THESIS:

TrkA AS A PHARMACOLOGICAL TARGET FOR MODULATION OF MEMORY FORMATION

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ABSTRACT

TrkA is the receptor for Nerve Growth Factor (NGF). Through TrkA, NGF conveys neuronal survival, proliferation, neuritogenesis and modulation of synaptic plasticity. Receptor TrkA decreases proportionally as cognitive deficits increase in Mild Cognitive Impairment, Alzheimer's disease and Down syndrome. Even more so, the decrease in function and density of TrkA precedes cholinergic neuron death in these pathologies. NGF transport is also impaired. These observations have led to the hypothesis that providing agonists of TrkA may rescue the cholinergic neurons, halt disease progression and even reverse symptoms. As endogenous molecules usually do not have desirable pharmacological properties, many advocate for small molecule agonists as potential therapeutics in neurotrophin-related pathologies. Among such small molecules, a compound named D3 has emerged as a promising therapeutic. It is a small, water-soluble, stable, specific partial agonist of TrkA. D3 binds and activates TrkA, and can potentiate the effect of NGF. D3 rescued spatial LTM in memory-impaired, aged rats and improved learning and STM in APP over-expressing mice. Conversely, it impaired LTM in wild type, healthy young mice. This follows a paradigm in which a system which is deficient in a receptor may benefit from an agonist, but a homeostatic system may be impaired by over-excitation. We now elucidate part of the mechanism of action of D3. It increases TrkA phosphorylation in the nuclei basalis and hippocampus, leading to increases in trophic signaling pathways downstream of the receptor. D3 leads to a decrease in basal dendritic arborisation in the CA1 region, possibly through receptor desensitization in the cholinergic basal forebrain neurons. This change in morphology is then translated into a specific, hippocampal-dependent consolidation, spatial memory impairment, sparing learning and different types of declarative memory such as novel object recognition. D3 does not affect habituation or exploration, implying little effects on diverse subtypes of behaviour. It would be important to assess whether the effects reported here, or different ones, are observed in the memory-impaired models. Such studies would enable D3 to progress as a potential therapeutic agent (for a specific patient population) in the treatment of dementia.

RÉSUMÉ

TrkA est le récepteur du facteur de croissance du tissu nerveux (NGF). Par l'entremise de TrkA, NGF promouvoit la survie neuronale, la prolifération, la neuritogenèse et la modulation de la plasticité synaptique. Les niveaux du récepteur TrkA diminuent proportionnellement avec l'amplification des troubles cognitifs dans le déficit cognitif léger, la maladie d'Alzheimer et le syndrome de Down. Plus encore, une diminution en fonction et en densité de TrkA précède la mort des neurones cholinergiques dans ces pathologies. Le transport de NGF est également altéré. Ces observations ont conduit à l'hypothèse que l'administration d'agonistes de TrkA pourrait favoriser la survie des neurones cholinergiques, freiner la progression de la maladie, et même inverser les symptômes. Comme les molécules endogènes ne disposent généralement pas de propriétés pharmacologiques désirables, l'usage de petites molécules comme agonistes potentiellement thérapeutiques pour traiter les pathologies liées aux neurotrophines a été proposé. Parmi ces petites molécules, un composé nommé D3 a émergé comme étant une thérapeutie prometteuse. Il s'agit d'un agoniste partiel de TrkA, stable et spécifique, soluble dans l'eau. D3 lie et active TrkA, et potentialise l'effet de NGF. D3 restaure la MLT spatiale chez les rats âgés et atteints de déficits de mémoire, et améliore l'apprentissage et la MCT chez les souris sur-exprimant la protéine APP. Paradoxalement, D3 altère la MLT chez les jeunes souris saines de type sauvage. Ceci obéit à un paradigme selon lequel un système qui est déficient en un certain récepteur peut bénéficier d'un agoniste, mais un système homéostatique peut être compromis par une surexcitation. Nous élucidons maintenant une partie du mécanisme d'action de D3. Il augmente la phosphorylation de TrkA dans le noyau basal de Meynert et l'hippocampe, conduisant à l'amplification de voies trophiques de signalisation en aval du récepteur. D3 conduit à une diminution de l'arborisation dendritique basale dans la région CA1, possiblement par la désensibilisation des récepteurs dans les neurones cholinergiques du cerveau antérieur basal. Ce changement de morphologie est ensuite traduit en une consolidation qui est dépendante et spécifique de l'hippocampe, ainsi qu'à des troubles de la mémoire spatiale, tout en épargnant l'apprentissage et différents types de mémoire déclarative, telle la mémoire de reconnaissance. D3 ne modifie pas l'accoutumance et l'exploration, ce qui suggère peu d'effets sur les divers sous-types du

comportement. Il serait important d'évaluer si les effets rapportés ici, ou d'autres effets, sont observés dans les modèles animaux de l'amnésie. Ces études permettraient au composé D3 d'être considéré comme un potentiel agent thérapeutique (pour une population spécifique de patients), dans le traitement de la démence.

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PREFACE AND CONTRIBUTION OF AUTHORS

This thesis reports original scholarship led and performed by myself with the collaboration of the following persons.

Mario Hernan Maira Arce collaborated with experimental design and the primary hippocampal cultures.

Iulia Pirvulescu performed the Western Blots on the brains of mice sacrificed after acute D3 treatment, assisted during the intracerebroventricular compound delivery surgeries, and collaborated with the Morris Water Maze and Novel Object Recognition Task.

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INTRODUCTION

Memory

Memory is the mechanism by which the brain receives input from a stimulus, processes it, stores it, and makes it available for later retrieval. There are several classifications of memory, the most conventional being the division between short and long-term memory (STM and LTM respectively). STM refers to information that will be used in the immediate term (minutes or hours). LTM refers to information that is consolidated, stored and retrieved, in longer periods of time (days, weeks, months or, years). LTM can also be divided into explicit or implicit memory. Explicit refers to information that is retrieved consciously whereas implicit refers to an unconscious process (such as priming, skills, habits, etc.) (Buchsbaum and D'Esposito, 2008, Kandel, 2013).

The formation of explicit memory is thought to follow a bidirectional pathway. It is first acquired and processed in one of three polymodal association cortices (prefrontal, limbic and parieto-occipital-temporal). The processed information is relayed to the parahippocampal and perirhinal cortices; then to the entorhinal cortex, dentate gyrus – hippocampus and subiculum for further consolidating. From these areas, the information "flows" back to the entorhinal cortex, the parahippocampal and perirhinal cortices and finally the polymodal association cortices where it is stored (Buchsbaum and D'Esposito, 2008) (Figure 1). It has not been fully elucidated how these circuits work and which brain regions are critical in the different steps of memory formation, but many theories and models have been proposed in this regard. Here, we follow mainly the Relational Theory, in which the hippocampus orchestrates memory acquisition, relations with existing memories and further retrieval upon prompting (Cohen and Eichenbaum, 1993, Bird and Burgess, 2008). A recent publication supports this theory, as well as the (until now hypothesized) electrophysiological changes that take place in the hippocampus during and after memory formation. This group showed how engram cells in the dentate gyrus of the hippocampus are sufficient and necessary for the consolidation and recall of declarative memory. They also showed how memory can be altered by affecting the physiology of these cells, both with protein synthesis inhibitors as with optogenetics (Ryan et al., 2015).

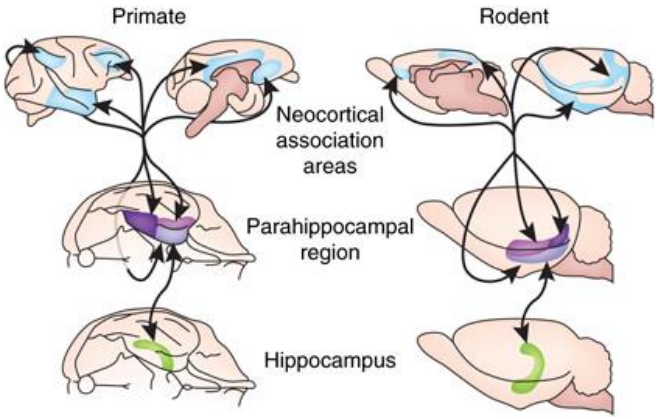


Figure 1. Analogies between primate and rodent memory anatomy. Stimuli are initially perceived in the cortex, undergo hippocampal processing and finally return to the cortical association areas where they are stored (Dickerson and Eichenbaum, 2010).

The hippocampus is a key relay organ for memory processing, consolidation, retention and retrieval. Consolidation mediates the transformation of STM into LTM (Kandel et al., 2014). There have been many studies performed regarding the time-points involved in such processes. Consolidation takes place in the first 24 hours after an event and there are many pharmacological, genetic and behavioural strategies that may be used to interfere with it. Retrieval is also time-sensitive in the sense that memories may be forgotten if they are not retrieved and re-consolidated in a variable time-frame. Even more so, memories may be retrieved and modified before their subsequent consolidation – transforming them into a different memory (Ambrogi Lorenzini et al., 1999, Goshen et al., 2011).

Several types of declarative memory rely on the hippocampus. These may be assessed in the laboratory by working with a vast variety of animal models. In rodents, the most classically tested is spatial memory, usually with the Morris Water Maze (MWM). Studies have shown that by damaging only 30% of the dorsal hippocampus, rats exhibit spatial memory defects. Another form of declarative memory is recognition memory, commonly tested with the Novel Object Recognition (NOR) test. Although it is partially processed in the hippocampus, it also requires the entorhinal and parahippocampal cortices (Eichenbaum, 2003, Broadbent et al., 2004).

Spatial memory has been widely studied. Its basis relies on a specific neuronal population in the hippocampus, called place cells. These cells fire depending on the individual's presence at a determined location in the environment, called a place field.

Place cells are not only influenced by the where, but also by the when and what of a new or known environment. Furthermore, they also keep track of sequences: past, current and future locations, building a map. While the individual maps the surrounding, synaptic plasticity mediates the formation of new and stable place fields. The circuit is thought to involve mainly the entorhinal cortex and the hippocampus (O'Keefe and Conway, 1978, Moser et al., 2015).

Place cells have been proposed to be crucial not only for spatial memory, but also for declarative memory. As an example, a group of humans was exposed to a virtual reality task in which they have to deliver an object to a specific location. When the subjects are asked to remember exclusively the objects, electrodes placed in the medial temporal lobe detect neuronal activity in the place cells corresponding to the places where those objects were delivered (Miller et al., 2013).

In rodents, the task used most frequently to assess spatial memory is the MWM. In this experiment, a mouse or rat is placed in a large water tank containing four orientation cues on its walls and a hidden escape platform. The mouse will initially randomly find the platform, but, over time, learns the visual cues and uses that memory in order to escape faster. The latency is measured as the time the mouse takes to find the platform, and it decreases significantly as the mouse learns and remembers where the platform is. After the learning phase, a probe trial is performed in which the platform is removed and the time that the mouse spends near the position where the platform had been is quantified (Morris, 1984).

Recognition memory is also traditionally tested in animal models. Rodents and primates have an innate preference for novelty. This behavioural feature may be used to assess memory by a simple experiment called NOR. The mice are exposed to a set of identical objects and allowed to explore and familiarize themselves with them. Next, after a variable amount of time, a novel object replaces one of the objects. Due to the novel object preference paradigm, the mice spend more time exploring the novel object, if they remember the familiar one. This type of memory is called recognition memory. It also involves hippocampal plasticity but is not solely dependent on it. The hippocampus is critical for the task only when spatial cues or a complex environment is used. Over

75% of this memory organ must be destroyed for rats to show impairment in the NOR (Dere et al., 2007, Ennaceur, 2010).

So far we have discussed the behaviour and anatomy associated with specific types of memory. But how does this all occur at the molecular and structural level? The main theories revolve around synaptic tagging, Long Term Potentiation (LTP) and Long Term Depression (LTD). They are all based on how neurons are connected to each other in circuits. Every neuron has tens of thousands of connections with other neurons, but each connection is independently regulated. Synaptic tagging refers to a process in which one axon (pre-synaptic) is lightly stimulated and consecutively a matching post-synaptic dendrite is strongly stimulated. These events release a series of downstream signalling pathways, with protein translation, structural actin changes, increases in kinase activity, receptor phosphorylation and receptor translocation to the pre and post-synaptic density – all of which collide in the strengthening of that specific connection (Martin and Kosik, 2002). In experimental terms, the process has been widely studied by means of LTP and LTD. LTP happens when there is strong stimulation and a resulting strengthening of the connection, whereas LTD occurs when there is a milder stimulation and corresponding weakening (Malenka and Bear, 2004).

This strengthening and weakening of connections are the basis of synaptic plasticity (Ruan et al., 2006). Synaptic plasticity refers to the concept that the nervous system is not static; it can make, destroy and reform the connections required for learning, forgetting and modulation of memories. Such plasticity depends on many factors, including spine density, spine morphology and dendrite arborisation (Drapeau and Nora Abrous, 2008). Spines can vary in quality and quantity: the greater the spine density, the greater the number of connections (excitatory and/or inhibitory); the larger the spine volume, the greater synaptic strength (Harms and Dunaevsky, 2007); and the greater the dendritic arborisation, the greater differentiation and connectivity (Roth et al., 2010). Synaptic plasticity is susceptible to factors such as the immune system (Richwine et al., 2008), exercise (Voss et al., 2013), and, relevant to this paper, pharmacological agents.

NGF and TrkA

All organs require growth factors to ensure cell survival and proper functioning, and the nervous system is no exception. Neurotrophins are the growth factors that promote neuronal survival and homeostasis. The first neurotrophin to be described was Nerve Growth Factor (NGF), by Rita Levi-Montalcini and Viktor Hamburger at the beginning of the 20th Century. This was followed by the discovery of Brain Derived Neurotrophic Factor, NT3 and NT4/5 (Bothwell, 2014, Lewin and Carter, 2014).

In the brain, NGF is made in the cortex, basal forebrain and in the hippocampus. It is produced mainly by neurons, but also by astrocytes and microglia throughout the nervous system. NGF binds to two receptors: TrkA and p75 (Niewiadomska et al., 2011). Different from the peripheral nervous system, the NGF-TrkA interaction in the central nervous system goes beyond survival: it also involves growth, differentiation and strengthening of synaptic transmission (Deinhardt and Chao, 2014).

Within the “memory pathway”, TrkA receptors are expressed in the basal forebrain cholinergic neurons (BFCN), which innervate the cortex and hippocampus (Niewiadomska et al., 2011). These TrkA^{+} cholinergic fibers play a key role in hippocampal neuronal survival and normal functioning, leading to normal hippocampal-dependent memory (Magno et al., 2011).

When NGF binds to TrkA, the receptor activates its kinase and triggers the phosphorylation of tyrosine residues (Y490 and Y785) adaptors for signaling cascades (Deinhardt and Chao, 2014). Through TrkA, NGF conveys neuronal survival, neuritogenesis, regulation of cell proliferation, cytoskeleton remodeling and assembly, and modulation of synaptic plasticity (Maliartchouk et al., 2000, Aboulkassim et al., 2011, Niewiadomska et al., 2011). Receptor p75 has classically been seen as pro-apoptotic since it does not contain a catalytic motif. Nonetheless, it is currently understood that p75 is also important for neuronal survival, differentiation and synaptic plasticity (Niewiadomska et al., 2011).

Memory and TrkA

As the population ages, memory-related diseases continue to increase in incidence and prevalence. Alzheimer's disease (AD) is the most common type of dementia, and its aetiology and pathophysiology remain elusive. Furthermore, there is no effective treatment, either to halt or to reverse the disease. There are currently many targets being studied for potential therapeutics in AD. In this regard, we will focus on the receptor TrkA.

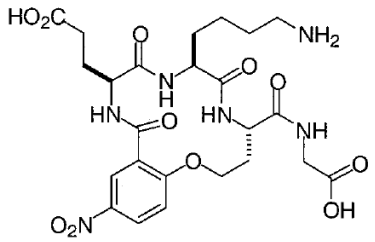
TrkA density and activity decrease as disease progresses in AD (Counts et al., 2004), mild cognitive impairment (Mufson et al., 2000), and Down syndrome (Sendera et al., 2000). The same decrease was found in aged, memory-impaired rats (Saragovi, 2005). Interestingly, receptor down-regulation preceded cholinergic neuron death (Mufson et al., 2000). One of the few features in which there is a consensus regarding AD pathology is the death of the cholinergic neurons. Such cholinergic neuron death underlies the memory-impairment phenotype of AD (Duan et al., 2014). Impaired NGF transport in AD is also postulated as one of the causes for cholinergic neuron death (Mufson et al., 1995, Scott et al., 1995). Consequently, we can exploit the possibility of rescuing $TrkA^{+}$ cholinergic neurons using agonists in order to prevent neuronal death.

NGF and other neurotrophins have been tested in clinical trials but have failed, due to their lack of specificity and poor pharmacokinetics. NGF, when administered intraventricularly to AD patients in order to improve cognition, conveyed lumbar pain and weight loss that necessitated repeatedly decreasing the dose administered (Eriksdotter Jonhagen et al., 1998). Due to the poor pharmacological properties of the endogenous ligands, synthetic specific agonists and antagonists are excellent alternatives. Small-molecule mimetics of NGF may agonize TrkA without binding or activating receptor p75. They also have improved pharmacokinetics as compared to NGF, which ultimately makes them better therapeutic candidates (Saragovi and Zaccaro, 2002, Longo and Massa, 2013).

A Small-Molecule Agonist of TrkA: Compound D3

TrkA activation by NGF conveys neurotrophic signals that promote neuronal survival, differentiation and neurogenesis. In 1999, the Saragovi laboratory designed a peptidomimetic agonist ligand of TrkA, termed D3. The ligand is a small, water-soluble, stable, specific partial agonist of TrkA. D3 binds and activates TrkA, and can potentiate the action of NGF (Maliartchouk et al., 2000).

The design of compound D3 was based on the pharmacophores of a human TrkA specific antibody (5C3) and several NGF peptide analogues. By screening these compounds, D3 was identified as a small, specific, stable, high-affinity agonist of TrkA (Figure 2). D3 binds to the Ig-like C2 region of the extracellular domain of TrkA at a site different than NGF. This prevents D3 from interfering with the binding and activating of TrkA by NGF (Maliartchouk et al., 2000).



D3

Figure 2. Compound D3 selectively activates receptor TrkA. Molecular structure of the D3 peptidomimetic.

The effects of D3 were initially tested *in vitro*, where it selectively enhanced the trophic protection mediated by TrkA and induced differentiation in dorsal root ganglia and fetal septal neuron cultures (Maliartchouk et al., 2000). When tested *in vivo*, D3 rescued spatial LTM in memory-impaired aged rats (Bruno et al., 2004). Equally, on a different memory impairment model (the APP over-expressing mice) it improved learning and STM. Conversely, it impaired LTM in wild type, healthy, young mice (Aboulkassim et al., 2011). This leads to the central question in my research project: what is the mechanism of action of D3? In particular, how does D3 convey improvement in the memory-impaired models but impairment in healthy mice? We believe this is due to trophic signaling pathways being activated and their effects on the hippocampus. Changes in hippocampal neurogenesis and/or changes in hippocampal neuronal differentiation

could benefit the memory-impaired models but could cause disarray in the healthy, young, wild type mice. New neurons in the hippocampus have increased synaptic plasticity which can overwrite existing connections; increasing the synaptic plasticity of existing neurons may have the same effect. On the contrary, these effects may benefit the memory-impaired models since they have decreased neurogenesis and synaptogenesis which could be compensated by D3. A decrease in neurogenesis or neuronal differentiation could also explain the memory impairment in the healthy, young mice; but in this case an opposing increase may be seen in the memory-impaired model due to compensation of their ligand and receptor deficiencies.

TrkA Signalling and LTM

In the quest to answer our research question, we studied proteins that may be downstream of D3 treatment (most likely TrkA-mediated) and have been shown to play a role in LTM. Activation of TrkA mainly involves three signaling pathways: Ras/Raf/Mitogen-activated protein kinase (MAPK), Phosphoinositide phospholipase C γ, and Phosphoinositide 3-kinase (PI3K)/Akt (Niewiadomska et al., 2011). From these pathways, several molecules have been demonstrated to play a role in LTM. We focused on and will further explain the following: MAPK, Extra-cellular-signal-regulated Kinase 5 (Erk5), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), Akt, cAMP response element-binding protein (CREB), Protein kinase C-ζ (PKCζ) and Protein Kinase M-ζ (PKMζ) (Figure 3).

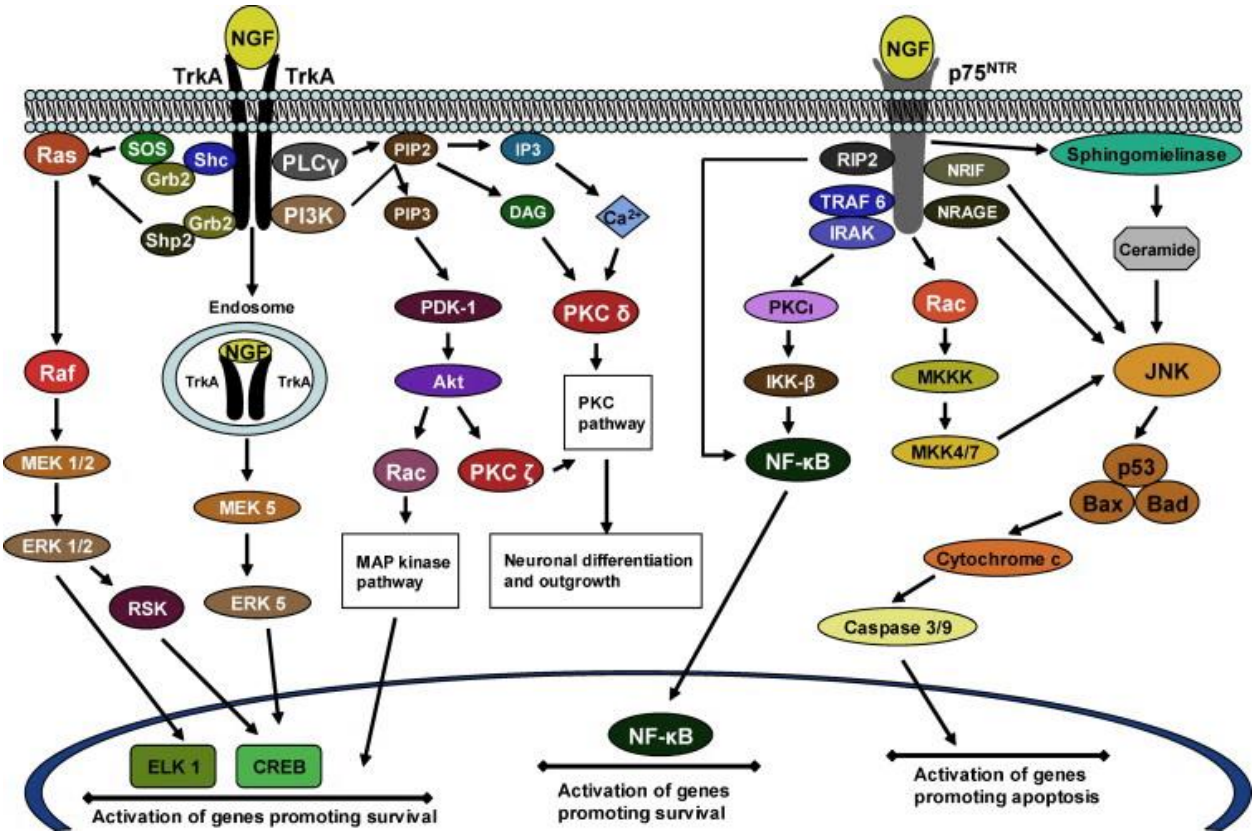


Figure 3. TrkA and p75 signalling pathways. The three main pathways downstream of TrkA correspond to MAPK, PLC γ and PI3K/Akt. Downstream of the TrkA-NGF endosome is Erk5. ELK1 and CREB are activated later promoting neuronal survival and differentiation, as final substrates of the activation of TrkA (Niewiadomska et al., 2011).

MAPK MAPK is a signaling molecule crucial for hippocampal-dependent LTM consolidation. In one pathway, synaptic activity increases intracellular calcium, which, through different kinases, activates MAPK. MAPK is then translocated to the nucleus where it triggers CREB phosphorylation and consequent gene transcription (Xia and Storm, 2012).

MAPK forms part of a rapid-activation system of TrkA by NGF. Whereas TrkA-NGF endosomes take longer to be translocated to the neuronal soma, intermediate signaling molecules such as MAPK deliver the signal within minutes to the cell body. In aged rats, there is a decrease in both TrkA and MAPK activation. Furthermore, there is a

correlation between the amount of p-MAPK and the strategy used by aged rats in the MWM (Williams et al., 2006).

Erk5 Another extra-cellular-signal-regulated kinase (Erk) is Erk5, which is also NGF/TrkA-induced. It promotes neuronal proliferation during embryonic development as well as adult neurogenesis in the subventricular zone and the dentate gyrus. When the TrkA receptor is stimulated at a distal axon, the ligand and receptor undergo endocytosis and phosphorylate MEK-5, which in turn phosphorylates Erk-5, leading to CREB Ser133 phosphorylation by Rsk. This pathway is exclusively mediated by Erk5. In contrast, when a TrkA receptor is stimulated in the neuronal soma, it also triggers MAPK-mediated CREB phosphorylation (Watson et al., 2001).

Inhibiting or knocking out Erk5 impairs hippocampal neurogenesis and hippocampal-dependent memory. Consistently, over-expression of Erk-5 increased hippocampal neurogenesis and extension of long-term spatial memory (Pan et al., 2013). The same group, in a more recent publication, showed how a MEK-5 knock-in exhibited significantly more dendrites, dendrite crossings and spine density than controls — proving also Erk5's role in neurito- and synaptogenesis (Wang et al., 2014).

Akt When TrkA is activated, it phosphorylates and forms a complex with PI3K. PI3K indirectly activates the serine/threonine kinase Akt — another signalling molecule which then "turns on" the MAPK and PKC pathways (Niewiadomska et al., 2011).

Akt phosphorylates AMPA receptors, increasing their density at the post-synaptic membrane and leading to increased synaptic strength (Wang et al., 2003). Akt has also been shown to play a central role in neuronal survival downstream of neurotrophic factors (Hers et al., 2011).

CaMKII CaMKII is found at the post-synaptic density and is necessary for the initiation of LTM in the hippocampus. CaMKII remains anchored at the spines and is continuously available for incoming stimuli. LTP induction triggers calcium influx, which binds to calmodulin which in turn activates CaMKII; it then self-phosphorylates and becomes an autonomous kinase. Calcium influx also induces the association between CaMKII and the NMDA receptors. CaMKII, through S831 phosphorylation of GluA1,

increases conductance through AMPA receptors. CaMKII isoforms α and β are primarily expressed in the brain. Enhanced activity promotes the α isoform, whereas decreased activity enhances the β isoform (Lucchesi et al., 2011, Souza et al., 2012, Sanhueza and Lisman, 2013, Hell, 2014).

CREB LTM requires gene transcription and protein synthesis. In this aspect, the transcription factor CREB plays a crucial role. It is phosphorylated at Ser133 by many different protein kinases, and thereafter controls the transcription of genes corresponding to structural proteins, signaling factors and growth factors. It has proved to be indispensable for learning and memory (Kandel, 2012, Souza et al., 2012).

The genes expressed through CREB remain available at the synaptic terminals awaiting activation. In this manner, CREB acts upstream and downstream of synaptic tagging, conveying time and place specificity to memory (Kandel et al., 2014).

PKCζ When thinking about the molecular mechanisms of LTM, several groups have postulated a role for proteins, like CaMKII, once active, can remain so indefinitely. Another protein to be considered in this respect is the PKC isoform PKMζ. PKMζ is synthesized via transcription starting at a specific intron within the PKCζ gene and lacks the regulatory domain of PKC. Under basal conditions, PKC is maintained inactive by the interaction of its catalytic C-terminal domain and its inhibitory N-terminal domain. In LTP, the influx of Ca^{+2} binds to the regulatory domain, thereby activating the enzyme (Sacktor, 2008, Bougie et al., 2009, Sacktor, 2011). PKMζ, through continuous phosphorylation, doubles the amount of post-synaptic AMPAR and therefore perpetuates LTP and maintains LTM. During LTP induction, NMDAR, CaMKII, PI3K, MAPK, PKA and mammalian target of rapamycin (mTOR) are required to release the translational block on PKMζ synthesis (Sacktor, 2011).

Many of the initial studies were based on ZIP, an inhibitor of PKMζ. Several groups have debated the essential presence of PKMζ, since a knockout mouse does not exhibit the same deficits as those mice treated with ZIP (Lee et al., 2013, Volk et al., 2013). This has been proposed to be due to lack of specificity of the inhibitor. It may also block

another atypical PKC, the PKCι, which could act as a compensatory mechanism in the mouse PKMζ knockouts.

We started unveiling the mechanism of action of D3 by exploring it in healthy, wild type mice (with memory impairment). We focused on these questions: what types of memory, and which processes within memory formation, does D3 affect? Biochemical, neurophysiological, and anatomical methods were applied. For further studies, we aim to assess whether the same mechanisms of action apply to both the healthy and the memory-impaired models.

MATERIALS AND METHODS

1. Mice

C57BL/6 wild-type male mice, 4-6 months old, were obtained from Charles River or Harlan Laboratories. All animal procedures respected the American Association for Laboratory Animal Science guidelines for use of animals in research, and all protocols were approved by the McGill University Animal Welfare Committees. All animals were housed on 12-hour dark-light cycle with food and water ad libitum. The mice used for the Novel Object Recognition tests were housed in inverse cycle facilities. Female C57BL/6 mice were ordered from Charles River for timed pregnancies (E15). A Thy-1 GFP line M mouse was obtained from Dr. David Stellwagen from McGill University, and then bread with C57BL/6 wild-type females obtained from Jackson Labs. From the offspring, the male Thy-1 GFP were used for the acute ICV experiments, and the females for the 2-week compound delivery.

2. Primary Hippocampal Cultures

Cultures were performed based on existing publications (Jones et al., 2012). Briefly, in sterile conditions, hippocampi of E15 C57BL/6 mice were dissected in HHGN (HBSS, Hepes, Glucose) on ice. Trypsin was added to a final concentration of 0.125% and placed at 37^{o}C for 20 minutes. Fetal bovine serum 1/10 dilution was used to stop trypsinization. Cells were spun, re-suspended in culture medium (Neurobasal supplemented with B27, penicillin/streptomycin and Glutamax), triturated, counted and plated at a 20 000 – 50 000 cells/well density.

The cultures were treated with NGF at 2 nM and 500 pM, D3 at 10 μM and 1 μM, or controls, replacing the medium twice a week. After 2 weeks, the cultures were stopped, washed with PBS, fixed with 4% paraformaldehyde at room temperature for 10 minutes, and membranes were permeabilized by incubating with 0.2% Triton X-100 PBS. Blocking was performed with 3% bovine serum albumin (BSA) and 5% normal goat serum in 0.2% Triton X-100 PBS. Primary antibodies: MAP2 (1:1000 dilution, Chemicon AB5622) and Ki-67 (1:100 dilution, BD pharmingen 556003) were incubated overnight

at 4^{o}C. Secondary antibodies 488 goat anti-rabbit and 594 goat anti-mouse were incubated at a dilution of 1:1000 at room temperature for 45 minutes.

3. Intracerebroventricular (ICV) Compound Delivery

Vehicle (aCSF) and D3 were delivered in two routes: ICV acute injection for the acute delivery studies and Alzet osmotic pumps for the 2-week delivery studies.

a. Acute ICV Injections

An adapted protocol from (DeVos and Miller, 2013) was used. Briefly, mice were anesthetized with isoflurane and kept warm with bubble wrap and a heat lamp when necessary. The mice were fixed in a stereotactic frame (Kopf Instruments), their hair was cut or shaved and the skin was cleaned with chlorhexidine. Carprofen was administered subcutaneously as analgesia and artificial tears were used to protect their eyes. An incision was made at the midline. A small hole was drilled at 0.46 mm posteriorly and 1 mm laterally from bregma. A Hamilton syringe connected to a microinjection unit was inserted 2 mm vertically in the brain targeting the lateral ventricle. 5 minutes were allowed for the compound or artificial cerebrospinal fluid (aCSF: 150mM NaCl, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 2 mM K_2HPO_4, 10mM glucose, 0.001% mouse serum) to diffuse before extracting the Hamilton syringe. Either 9 μg of D3, 15 μg of D3, or the equivalent volume of vehicle (aCSF) was injected for a maximum of 5 μL. The wound was closed with stitches and surgical glue, and polysporin was placed on the outside of the wound. Mice were allowed to recover in an incubator at 34^{o}C with wet food and water in the cage. They were monitored and injected with carprofen for the next two days following the surgery.

b. Alzet Osmotic Pumps

A modified protocol from (DeVos and Miller, 2013) and (Aboulkassim et al., 2011) was used. Briefly, Alzet pumps were loaded with 100 μL of either D3 (corresponding to 40μg) or aCSF. They were primed in sterile PBS overnight at 37^{o}C. The cannula was placed with a cannula holder (Kopf Instruments) at the same coordinates as described

above (targeting the right lateral ventricle) and glued with Loctite superglue. The pump was positioned subcutaneously on the back of the mice.

4. Western Blots

Hippocampus, cortex and nuclei basalis were dissected and analyzed separately. Whole cell lysates were prepared in ice-cold lysis buffer (20 nM Tris HCl pH 7.5, 137 mM NaCl, 2 mM EDTA pH 8, 4% NP40 detergent, ROCHE protease and phosphatase inhibitors). Samples were reduced with beta-mercaptoethanol and resolved by tricine/SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto activated PVDF membranes and blocked with BSA in Tris-Buffered Saline/Tween 20 (TBST). Primary antibodies at the following dilutions were incubated at 4^{o}C overnight. pCREB 1:1500 (Cell Signaling 9198 or Millipore 556003), CREB 1:1500 (Cell Signaling 9104), PKCζ 1:1000 (Invitrogen 38-1400), pMAPK 1:2000 (Cell Signaling 4370), MAPK 1:2000 (Cell Signaling 4695), pCaMKII 1:1000 (Cell Signaling 3361), CaMKII 1:3000 (Cell Signaling 4436), Actin 1:4000 (SIGMA A2066), pAkt 1:1000 (Cell Signaling 4060), Akt 1:1000 (Cell Signaling 9272), pErk5 1:1000 (Millipore 07-507), Erk5 1:1000 (Cell Signaling 3372), β-Tubulin-III 1:10 000 (SIGMA T2200). Horseradish peroxidase-conjugated secondary antibodies were incubated at 1:10 000 for one hour at room temperature. Proteins were visualized through enhanced chemiluminescence.

5. Immunoprecipitation

As per Bonifacino et al. (2001), whole cell lysates were left shaking overnight at 4^{o}C with TrkA antibody (Santa Cruz sc-118). Next, agarose beads were added and left shaking at 4^{o}C for 4 hours. Washes were done with lysis buffer and the proteins were eluted with loading buffer containing mercaptoethanol and boiling. Samples were resolved and transferred following the same Western Blotting procedures. Membranes were initially blotted for 4G10 (pan-phosphotyrosine antibody, Upstate 16-103) and then for total TrkA.

6. BrdU Delivery and Analysis

BrdU was delivered at a concentration of 1 mg/mL in 1% glucose in drinking water (UCSF IACUC 2009, http://www.iacuc.ucsf.edu/Policies/awSPBrdu.asp). The fluid each mouse drank was quantified to ensure there were no differences in consumption among them. No differences were observed between D3 and the controls. BrdU was administered for the 2 weeks that the mice were being treated with D3 or aCSF. The mice were then perfused and their brains were fixed by submerging in 4% PFA for 2 hours at 4^{o}C. Next, they were submerged for 5 days in 30% sucrose, changing the solution twice in this time interval. The brains were placed in OCT and left at -80^{o}C at least overnight.

The brains were cryo-sectioned into 12 μm thick slices using a LEICA cm 3050s cryostat. BrdU was exposed by submerging slides in 1N HCl at 45^{o}C for 30 minutes. Membranes were permeabilized with 0.4% Triton X-100 PBS for 15 minutes. Tissues were blocked with 5% NGS/3% BSA for 1 hour at room temperature. Slides were incubated with BrdU antibody 1:300 (abcam ab6326) and NeuN 1:500 (mouse, Millipore mab-N78) in 0.2% Triton X-100 PBS overnight at 4^{o}C. Washes were made with 0.2% Triton X-100 PBS. Secondary antibodies (goat anti-rat FITC 1:1000 and goat anti-mouse Alexa 594 1:1000) were incubated in 0.2% Triton X-100 PBS at room temperature for 45 minutes and washed. Sections were covered with VECTASHIELD hardset mounting medium and visualized under an epifluorescence microscope. Approximately 30 sections per brain were quantified.

7. Neuronal Branching and Spine Density Analysis

Thy-1 GFP line M mice were used to assess neuronal branching, spine density and spine morphology (Feng et al., 2000). Two treatment paradigms were considered: acute ICV injections and 2-week delivery with an Alzet osmotic pump. From the offspring of the Thy-1 mouse, male mice, on average 4 months old, were used for assessments post-ICV injections. Whereas female mice, on average 5 months old, were used for the 2-week treatment.

Treatments were administered as described above (see section Intracerebroventricular Compound Delivery).

The mice that were treated with acute ICV injections also underwent MWM in order to confirm behavioral impairment caused by the compound. These mice were sacrificed 2 weeks after treatment, after the second probe trial of the MWM. The mice that were treated with D3 for two weeks were sacrificed immediately after treatment completion.

The mice were perfused and the brains were dissected and fixed as described above (BrdU Delivery and Analysis Section). The brains were cryo-sectioned into 50 μm thick slices using a LEICA cm 3050s cryostat. The slides were then covered with VECTASHIELD antifade mounting medium with DAPI. Confocal images were then obtained at a magnification of 63X plus a 5X digital zoom for the spine analyses.

The Imaris software (http://www.bitplane.com/) was used to perform the analysis. Filament tracing tools were used to determine total branching points and Sholl analysis on basal and apical branching of neurons in the CA1 and CA3 region of the hippocampus. The guidelines established in Swanger et al. (2011) were followed in order to quantify spine density, mean spine length and mean spine volume in tertiary dendrites in the same hippocampal regions.

Measurements of arborisation were performed by analyzing branching points, Sholl intersections and mean Sholl intersections. Spines were analyzed forspine density, mean spine volume and mean spine length. Each treatment paradigm was analyzed separately. The number of mice in each group was 4 pump-controls, 5 pump-D3s, 4 ICV controls and 3 ICV D3s. The differences in n derive from lack of expression of GFP in some of the mice which precluded their use. At least 2 neurons/mouse were quantified in the CA1 region and at least 1 neuron/mouse in the CA3 region.

8. Electrophysiological Recording in Hippocampal Slices.

As per (Aboulkassim et al., 2011), groups of vehicle-treated and D3-40-treated mice were used, and hippocampal slices were prepared 6 days after acute D3 ICV injection. In brief, under deep anesthesia, brains were rapidly removed, and coronal slices (350

μm thickness) were cut in hyperosmotic, ice-cold, and carbogenated (bubbled by 95% O2/5% CO_2 to maintain the pH at 7.4) solution (252 mM sucrose, 2.5 mM KCl, 4 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1.25 mM KH_2PO_4, 26 mM $NaHCO_3$ and 10 mM glucose) using a Vibratome. Freshly cut slices were placed in an incubating chamber with carbogenated aCSF (~310 mOsM) consisting of 125 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, and 25 mM glucose. Slices were recovered at 32°C for 1 h and subsequently maintained at room temperature. Carbogenated aCSF containing bicuculline methobromide (5 μM) to block GABAA receptor-mediated inhibitory synaptic currents was used to perfuse slices in all recordings. Postsynaptic responses, evoked by stimulating the Schaffer collateral-commissural pathway via constant current pulses (0.08 ms) delivered through a tungsten bipolar electrode were recorded from the hippocampal CA1 region, amplified by a Multiclamp 700B, and stored in a personal computer for offline analysis using Clampfit (Molecular Devices, Sunnyvale, CA). Field excitatory postsynaptic potential (fEPSP) was evoked at 0.05 Hz and detected by an aCSF-filled glass electrode placed in the stratum radiatum of the hippocampal CA1 region. Long-term plasticity of fEPSP such as long-term potentiation (LTP) was induced by high-frequency (100 Hz, 100 pulses) tetanus. Recording and analysis of electrophysiological data were performed blind to the identity and treatment of the mice.

9. Morris Water Maze

As previously described (Aboulkassim et al., 2011), the MWM consisted in an 8-day training in a water tank, divided virtually into four quadrants landmarked with visual cues on the surrounding wall, and filled with opaque water. In the first 3 days of training, the platform was held visible in one of the quadrants, 1 cm above the water-level with a dark cue on top, to exclude animals with visual or motor deficits. After these three days, the mice were treated with aCSF or D3 at 9 μg or 15 μg in aCSF. They were allowed to rest and recover for one day. For the next five days (learning), the visual cues were changed position only at the start, the platform was placed in a different quadrant and was submerged 1 cm below the water level. Each day, escape latencies were recorded from three different directions for each mouse, with an intertrial time not exceeding 45

minutes. On the last day of training, a minimum of 2 hours were allowed for the mice to rest, after which the platform was removed and the mice were allowed to swim freely for 60 s (probe trial 1, STM). One week later, the groups underwent a second probe trial (probe trial 2, LTM). Both probe trials were quantified as the percentage of the time the mice spent swimming in the target quadrant (where the platform had been).

This task, starting at the learning phase, was repeated at 2 and 3 months after treatment.

HVS Image Software for Morris Water Maze was used to quantify the latencies and tracking during the probe trials.

10. Novel Object Recognition

Based on an existing protocol (Leger et al., 2013), a square 80 x 80 cm transparent box was used to perform the tests. The HVS Image Open Field was used to track the mice and exploration was manually recorded. Exploration (hits) was defined as approximation and nose-poke, backing up, sniffing and grabbing the objects.

Mice underwent intra-cerebroventricular injections and were allowed to rest and recover for one day. Next, they were individually allowed to habituate to the apparatus described above. The distance traveled by the mice, time they spent moving, percentage of the field they covered and number of entries to the different sections in the apparatus were recorded. After habituation, the test phase corresponded to 2 days. On the first day, the mice were exposed to two identical objects (randomly assigned pair) on opposite corners of the apparatus. The objects corresponded to a tower of blocks and a cell-culture flask filled with sand. The objects were previously tested in naïve mice in order to rule out object preference. The test mice were allowed to explore the objects for five minutes and two blinded researchers manually counted the amount of times each mouse explored each object. The second day, this task was repeated with the same two objects. Three hours after the latter, the mice were exposed to a novel object, randomly replacing one of the two previous objects. The same manual quantification was performed.

11. Imaging

Imaging work was performed at the Lady Davis Research Institute Cell Imaging Facility.

Epifluorescence images were collected using the Leica DM LB 2 microscope equipped with the LAS acquisition software and a Leica DFC480 camera for detection.

Confocal images for the *in vitro* studies were collected using a Leica DMI6000 B microscope equipped with the Quorum technologies WaveFX spinning disk confocal microscopy system the Volocity software, and a high dynamic ImagEM EM-CCD camera for detection.

Confocal images for the differentiation studies were collected using a Zeiss Axiovert 200M inverted microscope equipped with the LSM 5 Pascal point laser module, the LSM AIM acquisition software, and 2 PMT detectors for spectral detection.

Analysis and quantification were performed with the Image J, Volocity and Imaris softwares.

12. Statistical Analysis

All measures are reported as mean ± SEM. All measurements were assessed for normality using the Shapiro-Wilks test. All comparisons between two groups were performed with unpaired Student's t-test; all comparisons between three or more groups were performed with between/within analysis of variance and Newman-Keuls post-hoc pairwise comparison tests. $p \leq 0.05$ was considered significant.

RESULTS

D3 does not alter recognition memory

Mice were treated through acute ICV injections, either with aCSF vehicle (n=6) or 15 μg of D3 in aCSF (n=7). The mice were allowed to habituate to the experimental setting. When they were exposed to identical objects in the familiarization stage, the controls and the D3-treated mice explored both objects equally. In the novel object trial, both groups spent a significantly greater amount of time exploring the new object ($p<0.01$). No differences were observed between the vehicle and the D3-treated groups. Object preference was excluded because exposure of naïve mice to both objects resulted in equal exploration, and by randomizing the familiar and novel objects for both experimental groups (Figure 4). In summary, there were no differences observed with D3 treatment in the NOR task.

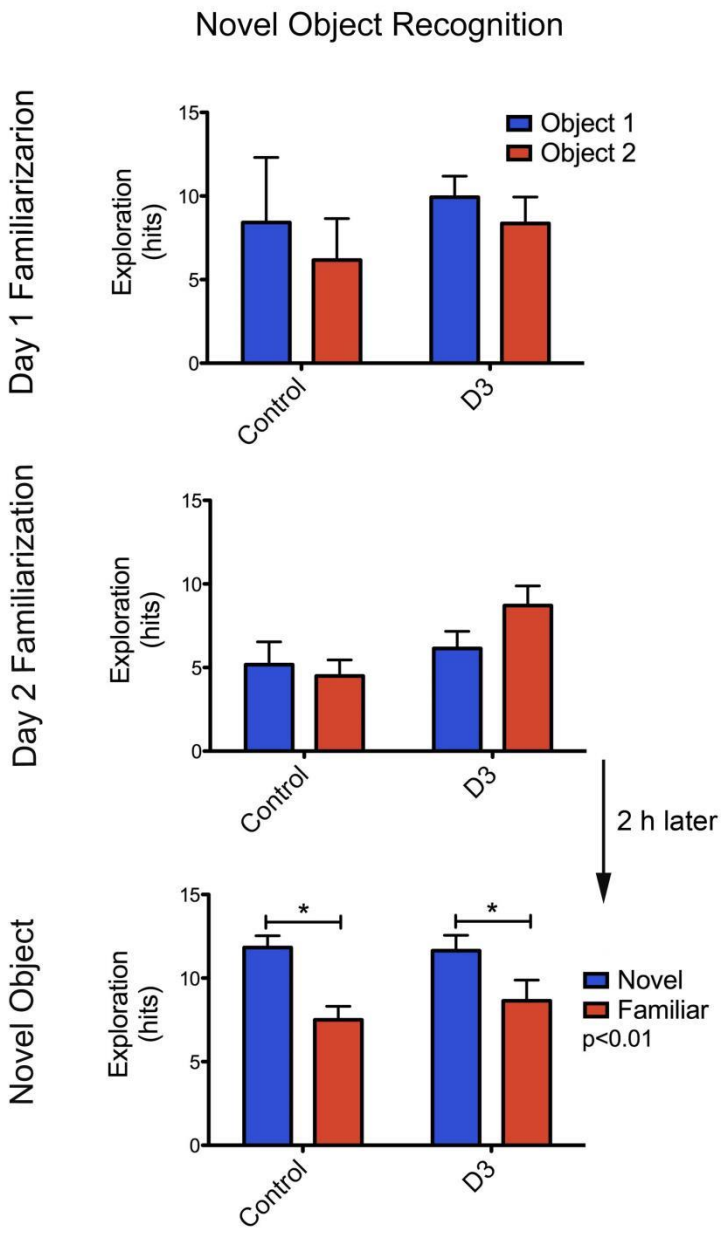


Figure 4. Compound D3 does not impair recognition memory. Mice explore objects by approximation, nose-pokes, backing up, sniffing and grabbing them. Such explorations were quantified in vehicle and D3-treated mice. Both groups explored equally the two identical objects and subsequently explored significantly more the novel object ($p<0.01$, controls n=6, D3 n=7). There were no differences between groups.

D3 has no effects on habituation in open field or object exploration

Before the NOR, mice were allowed to habituate to the testing environment. We observed no differences between the controls and the D3-treated mice in the distance (path) traveled, the time spent exploring, the percentage of the space the mouse covered or the amount of times the mouse entered the different sections of the field. The same measurements were performed during object exploration and again we observed no differences between the two experimental groups. Both groups increased

exploration when exposed to the objects for the first time and when exposed to the novel object (Controls n=6, D3 n=7) (Figure 5A, B, C, D).

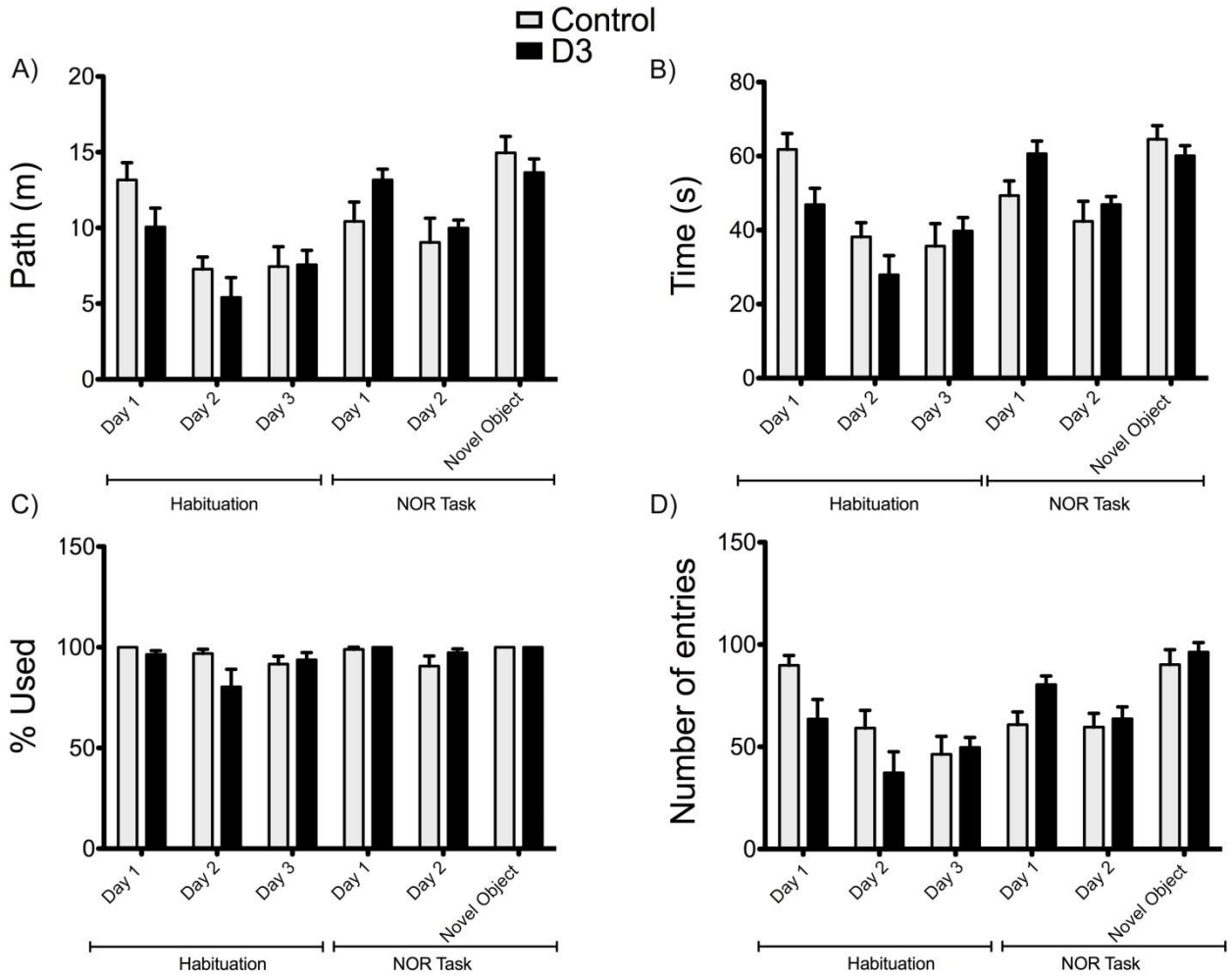


Figure 5. D3 does not have an effect on habituation or object exploration. In all graphs, the first three days correspond to habituation, the following two days to familiarization (identical objects) and finally on the fifth day the novel object trial. A) The distance travelled by the mice was recorded in meters (p=0.7). B) The time spent moving was recorded in seconds (out of a total of 300 s, p=0.4). C) The percentage of the field explored by the mice (p=0.9). D) The number of entries to the different quadrants in the field (p=0.7). No significant differences were observed in any of the measurements (Controls n=6, D3 n=7).

D3 acute delivery conveys reversible, short-term, spatial memory impairment, assessed through the Morris Water Maze

No differences were noted between the vehicle-treated (controls) and the D3-treated mice in visual and motor function assessed with the visible platform test and throughout the hidden platform task by ensuring the mice could swim properly and were focussing on the cues inside the tank (data not shown).

Starting one day after acute intraventricular injection, no differences were found, in the learning phase, between the controls (n=5), D3 9 μg (n=3) and D3 15 μg (n=5). Two hours after the last trial on the fifth day (Probe Trial 1), the control mice spent significantly more time in the target quadrant, whereas the D3-treated mice in a dose-dependent manner did not (supplementary figures). Likewise, there was a significant dose-dependent decrease in the percentage of the time that the D3-treated mice (low dose p=0.12, high dose p=0.046) spent in the target quadrant as compared to the controls. One week later, in the Probe Trial 2, a trend towards a similar change was observed (low dose p=0.057, high dose p=0.062) (Figure 6A). This indicates that the D3-treated mice exhibited memory impairment as soon as 2 hours after the last learning trial (which is 6 days after treatment) and this impairment was likely maintained at 1 week.

As reported previously (Aboulkassim et al., 2011), 2-week delivery of D3 conveys LTM impairment that is persistent over a two-month period. Therefore, the above mentioned experiment was repeated two and three months after acute treatment. There was no learning impairment at two and three months after treatment (Figure 6B and 6C). There were no differences between the controls and D3-treated mice (low and high dose) when tested in the Probe Trials either. All groups spent significantly more time in the target quadrant as compared to the other quadrants.

These data indicate that, while a two-week treatment caused memory impairment that was sustained even two months later, an acute D3 intraventricular injection conveys memory impairment that is reversible within 2 months or earlier.

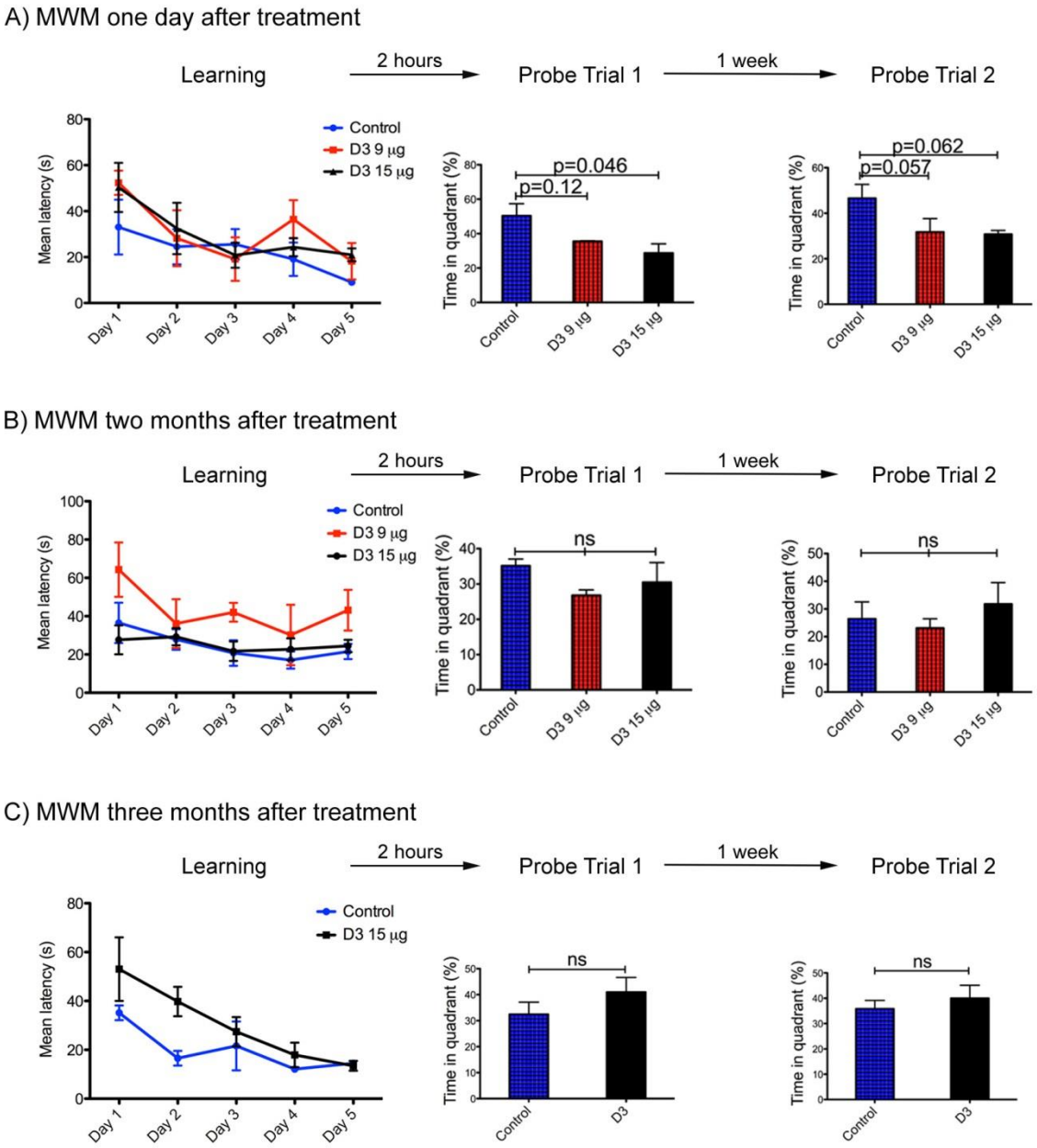


Figure 6. D3 conveys reversible STM impairment after acute ICV injection. A) Mice were trained in the Morris Water Maze (MWM) with three trials per day for 5 consecutive days. Values are shown as mean latency to find the platform (seconds). In the probe trials, values are displayed as the percentage of time the mice spent in the target quadrant. Probe Trial 1 (PT1) was performed two hours after the last trial on day 5 and Probe Trial 2 one week after PT1. Controls (n=5), D3 9 μg (n=3) and D3 15 μg (n=5). B)

No learning or memory deficits were observed two months after treatment. C) No learning or memory deficits were observed three months after treatment, implying reversibility of the effects. This experiment was performed twice — independently obtaining equal results.

D3 increases TrkA phosphorylation and activates pathways downstream in the nuclei basalis and hippocampus

We studied pathways activated by D3. After acute ICV, three different time points were studied: 1 hour (n=1, not quantified), 5 hours (n=4) and 24 hours (n=4). Mice were treated with aCSF (n=8) or 15 μg of D3 and sacrificed at the above mentioned lapses. The brains were dissected (cortex, hippocampi and nuclei basalis).

In order to assess TrkA activation, immunoprecipitations were performed with TrkA and then blotted with a pan-phosphotyrosine antibody (4G10). A one-between ANOVA showed an increase in TrkA phosphorylation in the hippocampus ($p=0.03$; n=8, 2 repeats). Newman-Keuls pairwise comparison confirmed and increase in the nuclei basalis at 5 and 24 hours (not shown, only one repeat); and an increase in the hippocampus at 5 ($p=0.02$; n=8; 2 repeats) and a trend to an increase at 24 hours ($p=0.11$; n=8; 2 repeats) (Figure 7). Our group had previously shown (Bruno et al., 2004) the diffusion of D3 within the Central Nervous System, which is compatible with the activation shown here.

Western Blots were performed on whole cell lysates at different time-points after D3 treatment. At 5 hours, an non-statistically significant increase in CaMKII was observed in the nuclei basalis as well as a non-statistically significant increase in Erk5 in the hippocampus (data not shown). At 24 h post-treatment, an increase in pErk5 and pMAPK was observed in the nuclei basalis (Figure 8) as well as an increase in CREB in the hippocampus (Figure 9). No significant changes were observed in any of the remainder signaling molecules studied.

Similar studies were carried out. After a 2-week ICV infusion, a 5 day time-point was studied (D3 n=3, vehicle n=3). When assessing the pathways which were activated, an increase in trophic signals: Erk5 (unpaired t-test $p=0.0089$; 2 repeats), CREB (unpaired

t-test p=0.0086; 2 repeats) and pAkt (unpaired t-test p=0.002; 2 repeats), specifically in the hippocampus, was observed (Figure 10). No changes were observed in any of the other proteins (MAPK, PKCζ, CaMKII) or anatomical regions (cortex and nuclei basalis, data not shown).

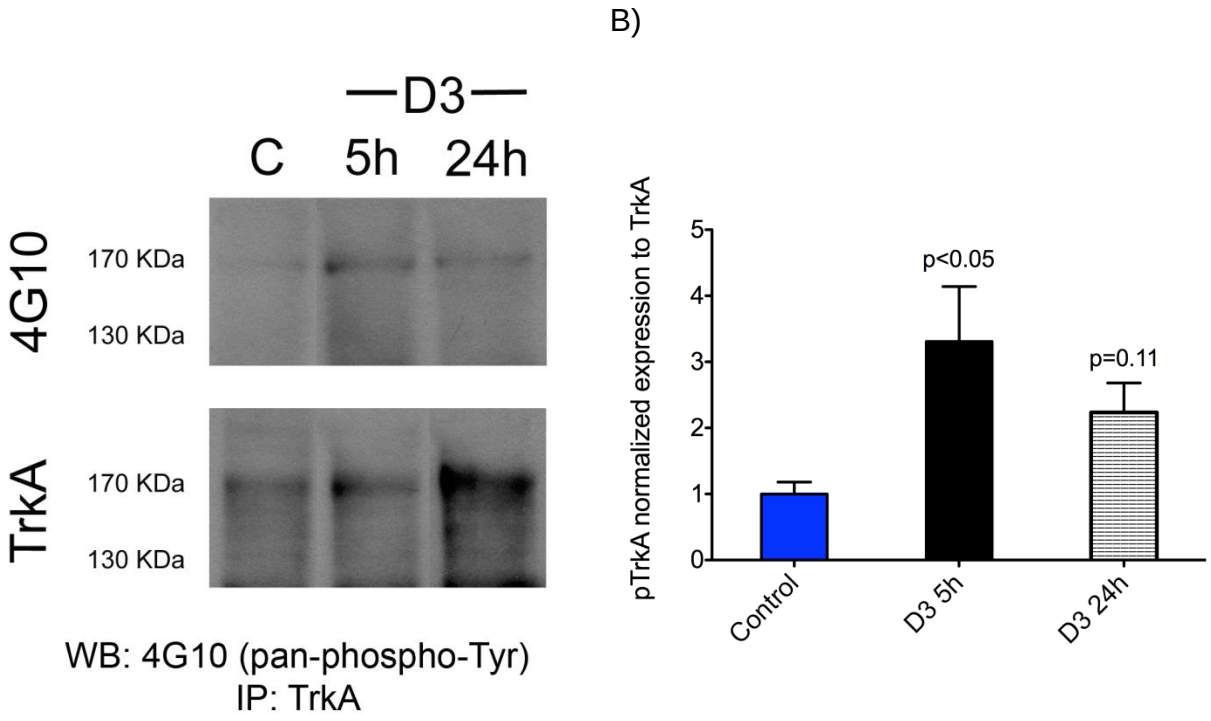


Figure 7. Acute D3 ICV injection increases TrkA phosphorylation in the hippocampus. A) Immunoprecipitation with TrkA and blotting with pan-phospho-tyrosine antibody 4G10 shows increased phospho-TrkA at 5 (n=3) and 24 hours (n=2) in the hippocampus, as compared to controls (n=3). Two repeats of this experiment were performed obtaining identical results. B) Quantification of the 170 kDa band standardized to total TrkA confirms a significant increase in pTrkA at 5 hours (p<0.05) and 24 hours (p=0.11) in the hippocampus.

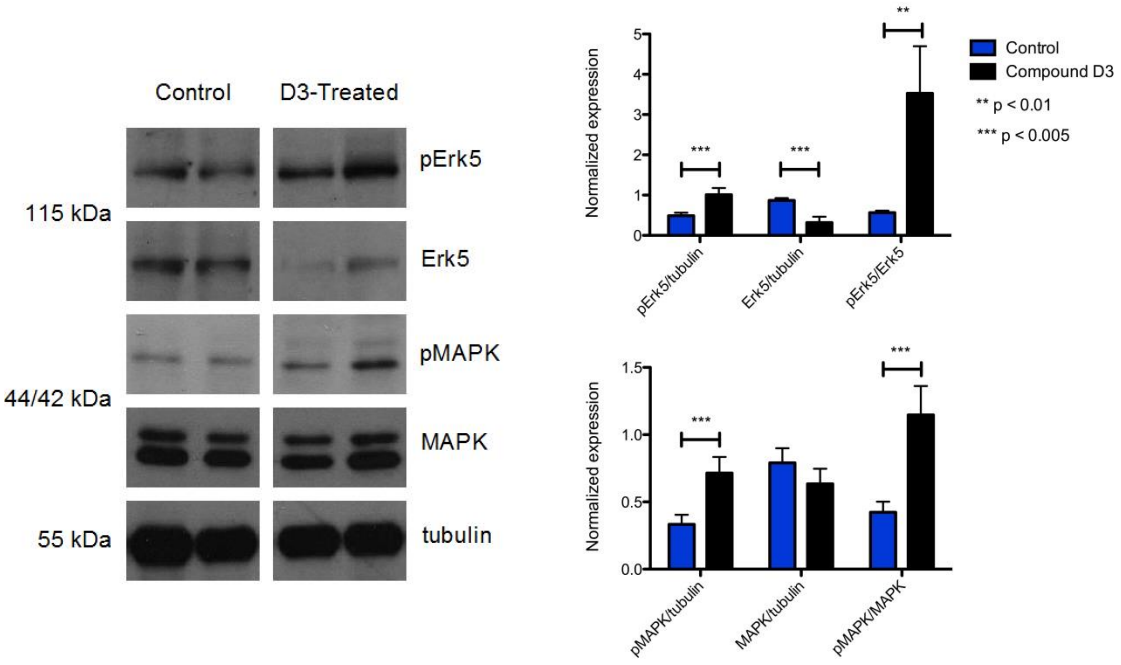


Figure 8. D3 treatment increases pErk5 and pMAPK in the nuclei basalis 24 h after D3 treatment. Western Blots from protein extracts from the nuclei basalis of mice sacrificed 24 hours after D3-treatment (n=4) or vehicle (Control, n=3), along with their optical densitometry analysis show a significant increase in pErk5 and pMAPK ($p<0.01$).

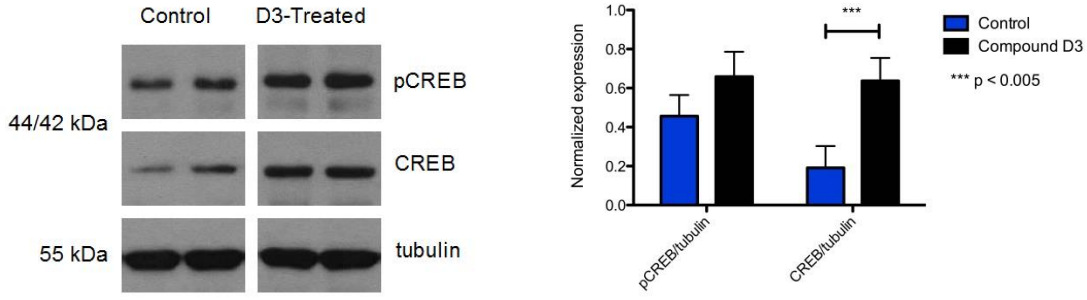
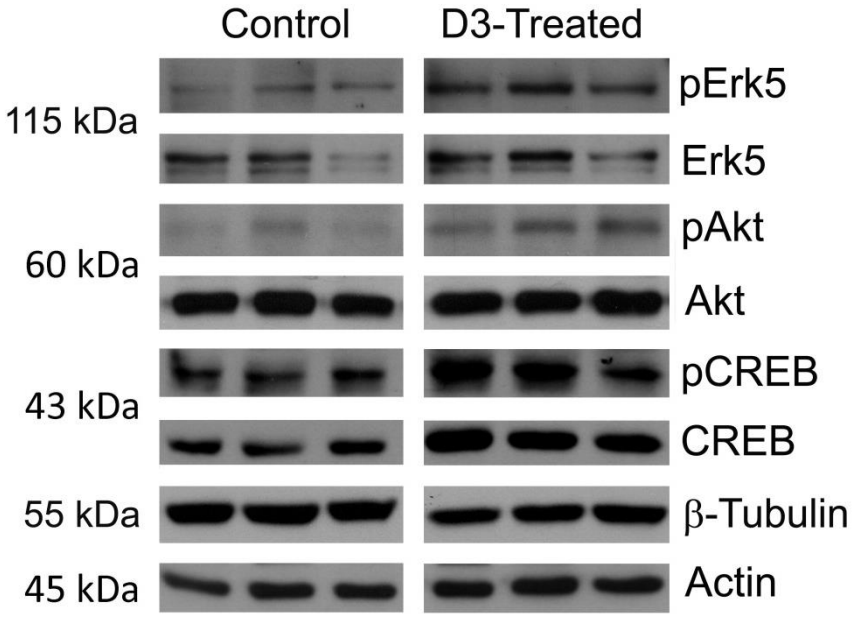


Figure 9. D3 treatment increases CREB in the hippocampus 24 h after D3 treatment. Western blots from protein extracts from the hippocampus of mice sacrificed 24 hours after D3-treatment (n=4) or vehicle (Control, n=3), along with their optical densitometry analysis show a significant increase in CREB ($p<0.005$).

A)



B)

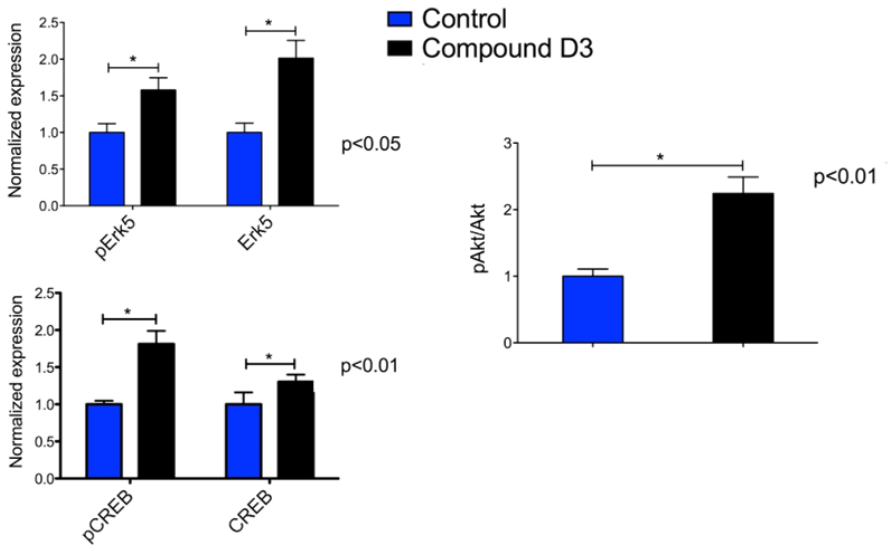


Figure 10. pAkt, Erk5 and CREB are increased specifically in the hippocampus after a 2-week D3 treatment. A) A representative sample of proteins tested in the hippocampus. An increase in pAkt, total CREB and total Erk5 is observed. B)

Densitometry quantification of the bands shows a significant increase in pAkt (p<0.01), total Erk5 (p<0.05) and total CREB (p<0.01). An increase in pErk5 and pCREB is observed, but this is proportional to the increase observed in the inactive form of the proteins.

D3 affects hippocampal-dependent memory consolidation

D3 may be targeting directly or indirectly the hippocampus because: (a) D3 has an effect on spatial but not recognition memory; (b) the changes take place as soon as six days after treatment; and (c) many of the biochemical pathways are being altered in the hippocampus. Consequently, D3 is most likely either impairing hippocampal-dependent memory consolidation or retrieval. Healthy, wild-type mice with normal STM were trained and allowed to consolidate memory for 48 hours. After being randomized into a group that received aCSF (n=6) and one that received 15 μg of D3 (n=7), they were re-tested. No difference was observed in memory between the controls and the D3-treated mice suggesting D3 is impairing mainly hippocampal memory consolidation (Figure 11).

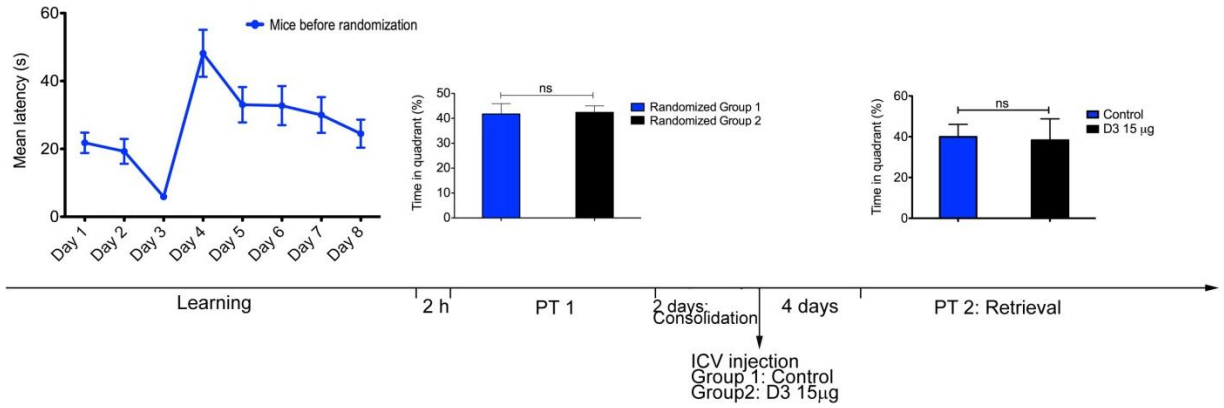


Figure 11. D3 affects mainly hippocampal-dependent spatial memory consolidation. Mice were trained as previously described and underwent PT1. They were not manipulated for the following 48 hours to allow for memory consolidation. Next, they were randomly placed in one of two groups: mice treated with aCSF or those treated with acute D3 (15 μg). Four days later, they were re-tested (PT2) to assess retrieval, and no significant differences between controls and the D3-treated mice were observed.

D3 decreases dendrite branching predominantly in the basal dendrites of neurons in the CA1 region of the hippocampus

A decrease in branching points in the CA1 region was observed in the mice treated with D3 for 2 weeks (ANOVA p=0.006). When performing Newman-Keuls pairwise comparison, there is a significant decrease mainly in the basal branching in CA1 neurons (p=0.021) and a lesser not significant decrease at the apical branching (p=0.10). Consistently, a trend of lesser number of Sholl intersections (ANOVA p=0.0598), were detected mainly at the basal region (p=0.10) as opposed to the apical (p=0.27). When the mean number of Sholl interesections were quantified, a significant decrease was observed in the D3-treated mice (ANOVA p=0.035), accountable mainly to the basal region (p=0.042) rather than the apical (p=0.3) (Figure 12 A, B, E, F; quantified in I, J and K).

Importantly, no changes in the amount of branching points we observed in the CA3 region of the hippocampus with the 2-week D3 treatment (p=0.796) (Figure 13 A, B, E, F; quantified in I). Also, no changes in the amount of branching points were observed in either the CA1 region (p= 0.888) or CA3 region (p=0.857) of the D3-acute ICV treated mice (data not shown). It is important to mention that these mice (treated with acute D3) did not show evidence of memory impairment as compared to controls, although the controls did no act as a good point of comparison in the MWM (data not shown).

Furthermore, no differences were observed overall or after pairwise comparisons, with either of the treatment paradigms (chronic and acute), in either of the hippocampal regions (CA1 and CA3) and sub-regions (basal and apical) for spine density (p=0.33, Figures 12 C, D, G, H; quantified in L and 13 C, D, G, H; quantified in J), mean spine length (p=0.25) or mean volume (p=0.13) (data not shown).

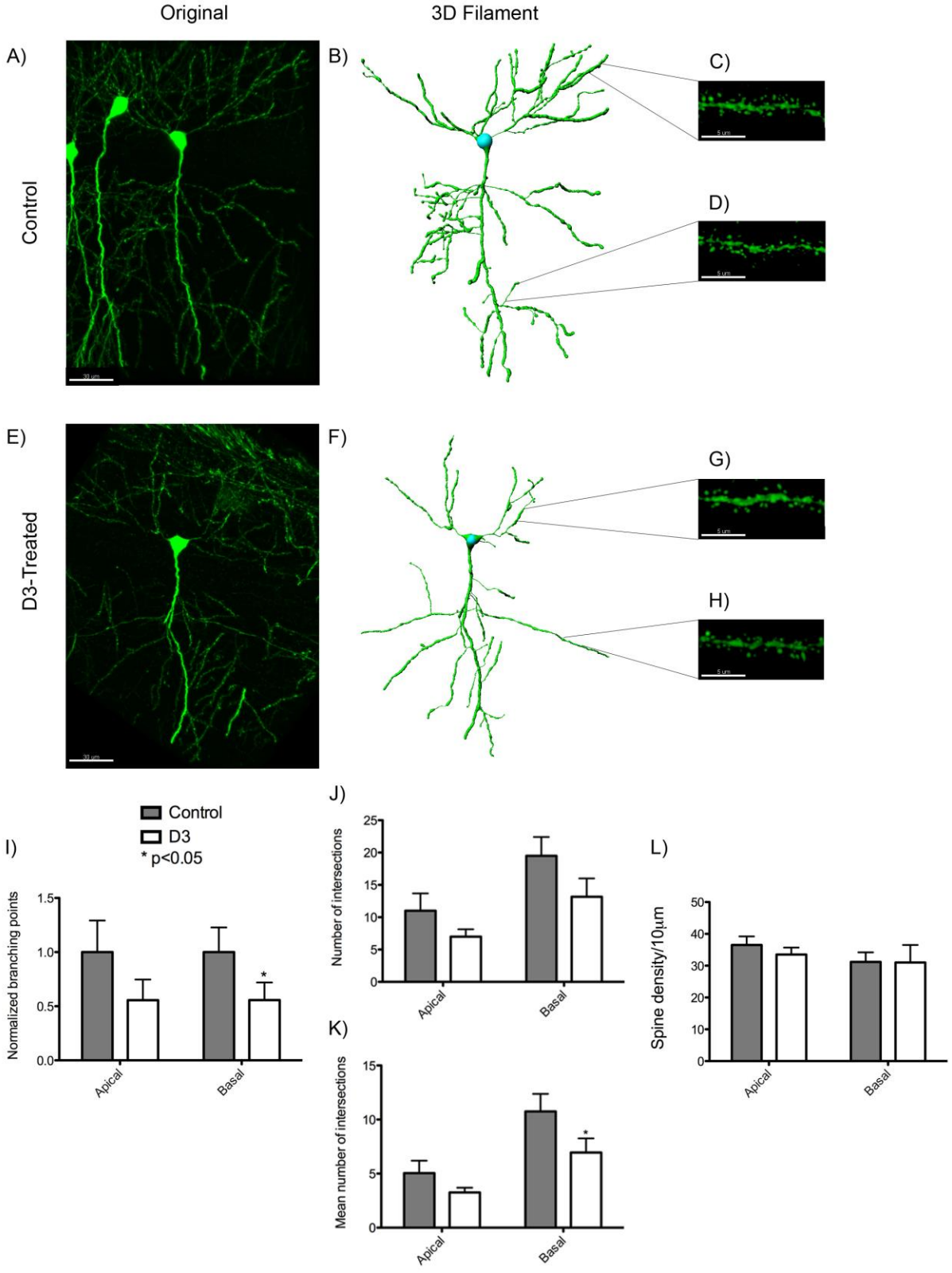

Original
3D Filament
A)
B)
C)
D)
Control
E)
F)
G)
H)
D3-Treated
I)
Control
D3
* p<0.05
Normalized branching points
Apical
Basal
J)
Number of intersections
K)
Mean number of intersections
L)
Spine density/10µm

Figure 12. D3 reduces dendritic arborisation in the basal CA1 region. A) Representative confocal image of neurons in the CA1 region of a control mouse. B) 3D filament of an isolated neuron from panel A. C) Representative magnified segment from a basal CA1 tertiary dendrite in a control mouse. D) Representative magnified segment from an apical CA1 tertiary dendrite in a control mouse. E) Representative confocal image of neurons in the CA1 region of a 2-week D3-treated mouse. F) 3D filament of an isolated neuron from panel E demonstrating the significant decrease in arborisation ($p=0.006$) after D3 treatment. G) Representative magnified segment from a basal CA1 tertiary dendrite in a 2-week D3-treated mouse. H) Representative magnified segment from an apical CA1 tertiary dendrite in a 2-week D3-treated mouse. I) Quantification of branching in the apical and basal region show a decrease in branching points mostly in the basal dendrites of mice treated with D3 ($p=0.021$). A trend to a decrease is also observed in the apical region ($p=0.10$). The values are shown as a fold decrease from control ± SEM. J) Sholl analysis performed in the apical and basal regions did not yield any statistically significant differences, but also yielded a trend to a decrease in arborisation in the basal region ($p=0.1$). Values are showed as the average ± SEM of the total number of intersections. K) Mean Sholl analysis performed in the apical and basal regions yielded a significant decrease in arborisation in the basal region ($p=0.042$). Values are showed as the average ± SEM of the mean number of intersections. L) Spine density was quantified in the CA1 region and is expressed in spines/10µm, no significant differences were observed with D3 treatment in any of the regions.

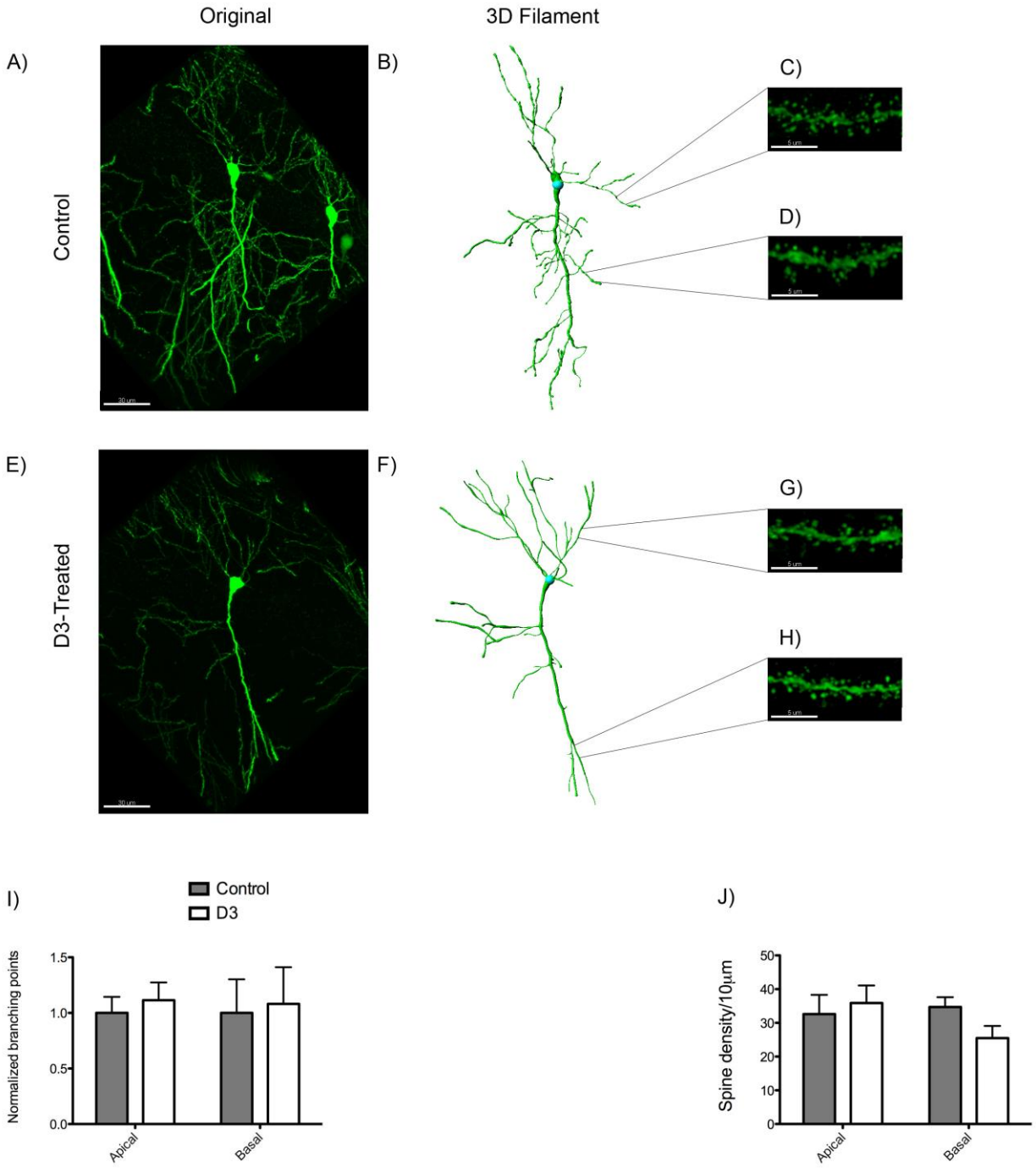


Figure 13. D3 has no effect on neuronal arborisation in the CA3 region. A) Representative confocal image of neurons in the CA3 region of a control mouse. B) 3D filament of an isolated neuron from panel A. C) Representative magnified segment from

a basal CA3 tertiary dendrite in a control mouse. D) Representative magnified segment from an apical CA3 tertiary dendrite in a control mouse. E) Representative confocal image of neurons in the CA3 region of a 2-week D3-treated mouse. F) 3D filament of an isolated neuron from panel E. G) Representative magnified segment from a basal CA3 tertiary dendrite in a 2-week D3-treated mouse. H) Representative magnified segment from an apical CA3 tertiary dendrite in a 2-week D3-treated mouse. I) Quantification of branching in the apical and basal region show no difference between the control and the D3-treated mice ($p=0.796$). A trend to a decrease is also observed in the apical region ($p=0.10$). The values are shown normalized to their respective control ± SEM. J) Spine density in the CA3 region was quantified and is expressed in spines/10µm, no significant differences were observed with D3 treatment in any of the regions.

DISCUSSION

D3 and Recognition Memory

In previous publications (Bruno et al., 2004, Aboulkassim et al., 2011), the effects of D3 had been described exclusively for spatial memory. Thus, it was now relevant to test whether D3 affects at least one more type of memory. The NOR task enables an assessment of recognition and mainly STM. The fact that no changes were observed with D3 treatment suggests that D3's effects are not generalized over the memory circuit: the memory impairment conveyed by D3 is rather specific for (at least) spatial memory.

Recognition and spatial memory have different anatomical substrates. This could also imply that D3 is affecting particularly the anatomical sites that mediate spatial memory formation, i.e., the hippocampus. The degree of the effect also plays a role; the MWM may be more sensitive than the NOR since it requires attention, orientation and has a higher degree of complexity. Because the MWM is more challenging, defects are more easily perceived.

Our results are consistent with previous publications (Niewiadomska et al., 2006), where they delivered NGF intraventricularly and assessed rats for spatial and recognition memory, finding differences exclusively in spatial memory. This also supports the specificity of D3 on TrkA-mediated mechanisms, and to the best of our knowledge, it would be the first time that these same effects are reported with a small molecule agonist of TrkA.

When distinguishing short versus long-term memory, a previous publication from our group (Aboulkassim et al., 2011) had already reported improvement in the APP mouse model, induced by D3, taking place within two hours of the last training session. Therefore, it is unlikely that the lack of differences was due to a time-point effect. Also, both tests evaluate for explicit types of memory, so it cannot be concluded that D3 is affecting explicit versus implicit memory differently. Finally, it can be inferred that D3 is not having an effect on novel preference.

It remains to be seen whether the above mentioned effects in healthy mice apply also to the memory-impaired models. However, based on previous publications (Niewiadomska et al., 2006), it can be predicted that an improvement on the NOR task by treating memory-impaired models with D3 may not be observed.

Habituation and Exploration

The mice were allowed to habituate in a NOR apparatus. No changes were observed in habituation: the mice did not spend more time close to the walls, crossing the midfield and no changes were observed in locomotion. Thus, D3 does not convey increased anxiety, disinhibition or fear.

Regarding the memory impairment seen in the MWM, one possible explanation is that, in place of pharmacologically-induced memory impairment, the mice know where the platform is but instead wish to explore further the rest of the tank. Consequently, habituation and exploration were assessed with the NOR task in order to test for such behavioural effects. Since no changes were observed in exploration, the possibility that the mice are increasingly exploring the tank in the MWM is discarded and the conclusion that indeed the mice are memory-impaired prevails. Equally, one can say that the lack of differences in the NOR task is not due to a compensatory increase in exploration.

D3 and Spatial Memory

In a previous publication (Aboulkassim et al., 2011), two-week D3 treatment improved learning in an AD mouse model, but had no effects on learning in wild-type mice. When mice were treated with D3 though acute injections, no effects were observed on learning assessed with the MWM. Nonetheless, as early as 2 hours after the last learning trial, 6 days post-treatment, the mice displayed impairment in memory measured by the probe trials. If STM is considered as taking place in minutes to hours, it implies that D3 conveys short-term spatial memory impairment. A failure to remember where the platform was 2 hours after the last learning trial makes it impossible for the mice to remember 1 week later (in the probe trial 2). Pharmacodynamically and pharmacokinetically, different dosing regimens will have different medication effects; in

this case it is demonstrated by the manner in which a 2-week treatment with D3 impairs spatial LTM, whereas an acute injection impairs spatial STM.

The MWM requires many features of the memory formation process to be intact: attention, orientation, processing, consolidation, storage and retrieval. Since it requires mice to swim in temperate-cold water, it also imposes stress on the mice which prompts them further to find the escape platform. Therefore, there are many factors that can interfere with spatial memory formation assessed in these conditions.

Since the hippocampus (a) processes declarative memory, (b) is the most important organ for spatial memory, (c) contains TrkA and (d) is regulated by the cholinergic fibers (with the greatest TrkA density in the adult brain), it appears that D3's effects are mainly concentrated in this organ. The hippocampus appears to be sensitive to disruptions, which may be expressed as a spatial memory deficit. Only 30% of the hippocampus must be destroyed to show defects in spatial memory, as opposed to at least 75% that must be destroyed to affect recognition memory (Broadbent et al., 2004). Therefore, a small disruptive effect on the hippocampus would be more easily detected by spatial memory tasks.

Different mechanisms mediate learning and remembering. Why do the mice express impairment 2 hours after the last learning trial but not day after day in the learning phase? One possibility is that when the platform is present, the mice have an estimate of where it is, and therefore during the learning trials they are reassured of the proper location. During the first trial of the next day, the mice again are prompted to find the platform thus retrieving and reconsolidating the memory. They are not impaired within 45 minutes and they find the platform, which reassures them they are in the right location. Whereas in the probe trial (without the platform) the mice may arrive at the right location, but when they do not find the platform, they proceed to swim arbitrarily. As opposed to the controls, which remain in the target quadrant persistently, certain that they are in the right location.

With the MWM, the map-building capacities of the hippocampus are assessed. This requires the establishment of specific connections within a circuit between the

hippocampus and the associated cortices. Each time that a memory is retrieved, it is susceptible to modifications and even deletion. Therefore, when the D3-treated mice are placed in a scenario in which the platform is not there, the memory is retrieved but it is easily modified and disrupted, making it impossible to re-consolidate the memory in its original form. Since it is not re-consolidated, it remains absent even a week later during the second probe trial. Therefore, D3 may be having a detrimental effect on synapse stabilization. Synaptic tagging may take place during learning, and it is strengthened each time during the learning trials. But, when the synapse is challenged and the mouse does not find the platform, the synapse is not strengthened in the D3-treated mice, instead leading to a faster weakening or disruption of the synapses. This is supported by the fact that D3 increases LTD (Aboulkassim et al., 2011), without changes in LTP (tested both in chronic and acute D3 treatments, supplementary figures).

The memory impairment elicited by D3 treatment is reversible at 2 months after an acute injection, whereas it is sustained after a 2-week delivery. This could be due to functional as opposed to structural changes taking place. An acute delivery may modify the physiology of processing and consolidation of memory, affecting synapse formation and stability without major structural changes. Instead, a 2-week delivery may change connection morphology grossly, making these more permanent changes.

Biochemical Changes Induced by D3

Since compound D3 was designed to activate specifically TrkA, it is expected that most, if not all, signalling pathways activated are downstream of this receptor. Many of the proteins downstream of TrkA are also major players in memory formation, therefore TrkA activation may convey memory improvement or impairment phenotypes – or in the case of D3, both. So far, no evidence has been found of D3 activating associated receptors such as p75 (Maliartchouk et al., 2000, Bruno et al., 2004, Zaccaro et al., 2005, Aboulkassim et al., 2011) or different pathways unrelated to TrkA. Furthermore, D3 binds to TrkA at a different site than NGF (Maliartchouk et al., 2000), but still activates the main pathways triggered by NGF, downstream of the receptor dimer phosphorylation and endosome.

Since TrkA is mainly expressed in the hippocampus and the nuclei basalis, most of the changes were predicted to take place in these anatomical regions, and that was indeed the case. Interestingly, TrkA phosphorylation and the initial signaling pathways activated are first increased in the nuclei basalis and hippocampus. The apparent increase in TrkA as well as its phosphorylation, is likely due to the fact that D3 stabilizes the TrkA homo-dimers in the cell membrane (Maliartchouk et al., 2000); and as we prove here, thereafter increases its phosphorylation and signaling pathways. Though long term, after treatment completion and when the memory tests are underway, the increase in signaling pathways is only observed in the hippocampus. This organ is crucial in the memory phenotype described.

TrkA, like all growth factor receptors, plays a vital role in maintenance of homeostasis. In diseases such as AD, MCI and DS, TrkA's decrease is associated with memory impairment (Mufson et al., 2000, Sendera et al., 2000, Counts et al., 2004). In the respective animal models, which also have decreased TrkA (Saragovi, 2005), adding D3 conveys reversal of the memory impairment. On the other hand, in the wild-type, healthy, young mice, increasing the normal, baseline TrkA activity, may disrupt homeostasis and consequently lead to memory impairment.

At 24 hours after D3-treatment, an increase in pErk5 and pMAPK is noted in the nuclei basalis. This may be interpreted as D3 binding and activating TrkA directly in the cell body (Watson et al., 2001), leading to increase signaling proteins within minutes of TrkA phosphorylation. However, since D3 is injected intraventricularly, it does take at least two hours to penetrate and reach its target tissues (Bruno et al., 2004). Several days after a two-week treatment, the Erk5 increase is only seen in the hippocampus and the MAPK increase can no longer be detected. Therefore, it may be that this activation is downstream of the TrkA endosomes transported retrogradely from the synaptic terminals (Watson et al., 2001, Pan et al., 2013). Both the acute and long-term Erk pathways activity may be increased leading to disarrayed neuritogenesis and synaptogenesis (Wang et al., 2014), which could lead to the increased LTD and memory impairment. No changes were observed in hippocampal neurogenesis *in vivo*

after D3 treatment (supplementary figures); and therefore, this is an unlikely effect and mechanism for the observed memory impairment.

An increase in pCaMKII in the nuclei basalis after 5 hours would be consistent with an increase in LTP instead of LTD, but this increase is transitory since it is not seen at any other time-point or in any other anatomical region. In contrast, pAkt shows a later and persistently increased phosphorylation, especially in the hippocampus. This had already been described by our group (Aboulkassim et al., 2011), where it is stated that such an increase can lead to hippocampal LTD through deregulation of the PI3K/Akt pathway. Finally, CREB and its active phosphorylated form are persistently increased, particularly in the hippocampus. CREB is downstream from all of the mentioned signaling pathways and acts as a transcription factor for many proteins which mediate survival and differentiation. Another step in this project would be to delimit which genes are being transcribed by pCREB after D3 treatment. It is interesting, though, that D3 elicits increases in the total form of several proteins, not only phosphorylation, maybe due to the increase in transcription factors.

A decrease in pErk5 was observed in the hippocampus at 5 hours after treatment. It is hard to assess the physiological relevance of this finding since this decrease was not observed at 24 hours. And an opposite effect (increase in pErk5) was observed 5 days after the 2-week treatment with D3. It does yield the question whether it would be physiologically relevant in a sequential dosing setting. Endosome trafficking analysis could be performed in order to assess whether this transitional decrease in pErk5 is due to a decrease in soma endosome formation relatively soon after D3 treatment.

Similar signaling pathways activation as the ones reported in this document have been published *in vitro* with D3: an increase of pTrkA after D3-treatment of a cell line containing only TrkA and not P75 (Maliartchouk et al., 2000) as well as an increase in MAPK1/2 in conjunctival cells after treatment with D3 (Jain et al.,2011). Also, *in vivo*, Lui, *et al* (2014) showed that administering memantine (a NMDA receptor antagonist) to an Alzheimer's mouse model increased TrkA phosphorylation, MAPK and CREB among other signaling pathways.

The lack of changes observed in the signaling pathways in the cortex (after acute or long-term treatment) is most likely due to the fact that TrkA is not widely distributed in the cortex, but mostly in the basal forebrain. Therefore, it would be difficult to observe activation in total cell lysates of the cortex. Nonetheless, these findings support the idea that D3 does not have global effects on the CNS but that they are targeted to those specific regions containing TrkA. Also, it ascertains that the memory defects conveyed by D3 are not due to impaired memory storage.

Memory Processes and D3

It is important to assess the specific processes in which D3 modifies memory. Taking into consideration the LTM signalling pathways in the hippocampus and the specific defects in spatial, short-term memory conveyed by D3, one may conclude that D3's effects are taking place mostly in the hippocampus. The hippocampus has been widely studied in memory processes, specifically in memory consolidation and retrieval. Therefore, it is the logical next step to test how D3 elicits its effects in this context.

There are many ways in which memory can be disrupted. Disruption or modification of memories upon retrieval has been a widely studied topic. Pharmacological, genetic and behavioural interventions upon memory retrieval may modify or even delete an existing memory. Re-consolidation may take place in the original or different forms of the memory (Nadel and Moscovitch, 1997, Abel and Lattal, 2001, Blake et al., 2014).

In order to assess whether D3 affects memory consolidation or retrieval in the hippocampus, mice were trained and allowed to consolidate. By treating the mice with D3 after memory consolidation has taken place, D3's effects are no longer observed. Therefore, the most likely conclusion is that D3 is affecting memory consolidation, and not retrieval. This can also explain the above mentioned conclusions regarding D3's effect on STM without affecting learning: retrieval is unimpaired but the mice cannot properly consolidate or re-consolidate memories.

The effects of D3 on neuronal structure in the hippocampus

So far the different effects of D3 have been discussed in terms of biochemistry (mainly increasing signaling pathways in the hippocampus and nuclei basalis) and behaviour (it affects the consolidation of hippocampal-dependent spatial memory without effects on recognition memory or exploration). How are these effects linked? We studied hippocampal neurogenesis and hippocampal neuronal differentiation as candidates.

When studying hippocampal neurogenesis, no changes were observed between D3-treated mice and controls. Inversely, when assessing hippocampal differentiation, a decrease in dendrite arborisation in the basal region of the CA1 area of the hippocampus was observed after a 2-week D3 treatment, with a trend to a decrease in the apical region and no changes in the CA3 region. Interestingly, no changes were observed in spine density, mean spine volume or mean spine length in these areas.

This decrease in dendrite arborisation occurs in pyramidal neurons in the CA1 region which are most likely Place Cells, potentially explaining the memory defects observed. The decrease could be due to a D3-induced saturation of the TrkA receptor and signaling pathways in the cholinergic basal forebrain neurons , which are connected through the entorhinal cortex and then to the basal region of the CA1 area (Mufson et al., 2008). These neurons have a higher TrkA receptor density than the hippocampal neurons themselves; therefore this decrease could be an indirect effect of D3. A trend of decrease in arborisation was observed also in the apical region which is connected to the CA3 region of the hippocampus and mainly involved in pattern separation (Voss et al., 2013).

It was surprising to not find any differences in spine density, mean spine volume and mean spine length. This could be explained by asymmetric shaft synapses, with excitatory synapses shifting within the dendrite (Bourne and Harris, 2011). VGLU1/2 immunofluorescence could be a better assessment of spine density in such case. Nonetheless, even without a decrease in spine density, a decrease in dendritic arborisation implies a final decrease in the number of spines.

Also, it was unexpected to not observe the same or any other change in the mice treated with acute D3 ICV injections. This could be due to a different mean age of the mice, since they did not show the same memory impairment described before. It could also be due to a wash-out or interference effect: the mice that were treated with D3 for 2 weeks were sacrificed immediately after treatment and did not undergo training whereas the mice that received acute injections were sacrificed two weeks after treatment and underwent a MWM task.

CONCLUSION

Memory formation/retention is not a fixed process: it can be modified in pathological and normal states through molecular interventions. We present here part of the mechanism of action of a therapeutic with potent and promising effects. Through this project, we contributed with an overall assessment and demarcation of D3's physiological and anatomical targets, mediated by the biochemical targets previously published.

We confirm that D3 is a partial agonist of TrkA which has differing effects on memory depending of the animal model used as substrate. It benefits memory-impaired models, whereas it impairs memory in healthy, young, wild type mice. We now report that this impairment is specific for hippocampal-dependent spatial memory consolidation. This impairment takes places after trophic signaling pathways have been sequentially activated in the hippocampus and nuclei basalis.

We validate the hypothesis that over-activating a system in a homeostatic organism will not lead to a super-organism (with "super-memory"), but instead will generate disequilibrium and lead to harm. By showing that D3 affects neuronal morphology we prove that this pharmacological agent can influence the memory circuitry, positively or negatively, and both in STM as LTM. In the memory-impaired models, where there is a clear improvement in memory, an opposing effect may take place. Such outcome would manifest with an increase in dendritic arborisation, since that is a scenario in which the system would not be saturated--to the contrary, we would be supplying a potentially beneficial agonist to a deficient system.

The relevance of this work rests on the lack of appropriate therapeutics for most types of dementia, mainly Alzheimer's disease. Neurotrophin agonists have been major candidates in this regard. This work supports the main principle in medicine: "primum non nocere" (first, do no harm). Promising therapeutics must be widely assessed and specific patient populations must be carefully selected before entering clinical trials. Still, D3 persists as a promising therapeutic whose mechanism will soon be discovered, and safety in memory-impaired populations assured, moving forth on the clinical trial pathway.

SUPPLEMENTARY FIGURES

Morris Water Maze probe trials post-D3 treatment

6 days after acute ICV injection and training in the MWM, D3-treated mice were compared to vehicle-treated (control) in the probe trial 1. The control mice spent significantly more time in the target quadrant than in the remainder quadrants. In contrast, the D3-treated mice (in a dose-dependent fashion) did not (Figure 14A). One week later, the mice were assessed in the probe trial 2 obtaining equivalent results (Figure 14B).

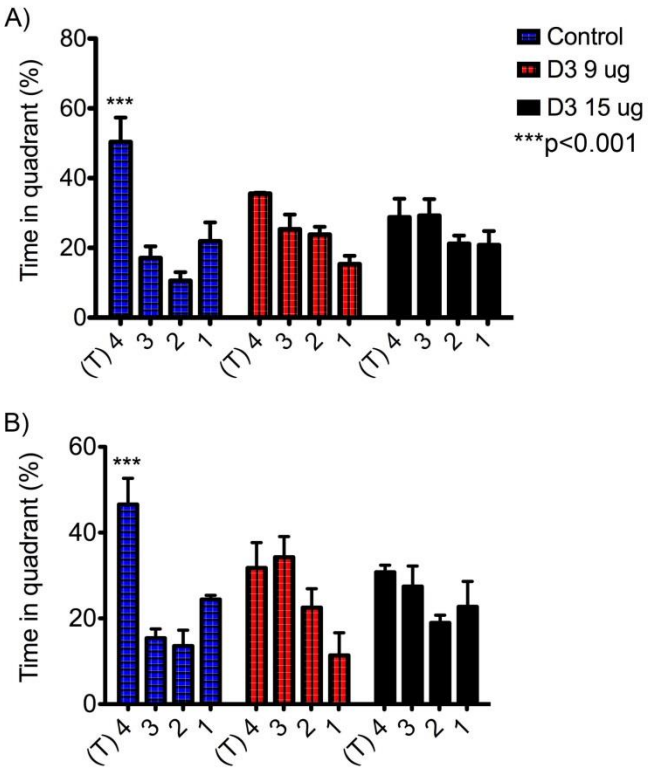


Figure 14. D3-treated mice do not spend significantly more time in the target quadrant during the probe trials. A) Probe Trial 1: Vehicle-treated (control) mice spend significantly more time in the target quadrant '4' than in the remainder quadrants (<0.001). Quadrants 1 and 3 were those adjacent to 4, and quadrant 2 was that opposite to 4. Neither the D3 9μg-treated mice nor the D3 15μg-treated mice spent significantly more time in the target quadrant (p=0.09 and p=0.34 respectively). B) Probe Trial 2: One week later, equivalent results were obtained. Controls p<0.001, D3

9μg p=0.01 but not corresponding to the correct quadrant and D3 15μg p=0.21. The statistical significance observed in the 9μg D3-treated mice in the probe trial 2 reinforces the fact that these mice had a milder impairment that the 15μg D3-treated mice, since they remained more time in a zone close to but not exclusively corresponding to the target quadrant.

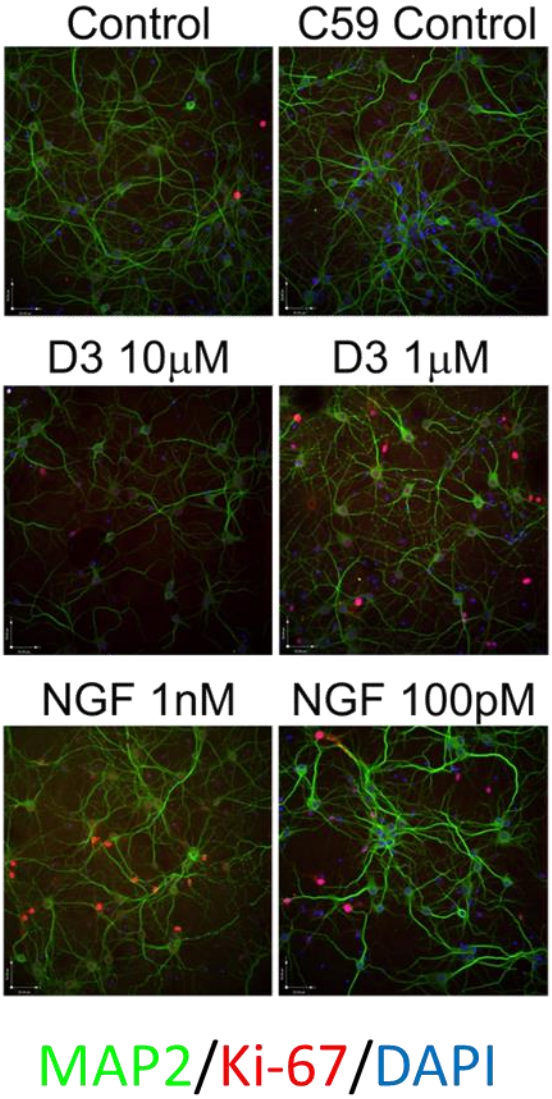
D3 and neurogenesis

The effects of D3 on hippocampal neurogenesis were assessed because: (a) TrkA mediates neuronal survival, (b) D3 affects spatial memory and (c) signalling pathways are activated in the hippocampus.

Initially, neurogenesis studies were performed in hippocampal primary cultures. There was a trend to an increase in Ki-67 positive neurons in the cultures treated with D3 at a high concentration (10 μM, p=0.06) and a statistically significant increase with a low concentration (1 μM, p<0.05), and also with NGF at a high concentration (2 nM, p<0.05). C59 is a similar molecule to D3 but with no biological action, which acted as a negative control (Figure 15, two repeats).

Next, neurogenesis was assessed *in vivo*. Mice received simultaneous D3 and BrdU treatments. No differences in neurogenesis were observed between the controls (n=2) and the D3-treated mice (n=2) in the dentate gyrus, CA3 or CA1 regions of the hippocampus (Figure 16).

A)



B)

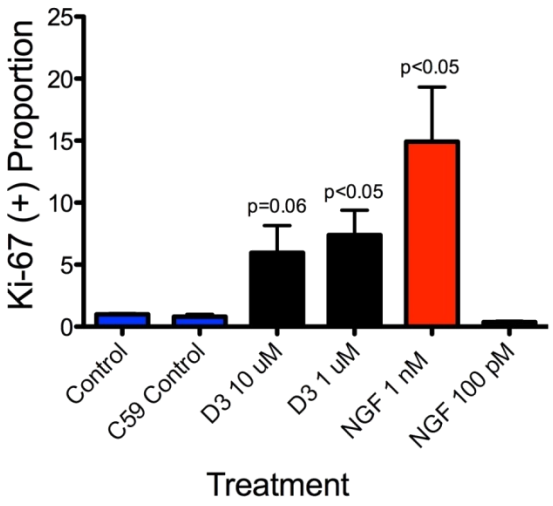


Figure 15. D3 significantly increases hippocampal neurogenesis *in vitro*. A) Primary hippocampal cultures were treated with supplemented Neurobasal medium (Control), and the following compounds in the same medium: C59 (an inert compound similar to D3), D3 at two different concentrations: 10 μM and 1 μM, and NGF at two different concentrations: 1 nM and 100 pM. Immunofluorescence was performed for Ki-67 (red) and MAP2 (green). B) Quantification of the proportion of Ki-67 positive neurons showed a trend to an increase with D3 at a high concentration (p=0.06), a significant increase in neurogenesis with D3 at a low concentration ($p<0.05$) and with NGF at 1 nM ($p<0.05$), two repeats.

A) B)

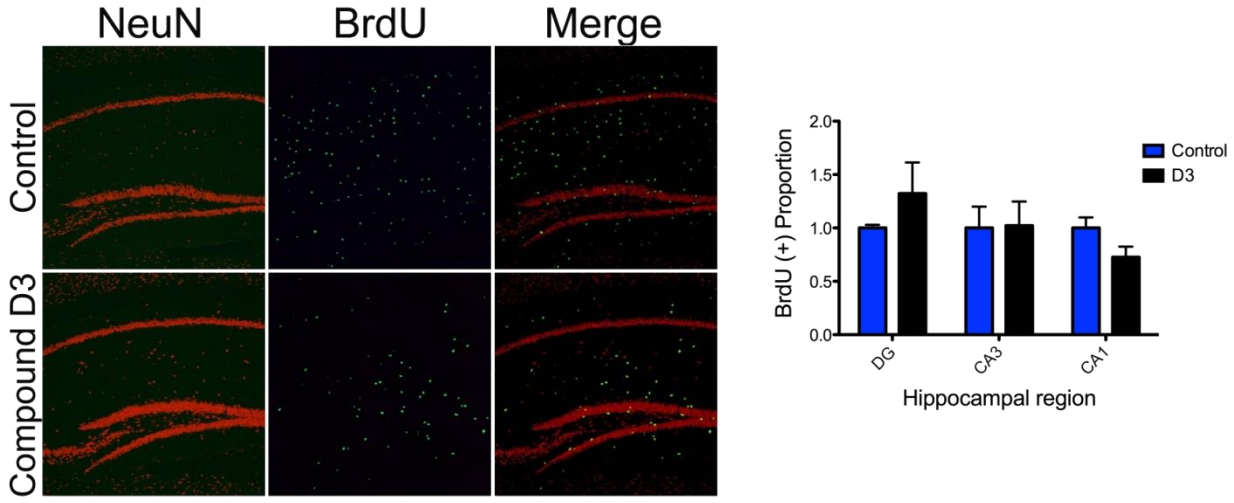
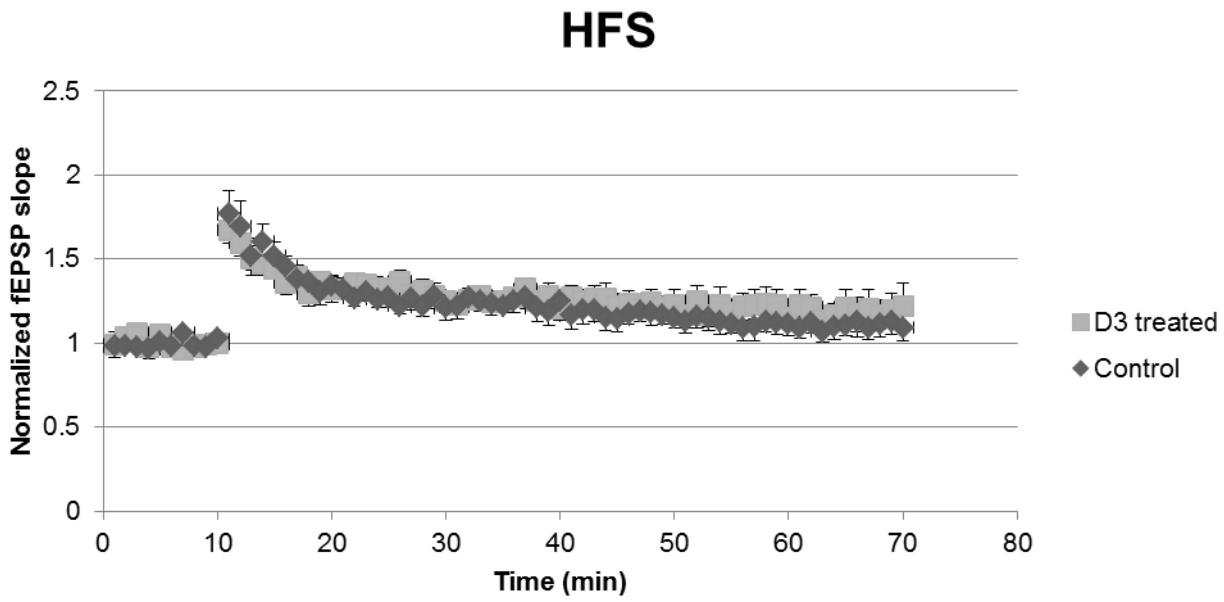


Figure 16. D3 does not increase neurogenesis *in vivo*. A) Simultaneously, mice were treated with aCSF or D3 (40 μg) ICV and BrdU PO. Immunofluorescence was performed for BrdU (green) and NeuN (red). B) The amount of BrdU positive neurons was quantified in the dentate gyrus, CA3 and CA1 regions standardizing them to the total amount of BrdU positive neurons. No significant differences were observed between the controls and the D3-treated mice.

Acute D3 and LTP

In order to test whether D3 conveyed changes in LTP and baseline connectivity after acute ICV injection, electrophysiological analysis was performed. No differences were observed in LTP between D3 and vehicle-treated wild type mice, 6 days after acute ICV injections (Figure 17). No differences were observed in the input/output analysis either (data not shown).

A)



B)

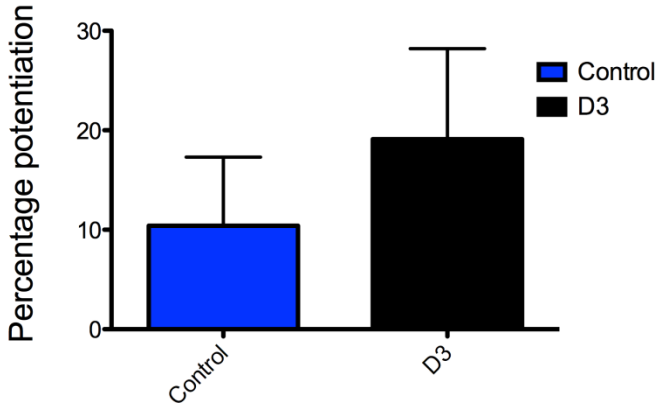


Figure 17. Acute D3 treatment has no effects on LTP. A) Electrophysiological recordings were performed in mice 6 days after acute ICV injection with D3 or vehicle. B) No significant differences were observed in percentage potentiation during the last 10 minutes, at 60 minutes after tetanus, a parameter corresponding to LTP (Controls n=7 recordings, D3 n=6 recordings).

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