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ORIGINAL ARTICLE

Leptin-based hexamers facilitate memory and prevent amyloid-driven AMPA receptor internalisation and neuronal degeneration

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Abstract

Key pathological features of Alzheimer's disease (AD) include build-up of amyloid β (Aβ), which promotes synaptic abnormalities and ultimately leads to neuronal cell death. Metabolic dysfunction is known to influence the risk of developing AD. Impairments in the leptin system have been detected in AD patients, which has fuelled interest in targeting this system to treat AD. Increasing evidence supports pro-cognitive and neuroprotective actions of leptin and these beneficial effects of leptin are mirrored by a bioactive leptin fragment (leptin$_{116–130}$). Here we extend these studies to examine the potential cognitive enhancing and neuroprotective actions of 8 six-amino acid peptides (hexamers) derived from leptin$_{116–130}$. In this study, we show that four of the hexamers (leptin$_{116–121,\ 117–122,\ 118–123}$ and $_{120–125}$) replicate the ability of leptin to promote α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor trafficking and facilitate hippocampal synaptic plasticity. Moreover, the pro-cognitive effects of the hexamers were verified in behavioural studies, with the administration of leptin$_{117–122}$ enhancing performance in episodic memory tasks. The bioactive hexamers replicated the neuroprotective actions of leptin by preventing the acute hippocampal synapto-toxic effects of Aβ, and the chronic effects of Aβ on neuronal cell viability, Aβ seeding and tau phosphorylation. These findings provide further evidence to support leptin and leptin-derived peptides as potential therapeutics for AD.

KEYWORDS
Alzheimer's disease, amyloid, hippocampus, leptin, memory, synaptic plasticity, tau

Abbreviations: AD, Alzheimer's disease; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; Aβ, Amyloid beta; ANOVA, analysis of variance; APP, amyloid precursor protein; DMEM, Dulbecco's modified eagle medium; DMSO, dimethyl sulphoxide; DR, discrimination ratio; ELISA, enzyme-linked immunosorbent assay; fEPSP, field excitatory postsynaptic potential; HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); HFS, high-frequency stimulation; IP, intra-peritoneal; LepR, Leptin receptor; LDH, lactate dehydrogenase; LTD, long-term depression; LTP, long-term potentiation; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NBF, neutral buffered formalin; OPC, object place context; PBS, phosphate buffered saline; SEM, standard error of the mean; TMB, 3,3′,5,5′-Tetramethylbenzidine.

1 | INTRODUCTION

Alzheimer's disease (AD) is a degenerative brain disorder that leads to pronounced cognitive impairments. Key features of AD are the accumulation of amyloid beta (Aβ) and hyper-phosphorylation of tau that result in the formation of amyloid plaques and neurofibrillary tangles, respectively. Toxic forms of Aβ are produced by proteolytic processing of amyloid precursor protein (APP). Acute exposure to oligomeric Aβ promotes disruption of hippocampal synaptic function and chronic Aβ treatment leads to neuronal death (Malekizadeh et al., 2017). Numerous studies indicate that soluble Aβ oligomers interfere with activity-dependent synaptic plasticity resulting in impairments in hippocampal-dependent memory (Morley & Farr, 2014; Shankar et al., 2008). Indeed, induction of long-term potentiation (LTP) at CA1 synapses is blocked after treatment with Aβ, whereas acute exposure to Aβ facilitates induction of hippocampal long-term depression (LTD; Walsh et al., 2002; Shankar et al., 2008). Insertion and removal of AMPA receptors from synapses are crucial for hippocampal synaptic plasticity (Collingridge et al., 2004), and Aβ also interferes with AMPA receptor trafficking (Hsieh et al., 2006; Liu et al., 2010), which is likely to contribute to Aβ-driven impairments in hippocampal synaptic plasticity.

The risk of developing AD increases significantly with age. However, clinical evidence indicates that lifestyle and dietary factors also influence AD risk (Cunnane et al., 2020; Livingston et al., 2020; Stranahan & Mattson, 2012), with AD risk elevated in individuals with mid-life obesity. Food intake and body weight are regulated by the hormone leptin and circulating leptin levels correlate directly with body fat content (Maffei et al., 1995). Thus, mid-life obesity and the associated increase in body fat content elevates plasma leptin levels ultimately resulting in leptin resistance (Friedman, 2014). Consequently, AD risk is likely to be markedly altered in the obese, leptin-resistant state. Alterations in leptin function occur in AD as significant reductions in leptin levels have been detected in AD patients (Power et al., 2001) and in transgenic mice with familial AD mutations (Fewlass et al., 2004). Prospective studies have found a link between low leptin levels and an increased risk of AD (Lieb et al., 2009). Strengthening the hypothesis that leptin function correlates with AD and the severity of cognitive impairment, a recent meta-analysis of the 48 epidemiological studies concluded that AD patients had lower blood leptin than cognitively normal individuals and that lower leptin was associated with higher degree of cognitive impairment (Garcia-Garcia et al., 2022). Collectively this suggests that failure to maintain circulating leptin levels within the normal physiological range and/or dysfunctions in the leptin system significantly elevates AD risk.

In addition to playing a role in the hypothalamic control of energy homeostasis, leptin has a major impact on hippocampal excitatory synaptic function. Rodents with insensitivity to leptin display impaired hippocampal synaptic plasticity and spatial memory (Li et al., 2002), whereas rodent performance in hippocampal-specific memory tasks is enhanced after leptin administration (Wayner et al., 2004). In cellular studies, rapid pro-cognitive effects of leptin occur, with significant alterations in glutamate receptor trafficking and excitatory synaptic strength at hippocampal synapses (Luo et al., 2015; Moult et al., 2010; Shanley et al., 2001). However, the effects of leptin extend beyond its cognitive enhancing actions, as leptin protects against various toxic stimuli including Aβ (Doherty et al., 2013; Guo et al., 2008). Leptin also inhibits the aberrant effects of Aβ on hippocampal synaptic plasticity and it blocks Aβ-driven synaptic removal of AMPA receptors (Doherty et al., 2013; Malekizadeh et al., 2017; Tong et al., 2015), suggesting that leptin-based treatments may be beneficial in AD.

As leptin is a large peptide with widespread actions, use of smaller molecules that mirror leptin action may be a better therapeutic approach. Indeed, specific fragments of the leptin molecule display CNS activity (Grasso et al., 1997; Rozhavskaya-Arena et al., 2000), and we have shown that one leptin fragment ($leptin_{116–130}$) mirrors the pro-cognitive and neuroprotective properties of whole leptin (Malekizadeh et al., 2017). Here we extend these studies to show that specific hexamer peptides derived from $leptin_{116–130}$ mirror the neuroprotective and pro-cognitive actions of leptin and $leptin_{116–130}$. These findings have important implications for the use of leptin-based peptides to treat AD.

2 | METHODS

2.1 | Preparation of amyloid β

The lyophilised powder was solubilised in dimethyl sulphoxide (DMSO) and diluted to a working concentration. For cell viability and biochemical assays, Aβ (ab120301; Abcam) was prepared as before as Aβ displays toxicity when in a β sheet conformation (Simmons et al., 1994). Briefly, lyophilised powder was solubilised in DH_20 to a concentration of 1 MM in PBS and incubated for 24 h at 37°C prior to further dilution to working concentration

2.1.1 | Human neural cell line SH-SY5Y

The human neuroblastoma cell line, SH-SY5Y (#94030304, 2017; RRID:CVCL_0019; ECACC) was maintained as before (Malekizadeh et al., 2017). These cells are not listed as commonly misidentified cells by the International Cell Line Authentication Committee and were purchased new for these experiments and therefore no verification was required. Cells (passage 10–18) were plated at 10 000 cells per well in a 96-well plate and at 5×10^5 cells in 60 mm dishes for protein extraction plates (153066158015; VWR). To induce differentiation, cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with glucose (4500 μg/L), 1% (v/v) cosmic calf serum (C8056; Fisher Scientific) and 10 μM retinoic acid (R2625, Sigma) for 5 days. Then incubated in DMEM (21 885 108; Fisher Scientific) supplemented with glucose (4500 μg/L), serum

replacement 2 (2%; S9388, Sigma) and 18 μM 5-fluorodeoxyuridine (F0501, Sigma) to inhibit undifferentiated cell proliferation. 50% of the medium was changed every 2–3 days and drug treatments carried out 7 days after differentiation. Reagents used were 0.1–10 nM human leptin (L4146, Sigma), $leptin_{116-130}$ (L6788, Sigma), $leptin_{116-121}$; $leptin_{117-122}$; $leptin_{118-123}$; $leptin_{120-125}$; $leptin_{121-126}$; $leptin_{123-128}$; $leptin_{124-129}$; or $leptin_{125-130}$; (all Severn Biotechnology); and 10 μM $A\beta_{1-42}$. Leptin and leptin peptides were dissolved in culture medium, aCSF or HBS.

2.1.2 | Cell survival assays

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) assays were carried out as before (Oldreive et al., 2008; Oldreive & Doherty, 2010). Data were expressed as percentage mitochondrial activity or LDH release relative to untreated cells.

Briefly, to prepare LDH chemical mixture, Iodonitrotetrazolium chloride (I8377, Sigma), phenozine methosulphate (P9625, Sigma) and β-nicotinamide adenine dinucleotide hydrate (N8129, Sigma) were mixed together at the ratio of 167:43:431(w/w/w). 16 mg LDH chemical mixture, 60 μl lactic acid (L1250, Sigma) and 12 μl Triton X-100 (X100, Sigma) were added to 12 ml pH 8.2 LDH assay buffer (0.2 M Tris in dH_2O) to prepare fresh LDH reaction mix. In a 96-well clear plate, 50 μl LDH reaction mix was mixed with 50 μl cell culture medium and plates were read at a wavelength of 490 nm every 5 min in Biohit BP100 pLate reader.

For MTT assay, 1 mg/mL (3-(4,5-dimethylthiazol-2-yl)-2,5-diph enyltetrazolium bromide) (MTT; M2003, Sigma) assay was added to cell culture plates at the end of their treatment period. These cells were incubated for a further 1.5 hours after which all medium was removed, and cells were solubilised with 100 μl Dimethyl sulfoxide (DMSO; D8418, Sigma). Absorbance was read at 570 nM on a Biohit BP100 plate reader.

2.1.3 | Quantification of expression of p-tau by ELISA

Protein was extracted into 500 μl tris-buffered saline containing protease inhibitor cocktail (Set II, Merck) as before (Ren et al., 2008). Bradford assay was used to determine protein concentration. Samples were diluted to give equal loading, and expression levels were determined using rabbit anti-p-tau (ser-396, 1:2000; RRID:AB1575880, Genscript) antibodies, followed by detection using an appropriate HRP-conjugated secondary antibody (RRID:AB_390191, Sigma) and 3,3′,5,5′-Tetramethylbenzidine (TMB) Liquid Substrate System (T8665, Sigma). Each protein sample was run in duplicate (technical repeats), and absorbance was measured on a Biohit BP100 plate reader. Experiments were carried out on five separate occasions, representing five separate plate downs and thus biological repeats.

2.1.4 | Quantification of amyloid after seeding

SY-SY5Y cells were plated onto 13 mm borosilicate glass coverslips (CC7672-7548, CC7672-7458; VWR) and differentiated, prior to seeding with 1 μM $A\beta_{1-42}$ and treated with 10 nM leptin, or fragments for 96 h and fixed in neutral-buffered formalin (NBF; HT501128, Sigma) for 15 min before washing with phosphate-buffered saline (PBS; P4417, Sigma). 500 μl of Thioflavin-S solution (T1892, Sigma; 0.05% in PBS) was added for 10 min before three 5 min washes with PBS. Coverslips were mounted and sealed on glass slides. Slides were imaged using a Zeiss Axio Imager M2 microscope with Apotome.2 at 63× magnification and analysed using Fiji (Schindelin et al., 2012). Five separate experimental plate downs were established with duplicate coverslips from which 5–10 fields of view across the 2 coverslips were imaged, thus 15–35 fields of view were collected for each condition in total.

2.1.5 | Hippocampal cultures

Hippocampal cultures were prepared as before (Moult et al., 2010). Briefly, neonatal Sprague–Dawley rats (1–3 days old; both sexes used) were killed by cervical dislocation in accordance with Schedule 1 of UK Animals Scientific Procedures Act, 1986 (Home Office project licence: P9198AAB7). The brains of three to four neonates were pooled and used for the preparation of each culture. Hippocampi were removed and washed in (4-(2-hydroxyethyl)-1-piperazineeth anesulfonic acid) (HEPES)-buffered saline comprising (in mM) 135 NaCl (S9888, Sigma), 5 KCl (P3911, Sigma), 1 $CaCl_2$ (C1016, Sigma), 1 $MgCl_2$ (M8266; Sigma), 10 HEPES (H3375, Sigma), and 25 D-glucose (G8270, Sigma, pH 7.4). Cells were treated with papain (1.5 mg ml^{-1}; P4762, Sigma Aldrich) for 20 min at 37°C, and dissociated cells plated onto sterile dishes (35 mm diameter; Greiner Bio-One Ltd.) treated with 20 μg ml^{-1} poly-d-lysine (P7886, Sigma; 1–2 h). Cultures were maintained in serum replacement medium (SR2; S9388, Sigma) in a humidified atmosphere of 95% O_2 and 5% CO_2 at 37°C for up to 3 weeks.

2.1.6 | Surface labelling of AMPA receptors

Immunocytochemistry was performed on 8–12 DIV-cultured hippocampal neurons. Neurons were washed with HEPES buffered saline containing glycine (0.01 mM) and treated with leptin hexamers at 21–23°C. To label surface GluA1, neurons were incubated with an antibody against the N-terminal region of GluA1 (sheep anti-GluA1; 1:100; Moult et al., 2010) at 4°C, then fixed with 4% paraformaldehyde (J19943.K2, Thermofisher) for 5 min. Surface GluA1 staining was visualised by the addition of an anti-sheep Alexa 488-conjugated secondary antibody (1:250; A-11015, Life Technologies) for 30 min. No labelling was observed after incubation with secondary antibodies alone.

A Zeiss LSM 510 confocal microscope was used for image acquisition and 488-nm laser line was used to excite the Alexa 488 fluorophore. Images were obtained in single-tracking using a 15-s scan speed and the intensity of staining was determined offline using LaserSharp software (Carl Zeiss). Analysis lines (50 μm) were drawn along randomly selected dendritic regions and mean fluorescence intensity of staining was calculated for each dendrite (McGregor et al., 2017). Data were obtained from at least four randomly selected cells for each condition, and all data were obtained from at least three different cultures from different animals. Within a given experiment, all conditions, including illumination intensity and photomultiplier gains were kept constant. To quantify data obtained from separate days, data were normalised relative to mean fluorescence intensity in control neurons.

2.1.7 | Hippocampal slice preparation and electrophysiology

Hippocampal slices (350 μm) were prepared from juvenile (P14-21) male Sprague–Dawley rats, as before (Luo et al., 2015; McGregor et al., 2017). Animals were killed using schedule 1 approaches under UK (Scientific Procedures Act) 1986 legislation 1986 (Home Office project licence: P9198AAB7). Brains were removed and placed in ice-cold artificial cerebrospinal fluid (aCSF) containing (in mM): 124 NaCl (S9888, Sigma), 3 KCl (P3911, Sigma), 26 $NaHCO_3$ (S6014, Sigma), 1.25 NaH_2PO_4 (S8282, Sigma), 2 $CaCl_2$ (C1016, Sigma), 1 $MgSO_4$ (M7506, Sigma) and 10 D-glucose (G8270, Sigma) and bubbled with 95% O_2 and 5% CO_2. Once prepared, parasagittal hippocampal slices recovered at room temperature (19–22°C) in oxygenated aCSF for 1 h before use. Slices were transferred to a submerged chamber maintained at room temperature (19–22°C) and perfused at 2 ml min^{-1}. Standard extracellular recordings were used to monitor evoked field excitatory postsynaptic potentials (fEPSP) from stratum radiatum. The Schaffer collateral-commissural pathway was stimulated (constant voltage; 0.1 ms) at 0.033 Hz, using a stimulus intensity that evoked a peak amplitude ~50% of maximum. Synaptic potentials were low pass filtered at 2 kHz and digitally sampled at 10 kHz. The fEPSP slope was measured and expressed relative to the baseline.

LTP was induced using a high-frequency stimulation paradigm (100 Hz, 1 s). Recordings were made using an Axopatch 200B amplifier and analysed using LTP v2.4 software (Anderson & Collingridge, 2007). For studies comparing the actions of leptin hexamers on synaptic transmission, the mean slope (average of 5 min recording) of fEPSPs obtained during the 5 min period immediately prior to hexamer addition was compared with that after 25–30 min exposure. In LTP studies, the degree of potentiation was calculated 30–35 min after HFS and expressed as a percentage of baseline ± standard error of mean (SEM). For all experiments, each *n* value represents an individual slice taken from a separate animal.

3 | BEHAVIOURAL EXPERIMENTS

3.1 | Subjects and design

41 C57/BL6 mice (6–8 months; 25 male and 16 female) were used. Sample sizes were determined using G-power and data from Malekizadeh et al. (2017) which examined the effects of leptin on similar types of memory. Malekizadeh et al. (2017) used group sizes of 14 per group which produced a η^2 of 0.181 for the critical group comparison in the ANOVA. G-power confirmed that this produced a large effect size (f = 0.47) and so the same group sizes were used in the current study. Mice were housed in groups of 3–5 and kept on a 12 h light/dark cycle (behavioural testing carried out during the light phase) with ad libitum access to food and water. All procedures were approved by the Animal Welfare Ethics Committee (University of St Andrews) and complied with national (Animal [Scientific Procedures] Act, 1986) and international (European Directive 2010/63/EU) legislation (Home Office project licence P53E784C4). Mice were randomly assigned to three treatment groups: saline (n = 14, 6 female), leptin (n = 13, 4 female) and $leptin_{117–122}$ (n = 14, 6 female). A random number generator was used to generate a random sequence of the numbers from 1 to 41. The first 14 numbered animals were assigned to the saline group, the next 13 to the leptin group and the final 14 to the hexamer group. Experiments were run blind to treatment group. 100 μl of either saline, leptin or $leptin_{117–122}$ (7.8 nM/ml; Malekizadeh et al., 2017) were administered daily by IP injection during the 20-day testing period. This consisted of 4 days Object-Place-Context (OPC) tests, 4 days rest, 1 day T-maze, 3 days elevated plus maze, 4 days rest and 4 days OPC testing (Figure 5a). No inclusion/exclusion criteria were used in these studies. Weight was monitored throughout experiments and did not differ significantly between groups.

3.2 | Episodic-like memory (Object-Place-Context; OPC)

Mice were habituated to the testing box (30 × 20 × 20 cm) for 3 days (1 group habituation, 2 days individual habituation). The box could be configured with 2 different sets of contextual features using floor and wall inserts (blue with a plain floor v green/white stripes with a mesh floor) and mice were habituated to both contexts (10 min sessions) on each day. Objects were household items approximately the size of the mice in at least 1 dimension. Mice then underwent object recognition, object–place and object–context training (4 days each) as before (Wilson et al., 2013). Mice received the IP injections 30 min prior to testing. OPC testing was carried out as before (Vandrey et al., 2020) and consisted of 3 stages; 2 sample phases and a test phase (Figure 5b). Each phase was 3 min duration and exploration behaviour was scored using in-house software (Observe, University of St Andrews). Between all phases, the box was cleaned. Testing was recorded to allow offline scoring of behaviour. The discrimination ratio (DR) was calculated by subtracting the amount of time exploring the familiar object from the time exploring the novel

object and dividing by the total exploration time. Total exploration in sample and test phases were compared between groups. Object location, object used at test, order of contexts and context at test were all counterbalanced.

3.3 | Spontaneous alternation T-maze

Mice were tested individually for 15 min on a T-maze with arms 16 cm in length. Each mouse was placed on the central arm and exploration was recorded and scored using custom-written software (Observe, University of St Andrews). In the spontaneous alternation task, a correct response was defined as animals choosing the arm that they have least recently visited (e.g. ABC or BAC), non-alternation is when the animal returns to an arm it has visited previously before completing a full sequence (e.g. ACA, BAB, AAB etc.). Performance is quantified as the proportion of alternation, which is the number of successful alternation sequences divided by the total number of attempts (successful + unsuccessful).

3.4 | Elevated plus maze

Mice were tested on a standard elevated plus maze consisting of a 4-arm (35 cm in length) maze arranged in a '+' cross. Two opposing arms had high walls (20 cm) while the other two had no walls. Mice were tested individually with each testing session lasting 5 min (Haleem et al., 2015). Testing started by placing a mouse in the centre of the maze facing an open arm. Exploration was recorded and scored using in-house software (Observe, University of St Andrews). Two measures of exploration were taken; number of entries into open and closed arms and time spent in open vs. closed arms.

3.5 | Statistical analyses

In immunocytochemical, ELISA and cell viability studies, all statistical analysis was carried out using GraphPad PRISM 9 (Graph Pad Inc.). In all experiments, data are expressed as mean ± SEM. After normal distribution test, statistical analyses were performed using one-way analysis of variance (ANOVA) with Tukey's post hoc test for comparisons between multiple groups. $p < 0.05$ was considered significant. In electrophysiological studies, all statistical analyses were performed using Sigmaplot 14.5 (Systat Software) and all analyses were performed using repeated measures ANOVA for comparison between multiple groups. All behavioural data were analysed using SPSS 26.0 (IBM). Following Shapiro–Wilk testing for normality, OPC data (DR, total exploration) was examined with mixed factorial ANOVA with treatment group as between-subject factor and testing block (1st 4 days, last 4 days) as within-subject factor. Tukey's tests were used for post hoc comparisons. One-sample t-tests were used to compare performance to 0. T-maze and Plus maze data were analysed using one-way ANOVA. Finally, weight was analysed using mixed factorial ANOVA with group and day as between- and within-subject factors respectively. No analysis for outliers was used and all statistical tests were two-tailed and used $p < 0.05$ significance threshold.

4 | RESULTS

4.1 | Leptin-derived hexamers have differential effects on hippocampal synaptic plasticity

Leptin enhances NMDA receptor function and facilitates hippocampal synaptic plasticity (Malekizadeh et al., 2017; Oomura et al., 2006; Shanley et al., 2001; Wayner et al., 2004) and these effects are mirrored by the leptin fragment, leptin$_{116-130}$ (Malekizadeh et al., 2017). To assess if the leptin-based hexamers also mirror these actions, the effects of 6 leptin hexamers (Figure 1a) were compared on LTP induced in juvenile hippocampal slices using a high-frequency stimulation (HFS) paradigm (100 Hz; 1 s). In control slices, HFS-induced LTP such that synaptic transmission was increased to 121 ± 8.5% of baseline at 40 min ($n = 5$; $F_{[1,4]} = 14.82$; $p = 0.031$; Figure 1b). Treatment with 10 nM leptin$_{116-121}$ for 50 min prior to HFS facilitated LTP as the magnitude of LTP was significantly enhanced to 162 ± 6.3% of baseline in leptin$_{116-121}$-treated slices ($n = 5$; $F_{[1,4]} = 17.63$; $p = 0.0079$; Figure 1b,h). Similarly, in slices exposed to leptin$_{117-122}$ (10 nM; 50 min), the magnitude of LTP was enhanced (to 191 ± 19.7% of baseline; $n = 6$; $F_{[1,5]} = 9.10$; $p = 0.0005$) compared to control LTP (125 ± 4.9%; $n = 5$; $F_{[1,4]} = 12.06$; $p = 0.024$; Figure 1c,h). Likewise, an increase in the magnitude of LTP was observed in slices treated with either 10 nM leptin$_{118-123}$ (158 ± 4.8%; $n = 7$; $F_{[1,6]} = 7.452$; $p = 0.0008$; Figure 1d,h) or leptin$_{120-125}$ (163 ± 8.8%; $n = 5$; $F_{[1,4]} = 10.92$; $p = 0.0092$; Figure 1e,h) relative to control LTP induced in interleaved slices (121 ± 7.7% of baseline; $n = 6$; $F_{[1,5]} = 10.08$; $p = 0.035$). In contrast, no facilitation of LTP was observed in slices exposed to either 10 nM leptin$_{124-129}$ or leptin$_{125-130}$, for 50 min as no significant difference in LTP magnitude was observed in leptin$_{124-129}$ (137 ± 5.6% of baseline; $n = 5$; $F_{[1,4]} = 0.156$; $p = 0.078$; Figure 1f,h) or leptin$_{125-130}$ (137 ± 2.4% of baseline; $n = 5$; $F_{[1,4]} = 1.627$; $p = 0.083$; Figure 1g,h)-treated slices compared to control slices (139 ± 5.8% of baseline; $n = 5$; $F_{[1,4]} = 9.284$; $p = 0.024$).

4.2 | Differential regulation of AMPA receptor trafficking by the leptin hexamers

Our previous studies indicate that leptin also regulates glutamate receptor trafficking as leptin stimulates insertion of the AMPA receptor subunit, GluA1 into synapses (Moult et al., 2010). Thus, to further verify hexamer bioactivity, the effects of the hexamers on AMPA receptor trafficking were examined by monitoring GluA1 surface expression in hippocampal neurons (Doherty et al., 2013; Moult et al., 2010). In agreement with previous studies

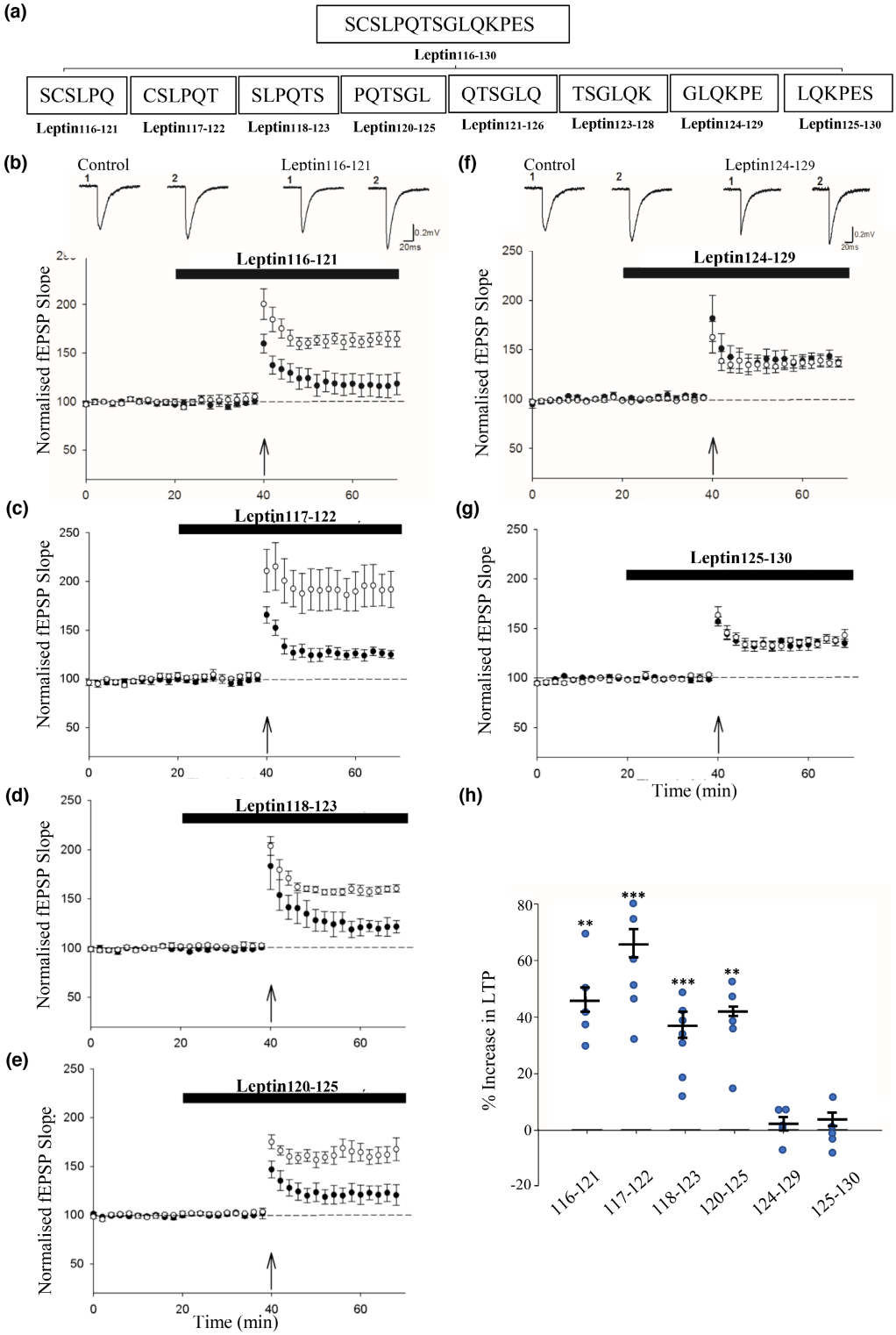

FIGURE 1 Leptin hexamers facilitate hippocampal synaptic plasticity and promote AMPA receptor trafficking. (a) Scheme illustrating the different leptin hexamers generated from leptin$_{116–130}$. (b) Pooled data showing the effects of leptin$_{116–121}$ (10 nM; $n = 5$; b), leptin$_{117–122}$ (10 nM; $n = 6$; c), leptin$_{118–123}$ (10 nM; $n = 7$; d), leptin$_{120–125}$ (10 nM; $n = 5$; e), leptin$_{124–129}$ (10 nM; $n = 5$; f) and leptin$_{125–130}$ (10 nM; $n = 5$; g) on long-term potentiation (LTP; filled circle) evoked by high-frequency stimulation (HFS; 100 Hz, 1 s) compared to control LTP (open circle) at Schaffer-collateral (SC)-CA1 synapses in juvenile hippocampal slices. Each point is the average of four successive responses, and representative field excitatory postsynaptic potentials (fEPSPs) are shown above each plot and for the time indicted. Scale bars for fEPSP traces represent 20 ms and 0.2 mV. H. Pooled data illustrating the effects of all the hexamers on hippocampal LTP. The addition of leptin$_{116–121}$, leptin$_{117–122}$, leptin$_{118–123}$ or leptin$_{120–125}$ enhanced LTP, whereas leptin$_{124–129}$ and leptin$_{125–130}$ had no significant effect on the magnitude of LTP.

(Malekizadeh et al., 2017), treatment of hippocampal neurons with leptin$_{116-130}$ (10 nM;15 min) significantly increased GluA1 surface expression (to 144 ± 10% of control; n = 36 dendrites; n = 3 cultures; $F_{[1,71]}$ = 11.59; p < 0.001, not shown). Similarly, exposure to either leptin$_{116-121}$ or leptin$_{117-122}$ (both 10 nM; 15 min) increased GluA1 surface expression to 146 ± 9% of control (n = 36 dendrites; n = 3 cultures; $F_{[1,71]}$ = 14.28; p < 0.001) and 167 ± 10% of control (n = 48 dendrites; n = 4 cultures; $F_{[1,95]}$ = 33.48; p < 0.001), respectively (Figure 2a,b). Elevations in GluA1 surface expression were also detected in leptin$_{118-123}$ (141 ± 8% of control; n = 48 dendrites; n = 4 cultures; $F_{[1,95]}$ = 49.55; p < 0.001) and leptin$_{120-125}$ (151 ± 5% of control; n = 36 dendrites; n = 3 cultures; $F_{[1,71]}$ = 51.91; p < 0.001) treated neurons (Figure 2b). In contrast, exposure to leptin$_{121-126}$ (103 ± 3% of control; n = 36 dendrites; n = 3 cultures; $F_{[1,71]}$ = 0.293; p = 0.59), leptin$_{123-128}$ (101 ± 4% of control; n = 48 dendrites; n = 4 cultures; $F_{[1,95]}$ = 0.337; p = 0.855), leptin$_{124-129}$ (89 ± 6% of control; n = 48 dendrites; n = 4 cultures; $F_{[1,95]}$ = 1.567; p = 0.214) or leptin$_{125-130}$ (98 ± 5% of control; n = 36 dendrites; n = 3 cultures; $F_{[1,71]}$ = 0.197; p = 0.889) had no effect on GluA1 surface labelling (Figure 2a,b). These data indicate that four hexamers, namely leptin$_{116-121}$, leptin$_{117-122}$, leptin$_{118-123}$ and leptin$_{120-125}$ mirror the ability of leptin to enhance GluA1 surface expression.

4.3 | Leptin$_{117-122}$ mirrors leptin and leptin$_{116-130}$ improvement of episodic-like memory

As our data show that leptin$_{117-122}$ enhances hippocampal synaptic plasticity, we next investigated whether leptin$_{117-122}$ influences hippocampal-dependent memory. Previous studies indicate that leptin improves spatial memory (Oomura et al., 2006; Haleem et al., 2015) while both leptin and leptin$_{116-130}$ improve episodic-like memory in mice (Malekizadeh et al., 2017). Here we compared performance on the episodic-like memory OPC task of mice given acute (1–4 days) or chronic (17–20 days) administration of leptin$_{117-122}$, leptin or saline. Both leptin$_{117-122}$ and leptin significantly improved DR performance on the OPC task relative to saline treatment (Figure 3b) in both the acute (leptin: 0.31 ± 0.03, leptin$_{117-122}$: 0.35 ± 0.02, saline: 0.1 ± 0.01) and chronic (leptin: 0.40 ± 0.02, leptin$_{117-122}$: 0.39 ± 0.03, saline: 0.09 ± 0.02) treatment conditions. This was confirmed by a significant effect of group ($F_{[2,38]}$ = 60.482, p < 0.001, η_p^2 = 0.761), and post hoc comparisons showing leptin$_{117-122}$ and leptin-treated animals performing significantly better in the OPC test than controls (p < 0.05) and not differing significantly from each other. There was no main effect of condition (acute v chronic; p > 0.05) although a significant interaction between group and condition was found ($F_{[2,38]}$ = 3.951, p = 0.022, η_p^2 = 0.172). This was driven by a smaller difference between the acute and chronic conditions in the leptin $_{117-122}$ group relative to the leptin group. However, comparison of the leptin vs. leptin $_{117-122}$ group across acute and chronic conditions revealed no significant differences. Total exploration time in the test and sample trials did not differ across groups with either

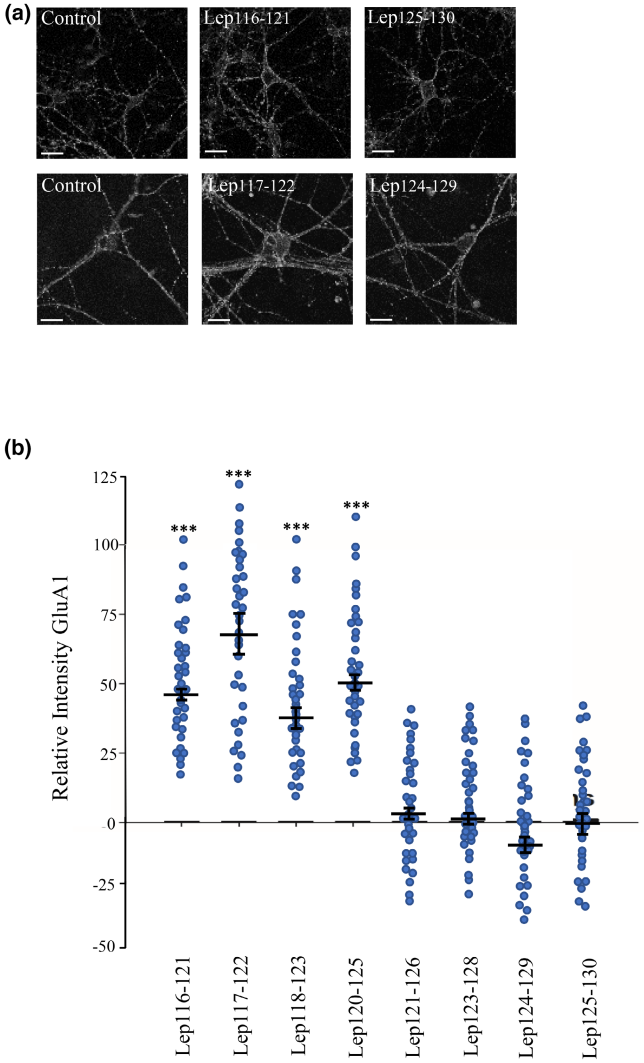

FIGURE 2 Leptin$_{116-121}$ and leptin $_{117-122}$ increase GluA1 surface expression. (a) Representative confocal images of GluA1 surface labelling in control (Hepes buffered saline (HBS)-treated) neurons (DIV7-12) and after the addition of leptin$_{116-121}$, leptin$_{117-122}$, leptin$_{124-129}$ (n = 36 dendrites; n = 3 cultures) or leptin$_{125-130}$ (n = 36 dendrites; n = 3 cultures). In this and subsequent images, the scale bars represent 10 μm. (b) Pooled data illustrating the relative effects of the hexamers compared to control on GluA1 surface expression in hippocampal neurons. The addition of 10 nM leptin$_{116-121}$ (n = 36 dendrites; n = 3 cultures), leptin$_{117-122}$ (n = 48 dendrites; n = 4 cultures), leptin$_{118-123}$ (n = 48 dendrites; n = 4 cultures), or leptin$_{120-125}$ (n = 36 dendrites; n = 3 cultures) significantly increased GluA1 surface expression, whereas leptin$_{121-126}$ (n = 36 dendrites; n = 3 cultures), leptin$_{123-128}$ (n = 48 dendrites; n = 4 cultures); leptin$_{124-129}$ (n = 48 dendrites; n = 4 cultures) and leptin$_{125-130}$ (n = 36 dendrites; n = 3 cultures) had no significant effect.

acute or chronic administration, showing that effects are not driven by changes in motivation to explore (Figure S1). This replicates the episodic memory-enhancing effects of leptin previously demonstrated and goes on to show that leptin$_{117-122}$, is equally effective at improving episodic-like memory. These findings also show that the

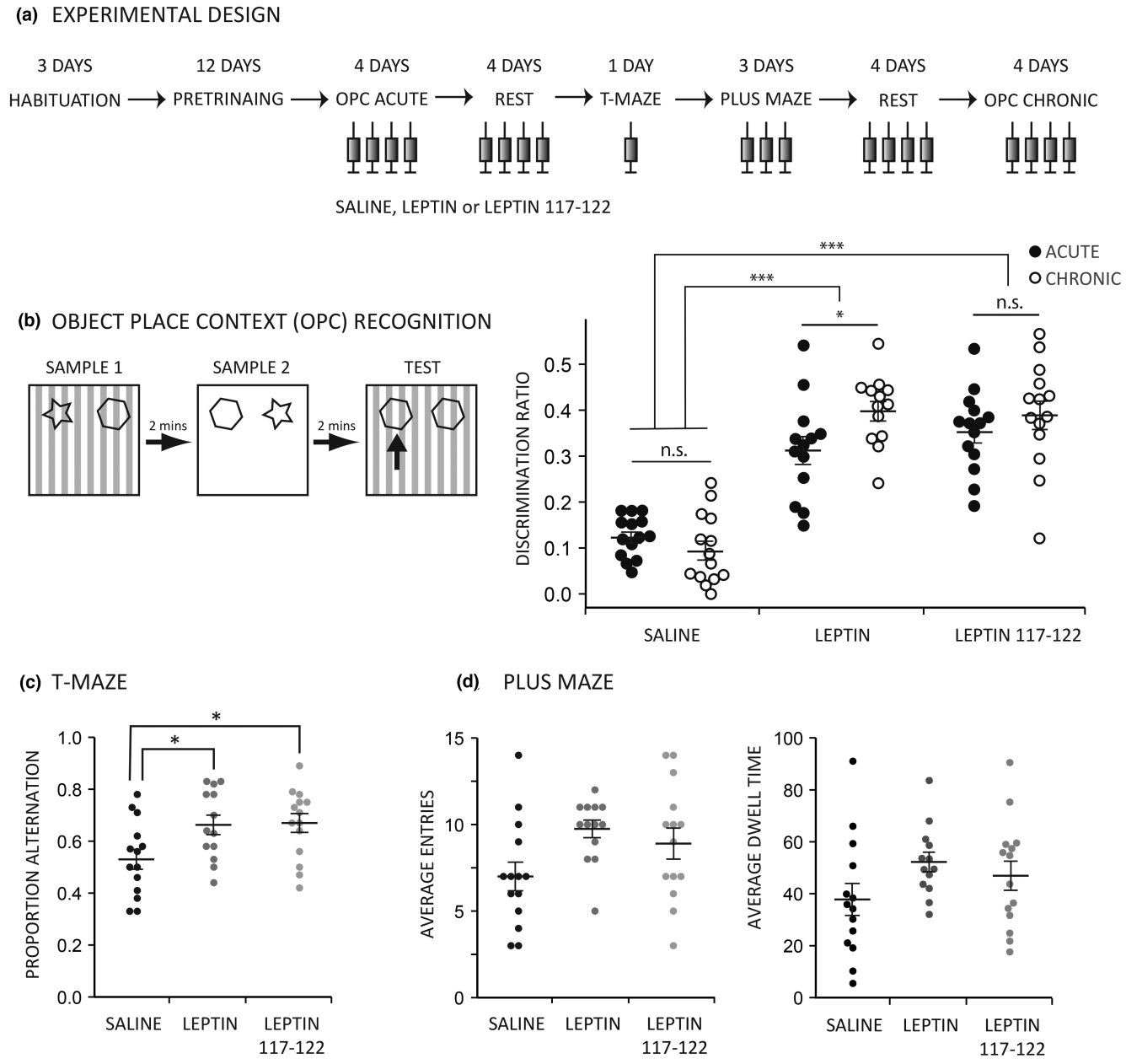

FIGURE 3 Leptin$_{117-122}$ mirrors the improvement of episodic-like memory induced by leptin. Schematic of the experimental design (a, b) for (object, place, context) OPC tests. Mice treated with leptin ($n = 13$) or leptin$_{117-122}$ ($n = 14$) showed enhanced performance in the OPC test (b) as well as in the T-maze (c) relative to mice treated with saline ($n = 14$). However, all groups of mice performed similarly in the elevated plus maze (d). Statistical significance is denoted as $p < 0.05$ (*) and $p < 0.001$ (***).

improvement in memory induced by leptin and leptin$_{117-122}$ is maintained over an extended period of administration.

We next determined whether leptin$_{117-122}$, mirrored leptin's known improvement in spatial memory (Oomura et al., 2006; Haleem et al., 2015), by examining spontaneous alternation in a T-maze task. The proportion alternation which is a measure of spatial memory on the T-maze, was higher in both leptin and leptin$_{117-122}$-treated mice relative to saline treated (Figure 5c; $F_{[2,38]} = 4.243$, $p = 0.02$, $\eta_p^2 = 0.183$) with post hoc tests confirming that leptin and leptin$_{117-122}$ treated mice performed better than controls ($p = 0.046$) but did not differ from each other ($p = 0.038$). Thus leptin$_{117-122}$ improves performance in a spatial memory task and is indistinguishable from leptin in doing so.

As differences in anxiety could underlie differences in novelty exploration, we investigated whether leptin or leptin$_{117-122}$ affects anxiety-like behaviour using an elevated plus maze task. Anxiety was measured by examining the number of entries and dwell time in the open arms. All treatment groups performed similarly (Figure 3d), confirmed by no significant differences in the number of open arm entries ($p > 0.05$) or the time spent on the open arm ($p > 0.05$). These data indicate that neither leptin nor leptin$_{117-122}$, had significant effects on anxiety.

4.4 | Leptin$_{116-121}$ and leptin$_{117-122}$ prevent Aβ-driven internalisation of GluA1

AMPA receptor trafficking is also detrimentally influenced in AD as Aβ causes GluA1 endocytosis (Hsieh et al., 2006; Liu et al., 2010); an effect alleviated by treatment with either leptin (Doherty et al., 2013) or leptin$_{116-130}$ (Malekizadeh et al., 2017). Thus, the neuro-protective actions of two of the bioactive hexamers, leptin$_{116-121}$ and leptin$_{117-122}$ were examined further, by probing the cell surface density of GluA1 in hippocampal neurons (Moult et al., 2010). In control neurons, treatment with Aβ_{1-42} (500 nM; 20 min) significantly reduced (to 57.87 ± 2.65%) GluA1 surface expression relative to inactive Aβ_{42-1} (n = 36 dendrites; n = 3 cultures; $F_{[1,71]}$ = 48.47; p < 0.001; Figure 4a,b). Application of 1 nM leptin$_{116-121}$ had no significant effect on GluA1 surface expression per se (101.4 ± 3.80% of control; n = 36 dendrites; n = 3 cultures; $F_{[1,71]}$ = 1.87; p = 0.98), but in leptin$_{116-121}$-treated neurons, Aβ_{1-42} failed to alter GluA1 surface expression (97.69 ± 2.93% of control; n = 36 dendrites; n = 3 cultures; $F_{[1,71]}$ = 0.145; p = 0.95; Figure 4C,D). Similarly, treatment with 1 nM leptin$_{117-122}$ had no significant effect on GluA1 trafficking (95.31 ± 4.64% of control; n = 36 dendrites; n = 3 cultures; $F_{[1,71]}$ = 0.831; p = 0.43), but it prevented Aβ_{1-42}-driven internalisation of GluA1 (99.10 ± 5.45% of control; n = 36 dendrites; n = 3 cultures; $F_{[1,71]}$ = 6.71; p = 0.43; Figure 4e,f). These data indicate that leptin$_{116-121}$ and leptin$_{117-122}$ replicate the neuroprotective actions of leptin, by preventing the aberrant effects of Aβ on AMPA receptor trafficking.

4.5 | Leptin$_{116-121}$ and leptin$_{117-122}$ mirror the neuroprotective actions of leptin

Multiple strands of evidence support a role for leptin in protecting against neurotoxic stimuli (Doherty et al., 2008; Doherty et al., 2013; Guo et al., 2008), but only part of the C-D loop region, leptin$_{116-130}$, is required to replicate this effect of the full-length molecule (Malekizadeh et al., 2017). Initially, we treated cultures with the peptides to ensure that they did not have any significant effects on cell viability in the absence of a stressor and no significant effects were observed (Figure S2). Thereafter, we determined if, like leptin$_{116-130}$, the leptin hexamers protect against neurotoxicity, by exposing SH-SY5Y neuroblastoma cells to Aβ_{1-42} (10 μM; 96 h) to induce cell death. The hexamers (10 nM) were then co-administered with Aβ_{1-42} and their effects compared to leptin and leptin$_{116-130}$. A one-way ANOVA revealed a significant effect of treatment ($F_{(7,32)}$ = 10.23, p < 0.0001). Post hoc comparisons (Tukey's) revealed that after treatment with Aβ_{1-42}, mitochondrial activity was reduced to 59.16 ± 5.80% of control (n = 5; p < 0.0001; Figure 5a) in MTT assays. In line with previous studies (Doherty et al., 2013; Malekizadeh et al., 2017), treatment with either 10 nM leptin or leptin$_{116-130}$ significantly protected against the Aβ_{1-42}-driven decrease in mitochondrial activity with 91.8 ± 5.2% and 100.1 ± 6.7% of control, respectively (n = 5; p = 0.0017 and p < 0.0001 relative to Aβ_{1-42} alone respectively; Figure 5a). Similarly, treatment with either leptin$_{116-121}$ or leptin$_{117-122}$ significantly protected against Aβ_{1-42}-triggered decrease in mitochondrial activity with 102.8 ± 4.0% and 99.7 ± 3.7% of control, respectively (n = 5; both p < 0.0001 relative to Aβ_{1-42}; Figure 5a). However, leptin$_{124-129}$-induced lesser protection with 86.5 ± 6.4% of control activity (n = 5; p = 0.0125 relative to Aβ_{1-42} alone; Figure 3a) and leptin$_{125-130}$ did not protect against 10 μM Aβ_{1-42} resulting in 69.08 ± 5.5% of control cell number (n = 5; p = 0.8593 relative to Aβ_{1-42}; Figure 5a). To verify these data reflect changes in cell viability, an LDH assay was used to measure lactate dehydrogenase release due to membrane rupture and cell death. A one-way ANOVA revealed a significant effect of treatment ($F_{(7,80)}$ = 6.537, p < 0.0001). Post hoc comparisons (Tukey's) revealed that in line with previous studies, both leptin and leptin$_{116-130}$ significantly prevented LDH release induced by 10 μM Aβ_{1-42} (124.8 ± 4.9% of untreated control; n = 11; p = 0.0005 comparing 10 μM Aβ_{1-42} to untreated control; Figure 5b) with 99.5 ± 2.5% and 100.8 ± 2.2% of untreated control, respectively after treatment (n = 11; p = 0.0004 and p = 0.0009 relative to Aβ_{1-42} alone respectively; Figure 5b). Similarly, leptin$_{116-121}$ and leptin$_{117-122}$ significantly protected against Aβ_{1-42}-triggered LDH release with 97.7 ± 3.1% and 97.4 ± 3.5% of control, respectively (n = 11; p = 0.0001 and p < 0.0001 relative to Aβ_{1-42} alone respectively; Figure 5b). In contrast, neither leptin$_{124-129}$ nor leptin$_{125-130}$ protected against Aβ_{1-42} mediated LDH release resulting in 113.3 ± 5.8% and 111.8 ± 5.5% of control, respectively (n = 11; p = 0.4205 and p = 0.2698 relative to Aβ_{1-42} alone respectively; Figure 5b). Together these data reveal that leptin$_{116-121}$, and leptin$_{117-122}$, but not leptin$_{124-129}$ or leptin$_{125-130}$ mirror the neuroprotective effects of leptin in preventing Aβ_{1-42}-induced cell death.

4.6 | Leptin$_{116-121}$ and leptin$_{117-122}$ show similar efficacy to leptin or leptin$_{116-130}$ in preventing Aβ_{1-42}-induced toxicity

To further examine the pharmacological profile of the hexamers, concentration-response relationships were generated for leptin, leptin$_{116-130}$, leptin$_{116-121}$ and leptin$_{117-122}$ and compared to leptin$_{124-129}$ and leptin$_{125-130}$. Cells were exposed to 10 μM Aβ_{1-42} (96 h) to induce cell death, together with various concentrations of leptin, and the fragments (range 0.0001–100 nM) and mitochondrial activity were measured via MTT assay. Comparable dose–response curves were observed for all four bioactive leptin hexamers (Figure 5c), whereas leptin$_{124-129}$ and leptin$_{125-130}$ were without effect. In parallel studies, analogous concentration–response profiles were obtained for leptin and the hexamers using an LDH assay (Figure 5d). These data indicate that leptin$_{116-121}$, and leptin$_{117-122}$ not only mirror the neuroprotective actions of leptin, but they also have similar efficacy to full-length leptin and leptin$_{116-130}$. In all cases, n = 14 separate plate downs with each assay run in triplicate for each condition in each plate down as a biological repeat.

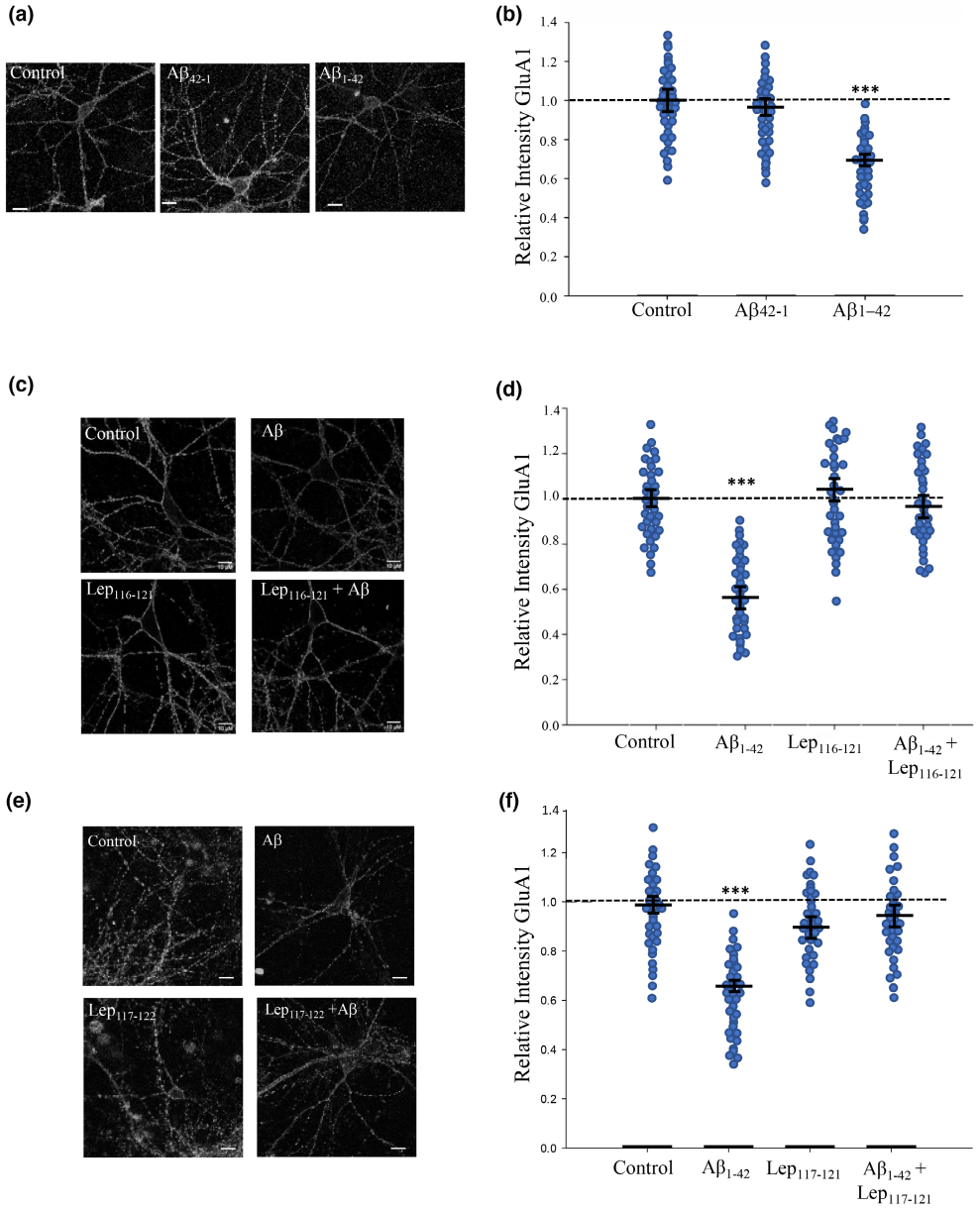

FIGURE 4 Leptin$_{116–121}$ and leptin$_{117–122}$ prevent Aβ-driven internalisation of GluA1. (a) Representative confocal images of GluA1 surface labelling in control hippocampal neurons (DIV7-12) and after the addition of 1 μM Aβ_{1-42} (20 min), which promotes GluA1 internalisation. (b) Pooled data showing the relative effects of Aβ_{1-42} on GluA1 surface expression in hippocampal neurons. (c) Representative confocal images of GluA1 labelling in hippocampal neurons in control conditions and after the addition of Aβ_{1-42}, leptin$_{116–121}$ and in the combined presence of Aβ_{1-42} and leptin$_{116–121}$. (d) Histogram of pooled data illustrating the relative effects of Aβ_{1-42}, leptin$_{116–121}$ and Aβ_{1-42} plus leptin$_{116–121}$ on surface GluA1 expression ($n = 36$ dendrites; $n = 3$ cultures). (e) Representative confocal images of GluA1 labelling in hippocampal neurons in control conditions and after the addition of Aβ_{1-42}, leptin$_{117–122}$ and in the combined presence of Aβ_{1-42} and leptin$_{117–122}$. (f) Histogram of pooled data illustrating the relative effects of A$\beta_{1-42,}$ leptin$_{117–122}$ and Aβ_{1-42} plus leptin$_{117–122}$ on surface GluA1 expression ($n = 36$ dendrites; $n = 3$ cultures).

4.7 | Leptin$_{116–121}$ and leptin$_{117–122}$ mirror leptin and leptin$_{116–130}$ prevention of amyloid seeding

Emerging evidence reveals that Aβ_{1-42} administration leads to the propagation of Aβ in cellular and animal models (Nath et al., 2012; Guo and Lee, 2014). Here, we treated cells with a sub-lethal concentration of Aβ_{1-42} (1 μM; 96 h) either alone or in combination with 10 nM leptin, leptin$_{116–130}$, leptin$_{116–121}$ or leptin$_{117–122}$ and labelled with thioflavin S to detect amyloid accumulation (Urbanc et al., 2002). A one-way ANOVA revealed a significant effect of treatment ($F_{(9,191)} = 21.17$, $p < 0.0001$). Post hoc comparisons (Tukey's) revealed that treatment with Aβ_{1-42} significantly increased thioflavin S fluorescence compared to control (225.1 ± 9.256% increase: Figure 6a,b; $p < 0.0001$). For all experiments, n = 15–35 fields of view per condition from five separate plate downs. In the absence of Aβ_{1-42}, thioflavin S-positive fluorescence was comparable across all

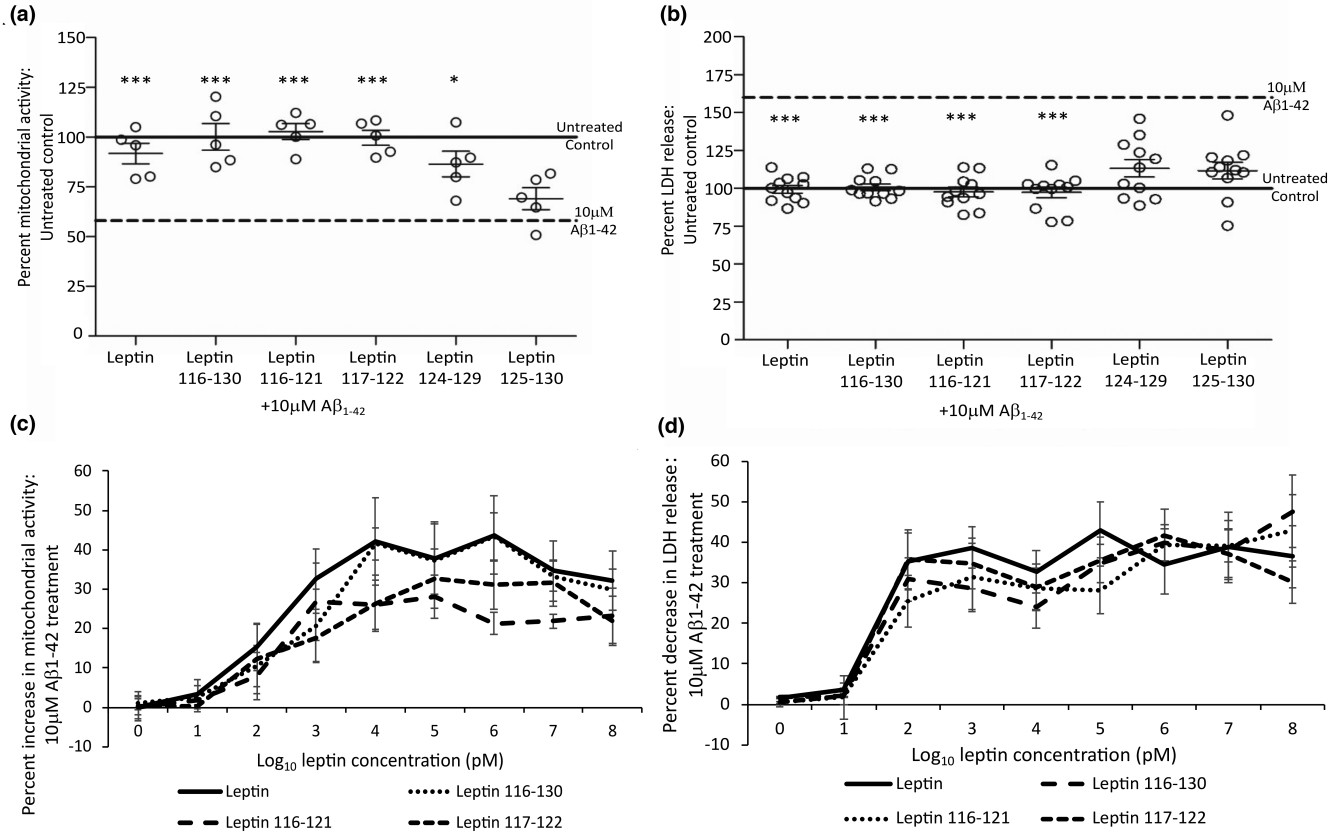

FIGURE 5 Leptin$_{116-121}$ and leptin $_{117-122}$ prevent Aβ-mediated cell death. (a) Pooled data from (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction (MTT) assays ($n = 5$) of SH-SY5Y cells demonstrating the protective effects of leptin, leptin$_{116-130}$, leptin$_{116-121}$, leptin$_{117-122}$ and leptin$_{124-129}$ against amyloid beta$_{1-42}$ (Aβ_{1-42}) on mitochondrial activity and of lactate dehydrogenase (LDH) assays ($n = 11$) (b) similarly exhibiting the protective effects of leptin, leptin$_{116-130}$, leptin$_{116-121}$ and leptin$_{117-122}$. Concentration effects of leptin, leptin$_{116-130}$, leptin$_{116-121}$, and leptin$_{117-122}$ on mitochondrial activity (c) and LDH release (d). In all cases, $n = 14$ separate plate downs with each assay run in triplicate for each condition in each plate down as a biological repeat. In (a) and (b) the control condition cited is an untreated control and in (c) and (d) data are compared to an Aβ_{1-42} only condition. Pooled data are shown and statistical significance denoted: $p < 0.05$ (*) and $p < 0.001$ (***).

treatment groups (Figure 6b; relative to untreated control $p = 0.9996$ for leptin; $p = 0.9742$ for leptin$_{116-130}$; $p = 0.9952$ for leptin$_{116-121}$; $p = 0.3100$ for leptin$_{117-122}$). However, treatment with 1 μM Aβ_{1-42} failed to significantly increase thioflavin S-positive fluorescence in cells co-treated with either 10 nM leptin, leptin$_{116-130}$, leptin$_{116-121}$ or leptin$_{117-122}$ (Figure 6a,b; relative to untreated control $p = 0.6707$ for leptin; $p > 0.9999$ for leptin$_{116-130}$; $p = 0.9985$ for leptin$_{116-121}$; $p > 0.9999$ for leptin$_{117-122}$), such that thioflavin S-positive fluorescence was not significantly different to control. These data suggest that treatment with a sub-lethal dose of Aβ_{1-42} upregulates thioflavin S-positive labelling which is indicative of Aβ accumulation, and this effect is inhibited by leptin, leptin$_{116-130}$, leptin$_{116-121}$ or leptin$_{117-122}$.

4.8 | Leptin$_{116-121}$ and leptin$_{117-122}$ reduce Aβ_{1-42}-induced upregulation of p-tau

A key feature of AD pathogenesis is the accumulation of p-tau and we have shown that leptin and leptin$_{116-130}$ inhibit Aβ_{1-42}-driven upregulation of p-tau in vitro (Malekizadeh et al., 2017). To assess if the leptin hexamers influence AD-related increase in p-tau, SH-SY5Y cells were exposed to 10 μM Aβ_{1-42} (96 h) alone, or in combination with 10 nM leptin, leptin$_{116-130}$, leptin$_{116-121}$, leptin$_{117-122}$, leptin$_{124-129}$ or leptin$_{125-130}$ and p-tau levels were detected using ELISA. A one-way ANOVA revealed a significant effect of treatment ($F_{(7,32)} = 8.225$, $p < 0.0001$). Post hoc comparisons (Tukey's) revealed that in line with previous studies, Aβ_{1-42} increased the expression of p-tau compared to control cells (42.8 ± 13.6% increase, $n = 5$; $p = 0.0033$; Figure 6c). Treatment with leptin or leptin$_{116-130}$ prevented Aβ_{1-42}-mediated p-tau upregulation, with leptin-treated cells displaying a 39.4 ± 8% decrease in p-tau levels relative to Aβ_{1-42} alone and leptin$_{116-130}$ exhibiting a 41.2 ± 4.6% decrease in p-tau (Figure 6c; $n = 5$; $p < 0.0001$ relative to Aβ_{1-42} alone for both). Leptin$_{116-121}$ and leptin$_{117-122}$ both inhibited Aβ_{1-42}-mediated p-tau upregulation in a manner not statistically different from leptin or leptin$_{116-130}$ (Figure 6c; $n = 5$; $p \geq 0.9999$ and $p = 0.9572$ relative to leptin respectively; $p \geq 0.9999$ and $p = 0.8741$ relative to leptin$_{116-130}$ respectively). Thus leptin$_{116-121}$ caused a 40.7 ± 3.9% decrease in p-tau expression compared to Aβ_{1-42} alone, whereas leptin$_{117-122}$ demonstrated a 31.9 ± 6.1% decrease in p-tau (Figure 6c; $n = 5$;

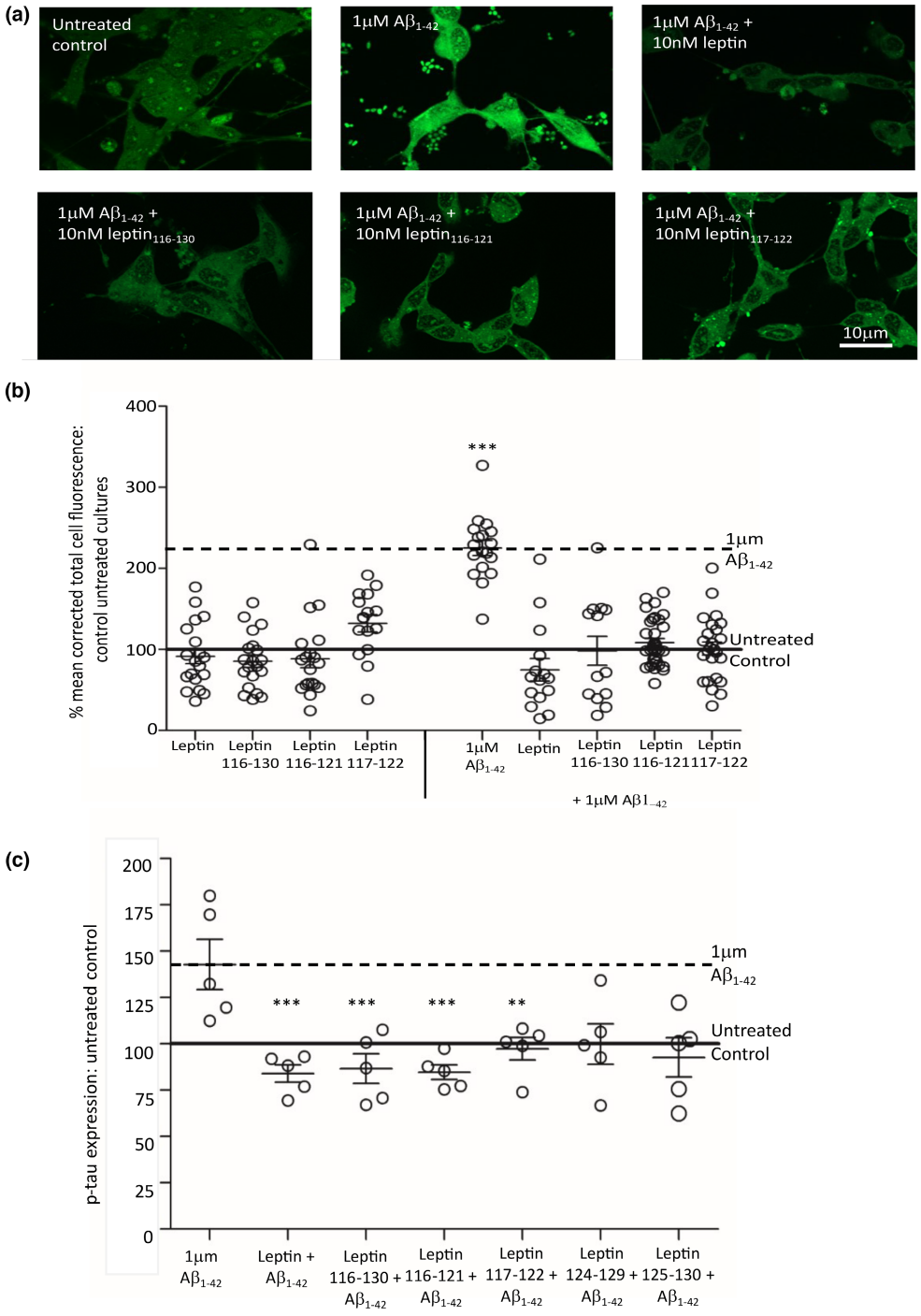

FIGURE 6 Leptin$_{116–121}$ and leptin$_{117–122}$ mirror leptin and leptin$_{116–130}$ prevention of amyloid seeding and p-tau upregulation. Photomicrographs (a) of thioflavin S-stained SH-SY5Y neurons untreated, or treated with a sub-toxic dose of A$\beta_{1–42}$ (Aβ_{1-42}) alone or in combination with leptin, leptin$_{116–130}$, leptin$_{116–121}$ or leptin$_{117–122}$. Pooled data of the relative fluorescence demonstrating that leptin, leptin$_{116–130}$, leptin$_{116–121}$ or leptin$_{117–122}$ prevent Aβ_{1-42}-mediated thioflavin S increase; $n = 13–35$ fields of view from five separate plate downs (b). The expression of p-tau was quantified using enzyme-linked immunosorbent assay (ELISA) and pooled data are shown demonstrating that leptin, leptin$_{116–130}$, leptin$_{116–121}$, leptin$_{117–122}$ attenuate Aβ_{1-42}-driven upregulation of phosphorylated tau (p-tau), $n = 5$ separate plate downs with each protein extract run in duplicate on each occasion (technical repeats; c). Statistical significance has been denoted: $p < 0.05$ (*) $p < 0.01$ (**) and $p < 0.001$ (***).

$p < 0.0001$ and $p = 0.0016$ relative to Aβ_{1-42} alone respectively). In contrast, leptin$_{124–129}$ and leptin$_{125–130}$ induced small but not significant decreases in p-tau expression ($n = 5$; $p = 0.1384$ and $p = 0.0960$ relative to Aβ_{1-42} alone respectively). Collectively, these data indicate that leptin$_{116–121}$ and leptin$_{117–122}$ inhibit Aβ_{1-42}-driven upregulation of p-tau to a similar degree as leptin or leptin$_{116–130}$.

5 | DISCUSSION

Although food intake and body weight are regulated by the hypothalamic actions of leptin (Spiegelman & Flier, 2001), the hippocampus is also a key CNS target for leptin. Increasing evidence indicates a pro-cognitive role for leptin within this brain region, as leptin facilitates hippocampal synaptic plasticity and hippocampus-dependent learning and memory (Irving & Harvey, 2021; McGregor & Harvey, 2018). In cellular studies, rapid effects of leptin on glutamate receptor trafficking and dendritic structure have been observed that likely contribute to the pro-cognitive properties of leptin (Irving & Harvey, 2014). Additionally, leptin significantly impacts neuronal viability, as leptin enhances the survival of central and peripheral neurons and protects neurons against various toxic stimuli (Weng et al. 2007; Doherty et al., 2008; Guo et al., 2008).

A correlation between plasma leptin levels and AD risk has been detected in clinical studies (Lieb et al., 2009; Power et al., 2001), and increasing evidence supports the notion that targeting the leptin system is beneficial in AD models (Doherty et al., 2013; Farr et al., 2006; Fewlass et al., 2004). However, other studies have found no link between leptin levels and the incidence of Alzheimer's disease or cognitive decline in AD patients (Teunissen et al., 2015). However, in this study, the average age of the AD patients was 63 and therefore this may reflect differences between the late and early onset forms of the disorder. However, the whole leptin molecule is not required for its protective actions as a smaller leptin fragment, $leptin_{116–130}$, is as effective as leptin in preventing the toxic effects of Aβ on hippocampal synapses and neuronal viability (Doherty et al., 2013; Malekizadeh et al., 2017). Here we extend those studies to provide compelling evidence that two specific leptin hexamers, $leptin_{116–121}$ and $leptin_{117–122}$, derived from $leptin_{116–130}$ also replicate the rapid effects of leptin on AMPA receptor trafficking and facilitation of hippocampal synaptic plasticity, suggesting that these hexamers mirror the synaptic actions of leptin. In contrast, the leptin hexamers $_{121–126}$, $_{123–128}$, $_{124–129}$ and $_{125–130}$ were found to be inactive. Further evaluation of two of the active hexamers ($leptin_{116–121}$ and $leptin_{171–122}$) established that these hexamers prevent the acute detrimental effects of Aβ on hippocampal synaptic function, as treatment with either hexamer prevents Aβ-driven internalisation of the AMPA receptor subunit, GluA1. Both hexamers replicate the neuroprotective characteristics of leptin as exposure to either $leptin_{116–121}$ or $leptin_{117–122}$ counteracts the chronic effects of Aβ on neuronal viability. This study highlights the cross-species potency of these neuroactive hexamers by using established in vitro models of AD and building on existing murine in vivo studies. This is essential for peptides where protein sequences per se diverge between species and even a single amino acid alteration could be crucial in determining whether efficacy is maintained. And yet early-stage testing of neuroactive compounds in humans is necessarily limited. Thus, we have shown potent neuroprotection following $leptin_{116–121}$ and $leptin_{1717–122}$ administration in amyloid-treated human neural cells coupled to the prevention of amyloid-driven synaptic deficits in rat hippocampal slices and improvements in episodic-like memory in mice. These data exhibit a robust cross-species response to $leptin_{116–121}$ and $leptin_{1717–122}$ across the mammalian taxon and validates the use of rodent models to further determine the neurobeneficial effects of these molecules. Collectively, these findings indicate that the beneficial actions of whole leptin are mirrored by $leptin_{116–121}$ and $leptin_{1717–122}$ as evidenced by the ability of both hexamers to prevent the aberrant effects of Aβ in various cellular models of AD.

Previous studies have demonstrated that leptin acts as a potential cognitive enhancer as it facilitates synaptic plasticity at hippocampal CA1 synapses (Malekizadeh et al., 2017; Oomura et al., 2006; Shanley et al., 2001). Similarly, treatment of hippocampal slices with four of the hexamers ($leptin_{116–121}$, $leptin_{117–122}$, $leptin_{118–123}$ or $leptin_{120–125}$) resulted in a significant increase in the magnitude of LTP compared to control LTP. It is well known that activity-dependent alterations in the synaptic density of AMPA receptors are key for maintaining the increase in synaptic efficacy associated with LTP (Collingridge et al., 2004). Moreover, we have shown that leptin regulates the movement of the AMPA receptor subunit, GluA1 to and away from hippocampal synapses (Moult et al., 2010); a process mirrored by $leptin_{116–130}$ (Malekizadeh et al., 2017). Here we show that the four hexamers that facilitate LTP, also increase the surface expression of GluA1 in hippocampal neurons. Conversely, treatment with $leptin_{121–126}$, $_{123–128}$, $_{124–129}$ and $_{125–130}$ had no effect on GluA1 trafficking as no significant change in GluA1 surface expression was detected after exposure to these hexamers. Overall, these findings indicate that four of the hexamers ($leptin_{116–121}$, $leptin_{117–122}$, $leptin_{118–123}$ and $leptin_{120–125}$) mirror the effects of leptin on glutamate receptor trafficking and hippocampal synaptic plasticity.

To assess whether the hexamer-induced changes in synaptic plasticity have a significant impact on learning and memory, the effects of one hexamer, $leptin_{117–122}$ were compared to whole leptin and a saline control on a series of behavioural tasks which assessed episodic-like memory, spatial memory and anxiety-like behaviour. Consistent with our previous findings (Malekizadeh et al., 2017) leptin improved performance on the OPC task. We extend these findings to show that $leptin_{117–122}$ produces a similar facilitation of episodic-like memory. Given recent findings showing that increased performance on object exploration tasks correlates with memory discrimination in humans (Sivakumaran et al., 2018), this provides evidence of improved memory in rodents. The OPC task assesses integration of object, place and context information and consequently provides a model of episodic memory. Given that a deficit in episodic memory is one of the first symptoms of AD, these data suggest that $leptin_{117–122}$ might be a useful therapeutic target. Future studies could aim to examine whether the hexamers rescue similar episodic-like memory deficits reported in transgenic models of AD (Davis et al., 2013). The fact that chronic administration of both leptin and $leptin_{117–122}$ improved memory over a 3-week period also suggests that the memory facilitation is persistent.

As previous studies have shown that leptin facilitates spatial memory (Oomura et al., 2006; Haleem et al., 2015), we assessed whether $leptin_{117–122}$ has a similar effect relative to leptin and a

saline control. We found a small but significant increase in spatial memory performance on a spontaneous alternation task which has been shown to be hippocampus-dependent (Ainge et al., 2007; Dudchenko 2004). This is consistent with the current findings of facilitation of hippocampal synaptic plasticity and suggests that one driving factor behind enhanced performance in the OPC task is an improved memory for spatial features of an environment. Although anxiety-like behaviour can impact performance on behavioural tasks, particularly those that are spontaneous, we found no significant difference between groups on plus-maze performance demonstrating that changes in memory performance are not attributable to changes in behaviour induced by anxiety. Although we have no direct evidence that the hexamers cross the blood brain barrier in our behavioural studies, given that previous studies have shown that leptin and fragments cross the blood brain barrier (Grasso et al., 1997; Lee et al., 2008), and the behavioural effects of leptin and the hexamers are strikingly similar, the most feasible explanation is that the hexamer also readily accesses the brain. This is also consistent with the facilitation of synaptic plasticity by the hexamers that we observed.

Aβ interferes with excitatory synaptic function at hippocampal CA1 synapses, such that acute exposure to Aβ promotes endocytosis of the AMPA receptor subunit GluA1 (Hsieh et al., 2006). Our previous studies indicate that leptin and $leptin_{116-130}$ prevent this synapto-toxic effect of Aβ in hippocampal neurons (Doherty et al., 2013; Malekizadeh et al., 2017). Likewise, in hippocampal neurons treated with $leptin_{116-121}$ or $leptin_{117-122}$ the ability of Aβ to internalise GluA1 was markedly reduced, suggesting that the hexamers replicate the protective actions of leptin by preventing the harmful effects of Aβ on AMPA receptor trafficking.

Accumulating evidence indicates a neuroprotective role for leptin in models that mirror the neuronal degeneration that occurs in diseases like AD (Doherty et al., 2008; Guo et al., 2008). For instance, in models that reproduce the toxic events associated with chronic exposure to Aβ, leptin attenuates the magnitude of cell death, by activating pro-survival signalling cascades such as PI3 kinase and STAT3 (Doherty et al., 2008; Doherty et al., 2013; Guo et al., 2008). In line with previous studies, we show that the viability of human SH-SY5Y cells is markedly reduced after chronic treatment with $Aβ_{1-42}$ (Malekizadeh et al., 2017), and treatment with either $leptin_{116-121}$ or $leptin_{117-122}$ markedly increased the viability of SH-SY5Y cells after chronic exposure to $Aβ_{1-42}$. In contrast, no reductions in the viability of human SH-SY5Y cells was detected after treatment with either $leptin_{124-129}$ or $leptin_{125-130}$. This provides further compelling evidence that $leptin_{116-121}$ and $leptin_{117-122}$ (but not $leptin_{124-129}$ or $leptin_{125-130}$) replicate the full spectrum of leptin action, including the ability to protect against the toxic actions of Aβ.

Key pathological features of AD include accumulation of hyperphosphorylated tau and amyloid deposition. Our data show that $leptin_{116-121}$ and $leptin_{117-122}$ are as effective as leptin or $leptin_{116-130}$ in preventing Aβ-driven upregulation of p-tau levels. Leptin and $leptin_{116-130}$ decrease tau phosphorylation (Greco et al., 2008; Malekizadeh et al., 2017; Zhang et al., 2016), via inhibition of GSK3β. Here we show that only a short six amino acid sequence derived from $leptin_{116-130}$ is sufficient to replicate these effects on tau phosphorylation. There is clear evidence that leptin modulates Aβ levels, as the activity of β secretase, a key enzyme in the production of $Aβ_{1-42}$, is reduced by leptin (Fewlass et al., 2004). In AD, amyloid deposits typically appear with a hierarchical spatial distribution, suggesting propagation of the peptide between different brain areas (Braak & Braak, 1991; Thal et al., 2002) and in support of this several studies have demonstrated transmission of Aβ between cells both in vivo and in vitro (Gouras et al., 2010; Nath et al., 2012). The release of intracellular amyloid is well established as the source of amyloid plaques (Calhoun et al., 1999; Zhao et al., 1996). Here we show that chronic treatment of SH-SY5Y cells with a low dose of $Aβ_{1-42}$ results in amyloid seeding, and that exposure to leptin, $leptin_{116-130}$, $leptin_{116-121}$ or $leptin_{117-122}$ prevented generation of more amyloid. Together these data present compelling evidence that $leptin_{116-121}$ and $leptin_{117-122}$ are as effective as leptin and $leptin_{116-130}$ in reducing tau phosphorylation and amyloid accumulation.

A key area that remains to be addressed is how the leptin-based peptides interact with the leptin receptor (LepR). Based on its similarity to the IL-6 receptor, it has been proposed that leptin has three possible binding domains that enable interaction with LepR. Binding site I lies within the C terminus of helix D and works in concert with residues from the connection loop between helices A and B. Binding site II consists of residues from helices A and C whereas binding site III contains residues at the N-terminus of helix D (Peelman et al., 2004). None of these hypothetical LepR interaction sites are located within the $leptin_{116-130}$ sequence, identified as bioactive by us and others (Grasso et al., 1999; Malekizadeh et al., 2017) and on which the leptin hexamers are derived. However, the exact locations of binding sites I and II remain controversial (Greco et al., 2021) and therefore detailed in silico and empirical studies are needed to model leptin fragment/s interactions with LepR and to clarify further how native leptin interacts with its receptor. Evidence suggests that the long LepR isoform (LepRb) may not be required for $leptin_{116-130}$-driven anti-obesity signalling as *db/db* mice that lack functional LepRbs still respond to $leptin_{116-130}$ (Grasso et al., 1999). The signalling pathways activated by $leptin_{116-130}$ mirror those activated by the full-length leptin molecule and include JAK/STAT, MAPK and PI3-kinase/Akt signalling (Jeremy et al., 2022; Lin et al., 2014; Malekizadeh et al., 2017). Consequently, this raises the question of how leptin-mimetic peptides are signalling and opens the possibility for involvement of other LepR isoforms.

As the emerging evidence that leptin and leptin-based peptides have a wide range of neuro-beneficial effects in empirical models of AD, their suitability as druggable entities should be considered. Leptin's multi-faceted protective effects against various elements of AD pathology are well known, with beneficial effects demonstrated at very early stages of the disease, including effects on synaptic dysfunction (Doherty et al., 2013) and mitochondrial aberrations (Cheng et al., 2020) through to behavioural changes (Greco et al., 2010) and ultimately the loss of neurons. This study explores the potential for the small hexamer peptides to elicit beneficial changes on synaptic function, biomarker expression, episodic memory to cell death. It is

prudent at this stage in the investigation of the neurological actions of these peptides to consider ways in which they can be modified to aid with the potential routes for administration and bioavailability, and to test the modified forms on the simpler models used here, prior to adopting more complex studies on transgenic animals. Peptide-based natural hormone analogues present a simple way to target hormonal receptors such as LepR for pharmacological benefit. However, peptide-based therapeutics present several challenges including delivery difficulties and in vivo instability. Significant progress has been made in advancing the use of peptide therapeutics, with numerous strategies to enhance bioavailability being developed including C-terminal amidation or N-terminal acetylation (Di, 2015), incorporation of synthetic enantiomer amino acids (Weinstock et al., 2012), and peptide cyclisation (Hayes et al., 2021). Therefore, emerging strategies will allow further development of leptin peptides towards therapeutic leads but will require robust evaluation to ensure efficacy is maintained. As different leptin fragments have been linked with different aspects of leptin's biological functions, there is the potential for hexamer-based fragments to have reduced off-target effects compared to the full-length molecule.

6 | CONCLUSION

In conclusion here we provide compelling evidence that the leptin hexamers, leptin$_{116–121}$ and leptin$_{117–122}$, mirror the synaptic effects of whole leptin, via their ability to rapidly regulate AMPA receptor trafficking and enhance synaptic plasticity at hippocampal CA1 synapses. In addition, leptin$_{116–121}$ or leptin$_{117–122}$ prevent the acute effects of Aβ at hippocampal synapses, as Aβ-driven internalisation of GluA1 is inhibited by the two leptin hexamers. Treatment with either leptin$_{116–121}$ or leptin$_{117–122}$ also prevents the neuronal damage caused by chronic exposure to A$\beta_{1–42}$ and reduces Aβ-driven accumulation of AD-linked biomarkers. Leptin$_{117–122}$ also improves episodic-like and spatial memory relative to controls. Overall, these findings add further weight to the possibility of targeting the leptin system to treat AD, and it identifies that two leptin-based hexamers are highly potent leptin-mimetics that may provide the basis for the development of AD therapeutic agents in the future.

AUTHOR CONTRIBUTIONS

JH, GD and JA supervised and designed the experiments and wrote the manuscript. AH, YM, CM, SP, IF, KH and BM all performed the experiments and analysed the data.

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All experiments were conducted in compliance with the ARRIVE guidelines.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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DATA AVAILABILITY STATEMENT

The data sets used and analysed during the current study are available from the corresponding authors upon reasonable request. Data for the cell culture studies, ELISAs and behavioural analyses are available at: https://doi.org/10.17630/63bc8b8e-40e7-4098-b137-edf8383e0699. Data for electrophysiology and immunocytochemical studies are available at DOI: 10.15132/10000189

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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