Dissecting the role of cyclic nucleotides in memory processes

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ISBN: 978-94-92679-80-2

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Cover design, typesetting, layout and printing by Printservice Ede

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DISSERTATION

To obtain the degree of Doctor at Maastricht University, on the authority of the Rector Magnificus, Prof. dr. Rianne M. Letschert in accordance with the decision of the Board of Deans, to be defended in public on Thursday 21 February 2019, at 12.00 hours

by

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Contents

Chapter 1

General Introduction

Synaptic plasticity and memory

Memories shape our personalities and define our daily life. Memory decline encountered in aging and in certain diseases constitute a burden for individuals, their families and the society in general. Thereafter, better understating regarding the brain structures and the molecular mechanism involved in memory has been the focus of scientific research for over forty years.

Based on their nature, memory could be subdivided in declarative and non-declarative memory. Alternatively, declarative memory is also called explicit, indicating that is the result of conscious recollecting, and non-declarative memory could be called implicit since it is the result of unconsciousness actions driven from experience. The main subdivision of implicit memory is procedural memory that represents acquired skills and habits, like learning how to play an instrument or cycling. The brain structure involved in this type of memory is the striatum.

Regarding declarative memory, it has two main subdivisions: i) episodic memory, representing personal experiences and events, like the brand of your first car or the name of your dog and ii) semantic memory that represents non-personal facts and concepts, like knowing that the grass is green or soccer is a sport. The brain area involved in declarative memory is the medial temporal lobe that includes the hippocampus and a group of other structures (entorhinal cortex, perirhinal cortex and parahippocampal cortex) that via connections with the neocortex participate in formation of declarative memories. In this thesis I will focus on episodic memories and more precisely in the different stages of mnemonic processes.

Declarative memories in general and episodic memories in particular are easier to form, but also more prone to disruption and forgetting. Most of the information that we receive on a daily basis is held in the brain only for a few minutes or hours. These short-term memories are not stable and can be easily disrupted and erased. With the process of consolidation a fraction of these memories is transformed into long-term memories that are stored in the brain for a longer period of time lasts from days to years. Except for memory consolidation, other mnemonic processes include the phase of acquisition that represents the encoding of sensory information in the brain and retrieval that refers to recall of a previously stored memory (1).

In the study of complex mnemonic processes, an important asset is the molecular correlate of memory termed as long-term potentiation (LTP), representing activity-dependent

enhancement of synaptic strength. Hippocampal LTP is thought to have an eminent role in memory formation and constitutes a widely studied model for synaptic plasticity. Induction of LTP requires activation of both pre- and post-synaptic cells. Arrival of an action potential to the presynaptic cell increases Ca^{2+} levels inside the cell that subsequently promotes exocytosis and release of neurotransmitters (e.g. glutamate) to synaptic cleft. In turn, glutamate binds to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) and kainite receptors, increasing inward flow of Na^{+} into the postsynaptic cell and efflux of K^{+}. The latter causes the depolarization of the postsynaptic neurons and the voltage-dependent relief of N-methyl-D-aspartate receptors (NMDARs) from Mg^{2+} blockage. Activation of NMDARs promotes Ca^{2+} influx through the channel and activation of Ca^{2+}/calmodulin that promotes activation of downstream kinases which activate processes that eventually result in enhancement of synaptic strength, as described in the next section (2).

As with memory, LTP is governed by distinct phases. The early phase of LTP (E-LTP) is labile and could last up to 3 hours. E-LTP is not dependent on synthesis of new proteins, but is mainly represented by trafficking of an already existing pool of AMPARs in the postsynaptic cell, as well as increased release probability of glutamate at the presynaptic cell. The late LTP (L-LTP) could last several hours (> 8 hours) and requires gene transcription and protein synthesis (3).

Cyclic nucleotide signaling and phosphodiesterase inhibitors

Transmission of extracellular signals to intracellular compartments is mediated by the second messengers cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). Since the initial discovery of cAMP in 1971 by Earl W. Sutherland Jr. and the subsequent description of cGMP signaling pathway in cardiovascular system in 1998 by Robert F. Furchgott, Louis J. Ignarro and Ferid Murad, it is evident that cyclic nucleotides participate is a myriad of functions, including synaptic transmission. Although both cAMP and cGMP signaling pathways share some common downstream effectors, including protein kinases, transcription factors, channels and receptors, several signalosome aspects differ. The cAMP pathway is initiated upon binding of a ligand (neurotransmitter or hormone) to the Gs-protein coupled receptors that further activate the enzyme adenylate cyclase (AC). The latter catalyzes the conversion of adenosine triphosphate to cAMP promoting multiple intracellular cascades. Similarly, cGMP production is catalyzed by the enzyme guanylate cyclase (GC) that is stimulated by the small gaseous molecule nitric oxide (NO) as will be describe more in detail below.

The action of cyclic nucleotide signaling pathways in signal transduction and synaptic strengthening involves the activation of their downstream effectors. The most common effector of cAMP signaling cascade is protein kinase A (PKA), while protein kinase G (PKG) is the most well-known effector of cGMP pathway. Activation of cAMP/PKA or cGMP/PKG pathways at the postsynaptic cell promotes among others activation of cyclic nucleotide-gated channels (CNGC), trafficking of AMPARs and phosphorylation of cAMP response element binding protein (CREB) either directly or indirectly (4-7). Phosphorylated CREB could bind to the cAMP response element (CRE), initiating the transcription of specific genes coding for receptors or neurotrophic factors. Additionally, at the presynaptic cell, activation of these two cyclic nucleotide pathways promotes the release of neurotransmitters like glutamate and dopamine.

Since cyclic nucleotide pathways orchestrate several intracellular responses, and fine-tuning between their synthesis and degradation is essential for proper neuronal functioning. Phosphodiesterases (PDEs) are the only enzymes that degrade cyclic nucleotides in response to intracellular stimuli (8). Therefore, PDE inhibition diminishes the degradation of cAMP and/or cGMP, promoting the elevation of one or both second messenger molecules. So far, they have been described over 100 human PDEs that are divided into 11 families with distinct expression pattern and catalytic properties (9). Some classes of PDE inhibitors could catalyze both cyclic nucleotides (PDE1, PDE2, PDE3, PDE10, PDE11), while other are specific either for cAMP (PDE4, PDE7, PDE8) or for cGMP (PDE5, PDE6, PDE9).

The past decades a growing body of studies showed that upregulation of cyclic nucleotide pathways could facilitate LTP and memory formation in adult, aged, pharmacologically impaired and transgenic animal models of AD (10), rendering them a promising target for cognition enhancement. Importantly, cGMP and cAMP are involved in different phases of plasticity and mnemonic process. More precisely it was shown that cGMP is activated at the E-LTP and early consolidation, whereas cAMP is activated at the L-LTP and late consolidation. Additionally, cGMP/PKG signaling at the early consolidation is a prerequisite for intact cAMP/PKG signaling at the late consolidation (11). The latter observation suggests that at least during the consolidation phase the action of the two nucleotides is sequential with cGMP activation preceding cAMP activation. Although the molecular mechanism underlying this relationship is yet unknown, it has been suggested that upregulation of cGMP/PKG pathway facilitates cAMP/PKA signaling via acting in other intracellular modulatory systems (e.g. activation of CNGC, release of Ca^{2+} from ryanodine stores) (12-14).

Nitrinergic signaling

NO is produced by L-arginine from the enzyme nitric oxide synthase (NOS), and it can be found in several types of cells including neurons, endothelial cells and macrophages. In mammals, there have been characterized 3 different genetic loci giving rise to 3 isoenzymes for NOS: i) the neuronal form (nNOS) or type I that is widely expressed in the brain and is mainly located in the striatum, nucleus accumbens, hippocampus and amygdala, ii) the inducible form (iNOS) or type II that is present is astrocytes, microglial cells, smooth cells and macrophages and is produced in response to inflammation or trauma and iii) the endothelial form (eNOS) or type III expressed in the endothelial cells in the central nervous system (CNS) and the periphery. The nNOS and the eNOS are classified as constitutive forms (cNOS) and require binding with calmodulin that is achieved upon increased Ca^{2+} levels. However the iNOS could act in a Ca^{2+}-independent manner and therefore produces NO even at low Ca^{2+} levels. Another difference between cNOS and iNOS is that the first produces low amount of NO transiently, while the latter produces high amounts of NO that last for hours to days. Balance between production of cNOS and iNOS defines the physiological properties of NO, since production of low levels is neuroprotective, while higher levels lead to neurotoxicity (for a review, see (15)).

In the CNS, NO acts as unconventional neurotransmitter that is not stored in vesicles and upon production acts as a retrograde messenger traveling from the post-synaptic to the pre-synaptic neuron, where promotes the release of neurotransmitters (16). Consistent with the above observation, NO activity was shown to affect LTP. In particular, diminishing of NO signaling, either by NOS inhibitors or by application of substances that absorb NO in the extracellular space, was shown to block induction of LTP in both *in vivo* and *in vitro* studies (17). On the contrary, low dose of NO could convert E-LTP to more stable L-LTP (18).

As mentioned above, the main action of NO is mediated by increase in cGMP levels and activation of the downstream effectors and CNGC that both regulate neurotransmission (19). Except for the cGMP-mediated action of NO, currently has been shown that NO could affect the action of proteins and channels with S-nitrosylation, inhibiting or up-regulating their activity. For example, nitrosylation could reduce the activity NMDARs (for a review, see (20, 21)). The latter inhibition of NMDAR activity is suggested as part of the neuroprotective action of NO. Excessive NMDAR activation leads to prolonged stimulation of NOS and subsequently NO production. In turn, NO nitrosylates NMDARs, preventing

abnormal elevation in Ca^{2+} that leads to excitotoxicity (22). The NO signaling is terminated by NO scavengers, inhibitors of NOS and PDE inhibitors.

AMPA receptors: subunit composition, dynamics and trafficking

AMPAR is an ionotropic, glutamate-gated channel that mediates fast excitatory neurotransmission in the central nervous system. Combination of four different subunits, GluA1-GluA4, give rise to tetrameric AMPARs with unique physiological characteristics and trafficking patterns. Although the ligand-binding domain is highly conserved, the extracellular N-terminal subunit and the intracellular C-terminal are disparate between the subunits (23). Combination of molecular techniques has shown that the majority of AMPARs in the adult hippocampus are comprised of GluA1/GluA2 and GluA2/GluA3 heterotetramers, while GluA1/GluA3 and homotetrameric GluA1 represent only a small percentage (~8%) (24). In addition, GluA4-containing AMPARs are abundant in the developing brain, while in the adult brain they are mainly expressed in the cerebellum, parvalbumin-positive fast-spiking interneurons and certain synapses of auditory neurons (25-27).

The assembly of AMPARs into heterotetramers [(dimers or (hetero)dimers)] takes place in the endoplasmic reticulum (ER), where GluA2 subunits undergo RNA editing to replace glutamine (Q) with arginine (R) (28). The Q/R editing defines the conductance of the channels, rendering the receptors Ca^{2+}-impermeable. The majority of AMPARs in the adult brain consists of GluA2 subunits that are edited and therefore are Ca^{2+}-impermeable with low channel conductance. AMPARs that lack the GluA2 subunit could be Ca^{2+}-permeable, but are scarce in basal conditions. Nevertheless, GluA1 homotetramers were shown to traffic to the membrane during the initial phase of LTP, where they promote Ca^{2+} influx inside the cell, facilitating synaptic transmission (29). Replacement of these types of receptors from Ca^{2+}-impermeable denotes transition of E-LTP to L-LTP (30).

AMPARs are characterized by high motility and their trafficking to the synapse involves several steps including exocytosis to extrasynaptic sites, lateral diffusion to the synapse and stabilization via interaction with the postsynaptic density (PSD) (31). Subunit composition of AMPARs and subsequent interaction with scaffolding proteins, and post-translation modification determine their trafficking to the synapse. Based on the C-terminal domain, AMPARs could be divided in short-tail and long-tail. The first group consists of GluA1, GluA4 and a splice variant for GluA2, while the second group consists of GluA2, GluA3 and a splice variant for GluA4. In general short-tailed subunits like GluA2/GluA3 traffic to the synapse constitutively, whereas trafficking of long-tailed subunits like

GluA1/GluA2 is activity-dependent (32).

As mentioned above, the C-terminal could undergo post-translational modifications, with phosphorylation being the most intensely studied. Several kinases including Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII), PKA, PKG and protein kinase C (PKC) could phosphorylate the GluA1 subunit (4). Specifically, phosphorylation by PKA or PKG at S845 triggers trafficking of GluA1-AMPARs to extrasynaptic sites (5, 33). Additional phosphorylation from CaMKII or PKC at S831 during induction of LTP promotes insertion of GluA1-AMPARs to synaptic sites (34). Phosphorylation at S845 and S831 also increases the channel-open probability and the conductance of the channel, respectively (35-37). The importance of these phosphorylation sites in plasticity was underscored by studies that utilized knock-in mutant mice that lack both or one phosphorylation sites. Lack of both phosphorylation sites impaired bidirectional synaptic plasticity (38). Nevertheless, lack of S831 alone did not affect LTP or long-term depression (LTD), whereas lack of S845 impaired only LTD (39). The latter comes in concordance with previous observations that dephosphorylation at S845 is related to endocytosis of AMPARs and LTD that represents weakening of the synaptic strength (40). Except for the above phosphorylation sites that attracted more interest, it was also shown that phosphorylation by PKC at S818 enhances activity-dependent insertion of GluA1-AMPARs at extrasynaptic sites, whereas blockage of S818 phosphorylation induced faster decline of LTP (41).

Different subunits of AMPARs interact with specific auxiliary subunits known as transmembrane AMPARs regulatory proteins (TARPs). The interaction between AMPARs and TARPs occurs via the PDZ domain, located in their C-termini. Stargazin is known as the prototypical TARP, first described in the mutant mouse stargazer that lacks functional AMPARs at synapses (42). Regarding the role of stargazin in synaptic delivery and clustering of AMPARs, it was shown that it mediates interaction between AMPARs and scaffolding proteins like PSD-95, since AMPARs could not bind directly to PSD-95 (43). Additionally, during LTP, CaMKII-mediated phosphorylation of stargazin increases its affinity or accessibility to PSD-95, promoting stabilization of AMPARs to the synapses (44).

Except for stargazin, several other TARPs have been identified with most well-known being synapse-associated protein-97 (SAP-97), reversion-induced LIM gene (RIL), glutamate receptor-interacting protein (GRIP) and protein interacting with C-kinase 1 (PICK1). SAP-97 and RIL are GluA1-binding TARPs that are involved in synaptic delivery and interaction of AMPARs with actin, respectively (45, 46). GRIP2 and PICK1 are binding to GluA2 and GluA3 subunits. Binding of GRIP and PICK1 in GluA2 receptors has an important role in

subcellular localization of AMPARs and LTD. Specifically, GluA2-GRIP interaction prevents insertion of AMPARs to the synapse, causing retention of AMPARs in intracellular pools and facilitation of LTD (47). Nevertheless, phosphorylation of GluA2 by PKC decreases its affinity for GRIP, but not for PICK1, allowing AMPARs to return to the synaptic surface (48).

Tau-related pathogenesis

Alzheimer's disease (AD) is one of the most common neurodegenerative disorders characterized by progressive memory decline. The distinguished pathological hallmarks characterizing AD include: tau positive neurofibrillary tangles (NFTs), amyloid plaques and neuronal loss. Initially, the pathogenesis of the disease was attributed to aggregation of Aβ into amyloid plaques (49). Nevertheless, recent studies suggest that tau protein also has a crucial role in the neuronal loss and cognitive deficits of the disease (50, 51). Upregulation of cyclic nucleotide signaling cascades have been shown to be promising targets in enhancing cognition or to restore memory impairments (10). Additionally, agents that elevate cyclic nucleotide signaling could compensate against Aβ-induced pathology and synaptic dysfunction (52, 53). Importantly, Aβ and tau share several common features, including structural similarity (54-56), tendency towards oligomerization (57, 58), activity-dependent release (56, 59-61), and association with the same membrane proteins (62-66). In a similar way, upregulation of the cyclic nucleotide cascade may protect against tau-induced pathology.

Tau belongs to the family of microtubule-associated proteins (MAPs) that is encoded by the MAPT gene. Alternative splicing results in six isoforms that consist of either zero, one or two N-terminal inserts (N), and three or four microtubule-binding repeats (R). The expression of tau isoforms differs during the developmental stages. For instance, the adult brain expresses all six isoforms, while the fetal brain only expresses the shortest isoform (3R/0N) (67-69). Additionally, it was shown that although pathological aggregates of tau are immunoreactive for both 4R and 3R isoforms (70, 71), the ratio of 4R/3R expression is increased in certain tauopathies, in which 4R isoforms accumulate in brain lesions (72).

The physiological role of tau in neurons involves assembly and stabilization of microtubules, maintenance of axonal stability (73), axonal transport, neurite outgrowth (74-77), targeting of glutamatergic receptors to postsynaptic sites (78-80) and docking of synaptic vesicles (81, 82). Although tau phosphorylation in the binding domain is important for its interaction with microtubules, hyperphosphorylation prevents its association with the cytoskeleton, inducing axonal transport deficits and synaptic dysfunction (83, 84). Under

physiological conditions, tau is soluble with no secondary structure and it is mainly localized in the axons. On the contrary, in its pathological state, tau forms insoluble fibrillar aggregates, known as paired helical filaments (PHF) or NFTs, and it is also localized in the cell soma (85).

Several pathological conditions promote conformational changes to tau molecule. Oxidative stress, hyperphosphorylation, mutations and truncation lead to structural changes of tau protein that makes it prone to aggregation into soluble pre-tangle molecules, known as oligomers (86, 87). Additionally, structurally modified tau could bind to native tau, causing changes and leading to propagation of tau pathology in a prion-like manner (88, 89). In the progress of tau pathology, tau oligomers are clustering to form PHFs and high order NFT aggregates. Recently, Lasagna-Reeves (90) showed that tau oligomers, independently of NFTs lead to the formation of annular protofibrils (APFs), pore-like structures in the membrane of the cell, nucleus or other organelles, causing ion leakage and changes in the cell permeability. These changes may lead to cellular damage promoting spreading of tau to the extracellular space and to non-affected brain areas.

Regarding the spreading of tau pathology, it is speculated that extracellular soluble tau passes from one neuron to neighboring neurons, spreading the disease from the hippocampus to the cortex (91, 92). The exact mechanism that leads to tau spreading is not yet completely understood; however, several mechanisms have been proposed. Tau may be released after its packing into synaptic vesicles, called exosomes (93). In the same line, it has been shown that Fyn receptor and markers related with autophagy may contribute to tau secretion (94). Transmission of tau to neighboring cells could be mediated by activation of the muscarinic M1/M3 receptors (95), or endocytosis (96), or direct cell penetration (97). Upon entering the cell, tau induces disruption in the microtubule transport system (98) and Ca^{2+} homeostasis (99, 100) that are critical for learning and memory processes. Additionally, it promotes caspase activation that leads to neuronal death (101).

Aim and Outline of the Thesis

The aim of the present dissertation is to investigate the molecular mechanisms underlying the temporal distinction in the action of cyclic nucleotides in vivo. Additionally, we examined the neuroprotective properties of upregulating the cyclic nucleotide signaling cascade against tau pathology.

In **chapter 2** we provide a thorough overview of the cyclic nucleotide pathways, as well as, their involvement in memory formation and in pathological situations in which memory is impaired. Since targeting the cyclic nucleotide pathways was proven to be promising approach for enhancing memory or ameliorate memory impairments, upstream and downstream molecules involved in the cAMP and cGMP pathways have been reviewed, including ACs, sGCs, PKA and PKG. We also underscored the action of PDEs since they do not only regulate the concentration of cyclic nucleotides within a cell, but also represent a point of cross-talk between cyclic nucleotides. Finally, we bring forward the notion of temporal compartmentalization in the action of cyclic nucleotides and we provide an overview of the expanded optical and genetic toolbox in detecting and manipulating several components of cyclic nucleotide signaling pathways.

In **chapter 3** we summarize studies showing the involvement of ionotropic (NMDARs and AMPARs) and metabotropic glutamate receptors (mGluRs) in object recognition and object location memory tests. The existence of multiple binding sites in the NMDARs and AMPARs led to the development of several compounds able to modulate glutamatergic transmission. Agonist for either NMDARs or AMPARs were mainly restricted to brain lesions since increased activation of the ionotropic glutamate receptors leads to excitotoxicity and cell death. Regarding NMDARs, agonists or antagonists of the glycine-binding site, modulators of the polyamine-binding site and competitive or non-competitive antagonists have been investigated in healthy and impaired animal models. In addition, most of the studies examining the action of AMPARs implemented antagonists and positive modulators known as ampakines. Finally, mGluRs were mainly studied by utilization of antagonists, negative and positive allosteric modulators.

Continuing to the experimental approaches, in **chapter 4** we research the underlying molecular mechanism of the differential action of cAMP/PKA and cGMP/PKG during memory formation. This study was inspired by previous findings showing that application of selective PDE5 and PDE4 inhibitors could enhance memory when they are applied within specific mnemonic phases. Considering that both PKA and PKG are involved in AMPAR

dynamics, we speculate that the differential effect of the above inhibitors could be mediated by changes in AMPAR synthesis and trafficking to the membrane.

In chapter 5 and 6, we investigate whether upregulation of cGMP and cAMP pathways could protect against tau-induced impairments in plasticity and memory formation. In **chapter 5** we assess several molecules involved in NO/sGC/cGMP/PKG pathway for their potential neuroprotective mechanism against tau-pathology. The study was inspired by the initial observation that intrahippocampal injection of tau oligomers could suppress CREB phosphorylation during memory formation. Considering that PKG leads indirectly to CREB activation, we speculate that upregulation of several components of the NO/sGC/cGMP/PKG pathway could rescue deficits caused by tau. In **chapter 6** we examine again pharmacological interventions that could combat tau pathology, but this time we focus on the cAMP/PKA signaling as potential target.

Finally, in **Chapter 7** we summarize and discuss the main findings of this dissertation.

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Chapter 2

Role of cyclic nucleotides and their downstream signaling cascades in memory function: being at the right time at the right spot

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Abstract

A plethora of studies indicate the important role of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) cascades in neuronal plasticity and memory function. In turn, altered cyclic nucleotide signaling has been implicated in the pathophysiology of mnemonic dysfunction encountered in several diseases. In the present review we provide a wide overview of studies regarding the involvement of cyclic nucleotides in physiological and pathological mnemonic processes. Additionally, upstream and downstream molecules involved in the cyclic nucleotide signaling cascade are reviewed, including adenylate cyclases (ACs), guanylate cyclases (GCs), protein kinase A (PKA), protein kinase G (PKG) and exchange protein activated by cyclic AMP (Epac). Next, we discuss the regulation of the intracellular concentration of cyclic nucleotides. In that respect, phosphodiesterases (PDEs) hold a prominent role, as they are the enzymes that degrade cAMP and/or cGMP, as well as A-kinase-anchoring proteins (AKAPs) that refine signal compartmentalization of cAMP signaling. We also provide an overview of the available data pointing to the existence of specific time windows in cyclic nucleotide signaling during neuroplasticity and memory formation. The latter resulted in the significance to target specific time windows in order to improve cognitive functioning including memory. Finally, we highlight the significance of emerging imaging tools like Förster resonance energy transfer (FRET) imaging and optogenetics in detecting, measuring and manipulating the action of cyclic nucleotide signaling cascades.

Introduction

Cyclic nucleotides are the "second messengers" that connect the extracellular environment to the intracellular environment and transduce the signal of the "first messenger" like hormones and neurotransmitters. The initial second messenger described was the cyclic adenosine monophosphate (cAMP) in 1971 by Earl W. Sutherland Jr. who won the 'Nobel Prize in Physiology or Medicine' for his discoveries concerning the "mechanism of the action of the hormones". The same prize was awarded 27 years later to Robert F. Furchgott, Louis J. Ignarro and Ferid Murad for their work regarding the role of nitric oxide (NO) and cyclic guanosine monophosphate (cGMP) as signaling molecules in the cardiovascular system. The importance of cAMP in signal transduction was highlighted by the work of Eric Kandel who described the importance of the synapses in memory formation, showing that activation of cAMP and its downstream kinases is essential for synaptic plasticity. Also Eric Kandel together with Arvid Carlsson and Paul Greengard received the 'Nobel Prize in Physiology or Medicine' for his seminal work on memory processes and function. Over the past decades, an abundance of studies emerged showing the role of cyclic nucleotides in cellular mechanisms related to memory function, including signal transduction, neuroplasticity, metabolism, gene transcription and cell growth.

Box1. Different stages of plasticity and memory

Synaptic potentiation represents an experimental model for examining the synaptic basis of learning and memory. Depending on the duration, synaptic potentiation can be divided into short-term potentiation (STP), lasting <1 h, and long-term potentiation (LTP). Subsequently, LTP can be divided in 3 phases with distinct underlying mechanisms; LTP1 that dependents on activation of kinases, LTP2 that is the long-lasting phase and dependents on protein translation form pre-existing mRNA and LTP3 which requires gene transcription and subsequent protein translation (1, 2). Early phase LTP (E-LTP) or LTP1 usually lasts less than 3 h while late LTP (L-LTP) or LTP3 could last up to 8 h. With respect to mnemonic stages, it is suggested that E-LTP is equivalent to short-term memory (STM) and L-LTP is related to long-term memory (LTM) (3), while an intermediate-term memory (IM) phase is similar to LTP2. It could be proposed that STM could be converted to IM via early consolidation and from IM to LTM via late consolidation (4).

The present review will summarize available data and discuss the role of cyclic nucleotides in mnemonic processes. Additionally, upstream and downstream molecules involved in the cyclic nucleotide signaling cascade are discussed. Accordingly, the existence of time windows and their action is discussed, and the resulting significance to target specific windows in order

to improve memory performance. Finally, we highlight the significance of emerging imaging tools in manipulating and determining the action of cyclic nucleotides signaling cascades.

Second messenger cascades

The cAMP pathway. The second messenger cAMP is synthetized from adenosine triphosphate (ATP) by adenylate cyclase (AC). ACs are transmembrane enzymes regulated by G-protein coupled receptors (GPCR). There have been identified nine unique membrane isoforms of AC, which are referred to as AC1-9, and one soluble form (sAC). Neurotransmitters can activate or inhibit AC signaling into the cells via GPCR. In basal conditions, GPCR are heterotrimers consisting of 3 subunits: α, β, γ. Activation of these receptors by their respective ligand (e.g. neurotransmitter or hormone) results in dissociation of the subunits into a free α subunit (G_α) and a free βγ subunit complex ($G_{\beta\gamma}$). The G_α can directly bind to AC, causing its activation ($G_{\alpha s}$) or inhibition ($G_{\alpha i}$). Increased production of cAMP triggers a multitude of cellular reactions coordinated by its downstream effectors, including protein kinase A (PKA), also named c-AMP dependent protein kinase (cAPK), cyclic nucleotide-gated channels (CNGC) and exchange protein directly activated by cAMP (Epac). Nevertheless, the most widely known and studied effector mediating intracellular cAMP signaling is PKA. Activated PKA can phosphorylate several cytosolic and nuclear substrates. A major event facilitating neuronal plasticity is phosphorylation and subsequent activation of cAMP response element binding protein (CREB) at Ser133 by PKA. Phosphorylated CREB (p-CREB) is an activated transcription factor that binds the cAMP response element (CRE), initiating the transcription of specific genes coding for neurotransmitter receptors such as ionotropic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors or growth factors including brain-derived neurotrophic factor (BDNF) (5). Termination of PKA activity occurs via a negative feedback mechanism. Among the substrates that undergo phosphorylation by PKA are the phosphodiesterase enzymes (PDEs) that degrade cyclic nucleotides. Upon activation, PDEs catalyze the conversion of cAMP to ATP, reducing cAMP levels and bringing PKA back to its inactive state. PKA provides an additional negative feedback in the AC/cAMP signaling cascade by inhibiting the enzymatic activity of AC5 and AC6 (6, 7).

Despite the traditionally described AC/cAMP/PKA pathway that leads to CREB phosphorylation, it has been shown that cAMP and PKA can independently lead to CREB phosphorylation via the extracellular signal-related kinase (ERK). In response to neurotransmitter release, the concentration of intracellular calcium (Ca^{2+}) is raised leading to

the increased phosphorylation of ERK via the traditional Ras/Raf/MEK/ERK pathway (8). Subsequently, CREB is phosphorylated by the ERK-activated Rsk family of protein kinases (9, 10). More precisely, Rsk2 is implicated in the Ca^{2+}-stimulated CREB phosphorylation in cell cultures (10). The impact of ERK on CREB phosphorylation is also supported by the observation that inhibition of ERK reduces CREB phosphorylation (22). Additionally, it has been shown in neuronal cell cultures that ERK could be activated in a Ras-independent and PKA-dependent fashion. Based on that model, PKA promotes activation of the small G-protein Rap1 and the downstream kinase B-Raf creating the Rap1/B-Raf signaling complex that results in ERK activation (11). This pathway could work in tandem with the previously described pathway in facilitating neuronal signaling upon an extracellular stimulus. Finally, except for the direct role of PKA in activated ERK, translocation of ERK to the nucleus requires PKA activity (6).

Additionally, there seems to be an interplay between the cAMP second messenger system and the phosphoinositol second messenger system. As with AC, binding of a ligand to the $G_{\alpha q}$-linked GPCR receptor leads to activation of phospholipase C (PLC) which hydrolyzes phosphatidylinositol 4,5-biphosphate (PIP2). The latter leads to the production of two intracellular mediators inositol-1,4,5,triphosphate (IP3) and diacylglycerol (DAG). IP3 diffuses rapidly into the cytosol and binds to the IP3 receptors of the endoplasmic reticulum, promoting the release of Ca^{2+}. DAG is retained in the membrane where it interacts with and activates protein kinase C (PKC) in the presence of Ca^{2+}. Of note, the source of Ca^{2+} could also be extracellular after stimulation of an ionotropic receptor [e.g. N-methyl-D-aspartate (NMDA) receptor]. Upon activation, PKC can activate ERK by acting on Raf as part of the Ras/Raf/MEK/ERK pathway (12). Additionally, PKC stimulates activation of AC2, AC4, AC7 (13-15) and inhibits AC9 (16) creating a regulatory loop between PKC and cAMP/PKA signaling pathway. Elevated intracellular Ca^{2+} could also result in the activation of specific Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), which can stimulate the insertion of AMPA receptors in the postsynaptic membrane (17). In addition, CaMKII can also activate PKA indirectly via activation of AC and subsequent production of cAMP (18).

Except for the above mechanisms that represent the postsynaptic action of the cAMP signaling pathway, a presynaptic role has also been identified. Presynaptically, cAMP is mainly involved in synthesis, release and metabolism of neurotransmitters like glutamate and dopamine (19, 20), most likely via a presynaptic CaMKII/AC/cAMP/PKA cascade (21).

The cGMP pathway. In the same line as cAMP, cGMP production is catalyzed by the enzyme guanylate cyclase (GC) from guanosine triphosphate (GTP). GC exists in two forms;

either a membrane bound particulate GC (pGC) or soluble GC (sGC) form. The seven isoforms of pGC are mainly expressed in the periphery and their distribution in the brain is restricted to the pineal gland, the cerebellum, the pituitary and the olfactory epithelium. sGC is widely distributed in the brain including the hippocampus, striatum, cerebral cortex and locus coeruleus (22). In the brain, intracellular responses to neurotransmitters are mainly attributed to activation of sGC, while the pGC responds to natriuretic peptides (23).

It has been proposed that several neurotransmitters could activate sGC for the production of cGMP. However the most potent activator is NO which is synthesized from the enzyme nitric oxide synthase (NOS) in response to elevated Ca^{2+} levels. Ca^{2+}/calmodulin binds to the catalytic domain of NOS which catalyzes the synthesis of NO from L-arginine. At nanomolar concentrations, NO binds to sGC and rapidly increases the catalytic conversion of GTP to cGMP (24, 25). The main intracellular effectors conveying the NO-sGC signal include cGMP-gated cation channels, protein kinase G (PKG; or cGMP-dependent protein kinase (cGK)) and phosphodiesterases (cGMP-specific phosphodiesterases and cGMP-regulated phosphodiesterase that have allosteric sites for cGMP). From the above effectors, PKG has been most extensively studied and several lines of evidence implicate the cGMP/PKG pathway in the modulation of synaptic transmission. NO acts as retrograde messenger and stimulates presynaptic sGC to induce production of cGMP and PKG. Additionally, in the presynaptic terminal, cGMP can influence the release of neurotransmitters, like glutamate and dopamine (26, 27). Postsynaptically it has been shown that upregulation of the cGMP/PKG pathway results in CREB phosphorylation (28, 29). The underlying mechanism of this relationship is yet elusive. *In vitro* studies conducted with cell lines showed that, although PKG could phosphorylate a synthetic peptide of CREB that contains the Ser133 sequence, the reaction occurs at a lower rate in comparison to PKA (30). Therefore, the general belief is that the cGMP/PKG pathway mediates CREB phosphorylation via an indirect mechanism, which is also supported by the fact that there is no evidence showing PKG nuclear localization. PKG could also facilitate CREB phosphorylation by stimulating the release of Ca^{2+} from ryanodine-sensitive stores (28). In that respect, CREB phosphorylation can be mediated by Ca^{2+}-sensitive ERK via the CREB kinase Rsk2 (10).

Adenylate cyclases: regulation, tissue distribution and participation in neuronal function

ACs are the key enzymes in the initiation of cAMP signaling since they catalyze the conversion of ATP to cAMP. Based on their regulation, membrane-bound ACs can be divided

in four groups. Group I consists of AC1, AC3 and AC8, which are activated by Ca^{2+} and CaMKII. Additionally, they are synergistically activated by $G_{\alpha s}$ (31-33), while they are inhibited by $G_{\beta\gamma}$ (31, 34, 35). Although AC3 is grouped along with AC1 and AC8 as Ca^{2+}-activated AC, this classification is based on one study showing that AC3 can be activated by high levels of Ca^{2+}, but only when the co-factors forskolin or GppNHP (a non-hydrolysable GTP analog) are present (36). The fact that in cell lines only AC1 and AC8 are stimulated in physiologically relevant concentrations of Ca^{2+}, questions the co-categorization of AC3 in the same group as AC1 and AC8 (37). Group II contains the Ca^{2+} insensitive AC2, AC4 and AC7, which are stimulated by $G_{\beta\gamma}$ subunits (31, 38-40). Group III contains AC5 and AC6 which are inhibited by Ca^{2+} and the $G_{\alpha i}$ (41-44) and group IV contains AC9, which is the only AC isoform that is not responsive to forskolin, and undergoes negative regulation by calcineurin (CaN) (45, 46). Finally, the sAC is located in the nucleus, mitochondria and centrosome during cell division and it is activated by bicarbonate. Changes in bicarbonate reflect changes in pH and carbon dioxide indicating that unlike membrane AC that responds to extracellular signal, sAC responds to intrinsic cellular signals (47).

A detailed analysis of the expression of the different AC isoforms in mouse brain, revealed that all isoforms are expressed in neuronal tissue with the exception of AC7, which is undetectable in the brain, and AC4 that is mainly expressed in vessels (48). With respect to their expression pattern in the hippocampus, it was shown that AC1, AC2 and AC8 are highly expressed in cornu Ammonis 1 (CA1), while AC1 and AC2 are also expressed in dentate gyrus (DG). AC5 and AC6 are mainly expressed in the CA2 subregion. Interestingly, AC9 is abundant in the hippocampus and it is the only isoform highly expressed in all three CA subregions and in the DG (46). Based on the above observation, the authors suggested regional co-localization and complementation in the expression pattern of ACs that share the same regulatory mechanism in the hippocampus. Additionally, there seems to be complementation in the expression of group I ACs, with AC1 expression prevailing in the DG, while AC8 is mainly restricted to CA1. Except for the distinct cellular localization, AC1 and AC8 also display distinguished patterns of subcellular expression. In particular, AC1 is abundantly expressed in the postsynaptic density and extrasynaptic sites, whereas AC8 is mainly expressed in the presynaptic active zone and extrasynaptic fractions (49). Despite the importance of the above findings regarding the expression of AC, they do not provide evidence for the expression of functional AC enzymes in the different subregions of the hippocampus (48, 50).

Role of ACs in synaptic plasticity and memory. ACs have been shown to be involved

in plasticity and hippocampus-dependent forms of memory. Consistent with their expression and distribution in the hippocampus, the involvement of the Ca^{2+}-stimulated AC1 and AC8 is extensively studied in LTP studies and behavioral paradigms. Mice that overexpress the AC1 encoding gene Adcy1 showed sustained LTP (e.g. L-LTP) after an induction protocol that normally induces only E-LTP (51), and additionally exhibited impairments in long-term depression (LTD) (52). This observation was accompanied by a pro-cognitive effect in the object recognition test (ORT), in which transgenic mice were able to remember the objects after a long interval between the two trials (51). These results indicate that increased activation of AC1 could convert the initial short-term memory (STM) into a more stable long-term memory (LTM) that lasts up to days. Additionally, the Adcy1-overexpressing mice exhibited lower rates of fear memory extinction. The authors attributed the latter finding to increased ERK and CREB phosphorylation that could result in enhanced initial memory formation and therefore hampered extinction (51). Intriguingly, another study showed that AC1-overexpressing mice showed better memory flexibility in a spatial memory task, as reflected by their ability to suppress previously learned information and relearn the task, indicating that the extinction mechanism appears to differ in fear and spatial memory (52).

Noteworthy is a study from Storm and colleagues in which they examined the effect of AC1 overexpression in both young and aged mice (53). As it was expected, young mice showed a superior mnemonic ability in comparison to their aged littermates in several mnemonic tasks including fear conditioning, object recognition and spatial memory. Interestingly, AC1 overexpression in aged mice did not affect their performance in the first two tasks and even resulted in memory deficits in spatial memory. Considering that basal levels of AC1 activity were downregulated with aging, the above finding seems initially counterintuitive. Nevertheless, it was also shown that in the aging brain PDEs are also downregulated leading to an enhancement in cAMP levels and PKA activity (54, 55). Therefore, the negative effect of AC1 overexpression is possibly due to hyperstimulation of the already disinhibited cAMP pathway in aged animals.

Except for overexpression of the gene, a reversed approach was also employed in order to investigate the role of Ca^{2+}-stimulated ACs in plasticity and memory. Early studies showed that knockout (KO) of AC1 impaired the mossy fiber (DG→CA3) LTP, while the perforant path (entorhinal area→DG) and the Schaffer collateral (CA3→CA1) LTP were unaffected (56). Despite the fact that AC8 contributes in lesser extent than AC1 to Ca^{2+}-stimulated ACs activity in the hippocampus, AC8 KO mice manifested the same level of impairments in mossy fiber LTP as AC1 KO mice (57). Although genetic depletion of either

AC1 or AC8 did not impair the Schaffer collateral LTP in the hippocampus, double KO (DKO) mice for both genes showed deficits in this type of LTP (58). DKO mice also failed to express LTD (59), suggesting that the action of both Ca^{2+}-stimulated ACs is important for bidirectional synaptic plasticity.

At the behavioral level, KO mice for either AC1 or AC8 displayed deficits in the Morris water maze (MWM) spatial learning task (60, 61). It appears to be a functional redundancy in these two isoforms since AC1 or AC8 KO mice did not exhibit memory impairments in passive avoidance learning and contextual fear conditioning, while DKO had impaired performance in these tasks (58). Importantly, although AC1 KO mice exhibited normal acquisition and retrieval of contextual fear memory, they were unable to sustain it for a long period in comparison to wild type mice, pointing out the importance of AC1 for memory stability (62). The differential effect of AC1 or AC8 KO mice in comparison with DKO mice in various learning tasks could be related to the involvement of different brains areas during each task, as well as with the distinct distribution of the different Ca^{2+}-stimulated ACs in the subregions of the hippocampus. For example, it was suggested that the mRNA of AC1 was increased in the subregion CA1-CA2 of the hippocampus during the acquisition phase of radial arm water maze (e.g. a derivative of MWM that assesses reference spatial memory), but not during the procedural version of the task (63). This regional specificity indicates that formation of spatial and non-spatial memory requires distinct distribution of Ca^{2+}-stimulated ACs in the hippocampus (63). Finally, impairments in DKO mice were accompanied by deficits in relearning and suppression of old spatial memory in the reversal platform test of MWM (59), outlining again the importance of balanced cAMP/PKA signaling for strengthening and weakening of the synapses.

Although studies examining the involvement of AC1 or AC8 isoforms in plasticity and memory are prevailing, AC3 seems to possess a unique role in mediating signal transduction. This notion was supported by its high expression in neuronal cilia (64). Specifically, in the hippocampus, AC3 is only expressed in the primary cilia of neurons (65). Although the exact role of neuronal cilia in memory remains elusive, it is hypothesized that neuronal cilia represent signaling platforms, sub-serving interactions between receptors (66). It was shown that AC3 KO mice display severe deficits in short-term object recognition memory. Additionally, the transgenic mice showed no impairment in a contextual fear conditioning paradigm, but were unable to dissociate the context with the foot-shock during the extinction paradigm (65). Therefore, the AC3 KO mice appear to have a similar behavioral phenotype with the AC1 and AC8 KO mice, showing impaired acquisition of spatial memory and

extinction of fear memory. Altogether, these findings suggest a novel role of AC3 in conjugation with the already established role of AC1 and AC8 in neuronal function underlying mnemonic processes.

Role of PKA in synaptic plasticity and memory

Each PKA is a holoenzyme, consisting of two regulatory (R) and two catalytic (C) domains. The PKA family is comprised of four R (RIα, RIβ, RIIα, RIIβ) and three C (Cα, Cβ, Cγ) subunits. The division of PKA into two classes, PKAI (consisting of RIα and RIβ dimers) and PKAII (consisting of RIIα and RIIβ dimers) has been attributed to the differences in the R subunits. Binding of cAMP in the two R domains, causes conformational changes leading to the dissociation of the C subunits from the R subunits (67, 68). Subsequently, the C subunits could translocate to the nucleus where they catalyze phosphorylation of serine and threonine residues of several proteins (69). In addition, recent evidence showed that there is a functional pool of PKA tetramers in the nucleus that contributes to fast nuclear PKA signaling (70). Since the pioneering work of Kandel in *Aplysia* showing the necessity of PKA for consolidation of long-term memories, several studies established the involvement of PKA signaling in plasticity and memory in rodents. More precisely, a great body of literature supports that PKA activity is important for L-LTP in hippocampal slices. This observation is supported by both pharmacological and genetic models showing that inhibition of PKA activity can suppress expression of L-LTP (71-73) and conversely PKA activation can elicit L-LTP (74, 75). Nguyen and Kandel showed that application of the two different PKA inhibitors KT-5720 (inhibits the catalytic subunit) and Rp-cAMPS (inhibits the regulatory subunit) during the induction of LTP leads to a rapid decline in the amount of potentiation (73). Nevertheless, LTP was unaffected when the inhibitors were applied 30 min after induction, indicating that PKA recruitment is essential during a defined time window (73). Additionally, LTP was declined in the transgenic mouse model *R(AB)*, that expresses a dominant inhibitory form of the regulatory subunit of PKA (76), while in mice lacking the $C\beta_1$ isoform of the Cβ subunit, L-LTP was attenuated 2.5 h after the induction (77). Although the above studies implicate the action of PKA at the L-LTP, the study of Otmakhova et al. supported the idea that the action of PKA at the postsynaptic cell is not only restricted to L-LTP, but it is also important for expression of E-LTP (78).

Based on these later observations PKA activity could be involved in both E-LTP and L-LTP and its action is mediated via phosphorylation of different substrates. Regarding its role in L-LTP, PKA actions seem to be mediated by CREB phosphorylation and subsequent

transcription of new proteins involved in plasticity (79, 80). The mechanism by which PKA is involved in E-LTP is more unclear, but is thought to require phosphorylation of existing substrates like AMPA and NMDA receptors. Bidirectional trafficking of AMPA receptors is important for hippocampal synaptic plasticity (81, 82). During LTP, PKA-dependent phosphorylation of GluA1-AMPA receptors at Ser845 is required for stable LTP and incorporation of GluA1-AMPA receptors at the synaptic site was blocked by PKA inhibitors (83). Similarly, PKA activity is also linked to NMDA receptor phosphorylation and it was shown that phosphorylation of NMDA receptors by PKA would alter Ca^{2+} influx via the channel (84).

Apart from the above substrates, activated PKA could facilitate signal transmission at E-LTP by modulating the activity of protein kinases and phosphatases. PKA indirectly promotes CaMKII phosphorylation by blocking the activity of protein phosphatase-1 (PP1) through activation of its endogenous blocker protein phosphatase inhibitor-1 (I-1) (85, 86). Negative feedback in the above cascade is provided by Ca^{2+}-activated CaN that inhibits I-1 activity and subsequently CaMKII phosphorylation (87).

As with LTP experiments, a variety of rodent behavioral studies verified that intact PKA signaling is important for formation of LTM. Disruption of PKA activity either via genetic or via pharmacological intervention resulted in impairments in long-term spatial memory (76, 88), contextual memory (76, 89-91) and aversive memory (92-94). It was also shown that PKA activity occurs immediately after training in certain mnemonic tests suggesting it to play a role in STM. Specifically, PKA and p-CREB immunoreactivity were increased in the hippocampus immediately, 3h and 6h after training in the step-down inhibitory test, while intra-hippocampus infusion of the PKA inhibitor KT5720 immediately or 3h or 6h post-training impaired the consolidation process of the task (92). Similarly, two different studies measured PKA levels in the hippocampus during spatial learning and showed that PKA immunoreactivity increases rapidly during training and remained high at later stages of the acquisition phase (95, 96).

A later study from Havekes et al. sought to determine the region specific changes in the RIIα,β subunits of PKA in the hippocampus during habituation, training and reversal training in the Y-maze task (97). Habituation increased PKAIIα,β non-specifically in all the regions of the hippocampus, suggesting that PKA expression is elevated at the initial storing phase of spatial memory. During training and reversal training (day 3) PKAIIα,β expression was increased in CA3 region and DG, while at the end of the procedures (day 7) immunoreactivity of PKAIIα,β returned to basal levels. These results indicate that PKA

expression in the hippocampus is essential for the acquisition and consolidation of new information, initiating molecular cascades that eventually will facilitate formation of stable LTM (97).

Despite the fact that PKA activation facilitates consolidation of memories in the hippocampus, it was shown that activation of the cAMP/PKA signaling cascade in the prefrontal cortex could have a differential effect (55). Although upregulation of the cAMP/PKA pathway could improve performance in memory-dependent tasks, infusion of a PKA activator in the prefrontal cortex of young rats induced a dose-dependent deficit in delayed-alternation performance (98). This observation was more profound in aged animals in which cAMP/PKA signaling is reduced in the hippocampus, while it is elevated in the prefrontal cortex. Consistent with this observation, a PKA activator (Sp-cAMPS) impaired working memory in aged rats, while low doses of a PKA inhibitor (Rp-cAMPS) had the opposite effect (54). Interestingly, the beneficial effect of PKA inhibition was more intense in animals with greater memory deficits due to aging. These observations suggest the importance of taking into consideration age-related and region-specific biochemical differences when examining the cognitive enhancing properties of drugs that act on the cAMP/PKA pathway.

Role of Epacs in modulating cAMP signaling

The discovery of Epacs brought a new perspective to the prevailing view that PKA is the only downstream effector for cAMP signaling. There are two isoforms of Epac, i.e. Epac1 and Epac2 (Epac2A and Epac2B), produced by their two respective genes. The C-terminal catalytic region is the same for both Epacs and it is composed of 3 domains: i) a Ras-exchange motif (REM) domain, ii) a Ras-association (RA) domain and iii) a GEF domain responsible for Epac exchange activity on Raf GTPases (99). The regulatory N-terminal contains a dishevelled, Egl-10, pleckstrin (DEP) domain responsible for membrane binding and a cyclic-nucleotide-binding (CNB) domain that binds cAMP with high affinity (99, 100). Although Epac1 and Epac2B have one CNB-B domain, Epac2A has an additional CNB-A domain at the N-terminal. Despite that difference, CNB-A binds cAMP with very low affinity in comparison to CNB-B, and does not contribute significantly to Epacs' regulation by cAMP (101). Similarly to PKA, binding of cAMP to the regulatory domain of Epacs leads to their activation. Subsequently, Epacs activate the small GTPases Rap1 and Rap2 that represent their main downstream effector (102). The tissue distribution of Epac1 and Epac2 differs since Epac1 appears to be widely distributed, while Epac2 is mainly expressed in the brain

and the adrenal glands. Regarding their pattern of expression in the rat brain, mRNA of Epac1 is abundantly expressed, but generally at low levels, whereas the mRNA of Epac2 is highly expressed in certain regions including cortex, hippocampus, habenula and cerebellum (103). At the subcellular level, Epacs are mainly localized in the nuclear membrane and mitochondria, while during cell division they are located to the mitotic spindle and centrosome (104).

Through their effectors, Epacs can orchestrate several cellular functions raging from gene transcription to cell proliferation and apoptosis, and additionally regulate several intracellular pathways and signaling processes. For example, downstream activation of ERK and P13-kinase-dependent PKB/Akt pathway via Epacs, introduces an important link between Epac action and memory processes (105). Both electrophysiological and behavioral studies suggest the importance of Epacs in learning and memory. Perfusion of mouse hippocampal slices with the Epac agonist 8-(4-chlorophenylthio)-2′-O-methyl-cAMP (8-pCPT) enhanced the maintenance of LTP induced by a weak tetanus, without affecting the initial magnitude of potentiation. This effect was shown to depend on ERK phosphorylation and protein synthesis, while blockage of transcription and PKA activity did not abolish 8-pCPT-dependent facilitation of LTP (106). These results suggest that Epac activation initiates ERK signaling that subsequently promotes local translation of a pre-existing pool of dendritic mRNAs (106). It appears that Epacs comprise a dual function in synaptic transmission since it was shown that, except for the observed enhancement of synaptic transmission, activation of Epacs could also induce LTD in hippocampal slices. This function was shown to be dependent on activation of the p38-mitogen activated protein (MAP) kinase pathway and on internalization of GluA2/3-containing AMPA receptors (107). Considering that PKA was shown to facilitate LTP in the hippocampus (74), the latter finding regarding Epac-dependent LTD provides an additional pathway in the already established role of cAMP signaling cascade in synaptic plasticity. Nevertheless, it still remains unanswered which cellular components determine which pathway will be activated in response to cAMP. Considering that PKA and Epacs have the same affinity for cAMP in living cells, it was hypothesized that subcellular compartmentalization or substrate availability determine which downstream effector will be activated by cAMP (107).

Consistent with the electrophysiological findings, the role of Epacs in memory formation was also supported by behavioral studies. Most importantly, it was shown that Epacs participate in distinct stages of memory formation. Intracerebroventricular (ICV) administration of 8-pCPT 1 h after the fear conditioning paradigm improved mice

performance (108). Similarly, intrahippocampal infusion of the Epac agonist immediately after fear conditioning facilitated memory formation and it was able to attenuate PKA antagonist-induced memory impairments (109). Another study, that tried to gain more insight into the distinct memory stages during which Epac activation can facilitate memory, supports the idea that intrahippocampal activation facilitates contextual fear memory when given at the stage of retrieval, but not at the acquisition phase (110). Additionally, it was shown that Epac2 is the responsible isoform for the enhancing effects of Epac's activation on memory retrieval, since silencing of Epac2 via intrahippocampal injection of siRNA impaired memory retrieval (110).

Although there is scarce of evidence, a few studies support a promising role of Epacs as a target in neurodegenerative diseases. For example, in neuronal cell cultures, Epac1 can exhibit neuroprotective action against the neuropathological features of Alzheimer's disease (AD). Specifically, activation of Epac1 after stimulation of the G_s-coupled serotonin receptor could promote activation of the small GTPases Rap1 and Rac1. This activation of the Epac1-Rap1-Rac1 signaling cascade increased the cleavage of the amyloid precursor protein (APP) via the α-secretase pathway (111, 112), leading to the release of soluble APPα (sAPPα) at the extracellular space (113). Importantly, it was shown that sAPPα has promising cognitive enhancing and neuroprotective properties (114). These observations are particularly important because they could extend our knowledge regarding AD pathology and suggest novel targets for the treatment of the disease. Regarding the role of Epac2 in neurodegenerative diseases, it is reported that Epac2 activation via 8-pCPT influences dendritic spine remodeling, and mutations in the EPAC2 gene could contribute to brain disorders (115). Nevertheless, there is no further evidence supporting the cognitive enhancing properties of Epac2 in AD.

Guanylate cyclases: regulation, tissue distribution and participation in neuronal function

GCs are widely distributed signal transduction enzymes involved in a variety of cellular processes including host defense reactions, cell growth and cell proliferation (for a review see 116). In response to various cellular stimuli, they convert GTP into the second messenger cGMP. In contrast to the transmembrane pGC that serves as a receptor for atrial, B-type and C-type natriuretic peptides, sGC is a receptor for gaseous ligands, especially NO. As a result, sGC is especially interesting in relation to brain neuroplasticity and memory formation. It can associate with the plasma membrane through protein-protein interactions in a constitutive or Ca^{2+}-dependent manner (117-120). sGC is typically found as a heterodimer,

consisting of a larger α-subunit and a smaller haem-binding β-subunit, although it also exists as homodimer (121). Four human sGC subunits have been identified: α1, α2, β1 and β2 of which the α1/β1 and α2/β1 dimers are the most well-known. The β-subunit contains an evolutionarily conserved amino-terminal haem-binding domain, which is crucial for the sensing of NO. Based on sequence homology with the crystallized catalytic domains of AC, the carboxy-terminal catalytic domains of both sGC subunits are assumed to be orientated in a head-to-tail fashion. The catalytic domains of both subunits are required for the formation of a catalytic active center (116, 122, 123).

The different human isoforms of sGC have been known for some time, however little is known about their overall tissue distribution (124). Studies in rat brains, have located sGC mRNA predominantly in the striatum, the olfactory system and layers II and III of the cerebral cortex. More recently, expression patterns in human brains and peripheral tissues have been determined. In all the regions of the human brain investigated, pituitary gland and placenta, expression of β1 is greater than α1. Most of the other tissues studied showed greater α1 expression compared to β1 especially in heart, prostate, small intestine and appendix. Some of the tissues displayed very low levels of expression of both subunits including skeletal muscle, bladder, testis and peripheral leukocytes. In contrast to the adult tissue, fetal brain showed similar levels of α1 and β1. Both adult and fetal heart displayed more α1 than β1. Fetal lung showed greater sGC expression than adult lung.

Role of sGCs in synaptic plasticity and memory. NO/sGC/cGMP signaling can be compromised either by reducing the bioavailability of NO or by altering the redox state of sGC itself, thereby making it unresponsive to endogenous NO and NO-releasing drugs (116). This led to the development of two drug classes to be able to overcome these obstacles: sGC stimulators (stimulate sGC directly and enhance sensitivity of the reduced enzyme to low levels of bioavailable NO) and sGC activators (activate the NO-unresponsive, haem-oxidized or haem-free enzyme). However, sGC stimulators and activators have mainly been investigated for their potential as treatment of arterial and pulmonary hypertension, heart failure, atherosclerosis, thrombosis, erectile dysfunction, renal fibrosis and failure, and liver cirrhosis. Targeting sGC to enhance multiple aspects of memory processes in particular or neuroplasticity in general, is a relatively new strategy in the field of sGC drug development, hence, the limited availability of literature. To investigate the potential of enhanced sGC signaling to improve memory function, studies use, next to stimulators and activators, GC inhibitors as negative control. In this respect, ODQ, a GC inhibitor (125), has shown to impair memory performance during the novel object recognition (NOR) test in mice when

administered 30 min before the retention trial (126). Furthermore, ODQ impaired performance on the passive avoidance test when administered 30 min before acquisition, but only when combined with the NO precursor 7-nitroindazol (7-NI). By itself neither drug had an effect. ODQ also significantly impaired olfactory memory as reflected by the decreased ratio 'percent cued food/percent total food eaten' in the social transmission of food preference test when compared to the control group. ODQ has also shown to suppress LTP in several studies (127, 128-130). Similarly, bilateral intrahippocampal administration of the sGC inhibitor LY 83583 caused full amnesia for inhibitory avoidance when given immediately after training, but not 30 min post-training (131).

Alternatively, the compound YC-1 which activates as well as stimulates sGC, was shown to significantly decrease the acquisition latency (1-4 days) of 24-month-old rats in the MWM when administered daily for 2 weeks (132). Additionally, the same 24-month-old rats showed a reduced time spent in the escape platform's quadrant when compared to 4-month-old rats. YC-1 reversed the reduction of the time spent in the escape platform's quadrant of the 24-month-old rats. YC-1 also reversed the diminished retention latency in 24-month-old rats on the second day during the passive avoidance task.

Additional support comes from Chien and colleagues, who also showed that YC-1 shortened the escape latency in the MWM during the test trial when injected 10 min before the first trial of each daily training session, and increased and decreased the retention scores in the passive and active avoidance task, respectively, when injected 10 min before foot-shock training. Administration of YC-1 30 min after foot-shock stimulation did not significantly affect retention scores in response to the passive avoidance task. Administration of scopolamine, a cholinergic muscarinic antagonist, markedly impaired memory acquisition. Pretreatment with YC-1 inhibited the scopolamine-induced learning deficit. The enhancement of learning behavior by YC-1 was antagonized by ICV injection of the NOS inhibitor L-NAME and PKG inhibitors KT5823 and Rp-8-Br-PET-cGMPS, indicating that the NO/cGMP/PKG pathway is involved in the action of YC-1 (133, 134).

Role of PKG in synaptic plasticity and memory

PKG exists in two forms, the soluble PKGI and the membrane-bound PKGII (135, 136). The two PKG families (PKGI and PKGII) are encoded by different genes and they are both homodimers consisting of two identical subunits, the C and the R. Each subunit has two binding sites for cGMP at the R domain, and allosteric binding of cGMP increases the catalytic kinase activity of the enzyme 3- to 10- fold. Binding of cGMP to the R site does not

lead to its dissociation from the C site.

Two isoforms of PKGI exist, PKGIα and PKGIβ, which differ in their N-terminal domains. PKGIα is activated at 10-fold lower cGMP concentrations compared to PKGIβ. Both exhibit different physiological functions because they interact with different substrates, vary in their regional and subcellular localization, and are expressed in different tissues. PKGIα is highly expressed in cerebellum, dorsal root ganglia and lung, whereas PKGIβ is found predominantly in hippocampus, olfactory bulb, smooth muscle and platelets. PKGIβ expressed in the hippocampus is therefore believed to be involved in LTP and spatial learning and memory. Hippocampus-specific PKGI KO mice showed reduced LTP, but, unexpectedly, no defect in spatial learning and memory tests (137). As a result, the authors suggest that PKGI might therefore be involved in more subtle types of learning in the hippocampus. Hippocampal PKGI signaling has shown to be mediated by vesicle trafficking regulated by RhoA and vasodilator-stimulated phosphoprotein (138). PKGII is localized at the plasma membrane and is expressed in the thalamus, septum, amygdala and olfactory bulb, as well as intestinal mucosa, kidney and chondrocytes. PKGII is much less investigated in relation to neuronal functions including learning and memory, although PKGII KO mice have shown to exhibit significant deficits in spatial learning in the MWM (139).

The critical role of PKG in especially the early stages of memory function was suggested by studies using PKG activators and PKG inhibitors (which do not differentiate between PKGI and PKGII). First support for a role of PKG in memory formation comes from the finding that PKG inhibitors block LTP, and PKG activators facilitate LTP in response to weak tetanic stimuli (128, 130, 140, 141). Also, rats submitted to memory paradigms showed a significant increase in PKG activity in the hippocampus (131). Interestingly, the increase in PKG was only observed immediately after training, whereas no changes were observed 30 min after training raising the idea that the hippocampal PKG cascade is involved in the early stages of memory formation. This is supported by the observation that the PKG activator 8-Br-cGMP, when administered bilaterally into the hippocampus of rats immediately after the learning trial in the ORT, showed a dose-dependent improvement in object recognition compared to saline condition (142). Similarly, intrahippocampal infusions of the PKG inhibitor KT5823 reversed an amyloid-β (Aβ)-induced increase in escape latency and traveled distance in the MWM (143).

Regulation of the intracellular concentration of cyclic nucleotides

The intracellular levels of cyclic nucleotides mainly depend on the fine-tuning between their rate of production by AC and GC and the rate of elimination. The latter is mainly achieved by PDEs and by energy-dependent transport of cyclic nucleotides to the extracellular space. Additionally, the intracellular concentration of cyclic nucleotides is regulated by their sequestration into functionally distinct compartments within the cell.

Energy-dependent transport of cyclic nucleotides. The concept of active transport of cyclic nucleotides into the extracellular space has been mainly studied in other tissues rather than the brain. Transport of cyclic nucleotides involves an organic anion transport process that utilizes multidrug resistant proteins (MRPs). Although there is scarce of evidence regarding the exact function of all the members of the MRP family, it has been shown that cyclic nucleotides can be substrates for MRP4, 5 and 8 (144-147). Although both cyclic nucleotides were shown to be substrates for these three MRP subfamilies, a later study conducted with human erythrocytes suggested that MRP5 mediates cGMP transport 20-times more efficiently than transport for cAMP, indicating that cGMP is a more favorable substrate for MRP5. Along the same lines, it was also shown that the cGMP-specific PDE inhibitor, sildenafil, can block MRP5-mediated export of cGMP (146).

Regarding the expression of MRP1-6 in the adult human brain, it was shown that only MRP1, 4, 5 are present in the brain, while the other members of the family are not detectable in brain tissue (148). Interestingly, MRP4 and 5 were both detected in astrocytes, while MRP5 was also present in pyramidal cells (148). Although MRPs seem to participate in the regulation of cyclic nucleotide concentration in neurons, this observation raises the question of the purpose of an energy-dependent transport of cyclic nucleotides into the extracellular space. A possible explanation could be that this mechanism could serve as a cell-cell communication. For example, in bacteria, cAMP at the extracellular space could bind to membrane receptors and promote their differentiation (149). Nevertheless, there is not a mechanism described in mammals that would explain this theory, especially considering that MRP-mediated transport is unidirectional.

The most prominent explanation is that MRPs facilitate the extracellular transport of cyclic nucleotides where they act as extracellular messengers promoting an autocrine and paracrine mechanism. The concept of extracellular cAMP and cGMP activity is not new (150) and it is thought to contribute to plasticity and memory-related functions of cyclic nucleotides (151). In particular, it was shown that upon extrusion to the extracellular space, cAMP is

converted to adenosine that subsequently mediates several functions throughout the body and the brain via the adenosine receptors A_1, A_{2A}, A_{2B}, and A_3 (152). More recent evidence showed that externally applied cGMP could improve spatial memory. Specifically, the study of Cabrera-Pastor et al. showed that extracellular cGMP modulates glycine receptors leading to an increase in intracellular Ca^{2+} and subsequently CaMKII activation (153).

Degradation of cyclic nucleotides by PDEs. Regulation of intracellular concentration of cyclic nucleotides through degradation by PDEs has been extensively studied. By hydrolyzing cAMP and cGMP, PDEs play an important role in regulating signal transduction mediated by cyclic nucleotides (154). PDEs are grouped into 11 families based on homology of the catalytic domain. Most of the families have more than one gene and each gene can consist of several different splice variants and isoforms (155). In total, there are estimated to be over 100 specific human PDEs, discretely localized to specific subcellular domains (156, 157). Additionally, the different families of PDEs differ in their tissue distribution, properties and substrate specificity with the latter constituting a fundamental distinction between the PDE families. PDE1, PDE2, PDE3, PDE10, PDE11 have a dual substrate specificity, hydrolyzing both cAMP and cGMP. PDE4, PDE7 and PDE8 are cAMP-specific, while PDE5, PDE6 and PDE9 are cGMP-specific. Except for their role in hydrolysis, PDEs mediate a more complex role in regulating and refining cyclic nucleotide signaling in the brain. In this respect, it was shown that cyclic nucleotides can regulate the activity of PDEs, providing a point of cross-talk in which cAMP signaling could influence cGMP signaling and vice versa. With the exception of PDE6 that is mainly found in the retina, all the other families are expressed in several brain areas including the hippocampus, stimulating studies investigating the potential of PDE inhibitors as cognitive enhancers. In the section below the 11 families of PDEs are described and a review is provided on the current studies regarding the role of PDE inhibitors in memory and memory-related diseases.

Dual substrate specificity phosphodiesterases. **PDE1** is a unique PDE family that is regulated by Ca^{2+} and activated by calmodulin. Three genes have been identified for the PDE1 family: PDE1A, PDE1B and PDE1C. All three are abundantly expressed in the brain with the highest levels found in the striatum and moderate level in the cortex and the hippocampus (158-160). The kinetic properties for the 3 isoenzymes differ with PDE1A and PDE1B hydrolyzing cGMP with a Km value lower (3 μM) than cAMP (50-100 μM), whereas PDE1C hydrolyzes both cAMP and cGMP with similar Km value (1 μM) (161, 162). Interestingly, the affinity of PDE1 for the Ca^{2+}/calmodulin complex is altered by phosphorylation. Specifically, phosphorylation of PDE1A1 and PDE1A2 by PKA (163), and

PDE1B1 by CaMKII (164), decreases their affinity for Ca^{2+}/calmodulin, resulting in decreased activity for PDE1. Due to its regulation mechanism, PDE1 constitutes an interesting point of crosstalk and integration of intracellular pathways that are mediated by cAMP and cGMP, and pathways that lead to increased Ca^{2+}. In terms of plasticity, the fact that phosphorylation by PKA decreases the activity of PDE1A1 and PDE1A2 indicates that a signal that increases cAMP would further prolong its action, imposing a paradigm of positive feedback that is reversed after increased Ca^{2+} (154). Additionally, it is reported that cGMP can inhibit PDE1-induced hydrolysis of cAMP (162). Although PDE1 activity significantly contributes to cGMP-mediated inhibition of cAMP hydrolysis in human myocardium, this relationship is not yet determined in neuronal cells. Regarding the action of PDE1 inhibition in the brain, a recent study from Snyder et al. showed that acute treatment with the selective PDE1 inhibitor ITI-214 can improve several stages of mnemonic process in heathy rats after acute treatment (165).

PDE2 is highly expressed in the brain and alternative splicing of the same gene results in 3 isoforms: PDE2A1, PDE2A2 and PDE2A3 (166-168). In the rat brain, PDE2 mRNA can be found in the hippocampus, cortex, amygdala, cerebellum, striatum and hypothalamus (169, 170). The different isoforms of PDE2 differ in their N-terminal sequence introducing differences in their hydrophobicity and subsequently cellular distribution. Additionally, the N-terminus has two cGMP-binding domains, GAF-A and GAF-B, participating in dimerization and binding of cGMP, respectively (171). Although, PDE2 has a dual substrate specificity, hydrolyzing both cAMP and cGMP with similar catalytic properties (Km=30 μM for cAMP and 10 μM for cAMP), cGMP seems to be a preferred allosteric modulator for PDE2, causing cGMP to be more effective in activating PDE2 (172). Specifically, binding of cGMP to one of the GAF domains induces an allosteric modification that lowers the Km for cAMP and promotes up to a 30-fold increase in cAMP hydrolysis (173, 174). Because of its function, PDE2 is part of a negative feedback mechanism and facilitates crosstalk between cGMP and cAMP pathways, implicating important physiological functions. For example, in the brain, signals that elevate intracellular cGMP levels promote PDE2-mediated cAMP hydrolysis, restoring basal levels of cyclic nucleotides (155). Particularly, at the presynaptic terminal of the CA1 area in the rat hippocampus, PDE2A can modulate cAMP levels in response to activation by cGMP, indicating a modulatory role of PDE2A in short-term synaptic plasticity (175). The latter was also highlighted in the study of Boyken et al. in which PDE2A expression appeared very high in the docked fraction of synaptosomes, suggesting an important role of PDE2A in neurotransmitter release from the presynaptic terminal (176).

The expression of PDE2 in brain areas that are involved in memory and cognition rendered it a promising target for the development of cognitive-enhancing drugs. The first available PDE2 inhibitor BAY 60-7550 has shown to enhance memory acquisition and consolidation in healthy mice and rats in the ORT when administered before and after the learning trial, respectively (177, 178). These memory enhancing effects of BAY 60-7550 are inhibited after co-administration of NOS or PKG inhibitors, indicating that the effect of BAY 60-7550 on memory seems to be mediated primarily through activation of the sGC/cGMP/PKG cascade (179). Additionally, it was shown that BAY 60-7550 could improve memory performance in a mouse model for AD, without combating the Aβ pathology of the disease (180).

PDE3 is encoded by two genes, PDE3A and PDE3B with three (PDE3A1, PDE3A2,PDE3A3) (181) and one splice variants (PDE3B1) (172), respectively. In the rat brain, PDE3A mRNA has been found in the cortex, cerebellum and brain stem (182), while PDE3B mRNA is mainly found in peripheral tissues with prominent expression in the cardiovascular system (183). PDE3 hydrolyzes both cAMP and cGMP in a mutual competitive manner, with same catalytic properties for both nucleotides (Km=0.2 μM for cAMP and Km=0.1 μM for cGMP). However, the velocity for cAMP hydrolysis is 4- to 10-fold higher than for cGMP and the substrate affinity for cGMP is higher (154). Therefore, increased production of cGMP through activation of sGC, could inhibit PDE3-mediated hydrolysis of cAMP, rendering it a cGMP-inhibited PDE. The different variants are distinguished by the different lengths of the N-terminal domain. The difference in length of the N-terminus between the variants determines their subcellular localization and their regulation by kinases. The longest variant, PDE3A1, has two N-terminal hydrophobic associated regions (NHR1 and NHR2). It also contains one phosphorylation site for protein kinase B (PKB) and two phosphorylation sites for PKA. The latter was shown to activate PDE3A1 (181), downregulating cAMP signaling through negative feedback. PDE3A2 lacks the NHR1 domain along with the PKB phosphorylation site, but contains NHR2 and the two phosphorylation sites for PKA. The shortest variant, PDE3A3, lacks all the above domains and phosphorylation sites (184).

There are several drugs that can inhibit both isoenzymes equally including cilostazol, cilostamide, enoximone and milrinone. Considering the role of PDE3 inhibitors in memory and cognition enhancement, most of the supportive evidence comes from studies conducted with cilostazol. Behavioral studies showed that the neuroprotective effect of cilostazol against Aβ-induced memory impairments can, at least in part, be attributed to reduction of oxidative

stress and prevention of Aβ accumulation (185, 186). A study conducted with a transgenic mouse strain that exhibits accelerating aging showed that administration of cilostazol for 3 months could have a neuroprotective effect that is reflected in the molecular level by increased p-CREB in the hippocampus and improved integrity of the blood-brain barrier (BBB) (187). Finally, it was shown that treatment with cilostazol for one month could protect against tau pathology and cognitive decline in the rTg4510 mouse line via increasing proteasome activity (188).

PDE10 and PDE11 are two distinct families that were characterized more recently. Regarding **PDE10**, the entire family is encoded by one gene, PDE10A, and alternative splicing generates 18 splice variants (PDE10A1-18) (189). In rat brain, PDE10A mRNA is highly expressed in the striatum, but it is also found in the hippocampus, cortex and cerebellum (54, 56, 57). PDE10A hydrolyzes both cyclic nucleotides with a significantly higher affinity for cAMP (Km=0.20 μM) than for cGMP (Km=1 μM). Because the maximum velocity for hydrolyzing cGMP is 5-fold higher than the one for cAMP, PDE10A hydrolysis of cGMP seems to be inhibited by cAMP (190, 191). Therefore, PDE10A may function as a cAMP-inhibited phosphodiesterase and as a result, stimuli that elevate cAMP could subsequently increase cGMP levels. This function is opposite from the one described for the PDE3 family (155). As with PDE2, PDE10A has 2 GAF domains in the N-terminus. *In vitro* studies have shown that the GAF domains at PDE10A have very low affinity for cGMP (191), and it is considered highly impossible for a cell to reach such high concentrations of cGMP in order to bind to each one of the GAF domains. Nevertheless, the possibility cannot be abolished that a certain regulation could increase the affinity of GAF domains for cGMP. Accordingly, it was recently shown *in vitro* that PDE10A allosterically binds cAMP in one of the GAF domains (192). Regarding its function in the hippocampus, it was reported that mRNA levels of specific PDE10A splice variants are increased after induction of LTP, providing a regulatory mechanism to dampen LTP-induced elevated levels of cGMP (193).

PDE10A1 and PDE10A2 represent the major PDE10A variants in humans (190, 194). A notable difference between the two isoforms is that only PDE10A2 has a PKA phosphorylation site at the N-terminal domain (195) that alters its localization from Golgi apparatus to cytosol (196). That change in PDE10A2 distribution may represent a negative feedback in response to elevated cytosolic levels of cAMP (196). In concordance with the abundant expression of PDE10A in the striatum, development of PDE10 inhibitors currently aims at the treatment of motor disorders. In addition, PDE10 inhibition was shown to have antipsychotic potential and improve cognitive symptoms in animal models of schizophrenia,

providing a new therapeutic approach for the disorder (197-200), though clinical trials with schizophrenia patients did not results in any positive effects.

PDE11 is the newest member of the PDE family which has similar affinities for both nucleotides (Km=1-2 μM for cGMP and Km=2-3 μM for cAMP), and catalyzes both substrates with similar velocity. Four splice variants of the PDE11A gene have been identified (PDE11A1-PDE11A4) that differ in the amino-terminal sequence. PDE11A2 and PDE11A3 have one complete and one incomplete GAF domain in the N-terminus (172), whereas PDE11A4 has two complete GAF domains with phosphorylation sites for PKA and PKG (201). Expression of PDE11 in the brain is relatively low, but it is profoundly distributed in the ventral hippocampus, CA1, subiculum and the amygdalohippocampal area (202). Nevertheless, there is a recent study showing that PDE11 KO mice exhibit particular behavioral and biochemical changes observed in psychiatric disorders including hyperactivity and deficits in tests related to social behavior (202). The involvement of PDE11 in social memory came from a later study in which PDE11 KO mice exhibited normal STM, but not LTM, for social recognition, while non-social odor recognition memory was unaffected. The impaired consolidation of social memory was shown to be associated with decreased protein synthesis in the ventral hippocampus (203, 204). Recently, the first potent PDE11 inhibitors have been identified in a high-throughput screen in a yeast-based assay, paving the way for the development of selective PDE11 inhibitors (205).

cAMP-specific phosphodiesterases. This category of PDEs contains three members that are all highly expressed in the brain; PDE4, PDE7 and PDE8. Among them, **PDE4** has a prominent position since it is the best characterized and inhibition of PDE4 constitutes a potent method for upregulating the cAMP/PKA (Km<10 μM) cascade. Additionally, PDE4 enzymes are distinguished from other PDEs due to their specific inhibition by rolipram. PDE4 is encoded by four different genes (PDE4A-PDE4D), and more than 25 different human isoforms have been described (172). PDE4A, PDE4B and PDE4D are highly expressed in the hippocampus, striatum, thalamus, cortex and cerebellum, while the expression of PDE4C in the brain is relatively low (206). PDE4A and PDE4B constitute the majority of membrane-bound PDE4, and PDE4D the majority of soluble PDE4 in the brain (207).

PDE4 enzymes have two unique highly conservatory domains at the N-terminal domain; upstream conserved region 1 and 2 (UCR1 and UCR2). The presence or absence of these domains divides PDE4 isoforms into three groups: the long, the short and the super short variants. Specifically, the long variants have both the UCR1 and UCR2 domains, the short variants have a complete UCR2 domain and the super-short variants have a truncated

UCR2 domain (208). UCR1 has a phosphorylation site for PKA and upon phosphorylation diminishes PKA's affinity to UCR2, and subsequently promotes PDE4 activation (209). Furthermore, PKA phosphorylation alters the sensitivity of PDE4 towards inhibition by rolipram (210), probably due to conformational changes in the catalytic region (209). The activity of PDE4 is also regulated by ERK phosphorylation in the catalytic domain. ERK phosphorylation promotes activation of the short PDE4 variants and inhibits the long PDE4 variants, while the ERK phosphorylation motif is absent in the super short variants (211). Therefore, phosphorylation by ERK and PKA is particularly interesting in the case of long isoforms and is considered to regulate cAMP signaling. Activation of ERK can inhibit the activity of the enzyme, elevating cAMP levels. Subsequently, PKA activation reliefs ERK-mediated inhibition and reactivates catalytic activity of PDE4, lowering cAMP to its basic levels (212). Although it is not shown yet in neuronal cells, CaMKII can also modulate PDE4D activity in cardiac myocytes. At both basal state and after stimulation of the cAMP pathway, CaMKII activates PDE4D via phosphorylation providing an additional mechanism, besides PKA-mediated PDE4 activation, for controlling intracellular cAMP levels (213).

Phosphorylation by kinases is one mechanism for regulating catalytic function of the different PDE4 isoenzymes. Crystallographic studies revealed multiple conformations for PDE4 isoenzymes that could also alter the catalytic activity of the enzyme (214). In one conformation, the α-helix-UCR2 from one monomer folds over and interacts with the catalytic site of the other monomer, resulting in >50% inhibition of the enzyme. The UCR2 autoinhibition is removed by PKA phosphorylation of UCR1. Conversely, phosphorylation by ERK stabilizes the UCR2-autoinhibition state (215). The sequence of the α-helix is the same for all the isoforms with the exception of a single amino acid that differentiates PDE4D from the other PDE4 isoforms. In fact, this polymorphism has been utilized for the development of isoform-selective inhibitors (214, 215). In a different PDE4 conformation, the helix of the C-terminus, which is called Control Region 3 (CR3), interacts with the catalytic site of the same monomer, causing complete autoinhibition of the enzyme. As with UCR2, a unique polymorphism in the CR3 helix distinguishes PDE4B and PDE4D isoforms and has been exploited for the development of selective PDE4B inhibitors with promising anti-inflammatory action (216, 217).

Since PDE4 is highly expressed in the hippocampus, PDE4 inhibitors have been extensively studied as cognitive enhancers. In addition to pro-cognitive action, rolipram was shown to reverse plasticity impairments and cognitive decline in several transgenic AD mouse models (218-221). The cognitive enhancing effects of rolipram were related to the attenuation

of decreased CREB phosphorylation, as observed in the disease (222). In the study of Vitolo et al. it was suggested that the Aβ peptide prevents AC activation leading to lower intracellular levels of cAMP that shifts the equilibrium towards the inactive form of PKA. Subsequently, treatment with rolipram restores the balance, promoting PKA dissociation and CREB phosphorylation (223). Except for restoring CREB phosphorylation, it was shown that activation of cAMP/PKA can enhance proteasome activity and subsequently promote tau clearance (224). Similarly, a newly synthetized PDE4 inhibitor FFPM was also shown to ameliorate cognitive decline in APP/PS1 mouse model by promoting cAMP/PKA/p-CREB/BDNF pathway and reducing the levels of inflammatory factors (225). Another possible mechanism by which PDE4 inhibition could be neuroprotective against AD pathology is via activation of the small heat-shock protein 20 (Hsp20). In particular, Hsp20 binds directly to PDE4 within a region of the catalytic domain, remaining in a non-phosphorylated state (226). Disruption of Hsp20-PDE4 complex for example via PDE4 inhibition promotes phosphorylation of Hsp20 by PKA/PKG at Ser16. Subsequently, phosphorylated Hsp20 interacts with Aβ preventing its aggregation into toxic oligomers (227). Although the latter finding suggests that PDE4 inhibition could protect against Aβ pathology, there is no animal study at the moment showing that PDE4 inhibition could combat amyloid deposition.

Despite the promising preclinical findings, clinical studies with PDE4 inhibitors have been hampered due to dose-limiting emetic effects. In this respect, recent studies indicate the importance of developing more selective PDE4 inhibitors in order to dissociate the pro-cognitive action from the emetic effect. The newly described PDE4 inhibitors GEBR-7b and GEBR-32a enhanced memory performance in healthy mice and an AD mouse model with doses being at least 100 times lower than the one inducing emesis (228, 229). Of note, it was shown that the cognitive enhancing effect of GEBR-7b does not involve changes in Aβ pathology (229). Roflumilast, a PDE4 inhibitor that is already clinically approved for the treatment of chronic obstructive pulmonary disease (COPD) (230), was shown to improve cognition in both healthy adult rodents as well as healthy adult volunteers at non-emetic doses (231, 232). Finally, development of allosteric modulators of PDE4 gained more interest the past few years. Accordingly, the allosteric modulator D159687 was shown to exert pro-cognitive action in cynomolgus macaques, at a dose that is 60 times lower than the one eliciting emesis (214).

When it comes to treatment with PDE4 inhibitors or other drugs that upregulate cAMP signaling, an important aspect is the transcriptional regulation of the PDE4 family by cAMP.

Several studies have shown that chronic treatment with antidepressants that stimulate the cAMP signaling pathway, could upregulate expression of PDE4A and PDE4B in the rat frontal cortex without affecting PDE4D levels (233, 234). Later studies that examined in more detail the expression of the different variants of PDE4A, PDE4B and PDE4D isoforms after chronic administration of fluoxetine showed a more complex regulation of their expression (235). Repeated treatment with fluoxetine increased PDE4D1/PDE4D5 in the mouse cerebral cortex, while it decreased expression of all the PDE4B variants tested. Additionally, PDE4D3 expression was increased in both the cerebral cortex and the hippocampus (235). Further studies showed that fluoxetine administration reduced PDE4D3 expression in the cingulate cortex, while PDE4D4 expression was increased in the frontal and frontoparietal cortex. Moreover, expression of PDE4D1 and PDE4D5 was decreased in several areas of the hippocampus (236). These studies indicate that the earlier results, showing unaltered levels of PDE4D expression after fluoxetine treatment, most likely represent the net result of opposing changes in different PDE4D splice variants. In cortical cell cultures, stimulation of cAMP by dibutyryl-cAMP promotes the expression of the short variants PDE4B2 and PDE4D1/PDE4D2, while PDE4A levels remain unaltered. This finding is particularly interesting since these variants were almost undetectable at basal conditions, and their expression was increased in a cAMP-activated state (237). Additionally, repeated treatment with rolipram differentially altered the expression of the PDE4 variants in the cerebral cortex and the hippocampus. PDE4D3 expression was increased in both brain areas examined. Nevertheless, chronic treatment with rolipram decreased PDE4A1/PDE4A5 levels in the hippocampus, while PDE4B1/PDE4B2/PDE4B3 expression was increased in the cerebral cortex. Taken together, all the above mentioned studies indicate that transcription of PDE4 variants in the brain is controlled by cAMP signaling in a isoenzyme and brain-specific manner (238).

The **PDE7** family includes two genes, PDE7A and PDE7B, and hydrolyses cAMP with high affinity (Km<0.2 μM). Alternative splicing gives rise to three splice variants for PDE7A (PDE7A1-3) (239, 240) and PDE7B (PDE7B1-3) (241) in rats. However, in humans only one variant has been described for PDE7B. Although the N-terminal domain does not contain regulatory sites, it interacts directly with the catalytic domain of PKA, inhibiting its action (242). The latter provides an alternative way in which PDE7 could downregulate the cAMP/PKA signaling pathway. Due to the high expression of PDE7A1 and PDE7A3 in a variety of human immune cells, specific inhibition of these variants seems a promising approach for preventing chronic inflammation (240, 243). Except for peripheral tissues, PDE7

is highly expressed in the brain and both PDE7A and PDE7B were found in the hippocampus, cortex and striatum in the rat brain (244, 245). PDE7B1 is ubiquitously expressed in the striatum and it was shown to possess a prominent role in regulating dopamine-mediated cAMP signaling in striatal neurons (238). Elevation of cAMP/PKA levels in the striatum promotes transcriptional activation of PDE7B1 providing a negative feedback that eventually down-regulates intracellular cAMP levels (238). Therefore, development of specific inhibitors for the PDE7B1 isoform could be an alternative strategy for the treatment of diseases related to loss of dopaminergic signaling. Although there are a few potent PDE7 inhibitors, there is a scarce of evidence regarding their effect on memory. A newly described compound VP1.15 that inhibits both PDE7 and glycogen synthase kinase 3 (GSK-3) showed pro-cognitive action in healthy mice (246). Additionally, chronic treatment with the PDE7 inhibitor S14 in AD mice ameliorated memory impairment and reduced pathological hallmarks of the disease, including Aβ deposition and tau phosphorylation (247). Clearance of Aβ deposits was mediated by astroglial phagocytosis, outlining the protective potential of PDE7 inhibition against neuroinflammation.

The **PDE8** family contains two genes, PDE8A and PDE8B, and shows the highest catalytic rates for cAMP (Km~0.8 μM) among all PDEs. PDE8A mRNA is expressed in a variety of peripheral tissues and with regard to its brain expression, it can be found in the substantia nigra, thalamus and to a lesser extend in the hippocampus and cortex (248). PDE8B mRNA is mainly expressed in the brain and more precisely in the hippocampus, cortex, striatum and midbrain (249). The N-terminal region contains REC (receiver) and PAS (Per, Arnst and Sim) regulatory domains that participate in a communication system and in protein interaction in prokaryotes. Their specific function in vertebrates remains unknown. Similarly to other PDE families, upon increased cAMP levels, PKA phosphorylation increases the catalytic activity of the enzyme, providing a negative feedback mechanism (250). Considering the high distribution of PDE8B in the hippocampus, it is considered a promising target for the treatment of memory-related disorders. To our current knowledge, one selective PDE8B inhibitor has been identified, but pre-clinical development was halted due to peripheral side effects in dogs (251).

cGMP-specific phosphodiesterases. This category of PDEs comprises PDE5, PDE6 and PDE9, with PDE5 representing the most well-studied family. **PDE5** is encoded by a single gene with different promoters resulting in three variants of the gene, i.e. PDE5A1, PDE5A2 and PDE5A3. The three variants differ in their N-terminal and tissue localization. Although PDE5 has two GAF domains (GAF-A and GAF-B) at the N-terminal, cGMP binds

only to GAF-A. Activation of the enzyme requires a well-orchestrated series of events that is initiated by elevated intracellular cGMP levels. Subsequently, cGMP interacts with the catalytic domain, facilitating binding of cGMP to the GAF-A domain (252, 253). The latter promotes phosphorylation of specific serine residual that increases by 10-fold the affinity of the enzyme for cGMP and additionally promotes its catalytic activity (254, 255). The main kinase that mediates this phosphorylation is PKG. Nevertheless, when cGMP reaches high levels, PKA can also phosphorylate the enzyme, prolonging its activation. This mechanism provides a negative feedback loop, ensuring that intracellular cGMP concentration will not exceed the appropriate levels (254). Additionally, both cyclic nucleotides were shown to promote transcriptional regulation of PDE5A via regulatory elements in the promoter of the gene. In rat cell cultures, a cAMP analogue stimulated transcription of PDE5A2 and in lesser extend PDE5A1, probably via association with CRE (256). Additionally, cGMP and cAMP were shown to facilitate PDE5A1 transcription via the AP-2 and Sp-1 sequences in the promoter of the gene (257).

Similar to PDE3 and PDE4 inhibitors, PDE5 inhibitors are well-known for their vascular effects. More specifically, PDE5 inhibition causes relaxation of smooth muscles in blood vessels, hence its importance for the treatment of erectile dysfunction. Sildenafil, vardenafil and tadalafil are examples of FDA approved PDE5 inhibitors for the treatment of erectile dysfunction. Because of their vasodilatory properties, sildenafil and tadalafil are also FDA approved for the treatment of hypertension of the pulmonary artery. Nevertheless, there is an extensive body of evidence indicating their promising cognitive enhancing properties in healthy, aged and AD animal models. Several studies showed that sildenafil improves memory formation in healthy mice, rats and cynomolgus macaque, either when given before or immediately after the mnemonic test (258-261). Additionally, sildenafil could compensate against memory impairments in AD and aged mice. In addition to restored CREB phosphorylation, treatment with sildenafil also resulted in reduction of pathological hallmarks related to AD and aging. For example, chronic treatment with sildenafil reduced Aβ deposition in APP/PS1 mice (262), and normalized the amyloidogenic APP cleavage pathway in aged mice (263). Accordingly, a recent study showed that as with rolipram, sildenafil could activate Hsp20 in cell cultures, preventing aggregation of toxic Aβ fibrils (264).

Despite the cerebrovascular effect of PDE inhibitor, animal studies showed that the cognitive enhancing properties of sildenafil cannot be attributed to increased cerebral blood flow (265) and metabolism in the brain, but are mainly related to underlying mechanisms of plasticity (262, 266). Nevertheless, four clinical studies have tested the effects of PDE5

inhibition on memory in healthy volunteers and none of these studies proved a therapeutic benefit on cognitive function (267). Cognitive ceiling effects may have been a limiting factor in these studies, since they were conducted in healthy volunteers. Another point of concern regarding the commercially available PDE5 inhibitors is their lack of selectivity. For example, sildenafil also inhibits PDE6 that is expressed in the retina and therefore chronic administration could cause visual disturbances. The past few years considerable efforts have been made for the development of more selective PDE5 inhibitors with optimal kinetic properties. In this respect, the newly synthetized compounds 7a and 6c represent optimized versions of the existing PDE5 inhibitors with higher selectivity for the enzyme and increased BBB permeability (268, 269). Additionally, both inhibitors were shown to exert neuroprotective mechanism against synaptic and memory deficits in APP/PS1 mice.

PDE9 family is encoded by one gene, PDE9A, and hydrolyses cGMP with the highest affinity (Km~0.17 μM) among the cGMP-specific PDEs, supporting the idea that it is the main regulator of cGMP signaling in the brain (270). Despite the identification of one gene, complex processing of its mRNA results in twenty-one splice variants (271). Unlike the other PDEs, the N-terminal domain of the PDE9A isoenzyme does not contain GAF domains or any other regulatory regions. Additionally, the sequence of the catalytic domain appears to be unique among the other PDEs. The latter results in unresponsiveness of PDE9A to common PDE inhibitors (272, 273). PDE9A mRNA is expressed in several peripheral tissues. Regarding PDE9A mRNA expression in the brain, studies with rodents showed diverse patterns of distribution of its transcripts, with high levels in the hippocampus, cortex, olfactory bulb, striatum, thalamus, amygdala and the highest expression in the cerebellum (170, 270). At the cellular level, PDE9A is mainly localized in neuronal cell bodies and dendrites (274). Additionally, PDE9A exhibits a distinct pattern of subcellular distribution that is region-specific. For example, in the cerebellum PDE9A is evenly distributed in the plasma and nucleus, while in the hippocampus it is mainly found in the nucleus (275). So far, three potent PDE9 inhibitors, BAY 73-6691, PF-04447943 and BI 409306, were shown to potentiate synaptic plasticity and enhance memory performance in healthy rodents. Additionally, these drugs were able to reverse scopolamine-induced memory impairments in both mice and rats, indicating a promising role for AD (276-278). In that respect, BAY 73-6691 was shown to protect against synaptic deficits induced by $A\beta_{42}$ oligomers and improve memory performance in an AD transgenic mouse model (279). Additionally, it was shown *in vitro* that PDE9 inhibition by either BAY 73-6691 or PF-04447943 could protect against Aβ-induced cytotoxicity (264). The same study showed that PDE9 inhibition offered a higher

level of neuroprotection and with an earlier onset in comparison to rolipram and sildenafil treatment (264). Notably, newly synthesized PDE9 inhibitors were able to prevent Aβ aggregation into toxic fibrils and this neuroprotective function was attributed to their antioxidant ability (280) or metal-chelating capacity (281). Nevertheless, the nuclear distribution of PDE9A suggests that PDE9 inhibition could not compensate for deficits in sGC signaling (282) that are encountered in both AD patients and models of AD pathology (283, 284).

Signal compartmentalization of cyclic nucleotides

The concept of signal compartmentalization has emerged as a need to understand the complex and specific spatiotemporal signaling processes in response to extracellular stimuli. Since cAMP participates in a plethora of cellular pathways, its signal sequestration is essential for proper regulation. Discrete compartments of cAMP gradients are shaped by A-kinase anchoring proteins (AKAPs) that act as a scaffold, binding several components of the cAMP signaling cascade, including ACs, PDE4, PKA, Epacs and phosphatases. The presence of several components and regulators of the cAMP signaling cascade in the same complex results into feedback loops in which initial activation of ACs and elevation of cAMP levels, activates PKA that eventually potentiates cAMP degradation via PDEs. The AKAP family is comprised of over 50 members (285, 286). Regulation of PKA compartmentalization into discrete intracellular compartments is mainly determined by the subunit composition of PKA. The two R isoforms, RI and RII, and subsequently PKAI and PKAII are differentially expressed in tissues and show distinct cellular distribution. Their interaction with AKAPs mainly contributes to the anchoring of the different PKA classes into specific subcellular structures. Although the majority of AKAPs show preference for the PKAII class (287), there have been identified AKAPs that are specific for both PKA classes. Considering that the AKAPs anchor together PKA molecules with their substrates or effectors, sequestration of cAMP/PKA signaling provides an advantageous mechanism ensuring fast intracellular responses to extracellular stimuli (for a review, see (288)).

AKAP5, also known as AKAP150 in rodents (orthologue of human AKAP79 and bovine AKAP75), is highly expressed in neural cells and is able to anchor PKAII in the postsynaptic terminal of neurons (289, 290). Except for the unique PKA binding domain, AKAP5 also contains three sequences at the N-terminal that facilitate targeting of the anchoring protein to the neuronal plasma membrane. In the mouse brain, AKAP5 is mainly distributed in the hippocampus, amygdala and cortex, suggesting a pivotal role in the

processes of learning and memory (291-294). Regarding its subcellular localization, AKAP5 is found in the dendrites and more specifically in the postsynaptic density (PSD) of dendritic spines, where it regulates their structure and function (295). Tethering of AKAP5 to PSD is achieved via interaction of structural components including PIP2, F-actin, cadherins (296, 297) and membrane-associated guanylate kinase (MAGUK) (295, 298). In the PSD, AKAP5 interacts with a plethora of signaling molecules including PKC (299), calmodulin (300), CaN (300), as well as receptors and ion channels including AC5, AC6 (301), L-type calcium channel (302, 303), potassium channel (304, 305) and β-adrenergic receptor (306). Additionally, AKAP5 binds indirectly to AMPA receptors via the MAGUK scaffolding protein SAP-97, and to NMDA receptors via PSD-95 (298), orchestrating glutamatergic neuronal transmission.

Except for the above interaction with structural and signaling molecules, AKAP5 was shown to mediate a well-orchestrated modulation of cAMP pools in neuronal cells via interaction with PDE4. The most well-studied paradigm represents the PKA/AKAP5-mediated regulation of the β2-adrenergic receptor (β2-AR) via PDE4D5 (156, 307). Specifically, β2-AR is coupled to both G_s and G_i proteins and phosphorylation of the receptor by PKA switches its coupling from G_s to G_i (308). Activation of the β2-AR by its agonist promotes activation of AC via G_s that subsequently elevates cAMP intracellular concentration. When this concentration reaches the threshold for promoting activation of AKAP5-anchored PKA, the latter phosphorylates β2-AR and switches its coupling to G_i. The dissociated $G_{\beta\gamma}$ subunit initiates a cascade of signaling molecules that lead to ERK activation (308). Recruitment of the βarrestin-bound PDE4D5 into the β2-AR signaling complex provides a negative feedback by lowering cAMP concentration and attenuating the ability of AKAP5-anchored PKA to phosphorylate β2-AR. In this signaling cascade, disruption of PKA-AKAP5 tethering prevents ERK activation, even when PDE4D5 was knocked-down (307), outlining the importance of sequestration of PKA and PDE4D5 in regulating cAMP concentration.

There is a growing body of evidence indicating that the AKAP5 signalosome is important for mediating neuronal signaling and facilitating mnemonic processes. An example of this action represents the regulation of AMPA receptor trafficking in the synapse. The GluA1 subunit of AMPA receptors is phosphorylated by PKA and CaMKII/PKC at serine residues 854 and 831, respectively. Phosphorylation of GluA1-AMPA receptors by PKA increases channel opening probability and promote their trafficking into the extrasynaptic sites (83, 309-313). In response to NMDA receptor activation and PKC phosphorylation,

extrasynaptic AMPA receptors move to the synapse facilitating LTP induction (314, 315). Subsequently, dephosphorylation by CaN induces removal of the synaptic GluA1-AMPA receptors via endocytosis during LTD (316-318). Anchoring of PKA, PKC and CaN by AKAP5 (319) facilitates rapid endocytosis and exocytosis of AMPA receptors in response to a signal.

As mentioned above, the brain distribution of AKAP5 suggests an eminent role in memory formation. In this respect, it was shown that AKAP5 null neurons exhibit altered synaptic plasticity and impaired LTD, due to exclusion of PKA and CaN from the dendritic spines (320). Additionally, induction of LTP *in vivo* caused upregulation of AKAP150 mRNA at the L-LTP (321). Since LTD is perceived to represent the cellular correlate of memory retention (322), AKAP KO mice also exhibited impairments in memory retention (321). Additionally, both LTP and LTD were abolished in a mouse strain (D36) carrying a truncated version of AKAP5 that prevents binding of PKA (323, 324). These results indicate that D36 mice exhibit a more severe electrophysiological impairment in comparison to AKAP KO mice. This observation was also confirmed in the study of Weisenhaus et al. that additionally examined whether the observed electrophysiological deficits are extended to the behavioral level (325). In their study, D36 mice appear to have more pronounced impairments in reversal learning in an operant conditioning paradigm for food reward in comparison to AKAP KO mice. The differences between the two strains indicate the importance of interaction between PKA and AKAP5 in controlling synaptic transmission.

Accordingly, several studies demonstrated that anchoring of PKA to AKAP5 is critical for maintaining compartmentalized cAMP signaling that subsequently coordinates learning and memory (326). In that respect, most of the animal studies indicate the importance of PKA-AKAP5 interaction utilizing the fear conditioning paradigm. In the study of Moita et al. the st-Ht31 peptide that competes for the binding site of PKA in the AKAP5 (327), was infused in the lateral amygdala of mice and resulted in impaired consolidation of fear memory (293). A later study extended these findings showing that AKAP5 is upregulated in the hippocampus of mice during exposure to a novel context, but also at the late consolidation period of fear conditioning memory (328). Along the same lines, disruption of PKA-AKAP5 anchoring in the hippocampus via the st-Ht31 peptide or the more specific superAKAP-IS peptide prevented late-consolidation, but not acquisition and retrieval of fear memories (329). Finally, KO of AKAP5 was accompanied with spatial memory retention deficits evaluated with the MWM test in mice (321).

Temporal distinction in cyclic nucleotide signaling: time windows of action

The molecular mechanism underlying different mnemonic stages is matter of investigation for several years and it still remains elusive. Interestingly, several experimental lines indicate that cyclic nucleotides participate in distinct time windows of memory processes. Specifically, the sGC/cGMP/PKG signaling pathway is involved in the early consolidation phase, while AC/cAMP/PKA signaling is implicated in the late consolidation stage (3). An important aspect of this temporal distinction is whether cyclic nucleotides act in parallel or sequential to activate CREB. In other words, does cGMP/PKG signaling at the early phase depend on cAMP/PKA signaling at the late phase of consolidation or do the two signaling cascades act independently during memory formation? Early work with LTP experiments showed that cGMP/PKG is involved in L-LTP by promoting CREB phosphorylation and Ca^{2+} release from ryanodine stores (28, 29). These experiments support the notion that the cGMP/PKG pathway acts independently of the cAMP/PKA pathway to promote CREB phosphorylation and eventually protein synthesis required for long-term plasticity. The parallel action of the two pathways was further underpinned by studies conducted in honeybees showing that an externally applied cGMP analog could synergistically activate PKA at the presence of nanomolar concentrations of cAMP (330). Nevertheless, there are studies showing that the two pathways are converging in order to produce long-term plasticity. A study conducted in crickets showed that activation of the NO/cGMP cascade precedes activation of the cAMP signaling pathway for the formation of long-term memory. In fact, it is suggested that NO/cGMP stimulates cAMP activation via CNGC and calmodulin (331). Based on the model that the authors proposed, cGMP promotes activation of CNGC leading to increased influx of Ca^{2+} that in turn activates calmodulin. Finally, the Ca^{2+}/calmodulin complex could directly activate ACs, providing the link between the two cyclic nucleotide cascades. Of note, in the cricket study, the cGMP analogs were applied externally. A follow-up study showed that cGMP produced in physiological conditions after a learning paradigm could not activate PKA even in the presence of low levels of cAMP (332). The authors suggested that the contradictory findings show that cGMP production and PKA activation occur in different subcellular compartments in the same or in different neurons. Therefore, in physiological conditions PKA is inaccessible to cGMP (332).

A later study from Bollen et al. in which they targeted cGMP/PKG and cAMP/PKA pathways during specific time windows of plasticity and memory formation, underscored the temporal dissociation of the signaling cascades and, additionally, the cGMP-mediated

modulation of the cAMP pathway (333). The electrophysiological study showed that activation of the cGMP/PKG pathway before or 10 min after a weak tetanus-induction protocol could convert E-LTP to L-LTP, suggesting that both acquisition and early consolidation are cGMP/PKG dependent. The same observation was possible for the cAMP/PKA pathway when it was activated either before or 90 min after the induction protocol indicating that cAMP/PKA pathway participates in acquisition and late consolidation processes. At the behavioral level, activation of the cGMP/PKG pathway at the early consolidation time window or the cAMP/PKA pathway at the late consolidation time window could extend STM into LTM as tested with rats in the ORT. The existence of defined time windows in the action of cyclic nucleotides was outlined from a later study showing that the pro-cognitive effect of upregulating the cGMP/PKG cascade is apparent when it takes place within 45 min after training in the ORT. However, elevation of cAMP/PKA cascade is only effective when it occurs between 3 and 5.5 h after training in this test (334). Additionally, the previous study of Bollen et al. showed that early activation of cGMP/PKG requires intact cAMP/PKA signaling at the late phase in order to promote L-LTP and LTM. In both electrophysiological and behavioral studies, the plasticity and memory enhancing properties of cGMP/PKG activation were abolished when PKA was blocked at the late phase of LTP or memory formation. On the contrary, PKG inhibition at the early phase of plasticity and memory did not affect the pro-cognitive effect of cAMP/PKA pathway activation at the late phase. These findings suggest that activation of cGMP/PKG at the early phase of plasticity or consolidation requires cAMP/PKA signaling at the late phase for maintaining L-LTP and LTM (333). Importantly, a temporal distinction in the action of cyclic nucleotides can be only made for the consolidation phase, since upregulation of either cGMP/PKG or cAMP/PKA at the acquisition phase has pro-cognitive effects.

Considering the various experimental procedures and the different complexities of the organisms that have been employed in these studies, it is difficult to draw a conclusion regarding the temporal relationship between cGMP/PKG and cAMP/PKA pathways. Nevertheless, the evidence so far points out that, at least in mammals, these two cyclic nucleotide signaling cascades act in parallel during the acquisition phase, while at the consolidation phase, activation of cGMP/PKG and cAMP/PKA is sequential. This observation raises several questions regarding the mechanistic relationship between the two cyclic nucleotide signaling cascades. For example, by which mechanisms the cGMP/PKG cascade promotes activation of the cAMP/PKA cascade? Considering that PKG inhibition at the early phase of memory consolidation did not affect the pro-cognitive effect of cAMP/PKA

at the late consolidation phase, it is unlikely that PKG acts directly on the cAMP/PKA cascade. Nevertheless, it is possible that cGMP promotes cAMP/PKA activation via another downstream effector, like cGMP-regulated ion channels. Additionally, if PKA inhibition at the early phase of LTP and memory consolidation does not affect the pro-cognitive action of cGMP/PKG upregulation, what is the relevance of the immediate peak in PKA activity at the very early phase of a memory task, as was reported in previous studies (92, 95, 96)? A possible explanation is that PKG and PKA share a common downstream effector at this very early phase and therefore concomitant inhibition of PKA and activation of PKG maintain an intact underlying mechanism. Both PKG and PKA phosphorylate the GluA1 subunit of the AMPA receptor at the same serine residue (S845), promoting its trafficking into the membrane (335). Thus, it is plausible that this common mechanism could explain the above finding regarding the role of cyclic nucleotides at the very early phase of plasticity and memory consolidation. Such common mechanism has been suggested to relate to the memory processes of encoding, which are active during acquisition and continue during a short period after acquisition, i.e. into the actual consolidation window (336). Finally, what is the therapeutic relevance of the temporal distinction in the function of cyclic nucleotides? Considering that activation of the cGMP/PKG and the cAMP/PKA cascade via, for example, specific PDE inhibitors induces unwanted side effects, combining cGMP-selective and cAMP-selective PDE inhibitors at low sub-efficacious doses could be a possible alternative effective treatment (337). In that case, the pro-cognitive effects of upregulating the cGMP/PKG and cAMP/PKA cascade will be maintained, while the unwanted side effects are circumvented.

New techniques in detecting, measuring and modulating cyclic nucleotide signaling

So far, we presented data of the last decades indicating the complexity of cyclic nucleotide signaling and the plethora of molecules that orchestrate cAMP and cGMP dynamics and their subcellular distribution. Better understanding of cyclic nucleotide signaling imposed the need for new ways to measure levels of cAMP and cGMP in cells. Biochemical techniques including radio- and immuno-assays can give a relative estimation of the amount of cAMP or cGMP in cell lysates. Nevertheless, these techniques require a large amount of cells or tissue, lack spatiotemporal resolution and cannot provide evidence for the real-time changes in cAMP/cGMP gradients in living cells. The development of optical biosensors based on Förster resonance energy transfer (FRET) significantly improved our

ability to measure and monitor cyclic nucleotide signaling (338).

Application of FRET imaging for detecting cAMP signaling. The first biosensors for detecting cAMP intracellular levels utilized the dissociation of the C and R regulatory subunits of PKA upon cAMP binding. The first cAMP biosensor named "FlCRhR" comprised a fluorescein-tagged C subunit and a rhodamine-labeled R subunit in which binding of cAMP to the R subunit caused dissociation of the subunits and reduction in FRET emission (339). A few years later, Zaccolo et al. developed a genetically encoded cAMP biosensor in which the R or the C subunit of PKA was fused with a fluorescent probe (340). Although utilization of FlCRhR provided information about the spatial distribution of cAMP/PKA during stimulation of sensory neurons in Aplysia (341) and the PKA-based biosensor was proven useful in unraveling cAMP signaling dynamics and compartmentalization in rat cardiac myocytes (342), their application was restricted due to technical difficulties. For example, transfection of the whole holoenzyme in the case of FlCRhR was not feasible in neurons, while PKA biosensors required transfection of both plasmids and equal subunit distribution in the cytosol.

The discovery of Epacs as additional downstream effectors of cAMP signaling, led to the development of singled-chained Epac-based biosensors. Both Epac1 and Epac2 were fused with cyan-fluorescent protein (CFP) at the N-terminus and yellow-fluorescent protein (YFP) at the C-terminus to generate the first Epac1 and Epac2 biosensors. Binding of cAMP to the biosensor introduces conformational changes reducing yellow to cyan emission and therefore decreasing FRET (343-345). Fusion of Epac1-camps to the N-terminal domain of the different PKA subunits led to PKA-RI- and PKA-RII-specific FRET biosensors (346). As it was shown before, PKA-RI and PKA-RII are tethered to distinct cellular compartments via AKAPs. These compartments generate spatially distinct modulation of intracellular signaling cascades in response to extracellular stimuli (346). Utilization of PKA-RI and PKA-RII biosensors in rat myocytes revealed a microdomain-specific regulation of cAMP levels mediated via different PDEs (347). In these cells, simulation of the β-AR generates a spatially restricted pool of cAMP that mainly activates PKA-RII and to lesser extend PKA-RI. Increased production of cGMP via stimulation of sGC promotes activation of PDE2 that is in close proximity to the PKA-RII pool and inhibition of PDE3 that resides close to PKA-RI reversing the PKA-defined cAMP gradient (347). Additionally, Epac2-camps tagged to AC8 (Epac2AC8^{D416N}) helped to identify distinct pools of cAMP microdomains associated with ACs activity in pituitary cells (346). Worth mentioning is that Calebiro et al. generated a transgenic mouse line (GAG-Epac1-camps) that expresses an Epac1 biosensor ubiquitously allowing examination of cAMP signaling in a more physiological context (348).

The most well-known Epac-based probes are called ICUE (indicator of cAMP using Epac), and so far there have been developed three versions of the construct (ICUE1-3) with progressively improved properties (344, 349, 350). ICUE1 constructs that were modified to be expressed in the plasma membrane, mitochondria and the nucleus revealed the dynamics and the propagation of cAMP signaling in the subcellular compartments in response to adrenergic stimulation (344). ICUE3 probes targeted to the nucleus provided a first indication of the role of the nuclear PKA holoenzyme, showing that it promotes signaling in response to activated sAC (70). Additionally, utilization of ICUE3 probe revealed a novel role of the actin binding protein coronin 1 in modulating synaptic plasticity and neurobehavioral processes via potentiation of the cAMP/PKA pathway (351).

An alternative approach to detect cAMP signaling is via the A-kinase activity reporter (AKAR). This family of biosensors contains a PKA substrate sequence and a phospho-binding domain sandwiched between 2 fluorescent proteins (eCPF and YFP/Venus). Increased PKA activity leads to phosphorylation of PKA substrate and subsequent binding to the phospho-domain increasing FRET. The study of Gervasi et al. in which they used AKAR2, showed the differential amplitude and time course of PKA signal integration from membrane to the nucleus in response to AC or GPCR stimulation (352). A modified version of AKAR4 that was targeted in lipid raft and non-raft regions of the plasma membrane gave first insight into the compartmentalization of PKA activity in the different microdomains in the membrane (353). Additionally, Epac1, Epac2 and AKAR2 biosensors revealed differential regulation of the cAMP/PKA pathway in response to β-AR stimulation in two different compartments of the cell; the bulk cytosol of the cell bodies and the submembrane domain of the thin dendrites (354). Specifically, the amplitude of the cAMP/PKA response in the cell soma was weaker in comparison to the thin dendrites, supporting modeling predictions showing that the surface to volume ratio affects cAMP dynamics (355). Accordingly, biosensor imaging in mouse brain slices showed that cAMP/PKA signaling differs between striatal and cortical neurons, with the first exhibiting faster and longer-lasting responses to stimuli that elevate cAMP/PKA pathway (356). This difference could be attributed to several parameters including enhanced PDE4 activity in the cortex and stronger AC activation in the striatum (356).

Application of FRET imaging for detecting cGMP signaling. The development of cGMP FRET probes was based on the utilization of a cyclic nucleotide binding domain derived from PKG or cGMP-specific PDEs fused within two fluorophores forming a FRET pair. A challenge in the development of cGMP probes was achieving high specificity for the

probes, because the levels of cGMP in a cell are lower than the levels of cAMP. The first PKG-based biosensor, called Cygnet-1 (cyclic GMP indicator using energy transfer), was comprised by a truncated version of PKGIα at the N-terminal fused between CFP and YFP, while Cygnet-2 was the catalytically inactive variant of Cygnet-1 (357). In both probes, binding of cGMP led to decreased FRET. The development of Cygnet biosensors contributes to our knowledge regarding the dynamics and the regulation of cGMP signaling in various cell types (358-360). In the brain, the use of Cygnet in thalamic neurons showed that although they express PDE1, 2, 9 and 10, basal cGMP concentration is mainly regulated by PDE2 activity (361). Additionally, Cygnet was used in combination with an Epac-based sensor (EPAC-SH^{150}) to disentangle the role of cyclic nucleotide signaling in medium spiny neurons (MSNs) in the striatum (362). The study showed that cGMP signaling could reduce cAMP signaling through activation of PDE2 in the MSNs (362).

Despite the fact that Cygnet biosensors enable the monitoring of cGMP levels and extend our knowledge regarding cGMP signaling, they exhibited low dynamic range and temporal resolution. Nikolaev et al. developed three shorter cGMP biosensors containing a single cGMP-binding domain from PKGIα (cGES-GKIB) or the GAF domain from PDE2 (cGES-DE2) or PDE5 (cGES-DE5) (363). Binding of cGMP decreases the FRET signal in case of the PKGIα-based biosensor, while the FRET signal increases in the case of PDE-based biosensors. Replacement of CFP/YFP in the cGES-DE5 sensor by a red (Dimer2) and green (T-Sapphire) fluorescent protein allowed simultaneous imaging of two FRET sensors (i.e. cAMP and cGMP biosensors) in the same cell (364). Surprisingly, the replacement in the fluorescent pair increased its affinity for cGMP by 40-fold, making it the most promising sensor for measuring real-time cGMP concentration in living cells (364). A few years later, a new series of cGMP biosensors was developed, i.e. cGi-500, cGi-3000, cGi-6000, with EC_{50} of 500 nM, 0.3 μM and 0.6 μM, respectively. These biosensors exhibit high selectivity for cGMP and a better dynamic range than the previous cGMP biosensors (365).

In an effort to increase the sensitivity for cGMP biosensors, Nausch et al. developed non-FRET biosensors named FlincGs (fluorescent indicators for cGMP). These biosensors (α-FlincG, β-FlincG, and δ-FlincG) contain a truncated cGMP binding domain from PKGIα or PKGIβ flagged with a circularly-permuted enhanced GFP (cpEGFP), and binding of cGMP increases the fluorescence emitted by cpEGFP (366). Finally, a recent cGMP biosensor was constructed from Oka containing the GAF-A domain of PDE5 fused between a blue fluorescent donor and a dark fluorescent acceptor. The development of this biosensor permitted triple parameter fluorescent imaging utilizing the blue fluorescent cGMP biosensor,

a CFP/YFP cAMP sensor and a red fluorescent probe for Ca^{2+} in a single cell (367).

Worth mentioning is that FRET sensors have been developed for detecting levels of cyclic nucleotides in close vicinity of PDEs. Specifically, Herget et al. fused Epac1-camps or cGES-GE2 to the N-terminus of PDE3A, PDE4A1 or PDE5A (368). These sensors allow detection of differences in cAMP and cGMP gradients around PDEs and contribute to a better understanding of PDE activity in cellular processes and in the compartmentalization of cyclic nucleotide signaling. Additionally, the sensors were used for evaluating the effect of selective PDE inhibitors in the local pools of cAMP gradients and compare their different pharmacokinetic properties (368).

Modulation of cAMP and cGMP signaling via optogenetics. Despite these techniques for detecting cyclic nucleotide signaling in living cells, the need to actively modulate neurons with high temporal resolution remained. The expanding toolbox in neuroscience techniques gave researchers the opportunity to modulate neurons via light. The technique of optogenetics is based on the presence of photosensory domains in light-sensing organisms. The initial optogenetic studies in the nervous system used the light-gated ion channels channelrhodopsin (ChR) and halorhodopsin (HR) to gain more insight into molecular cascades and networks that are activated during neuronal plasticity (369, 370). Coupling of a photoreceptor domain to different effector domains of cAMP or cGMP permits optogenetic manipulation of cyclic nucleotide signaling. Specifically, absorbance of light from the chromophore in the synthetic light-responsive system induces a conformational change that activates the effector domain. The responsiveness of the system in the different light spectra depends on the photoreceptor domain. Common chromophores used in the development of optogenetics tools are the light-oxygen-voltage-sensing (LOV) domain and the blue light utilizing flavin (BLUF) domain.

The first photoactivated adenylate cyclase (PAC), named euPAC, was identified in *Euglena gracilis* in which it was serving a role in photoavoidance. This AC has a heterotetrameric structure consisting of two PACα and PACβ that are activated by blue light and four catalytic domains homologous to group III ACs (371). The functional expression of PACα and PACβ was verified in different systems including *Xenopus laevis* oocytes, HEK293 cells, *Aplysia* and *Drosophila melanogaster* (372, 373). The large size and high basal activity in the dark after *in vivo* expression prevented the wide application of euPAC in other organisms. Another PAC, named BlaC, was constructed by Gomelsky and colleagues. The construct was containing the *blaC* gene encoding a group III AC isolated from *Beggiatoa sp.* and one BLUF domain, reducing the size significantly (374). Around the same time the group of Hegemann validated the efficacy of the same protein, which they named bPAC

(375). In *Escherichia coli* and *Xenopus* oocytes, bPAC showed low cyclase activity in darkness that is increased by 300-fold in the light. Additionally, the applicability of bPAC was validated in rat cortical neurons (375), *Drosophila* nervous system (375, 376) and zebrafish (377-379). More recently a blue light-regulated AC was identified in *Microcoleus chthonoplastes*, named mPAC. This enzyme contains a photoreceptive LOV domain and exhibits higher constitutive activity in comparison to euPAC and BlaC/bPAC, but also higher activity after blue light irradiation (380, 381). Although the promising dynamic range, extensive use of these PACs was restricted due to disadvantages in the use of blue light including low tissue penetration and photooxidative damage (382). The first synthetic PAC that is activated in the near-infrared window (NIRW) was engineered by the group of Gomelsky (383). The Ilac construct was containing a photosensory module from the *Rhodobacter sphaeroides* bacteriophytochrome DGC, BphG1 and a typre III AC domain from the Nostoc sp. CyaB1 protein. The effectiveness of Ilac was tested in cholinergic neurons of *Caenorhabditis elegans* in which exposure to red light altered the locomotor behavior of the animal indicating elevation in cAMP/PKA signaling (383). Importantly, generation of NIRW-activated ACs provides the opportunity of combinatory imaging techniques for detecting or manipulating cyclic nucleotide signaling.

The first photoactivated GC was constructed by multiple mutations in the *Beggiatoa* BlaC. The triple mutant, designated BlgC, was shown to exhibit GC activity *in vitro* and irradiation with blue light resulted in significant increase in cGMP production *in vivo* (374). The first natural light-activated GC was identified in fungus *Blastocladiella emersonii* by the group of Gomes (384, 385). The BeGC1, as it was named by the Gomes group, consists of rhodopsin fused to a GC catalytic domain and is activated by green light. The efficacy of the enzyme was confirmed in *in vitro* and *in vivo* assays including HEK293T cells, *Xenopus* oocytes, muscle cells of *Caenorhabditis elegans*, mammalian ovary cells and cortical neurons (385). Additionally, Gomelsky's group engineered a NIRW-activated construct for the production of cGMP (386). The chimeric construct was comprised of a bacteriophytochrome c-di-GMP synthase (diguanylate cyclase, DGC) originating from the *Rhodobacter sphaeroides* BphG1 protein and a constitutive c-di-GMP-specific PDE, YhjH from *E. coli*. DGC is not expressed in higher eukaryotes and could allow orthogonal regulation of c-di-GMP signaling in mammals (386).

A different angle in the attempt to manipulate the cyclic nucleotide signal constituted the engineering of light-activated PDEs (LAPD). The first photoactivated PDE was comprised of the photosensor module of *Deinococcus radiodurans* bacterial phytochrome and the

effector module of the human phosphodiesterase 2A (387). The photoactivated construct has dual substrate specificity and illumination with red-light could enhance the hydrolysis of cGMP and cAMP by 6-and 4-fold, respectively. Additionally, exposure of LAPD to far-red light could decrease its activity. The efficacy of LAPD in increasing hydrolysis of cyclic nucleotides was established in eukaryotic cell cultures and zebrafish embryos (387). Although LAPD appears promising for studies in living organisms, the dual specificity of the enzyme does not allow distinction between the action of cAMP and cGMP. An enzyme with PDE activity was isolated from the choanoflagellate *Salpingoeca rosetta* almost at the same time by the Kandori and the Oprian groups (388, 389). The Rh-PDE or RhoPDE, as it was named by the different groups, is a fusion of rhodopsin type I with PDE. The enzyme displays only a minimum amount of light-dependent PDE activity and can hydrolyze both cyclic nucleotides constitutively in the dark, but with higher selectivity for cGMP over cAMP (388, 389). Crystallography of the isolated PDE domain of the enzyme showed high resemblance in terms of sequence and structure to the human PDE9 (389). Future research will provide better insight in the function of this unique fusion protein and its potential use as optogenetic tool in modulating cyclic nucleotide signaling.

Discussion

The above studies indicate that development of treatments targeting the cyclic nucleotide signaling cascades could be a promising approach for combating memory impairments. Nevertheless, it is still a matter of intense investigation which components should be targeted in memory-related diseases. Proper memory formation is based on a well-orchestrated balance between production and degradation of cyclic nucleotides, as well as, positive and negative feedback mechanisms. In AD, the rationale for upregulating the AC-cAMP-PKA/Epac or the sGC-cGMP-PKG signaling cascades is based on the observation that CREB activation is negatively affected in the progression of the disease (222). Since these cascades promote CREB phosphorylation, they could represent a compensatory approach resulting in increased phosphorylation of CREB. However, upregulation of a system, even one that potentially possesses pro-cognitive function, does not result in memory improvement *per se*. For example, genetic deletion of G_{ia1}, that inhibits adenylate activity in the hippocampus, enhances LTP, but also results in severe memory deficits in several cognitive tasks (390). This result indicates that disrupting the “break” of AC activity causes a saturation of cAMP production in the hippocampus that leads to signal desensitization preventing formation of new memory (390). Similarly, overexpression of AC1 results in impaired

extinction of previously acquired memories which indicates synaptic deficit (51). On the other hand, pharmacological interventions like, for instance, preventing degradation of cyclic nucleotides by PDE inhibitors seems to represent a more balanced approach in modulating cyclic nucleotide concentration and improving cognitive function, yet caution is also needed here (see below; 53, 54). A possible explanation could be that in the case of a genetic approach there is complete depletion of a molecule, while in the case of pharmacological intervention there is a partial inhibition/stimulation, thus a more subtle influence on cellular signal transduction. Future studies will have to reveal the exact role of cyclic nucleotide signaling cascades in neuroplasticity and memory formation, as well as the full potential of targeting the cyclic nucleotide signaling cascades in pathological conditions.

Another important consideration is the altered biochemical signals in a diseased or aged brain. As it was reported before, the PKA signaling cascade appears over-activated in the prefrontal cortex of aged animals (55). Therefore, mild activation of the cAMP/PKA signaling cascade with cAMP-specific inhibitors or PKA activators could exacerbate the cognitive performance related to prefrontal cortex. Nevertheless, there is no evidence reporting a similar effect for Epacs. Another line of studies showed that sGC responsiveness to NO is decreased in the temporal lobe of AD patients (283). A possible approach to reduce the side effects of the disinhibited PKA signaling cascade in the PFC and other adverse side effects could be combination of drugs that act on different cascades. In this respect, it was recently shown that co-administration of sub-efficacious doses of PDE4 and PDE5 inhibitors could improve mnemonic process in physiological conditions, but also rescue memory impairments in an animal model for AD (337, 391). Additionally, the usage of sGC activators could overcome the lack of sGC responsiveness encountered in diseases like AD. These observations indicate the importance of taking into account age-related changes in expression and function of cyclic nucleotide signaling molecules when developing therapies for the treatment of memory diseases.

An integral part in the development of new therapeutic strategies is the in-depth understanding of the molecular mechanisms conveying memory processes. Development of new optical methods broadened our knowledge regarding the physiological action and compartmentalization of different molecules involved in the cyclic nucleotide cascades. Despite the sensitivity of these techniques, they are usually implemented in isolated cells or tissues. Although the cyclic nucleotide cascades are presented conventionally to be linear, the relationship of their signaling molecules deviates from linearity with the presence of several positive or negative loops. Additionally, several isoforms of the signaling components are

characterized by region and even sub-cellular specificity in their distributions. These characteristics impede accurate prediction of the exact molecules that would be activated in a specific brain domain upon administration of a drug that acts on the cyclic nucleotide pathways. Therefore, the implementation of computational models is eminent for integrating experimental data with other parameters including domain distribution, diffusional rates, interactions with other pathways and enzyme kinetics for the cyclic nucleotides signaling networks. For example, development of stochastic models was used to reveal the temporal patterns by which CaMKII and PKA are active during L-LTP and the importance of colocalization of PKA with its downstream effectors in subcellular microdomains for maintenance of L-LTP (392, 393).

Cyclic nucleotide signaling cascades modulate a vast array of physiological and pathophysiological processes via their regulation of the intracellular second messenger molecules, cAMP and cGMP. Despite a century of research into exactly how they exert their function in these processes, we are only starting to comprehend their multifactorial interactions and the complexity of signaling networks that they constitute in the brain. Emerging understanding of the functional roles of these cyclic nucleotide pathways in compartmentalized signaling brain networks, together with the ongoing development of the neuroscience tool box, including genetic models, optical techniques and predictive computational models, provides great potential for cyclic nucleotide signaling molecules to emerge as a promising target for the development of cognitive enhancing therapies for several brain disorders, including memory dysfunction as observed in AD patients.

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Chapter 3

Glutamatergic signaling in object recognition and object location memory

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Abstract

Glutamatergic neurotransmission is essential for a variety of cellular functions including synaptic plasticity and memory. As a result, aberrant glutamate signaling is observed in a variety of neurodegenerative disorders including Alzheimer's disease. Glutamate receptors are divided in ionotropic [mainly represented by N-methyl-D-aspartate (NMDA) and a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors] and metabotropic receptors. The development of receptor-specific pharmacological agents, as well as agents targeting different sites within each of the receptors, allowed the proper investigation of the role of these various receptors during memory formation. Widely used paradigms for this investigation are the object recognition and object location test. Critical consideration of the presented studies led us to the conclusion that distinct types of glutamate receptors are activated during the different mnemonic phases of acquisition, consolidation and retrieval. We additionally concluded that positive allosteric modulation of glutamatergic neurotransmission can facilitate memory formation in healthy animals, while inhibition by antagonists or negative allosteric modulators can be neuroprotective against excitotoxicity. These results suggest that pharmacological agents that act on the glutamatergic system could have promising clinical applications in memory-related disorders.

Introduction

Glutamate is the main excitatory neurotransmitter that is widely distributed in the central nervous system (CNS). It contributes to neurotransmission and plasticity changes that are important for learning and memory. Glutamate exerts its action via ionotropic and metabotropic receptors. Ionotropic glutamate receptors (iGluRs) act as ion channels, mediating fast excitatory neurotransmission and are mainly represented by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) and N-methyl-D-aspartate receptors (NMDAR), with kainate and delta receptors constituting a small percentage of this class of receptors. On the contrary, metabotropic glutamate receptors (mGluRs) transmit glutamatergic signaling in a slower fashion via second messenger proteins and ion channels. They belong to the group C family of G-protein-coupled receptors and eight different subtypes of mGluRs (mGluR1- mGluR8) are divided into 3 groups (I, II, III), with disparate physiological activity.

Functional NMDARs are heteromers consisting of combination of NR1 with NR2 or NR3 subunits. Different variants of these subunits give rise to different isoforms with distinguished distribution and functional properties. Activation of the receptor requires binding of its primary agonist, glutamate. Nevertheless, efficient opening of the channel requires additional binding of glycine, indicating that the co-agonist site is important for modulation of the receptor's function (1). Finally, the channel contains a polyamine-binding site in which polyamines could bind, exhibiting action of allosteric modulators. AMPARs mediate fast postsynaptic response and exhibit immediate desensitization. They are constituted by 4 different subunits (GluA1-GluA4), encoded by the same gene, and form homo-or hetero-tetramers. As with NMDARs, the binding sites of the AMPARs are not merely restricted in the glutamate binding site. In general, three distinct binding sites have been identified for the AMPARs including the agonist binding site, a desensitization-binding site and an intra-ion channel-binding site.

Metabotropic glutamate receptors mediate glutamate signaling via mechanisms including activation of second messenger proteins or ion channels. They have major role in synaptic transmission, mediating learning and memory processes, and they are distributed in several brain areas, including the hippocampus and the frontal cortex. The different groups of mGluRs are functionally distinguished. Group I mGluRs are associated with phospholipase C (PLC) in the membrane and their stimulation leads to activation of protein kinase C (PKC) and release of Ca^{2+} from intracellular compartments. Additionally, it is proposed that group I

mGluRs (mGlu1 and mGlu5) modulates NMDAR activation via PKC. In more detail, PKC increases NMDAR excitability by reducing Mg^{2+} blockage of NMDAR channel (2). Finally, both group II (mGlu2-3) and group III mGluRs (mGlu4-8) are negatively coupled to adenylate cyclase and their activation prevents formation of cyclic adenosine monophosphate (cAMP) and subsequently downregulates protein kinase A (PKA) activation.

The role of glutamatergic system in plasticity and cognition has been widely investigated with both *in vitro* and *in vivo* studies, respectively. Object recognition and object location tests (ORT and OLT, respectively) have been valuable assets for reconciling the effect of different compounds that act on glutamatergic system at different memory stages. We should stress out that both tests are purely mnemonic, based on animals' innate exploratory behavior, without involving exogenous reinforces and anxiogenic parameters. They consist of 2 phases: the trial phase (T1) in which an information is presented and the choice phase (T2) in which the animals should recall the above information. The time point in which a drug is given allows us to investigate different memory stages (3). Typically, administration of a drug before or after T1 influences the acquisition or consolidation phase, respectively. Retrieval is affected when the drug is administrated before T2. Although this is the general principal, an overlapping between the acquisition and the consolidation phase has been observed, suggesting that the first 4-6 min after T1 represent a transition phase between acquisition and consolidation in which the animals encode the presented information (4).

Targeting glutamatergic signaling via NMDARs

Considering the multiple binding sites of the NMDARs, several natural and synthetic compounds have been investigated for their ability to regulate the receptor and subsequently glutamatergic signaling. Although a few potent agonists have been characterized for the NMDARs, their application is mainly restricted to brain lesions since increased activation of the channel leads to excitotoxicity and cell death. Agonists or antagonists of the glycine-binding site, modulators of the polyamine-binding site and competitive or non-competitive antagonists are more promising and have been also implemented in animal models for their neuroprotective action is diseases related to excitotoxicity.

NMDAR antagonists in ORT. Antagonists of NMDARs have been extensively studied after systemic or central administration and in different time points of mnemonic processes, showing that action of NMDARs is temporally restricted to certain memory stages. Blockage of NMDARs in the perirhinal cortex (PRH) with the specific antagonist 2R-amino-5-

phosphonopentanoate (AP5; 30 mM), 15 min before T1, impaired object recognition memory only when the animals were tested after a long-(2 h) but not a short- retention interval (5 min), indicating that active NMDARs during the acquisition phase are important only for long-term object recognition memory (5). Similar results were obtained from another study in which intra-PRH infusion of AP5 (25 mM), 30 min before T1, impaired acquisition of object recognition memory when the animals tested after 24 h, but not after 20 min retention time (6). Along the same lines, intraperitoneal (i.p.) administration of MK801 (0.01 and 0.1 mg/kg), 20 min before T1, impaired object recognition memory tested after 1.5 h and 24 h retention interval (7). Taken together, the above results indicate that NMDAR-dependent plasticity processes are slow to develop, since at least 1.5 h delay is needed before the impairment is produced, and longer lasting, since it is apparent after 24 h.

Unlike the acquisition phase, NMDAR activity is not required for retrieval of stored memories. Systemic administration of MK801 (0.1 and 0.2 mg/kg; i.p.), 30 min before T2, did not alter object recognition memory tested after 1.5 h retention interval (8). Similarly, intra-PRH infusion of AP5 (25 mM), 15 min before T2, did not impair discrimination of the animals when tested after a retention delay of 2 h (6, 9).

Although the above studies allow us to draw a clear conclusion regarding the role of NMDAR activity in the mnemonic processes of acquisition and retrieval, confounding results have been obtained when NMDAR blockage has been induced during the consolidation phase. Intra-PRH infusion of AP5 immediately after T1 impaired memory consolidation tested after 25 min retention interval only for the high (60 mM), but not for a lower dose (30 mM) (10). In concordance to the above observation, a later study showed that intra-PRH infusion of AP 5 (25 mM), 2 min after T1, did not impair consolidation of object recognition memory tested either after a short (20 min) or a long retention interval (24 h) (6). On the contrary, Winters and Bussey showed that AP5 (30 mM) infusion into the PRH immediately, but not 40 min after T1, impaired animals performance in T2 conducted after 2 h retention interval (5).

The discrepancy of these findings could be attributed to the different experimental setups including strain of the animals, dosage used and/or delay between the end of the T1 phase and the injection of the drug. Notably, in the study of Abe et al. they implemented two doses and only the higher one showed impairment (10); the dose of 30 mM that is slightly higher than the dose used in the experiment of Barker et al. showed no impairment (6). Therefore, it seems that a higher dose is needed for inducing consolidation impairments via NMDAR blockage. Additionally, the fact that intra-PRH infusion of AP5 40 min after T1 did

not impair recognition memory could be attributed to the capacity of PRH to store memories shortly after acquisition. In this respect, lidocaine lesion in the PRH at several time points after T1 was shown to impair consolidation of object recognition memory only within a time frame of 20 min, whereas lidocaine lesion 40, 60 and 80 min after T1 did not affect recognition memory (11).

Peripheral administration of MK801 at the consolidation phase resulted also in conflicting results. Systemic injection of MK801 (0.01, 0.1 mg/kg; i.p.) immediately after T1 impaired recognition memory after 1.5 and 24 h retention interval (7), whereas in a different study, administration of MK801 (0.1, 0.2 mg/kg; i.p.) immediately after T1 did not impair object recognition memory tested after 1.5 h retention interval (8). Worth mentioning is that the two studies differ in animal species and experimental manipulation. For example in the first study, the animals underwent habituation 24 h before T1, while that was not the case in the later study. Moreover, we should also take into account that systemic administration of MK801 could have an effect in other brain regions masking the genuine effect of the drug on object recognition memory. For instance, the observed preference of the animals towards the novel object could be attributed to possible psychotropic or anxiolytic effects of the drug (8).

With respect to dosing, the relationship between NMDARs and its primary agonist, glutamate, represents a typical example of hormesis. Increased activation of NMDARs via glutamate leads to excitotoxicity due to pathological increase of intracellular Ca^{2+} influx, whereas low doses could promote synaptic transmission and plasticity. This concept has been utilized for the use of NMDAR antagonists as cognitive enhancers. For example, memantine, a low- to moderate- non-competitive NMDAR antagonist, is licensed for the treatment of Alzheimer's disease (AD) (12). At the preclinical level, chronic administration of memantine showed promising cognitive action, in two different transgenic models of AD. In the study of Scholtzova et al., chronic administration of memantine (4 months,10 mg/kg; i.p.) in APP/PS1 mice, improved object recognition memory tested after a short retention interval of 30 min (13). A following study of Martinez-Coria et al., extended the above finding, showing that chronic administration of memantine [3 months, 30 mg/kg; per os (p.o.)] could improve both short- and long-term recognition memory in 3xTg-AD mice tested after 1.5 h and 24 h retention interval, respectively (14).

Similarly, memantine was shown to compensate against age-related cognitive impairments. Chronic administration of memantine (3 weeks of treatment with last injection occurring 1 week before the test, 20 mg/kg; i.p.) improved recognition memory in aged rats tested after a long- but not a short-retention delay (15). The positive effect of NMDAR

blockage in aged animals could be attributed to altered synaptic plasticity occurring in aging. Aged animals exhibit shift in synaptic processes in favor of synaptic depression. Therefore, NMDAR blockage might facilitate memory consolidation via disrupting long-term depression that appears enhanced during aging (16).

NMDAR agonists and antagonists for the glycine binding site in ORT. The partial agonists for the glycine modulatory site, D-serine and D-cycloserine (DCS), were shown to facilitate long-term synaptic plasticity in the hippocampus, rendering them promising agents for cognition enhancement (17, 18). Specifically, administration of D-serine (50 mg/kg; i.p.) or DCS (20 mg/kg; i.p.), 30 min before T1, improved long-term recognition memory tested after 24 h interval (19). Similarly, D-serine improved long-term memory consolidation, tested after 24 h, when it was given 30 min, but not 6 h after T1, indicating that the cognitive enhancing effect of D-serine is exerted within the time window of early consolidation (19).

Regarding compounds that antagonize the glycine-binding site, kynurenic acid (KA) is probably the most well-known. A study conducted with macaque showed that administration of KA impaired object recognition memory, when it was focally injected into the medial, but not the lateral, PRH. Additionally, this impairment was significant when 30 and 60 sec delay was imposed between trials, but not after a shorter 10 sec delay (20). The above results come in agreement with previous studies conducted with NMDAR antagonists in rodents, showing a delay-dependent effect of NMDAR blockage. Additionally, the study outlines the importance of targeting a specific area into the PRH in order to examine NMDAR function.

NMDAR positive and negative modulators of the polyamine binding site in ORT. The activity of NMDARs is also regulated by several compounds that act on the polyamine binding site. Common positive modulators include spermine and spermidine, and negative modulators include arcaine, traxropodil, ifenprodil (21). Although there is scarce of evidence regarding the role of this class of drugs in memory processes, there are a few studies showing their effect on object recognition memory. In an animal model that exhibits AD-like cognitive impairment, intracerebroventricular (i.c.v.) infusion of the negative modulators, arcaine and traxropodil, immediately after T1 improved long-term memory consolidation tested after 24 h. In the same experiment, co-administration of the positive modulator spermidine with the negative modulator arcaine abolished the cognitive enhancing effect of the latter in ORT (22). Therefore, inhibition of the polyamine binding site, like NMDAR antagonism, could improve excitotoxicity-induced cognitive impairments. Importantly, a later study showed that combination treatment with DCS and ifenprodil could compensate against amyloid beta (Aβ)-

induced impairments in object recognition memory. A single i.c.v. administration of Aβ, followed by chronic treatment with either DCS [30 mg/kg; intragastrically (i.g.)] or ifenprodil (30 mg/kg; i.g.) or the two compounds in combination (7 days with the last injection given 3 min prior to T1) showed that only the combination treatment improved object recognition deficits, tested after 2 h (23). The authors concluded that ifenprodil and DCS act in different NMDARs subunits and only concomitant administration of both drugs could combat efficiently excitotoxicity.

NMDARs in OLT. Regarding the role of NMDARs in spatial memory, the general accepted theory is that unlike PRH, NMDAR activity in the hippocampus is important for the consolidation of a new memory, but not for the initial step of acquisition. The above concept is mainly supported by studies in which intrahippocampal infusion of NMDAR antagonists impaired consolidation, but not acquisition, of spatial memory (24, 25). Nevertheless, systemic administration of the antagonists 3-(2-Carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP) and MK801 showed to prevent memory acquisition in the OLT. Administration of CPP (10 mg/kg; i.p.), 30 min before, but not immediately after T1, impaired performance of the animals tested after 24 h inter-trial interval (26). Similarly, MK801 given 30 min prior T1 (0.05, 0.1; i.p.) impaired spatial memory of animals tested after 2 h delay (27). It is important to notice that MK801 has a half-live of around 30-120 min and therefore administration before T1 could have an effect at the early stage of consolidation (28). On the contrary CPP has a shorter half-life lasting around 14 min and the effect of the drug could be more restricted to the acquisition phase (29). Additionally, in both cases, CPP and MK801 were given systemically, affecting several brain areas. Intrahippocampal administration of NMDAR antagonists during OLT will elucidate the involvement of hippocampal NMDAR activity at different mnemonic stages.

In addition to NMDAR antagonists, evidence regarding the role of glutamatergic signaling in spatial memory was reported from studies that utilized the positive modulator, DCS. Upregulation of NMDAR activity after peripheral administration of DCS (20 mg/kg; i.p.), 30 min before T1, ameliorated natural forgetting occurring after 4 h in the OLT (27). A later study shade more light into the effect of DCS on different stages of spatial memory. Specifically, administration of DCS (7.5, 15, 30 mg/kg; i.p.) in three different time points, targeting the processes of acquisition, consolidation and retrieval (30 min before T1, immediately after T1 and 30 min before T2, respectively), could compensate natural forgetting in animals tested after 24 h retention interval. Significantly, the cognitive

enhancing effect of the drug followed an inverted U-shaped pattern for all the mnemonic processes, with 15 mg/kg being the optimal dose (30). The above results confirm *in vitro* studies conducted with DCS (31, 32) and underscore the observation that its therapeutic window lies in lower doses.

Targeting glutamatergic signaling via AMPARs

As with the NMDARs, the multiple binding sites in the AMPARs stimulate development of pharmacological agents that would target these sites. Although the role of AMPARs on memory is well established, the literature regarding the effect of pharmacological manipulations of AMPARs on memory is scarce. This is due to the fact that AMPAR agonists are toxic even at low doses. Additionally, interpretation of the results obtained with AMPAR antagonists remain still controversial, since blockage of AMPAR activity reduces synaptic depolarization and subsequently impedes NMDAR signaling.

AMPAR antagonists in ORT. One of the first drugs used for blocking AMPARs activity is 6-Cyano-2,3-dihydroxy-7-nitro-quinoxaline (CNQX). Although CNQX is not a specific AMPAR antagonist and modulates negatively NMDAR activity by competitive binding in the glycine-site of the receptor (33), its administration into the PRH showed temporal differences between AMPAR and NMDAR activity in that specific brain area. Intra-PRH administration of CNQX (3 mM), 15 min before T1, impaired memory acquisition of both short-and long-term object recognition memory, tested after a retention interval of 5 min and 2 h, respectively. Similarly, intra-PRH injection of CNQX, 15 min before T2, diminished retention of object recognition memory in animals tested after 2 h retention interval (5). As mentioned above, NMDAR activity during the acquisition phase is required only for long-term object recognition memory, and blockage of NMDARs before retention had no impact on memory. Considering that CNQX blocks indirectly NMDAR activity, a solid argument is that the above results are due to inhibition of NMDAR signaling. Having said this, it seems that AMPAR activity in the PRH is important for acquisition of short-term memory and retention. The latter comes in agreement with previous studies regarding the involvement of PRH at the stage of retrieval and in addition indicates the importance of AMPAR-mediated glutamatergic signaling in PRH for that mnemonic stage (11).

Unlike the above results, intra-PRH injection of CNQX at the consolidation phase followed the same pattern as with NMDAR blockage. Specifically, object recognition memory was impaired after immediate post-T1 administration of CNQX, but not when 40 min delay was applied between T1 and drug administration (5). Again, the lack of impairment

when CNQX was administered 40 min after T1, could be related to the previous observation suggesting that the involvement of perirhinal cortex in memory consolidation is limited to 20 min after T1 (11). The controversy regarding the role of AMPAR-mediated glutamatergic signaling in different stages of menmonic processes is intensified by studies conducted with 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(F)quinoxaline (NBQX), a selective AMPAR antagonist. A few studies, employing learning paradigms other than ORT and OLT, reported contradictory effects of NBQX administration on memory acquisition (34-36). In the ORT, a study from Pitsikas et al., showed that administration of different doses of NBQX (3.5, 7 and 10 mg/kg; i.p.), 15 min before T1, did not affect memory acquisition of the animals tested after 1 h inter-trial interval (37). On the contrary, a later study showed that administration of NBQX (5 mg/kg; i.p.), 15 min before T1, impaired animals recognition memory tested after 3 h (38).

AMPAR positive modulators ("ampakines') in ORT. Positive modulators for AMPARs have been examined as potentials cognitive enhancers. At the early 90's, the prototype drug of this class, aniracetam, was shown to potentiate AMPAR response without affecting NMDARs (39). Further *in vivo* studies with the drug were hampered due to its low potency and high metabolism. Additional work led to the development of new drugs ('ampakines') with higher selectivity that decrease the rate of receptor desensitization and show promising action in facilitating synaptic transmission (40). A wide range of behavioral paradigms examined the role of ampakines in memory, proving their cognitive enhancing properties (40-43).

In the ORT, oral administration of (S)-2,3-dihydro-[3,4]cyclopentano-1,2,4-benzothiadiazine-1,1-dioxide (S 18986-1), 60 min before habituation, T1 and T2 sessions, improved animals' performance tested after 24 h inter-trial interval. The experimenters tested six different doses (0.3, 1, 3, 10, 30, 100 mg/kg; p.o.) and only the dose of 10 mg/kg showed no improvement in animals performance, while the higher doses of 30 and 100 mg/kg impaired locomotor activity of the animals (44). Employing a similar experimental setup, it was found that sub-chronic treatment for 7 days with the last injection given 60 min before each session, also improved object recognition memory. Thus, S 18986-1 improves object recognition memory of the animals either after 3- or 7-day protocol of oral administration, without developing tolerance. In order to examine the stage of mnemonic processes in which S 18986-1 exerts its action, the drug was administered either before habituation or T1 or T2 (0.3 mg/kg; p.o.) (44). Only the animals that received the drug before T2 showed an improvement in object recognition memory similar with the one observed when the animals

received the treatment before each session (habituation+T1+T2) (44). These results suggest that S 18986-1 facilitates retention, but not acquisition, of object recognition memory.

Treatment with the positive modulator 5-(1-piperidinylcarbonyl)-2,1,3-benzoxadiazole (CX-691) showed that acute (0.1, 0.3, 1 mg/kg; p.o. 1 h before T1 and T2) or sub-chronic administration (0.03, 0.1, 0.3, 1 mg/kg; p.o. for seven days and 1 h before T1 and T2) of the drug improves object recognition memory tested after 24 h inter-trial delay, with lack of tolerance due to chronic treatment (45). In addition, administration of the lowest efficacious dose from the acute experiment (0.1 mg/kg; p.o.), 1 h before T1 or 1 h before T2, improved objected recognition memory after 24 h retention interval, whereas administration of the drug 30 min post-T1, at the consolidation phase, had no effect on animal's performance (45). The above results come in agreement with the previously suggested implication of AMPAR activity at the stage of memory acquisition and retrieval (5), but are in contrast with the study of Lebrun et al. in which S 18986-1 had a pro-cognitive effect only on retrieval, but not at the acquisition phase (44).

Finally, a study with young macaques with the positive modulator 7-chloro-3-methyl-3,4-dihydro-2H-1,2,4-benzothiadiazine 1,1-dioxide (IDRA 21) also proved the efficacy of these types of drugs as cognitive enhancers. The animals were tested in a lower (1 object) and a higher demanding version (6 objects) of the visual object recognition test with varying delays between T1 and T2 (10, 30, 60, 90 sec). Administration of IDRA 21 (15mg/kg; p.o.), 1 h before T1, improved recognition memory of the animals in both versions of the test, reaching statistically significant difference for the control animals at the longest delay (46). Considering that the above experiment was conducted with young macaques, it also provides evidence that modulation of AMPARs could improve cognition beyond the baseline performance.

Targeting glutamatergic signaling via mGluRs

mGluR antagonists in ORT and OLT. As with the iGluRs, there is a growing body of studies aiming to identify the role of mGluRs in memory processes. The first studies were conducted with compounds that were not exhibiting group- or subtype-specificity. In the ORT, co-administration of 2-methyl-6-(phenylethynyl)-pyridine (MPEP; 3 and 10 mg/kg; i.p.), a mGlu5-specific antagonist, with 2S-2-amino-2-(1S,2S-2-carboxycyclopropyl-1-yl)-3-(xanth-9-yl)propanoic acid (LY341495; 3mg/kg; i.p.), a mGlu2/3-weak antagonist, 30 min before T1, impaired acquisition of object recognition memory tested after a long- (24 h), but not a short- (15 min) retention interval. On the contrary consolidation and retrieval were unimpaired for

both short and long intervals, when combination of the antagonists was given immediately after T1 or before T2, respectively. The importance of group I/II mGluRs activity for memory acquisition was proven after intra-PRH administration of the above antagonists. Specifically, co-perfusion of MPEP (100 μM) with LY341495 (5 μM) and in addition co-perfusion of MPEP with EGlu (10 mM), a more specific antagonist for group II mGluR, 15 min before T1, impaired animals' performance tested after a long retention interval of 24 h (47).

Considering that group I and group II mGluRs are associated with distinguished molecular cascades, it would be hypothesized that inhibition of PKC signaling pathway in combination with upregulation of the PKA pathway is necessary for blocking long-term recognition memory in the PRH. Initially it seems counterintuitive that upregulation of the PKA signaling pathway could cause memory impairment, since its role in plasticity and memory is well established. Nevertheless, it has been also shown that the relationship between cAMP/PKA pathway and memory performance follows an inverted U-shaped pattern and upregulation of cAMP beyond physiologically normal levels could also induce memory impairments (48).

Although the above results support the notion that blockage of both group I/II mGluRs is required for impairing acquisition of long-term memory, administration of either MPEP (3 and 10 mg/kg; i.p.) or LY341495 (3 mg/kg; i.p.) alone did not affect recognition memory at the stages of acquisition, consolidation or retrieval. The same results were also obtained after intra-PRH administration (47). Similarly, i.c.v. administration of the specific group I mGluR antagonist, (S)-3,5-Dihydroxyphenylglycine [(S)-3,5-DHPG; 25, 50, 100 nmol], before or immediately after T1, or 30 min before T2, did not impair object recognition memory tested after 1 h inter-trial interval (49).

Later studies challenged the above observation, showing that blockage of group I mGluRs or group II mGluRs, independently, could impair recognition memory. For example, Christoffersen et al., showed that administration of MPEP before T1, either systemically (2, 5, 10 mg/kg; i.p.) or bilaterally, into the prelimbic cortex (1, 5, 10 ug/side) impaired short-term acquisition memory tested after 5 min retention interval (50). The dose that induced object recognition impairment after systemic administration (10 mg/kg) caused additionally exploratory suppression in both T1 and T2. However, the fact that prelimbic administration impaired memory without any further effect on exploratory behavior indicates that activity of mGluR5 in the limbic cortex is important for acquisition of short-term recognition memory. Additionally, a study from Pitsikas et al., showed that systemic administration of high doses (0.3, 1, 3 mg/kg; i.p.) of LY341495 immediately after T1 impaired memory consolidation

tested after a short retention interval of 1 h (51). On the contrary, the lower doses (0.05 and 0.1mg/kg; i.p.) did not affect object recognition memory in the above experimental setup and in addition compensated against natural forgetting occuring after 24 h (51).

Regarding group III mGluRs, intra-PRH infusion of group III mGluR specific antagonist (RS)-a-Methylserine-O-phosphate (MSOP; 50 mM), 15 min before T1, had no effect on animals' object recognition memory tested after 1 h (47). Nevertheless, specific antagonists for mGlu7 had an opposite effect. A specific negative allosteric modulator of mGlu7, 6-(4-methoxyphenyl)-5-methyl-3-pyridin-4-ylisoxazonolo [4, 5-c] pyridin-4 (5H)-one (MMPIP), was shown to impair short-term (2 h) acquisition of both object recognition and object location memory [3, 10, 30 mg/kg; subcutaneously (s.c); 30 min prior to T1] in a dose-dependent manner, with minimum effective dose being 3 mg/kg and 10 mg/kg, respectively (52). The observed impairments in ORT and OLT are in agreement with electrophysiological studies showing the importance of mGlu7 in memory formation (53).

mGlu5 antagonists and negative modulators in ORT. Antagonists and negative modulators of group I mGluRs counteract excessive increase in glutamate signaling, and thus protect against excitotoxicity. Recent studies showed that activation of mGlu5 potentiates NMDARs activity in both *in vitro* (54) and *in vivo* studies (55). Therefore, inhibition of group I mGluRs could decrease excitotoxicity via blockage of NMDARs. In this respect, blockage of mGluR5 has been studied in diseases related to excitotoxicity, like Parkinson's disease (PD) and AD.

Chronic administration of 2-methyl-6-(phenylethynyl)-pyridine (MPEP) for 14 days (2 mg/kg; i.p.), with ORT taking place on days 12-14, improved short-term object recognition memory tested after 5 min retention interval, in a rat model of PD (56). Additionally, a selective negative mGlu5 modulator, 2-chloro-4-((2,5-dimethyl-1-(4-(trifluoromethoxy)phenyl)-1H-imidazol-4-yl)ethynyl) pyridine (CTEP), was shown to ameliorate cognitive deficits in two transgenic mouse models of AD, APPswe/PS1ΔE9 and 3xTg-AD, after prolonged, but not short-term, administration. Specifically, administration of CTEP (2 mg/kg; i.p.) for 5 days resulted in no significant difference between the treated and untreated AD mice of both strains. On the contrary, chronic administration of CTEP for 3 months (2 mg/kg; i.p.; every 48 h) ameliorated object recognition deficits when the animals tested after 3 h retention interval. Of note, CTEP reduced deposition of amyloid-containing plaques in the cortex and the hippocampus after implementation of chronic treatment (57).

mGlu5 positive allosteric modulators in ORT. Positive allosteric modulators (PAMs) of mGluR5 have been tested in the ORT as promising cognitive enhancers in healthy animals or

in impaired animal models. Acute treatment with the novel PAM S-(4-fluoro-phenyl)-{3-[3-(4-fluoro-phenyl)-[1, 2, 4]-oxadiazol-5-yl]-piperidin-1-yl}-methanone (ADX47273; 0.1, 1, 10, 30, 50 mg/kg; i.p.), 30 min before T1, improved acquisition of long-term object recognition memory tested after 48 h retention time (60). Although ADX47273 was given 30 min before T1, an effect in early consolidation cannot be excluded due to its half-life lasting approximately 2 h.

Another PAM, 3-Cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide (CDPPB), was tested in the ORT in naïve, MK801- and phencyclidine (PCP) -impaired animals. In the study of Uslaner et al. administration of CDPPB (10 and 30 mg/kg for the healthy animals and 3, 10, 30 mg/kg for the impaired animals; i.p.), 30 min before T1, improved object recognition memory tested after 24 h in both healthy and MK801-impaired animals (61). Importantly, the memory enhancing properties of CDPPB were exhibited only for the lower doses, suggesting that the effect of CDPPB follows an inverted U-shaped dose-response curve (61). In a later study of Horio et al., chronic, but not acute, treatment with CDPPB for 10 days (10 mg/kg/once per day; s.c.) was able to reverse cognitive impairment induced after administration of the NMDAR antagonist PCP (62). Specifically, acute treatment with CDPPB (10 mg/kg; i.p.), 1 h before T1, was not able to alleviate PCP-induced memory impairments. On the contrary, chronic treatment with CDPPB (1 and 10 mg/kg; i.p.) for 14 days, with the last injection given 24 h before T1, improved long-term object recognition memory tested after 24 h retention interval. Finally, a different study showed the neuroprotective properties of CDPPB in a mouse model of Huntington's disease. Chronic administration of CDPPB (5 mg/kg; i.p.) for 7 days was able to compensate against object recognition memory impairments tested after 1.5 h retention interval (63).

Efforts have been made for the development of mGluR5 PAMs with signaling bias that promote mGluR5 association with other signaling pathways, but without enhancing NMDAR activity. In this respect, the "biased" mGluR5 PAM, [6,7-Dihydro-2-(phenoxymethyl)oxazolo[5,4-c]pyridin-5(4H)-yl](fluorophenyl)methanone (VU0409551), was shown to have promising pro-cognitive action in ORT. In particular, administration of VU0409551 (1, 3, 10 mg/kg; p.o.), 30 min before T1, improved object recognition memory of animals tested after 24 h retention interval in a dose-dependent manner with minimum effective dose being 3 mg/kg (64). Although, "biased" PAMs seem optimal cognitive enhancers with improved safety profile due to reduced possibility for excitotoxicity, further investigation is still on a primary stage.

Conclusions

Glutamate signaling is mainly mediated by NMDARs, AMPARs and mGluRs. The continuous development of compound targeting the above receptors promoted studies regarding the role of glutamatergic transmission in memory. In this respect, ORT and OLT have been valuable assets for reconciling the effects of different compound that act on glutamatergic system at different memory stages. Importantly these studies revealed that the different types of receptors mediate glutamate signaling at temporally distinct stages of mnemonic processes. Specifically, it was shown that activation of NMDARs and group I/II mGluRs at the acquisition stage is required for storage of long-term memories, while activation of AMPARs and mGlu7 during acquisition processes facilitates storage of short-term memories. In addition, blockage of NMDARs or AMPARs could disrupt early, but not late, consolidation of object recognition memory, while only blockage of AMPARs could induce impairments at the stage of retention. Further investigation showed that positive modulation of the glutamatergic system could have pro-cognitive action, compensating against natural forgetting in healthy animals or counteracting memory deficits in impaired animal models. The latter was proven by studies that utilized NMDAR agonists for the glycine-binding site, positive modulators for AMPARs and mGluRs. Importantly, inhibition of glutamate transmission by NMDAR antagonists and negative modulators for the glycine-binding site or mGlu5 antagonists could rescue memory deficits in diseases related with excessive glutamate production. Considering the clinical application of the above observations, development of more specific targets probably represents the most promising option for further investigation.

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Chapter 4

The pro-cognitive effect of upregulating cGMP signaling during memory acquisition or early consolidation is mediated by increased AMPA receptor trafficking

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Abstract

The mnemonic phases of acquisition, early and late consolidation are characterized by distinct molecular processes. Although both cyclic nucleotide signaling cascades are implicated in acquisition phase, a distinction can be made for the consolidation phase. Early consolidation is related to upregulation of the cyclic guanosine monophosphate (cGMP)/ protein kinase G (PKG) pathway, whereas late consolidation is mediated by increase in cyclic adenosine monophosphate AMP (cAMP)/ protein kinase A (PKA) pathway. Accordingly, administration of the cGMP-selective phosphodiesterase (PDE) 5 inhibitor vardenafil or the cAMP-selective PDE4 inhibitor rolipram can improve memory when they are applied in specific time windows, yet the molecular mechanism of this phenomenon remains elusive. Considering that the glutamatergic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) could mediate the effects of nucleotide signaling on memory processes, we hypothesized that the differential action of the above inhibitors is related to changes in AMPAR dynamics. In the present study we showed that intraperitoneal administration of either vardenafil or rolipram in mice causes rapidly changes in AMPARs over time. Specifically, we observed a transient increase in synthesis of GluA1-AMPARs at 40 min after drug administration followed by increased membrane levels of GluA1-AMPARs at 60 min after drug administration. In addition, treatment with vardenafil during the acquisition or early consolidation of object location memory resulted in increased surface levels of GluA1- and GluA2-AMPARs which were still augmented 24 h after learning. Nevertheless, the membrane levels of AMPARs were not affected anymore 24 h after learning when rolipram was administrated either at the acquisition or late consolidation phase. These results suggest that dissociative molecular mechanisms could mediate the pro-cognitive function of different classes of PDE inhibitors and that in case of vardenafil this phenomenon could be explained by changes in AMPAR dynamics.

1. Introduction

Memory is a complex cognitive process by which the brain stores and retrieves information. When discussing the concept of memory a distinction can be made between the different subtypes of memory on the one hand, and the different memory phases (or processes) on the other. The different subtypes of memory include short-term, intermediate and long-term memory. Additionally, the different memory phases can be distinguished in the acquisition, the consolidation and the retrieval phase. During the acquisition phase, sensory information can be processed and encoded in the brain, while retrieval is the ability to access and retrieve this information from memory storage. Consolidation represents transformation of memories or information from a labile state to a more stabilized form. Memory consolidation can be further divided in early and late consolidation. It is suggested that conversion from short-term memory to intermediate memory and from the latter to long-term memory are mediated be early and late consolidation, respectively (1). Importantly, each memory phase is governed by distinct molecular cascades (2). In this respect, cyclic nucleotides, like cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP), have a prominent role in memory formation (3-5).

In the study of the involvement of cyclic nucleotides in mnemonic processes, the phosphodiesterase (PDE) inhibitors are important assets. PDEs are the enzymes that hydrolyze cGMP and/or cAMP, prolonging their action. Therefore, application of PDE inhibitors gained particular interest for having potential memory enhancing effects. The PDE superfamily exists out of eleven subfamilies of which especially the PDE4 and PDE5 subfamilies are highly expressed in the rodent and human hippocampus (6). As a result, PDE4 and PDE5 inhibitors are abundantly tested for their memory enhancing potential (1). Importantly, it was shown in rats that administration of the cGMP-specific PDE5 inhibitor vardenafil at the early consolidation time window or the cAMP-specific PDE4 inhibitor rolipram at the late consolidation time window could extend short-term memory into long-term memory (7-9). The existence of these defined time windows in the action of the different cyclic nucleotides during memory consolidation was further outlined in a study in rats showing that the cognitive enhancing effect of PDE5 inhibition was apparent when vardenafil was administered up to 45 min after the learning trial, whereas PDE4 inhibition via rolipram was effective when administered between 3 and 5.5 h after the learning trial of the object recognition task (ORT) (10). Additionally, both vardenafil and rolipram were shown to

enhance memory function by improving memory acquisition when administered before the learning trial.

In the hippocampus, common downstream effectors for cGMP and cAMP are protein kinase G (PKG) and protein kinase A (PKA), respectively. In turn, both PKG and PKA share the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) as common downstream effector, which represents one of the main types of receptors in excitatory synapses (11, 12). AMPARs are mainly heterotetramers consisting of various combinations of four subunits, designated as GluA1-4 (13-15). Despite the existence of several subtypes, most of the AMPARs in the hippocampus are heteromers of GluA1/GluA2 or GluA2/GluA3 (16). There is a plethora of evidence showing the importance of GluA1/GluA2 heteromers in synaptic transmission and memory formation (17, 18), however it has been shown only recently that GluA2/GluA3 receptors participate in homeostatic scaling in the absence of activity and are involved in hippocampal synaptic plasticity (19, 20). Accordingly, rapid trafficking and synaptic incorporation of AMPARs has an eminent role in these processes.

Phosphorylation of AMPARs by PKG and PKA (11, 12) promotes trafficking of already existing AMPARs to extrasynaptic sites in the membrane (21) and additionally increases channel opening probability (22). Based on the model proposed by Soderling and his group, incorporation of AMPARs to extrasynaptic sites primes their delivery to synapses during induction of long-term potentiation (LTP) (21); the proposed molecular correlate of memory. Importantly, the different AMPAR subunits also participate in a biphasic process that mediates plasticity-induced trafficking of receptors to synaptic sites. Neuronal activity involves trafficking of GluA1/GluA2 heteromers to the synapses (23, 24). Later on, these receptors are replaced by the constitutively trafficking GluA2/GluA3 heteromers, maintaining long-lasting synaptic strengthening (25). Considering that upregulation of cAMP or cGMP pathways has an essential role in promoting trafficking of AMPARs to the extrasynaptic site and subsequently enhancing synaptic plasticity, we hypothesize that the pro-cognitive action of PDE inhibitors during the specific time windows can be explained by changes in AMPAR dynamics.

In order to investigate this hypothesis, we initially confirmed the previously observed pro-cognitive effect of vardenafil and rolipram when administered within specific time-windows using the object location task (OLT) in mice. Thereafter, we examined whether intraperitoneal (i.p.) administration of vardenafil or rolipram could have an effect on AMPAR trafficking or synthesis in mice sacrificed shortly after drug administration or 24 h after the OLT.

2. Materials and Methods

2.1 Animals

All experimental procedures were approved by the local ethics committee of Maastricht University for animal experiments and met governmental guidelines. In total 138 male C57BL/6 mice (Charles River, Sulzfeld, Germany) were tested between 4-5 months of age. Specifically, 48 mice were used for the study that involved only treatment and 69 mice were used for the study that involved treatment and behavioral testing. All animals were housed individually in standard green line Tecniplast individually ventilated cages (IVC) on sawdust bedding. The animals were housed on a reversed 12/12-h light/dark cycle (lights on from 19:00h to 07:00h) and received food and water *ad libitum*. The mice were housed and tested in the same room. A radio, playing softly, provided background noise in the room.

2.2 Drug preparation

We administered two selective PDE inhibitors: PDE5 inhibitor vardenafil (kindly donated by BAYER, Wuppertal, Germany) and PDE4 inhibitor rolipram (Sigma Aldrich, Zwijndrecht, the Netherlands). Both inhibitors were previously shown to cross the blood-brain barrier (10). Both PDE inhibitors were dissolved in the same vehicle (98% methyl cellulose tylose solution (0.5%) and 2% tween80) and administered in a volume of 4 ml/kg. The drugs were given i.p. at a dose of 0.3 mg/kg for vardenafil and 0.03 mg/kg for rolipram. Dosages, injection volumes and time of injection are based on extensive previous experience of the lab with the current drugs (7-9). The solutions were prepared freshly each testing day.

2.3 Object location task

The OLT is a hippocampus-dependent spatial memory task that has been derived from the ORT (26). The OLT has been performed as previously described (27). In short, the apparatus consists of a circular arena, 40 cm in diameter and 40 cm in height. The back half of the wall was made of white polyvinyl chloride (PVC) and the front was made of transparent PVC. Fluorescent red tubes and a light bulb provided a constant illumination of about 20 lux on the floor of the apparatus. We used two different sets of two identical objects, which were divided in a semi-random manner between animals and over all treatment conditions to avoid object preferences. The objects consisted of a massive metal rectangular prism (2.5 cm × 5 cm × 7.5 cm) containing two holes (diameter 1.5 cm) and a massive aluminum cube with a tapering top (4.5 cm × 4.5 cm × 8.5 cm). Prior to testing, the animals were habituated to the arena, the objects and the injections. The test session was comprised of two trials; the learning trial (T1) and the test trial (T2), each lasting 4 min. Prior to the experimental trials the mice

were put in an empty cage for 4 min to increase arousal during testing. In both trials mice were placed into the arena facing the transparent wall. During T1, two identical objects were placed inside the apparatus on a horizontal line in the middle of the arena (object a1 and a2). At the end of the test, the mice were returned to their home cage for a predetermined interval of 24 h. After this interval, the mice were put back into the arena for T2 in which one of the two objects from T1 was moved to a different position on a vertical line, to the front or back of the arena (b), while the other object was at the same position as in T1. Between the trials, the objects and arena were cleaned with 70% ethanol, in order to avoid olfactory cues.

The readout parameters of the OLT are similar to the ORT (28) and are referring to the exploration time for each object during T1 and T2. Exploration was defined in the following manner: directing the nose to the object at a distance of no more than 2 cm and/or touching the object with the nose. Sitting on the object was not considered exploratory behavior. The exploration time (in seconds) of each object during T1 are presented as 'a1' and 'a2'. The time spent exploring the familiar and the displaced object in T2 are represented as 'a3' and 'b', respectively. Using this information, the following variables were calculated: the total exploration time during T1 [e1 (=a1+a2)], the total exploration time during T2 [e2 (=a3+b)] and the discrimination index [d2 (=b-a3/e2)]. The d2 index is a relative measure of discrimination corrected for exploratory activity and could range from -1 to 1. A significant difference from zero, i.e. chance level, indicates that the mice remembered the object locations from T1, and a difference from the vehicle condition signifies an actual memory improvement. Considering that mice require a minimum amount of exploration in order to show reliable memory performance (28), mice exploring less than 10 sec during T1 or less than 7 sec during T2 were excluded from the analysis.

For the behavioral test, the mice were divided into three groups: the "acquisition group" in which either vardenafil or rolipram were administrated 30 min before T1, the "early consolidation group" in which the animals received the treatment 20 min after T1 as not to influence acquisition/encoding (9), and the "late consolidation group" in which drug administration was performed 3 h after T1. All three groups were tested in T2 after a 24 h inter-trial interval in order to assess the efficacy of the treatment in improving long-term spatial memory. The experimenter was always blind to the experimental conditions.

2.4 Biotinylation assay and sample preparation

After completion of the behavioral testing and a sufficient washout period, the animals again received treatment at the abovementioned time-points, but this time they were sacrificed

after 24 h for biochemical analysis, without undergoing T2. In order to examine the effect of vardenafil or rolipram in AMPARs dynamics *per se*, a different cohort of mice were treated with either vardenafil or rolipram and sacrificed at different time points, i.e. 15 min, 40 min and 60 min after treatment. Animals were sacrificed by means of cervical dislocation, the brains were excised and both hippocampi were isolated. Coronal hippocampal slices of 400 µm thickness were obtained using a McIlwain tissue chopper. The slices were transferred in ice-cold ACSF (124 mM NaCl, 4.4 mM KCl, 1 mM Na2HPO4, 25 mM NaHCO3, 2 mM CaCl, 2 mM MgSO4 and 10 mM glucose) and incubated with 1mM sulfo-NHS-SS-biotin (Thermo Scientific,#21328 Bleiswijk, The Netherlands) for 60 min on ice. Following biotin incubation, slices were washed with cold 100 mM glycine to remove the excess of biotin and flash-frozen in liquid nitrogen. Frozen hippocampal slices were mechanically dissociated in lysis buffer (1 mM EDTA, 1 mM EGTA, 1% glycerol, 0.1% triton and 1% IGEPAL CA-630 in PBS), containing protease and phosphatase inhibitors. Protein concentration was determined with Lowry protein assay (Bio-Rad Laboratories, Veenendaal, The Netherlands). For the membrane fractions, protein lysates (60 µg) were incubated overnight with streptavidin-coated Dynabeads (Thermo Scientific, #65601, Bleiswijk, The Netherlands) at 4 °C under constant rotation. Dynabeads containing surface biotinylated proteins were separated from cytosolic proteins by magnetic precipitation. Biotinylated proteins were eluted from streptavidin beads with 1xSDS loading buffer (1 M Tris HCL, 75% glycerol, 6% SDS, 15%-β-mercaptoethanol and 0.025% brome phenol blue in milliQ) at 95 °C for 5 min.

2.5 Western blotting

Surface protein fractions (60 µg) and their corresponding total protein samples (8 µg) were resolved in 10% SDS-PAGE and then transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Veenendaal, The Netherlands). The membranes were blocked (50% Odyssey blocking buffer in PBS, Li-Cor, Lincoln, NE, USA) for 1 h at room temperature, followed by overnight incubation with the primary antibodies at 4 °C. The primary antibodies consisted of mouse anti-glutamate receptor 1 N-terminus (1:1000, #MAB2263, Merck Millipore, Burlington, MA, USA), rabbit anti-GluA2 (1:1.000, #MAB5306S, Cell Signalling, Danvers, MA, USA) and mouse anti-GAPDH (1:1.000.000, #10R-G109A, Fitzgerald Industries, Acton, MA, USA) as loading control. Membranes were subsequently incubated with secondary antibodies for 1 h at room temperature: goat anti-rabbit IRDye 800 (1:10.000, Li-Cor, Lincoln, NE, USA) and donkey anti-mouse IRDye 680 (1:10.000, Li-Cor, Lincoln, NE, USA). Membranes were visualized using the Odyssey Infrared Imaging System (Li-Cor,

Lincoln, NE, USA) and protein bands were quantified by ImageJ (https://imagej.nih.gov/ij/). Raw intensity measures were normalized to GAPDH to control for loading differences.

2.6 Statistics

For the behavioral test, one sample t-tests were performed for comparing the d2 index of vardenafil or rolipram to zero (i.e. chance level). For both the behavioral experiments and western blots, statistical differences were evaluated with one-way ANOVA followed by post-hoc Dunnett's t-tests. Outliers were excluded based on a Dixon Q-test for outliers.

3. Results

3.1 Treatment with vardenafil or rolipram improves long-term spatial memory in mice when administered within specific time-frames

Animals treated with vehicle 30 min before T1, to target the acquisition process, were not able to remember the location of the new object when tested after 24 h as their respective d2 value did not significantly differ from zero, i.e. chance level (Figure 1A). When vardenafil or rolipram were given 30 min before T1 both treatments were effective in improving the animals' spatial memory when tested after 24 h (Figure 1A). The d2 values of the animals treated with vardenafil or rolipram differed significantly from chance level as measured with one-sample t-tests (vardenafil: $p<0.0001$; rolipram: $p=0.005$), indicating improved spatial memory. Additionally, a one-way ANOVA comparing the d2 value of every group showed a significant difference between group performance ($F_{2,51}=7.838$; $p=0.001$). The post-hoc Dunnett's t-tests, comparing every condition to vehicle treatment, indicated that treatment with vardenafil ($p=0.001$) or rolipram ($p=0.013$) significantly enhanced mice OLT performance.

Administration of vardenafil or rolipram 20 min after T1, at the early consolidation phase, resulted in improved spatial memory only for the vardenafil-treated animals ($p<0.001$) (Figure 1B). The d2 value of the animals that received either vehicle or rolipram did not differ from chance level. A one-way ANOVA revealed a significant difference between the experimental groups ($F_{2,51}=11.239$; $p<0.0001$). Furthermore, a post-hoc Dunnett's t-test confirmed that mice treated with vardenafil performed significantly better than vehicle animals ($p<0.0001$), while the performance of rolipram-treated animals was not different from vehicle.

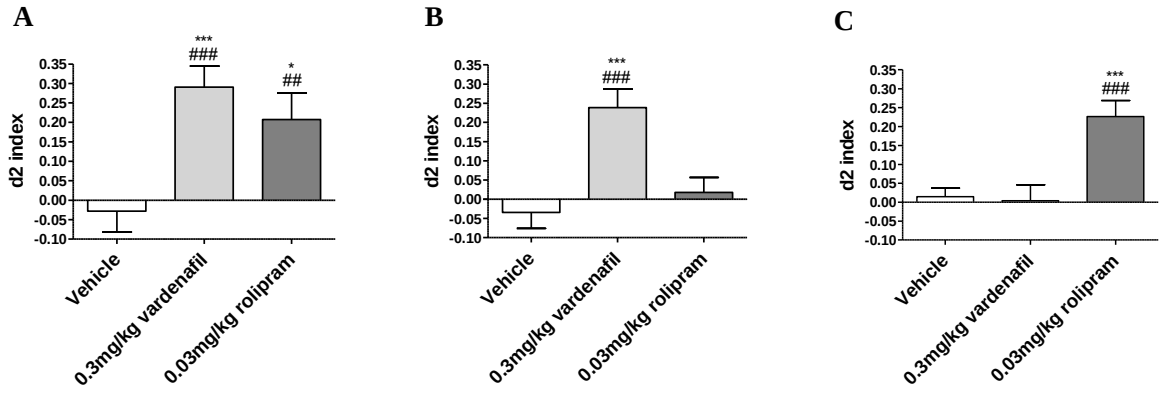


Figure 1. The effect of vardenafil and rolipram treatment (i.p.) in the OLT at different memory stages. (A) OLT performance after treatment with vardenafil or rolipram 30 min before T1 and with 24 h retention interval showed that both treated groups are able to discriminate between the old and the new location of the object as compared to zero (chance level). Additionally, treated animals exhibit improved d2 index in comparison to that of the vehicle. N=18 for all three groups. (B) OLT performance when the treatment was given 20 min after T1 and the animals tested after 24 h inter-trial interval showed that only treatment with vardenafil was able to improve the animals' performance in comparison to chance level as well as the d2 index in comparison to that of the vehicle. N=18 for all the groups. (C) OLT performance when treatment was administered 3 h after T1 showed that only treatment with rolipram improved the animals' performance in comparison to the chance level. Additionally, the d2 index differs significantly from that of the vehicle treated group. Vehicle group: N=17, vardenafil group: N=19, rolipram group: N=20. Data are shown as mean ± SEM. A significant difference from zero is depicted with hashes (one sample t-tests, ##: $p < 0.01$; ###: $p < 0.001$). A significant difference from the vehicle condition is depicted with asterisks (one-way ANOVA followed by post-hoc Dunnett's test, *: $p < 0.05$; ***: $p < 0.001$).

Treatment with vardenafil or rolipram 3 h after T1, at the late consolidation phase, showed that only rolipram ($p<0.0001$) could enhance spatial memory at this time point, while there was no statistically significant difference between the d2 value for vardenafil-treated animals when compared to chance level (Figure 1C). A one-way ANOVA comparing the performance between the different treatment groups showed a significant treatment effect ($F_{2,53}=11.285$; $p<0.0001$). A post-hoc Dunnett's t-test additionally confirmed that that rolipram-treated animals performed significantly better in comparison to the vehicle condition ($p=0.001$), whereas no difference was observed for the vardenafil condition.

3.2 Administration of vardenafil or rolipram results in a time-dependent differential effect on GluA1-AMPAR dynamics

Before investigating the underlying mechanism of the temporally-distinct action of vardenafil and rolipram, we first examined the effect of the compounds on GluA1-AMPAR dynamics over time. When the animals were sacrificed 15 min after drug administration, we observed a significant treatment effect for the surface expression ($F_{2,13}=4.918$; $p=0.026$) (Figure 2A) and trafficking of GluA1-AMPARs ($F_{2,10}=12.445$; $p=0.002$) (Figure 2C).

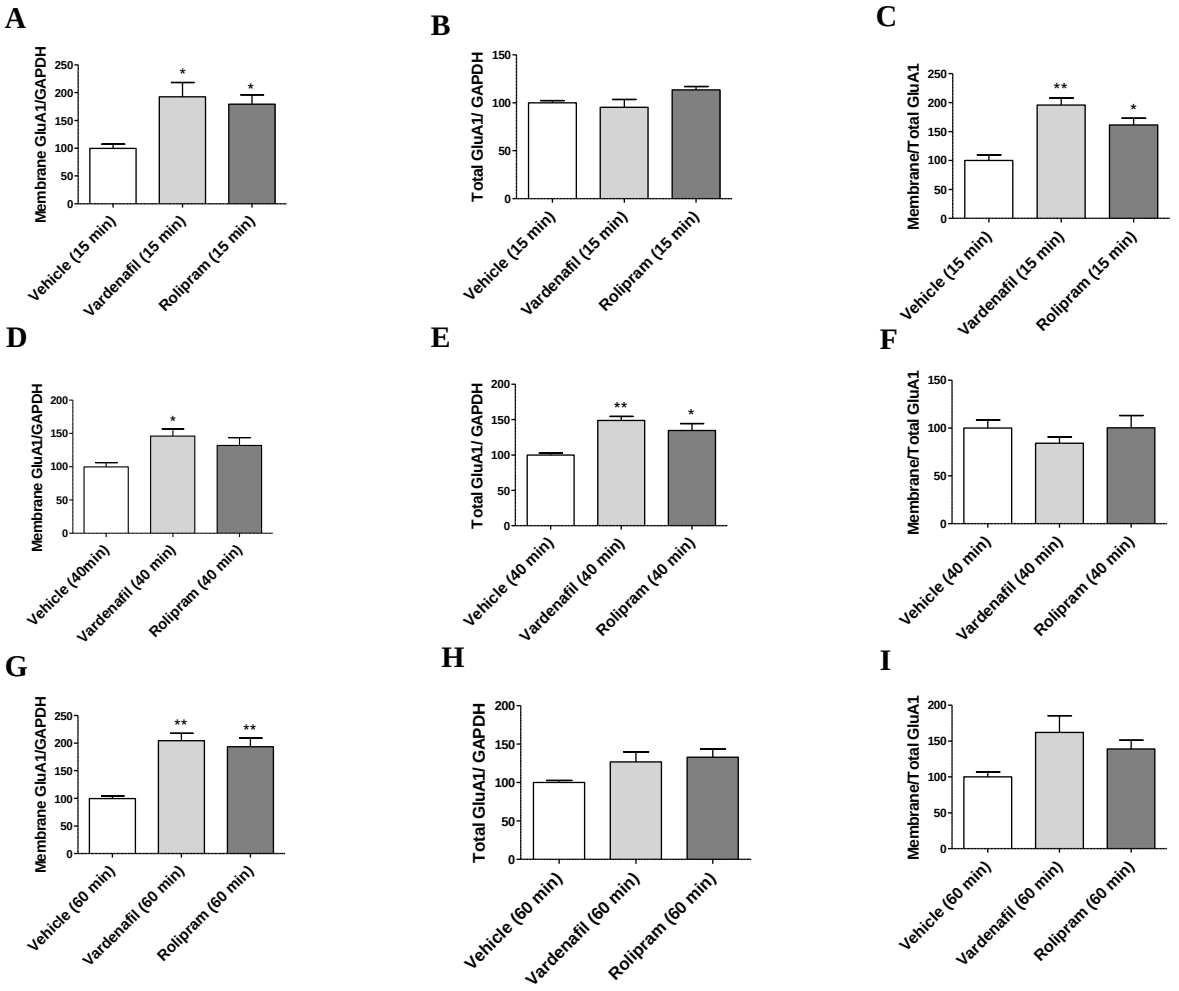
Specifically, treatment with both vardenafil (p=0.019) and rolipram (p=0.041) significantly increased the amount of GluA1-AMPARs in the membrane. In addition, treatment with both drugs upregulated trafficking of GluA1-AMPARs (vardenafil: p=0.001; rolipram: p=0.011). Unlike these measurements, administration of either drugs did not affect the total levels of GluA1-AMPARs ($F_{2,11}$=2.265; p=0.150) (Figure 2B).

Harvesting the brains 40 min after treatment administration, resulted in significant treatment effects for the surface ($F_{2,13}$=4.011; p=0.044) and total levels ($F_{2,12}$=8.965; p=0.004) of GluA1-AMPARs (Figure 2D-E), whereas there was no significant treatment effect for the ratio membrane/total GluA1 ($F_{2,12}$=0.774; p=0.483), i.e. trafficking (Figure 2F). Although both treatments upregulated the total levels of GluA1-AMPARs (vardenafil: p=0.002; rolipram: p=0.018), only treatment with vardenafil resulted in increased surface levels of GluA1-AMPARs (vardenafil: p=0.026; rolipram: p=0.124).

Finally, sacrificing the mice 60 min after treatment revealed a significant effect only for the membrane levels of GluA1-AMPARs ($F_{2,12}$=11.293; p=0.002) (Figure 2G). Specifically, administration of both vardenafil (p=0.001) and rolipram (p=0.003) promoted the surface expression of GluA1-AMPARs. On the contrary, there was no significant treatment effect for the total levels ($F_{2,12}$=1.6; p=0.242) or trafficking of GluA1-AMPARs ($F_{2,13}$=2.821; p=0.096) (Figure 2H-I).

3.3 The effect of vardenafil or rolipram administration on GluA1- and GluA2-AMPAR dynamics at the acquisition phase

To determine the molecular basis of the pro-cognitive effect of vardenafil or rolipram at the acquisition phase of spatial memory, animals were treated with one of the drugs 30 min before T1 and their brains were collected after 24 h. A one-way ANOVA showed a significant treatment effect for the surface expression ($F_{2,18}$=5.5; p=0.014) (Figure 3A) and trafficking ($F_{2,18}$=16.72; p<0.0001) of GluA1-AMPARs (Figure 3C), while no significant difference was detected for the total levels ($F_{2,18}$=2.232; p=0.136) (Figure 3B). Accordingly, post-hoc Dunnett's t-tests indicated that only vardenafil, but not rolipram, led to increased surface expression (vardenafil: p=0.021; rolipram: p=0.991) and trafficking of GluA1-AMPARs (vardenafil: p<0.0001; rolipram: p=0.526).



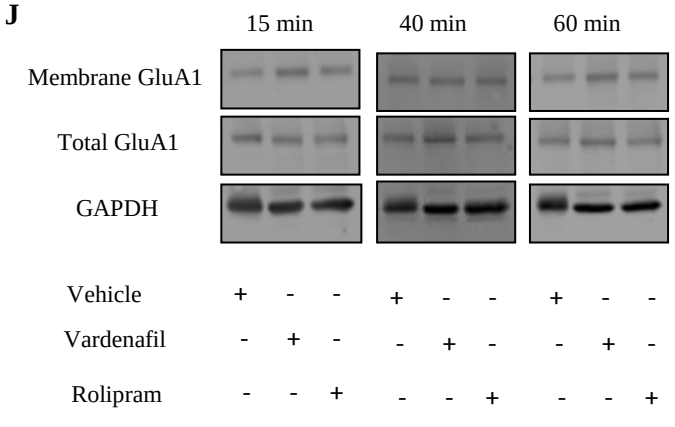


Figure 2. Effects of vardenafil or rolipram treatment on GluA1-AMPAR dynamics at different time points. (A-C) Administration of either vardenafil or rolipram resulted in increased GluA1-AMPAR membrane/total ratio in the hippocampus of mice sacrificed 15 min after treatment, while total levels of GluA1-AMPARs remain unaffected. Additionally, increased surface expression of GluA1-AMPARs was observed for the vardenafil-treated animals in comparison to the vehicle. A: N=4,6,6 for vehicle, vardenafil and rolipram, respectively; B: N=3,6,5 for vehicle, vardenafil and rolipram, respectively; C: N=3;4;6 for vehicle vardenafil and rolipram respectively. (D-F) When the brains were harvested 40 min after drug administration, both treatments resulted in increased total levels of GluA1-AMPARs and a concomitant upregulation of the surface fraction of the receptors only for the vardenafil-treated animals. At this time point, there was no difference in the membrane/total ratio of GluA1-AMPARs between the groups. D-E: N=4,6,6 for vehicle, vardenafil and rolipram, respectively; F: N=4,5,6 for vehicle, vardenafil and rolipram, respectively. (G-I) Waiting 60 min before harvesting the brains resulted in upregulation of membrane levels of GluA1-AMPARs for both treatments, while the total levels and the membrane/total ratio did not differ from the vehicle. G-H: N=3,6,6 for vehicle, vardenafil and rolipram, respectively; I: N=4,6,6 for vehicle, vardenafil and rolipram, respectively. (J) Representative blots for each time point. Data are shown as mean ± SEM. A significant difference from the vehicle condition is depicted with asterisks (one-way ANOVA followed by post-hoc Dunnett's test, *: $p < 0.05$; **: $p < 0.01$).

Regarding GluA2-AMPARs, a significant treatment effect was observed for the surface levels ($F_{2,18}$=10.014; p<0.0001) (Figure 3D) and the ratio membrane/total GluA2 ($F_{2,18}$=41.447; p=0.000) (Figure 3F). At both cases, post-hoc Dunnett's t-test revealed a significant upregulation in the membrane levels (p<0.0001) and trafficking of GluA2-AMPARs (p<0.0001) for the vardenafil-treated animals. However, no difference was observed for the rolipram-treated animals in comparison to the vehicle conditions (membrane GluA2/GAPDH: p=0.994; membrane/total GluA2: p=0.371). Additionally, a significant treatment effect was detected for the total levels of GluA1-AMPARs ($F_{2,18}$=7.316; p=0.005) (Figure 3E).

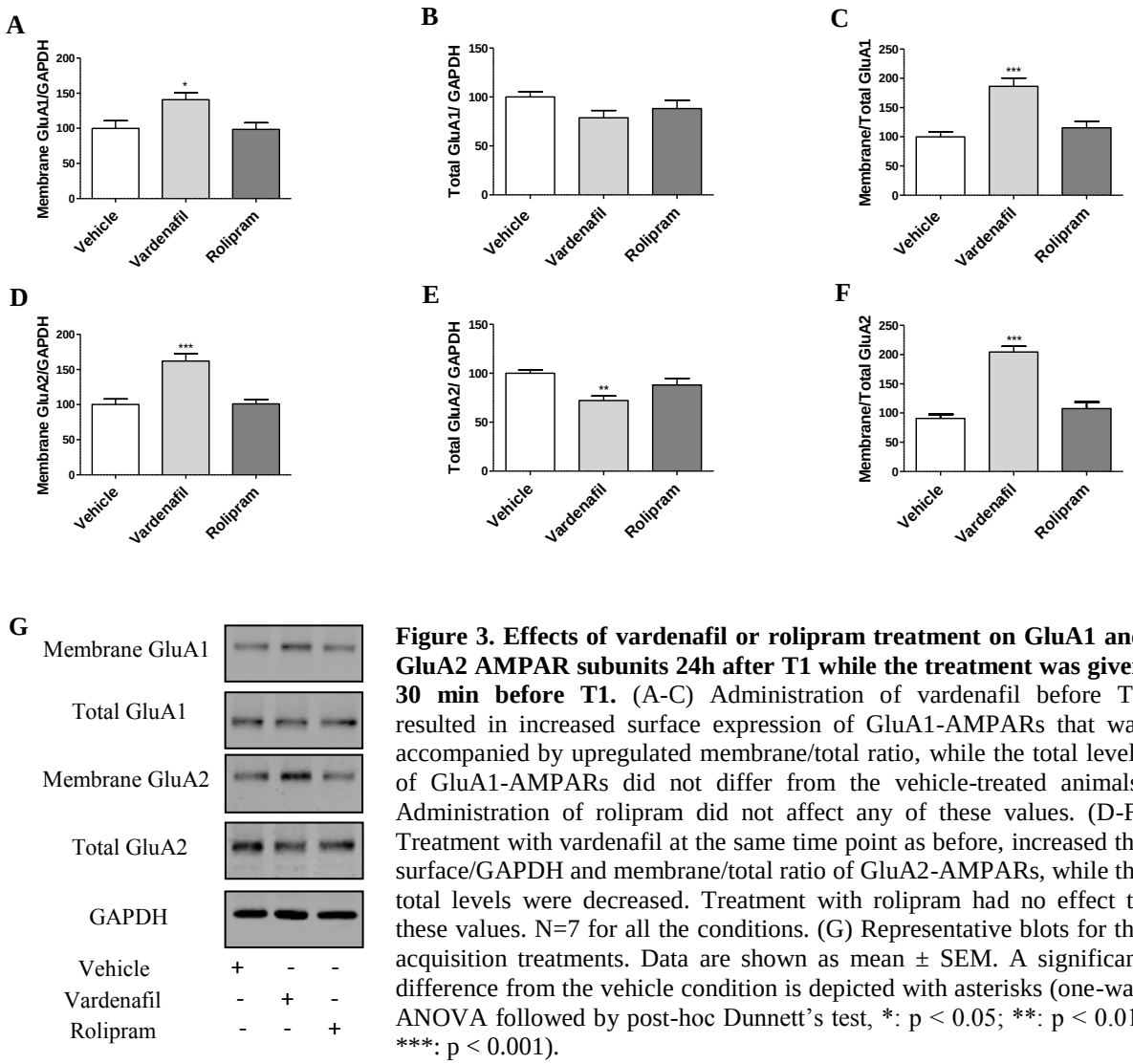


Figure 3. Effects of vardenafil or rolipram treatment on GluA1 and GluA2 AMPAR subunits 24h after T1 while the treatment was given 30 min before T1. (A-C) Administration of vardenafil before T1 resulted in increased surface expression of GluA1-AMPARs that was accompanied by upregulated membrane/total ratio, while the total levels of GluA1-AMPARs did not differ from the vehicle-treated animals. Administration of rolipram did not affect any of these values. (D-F) Treatment with vardenafil at the same time point as before, increased the surface/GAPDH and membrane/total ratio of GluA2-AMPARs, while the total levels were decreased. Treatment with rolipram had no effect to these values. N=7 for all the conditions. (G) Representative blots for the acquisition treatments. Data are shown as mean ± SEM. A significant difference from the vehicle condition is depicted with asterisks (one-way ANOVA followed by post-hoc Dunnett's test, *: $p < 0.05$; **: $p < 0.01$, ***: $p < 0.001$).

This effect was attributed to decreased total levels of GluA2-AMPARs in the vardenafil condition (p=0.002), while the rolipram condition did not differ from vehicle (p=0.2015).

Finally, no treatment effect was detected for the ratio membrane GluA2/GluA1 ($F_{2,18}$=1.525; p=0.244; data not shown) and total GluA2/GluA1 ($F_{2,18}$=10.453; p=0.643; data not shown).

3.4 The effect of vardenafil or rolipram administration on GluA1- and GluA2-AMPAR dynamics at the early consolidation phase

In order to examine the cognitive enhancing effects of vardenafil or rolipram administration at the early consolidation phase, mice received one of the drugs 20 min after T1 and their brains were excised 24 h after T1.

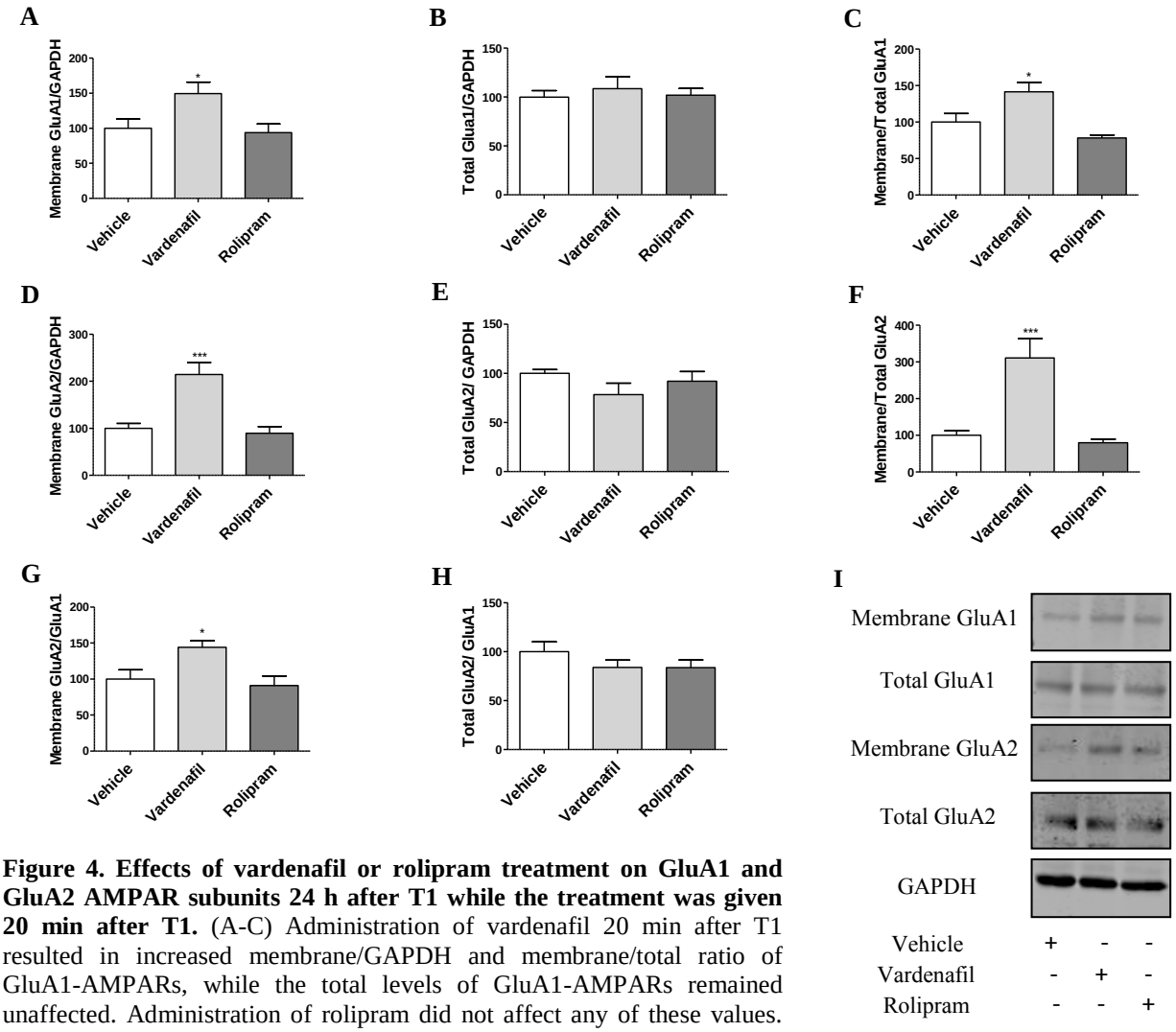


Figure 4. Effects of vardenafil or rolipram treatment on GluA1 and GluA2 AMPAR subunits 24 h after T1 while the treatment was given 20 min after T1. (A-C) Administration of vardenafil 20 min after T1 resulted in increased membrane/GAPDH and membrane/total ratio of GluA1-AMPARs, while the total levels of GluA1-AMPARs remained unaffected. Administration of rolipram did not affect any of these values. A-B: N=8 for all the groups; C: N=8,8,7 for vehicle, vardenafil and rolipram, respectively. (D-F) Similar to GluA1-AMPARs, treatment with vardenafil at the same time point upregulated both the membrane/GAPDH and membrane/total ratio of GluA2-AMPARs, without changing the total levels. Treatment with rolipram did not affect these values. D-E: N=8 for all the groups; F: N=8,8,7 for vehicle, vardenafil and rolipram, respectively. (G-H) Vardenafil-treated animals exhibited increase in membrane GluA2/GluA1 ratio, whereas no changes are observed in the total GluA2/GluA1 ratio. G: N=8 for all the groups; H: N=8,7,8 for vehicle, vardenafil and rolipram, respectively. (I) Representative blots for the early consolidation treatments. Data are shown as mean ± SEM. A significant difference from the vehicle condition is depicted with asterisks (one-way ANOVA followed by post-hoc Dunnett's test, *: $p < 0.05$; ***: $p < 0.001$).

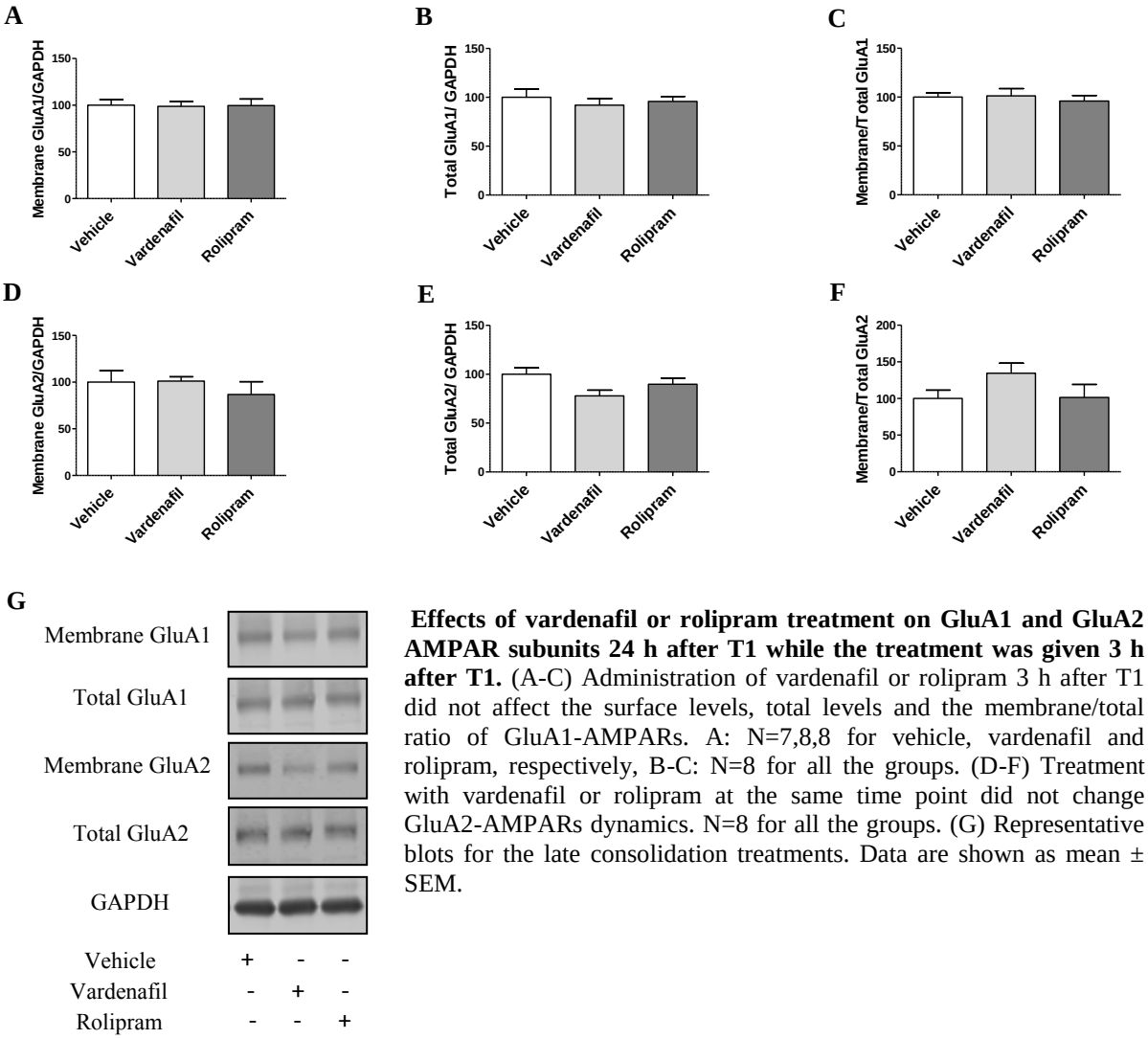
At this time point, we detected a significant treatment effect for the membrane levels (Figure 4A, D) and trafficking (Figure 4C, F) for both GluA1- and GluA2-AMPARs (one-way ANOVA; membrane GluA1/GAPDH: $F_{2,21}$=4.697; p=0.021, membrane/total GluA1: $F_{2,20}$=8.885; p=0.002, membrane GluA2/GAPDH: $F_{2,21}$=14.622; p<0.0001, membrane/total GluA2: $F_{2,20}$=15.187; p<0.0001). There was no significant treatment effect for the total levels for both GluA1-and GluA2-AMPARs ($F_{2,21}$=0.255; p=0.777, $F_{2,21}$=1.450; p=0.257) (Figure 4B, E).

Subsequently, post-hoc Dunnett's t-tests showed a significant increase in the membrane levels (vardenafil: p=0.04; rolipram: p=0.930) and trafficking (vardenafil: p=0.021; rolipram: p=0.289) for the vardenafil, but not for the rolipram condition. Similarly, a significant difference was detected between vehicle- and vardenafil-treated animals with regard to the surface expression (vardenafil: p<0.0001; rolipram: p=0.889) and trafficking (vardenafil: p<0.0001; rolipram: p=0.878) of GluA2-AMPARs, while treatment with rolipram did not affect these values.

Additionally, we detected a significant treatment effect for the ratio membrane GluA2/GluA1 ($F_{2,21}$=5.774; p=0.010) (Figure 4G). Post-hoc Dunnett's t-test revealed a significant difference between the vehicle and vardenafil condition (p=0.028), whereas no difference was observed for the rolipram condition (p=0.818). There was no significant treatment effect for the ratio total GluA2/GluA1 ($F_{2,20}$=1.190; p=0.325) (Figure 4H).

3.5 The effect of vardenafil or rolipram administration on GluA1- and GluA2-AMPAR dynamics at the late consolidation phase

The cognitive enhancing effect of vardenafil or rolipram at the late consolidation phase was examined in mice that received the treatment 3 h after T1 and were sacrificed 24 h after the test. A one-way ANOVA showed no difference for the surface ($F_{2,20}$=0.012; p=0.988), total levels ($F_{2,21}$=0.349; p=0.349) and trafficking ($F_{2,21}$=0.21; p=0.812) of GluA1-AMPARs (Figure 5A-C). Similarly, there was no significant treatment effect for the surface ($F_{2,21}$=0.526; p=0.597), total levels ($F_{2,21}$=3.144; p=0.064) and trafficking of GluA2-AMPARs ($F_{2,21}$=1.799; p=0.19) (Figure 5 D-F). Finally, no significant treatment effect was observed for the ratios membrane GluA2/GluA1 ($F_{2,21}$=0.893; p=0.567; data not shown) and total GluA2/GluA1 ($F_{2,21}$=0.584; p=0.424; data not shown).



Effects of vardenafil or rolipram treatment on GluA1 and GluA2 AMPAR subunits 24 h after T1 while the treatment was given 3 h after T1. (A-C) Administration of vardenafil or rolipram 3 h after T1 did not affect the surface levels, total levels and the membrane/total ratio of GluA1-AMPARs. A: N=7,8,8 for vehicle, vardenafil and rolipram, respectively, B-C: N=8 for all the groups. (D-F) Treatment with vardenafil or rolipram at the same time point did not change GluA2-AMPARs dynamics. N=8 for all the groups. (G) Representative blots for the late consolidation treatments. Data are shown as mean ± SEM.

4. Discussion

The present study replicated previous findings showing that administration of PDE4 or PDE5 inhibitors within specific time windows of spatial memory can enhance memory performance in rodents (8-10). In more detail, administration of the cGMP-specific PDE5 inhibitor vardenafil or the cAMP-specific PDE4 inhibitor rolipram enhanced long-term object location memory when the drugs were given during the acquisition (i.e. 30 min before T1) phase. Despite the similar effect at the acquisition phase, a temporal distinction in the action of the above inhibitors was observed when they were given at the consolidation phase. Administration of vardenafil at the early (20 min after T1), but not late (3 h after T1), consolidation phase and rolipram at the late, but not early, consolidation phase counteracted natural forgetting in the OLT.

It is suggested that the time-dependent effect in the action of PDE inhibitors is related to the differential involvement of their corresponding cyclic nucleotide signaling cascade in memory consolidation. Intrahippocampal infusion of cAMP or cGMP analogues in different behavioral paradigms resulted in similar temporal dissociation in the action of cyclic nucleotides (4, 29, 30). Additionally, it was shown that the cognitive enhancing properties of PDE4 or PDE5 inhibitor administration at the specific mnemonic phases during consolidation could be abolished by intrahippocampal inhibition of PKG or PKA, the main downstream effectors of cyclic nucleotides (9).

In line with these previous observations, a temporally differential effect was observed in the effectiveness of vardenafil and rolipram at the consolidation phase, whereas both drugs are equally effective on memory acquisition when given before the learning trial. Although the underlying mechanism for this phenomenon is yet unknown, it is possible that the effect at acquisition is predominantly related to changes in cyclic nucleotide signaling in the presynaptic cell, while the effect during the consolidation phase is mainly induced postsynaptically. In this respect, it was found that presynaptic activation of cGMP or cAMP promotes the synthesis and/or release of neurotransmitters including glutamate (31, 32), increasing the release probability of the synapses. On the other hand, administration of either vardenafil or rolipram at the consolidation phase probably enhances ongoing postsynaptic events including trafficking of preexisting AMPARs to the synaptic sites and/or synthesis of new proteins (e.g. AMPAR's) via activation of cAMP response element-binding protein (CREB). Of note, the distinction between presynaptic/postsynaptic and acquisition/consolidation, respectively, is more a continuous than a discrete categorization as AMPAR trafficking could also be involved in acquisition processes. Of note, the mechanism that restrains the activity of the cAMP/PKA signaling cascade at the early consolidation and cGMP/PKG signaling pathway at the late consolidation remains still elusive. Nevertheless, it was recently shown that activation of cAMP/PKA pathway at the late consolidation phase of object recognition memory requires intact cGMP/PKG signaling at the early consolidation phase during formation of long-term memories (9). This observation supports the notion of a sequential relationship between cGMP and cAMP at least during consolidation processes.

Due to the apparent relationship between GluA1-AMPARs and cyclic nucleotide signaling, we sought to determine whether upregulation of cAMP/PKA or cGMP/PKG signaling via rolipram of vardenafil, respectively, could affect GluA1-AMPAR dynamics. Interestingly, administration of either vardenafil or rolipram had a distinguished effect on AMPARs over time. More specifically, 15 min after treatment with either of the drugs there

was an increase in surface expression of GluA1-AMPARs that was also depicted in increased trafficking; 40 min after treatment the total levels of GluA1-AMPARs were upregulated, resulting in increased surface expression for the vardenafil-treated mice; and 60 min after treatment there was an increase in the surface expression of GluA1-AMPARs for both treatments. The above findings suggest that there is a "wave" in the trafficking-synthesis of GluA1-AMPARs.

The initial upregulation in trafficking and surface expression of GluA1-AMPARs could be the result of increased mobilization of receptors that reside in synaptic endosomes (33). Later on, the observed increased synthesis of GluA1-AMPARs for both vardenafil- and rolipram-treated animals could be mediated via CREB-dependent transcription or via translation of local pools of GluA1 mRNAs. Several studies have shown that CREB is the convergent point between cAMP/PKA and cGMP/PKG signaling and an increase in CREB phosphorylation is a critical step in the commencement of protein transcription (34, 35). Additionally, there are several lines of research indicating that mRNA of AMPARs subunits can be found in hippocampal dendrites (36-38), where it is locally translated and subsequently incorporated into or close to the synapse (39, 40). Further strength to this observation was provided by a study showing that local translation of GluA1- and GluA2-containing AMPARs could be triggered be pharmacological manipulations that could elicit LTP, like activation of metabotropic glutamate receptors (mGluRs) or application of high levels of potassium (39). Finally, 60 min after upregulation of cAMP/PKA or cGMP/PKG cascades a significant increase in surface expression of GluA1-AMPARs was observed. Considering the previously observed increase in the total levels of GluA1-AMPARs at 40 min, this indicates that the synthesis of new receptors started mitigating and more receptors, most likely newly synthetized, are inserted into the membrane.

In addition to the time-dependent effect of PDE4 and PDE5 inhibition on GluA1-AMPAR dynamics, our study demonstrates that increased surface expression and trafficking of GluA1- and GluA2-AMPARs could explain the pro-cognitive effect of vardenafil when administrated either at the acquisition or at the early consolidation phase, but not at the late consolidation phase, of spatial memory. The ratio of surface GluA2/GluA1 receptors did not differ when vardenafil treatment was given during the acquisition phase. However, there was a significant increase in GluA2-AMPARs in comparison to GluA1-AMPARs when vardenafil was administered at the early consolidation phase. These findings indicate that different types of receptors are upregulated when the cGMP/PKG pathway is stimulated at different phases of the mnemonic process. Considering that GluA1/GluA2 and GluA2/GluA3 heteromers are

the prevailing types of AMPARs in pyramidal hippocampal neurons (16), we could hypothesize that at the acquisition phase there is mainly an increase in surface expression of GluA1/GluA2 heterotetramers, while at the early consolidation phase there is an additional increase in GluA2/GluA3 heterotetramers. Nevertheless, we cannot be conclusive about whether the receptors are incorporated at the synapse or they still reside at extrasynaptic sites.

Unlike vardenafil, administration of rolipram during the two mnemonic phases (i.e. acquisition and late consolidation) in which it exerts pro-cognitive function, did not affect total levels or trafficking of AMPARs. The distinguished function between vardenafil and rolipram should be related to their downstream effectors, which, in turn, impact on AMPAR trafficking. Although both inhibitors increased surface expression of GluA1-AMPARs 60 min after their administration, only for vardenafil this effect was still apparent after 24 h. This finding was particularly surprising for rolipram since several studies showed that activation of cAMP/PKA pathway promotes trafficking of AMPARs (22, 24, 41-43). Importantly, the majority of these studies has been conducted in *in vitro* or *ex vivo* systems and trafficking of AMPARs has been monitored for only a few hours after plasticity-inducing pharmacological treatments (24, 43). In our study the effect of treatment in AMPAR dynamics was examined 24 h after the mnemonic test. Thereafter, despite the initial effect of rolipram on AMPARs trafficking, another mechanism seems to be responsible for its long-term cognitive enhancing properties.

Upregulation of the cAMP pathway via the PDE4 inhibitor rolipram could also result in activation of the exchange protein activated by cAMP (Epac) (44). Epacs have a multifactorial role in plasticity, enhancing release of neurotransmitter and facilitating both LTP and long-term depression (LTD) (45-47). Interestingly, in cell cultures of rat cortical neurons, activation of Epac2 induced spine shrinkage by promoting endocytosis of GluA2/GluA3 AMPARs (48). Considering this finding, it is possible that activation of the cAMP pathway via rolipram leads to activation of several intracellular pathways that promote the initial trafficking of AMPARs to the synapse and, later on, their endocytosis into the cell. In turn, other receptors than AMPARs could be inserted into the synapse maintaining the increased synaptic size.

In conclusion, our study showed that upregulation of the cGMP/PKG or cAMP/PKA signalling cascades via the PDE5-specific inhibitor vardenafil and the PDE4-specific inhibitor rolipram causes immediate and versatile changes in GluA1-AMPAR dynamics. Additionally, administration of vardenafil at the acquisition and early consolidation phase of object location memory results in enhanced long-term memory that could be explained by increased surface

levels and trafficking of GluA1-and GluA2-AMPARs. Nevertheless, the long-lasting pro-cognitive effect of rolipram, when administered either at the acquisition or late consolidation phase, is not related to changes in AMPARs. Collectively, these results suggest that there is a differential underlying mechanism mediating the cognitive enhancing properties induced by upregulation of cyclic nucleotide signalling. Future studies are required to determine the molecular components of these pathways.

Acknowledgements

PRAH and BTJvH were financially supported by the Human Enhancement and Learning (HEaL) initiative of Maastricht University.

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Chapter 5

Synaptic and Memory Dysfunction Induced by Tau Oligomers is Rescued by Up-regulation of the Nitric Oxide Cascade

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Abstract

Soluble aggregates of oligomeric forms of tau protein (oTau) have been associated with impairment of synaptic plasticity and memory, therefore representing a critical hallmark in the etiopathogenesis of Alzheimer's disease. However, the molecular mechanisms underlying the synaptic and memory dysfunction induced by elevation of oTau are still unknown. Using a combination of biochemical, electrophysiological and behavioral techniques, phosphorylation of the cAMP-responsive element binding (CREB) protein, a transcriptional factor involved in memory, as well as long-term potentiation (LTP), a type of synaptic plasticity thought to underlie memory formation, and both short-term spatial and associative memory, were examined following oTau elevation following up-regulation of the nitric oxide (NO) cascade. Phospho-CREB was found to be reduced after oTau elevation during memory formation. This lead us to explore whether upregulation of various components of the NO signaling pathway impinging onto CREB is capable of rescuing oTau-induced impairment of plasticity, memory and CREB phosphorylation. Increased NO levels protected against oTau-induced impairment of LTP throughout the activation of soluble guanylyl cyclase and elevation of cGMP levels, which stimulate cGMP-dependent protein kinases (PKG). Pharmacological inhibition of cGMP degradation through inhibition of phosphodiesterase 5 rescued oTau-induced LTP reduction. Activation of PKG rescued oTau-induced LTP and memory impairments. Finally, elevation of cGMP levels re-established normal CREB phosphorylation after LTP induction in the presence of oTau. Thus, up-regulation of CREB activation through agents acting on the NO cascade might be beneficial against tau-induced synaptic and memory dysfunctions.

1.Introduction

An increased interest in Alzheimer's disease (AD) research is now directed towards tau protein, a hallmark of the disease. Insoluble aggregates of tau are responsible for the formation of neurofibrillary tangles (NFTs). However, growing evidence is pointing at very soluble small tau aggregates in the etiopathogenesis of the disease, as they emerge as more acutely toxic than large insoluble aggregates. Extracellular oligomeric forms of tau (oTau) have been shown to affect memory and its cellular correlate, long-term potentiation (LTP) (1, 2). However, despite the strong correlation between oTau and AD pathology (3, 4), the molecular mechanism by which tau protein induces synaptic dysfunction and memory impairment remains unidentified.

There is wide consensus that cyclic adenosine monophosphate (cAMP) responsive element binding (CREB) protein plays a key role in memory consolidation. Modification of CREB phosphorylation is a post-translational modification involved in gene transcription mechanisms leading to synaptic plasticity and memory formation (for a review see (5), and is likely to be affected in AD (6-23). CREB is at the crossroads of several molecular pathways and mechanisms that have been proposed as potential therapeutic targets against AD, including the nitric oxide (NO)/cyclic guanosine monophosphate (cGMP) dependent protein kinases (PKG)/CREB pathway, the cAMP dependent protein kinases (PKA)/CREB pathway and the mitogen-activated protein kinase/extracellular regulated kinase (MAPK/ERK) pathway (24). To this regard, the NO/cGMP/PKG/CREB cascade is particularly attractive because drugs boosting it, especially phosphodiesterase 5 (PDE5) inhibitors, are widely used for the therapy of erectile dysfunction and pulmonary hypertension (25), and it is therefore plausible that their administration is compatible with therapeutic usage.

NO, a gaseous molecule produced by the enzyme NO-synthase, is involved in various steps of brain physiology, from development to synaptic plasticity and memory (26-28). NO activates soluble guanylyl cyclase (sGC) which, in turn, produces cGMP (29), a cyclic nucleotide whose levels are also downregulated by PDE5, an enzyme that specifically hydrolyzes the nucleotide. Following its production, cGMP activates PKGs, a family of kinases that have been implicated in the modulation of neurotransmission, LTP and memory (30-33), and are capable of phosphorylating CREB. Intriguingly, proteomic and metabolomic studies have revealed a disrupted NO homeostasis in AD (34). Moreover, up-regulation of the NO cascade through drugs acting on its various molecular components has provided very promising results in studies aimed at finding strategies to counteract the damage of synaptic

plasticity and memory by oligomers of Aβ (35-37), another toxic protein in AD. Given that oTau share a common molecular mechanism with Aβ oligomers when they impair memory and LTP in mice (38), we investigated whether the oTau-induced damage of synaptic function and memory can be rescued via up-regulation of the various elements of the NO cascade.

2. Materials & Methods

2.1 Animals

All the experiments were performed using 3-4 month-old male and female C57BL/6 mice hosted at the Columbia University animal facility. The mice were maintained on a 12h light/dark cycle in stable conditions in terms of temperature, humidity and ventilation. Water and food were offered *ad libitum.* All animal experiments were approved by the ethical committee of Columbia University (IACUC #: AC-AAAO5301).

2.2 Preparation of recombinant tau

The tau 4R/2N construct was prepared in expression vector pET29a (Bioclone) in the bacterial strain BL21 (DE3) for protein expression, as previously described (1). Cells were streaked on LB agar ampicillin plates and a single colony was picked and grown overnight in a mixture of overexpression and expansion broth (Zymo Research). Cells were pelleted by centrifugation at 6000 g for 30 min in a GS3 rotator at 4 ºC. Cell lysates were lysed in a 2% Triton-X-100 phosphate-buffered saline with a protein inhibitor mixture. Streptomycin sulfate was added to precipitate DNA. After centrifugation the supernatant was heated at 100 ºC for 15 min and the precipitate was removed by centrifugation. After adding TCEP and 1% PCA, the pH of the supernatant was neutralized by using 1N NaOH. For the first purification step, the supernatant was transferred to a slide-A-lyser cassette (20 MWCO) and buffered exchanged to remove excess chemicals. Afterwards the supernatant was loaded on His-Spin Protein Miniprep columns (Zymo Research) and eluted with phosphate buffer containing 250 mM imidazole. For oligomerization, tau was transferred to a slide-A-lyser cassette and buffer exchanged with oligomerization buffer following incubation with 1 mM H_2O_2 at room temperature for 20 hours for introducing disulfide bonds. Tau protein concentration was determined from the absorption at 280 nm with an extinction coefficient of 7450 cm-1M-1 and oligomers were visualized through Western blotting. Oligomers were transferred in Tris-Acetate gels and then immunoblotted on nitrocellulose membrane. The primary antibody was diluted to a final concentration of 1:1000 for immunoblotting (anti-tau antibody; EP2456Y;

RabMad). The secondary goat anti-rabbit antibody (1:10000) was purchased from ThermoScientific.

2.3 CREB Western Blotting on mouse brain

Hippocampal lysates were prepared as previously described (8). Briefly, hippocampal tissue was homogenized in lysis buffer (62.5 mM Tris-HCl pH 6.8, 3% SDS) and incubated at 4 ºC for 10 min, then sonicated before centrifugation at 16000 rpm for 5 min. p-CREB antibodies were from Millipore, t-CREB antibodies were from Santa Cruz Biotech, and β-III-Tubulin antibodies were from Promega. Antibodies were used at a 1:1000 dilution. For quantitative immunoblot analysis, equal amounts of proteins were loaded into each lane. To confirm equal loading, blots were re-probed with corresponding pan-antibodies and antibody for tubulin. For quantification, we used a signal in the linear range. Immunoblot data were quantified by measuring the band intensity using imaging software (NIH ImageJ).

2.4 CREB Western Blotting on hippocampal slices

Western blot analysis was performed as previously described (39). Hippocampal slices were collected from the recording chamber 120 min after LTP induction, homogenized in RIPA buffer in presence of phosphatase and protease inhibitors, and sonicated 3 times for 10 min. Four tetanized slices for each experimental condition (vehicle, oTau, oTau + 7a) were used. Membranes were incubated overnight at 4 °C with rabbit anti-p-CREB(ser133) (Millipore, 1:1000) and mouse anti-GAPDH (Millipore, 1:5000), used as loading control. Protein detection was performed by using a secondary infrared fluorescent dye conjugated antibody absorbing at 800 (ThermoFisher, Goat anti-rabbit 800 nm; 1:10000) or 700 nm (Thermo fisher, Goat Anti-mouse 680 nm; 1:10000). Blots were visualized using an Odyssey Infrared Imaging Scanner (Li-Cor Science Tec, Milan, Italy) and quantified by densitometric analysis performed after normalization with loading controls by using imageJ software.

2.5 Drug preparation

For the LTP experiment, all the compounds were dissolved in artificial cerebrospinal fluid (ACSF) to achieve the final concentration required. DEA/NO, ODQ, BAY41-2272, and 8-Br-cGMP were diluted in 0.1% DMSO; sildenafil and compound 7a in 0.05% DMSO. DEA/NO was stored for 24 hours in alkaline solution (0.01 m NaOH) and diluted in ACSF immediately before use. For the behavioral experiments, compound 7a and 8-pCPT-cGMP were dissolved in 2% DMSO and 2% Tween. Compound 7a was synthesized in six steps (36), while DEA/NO and BAY41-2272 were purchased from Enzo life Science (Farmingdale, NY, USA), 8-Br-cGMP from Biolog Life Science Institute (Bremen, Germany), ODQ from Cayman

Chemical (Ann Arbor, MI, USA), sildenafil and 8-pCPT-cGMP from Sigma-Aldrich (St. Louis, MO, USA).

2.6 Electrophysiological recordings

Mice were sacrificed through cervical dislocation and hippocampus was removed immediately after decapitation. Transverse hippocampal slices (400 μm) were cut on a tissue chopper and transferred to the recording chamber where the physiological conditions in the brain were maintained by perfusion of ACSF continuously bubbled with 95% O_2 and 5% CO_2. The ACSF consisted of (in mM): NaCl (124.0), KCl (4.4), Na_2HPO_4 (1.0), $NaHCO_3$ (25.0), $CaCl_2$ (2.0), $MgCl_2$ (2.0), and glucose (10.0). Slices were allowed to recover for at least 90 min before commencing the extracellular field recordings. A bipolar tungsten electrode and a glass electrode filled with ACSF were placed in the Schaeffer collateral fibers and the CA1 stratum radiatum, respectively. An input-output analysis was utilized in order to determine the maximal slope and the baseline was recorded every minute at approximately 35% of the maximum evoked slope (40). After establishing a 30 min stable baseline, LTP was induced using a theta-burst stimulation and was recorded for 2 hours after tetanization. LTP was measured as field-EPSP (fEPSP) slope expressed as percentage of the baseline and the results were represented as mean ± SEM.

2.7 Stereotaxic surgery and infusion method

Before fixing the mice in the stereotaxic device, anesthesia was induced by intraperitoneal injection with Avertin (500 mg/Kg). Animals were injected with analgesic (Carprofen subcutaneously on the back, 5 mg/Kg) and local anesthetic (Marcaine subcutaneously under the scalp 3 mg/Kg). A midline incision was made in the skull and the underlying area was cleared of tissue by using H_2O_2. The coordinates of the dorsal hippocampus were 2.46 mm posteriorly and 1.5 mm laterally from Bregma to a depth of 1.30 mm (41). A 26-gauge guide cannulas (PlasticOnes) was fixed to the skull using acrylic dental cement (Paladur). After 6-9 days of recuperation period, the mice were injected bilaterally with oTau (500 nM) or vehicle to a final volume of 1 μl. For the injections, we utilized Hamilton syringes connected with a polyethylene tube at the end of which an internal microsyringe was fixed. For all the behavioral tests, mice were injected intrahippocampally, twice at 3 hours and 20 min prior to the task. After the infusion, the needle was held in place for 1 min to ensure complete diffusion.

2.8 Spatial short-term memory testing with radial arm water maze (RAWM)

The RAWM consists of a white circular pool, 120 cm in diameter, filled with non-toxic white paint to make the water opaque. Within the pool there is an apparatus consisting of six arms radiating from the central area, forming six arms. Spatial cues were present on the walls of the room. Throughout the test, the water temperature was maintained stable at 24 ± 2 °C. The platform was positioned at the end of one of the arms, submerged in the water. The location of the platform (10 cm diameter) was kept constant for each mouse, while the starting position differed between the trials. The test took place for two consecutive days, and each mouse underwent 15 trials per day. On the first day, mice were trained for 15 trials, with the first 12 trials alternating between visible (platform flagged) and hidden (platform 1 cm beneath the water surface). The last 3 trials of the first day and all the 15 trials of the second day were done with hidden platform. In each trial, the mouse was allowed to swim freely for 60 sec in the maze to find the platform. Once on the platform, the mouse was allowed to rest for 20 sec and to observe the visual cues. If a mouse was unable to find the platform within 60 sec, the experimenter guides it towards the platform for the 20 sec stay. During the 1 min trial, each time the mouse entered an arm other than the goal arm (in which the platform was located) or if the mouse did not take any decision regarding which arm to explore within 10 sec, an error was registered. Entry into an arm was defined as the entry of all the four paws of the mouse into the particular arm. After completing each trial, the mouse was removed from the pool, gently towel dried and placed back into its cage under a heat lamp. To avoid the learning limitations imposed by over practice and to avoid fatigue that may result from consecutive trials spaced practice training was established by running the mice in cohorts of 4 or 5 and alternating different cohorts through the 15 trials over the testing period each day. The result is shown by dividing the 30 trials into 10 blocks. Each block represents the error average of 3 consecutive trials. Both days mice were injected with oTau intrahippocampally. Treatment was given after the second and the forth block of trials each day.

2.9 Visible platform testing

The test has been utilized for the assessment of visual and/or motor, and/or motivational deficits (37). It was performed in the same pool with the RAWM. The test takes place in 2 consecutive days and mice underwent 2 sets of trials each day. Every set consisted of 3 trials in which the mouse trained to find the visible escaping platform, flagged with a bottle cup on the top. During one set of trials the platform was located in one of the three quadrants of the pool. The starting position of the mouse was kept constant for a specific position of the

platform. The mice were placed gently on the water, facing the walls and each trial lasted until the mouse had found the platform until the maximum time of 60 min. After the end of the trials mice were guided to the platform and allowed to observe environmental cues for 20 sec. Time between entering the pool and reaching the platform (latency) and velocity were analyzed by using a video tracking system (Ethovision XT). The results were shown in 4 blocks and each block represents the average of one set of experiment. oTau was infused both days. Treatment was administered after the first set of three trials each day.

2.10 Fear conditioning

The fear conditioning test was used for evaluating associative fear memory in rodents. The test consists in total of 3 days, in which the first day the animals are placed in the fear conditioning chamber (Noldus) for 2 min before the presentation of the conditional stimulus (tone; 2880 Hz at 85 Db). In the last 2 sec of the tone mice received the unconditional stimulus (foot shock; 0.8 mA). After the pairing of the 2 stimuli, mice were left in the chamber for another 30 sec in the absence of stimulus. The second day mice were returned to the same conditioning chamber for another 5 min without the presence of tone or shock. Freezing behavior, distinguished by the absence of movement except breathing, was monitored during the test using a vision tracking and analysis system (Ethovision XT, Noldus). The third day, the cued fear memory was evaluated. For that, mice were placed in the same chamber with modified walls, floor and vanilla odor, which represents a novel context. In the course of 5 min, 2 min of freely exploration was followed by exposure for 3 min in the conditional stimulus. For the fear conditioning, the mice were injected with the drug following the above regimen only the first day of the behavioral experiment. Treatment was given immediately after the foot shock in order to examine the effect of the treatment in the consolidation of the contextual fear memory. Additionally, administration of the treatment after the foot shock excludes the possibility of interference with the perception of the pain.

2.11 Open field

The test has been used for assessing the exploratory behavior and anxiety levels (42). Mice were placed in a novel open environment consisting of Plexiglass transparent walls (model ENV- 520; Med Associates, St. Albans, Vermont) (43.2 cm long x 43.2 cm width x 30.5 cm high). Mice were placed in the open field. Their activity was automatically recorded for 10 min, on two consecutive days. oTau was infused both days. Mice were treated with the compound each day after the end of the test.

2.12 Sensory threshold assessment

The test was used for evaluating animal perception of the shock. The test was performed the last day of experiments, and the animals were placed in the same chamber that the fear conditioning test took place. Animals subjected to 1 sec foot shocks of increasing intensity from 0.1 to 0.7mA at 0.1 mA increments every 30 sec. Behavior was recorded by video capture software (Ethovision XT) and was evaluated manually. The graphs represent the average of the foot shock intensity that elicited the first visible response (flinching), the second motor response (jumping), and the first audible response (vocalization). Mice were injected with oTau prior to the experiment as described before.

2.13 Statistical Analysis

For electrophysiological recordings the whole trace was analyzed by two-way ANOVA for repeated measures comparing traces after tetanic stimulation with treatment condition as main effect. Additionally, the last 5 points of the trace were analyzed by one-way ANOVA followed by Bonferroni post-hoc comparisons. For the behavioral tests, animals were run in cohorts in which sex of mice was kept balanced across groups. Results were analyzed with ANOVA for repeated measures (RAWM errors and latency) or one-way ANOVA with Bonferroni post-hoc correction. For western blotting, conditions were compared by using unpaired t-test or one-way ANOVA. Statistical analysis was performed by using Systat 9 software (Chicago, IL, USA). Differences were considered significant at a p value less than 0.05. Results were expressed as Standard Error of the Mean (SEM).

3. Results

3.1 oTau affects molecular mechanisms underlying memory formation

Considering the profound effect that oTau exposure has on synaptic plasticity and memory (1, 2), we decided to determine whether the molecular mechanisms underlying memory formation, including CREB phosphorylation, are affected by oTau. Foot-shock, a stimulus that is normally used for training in fear conditioning tests, increased phosphorylation of the memory-related molecule CREB after 60 min (Figure 1A-B). However, the presence of oTau (22.95 µg/ml, two intrahippocampal injections at 180 min and 20 min prior to applying the electric shock) reduced the levels of phosphorylated CREB (pCREB) compared to vehicle-treated control mice (Figure 1C-D). Tau did not produce a modification of phosphorylated CREB without foot-shock stimulation (data not shown). Taken together, these data demonstrated that, similar to extracellular Aβ oligomers (7),

molecular mechanisms involved in gene transcription and memory are inhibited by extracellular oTau.

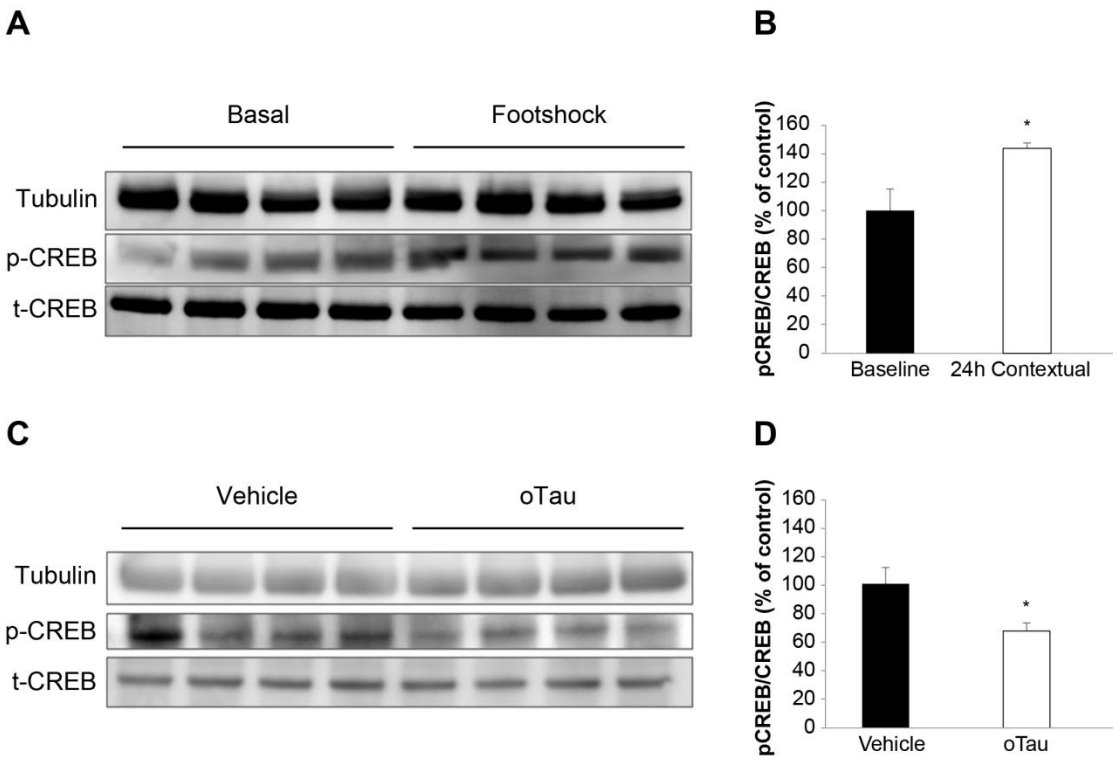


Figure 1. oTau impairs CREB phosphorylation during memory formation. A) Immunoblots of mouse hippocampal homogenates of mice previously treated with/without a foot-shock to induce fear memory. Hippocampi were harvested 1 hr after the foot-shock. **B**) Average ratio of p-CREB/t-CREB for experiments shown on panel A (unpaired t-test: $t_{(6)} = 2.807$; $p = 0.031$; $n = 4$ per each group). **C)** Immunoblots of mouse hippocampal homogenates of mice treated with vehicle (Veh) or oTau (22.95 µg/ml). **D)** Average ratio of p-CREB/t-CREB for experiments shown on panel C (unpaired t-test: $t_{(6)} = 2.541$; $p = 0.044$; $n = 4$ per each group). * $p < 0.05$.

3.2 Increase in NO levels through the NO donor, DEA/NO, protects against oTau-induced impairment of LTP

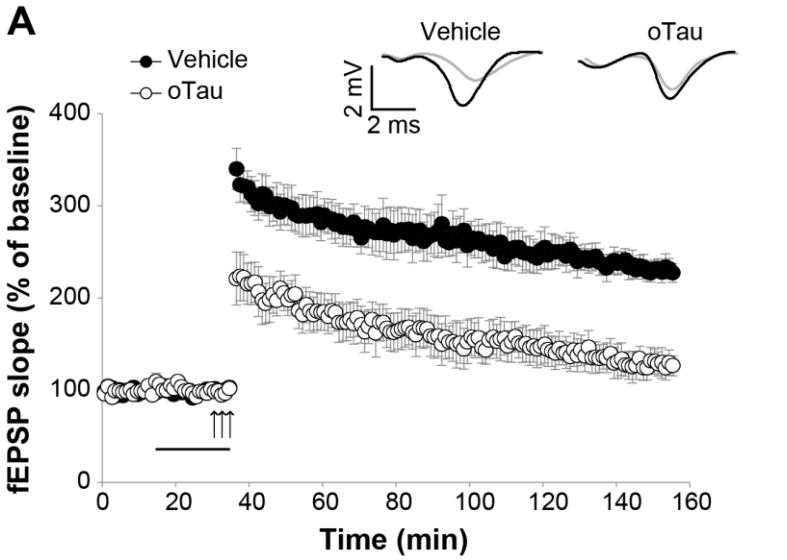
Given that CREB phosphorylation can be enhanced through up-regulation of the NO cascade, we determined whether an increase in NO levels is capable of counteracting the oTau-induced defect in the memory electrophysiological surrogate, LTP (1, 2). As previously shown (1), LTP was suppressed in hippocampal slices exposed to oTau (100 nM) for 20 min prior to tetanization compared to vehicle-treated slices (Figure 2A). However, a brief perfusion of 5 min with the NO donor, 2-(*N*,*N*-dethylamino)-diazenolate-2-oxide diethylammonium salt (DEA/NO), at a concentration of 3 µM was sufficient to rescue the oTau-induced LTP suppression (Figure 2B). Moreover, DEA/NO alone did not enhance LTP *per se*, nor affected basal neurotransmission during perfusion (Figure 2B). These slices showed similar levels of potentiation as tetanized slices treated with DEA/NO paired with

oTau or slices treated with vehicle (Figure 2C). Taken together, these results demonstrate that NO protects against oTau-induced inhibition of LTP.

3.3 sGC is involved in the beneficial effect of NO elevation against oTau-induced impairment of LTP

NO is a signaling molecule that binds to and stimulates sGC, among other substrates (43). sGC, in turn, catalyzes the conversion of GTP in cGMP. To investigate whether cGMP production is needed by DEA/NO to rescue oTau-induced impairment of LTP, we treated hippocampal slices with 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), an irreversible inhibitor of NO-sensitive sGC (44). Perfusion with 10 μM ODQ for 10 min prior to tetanization prevented the protective effect induced by DEA/NO paired with 100 nM oTau for 20 min (Figure 3A). Moreover, ODQ alone reduced LTP to levels equal to those obtained with oTau without affecting baseline transmission during perfusion (Figure 3A).

The block of the positive outcome of the administration of DEA/NO against oTau-induced damage of LTP by ODQ is consistent with the interpretation that sGC is in the pathway of the NO beneficial effect. However, alternative explanations are also possible including the possibility that ODQ might have acted by simply disrupting the physiological mechanisms needed to support LTP. Thus, to directly define whether activation of sGC at the downstream level of NO can be beneficial against oTau-induced damage of synaptic plasticity, in interleaved experiments with those shown on Figure 3A, we perfused hippocampal slices with the sGC stimulator 3-(4-amino-5-cyclopropylpyrimidine-2-yl)-1-(2-fluorobenzyl)-1H-pyrazolo[3,4-b]pyridine (BAY41-2272), targeting different sGC isoforms without affecting PDE activity (45). When BAY41-2272 (100 nM, 10 min) was paired with oTau (100 nM, 20 min), LTP reduction caused by oTau was no longer present (Figure 3B). BAY41-2272 (100 μM) alone, 10 min prior tetanization, did not modify the amount of potentiation, nor affected baseline transmission during perfusion (Figure 3B). No significant difference was found between tetanized slices treated with BAY41-2272 paired with oTau compared with slices treated with BAY41-2272 alone (Figure 3C). Altogether, these findings indicate that stimulation of sGC might play a beneficial role against oTau-induced LTP reduction.


A
Vehicle
oTau
Vehicle
oTau
2 mV
2 ms
fEPSP slope (% of baseline)
400
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20
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60
80
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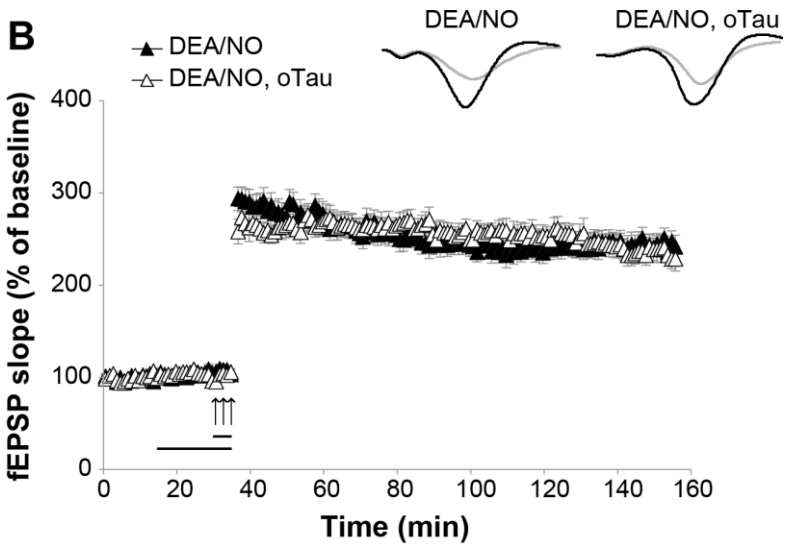
120
140
160
Time (min)


B
DEA/NO
DEA/NO, oTau
DEA/NO
DEA/NO, oTau
fEPSP slope (% of baseline)
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Time (min)

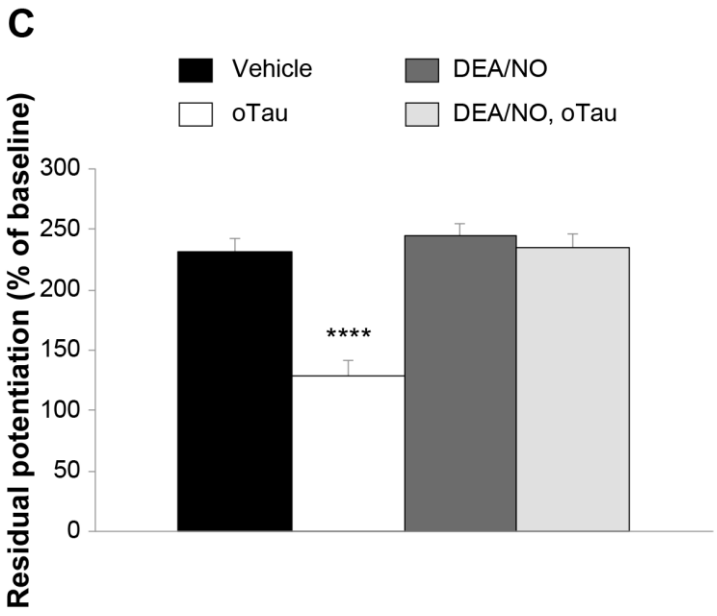
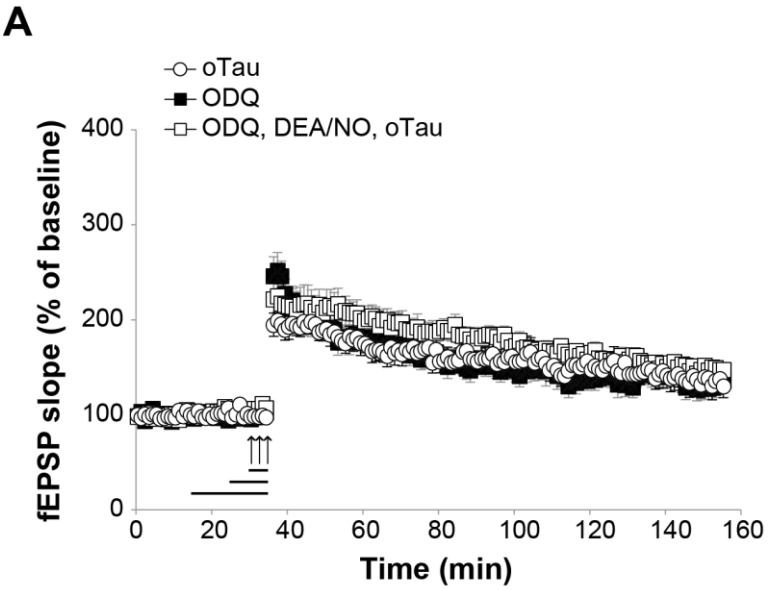

C
Vehicle
oTau
DEA/NO
DEA/NO, oTau
Residual potentiation (% of baseline)
300
250
200
150
100
50
0
****

Figure 2. oTau induced LTP impairment was rescued by the NO donor DEA/NO. A) LTP was impaired in hippocampal slices from WT mice perfused for 20 min before tetanus with oTau (100 nM) compared to vehicle-treated slices (n = 8/7; ANOVA for repeated measures: $F_{(1,13)} = 18.502$, $p = 0.001$). Upper panel: representative traces of fEPSP before (gray) and after tetanus (black) here and in B. **B)** A perfusion with DEA/NO (3 μM, 5 min) rescued the impairment of LTP in slices concomitantly treated with oTau (100 nM, 20 min) in experiments that were interleaved with those shown in A (n=12; ANOVA for repeated measures: $F_{(1,18)} = 29.129$, $p < 0.0001$ comparing tetanized slices treated with oTau + DEA/NO vs. oTau). No differences were found between tetanized slices treated with DEA/NO alone (n = 12) vs. DEA/NO + oTau ($F_{(1,22)} = 0.016$, $p = 0.9$ and DEA/NO alone did not modify potentiation (DEA/NO vs. vehicle: $F_{(1,17)} = 0.374$, $p = 0.549$). **C)** Quantification of the residual potentiation of the last 5 min recording from LTP data shown in A and B. One-way ANOVA among all: $F_{(3,35)} = 18.965$, $p < 0.0001$; Bonferroni's: $p < 0.0001$ between oTau and other experimental conditions. The horizontal bars indicate the period during which drugs were added to the bath solution, and arrows indicate tetanus delivery here and in the following figures. **** $p < 0.0001$.

3.4 Elevation of cGMP levels protects against oTau-induced LTP impairment

sGC is known to mediate LTP and memory formation through elevation of intracellular cGMP levels (43). Thus, we hypothesized that the administration of cGMP analogs might protect against oTau-induced impairment of LTP. We used 8-Br-cGMP, a permeable cGMP analog that specifically activates PKG at low concentrations (K_a, 0.01-0.21 μM for PKG, 12 μM for PKA) (46, 47). When hippocampal slices were perfused with oTau (100 nM, 20 min) paired with 8-Br-cGMP for 10 min (1 μM) before tetanus, LTP was no longer reduced (Figure 4A-B). Furthermore, the protection was not caused by an effect of 8-Br-cGMP on LTP, because perfusion with the analog alone did not enhance the amounts of potentiation (Figure 4A-B). Finally, 8-Br-cGMP alone did not affect baseline transmission during perfusion (Figure 4A-B).

cGMP level is maintained through a balance between its production, catalyzed by sGC, and its degradation, catalyzed by PDEs. Therefore, another strategy to increase cGMP level is to use PDE inhibitors. Specifically, we used two inhibitors of PDE5, sildenafil and compound 7a. Sildenafil is a well-known and extensively studied PDE5 inhibitor with an IC_{50} of 6.0 nM and *in vivo* half-life of 0.4 hours in rodents (~4 hours in humans) (48, 49). However, the selectivity ratio for PDE1 and PDE6 is 180 and 12, respectively (50). Compound 7a possesses higher selectivity for PDE5 (PDE5/PDE6 >1000) with an IC_{50} of 0.27 nM and plasma half-life of 1.33 hours in rodents (36). We found that a 10 min perfusion with sildenafil (50 nM) in the presence of oTau counteracted the LTP reduction (Figure 4C-D). Similarly, a 10 min perfusion with 7a (50 nM) rescued the LTP defect in slices concomitantly perfused with oTau (Figure 4C-D).


A
oTau
ODQ
ODQ, DEA/NO, oTau
fEPSP slope (% of baseline)
400
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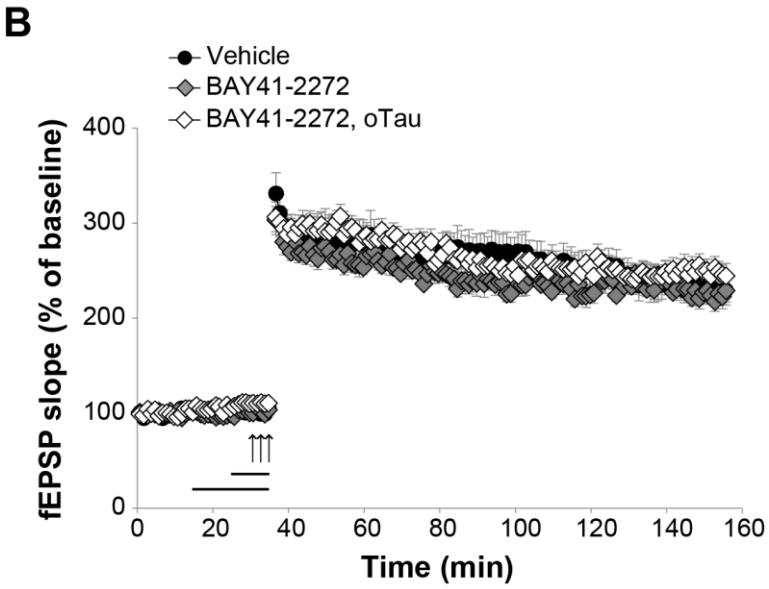
Time (min)


B
Vehicle
BAY41-2272
BAY41-2272, oTau
fEPSP slope (% of baseline)
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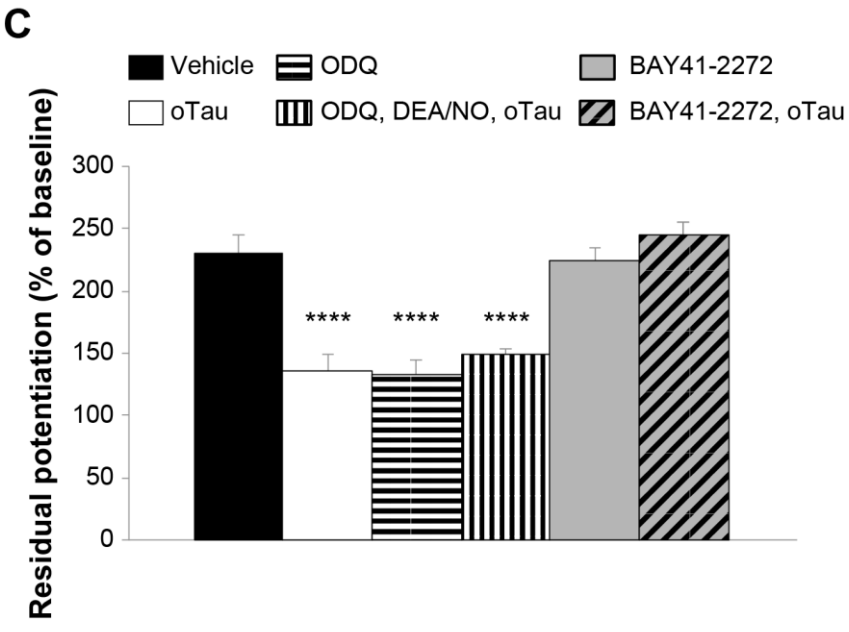
Time (min)


C
Vehicle
ODQ
BAY41-2272
oTau
ODQ, DEA/NO, oTau
BAY41-2272, oTau
Residual potentiation (% of baseline)
300
250
200
150
100
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0
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Figure 3. sGC activation protects against the detrimental effect of oTau onto LTP. A) The sGC inhibitor ODQ (10 μM, 10 min) blocks the rescue of oTau (100 nM, 20 min) induced LTP reduction by DEA/NO (3 μM, 5 min). Potentiation in slices treated with ODQ + DEA/NO + oTau was similar to those treated with ODQ alone, or oTau alone (ODQ + DEA/NO + oTau: n = 13; ODQ alone: n = 9; oTau alone n = 8; ANOVA for repeated measures: $F_{(1,20)} = 2.346$, p = 0.141 comparing ODQ + DEA/NO + oTau vs. ODQ; $F_{(1,15)} = 0.004$, p = 0.954 vs. oTau). **B)** Perfusion with BAY41-2272 (100 μM, 10 min) rescued the impairment of LTP in slices concomitantly treated with oTau (100 nM, 20 min) in experiments interleaved with those shown in A (BAY41-2272 + oTau: n = 11; ANOVA for repeated measures: $F_{(1,17)} = 78.187$, p < 0.0001 comparing BAY41-2272 + oTau vs. oTau). BAY41-2272 alone did not modify potentiation (BAY41-2272 alone: n = 11, vs. vehicle: n = 7; $F_{(1,16)} = 1.089$, p = 0.312). No significant differences were found between tetanized slices treated with BAY41-2272 + oTau or BAY41-2272 ($F_{(1,20)} = 4.274$, p = 0.052 comparing BAY41-2272 + oTau with BAY41-2272). **C)** Quantification of the residual potentiation from LTP curves shown in A and B. One-way ANOVA among all: $F_{(5,53)} = 25.571$, p < 0.0001; Bonferroni's: p < 0.0001 between oTau or ODQ or ODQ + DEA/NO + oTau and other experimental conditions. **** p < 0.0001.

This phenomenon could not be attributed to an effect of PDE5 inhibition on LTP *per se*, since perfusion with sildenafil or 7a alone did not affect the amount of potentiation, nor to an effect on basal neurotransmission since the two inhibitors did not affect basal synaptic transmission during perfusion (Figure 4C-D). Altogether, these experiments suggest that elevation of cGMP levels protects against oTau-induced inhibition of LTP.

3.5 PKG activation rescues oTau-induced LTP impairment.

PKG is activated by cGMP. We therefore used the specific PKG activator, 8-pCPT-cGMP, to determine whether activation of the kinase protects against oTau-induced impairment of LTP. This compound has higher lipophilicity and membrane permeability than 8-Br-cGMP, and is selective for activation of the two isoforms of PKG, PKGI (K_a of 0.05 μM) and PKGII (K_a of 0.0035-0.08 μM) compared with other cGMP targets such as PDEs (51). Initially, we confirmed that 20 min perfusion with 100 nM oTau blocked LTP (Figure 5A-B). Additionally, 10 min perfusion with 8-pCPT-cGMP (1 μM) before potentiation, in the presence of oTau, abolished LTP suppression. The phenomenon could not be attributed to an effect of PKG activation on LTP *per se*, since perfusion with 8-pCPT-cGMP alone did not affect the amount of potentiation (Figure 5A-B), nor to an effect on basal neurotransmission since the activator did not affect basal synaptic transmission during perfusion (Figure 5A-B). Altogether, these experiments suggest that PKG activation protects against oTau-induced inhibition of LTP.

3.6 Elevation of cGMP levels and activation of PKG rescue memory impairment in mice injected with oTau.

Because the LTP experiments indicate that up-regulation of the NO cascade ameliorates oTau-induced reduction of the memory surrogate LTP, we aimed to extrapolate these findings to memory by using compound 7a and 8-pCPT-cGMP to elevate cGMP levels

and activate PKG. At first, we examined spatial working memory through the 2-day radial-arm water maze (RAWM). The task requires short-term reference memory (52). Based on previous studies (1), oTau (22.95 µg/ml) was administrated through intra-hippocampal cannulas 180 and 20 min before training.

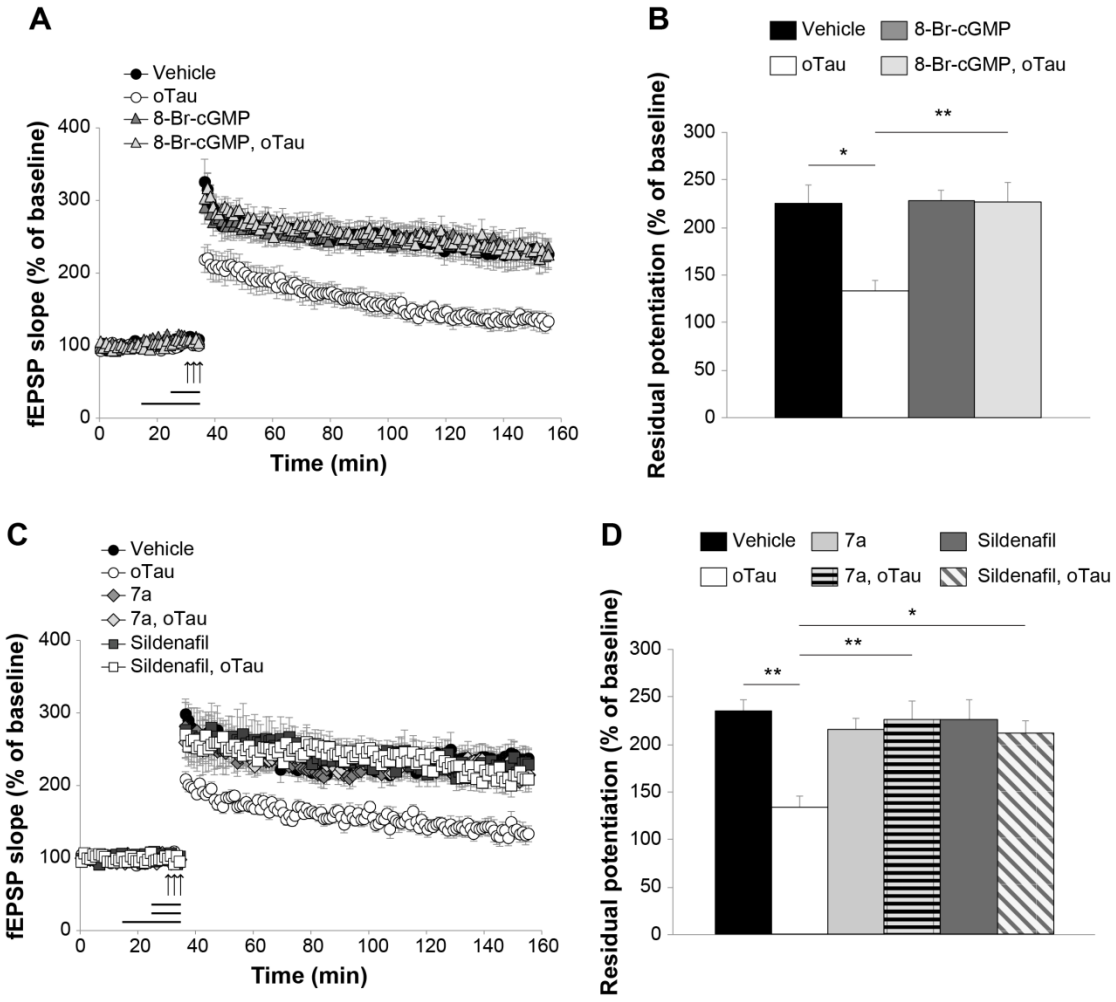

Figure 4: cGMP elevation protects against oTau-induced LTP impairment. A) Slice perfusion with 8-Br-cGMP (1 µM, 10 min, n = 13) rescued oTau-induced LTP impairment (100 nM, 20 min, n = 8; ANOVA for repeated measures: $F_{(1,19)} = 18.537$, $p < 0.0001$). 8-Br-cGMP alone did not modify potentiation (n = 13 vs. 7 in vehicle-treated slices; $F_{(1,18)} = 0.001$, p = 0.974 compared to vehicle). No difference was found between tetanized slices treated with 8-Br-cGMP vs. 8-Br-cGMP + oTau ($F_{(1, 24)} = 0.065$, p = 0.802). **B)** Residual potentiation from data shown in A. One-way ANOVA: $F_{(3,37)} = 6.670$, p = 0.001; Bonferroni's: p = 0.011 between oTau and vehicle; p = 0.002 between oTau and 8-Br-cGMP + oTau. **C)** Perfusion with compound 7a (50 nM) or sildenafil (50 nM) for 10 min rescued the LTP impairment in oTau-treated slices (7a + oTau: n = 10; sildenafil + oTau: n = 11; oTau: n = 10; ANOVA for repeated measures: $F_{(1,18)} = 20.747$, $p < 0.0001$ and $F_{(1,19)} = 34.688$, $p < 0.0001$ compared with oTau-treated slices, respectively). 7a or sildenafil alone did not modify potentiation (7a: n = 11; sildenafil: n = 11; vehicle: n = 9; $F_{(1,18)} = 0.156$, p = 0.697 and $F_{(1,18)} = 0.016$, p = 0.900). No significant differences were found between tetanized slices treated with 7a vs. 7a + oTau ($F_{(1,19)} = 0.060$, p = 0.809) and between tetanized slices treated with sildenafil vs. sildenafil + oTau ($F_{(1,20)} = 0.013$, p = 0.910). **D)** Residual potentiation from data shown in C. One-way ANOVA: $F_{(5,56)} = 5.673$, $p < 0.0001$; Bonferroni's: p = 0.001 between oTau and vehicle; p = 0.002 between oTau and 7a + oTau; p = 0.01 between oTau and sildenafil + oTau. * $p < 0.05$, ** $p < 0.005$.

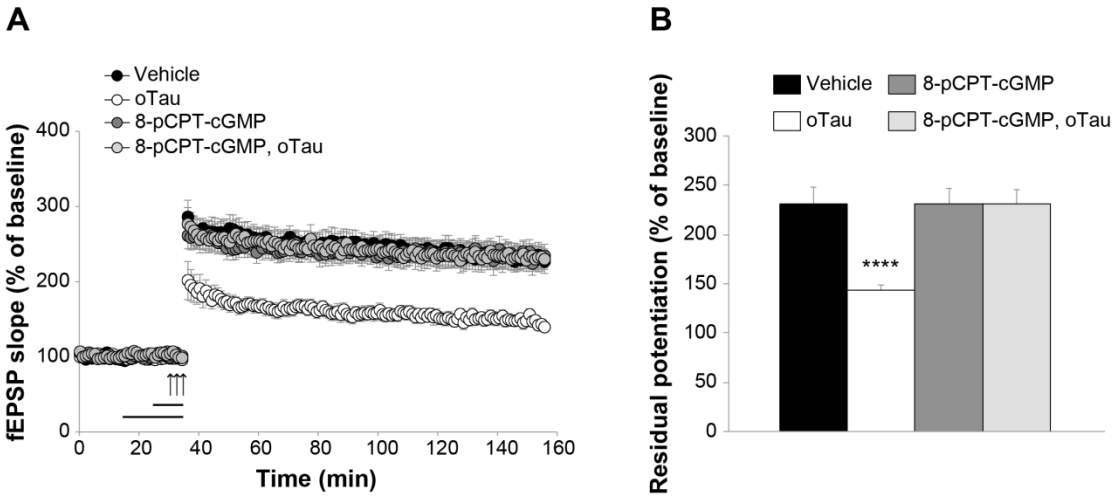

Figure 5. PKG activation counteracts LTP impairment in oTau-treated slices. A) Perfusion for 10 min with 8-pCPT-cGMP (1 μM) before LTP induction rescued the oTau-induced LTP reduction (n = 10; ANOVA for repeated measures: $F_{(1,19)} = 24.037$, $p < 0.0001$ compared with slices perfused with oTau). Perfusion with oTau 20 min prior to tetanization diminished LTP (oTau: n = 10; vehicle: n = 11; $F_{(1,19)} = 26.310$, $p = 0.0001$ comparing oTau vs. vehicle) while 8-pCPT-cGMP alone did not affect potentiation (n = 11; $F_{(1,20)} = 0.100$, $p = 0.755$, compared with vehicle). **B)** Quantification of the residual potentiation of the last 5 min recording from LTP curves shown in A. One-way ANOVA among all: $F_{(3,38)} = 9.964$, $p < 0.0001$; Bonferroni's: $p < 0.0001$ between oTau and other experimental conditions. **** $p < 0.0001$.

Compound 7a (3 mg/Kg) and 8-pCPT-cGMP (40 μg/Kg) were given i.p. after the 2nd, 4th, 7th and 9th block of trials. We first confirmed that administration of oTau significantly reduced spatial working memory (Figure 6A). Administration of compound 7a or 8-pCPT-cGMP reversed cognitive decline induced by oTau, since mice performance resembled that of the vehicle-treated mice (Figure 6A). Additionally, administration of compound 7a or 8-pCPT-cGMP alone did not improve memory performance in vehicle-treated animals (Figure 6A). Control experiments with the visible platform test excluded that the outcome of these experiments was influenced by an effect on visual, motor and motivational skills, since the different groups showed similar swimming speed or time to find the visible platform (Figure 6B, C). Thus, PKG activation is beneficial against oTau-induced impairment of spatial working memory.

Next, we examined the effect of compound 7a and 8-pCPT-cGMP on contextual fear memory. This task depends upon hippocampus and amygdala function (53) and assesses associative memory, a type of memory that is affected in AD patients (54). We used the same concentration of oTau and drugs as for RAWM experiments. Mice were injected with oTau (180 min and 20 min prior to the training session on the first day), and 7a or 8-pCPT-cGMP immediately after training. There was no significant difference between groups during baseline recording (Figure 6D).

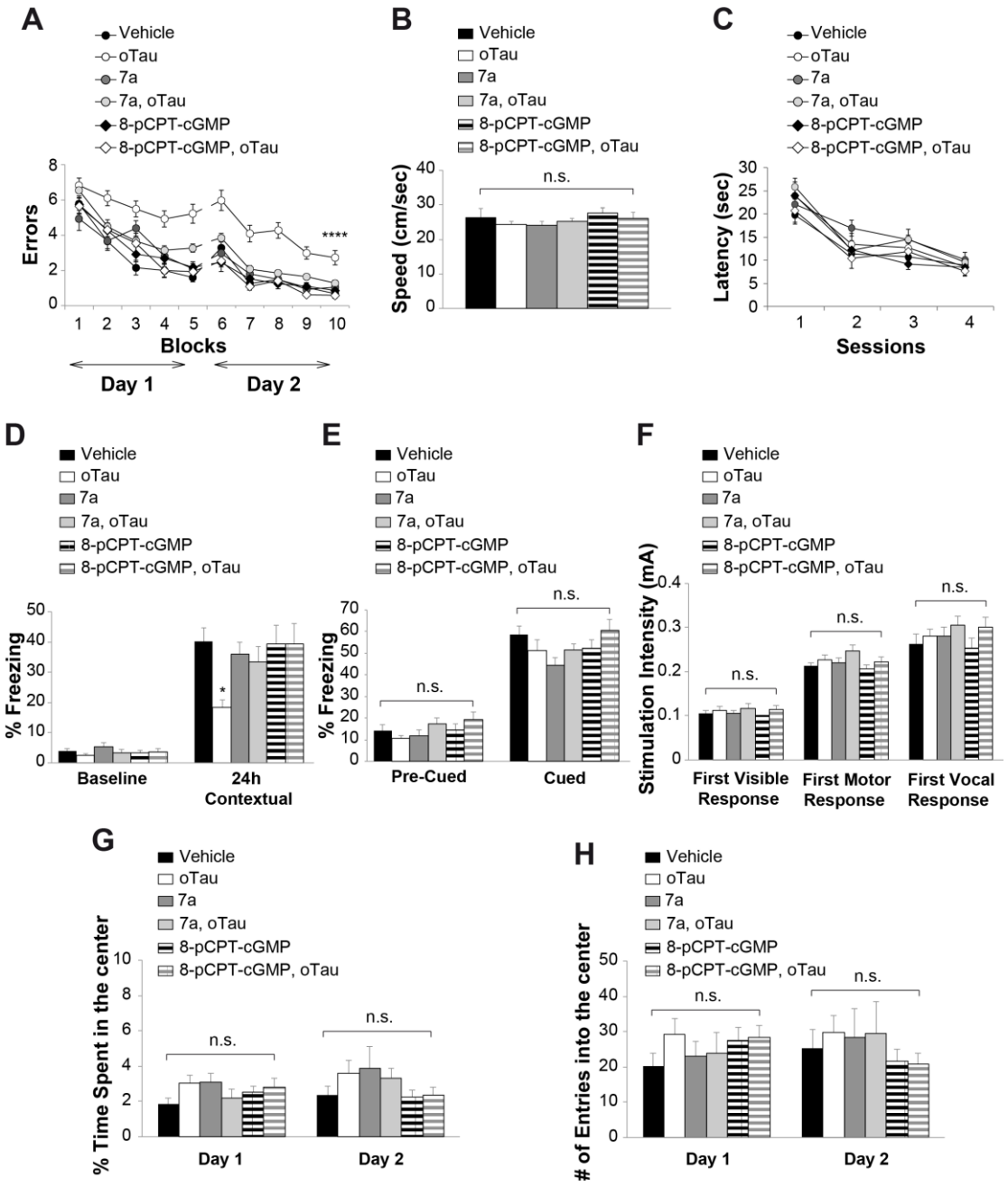

Figure 6. cGMP elevation ameliorates oTau-induced memory impairment. A) Administration of the PDE5 inhibitor 7a (3 mg/kg, i.p.) or the PKG activator 8-pCPT-cGMP (40 µg/Kg, i.p.) protected against the impairment of RAWM performance induced by oTau (22.95 µg/ml). ANOVA for repeated measures among all (day 2): $F_{(5,83)} = 17.973$, $p < 0.0001$. One-way ANOVA for block 10: $F_{(5,83)} = 9.016$, $p < 0.0001$; Bonferroni's $p < 0.0001$ oTau vs. vehicle or oTau+8-pCPT-cGMP and $p = 0.003$ vs. oTau+7a. 7a or 8-pCPT-cGMP alone do not modify memory (Bonferroni's $p = 1$ compared to vehicle for block 10). Vehicle: n = 15, oTau: n = 17, oTau+7a: n = 16, oTau+8-pCPT-cGMP: n = 14, 7a: n = 14, 8-pCPT-cGMP: n = 13. **B-C)** Testing with the visible platform task for assessment of visual-motor-motivational deficits for animals shown in A did not reveal any difference in average speed (ANOVA: $F_{(5,83)} = 0.570$, $p = 0.723$) (B) and time to reach the visible platform (ANOVA for repeated measures: $F_{(5,83)} = 1.243$, $p = 0.297$) (C) among the six groups. **D)** Administration of the 7a (3 mg/kg) or 8-pCPT-cGMP (40 µg/Kg) protected against the impairment of contextual memory induced by oTau (22.95 µg/ml), without modifying memory *per se* [24 hours: ANOVA $F_{(5,83)} = 2.699$, $p = 0.026$; Bonferroni: vehicle vs. oTau: $p = 0.036$; vehicle vs. oTau+7a or oTau+8-pCPT-cGMP or 7a or 8-pCPT-cGMP: $p = 1$]. No differences

were detected during baseline assessment (ANOVA among all: $F_{(5,83)} = 0.978$, $p = 0.436$). Vehicle: n = 16, oTau: n = 14, oTau+7a: n = 17, oTau+8-pCPT-cGMP: n = 14, 7a: n = 15, 8-pCPT-cGMP: n = 13. **E)** Freezing responses before (Pre) and after (Post) the auditory cue were the same among the six groups shown in D in the cued conditioning test. ANOVA: Pre-cued: $F_{(5,83)} = 1.223$, $p = 0.306$; Cued: $F_{(5,83)} = 2.010$, $p = 0.086$. **F)** No difference was detected among the groups shown in D during assessment of the sensory threshold. ANOVA among all: for visible response $F_{(5,83)} = 0.683$, $p = 0.637$; for motor response $F_{(5,83)} = 1.756$, $p = 0.131$ and for audible response $F_{(5,83)} = 0.933$, $p = 0.464$. **G-H)** Open field testing showed a similar percentage of time spent in the center compartment ($F_{(5,83)} = 7.037$, $p = 0.407$) (G) and the number of entries into the center compartment ($F_{(5,81)} = 0.297$, $p = 0.850$) (H) among all conditions at day 2, indicating no differences in exploratory behavior. Vehicle: n = 15, oTau: n = 16, oTau+7a: n = 17, oTau+8-pCPT-cGMP: n = 14, 7a: n = 12, 8-pCPT-cGMP: n = 13.

However, as previously demonstrated (1, 38), administration of oTau interfered with memory formation, since mice did not remember the context in which they received the foot shock 24 hours after training (Figure 6D). Administration of compound 7a or 8-pCPT-cGMP rescued contextual memory impairment caused by oTau (Figure 6D). Compound 7a or 8-pCPT-cGMP did not modify memory *per se*, since freezing did not significantly differ in animals treated with vehicle or compound 7a or 8-pCPT-cGMP alone (Figure 6D). We also examined cued fear conditioning, which is an amygdala dependent and hippocampus-independent task (53), without finding differences between groups before or after the cued stimulus (Figure 6E). Control test showed that different treatments did not change perception of pain, as determined through sensory threshold assessment (Figure 6F). Finally, administration of oTau, 7a, or 8-pCPT-cGMP did not affect exploratory activity, locomotor function and anxiety, assessed by the open field test (Figure 6G, H). Altogether, these findings indicate that upregulation of the NO cascade could ameliorate oTau-induced memory loss.

3.7 Elevation of cGMP levels rescues oTau-induced impairment of CREB phosphorylation induced by tetanic stimulation.

To determine whether the beneficial effects of upregulation of the NO cascade onto memory formation occurs through a rescue of the reduction in pCREB following oTau exposure, we evaluated the effect of elevation of cGMP levels onto pCREB in the presence of the PDE5 inhibitor compound 7a. Hippocampal slices were treated as described in the electrophysiological experiments and stored 120 min after treatment to analyze pCREB expression by western blotting. We found that treatment with oTau (100 nM) for 20 min before tetanic stimulation blocked the increase in pCREB due to tetanic stimulation (Figure 7). Addition of the PDE5 inhibitor 7a (50 nM, 10 min) produced a significant protection against oTau effect on pCREB in tetanized slices (Figure 7). Thus, these results indicate that an increase in cGMP levels can protect against oTau suppression of the enhancement of CREB phosphorylation occurring during LTP.

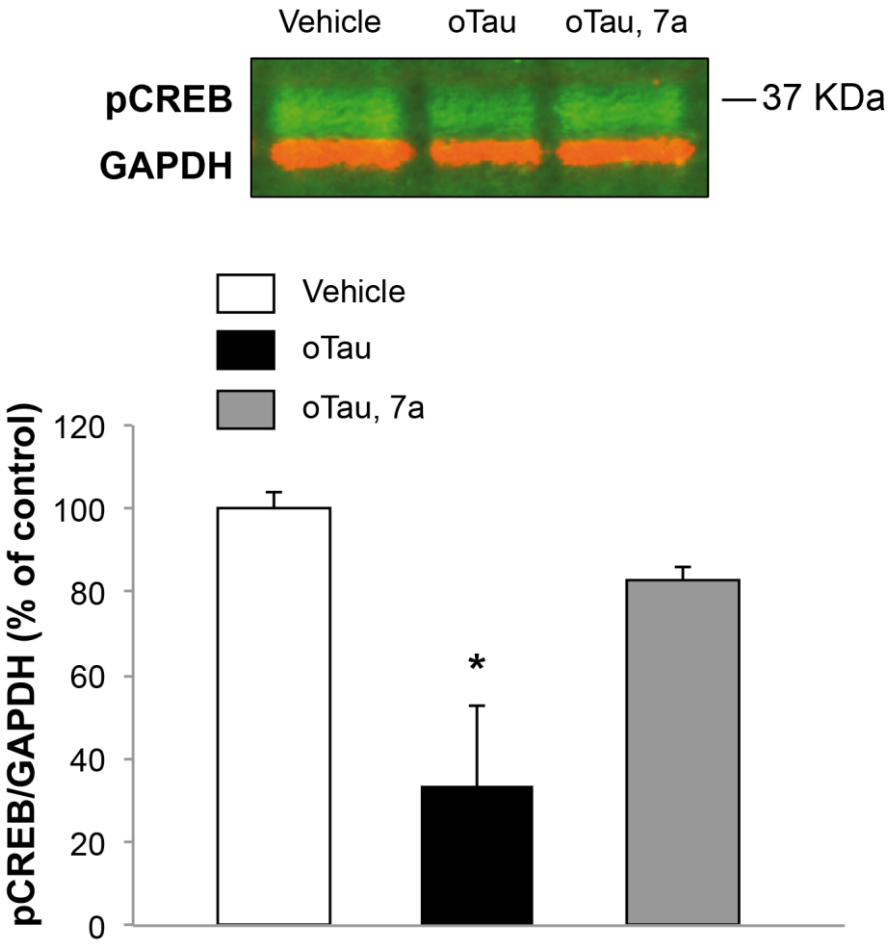

Figure 7. cGMP elevation rescues oTau-induced suppression of CREB phosphorylation in tetanized hippocampal slices. WB performed on hippocampal slices treated for electrophysiological experiments and stored at 120 min after tetanus. Bar graph shows that oTau significantly decreases pCREB expression in tetanized slices, whereas a concomitant treatment with the PDE5 inhibitor 7a is capable of rescuing pCREB expression (one-way ANOVA: $F_{(3,8)} = 10.263$; $p = 0.004$; Bonferroni's post-hoc: $p = 0.035$ between vehicle and oTau; $p = 0.006$ between oTau and oTau+7a). GAPDH expression was used as an internal control.

4. Discussion

In the present study, we report the effects of compounds mimicking different components of the NO cascade or interfering with the cascade, onto oTau-induced impairment of LTP and memory. The initial observation that inspired this work was the finding that enhancement of CREB phosphorylation during memory formation was suppressed in animals exposed to oTau. This finding is consistent with a recent study suggesting that tau is a target gene of CREB and negatively regulates its transcription (55). Overexpression of CREB significantly reduced mRNA levels of tau by acting on the CRE1 site of the tau promoter to inhibit the transcription of the tau gene (55). Other studies have demonstrated a similar relationship between phospho-CREB and tau. It was for example observed that upregulating the expression of CREB and phospho-CREB attenuates the level of hyperphosphorylated tau

in ischemic neurons of the parietal cortex in rat brains (56). Additionally, it has been shown that the tau/Fyn/NR2B signaling pathway might interfere with CREB activity and expression (57). Post-translationally modified and hyperphosphorylated tau proteins cause a reduction and a default of activity and phosphorylation of Fyn (tyrosine protein kinase), NR2B (receptor unit of NMDA receptor) and CREB. Impairment of CREB phosphorylation by oTau led us to hypothesize that up-regulation of the NO/cGMP/PKG/CREB pathway that is known to impinge on CREB can be beneficial in AD. To investigate our hypothesis and provide novel insights into the molecular mechanisms underlying oTau-induced defects of learning and memory, we examined the individual components of the NO/cGMP/PKG/CREB signaling pathway in relation to the elevation of oTau levels in mouse hippocampus.

We first investigated the effect of NO on LTP reduction caused by oTau through the NO donor DEA/NO. Our findings suggested that elevation of NO is able to rescue the LTP impairment, providing proof that NO has a protective effect on impaired synaptic strengthening and corroborating other studies showing that NO is involved in hippocampal plasticity processes (58-60). Nevertheless, the role of NO has been controversial as diverse studies have shown that NO is both neuroprotective and neurotoxic. NO has been correlated with neurodegenerative diseases through its formation of reactive nitrogen species (61), but it has correspondingly been shown that NO is able to reduce tau pathology and decrease cell loss, acting as a junction point between Aβ peptides, caspase activation, and tau aggregation (62). Noticeably, it would depend where NO is produced that attributes to its role in AD. The neurotoxic NO is produced by microglia inducible NOS (iNOS) which causes synaptic dysfunction through the production of peroxynitrite (63-65). NO produced by the other isoforms; endothelial NOS (eNOS) and/or neuronal NOS (nNOS) seems to be linked with neuroprotective mechanisms (66). Interestingly, a recent study has investigated the controversial roles of NO and its effects on synaptic plasticity in 3xTg-AD mice. Based on the evidence that these animal models often show evidence of dysfunctional calcium-regulated synaptic plasticity before the onset of cognitive deficits occur, it was suggested that there must be some compensatory mechanism which allows the hippocampus to maintain its normal net output, whilst there is already evidence of synaptic dysfunction (67). The work suggested that there is a relationship between the increased calcium release seen in pre-symptomatic AD mice and NO, since NO is calcium-regulated. Block of NO synthesis resulted in a markedly augmented synaptic depression in the AD mice. This would explain why AD mice and AD patients have elevated nNOS and ryanodine receptor levels (66, 68-70), as a mechanism to boost the NO cascade to compensate for the synaptic dysfunction they

experience. At later stages of AD, the cumulative NO levels would reach a level of neurotoxicity and convert the role of the gas molecule from neuroprotective to neurotoxic, demonstrating that NO acts as a Jekyll-Hyde molecule depending on its concentrations (67).

To evaluate the downstream effects of NO we used the irreversible sGC inhibitor ODQ, which is capable of reducing LTP levels as low as hippocampal slices perfused with tau. Most importantly, pairing oTau with ODQ and DEA/NO blocked the neuroprotective role of the NO donor supporting previous evidence that sGC is an important feature in the NO signal pathway involved in the rescue of synaptic dysfunction (35, 43). To exclude the possibility that ODQ might have disrupted some other mechanism involved in the induction of LTP, we used an alternative strategy to investigate the importance of sGC in rescuing oTau-induced synaptic impairment. We utilized the sGC stimulator BAY41-2272 and obtained similar results as those found with DEA/NO, namely BAY41-2272 was able to re-establish normal LTP after exposure to oTau. Interestingly, AD patients were found to have approximately 50% less sGC activity in the superior temporal cortex compared to controls (71). These observations provide evidence that sGC is highly important for the NO cascade and plays a direct role in the etiopathology of AD. Thus, our findings on activation of sGC and oTau-induced damage of LTP and memory are consistent with this scenario. Given that sGC is responsible for producing cGMP from GTP (43), we assumed that increasing cGMP levels would be beneficial and counteract the oTau-induced LTP impairment. In agreement with our hypothesis, both cGMP-analogs 8-Br-cGMP and 8-pCPT-cGMP rescued oTau-induced synaptic impairment to normal physiological levels. Furthermore, we observed that oTau-impaired LTP is restored after perfusion of hippocampal slices with two PDE5 inhibitors, sildenafil and compound 7a, that elevate cGMP levels.

Various PDE enzymes are able to hydrolyze cGMP regulating its intracellular levels. We and other groups highlighted the importance of PDE5 in modulating the NO-cGMP signal transduction pathway, and thus its effect on synaptic plasticity and memory (36, 37, 72-75). We previously reported that administration of PDE5 inhibitors sildenafil and compound 7a in a mouse model of amyloid deposition not only increased cGMP levels but also exerted an immediate and long-lasting amelioration of synaptic function and memory (36, 37). However, a possible point of contention could regard the genuine efficacy of PDE5 inhibitors on improving cognitive aspects, since their effect could have been attributed to the increased blood flow and glucose metabolism (as mentioned above, the initial use of PDE5 inhibitors was for treating hypertension and erectile dysfunction), due to the effect of PDE5 inhibitors on vasodilatation (76). This is unlikely because a previous study showed that the effect of

PDE5 inhibition on memory and cognition is not related with the cerebrovascular effects (77). Most importantly, in our study, *in vitro* application of sildenafil and compound 7a, in which cerebrovascular effects can be excluded, increased the amount of potentiation in slices perfused with oTau. Therefore, the above observations as well as the design of our study exclude the possibility that PDE5 inhibitors exert their action through increased vasodilatation.

A relevant finding of this study is the reversal of spatial and associative memory impairment followed by intra-cerebral administration of oTau in mice after acute treatment with PDE5 inhibitors. Both types of memory are impaired at early stages of AD (54, 78). Thus, up-regulation of cGMP via inhibition of PDE5 exerts a protective effect not only on synaptic plasticity but also on memory showing to improve several aspects of cognitive performance in oTau pathologies.

As with PDE5 inhibitors, the role of PKG in spatial and associative memory has been investigated in oTau-injected mice. In the present study, we demonstrate that intraperitoneal administration of the PKG activator 8-pCPT-cGMP in mice treated with oTau ameliorates memory deficits in the RAWM and fear conditioning tasks. Thus, we provide evidence that PKG activation enhances memory impairment induced by oTau. This finding is consistent with the observation that PKG inhibitors block potentiation, and exogenous administration of PKG produces activity dependent potentiation that mimics the one induced by tetanic stimulation (79). Accordingly, inhibition of PKG activity post-training for the inhibitory avoidance task prevents memory formation (80). Thus, we provide evidence that PKG activation ameliorates memory impairment induced by oTau.

Dysregulation of the NO cascade in AD has been shown in proteomic and metabolomic studies (34). Consistent with this finding, the NO/cGMP/PKG/CREB signaling pathway has been demonstrated to be down-regulated in mouse models of amyloid deposition (35, 37) and its down-regulation has been mainly attributed to the presence of amyloid-beta (Aβ) oligomers (35, 81). Within the present work, we have extended this observation to soluble forms of tau. Such a parallelism can be due to numerous common features of tau and Aβ oligomers, including beta-sheet structure, aggregation status (1, 82, 83), activity-dependent release (1, 84-86), capability of entering neurons (1, 38, 87-89) and affecting astrocytic intracellular calcium signaling (90-94), and binding to the same cell surface protein, amyloid precursor protein (APP) (95-99).

The discovery of soluble tau aggregates and their involvement in the pathogenesis of AD triggered the development of therapeutics aimed to halt tau aggregation (100-104) or

induce tau clearance by immunotherapy (105). However, concerns have been raised regarding the efficacy of those treatments, since they could reduce the overall NFT load without reducing oTau. Moreover, considering the fact that NFTs may act as a protective mechanism (106), preventing the spreading of the pathology, the above approaches may be harmful. Additionally, tau plays a key role in physiological cell function; thus therapies directly affecting tau levels might interfere with the normal physiological role of tau. Immunotherapy is additionally burdened, because most of the antibodies exhibit high affinity for tau, without binding to the specific tau conformers involved in tau seeding (107). Thereby, we suggest that an alternative strategy to protect against tau-induced memory deficits would be mediated by drugs acting downstream of the NO production. To this regard, the outstanding safety profile of PDE5 inhibitors makes them particularly attractive, as a viable mean to counteract AD.

Concluding, our findings provide a novel view on how oTau affects synaptic plasticity and memory, pointing at the NO cascade as a second messenger pathway that can be exploited to counteract tau-induced damage of synaptic plasticity and memory, and offering a new window of therapeutic opportunities against AD and other neurodegenerative diseases characterized by an increase in oTau.

Competing interests

OA is the founder of Neurokine Therapeutics. JF, EKA, MVdB, WG, MF, SXD, DWL, and PD declare that they have no competing interests.

Funding

Work on this manuscript was supported by NIH grant R01AG049402 to OA.

Acknowledgements

We thank I. Francis for advises with CREB experiments.

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Chapter 6

Upregulation of cAMP pathway counteracts oligomeric tau-induced impairments of synaptic plasticity, spatial memory and GluA1 receptor trafficking

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Chapter 7

General discussion

Aim of the thesis

In the present dissertation we aimed to elucidate the molecular mechanism underlying the time-dependent activation of cyclic nucleotides and additionally examine the neuroprotective effect of upregulating the cyclic nucleotide pathways against tau pathology. Considering that common downstream effector for both cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) signaling pathways is the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR), we examined whether the temporally defined pro-cognitive action of the cGMP-specific phosphodiesterase (PDE) 5 inhibitor vardenafil and the cAMP-specific PDE4 inhibitor rolipram is explained at the molecular level by differential changes in AMPAR synthesis and trafficking (chapter 4). Along the same lines, we investigated whether upregulation of the cGMP/protein kinase G (PKG) pathway and upstream molecules that comprise the nitrinergic signaling could ameliorate plasticity and memory deficits induced by externally applied tau oligomers (oTau) (chapter 5). Finally, we sought to determine whether upregulation of the cAMP/protein kinase A (PKA) pathway could protect against oTau-induced impairments in long-term potentiation (LTP), hippocampus-dependent memory and AMPAR trafficking (chapter 6).

Role of cyclic nucleotide signaling on AMPARs trafficking

The role of cyclic nucleotides in synaptic plasticity and behavior it is supported by a vast literature. Additionally, several studies from us and others have shown the temporal specificity in the action nucleotides, suggesting that cGMP is involved at the early and prone to disruption phase of consolidation, while cAMP is involved at the late and more stable phase of consolidation. Accordingly, application of the cGMP-specific PDE5 inhibitor vardenafil and the PDE4-specific inhibitor rolipram at confined mnemonic phases of early and late consolidation respectively, was exerting a pro-cognitive effect (1-3). A study in rats, has shown that the cognitive enhancing properties of vardenafil are apparent when given within 45 min after the mnemonic test, while rolipram is effective when given between 3 and 5.5 h after the learning trial (4). In chapter 4, we confirm these findings in mice by showing that application of vardenafil 20 min after a mnemonic paradigm of spatial memory is able to convert short-term memory to long term memory. The same outcome was observed for rolipram when it was given 3 h after the learning trial. Additionally, both inhibitors were able to enhance memory formation when they were given 30 min before the learning trial, at the acquisition phase. Despite the fact that our study confirmed previous studies, the most

important finding was that the cognitive enhancing properties of vardenafil administrated either at the acquisition or at the early consolidation phase were accompanied by increased surface expression of both GluA1- and GluA2-containing AMPARs. As it was expected, application of vardenafil at the late consolidation phase had not effect on AMPAR dynamics. A surprising outcome was that rolipram did not affect surface expression or trafficking of AMPARs when it was given at the acquisition and late consolidation phase, despite its promising pro-cognitive action at these mnemonic phases.

An interesting finding is that application of vardenafil at the acquisition phase has a similar effect in upregulating both GluA1- and GluA2-containg AMPARs, while during the early consolidation phase the number of surface GluA2-AMPARs is significantly higher in comparison to GluA1-AMPARs. Considering the subunit composition of AMPARs, it is possible that administration of vardenafil at the acquisition phase results in increased GluA1/GluA2 receptors, while its administration at the early consolidation phase upregulates mainly GluA2/GluA3 receptors.

Our study suggests that the long-lasting memory enhancing properties of upregulating the cGMP or cAMP signaling pathway are dissociative at the molecular level. The initial assumption of a similar downstream mechanism was due to the fact that the cGMP and cAMP signaling cascades share several common downstream effectors including AMPARs (5, 6). Nevertheless this is based on a simplified model in which cyclic nucleotides activate their downstream kinases promoting insertion of AMPARs to perisynaptic sites (7). Nevertheless, as it was discussed in chapter 2, the relationship between signaling molecules in the cyclic nucleotide cascade deviate from linearity with the existence of several positive and negative loops, and downstream effectors. In this respect, activation of cAMP could also result in activation of exchange protein activated by cAMP (Epacs) that are shown to participate in bidirectional synaptic plasticity modulating both LTP and long-term depression (LTD). The latter involvement of Epacs in LTD is facilitated by increased endocytosis of GluA2/GluA3 receptors (8, 9). Future research could unravel the molecular mechanism that mediate the memory enhancing action of upregulating the cAMP pathway. For example, PKA activity was shown to modulate Ca^{2+} influx through N-methyl-D-aspartate receptors (NMDARs) in hippocampal cell cultures (10), as well as trafficking of NMDARs to the synapse (11). These processes are essential for NMDAR-dependent LTP and subsequent synaptic strengthening (12, 13). Thereafter, investigating the effects of PDE4 inhibition on NMDARs trafficking could be an interesting addition to our data.

Although PDE4 inhibition does not result in increased surface expression of AMPARs

after a long interval between treatment and collection of the brains (24 h), in chapter 4, we also showed that both PDE4 and PDE5 inhibitors affect dynamics of GluA1-AMPARs after shorter intervals. Specifically, both drugs increased surface expression and trafficking of GluA1-AMPARs 15 min after treatment. Considering the fact that AMPARs are stored in endosomes, close to the membrane, this fast effect could be due to trafficking of already existing AMPARs (14). When we wait 40 min before collecting the brains, we observed an increase in total levels of AMPARs that would be the result of increased transcription (15, 16) or local translation (17, 18). Finally, 60 min after treatment, there was significant increase only in the surface expression of GluA1-AMPARs, while the total levels were intermediate and non-significantly higher from the control group. This finding probably suggests that the newly produced AMPARs (40 min after treatment) are trafficking to the membrane (60 min after treatment). Even though the main objective of chapter 6 was not related to the effects of PDE4 inhibition on AMPARs dynamics *per se*, we cannot neglect the fact that treatment with the PDE4 inhibitor roflumilast alone did not affect trafficking or total levels of GluA1-AMPARs. In that study the interval between treatment and collection of the brains was 85 min. In both studies we used the same experimental design, while the only difference was between the PDE4 inhibitors used. Without drawing certain conclusions, we could speculate that the effect of PDE4 inhibition on AMPARs trafficking is transient. This could also corroborate the finding that treatment with rolipram did not result in sustainable increase in surface expression of AMPARs after 24 h.

In both chapter 4 and chapter 6, we utilize the surface biotinylation method in order to examine changes in surface expression of AMPARs. However this method does not provide evidence regarding the functional role of AMPARs and their specific position in the membrane. It is known that AMPARs enter the membrane at perisynaptic sites and subsequently move to the membrane via lateral diffusion, resulting in a "priming effect". We acknowledge that in our studies we did not provide an actual link between the cyclic nucleotide signaling cascades and functional AMPARs. Further studies could provide a better insight into this relationship.

Cyclic nucleotide signaling and tau pathology

The aim of both chapters 4 and 5 was to examine the effects of upregulating the cyclic nucleotide pathways against oTau toxicity. An important aspect of both studies is that we chose to apply oTau externally in order to model deficits in synaptic plasticity and memory, characterizing tauopathies. As it was mentioned, due to the fact that neurofibrillary tangles

(NFTs) represent one of the main pathological hallmarks in Alzheimer's disease (AD), the initial belief was that they are the eventual major toxic entities in the disease. Recent findings seem to challenge this idea. For example, animal studies with a conditional mouse model of tauopathy (rTg4510) showed that formation of NFTs is not correlated with neuronal dysfunction and they could accumulate without affecting memory function (19). Furthermore, observations with an animal model expressing all six isoforms of human tau showed that NFTs are not associated with cell death (20). The above studies in combination with the observation that inhibition of abnormal tau hyper-phosphorylation could ameliorate motor deficits in the P301L mouse model, without reducing NFTs (21), strongly suggest that NFTs are not the mediators of toxicity in tauopathies. In fact, it is also considered that NFT may act as a protective mechanism, preventing the spreading of the pathology (22).

As it was underscored in both chapters there is a growing body of literature suggesting that actually low order oTau constitutes the toxic species in AD, responsible for the amnestic symptoms. For instance, oTau has been shown to affect synaptic markers, mitochondrial function (23), axonal transport (24) and prevent formation of new memories in the object recognition task (23). Our study confirms and gives strength to those observations, suggesting that perfusion of oTau before LTP induction reduces the amount of potentiation in hippocampal slices. Next, infusion of oTau in the hippocampus of wild-type mice impairs memory formation, as examined with several behavioral tasks requiring hippocampal activity.

Additionally, we showed that upregulation of either nitric oxide (NO)/soluble guanylate cyclase (sGC)/cGMP/PKG pathway or cAMP/PKA pathway could protect against plasticity and memory deficits induced by oTau. Both signaling cascades are negatively affected during AD (25-28), and their enhancement via several pharmacological agents could be a promising approach in combating AD-related memory decline. Additionally, we showed in chapter 5 and 6 that upregulation of these signaling pathways could reverse oTau-induced reduction in plasticity markers including cAMP response element-binding protein (CREB) and GluA1-AMPARs, respectively. This does not necessarily mean that upregulation of the different cyclic nucleotide cascades combats oTau-induced deficits via a different mechanism. Due to the fact that both CREB and GluA1-AMPARs are downstream effectors of cGMP and cAMP pathways, it is possible that 1) upregulation of cAMP pathway could also compensate against oTau-induced impairments in CREB phosphorylation and 2) upregulation of cGMP could reverse oTau-reduction in GluA1-AMPARs trafficking.

Regarding the first hypothesis, previous studies have shown that cAMP signaling could promote CREB phosphorylation via PKA activation (29, 30). Along the same lines,

sub-chronic administration of rolipram enhanced CREB phosphorylation in healthy rats during contextual fear conditioning. Additionally, rolipram could compensate against pathological reduction of pCREB induced either after Aβ application in neuronal cells cultures (31) or in transgenic AD animal models (32). The latter findings were also confirmed with another PDE4 inhibitor FFPM that was shown to restore memory impairments and CREB phosphorylation levels in APP/PS1 transgenic mice, after chronic treatment (33). Based on the well-established relationship between cAMP signaling and CREB phosphorylation, it is possible that in addition to PDE5 inhibition, PDE4 inhibition could also compensate against oTau-induced reduction in pCREB.

The second hypothesis is also supported by our results in chapter 4. In chapter 6 we collect the brains 85 min after treatment with roflumilast, a time point that it was not investigated in chapter 4. Nevertheless, the fact that PDE5 inhibition resulted in increased surface expression of AMPARs after 24 h, indicates that administration of vardenafil has a sustainable effect in AMPAR trafficking. Therefore, it could possibly compensate against oTau-induced impairments of AMPARs trafficking in the time point that we investigated in chapter 6.

In chapter 5, except for cGMP analogues and PDE5 inhibitors, we also investigated the neuroprotective action of drugs that act upstream of the cGMP pathway, including NO donors and sGC stimulators, in counteracting oTau-induced synaptic dysfunction. Extending these observations at the behavioral level is a future goal. Although previous studies have shown that treatment with either NO donors or sGC stimulators have pro-cognitive effects, their action against oTau-induced memory impairments has not been investigated yet. So far, studies indicate that treatment with different NO donors was able to reverse natural forgetting in object recognition task (34, 35). Additionally, administration of sGC stimulators enhanced spatial memory and passive avoidance in young and aged rats (36-38).

Our results provided first evidence that intrahippocampal injection of oTau could result in decreased trafficking of GluA1-AMPARs, without affecting their total levels and the phosphorylation at the s845 site. Subsequently upregulation of the cAMP signaling pathway with PDE4 inhibition was able to reverse this impairment in GluA1-AMPAR trafficking. Despite the impact of oTau on GluA1 receptors, we did not measure the effect of oTau on other types of AMPARs, such as GluA2 and GluA3. Based on previous studies, oAβ could also affect GluA2/GluA3 receptors that represent the other predominant type of receptors in the hippocampus along with GluA1/GluA2. Specifically, it was shown that application oAβ leads to removal of GluA2/GluA3 receptors from the synapses (39). The increased

internalization of GluA2/GluA3 was dependent on interaction with protein interacting with C-kinase 1 (PICK1) protein (40). Importantly, it was recently shown that tau facilitates interaction between PICK1 and GluA2, and subsequent endocytosis of GluA2-AMPARs (41). Therefore, an interesting addition to our study would be to examine the effects of oTau, as well as, cAMP and cGMP upregulation on GluA2-AMPAR trafficking.

Critical considerations

As with the majority of pharmacological agents, several points of controversy exist. For instance, the occurrence of unwanted side effects. As such, clinical application of NO donors and PDE inhibitors is hampered due to cardio-and cerebrovascular effects. However, it was shown that the cognitive enhancing doses of PDE4 and PDE5 inhibitors do not correlate with changes in blood flow and glucose utilization in the brain (42). Additionally, several studies suggest that the cognition enhancing action of drugs that act on the nitrinergic system can be dissociated from their cerebrovascular effects (43-46). These findings, in combination with the *ex vivo* experimental design of our studies in which NO donors and PDE inhibitors were shown to affect plasticity mechanisms, exclude the possibility that they exert their pro-cognitive effect through increased vasodilatation at the doses used in our studies.

Except for cardio- and cerebrovascular effects, the clinical application of PDE4 inhibitors remains also controversial due to emetic side effects by activating the vomiting centers in the brain stem. These emetic issues could be overcome with application of selective PDE4 inhibitors that are already effective on memory at very low doses (47). Though still being a rather nonselective PDE4 inhibitor, the memory enhancing dose of roflumilast, as shown is previous studies and also used in the current study, was disparate from the dose inducing emesis as shown in both heathy animals and humans (48, 49). In addition, roflumilast has already been clinically approved for the treatment of chronic obstructive pulmonary disease (COPD) mainly based on its reduced emetic potential (50), thus adding to the translational value of our findings.

Another point of concern is the translational value of studies using PDE5 inhibitors, since issues have been raised regarding their efficacy in elderly including AD patients. Although the PDE5 mRNA was present in the hippocampus of rodents (51, 52), its expression was found to be very low in the human hippocampus and other cortical areas (53). Moreover, it was indicated that the mRNA of PDE5 was non-detectable in the human brain of elderly people or patients with AD (54). Nevertheless, a more recent study from Teich et al. showed

that PDE5 mRNA and protein are certainly present in the human brain and particularly in the hippocampus (55). The authors also attributed the previously reported negative data to the utilization of incorrect primers (55). Despite the promising results of PDE5 inhibitors at the preclinical level, there are a few studies examining the therapeutic potential of PDE5 inhibitors in humans. These clinical studies showed that the cognitive enhancing properties of PDE5 inhibitors are present but only exerted after chronic, yet not acute treatment (56-58). Finally, currently available PDE5 inhibitors are also characterized by other peripheral side effects, like visual disturbances, due to their additional inhibition of PDE6 and PDE1. These side effects could be mitigated by the development of more selective inhibitors. For example the PDE5 inhibitor 7a that was used in our study was shown to exhibit higher selectivity for the PDE5 enzyme and increased permeability of the blood-brain barrier (59), rendering it a promising and improved agent.

Conclusions

In the present thesis we expand our knowledge regarding the molecular basis of pharmacological agents that act on the cyclic nucleotide signaling cascades. Although the pro-cognitive effect of upregulating the cGMP pathway at specific mnemonic process could be attributed to increases in AMPARs trafficking, the molecular mechanisms underlying the cognitive enhancing effects of increasing the cAMP pathway requires further investigation. Additionally, we demonstrated that upregulating either the GMP or the cAMP pathway could be a promising neuroprotective approach in ameliorating synaptic plasticity and memory decline encountered in tauopathies.

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Chapter 8

Summary

The literature background and the main rationale of the thesis are introduced in **chapter 1.**

The current literature summarized in **chapter 2** suggests that targeting several components of the cAMP or the cGMP pathway could enhance synaptic plasticity and memory formation. Despite the overwhelming body of literature showing a pro-cognitive action in upregulating either cAMP or cGMP signaling, it is still controversial which components should be targeted in memory-related diseases. In this respect, pharmacological interventions like PDE inhibitors seem to represent a more balanced approach in comparison to genetic interventions that lead to complete depletion of a molecule. Additionally, a possible strategy to reduce the unwanted side effects of pharmacological agents could be via combination of sub-optimal doses of drugs that act on different cascades. Finally, we suggest that application of optical and genetic techniques for better understanding of the complex mechanisms participating in the spatiotemporal regulation of cyclic nucleotide signaling, and implementation of computational models for integrating experimental data would be important assets in the development of new therapeutic strategies.

In **chapter 3** we summarize studies regarding the role of glutamatergic signaling in object recognition and object location memory. As in every system, balance in the action of glutamatergic transmission is the key in neuronal functioning, since excessive activation via agonists or blockage via antagonists impairs memory. Nevertheless, the latter studies revealed that activation of different types of receptors is responsible for glutamatergic signaling at different mnemonic phases. Importantly, it was shown that inhibition of glutamatergic neurotransmission by antagonist or negative modulators could ameliorate cognitive deficits in pathological conditions characterized by excitotoxicity, like Alzheimer's disease. Notwithstanding, administration of agonists for the glycine-binding site of NMDARs and positive modulators of AMPARs and mGluRs could have pro-cognitive effect in healthy animals. Collectively, these results suggest that pharmacological agents acting on glutamatergic system could have promising clinical applications.

In **chapter 4** we showed that administration of vardenafil or rolipram at the acquisition phase of spatial memory could compensate against natural forgetting occurring after 24h in mice. Importantly, at the consolidation phase we were able to distinguish the action of these drugs. In particular, memory enhancement was achieved when vardenafil was administrated at the early consolidation phase or when rolipram was given at the late consolidation phase.

Subsequently, we assessed whether the pro-cognitive effect of the above inhibitors could be related to changes in AMPAR dynamics. In short, upregulation of cGMP signaling during the acquisition and early consolidation phase via vardenafil administration improved long-term memory and this phenomenon could be explained by increased surface expression of GluA1- and GluA2-containing AMPARs. Additionally, although administration of rolipram at the acquisition and late consolidation phase enhanced long-term memory in mice tested in the object location test, the underlying mechanism does not involve changes in AMPAR trafficking or synthesis.

In the study presented in **chapter 5** we showed that increase in NO levels could protect against oTau-induced suppression of LTP via activation of sGC that in turn stimulates production of cGMP and subsequent activation of PKG. The above results were extended at the behavioral level, showing that upregulation of PKG either indirectly via PDE5 inhibition or directly via a PKG activator could rescue deficits of short-term reference memory and contextual memory resulted from intrahippocampal injection of oTau. Importantly, elevation of cGMP levels restored CREB phosphorylation after LTP induction in the presence of oTau. These results provide novel evidence showing that agents acting on the NO cascade could be beneficial in counteracting plasticity and memory decline encountered in tauopathies.

Finally in **chapter 6**, despite of confirming the previous observation that oTau could cause faster decline in synaptic plasticity and blockage of memory formation, we also showed that upregulation of cAMP/PKA signaling could ameliorate LTP and spatial memory impairments. At the molecular level, we demonstrated that intrahippocampal administration of oTau hampered trafficking of GluA1-AMPARs, while concomitant increase in cAMP signaling via PDE4 inhibition was able to compensate against oTau-induced impairments in AMPARs trafficking. In conclusion, our study provides a link between tau pathology and glutamatergic system and additionally suggests that interventions that target the cAMP/PKA signaling pathway could be promising in diseases characterized by tau aggregates.

Appendices

Valorization addendum

About the author

List of publications

Acknowledgements

Dementia is a decline in mental ability severe enough to interfere with daily life functioning. One of the most commonly encountered forms of dementia is Alzheimer's disease (AD) accounting for 60 to 80% of the cases. AD is characterized by aggregation of amyloid plaques (Abeta) and neurofibrillary tangles (tau) in the brain leading to memory loss and spatiotemporal confusion. Worldwide evidence reveals that 6% of the population over the age of 65 and 35% of those over 85 are affected. In real numbers, it is estimated that in 2025 7.1 million people will be affected by the disease. As the proportion of elderly persons is predicted to increase dramatically in the next decades, AD is estimated to affect 13.8 million in 2050 (1). AD and other types of dementia impose a burden not only for the patients, but for society as a whole. Important financial resources are spent every year for the caregiving of the patients. The total health care cost for all individuals with AD and other dementias is estimated at $277 billion in 2018, while this cost is expected to exceed $1 trillion in 2050. Additionally, caregivers and relatives of the patients report high emotional stress and exhibit increased risk for depression. As a result therapeutic interventions that could even modestly alleviate the cognitive deficits would have a major public health benefit. Since AD was first described in 1906, there is an abundance of ongoing research aiming to understand the underlying mechanism of the disease and to propose possible therapeutic interventions (2). Despite the extensive scientific research there is no cure for the disease and the commercially available treatments to date have limited efficacy and several side effects. The significance of the present dissertation is imprinted in its foremost goal to contribute to the better understanding of molecular mechanisms underlying memory formation, and additionally identify a possible mechanism leading to protection against memory impairment induced by tau pathology. An effective therapeutic strategy for combating the cognitive decline observed in the disease would have a great impact on the patients' quality of life.

In chapter 4 we examined the molecular basis underlying the cognitive enhancing action of drugs that act on the cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) signaling cascades. Although upregulation of cyclic nucleotide signaling cascades was shown to be promising in alleviating memory decline characterizing dementias (3), the mechanistic basis of their effects are largely unknown. In the present thesis we showed that upregulation of cGMP pathway during different mnemonic phases is related to increased surface levels of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) receptors; one of the main excitatory receptors contributing to neurotransmission. However, the pro-cognitive effect of upregulating the cAMP pathway was not depicted through changes in AMPA receptor dynamics; neither trafficking nor synthesis.

These findings suggest that although the cAMP and cGMP pathways converge in several common downstream effectors and their upregulation results in a similar behavioral output, the mechanistic basis of their cognitive enhancing action is disparate. This observation contributes significantly to the better comprehension of the signaling pathways governing memory formation and could have important implications in therapeutic interventions targeting the cyclic nucleotide cascades.

In both chapter 5 and 6, we examined the pro-cognitive action of agents that act on the nitrinergic and cyclic nucleotide pathways against tau pathology. The innovative aspect of both chapters is that we utilized exogenously applied tau oligomers for modeling memory impairments encountered in tauopathies. Tau oligomers have recently emerged as one of the underlying causes inducing neuronal loss and memory deficits at the early onset of AD, before the appearance of other pathological hallmarks. Importantly, we showed that pharmacological agents that upregulate nitrinergic or the cyclic nucleotide signaling could compensate for oligomeric tau-induced impairments of synaptic strengthening and consequently memory formation. In addition, the cognitive enhancing action was mediated by an increase in plasticity markers that have been consistently shown to be downregulated in AD, like cAMP response element-binding protein (CREB) and AMPA receptors. Our results could also be extended to other disorders that are characterized by tau pathology including corticobasal degeneration, supranuclear palsy and fronto-temporal dementia with Parkinsonism linked to chromosome 17. Although each of the above disorders has an unique biochemical signature related to tau aggregates, they are all characterized by increased tau phosphorylation and formation of tau oligomers (4).

An important aspect of all the chapters is that, among other agents for upregulating the cAMP and cGMP signaling pathways, we utilize phosphodiesterase (PDE) inhibitors that have been studied both at the preclinical and clinical level for their therapeutic properties. The currently approved pharmacological treatments for AD act either on the cholinergic system by prolonging the procognitive action of the neurotransmitter acetylcholine in the synapse or in the glutamatergic system by reducing neurotoxicity due to the excessive activation of N-Methyl-D-aspartic acid (NMDA) receptors. The low efficacy and the severe side effects of these treatments impose a need for the development of new therapeutic strategies.

Several cAMP-specific PDE4 inhibitors have been tested in clinical trials up to Phase II for their cognitive enhancing action. However, the results of those trials have not yet been disclosed (5, 6). The highly potent PDE4 inhibitor roflumilast, that we showed in chapter 6 to compensate against tau-induced plasticity and memory impairments, is already on the market

under the name Daxas or DailyResp for the treatment of Chronic Obstructive Pulmonary Disease (COPD) (7). Importantly, roflumilast was recently shown to improve several aspects of cognition, including memory in healthy volunteers (8) at a dose that is 5 times lower than the approved dose for the treatment of COPD. As a result, it is devoid of the common emetic side effects of PDE4 inhibitors (5).

In addition to roflumilast, the cGMP-specific PDE5 inhibitors sildenafil and vardenafil are FDA-approved for the treatment of erectile dysfunction with the tradenames Viagra and Levitra, respectively, because they induce smooth muscle relaxation of blood vessels. Additionally, sildenafil is also commercially available as Revatio for the treatment of hypertension of the pulmonary artery. Despite the memory improving action of PDE5 inhibitors, as reported in animal studies, three clinical studies with healthy volunteers found no effect of vardenafil or sildenafil on memory performance after a single dose (9-11). Nevertheless, another study showed that chronic administration of the PDE5 inhibitor udenafil (Zydena) did improve cognition and executive function. This may suggest that therapeutic properties of PDE5 inhibitors require chronic treatment to be induced (12). Additionally, PDE5 inhibitors exhibit a safe pharmacological profile since their side effects in humans are mild and have only been reported by a minority of users (13). Having said this, further work and development of PDE5 inhibitors with optimized pharmacokinetic properties for CNS delivery is needed before their utilization as a treatment in neurodegenerative disorders (14). In that respect, our findings in chapter 4 and 5 contribute to the current knowledge regarding the action of PDE5 inhibitors *ex vivo* and *in vivo*, by showing the molecular mechanism responsible for their pro-cognitive action and providing first evidence of their neuroprotective action against tau pathology.

We acknowledge that all the studies presented in the current thesis have been conducted in rodents and do not provide direct evidence for clinical efficacy on the read-out parameters of the drugs as described above. Although animal experiments are not always translatable to humans, animal research still represent one of the initial and integral steps of scientific research into pathophysiological mechanisms and how to combat them. We hope that our results will inspire further interest into the development and eventual clinical testing of drugs that act in the cyclic nucleotide pathways, while hopefully demonstrating their therapeutic potential in memory-related disorders.

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Appendices

Valorization addendum

About the author

List of publications

Acknowledgements

Eleni Konstantina Argyrousi was born in Trikala, Greece on December 18th 1990. In 2008 she finished her high school education in Trikala. Being fascinated by the field of human biology she enrolled in the Bachelor program of Biology at University of Patras in September 2008. In 2013 she obtained her Bachelor's degree in 'Biology' and the same year she enrolled in the Research Master program Cognitive and Clinical Neuroscience, specialization Fundamental Neuroscience, at Maastricht University. The second year of the program she started the internship for her Master thesis at Columbia University under the supervision of Prof. Dr. Arancio and Prof. Dr. Prickaerts. During this period she was mainly trained in the techniques of electrophysiology and behavioral testing and her scientific interest was focused on investigating the therapeutic properties of phosphodiesterase inhibitors against synaptic and memory decline. In 2015 she obtained her Master's degree and worked briefly at the same group at Columbia University as Staff Associate. In November 2015 she started working as PhD candidate at Maastricht University under the supervision of Prof. Dr. Prickaerts, aiming to gain more knowledge regarding the molecular mechanisms of mnemonic processes. During the first 8 months of her PhD she worked as visiting scholar at Mount Sinai in the group of Dr. Neves and the last 6 months of her PhD she visited again the lab of Prof. Dr. Arancio at Columbia University, where she completed her research projects. The studies conducted during her 3-year PhD program are presented in this dissertation.

Appendices

Valorization addendum

About the author

List of publications

Acknowledgements

Published in peer-reviewed journals:

- Prickaerts J, Van Goethem NP, Gulisano W, **Argyrousi EK**, Palmeri A & Puzzo D (2017) Physiological and pathological processes of synaptic plasticity and memory in drug discovery: Do not forget the dose-response curve. *European journal of pharmacology, 817*, 59-70
- Ricciarelli R, Brullo C, Prickaerts J, Arancio O, Villa C, Rebosio C, Calcagno E, Balbi M, Van Hagen BTJ, **Argyrousi EK**, Zhang H, Pronzato MA, Bruno O & Fedele E (2017) Memory-enhancing effects of GEBR-32a, a new PDE4D inhibitor holding promise for the treatment of Alzheimer's disease. *Scientific reports, 7*, 46320

Submitted or in preparation:

- **Argyrousi EK**, de Nijs L, Lagatta DC, Schlütter A, Weidner MT, Zöller J, van Goethem NP, Joca SRL, van den Hove DLA & Prickaerts J (2019) Effects of DNA methyltransferase inhibition on pattern separation performance in mice (under review)
- **Argyrousi EK**, Fiorito J, Van den Berg M, Gulisano W, Fa' M, Deng SX, Landry DW, Puzzo D & Arancio O Synaptic and Memory Dysfunction Induced by Tau Oligomers is Rescued by Up-regulation of the Nitric Oxide Cascade (submitted)
- **Argyrousi EK**, Heckman PRA, van Hagen BTJ, Muysers H, van Goethem NP & Prickaerts J The pro-cognitive effect of upregulating cGMP signaling during memory acquisition or early consolidation is mediated by increased AMPA receptor trafficking (submitted)
- **Argyrousi EK**, Arancio O & Prickaerts J (2019) Upregulation of cAMP pathway counteracts oligomeric tau-induced impairments of synaptic plasticity, spatial memory and GluA1 receptor trafficking (in preparation)
- **Argyrousi EK**, Heckman PRA & Prickaerts J Role of cyclic nucleotides and their downstream signaling cascades in memory function: being at the right time at the right spot (in preparation)

Invited book chapters:

- **Argyrousi EK**, Heckman PRA & Prickaerts J (2018) Glutamate signaling in Object Recognition and Object Location Memory Tests. In A. Ennaceur (Ed.), *Handbook of Object Novelty Recognition* (pp. 541-51). Amsterdam: Academic press
- Heckman PRA, **Argyrousi EK** & Prickaerts J (2018) Phosphodiesterase Inhibitors in Object Recognition and Object Location Memory Tests. In A. Ennaceur (Ed.), *Handbook of Object Novelty Recognition* (pp. 567-74). Amsterdam: Academic press
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Appendices

Valorization addendum

About the author

List of publications

Acknowledgements

I would like to start this section by thanking my promotion team: Prof. Dr. Jos Prickaerts and Prof. Dr. Ottavio Arancio, who helped me during my Master and PhD projects, gave me the opportunity to work in their labs and who are my scientific role models.

Jos, I still remember the Skype meeting that we had during the selection process for the Master program in Fundamental Neuroscience. More than five years ago, I told you that I wanted to be enrolled in the FN program, because I was intrigued by your scientific work with phosphodiesterase inhibitors. I know that back then you didn't believe me, but the future showed that I wasn't joking at all. I would like to thank you for giving me the opportunity to attend the Master program at Maastricht University that shaped my scientific interest and for being such a great mentor for me. Additionally, I would like to express my gratitude for giving me a PhD position in your group that helped me to go one (or a few) step(s) further in my scientific career. Your optimism, your cheerfulness and your passion for science will be really unforgettable to me.

Ottavio, our paths crossed for the first time in November 2014 when you welcomed me in your lab at Columbia University for my Master internship. I never thought that I would be able to learn so many things in such a short time and yet it happened. I would like to thank you for giving me the opportunity to work with you during my Master and my PhD projects, for convincing me that I can do stuff that I never thought I would, for making me meet impossible deadlines and for being next to me for any difficulties that I had during my stay in New York.

I would also like to thank Dr. Susana Neves that gave me the opportunity to work at her lab at Mount Sinai during my first PhD internship. During this internship I was able to acquaint myself with several biochemical and genetic engineering techniques. Needless to say, I was able to travel daily through Central Park for eight months which by itself is an memorable experience.

Along with my promotion team I would like to thank all the past and current members of the group in Maastricht. Nick, you are one of the most resourceful, hardworking and smart persons that I ever met. Thank you for showing me the way around the animal facilities and for being such a great co-worker. Britt, I have the feeling that we have such different, but also similar personalities at the same time. Thank you for picking me up at the P&S building, for helping me with several projects and appreciating my weirdness in several aspects. Dean, you have one of the most "Greek" personalities that I ever met, and I say that as a compliment.

Thank you for all the scientific and (maybe more importantly) non-scientific conversations that we had, for all the great moments in Nice, Lunteren, Crete and Efteling, but most importantly for being the "D" in the "P.D.E. group". Ellis, you've been my co-traveler literally and figuratively to Hasselt and during the *ex vivo* experiments. Those were truly bonding experiences. I wish you the best for the future and I am pretty sure all your goals will be achieved. Manon, we didn't spend so much time together, since soon after you joined the group, I departed again. Nevertheless, I wish you all the best trying to juggle no less than 3 supervisors at the same time.

Additionally, I would like to thank my former students Niek, Sjoerd, and Hannah that decided to conduct their Bachelor or Master internships in our group and assist me with my projects. Chronologically speaking I should start with Niek that was my first Master student and student in general. You are such a kind and hardworking person, and all this work paid off with a great position in Amsterdam. Simeon and Sjoerd, Sjoerd and Simeon, you were such an amazing team. You spend only a few months in the lab, but I have the most cheerful and fun memories about you. Hannah, you've been my last student when I was in Maastricht and probably the one that I will remember the most. Without your contribution, several projects would never have been finished in time. You grew into an independent researcher and you were able to coordinate everything during my absence. Except for a good researcher you are also a truly bright person, and I am pretty sure you will excel in your new position as a PhD student.

I cannot forget the technical team at Maastricht University: Hellen, Marjan, Denise, Rachelle and Anne that shared with me their experience in several experimental aspects either at the Wet lab or during the behavioral experiments. Many thanks as well to the technical team at Columbia University: Hong and Agnes that taught me electrophysiology and behavioral testing, respectively, and for being always an amazing company, while working at the lab.

Pim, you are one of the first people that I met when I moved back to Maastricht in 2016. You assisted me in so many projects, we wrote papers together, you became my best friend, the best company that I ever had and my family away from family. Thank you for all the support, for believing so much in me and for always finding a way to cheer me up.

My little brother George, thank you for all the support and for being always a source of optimism. Although you are six years younger, I always look up to you, admire your mind

and your spirit and ask for your advice. I hope that one day you will manage to instill some of your programming skills to me.

Finally, in both English and Greek, I would like to thank my parents.

Mom and Dad, I definitely owe everything to you. I couldn't write this thesis if I didn't have your constant support and help at every level possible. You are the people that I could trust the most and you are always there to give me advice and strength. Words cannot express how much you mean to me. Thank you for making all my dreams come true!

Μαμά και μπαμπά, σίγουρα χρωστάω τα πάντα σε εσάς. Δεν θα μπορούσα να γράψω αυτή τη διατριβή αν δεν είχα τη σταθερή σας υποστήριξη και βοήθεια σε όλα τα επίπεδα. Είστε οι άνθρωποι που μπορω να εμπιστευτώ περισσότερο και είστε πάντα εκεί για να μου δινετε συμβουλές και δύναμη. Οι λέξεις δεν μπορούν να εκφράσουν πόσο πολλά σημαίνετε για μένα. Σας ευχαριστώ που κάνατε όλα τα όνειρά μου πραγματικότητα!