the measurement of NT (n=5 embryos). It was found that the length of the NT in ISL-injected embryos was significantly smaller than in vehicle controls (Fig. 24).

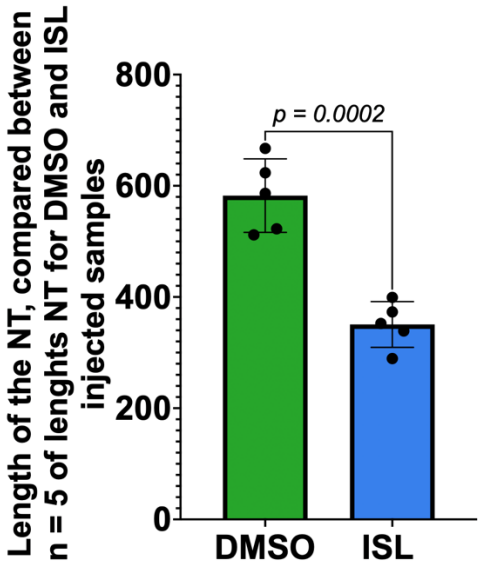


Figure 24. *Histogram comparing length of neural tube between ISL injected and vehicle controls (n=5 embryos).*

Statistical analysis was unpaired student -t test. Statistical analysis was performed using an unpaired t-test., p values are shown,p<0.05.

3.8 Isoliquirtigenin injections at stage 16 *in ovo* increased area and intensity of Smad 1/5/9 at level of stage 20 FLB.

As the next step, IHC was performed to investigate Smad1,5,9 antibody staining and the number of dorsal interneurons using various dorsal interneuron-specific antibodies. The area, length, and intensity of Smad 1/5/9 were measured and analysed (Fig. 12).

It was found that ISL injection significantly increased the length of the Smad 1/5/9 as well as the area (Figure 25 A – D). This can be seen in graphs, as well as in IHC staining of the sectioned embryos. Smad 1/5/9 in ISL-injected embryos is much broader, also a stripe of Smad 1/5/9 can be seen on the ventral side of the NT. Additionally, the intensity of Smad 1/5/9 was increased in ISL-injected embryos, which suggests higher levels/activity of BMP signalling (Fig.26).

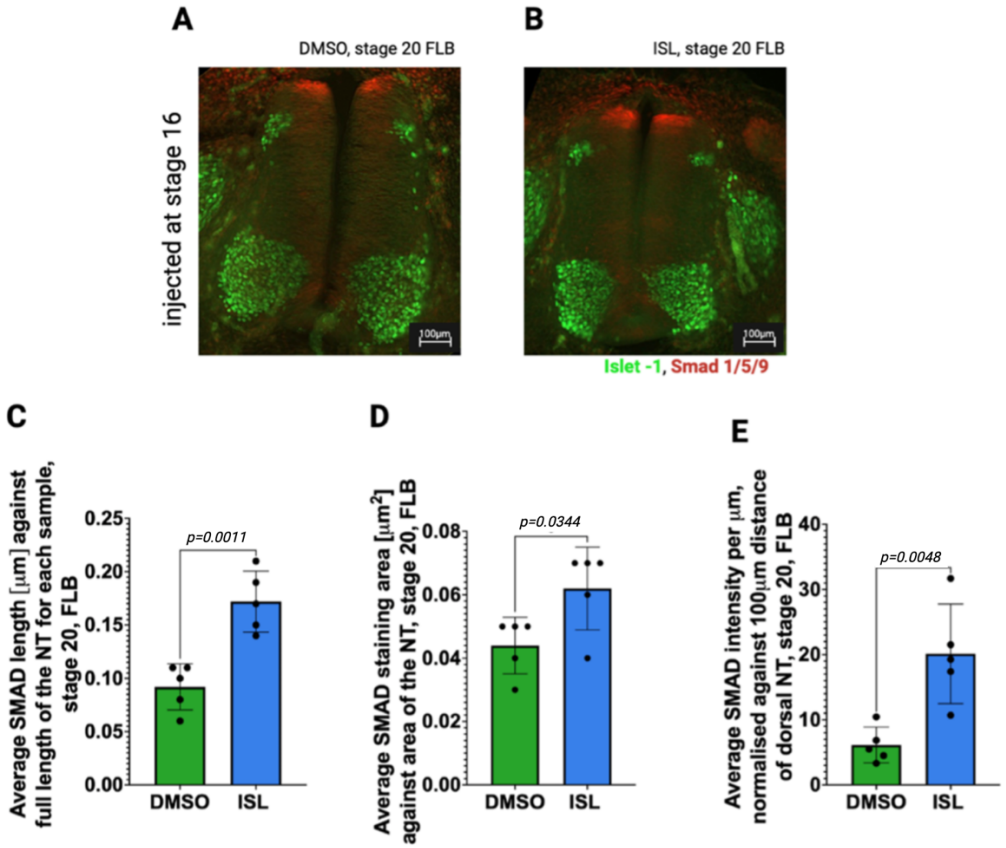


Figure 25. *IHC analysis of chick embryo neural tube, stage 20, ISL injection, Smad 1/5/9.*

Representative photographs from control DMSO (A) and ISL (B) injections at stage 16 (n=5 embryos), scale bars are shown. (C, D, E) Histograms of Smad 1/5/9 staining area, length, and intensity for embryos (n=5 embryos). Statistical analysis was performed using an unpaired t-test., p values are shown, ns – nonsignificant, $p<0.05$.

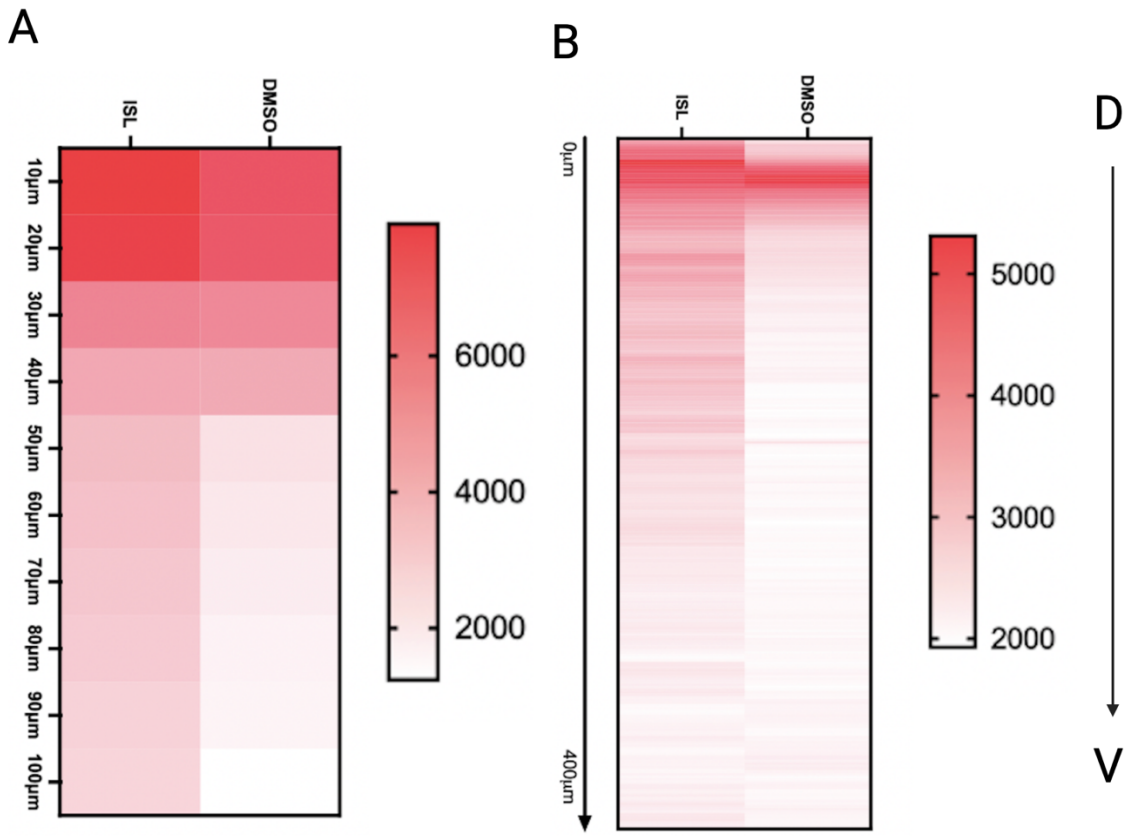


Figure 26. *Heat maps presenting Smad 1/5/9 intensity along 100 µm of the neural tube, stage 20, ISL. Standardised length - 400µm of the neural tube (B). Injections of the drugs were done at stage 16 and embryos were allowed to develop until stage 20. Measurements were taken using ZEN ZEISS software, and presented results are an average on n=5 embryos. Graphs generated in PRISM GraphPad. Presented results show changes in intensity of Smad 1/5/9 alongside DV axis for DMSO and ISL injected embryos.*

3.9 Isoliquirtigenin injections at stage 16 *in ovo* had no impact on number of dI3 neurons at stage 20 but significantly increased number of dI1 and dI6 neurons as well as number of dorsal rootlets.

As shown in Fig.27, injection of ISL had no significant impact on the number of dI3 neurons (Fig. 27 A–C) as well as the area of MN (Fig. 27 D). Investigation of the impact of ISL injections on DREZ position and size also revealed no significant impact on DREZ size and position, however, it was observed that the number of rootlets innervating the DREZ area appeared to be increased significantly. The next step was to investigate the impact of ISL injections on other dorsal neuron classes: dI1, dI2, dI4, and dI6. Embryo sections were stained with Lhx2 marking for dI1 neurons and

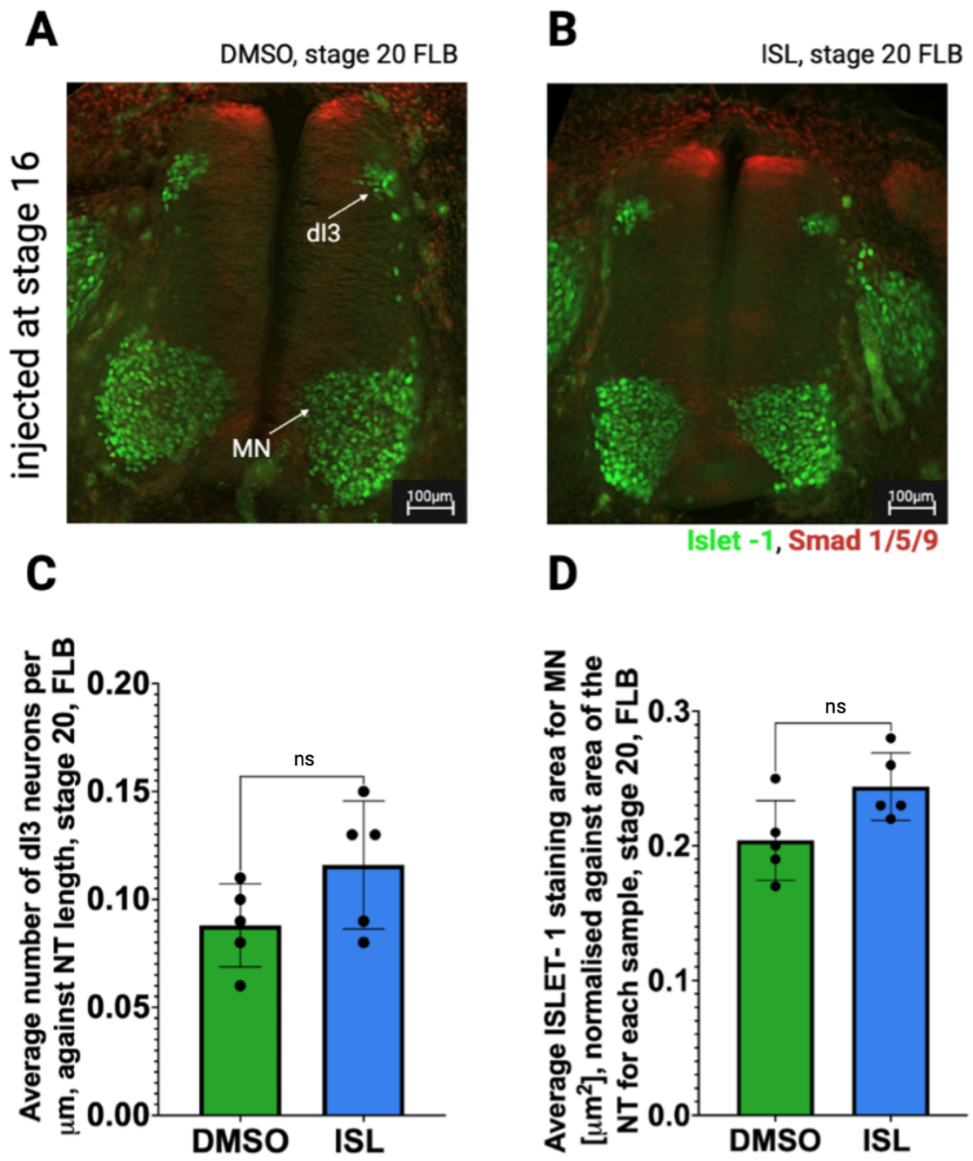


Figure 27. *IHC analysis of chick embryo neural tube, stage 20, ISL injected, Islet-1.*

Representative photographs from control DMSO (A) and ISL (B) injections at stage 16 (n=5 embryos), scale bars are shown. (C) Histogram of Islet-1 staining for dI3 FLB embryos (n=5 embryos). (D) Histogram of Islet-1 staining for motor neuron (MN) area (n= 5 embryos). Statistical analysis was performed using an unpaired t-test., ns – nonsignificant, $p<0.05$.

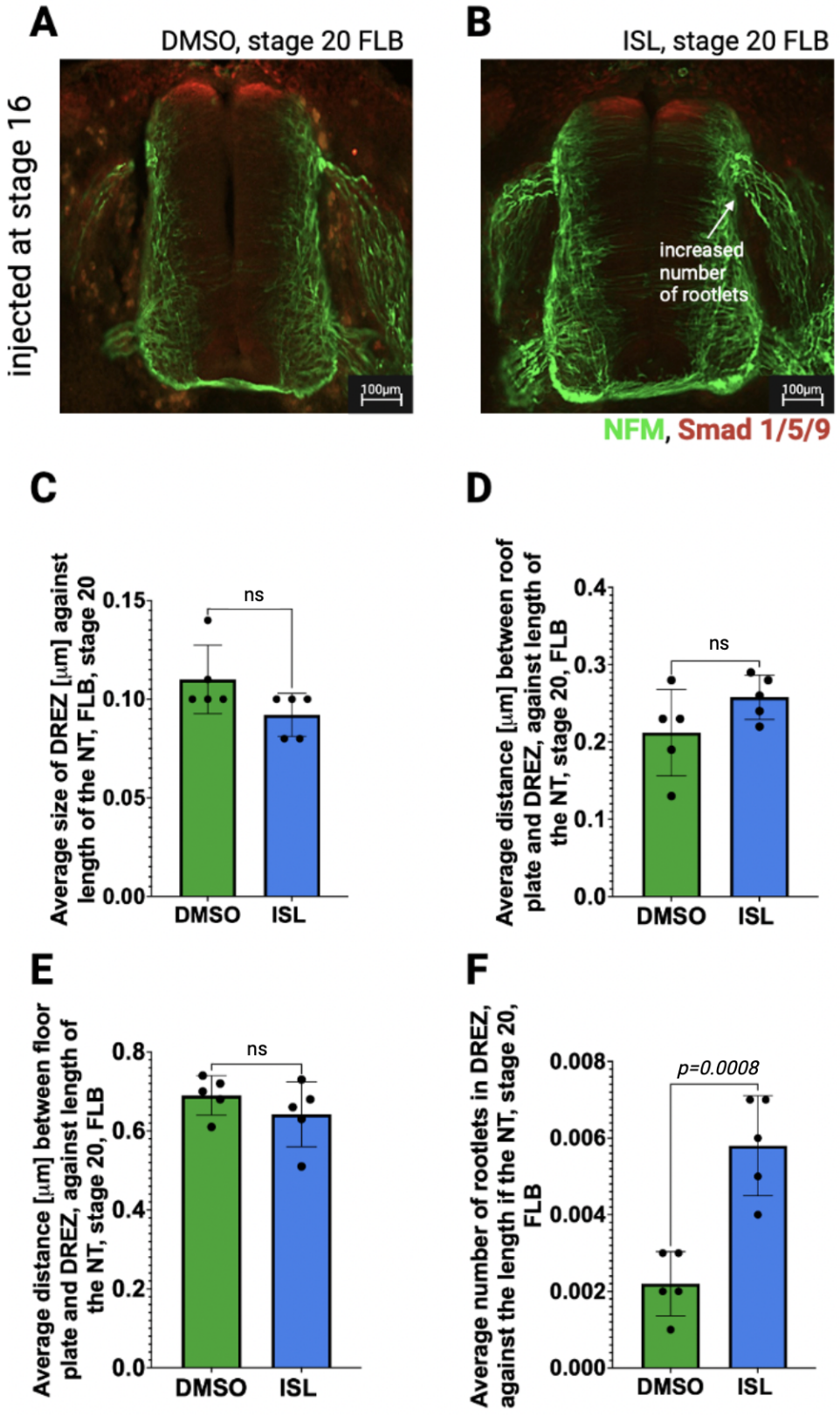


Figure 28. *IHC analysis of chick embryo neural tube, stage 20, ISL injected, NFM.*

Representative photographs from control DMSO (A) and ISL (B) injections at stage 16 (n=5 embryos), scale bars are shown. (C) Histogram of DREZ length (n=5 embryos). (D) Histogram presenting distance between RP and start of DREZ (n= 5 embryos). (E) Histogram presenting distance between FP and start of DREZ (n= 5 embryos). (F) Histogram presenting differences in number of rootles between DMSO and ISL injected embryos (n = 5embryos). Statistical analysis was performed using an unpaired t-test., p values are shown, ns – nonsignificant, $p<0.05$.

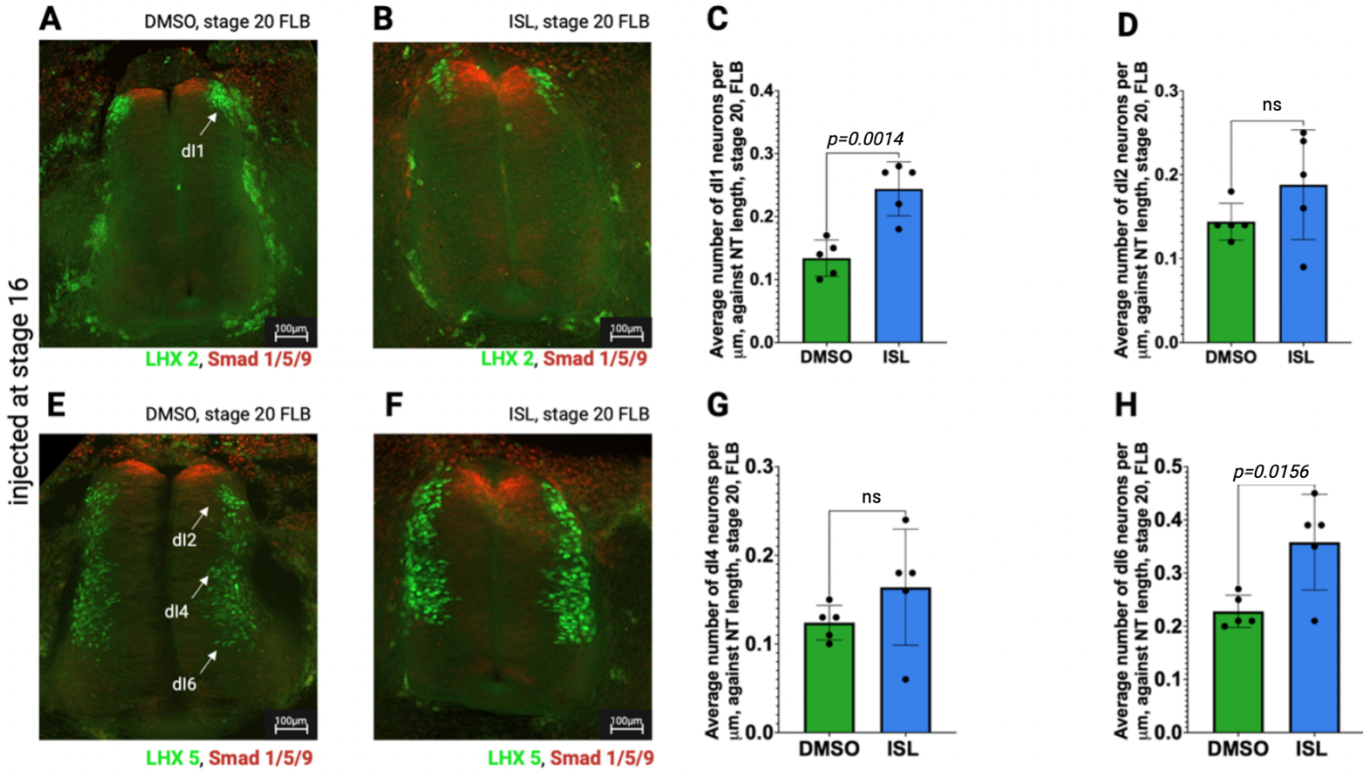


Figure 29. *IHC analysis of chick embryo neural tube, stage 20, ISL injected, Lhx2, Lhx5*

Representative photographs of DMSO (A, E) and ISL (B, F) injected at stage 16 (n=5embryos) scale bars are shown. (C) histogram representing Lhx2 staining for dI1 neurons FLB. (D, G, H) histograms representing Lhx5 staining for dI2, dI4 and dI6 neurons, respectively (n = 5 embryos). Statistical analysis was performed using an unpaired t-test., p values are shown, ns – nonsignificant, p<0.05.

Lhx5 antibody marking for dI2, dI4, and dI6 neurons. Each class of neurons was counted manually and then the number was divided by the length of the NT (Fig. 12).

It was found that injection of ISL significantly increased the number of dI1 neurons (Fig. 29, A–C) but not classes dI2 and dI4 (Fig.29 D – G). Interestingly, a significant increase in number of dI6 neurons was observed. This latter change could be related to ectopic Smad 1/5/9 staining on the ventral side of the neural tube, which was not observed in DMSO-injected samples (Fig. 29B, F).

4 Discussion

In this study, we aimed to investigate the temporal role of BMP signalling in the birth of dI3 dorsal interneurons using the developing chick embryo as a model. This study also explored how BMP signalling influences the integrity of the dorsal root entry "gateway," a structure that originates from the same region of the dorsal neural tube as the dI3 neurones. The “gateway” permits axon innervation into the CNS while preventing neuronal migration out of the CNS. A key finding was that injection *in ovo* of DMN broadens and dampens Smad 1/5/9 levels. This effect is correlated with the increased number of dI3 neurons at the expense of dI1 and dI2 numbers and area of the MN. However, the injection did not impact DREZ formation or anatomy. In addition, we found that ISL, as expected. Increased Smad 1/5/9 levels but did not affect the dI3 interneuron number. Injection of ISL increased the number of dorsal rootlets innervating the DREZ at stage 20.

4.1 DMN alters dorsal interneuron neurogenesis in avian embryos from stage 16 to stage 18.

Data presented in this study is consistent with the expected timing of BMP/Smad signalling in the avian NT and the effect of BMP/Smad signalling inhibition on dI1 and dI2 interneuron generation, it will be essential in the future to investigate the effects of DMN to examine whether this is specific to BMP signalling by staining for any changes in expression of other downstream signalling effectors of BMP signalling such as Msx1, ATF2, CREB and Id proteins (Boergermann et al. 2010; Yang et al. 2013; Le Dreau, Marti; 2013; Muroyama et al.2002; Tribulo et al. 2003).

In this study, we established, that injection *in ovo* of DMN leads to broadening and reducing Smad signalling. This is consistent with the study, where the effects of BMP blockade in Id expression were investigated, by isolation of otic vesicles from stage 20 chick embryo using microsurgery techniques, then incubation of the culture for 24 hours. Otic vesicles were incubated with either DMN or Noggin. The results presented a significant reduction in the expression of Id genes. The findings suggested that Bmp and its receptor with Noggin or inhibiting receptor phosphorylation with Dorsomorphin led to decreased levels of Id1-3 mRNAs. This observation implies that during this

developmental stage, the expression of Id genes in the otic vesicle is strictly dependent on Bmp activity (Kamaid & Neves & Giraldez; 2010).

BMP signalling is crucial in the establishment of the DV axis (Antebi et al. 2017). Its gradient is generated during DV patterning, however molecular mechanisms regarding BMP gradient formation remain unclear (Yan & Wang; 2021). It is believed that mature oocyte maintains symmetry alongside the DV axis prior to fertilisation (Yan & Wang; 2021; Miyazono et al., 2010). After fertilisation, maternal factors trigger the expression of zygotic BMP (Yan & Wang; 2021; Plouhinec et al., 2013b). Subsequently, BMP inhibitors such as Noggin and Chordin are being produced. These inhibitors bind specifically to BMP proteins, preventing their diffusion to the dorsal half of the embryo, resulting in a gradient of BMP concentration with higher levels dorsally and lower levels ventrally (Yan & Wang; 2021; Plouhinec et al., 2013b; Miyazono et al., 2010; Little and Mullins, 2009). Thus, the future experiments of this study should involve the investigation of the position of BMP inhibitors such as Noggin, which would allow us, to see possible changes in the BMP gradient within the NT, as well as to investigate if injection of DMN leads to any changes in Noggin position within the NT. Additional experiments, e.g. assay of how long DMN is active within the chick embryo following sub-blastodermal injection would be beneficial; DMN is known to have half-life of 1.6-4.3 hours depending on vehicle used for administration (Hong & Yu; 2010). This would enable the time-window of its impact to be refined and to provide further evidence for its specific effect on BMP signalling and dI3 interneuron birth.

Presented results are consistent with previous MRes student findings, C.J. Arnell, 2020 "Understanding the Origins of the DREZ: a Possible New Route for Spinal Cord Injury Therapy" who showed that Smad1/5/9 staining around stage 16 is broad in the dorsal NT restricting by stage 17/18 to a transient stripe of expression at the level of the DREZ and a more stable stripe of expression adjacent to the roof plate which was still present at stage 22. Also, presented were noggin mRNA staining patterns which were complementary to Smad 1/5/9: Smad1/5/9 stripe at stage 17/18 was in a

similar location to stripe which showed an absence of noggin mRNA. In the future therefore it will be important to analyse noggin mRNA levels in DMN-injected embryos.

As much as it is known that BMP signalling drives patterning and formation of neural circuits there is no information regarding the specificity of timings of dorsal interneurons being born and BMP activity, however, there is evidence that different types of BMP ligands stimulate the birth of different classes of interneurons (Rekler & Kalcheim; 2021). It was found, that in avian and mice, BMP7 is required for the generation of dI1-dI3-dI5 interneurons. Within the BMP signalling pathway, Smad7, which inhibits the BMP pathway, is expressed in the NT and is reported to restrict Smad 1/5/9 to the very dorsal part of the mouse NT. In mice, ectopic misexpression of Smad7 suppressed dI1 and dI3 neural fates and increased the number of dI4–dI6 spinal neurons. Thus, future experiments for this project could be an investigation of the expression of Smad7 within the NT following DMN injection at stage 16 which will as mentioned above, provide more insight into BMP pathway regulation by DMN but also supporting evidence that the DMN effects that we have observed are BMP-specific. To this same effect, further experiments are needed which investigate the effect of DMN on BMP receptor expression (Boergermann et al. 2010; Yang et al. 2013). These studies should also include targets suggested to be affected by DMN i.e. Akt/mTOR/Wnt pathways, where it was found that DMN targets these pathways and reduces the proliferation and migration of colorectal cancer cells (Ghanaatgar-Kasbi et al. 2019).

Evidence, that BMP4 stimulates the birth of dI1 and dI2 neurons was tested by Andrews et.al, 2017, by assessing timings on different BMPs in the chick embryo spinal cord. Markers for dorsal patterning i.e. Pax3, were used, as it's expressed in all dorsal progenitors in VZ prior to HH14. Their work showed that dorsal interneurons arise 12-24 hours post Pax3 expression, after HH14. dI1 neurons arose around stage 18, similar to dI2. Islet–1–positive MNs arose around stage 16, and dI3 between stages 18-21 (Andrews et al.2017). These latter findings are consistent with results presented here: dI1-3 interneuron birth and MN birth can be influenced from stage 16 to stage 18 with a possible limit for dI3 neurons to stage 18/19 given that increase in dI3 neurons at FLB level in stage 18 injected

embryos showed lower significance compared to HLB level. Further extending experiments for this part of the study could be IHC for Pax3 after injecting DMN/ISL to investigate if Pax3 expression is affected. Pax3 labels the dorsal progenitor domain, indeed overall it will be necessary for the future to assess markers of dorsal progenitors to ask whether the changes in dorsal interneuron populations observed in this study are because of effects of DMN in dorsal progenitor populations or on neuronal birth more directly.

4.2 How does Dorsomorphin influence dI3 interneuron development?

Experiments presented in this study have shown that Smad1/5/9 levels were broadened and dampened by DMN which suggests a decreased amount of BMP signalling, supported by an expected decrease in the number of dI1 and dI2 neurons. dI1 and dI2 neuronal birth are known to require high levels of BMP signalling (Duval et al.2019; Andrews et al, 2017). However, as mentioned above, an effect on BMP signalling is inferred, but not yet proven. Injections of DMN between stages 16-18 resulted in increased numbers of dI3 neurons together with decreased area of motor neurons and decreased dI1 and dI2 neurons. The impact on MNs is interesting given that ventral interneurons dI4 and dI6 were unaffected, but this may reflect the common expression of Islet-1 by dI3 neurons and MNs. The increase in dI3 neurons appears to be at the expense of dI1 and dI2 interneurons, thus, DMN has not caused dorsalisation of the NT, possibly because noggin is expressed dorsally at the stages analysed. Loss of dI1 and dI2 and increase of dI3 interneurons raises the hypothesis that DMN expands the dI3 progenitor domain but restricts the progenitor domains of dI1 and 2 interneurons. Future experiments should test this hypothesis by analysis of the expression of specific transcription factors that mark specific dorsal progenitors i.e. Atoh1, Ngn1, and Ascl1 (see Fig.7). dI3 neuron number can increase by broadening and dampening Smad 1/5/9 staining in the dorsal avian NT, thus this effect could be mirrored by broadening and dampening the BMP ligand gradient. While a gradient of BMP activity has been proposed as a determinant of patterning interneuron cell type identity *in vivo*, its sufficiency for pattern formation in vitro remains unclear. Duval et al. 2019, illustrated that exposure to BMP4 triggers the expression of pSmad within self-emerging epithelia in both mouse and human

pluripotent stem cell-derived spinal organoids. Elevating BMP4 concentration, in mouse spinal organoids, correlated with an increase in the quantity of pSmad-expressing cells, elevated mean levels of pSmads, and prolonged duration of Smad activity. Likewise, altering the duration of BMP4 exposure affected the timing of signalling events. Crucially, adjusting BMP4 concentration or exposure duration influenced pSmad dynamics, resulting in an extended generation of BMP-dependent cell types, i.e. dI1 and dI2, and a bias towards dorsal cell types over ventral ones (Duval et al. 2019, Tozer et al. 2013). Additionally, species-specific differences in the duration of competence time windows between mice and humans contribute to the tempo of neural differentiation (Andrews et al.2017). Likewise, altering the duration of BMP4 exposure, in mice spinal organoids, affected the temporal dynamics of signalling. Following the removal of BMP4, nuclear pSmad levels declined within 6 hours, suggesting the absence of long-term intracellular signalling memory induced by BMP exposure. Adjusting the timing of BMP4 exposure significantly influenced cell identities, with cells exhibiting a 12-hour competence time window for generating specific BMP4-dependent cell types. The most dorsal cell types required earlier exposure times compared to more ventral ones. (Duval et al., 2019; Andrews et al., 2017; Rekler et al.2021). These published data suggest that not only the amount of pSmad but also the prolonged exposure to pSmad could lead to an increase in dI3 neurons. These findings are consistent with the experiments reported here. In addition, Valizadeh-Arshad et al. 2018 reported on their work on hESCs-derived neurons. Here DMN was used in a cocktail of small molecules to control signalling pathways, including the BMP pathway, during the neural differentiation of hESCs. This method improved the enhanced neural differentiation and was described as an effective and affordable approach to regulating the differentiation of stem cells into neural cell types, however, there was no data provided on whether this increased the number of dI3. This data supports the hypothesis that DMN impacts neural differentiation and highlights how the work reported in the chick model is relevant to human studies.

Tozer et al. 2013, assessed the consequences of blockading the BMP pathway, by overexpression of Smad 6 on dP1-3 progenitors and their corresponding neurons. Electroporation of

Smad 6 at HH8 caused a reduction of the entire dP1-3/dI1-3 population, by possibly disrupting the formation of the Pax3 progenitor domain (Tozer et al., 2013). Moreover, it was found, that number of dI3 neurons was reduced, but only when Smad 6 was transfected at HH8. Later, around HH16, transfections of Smad 6 resulted in the recovery of the dI3 population. This suggests that Smad 6 over-expression likely specifies the dorsal progenitor domain early as it does not impact interneuron birth at later stages.

In experiments performed on mice, knockdown of Cadherin 6B, by electroporation of DNA constructs, inhibited BMP signalling and disrupted generation of Islet-1 expressing cells (dI3 neurons) in the dorsal neural tube (Park &Gumbiner, 2015). This may suggest that complete inhibition of the BMP signalling pathway, e.g. by Cadherin-6B deficiency leads to a decrease in dI3 neurons. Thus, from this work, DMN might act by dampening the BMP pathway together with increased longevity of BMP signalling on dI3 progenitors as evidenced by the broadening of Smad1/5/9 staining and, in this way, leading to an increase in the number of dI3 neurons. Thus, the mechanism of DMN may be decrease and prolong BMP signalling in dI3 progenitors which expands their domain or increases their ability to generate neurons. As discussed above the specificity of DMN for BMP signalling and its effect on BMP receptors and inhibitors needs to be explored in the future to provide evidence for this hypothesis.

Another approach to analyse interactions of DMN/BMP could be analysis of the impact of activin signalling on birth of dI3 neurons and investigation of whether DMN injection has any impact on the activin pathway. Timmer, Chesnutt & Niswander in 2005, showed, that activin signalling specifically promotes dI3 interneuron formation without altering surrounding cell populations. Moreover, the dI3 population either expanded or remained unaffected, regardless of Alk4 activation strength (Alk4 is the receptor for activin), suggesting that activin ligands in the neural tube do not function via an activity gradient. While BMP signalling is essential for dorsal neural tube patterning and can promote dI3 formation at low BMP activity levels, dI3 interneurons still form when BMP

signalling is inhibited by Noggin misexpression (Chizhikov and Millen, 2004a; Chesnutt et al., 2004) or conditional BMP receptor inactivation (Wine-Lee et al., 2004).

As a result of this study and combined with the study of CJ Arnell (2020), a new hypothesis can now be raised. dI3 interneuron birth is promoted following extended exposure to extended exposure to lower levels of BMP signalling which is provided by a further short phase of Smad1/5/9 signalling in the dorsal NT at stage 17/18. Possibly in this scenario BMP signalling supports the competence of neural progenitors to generate dI3 interneurons.

4.3 Efficacy and specificity of isoliquirtigenin.

Isoliquirtigenin is reported to increase BMP signalling (Alvarado et al. 2016; Riege et al., 2023; Genthe et al., 2017; Vrijens et al., 2013). The data presented in this study, where the intensity and area of Smad 1/5/9 significantly increased is consistent with ISL as an activator of BMP signalling, however, as outlined above for DMN, additional study of other BMP effectors and regulators are required to provide further evidence. ISL activity from stage 16 to stage 20 was associated with an increased number of dI1 and dI2 neurons, the most dorsal classes of neurons and thus the most susceptible to BMP signalling. ISL had no impact on dI3 neurons. There is a lack of research on the impact of ISL on the development of dorsal neurons, the mechanism of action of ISL on neurogenesis is unknown and future work should investigate the effect of ISL on the expression of BMP receptors, ligands, and endogenous inhibitors such as noggin, as well as confirmation to specify ISL for BMP signalling by the study of its effect on Pax7 and Msx1 expression. However, it is known, that orally administered ISL halftime is 4.6hours in rats (Qiao et al., 2014). This should be further investigated using our *in ovo* model but suggests that the ISL drug works for very specific and short periods of time.

Experiments performed in zebrafish have investigated the impact of ISL treatment on craniofacial cartilage formation. Wild-type and wdr68hi3812/hi3812 zebrafish mutants were treated with ISL. It was shown that ISL treatment partially restored craniofacial cartilage formation in mutant zebrafish. This is important, as defects in BMP signalling contributes to the wdr68 mutant phenotype

in zebrafish, and many craniofacial syndromes are caused by defects in signalling pathways that pattern the cranial neural crest cells along the dorsal-ventral axis (Alvarado et al. 2016).

Understanding how ISL impacts BMP ligand-specific signalling would be a crucial next stage in the development of this research, as both, BMP4 and BMP7 can increase the numbers of RP, dI1, and dI3 cells, however, they do so to markedly different extents (Andrews et al.2017).

Injections of ISL at stage 16 led to an increase of dI6 neurons which correlated with an ectopic stripe of Smad 1/5/9 on the ventral side of the NT at stage 20. This is consistent with Lupo, Harris, and Lewis (2006), who in their review acknowledged that overexpression of BMP represses the specification of ventral progenitor domains while enhancing the specification of dorsal fates. Downregulation of BMP signalling in the ventral spinal cord might be an essential step in the specification of ventral fates.

Another route to increase the BMP signalling could be by inhibition of Noggin, a BMP antagonist. In mice embryos, it was shown that lack of Noggin caused increased BMP/Smad 1/5/9 signalling in muscle tissue, together with the induction of BMP target genes such as Id1, 2, 3 as well as Msx1 (Costamagna et al. 2016). Thus, in relation to this study, future experiments could investigate strategies to inhibit Noggin and analyse its impact on neural cell dorsal fates. For this study, also investigating the RNA and protein expression patterns of noggin, after injection with ISL could be informative.

Injections of ISL led to increased number of rootlets in DREZ area. Mechanism of action of that event is not known, however it could be due to an effect of increased BMP signalling on neuroepithelial cells forming the DREZ around stage 16 (Golding Shewman, Cohen; 1997). Another possibility is that increased BMP could affect expression of the transcription factor Krox-20, which is expressed in boundary cap cells. Boundary cap cells form temporary clusters at the entry and exit points for all cranial and spinal nerves within the developing spinal cord, which form the interfaces between the CNS and PNS. Increased BMP signalling within neural crest cells which generate the

boundary cap cells could affect the formation or structure of the dorsal root entry points, however, potential mechanisms are yet to be investigated (Gerschenfeld et. al, 2024; Gresset at al.2015).

4.4 Stem cell therapies for SCI

Cellular replacement therapies aimed at treating neurological conditions involve the use of neurons derived from hESCs or iPSCs to replace damaged or diseased neurons. In the context of spinal cord injuries, considerable progress has been made in generating *in vitro*-derived motor neurons, which are crucial for restoring coordinated movement. However, a significant gap remains in the development of protocols to generate *in vitro*-derived sensory interneurons (INs), which are essential for perceiving the environment (Gupta et al.; 2017). This study showed that both a BMP inhibitor or activator alters the number of dorsal sensory interneurons in the avian NT. Especially, the injection of DMN, which broadened and dampened Smad1/5/9 staining, increased the number of dI3 neurons. This finding is important to investigate further, as dI3 neurons are deemed necessary for stable recovery of locomotor activity post-injury (Bui et al. 2016).

In a recent study by Xu et al. (2023), early embryonic neural stem/progenitor cells (NSPCs) were isolated and cultured on a collagen scaffold to generate spinal cord-like tissues. These tissues were then transplanted into rats and rhesus monkeys with SCI. The findings demonstrated that NSPCs can differentiate into mature cells with specific neuron identities upon transplantation into monkeys, facilitating the recovery of damaged neuronal circuits and promoting hindlimb motor recovery. Understanding how dI3 neurons can be generated efficiently from NSPCs could support the development of similar strategies with a goal to eventually reconstitute proprioceptive circuits in humans with SCI. Translation of this work to stem cell therapies and human organoids would require more knowledge on DMN, noggin and BMP, however the requirements might be different in avian NT than in human.

Despite stem cell therapies for SCI being very promising, they are facing challenges. At first, there is difficulty in recruiting enough SCI patients for trials. This is due to the severity of injury and

patient characteristics. Additionally, there are concerns regarding the safety of stem cell characteristics and transplantation procedures. With ESCs and iPSCs tumorigenesis is a major concern (Gao et al., 2020; Gazdic et al., 2018; Mothe & Tator, 2012; Shang et al., 2022).

Conclusion

This study aimed to investigate the temporal role of BMP signalling in the birth of dI3 dorsal interneurons, using the developing chick embryo as a model, as well as the impact of BMP signalling on the integrity of the dorsal root entry 'gateway', which allows sensory axon innervation into the CNS but not neuronal migration out of the CNS. This "gateway" develops from the same area of the dorsal neural tube as dI3 neurons. The key findings were that DMN inhibition appeared to dampen BMP signalling and extend its activity within dI3 progenitors and that this effect was evident from stage 16 to stage 18. This therefore identified a 'time window' for BMP signalling strength and duration for dI3 interneuron generation in avian embryos. Future work will confirm the specificity of DMN and ISL for BMP signalling in the dorsal avian neural tube. Their impact on dorsal progenitor transcription factor expression and the location of BMP receptor and Noggin expression at different developmental stages. This research offers further insights into potential stem cell therapy approaches for SCI, involving the regeneration of dI3 neurons

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