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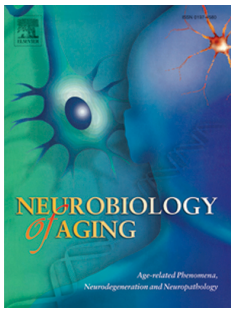

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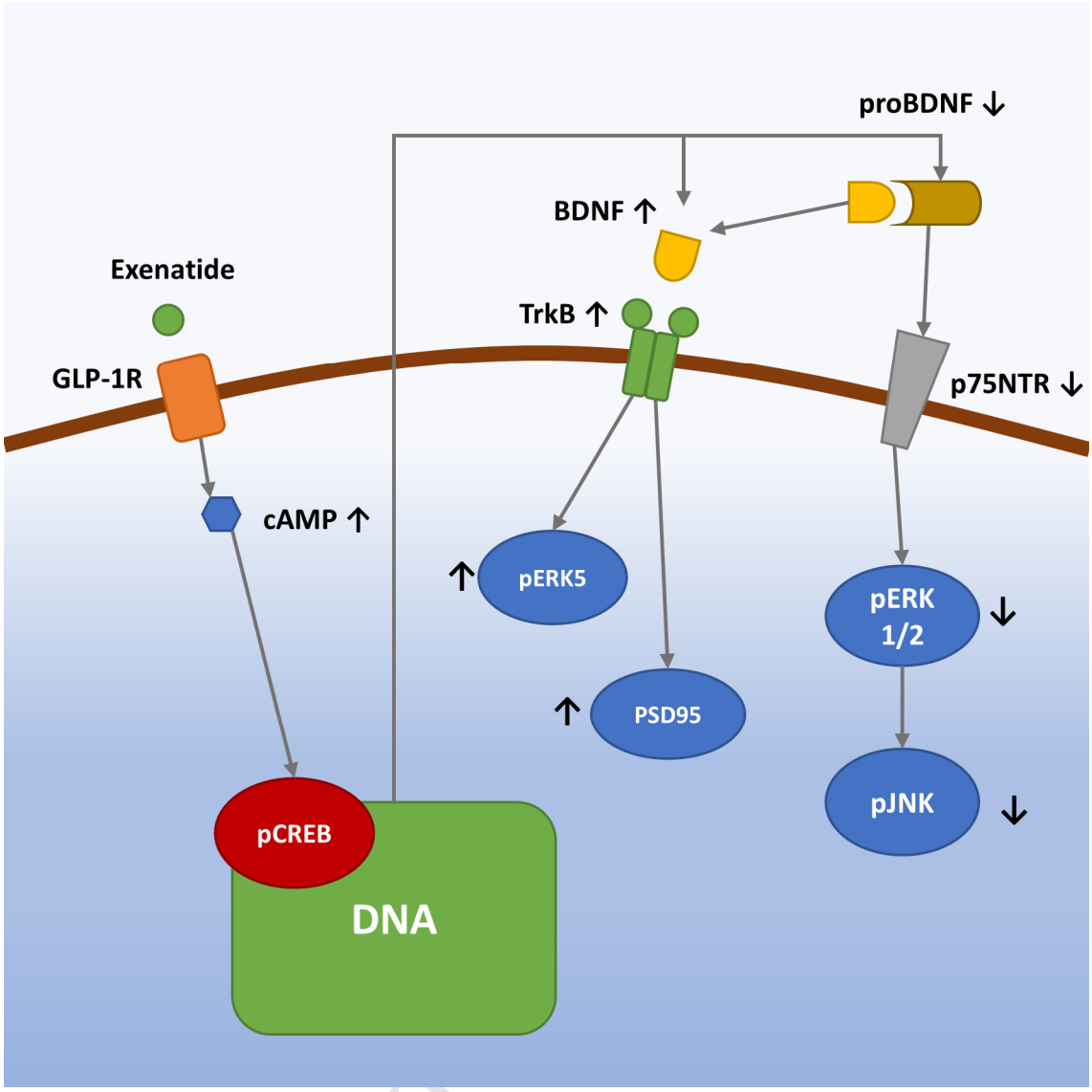
proBDNF ↓
BDNF ↑
Exenatide
TrkB ↑
GLP-1R
p75NTR ↓
cAMP ↑
↑ pERK5
pERK 1/2 ↓
↑ PSD95
pJNK ↓
pCREB
DNA

Exenatide exerts cognitive effects by modulating the BDNF-TrkB neurotrophic axis in adult mice

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Abstract

Modulation of insulin-dependent signalling is emerging as a valuable therapeutic tool to target neurodegeneration. In the brain, the activation of insulin receptors promotes cell growth, neuronal repair, and protection. Altered brain insulin signalling participates in the cognitive decline seen in Alzheimer's disease (AD) patients and the ageing brain. Glucagon-like peptide-1 (GLP-1) regulates insulin secretion and, along with GLP-1 analogues, enhances neurotrophic signalling and counteracts cognitive deficits in preclinical models of neurodegeneration. Moreover, recent evidence indicates that GLP-1 modulates the activity of the Brain-Derived Neurotrophic Factor (BDNF). In this study, in adult wild-type mice, here employed as a model of mid-life brain ageing, we evaluated the effects of a 2-month treatment with exenatide, a GLP-1 analogue. We found that exenatide promotes the enhancement of long-term memory performances. Biochemical and imaging analysis show that the drug promotes the activation of the BDNF-TrkB neurotrophic axis and inhibits apoptosis by decreasing p75NTR-mediated signalling. The study provides preclinical evidence for the use of exenatide to delay age-dependent cognitive decline.

Keywords: GLP-1, GLP-1 receptor, type 2 diabetes mellitus, long-term potentiation, cognitive enhancement

1. Introduction

Insulin is emerging as a critical modulator of brain signalling, and the use of the hormone has been explored in several neurodegenerative disorders (Aviles-Olmos et al., 2013; Bassil et al., 2017a; Craft, 2012). Insulin is primarily secreted by the pancreas but also synthesized within the Central Nervous System (CNS) (Schechter et al., 1996). Brain insulin contributes to synaptic maintenance, neuronal outgrowth and survival and impinges on the molecular pathways underlying learning and memory processes (Benedict et al., 2007; Craft and Stennis Watson, 2004; Ghasemi et al., 2013; Schulingkamp et al., 2000). Epidemiological studies on patients affected by Type 2 Diabetes Mellitus (T2DM) have shown that defective insulin signalling, a phenomenon also known as insulin resistance, is associated with the development of cognitive impairment (Kullmann et al., 2016). Defective glucose metabolism and utilization have also been reported in subjects affected by Mild Cognitive Impairment (MCI) (Arnáiz et al., 2001; Kantarci et al., 2010). Insulin-related deficits have been described in carriers of the apolipoprotein E4 allele, a risk factor for Alzheimer's disease (AD) (Frölich et al., 1998). In addition, insulin resistance, along with altered insulin-related signalling, have been described in the brain of AD patients (Craft, 2012, 2007; De Felice, 2013; De Felice et al., 2014; Talbot et al., 2012). Finally, brain aging is associated with decreased insulin sensitivity, a phenomenon caused by the gradual loss of insulin secretion and the reduced expression or function of insulin receptors (IRs) within the CNS (Carro and Torres-Aleman, 2004; Fink et al., 1983; Frölich et al., 1998; Hoyer, 2002).

Glucagon-like peptide-1 (GLP-1) is an endogenous insulinotropic hormone that participates in the homeostatic regulation of peripheral levels of insulin and glucose (Campbell and Drucker, 2013; Holst, 2007). In pancreatic β-cells, GLP-1 and GLP-1 analogues stimulate insulin secretion and are efficiently employed for T2DM treatment (Lin et al., 2015; Salehi et al., 2010). The activation of the GLP-1 receptor (GLP-1R) results in the production of intracellular cyclic adenosine monophosphate (cAMP) and followed by the activation of protein kinase A (PKA) and phosphoinositide 3-kinase (PI3K). These processes lead to the enhancement of protein synthesis, cellular proliferation, mitochondrial biogenesis and functioning (Athauda and Foltynie, 2016). This signalling cascade also inhibits apoptotic and pro-inflammatory pathways, ultimately favouring cell survival (Athauda and Foltynie, 2016). GLP-1Rs are expressed in the brain (Campbell and Drucker, 2013; Hamilton and Hölscher, 2009) and contribute to the protection, proliferation, and differentiation of progenitor cells into neurons (Hamilton et al., 2011; Isacson et al., 2011; Li et al., 2009). The receptors also regulate neuronal excitability, synaptic plasticity, and memory formation (Abbas et al., 2009; Gault and Hölscher, 2008a; Holscher, 2014; Liu and Pang, 2016). Furthermore, a growing body of evidence indicates that GLP-1, through the activation of the cAMP response element-binding protein (CREB), induces the expression of the Brain-Derived Neurotrophic Factor (BDNF), a critical

neurotrophic factor acting through its tropomyosin-related kinase B receptor (TrkB) (Mattson, 2012). GLP-1 analogues cross the blood-brain barrier and have been tested in several preclinical models of neurodegenerative conditions and clinical trials (Bassil et al., 2014; Calsolaro and Edison, 2015). Exendin-4, a GLP-1R agonist, approved for T2DM treatment, is currently under evaluation in trials targeting AD and Parkinson's disease (PD; NCT01255163, NCT01174810, NCT01971242) (Athauda et al., 2017; Aviles-Olmos et al., 2013; Bassil et al., 2017b, 2014).

We have recently shown that a 6-month treatment with exenatide, a synthetic, long-lasting GLP-1 analogue, promotes beneficial effects on Short- and Long-Term Memory (STM and LTM, respectively) performances in Presenilin-1 Knock-In (PS1-KI) mice, a preclinical model of amyloid-independent neuronal dysfunction (Bomba et al., 2013). In the present study, we tested the cognitive effects of a 2-month treatment with exenatide in adult mice enrolled at 10 months of age (m.o.a.). The timeframe of the intervention (10-12 m.o.a.) was chosen as, in mice, this age bracket is comparable to the mid-life stage of humans, a period that is crucial for the onset of either age-related cognitive decline or dementia (Winblad et al., 2016). Endpoints of the study were drug-related effects on cognitive performances, glucose metabolism, mitochondrial and synaptic functioning as well as modifications of structural plasticity. Drug effects were compared with a group of age-matched mice treated with vehicle.

2. Materials and Methods

2.1. Animals and treatment paradigm

All the procedures involving the animals and their care were approved by the Institutional Ethics Committee (CEISA protocol no. 17; Min. IDD: DGSAF/14264). Animal handling was performed by institutional guidelines and compliance with national and international laws and policies. Efforts were made to minimize the number of employed animals and their suffering. Mice were housed on a 12 h light/dark cycle, provided with, *ad libitum,* access to food and water, and bred in the CeSI-MeT animal facility. Behavioral tests were performed during the light cycle. Twenty-two animals (with a B6;129 background; 16 females and 6 male mice) were enrolled at 10 m.o.a. and randomly assigned to a 2-month exenatide (8 females and 4 males) or vehicle (PBS; 8 females and 2 males) administration. Animals received daily intraperitoneal injections of exenatide (500 μg/kg body weight) or vehicle five days per week. Animals were also subjected to vehicle or drug administration during the behavioural testing phase. Exenatide was generously provided by Eli Lilly. Fasting glycemia was measured at the end of the 2-month treatment period with a strip-operated digital glucometer (Freestyle Lite, Abbott).

2.2 Morris Water Maze

The Morris Water Maze (MWM) test was performed as previously described (Bomba et al., 2013; Isopi et al., 2015). In brief, the MWM apparatus (Panlab) is a circular pool (1.2m diameter) filled with warm (22±1° C) water. The pool is located in a room containing several intra- and extra-maze visual cues. Mice were trained to swim in the pool and climb on a circular platform (12 cm diameter) submerged (2 cm) beneath the water surface. Mice were placed on the platform 10 s before the beginning of the first training session to reduce the stress related to the task. Mice that failed to find the platform within 90 s were manually guided to it and allowed to remain there for 10 s. Mice underwent four trials per day for 4 consecutive days. Between trials, mice were, placed back into their cages warmed with a fan for 20 min. Spatial memory performances were assessed 1.5 and 24 h after the end of the last training trial. The two probe tests consisted of a 60 s free swim in the pool in which the platform has been removed. Performances were evaluated in terms of time taken to reach the location where the platform used to be (escape latency), number of crosses over the platform location, and time spent in the target (T target) or opposite (T opposite) quadrants.

2.3. Tissue collection and reagents employed

At the end of the 2-month treatment and following MWM test, mice were killed with carbon dioxide and tissue samples collected for biochemical analysis. Brains were halved into two hemispheres, immediately frozen by liquid nitrogen, and kept at −80 °C for subsequent analysis. For Western blot (WB) analysis, each hemisphere was dissected into sub-regions (hippocampus and whole cortex), snap frozen in liquid nitrogen, and stored at −80 °C until sampling. All chemicals and reagents were, unless otherwise specified, purchased from Sigma-Aldrich.

2.4. Cytochrome c oxidase activity

Cytochrome c oxidase (COX) activity was determined as previously described (Bomba et al., 2013). Briefly, whole-brain samples were minced and homogenized in a modified Chappel–Perry medium containing (in mM): 1 adenosine 5'-triphosphate (ATP), 100 KCl, 5 MgCl2, 1 ethylenediamine N, N, N', N'-tetraacetic acid (EDTA), 5 ethylene glycol tetraacetic acid (EGTA), and 50 HEPES buffer adjusted to pH 7.4. Homogenates were further diluted 1:2 (v/v) in the same medium with polyethylene glycol ether W-1 (100 mg/g tissue) and then maintained on ice for 30 min. COX activity was assayed, using a Clark oxygen electrode (Rank Brothers), in 1.5 ml of reaction medium containing (in mM): 30 cytochrome *c*, 4 rotenone, 0.5 dinitrophenol, 10 sodium malonate, and 75 HEPES buffer at pH 7.4. COX activity was measured as the difference between the rate of oxygen consumption in homogenates after addition of substrate (4mM sodium ascorbate with 0.3 mM N, N, N', N'-tetramethyl-p-phenylenediamine) and the rate of oxygen consumption observed in medium containing only the substrate (to take in account ascorbate auto-oxidation). Results are reported as $nmolO_2/mg_{protein}/min$.

2.5. Cytosolic LDH activity

Forward and reverse lactate dehydrogenase (LDH) activities were spectrophotometrically assayed as previously described (Bomba et al., 2013). Briefly, brain tissues were homogenized in a phosphate buffered solution (PBS, 20 ml/g of tissue) and centrifuged to remove cellular debris. The supernatant was collected and protein concentration determined by the Bradford assay following manufacturer instructions. Forward LDH (pyruvate to lactate; Pyr → Lac) activity was measured by adding pyruvate (2.50 g/l) and reduced nicotinamide adenine dinucleotide (NADH 0.3 g/l) to the sample. Similarly, reverse LDH (lactate to pyruvate; Lac → Pyr) activity was measured by adding lactate (8.6 g/l) and oxidized nicotinamide adenine dinucleotide (NAD^+, 3.5 g/l) to the sample after pH adjustment to 8.8 with 0.05M sodium pyrophosphate. Changes in NADH absorbance, evaluated at 340 nm, were measured at room temperature with a microplate reader (Spectra max 190, Molecular Devices). Forward (Pyr → Lac) and reverse (Lac → Pyr) LDH activities are calculated as $\mu mol/mg_{protein}/min$ and expressed as a percentage relative to control.

2.6. Western blot analysis

Specimens were collected and lysed in ice-cold RIPA buffer [phosphate buffer saline pH 7.4 containing (in %): 0.5 sodium deoxycholate, 1 Nonidet P-40, 0.1 SDS, 1 protease and phosphatase inhibitor cocktails, and 5mM EDTA]. Protein lysates (10 µg) were separated on 9-13% SDS–polyacrylamide gel and electroblotted onto polyvinyl difluoride membrane (PVDF). Nonspecific binding sites were blocked with 5% non-fat dry milk (Bio-Rad Laboratories) in Tris-buffered saline [TBS; containing (in mM): 20 Tris–HCl, 150 NaCl, pH 7.4] for 30 min at RT. Membranes were then incubated overnight at 4°C with the primary antibodies, diluted with TBS containing 0,1% Tween 20 (TBS-T) and 5% non-fat dry milk. A full list of the employed antibodies is available in a supplementary table (Table S1). Peroxidase-conjugated anti-rabbit or anti-mouse IgG (1:10000; Vector Laboratories) were employed as secondary antibodies. Chemiluminescent signals were visualized by ECL (Euroclone), according to the manufacturer instructions. The relative densities were determined and normalized to actin by using ImageJ software. Values are given as either relative units (RU) or phospho-protein/total protein ratio. The latter was calculated as follows: (phospho-protein/loading control)/(total protein/loading control).

2.7. Neuronal hippocampal cultures

Neuronal hippocampal cultures were obtained from fetal CD1 mice at 14 days of gestation, as previously described (Frazzini et al., 2016; Sensi et al., 1999). Briefly, fetuses were decapitated, brains extracted, and dissected in an ice-cold dissecting medium; after meninges removal hippocampi were collected, minced with forceps, and transferred in a 0.25% trypsin solution for 15 min at 37 °C. Hippocampi were then centrifuged and pellet dissociated with a fire-polished glass pipette. Cell suspensions were then

transferred in a Neurobasal medium (ThermoFisher), supplemented with 0.5 mM L-glutamine, 5% fetal bovine serum (ThermoFisher), 5% horse serum (ThermoFisher), 1 × B27 (ThermoFisher) and 0.2% penicillin/streptomycin, and plated onto pre-treated laminin/poly-DL-lysine coated Petri or optical glass dishes. Three days after plating, cytosine arabinofuranoside (5 µM) was added to the culture medium to obtain near-pure hippocampal cultures. Every three days, 25% of the medium was replaced with fresh Neurobasal.

2.8. Neuronal transfection and dendritic spine imaging

Hippocampal neurons were transfected at 7 DIV with a TurboGFP expressing vector (pSIH-H1-copGFP) using Lipofectamine2000 (ThermoFisher) according to the manufacturer protocol. Neurons were pharmacologically treated at 12 DIV and employed for experiments at 15 DIV.

TurboGFP-transfected neurons were imaged with a Zeiss LSM800 confocal microscope (Zeiss) equipped with a 488nm LED laser line, an Airyscan detector, and a 63x oil immersion objective (1.5 NA, Zeiss). Images were acquired in the Airyscan mode. Some additional experiments were performed with a Zeiss LSM7 MP microscope, equipped with an IR 2-photon laser line (Coherent), a BiG detector, and a 63x water immersion objective (1.0 NA, Zeiss). In all cases, images were acquired with the ZEN software (Zeiss) and then stored for offline analysis.

Data analysis was performed as follows: images were exported in TIFF format and evaluated with Fiji (Schindelin et al., 2012). Primary and secondary proximal dendrites (within 150 µm from the soma) were studied and dendritic spines manually counted by a trained experimenter blind to treatment. Data were normalized as number of spines per µm of dendrite length.

2.9. Data analysis

No statistical methods were used to predetermine sample size. Statistical analysis was performed by two-tailed Student's *t*-test method. As for the study of dendritic spine density one-way ANOVA followed by Fisher LSD post hoc test was performed. The level of significance was set at $P<0.05$. Data were expressed as mean ± SEM.

3. Results

3.1. Exenatide improves long-term memory performance in treated mice

Exenatide- or the vehicle-related effects on memory performance were assessed at the end of the 2-month treatment by employing the MWM task, a setting that evaluates the functioning of hippocampus-dependent spatial memory (Morris et al., 1982; Vorhees and Williams, 2006).

MWM results showed no statistically significant differences between the two study groups when comparing task learning performance (Fig. 1A). On the contrary, exenatide-treated mice performed significantly better in parameters related to LTM activities. The treated group, assessed 24 h after the last training session, showed reduced time spent to reach the platform location (Fig. 1B-C, escape latency; P=0.039) and time spent in the opposite quadrant (Fig. 1F, P=0.020). Treated mice also showed increased time spent on the target quadrant (Fig. 1E, P=0.004). No differences between the two study groups were found in STM performances (Fig. 1B-F, P>0.05) or the number of platform crosses (Fig. 1D, P>0.05). Additional analysis revealed no differences regarding the swimming speed, distance travelled, or time spent in the remaining quadrants (Fig. 1H-L, P>0.05). These findings rule out the possibility that the improved cognitive performances of exenatide-treated mice are due to enhanced swimming abilities or motivation.

3.2. Exenatide treatment has no effects on the body weight and fasting glycemia of treated mice

Exenatide is known to increase insulin sensitivity and improve glucose homeostasis in T2DM patients (DeFronzo et al., 2005). We have previously shown that the molecule exerts an overall increase in the brain anaerobic glycolysis rate in PS1-KI mice (Bomba et al., 2013). We, therefore, evaluated exenatide- or vehicle-related effects on changes in the body weight and glycemia of the two study groups (Rodriquez de Fonseca et al., 2000; Wadden et al., 2013). The two study groups showed no statistically significant differences for these parameters (Fig. 2A-B, P>0.05).

3.3. Exenatide treatment does not affect the brain energy metabolism of treated mice

Effects on anaerobic and aerobic glycolytic pathways were also studied as exenatide treatment has been found to be associated with a metabolic shift to anaerobic glycolysis (Bomba et al., 2013). Forward LDH activity ($LDH_{Pyr-Lac}$; an index of anaerobic glycolysis) of whole brain samples homogenates was found to be similar in the two cohorts (Fig. 3A, P>0.05). Aerobic glycolysis, assayed by measuring the whole brain reverse LDH activity ($LDH_{Lac-Pyr}$) was also found to be similar in the two study groups (Fig. 3B, P>0.05). Incretins improve mitochondrial functioning and counteract the production of injurious Reactive Oxygen Species (ROS) of mitochondrial origin (Lee et al., 2017; Petersen et al., 2016; Tews et al., 2009). In addition, exenatide prevents mitochondrial DNA damage and stimulates mitochondrial biogenesis (Fan et al., 2010). Thus, whole brain COX activity, a marker of mitochondrial functioning (Bomba et al., 2013), was assessed in

the two study groups and the study showed no statistically significant differences between the two cohorts (Fig. 3C, P>0.05).

3.4. Exenatide treatment promotes BDNF signalling in treated mice

Exenatide promotes the CREB-dependent transcription of genes, including *BDNF* (Ohtake et al., 2014), that are involved in the modulation of synaptic plasticity and cognition. WB analysis, performed on *ex vivo* brain samples of the two study groups, revealed increased phosphorylation (and thus activation) of CREB (Fig. 4A-B) as well as increased expression levels of BDNF in exenatide-treated mice (Fig. 4A and 4C). The increases were found to occur in the hippocampus and are in line with a trend toward enhanced activation of the BDNF receptor, TrkB (pTrkB, Fig. 4A and 4D). Conversely, in the cortex, drug treatment did not affect CREB nor TrkB activation but increased BDNF levels (Fig. 4A-D).

To address whether exenatide affects the BDNF-TrkB signalling cascade, we investigated effects on BDNF-related extracellular signal-regulated kinase 5 (ERK5) and PSD95 (Alonso et al., 2004; Su et al., 2014; Subramaniam and Unsicker, 2010; Yoshii and Constantine-Paton, 2007). These proteins are involved in neuronal survival (Cavanaugh, 2004; Parmar et al., 2014), neuronal differentiation (Nishimoto and Nishida, 2006), adult neurogenesis, and hippocampus-dependent LTM performances (Wang et al., 2014) as well as the strengthening and stabilization of spines (Yoshii and Constantine-Paton, 2007). WB analysis of these signalling proteins showed that exenatide treatment increased the levels of phosphorylated ERK5 (pERK5) and PSD95 (Fig. 4A, 4F and 4G, respectively).

3.5. Exenatide reduces p75NTR signalling in treated mice

Biologically active BDNF originates from the proteolytic cleavage of proBDNF, the neurotrophic factor immature form. ProBDNF counteracts the BNDF trophic effects and activates apoptotic signalling by binding to the p75 neurotrophin receptor (p75NTR). The analysis of the proBDNF signalling cascade in the two study groups indicated that exenatide decreased the expression levels of proBDNF, p75NTR, phosphorylated $ERK_{1,2}$ ($pERK_{1,2}$) and JNK (pJNK) in treated mice (Fig. 4A, 4H-I, 4E, and 4J, respectively) (Hibi et al., 1993).

GLP-1 has been shown to activate insulin-related pathways; we, therefore, investigated serine phosphorylation of the insulin receptor substrate 1 (IRS1), a modification known to block the downstream insulin signalling cascade (Bomfim et al., 2012). The analysis revealed that exenatide administration promotes a reduction of phosphorylation at Ser1101 in the cortex but not in the hippocampus (Supplementary Fig. 1).

3.6 Exenatide increases spine density in vitro

BDNF signalling promotes structural plasticity and synaptic pruning, two correlates of learning and memory (Harward et al., 2016; Minichiello et al., 1999). To test whether exenatide promotes synaptogenesis and structural plasticity through the activation of the BDNF-TrkB axis, we evaluated synaptic spine density in hippocampal neurons. To that aim, cells were transfected with a TurboGFP expressing vector and treated, for three days, with the vehicle, exenatide (500 nM), or exenatide and ANA-12 (10 µM), a selective, high-affinity inhibitor of TrkB (Cazorla et al., 2011). Data analysis indicated that exenatide-treated neurons show increased dendritic spine density (Fig. 5) when compared to vehicle-treated sister cultures. Exenatide-driven neurotrophic effects were blunted by co-application of ANA-12 (Fig. 5). Taken together, this set of experiments indicates that exenatide effects on structural plasticity are, at least in part, mediated by a drug-dependent potentiation of the BDNF-TrkB axis.

4. Discussion

In this study, we investigated the cognitive effects of exenatide in a preclinical model of mid-life brain ageing. The main finding of the study is that a 2-month exenatide treatment produces LTM enhancement (Fig. 1). These results are in line with several studies showing that GLP-1R activation promotes beneficial effects on learning- and memory-related tasks (During et al., 2003). These findings also parallel previous evidence indicating a positive impact of the compound on the cognitive performances of the PS1-KI mouse (Bomba et al., 2013).

The investigation of the mechanisms involved in these cognitive activities revealed a composite picture. Beneficial activities on cognition were not paralleled by drug-dependent effects on peripheral metabolism as treated mice showed no difference in overtime changes in body weight or fasting glycemia compared to vehicle-treated animals (Fig. 2). We also evaluated drug effects on the overall brain metabolic status, as our previous findings in a mouse model of neurodegeneration indicated that exenatide promotes a brain metabolic shift towards anaerobic glycolysis (Bomba et al., 2013). This shift impacts cognition as anaerobic glycolysis, along with the activation of the astrocyte-neuron lactate shuttle system, is intertwined with the modulation of neuronal plasticity and the maintenance of long-term potentiation (LTP) (Bouzier-Sore and Pellerin, 2013; Itoh et al., 2003; Pellerin et al., 1998; Suzuki et al., 2011). However, analysis of the brain anaerobic and aerobic glycolysis of the two study groups showed no exenatide-dependent effects on CNS metabolism (Fig. 3). These results are not in line with the well-known exenatide effects on glucose homeostasis and utilization (Bomba et al., 2013; Drucker et al., 2006; Klonoff et al., 2008). The discrepancy can be explained by taking into account the specificity of our experimental setting. For instance, in our paradigm, anaerobic and aerobic metabolic indices were evaluated in whole brain samples, a setting that may mask changes that occur at subregional and/or intercellular levels (Bomba et al., 2013). Moreover, the

hypoglycemic action of GLP-1 requires an underlying diabetic condition and our animal model, as previously shown (Masciopinto et al., 2012), is devoid of patent metabolic deficits and/or subtle energetic alterations (Samson and Garber, 2013).

A possible mechanism for the cognitive effects exerted by exenatide revolves around the neurotrophic properties of insulin. The hormone acts as a neurotrophic factor, improves synaptic activity and plasticity, positively modulates memory formation and storage, and also promotes neuroprotection (Benedict et al., 2004; Biessels et al., 1996; Dudek et al., 1997; Zhao and Alkon, 2001). In our setting, however, we did not observe an exenatide-driven effect on insulin signalling in the hippocampus (Fig. S1), the brain region primarily investigated by our behavioural task.

In addition, activation of GLP-1Rs increases phosphorylation of CREB protein (Wu et al., 2017), thereby inducing the transcription of critical genes including *BDNF* (Ohtake et al., 2014). Recent *in vitro* evidence has shown that exendin-4 increases BDNF levels in a CREB-dependent manner (Ohtake et al., 2014) and indicates that GLP-1 analogues act as neurotrophic factors (Luciani et al., 2010; Perry et al., 2002). Given the role of GLP-1R agonists in the regulation of neurotrophic signaling, it is conceivable that some of the exenatide-related effects relate to the modulation of synaptic plasticity and the enhancement of a neurotrophic drive (During et al., 2003; Gault and Hölscher, 2008b; Gumuslu et al., 2016; Ohtake et al., 2014).

Lending experimental support to this hypothesis, we found that the drug treatment induces activation of CREB which eventually promotes the elevation of BDNF and TrkB expression levels in the hippocampus (Fig. 4B-D). The idea that CREB is specifically involved in BDNF signalling and LTM originates from findings showing that *BDNF* expression occurs through a CREB-dependent transcriptional mechanism (Pasini et al., 2015; Xu et al., 1998). Our data are in line with these findings and a large number of studies showing that, in the adult hippocampus, the BDNF-TrkB axis plays an essential role in learning (Minichiello, 2009; Minichiello et al., 1999), maintenance of LTP (Minichiello et al., 2002; Patterson et al., 1996), and memory (Bekinschtein et al., 2008). Furthermore, activation of TrkB promotes the downstream induction of pro-survival pathways such as PI3K-Akt, phospholipase C-γ (PLCγ), and ERK (Minichiello, 2009). Further supporting the idea of an exenatide-dependent modulation of the BDNF-TrkB axis, we found that the 2-month treatment increased levels of pERK5, a TrkB-activated kinase implicated in neuronal survival (Liu et al., 2003; Su et al., 2014). Exenatide treatment also increased levels of PSD-95, a post-synaptic density marker associated with learning-related synapse maturation (Chen et al., 2011). Supporting this notion, we observed, in cultured hippocampal neurons, an exenatide-driven increase in dendritic spine density. The phenomenon is reduced by the administration of ANA-12, a TrkB inhibitor (Cazorla et al., 2011). Overall, these findings are in line with evidence indicating that BDNF-TrkB signalling facilitates PSD-95 transport and the structural and functional potentiation of synapses (Ji et al., 2005; Tanaka et al., 2008; Yoshii and Constantine-Paton, 2007).

It should be underlined that pharmacological inhibition of TrkB activity did not entirely prevent exenatide-driven neurotrophic action, thereby opening the possibility that the compound also promotes synaptic pruning through BDNF-independent pathways as previously reported (Acuna-Goycolea and van den Pol, 2004; Liu et al., 2017). This notion is also supported by the cortical effects of the treatment (Fig.4). In the cortex, the beneficial effects of exenatide seem independent of transcriptional activity. On a speculative note, the exenatide effects on the cortex may revolve around insulin-centred mechanisms related to the drug-driven decreased phosphorylation (and thus inactivation) of IRS-1 (Fig. S1).

Surprisingly, exenatide was not found to induce the activation of $ERK_{1,2}$, two kinases that act downstream of the BDNF-TrkB axis (Minichiello, 2009). The discrepancy may be explained by evidence indicating that the two kinases affect STM but not LTM performances (Alonso et al., 2002; Tyler et al., 2002), and the fact that only the latter were found enhanced in the exenatide group (Fig. 1). Moreover, the overall pro-survival role of $pERK_{1,2}$ has been recently questioned as novel findings indicated that $ERK_{1,2}$ activation could also participate in a variety of death-related signaling pathways (Subramaniam and Unsicker, 2010), a dichotomy that depends on the duration of $pERK_{1,2}$ signaling and the net balance in the activation of divergent paths (Subramaniam and Unsicker, 2010).

Neurotrophins are synthesized as pro-neurotrophins. Pro-neurotrophins are immature forms that require being proteolytically cleaved into their mature counterparts and bind with high affinity to p75NTR (Lee et al., 2001). Pro-neurotrophins mediate apoptotic signaling cascades (Dechant and Barde, 2002; Teng et al., 2005) and p75NTR activation induces cell death via $ERK_{1,2}$ (Subramaniam and Unsicker, 2010) and JNK (Numakawa et al., 2010) which, in turn, promote the synthesis of pro-apoptotic factors (Harrington et al., 2004; Kraemer et al., 2014; Lebrun-Julien et al., 2010; Teng et al., 2005). It is, therefore, conceivable that the exenatide-driven increases of BDNF may go along with a reduction of proBDNF levels and an overall reduction of apoptotic signalling. Supporting this idea, we have found that exenatide treatment reduced levels of proBDNF levels, p75NTR, $pERK_{1,2}$ and pJNK (Fig. 4A, 4E-F, 4I-K). Changes in the balance between the amount of mature and immature neurotrophins promote or rescue from neurodegenerative processes (Dechant and Barde, 2002; Meeker and Williams, 2014). Within that conceptual framework, one can therefore speculate that the exenatide-driven positive effects result from the convergent activation of BDNF-dependent neurotrophic pathways as well as the reduction of the detrimental p75NTR-related signaling cascade (Fig. 6).

5. Conclusions

Brain ageing and ageing-related neurodegenerative disorders are posing a significant challenge for health systems worldwide. Most of the therapeutic efforts aimed at counteracting neurodegenerative processes, like the ones occurring in AD or PD, have addressed putative determinants of the diseases, but did neglect pharmacological interventions aimed at restoring or promoting synaptic plasticity. Ageing is

associated with decreased neurotrophic signalling, and recent clinical evidence indicates that modulation of BDNF may slow cognitive decline and protect against dementia (Buchman et al., 2016; Weinstein et al., 2014). Our data support the idea of a strategic role played by exenatide in the modulation of BDNF signalling and memory. Our results indicate that exenatide, or GLP-1 analogues, may exert beneficial effects against age-related cognitive decline. Our findings may have translational implications. In that regard, exenatide can be part of a therapeutic armamentarium targeted to mid-life, a period when optimal levels of neural plasticity are still available, thereby offering the possibility to maximize the effects of pharmacological interventions.

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Author contributions

SLS conceived and designed the experiments and supervised the study. MB performed *in vivo* treatment, behavioural testing, and LDH assay analysis. ES and LMC performed and analyzed COX activity. VC and AC performed and analyzed WB experiments. AG performed primary hippocampal cultures and neuronal transfection. AG and NM acquired and analyzed confocal images. MB, AG, and SLS analyzed and interpreted the data. MB, AG, and SLS wrote the manuscript. All authors discussed, revised, and approved the final version of the manuscript.

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Figure legends

Figure 1. Effects of exenatide or vehicle on Short-Term Memory and Long-Term Memory of adult mice. Memory performances were evaluated, in the two study groups with the MWM test. (A) The pictogram illustrates the experimental setting of the study. (B) The graph illustrates the learning curve of vehicle- (n=12) and exenatide-treated (n=10) mice as assessed upon the 4-day training session. MWM analysis reveals no statistically significant differences in learning performances between the two groups (P>0.05). (C) Representative pictogram of typical MWM probe trials recorded 1.5 h (for STM, left) or 24 h (LTM, right) after the last training session. Traces depict representative paths followed by a mouse trying to reach the original platform location. (D) The bar graph shows exenatide-driven decreases of latency (the time spent to reach the location where the platform used to be) in the LTM (P=0.039) but not the STM trials (P=0.860). (E) The bar graph shows no drug-related changes in the number of crosses (the number of times each mouse crosses the location where the platform used to be) in the STM or LTM trials (P=0.117 and P= 0.313, respectively). (F) The bar graph shows exenatide-driven increases of the time spent in the target quadrant (the quadrant where the platform used to be) in the LTM (P=0.001) but not in the STM trials (P=0.420). (G) The bar graph shows drug-related decreases of the time spent in the opposite quadrant (the quadrant opposed to the one where the platform used to be) in the LTM (P=0.008) but not in the STM trials (P=0.409). (H) The bar graph shows no drug-related changes in swimming speed in the LTM trial (P=0.98). (I) The bar graph shows no drug-related changes in distance travelled in the LTM trial (P=0.89). (I) The bar graph shows no drug-related changes time spent in the remaining Q2 and Q3 quadrants during LTM trial (P=0.37 and P=0.92, respectively). Data show the mean ± standard error of the mean (SEM). "*" indicates P<0.05, "**" indicates P<0.01.

Figure 2. Effects of exenatide or vehicle on metabolism. (A) The graph depicts effects of exenatide or vehicle treatment on body weight (data are expressed as percentage variations compared to baseline). Results show no differences between exenatide and vehicle treatment in terms of time-dependent changes in body weight occurring in the 2-month treatment period (P>0.05). (B) The bar graph shows analysis of fasting glycemia tested in a subset of animals of the two study groups at the end of the 2-month treatment period (n=7 exenatide-treated and n=5 vehicle-treated mice). Results show no differences between exenatide and vehicle treatment in terms of blood glucose concentrations (P>0.05). Data show mean ± SEM.

Figure 3. Effects of exenatide or vehicle on anaerobic and aerobic brain metabolism. (A, B) Bar graphs show effects of exenatide or vehicle treatment on LDH activities in whole brain homogenates. Exenatide

treatment showed no differences for changes in (A) forward LDH ($LDH_{Pyr \rightarrow Lac}$; P=0.267) or (B) reverse LDH activities ($LDH_{Lac \rightarrow Pyr}$; P=0.637) when compared to vehicle. LDH activities are measured as µmol/$mg_{protein}$/min and results are expressed as percentage variations compared to values from vehicle-treated mice used as a control. (C) The bar graph shows effects of exenatide or vehicle treatment on mitochondrial COX activity assessed in whole brain homogenates. Exenatide showed no significant difference compared to vehicle in terms of effects on mitochondrial respiration (P>0.05). Results are reported as nmolO_2/$mg_{protein}$/min. Data show mean ± SEM.

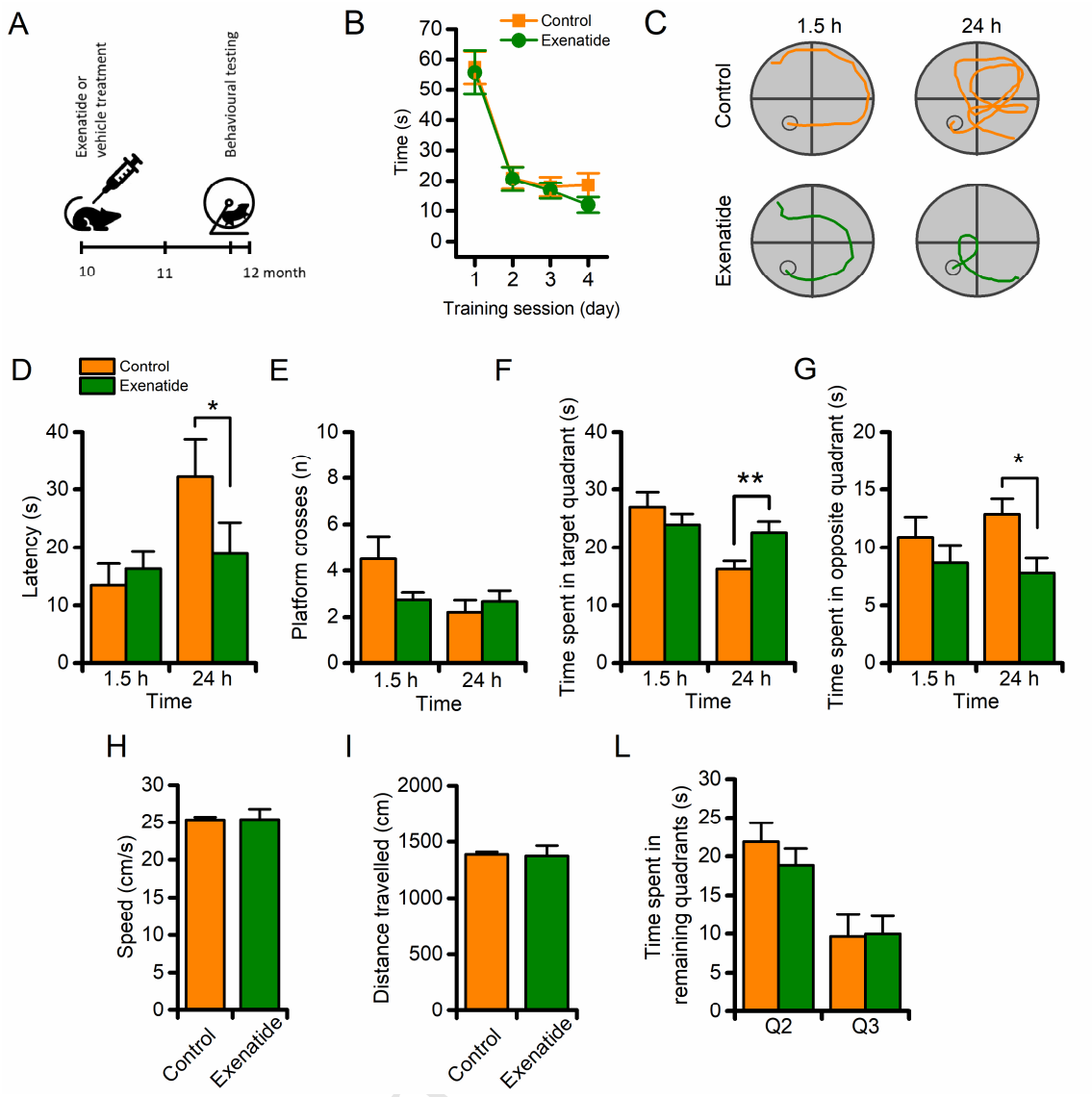
Figure 4. Effects of exenatide or vehicle on BDNF and p75NTR signalling. (A) Western Blots show exenatide- or vehicle-driven effects on BDNF and p75NTR signalling in the hippocampus (Hipp) and in the cortex (Ctx); images are representative of three independent experiments. (B) Bar graphs depict levels of pCREB (Hipp, P=0.0013; Ctx, P=0.15) in the two study groups. (C-D, F) Bar graphs depict expression levels of BDNF (Hipp, P=0.0001; Ctx, P=0.0013) and levels of pTrkB (Hipp, P=0.055; Ctx, P=0.52) or pERK5 (Hipp, P=0.004; Ctx, P=0.0002) in the two study groups. (E) Bar graphs show levels of $pERK_{1,2}$ (Hipp, P=0.06; Ctx, P=0.0079) in the two study groups. (G) Bar graphs show expression levels of PSD95 (Hipp, P=0.001; Ctx, P=0.042) in the two study groups. (H-J) Bar graphs depict expression levels of proBDNF (Hipp, P=0.004; Ctx, P=0.004), p75NTR (Hipp, P=0.0007; Ctx, P=0.0002), or levels of pJNK (Hipp, P=0.0015; Ctx, P=0.0001) in the two study groups. Data show mean ± SEM of relative units (R.U.). "#" indicates P≤0.06, "*" indicates P<0.05, "**" indicates p<0.01, "***" indicates p<0.0005.

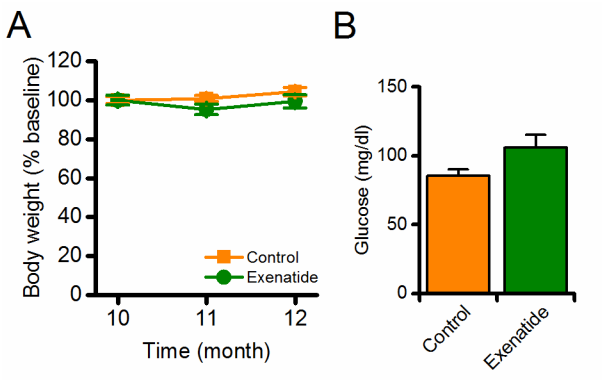
Figure 5. Neurotrophic effects of exenatide is mediated by TrkB signalling *in vitro*. (A) Representative images of dendrites obtained from TurboGFP-transfected hippocampal cultured neurons treated with vehicle (left), exenatide (500 nM; middle), or exenatide + ANA-12 (500 nM + 10 µM, respectively; right). Images are representative of at least three independent experiments. (B) Bar graph depicts quantification of dendritic spine density observed after a 3-day treatment with vehicle (0.33 ± 0.017 spines/µm, n=27), exenatide (0.42 ± 0.027 spines/µm, n=21), or exenatide + ANA-12 (500 nM and 10 µM, respectively; 0.37 ± 0.018 spines/µm, n=32; Control vs exenatide P=0.005, Control vs exenatide + ANA-12, P=0.12). "**" indicates P<0.01.

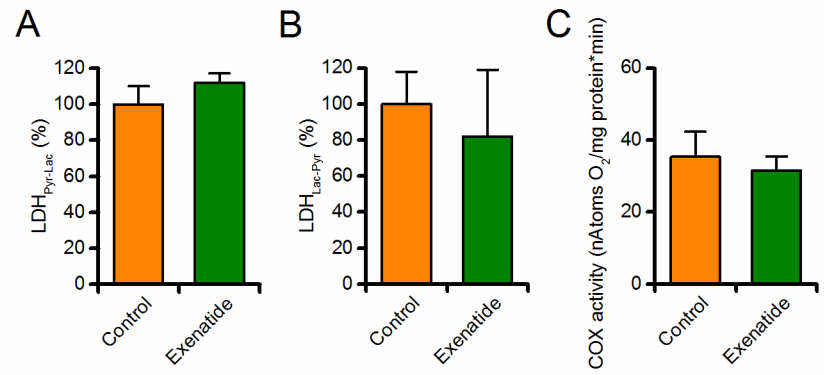
Figure 6. Exenatide-driven modulation of neurotrophic and apoptotic pathways. The pictogram illustrates a schematization of the exenatide-driven effects. Exenatide binds to GLP-1Rs and triggers cAMP elevation which in turn activates the transcription factor CREB. CREB increases BDNF levels, thereby promoting the enhanced activation of the neurotrophic BDNF-TrkB axis. Activation of BDNF-TrkB leads to ERK5 activation, a pro-survival kinase, and to increased expression of PSD95, a protein highly involved in synaptic potentiation. Exenatide also promotes anti-apoptotic effects by reducing the proBDNF-p75NTR mediated

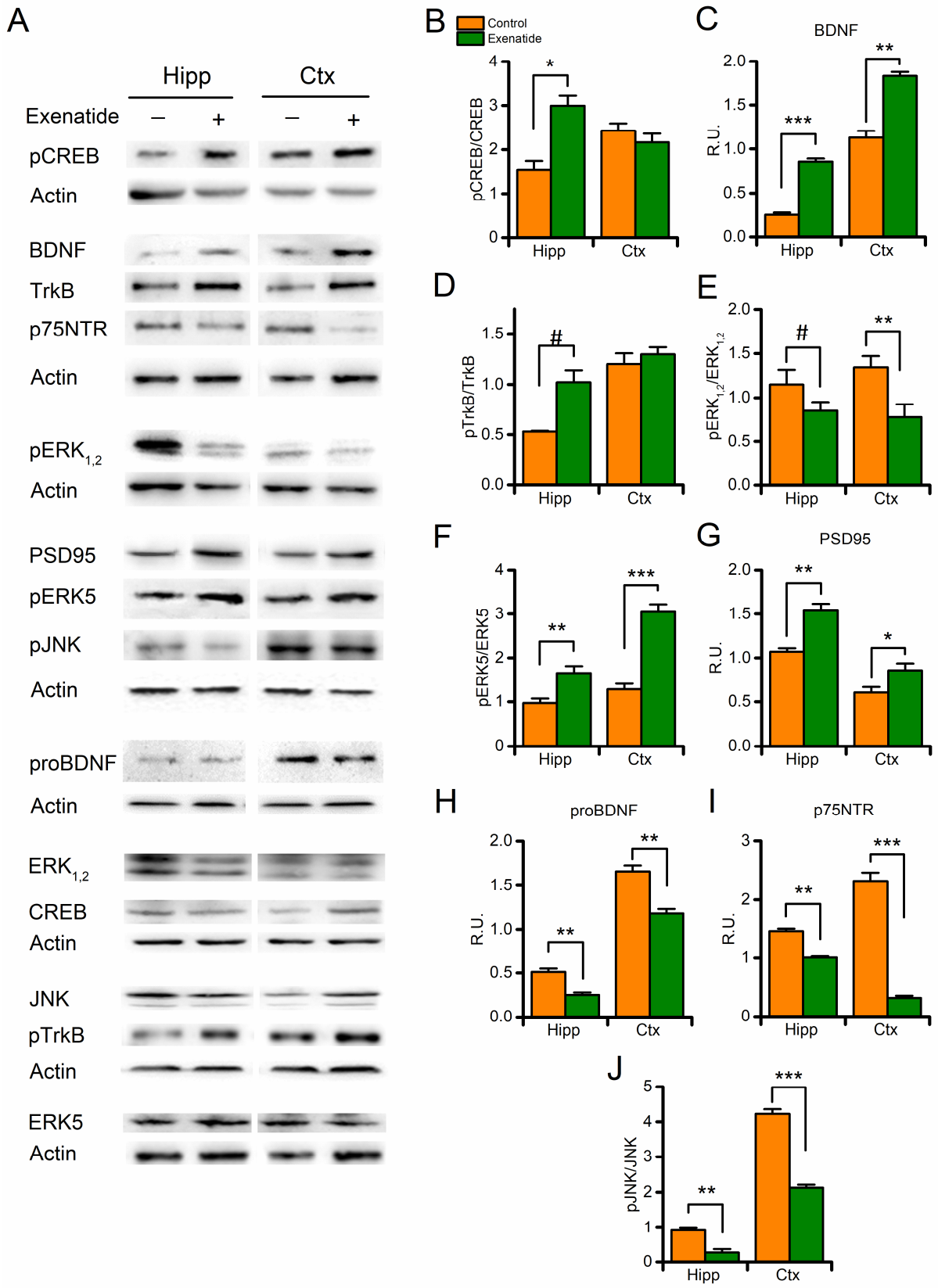
signalling cascade. Reduction in proBDNF-p75NTR signalling results in decreased activation of a set of kinases, $ERK_{1,2}$ and JNK, that participate in neuronal demise.

Figure S1. Effects of exenatide or vehicle on insulin signaling. (A) Western Blots show exenatide- or vehicle-driven effects on IRS1 phosphorylation at Ser1101 in the hippocampus (Hipp) and in the cortex (Ctx). (B) Bar graph depicts levels of pIRS1 (Hipp, P=0.13; Ctx, P=0.0011) in the two study groups. "**" indicates P<0.01.





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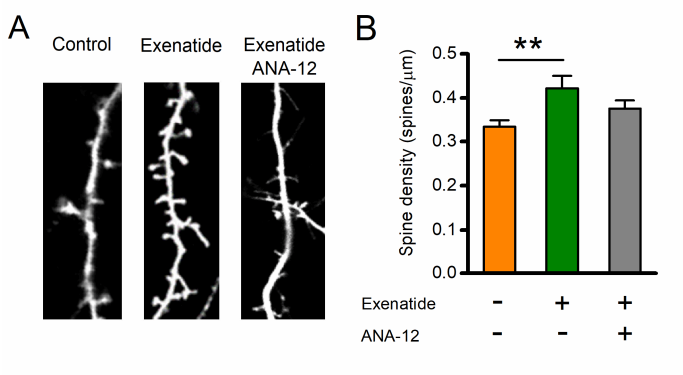

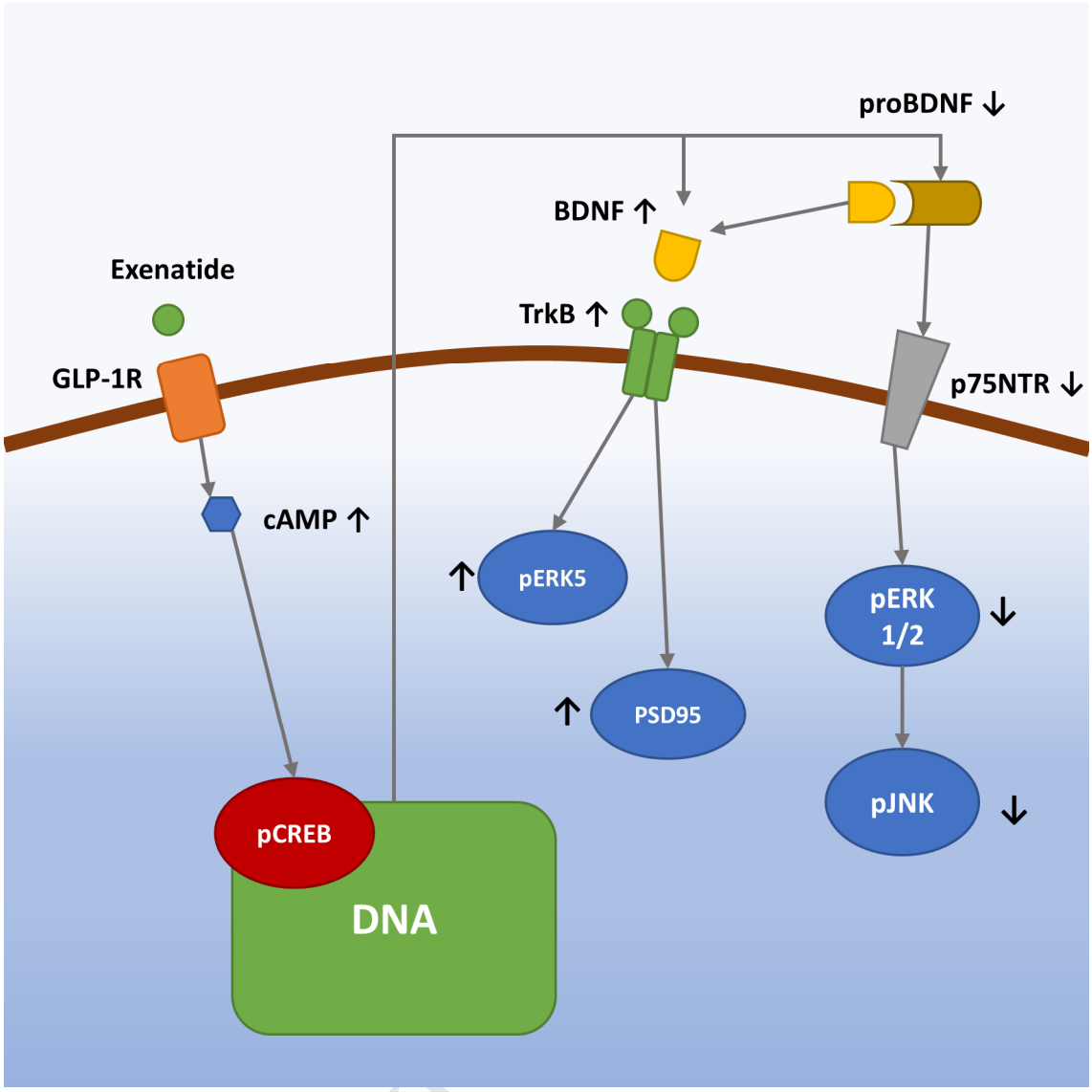
proBDNF ↓
BDNF ↑
Exenatide
TrkB ↑
GLP-1R
p75NTR ↓
cAMP ↑
↑ pERK5
pERK 1/2 ↓
↑ PSD95
pJNK ↓
pCREB
DNA

Highlights

- Brain insulin plays a critical role in neuronal functioning and synaptic plasticity
- Defective insulin signaling participates to cognitive decline and brain aging
- GLP-1 and its synthetic analogs modulate peripheral and brain insulin signaling
- Exenatide, a GLP-1 analog, exerts cognitive effects in a mouse model of mid-life aging
- Exenatide modulates the BDNF-TrkB neurotrophic axis and reduces apoptotic signaling

Verification

1. The authors declare that:

(a) any competing financial interests exist.

(b) any author's institution has contracts or financial interests relating to this research.

(c) any other agreement that could be seen as involving a financial interest in this work exist.

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3. The authors declare that the data contained in the manuscript being submitted have not been previously published, have not been submitted elsewhere and will not be submitted elsewhere while under consideration at Neurobiology of Aging.

4. The authors declare that all the procedures involving the animals and their care were approved by the Institutional Ethics Committee (CEISA protocol no. 17; Min. IDD: DGSAF/14264). Animal handling was performed in accordance with institutional guidelines and in compliance with national and international laws and policies.