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Level of Individual Differences in a Population of Sprague Dawely Rats in a Behavioral Test Battery and Effect of Dopaminergic Signaling Modulation on Spatial Reference Memory as well as Prefrontal Cortex Protein Expression

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This is to certify that the thesis prepared by Daniel Daba, entitled: “Level of Individual Differences in a Population of Sprague Dawely Rats in a Behavioral Test Battery and Effect of Dopaminergic Signaling Modulation on Spatial Reference Memory as well as Prefrontal Cortex Protein Expression” and submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy (Pharmacology) complies with the regulations of the University and meets the accepted standards with respect to originality and quality

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Abstract

Level of Individual Differences in a Population of Sprague Dawely Rats in a Behavioral Test Battery and Effect of Dopaminergic Signaling Modulation on Spatial Reference Memory as well as Prefrontal Cortex Protein Expression

Daniel Daba Feyissa,

Addis Ababa University, 2019

Spatial reference memory is known to be modulated by the dopaminergic system involving different brain regions with projections to the hippocampus. The roles of dopamine type 1 and 2 receptors are reported to be different upon learning or memory in an activation and task dependent manner. While the hippocampus is traditionally considered a key structure in the formation of spatial memories, recent data has converged on the importance of its connections to the prefrontal cortex supporting memory consolidation, as well as controlling memory retrieval. However, the role of dopamine signaling on prefrontal cortex-mediated spatial reference memory consolidation, and especially on proteomic changes after learning tasks, remains poorly investigated. On the other hand, different early pre-and postnatal experiences and environmental complexity support individual behavior, physiology, and molecular processes during adulthood. For these reasons, it is difficult to generalize between different laboratories but individuality should be estimated for each local population of animals and may then provide more reliable results in animal models of mental and cognitive diseases and individual vulnerability.

The current research is focused on investigation of the role of dopamine type 1 and 2 like receptor signaling on spatial reference memory in “simple” and “difficult” hole-board paradigm as well as the change in proteomic landscape in prefrontal cortex. Furthermore, the current research helps to investigate the level of individual differences in a given population of Sprague-Dawley rats which may indirectly provide some evidences about whether dopaminergic signaling improves spatial reference learning and memory through direct effect or through increasing motivation of animals for search of the reward.

Briefly for the investigation of the level of individual differences in a population of Sprague-Dawley rats, 162 naïve rats were underwent a behavioral test battery including commonly used test paradigm for spatial learning and memory (hole-board) and different behavioral patterns test paradigms such as open field, elevated plus maze, forced swim test as well as rota rod for motor abilities. And to assess the contribution of D_1 and D_2 -like dopamine receptor signaling on spatial learning and memory in a food rewarded hole-board task, a canula was implanted surgically into right side lateral ventricle of 60 rats' age 12-13 weeks. The rats were grouped into 6 groups with 10 rats each. The first four groups were treated with SKF-81297 (1µg and 5µg) and Sumanirole (1µg and 5µg) through intracerebroventricular infusion once 30 min prior to daily training sessions. The other two groups were treated with 1µg of SCH 23390 and Remoxipride following similar procedure. D1 agonist at one dosage (5µg) was used in a more demanding hole-board task. In addition, proteomics studies were carried out to identify proteins that could be influenced by the dopaminergic signaling during the spatial reference memory acquisition and consolidation in a more demanding hole-board task.

The high dimensional behavioral results from behavioral test batteries mentioned above were reduced to fewer components associated with spatial cognition, motivation, anxiety and depression-like behaviors. The behavioural battery test indicated that, 24 % showed a high, 19 % a low and 57 % an intermediate intrinsic state of motivation. The largest 'low' portion (41%) was given for the factor cognition and the largest 'high' portion (29%) for the intrinsic anxiety. Thus, almost every third rat was intrinsically at a high level in the population. Because the animals were intact, untreated and experimentally naïve the results reflect trait patterns of behavior and thus individuality. On the other hand, the hole-board spatial reference memory assessment revealed that D1R agonism induced persistent enhancement of performance, whereas D1R antagonism had no significant effect on spatial reference memory formation. D2R agonist and antagonist exerted no effects. Phase specific comparisons revealed an enhancement of spatial acquisition in the presence of the D1R but not D2R agonism on acquisition, but not during retention. The D1R agonist tested in the hole-board task with increased "difficulty" revealed that D1R agonist treated animals performed significantly better during all training phases, with results better resolved than in the "easy" task. Additionally, proteomic analysis of the prefrontal cortex revealed ninety six proteins to be regulated by D1R agonism, from which 35 were

correlated with behavioral performance. Obtained targets were grouped by function, showing synaptic transmission, synaptic remodeling, and dendritic spine morphology as the major functional classes affected.

The behavioral test batteries pointed out that an experimenter recruiting experimental samples can expect about ≥24 % probability to have intrinsically high animals in terms of anxiety, cognitive, motivation and depressive like behaviours in the sample. Furthermore, activation of D1R signaling during spatial acquisition and consolidation improved reference memory index depending on the task difficulty, with greater effect in a “difficult” task and altered the proteome landscape of the prefrontal cortex indicative of massive organizational synaptic restructuring.

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I am deeply indebted to my advisor Professor Ephrem Engidawork for his guidance, advice on different scientific and methodological aspects of this project, thorough reviewing of my dissertation and making collaboration with Lubec’s proteomics laboratory where I have conducted my research work. I am also thankful to him for accepting me as his PhD student. My sincere gratitude also goes to Professor Gert Lubec, Medical University of Vienna, for giving me the opportunity to carry out this research project in his laboratory and expert advices. I also want to thank Professor Volker korz, Medical University of Vienna, for sharing with me his expertise on different laboratory techniques related to this research project and others. Finally, I would like to pass my acknowledgment to Jimma University for sponsoring my study at Addis Ababa University.

At last, I would like to dedicate my work to my family who has constantly given me encouragement during all difficult times and also to rats whose lives were sacrificed in our efforts to aid biomedical research which might contribute for the betterment of human health.

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This dissertation is based on the following papers

1. Dopamine type 1- and 2-like signaling in the modulation of spatial reference learning and memory; published on *Behavioural Brain Research* (2019)
2. Individual Differences in Male Rats in a Behavioral Test Battery: A Multivariate Statistical Approach; published on *Front. Behav. Neurosci* (2017)

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List of Abbreviations

ADHD	Attention deficit hyperactivity disorder
CA	Cornus Ammonis
CaMKII	Calcium calmodulin kainase II
CRE	Cyclic AMP response element
DCM	Dynamic Causal Modeling
DTT	Dithiothreitol
EC	Entorhinal cortex
EPM	Elevated plus-maze
EPSP	Excitatory postsynaptic potential
fMRI	functional magnetic resonance imaging
FST	Forced swim test
GO	Gene ontology
GOA	Gene ontology Annotation
HCD	High energy Collisional dissociation
HCP	Hippocampus
HEPES	Hydroxyethyl-piperazineethane-sulfonic acid buffer
LTD	Long-term depression
LTM	Long term memory
MAPK	Mitogen activated protein kinase
mPFC	Medial prefrontal cortex
NMDA	N-methyl D-Aspartate
OTT	One-trial tolerance
PCA	Principal component analysis

PET	Positron emission tomography
PHA-L	*Phaseolus vulgaris*-leucoagglutinin
PKA	Protein kinase A
PKC	Protein kinase C
PP1	protein phosphatase-1
RE	Thalamic nucleus reunions
RMI	Reference memory index
STEP	Striatal-Enriched protein tyrosine Phosphatase
TA	Temporoammonic pathway
TEAB	Triethylamoniumbicarbonate
TFA	Trifluoroacetic acid
VH	Ventral hippocampus
VTA	Ventral tegmental area
WM	Working memory

1. Introduction

1.1 Learning and Memory

Memory is essential to behavior, enabling organisms to draw on past experience to improve choices and actions. It can be broadly defined as lasting alterations of a behavioral output produced in response to a transient environmental input (Sweatt, 2010). In order for a transient stimulus to induce a lasting change in behavior, cells must undergo a complex set of stimulus-specific cellular and molecular changes that will consolidate a memory into an everlasting trace. Since the 1960s, memory researchers have recognized the importance of gene transcription and protein synthesis in long-term memory formation in a variety of experimental memory paradigms (Squire et al., 1980).

Learning may be described as the mechanism by which new information about the world is acquired, and memory as the mechanism by which that knowledge is retained. It is convenient to categorize memory as being declarative (explicit), which is defined as that involved in the conscious recall of information about people, places, and things or non-declarative (implicit), which is characterized by the non-conscious recall of tasks such as motor skills. Explicit memory depends on the integrity of temporal lobe and deep lying sub-cortical structures such as the hippocampus, subiculum, and entorhinalcortex. Implicit memory includes simple associative forms of memory, such as classical conditioning, and non-associative forms, such as habituation, and relies on the integrity of the cerebellum and basal ganglia (Squire, 1992).

Memories are subdivided into short- (up to 30 min), intermediate- (hours) and long-term (days to years) memories (Kandel et al., 2014). Short-term memories do not depend on de novo gene expression and protein synthesis, whereas long-term memories depend on de novo transcription and, presumably, on the establishment of new functional synapses (Caroni et al., 2014; Kandel et al., 2014).

Another way of memory classification, may be functional, is working and reference memory. Reference memory represents knowledge for aspects of a task that remain constant between trials. Originally, the term was introduced to distinguish two types of knowledge rats may retain in a radial-arm maze task: knowledge about which arms of the maze always contain a food reward in each trial (reference memory) and memory for the arms that have already been visited in search for food in the current trial (Working memory, WM). Reference memory, unlike WM, is subject to memory consolidation (system), that is, progressive stabilization over time that requires the synthesis of new RNA and proteins, and the implementation of long-lasting morphological changes of synapses in neurons participating in memory representation.

Although introduced and to this day mostly used to describe behavior and task requirements for spatial tasks (mainly radial arm maze, holeboard and the Morris water maze), reference memory represents an operational definition that cannot be exclusively tied to a specific experimental paradigm. Reference memory represents, like any other form of LTM, the end point of a series of processes that, beginning with sensory transduction, attention, and encoding, result in long-lasting behavioral changes, from which the existence of memory is inferred. Consequently, pharmacological interventions at any point in this series of processes can affect performance in memory tests (Barnes, 1988).

Early studies in spatial reference memory in the radial-arm maze (e.g., Olton et al, 1979) suggested that the hippocampus was required for WM but not for reference memory. However, this anatomical distinction had to be abandoned in light of evidence that animals with pre-training lesions to the hippocampus were impaired in acquiring reference memory in the radial-arm maze, and in view of results showing that it is the spatial nature of the radial-arm task, rather than the working or reference memory requirement, that determines hippocampal involvement (Nadel and MacDonald, 1980). It seems, however, that while the initial acquisition of reference memory requires the hippocampus, long-term retention of successfully acquired reference memory may critically depend instead on cortical structures (Barnes, 1988).

1.1.1 Memory Consolidation

Synaptic plasticity can be triggered in seconds, LTP of synaptic transmission in minutes and the emergence of new synapses in 1-2 h, but long-term consolidation of memories occur about 12 h after acquisition (Redondo and Morris, 2011; Bekinschtein et al., 2007). Memory consolidation is the progressive postacquisition stabilization of long-term memory. The term is commonly used to refer to two types of processes, namely, synaptic consolidation and system consolidation. Synaptic consolidation (also cellular consolidation, local consolidation) refers to the post-encoding transformation of information into a long-term form at local synaptic and cellular nodes in the neural circuit that encodes the memory. The current central dogma of synaptic consolidation is that it involves stimulus induced activation of intracellular signaling cascades, resulting in postranslational modifications, modulation of gene expression and synthesis of gene products that alter synaptic efficacy. Synaptic consolidation is traditionally assumed to draw to a close within hours of its initiation, at the end of which it becomes resistant to a number of agents that otherwise can prevent the memory from being converted into the long-term form (‘‘amnesic agents,’’ among them distracting stimuli and pharmacological agents). Synaptic consolidation exists throughout the animal kingdom and occurs in all memory systems studied so far (Dudai, 2004).

Systems consolidation refers to the post-encoding time dependent reorganization of LTM representations over distributed brain circuits (Dudai and Morris, 2000). It is assumed that systems consolidation involves recurrent waves of synaptic consolidation in the new brain locales that receive new or reprocessed experience-dependent information, i.e., synaptic consolidation could be regarded as subroutines in systems consolidation (Dudai, 2012). Systems consolidation may last days to months and even years, depending on the memory system and the task.

Mechanistic studies of learning and memory have focused on sequences of synaptic and cellular plasticity processes, suggesting that cascades of memory consolidation processes starting at the time of acquisition might gradually lead from short- to intermediate- and long-term consolidation of memories (Kandel et al., 2014; Redondo and Morris, 2011). In parallel with plasticity processes initiated at the time of acquisition at individual synapses and neurons, ensembles of synapses and neurons specifically involved in learning can be recruited again through local and system wide network events that are thought to involve replay of learned sequences (Girardeau et al., 2014). Replay processes have been shown to occur during quiet wakefulness and during non-REM sleep, and they are thought to have important roles in memory consolidation (Hsiang et al., 2014).

Mechanism of Memory consolidation

Hippocampus-dependent memory consolidation depends upon Ca^{2+}activation of the cAMP/MAPK/CRE transcriptional pathway (Athos et al., 2002; Sindreu et al., 2007) and de novo protein synthesis (Figure 4) (Kelleher et al., 2004). Stimulation of MAPK activity positively regulates CRE-mediated transcription (Sindreuet al., 2007) and protein synthesis (Kelleher et al., 2004). Activation of CRE-mediated transcription in the hippocampus during consolidation depends upon the calmodulin (CaM)-stimulated adenylyl cyclases (AC1 and AC8), which generate a cAMP signal required for the activation and nuclear translocation of MAPK (Sindreu et al., 2007). Furthermore, the cAMP/MAPK/CRE transcriptional pathway undergoes a circadian oscillation in the hippocampus (Eckel-Mahan et al., 2008) and disruption of this signaling oscillation impairs memory persistence (Eckel-Mahan et al., 2008; Phan et al., 2011).

The CRE integrates Ca^{2+} and cAMP signals (Impey et al., 1998a) and the CRE-binding protein, CREB, is implicated in LTM and other forms of neuroplasticity in mice (Pittenger et al., 2002) and drosophila (Yin et al., 1994). Stimulation of CRE-mediated transcription during training depends on activation of NMDA receptors and MAPK (Athos et al., 2002). This effect was observed by IP administration of a MEK inhibitor, SL327 which blocks fear-associated memory (Atkins et al., 1998) as does administration of PD98059 directly to area CA1 (Athos et al., 2002).

Training for contextual memory activates PKA in a subpopulation of neurons in area CA1 of the hippocampus that also show coactivation of MAPK and MSK-1 (Sindreu et al., 2007). Mice deficient in type 1 adenylyl cyclase (AC1) are deficient in spatial memory (Wu et al., 1995), mossy fiber LTP (Villacres et al., 1998) and cerebellar LTP (Storm et al., 1998). Furthermore, mice lacking both AC1 and AC8, double knockout mice do not exhibit LTM for hippocampus dependent memory (Wang et al., 2004). Interestingly, a transgenic mouse strain in which AC1 is over expressed in the forebrain of mice (AC1 + mice) exhibit enhanced memory for novel objects (Wang et al., 2004) and more persistent remote contextual memory (Shan et al., 2008).

The CRE mediated transcription that depends on activation of NMDA receptor and MAPK is regulated by Striatal-Enriched protein tyrosine Phosphatase (STEP).STEP regulates NMDA receptor and AMPA receptor trafficking as well as MEK downstream proteins such as ERK1/2, p38, Fyn, and Pyk2 activity (Xu et al., 2014). The activity of STEP is controlled by its dephosphorylation by protein phosphatase-1 (PP1). Activation of PKA through cAMP signaling cascade leads to phosphorylation and inactivation of PP1 and the consequent inactivation of STEP (Valjent et al., 2005). This modification of the equilibrium of STEP and MEK activity after cAMP/PKA activation allows for the activation of ERK by MEK (Figure 4).

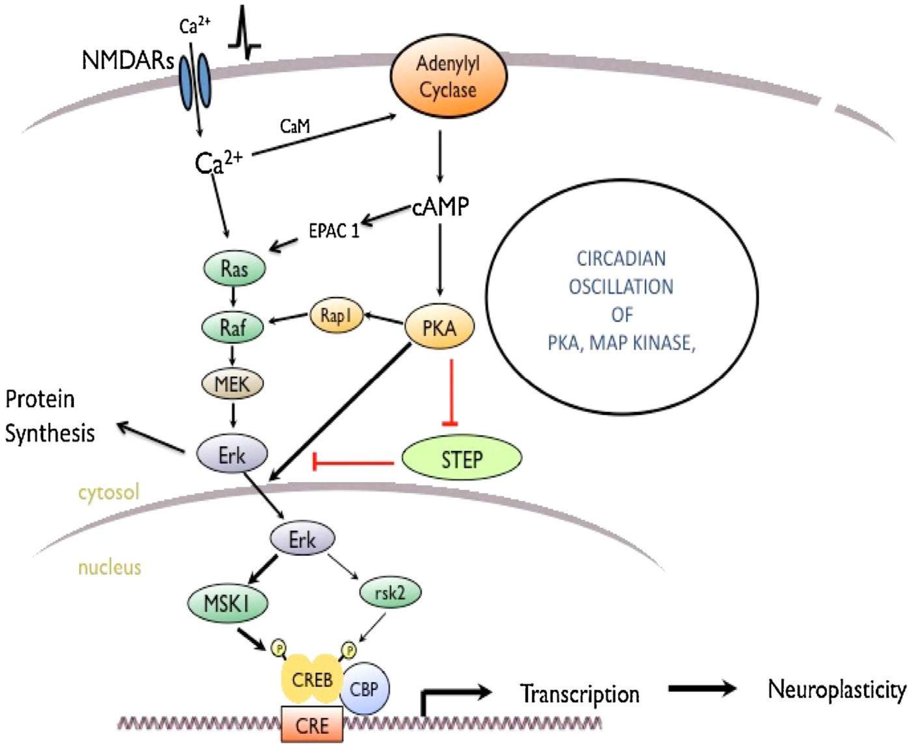


Figure 1: Hypothesis for the persistence of hippocampus-dependent memory: **It is hypothesized that MAPK is activated during training because of upstream cAMPstimulationofRas and/or Rap1 coupled with PKA inhibition of STEP phosphatase. The eventual decrease in pMAPK may be due to calcineurin reactivation of STEP. It is proposed thatthe persistence of hippocampus-dependent memory is dependent on the CaM-stimulated adenylyl cyclases and that memory is maintained by a circadian oscillation ofthis pathway in the hippocampus. It is hypothesized that the cAMP/MAPK/CREB transcriptional pathway reaches a maximum during the dark phase of the circadian cycle, specifically in REM sleep.**

1.1.2 Memory Retrieval

Memory is often considered to be a process that has several stages, including acquisition, consolidation and retrieval (Abel and Lattal, 2001). Understanding mechanism of memory retrieval is very crucial since most patients with memory deficits consult because they cannot remember, not because they cannot learn.

When humans and animals form new memories, contextual information associated with the experience is also routinely encoded without awareness (Tulving and Thomson, 1973). Contextual information plays an important role in memory retrieval since the content of *what* is often critically dependent on *where* that memory is retrieved (Maren and Holt, 2000; Bouton, 2002). This ‘‘contextual retrieval’’ process allows the meaning of a cue to be understood according to the context in which it is retrieved (Maren et al., 2013). For example, encountering a lion in the wild might be a life- threatening experience to someone, but seeing the same lion kept in its cage in the zoo might be an interesting (andnon-threatening) experience. Therefore, the same cue in different contexts has totally different meanings. Contextual processing is highly adaptive because it resolves ambiguity during memory retrieval (Bouton, 2002; Maren et al., 2013; Garfinkel et al., 2014).

Mechanism of Memory Retrieval

Initial events leading to long-term memory formation in different memory tasks involve the activation of glutamate AMPA, NMDA, and metabotropic (mGluR) receptors in the hippocampus (McGaugh, 2000; Steele and Morris, 1999; Riedel et al., 1999). Similarly, but not equally, memory retrieval seems to require activation of the glutamate receptors. Blockade of hippocampal AMPA/kainate receptors 10 min before testing impairs retrieval of inhibitory avoidance response (Szapiro et al., 2000). Similarly, inactivation of AMPA/kainate receptors before testing of a water maze task, a multi-trial spatial learning, diminished memory retrieval as well (Riedel et al., 1999). In addition, blockade of mGluRs by a general antagonist as well as systemic administration of a specific selective mGluR5 antagonist before testing, both prevent retrieval of contextual fear memories (Szapiro et al., 2000; Schulz et al., 2001). In contrast, pretest administration of NMDA receptors antagonists, at doses that blocked memory formation in two learning tasks when given at the time of training (Steele and Morris, 1999), did not alter memory retrieval (Szapiro et al., 2000; Steele and Morris, 1999). However, several downstream protein kinases have been shown to participate in memory retrieval. Protein kinase C, the calcium-dependent isoforms alpha and beta I, (Vianna et al., 2000), MAPKs (p42 and p44) and PKA pathways (Szapiro et al., 2000) are necessary for memory retrival. Therefore, the tenet that retrieval is or must be a function of consolidation or at least involve mechanisms similar to it (Riedel et al., 1999), might only be partially true.

1.1.3 Memory and Hippocampus

The hippocampus comprises distinct sub-regions (i.e., CA1 to CA3, dentate gyrus and subiculum) and is part of the hippocampal formation, which also includes the parahippocampal, perirhinal and entorhinal cortices (Simons and Spiers, 2003). Figure 1 gives an overview of the input and output pathways of the hippocampal formation as described inMilner et al. (1998).

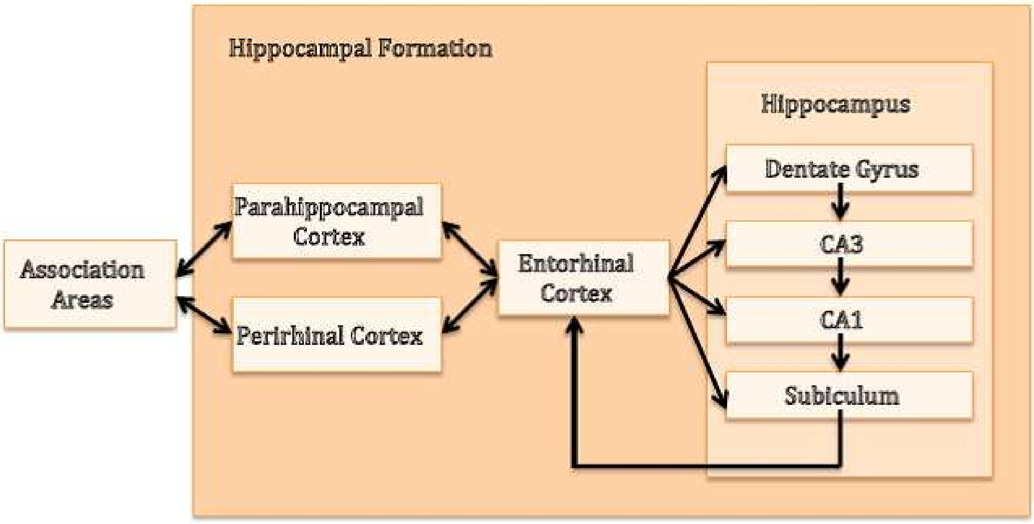


Figure 2: Input and output pathways of the hippocampal formation.(adapted from Milner et al., 1998).

Although several areas of the brain play a part in encoding, consolidation and retrieval of several forms of memory, the hippocampus has been recognized as playing a vital role in formation of declarative memory in particular, which describes the synthesis of episodic and semantic memories. The observations of Scoville and Milner in 1957, showing that bilateral hippocampal removal as a treatment of epilepsy for patient H.M. resulted in anterograde amnesia, explicitly identified the importance of the role of the hippocampus and temporal lobe structures in memory. Another relevant case study is patient R.B. who suffered from anterograde amnesia after a lesion of hippocampal CA1 region (Zola-Morgan et al., 1986). Additional evidence of the hippocampal role in episodic memory provide case studies of three children who suffered brain injuries at several ages (birth, four years old, and nine years old) in which hippocampus was affected (Vargha-Khadem et al., 1997).Since these findings, studies in humans (Squire et al., 1984) and animals (Morris et al., 1990) have consolidated the essential role of hippocampus in declarative memory.

Non-invasive methods using direct brain imaging techniques such as magnetic resonance imaging and positron emission tomography (PET) that characterized blood flow and oxygen use in the hippocampus have identified fluctuations in these parameters during learning tasks (Squireet al., 1990; Squire, 1992). Furthermore, several works studying lesions in animals (Anagnostaras, 1999; Zola and Squire, 2001), patients (Teng and Squire, 1999) and transgenic approaches (Minichiello et al., 1999) suggested that the hippocampal formation plays a crucial role in various types of memory.

It appears that the key role of the hippocampus in spatial learning is synthesis of the configuration of spatial cues, which is governed, at least to some extent, by temporal events (Teng and Squire, 1999**)**. Spatial ability is broadly defined as the ability to perceive, encode, store, retrieve, transform and integrate spatial information drawn from two- or three-dimensional space (Ecuyer-Dab and Robert, 2004). Spatial learning and memory help animals find locations that provide, among other things, food and safety, and therefore are crucial for survival. Rats displaying extraordinary spatial abilities are commonly used in studies examining animals' cognitive capacity in spatial tasks that comprise a variety of mazes (Cimadevilla et al. 2000).

In addition to the projection to subiculum, CA1 neurons project to the perirhinal, postrhinal, and entorhinal cortices, and a number of studies have suggested that these pathways play a role in various forms of learning and memory (O'Mara et al., 2000). Evidence suggests that positional information relies on hippocampal-subicular interaction, directional information on the interaction between postsubiculum and subiculum, and sensory information on the interaction between entorhinal cortex and subiculum (O'Mara et al., 2000).

While recognizing the primary role of the hippocampus in memory formation, the interaction with cortical structures, particularly in the context of long-term storage of memories, remains an issue of debate, and it has been proposed that sequential activation of the hippocampus and neocortex may be involved in consolidation of memory. Data from a systematic study on individuals with lesions of the hippocampus, in some cases extending to the temporal cortex, revealed that the extent of the lesion (from that affecting only the CA1, CA3, and dentate gyrus to that involving the entire hippocampal complex and temporal lobe) dictated the degree of impairment in recall and, to some extent, the remoteness of the memory. The development of neuroimaging techniques has allowed further assessment of the role of the hippocampal complex in retrieval of distant memories, and the evidence suggests that activation of hippocampal circuits occurs even when very remote memories are elicited (Nadel et al., 2000). Analysis of this question in animals has revealed that sectioning the fornix, or damaging the hippocampus or entorhinal cortex, typically impaired very recent memory, but generally spared more remote memory. This suggests that the hippocampus is necessary for memory storage and retrieval for only a limited time after learning and that time-related modification of cortical connections allows for memory retrieval independent of the hippocampus (Squire and Alvarez, 1995). However, it has been pointed out that this might also be explained if representation of older memories was more diffusely distributed in hippocampus. In this case, temporally graded retrograde amnesia could be explained because a partial lesion of the hippocampus will spare a remote memory more than a recent memory, whereas complete hippocampal lesions will affect recent and remote memories equally (Nadel and Moscovitch, 1997).

The pyramidal neurons in hippocampal CA1 receive two types of input from the entorhinal cortex (Figure 2). Neurons in layer II of the entorhinal cortex project indirectly to CA1 via the dentate gyrus and CA3 region through the perforant path. While neurons in entorhinal layer III primarily terminate on the distal dendrites of pyramidal neurons in CA1 and the subiculum. This monosynaptic pathway from the entorhinal cortex to CA1 is called the temporoammonic pathway (TA). Studies show that lesioning the TA inputs from the entorhinal cortex to CA1 region did not affect the formation of hippocampal memories, but prevented them from becoming consolidated in cortical networks (Remondes and Schuman, 2002; Burgess et al., 2002). When inputs from area CA3 were removed to isolate the TA-CA1 circuit, place fields of CA1 neurons were preserved, and rats with an isolated CA1 area performed normally in spatial recognition tasks, but were impaired in spatial navigation (Brun et al., 2002).These observations suggest that the direct entorhinal-hippocampal connections play a unique and pivotal role in spatial learning and memory.

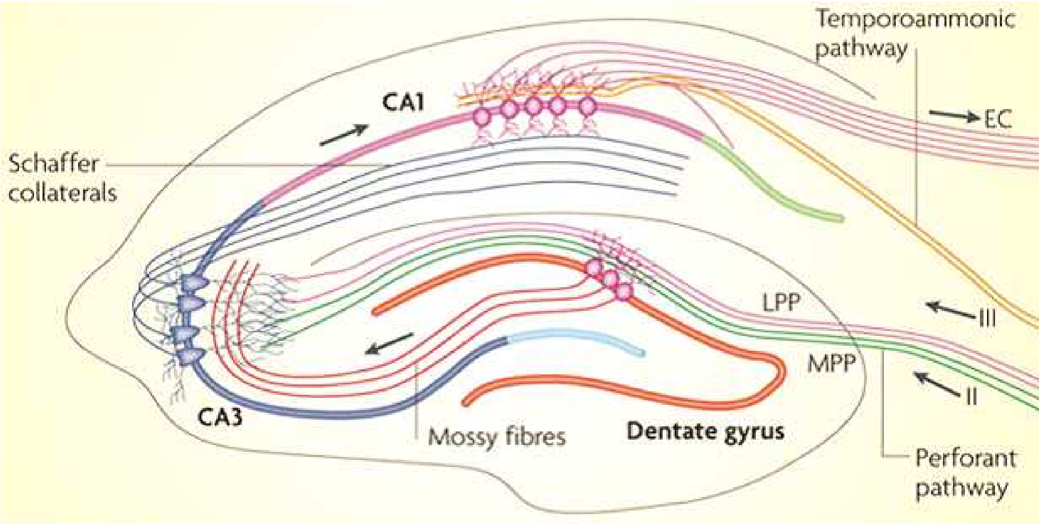


Figure 3: Diagram of the hippocampal neural network (Deng. et al., *Nat Rev Neurosci*. 2010)

1, 1.4 Memory and Prefrontal Cortex

The empirical literature on the medial prefrontal cortex (mPFC) is dominated by studies of its role in decision making, including conflict monitoring (Botvinick et al., 2004), error detection (Holroyd et al., 2002), executive control (Posner et al., 2007), reward-guided learning (Rushworth et al., 2011), and decision making about risk and reward (Bechara and Damasio, 2005). However, the mPFC also plays a key role in memory, as highlighted by its selective involvement in the retrieval of "remote" memories (i.e., items learned several weeks earlier) (Frankland et al., 2004; Takashima et al., 2006). Other studies implicate mPFC in "recent" memory, learned 1-2 days earlier. For example, inactivation of mPFC impairs the recall of fear memory learned the previous day (Corcoran and Quirk, 2007). Hence, the mPFC plays a role in both recent and remote memory. Other studies have emphasized the role of mPFC in the consolidation of memories, in that interfering with mPFC immediately after learning disrupts subsequent recall in many tasks (Tronel and Sara, 2003). All of these studies implicate mPFC in what might be defined as "long-term" memory (i.e., memory spanning several hours or longer). There is also evidence that mPFC is important for "short-term" memory, spanning seconds to minutes. For example, rats with mPFC lesions have difficulty recalling place-reward associations over a 30 min delay (Seamans et al., 1995) or waiting for a response cue over a 30 second delay

(Narayanan et al., 2006). In summary, there is evidence that the mPFC playsa critical role in remote, recent and short-term memories over a broad range of tasks.

1.1.5 Interaction of Prefrontal Cortex with Hippocampus

Given the prominent role of hippocampus in memory, it is no surprise that the hippocampus and mPFC are anatomically related. Compared to other cortical areas, projections from the ventral half of the hippocampus and subiculum to mPFC are particularly strong (Cenquizca and Swanson, 2007; Jay and Witter, 1991). The pathway is unidirectional but may be reciprocated via a bi-synaptic route through the nucleus reunions (RE) or lateral entorhinal cortex (Figure 3) (Vertes et al., 2007).

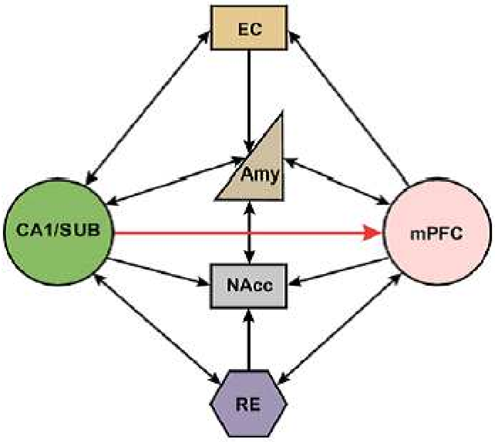


Figure 4: Schematic representation of direct and indirect neural circuits between the medial prefrontal cortex and hippocampus/subiculum:**Hippocampal area CA1 and the subiculum (SUB) have strong direct projections to the mPFC, but there are no direct projections from the mPFC back to the HPC. The reuniens (RE) and amygdala has reciprocal connections with both the mPFC and HPC. NAcc receives inputs from mPFC, HPC, RE and amygdala. mPFC also project to entorhinal cortex (EC) which in turn has reciprocal projections with HPC. SUB, subiculum; EC, entorhinal cortex; Amy, amygdala; NAcc, nucleus accumbens; RE, nucleus reuniens; mPFC, medial prefrontal cortex.**

Researchers have started investigating the interplay of HPC and vmPFC in several related contexts, such as retrieval-mediated learning (Zeithamova et al., 2012), learning and choosing based on conceptual (episodic) knowledge (Kumaran et al., 2009), deliberative decision-making (Yu and Frank, 2015) or memory-based preferential choices (Gluth et al., 2015). Although more and more research focus on the connections between these two regions, the neural mechanism underlying the coordinated action of HPC and PFC are still unclear (Shin and Jadhav, 2016). However, there are several lines of evidence for functional interaction between mPFC and HPC.

To mention some, hippocampal theta-band (5-10 Hz or 4-8 Hz) oscillations (Buzsáki, 2002), which is associated with spatial learning and memory consolidation in rats (Benchenane et al., 2010; Rangel et al., 2016) is synchronized with prefrontal region in rodent memory studies (Colgin, 2011) and in human decision making (Guitart-Masip et al., 2013). Similarly, Dynamic Causal Modeling (DCM) (Friston et al., 2003) of the functional magnetic resonance imaging (fMRI) data showed that the bidirectional flow of memory-related information observed between HPC and PFC supports memory formation (consolidation), contextual memory retrieval and memory-guided decisions (Shin and Jadhav, 2016). Furthermore, functional disconnection studies which involves inactivation of hippocampus and mPFC in either of the hemisphere (Floresco et al., 1997)demonstrate that mPFC-hippocampal communication is necessary for short-term memory in paradigms including the water maze (Wang and Cai, 2008), the T-maze (Wang and Cai, 2006), spatial win-shift on the radial arm maze (Floresco et al., 1997; Goto and Grace, 2008), and the Hebb-Williams maze, a spatial maze requiring a specific set of turns to reach reward (Churchwell et al., 2010). In fact, the effects of disconnection are nearly the same as those seen after bilateral mPFC inactivation; supporting the claim that mPFC is dependent upon the hippocampal-mPFC pathway either for context or for rapid learning.

i) Anatomy of Prefrontal Cortex-Hippocampus Projection

It has long been appreciated that there are both direct monosynaptic projections, as well as indirect polysynaptic projections between the HPC and the mPFC (Hoover and Vertes, 2007). In rats, injections of retrograde tracers into different areas of the mPFC robustly label neurons in the ventral hippocampus (VH) and subiculum (Hoover andVertes, 2007). In addition, injections of the anterograde tracer, *Phaseolus vulgaris*-leucoagglutinin (PHA-L), into the HPC reveal direct projections to the mPFC (Jay and Witter, 1991). Hippocampal projections to the mPFC originate primarily in ventral CA1 and ventral subiculum; there are no projections to the mPFC from the dorsal hippocampus or dentate gyrus. Hippocampal projections course dorsally and rostrally through the fimbria/fornix, and then continue in a rostro-ventral direction through the septum and the nucleus accumbens (NAcc), to reach the infra limbic prefrontal cortex (IL), prelimbic prefrontal cortex (PL), medial orbital cortex, and anterior cingulated cortex (Jay andWitter, 1991; Cenquizca and Swanson, 2007). Afferents from CA1 and the subiculum are observed throughout the entire rostro-caudal extent of the mPFC, with only sparse projections to the medial orbital cortex.

Indirect multi-synaptic pathways from the HPC to mPFC include projections through the NAcc and ventral tegmental area (VTA), amygdala, entorhinal cortex (EC), and midline thalamus (Russo and Nestler, 2013; Wolff et al., 2014). These complex multi-synaptic pathways from both subcortical and cortical areas are critically involved in higher cognitive functions that are related to several major psychiatric disorders. For example, it has been reported that NAcc receives convergent synaptic inputs from the PFC, HPC and amygdale (Groenewegen et al., 1999). This cortical-limbic network has been shown to mediate goal-directed behavior by integrating HPC-dependent contextual information and amygdala-dependent emotional information with cognitive information processed in the PFC (Goto and Grace, 2008). In addition, the mPFC projects to the thalamic nucleus reunions (RE), which in turn has dense projections to the HPC (Varela et al., 2014). Importantly, this projection is bidirectional, which provides another route for the HPC to influence the mPFC (Figure 3). Interestingly, it has been shown that single RE neurons send collaterals to both the HPC and mPFC (Hoover and Vertes, 2012; Varela et al., 2014). This places the RE in a key position to relay information between the mPFC and HPC to coordinate their functions (Hoover andVertes, 2012; Varela et al., 2014; Griffin, 2015; Ito et al., 2015). The mPFC also has strong projections to the EC, which in turn has extensive reciprocal connections with hippocampal area CA1and the subiculum (Cenquizca and Swanson, 2007). Interestingly, the CA1 and subiculum send direct projections back to the mPFC, allowing these areas to form a functional loop that enables interactions between cortical and subcortical areas during memory encoding and retrieval (Preston and Eichenbaum, 2013).

ii) The physiology of Hippocampus-Prefrontal Cortex Projections

The physiology of projections between the HPC and mPFC has been extensively investigated in rodents. These projections consist of excitatory glutamatergic pyramidal neurons that terminate on either principal neurons or GABAergic interneurons within the mPFC (Carr and Sesack, 1996; Tierney et al., 2004). Electrical stimulation in hippocampal area CA1 or the subiculum produces a monosynaptic excitatory postsynaptic potential (EPSP) followed by fast and slow inhibitory postsynaptic potentials (IPSPs); the latter are due to both feed-forward (Tierney et al., 2004) and feedback inhibition (Dégenètais et al., 2003). Excitatory responses evoked in mPFCneurons by electrical stimulation of the HPC are antagonized by CNQX but not by AP5, indicating that these responses are AMPA-receptor dependent (Jay et al., 1992). Hippocampal synapses in the mPFC exhibit activity-dependent plasticity including long-term potentiation (LTP), long-term depression (LTD), and depotentiation (Laroche et al., 2000; Takita et al., 1999). These forms of plasticity are NMDA receptor-dependent and involve activation of serine/threonine kinases such as CaMKII, PKC, and PKA (Burette et al., 1997; Takita et al., 1999).

Within the indirect mPFC-RE-HPC pathway, a large proportion of RE projection neurons are glutamatergic (Bokor et al., 2002). Nucleus reunion stimulation produces strong excitatory effects on both HPC and PFC neurons (McKenna and Vertes, 2004), suggesting that the RE is capable of modulating synaptic plasticity in both the HPC and mPFC (Eleore et al., 2011).

1.2 Roles of Dopamine in Learning and Memory

1.2.1 Dopamine System

Since the discovery of the physiological functions of 3-hydroxytyramine (dopamine), a metabolite of the amino acid tyrosine, more than 60 years ago (Carlsson et al., 1957), this catecholaminergic neurotransmitter has attracted an enormous amount of attention. In a similar manner to other monoamine neurotransmitters, dopamine generally exerts its actions on neuronal circuitry via a relatively slow modulation of the fast neurotransmission mediated by glutamate and GABA. Dopaminergic innervations are the most prominent in the brain. Four major dopaminergic pathways have been identified in the mammalian brain; the nigrostriatal, mesolimbic/mesocortical and tuberoinfundibular systems that originate from the A9 (nigrostriatal), A10 (mesolimbic and mesocortical, often collectively termed the mesocorticolimbic pathway), and A12 (tuberoinfundibular) groups of dopamine-containing cells (Anden et al., 1964; Dahlstroem and Fuxe, 1964), respectively. These neurons are critically involved in various vital central nervous system functions, including voluntary movement, feeding, affect, reward, sleep, attention, learning and memory. In the periphery, dopamine plays important physiological roles in the regulation of olfaction, retinal processes, hormonal regulation, cardiovascular functions, sympathetic regulation, immune system, and renal functions, among others (Carlsson, 2001; Iversen and Iversen, 2007).

1.2.2 Dopamine Neurons Activity

DA neurons exhibit two distinct modes of spike firing: tonic single spike activity and burst spike firing (Grace and Bunney, 1984a, b). Tonic firing refers to spontaneously occurring baseline spike activity and is driven by pacemaker-like membrane currents of DA neurons (Grace and Onn, 1989). However, these DA neurons are under the influence of a very potent GABAergic inhibition, preventing some DA neurons from firing spontaneously in the basal condition (Grace and Bunney, 1984b). Tonic firing of DA neurons has been shown to underlie the baseline tonic level of DA concentration within the striatum (e.g. 10-20 nM within the striatal region (Keefe et al., 1993). Studies suggest that this is mediated by an escape of DA from the synapse into the extra synaptic space (Floresco et al., 2003). Therefore, the concentration of tonic extracellular DA is dependent on the number of DA neurons demonstrating spontaneous tonic spike activity (Floresco et al., 2003).

The functional relevance of this tonic DA release is unknown. However, transient suppression of tonic spiking in DA neurons follows the omission of expected reward, somehow implicating this spiking pattern in reward-based learning (Tobler et al., 2003). DA release in the striatum has been shown gradually increase (ramps up) as rats expect distant reward, perhaps providing motivational drive (Howe et al., 2013). Studies have also revealed that a transient suppression of tonic spike firing in DA neurons occurs in response to aversive stimuli (Ungless et al., 2004). In contrast, phasic activation of the DA system represented by the burst spike firing pattern is dependent on glutamatergic excitatory synaptic drive onto DA neurons from a number of areas, including the pedunculopontinetegmentum (PPTg) (Floresco et al., 2003) and the subthalamic nucleus (Smith and Grace, 1992). Burst spike firing triggers a high amplitude (e.g. mM to hundreds of mM levels), transient, phasic DA release intrasynaptically within the targeted areas (Floresco et al., 2003). This high amplitude DA release is nonetheless suggested to be subject to powerful, immediate reuptake into pre-synaptic terminals via DA transporters (Suaud-Chagny et al., 1995), and therefore, phasic DA release would act transiently within the synaptic cleft and in very close proximity to the synapse (Floresco et al., 2003; Venton et al., 2003).

Dopamine neurons show phasic activations following unpredicted reward coding a quantitative “prediction error” signal, namely the difference between received and predicted reward value. A reward that is better than predicted elicits an activation (positive prediction error response), a fully predicted reward draws no response, and a reward that is worse than predicted induces a decrease in activity (negative error response) (Schultz,2007,2013). These prediction error responses of DA cells have been closely related to reinforcement learning models which assign a functional role of DA in modulating cortico-striatal inputs through a reward-prediction error teaching signal (Morris et al., 2006; Pan et al., 2008). In fact, fast DA release consistent with these reward predicting signals of DA neurons has been measured in nucleus accumbens during associative learning (Day et al., 2007). Besides “classic” reward-prediction error responses, phasic DA cell firing patterns also include responses to salient and aversive sensory stimuli (Brischoux et al., 2009; Matsumoto and Hikosaka, 2009).

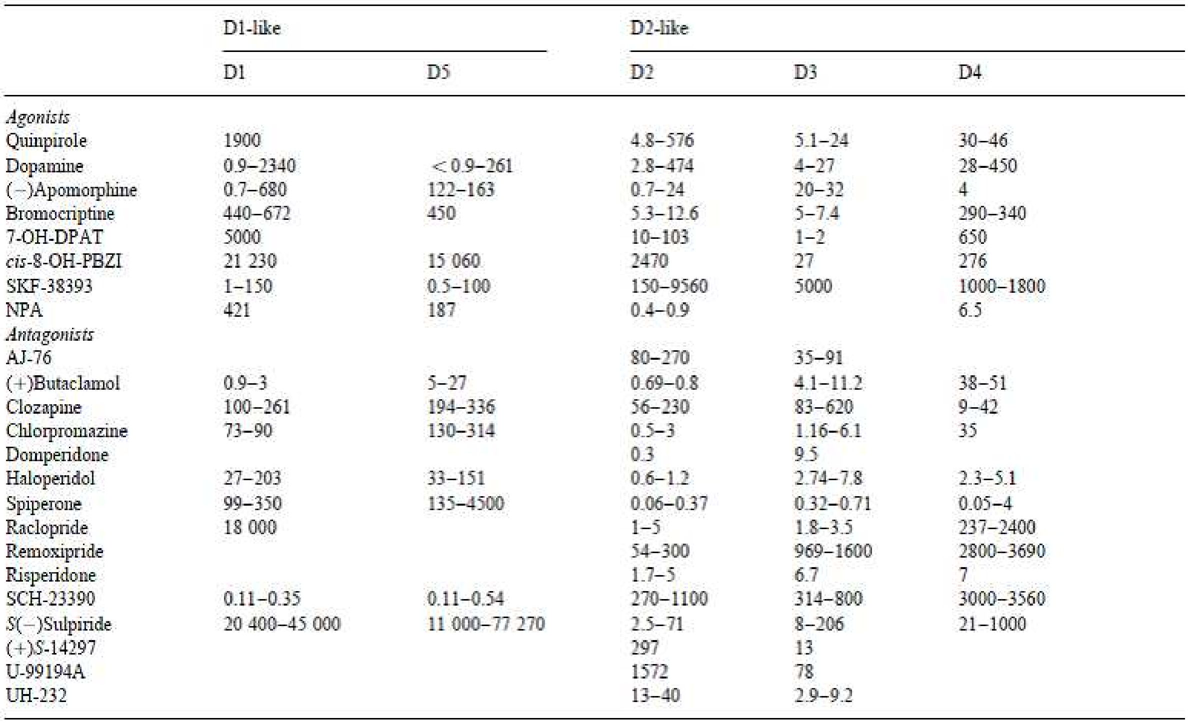
1.2.3 Dopamine Receptors

The physiological actions of dopamine are mediated by binding to five distinct but closely related membrane receptors, which belong to the family of seven transmembrane domain (7TM) G-protein coupled receptors. They are divided into two major groups: the D1 like (D1 and D5) and D2 like (D2, D3 and D4) classes of dopamine receptors (Vallone et al., 2000).

Individual members of the subfamilies of the D1- and D2-class receptors share a high level of homology at their transmembrane domains and have distinct pharmacological properties. It is commonly accepted that the D1-class dopamine receptors (D1 and D5) activate the Gαs/olf family of G proteins to stimulate cAMP production by AC and are found exclusively postsynaptically on dopamine-receptive cells, such as GABA-ergic medium spiny neurons (MSNs) in the striatum. The D2-class dopamine receptors (D2, D3, and D4) are coupled to the Gαi/o family of G proteins and thus induce inhibition of AC. In contrast to the D1-class dopamine receptors, D2 and D3 dopamine receptors are expressed both postsynaptically on dopamine target cells and presynaptically on dopaminergic neurons (Rankin et al., 2010; Rondou et al., 2010).

Dopamine activates D1 to D5 dopamine receptors with various affinities ranging from nanomolar to micromolar range (Table 1). In general, different subtypes of dopamine receptors vary significantly in their sensitivity to dopamine agonists and antagonists (Sokoloff et al., 2006; Rankin et al., 2010; Rondou et al., 2010).

Table 1: Pharmacological properties of dopamine receptors



	D1-like		D2-like		
	D1	D5	D2	D3	D4
Agonists					
Quinpirole	1900		4.8–576	5.1–24	30–46
Dopamine	0.9–2340	< 0.9–261	2.8–474	4–27	28–450
(−)Apomorphine	0.7–680	122–163	0.7–24	20–32	4
Bromocriptine	440–672	450	5.3–12.6	5–7.4	290–340
7-OH-DPAT	5000		10–103	1–2	650
cis-8-OH-PBZI	21 230	15 060	2470	27	276
SKF-38393	1–150	0.5–100	150–9560	5000	1000–1800
NPA	421	187	0.4–0.9		6.5
Antagonists					
AJ-76			80–270	35–91	
(+)Butaclamol	0.9–3	5–27	0.69–0.8	4.1–11.2	38–51
Clozapine	100–261	194–336	56–230	83–620	9–42
Chlorpromazine	73–90	130–314	0.5–3	1.16–6.1	35
Domperidone			0.3	9.5	
Haloperidol	27–203	33–151	0.6–1.2	2.74–7.8	2.3–5.1
Spiperone	99–350	135–4500	0.06–0.37	0.32–0.71	0.05–4
Raclopride	18 000		1–5	1.8–3.5	237–2400
Remoxipride			54–300	969–1600	2800–3690
Risperidone			1.7–5	6.7	7
SCH-23390	0.11–0.35	0.11–0.54	270–1100	314–800	3000–3560
S(−)Sulpiride	20 400–45 000	11 000–77 270	2.5–71	8–206	21–1000
(+)*S*-14297			297	13	
U-99194A			1572	78	
UH-232			13–40	2.9–9.2	

The *K*i values (nM) of several compounds are listed. For many ligands the lowest and the highest *K*i found in the literature are given. Discrepancies among the values are due to differences among the cell types used for the binding experiments or to the affinity state of the receptor. (7-OH-DPAT, 7-hydroxy-*N*-*N*-di-*n*-propyl-2-aminotetralin; *cis*-8-OH-PBZI,*cis*-8-hydroxy-3-(*n*-propyl)1,2,3a,4,5,9b-hexahydro-1H-benz[e]indole NPA: *N*-propylnorapomorphine) Dopamine Receptor

Dopamine Receptors Signaling

The complexity of the signaling network that is regulated by cAMP downstream from dopamine receptors also provides mechanisms for a context-dependent regulation of cellular responses to dopamine. For example, it is possible that many of the consequences of dopamine receptor stimulation occur only under specific conditions that require the coactivation of other types of receptors. These types of cellular responses that are dependent on the co-occurrence of different forms of stimulation are designated as coincidence detectors and are known to play a central role in the regulation of synaptic plasticity. MAP kinases have been shown to act as important coincidence detectors that integrate the actions of dopamine with those of other neurotransmitter systems.

Many MAP kinases have been shown to be signaling intermediates that are involved in the regulation of dopamine associated behaviors (Berhow et al., 1996). D1 dopamine receptors are essential for the activation of these kinases in medium spiny neurons (MSNs) (Liu et al., 2006; Valjent et al., 2005; Zhang et al., 2004). In contrast, D2-class dopamine receptors, in particular D3 dopamine receptors, have been shown to mediate the inhibition of ERK-mediated signaling in the striatum (Zhang et al., 2004).

The activation of ERK by D1 dopamine receptors in response to cocaine administration has been shown to be dependent upon the stimulation of ionotropic glutamate receptors and to be preventable by the NMDA receptor antagonist, MK-801 (Valjent et al., 2005). The regulation of ERK phosphorylation by D1 dopamine receptors and NMDA receptors may result from the convergence of several signaling modalities that are regulated by these two types of receptors. NMDA receptor stimulation results in activation of the ERK kinase by MEK. In the absence of D1 dopamine receptor stimulation, the action of MEK on ERK activity is counteracted by the activity of STEP, resulting in a zero-sum equilibrium in which the overall activity of ERK remains unchanged. The activity of STEP depends on its dephosphorylation by PP1. Activation of the D1 dopamine receptor/PKA/DARRP-32 signaling cascade leads to the inactivation of PP1 and the consequent inactivation of STEP (Valjent et al., 2005). This modification of the equilibrium of STEP and MEK activity after D1 dopamine receptor stimulation allows for the activation of ERK by MEK (Figure5A). The co-regulation of ERK by D1 dopamine receptors and NMDA receptors has indicated that ERK might act as a signal integrator for dopamine and glutamate neurotransmission during development of the behavioral responses mediated by these two neurotransmitters.

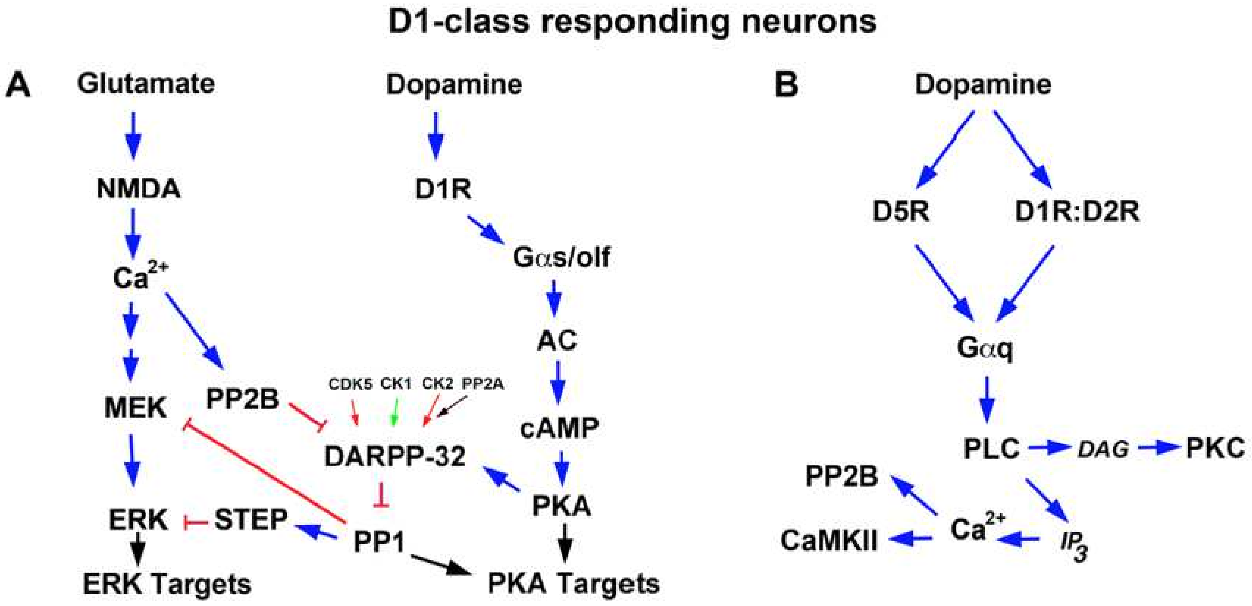


Figure 5: Signaling networks regulated by dopamine in D1-class dopamine receptor responding neurons: **Regulation of G_s/cAMP/PKA signaling by D1-class dopamine receptors (A); Regulation of Gq/PLC signaling by D1-class dopamine receptors or D1:D2 dopamine receptor heterodimers (B). The action of other neurotransmitters, growth factors, and neurotrophins has been included to illustrate the role of these intermediates as signal integrators. Blue arrows indicate activation, red T-arrows indicate inhibition, and green arrows indicate the amplification of an already activated function. Black arrows indicate actions that can either be activatory or inhibitory on the function of specific substrates. D1R, D1 dopamine receptor; D2R, D2 dopamine receptor; D5R, D5 dopamine rceptor.**

There is also evidence that, in addition to their effects on cAMP regulated signaling, dopamine receptors can also couple to Gq to regulate phospholipase C (PLC) (Figure5B). Activation of PLC results in the activation of PKC by DAG and an increased mobilization of intracellular calcium in response to IP3. Dopamine regulate Gq mediated signaling through activation of either D5 receptor (Sahu et al., 2009; So et al., 2009) or D1/D2 receptors heterodimers (Lee et al., 2004; Perreault et al., 2010; Zhang et al., 2010).

D2-class receptors also modulate intracellular calcium levels (Figure 6, A and B) by acting on ion channels or by triggering the release of intracellular calcium stores (Nishi et al., 1997; Missale et al., 1998).

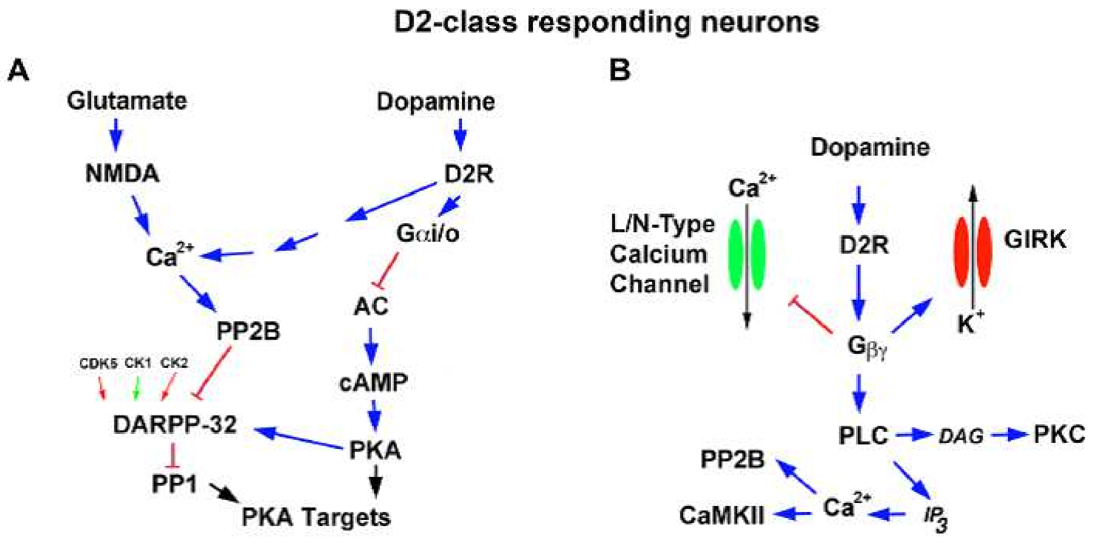


Figure 6: Signaling networks regulated by dopamine in D2-class dopamine receptor responding neurons: (**A): Regulation of Gis/cAMP/PKA signaling by D2-class receptors (B): Regulation of Gβγ signaling by D2-class receptors. Blue arrows indicate activation, red T-arrows indicate inhibition, and green arrows indicate the amplification of an already activated function. Black arrows indicate actions that can either be activatory or inhibitory on the function of specific substrates.**

Some of these actions of D2-class receptors on calcium signaling seem to be related to signaling responses that are mediated by the Gβγ subunits of heterotrimeric G proteins. Gβγ subunits that are regulated by D2 dopamine receptors have been shown to activate PLC and to increase the cytoplasmic calcium concentration in MSNs (Hernandez-Lopez et al., 2000). It is noteworthy that this mechanism also reduces the level of activity of L-type calcium channels in these cells, indicating that stimulation of D2 dopamine receptors has complex effects on calcium-mediated biological processes in these neurons (Figure 6B). It is worth mentioning that D2 dopamine receptor-regulated Gβγ subunits are also involved in the regulation of N-type calcium channels in striatal interneurons (Yan et al., 1997. The Gβγ are also involved in the association/regulation of the D2 dopamine receptor and G protein-coupled inwardly rectifying potassium channels (GIRKs) (Kuzhikandathil et al., 1998; Lavine et al., 2002). The activation of GIRKs after activation of the D2 dopamine receptor (Figure 6B) and other GPCRs has an inhibitory effect in neurons and could potentially mediate several functions of dopamine in vivo.

1.2.4 Dopaminergic modulation of Learning and Memory

Numerous reports suggest that the VTA is the main source of dopamine to the dorsal hippocampus (McNamara et al., 2014; Gasbarri et al., 1997). The observation of an increase in VTA firing rate when an animal is presented with novel stimuli (Horvitz et al., 1997; Kitchigina et al., 1997) and the subsequent increase of dopamine levels in the hippocampus during exploration of a novel environment (Ihalainen et al., 1999) substantiated the reports. However, the dense dopamine receptor expression and sparse VTA dopamine axon networks in the dorsal hippocampus creates discrepancy about the source of dopamine in the hippocampus. There is a strong projection of neurons from locus coeruleus (LC) to the dorsal hippocampus. This led to the assumption that the LC may supply the hippocampus with dopamine (Devoto and Flore, 2006; Lemon and Manahan-Vaughan, 2012; Smith and Greene, 2012). The current research proves that neurons of the LC contribute to the strong dopaminergic tone in the dorsal hippocampus (Kempadoo et al., 2016).

In the hippocampus, activation of dopamine D1/D5 receptors reduces the threshold for induction of synaptic plasticity that lasts for very long (>24 h) periods of time in rodents (Hansen and Manahan-Vaughan, 2014; Hagena et al., 2016). Correspondingly, weak synaptic potentiation is prolonged into persistent long-term potentiation if patterned afferent stimulation occurs in the presence of dopamine D1/D5 receptor agonists (Tenorio et al., 2010; Hagena et al., 2016; Hansen and Manahan-Vaughan, 2015). Moreover, hippocampal LTP is impaired by dopamine D1/D5 receptor antagonists (Straube and Frey, 2003; Hansen and Manahan-Vaughan, 2015). LTP is tightly associated with spatial learning and exploration of a novel environment results in the strengthening of STP into persistent (>24 h) LTP in rodents in vivo (Kemp and Manahan-Vaughan, 2008a; Hagena et al., 2016). This facilitation of LTP through novel learning requires activation of dopamine D1/D5 receptors (Kemp and Manahan-Vaughan, 2008b; Hagena et al., 2016).

When released into the dorsal hippocampus, dopamine binds to D1/D5 receptors to promote attention, episodic memory formation, spatial learning, and synaptic plasticity (Xing, 2010; da Silva et al., 2012). Successful spatial learning requires that hippocampal place cells, location-encoding pyramidal neurons (O’Keefe, 1999), display consistent and stable patterns of neural activity, a process that can be enhanced by selective attention to spatial cues and by dopamine agonists (Muzzio et al., 2009; Kentros et al., 2004). Conversely, dopamine receptor blockade attenuates the ability of spatial attention to stabilize the firing pattern of hippocampal place cells (Kentros et al., 2004). The role of dopamine in driving attentional processes is highlighted by the fact that methylphenidate, one of the most common treatments for attention-deficit hyperactivity disorder (ADHD), improves attention by increasing synaptic availability of dopamine in the hippocampus, as well as in the prefrontal cortex and striatum(Volkow, 2012; Carmack et al., 2014). In addition, the dopaminergic system is involved in reward-learning behavior (Ungless, 2004). These all findings suggest that dopamine is critical for the selective attention underlying spatial learning and memory.

Furthermore, Dopamine receptors are differently involved in spatial learning and memory, probably by modulating different memory systems that contribute to the processing of spatial information (Amico et al., 2007) in an activation, task, and brain region dependent manner. A predominant role of the D1 receptor is generally considered (El-Ghundi et al., 2007). Cocurello et al. (2000) found a more selective role of nucleus accumbens D1 than D2 receptors in the acquisition of spatial information.

Similarly, striatal D1R deficient mice showed impairments in the acquisition of a standard and memory deficiencies in the reversal water maze task (Sariñana and Tonegawa, 2016). These effects are probably based on the necessity of interaction between D1R and NMDA receptors in the striatum (Song et al., 2016) and also in the nucleus accumbens (Ferretti et al., 2005). D2 receptor activation in the ventral pallidum in rats (Péczely et al., 2016) improves and blockade of hippocampal D2 receptors in mice (Rochetti et al., 2015) impaired spatial reference memory acquisition and consolidation in a water maze task, whereas brain-wide activation improves acquisition but not consolidation of spatial memories in a Hebb-Williams complex maze (Raut et al., 2014). Besides effects of agonist applications before or during training, post-training application of specific D1R and D2R agonists also resulted in improved spatial memory consolidation in hippocampal dependent tasks, that could be dissociated from similar processes involving the caudate nucleus (Packard and White, 1991; Packard and McGaugh, 1994).

Dopamine regulates CA1 synaptic plasticity, novelty perception, and spatial memories (Li et al., 2003; McNamara et al., 2014). Dopamine via activation of D1 receptors is involved in spatial reference memory, novel object recognition and dentate gyrus synaptic plasticity (Korz and Frey, 2007; Hamilton et al., 2010; Twarkowski and Manahan-Vaughan, 2016; Yang et al., 2017). Thus, dopaminergic receptors act similarly or differently in a brain region and task dependent manner upon learning and memory.

1.3 Cognitive and Behavioral Test Batteries

Behavioral tests are common in various fields of basic and preclinical research such as pharmacology i.e., to test drug effects upon learning and memory and behavioral patterns. For this reason, a variety of specific tests have been developed to assess the degree of anxiety- or depression-like behavior as well as cognitive abilities. In practice, relatively small sample sizes of experimental animals are compared, i.e., in pharmacological studies. Furthermore, the animals often undergo a battery of tests in order to gain comprehensive results for different behavioral patterns. The results of these tests indicate that different behavioral dimensions proved not to be highly correlated (Steptoe, 2010). However, inter-task correlations in test batteries have been repeatedly found (Arendash and King, 2002; Learmonth et al., 2015).

The relevance of behavioural test models for cognitive studies is very crucial. Some of the models such as hole-board have a direct relevance since it helps for direct evaluation of the inherent cognitive ability of the experimental animals or cognitive enhancing effect of the pharmacological products. Other behavioral test batteries such as forced swim test, elevated plus maze, open field and Rota rod are indirectly used for measurement of the cognitive abilities. They help to evaluate depression-like behaviors, anxiety, motivation and motor abilities, which affects quality of the outcome of cognitive assessment. Because behavioral disorders such as depression, anxiety and lack of motivation as well as motor dysfunction results in false outcome about the inherent cognitive ability of the animals and cognitive enhancers.

1.4 Rationale for the Study

Animal models including behavioral test batteries have been useful for learning about disease mechanisms, but their utility in predicting treatment efficacy in clinical trials has frequently been challenged (Worp*et al*., 2010; Begley and Ellis, 2012; Landis, *et al*., 2012). Numerous “promising” preclinical studies are published each year; however, very few of the proposed interventions show positive results when moved to clinical trial. These discrepancies are often attributed to publication bias, overoptimistic conclusions, and poorly designed and executed pre-clinical studies (Worp*et al*., 2010; Begley and Ellis, 2012; Landis, *et al*., 2012).

Most researches related to learning and memory show hippocampus as the center for spatial memory acquisition, consolidation and retrieval. There are dopaminergic projections into the hippocampus. This leads to an assumption that dopamine modulates hippocampal spatial learning and memory. As a result, most of the researches involving investigation of the roles of dopamine in learning and memory target hippocampus. These researches have so far proved that hippocampus is the center for memory and identified several proteins that are regulated by dopaminergic modulation (D’Amico et al., 2012, Smith et al., 2005).This means that the modulatory role of dopamine in hippocampal learning and memory is more or less well addressed.

On the other hand, the interaction between hippocampus and PFC during memory formation, consolidation and retrieval is also well understood (Cenquizca and Swanson, 2007; Jay and Witter, 1991). For example, the essentiality of bidirectional interactions between the HPC and PFC for mediating the behaviorally relevant spike patterning to optimally utilize retrospective and prospective memory processes is well understood (Goto and Grace, 2008). Accumulating evidence also suggests that the mesocortical dopamine projection to the PFC plays a critical role in the modulation of information processing by HPC–PFC interactions. Furthermore, functional human imaging study has revealed that genetic variance of catechol-O-methyl transferase, a major enzyme involved in clearance of dopamine in the PFC, affects HPC--PFC interactions during memory processing (Bertolino et al., 2006). Electrophysiological recordings and behavioral assessments in rats also provide evidence that the ability to utilize previous experience toward achievement of a specific goal is mediated by the dopamine dependent exchange of information between the HPC and PFC through both direct (from the HPC to the

PFC) and indirect (from the PFC to the HPC) reciprocal connectivity between these regions (Goto and Grace, 2007).

The PFC expresses both dopamine D1 and D2 receptors (Vincent et al. 1993). D1 activation could facilitate the use of episodic memory by facilitating PFC feedback onto the HPC via an indirect pathway, where as D2 receptor activation could trigger a switch from the use of HPC-based episodic memory to a PFC-based employment of future planning strategies via suppression of HPC activity (Goto and Grace, 2008).

Neuromodulation by means of the cathecholeminergic system is a key component of motivation-driven learning and behaviorally modulated hippocampal synaptic plasticity. In particular, dopamine acting on D1/D5 receptors exert a very potent regulation of forms of hippocampal synaptic plasticity that last for very long-periods of time (>24 h), and occur in conjunction with novel spatial learning. Antagonism of these receptors not only prevents long-term potentiation (LTP) and long-term depression (LTD), but prevents memory of the spatial event that, under normal circumstances, leads to the perpetuation of these plasticity forms (Twarkowski and Manahan-Vaughan, 2016). Furthermore, drugs such as methyilphenidate that are used to treat attention deficit hyperactivity disorder (ADHD) through non-selective inhibition of catecholamine reuptake improve prefrontal cortex dependent cognitive function. On the other hand, different early pre-and postnatal experiences and environmental complexity support individual behavior, physiology, and molecular processes during adulthood. For these reasons, it is difficult to generalize between different laboratories but individuality should be estimated for each local population of animals and may then provide more reliable results in animal models of mental and cognitive diseases and individual vulnerability.

As indicated above, the HPC interacts with PFC during spatial learning and memory through dopamine projection. Furthermore, like hippocampus, prefrontal cortex contributes in memory formation, consolidation and retrieval (Magnusson et al., 2007; Yo et al., 2007). However, the role of dopamine signaling on prefrontal cortex-mediated spatial memory consolidation, and especially on proteomic changes after learning tasks, remains poorly investigated. And also, many current researches targeting dopamine transporter inhibition to enhance cognitive ability doesn’t produce promising results. This might be due to incomplete understanding of the role of dopaminergic signaling during memory aquesition and consolidation. Furthermore, the individual differences between experimental animals would affect the outcome of the experiment due to shortage of knowledge about the level of individual differences. The current research would also fill this gap through estimation of the individual differences in a population of Sprague-Dawely rats which is commonly used experimental animal for cognitive assessment. The outcome of behavoural battery test for estimation of individual differences in a population of Sprague-Dawely could also give a clue about whether the improvement in spatial learning and memory by dopaminergic modulation is due to its direct cognitive enhancing effect or by increasing motivational status of experimental animals.

In conclusion, the current research was aimed to contribute knowledge regarding prefrontal cortex protein expression regulated through dopaminergic signaling during reward motivated holeboard task and provide evidence how dopaminergic modulation improves the cognitive of experimental animals besides estimating the level of individuality in a population of experimental animals mentioned above. Furthermore, the outcome will pave the way for elucidation of molecular mechanism by which dopamine modulates spatial memory in prefrontal cortex which further helps to solve the shortage of promising results in researches involving dopamine transporter inhibition to enhance cognitive ability.

2. Objectives

2.1 General Objective

To investigate level of individual differences in a population of experimental animals and effects of dopaminergic modulation in prefrontal cortex proteins expression during spatial reference memory acquisition and consolidation

2.2 Specific Objectives

- To assess cognitive features, level of anxiety, depression like behavior and motivation in a population of Sprague Dawley rats
- To investigate the effect of D1 like and D2 like receptor activation on spatial learning and memory
- To compare the effects of D1 like receptor activation on spatial learning and memory in simple and “difficult” holeboard tasks
- To investigate the effect of dopamine receptors blockade on spatial learning and memory
- To profile the protein expressed in prefrontal cortex during spatial reference memory aquesition and consolidation after selective D1 receptor activation

3. Material and Methods

3.1 Drugs and Chemicals

The following drugs and chemicals were used for the experiment The receptor agonists D_1: (±)-6-CHLORO-PB hydrobromide (SKF-81297), Sigma-Aldrich, S143, K_i D1: 2.2 nM, (Andersen and Jansen, 1990); D_2: Sumanirole maleate; Sigma-Aldrich SML1087, $K_{i:}$ D2: 9.0 ± 1, D3: 1940±142; D4:>2190, D1: >7140, nM, (McCall et al., 2005) and antagonists D_1: (R+)-SCH hydrochloride, Sigma-Aldrich D054, $K_{i:}$ D1: 0.11-0.35; D5: 0.11-0.54; nM, (Vallone et al., 2000); D_2: Remoxipride hydrochloride, Tocris 0916, $K_{i:}$ D2: 54-300; D3: 969-1600; D4:2800-3690 nM, (Vallone et al., 2000) Nembutal (Sigma-Aldrich), 3-[(3- Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS, Sigma-Aldrich), Dithiothreitol (DTT, Sigma-Aldrich), Triethyl ammonium bicarbonate (TEAB, Thermo Scientific), hydroxyethyl-piperazineethane-sulfonic acid buffer (HEPES, Biochrom GmbH), Trifluoroacetic acid (TFA, Sigma-Aldrich), Acetonitrile (ACN, Sigma-Aldrich).

3.2 Animals

Male Sprague Dawley rats, aged 12-13 weeks and 5-6 months, bred and maintained in standard Makrolon cages (length: 60 cm, width: 34, height 20 cm; 1 rat per cage for 12-13 weeks old and 3 animals per cage for 5-6 months old) filled with autoclaved woodchips in the Core Unit of Biomedical Research, Division of Laboratory Animal Science and Genetics, Medical University of Vienna, were used. Food (sniffR, Soest; Germany) and tap water were available ad libitum. Facility conditions were: temperature: 22±2C; humidity: 55±5%; 12 h artificial light/12 h dark cycle: light on at 7:00 a.m.). All procedures were carried out according to the guidelines of the Ethics committee, Medical University of Vienna, and were approved by Federal Ministry of Education, Science and Culture, Austria.

3.3Surgery

The rats (300-350g) aged 12-13 weeks were anesthetized with Nembutal (40 mg/kg, i.p.). An intracerebroventricular cannula (4.5 mm in length) was stereotactically implanted into the lateral ventricle of the right hemisphere (coordinates: AP — 0.8; L 1.5 from bregma). Together with an anchor screw the cannula was fixed with dental cement (Paladur, HeraeusKulzer, Hanau, Germany). After surgery, the animals were single housed and were allowed to recover from surgery for at least 4 days. The correct placement of the cannulas was verified by an angiotensin

II (70 ng/µl; 5µl volume) water drinking test. Correct placement was defined if the animals drunk within 3 min after injection. A failure rate of less than 20% was observed.

3.4 Pharmacological Treatment

SKF-81297 and others were dissolved in saline (NaCl 0.9%, pH 4.5-7.0; Fresenius Kabi Austria GmbH, Graz, Austria) and applied at a volume of 5 µl at a rate of 1 µl/min using a Hamilton syringe (CR700-20). Saline treated rats served as controls. For each drug treatment, time matched saline treated rats (1 – 2 with each group) were used and since there was no difference between these controls, saline treated rats were pooled for statistical comparisons. Performance between individual control rats were defined as unaltered when the RMI was not exceeding the mean ± one standard deviation from that of previous control groups. For the difficult task a second, independent control group was tested. Substances were applied at a dosage of 1µg and 5µg. The dosages were chosen from previous experiments (Ahmed et al., 2006; Korz and Frey, 2007) and related literature (Hagena and Manahan-Vaughan, 2016) showing that basal synaptic plasticity is unaffected by intracerebroventricular application even at higher dosages and higher dosages were used to discriminate the roles of these compounds upon learning and memory (André and Manahan-Vaughan, 2016). Similar dosages were given locally in the nucleus acumbens (Yates and Bardo, 2017) or the basolateral amygdale (Larkin et al., 2016) for studying receptor specific regulation of behavior.

3.5 Behavioral Tests

Behavioral tests were performed on adult rats aged 5-6 monthsin the following sequence: Elevated plus maze, open field, rota-rod, forced swim test, holeboard test. Only Holeboard test was carried outon rats aged 3-4 weeks. The apparatuses (Bilek+Schüll GMBH; Vienna, Austria) were custom made as well as the software (TIBE V.1.0; Vienna, Austria) for analysis. Hole visits in the holeboard were recorded manually from videos. The between tests interval was 1 week for rats aged 5-6 months and the inter trial interval during holeboard test was 20 min for both age groups. Because the ratio between the number of analyzed variables and number of individuals is critical for the validity of PCA results, a one common test was chosen for each feature. Also the number of variables within each test was minimized to those with the highest variance between individual rats. These tests represent a standard test battery used for the evaluation of side effects in pharmacological studies (Sunyer et al., 2007, 2008).

3.5.1 Elevated plus Maze

The elevated plus maze is a common test to assess anxiety (Belzung and Griebel, 2001). It consists of a cross of two closed arms with side walls and two open arms. Rats and mice usually prefer to stay in closed arenas and avoid the exploration of open arenas (Carobrez and Bertoglio, 2005; Arantes et al., 2013). Thus, the longer the animals stay on open arms the lesser they are considered to be anxious. The elevated plus maze consists of two plus shaped plastic lanes (10 cm in width, 1.10m in length at a height of 62 cm), with one lane (closed arm) surrounded by black plastic walls (40 cm in height), except at the crossing point with a gap of 10 cm, so that each arm had a length of 50 cm. The animals were placed in this center and allowed to explore the maze for 5 min. The movements were recorded by a tracking system and stored on a computer. The ratio of the time (s) the animals spent in the closed and the open arms (EPMRCO), the ratio of distances traveled and the rest in closed and open arms (EPMRDOC), the local movement (EPMlocal, <10 cm/s) and the number of entries into the open arms were considered for the analysis. The last three variables were chosen in order to compare it with corresponding variables in the open field (Carola et al., 2002) with regard to locomotion and the motivation to explore open arenas (the exploration of the central arena in the open field was almost invariant between animals).

3.5.2 Open Field

The open field test is widely used to test exploratory behavior and general activity of mice and rats (Crusio, 2001). The open field consists of a black plastic board (1.20m× 1.20m) surrounded by black plastic walls (50 cm in height). The rats were placed in the arena and allowed to explore for 10 min. The movements were recorded by a video tracking system and stored on a computer. Considered were the ratio between distance traveled (m), and the time the animals did not move (s) OFRDR, local movements (OFlocal, <10 cm/s) and the average velocity of movement (OFveloc; m/s). The usually used variables of the ratio of the time spent in the central and peripheral part of the arena in order to assess the motivation to voluntarily explore unsafe areas were not considered because there was minimal variability between animals, thus they do not provide information about individuality and unnecessarily increases the number of variables in the PCA. In order to provide information about the readiness to enter unsafe areas, the number of entries in open arms of the elevated plus maze test has been considered (Carola et al., 2002).

3.5.3 Rota Rod

The rota-rod is a widely used test to assess motor disabilities of rodents. The time-length the animals are able to stay on a rotating rod is considered to be a correlate of motor abilities (Brooks and Dunnett, 2009). The apparatus consists of four adjacent rods (10 cm in width at a height of 40 cm) separated with plastic barriers. The animals were placed on the rods which rotated with increasing speed. Rats were tested three times. The time until the rats fall off the rods was measured and the mean time (RRtime; s) for the three tests was used for analysis.

3.5.4 Forced Swim Test

The forced swim test is a common test to assess depressive-like behavior. Therefore the time the animals are immobile and not struggling to escape fromthe water is considered to be a correlate of depressive-like behavior (Slattery and Cryan, 2012). The test consists of training and a test session on two consecutive days. The rats were placed in a translucent plastic cylinder (20 cm in diameter and 45 cm in height filled with water up to a 33 cm level at a temperature of 25 ∘ C). The rats were placed in the cylinder for 10 min during the training and 5 min during the test session. Movements in the cylinder were recorded by a video tracking system and stored on a computer. The time (s) the animals spent immobile during training (FSIMTR) and during test (FSIMT) were used for the analysis.

3.5.5 Holeboard

The holeboard is a test of spatial learning and memory and has been found to be reliable and highly sensitive to pharmacological interventions, induction, and maintenance of neuronal plasticity, and discrimination between physiological states of animals (Uzakov et al., 2005; Korz and Frey, 2007; Schulz and Korz, 2010; Meyer and Korz, 2013).

The holeboard board (1 m × 1 m) was made of black plastic surrounded by translucent plexi glass walls. Each sides of the wall were equipped with proximal spatial cues. Room structures visible outside the board served as distal cues. Four out of sixteen regularly arranged holes (diameter and depth 7 cm) were baited (dustless precision pellets, 45 mg, Bioserv®, Somerville, NJ, USA). The pattern of baited holes remained the same during the entire test. A second board below the first was provided with scattered food pellets to avoid olfactory cues. The rats were familiarized to the experimenter through 15 min handling sessions per day for three days prior to the experiment, followed by two days of habituation to the hole-board during which the animals were allowed to explore the maze for 15 min each day with access to food pellets. Controlled food restriction reduced the weight of the rats to reach 85% of its initial body weight. Tap water was given *ad libitum*. Thereafter, the rats were trained for three days (five trials on day one, four trials on day 2 and a retention trial at day 3). The interval between two successive trials for an individual was 20 min. Every trial lasted for 120 s or until all four pellets were eaten. The apparatus was cleaned with 1% Incidin between trials in order to remove odor cues of individual rats. Performance of the rats in the maze was recorded by a video camera and stored on a computer.Starting position was always the same during trials. The hole visits and removals of pellets were noted for each trial. In order to compare rats with similar levels of motivation, rats with less than 40-holes visits in total over the ten trials were excluded from the analysis.

Reference memory errors were noted as the number of visits to the unbaited holes. Reference Memory Index (RMI) was calculated using the formula (first + revisits of baited holes)/total visits of all holes. All behavioral training/testing was performed during the light phase of the light–dark cycle. Drugs were applied 30 min prior to trial 1, trial6 and trial 10 at days 6, 7 and 8. The “easy” task consists of a four-connected neighbourhood of baited holes, whereas in the “difficult” task the baited holes were more scattered over the board (Figure 10). One hour after the 10th trial, the rats were sacrificed, the brains rapidly removed and dissected at 4°C. The prefrontal cortex and hippocampal subregions (CA1, CA3 and Dentate gyrus) tissues were then stored at -80 °C until use.

3.6 Quantitative Proteomics

3.6.1 Enrichment of Membrane Protein Fraction

Membrane protein fractions from prefrontal cortex were prepared as previously described with minor modifications (Shanmugasundaram et al. 2015). Briefly, the pre-frontal cortices were dissected and stored at -80^{o}C. Tissues were homogenized in a buffer containing 0.32 M sucrose, 10 mM HEPES pH 7.4, 0.1mM $CaCl_2$ supplemented with protease and phosphatase inhibitors on ice. The homogenate was centrifuged at 1000 × *g* for 10 min at 4^{o}C. The supernatant was further centrifuged at 15,000 × *g* for 30 min at 4^{o}C to obtain membrane (crude synaptosome) pellet.

3.6.2 Proteolytic Digestion

The proteomic samples preparation was performed as previously described (Sialana et al. 2016). Briefly, membrane pellets were reconstituted in strong-chaotropic extraction buffer (SCEB) that contains 7 M urea, 2 M thiourea, 4% CHAPS, 100 mM DTT and 50 mM TEAB supplemented with protease inhibitors. Protein amounts were estimated using Pierce 660 protein assay (Pierce, USA). Protein samples (75µg) were digested with trypsin (1:100 w/w) using the filter-aided sample preparation (FASP) as previously described with minor modifications (Wisniewski et al. 2009). All digests were desalted and concentrated by solid phase extraction using C18 material (Nest Group, USA). Lyophilized peptides were reconstituted in 2% ACN and 0.1% TFA and the peptide concentrations were normalized to 0.1µg/µL using Fluorometric Peptide Assay (Pierce, USA).

3.6.3 Liquid Chromatography Tandem Mass Spectrometry

LCMS analyses was performed using a single-shot LCMS approach with 4-h gradient using a Dionex Ultimate 3000 system (Thermo Fisher Scientific) coupled to a Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific, Germany) with LCMS parameters as described previously (Stojanovic et al. 2017). Software versions used for the data acquisition and operation of the Q-Exactive were Tune 2.8.1.2806 and Xcalibur 4. The chromatographic solvents were as follows: solvent A consisted of 0.1% formic acid in water and solvent B consisted of 0.1% formic acid in 80% acetonitrile. From a thermostated autosampler, 10 µL that correspond to 1 µg of the peptide mixture was automatically loaded onto a trap column (PM100-C18 3µm, 75 µm × 20 mm, Thermo Fisher Scientific, Austria) with a binary pump at a flow rate of 5 µL/min using 2% acetonitrile in 0.1% TFA for loading and washing the pre-column. After washing, the peptides were eluted by forward-flushing onto a 50 cm analytical column with an inner diameter of 75 µm packed with 2 µm-C18 reversed phase material (PepMap-C18 2 µm, 75 µm x 500 mm, Thermo Fisher Scientific, Austria). Peptides were eluted from the analytical column with a 220 min gradient ranging from 10 to 37.5% solvent B, followed by a 20 min gradient from 37.5 to 50% solvent B and finally, to 90% solvent B for 5 min before re-equilibration to 5 % solvent B at a constant flow rate of 300 nL/min.

The LTQ Velos ESI positive ion calibration solution (Pierce, IL, USA) was used to externally calibrate the instrument prior to sample analysis and an internal calibration was performed on the polysiloxane ion signal at m/z 445.120024 from ambient air (Olsen et al. 2005). MS1 scans were performed from m/z 400-2000 at a resolution of 70,000. Using a data-dependent acquisition mode, the 20 most intense precursor ions of all precursor ions with +2 to +6 charge were isolated (within a 1.6 m/z window) and fragmented to obtain the corresponding MS2 spectra. The fragment ions were generated in a higher-energy collisional dissociation (HCD) cell at an NCE of 27% with a fixed first mass fixed automatically and were detected in an Orbitrap mass analyser at a resolution of 17,500. The dynamic exclusion for the selected ions was 20s. Maximal ion accumulation time allowed in MS1 and MS2 mode was 30 and 50 ms, respectively. Automatic gain control was used to prevent overfilling of the ion trap and was set to 1 x 10^6 ions and 5 x 10^4 ions for a full Fourier transform MS1 and MS2 scan, respectively.

3.6.4 Protein Identification

All MS1-MS2 spectra were matched against UniProtKB/Swiss-Prot rat protein database version 2017.07.05 (31,560 sequences, including isoforms) using the Mascot 1.5.3.30 via Proteome Discoverer 2.3 platform (Thermo Scientific, USA).The search parameters were as follows: two tryptic missed cleavage sites, mass tolerances of 10 ppm and 0.02 Da for the precursor and fragment ions, respectively. Oxidation of methionine was set as variable modification, whilst carbamidomethylation of cysteine residues were set as fixed modifications. The data was also matched against a decoy reverse database. Peptides and protein identifications with 1% FDR are reported implementing the Percolator algorithm. Protein identifications requiring a minimum of two peptides sequences were reported.

3.6.5 Label-free Quantification

Label-free quantification was implemented using the Minora feature of Proteome Discoverer 2.2. The following parameters were used: maximum retention time alignment of 10mins with minimum of S/N of 5 for feature linking mapping. Abundance was based on precursor/peptide area intensities. Normalization was performed such that the total sum of the abundance is the same for all sample channels. Imputation was performed by replacing the missing values with random values from the lower 5% of the detected values.

3.6.6 Functional Annotation of Proteins

Enrichment of GO annotations was performed on the significant proteins using GOA database (v30.08.2017) using the Clue GO via the Cyto scape platform (Bindea et al. 2009; Huntley et al. 2015). To reduce redundancy of GO terms the fusion option was selected. Enriched GO terms (Benjamini–Hochberg P-value <0.05) were functionally grouped into networks linked by their kappa score level (≥0.40). Functionally related groups partially overlap and only the most significant terms per group were labeled.

3.7 Statistics

Each variable was separately analyzed for minimum and maximum values, median, 25 and 75% percentiles, arithmetic mean, standard deviation, and standard error, the lower and the upper 95% confidence interval. Correlations between the measures of each test were detected by the Pearson product moment correlation (two-tailed). In order to reduce the number of dimensions a Factor analysis (principal component with Varimax rotation and Kaiser normalization, maximally 25 iterations to convergence) based on the correlation matrix was performed for all variables. Factor rotations simplify the interpretation of the factors. The coordinate axis rotation allows a better distribution of the loadings on the factors. The varimax rotation was chosen because it is an orthogonal rotation method so that the different factors do not intercorrelate, thus each factor represents an independent behavioral pattern. Varimax rotation reduces the number of variables with high multiple factor loadings. Extracted components with eigen values higher than one were considered for further interpretation. In order to separate the animals according to their individual factor loadings (-1 to 1) on the components, loadings lower than -0.7 and higher than 0.7 were considered as border. Factor and correlation analyses were done with SPSS Version 20, all other analyses by using GraphPad Prism (Version 5.02).

A repeated measure twoway ANOVA (general linear model) with trial and treatment as factors and Tukey post-hoc tests for the differences over the entire training, or separately for day 1 and 2, and a oneway ANOVA for the retention trial was used. Border of significance was set at $p \leq 0.05$. Statistical analysis in protein levels was performed using a two-sided t-test with $p < 0.05$ (Students' or Welch's). Proteins are reported as significant between D_1 agonist-treated and vehicle controls when $P < 0.05$ and the protein ratios are greater than 1.5 obtained from the medians of summed sample abundances of biological replicates.P-values of the t-tests were not corrected to multiple comparisons (which is common in the analysis of proteomic data) because of the high conservatism of this procedure due to the very high numbers of comparisons, which highly increases the probability of false negative results. In order to evaluate the effect of the different comparisons, the effect size was calculated after Cohen. The thresholds for interpreting effect sizes are d=2; small, d=0.5; medium, d=0.8; large and d≥1.30; very large.Correlations between protein levels and reference memory indices were performed by using the Bayesian statistics Pearson correlation using SPSS version 25.The Bayes factor provided the evidence for the degree of correlation and thereby reduced the chance of false positive results, that may be observed by using p values of Pearson correlationsin multiple tests. The commonly used threshold of Bayes factor to define significance of evidence for correlation are extreme (<1/100); very strong (1/100-1/30); strong (1/30-1/10); moderate (1/10-1/3); anecdotal (1/3-1) and no evidence (=1). Only those proteins with Bayes factor less than 1/3 were reported as having correlation to the RMI and the rest were rejected even if their p values were significant.

4. Results

4.1 General Measures of Behavioral Test Batteries and Linear Regression

All the studied behavioral variables were not Gaussian-distributed (Shapiro-Wilk normality test): HB-trial 1, $W=0.82$, $p<0.001$; HB-trial 6; $W=0.67$, $p<0.001$; HB-trial 10; $W=0.70$, $p<0.001$; OF-distance; $W=0.94$, $p<0.001$; OF-rest; $W=0.97$; $p<0.001$; OF-velo; $W=0.81$; $p<0.001$; RR-time; $W=0.97$; $p<0.001$; FS-immobility; $W=0.51$; $p<0.001$; EPM-open; $W=0.95$; $p<0.001$; EPM-closed; $W=0.95$; $p<0.001$. Minimum and maximum values, percentiles, arithmetic means, standard deviation and error as well as confidence intervals are given in Table 2. The different number of values resulted from some missing values for the different tests.

Table 2: Statistical measures and number of valid cases for each behavioral variable along the entire cohort

	Trial, 1	Trial, 6	Trial, 10	OF-distance	OF-rest	OF-velocity	RR-time	FS-immobil	EPM-open	EPM-closed
Number of values	162	162	162	160	160	160	147	159	160	160
Minimum	0.0	0.0	0.0	0.2100	52.68	0.0	8.333	0.5400	0.0	81.86
25% Percentile	0.0	0.0	0.0	4.203	76.68	0.0100	26.33	1.417	26.07	206.8
Median	0.1429	0.0	0.0	5.825	82.43	0.0100	32.67	2.130	50.68	243.7
75% Percentile	0.4000	0.5000	0.7569	8.448	88.79	0.0100	38.00	2.880	85.96	271.7
Maximum	1.000	1.000	1.000	20.69	99.32	0.0400	72.00	19.73	177.6	299.9
Mean	0.2155	0.2375	0.3178	6.492	82.08	0.01144	33.14	2.366	57.35	236.9
Std. Deviation	0.2490	0.3597	0.4146	3.699	9.045	0.007341	10.18	1.750	40.14	41.78
Std. Error	0.01957	0.02826	0.03258	0.2925	0.7150	0.0005804	0.8399	0.1388	3.174	3.303
Lower 95% CI of mean	0.1769	0.1817	0.2534	5.914	80.67	0.01029	31.48	2.092	51.08	230.4
Upper 95% CI of mean	0.2542	0.2933	0.3821	7.070	83.50	0.01258	34.80	2.640	63.62	243.4

The results of the linear regression analysis using the Pearson coefficient and the resulting p-values are given in Table 3.

Table 3: Correlationmatrix (Coefficient r after Pearson), and p-value (two-tailed) of the between and within correlations of the different variables.

	HB-trial1	HB-trial6	HB-trial10	OF-distance	OF-rest	OF-velo	RR-time	FS-immobil	EPM-open
HB-trial 6	.497**								
p	**.000**								
HB-trial10	.429**	.732**							
p	**.000**	**.000**							
Of-distance	.123	.172*	.168*						
p	.121	**.029**	**.033**						
Of-rest	-.138	-.169*	-.167*	-.983**					
p	.082	**.032**	**.035**	**.000**					
Of-velo	.109	.182*	.170*	.932**	-.917**				
p	.169	**.021**	**.032**	**.000**	**.000**				
RR-time	-.075	.079	.002	-.096	.118	-.096			
p	.365	.339	.981	.247	.155	.246			
FS-immobil	-.012	-.055	-.077	-.025	.028	-.043	-.088		
p	.881	.495	.334	.753	.726	.588	.287		
EPM-open	-.073	.034	.066	.071	-.063	.102	-.019	.002	
p	.361	.673	.408	.374	.427	.200	.819	.983	
EPM-closed	.099	-.018	-.075	-.077	.075	-.104	.009	.008	-.971**
p	.213	.817	.343	.336	.347	.191	.917	.921	**.000**

Significant correlation coefficients are tagged with asterisks and the corresponding significance level (p) is given in bold. HB-trial 1, 6, 10: Holeboard reference memory index for trial 1, 6 and 10; respectively. OF-distance, -rest, -velo: Distance travelled, resting, and mean velocity in the open field; respectively. RR-time: time to be on the rotarod. FS-immobil: time in percent spent immobile in the forced swim task. EPM-open, - closed: time spent in open and closed arms on the elevated plus-maze; respectively.

Most of the results of the different tests were not correlated, whereas different measures within the tests were correlated. The open-field measures, distance travelled and mean velocity, were positively and OF-rest was negatively correlated with the RMI of holeboard trial 6 and 10 but not with those of trial 1.

4.2 Factor analysis (Principal component)

The main result of the principal component analysis is given in Table 4. Considered were only components with an eigenvalue higher than 1. The first three dimensions and the euclidean distances of the variables are also given in Figure 7.

Table 4: Rotated (Varimax-Kaiser normalization) matrix of extracted components with eigenvalues greater than 1

	Rotated matrix of components (eigenvalues > 1)			
	1 (28.9%)	2 (21.3%)	3 (19.9%)	4 (11.1%)
	Motivation	Cognition	Anxiety	Depression
HB-trial1	.062	**.741**	-.097	-.161
HB-trial6	.092	**.890**	.037	.120
HB-trial10	.125	**.870**	.040	.105
OF-distance	**.984**	.100	.035	-.028
OF-rest	**-.979**	-.103	-.022	.046
Of-velo	**.960**	.108	.060	-.010
RR-time	-.105	-.022	-.017	**.741**
FS-immobil	-.048	-.056	-.012	**-.715**
EPM-open	.041	-.006	**.992**	-.010
EPM-closed	-.053	.020	**-.992**	-.006

Percent values give the portion of explained variance for each factor. Significant factor loadings (>0.7, <-0.7) are given in bold. HB-trial 1, 6, 10: Holeboard reference memory index for trial 1, 6 and 10; respectively. OF-distance, -rest, -velo: Distance travelled, resting, and mean velocity in the open field; respectively. RR-time: time to be on the rotarod. FS-immobil: time in percent spent immobile in the forced swim task. EPM-open, - closed: time spent in open and closed arms on the elevated plus-maze; respectively.

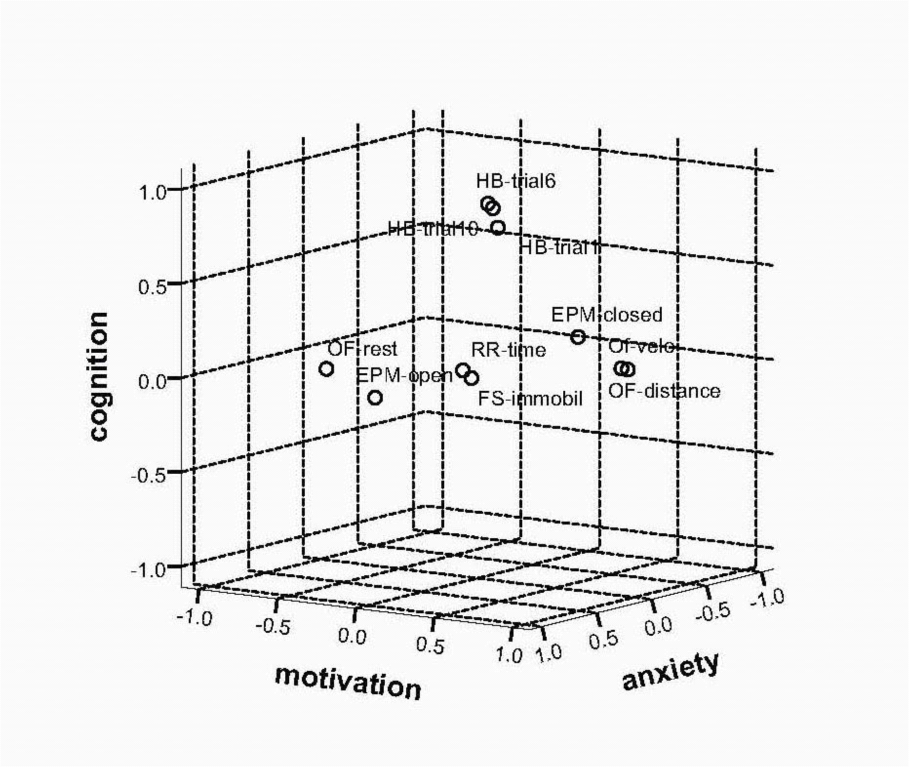


Figure 7: Diagram of the first three components in rotated space

HB-trial 1, 6, 10: Holeboard reference memory index for trial 1, 6 and 10; respectively. OF-distance, -rest, -velo: Distance travelled, resting, and mean velocity in the open field; respectively. RR-time: time to be on the rotarod. FS-immobil: time in percent spent immobile in the forced swim task. EPM-open, - closed: time spent in open and closed arms on the elevated plus-maze; respectively.

The first component explains 28.9 % of the total variance (after rotation) and is positively highly loaded by the open-field variables (OF-distance and OF-velo) and negatively by OF-rest. Because the variables distance and velocity reflects motivation this component was considered as representing the mental state of motivation. The second component, explaining 21.3 % of the variance is loaded by the holeboard reference memory indices and therefore considered as the cognition component. The third component explaining 19.9 % represents the mental state anxiety as it is characterized by EPM measures, the classic test for anxiety. The 4th component reflects depression.Because the results of the classic test for depression, the forced swim immobility, loads negatively and the rotarod results, representing motoric abilities but also requires a will to struggle, are positively loaded on this component. Thus, high depression is characterized by high immobility in the FS- and a long period of time spent on the rotating rod. For that reason, a high positive loading of individual rat scores on this component reveals low depression, whereas high negative loadings indicated high depressive states.

In general loading values of -7 and 7 are considered as significant, whereas all values between these borders are considered as intermediate, thus may represent 'normal' states regarding depression. Because the animals are naive and not treated in except the tests, these mental states are considered as intrinsic. Similar high positive (> 0.7) or negative (< -0.7) loadings of individual rat scores on component 1 reveal high vs. low intrinsic motivation with the rest representing normal motivation within the population. By doing this also for the other components a pattern of the distribution of intrinsic cognitive abilities and mental states for this population of rats can be calculated and is depicted in Figure 8.

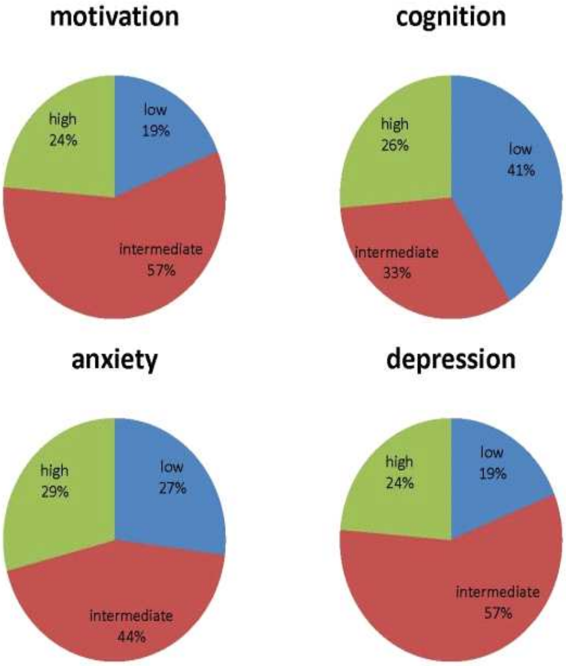


Figure 8: Diagram of the distribution of individual rats with high, low or intermediate innate states of motivation, cognition, anxiety and depression in the population of rats.

As indicated in Figure 8, 24 % showed a high, 19 % a low and 57 % an intermediate intrinsic state of motivation. The largest 'low' portion (41%) was given for the factor cognition and the largest 'high' portion (29%) for the intrinsic anxiety. Thus, almost every third rat was intrinsically at a high level in the population. Because these results were based on a large cohort of rats, it can be assumed that this is a characteristic feature for male Sprague-Dawley rats of this age and that an experimenter recruiting experimental samples can expect about 30 % probability to have an intrinsically highly anxious animal in the sample.

4.3 Effects of Dopamine Receptor Agonists and Antagonists on Spatial Reference memory

4.3.1 Effects of dopamine receptor agonists and antagonists on the reference memory index in the “simple” holeboard task

In order to understand the contributions of D_1 and D_2 receptor signaling to learning and memory in hole-board task, selective D_1 agonist SKF-81297 and D_2 agonist sumanirole (both at 1 and 5μg), D1 antagonist SCH hydrochloride and D2 antagonist remoxipride hydrochloride were infused into the lateral ventricle of young rats before training.

Entire Training

The overall comparison revealed a trial effect ($F_{9,639}$ =48.63, p<0.001), a trial x group interaction ($F_{54,639}$ = 2.18, p<0.001) and a treatment effect ($F_{6,71}$ = 9.55, p<0.001). Tukey-HSD post hoc tests revealed a significant difference of rats treated with SKF-81297 at both dosages showing a significantly higher RMI (1μg: p=0.003; 5μg: p=0.032) as compared with control animals (Fig. 9A), whereas sumanirole treated rats showed no difference compared with controls at both doses (Fig. 9C). Treatment with the lower dose of SKF-81297 resulted in better overall performance than both dosages of sumanirole (1μg: p=0.018; 5μg: p=0.002), whereas the higher dose of SKF-81297 resulted in better performance compared with 5μg (p=0.027) but not 1μg (p=0.127) dose of sumanirole, suggesting diminishing returns with higher SKF-81297 concentrations. D2 antagonist did not significantly affect the RMI performance as compared to controls (Fig. 9D). However, D1 antagonist treated rats overall displayed a lower RMI than normal saline treated animals (Fig. 9B), reinforcing the importance of D_1 receptor signaling in memory consolidation.

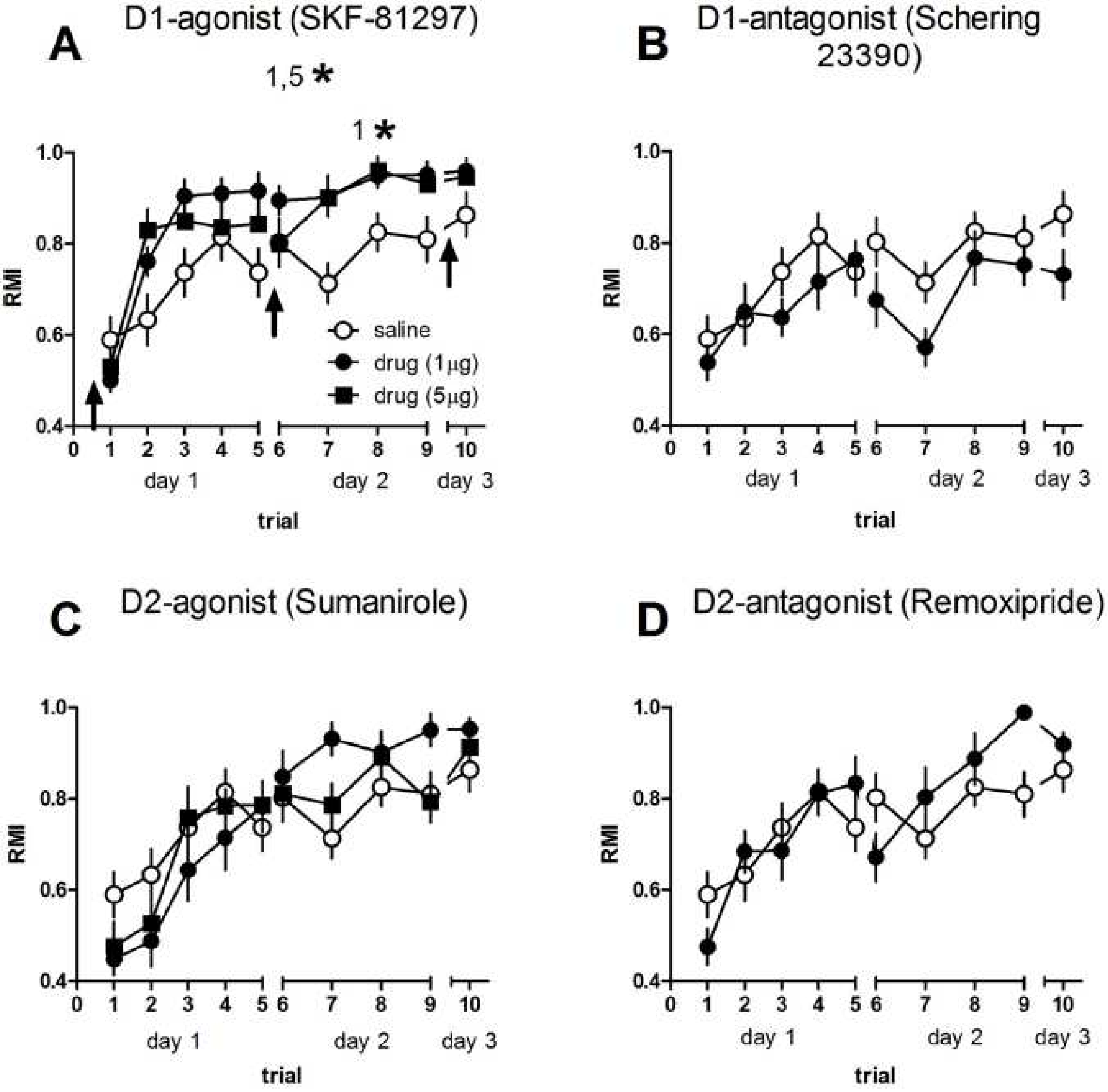


Figure 9: Reference memory indices of animals treated with the dopamine receptor

Agonists (left panel) and antagonists (right panel) for D1-like receptors (upper panel) and D2-like receptors (lower panel) over the entire training. Significant differences between groups are indicated above the figures by a horizontal bar. Numbers show the doses that significantly differ as compared to saline treated rats. Data are presented as mean values and SEM (n=10 for each group).

Day 1

The overall comparisons at day 1 revealed a trial effect ($F_{4,284}$ =51.68, p<0.001), a trial x group interaction ($F_{24,284}$ = 1.68, p=0.027) and a significant treatment effect ($F_{6,71}$ = 5.12, p<0.001). Post hoc tests revealed that neither of the drugs at any dosage induced a difference when compared with control animals ($p>0.1$ each) but SKF-81297 treated rats at 1µg showed higher RMI when compared with sumanirole at both dosages (1µg: $p=0.001$; 5µg: $p=0.021$). However, SKF-81297 treated rats at 5µg performed better than sumanirole treated rats at the lower dose (1µg: $p=0.004$; 5µg: $p=0.085$).

Day 2

On the second day, there was a significant trial effect ($F_{3,213} = 10.41$, $p<0.001$), a trial x group interaction ($F_{18,213} = 1.89$, $p=0.018$) and a treatment effect ($F_{6,71} = 8.68$, $p<0.001$). SKF-81297 treated rats at the lower (1µg: $p=0.014$) but not the higher dosage (5µg: $p=0.085$) performed better than controls. Sumanirole treated rats at both dosages did not show a difference as compared to the controls (1µg: $p=0.056$; 5µg: $p>0.1$).

Day 3

A significant difference was found between groups on the third day ($F_{6,71} = 4.60$, $p=0.001$). However, neither of the groups showed a significant difference compared to the saline control group as revealed by post-hoc tests.

In order to gain additional information whether the agonist affects more the learning or the consolidation process, trial specific post hoc tests on the first and last trial days were performed. SKF-81297 treated rats at 1µg showed a significant difference at trial 5 ($p<0.05$) but not at trial 1 at day 1, whereas both trial 6 and trial 9 at day 2 were not significantly different from controls. Rats treated with SKF-81297 treated rats at 5µg showed no differences during these trials as compared to controls.

4.3.2 Effects of dopamine receptor agonist on the reference memory index in the "difficult" hole-board task

Since behavioral performances requiring a simple task may reach ceiling levels, especially in control animals during memory retention, it can be difficult to discriminate the full effects of D_1 receptor mediated RMI improvements observed above. Therefore, we trained animals in a more demanding task. Due to the lack of effects of the D2 agonist and the similar overall effect of the two dosages of the D1 agonist, only the D1 agonist at one dosage (5 µg) was used for training in a more demanding task (Fig. 10).

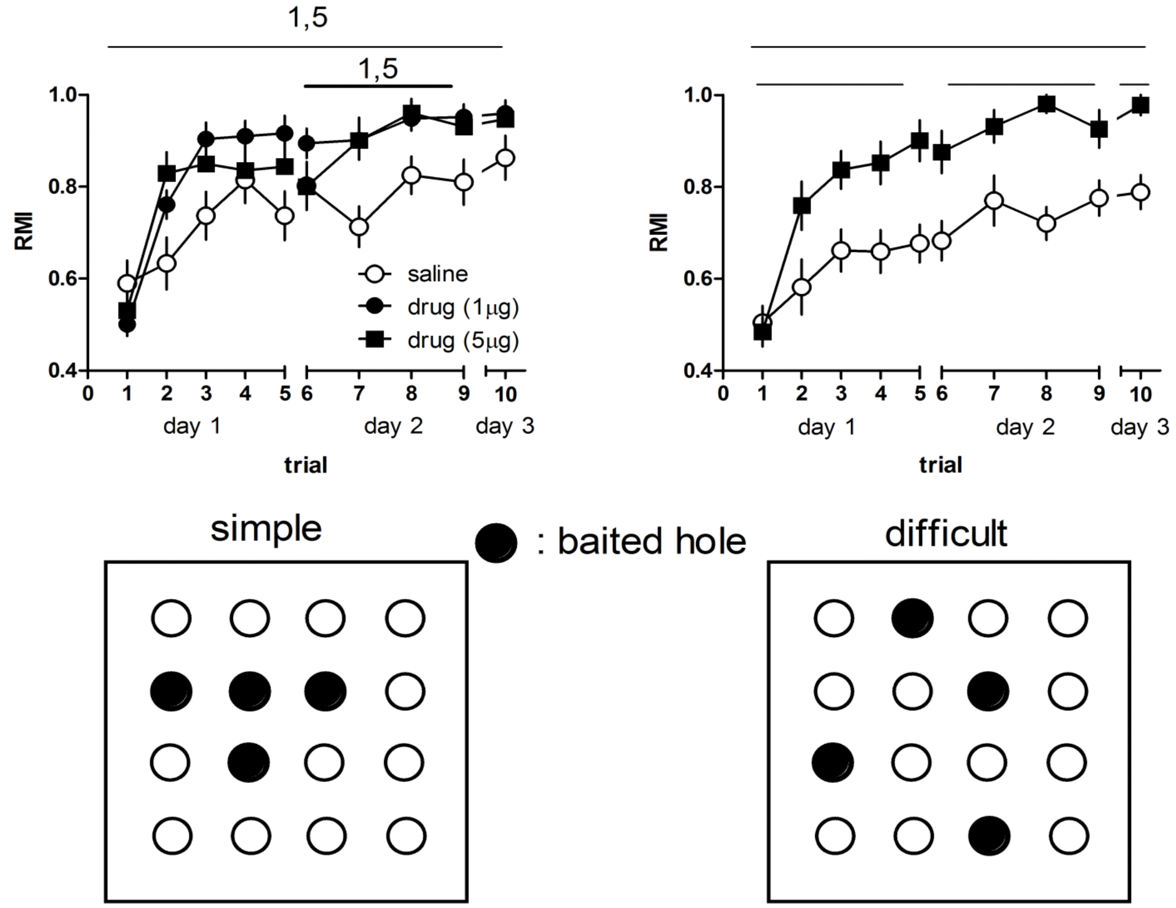


Figure 10: Reference memory indices of animals treated with the D1R-like receptor agonist of a simple (left) and a difficult (right) task. **Significant differences between groups are indicated by a horizontal bar. Given are the mean values and SEM (n=10 for D1 agonist and 9 for saline control). At the bottom the baited holes for the "simple" and the "difficult" task are indicated.**

A significant trial effect ($F_{9,171}$=19.24, p<0.001) was observed, revealing improved learning and memory consolidation. Second, a significant trial x treatment interaction ($F_{9,171}$=1.98, p=0.044) was seen, indicating that hole-board training and D_1 receptor agonism together can predict behavioral performance. Third, a significant treatment effect ($F_{1,19}$ = 25.14, p<0.001) could also be determined. At day 1 ($F_{1,19}$= 12.09, p=0.003), day 2 ($F_{1,19}$ = 29.36, p<0.001) and day 3 (F= -4.26, df=19, p<0.001) treated rats performed better than controls. In sum, our data reveals that D1 receptor signaling is necessary for learning in our simple and for learning and memory consolidation in the difficult hole-board task.

4.5 Effect of Dopamine on Prefrontal Protein Expression

To investigate D_1 mediated learning mechanisms in the prefrontal cortex, an LCMS-based quantitative proteomics approach was employed to determine the *in vivo* protein changes between SKF-81297 and normal saline treatment. After the 3-day training, prefrontal cortices of animals from the "difficult task" that showed a marked difference in the reference memory index (RMI) between treatment and control group were used for the proteomic comparison. Ten biological replicates of SKF-81297 treated and nine saline-treated animals were selected for proteomic analysis. From the analyses, a total of 5,223 protein groups were identified of which 4,116 proteins were used for label free quantitation. Of these, 96 proteins were significantly altered by SKF-82197 ($P < 0.05$; median ratio >1.5; D1agonist *c.f.* saline control). The proteins regulated by SKF-81297 treatment (48 up regulated, 48 down regulated) are listed in Table 5&6.

Table 5: D1 agonist upregulated proteins in the Prefrontal cortex

Acession	Description	Protein	P-value	Ratio, log$_2$	Cohen's D	Effect factor
Q6AY99	Aldo-ketoreductase family 1 member B10	AKR1B10	5.1E-03	2.06	1.41	Very large
A0A0G2K302	Adaptor-related protein complex 3, sigma 2 subunit	AP3S2	4.3E-02	3.75	0.96	Large
Q5MJ12	F-box/LRR-repeat protein 16	FBXL16	6.4E-04	1.31	1.83	Very large
D3ZW08	Adenyluccinatelyase	ADSL	2.1E-03	1.28	1.59	Very large
A0A0G2K1E1	RT1 class Ia, locus A1	RT1-A1	2.9E-02	3.15	1.05	Large
Q5M868	Non-lysomalglucylceramidase	GBA2	6.4E-04	3.47	1.83	Very large
A0A0G2JZA1	Roundabout guidance receptor 2	ROBO2	2.0E-02	1.40	1.13	Large
P52925	High mobility group protein B2	HMGB2	5.7E-03	2.96	1.39	Very large
P31421	Metabotropic glutamate receptor 2	GRM2	4.8E-02	0.67	0.93	Large
B5DF51	Membrane magnesium transporter 1	MMGT1	2.5E-02	1.50	1.08	Large
A0A0G2K2T2	Mitoguardin 1	MIGA1	3.7E-04	1.89	1.94	Very large
D4A3X1	Putative uncharacterized protein RGD1308601_predicted	RGD1308601	3.7E-04	2.50	1.94	Very large
P11505-2	Plasma membrane calcium-transporting ATPase 1	ATP2B1	4.7E-03	1.46	1.43	Very large
Q4QQV0	Tubulin beta chain	TUBB6	1.8E-03	2.12	1.62	Very large
F1LNZ5	Gamma-aminobutyric acid receptor subunit alpha-3	GABRA3	3.9E-02	0.77	0.98	Large
A0A0G2K2Y8	Multiple PDZ domain protein	MPDZ	4.7E-02	2.09	0.94	Large
G3V8U8	Branched-chain-amino-acid aminotransferase	BCAT2	4.4E-02	1.02	0.96	Large
A1L1J9	Lipase maturation factor 2	LMF2	2.1E-02	1.14	1.11	Large
P54748	cAMP-specific 3',5'-cyclic phphodiesterase 4A	PDE4A	2.8E-02	1.67	1.05	Large
F1LSC3	Splicing factor 1	SF1	2.4E-02	1.00	1.09	Large
P35286	Ras-related protein Rab-13	RAB13	4.8E-02	0.92	0.93	Large
D3Z9K2	Mitochondrial ribomal protein L54	MRPL54	1.6E-02	1.72	1.17	Large
A0A0G2K1A2	Myeloperoxidase	MPO	3.0E-02	1.42	1.04	Large
F1M9I6	Tetraspanin	TSPAN7	1.9E-02	0.59	1.14	Large
F1LZ38	Teneurintransmembrane protein 4	TENM4	3.1E-02	0.71	1.03	Large
Q5XHZ8	Component of oligomericgolgi complex 3	COG3	4.5E-02	1.89	0.95	Large
D3ZZB0	Disco-interacting protein 2 homolog C	DIP2C	4.1E-02	0.64	0.97	Large
Q5HZA4	LysM and putative peptidoglycan-binding domain-containing protein 1	LYSMD1	1.3E-02	0.84	1.22	Large
O08773	Regulator of G-protein signaling 14	RGS14	3.8E-03	0.76	1.47	Very large
D3ZR52	Lysophphatidylcholineacyltransfer	LPCAT4	4.7E-02	0.75	0.94	Large

	ase 4					
F1M790	Prtaglandin F2 receptor negative regulator	PTGFRN	4.7E-02	1.10	0.94	Large
F1M0F4	Exocyst complex component	EXOC6	1.3E-03	0.90	1.69	Very large
F1M4N6	Dedicator of cyto-kinesis 3	DOCK3	3.9E-02	0.62	0.98	Large
D4A857	Importin 9	IPO9	3.0E-02	0.75	1.04	Large
D4A7Y4	Uncharacterized protein	FAM171 A1	4.4E-04	1.16	1.91	Very large
A0A096MJT6	Similar to RIKEN cDNA D630029K19	RGD1307 100	2.6E-03	1.60	1.55	Very large
P15387	Potassium voltage-gated channel subfamily B member 1	KCNB1	4.1E-02	0.62	0.97	Large
Q5FVM6	Myotubularin-related protein 12	MTMR12	7.1E-03	0.84	1.34	Very large
F1M7N7	Ribomal protein S6 kinase	RPS6KA2	3.6E-02	0.94	1.00	Large
Q5RK25	Phphomannomutase	PMM1	4.0E-02	0.63	0.98	Large
A0A0G2KAT 5	Focal adhesion kinase 1	PTK2	1.7E-02	0.61	1.16	Large
D3ZQT0	Uncharacterized protein		4.4E-02	0.88	0.95	Large
Q9ES53	Ubiquitin recognition factor in ER-associated degradation protein 1	UFD1	9.0E-03	0.64	1.29	Large
B1WC49	Api5 protein	API5	3.4E-02	0.76	1.01	Large
D4ABN8	5'-3' exoribonuclease 1	XRN1	4.8E-02	1.25	0.94	Large
Q63553	SNF-related serine/threonine-protein kinase	SNRK	1.3E-04	0.87	2.15	Very large
P35559	Insulin-degrading enzyme	IDE	3.3E-02	0.61	1.02	Large
D4AAD6	Neuropilin and tolloid-like 1	NETO1	5.0E-02	0.74	0.93	large

Table 6: D1-agonist downregulated proteins in the prefrontal cortex

Accession	Description	Protein	P-value	Ratio, log$_2$	Cohen's d	Effect factor
P10818	Cytochrome c oxidase subunit 6A1, mitochondrial	COX6A1	7.5E-03	-1.62	1.33	Very large
A0A0G2KAU2	Armadillo repeat-containing X-linked protein 3	ARMCX3	9.9E-03	-1.91	1.27	Large
Q8VHI8	Vesicle transport protein SEC20	BNIP1	4.3E-03	-3.47	1.45	Very large
F1LQP8	Cadherin-6	CDH6	2.9E-02	-1.93	1.04	Large
G3V8Y9	Glutamate receptor 3	GRIA3	6.1E-03	-5.43	1.37	Very large
Q4KLL7	Vacuolar protein sorting 4 homolog B	VPS4B	6.6E-03	-2.03	1.36	Very large
Q5XIG9	Mitochondrial fission process 1	MTFP1	8.2E-04	-1.25	1.78	Very large
D3ZZI0	NIPA-like domain-containing 3	NIPAL3	2.0E-02	-2.78	1.12	Large
B2GV87	Receptor-type tyrine-protein phphatase epsilon	PTPRE	3.8E-02	-2.05	0.99	Large
F1M3D2	Dystrophiamyotonica, WD repeat-containing	DMWD	6.7E-03	-1.40	1.35	Very large
P51799	H(+)/Cl(-) exchange transporter 7	CLCN7	4.7E-04	-1.99	1.89	Very large
A0A0G2JZ52	Heterogeneous nuclear ribonucleoprotein U	HNRNPU	1.1E-02	-0.68	1.25	Large
P81799	N-acetyl-D-glucamine kinase	NAGK	1.7E-02	-1.48	1.16	Large
B2GV15	Dihydrolipoamideacetyltransferase component of pyruvate dehydrogenase complex	DBT	1.8E-02	-0.62	1.15	Large
D3ZGJ1	Immunoglobulin-like domain-containing receptor 2	ILDR2	3.0E-03	-0.82	1.52	Very large
M0RAT6	Uncharacterized protein	TMED8	1.8E-02	-1.85	1.15	Large
Q5PPI4	Lysome-associated membrane glycoprotein 5	LAMP5	1.6E-02	-0.91	1.17	Large
P19490	Glutamate receptor 1	GRIA1	1.2E-03	-0.63	1.70	Very large
D3ZC56	Dystonin	DST	3.3E-02	-0.63	1.02	Large
D3ZHK4	RB1-inducible coiled-coil 1	RB1CC1	4.4E-02	-1.30	0.96	Large
Q4V8J6	YTH N(6)-methyladenine RNA-binding protein 1	YTHDF1	3.4E-02	-1.22	1.01	large
D4A7I6	Uncharacterized protein	RGD1309995	4.0E-02	-1.75	0.97	Large
M0RDU0	Coagulation factor VIII-associated 1	F8A1	3.5E-03	-0.83	1.49	Very large
A0A0G2KAN5	Enhancer of rudimentary homolog (Drophila)	ERH	7.2E-03	-0.97	1.34	Very large
D4A4W6	RCG20695, isoform CRA_b	SLIRP	1.0E-02	-0.78	1.26	Large
B2RZB5	Charged multivesicular body protein 2A	CHMP2A	4.6E-02	-0.74	0.94	Large
D3ZIV8	YTH domain-containing 2	YTHDC2	1.2E-02	-1.89	1.23	Large
Q5U2R6	Putative monooxygenase p33MONOX	P33MONOX	1.8E-02	-0.70	1.15	Large
A0A0G2K0T6	Gamma-synuclein	SNCG	2.4E-02	-1.12	1.09	Large

G3V6C3	Polysaccharide biynthesis domain-containing 1	PBDC1	3.1E-03	-0.79	1.51	Very large
D3ZCT7	Sec23 homolog B, coat complex II component	SEC23B	2.5E-02	-1.37	1.08	Large
O70352	CD82 antigen	CD82	4.0E-03	-0.89	1.46	Very large
D4A604	Phphatidylinitol glycan anchor biynthesis, class T	PIGT	3.0E-03	-0.59	1.52	Very large
P20611	Lysomal acid phphatase	ACP2	4.5E-02	-0.60	0.95	Large
Q6PEC1	Tubulin-specific chaperone A	TBCA	1.3E-02	-0.60	1.21	Large
Q76MV3	COX17 cytochrome c oxidase copper chaperone	COX17	1.3E-03	-0.84	1.68	Very large
D3XAM6	Dual-specificity mitogen-activated protein kinase kinase 7	MAP2K7	2.5E-03	-1.14	1.55	Very large
D3ZPN0	Protocadherin 17	PCDH17	4.6E-02	-1.10	0.95	Large
D3ZCI2	Solute carrier family 9 (Sodium/hydrogen exchanger), member 7 (Predicted)	SLC9A7	3.2E-02	-1.10	1.03	Large
Q66H54	FTS and Hook-interacting protein	FAM160A2	3.3E-03	-1.17	1.50	Very large
D3ZC89	Uncharacterized protein	FAM114A2	3.8E-02	-1.02	0.99	Large
F1M3A4	Uncharacterized protein	NEWGENE_1305560	7.5E-03	-0.70	1.33	Very large
D3ZUL1	Coiled-coil domain-containing 124	CCDC124	2.8E-02	-1.24	1.05	Large
Q99PS2	Casein kinase 1, epsilon	CSNK1E	3.0E-02	-0.76	1.04	Large
F1M6D0	Mitochondrial ribomal protein S6	MRPS6	3.1E-02	-0.64	1.03	Large
Q00566-2	Isoform B of Methyl-CpG-binding protein 2	MECP2	2.4E-02	-0.67	1.09	Large
P38659	Protein disulfide-isomerase A4	PDIA4	3.8E-02	-0.80	0.99	Large
A0A0G2K847	ArfGAP with GTPase domain, ankyrin repeat and PH domain 1	AGAP1	2.3E-02	-0.67	1.10	Large

The biological function of the significantly altered proteins was explored by using a functional annotation GO enrichment (Fisher’s Exact test). Proteins identified after SKF-81297 treatment were associated with chemical synaptic transmission, synaptic plasticity, synaptic translation processes, neuronal differentiation and learning and memory (Table 7). Of particular interest are the proteins associated with visual learning and memory (GRIA1, MECP2, NETO1, RGS14) and catecholamine secretion (KCNB1, MECP2, SNCG).

Table 7: Biological processes of the D1-agonist regulated proteins

GOID	GOTerm	p-Value	p-Value Bonferroni	Associated Proteins Found
GO:0050805	negative regulation of synaptic transmission	8.20E-03	2.46E-02	[GRIA1, KCNB1, PCDH17]
GO:0017148	negative regulation of translation	3.15E-02	3.15E-02	[MECP2, PTGFRN, XRN1]
GO:0021953	central nervous system neuron differentiation	9.85E-03	1.97E-02	[CSNK1E, GBA2, PTK2, ROBO2]
GO:0050432	catecholamine secretion	4.34E-03	2.60E-02	[KCNB1, MECP2, SNCG]
GO:0007051	spindle organization	4.85E-03	2.43E-02	[CHMP2A, MECP2, RGS14, VPS4B]
GO:0008542	visual learning	2.87E-03	2.29E-02	[MECP2, NETO1, RGS14]
GO:0007051	spindle organization	4.85E-03	2.43E-02	[CHMP2A, MECP2, RGS14, VPS4B]
GO:0007613	memory	4.14E-03	2.90E-02	[GRIA1, MECP2, NETO1, RGS14]
GO:0050804	modulation of chemical synaptic transmission	7.57E-05	9.84E-04	[GRIA1, GRM2, KCNB1, MECP2, NETO1, PCDH17, PTK2, RGS14, SNCG]
GO:0008542	visual learning	2.87E-03	2.29E-02	[MECP2, NETO1, RGS14]
GO:0048167	regulation of synaptic plasticity	2.64E-03	2.37E-02	[GRIA1, KCNB1, MECP2, NETO1, RGS14]
GO:0050806	positive regulation of synaptic transmission	1.39E-03	1.53E-02	[GRIA1, MECP2, NETO1, PTK2, RGS14]
GO:0007268	chemical synaptic transmission	3.52E-05	4.93E-04	[ACP2, GABRA3, GRIA1, GRIA3, GRM2, KCNB1, MECP2, NETO1, PCDH17, PTK2, RGS14, SNCG]
GO:0060079	excitatory postsynaptic potential	2.06E-03	2.06E-02	[GRIA1, GRIA3, MECP2, NETO1]

Among the D1 agonist regulated proteins of the cellular components (Table 8), those heavily associated with dendritic spine morphology and synaptic remodeling are of particular interests since they are associated with learning and memory.

Table 8: Cellular components of the D1-agonist regulated proteins

GOID	GOTerm (Cellular Components)	PValue	p-Value Bonferroni	Associated Proteins
GO:0043197	dendritic spine	1.62E-03	3.24E-03	[ATP2B1, GRIA1, GRIA3, PTK2, RGS14]
GO:0031252	cell leading edge	1.16E-03	3.48E-03	[ATP2B1, GRIA1, KCNB1, LAMP5, PTK2, RAB13, ROBO2]
GO:0032589	neuron projection membrane	4.53E-06	3.17E-05	[ATP2B1, GRIA1, KCNB1, LAMP5, ROBO2]
GO:0098794	postsynapse	6.81E-05	3.41E-04	[ATP2B1, GABRA3, GRIA1, GRIA3, KCNB1, MECP2, MPDZ, NETO1, PTK2, RGS14]
GO:0014069	postsynaptic density	5.96E-03	5.96E-03	[GRIA1, GRIA3, MPDZ, NETO1, RGS14]
GO:0097060	synaptic membrane	8.77E-06	5.26E-05	[ATP2B1, GABRA3, GRIA1, GRIA3, GRM2, KCNB1, MPDZ, NETO1, RGS14]
GO:0045211	postsynaptic membrane	7.21E-05	2.88E-04	[GABRA3, GRIA1, GRIA3, KCNB1, MPDZ, NETO1, RGS14]

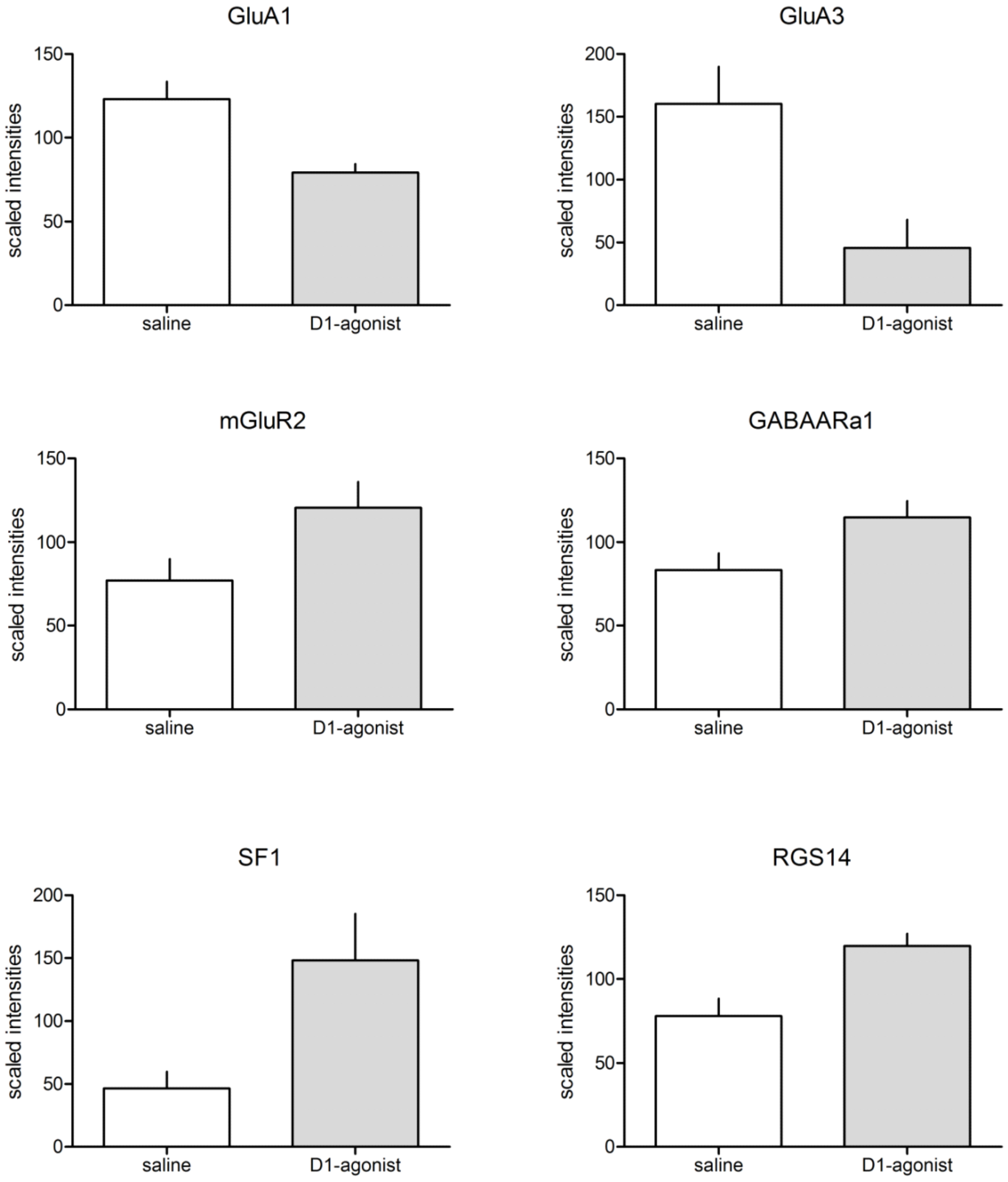


Figure 11: Graphic comparison of some of the proteins significantly regulated by the treatment with the D1-like receptor agonist.Given are the mean and standard error of the mean.

Strikingly, we unexpectedly found receptors traditionally associated with axonal growth and synaptogenesis in the developing brain (ROBO2, GABRA3), suggesting novel functions for these receptors in altering adult brain circuitry upon D$_1$-induced memory consolidation. In sum, these data reveal that D$_1$ receptor-mediated hippocampal dependent learning and memory tasks leads to massive restructuring of prefrontal cortex signaling networks by opening up the synaptic plasticity window.

Of the proteins’ expression regulated by dopamine some were positively and others were negatively correlated with the reference memory indices of day 3 (Table 9). Correlations were done over both groups. Byesian statistics Pearson correlationis presented separately with ascending p-values and Bayes factor in Table 9. Related biological processes and molecular functions are also presented (UniProt).

Table 9: Correlations of reference memory indices of individual animals with significantly D1-agonist regulated proteins.

Positive Correlation						
Protein	**Description**	**Pearson Coeff.**	**P-value**	**Bayes factor**	**Evidence for H1**	**Biological process**
ATP2B1	Plasma membrane calcium-transporting ATPase 1	0.654	0.002	0.059	strong	calcium transport out of cell
FAM171A1	Family with sequence similarity 171, member A1	0.644	0.003	0.072	strong	integral component of membrane
AKR1B10	aldo-keto reductase family 1 member B10	0.635	0.004	0.084	strong	reduction of aliphatic and aromatic aldehydes
IPO9	Importin-9	0.633	0.004	0.086	strong	nuclear import receptor
HMGB2	High mobility group protein B2	0.621	0.005	0.107	moderate	multifunctional, negative regulation of gene expression
TSPAN7	Tetraspanin-7	0.614	0.005	0.119	moderate	leukocyte migration, neurite outgrowth
RGD1308601	Putative uncharacterized protein RGD1308601_predicted	0.606	0.006	0.136	moderate	ubiquitin dependent protein catabolic process
FBXL16	F-box/LRR-repeat protein 16	0.571	0.011	0.225	moderate	Ubiquitin ligase conjugation pathway
RGS14	Regulator of G-protein signaling 14	0.553	0.014	0.286	moderate	learning, long-term memory, long-term potentiation
MIGA1	Mitoguardin 1	0.511	0.025	0.477	anecdotal	mitochondrial fusion
SNRK	SNF-related serine/threonine-protein kinase	0.508	0.026	0.492	anecdotal	intracellular signal transduction
GBA2	Non-lysosomal glucosylceramidase	0.507	0.027	0.502	anecdotal	formation of motor neuron axons
MPO	Myeloperoxidase	0.495	0.031	0.571	anecdotal	response to food, aging
RAB13	Ras-related protein Rab-13	0.485	0.035	0.632	anecdotal	multifunctional, cortical actin cytoskeleton organization
EXOC6	Exocyst complex component 6	0.483	0.036	0.649	anecdotal	protein transport, exocytosis

SF1	Steroidogenic factor 1	0.478	0.038	0.68	anecdotal	steroid hormone mediated signaling pathway
PTGFRN	Prostaglandin F2 receptor negative regulator	0.468	0.043	0.754	anecdotal	inhibition of both prostaglandin F2-alpha binding to its receptor
Negative Correlation						
NAGK	N-acetylglucosamine kinase	-0.8	0.000	0.001	Extreme	glutamate metabolism
AGAP1	Arf-GAP with GTPase, ANK repeat and PH domain-containing protein 1	-0.668	0.002	0.046	strong	membrane traffic, actin cytoskeleton dynamics
ILDR2	Immunoglobulin-like domain-containing receptor 2	-0.653	0.002	0.061	strong	lipid homeostasis
CD82	CD82 antigen	-0.647	0.003	0.068	strong	cell surface receptor signaling pathway
SEC23B	Protein transport protein Sec23B	-0.639	0.003	0.078	strong	intracellular protein transport
CLCN7	H(+)/Cl(-) exchange transporter 7	-0.611	0.005	0.125	moderate	chloride transmembrane transpor
COX17	COX17 cytochrome c oxidase copper chaperone	-0.59	0.008	0.172	moderate	positive regulation of cell proliferation
GRIA1	glutamate receptor 1	-0.587	0.008	0.180	moderate	negative regulation of synaptic transmission
F8A1	coagulation factor VIII-associated 1	-0.583	0.009	0.191	moderate	unknown
CDH6	Cadherin-6	-0.558	0.013	0.27	moderate	calcium-dependent cell-cell adhesion via plasma membrane cell adhesion molecules
FAM114A2	Protein FAM114A2	-0.551	0.015	0.295	moderate	unknown
TBCA	Tubulin-specific chaperone A	-0.548	0.015	0.305	moderate	tubulin complex assembly
DBT	Dihydrolipoyl-Acyltransferase	-0.546	0.016	0.316	moderate	metabolic process
YTHDC2	YTH domain-containing 2	-0.536	0.018	0.356	anecdotal	oocyte development
DMWD	Dystrophiamyotonica, WD repeat-containing	-0.525	0.021	0.406	anecdotal	unknown
COX6A1	Cytochrome c oxidase subunit 6A1, mitochondrial	-0.522	0.022	0.419	anecdotal	mitochondrial electron transport, cytochrom c to oxygen

NYAP2	Neuronal tyrosine-phosphorylated phosphoinositide-3-kinase adaptor 2	-0.522	0.022	0.421	anecdotal	neuron projection morphogenesis
ARMCX3	Armadillo repeat-containing X-linked protein 3	-0.513	0.025	0.468	anecdotal	cellular protein localization
GRIA3	gluatamate receptor 3	-0.508	0.026	0.494	anecdotal	chemical synaptic transmission
P33MONOX	Putative monooxygenase p33MONOX	-0.498	0.03	0.551	anecdotal	May be involved in the regulation of neuronal survival, differentiation and axonal outgrowth
PDIA4	Protein disulfide-isomerase A4	-0.479	0.038	0.673	anecdotal	cell redox homeostasis and protein folding
SLIRP	RCG20695, isoform CRA_b	-0.465	0.045	0.777	anecdotal	negative regulation of mitochondrial RNA catabolic process
PBDC1	Polysaccharide biosynthesis domain-containing 1	-0.462	0.047	0.799	anecdotal	unknown

Proteins	**Molecular Function**	**Proteins**	**Molecular Function**
ATP2B1	ATP binding, calcium transporting ATPase activty	CD82	costimulatory signals for the TCR/CD3 pathway
FAM171A1	unkown	SEC23B	zinc ion binding
AKR1B10	NADP (H) binding	CLCN7	antiporter acivity, voltage gated chloride channel activity
IPO9	protein transporter activity, Ran GTPase binding	COX17	copper chaperone activity, cuprous ion binding
HMGB2	DNA- , lipid-, drug-, transcription factor- binding	GRIA1	AMPA glutamate receptor activity
TSPAN7	unknown	F8A1	Unknown
RGD1308601	ubiquitin protein ligase activity	CDH6	calcium-dependent cell-cell adhesion via plasma membrane cell adhesion molecules
FBXL16	substrate recognition component SCF-type E3ubiquitin ligase complex	FAM114A2	Unknown
RGS14	G-protein alpha-subunit binding,	TBCA	beta tubulin binding

	GTPase activator activity		
MIGA1	protein hetero/homo-dimerization activity	DBT	Ubiquitin protein ligase binding
SNRK	protein serine/threonine kinase activity	YTHDC2	YTH domain-containing 2
GBA2	Non-lysosomal glucosylceramidase	DMWD	Dystrophiamyotonica, WD repeat-containing
MPO	heme-, heparin- binding, peroxidase activity	COX6A1	cytochrome-c oxidase activity, enzyme regulator activity
RAB13	GTPase activity, GTP binding	NYAP2	neuron projection morphogenesis
EXOC6	docking of excocytic vesicles on plasma membrane	ARMCX3	Armadillo repeat-containing X-linked protein 3
SF1	steroid hormone receptor activity	GRIA3	AMPA glutamate receptor activity
PTGFRN	Prostaglandin F2 receptor negative regulator	P33MONOX	Putative monooxygenase p33MONOX
NAGK	ATP binding, N-acetylglucosamine activity	PDIA4	Protein disulfide-isomerase A4
AGAP1	GTPase- activator- activity, GTP binding	SLIRP	RNA binding
ILDR2	Immunoglobulin-like domain-containing receptor 2	PBDC1	Polysaccharide biosynthesis domain-containing 1
		CD82	costimulatory signals for the TCR/CD3 pathway

5. Discussion

5.1. BehavioralTest Batteries Associated withLearning and Memory

Discrete distributions of cognitive abilities and intrinsic mental states were found in a large cohort of 5-6 months old male Sprague-Dawley rats. The lowest portions that exceed intermediate levels of mental states could be determined for motivation and depression. Thus, for experiments focusing on modulations of these states by behavioral experience or pharmacological treatment, the probability to include animals with an intrinsically high or low background is minimal. The most critical intrinsic state is cognition with a high portion of animals with low cognitive abilities, whereas that of high abilities is comparable to the other variables. This may partly be based on the age of the animals since often younger animals are used for experiments, which may be still more flexible and adaptive to task demands. However, only at this age male rats are fully adult in terms of social competence, brain development and musculoskeletal maturity (Adam and Boice, 1989; Sengupta, 2011; Mengler et al., 2014). The use of adult animals is of special interest because a lot of physiological and molecular phenomena change even over the first three months of age (McCutcheon et al., 2009).

Sprague-Dawley is an outbreed rat strain with broader genetic variability that may reflect the situation in a human population more realistic, and most of the studies in rodents are conducted as an animal model for mechanisms in human populations. However, in inbred strains with a narrow genetic variance, genetic similarity can only explain a part of behavioral variability in home cage behavior (Loos et al., 2014) and behavioral tests (Vorhees, 1983; van der Staay and Blokland, 1996; Lahmame and Armario, 1996). Moreover, not only in rodents but also in inbred human populations (Fareed and Afzal, 2014) such individuality in behavior and intrinsic mental states is a considerable factor influencing the variance in experimental results (Sequeira-Cordero et al., 2014; Shumake, 2014). Furthermore, different early pre-and postnatal experiences and environmental complexity support individual behavior, physiology, and molecular processes during adulthood (Oitzl et al., 2000; Braun and Champagne, 2014; Sarro et al., 2014). For these reasons, it is difficult to generalize between different laboratories but individuality should be estimated for each local population of animals and may then provide more reliable results in animal models of mental and cognitive diseases and individual vulnerability (Koolhaas et al., 2010). Environmental standardization of test procedures has been found to be more the cause than the remedy of low reproducibility of behavioral experimental outcomes between laboratories (Richter et al., 2009). The main reason is that the local conditions are stressed and the validity to other laboratories impaired. A characterization of local populations as suggested here may improve the external validity.

The second focus was on possible interactions between the testing procedures. No correlations were found between the performance in the different tasks, except between the open field and the hole-board procedure. Interestingly, a difference was found only for later trials but not for the first trial. The similarity in shape and size between open-field and holeboard and the associated familiarity with the arena might be one reason for these correlations. However, two habituation sessions preceded the holeboard test and habituation experience is similar for all rats, so that habituation as such should play a minor role. The enhanced motivation to explore is more likely to support holeboard learning and spatial memory, since the distance travelled and the velocity of travelling in the open-field correlates positively with the holeboard performance. Further, correlations could be found only when learning plays a major role, namely at the second day, and when a memory trace has been established at day 3 during the retention trial. Forced swim and Rotarod performance were not correlated in the Pearson test but were highly and oppositely loaded on the depression factor. Thus, immobility in the forced swim task was associated with short time periods on the rod. This association might be related to the fact that general locomotor abilities and activity also reduce the time of immobility in the forced swim test leading to false positive results in pharmacological studies (Slattery and Cryan, 2012). The factor as well as the linear Pearson regression analysis revealed no correlation between performance of the battery tests that are not explainable by underlying behavioral and physiological states. This is in contrast to other studies in which a positive correlation between the time spent in the open-arm of the elevated plus maze and the duration of immobility in the forced swim test (Estanislau et al., 2011) or a negative correlation between FS-immobility and open-field locomotor activity (Ho et al., 2002) in rats has been found.

The assessment of individual features early in life or during adulthood provides more reliability of results in studies focused on age dependent dementia or depression, because it allows the discrimination between intrinsic and age dependent mental states and cognitive abilities.

5.2 Dopaminergic Modulation of Learning and Memory

In this study, it was sought to identify the contribution of D_1 and D_2 receptor signaling to learning and memory formation in the hippocampal-prefrontal cortex axis. Agonism of D_1 receptors improved RMI in the learning task. D1 antagonist's effect wasn't statistically significant. The lack of significant effect of D2 agonist indicated that D1 receptor signaling, but not D_2 signaling, is required for proper learning and memory formation in the hole-board setup. This is in line with data revealing that D_1 receptors are predominately involved in learning, but not reference memory consolidation, in hole-board paradigms.

Experiments based on reward motivated tasks such as holeboard, depends on the level of motivation of the animals to search for the reward. The motivation of experimental animals is affected by the inherent motivational state of the animals and the degree of food restriction during the experiment. The degree of food restriction is similar for both treatment and control groups. The reference memory index and latency to search for food relies on the motivational states as well as the cognitive ability of the animals. Animals with higher cognitive ability and better motivation have higher RMI and smaller latency. In the present experiment, it was observed that D1 agonist treatment group had a relatively higher RMI and smaller latency as compared to control group. And also, the total number of holes visited by D1 receptor agonist treatment group is fewer than the control group. These two findings together indicate that D1 receptor agonism improves spatial reference memory rather than motivation. Furthermore, the recent study by Feyissa et al., 2017 indicated that the percent of rats with higher inherent motivational status in a population of Sprague Dewely rats is about 60% while 40% of the population had low inherent cognitive abilities. These individual differences in a population of Sprague-Dewely rats might also be an evidence for direct cognitive enhancing effect of dopamine through D1 receptor activation not by increasing the motivation of the animal to search for the reward. However, further investigation should be carried out in order to rule out the contribution of enhancing motivation by dopamine on spatial learning and memory through conducting an open field test.

Since the lack of an effect upon memory consolidation may be due to the cognitive load of the task, animals were tested in both easy and difficult tasks. It was hypothesized that in easy tasks, control animals learn and memorize faster, such that additional dopaminergic activation does not support better memory formation. However, upon increasing complexity of the task, additional dopaminergic signaling could increase behavioral performance. Indeed, it was found that D_1 agonist induced changes in RMI were more obvious with increased difficulty in the hole-board paradigm, reinforcing that task difficulty could skew the results in D_1-mediated learning and memory tasks.

The findings in the simple task are in line with other studies in which improvement effects of D_1 agonists on acquisition, but not consolidation, have been found (Coccurello et al., 2000; Sariñana and Tonegawa, 2016). In contrast to these studies, it was also observed that low levels of D_2 receptor activation to improve learning at acquisition phase on the second trial day. This might be based on individual contributions of dopamine receptor subtypes to acquisition and consolidation as well as differential activation of separate brain regions. For instance, D_1 but not D_2 receptors have been found to be involved in the acquisition of spatial information in the nucleus accumbens(Coccurello et al., 2000), whereas D_2 receptor activation in the ventral pallidum improves (Péczely et al., 2016) and hippocampal D_2 inactivation impairs spatial learning (Rocchetti et al., 2015).

In addition, dopaminergic effects on learning and memory are task specific, as acquisition, but not consolidation, effects of dopamine receptor activation have mainly been found in low to intermediate stressful training paradigms such as holeboardand land mazes, whereas consolidation effects appear mainly in stressful training paradigm such as water maze experiments (Péczely et al., 2016; Rocchetti et al., 2015). In line with this, interactions between the dopamine system and stress related activation of glucocorticoid receptors have been reported (Uban et al., 2013). Mineralocorticoid receptors in the ventral tegmental area can regulate dopamine efflux in the basolateral amygdala during conditioned fear (de Oliveira et al., 2014), while corticosterone improves conditioned fear in a hippocampal D_1 receptor dependent manner (Liao et al., 2013). Retrieval of long-term memories in an inhibitory avoidance task were found to be regulated by a D_2 and glucocorticoid interaction (Pakdel and Rashidy-Pour, 2006), indicating that stress is an important factor in dopamine-mediated learning and memory.

Although no D_1-mediated improvement of RMI was observed during the retrieval trials at day 3, one cannot rule out that consolidation has taken place since behavioral performances on the first and second day were similar. The lack of improvement on the third day may be limited by a ceiling effect in performance. This would suggest that early consolidation processes may be supported by both dopamine receptors in tandem with a balanced strength of activation for D_2receptors. The early processes may be related to synaptic consolidation and sleep dependent enhancement, whereas the retrieval trial represents the behavioral performance of systems consolidation or even reconsolidation (Walker et al., 2003).

Differences between dopaminergic signaling mechanisms and activity during spatial reference memory tasks of different cognitive demands are rare. However, in humans, there is some evidence that differences in spatial task difficulty (Bullmore et al., 2003) or different visual discrimination task loads (Boehler et al., 2011) are related to the activation of different dopaminergic brain regions as well as task load related levels of activity within the same brain area. The present results suggest that the involvement of dopaminergic activity upon behavioral performance was more pronounced in a difficult spatial reference memory task than simple tasks. Increased dopaminergic transmission (e.g. higher levels of dopamine) activity in the prefrontal cortex has been found in gerbils during learning of novel behavioral strategies in an active avoidance paradigm in a shuttle box (Stark et al., 2004). Similar processes, as well as the relative involvement of different brain areas, in the simple and difficult task can be hypothesized to underlie the behavioral performance as described in the present study.

The data obtained in proteomic screening of the prefrontal cortex, although at present only correlative, support some of the abovementioned possible dopamine related underlying mechanisms of memory and learning. For instance, the negative correlation of the AMPA receptor subunits GluA1 and GluA3 point to an adjustment of AMPAR composition related to learning and memory processes. Regulation of AMPAR and NMDAR composition in the entorhinal cortex by sleep deprivation has been found to affect reference memory in the water maze in rats (Xie et al., 2016). Generally, the regulation of glutamate receptor subunits and glutamate metabolism related proteins point to synaptic changes in adjustment with other brain regions involved in spatial long-term memory like the hippocampus (Kim et al., 2011; Lega et al., 2016; Magnusson et al., 2007). Glutamatergic mechanisms may also be related to steroid hormone mediated mechanisms. The positive correlation of SF-1 expression with RMI suggest a D_1 receptor mediated dopaminergic modulation of steroid hormone receptor activity, and/or in conjunction with mitochondrial local steroid hormone synthesis (Miller, 2013), suggested by a number of correlated proteins involved in mitochondrial mechanisms, may contribute to enhanced glutamatergic release (Treccani et al., 2014) and strengthened reference memory. Especially, mitochondrial fusion is required for local steroid hormone synthesis (Duarte et al., 2012), which is here suggested by upregulation of the fusion protein, Mitoguardin 1. Synapse restructuring related to learning is also suggested by the correlation of proteins of the ubiquitin proteasome system, which is involved in memory (Artinian et al., 2008; Chain et al., 1999) and in synaptic plasticity (Fonseca et al., 2006; Jarome et al., 2013). However, most striking is the detection of signaling systems traditionally investigated in developmental processes. For example, ROBO2 receptors are heavily involved in axonal guidance decisions and synaptic targeting in the developing cortex (Blockus and Chédotal, 2016), but their role in adult signaling systems is unknown. Additionally, GABRA3 receptor subunits are heavily expressed during cortical development and are downregulated during postnatal stages (Milenkovic et al., 2017), where its function remains elusive. To sum up, a plethora of D_1-induced alterations would contribute to the complex proteomic landscape of the prefrontal cortex in hippocampal-mediated learning and memory tasks, which can be used as a platform for detailed molecular studies on novel undescribed targets in the underpinnings of memory consolidation.

6. Conclusion

The present study pointed out that dopamine receptor type 1 signaling modulates spatial reference learning and memory in the prefrontal cortex through regulation of a plethora of proteins expression. Moreover, Dopamine receptor type 1 signialing modulation of spatial reference learning and memory is task dependent and more significant during performing difficult than simple tasks. On the other hand, dopamine receptor type 2 activation as well as antagonism had shown no significant differences in spatial reference learning and memory, ruling out the role of this receptor in learning behavior involving hole-board task. Furthermore, the study has provided evidence that observational test batteries increase the quality of spatial reference learning and memory test paradigms through providing information about the level of inherent motivation, anxiety or depression like behaviors in a population of experimental animals.

7. Future perspective

The current research identified plethora of proteins that are either up regulated or down regulated during dopaminergic signaling modulation of spatial memory acquisition and consolidation. However, further investigation is needed on detailed molecular studies on novel undescribed targets in the underpinnings of memory consolidation. For example, ROBO2 receptor, which is heavily involved in axonal guidance decisions and synaptic targeting in the developing cortex was upregulated. Its role in adult signaling system is unknown. Therefore, further investigation of the roles of this receptor in adult brain should be undertaken.

Hippocampal-prefrontal cortex interaction during spatial reference memory acquisition and consolidation was reported. The current studies identified several proteins regulated by dopaminergic modulation in prefrontal cortex. However, the proteins regulated in the HPC through such modulation weren’t carriedout in this study. Therefore, investigation of proteins regulated in the HPC, which helps to investigate how the change in protein profile in these two brain regions affects each other should be carried out.

Furthermore, gel electrophoresis followed by western blotting is necessary to be carried out for proteins regulated by dopaminergic modulation in order to conduct further investigation how each protein affect spatial memory acquisition and consolidation. Moreover, immuno-histochemistry should be conducted for proteins proven to modify the spatial memory acquisition and consolidation in order to map the brain regions affected by these proteins. Finally, the mechanism by which dopaminergic modulation monitors the hippocampal prefrontal cortex interaction needs to be investigated.

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