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Effects of Pharmacological Interventions on Rodent Behavior and Neuroplasticity

Britt T.J. van Hagen

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PROEFSCHRIFT

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Chapter 1

General Introduction

Cognitive enhancement

Cognition or cognitive function encompasses a series of mental functions that are involved in gaining knowledge and comprehension of the world around us. Cognitive functions include attention, reasoning, planning, behavioral flexibility, learning and different forms of memory, processes which are all highly important for every-day functioning (Nehlig, 2010). In general, these processes are interrelated, relying on multiple brain area's and signaling pathways for its functioning. However, due to the intricate nature of cognitive processes many of the mechanisms serving cognitive function are not fully understood yet.

A wide range of psychiatric diseases have cognitive impairments as one of their core symptoms, which have a negative effect on patients overall functioning, their recovery and long-term outcome (Millan et al., 2012). Therefore, it is of importance that the mechanisms underlying these different cognitive functions can be unraveled in order to develop targeted treatments which can ameliorate cognitive impairments. Treatments or drugs which have the specific goal of improving performance on cognitive functions are called 'cognitive enhancers'. These drugs are not designed to cure the psychiatric disease from which the patient is suffering but specifically target the cognitive symptoms that result from their condition, thereby improving their every-day functioning and subsequently their quality of life (Froestl et al., 2012).

The study of cognitive enhancement poses some challenges for researchers, since higher order cognition is mainly a human trait heavily supported by the use of langue. Yet the more invasive mechanistic studies and screening of new drugs can only be performed in animals, which are unable to use (our) langue as a demonstration of their cognitive skills. Therefore, the need to have well-validated animal models and cognitive tests which resemble the human situation as closely as possible is of uttermost importance to be able to study different aspects of cognition. Animal research performed with the aim to resemble the human situation as closely as possible is called 'translational research'. Apart from trying to closely mimic human situations, this research also has to take into account the nature of the animals the study is performed on and design the models and tasks to incorporate and use the differences that these species show from humans, in order to obtain the best possible translational outcome. In drug-screening studies cognitive deficits can be induced experimentally in animals pharmacologically, which can result in both temporary or chronic

deficits depending on the nature of the given drug and the treatment regimen. Secondly, genetic mutations can be introduced into an animal which will lead to the development of the disease or part of the symptoms. This dissertation focuses on the development and validation of a novel behavioral test to measure pattern separation performance in rodents and the potential of novel treatment strategies to enhance cognition and in particular pattern separation function in a translational manner.

Pattern separation & spatial memory

Behavioral pattern separation is a process that serves the ability to recognize and remember two highly similar, yet slightly different situations. Stemming from computational theories, this hippocampal process has just recently been studied in both humans and rodents (Bakker et al., 2008; Kirwan et al., 2012; Mc Hugh et al., 2007) and has gained recognition as a fundamental aspect of memory formation and retrieval. Pattern separation has been described as a process which reduces interference or overlap between highly similar input patterns in order for representations to be stored and retrieved separately (Sahay et al., 2011; Kent et al., 2016). In an every-day life example this means that (spatial) patterns separation processing is the reason that you are able to walk right to your car at the over-crowded parking lot after a day of work, (almost) each subsequent day. Even though circumstances in which you park in the morning barely differ from day-to-day, while the available parking spot on which you park does.

Pattern separation processing is centered in the hippocampus (Kirwan et al., 2012). The dentate gyrus (DG) sub-region is essential to pattern separation (Sahay et al., 2011), but in order to distinguish between different spatial locations the CA3 sub-region is also involved (Leutgeb et al., 2007). The majority of cortical input received by the DG stems from the entorhinal cortex, which is subsequently sent through to the CA3 along mossy fiber projections (Morris et al., 2012). Although it has been argued that other types of pattern separation processing may also take place in different brain regions to support other types of processing (Kent et al., 2016) this dissertation focusses on the memory-related behavioral pattern separation. Hippocampal neurogenesis – the generation of new neurons in the DG and their functional integration in the adult mammalian brain - has been implicated to contribute to pattern separation processing by the majority of studies investigating its underlying mechanisms (Celland et al., 2009; França et al., 2017). Recent studies have shown that pattern separation performance is impaired in a variety of psychiatric diseases, like Alzheimer's disease, post-traumatic stress disorder (PTSD) and schizophrenia (Das et al., 2014; Leal & Yassa, 2018). Due to the fundamental nature of pattern separation processing it is believed to be one of the first functions to be affected in the early-onset of these diseases, which implicates pattern

separation tests can be of high importance to early detection in cognitive screenings. Furthermore, pattern separation impairments can seriously affect every-day functioning and compromise the patient's recovery and long-term functional outcome. It can even be argued that pattern separation impairments are the cause of some of the more pronounced symptoms seen in PTSD and schizophrenia, due to the disability to judge situations and overgeneralizing of potential threats (Kheirbek et al., 2012; Faghihi et al., 2015). Therefore, it is of importance to gain insight on pattern separation processing and its underlying mechanisms to find a potential treatment for pattern separation deficits. Enhancing pattern separation performance can beneficially impact the quality of life of the different patient groups suffering from dysfunction in pattern separation processing.

The increased interest and importance of pattern separation studies also raises the need for a translational task designed to measure pattern separation performance in rodents. Pre-existing studies measuring pattern separation in rodents used tasks with a reward or punishment strategy (see chapter 2). Therefore, these studies incorporate emotional or anxiety components which are difficult to properly control for and translate to the human situation. In everyday-life situations humans need to utilize pattern separation processes without receiving a direct punishment or reward. Without this direct motivational importance, the process occurs spontaneously. Furthermore, emotional pattern separation involves additional, complicated circuitry (Leal et al., 2014) which complicates the correct interpretation of any specific attribution of individual processing circuits to the cognitive task performance per se.

To test spontaneously occurring pattern separation in rodents, we developed the spatial object pattern separation (OPS) task, which is based on showing only a slight variation in spatial locations. The task has been derived from the object location task (OLT) which is already widely used as a successful task to measure hippocampal dependent spatial memory (Ennaceur & Delacour, 1988). The OLT relies on naturally occurring exploratory behavior of rodents, hence there is no need for a direct reward or punishment to examine spatial memory. In this task, the animals are allowed to explore an arena with two similar objects placed in the middle of the arena. After a pre-determined interval, the animal is placed back in the same arena with the same set of objects yet one of the objects will be displaced to a novel location. Do to their natural curiosity a mouse or rat will spend more time exploring the object in the novel location, given that they remember and recognize that the object was formerly in a different location. Therefore, assessment of differences between the exploration times of both the 'moved'- and 'stationary' object will give a measure of spatial memory (Ennaceur & Delacour, 1988; van Goethem et al., 2012; Sierksma et al., 2014; Vanmierlo et al., 2016). The OPS task uses the same paradigm as the OLT but uses a set of gradually increasing displacement distances, in order to determine the point at which the animals need to utilize pattern

separation processes to recognize the novel location (van Hagen et al., 2015; van Goethem et al., 2015). Due to the similarity to the well-established OLT, the OPS task can be easily implemented across different labs and adapted to different experimental needs. From lesion studies performed with the OLT we can extrapolate that this task measures DG and CA3 centered processing in the hippocampus (van Hagen et al., 2015). The absence of any direct form of punishment or reward reduces stress and anxiety which could affect task performance outcome. Its reliance on naturally occurring exploratory behavior also reduces the need for lengthy strategy-learning training sessions before the start of the experiment. Further background, rationale and methods of this novel OPS tasks will be discussed in detail in chapter 2 and 6.

The serotonin 1A receptor

Serotonin or 5-hydroxytryptamine (5-HT) is a neurotransmitter that is widespread throughout the central- and peripheral nervous system. Since its first discovery in 1948 the serotonin system has been widely studied and shown to play an important role in a wide variety of essential normal brain functions like modulation of hunger, sleep, sex, memory, mood states, anxiety and many more (Hirst et al., 2008). Serotonin receptors are wide spread throughout the brain and highly expressed in many brain structures that are essential to cognition (Fiorino et al., 2013). The 5-HT system plays an important role in different forms of neuroplasticity and therefore dysfunctions or abnormal expression of 5-HT receptors may cause abnormal brain function which could lead to psychiatric diseases (Terry et al., 2008; Rojas & Fiedler, 2016).

Multiple different subtypes of 5-HT receptors have been identified, named 5-HT_1 to 5-HT_7 and some of these are further subdivided into additional subclasses. Apart from the 5-HT_3 subtype receptor 5-HT receptors are G-protein coupled receptors (GPCR's). The functional differences between the different sub-types are based on the inhibitory or stimulatory effects that the second messengers exert through mediation of the G-proteins (Fiorino et al., 2013).

This dissertation will focus on the serotonin 1A subtype receptor (5-HT_{1A}R). This receptor was among the first subtypes to be pharmacologically characterized and is the most widely studied in relation to cognition (Fiorino et al., 2013). 5-HT_{1A}R's have a high distribution in the hippocampus (especially in the DG and CA1) and the frontal cortex which are important to learning/memory and set-shifting/executive function, respectively (Hirst et al., 2008 Polter & Li, 2010). Therefore, these receptors can both directly and indirectly influence the neurotransmitter systems which are involved in cognitive impairments in different psychiatric diseases (Rojas & Fiedler, 2016). There are multiple cognition/memory enhancing drugs available that (partially) target the 5-HT_{1A}R. However, up to date most of these drugs show only moderate results in their memory and cognition enhancing effects.

This might be due to the differential effect that 5-HT_{1A}R's can exert on brain activity/function based on their cellular and spatial location in the brain.

Two types of 5-HT_{1A}R's can be discriminated based on their location, one type is expressed by serotonergic neurons in the dorsal raphe nucleus (DRN) where they act as an autoreceptor (Courtney & Ford, 2016). Activation of these receptors induces cell hyperpolarization that reduces 5-HT release (Polter & Li, 2010). 5-HT_{1A}R's are also expressed by many types of non-serotonergic neurons, eg. glutamatergic pyramidal neurons, GABAergic and cholinergic neurons. Activation of these so-called post-synaptic heteroreceptors mostly leads to a reduction in neuronal excitability and firing (Polter & Li, 2010), but indirect enhancing signaling effects on glutamate and dopamine have also been reported (Fiorino et al., 2014). Especially the activation of the latter receptor-type could lead to improved cognition in both healthy- and cognitively impaired individuals through its modulatory effects on other neurotransmitters (Fiorino et al., 2013). Furthermore, it has been found that 5-HT_{1A}R's play a crucial role in synaptic- and neuronal plasticity in both the developing- and adult brain (Polter & Li, 2010; Rojas & Fiedler, 2016). Adult hippocampal plasticity and neurogenesis is important for pattern separation processing, which has led to our hypothesis that stimulating 5-HT_{1A}R activity in the hippocampus and cortical regions could be beneficial to pattern separation performance. Further details on possible mechanisms by which 5-HT_{1A}R activation can improve memory and especially pattern separation processing are discussed in detail in chapter 3 & 4.

Aim & outline

The aim of this dissertation was to gain more insight in the neuronal signaling pathways involved in behavioral pattern separation processing and spatial memory, through investigation of the potential of different pharmacological agents to either impair or enhance these processes. In order to investigate pattern separation in rodents a novel behavioral task was developed and validated with the goal of creating an easily applicable rodent task to efficiently evaluate spatial pattern separation. The role of 5-HT_{1A}R signaling and possible enhancement of pattern separation was evaluated behaviorally and biochemically in both healthy rodents and pharmacological disease models. Additionally, the role of intra-cellular signaling pathways was investigated for different forms of spatial memory, using a relatively unknown alternative pharmacological impairment model in mice.

Chapter 2 starts with a general introduction on pattern separation processing and the general background of the development of the object pattern separation (OPS) task. This chapter gives a very detailed account of the entire rational behind the task and shows the data of our initial

validation studies in both healthy mice and rats. In the discussion of this chapter the various strengths and weaknesses of the tasks are elaborately described, along with the benefits of using the task compared to pre-existing pattern separation tasks.

In **chapter 3 & 4** the OPS task is used to investigate the role of 5-HT_{1A}R signaling in pattern separation processing. Building on a study performed by van Goethem et al. (2015), two 'biased' 5-HT_{1A}R agonists are used in **chapter 3** in order to differentiate between the chronic effects of either hetero- or auto- 5-HT_{1A}R activation on pattern separation performance and neuronal plasticity in healthy rats. This chapter provides more insight of the differential roles of pre- and post-synaptic 5-HT_{1A}R signaling and its effects on spatial pattern separation and (memory related) neuronal plasticity in healthy animals. Subsequently, **Chapter 4** further elaborates on the role of 5-HT_{1A}R's in pattern separation in relation to schizophrenia-like cognitive impairment. This chapter investigates the possibility to measure schizophrenia-related pattern separation impairments using a pharmacological, i.e. ketamine, rat model of schizophrenia in the OPS task. Ketamine-treated rats were subjected to the OPS task to measure their pattern separation performance. Secondly, a set-shifting task was used to measure behavioral flexibility, which is a pre-frontal centered cognitive ability that is known to be impaired in both schizophrenia patients and pharmacological rodent models of schizophrenia. The effect of acute 5-HT_{1A}R activation was measured in both these tasks to evaluate its effects on these schizophrenia-related impairments.

Different forms of spatial memory and memory impairment are evaluated in **chapter 5** using the OLT in both healthy mice and a pharmacologically induced memory impairment. This chapter is focused on evaluation of the potential of a market-approved soluble guanylate cyclase (sGC) stimulator, riociguat, to enhance spatial memory performance in mice. At the same time, an alternative pharmacological, i.e. biperiden, model was evaluated on its potential to acutely impair different forms of spatial memory in mice. Acute treatment with riociguat and a combination treatment with sub-optimal dosages of both riociguat and donepezil were evaluated on their potential to enhance memory performance in healthy mice and the biperiden-induced mice model for memory impairment.

Finally, **chapter 6** provides a full protocol for the OPS tasks with a highly detailed step-by-step guideline for researchers wanting to perform the tasks in their labs. This chapter provides all the necessary tools, explanation and considerations needed to plan, perform and interpreted the OPS task successfully within any behavioral rodent laboratory.

References

Bakker, A., Kirwan, C. B., Miller, M., & Stark, C. E. (2008). Pattern separation in the human hippocampal CA3 and dentate gyrus. *Science, 319(5870), 1640-1642.*

Clelland, C. D., Choi, M., Romberg, C., Clemenson, G. D., Fragniere, A., Tyers, P., ... & Bussey, T. J. (2009). A functional role for adult hippocampal neurogenesis in spatial pattern separation. *Science, 325(5937), 210-213.*

Courtney, N. A., & Ford, C. P. (2016). Mechanisms of 5-HT1A receptor-mediated transmission in dorsal raphe serotonin neurons. *The Journal of physiology, 594(4), 953-965.*

Das, T., Ivleva, E. I., Wagner, A. D., Stark, C. E., & Tamminga, C. A. (2014). Loss of pattern separation performance in schizophrenia suggests dentate gyrus dysfunction. *Schizophrenia research, 159(1), 193-197.*

Ennaceur A. and Delacour J. (1988) A new one-trial test for neurobiological studies of memory in rats. 1: behavioral data. *Behav. Brain Res. 31, 47–59.*

Faghihi, F., & Moustafa, A. A. (2015). A computational model of pattern separation efficiency in the dentate gyrus with implications in schizophrenia. *Frontiers in systems neuroscience, 9, 42.*

Fiorino, F., Severino, B., Magli, E., Ciano, A., Caliendo, G., Santagada, V., ... & Perissutti, E. (2013). 5-HT1A receptor: an old target as a new attractive tool in drug discovery from central nervous system to cancer. *Journal of medicinal chemistry, 57(11), 4407-4426.*

Froestl, W., Muhs, A., & Pfeifer, A. (2012). Cognitive enhancers (nootropics). Part 1: drugs interacting with receptors. *Journal of Alzheimer's disease, 32(4), 793-887.*

Hirst, W. D., Andree, T. H., Aschmies, S., Childers, W. E., Comery, T. A., Dawson, L. A., ... & Hughes, Z. A. (2008). Correlating efficacy in rodent cognition models with in vivo 5-hydroxytryptamine1a receptor occupancy by a novel antagonist, (R)-N-(2-methyl-(4-indolyl-1-piperazinyl) ethyl)-N-(2-pyridinyl)-cyclohexane carboxamide (WAY-101405). *Journal of Pharmacology and Experimental Therapeutics, 325(1), 134-145.*

Kent, B. A., Hvoslef-Eide, M., Saksida, L. M., & Bussey, T. J. (2016). The representational–hierarchical view of pattern separation: Not just hippocampus, not just space, not just memory? *Neurobiology of learning and memory, 129, 99-106.*

Kheirbek, M. A., Klemenhagen, K. C., Sahay, A., & Hen, R. (2012). Neurogenesis and generalization: a new approach to stratify and treat anxiety disorders. *Nature neuroscience, 15(12), 1613*.

Kirwan, C. B., Hartshorn, A., Stark, S. M., Goodrich-Hunsaker, N. J., Hopkins, R. O., & Stark, C. E. (2012). Pattern separation deficits following damage to the hippocampus. *Neuropsychologia, 50(10), 2408-2414.*

Leal, S. L., Tighe, S. K., Jones, C. K., & Yassa, M. A. (2014). Pattern separation of emotional information in hippocampal dentate and CA3. *Hippocampus*, *24*(9), 1146-1155.

Leutgeb, J. K., Leutgeb, S., Moser, M. B., & Moser, E. I. (2007). Pattern separation in the dentate gyrus and CA3 of the hippocampus. *science, 315(5814), 961-966.*

McHugh, T. J., Jones, M. W., Quinn, J. J., Balthasar, N., Coppari, R., Elmquist, J. K., ... & Tonegawa, S. (2007). Dentate gyrus NMDA receptors mediate rapid pattern separation in the hippocampal network. *Science, 317(5834), 94-99.*

Millan, M. J., Agid, Y., Brüne, M., Bullmore, E. T., Carter, C. S., Clayton, N. S., ... & Dubois, B. (2012). Cognitive dysfunction in psychiatric disorders: characteristics, causes and the quest for improved therapy. *Nature reviews Drug discovery, 11(2), 141.*

Nehlig, A. (2010). Is caffeine a cognitive enhancer? *Journal of Alzheimer's Disease, 20(s1), S85-S94.*

Polter, A. M., & Li, X. (2010). 5-HT1A receptor-regulated signal transduction pathways in brain. *Cellular signalling, 22(10), 1406-1412.*

Rojas, P. S., & Fiedler, J. L. (2016). What do we really know about 5-HT1A receptor signaling in neuronal cells? *Frontiers in cellular neuroscience*, *10*, 272.

Sahay, A., Wilson, D. A., & Hen, R. (2011). Pattern separation: a common function for new neurons in hippocampus and olfactory bulb. *Neuron, 70(4), 582-588.*

Sierksma, A. S. R., Van Den Hove, D. L. A., Pfau, F., Philippens, M., Bruno, O., Fedele, E., ... & Prickaerts, J. (2014). Improvement of spatial memory function in APPswe/PS1dE9 mice after chronic inhibition of phosphodiesterase type 4D. *Neuropharmacology, 77, 120-130.*

Terry Jr, A. V., Buccafusco, J. J., & Wilson, C. (2008). Cognitive dysfunction in neuropsychiatric disorders: selected serotonin receptor subtypes as therapeutic targets. *Behavioural brain research, 195(1), 30-38.*

van Hagen, B. T. J., Van Goethem, N. P., Lagatta, D. C., & Prickaerts, J. (2015). The object pattern separation (OPS) task: A behavioral paradigm derived from the object recognition task. *Behavioural brain research, 285, 44-52.*

van Goethem, N. P., Rutten, K., van der Staay, F. J., Jans, L. A., Akkerman, S., Steinbusch, H. W., ... & Prickaerts, J. (2012). *Object recognition testing: rodent species, strains, housing conditions, and estrous cycle. Behavioural brain research, 232(2), 323-334.*

van Goethem, N. P., Schreiber, R., Newman-Tancredi, A., Varney, M., & Prickaerts, J. (2015). Divergent effects of the 'biased'5-HT 1 A receptor agonists F15599 and F13714 in a novel object pattern separation task. *British journal of pharmacology, 172(10), 2532-2543.*

Vanmierlo, T., Creemers, P., Akkerman, S., van Duinen, M., Sambeth, A., De Vry, J., ... & Prickaerts, J. (2016). The PDE4 inhibitor roflumilast improves memory in rodents at non-emetic doses. *Behavioural brain research, 303, 26-33.*

Chapter 2

The object pattern separation (OPS) task; a behavioural paradigm derived from the object recognition task

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Abstract

The object recognition task (ORT) is widely used to measure object memory processes in rodents. Recently, the memory process known as pattern separation has received increasing attention, as impaired pattern separation can be one of the cognitive symptoms of multiple neurological and psychiatric disorders. Pattern separation is the formation of distinct representations out of similar inputs. In the search for an easily implemented task for rodents that can be used to measure pattern separation, we developed a task derived from the ORT and the object location task (OLT), which we called the object pattern separation (OPS) task. This task aims to measure spatial pattern separation per se, which utilizes memory processes centered in the DG and CA3 region of the hippocampus. Adult male C57BL/6 mice and adult male Wistar rats were used to validate different object locations which can be used to measure spatial pattern separation. Furthermore, different inter-trial time intervals were tested with the most optimal object location, to further evaluate pattern separation-related memory in mice. We found that specific object locations show gradual effects, which is indicative of pattern separation, and that the OPS task allows the detection of spatial pattern separation bi-directionally at intermediate spatial separations. Thus, object locations and time intervals can be specifically adjusted as needed, in order to investigate an expected improvement or impairment. We conclude that the current spatial OPS task can be best described as a specific version of the ORT, which can be used to investigate pattern separation processes.

1.1 Introduction

The object recognition task (ORT), as first described by Ennaceur and Delacour [1], is widely used to measure object memory in rodents. This task provides a relatively simple and well-established way of measuring object memory over different time intervals in both rats and mice [2, 3]. In search of a translational task that can measure the complex process of pattern separation in rodents, we developed a task derived from the well-validated ORT. While the ORT measures object memory, this task, which we named the object pattern separation (OPS) task, measures a memory process known as pattern separation. Pattern separation is the formation of distinct representations out of similar inputs [4]. As shown by our data, this task, which can be used for both mice and rats, uses a similar setup (and objects) to that of the ORT. However, instead of changing one object in the second trial, one of the objects is moved, along a straight line, to a distance adjacent to the old position. Rodents with impaired pattern separation will not recognize this new position, while rodents with intact pattern separation will recognize that the object has been moved. In essence, it is a more refined version of the object location task (OLT), which was also derived from the ORT (for an example see [5] for mice; and [6] for rats). The OPS task will be described in more detail later. First, an overview of what is currently known about pattern separation will be given, followed by a definition and description of the type of pattern separation that is measured by the OPS task.

In the literature, the term pattern separation can be found in multiple contexts, and different definitions of this process have been proposed. Here, the focus will be on cognitive pattern separation, which can be defined as a type of memory processing whereby separate representations are made out of highly overlapping information [7], by reducing interference from the overlapping information [8]. Pattern separation involves multiple processes; when seeing a similar, but new, environment or situation, one has to retrieve the old one correctly in order to make a good distinction between the two, and subsequently store both situations separately as distinct memories of similar events [9].

1.2 Pattern separation in rodents

The concept of pattern separation has been derived from computational neural-network theories concerning hippocampal function [10]. Multiple mechanisms of action have been proposed for pattern separation, but the exact process is not yet fully understood. What *is* known is that the hippocampus, and especially the dentate gyrus (DG), is highly involved in pattern separation [7]. The enthorinal cortex (EC) is the main source of input to the hippocampus, through its connections to the DG. From the DG, information is sent to the CA3 region, and from there it is sent on to CA1. In turn, CA1 projects to the subiculum, and sends hippocampal output back to the EC [11]. Computational models

suggest that pattern separation is achieved through dispersion of EC input onto the DG's granule cells which have small place fields. These in turn, sparsely send the information to the CA3 pyramidal cells via the mossy fiber synapses [4, 10]. Subsequently, similar or interfering inputs can be processed separately. Evidence to support these computational theories comes from electrophysiological and lesion studies performed on rodents. These studies show that there is a role for both the DG and CA3 region, depending on the nature of the variation between stimuli. Therefore, results depend on the nature of the task that is being used, demonstrating the need for a standardized task which measures specific pattern separation processes.

In studies of rodents with dorsal DG lesions, it has been shown that this part of the hippocampus is particularly sensitive to manipulations in spatial distances of stimuli. Rodents suffering from dorsal DG lesions were less able to discriminate between objects in close proximity to one another than between objects that were spatially further apart [7, 12, 13]. Gilbert et al. [14] showed impaired performance on a spatial separation cheese-board task for rats with a DG lesion, only on trials with low spatial separation. They performed the same task with CA3 lesioned rats and found that these rats were impaired equally at low as well as high spatial separations. This shows that the hippocampal region CA3 is important for spatial memory as a whole, while the DG is specific for pattern separation processes [15]. An experiment by Hunsaker et al. [16] measured the effects of both DG and selective CA3 lesions on two tasks with either an environmental (shape of the test arena) or a spatial (object location) change, while everything else was kept constant. They found that rats with DG lesions did not discriminate between changes in either environment or space, suggesting that the DG is necessary for pattern separation processes involving multiple types of stimuli. Interestingly, a specific lesion of CA3 resulted in an impaired performance on spatial change detection, but not on environmental change detection. This suggests that the CA3 region, which specifically supports spatial encoding and retrieval [17, 18], is also needed for pattern separation processes. As a result of the sparse encoding, the CA3 region can support the DG in pattern separation with regard to spatial differences.

Further insight into the pattern separation mechanism comes from electrophysiology studies. The first study to investigate the pattern separation abilities of hippocampal place cells in rats found that two different but visually identical environments could trigger distinct firing patterns in hippocampal place cells, even when external cues were controlled for [19]. A study focusing on correlated activity patterns in the place-modulated granule cells in the DG found results implicating a dual mechanism for pattern separation, involving both the DG and CA3 region [20]. When the explored environments were dissimilar, new cell populations were recruited from the CA3 region, but when the

environments were similar, DG granule cells were recruited. This implies that when there are only small spatial differences, the DG augments the incoming signal before it is sent to the associative network in the CA3 region. This means that the pattern of correlated activity changes, but no new cells are recruited. More dissimilar stimuli can be separated by the CA3 region itself, because there is a remapping of the activity pattern which is accompanied by different processing and storage procedures, making the distinct stimuli easier to retrieve later on. When there are only small differences in incoming stimuli from the EC, these stimuli are dispersed by sparsely firing granule cells in the DG [4]. Therefore, very specific, small amounts of information can be processed separately before entering the CA3 region.

What is learned from these studies is that the DG in rodents is imperative for proper pattern separation. In addition, because of its sparse encoding, the CA3 region is needed specifically for the detection of small spatial differences. When sparse encoding is not necessary, the DG itself is a sufficient pattern separator. In other words; the DG is the main pattern separator, and the CA3 region adds another level of pattern separation, which distinguishes between different locations by activating different neuronal subpopulations [21]. Therefore, both regions are likely to be actively involved when performing a spatial separation task within the same environment, as in the OPS task.

1.3 Pattern separation in healthy humans

Research on pattern separation processes has primarily been performed on rodents, but there are a few studies which have investigated these processes in humans. Most of the evidence comes from imaging studies [9, 22, 23], which have considerable methodological limitations compared to the more invasive techniques used in rodents. An fMRI study by Kirwan and Stark [9] examined BOLD signals during the encoding phase of pattern separation processes. Activity in both the hippocampus and parahippocampal cortex (PHC) was measured. The PHC (postrhinal cortex in rodents) provides input through the EC, and subsequently converges this in the hippocampus [24]. The PHC is believed to be involved in spatial recognition memory [25]. To measure pattern separation-related activity, they used a task in which pictures of similar objects were shown but sometimes with a slight variation. Participants were asked to rate the pictures as “new” (a completely new picture), “old” (exactly the same as a previous picture) or “similar” (similar object but a different picture). They found that activation of the PHC and hippocampus during initial encoding of new pictures predicted later success. During trials where interference was very high (i.e. a high emphasis on pattern separation), most activity was seen in the hippocampus and not the PHC, showing that, in humans, initial encoding is centered in both structures, but pattern separation is specific to the hippocampus. Furthermore, when there was a correct “hit” response, activity-related signals in the hippocampus were significantly higher, implying

that when there is a hit, extra cognitive effort is needed to match these stimuli. Bakker and colleagues [23] repeated this same task but at first only with similar pictures; subsequently, decreased activity was found the second time a picture was shown. They found that this lowered activity was identical for pictures that were considered old by the participant but were in fact different. When the participant detected a difference, the signal was as strong as if a new picture was shown, and this activity was centered in the DG and CA3 region of the hippocampus.

The above described studies show that a slight variation in presented stimuli is specifically detected by the DG and CA3. This provides evidence that the same mechanism that has been found in rodents also applies to human pattern separation, which suggests translatability of rodent studies to other mammalian species. Unfortunately, current imaging techniques cannot properly differentiate between the DG and the CA3 region. Therefore, separate functions of these regions, as shown in rodents, cannot yet be supported in human research [21].

1.4 Adult neurogenesis in the DG

Multiple studies have shown a connection between adult neurogenesis and pattern separation [4, 26, 27]. The hippocampus, and especially the DG, is one area of the brain where ongoing adult neurogenesis occurs [28]. It has been suggested that the addition of new granule cells in the DG is necessary for pattern separation, because it reduces interference with old information. By storing similar but new information separately, the information can easily be retrieved separately, and without interference [29]. If neurogenesis is down-regulated, new information will be stored in overlapping cell groups, and therefore memories could interfere with each other, leading to impaired pattern separation. This suggests that groups of adult granule cells in the DG are especially dedicated to a specific event. In addition, there is evidence suggesting that new neurons of a certain age show more plasticity than older neurons [30], allowing new neurons to encode new information, and leaving the previously stored information intact. Therefore, neurogenesis is believed to be crucial for pattern separation.

Evidence supporting such claims comes from studies where neurogenesis in the hippocampus was manipulated. Clelland et al. [4] ablated neurogenesis in the hippocampus of C57Bl/6 mice, using low-dose x-irradiation. Subsequently, the mice were tested in a delayed nonmatching to place radial arm maze task in order to measure pattern separation-dependent memory. Mice with ablated neurogenesis showed an impairment in spatial pattern separation when the target arms were in close proximity to each other (low separation). They performed equally to sham treated mice when the target arms were further apart (high spatial separation). The task was also performed with mice that had a

reduced number of new neurons in the DG. Again, an impaired performance on trials with low spatial separation was found. Another study which investigated the involvement of neurogenesis in pattern separation processes reported that transgenic mice overexpressing SREB2, a gene highly expressed in the hippocampus and which is connected to neurogenesis, showed impairment on a pattern separation task as well as a decreased number of new, differentiating cells [26]. Garthe et al. [31] suppressed neurogenesis in mice by using temozolimide; a pharmacological treatment that acts as a DNA-alkylating agent. Mice with suppressed neurogenesis were less able to learn a new location in the same environment of the water maze task. This particular finding illustrates that mice with suppressed neurogenesis do not have the ability to make small adjustments in their representation of a similar environment.

On the other hand, mice with increased exercise-induced DG neurogenesis showed enhanced pattern separation performance on a touch screen task when compared to control mice [27]. It was also found that measures of neurogenesis positively correlated with task performance. Finally, mice KO for SREB2 showed improvement on a pattern separation task, although there were no differences in neurogenesis measures when compared to controls [26]. The studies described in this section support the claim that improvements in pattern separating abilities are due to enhanced neurogenesis, and in particular the theory that newly formed cells in the DG are needed to process overlapping information. A clear, well-validated task designed to measure pattern separation could help to further evaluate this claim.

1.5 Pattern separation impairments in mental disorders

Impaired pattern separation can be part of the cognitive symptoms of multiple neurological and psychiatric disorders [32]. For instance, in Alzheimer's disease (AD) mice models, it has been shown that at a very early stage of AD progression, neurogenesis in the hippocampus was decreased. This occurred before other AD symptoms that could influence neurogenesis were visible, such as amyloid deposition and neuronal loss [33]. This implies that pattern separation is one of the first cognitive processes that can be affected in AD. Taking such findings into account could facilitate the early diagnosis and treatment of AD.

As a result of reductions in gray matter during normal aging, especially in the hippocampus, decreased pattern separation ability could also be implicated in normal aging. Accordingly, Holden et al. [34], using a spatial pattern separation task, showed that that older (+75 years old) healthy individuals had significantly lower scores as compared to healthy adults, even when general memory impairments

were taken into account. On a visual pattern separation task (originally developed by [9]), older non-demented adults (+65 years old) showed impairment in distinguishing previously shown stimuli from similar lures, while healthy young adults did not [35]. The same was found in a rodent study, in which aged mice (22 month old) performed significantly worse than control mice on a touch screen task that assessed spatial pattern separation. The mice could only be tested at an intermediate separation because the testing criterion was not met in order to proceed to low separation [27]. This implies that the pattern separation process is completely impaired in situations with low separation, which reflect true pattern separation.

Pattern separation is also hypothesized to play an extensive role in cognitive impairments associated with schizophrenia (CIAS) [36]. As a result of hippocampal dysfunction, schizophrenic patients show a decrease in the ability to distinguish between similar situations. This, in turn, could contribute to psychotic symptoms whereby memories are stored incorrectly and wrong associations with similar situations are made, otherwise known as illusory associations [37]. This theory is supported by a recent study showing pattern separation deficits in schizophrenic patients [38]. If this impairment could be treated, it might be argued that this not only affects the cognitive symptoms of schizophrenia, but also influences the frequency and severity of the positive and negative symptoms. To elaborate on this further, pattern separation is essential for assessing everyday situations and it is likely that positive symptoms could get triggered less frequently and less severely when a patient is able to interpret these situations correctly.

Research investigating pattern separation in respect to other mental illnesses suggests that impaired pattern separation could also be one of the underlying mechanisms of anxiety disorders such as post-traumatic stress disorder (PTSD) [10]. If similar events cannot be distinguished from one another, anxiety may be triggered by a greater array of events, i.e. harmless everyday stimuli might get wrongly associated with traumatic experiences. Pharmacotherapy developed with the aim of improving pattern separation could have great therapeutic benefits for people suffering from an anxiety disorder. Patients with an anxiety disorder show an adverse response to cues associated with danger, often ignoring cues that convey safety [39]. Healthy individuals have the ability to distinguish these cues from danger by making an appropriate representation.

1.6. From OLT to OPS

When finding an appropriate task to assess spatial pattern separation in rodents, it is important to present two similar situations which contain the same stimuli but with a minor spatial change, in order to assess if the rodents can distinguish between these situations. Ideally, the difference should be just big enough for the rodents to detect it, so that it can be certain that pattern separation processes in the DG are utilized. In the current study, we investigated how both rats and mice performed in the OPS task. This task is distinguishable from existing tasks using objects [7,12,15,40,41], in that multiple subtle differences in spatial separation can be tested. This provides the opportunity to select the best possible distance for measuring both impairment and improvement in spatial pattern separation. As a result of this refinement, the current OPS task is suited to measure small variations processed by the DG, and the CA3 region because of the spatial component.

2. Materials and methods

2.1 Animals

In the current OPS study, 35 three-month-old adult male C57BL/6 mice (average weight: 24.1 grams at the beginning of the study) and 47 three-month-old Wistar rats (average weight: 325.4 grams) were used (Charles River Laboratories International, Inc., Sulzfeld, Germany). All experimental procedures were approved by the local ethics committee for animal experiments at Maastricht University, and met governmental guidelines. The animals were housed individually in standard Tecniplast IVC system greenline cages, on a sawdust bedding, with a reversed light/dark cycle of 12/12 hours (lights off from 7:00 to 19:00). Food and water were available ad libitum. Background noise was provided by a radio, to mask noises in the room. All testing was performed during the dark phase of the light/dark cycle, under low illumination (20 lux).

2.2 Apparatus and objects

For the OPS task, similar apparatus and similar procedures as described previously for the object recognition task (ORT) were used [2,3]. The circular arena made of PVC had a diameter of 40cm (for mice) or 83cm (for rats), and was 40cm high. The front of the arena (facing the experimenter) consisted of see-through PVC and the other half was colored white or grey. In this task, two identical objects were used per trial. For mice, these objects were either two massive metal cubes (2.5cm x 5cm x 7.5cm) with two holes (diameter 1.5cm), or two massive aluminum cubes with tapering tops (4.5 x 4.5 x 8.5cm). For rats, the objects were either two massive metal blocks (10.0 cm x 5.0 cm x 7.5 cm) with two holes (diameter 1.9cm), or two massive aluminum cubes with square bases and tapering tops (13.0 cm x 8.0 cm x 8.0 cm). The animals were unable to move these objects.

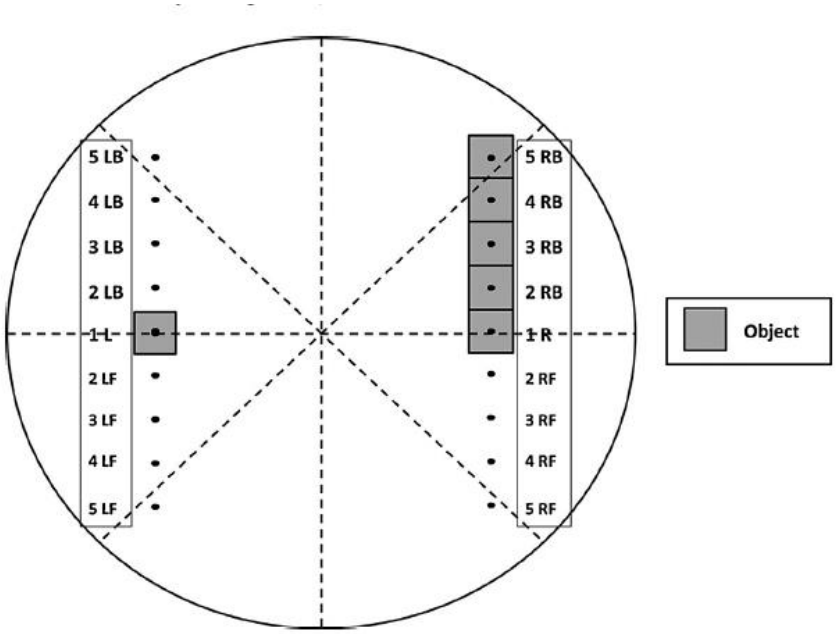

Figure 1. The Object Pattern Separation Task (OPS).
Schematic representation of the arena with the possible new locations for an object in T2 (indicated on the right). In the schema, L and R refer respectively to 'left' and 'right'. Furthermore, B and F refer to 'back' and 'front' respectively, indicative of the general direction of the displacement of an object. The number indications (1-5) represent the 5 possible locations where an object can be displaced to. Therefore, Position 5 (in each direction) represents the farthest possible displacement, and Position 1 represents no displacement. In T1 the placement of objects is always '1L' and '1R', in T2; one of the objects could get displaced to one of the new locations along the vertical axis (Position 1-5).

2.3 Behavioural paradigm

The OPS task is a modified version of the object location task OLT [6, 42], which in turn was developed from the ORT [1,2,3] and assesses especially the cognitive, i.e. learning and memory, aspect of pattern separation (no emotional component is involved). In the present study, the task consisted of two trials of free exploration with a time interval in-between. The first trial (T1) was the acquisition trial, in which two identical objects were placed symmetrically on a horizontal line in the arena, approximately 5cm (mice) or 15cm (rats) from the wall (position 1). In T1, the animals were always placed in the front of the arena facing the wall, and were allowed to explore the objects for three (rats) or four (mice) minutes, after which they were put back into their home cage. The second trial (T2) took place after a predetermined delay interval (0.5h / 1h / 2h / 3h / 4h or 6h). In this trial, one of the objects was displaced along a straight line to a position that was one of four different distances away from the previous location, i.e. position 1 (no change) to position 5 (maximum change). The object that was moved (either left or right), the direction (front/back), and the objects themselves, were all counterbalanced to avoid place and object preferences. The rodents were again allowed to explore this new spatial arrangement for three (rats) or four (mice) minutes. Over the two trials, the objects orientation must be kept the same. Between animals the objects were cleaned with a 70% ethanol solution to avoid the presence of olfactory cues. For a schematic overview of the OPS task set-up, and different positions used, see Figure 1.

The exploration time of each object was scored manually by the experimenter, using a computer. Exploration was defined as follows: directing the nose to the object at a distance of no more than 1cm (mouse) or 2cm (rat) and/or touching the object with the nose. Sitting on, or leaning towards, an object was not considered to be exploratory behaviour.

2.4 Animal handling and OPS testing

Before starting the experiments, the animals were habituated to the experimenter, the set-up, and the objects. This habituation period consisted of the animals being handled by the experimenter, and the animals being placed in the arena with the different objects, to get them acquainted with both the procedures and the environment [43]. After two weeks of handling, and of exploration of the arena and the objects therein, testing started. Before T1, the mice were placed in an empty cage for 4 minutes to increase their natural exploration behaviour during testing [42].

For the first part of the experiment, both mice and rats were randomly divided over one of the five different positions in T2. In total, 12 or 11 mice and 28 rats were tested per position, with a 1h

interval (except for position 3 where 29 rats were tested). It has been reported that at this interval, rodents are able to remember objects [3], and with this task our aim was to determine the cut-off position from which the mice and rats are able to discriminate between two spatial locations. Determining this position is important, because performance can subsequently vary in a bi-directional manner, i.e the discrimination performance can either decrease or increase depending on the experimental condition that is being tested. After determining the memory for adjacent locations, the cut-off position showing intermediate performance was used for the second experiment with mice, in order to determine the time-span in which they are able to remember and distinguish between spatial information. For this purpose, different time intervals of 0.5h / 1h / 2h / 3h / 4h and 6h were tested with the cut-off position. Validating the different intervals in healthy mice gives us insight into which time interval could be used for measuring both increases and decreases in performance.

2.5 Outcome measurements

The OPS task provides measures for exploration time and discrimination (see [43, 44]). The measures are the times spent exploring each object location during T1 and T2. The time spent exploring the two symmetrically placed objects in T1 are represented by 'a1' and 'a2', respectively. The time spent in exploring the stationary and the moved object in T2 are represented by 'a3' and 'b', respectively. From these exploration times, the following variables were calculated: e1, e2, d1, and d2. e1 and e2 are the total exploration times of both the objects together in T1 (e1=a1+a2) and T2 (e2=a3+b). Total exploration times in both trials should be sufficient in order to be able to reliably assess pattern separation/ discrimination [3, 43]. If an animal did not show sufficient exploration time, i.e. below 10 sec, in T1, T2, or both, the animal was excluded from the dataset. The d1 index is the difference in exploration time between the stationary and the moved object in T2 (d1=b-a3). The d2 index is a relative measure of discrimination, corrected for exploratory activity (d2= d1/e2) [44]. The d2 index can range from -1 to 1, with -1 or 1 indicating complete preference for the familiar or novel object location, respectively, and 0 signifying no preference for either object location. One-sample t-statistics were performed in order to assess whether the d2 index, for each experimental condition separately, differed significantly from zero (i.e. chance level). Different positions and different time intervals were compared using one-way ANOVAs. When the overall ANOVA was significant, Dunnett's post-hoc analyses were performed. To evaluate whether overall differences in exploration times between conditions did not influence the results, one-way ANOVAs between conditions were performed for both e1 and e2. When an ANOVA comparing total exploration time was significant, LSD post-hoc tests were performed. An α level of 0.05 was considered significant.

3. Results

3.1 Rat OPS performance

Figure 2 depicts the rat performance in the OPS task for the five different positions. The d2 index (discrimination corrected for the total exploration time) is given as a measure for pattern separation memory. A gradual increase in pattern separation performance is shown as the distance between the objects increases. Table 1 gives an overview of the different outcome measures of rat OPS performance. No animals were excluded from the statistical analysis. There were no significant differences in overall exploration time for T1 (e1) or T2 (e2) between the positions, as indicated by a one-way ANOVA (T1(e1): $F_{4,136}$=2.288, P=0.63; T2(e2): $F_{4,136}$=2.249, P=0.067). This implies that overall exploratory behaviour did not differ between positions, and therefore did not influence the difference in performance. One-sample t-tests comparing the d2 index to zero showed that positions 1 and 2 did not differ from chance level performance, but positions 3, 4 and 5 did significantly differ from zero (P=0.0036; P=0.0007 and P<0.001, respectively). This indicates that the rats did recognize the new position from position 3 onwards. For the d2-index, a one-way ANOVA comparing all conditions was significant ($F_{4,136}$=9.117, P<0.001), and post hoc Dunnett t-tests showed that positions 3, 4 and 5 significantly differed from position 1 (P=0.004; P=0.003 and P<0.001, respectively).

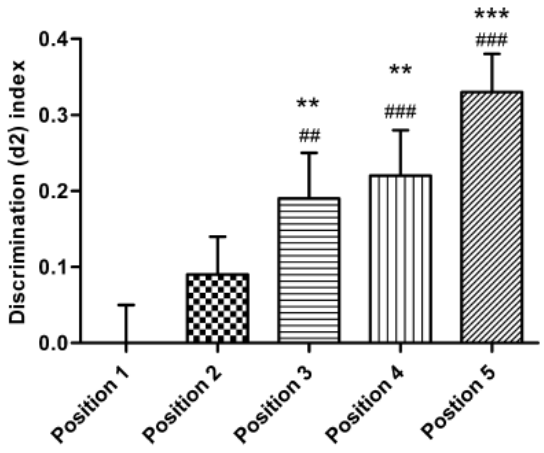

Figure 2. Rat OPS performance on different positions*: Object Pattern Separation performance of rats, at a 1 hour interval, for the different object positions. The d2 index reflects the difference in exploration of the stationary and the moved object, relative to the total exploration time. A significant difference from zero is depicted with hashes (one sample t-tests, ##: P<0.01; ###: P<0.001). A difference from position 1 is depicted with asterisks (Dunnett t-tests, **: P<0.01; ***: P<0.001).*

Table 1
Mean OPS outcome measures per position for rats.

Group/position	*N*	*e*1 (SEM)	*e*2 (SEM)	*d*1 index (SEM)	*d*2 index (SEM)
Position 1	28	30.75 (1.67)	27.56 (1.72)	−0.48 (1.53)	0.00 (0.05)
Position 2	28	29.56 (1.94)	25.32 (1.37)	2.30 (1.12)	0.09 (0.05)
Position 3	29	29.33 (1.54)	28.58 (1.38)	5.55 (1.82)	0.19 (0.06)
Position 4	28	31.10 (1.83)	27.00 (1.79)	6.16 (1.69)	0.22 (0.06)
Position 5	28	25.48 (1.64)	29.51 (1.36)	9.52 (1.53)	0.33 (0.05)

3.2 Mice OPS performance

In Figure 3, the performance of mice (d2 index) in the OPS task with different positions is depicted (see also table 2). The same trend as shown in the rat data was also seen in the mouse data. Exploration time of T1 (e1) between conditions, as tested by a one-way ANOVA, revealed a significant difference ($F_{4,53}$ =3.613; P=0.011). Post-hoc LSD analyses showed significantly higher exploration for position 2 when compared to the other four positions, implying a difference in exploration unrelated to the experimental conditions. There was no significant difference in exploration time between conditions for T2 (e2: $F_{4,53}$ =2.523; N.S.). One-sample t-tests showed that the d2 index of positions 1 and 2 were not significantly different from zero, but positions 3, 4 and 5 were (P=0.025; P=0.024 and P=0.001, respectively). Furthermore, a one-way ANOVA showed an effect for the d2 index between positions ($F_{4,53}$=3.302; P=0.017). Post hoc Dunnett t-tests revealed that position 4 showed a trend toward significance (P=0.086), and position 5 significantly differed from position 1 (P=0.008).

For both rats and mice, position 3 was the closest possible position from position 1 that was significantly different from zero. However, as shown by data on positions 4 and 5, there is still room for improvement in terms of pattern separation performance from position 3, which only has an intermediate effect [44]. The d2 indices of positions 1 and 2 show that it is also possible that performance in the OPS task can decrease. Therefore, position 3 was chosen to measure pattern separation in the second part of the experiment. On this position, performance could either improve to a full effect, or impair to no effect, and hence resemble performance on position 2. This makes position 3 the most appropriate to measure differences between groups with experimental manipulations that have putative pattern separation enhancing or impairing results.

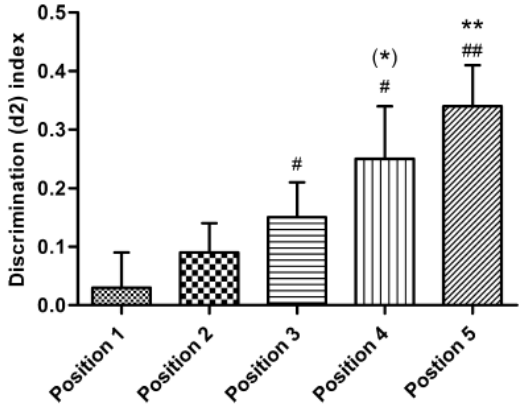

Figure 3. Mice OPS performance on different positions*: Mice Object Pattern Separation performance at a 1 hour interval for the different object positions. The d2 index reflects the difference in exploration of the stationary and the moved object, relative to the total exploration time. A significant difference from zero is depicted with hashes (one sample t-tests, #: P<0.05; ##: P<0.01). A difference in d2 from position 1 is depicted with asterisks (Dunnett t-tests, (*): P<0.10; **: P<0.01).*

Table 2
Mean OPS outcome measures of mice per position.

Group/position	*N*	*e*1 (SEM)	*e*2 (SEM)	*d*1 index (SEM)	*d*2 index (SEM)
Position 1	12	39.95 (4.25)	37.51 (6.16)	1.24 (2.15)	0.03 (0.06)
Position 2	12	57.39 (5.57)	57.47 (3.50)	5.40 (2.91)	0.09 (0.05)
Position 3	12	34.92 (4.63)	44.37 (6.94)	5.41 (3.67)	0.15 (0.06)
Position 4	11	36.37 (5.10)	52.10 (7.79)	10.08 (5.06)	0.25 (0.09)
Position 5	11	33.78 (6.23)	35.18 (4.39)	10.09 (2.78)	0.34 (0.07)

Table 3
Mean OPS outcome measures of mice per inter-trial interval.

Group/interval duration (h)	*N*	*e*1 (SEM)	*e*2 (SEM)	*d*1 index (SEM)	*d*2 index (SEM)
0.5	11	43.26 (6.53)	42.19 (5.73)	18.72 (4.88)	0.39 (0.07)
1	12	34.92 (4.63)	44.37 (6.94)	5.41 (3.67)	0.15 (0.06)
2	11	38.76 (4.68)	47.30 (5.80)	8.25 (2.39)	0.17 (0.05)
3	12	47.66 (4.75)	46.12 (4.05)	1.20 (4.80)	0.03 (0.09)
4	12	48.43 (5.50)	39.59 (5.35)	1.96 (2.79)	0.07 (0.07)
6	12	40.81 (4.07)	45.08 (4.45)	−2.58 (2.66)	−0.05 (0.05)

3.3 Inter-trial intervals

To further investigate the memory for the displacement on position 3, different inter-trial intervals were tested. Pattern separation was measured at time intervals ranging from 0.5h to 6h (table 3). The different time intervals were counterbalanced. There were no differences in overall exploration times for T1 (e1) or T2 (e2) between the different time intervals, as revealed by a one-way ANOVA (T1(e1): $F_{5,64}$ =1.081; P=0.379; T2(e2): $F_{5,64}$ =0.264; P=0.931). One-way ANOVA between conditions revealed a significant effect for the d2 index ($F_{5,64}$ =5.351; P<0.001). Figure 4 shows the d2 indices of the different intervals. Post hoc Dunnett t-tests revealed that that the 0.5h interval differed significantly with other inter-trial intervals, except for the 2h interval which showed a trend towards significance (1h: P=0.042; 2h: P=0.083; 3h: P=0.001; 4h: P=0.005; 6h: P<0.001). Accordingly, t-tests comparing the d2 indices to zero showed that pattern separation performance was best after a 0.5h interval (P<0.001). After 1h (P=0.025) and 2h (P=0.006), mice could still discriminate between positions 1 and 3. From 3 hours onwards, the discrimination index did not significantly differ from zero anymore, indicating that untreated mice no longer remembered the spatial arrangement after 3 hours.

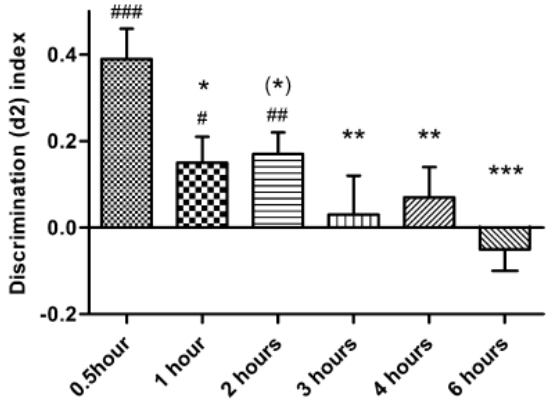

Figure 4. OPS performance on different inter-trial intervals*: Mice Object Pattern Separation performance at position 3 for different T1-T2 inter-trial intervals. The d2 index reflects the difference in exploration of the stationary and the moved object, relative to the total exploration time. A significant difference from zero is depicted with hashes (one sample t-tests, #: P < 0.05; ##: P < 0.01 ### < 0.001). A significant difference from the 0.5h inter-trial interval is depicted with asterisks (one-way ANOVA followed by post-hoc Dunnett t-tests, (*): P<0.10 *: P<0.05; **: P<0.01; ***: P<0.001).*

4. Discussion

4.1 Applicability

Our results show that the OPS task is a suitable behavioural paradigm to measure spatial pattern separation in rodents. Our specific aim was to measure the cognitive (i.e. memory) component of pattern separation, which involves memory processing with the goal of making separate representations out of highly similar input [7]. We established that the ability of rodents to make separate representations out of similar input can indeed be measured with this task. Firstly, OPS performance on the different positions showed a gradual increase when the difference between the old and the new situation increased. The rat data showed that from position 3 onwards, they recognized the new position, as revealed by both one sample t-tests and a one-way ANOVA. The mouse data showed that position 3 had an intermediate effect, as determined by a one sample t-test. Position 5 differed significantly from position 1, and position 4 showed a trend towards significance. The mouse study showed an effect of exploration time of T1 for position 2 as compared to the other groups. Despite this difference, the exploration times in T1 were always sufficient (>30 sec) to draw reliable conclusions [43]. Sporadic changes in exploratory behaviour can occur during extensive behavioural testing, and since no experimental manipulations were used, we interpret this difference to be incidental.

The data shows that the range of ability in terms of pattern separation in healthy normal rodents can be used as a starting point for testing impairment as well as improvement in performance under different experimental conditions. The location for the latter can be carefully chosen and validated from a range of different spatial separations (see also Figure 1). Improvement on position 1 is obviously impossible, since the conditions in T1 and T2 are exactly the same, hence the d2 value should be (not significantly different from) zero. This demonstrates a floor effect, and it reflects the ultimate score that rodents with full pattern separation impairment will show, even with higher separation of the object locations. Position 3 is the closest distance from position 1 to be discriminated from zero. Therefore, it is the best position from which to measure pattern separation. To further elaborate on this, position 3 in T2 has the highest similarity with T1, and will thus interfere the most with the memory of T1, therefore utilizing pattern separation processes to distinguish between positions. Positions 4 and 5 show higher discrimination performances, ultimately resulting in ceiling effects. The highest separation is easiest to discriminate and therefore there is no more room for improvement. When an improvement in pattern separation performance is found on position 3 for a specific experimental condition, the experimenter could consider testing the experimental condition on position 2 as well. On position 2, the displacement is very minimal, and normally rodents show no, or only very slight, pattern separation on

this position. When experimentally increasing pattern separation strongly, performance might increase on position 2 as well. Likewise, when no effect or only a slight effect is found on position 3, the experimenter could consider testing the experimental condition on position 4, to evaluate whether performance here is enhanced.

The considerations outlined above also apply to the different time intervals tested. Normal healthy mice showed pattern separation for up to two hours after T1. Thus, the described improvement and impairment in performance on position 3 can only be measured when using a short inter-trial interval. When a 3 hour inter-trial interval is used, discrimination between objects is not measured anymore, suggesting that pattern separation processes can no longer be utilized. Therefore, from this interval onwards, only performance improvements can be tested. Our aim was to be able to measure pattern separation processes bi-directional, as this makes it possible to have an extra control/reference group in experiments and hence increase the suitability of the test. It is therefore recommended that a 1h inter-trial interval is used, because this allows for increasing as well as decreasing OPS performance. This also adds to the translational value of the task, because human pattern separation processes should also be bi-directional as well, when given enhancing [45] or impairing drugs [46].

Most tasks used to measure spatial pattern separation only distinguish between low and high separation, without assessing intermediate pattern separation [4, 7, 16, 41]. When assessing low and high separations without checking for multiple intermediate distance effects, it cannot be exactly determined to what extent pattern separation has been measured. Subsequently, it cannot be known whether the chosen distances have floor or ceiling effects, and this can seriously affect results when testing for impairment or enhancement in performance. The one rat study which included a task that did make use of a range of distances to separate between an object paired with a food reward (always at the same location) and a similar object not paired with a food reward, used spatial separations between 15cm and 105cm[40]. The minimum distance used in this study is equal to our highest separation (15cm) when testing rats, and therefore may still not exploit the subtle variations needed to measure pattern separation. This is also evident from the finding that CA3 lesions impaired all spatial separations, i.e. there was no gradual decline in performance with decreasing spatial separation [40]. To be able to tap more specifically into pattern separation processes, the difference between two situations should therefore be as low as possible, i.e. smaller than 15cm. Very recently, a task has been described that is similar to ours, and which could very likely also allow for the assessment of pattern separation on a gradual scale, although to our knowledge, the authors have not yet done this [41,47]. The major difference between this study and our own study is that three objects were used in the first

trial, while in the second trial only two objects were used, with one object at a new location. Thus, an animal could relate the change in the location of that one object to the three other object locations, instead of just two, as in the present study. One might argue that using three instead of two objects in an OPS task involves a lower degree of pattern separation, since more spatial information is available that can be used to separate similar object information.

4.2 Translational value

One of the positive features of the OPS task is that all the measurements and parameters have already been used and validated extensively for the ORT [1-3]. As in the ORT, only one of the objects is changed, or in this case moved (as in the OLT), and it can therefore be argued that by measuring the difference in exploration times, we are still measuring recognition. This differs from some of the existing spatial separation paradigms in which multiple objects change position in T2 [16, 26]. The use of more than one change results in uncertainty about what is actually measured with exploration times, i.e. object and/or spatial memory. Furthermore, exactly the same discrimination index and statistics as used in the ORT can be used to evaluate the performance in this task. Therefore, using the OPS task should be a logical transition for anyone already familiar with the ORT. Moreover, the moved object can be alternated from front to back with the left or right object. Thus, when testing a second time, the animal can be presented with totally different objects and/or locations, i.e. the opposite object (left/right) can be moved and also the direction (front/back) can differ. This allows different situations to be created on every test trial, enabling multiple experimental conditions to be tested without memory interference from previous trials. This makes the task more reliable as within-subjects designs can be used without the risk of behavioural carry-over effects. In addition, fewer animals are needed with this set-up. Like the ORT, the OPS task does not use food deprivation or punishment (e.g. electrical shocks). This is positive from an ethical point of view as it reduces discomfort, but more importantly this makes the task more translational to cognitive tasks for humans (since these tasks do not use positive or negative reinforcements either). The spatial component in the task also enables easy translatability from animal to human research. There is a pattern separation task currently used in humans which uses circles moving over a horizontal axis in different separations [34]. There is also a picture task designed by Kirwan and Stark [9], which has mostly been used in fMRI research, which could easily be translated into the paradigm of the OPS task. For this purpose, instead of using the spatial separation in the OPS task, it would be possible to use highly similar objects to replace one of the objects with in T2. This task would then be the same as the ORT, except that the objects would have small adjustments made to them, for

example drilled holes or slots could be added at a specific object location. This task would measure object pattern separation without utilizing the spatial component of pattern separation.

Based on previous findings concerning pattern separation processes (see section 1.2), we purposely chose to add the spatial component to the OPS task in order to implement both DG and CA3 functioning. Furthermore, this allows for the investigation of the role of neurogenesis in pattern separation. It has been established that new neurons in the DG contribute to spatial pattern separation performance [26, 27]. In addition, spatial memory is processed in the dorsal hippocampus in rodents [48], and learning and memory are strongly influenced by hippocampal neurogenesis [49]. Thus, by using a spatial pattern separation task, neurogenesis and manipulations that affect neurogenesis can be assessed bi-directionally in this paradigm. This makes the OPS task suitable for the investigation of a wide array of different experimental questions related to object and/or spatial pattern separation.

To further evaluate the central pattern separation mechanisms within this paradigm, future studies should investigate plasticity and activity measures. Although there are indications from the behavioural data that the memory performance in the OPS task uses pattern separation mechanisms, this could be further supported by showing which central mechanisms are involved in completing the task. For example, the differentiation of newly-born granule cells in the DG should be mapped. If these new-born cells show differentiation into adult neurons after performing the OPS task, this would further confirm the validity of the theory that neurogenesis underlies pattern separation processes. Likewise, ablation of neurogenesis should impair performance on this task. At the same time, studying the mechanisms utilized by healthy rodents in this behavioural task could further increase our understanding of the role of the hippocampal formation in pattern separation processes. The OPS task offers the possibility to distinguish, and thus compare, between brain activity measures in situations with no differences between T1 and T2 (position 1) versus an adjacent distance (position 3). This is comparable to the fMRI study of Kirwan and Stark [9], who used similar and slightly altered pictures. Therefore, results from rodent studies using the OPS task in combination with more invasive methods could be more directly compared to human data. This would make the task useful for investigating the mechanisms underlying psychiatric or neurological conditions in which pattern separation processes are impaired, and possible treatments for these conditions.

4.3 Conclusion

In conclusion, the construct validity of the OPS task designed to measure spatial pattern separation in both mice and rats is based on the assumption that it offers a gradual scale of separation between locations of objects which have to be remembered. The OPS task is easy to perform, especially

when experience with the OLT is already available. Likewise, the brain structures involved in the performance of this task suggest that its face validity appears to be the same as that of the OLT. Due to the gradual scale of pattern separation, the OPS task potentially has many experimental uses and could be adapted to investigate multiple research questions involving impaired or enhanced pattern separation. For example, this task could be used to evaluate pure spatial memory pattern separation impairments in rodent models of various mental disorders and subsequently investigate possible treatments for these impairments. However, its face validity and exact predictive validity need to be established in future lesion and pharmacological studies, respectively, and in rodent models for aging and neuropsychiatric disorders.

5. References

[1] Ennaceur A, Delacour J. A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data. Behavioural Brain Research, 1988; 31: 47-59.

[2] van Goethem NP, Rutten K, van der Staay FJ, Jans LA, Akkerman S, Steinbusch HW, Blokland A, van't Klooster J, Prickaerts J. Object recognition testing: rodent species, strains, housing conditions, and estrous cycle. Behavioural Brain Research, 2012; 232: 323-334.

[3] Şık A, van Nieuwehuyzen P, Prickaerts J, Blokland A. Performance of different mouse strains in an object recognition task. Behavioural Brain Research, 2003; 147: 49-54.

[4] Clelland C, Choi M, Romberg C, Clemenson G, Fragniere A, Tyers P, Jessberger S, Saksida L, Barker R, Gage F, Bussey T. A functional role for adult hippocampal neurogenesis in spatial pattern separation. Science, 2009; 325: 210-213.

[5] Murai T, Okuda S, Tanaka T, Ohta H. Characteristics of object location memory in mice: behavioral and pharmacological studies. Physiology & Behavior, 2007; 90: 116-124.

[6] Bruno O, Fedele E, Prickaerts J, Parker L, Canepa E, Brullo C, Cavallero A, Gardella E, Balbi A, Domenicotti C, Bollen E, Gijselaers HJ, Vanmierlo T, Erb K, Limebeer CL, Argellati F, Marinari UM, Pronzato MA, Ricciarelli R. GEBR-7b, a novel PDE4D selective inhibitor that improves memory in rodents at non-emetic doses. British Journal of Pharmacology, 2011; 164: 2054-2063.

[7] Morris AM, Churchwell JC, Kesner RP, Gilbert PE. Selective lesions of the dentate gyrus produce disruptions in place learning for adjacent spatial locations. Neurobiology of Learning and Memory, 2012; 97: 326-331.

[8] Gilbert PE, Brushfield AM. The role of the CA3 hippocampal subregion in spatial memory: a process oriented behavioral assessment. Progress in Neuro-Psychopharmacology and Biological Psychiatry, 2009; 33: 774-781.

[9] Kirwan CB, Stark CE. Overcoming interference: An fMRI investigation of pattern separation in the medial temporal lobe. Learning & Memory, 2007; 14: 625-633.

[10] Kheirbek MA, Klemenhagen KC, Sahay A, Hen R. Neurogenesis and generalization: a new approach to stratify and treat anxiety disorders. Nature Neuroscience, 2012; 15: 1613-1620.

[11] Myers CE, Scharfman HE. Pattern separation in the dentate gyrus: a role for the CA3 backprojection. Hippocampus, 2011; 21: 1190-1215.

[12] Goodrich-Hunsaker NJ, Hunsaker MR, Kesner RP. The interactions and dissociations of the dorsal hippocampus subregions: how the dentate gyrus, CA3, and CA1 process spatial information. Behavioral Neuroscience, 2008; 122: 16.

[13] Kesner RP. A behavioral analysis of dentate gyrus function. Progress in Brain Research, 2007; 163: 567-576.

[14] Gilbert PE, Kesner RP, Lee I. Dissociating hippocampal subregions: a double dissociation between dentate gyrus and CA1. Hippocampus, 2001; 11: 626-636.

[15] Gold AE, Kesner RP. The role of the CA3 subregion of the dorsal hippocampus in spatial pattern completion in the rat. Hippocampus, 2005; 15: 808-814.

[16] Hunsaker MR, Rosenberg JS, Kesner RP. The role of the dentate gyrus, CA3a, b, and CA3c for detecting spatial and environmental novelty. Hippocampus, 2008; 18: 1064-1073.

[17] Scharfman HE. The CA3 "backprojection" to the dentate gyrus. Progress in Brain Research, 2007; 163: 627-637.

[18] de Almeida L, Idiart M, Lisman JE. Memory retrieval time and memory capacity of the CA3 network: role of gamma frequency oscillations. Learning & Memory, 2007; 14: 795-806.

[19] Tanila H. Hippocampal place cells can develop distinct representations of two visually identical environments. Hippocampus, 1999; 9: 235-246.

[20] Leutgeb S, Leutgeb JK. Pattern separation, pattern completion, and new neuronal codes within a continuous CA3 map. Learning & Memory, 2007; 14: 745-757.

[21] Leutgeb S. Detailed differences. Science, 2008; 319: 1623.

[22] Lacy JW, Yassa MA, Stark SM, Muftuler LT, Stark CE. Distinct pattern separation related transfer functions in human CA3/dentate and CA1 revealed using high-resolution fMRI and variable mnemonic similarity. Learning & Memory, 2011; 18: 15-18.

[23] Bakker A, Kirwan CB, Miller M, Stark CE. Pattern separation in the human hippocampal CA3 and dentate gyrus. Science, 2008; 319: 1640-1642.

[24] Van Strien N, Cappaert N, Witter M. The anatomy of memory: an interactive overview of the parahippocampal–hippocampal network. Nature Reviews Neuroscience, 2009; 10: 272-282.

[25] Eichenbaum H, Yonelinas A, Ranganath C. The medial temporal lobe and recognition memory. Annual Review of Neuroscience, 2007; 30: 123.

[26] Chen Q, Kogan JH, Gross AK, Zhou Y, Walton NM, Shin R, Heusner CL, Miyake S, Tajinda K, Tamura K. SREB2/GPR85, a schizophrenia risk factor, negatively regulates hippocampal adult neurogenesis and neurogenesis-dependent learning and memory. European Journal of Neuroscience, 2012; 36: 2597-2608.

[27] Creer DJ, Romberg C, Saksida LM, van Praag H, Bussey TJ. Running enhances spatial pattern separation in mice. Proceedings of the National Academy of Sciences, 2010; 107: 2367-2372.

[28] Zhao C, Deng W, Gage FH. Mechanisms and functional implications of adult neurogenesis. Cell, 2008; 132: 645-660.

[29] Frankland PW, Köhler S, Josselyn SA. Hippocampal neurogenesis and forgetting. Trends in Neurosciences, 2013: article in press.

[30] Aimone JB, Deng W, Gage FH. Adult neurogenesis: integrating theories and separating functions. Trends in Cognitive Sciences, 2010; 14: 325-337.

[31] Garthe A, Behr J, Kempermann G. Adult-generated hippocampal neurons allow the flexible use of spatially precise learning strategies. PLoS One, 2009; 4: e5464.

[32] Hanson JE, Madison DV. Imbalanced pattern completion vs. separation in cognitive disease: network simulations of synaptic pathologies predict a personalized therapeutics strategy. BMC Neuroscience, 2010; 11: 96.

[33] Mu Y, Gage FH. Adult hippocampal neurogenesis and its role in Alzheimer's disease. Mol Neurodegener, 2011; 6: 85.

[34] Holden HM, Hoebel C, Loftis K, Gilbert PE. Spatial pattern separation in cognitively normal young and older adults. Hippocampus, 2012; 22: 1826-1832.

[35] Toner CK, Pirogovsky E, Kirwan CB, Gilbert PE. Visual object pattern separation deficits in nondemented older adults. Learning & Memory, 2009; 16: 338-342.

[36] Schreiber R, Newman-Tancredi A. Improving cognition in schizophrenia with antipsychotics that elicit neurogenesis through 5-HT_{1A} receptor activation. Neurobiology of Learning and Memory, 2014; 110: 72-80.

[37] Tamminga CA, Stan AD, Wagner AD. The hippocampal formation in schizophrenia. American Journal of Psychiatry, 2010; 167: 1178-1193.

[38] Das T, Ivleva EI, Wagner AD, Stark CEL, Tamminga CA. Loss of pattern separation perfomance in schizophrenia suggests dentate gyrus disfunction. Schizophrenia Research, 2014; 159: 193-197.

[39] Mineka S, Zinbarg R. A contemporary learning theory perspective on the etiology of anxiety disorders: it's not what you thought it was. American Psychologist, 2006; 61: 10.

[40] Gilbert PE, Kesner RP. The role of the dorsal CA3 hippocampal subregion in spatial working memory and pattern separation. Behavioural Brain Research, 2006; 169: 1: 142-149.

[41] Bekinschtein P, Kent BA, Oomen CA, Clemenson GD, Gage FH, Saksida LM, Bussey TJ. BDNF in the Dentate Gyrus Is Required for Consolidation of "Pattern-Separated" Memories. Cell Reports, 2013; 5; 3; 759-768.

[42] Vanmierlo T, Rutten K, Dederen J, Bloks VW, van Vark-van der Zee LC, Kuipers F, Kiliaan A, Blokland A, Sijbrands EJ, Steinbusch H, Prickaerts J, Lutjohann D, Mulder M. Liver X receptor activation restores memory in aged AD mice without reducing amyloid. Neurobiology of Aging, 2011; 32: 1262-1272.

[43] Akkerman S, Blokland A, Reneerkens O, van Goethem NP, Bollen E, Gijselaers HJ, Lieben CK, Steinbusch HW, Prickaerts J. Object recognition testing: Methodological considerations on exploration and discrimination measures. Behavioural Brain Research, 2012; 232: 335-347.

[44] Akkerman S, Prickaerts J, Steinbusch HW, Blokland A. Object recognition testing: Statistical considerations. Behavioural brain research, 2012; 232: 317-322.

[45] Bostrom N, Sandberg A. Cognitive enhancement: Methods, ethics, regulatory challenges. Science and Engineering Ethics, 2009; 15: 311-341.

[46] Newcomer JW, Farber NB, Jevtovic-Todorovic V, Selke G, Melson AK, Hershey T, Craft S, Olney JW. Ketamine-induced NMDA receptor hypofunction as a model of memory impairment and psychosis. Neuropsychopharmacology, 1999; 20: 106-118.

[47] Bekinschtein P, Kent BA, Oomen CA, Clemenson GD, Gage FH, Saksida LM, Bussey TJ. Brain-derived neurotrophic factor interacts with adult-born immature cells in the dentate gyrus during consolidation of overlapping memories. Hippocampus, 2014; 24; 8; 905-911.

[48] Morris R, Garrud P, Rawlins J, O'Keefe J. Place navigation impaired in rats with hippocampal lesions. Nature, 1982; 297: 681-683.

[49] Van Praag H, Christie BR, Sejnowski TJ, Gage FH. Running enhances neurogenesis, learning, and long-term potentiation in mice. Proceedings of the National Academy of Sciences, 1999; 96: 13427-13431.

Chapter 3

Differential effects of acute and chronic 5-HT_{1A} auto- and hetero-receptor stimulation with the biased agonists F13714 and F15599 on pattern separation and neuronal plasticity in rats

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Abstract

Background and Purpose

The serotonin 1A receptor (5-HT_{1A}R) can exert differential effects on cognition-related neurotransmission depending on the location of the receptors. 5-HT_{1A} autoreceptors are located in the raphe nuclei, such as the dorsal raphe nucleus (DRN), and exert inhibitory effects on serotonergic transmission, whereas 5-HT_{1A} heteroreceptors are mainly located in limbic regions, such as the hippocampus and cortical regions, and can exert indirect stimulatory effects. This study aims to identify how pre- and post-synaptic 5-HT_{1A}R activity mediates cognition, specifically spacial pattern separation performance, following acute and chronic stimulation.

Experimental Approach

Male Wistar rats were treated with either F13714, a biased agonist which preferentially activates 5-HT_{1A} autoreceptors, or F15599, a biased agonist that preferentially activates 5-HT_{1A} heteroreceptors, both acutely and chronically for 14 days. Body temperature measurements were taken daily. Object pattern separation (OPS) performance was measured directly after acute treatment and at day 15 of chronic treatment. Animals were sacrificed after behavioral testing to measure 5-HT_{1A}R density and cognition-related markers in the hippocampus and DRN.

Key Results

Acute treatment with F13714 impaired OPS performance, whereas chronic treatment increased performance to vehicle levels. Body temperature was measured as a functional correlate of 5-HT1A receptor stimulation. It dropped from day 4 onwards and in parallel the number of 5-HT_{1A} receptors decreased in the DRN. F15599 enhanced OPS performance both acutely and chronically and caused an acute drop in body temperature, which rose again during chronic treatment. Furthermore, BDNF levels and doublecortin positive newborn neurons increased in the dorsal hippocampus after chronic F15599 treatment.

Conclusions and Implications

Chronic treatment with F13714 may result in desensitization of 5-HT_{1A} autoreceptors. Which causes a reversal of the initial impairment measured in OPS performance. Chronic post-synaptic stimulation of 5-HT_{1A} heteroreceptors with F15599 may have therapeutic potential to treat pattern separation impairments.

1. Introduction

Pattern separation is a type of memory processing that enables generation of separate representations from highly similar, but slightly different stimuli or events (Clelland et al., 2009). This is thought to be achieved by reducing the interference between overlapping patterns of activation so that similar patterns can be retrieved separately, enhancing memory accuracy (Holden and Gilbert, 2012, Kesner, 2013). Evidence from both human and rodent studies suggests pattern separation occurs mainly in the dentate gyrus (DG) of the hippocampus (Yassa and Stark, 2011, Morris et al., 2012). Yet other structures are also crucial for specific pattern separation processing, like the hippocampal CA3 region, which is necessary for the integration/separation of spatial components (Gilbert and Kesner, 2006, Leutgeb et al., 2007, Hunsaker et al., 2008, Lee et al., 2015). The underlying mechanism that supports cognitive pattern separation has not yet been unraveled completely, but adult neurogenesis in the dentate gyrus (DG) of the hippocampus seems crucial for pattern separation (Clelland et al., 2009, Franca et al., 2017). It is thought that by storing similar but new information in newborn cells, the information can be easily retrieved separately without interference (Frankland et al., 2013). Increasing evidence suggests that pattern separation processes are affected in multiple psychiatric diseases, including schizophrenia, dementia, PTSD and depression (Kheirbek et al., 2012, Eisch and Petrik, 2012, Stark et al., 2013, Ally et al., 2013, Das et al., 2014, Miller and Hen, 2015, Schreiber and Newman-Tancredi, 2014, Martinelli and Shergill, 2015, Faghihi and Moustafa, 2015). A targeted treatment to enhance pattern separation could therefore be of high value for the functional outcome and quality of life of patients.

The serotonin 1A receptor (5-HT_{1A}R) can exert differential effects on cognition-related neurotransmission, dependent on the location of the receptors. Somatodendritic 5-HT_{1A}Rs are located on serotonergic neurons in the raphe nuclei, such as the dorsal raphe nucleus (DRN), and act as autoreceptors, inhibiting 5-HT release and its cortical projections upon activation. Post-synaptic 5-HT_{1A} heteroreceptors are distributed across cortical and limbic brain areas, with high numbers in the hippocampus innervating different types of neurons (Hensler, 2003, Rojas and Fiedler, 2016). 5-HT_{1A}R activity is an important regulator of cognitive processes and memory related plasticity (Seyedabadi et al., 2014) and post-synaptic 5-HT_{1A}R activation has been linked to increased adult neurogenesis (Arnold and Hagg, 2012, Soumier et al., 2010). Pre- and post-synaptic 5-HT_{1A}Rs couple to different G_o/G_i proteins that influence the signaling cascade that is activated (Valdizan et al., 2010, Mannoury la Cour et al., 2006). Which G-protein is activated also depends on the nature of the agonist that activates the receptors (Newman-Tancredi, 2011).

The differential effects of 5-HT_{1A}R activation on cognition, specifically on pattern separation processes, has been previously studied in our lab; van Goethem et al. (2015) investigated the effects of two 'biased' 5-HT_{1A}R agonists on rat performance in a spatial object pattern separation (OPS) task.

The biased agonist F15599 (3-Chloro-4-fluorophenyl-(4-fluoro-4-(((5-methylpyrimidin-2-ylmethyl)-amino)-methyl)-piperidin-1-yl)-methanone) preferentially activates post-synaptic *cortical* heteroreceptors (Newman-Tancredi et al., 2009; Becker et al., 2016). F15599 likely does not interact much with *hippocampal* 5-HT1A receptors, where it shows a low level of cFos expression as compared with cFos expression in cortical areas (Newman-Tancredi et al., 2009). Therefore, its hippocampal effects, such as increases in adult neurogenesis and BDNF levels, are very likely indirect, resulting from cortical activation. F13714 (3-chloro-4-fluorophenyl-(4-fluoro-4-(((5-methyl-6-methylaminopyridin-2-ylmethyl)-amino)-methyl)-piperidin-1-yl-methanone) shows a preference for activation of 5-HT_{1A} somatodendritic autoreceptors (Koek et al., 2001, Yokoyama et al., 2016; see Newman-Tancredi, 2011 for review). Both of these 'biased' agonists possess exceptional selectivity for 5-HT_{1A}R versus a wide range of other receptors, transporters and enzymes (Vacher et al., 1998, Assie et al., 2006, Assie et al., 2010, Llado-Pelfort et al., 2010, Newman-Tancredi et al., 2009). Previous studies investigating the effect of F15599 administration on different aspects of memory and cognition have shown a high efficacy for alleviation of cognitive impairments, when compared to the prototypical 'unbiased' 5-HT_{1A}R agonist, 8-OH-DPAT (Depoortere et al., 2010, Assie et al., 2010). Therefore, selective targeting of specific receptor sub-populations using 'biased' agonists might prove an effective treatment strategy to improve cognitive functions. Indeed, unlike F15599, currently available (non-specific) 5-HT_{1A} agonists activate multiple signaling cascades which may (partly) cancel out the potentially beneficial effects of activating specific subpopulations of 5-HT_{1A} receptors.

The study performed by van Goethem et al. (2015) showed that acute administration of F15599 enhances OPS performance of rats, while acute F13714 impaired OPS performance, when compared to vehicle treated rats. These findings illustrate the therapeutic potential of specifically targeting 5-HT_{1A}R sub-populations to influence pattern separation function, and the value of biased agonists as a scientific tool to separately characterize the function of different 5-HT_{1A}R sub-populations.

The current study was designed to further elucidate the underlying mechanisms of differentially located 5-HT_{1A}R on pattern separation performance, by investigating the effects of chronic F15599 and F13714 treatment on OPS task performance and plasticity changes in the rat brain. A study by Assie et al. (2006) showed that F13714 induced rapid autoreceptor desensitisation during chronic threatment, indicated by recovery of extracellular 5-HT levels in the hippocampus. Other (non-biased) agonists showed desensitisation of somatodendritic 5-HT_{1A} autoreceptors on a

neurochemical level after 7 days of treatment (Dong et al., 1997, Blier and Ward, 2003), although the behavioural outcome remained unaffected after/by this autoreceptor desensitisation.

Since 5-HT_{1A}Rs originate from a single intronless gene without splice variats or subtypes, they are identical in amino acid sequence, regardless of their expression in different brain regions., Nevertheless, 5-HT_{1A}Rs can couple to different G-proteins depending on brain region and agonist activation. This implies that the preferential effects shown by F13714 and F15599 are due to a differential interaction of the compounds with the receptor coupled G-proteins, and not to differences in binding affinity per se. Thus, the compounds have been shown to preferentially activate specific G-protein subtypes (Newman-Tancredi et al., 2009; 2017). Taken together, this led to the hypothesis that F13714-induced desensitisation of autoreceptors in the dorsal raphe nucleus (DRN) would not only restore endogenous 5-HT release, but also thereby cause an additional shift to post-synaptic 5-HT_{1A}R activation by F13714. Therefore, it might be possible that chronic F13714 treatment leads to enhanced pattern separation performance compared to baseline performance, after desensitisation of the raphe-located 5-HT_{1A} autoreceptors has occurred. In contrast, post-synaptic 5-HT_{1A}R activation does not lead to desensitization when measured by electrophysiology and neuroendocrine levels with (non-biased) agonists (Hensler, 2003, Dong et al., 1997). This leads to the hypothesis that F15599-treated rats would maintain their enhanced OPS performance 24h after the last chronic treatment, when compared to vehicle treated animals.

Hypothalamic post-synaptic 5-HT_{1A}Rs regulate body temperature the in rats and agonist stimulation of these receptors leads to a hypothermic response (Rausch et al., 2006, Hensler, 2003). Therefore, body temperature can serve as an accurate mechanistic readout to measure post-synaptic receptor activation and desensitization changes *in vivo* (Zuideveld et al., 2004, Millan et al., 1993). During chronic treatment with F15599, a constant hypothermic response is expected, while somatodendritic receptor activation by F13714 will not induce a hypothermic response. However, if pre-synaptic receptors desensitize, the net-effect of F13714 could shift to post-synaptic activation thus producing a hypothermic response once this occurs.

To investigate the underlying molecular and cellular mechanisms underlying pattern separation performance and how these are affected by 5-HT_{1A}R activity, a series of memory-related neuronal plasticity measurements were included in the study. The brains taken from chronically treated animals allow for direct comparison of differential effects of either 5-HT_{1A} auto- or heteroreceptor activation on hippocampal plasticity and known memory related signaling pathways. Furthermore, evaluation of 5-HT_{1A} receptor density in the dorsal raphe nucleus (DRN), hypothalamus and hippocampus allow comparisons of region-specific changes in receptor population, caused by the differential 5-HT_{1A}R activation after F15599 and F13714 administration.

In summary, chronic treatment with F15599 was expected to lead to an upregulation of neurogenesis and related plasticity markers in the hippocampus, which could support pattern separation functioning in rats. Although F13714 shows a decreased effect on OPS performance acutely, it was possible that 5-HT_{1A} autoreceptor desensitization could take place causing OPS performance to be improved, supported by restored and eventually enhanced neuronal plasticity.

2. Methods

2.1 Animals

In total, 48 three-month-old male Wistar rats (Charles River Laboratories International, Inc., Sulzfeld, Germany) were used (average weight of 325.4 grams at the beginning of the study). All experimental procedures were approved by the local animal ethical committee of Maastricht University (licensed animal ethical committee: Min.VWS, GZBIVVB981845) under the Dutch Experiments on Animals Act (EAA, amended 1996), in accordance with the European Directive (2010/63/EU) for animal experiments. These experiments were registered under the protocol number DEC2013-013. All experimental results are reported in accordance with ARRIVE guidelines (Kilkenny et al., 2010).

Rats were housed individually in standard Tecniplast IVC system greenline cages, on sawdust bedding, and with a reversed light/dark cycle of 12/12 hour (lights off from 7:00h to 19:00h). Animals were housed in a consistently temperature- (22 ±1°C) and humidity- (55% ±10%) controlled environment. Food and water were available ad libitum. Background noise was provided by a radio playing softly. All testing was performed during the dark phase of the light/dark cycle, under low illumination (20lux). One of the animals deceased prior to the start of habituation; therefore, the entire experiment was performed with 47 rats.

2.2 Treatments

Animals were randomly assigned to one of the treatment conditions at the start of the study. N=16 rats received F15599 (also known as NLX-101) treatment, N=16 F13714 and N=15 were given vehicle (saline) treatment. All injections were administered intra peritoneal (IP) with a volume of 2ml/kg. As a baseline measurement for OPS performance, we first aimed to replicate the acute drug effects on OPS (van Goethem et al., 2015). We found similar behavioral effects with 0.08 mg/kg F15599 and 0.0025 mg/kg F13714, when given 30min before T1 of the OPS. After the acute study, there was a 2-week wash-out period, but animals stayed assigned to the same treatment groups. Chronic treatment consisted of daily injections (24h intervals) over a 14-day period with either 0.32 mg/kg F15599, 0.02 mg/kg F13714 or vehicle. These dosages were chosen to accomplish maximum receptor occupancy over the 24h period without inducing serotonergic symptoms (Assie et al., 2010). The drugs were a kind donation of Neurolixis Inc. (Dana Point, CA, USA) and provided to us as a dry powder, which was subsequently dissolved in saline. All solutions were prepared fresh on the day of administration.

Of note; the limited time window to perform OPS testing and subsequent harvesting of brain material at the end of the experiment did not allow all animals to be tested on the same day. To keep everything as similar as possible between animals, it was decided to divide the rats over 4

subsequent start days of the first treatment, with each condition equally represented on each day. This experimental planning has led to the advantage that possible fluctuations due to subtle day-differences have been balanced out of the behavioral and temperature data.

2.3.1 Behavioral OPS task apparatus and objects

Spatial pattern separation performance was measured in the object pattern separation (OPS) task (van Hagen et al., 2015). The apparatus and procedures followed were similar to those previously described in the study by van Goethem et al. (2015). OPS testing was performed in a circular arena with a diameter of 83cm and 40cm in height. One half consisted of see-through PVC and the other half of grey PVC. Two sets of identical objects were used, consisting of either two rectangular metal blocks (10.0cm x 5.0cm x 7.5cm) containing two holes (diameter 1.9cm), or two aluminum cubes with square bases and tapering tops (13.0cm x 8.0cm x 8.0cm). The rats were unable to displace these objects. The arena was dimly lit by fluorescent red tubes and a small desk lamp, providing illumination of about 20lux.

2.3.2 OPS testing procedure and habituation

The OPS task has been adapted from the object location task (OLT) (Ennaceur and Delacour, 1988), to asses spatial pattern separation processes in rodents based on the novelty-preference paradigm. A detailed description of the entire protocol and rationale can be found in the article by van Hagen et al. (2015). In short, the task was performed over two 3-min-trials in the arena, separated by a 1h interval. In the acquisition trial (T1), two identical objects were placed symmetrically on a horizontal line in the arena (position 1) to be explored by the rats. After the inter-trial-interval spent in the home cage, the second, test trial (T2) contained the same set of objects, but now one of the objects was displaced by a small separation from the previously explored location (position 3). Normal, untreated rats showed an intermediate discrimination for this position, in between that of completely similar (position 1) or highly different positions (position 5). This allowed measuring both increases and decreases in pattern separation performance (van Hagen et al., 2015).

Object orientation was kept the same over the two trials, displacement variations (left or right object, to front or back) and different object-sets were divided in a balanced manner over conditions and varied across test-days for individual animals. After each trial the objects and arena were cleaned with a 70% ethanol solution. Exploration times of each object were recoded manually by the experimenter on a desktop computer, using in-house developed scoring software. Exploration was defined as directing the nose to the object from a distance no more than 2cm or touching the object with the nose. Sitting on, or leaning to, an object was not scored as exploratory behavior.

Before the start of the experiment, the rats were habituated to the experimenter, the set-up and injections. This was done over a three-week period in which animals were gradually getting accustomed to handling procedures by the experimenter, habituated to the (empty) arena and objects and finally to the entire testing procedure. After habituation, the rats were assigned to a treatment condition and acute treatment was tested, followed by a two-week wash-out period before the chronic treatment. The OPS task was performed again on day 15, 24h after the last treatment.

2.4 Body temperature

Body temperature or more specifically the induction of hypothermia, is a highly specific and sensitive measure of (hypothalamic) post-synaptic 5-HT_{1A}Rs activation (Millan et al., 1993) in rats. Core body temperature was measured daily for each animal, as a model for pharmacological post-synaptic binding of F15599 and F13714. Measurements were always taken exactly 30-min after the daily treatment administration, using a digital rectal thermometer with an accuracy of 0.01°C (Onbo Electronics Co. Ltd., Shenzhen, China). The thermometer was cleaned with paper towel and lubricated with Vaseline (Unilever, the Netherlands) before each measurement. Temperature was always measured on the same time in the morning for each of the animals, to prevent individual differences due to circadian rhythm fluctuations.

Furthermore, additional baseline temperature measurements were taken on day 7 and 14 of the chronic treatment, 30-min prior to injection. These measurements functioned mainly as a control for chronic treatment effectiveness, i.e. constant body temperature up to 24h after the last treatment would signify constant drug activity in between daily injections and no interference of acute treatment effects with the post-injection temperature measurements.

2.5 Statistical analysis behavioural experiment (OPS task and temperature)

The statistical analysis for the entire study comply with the recommendations on experimental design and analysis in pharmacology research (Curtis et al., 2015). Total exploration times for each of the objects were calculated for both T1 and T2. Total time spent exploring both symmetrically placed objects in T1 (‘a1’ + ‘a2’ = ‘e1’) and total exploration times of the moved (‘b’) and stationary (‘a3’) object in T2 (‘a3’ + ’b’ = ‘e2’) were calculated exploration differences between treatment conditions. The main performance measure was the discrimination index or ‘d2’, calculated through (‘a3’-‘b’)/’e2’, eg. the difference in exploration time between objects relative to the total exploration in T2. See van Hagen et al. (2015) and (Akkerman et al., 2012a)for more detailed information on these measures. Total exploration times in T1 and T2 should be sufficient to reliably assess pattern separation memory. Therefore, animals that spend less than 9s exploring both objects in T1 or T2, were excluded from the dataset (Akkerman et al., 2012a).

One-sample t-statistics were used to compare the mean d2 index for each condition to zero (i.e. chance level performance)(Akkerman et al., 2012b), a significant difference from chance level signified that the animals recognized the moved object in T2 as being in a different position than in T1. Differences in d2 between conditions were evaluated by one-way ANOVAs separate for each test day. When the overall ANOVA was significant, Bonferroni post-hoc tests were performed.

The average temperature of each condition was calculated per treatment day, to show temperature changes over time. A two-way repeated measures ANOVA for treatment and day was performed. Bonferroni's multiple comparisons were used to evaluate treatment effects on each day. To measure receptor activation changes within a treatment condition, a repeated measures ANOVA over all days was performed for treatment condition. If significant, LSD t-tests were performed to compare day 1 temperature (baseline response) to all subsequent days. To analyze differences in temperature before and after treatment, pre-treatment measurements taken on day 7 and 14 were compared to the corresponding post-treatment measurement for each condition, using a paired samples t-tests. Additional paired samples t-tests were performed between day 7 and 14, for both the pre-treatment and post-treatment measurements.

2.6.1 Brain tissue collection

Brain samples from all animals were obtained ±1h after OPS testing on day 15. For immunohistochemical (IHC) measurements, half of the animals (N=8 per condition) were put under deep anesthesia (100mg/kg sodium pentobarbital, IP) and transcardially perfused with PBS (0.1M phosphate buffered saline, pH7.4) followed by 4% paraformaldehyde (PFA). Entire brains were taken out and post-fixed overnight in 4% PFA, and subsequently washed in PBS for 24h. The samples incubated in 10% sucrose (48h), followed by 3 days in 20% sucrose. 30µm thick coronal sections were cut using a cryostat (CM3010, Leica Biosystems) in areas of both the dorsal hippocampus (-1.72mm to -5.52mm from Bregma) and the DRN (-6.96mm -8.76 mm to Bregma) according to Paxinos and Watson (2007). The sections were collected in series of every 8th section and stored at -80 °C until further analysis.

The other half of the rats (N=8 for F15599 and F13714, N=7 for vehicle treated animals) were used for western blot analysis. Animals were decapitated, after which the right and left hippocampus were dissected and divided in the dorsal- and ventral-part before storage in separate tubes. The samples were snap-frozen in liquid nitrogen and stored at -80 °C until further analysis.

2.6.2. Immunofluorescent labeling of doublecortin

Fluorescent labeling of doublecortin (DCX) was performed on hippocampal sections to quantify ongoing cell division, differentiation and migration of neuronal precursor cells in the sub-granular (SGZ) and granular cell layer (GCL) in the DG of the hippocampus (von Bohlen Und Halbach,

2007). IHC labeling was performed on free floating hippocampal sections, frozen sections were washed in TBS (0.1 M Tris–HCl, pH 7.4, 0.9% NaCl), transferred to glass containers with pre-heated sodium citrate solution (10mM Sodium Citrate, 0.05% Tween 20 pH 6.0). The sections incubated for 20min in an 80°C water bath for antigen retrieval, followed by 20min cool down at room temperature (RT) and washing in TBS (2x 15min) and TBS-T (TBS containing 0.3% Triton X-100; 15min). Endogenous epitope blocking was performed for 1h in 3% donkey serum (Sigma Aldrich # D9663), followed by overnight incubation at 4°C with goat anti-DCX (1:100, Santa Cruz SC-8066) with 0.3% donkey serum in TBS-T. Washing in TBS-T, TBS and TBS-T (15min) was performed followed by 2h incubation with donkey anti-goat Alexa 488 (Invitrogen) at RT. After final washing steps (15min TBS-T, 2x 1h TBS) slices were carefully mounted on gelatin-chromaluin coated glasses, air dried and coverslips were mounted with 80% glycerol.

2.6.3 5-HT_{1A} receptor immuno-labeling in the dorsal raphe nucleus, hippocampus and paraventricular nucleus

5-HT_{1A} receptors were visualized using DAB immunohistochemistry, in sections containing the DRN, hippocampus and the PVN of the hypothalamus. Stored free-floating sections were washed in TBS (3x 10 min). Subsequent washing was performed after each incubation step with TBS-T, TBS and TBS-T (10min). Endogenous epitopes were blocked by 30min incubation with 0.3% H_2O_2 in TBS, followed by washing. Rabbit anti- 5-HT_{1A}R antibody (1:500, GTX104703, Genetex, Irvine, CA, USA) incubated overnight at 4°C in TBS-T. Biotinylated donkey anti-rabbit (1:500, Jackson Laboratories, West Grove, PA, USA) incubated for 2 hours at RT, followed by the avidin-biotin enzyme complex for 1h (ABC-kit, Vector Laboratories, Burlingame, Calif, USA). The immunoperoxidase reaction was carried out using a DAB solution (0.01% 3,3'diaminobenzine, 0.025% NiCl2, 0.003% H_2O_2 in 0.05 M Tris–HCl), the reaction was stopped after ±10min by rinsing in TBS (3x 10min). Sections were mounted on gelatin-chromaluin coated glasses and air-dried overnight. Dehydration was performed in ascending ethanol concentrations and coverslips were mounted over the sections using depex.

2.6.3 Protein gel electrophoresis

To compare relative protein quantities of memory related plasticity markers, western blots were performed with homogenized brain sample of the dorsal hippocampus. Membranes were stained for; mature and pro- brain derived neurotropic factor (BDNF), the transcription factor cAMP response element binding protein (CREB) and its phosphorylated state pCREB, synaptophysin, and post-synaptic density protein 95 (PSD95).

The dissected dorsal hippocampus samples were homogenized using a lysis buffer (100mM Tris, 200mM NaCl, 1mM EDTA, 2 mM DTT, 0.05% Triton) with added phosphates and protease inhibitors (protease inhibitor mix and phosphostop, Roche, Basel, Switzerland). Protein

concentration was determined (BioRad concentration assay, Hercules, CA, USA) to load equal amounts of protein from each sample into a 10% SDS-polyacrylamide gel (14% for BDNF) and separate them through gel electrophoreses. Subsequently, proteins were transferred into a PVDF membrane (Li-Cor Biosciences, Lincoln, NE, USA). Membranes were blocked with 1:1 diluted Odyssey blocking buffer (Li-Cor Biosciences, Lincoln, NE, USA) in PBS. Primary antibody solutions incubated overnight at 4°C, on separate blots for; rabbit anti-BDNF (1:600, #F0110, Santa Cruz, CA, USA); mouse anti-synaptophysin (1:1000, MAB5258, Merck Millipore, Burlington, MA, USA); mouse anti-CREB + rabbit anti-pCREB (1:3000, #9104; 1:100 #91988, Cell Signaling Technologies, Beverly, MA, USA) and mouse anti-PSD95 (1:2000, #0711, QED Bioscience, San Diego, CA, USA). Mouse anti-GAPDH (1:2000000, #10R-G109A, Fitzgerald, Huissen, NL) was used as a loading control. After washing in PBS and PBS-T (PBS + 0.1% Tween) secondary antibodies (1:10000 donkey anti-mouse IRDye680; 1:5000 goat anti-rabbit IRDye800, Li-Cor Biosciences, Lincoln, NE, USA) incubated for 1h. Membranes were air dried and imaged with an Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE, USA).

2.7 Imaging and analysis of plasticity measures

DCX labeled neurons were counted in the SGZ and GCL of the DG, the hippocampal region in which adult born immature neurons are known to develop and integrate into (von Bohlen Und Halbach, 2007). Dorsal hippocampal sections from bregma-2.16 to -4.68 (Paxinos and Watson, 2007) were imaged with an Olympus BX50 microscope and DP70 camera (Olympus, Tokyo, Japan), quantification was performed using StereoInvestigator® (version 11, MBF biosciences, Williston, VT, USA). Both hemispheres of each hippocampal section were analysed and summed up. The DG area was delineated at 10x magnification, to define the region of interest (ROI) and calculate the volume based on section thickness (Sahay et al., 2011). DCX positive neurons were counted in the entire ROI at 40x magnification using the optical fractionator method. Estimated total DCX cell numbers were obtained for each animal, by multiplying the total count of each section by 8 (number of section series). To control for volume differences, the total number of cells was divided by the estimated volume of each sample, giving the cell to surface ratio. The mean DCX ratios of the treatment conditions were compared by a one-way ANOVA, followed by Bonferroni's post-hoc comparisons to vehicle.

5-HT_{1A}R differences in optical density were semi-quantified and compared between treatment conditions in 3 separate areas; the DRN, the dorsal hippocampus and the PVN of the hypothalamus. Using a bright-field AX70 microscope and XM10 camera (Olympus, Tokyo, Japan), multiple photographic images were obtained at 20x magnification, to visualize the entire ROI in both hemispheres. Lighting conditions and settings were kept constant during imaging for all sections, to

avoid differences in pixel values. First, multiple obtained images from one section were stitched together using the ImageJ NIH () software extension MosaicJ®. Subsequently, the entire DRN, hippocampus or PVN of each section could be delineated and analyzed in ImageJ® using their corresponding 8-bit grayscale values. Area and gray-value measures of each region of interest were normalized for individual differences in background DAB reactivity by obtaining the mean gray value of non- DAB reactive tissue within the same image and set the threshold to 2x of the background value (Yagi et al., 2016). To calculate the relative receptor density the DAB immunoreactive area (above threshold) of each section was divided by the total area of the corresponding ROI selection (Yagi et al., 2016). For the DRN a minimum of 3 sections per animal were used, 5 sections for the dorsal hippocampus and 3 for the PVN (Madden and Zup, 2014). Mean ratios per animal were compared between treatment groups using a one-way ANOVA, followed by post-hoc Bonferroni tests between groups. Outliers were removed per condition using a Dixon test for outliers.

Western blot protein markers were semi-quantified using grey value intensities. Obtained fluorescent images of both the green (800) and red (700) channel of each membrane were analyzed with imageJ. After conversion to 8-bit greyscale images, intensity scores of the selected bands of each sample were generated in a gray value histogram. The background signal was removed using the same intensity cut off for each marker, determined by evaluation of the histogram of gray-value pixel distribution. To control for loading differences, target proteins were normalized by dividing them by the intensity of the corresponding GAPDH band. Before calculating the average ratio per treatment condition, outliers were removed per condition using the Dixon test for outliers. F15599 and F13714 treatment conditions were compared to vehicle using a one-sided t-test. A α level of 0.05 was considered significant for all statistical comparisons.

3. Results

3.1 OPS performance

An overview of main OPS task outcome measures are listed in Table 1A and 1B. Total exploration times of each test-day were compared between the groups for both T1 and T2. For T1 ($F_{2,44}$=0.684, n.s.) and T2 ($F_{2,44}$=2.504, n.s.) after acute treatment there were no significant differences in exploration times between conditions. Neither any differences were found for T1 and T2 exploration times after the chronic treatment (T1: $F_{2,43}$=1.143, n.s.; T2: $F_{2,43}$=0.770, n.s.), indicating that exploratory behavior is not affected by acute or chronic F15599 or F13714 administration.

Table 1. Mean OPS measures and their SEM for each acute treatment condition

A	**Acute treatment**	**Vehicle**	**F15599**	**F13714**
e1 (SEM)		36.44 (2.76)	32.87 (2.03)	34.44 (1.58)
e2 (SEM)		20.70 (1.93)	26.83 (1.94)	22.02 (2.23)
d2 (SEM)		0.20 (0.07) #	0.33 (0.08) ###	0.02 (0.08)
B	**Chronic treatment**	**Vehicle**	**F15599**	**F13714**
e1 (SEM)		30.82 (2.70)	26.18 (2.58)	26.09 (2.14)
e2 (SEM)		25.59 (2.61)	21.45 (2.14)	24.02 (2.38)
d2 (SEM)		0.16 0.07) #	0.27 (0.06) ###	0.17 (0.07) #

Mean OPS task outcome measures and their SEM for each of the treatment conditions; e1= total exploration time during T1, e2= total exploration time during T2, d2= discrimination index. A significant difference between d2 and chance level performance are indicated by hashes (one-sample t-test, #: P<0.05; ###: P<0.001). ***A)*** *Acute treatment OPS task Vehicle N=15, F15599 (0.08 mg/kg IP) N=16, F13714 (0.0025 mg/kg IP) N=16.* ***B)*** *OPS task measures 24h after the last chronic treatment. Vehicle N=14, F15599 (0.32 mg/kg/day IP) N=16, F13714 (0.02 mg/kg/day IP) N=16.*

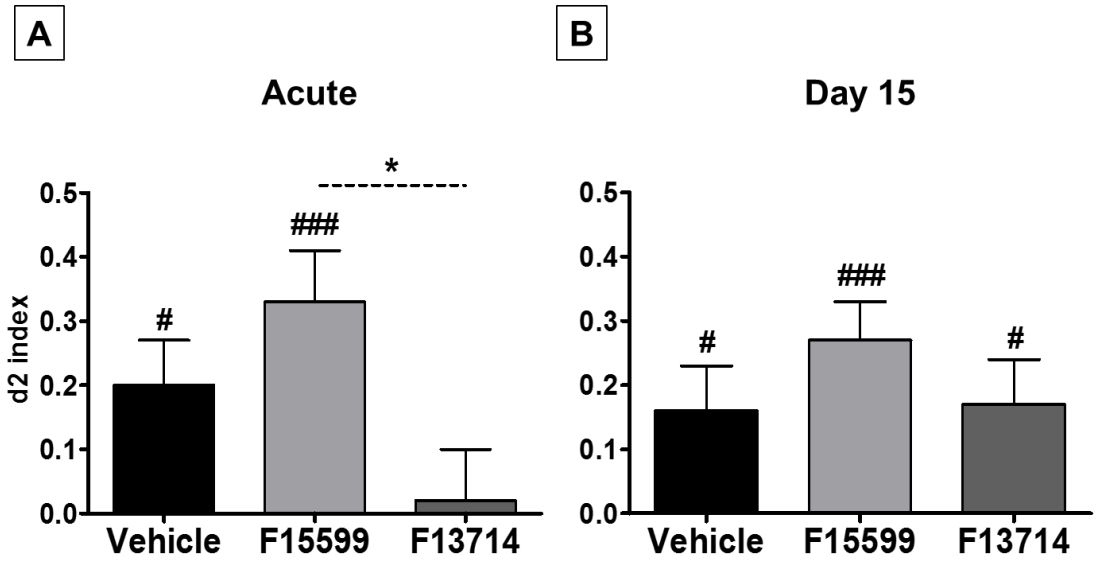

Figure 1 - Acute and chronic effects of F15599 and F13714 treatment on pattern separation (OPS) task performance in rats. *Mean OPS task scores are depicted as d2 index (+SEM) for each of the treatment conditions.* ***A)*** *d2 index after acute F15599 (0.08mg/kg IP), F13714 (0.0025mg/kg IP) and vehicle (saline) treatment. A significant difference between groups is indicated by an asterisk (post-hoc Bonferroni, *: P<0.05). Vehicle N=15, F15599 N=16, F13714 N=16.* ***B)*** *Chronic treatment effects on OPS task d2 performance measured 24h after the last treatment with either F15599 (0.32mg/kg/day IP), F13714 (0.02mg/kg/day IP) and vehicle (saline). Vehicle N=14, F15599 N=16, F13714 N=16. A significant difference between d2 and chance level performance are indicated by hashes (one-sample t-test, #: P<0.05; ###: P<0.001).*

The mean d2 performance for each treatment condition is visualized in Figure 1. One-sample t-statistics were performed comparing each condition to zero (eg. chance level performance). The mean d2 index of both acute and chronic vehicle treated rats differed from chance level performance, indicating functioning pattern separation memory. Comparison of the d2 index from acute F15599 treated animals with chance level also indicated a high significant pattern separation ability, while F13714 treated rats did not show any pattern separation ability (Table 1A). The d2 performance of the F15599 treatment group was significantly higher than that of the F13714 treatment, as shown by a one-way ANOVA between conditions ($F_{2,44}$=4.301; P=0.020), followed by Bonferroni's pairwise comparisons (P=0.017) (Figure 1A). However, no significant difference was found between the d2 index of vehicle treated and both drug treated groups.

After 14 days of chronic treatment, similar d2 performance was measured in F15599 treated animals, indicating that the enhancing effect of F15599 was maintained after 2 weeks of treatment (Figure 1B). F13714 treatment displayed different chronic effects on OPS performance, now the d2 index was significantly higher than chance level, showing improved pattern separation ability. Thus, findings show that acute activation of the pre-synaptic 5-HT_{1A}Rs by F13714 leads to impaired pattern separation, but this is restored after 14-days of chronic activation.

3.2 Temperature measurements

Daily body temperatures of each treatment condition are depicted in Figure 2**.** The two-way repeated measures ANOVA for both treatment condition and treatment day showed highly significant differences for treatment condition (*49.73%*; $F_{2,572}$=143.2, P<0.0001) and over time (2.3%; $F_{13,572}$=3.176, P=0.0001), as well as their interaction (*8.44%;* $F_{26,572}$=5.834, P<0.0001). Significant differences from the vehicle condition were evaluated separately for each treatment day using post-hoc Bonferroni t-tests, these results are indicated in Figure 2. Comparison to vehicle treatment, post-synaptic 5-HT_{1A}R activation by F15599 caused a significantly lower body temperature (p<0.001 for each day). Although the figure shows a gradual loss of hypothermic response to F15599, it was nevertheless maintained over the course of the 14-day treatment when compared to vehicle. In contrast, body temperature was not initially affected by F13714: temperature was similar to vehicle-treated rats for the first 4 days of treatment. However, from treatment day 5 onwards, F13714-treated animals displayed a lower body temperature from vehicle treatment, indicating a hypothermic response due to hypothalamic 5-HT_{1A}R activation (see figure 2).

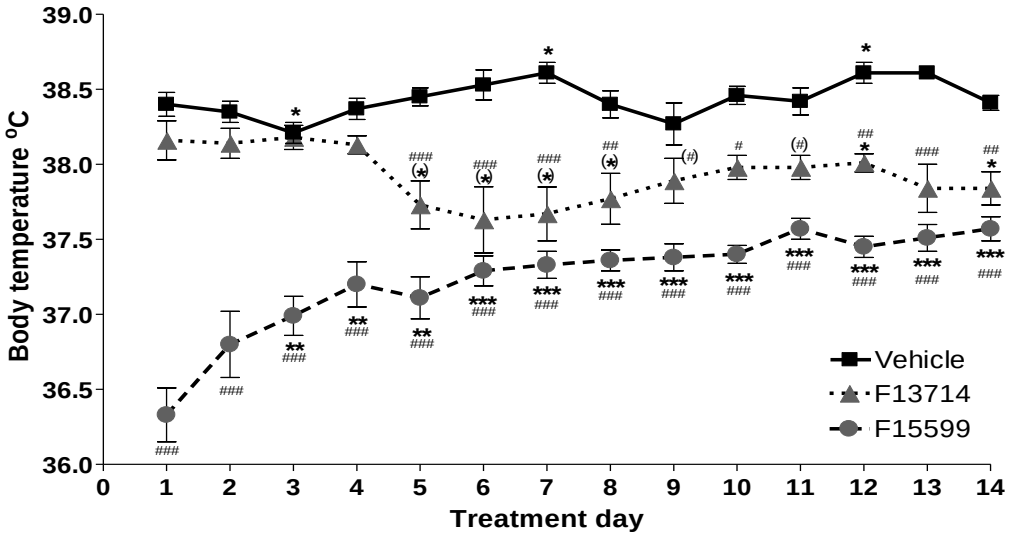

Figure 2 - Mean daily body temperature and SEM per day for each treatment condition.
*The average body temperature (+SEM) in °C for each treatment condition is plotted for all subsequent days. N=15 for vehicle and for both F15599 and F13174 N=16 for each of the observations. Significant differences from vehicle on the same day are indicated by hashes (Bonferroni's t-tests, #: P < 0.05; ##: P < 0.01; ###: P < 0.001). Significant differences compared to day 1 of the same treatment are indicated by asterisk (LSD t-tests, *: P < 0.05; **: P < 0.01; ***: P < 0.001).*

Treatment fluctuations over time were also analyzed with a repeated measures ANOVA for each treatment group and post-hoc LSD t-tests with day 1 (baseline). Chronic F13714 treatment resulted in a decrease in temperature over time ($F_{13,195}$=2.051, P=0.0187) with a significantly lower temperature on days 12 and 14 than on the first days. This supports the assertion that an initial modest drop in temperature after 4 days of treatment develops into a stable response after 14 days of F13714 treatment. Within repeated measure analyses of the F15599 treated rats ($F_{13,195}$=10.29, P<0.0001) indicates that the initial hypothermic response induced by chronic post-synaptic 5-HT_{1A}R activation decreases over time.

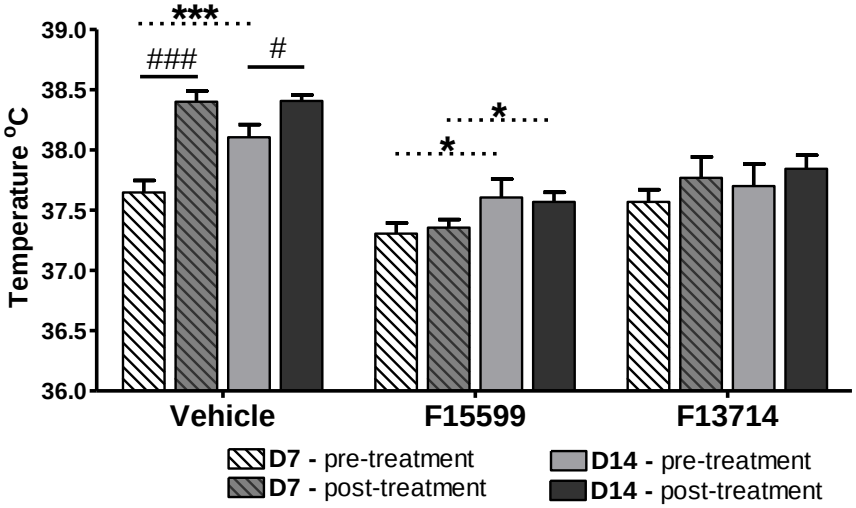

Figure 3 - Mean body temperature per condition, measured before and after treatment on day 7 and 14.
*For each of the treatment conditions average body temperature (+SEM) measured 30 minutes before, and 30 minutes after treatment are depicted separately for both day 7 (D7, striped bars) and day 14 (D14, plain bars). Treatment condition is indicated on the x-axis, with the 4 different bars representing repeated measures in the same animals (F155991 and F13714, N=16; Vehicle, N=15). Significant differences between pre- and post-measurement per day are indicated by hashes (paired samples t-test, P < 0.05: #; P < 0.001: ###). Asterisks indicate a significant difference between day 7 and 14 measurements of a treatment condition (paired samples t-test, *: P < 0.05).*

Temperature was also measured 30min before injections on both day 7 and 14. Figure 3 illustrates the differences between the pre- and post-treatment temperature for each treatment condition on both days. Only the vehicle treated group showed a significant increase in temperature between the pre- and post-treatment measurement on both day 7 and 14. The lack of a difference between pre- and post-treatment temperature in both drug treatment conditions may be due to sustained post-synaptic 5-HT_{1A}R activation by the agonists at the doses tested. Additional paired samples t-tests were performed between day 7 and 14, separated for pre- and post-treatment of each condition (figure 3). This revealed an increase in day 14 pre- and post-treatment temperatures compared to day 7 within the F15599 group (P=0.0467 and P=0.0117). No differences were found between day 7 and day 14 measurements of F13714 treated animals. Interestingly, the vehicle condition showed an increase in pre-treatment temperature between day 7 and 14, but no difference between both post-treatment temperatures.

3.3 Doublecortin positive cells in the DG

DCX positive cells were counted in the DG of the dorsal hippocampus to evaluate quantitative differences in adult born neurons. Figure 4 shows the mean ratio of DCX cells per surface for each condition. A one-way ANOVA ($F_{2,18}$=6,566, P=0.0072) showed that F15599 treatment significantly increased the DCX cell ratio compared to vehicle (Bonferroni t-test, P<0.01).

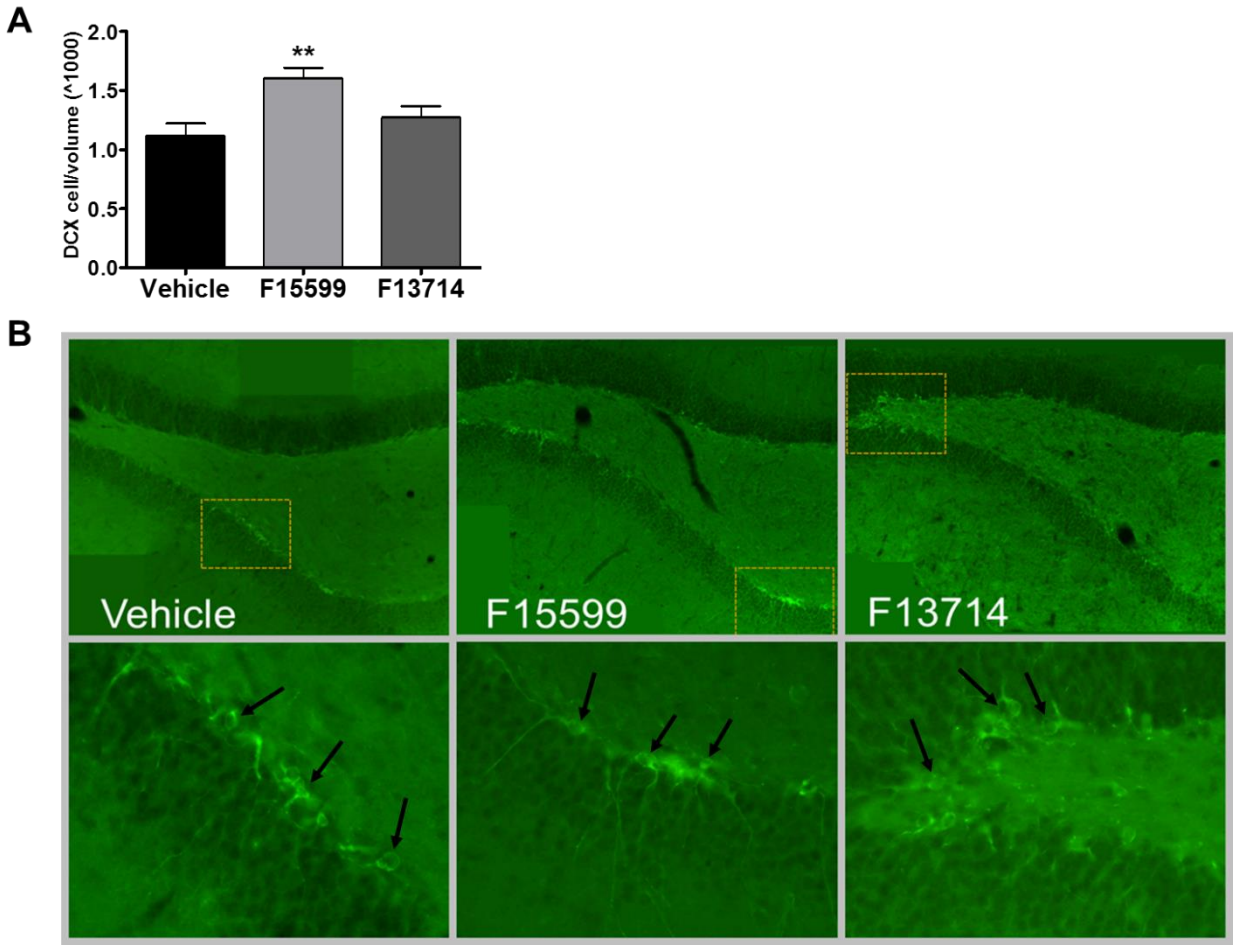

Figure 4 - Mean DCX ratio per condition and corresponding microscopy images.
A) *Given is the mean ratio of estimated total DCX-positive cells in the DG divided by the total volume of the region analyzed, obtained from dorsal hippocampus sections of N=8 chronically treated animals for each treatment condition. A significant difference from vehicle is indicated by hashes (Bonferroni's multiple comparisons: ##: P<0.01).* ***B)*** *Example images of the analysed DCX labeled sections from each treatment condition. Above: image at 10x magnification showing the granule cell layer of the DG. Below: close up of the 10x images indicated by the yellow box, showing DCX-positive cells in more detail. Arrows indicate examples of counted DCX cells.*

3.4 5-HT$_{1A}$R density

An overview of the results obtained from the 5-HT$_{1A}$R density analysis is shown in figure 5. A one-way ANOVA showed that 5-HT$_{1A}$R labeled DRN sections differed significantly in density ratios between the different treatment conditions ($F_{2,14}$=7.124, P=0.0073). Bonferroni's post-hoc tests revealed that both drug-treatment conditions have a significantly lower 5-HT$_{1A}$R distribution compared to vehicle treatment (F15599, P<0.05; F13714, P<0.01). In contrast, hippocampal 5-HT$_{1A}$R density ratios did not show any differences between the treatment conditions ($F_{2,16}$=0.03368, N.S.)

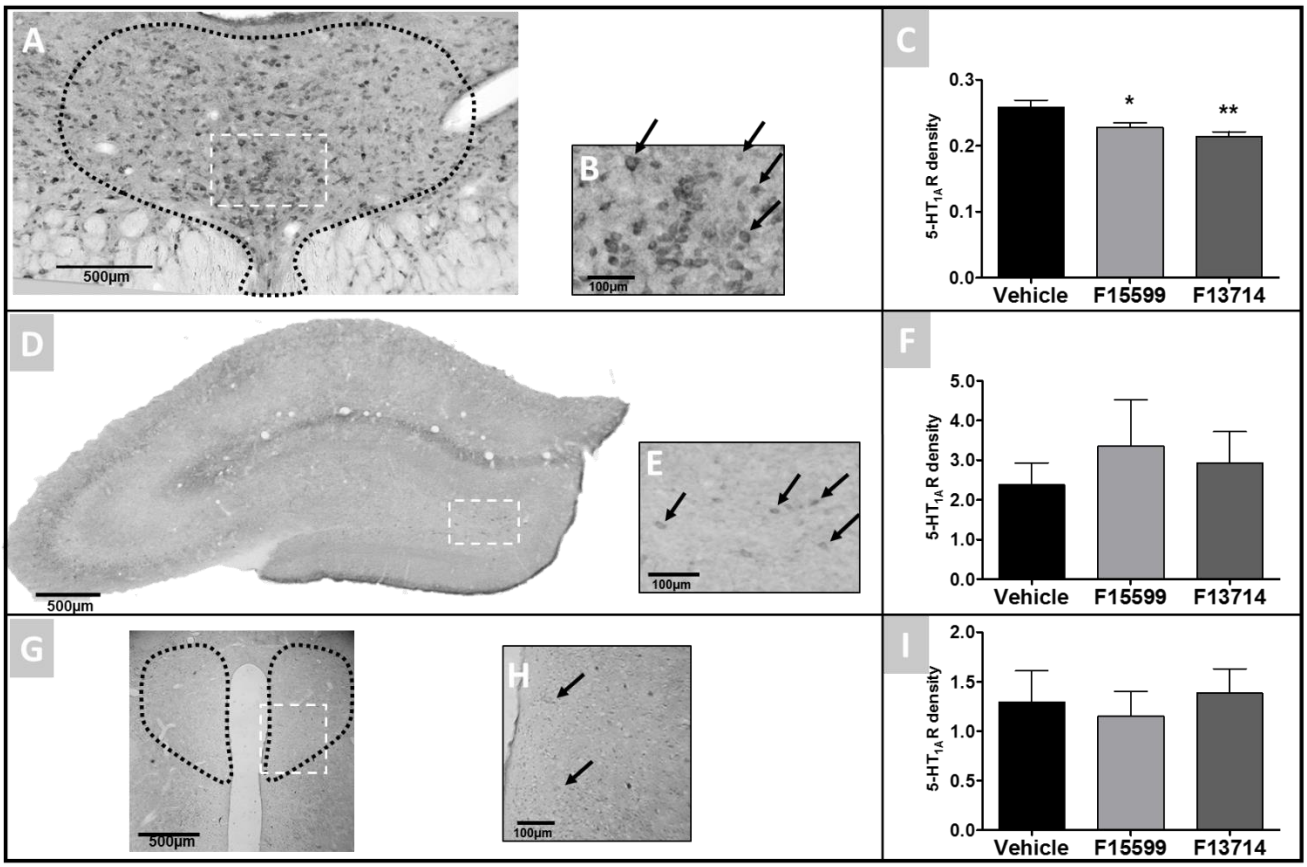

Figure 5 - Summary of 5-HT$_{1A}$R distribution measures in DRN and dorsal hippocampus sections.
A) *Microscopically obtained image of a 5-HT$_{1A}$R labeled DRN section from a vehicle treated animal, with the region of interested used for analysis delineated by the black dotted line.* ***B)*** *Close up of image A (indicated by the white square), showing dense 5-HT$_{1A}$R presence across cells of the DRN. Arrows indicate examples of cells which are labeled entirely of high enough intensity (above threshold) to be included in the measurements.* ***C)*** *Graph illustrating the mean ratio between 5-HT$_{1A}$R positive labeled surface and the remaining surface of the region analyzed. N=7 for each treatment condition.* ***D)*** *Microscopically obtained image of a 5-HT$_{1A}$R labeled hippocampal section from a vehicle treated animal, entire region which is shown was included as the region of interest.* ***E)*** *Close up of image D (indicated by the white delineation), showing 5-HT$_{1A}$R distribution in the DG region. Arrows indicate examples of hippocampal cells with enough 5-HT$_{1A}$R labeling to show the entire cell surface, at intensity above threshold for analysis.* ***F)*** *Graph illustrating the mean ratio between 5-HT$_{1A}$R positive labeled surface and the remaining surface of the dorsal hippocampus, N=7 for each treatment condition.* ***G)*** *Microscopically obtained image of a 5-HT$_{1A}$R labeled hypothalamic section from a vehicle treated animal. The region of interested containing the PVN is indicated by the black dotted line.* ***H)*** *Close up of image G (indicated by the white square), showing sparse 5-HT$_{1A}$R labeling across cells of the PVN. Arrows indicate examples of cells which are labeled at an intensity above the threshold to be included in the measurements.* ***I)*** *Graph illustrating the mean ratio between 5-HT$_{1A}$R positive labeled PVN surface and below threshold labeled PVN surface. N=7 for each treatment condition.*

3.5 Plasticity markers

Relative quantities of several protein markers were evaluated in dorsal hippocampus tissue from chronically treated animals, the results are summarized in Figure 6. For PSD95, synaptophysin and pCREB/CREB ratio no significant differences were found. However, an increase for mature BDNF and also its ratio to pro-BDNF was found for F15599 treated animals compared to vehicle (t-test, BDNF P=0.028; ratio P=0.025). The F13714 condition showed a trend for an increase in BDNF quantity, yet this failed to be statistically different from vehicle**.** BDNF intensity measurements are visualized in figure 6.

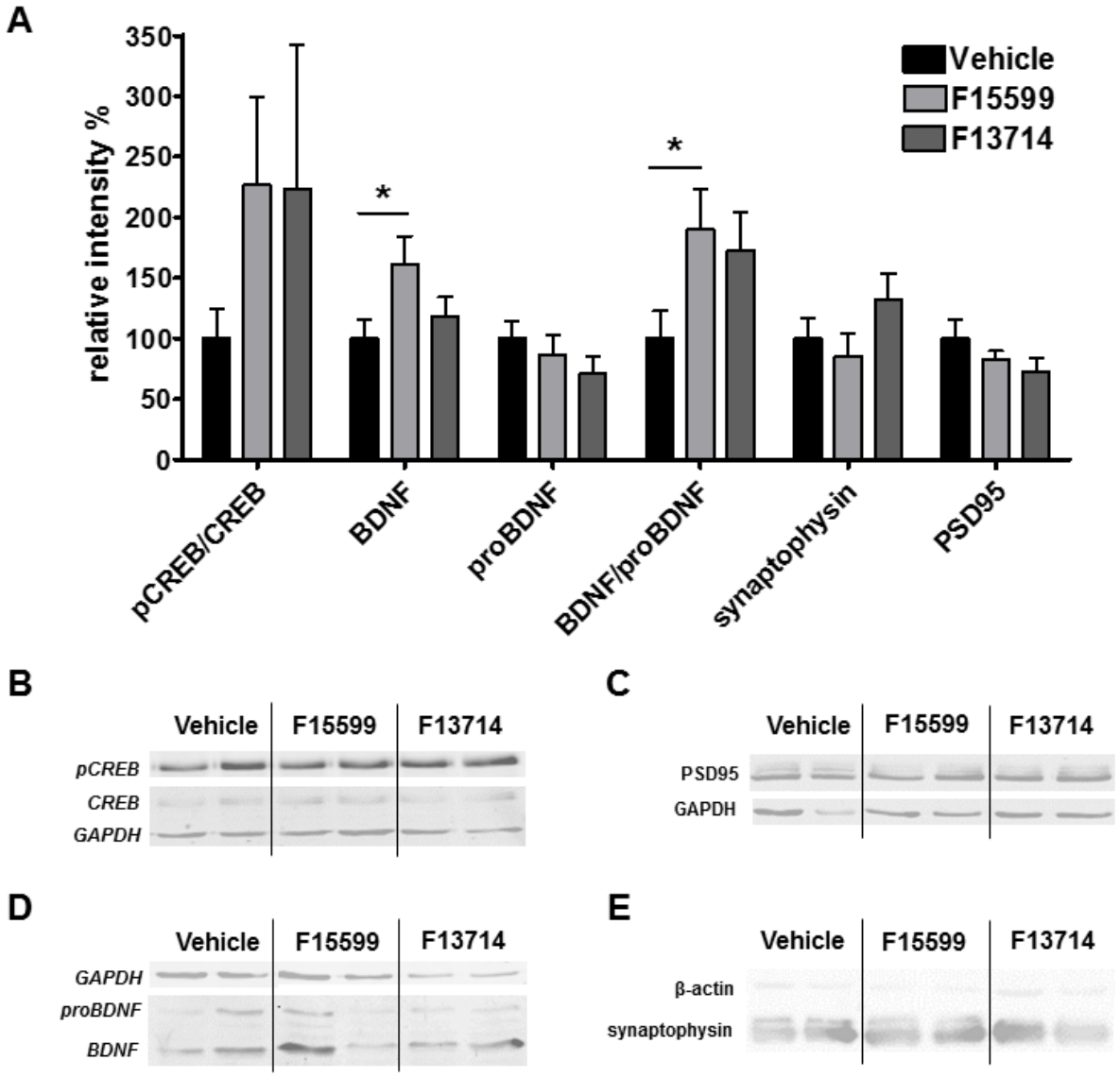

Figure 6 - Mean quantity percentages and SEMs of all plasticity markers and their corresponding western blot gels.
A) *Mean relative CREB, BDNF, synaptophysin and PSD95 quantity in dorsal hippocampus tissue of rats subjected to chronic treatment with F15599 (N=8), F13714 (N=8) and vehicle (N=7). of each condition. Mean differences are expressed percentages, with vehicle set to 100% for each of the markers. Asterisk indicate a significant difference from the vehicle condition (t-test; *: P<0.05).* ***B****) Analyzed lanes for each condition taken from the gel image labeled for pCREB, CREB and GAPDH* ***C****) Examples for each condition showing lanes from the gel labeled for PSD95 and GAPDH.* ***D)*** *Lanes per condition from the gel image labeled for (pro)BDNF and GAPDH.* ***E)*** *examples for each condition, of the gel image labeled and analyzed for synaptophysin and β-actin.*

4. Discussion

4.1 Differential effects of chronic pre- and post-synaptic 5-HT_{1A}R stimulation on pattern separation performance in rats.

The OPS task results showed that both acute and chronic (14 days) post-synaptic 5-HT_{1A}R activation with F15599 had beneficial effects on pattern separation performance in rats. Since OPS performance relies on hippocampal processes, this effect is most likely mediated through enhanced cortico-hippocampal signaling due to continuous post-synaptic 5-HT_{1A}R activation in the cortical region. Therefore, specifically targeting post-synaptic 5-HT_{1A}R activation with biased agonists such as F15599 could be a beneficial treatment therapy to enhance pattern separation deficits in psychiatric disorders.

While acute treatment with F13714 initially showed an impairment in pattern separation performance compared to the F15599 treatment group, performance ameliorated after 14 days of chronic treatment. This is shown by the one-sample statistics comparing the treatment groups to chance level performance (see Figure 1). Initially, pre-synaptic 5-HT_{1A}R activation by acute F13714 treatment did not lead to improved pattern separation performance in the rats. In contrast, after 14 days of chronic F13714 treatment, the OPS performance did improve, showing similar OPS performance to vehicle treated animals and thus well-functioning pattern separation. This shift in performance could be explained by desensitization of pre-synaptic 5-HT_{1A}R's in the DRN due to the continuous stimulation of these receptors (Assie et al., 2006). The inhibition of cortical 5-HT release after acute F13714 administration leads to the initial impairment in OPS performance (van Goethem et al., 2015), but through desensitization of the auto-receptors this inhibition is decreased. Therefore, we can conclude that chronic activation of pre-synaptic auto-receptors by F13714 will restored cortical 5-HT signaling to normal levels, subsequently leading to normalized OPS performance.

It could be expected that F13714 treatment would eventually mimic the effects of F15599 on OPS performance via its remaining post-synaptic activity. However, during the 14-day treatment, the effect on post-synaptic 5-HT_{1A}R's by F13714 still appears to be less than that of direct stimulation with F15599, as OPS task performance only shows amelioration of the initial impairment but no improvement from vehicle treated animals after 14-days of treatment with F13714. A similar trend is shown for the core-body temperature measurements in the F13714 group. Probably, post-synaptic receptor activation by F13714 at this dosage lacks the potential to activate cortical or hypothalamic 5-HT_{1A}R's to the same degree as F15599 (Newman-Tancredi et al., 2009), and therefore does not improve OPS performance or decrease core-body temperature, respectively. It could be possible that prolonged treatment with F13714 (i.e. >14 days) would have led to enhanced OPS performance,

since cortical 5-HT signaling possibly requires more time to normalize and subsequently increase after receptor desensitization. To further investigate this claim, future studies could investigate prolonged F13714 treatment at different dosages.

4.2 Differences in pre-synaptic 5-HT$_{1A}$ autoreceptor and post-synaptic heteroreceptor responses to chronic stimulation

The core-body temperature measurements illustrate the differential effects of chronic stimulation by F15599 and F13714 on the hypothalamic 5-HT$_{1A}$R subpopulation over time. The data support desensitization of the DRN somatodendritic autoreceptors by F13714 by showing a drop in temperature after 4 days of treatment, reflecting increased activation of post-synaptic 5-HT$_{1A}$R's in the PVN by F13714. Hypothermia did not occur during the first 4 days, presumably because of inhibition of 5-HT release by F13714 in this brain area and the corresponding decrease in activation of post-synaptic 5-HT1A receptors I the PVN. This rapid desensitization of pre-synaptic autoreceptors in rats was shown previously by measuring cortical 5-HT release after 0.63 mg/kg F13714 treatment (Assie et al., 2010). In the current study, desensitization of pre-synaptic autoreceptors occurs equally fast when using a dose of 0.02 mg/kg F13714, indicating that optimal receptor occupancy of F13714 is already achieved at these lower doses.

Furthermore, 5-HT$_{1A}$R density measurement was performed after (sub) chronic treatment of both compounds to investigate treatment effects on receptor population in the DRN, PVN and hippocampus and provide further insight into the possible desensitization mechanism. DRN receptor density ratio showed that animals who received chronic treatment with F13714 had significantly lower receptor density compared to vehicle treated animals. The antibody used for these measurements specifically binds to an intra-membrane loop of the 5-HT$_{1A}$R and therefore a decrease in antibody binding signifies a decrease of receptors expressed on the cell membranes. Therefore, we can conclude that desensitization of 5-HT$_{1A}$Rs in the DRN takes place through either decreased receptor expression or through internalization of these receptors. The latter is supported by a study by Riad et al. (2001), who demonstrated that receptors in the DRN internalized from the plasma membrane into the cytoplasm and by experiments using recombinant cell lines, in which F13714 was found to potently and efficaciously elicit 5-HT$_{1A}$ receptor internalization (Newman-Tancredi et al., 2009). Internalized receptors are uncoupled to the G-proteins which renders them unable to activate a signaling cascade. Furthermore, it has been shown that chronic agonistic stimulation caused a reduction of G protein binding in the DRN (Hensler, 2003).

In F15599 treated animals, a gradual loss of hypothermic activity over time was observed after an initial acute drop in temperature. This indicates a possible desensitization occurring in the post-synaptic hypothalamic receptors which reduced the initial hypothermic response. Since this

effect was not reflected in the behavioral data, i.e. no gradual decrease in OPS performance was observed, it might be possible that post-synaptic 5-HT_{1A}Rs in the PVN have a differential response to chronic activation than those located in the hippocampus. Previous studies have suggested desensitization in the PVN (Zhang et al., 2001, Zhang et al., 2004), but not in the hippocampus (Riad et al., 2001, Assie et al., 2006). Thus, our behavioral and core-body temperature data confirm the existence of a differential regulation of post-synaptic 5-HT_{1A}R subpopulations in different brain areas for the post-synaptic receptor population, induced simultaneously within one experimental group.

Nevertheless, the 5-HT_{1A}R density measurements in both the hippocampus and PVN fail to provide a further explanation of the discrepancy found between the behavioral and core-body temperature results, as the receptor densities in both brain areas remain unaffected after chronic drug treatment. The lack of difference in hippocampal 5-HT_{1A}R density after chronic F15599 treatment is in accordance with previous findings (Hensler, 2003) and our behavioral data, indicating that hippocampal 5-HT_{1A}Rs are more resistant to desensitization after agonist administration. 5-HT_{1A}R density in the PVN revealed no differences between both drug treatments and vehicle treated animals, indicating no desensitization at the level of receptor expression in the PVN. Therefore, a possible explanation for the gradual rise in core-body temperature during F15599 treatment could be due to compensatory mechanisms being activated in order to maintain homeostasis. Furthermore, 5-HT_{1A}R signaling directly influences hypothalamic-pituitary signaling and has an effect on peripheral hormone levels (Zhang et al., 2004, Locatelli et al., 2010), influencing many other signaling cascades. Therefore, many factors that are independent of direct 5-HT_{1A}R activity could influence core-body temperature. The initial hypothermic response induced by F15599 administration could trigger signaling cascades that either compensate for the enhanced 5-HT_{1A}R activation or suppresses the 5-HT_{1A}R activation in the PVN but not the hippocampus, leading to the gradual restoration of optimal body temperature. Alternatively, the lack of reduced receptor density after F15599 treatment does not exclude the possibility of receptor desensitization occurring in the PVN through an alternative mechanism. Hypothalamic 5-HT_{1A}Rs have different properties than other heteroreceptors with regard to their coupled G-protein (Serres et al., 2000) and reductions in the coupled G_z protein have been found to occur at the same time of desensitization after SSRI treatment (Serres et al., 2000). Therefore, it is possible that chronic F15599 stimulation could cause a 'functional' desensitization in the PVN, caused by either a reduced capacity of the receptor to activate the G-coupled proteins or by changes at the level of the G protein itself. Unlike structural desensitization through receptor changes, this type of desensitization would not affect receptor density, while affecting receptor function and thus downstream effects such as core-body

temperature. Future studies focusing on selective hypothalamic 5-HT_{1A}R activation and changes in its G-coupled protein function could further elaborate on these region-specific differences.

Unexpectedly, the density of pre-synaptic autoreceptors in the DRN was also decreased after 14 days of treatment with F15599, when compared to vehicle. Binding studies did not indicate that direct binding of F15599 in the DRN could to lead to receptor desensitization (Newman-Tancredi et al., 2009), so the mechanism underlying this effect remains to be elucidated. The enhanced OPS performance after F15599 treatment was maintained over 2 weeks of treatment, so it is likely that some degree of desensitization took take place in DRN over the course of this treatment. This would prevent autoreceptor inhibition of 5-HT release to decrease the beneficial effects of direct post-synaptic 5-HT_{1A}R activation. If this hypothesis is true, there should be a measurable drop in OPS performance for at least several days in between day 1 and day 14, due to the activation of pre-synaptic autoreceptors which exert inhibition of cortical 5-HT levels. This would be consistent with the 'bell-shape' dose-response curve observed for OPS performance in our previous study (Van Goethem et al., 2014), this is likely due to the fact that, at high doses of F15599, 5-HT_{1A} autoreceptors are activated, as seen for F13714. As it takes at least 4 days for the pre-synaptic receptors to desensitize (based on the observations above for F13714) and restore the beneficial effect of post-synaptic activation by F15599, OPS performance would be affected during a time-window within the first week of treatment. A future study which evaluates OPS performance on different time-points during chronic treatment could confirm if F15599 induced DRN desensitization is mediated through such proposed mechanism.

Comparison of the pre-treatment temperature to the post-treatment temperature measurements, taken on day 7 and 14 of treatment, revealed no differences for either drug treatment conditions. This suggests that a constant level of receptor occupancy/stimulation was obtained over the 24h period in between treatment administration. Furthermore, the lack of difference in temperature before and after treatment also shows that no acute treatment effects influenced the daily temperature measurements. Notably, the vehicle treated animals showed a difference in temperature between pre- and post- administration measurements on both day 7 and 14. This rise in temperature is most likely caused by injection stress and anticipation stress for the daily post-treatment temperature measurements (Olivier et al., 2003). The contribution of anticipation stress is nicely reflected in the comparison of both pre-treatment temperatures measured on day 7 and 14 in the vehicle groups. At day 7 rats were not anticipating a pre-treatment measurement while they possibly did at day 14, resulting in a significant increase in temperature for the day 14 pre-treatment measurement compared to day 7 for the vehicle group. The remaining increase between pre- and post-treatment temperature, reflects the added stress of solely the

injection. This normal response was being suppressed in both drug treatment conditions by the strong regulatory effect of 5-HT_{1A}R activation on temperature.

4.3 Effects of chronic F15599 and F13714 treatment on hippocampal plasticity

According to a majority of the current literature, neurogenesis in the DG is necessary for proper pattern separation function (Franca et al., 2017). It has been previously demonstrated that both acute and chronic 5-HT_{1A}R activation can increase hippocampal neurogenesis by promoting precursor proliferation and survival of late differentiating cells (Huang and Herbert, 2005, Klempin et al., 2010, Soumier et al., 2010). In order to investigate if enhanced OPS performance by F15599 administration is regulated through enhanced plasticity in the DG, DCX positive cell numbers were compared between conditions. Expression of DCX occurs during active division by neuronal precursor cells and keeps on being expressed for 2-3 weeks by their daughter cells (von Bohlen Und Halbach, 2007). Therefore, DCX labels all immature neuronal cells which started differentiating over the entire course of the chronic treatment. Evaluation of group differences between DCX positive cell quantities in the SGZ of dorsal hippocampal sections revealed a strong increase of immature neurons in F15599 treated animals, when compared to vehicle. In contrast, F13714 treatment did not show a significant increase in DCX when compared to vehicle. This data is in accordance with the OPS task results for both groups, supporting the claim that the biased agonism activity of F15599 preferentially elicits neuronal plasticity.

Several protein markers related to neuronal plasticity and memory were semi-quantified in dorsal hippocampal tissue and compared between conditions. Although both F13714 and F15599 treatments showed a more than doubled ratio when compared to vehicle, the difference between groups in the phospho-CREB to CREB ratio did not reach statistical significance. The high SEM within the groups show that this effect is not homogenous among individual animals and a larger group size to increase the power might have led to more conclusive results.

PSD95, which is a marker for post-synaptic spine and synapse maturation and has previously been found to mediate 5-HT_{1A}R synaptogenesis in the hippocampus (Dong et al., 1997), did not differ between treatment groups in our study. Similarly, synaptophysin, which is a protein present in all presynaptic synapses, did not differ between treatment conditions. Together, these observations indicate that F15599 and F13714 did not affect synapse quantity and that synaptogenesis is not needed for pattern separation improvement. However, this does not exclude the possibility of structural changes in synapse architecture or pruning which could have influenced OPS performance. Finally, both the quantity of (mature) BDNF and the ratio between BDNF and its pro-form were significantly higher in dorsal hippocampus tissue of F15599 treated animals when

compared to vehicle treated animals. BDNF is a common growth factor that has been linked to neuronal plasticity through stimulation of neuronal proliferation, survival, migration, phenotypic differentiation, axonal and dendritic growth, and synapse formation (Martinowich and Lu, 2008). Increases in its expression have been found to be mediated by 5-HT signaling (Mattson et al., 2004). Therefore, the increase in BDNF for F15599 treated animals further confirms the role of post-synaptic 5-HT_{1A}R activation in hippocampal plasticity. In line with the DCX data, these results suggest that the positive chronic effects of F15599 treatment on OPS performance are supported by neuronal plasticity in the dorsal hippocampus.

4.4 Conclusion

Taken together, this study underlines the complexity of 5-HT_{1A}R functioning, which appears to be highly dependent on the localization of the receptors within different brain structures. More research into these differential effects as well as different signaling cascades is needed to further elucidate the role of 5-HT_{1A}Rs. We can conclude that selective post-synaptic 5-HT_{1A}R stimulation by F15599 enhances pattern separation performance in rats, and this was associated with enhanced hippocampal plasticity. In contrast to pre-synaptic DRN (and probably also post-synaptic PVN receptors), cortical 5-HT_{1A}Rs are resistant to desensitization induced by chronic stimulation. Therefore, selective targeting of post-synaptic 5-HT_{1A}Rs with F15599 could be considered as a potential therapy for the treatment of pattern separation impairments. Selective pre-synaptic 5-HT_{1A}R targeting leads to rapid desensitization of DRN receptors, most likely due to internalization of the receptor protein. After desensitization, autoreceptor mediated inhibition of cortical 5-HT release decreases producing a net stimulation of post-synaptic receptors, leading to restored behavioral performance and hippocampal plasticity to the level of vehicle treated animals. Overall, the present study provides compelling evidence of the differential regulation of 5-HT_{1A}Rs by two selective biased agonists targeting different receptor subpopulations. The data support the development of biased agonists that target post-synaptic 5-HT_{1A} receptors as potential treatments for disorders involving pattern separation deficits and/or for which stimulation of hippocampal neurogenesis or plasticity would be desirable.

5. References

AKKERMAN, S., BLOKLAND, A., RENEERKENS, O., VAN GOETHEM, N. P., BOLLEN, E., GIJSELAERS, H. J., LIEBEN, C. K., STEINBUSCH, H. W. & PRICKAERTS, J. 2012a. Object recognition testing: methodological considerations on exploration and discrimination measures. *Behavioural brain research,* 232, 335-347.

AKKERMAN, S., PRICKAERTS, J., STEINBUSCH, H. W. & BLOKLAND, A. 2012b. Object recognition testing: statistical considerations. *Behavioural brain research,* 232, 317-322.

ALLY, B. A., HUSSEY, E. P., KO, P. C. & MOLITOR, R. J. 2013. Pattern separation and pattern completion in Alzheimer's disease: evidence of rapid forgetting in amnestic mild cognitive impairment. *Hippocampus,* 23, 1246-58.

ARNOLD, S. A. & HAGG, T. 2012. Serotonin 1A receptor agonist increases species- and region-selective adult CNS proliferation, but not through CNTF. *Neuropharmacology,* 63, 1238-47.

ASSIE, M. B., BARDIN, L., AUCLAIR, A. L., CARILLA-DURAND, E., DEPOORTERE, R., KOEK, W., KLEVEN, M. S., COLPAERT, F., VACHER, B. & NEWMAN-TANCREDI, A. 2010. F15599, a highly selective post-synaptic 5-HT(1A) receptor agonist: in-vivo profile in behavioural models of antidepressant and serotonergic activity. *Int J Neuropsychopharmacol,* 13, 1285-98.

ASSIE, M. B., LOMENECH, H., RAVAILHE, V., FAUCILLON, V. & NEWMAN-TANCREDI, A. 2006. Rapid desensitization of somatodendritic 5-HT1A receptors by chronic administration of the high-efficacy 5-HT1A agonist, F13714: a microdialysis study in the rat. *Br J Pharmacol,* 149, 170-8.

BECKER G., BOLBOS R., COSTES N., REDOUTÉ J., NEWMAN-TANCREDI A., ZIMMER L. 2016. Selective serotonin 5-HT1A receptor biased agonists elicit distinct brain activation patterns: a pharmacoMRI study. *Sci Rep, 23;6:26633.*

BLIER, P. & WARD, N. M. 2003. Is there a role for 5-HT1A agonists in the treatment of depression? *Biological psychiatry,* 53, 193-203.

CLELLAND, C. D., CHOI, M., ROMBERG, C., CLEMENSON, G. D., FRAGNIERE, A., TYERS, P., JESSBERGER, S., SAKSIDA, L. M., BARKER, R. A., GAGE, F. H. & BUSSEY, T. J. 2009. A Functional Role for Adult Hippocampal Neurogenesis in Spatial Pattern Separation. *Science,* 325, 210.

CURTIS, M. J., BOND, R. A., SPINA, D., AHLUWALIA, A., ALEXANDER, S., GIEMBYCZ, M. A., GILCHRIST, A., HOYER, D., INSEL, P. A. & IZZO, A. A. 2015. Experimental design and analysis and their reporting: new guidance for publication in BJP. *British journal of pharmacology,* 172, 3461-3471.

DAS, T., IVLEVA, E. I., WAGNER, A. D., STARK, C. E. & TAMMINGA, C. A. 2014. Loss of pattern separation performance in schizophrenia suggests dentate gyrus dysfunction. *Schizophr Res,* 159, 193-7.

DEPOORTERE, R., AUCLAIR, A. L., BARDIN, L., COLPAERT, F. C., VACHER, B. & NEWMAN-TANCREDI, A. 2010. F15599, a preferential post-synaptic 5-HT1A receptor agonist: activity in models of cognition in comparison with reference 5-HT1A receptor agonists. *Eur Neuropsychopharmacol,* 20, 641-54.

DONG, J., DE MONTIGNY, C. & BLIER, P. 1997. Effect of acute and repeated versus sustained administration of the 5-HT1A receptor agonist ipsapirone: electrophysiological studies in the rat hippocampus and dorsal raphe. *Naunyn-Schmiedeberg's archives of pharmacology,* 356, 303-311.

EISCH, A. J. & PETRIK, D. 2012. Depression and hippocampal neurogenesis: a road to remission? *Science,* 338, 72-75.

ENNACEUR, A. & DELACOUR, J. 1988. A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data. *Behavioural Brain Research,* 31, 47-59.

FAGHIHI, F. & MOUSTAFA, A. A. 2015. A computational model of pattern separation efficiency in the dentate gyrus with implications in schizophrenia. *Front Syst Neurosci,* 9, 42.

FRANCA, T. F. A., BITENCOURT, A. M., MAXIMILLA, N. R., BARROS, D. M. & MONSERRAT, J. M. 2017. Hippocampal neurogenesis and pattern separation: A meta-analysis of behavioral data. *Hippocampus,* 27, 937-950.

FRANKLAND, P. W., KOHLER, S. & JOSSELYN, S. A. 2013. Hippocampal neurogenesis and forgetting. *Trends Neurosci,* 36, 497-503.

GILBERT, P. E. & KESNER, R. P. 2006. The role of the dorsal CA3 hippocampal subregion in spatial working memory and pattern separation. *Behavioural Brain Research,* 169, 142-149.

HENSLER, J. G. 2003. Regulation of 5-HT1A receptor function in brain following agonist or antidepressant administration. *Life Sciences,* 72, 1665-1682.

HOLDEN, H. M. & GILBERT, P. E. 2012. Less efficient pattern separation may contribute to age-related spatial memory deficits. *Front Aging Neurosci,* 4, 9.

HUANG, G. J. & HERBERT, J. 2005. The role of 5-HT1A receptors in the proliferation and survival of progenitor cells in the dentate gyrus of the adult hippocampus and their regulation by corticoids. *Neuroscience,* 135, 803-13.

HUNSAKER, M. R., ROSENBERG, J. S. & KESNER, R. P. 2008. The role of the dentate gyrus, CA3a,b, and CA3c for detecting spatial and environmental novelty. *Hippocampus,* 18, 1064-73.

KESNER, R. P. 2013. Role of the hippocampus in mediating interference as measured by pattern separation processes. *Behavioural Processes,* 93, 148-154.

KHEIRBEK, M. A., KLEMENHAGEN, K. C., SAHAY, A. & HEN, R. 2012. Neurogenesis and generalization: a new approach to stratify and treat anxiety disorders. *Nat Neurosci,* 15, 1613-20.

KILKENNY, C., BROWNE, W. J., CUTHILL, I. C., EMERSON, M. & ALTMAN, D. G. 2010. Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. *PLOS Biology,* 8, e1000412.

KLEMPIN, F., BABU, H., DE PIETRI TONEL, D., ALARCON, E., FABEL, K. & KEMPERMANN, G. 2010. Oppositional effects of serotonin receptors 5-HT1a, 2, and 2c in the regulation of adult hippocampal neurogenesis. *Frontiers in molecular neuroscience,* 3, 14.

KOEK, W., VACHER, B., COSI, C., ASSIÉ, M.-B., PATOISEAU, J.-F., PAUWELS, P. J. & COLPAERT, F. C. 2001. 5-HT1A receptor activation and antidepressant-like effects: F 13714 has high efficacy and marked antidepressant potential. *European Journal of Pharmacology,* 420, 103-112.

LEE, H., WANG, C., DESHMUKH, S. S. & KNIERIM, J. J. 2015. Neural Population Evidence of Functional Heterogeneity along the CA3 Transverse Axis: Pattern Completion versus Pattern Separation. *Neuron,* 87, 1093-105.

LEUTGEB, J. K., LEUTGEB, S., MOSER, M. B. & MOSER, E. I. 2007. Pattern separation in the dentate gyrus and CA3 of the hippocampus. *Science,* 315, 961-6.

LLADO-PELFORT, L., ASSIE, M. B., NEWMAN-TANCREDI, A., ARTIGAS, F. & CELADA, P. 2010. Preferential in vivo action of F15599, a novel 5-HT(1A) receptor agonist, at postsynaptic 5-HT(1A) receptors. *Br J Pharmacol,* 160, 1929-40.

LOCATELLI, V., BRESCIANI, E., TAMIAZZO, L. & TORSELLO, A. 2010. Central nervous system-acting drugs influencing hypothalamic-pituitary-adrenal axis function. *Pediatric Neuroendocrinology.* Karger Publishers.

MADDEN, A. M. & ZUP, S. L. 2014. Effects of developmental hyperserotonemia on juvenile play behavior, oxytocin and serotonin receptor expression in the hypothalamus are age and sex dependent. *Physiol Behav,* 128, 260-9.

MANNOURY LA COUR, C., EL MESTIKAWY S., HANOUN N., HAMON M., LANFUMEY L. 2006. Regional differences in the coupling of 5-hydroxytryptamine-1A receptors to G proteins in the rat brain. *Mol Pharmacol, 70,1013-21.*

MARTINELLI, C. & SHERGILL, S. S. 2015. Clarifying the role of pattern separation in schizophrenia: the role of recognition and visual discrimination deficits. *Schizophr Res,* 166, 328-33.

MARTINOWICH, K. & LU, B. 2008. Interaction between BDNF and serotonin: role in mood disorders. *Neuropsychopharmacology,* 33, 73-83.

MATTSON, M. P., MAUDSLEY, S. & MARTIN, B. 2004. BDNF and 5-HT: a dynamic duo in age-related neuronal plasticity and neurodegenerative disorders. *Trends Neurosci,* 27, 589-94.

MILLAN, M. J., RIVET, J.-M., CANTON, H., LE MAROUILLE-GIRARDON, S. & GOBERT, A. 1993. Induction of hypothermia as a model of 5-hydroxytryptamine1A receptor-mediated activity in the rat: a pharmacological characterization of the actions of novel agonists and antagonists. *Journal of Pharmacology and Experimental Therapeutics,* 264, 1364-1376.

MILLER, B. R. & HEN, R. 2015. The current state of the neurogenic theory of depression and anxiety. *Current Opinion in Neurobiology,* 30, 51-58.

MORRIS, A. M., CHURCHWELL, J. C., KESNER, R. P. & GILBERT, P. E. 2012. Selective lesions of the dentate gyrus produce disruptions in place learning for adjacent spatial locations. *Neurobiology of Learning and Memory,* 97, 326-331.

NEWMAN-TANCREDI, A. 2011. Biased agonism at serotonin 5-HT1A receptors: preferential postsynaptic activity for improved therapy of CNS disorders. *Neuropsychiatry,* 1, 149-164.

NEWMAN-TANCREDI, A., MARTEL, J. C., ASSIE, M. B., BURITOVA, J., LAURESSERGUES, E., COSI, C., HEUSLER, P., BRUINS SLOT, L., COLPAERT, F. C., VACHER, B. & CUSSAC, D. 2009. Signal transduction and functional selectivity of F15599, a preferential post-synaptic 5-HT1A receptor agonist. *Br J Pharmacol,* 156, 338-53.

NEWMAN-TANCREDI, A., MARTEL, J. C., COSI, C., HEUSLER, P., LESTIENNE, F., VARNEY, M.A., CUSSAC, D. 2017. Distinctive in vitro signal transduction profile of NLX-112, a potent and efficacious serotonin 5-HT1A receptor agonist. *J Pharm Pharmacol,69,1178-1190.*

OLIVIER, B., ZETHOF, T., PATTIJ, T., VAN BOOGAERT, M., VAN OORSCHOT, R., LEAHY, C., OOSTING, R., BOUWKNECHT, A., VEENING, J. & VAN DER GUGTEN, J. 2003. Stress-induced hyperthermia and anxiety: pharmacological validation. *European journal of pharmacology,* 463, 117-132.

PAXINOS, G. & WATSON, C. 2007. *The Rat Brain in Stereotaxic Coordinates in Stereotaxic Coordinates*, Elsevier.

RAUSCH, J. L., JOHNSON, M. E., KASIK, K. E. & STAHL, S. M. 2006. Temperature regulation in depression: functional 5HT1A receptor adaptation differentiates antidepressant response. *Neuropsychopharmacology,* 31, 2274-80.

RIAD, M., WATKINS, K. C., DOUCET, E., HAMON, M. & DESCARRIES, L. 2001. Agonist-induced internalization of serotonin-1a receptors in the dorsal raphe nucleus (autoreceptors) but not hippocampus (heteroreceptors). *Journal of Neuroscience,* 21, 8378-8386.

ROJAS, P. S. & FIEDLER, J. L. 2016. What Do We Really Know About 5-HT1A Receptor Signaling in Neuronal Cells? *Front Cell Neurosci,* 10, 272.

SAHAY, A., SCOBIE, K. N., HILL, A. S., O'CARROLL, C. M., KHEIRBEK, M. A., BURGHARDT, N. S., FENTON, A. A., DRANOVSKY, A. & HEN, R. 2011. Increasing adult hippocampal neurogenesis is sufficient to improve pattern separation. *Nature,* 472, 466-70.

SCHREIBER, R. & NEWMAN-TANCREDI, A. 2014. Improving cognition in schizophrenia with antipsychotics that elicit neurogenesis through 5-HT(1A) receptor activation. *Neurobiol Learn Mem,* 110, 72-80.

SERRES, F., LI, Q., GARCIA, F., RAAP, D. K., BATTAGLIA, G., MUMA, N. A. & VAN DE KAR, L. D. 2000. Evidence that Gz-proteins couple to hypothalamic 5-HT1A receptors in vivo. *Journal of Neuroscience,* 20, 3095-3103.

SEYEDABADI, M., FAKHFOURI, G., RAMEZANI, V., MEHR, S. E. & RAHIMIAN, R. 2014. The role of serotonin in memory: interactions with neurotransmitters and downstream signaling. *Exp Brain Res,* 232, 723-38.

SOUMIER, A., BANASR, M., KERKERIAN-LE GOFF, L. & DASZUTA, A. 2010. Region- and phase-dependent effects of 5-HT(1A) and 5-HT(2C) receptor activation on adult neurogenesis. *Eur Neuropsychopharmacol,* 20, 336-45.

STARK, S. M., YASSA, M. A., LACY, J. W. & STARK, C. E. 2013. A task to assess behavioral pattern separation (BPS) in humans: Data from healthy aging and mild cognitive impairment. *Neuropsychologia,* 51, 2442-9.

VACHER, B., BONNAUD, B., FUNES, P., JUBAULT, N., KOEK, W., ASSIÉ, M.-B. & COSI, C. 1998. Design and Synthesis of a Series of 6-Substituted-2-pyridinylmethylamine Derivatives as Novel, High-Affinity, Selective Agonists at 5-HT1A Receptors. *Journal of Medicinal Chemistry,* 41, 5070-5083.

VALDIZAN, E. M., CASTRO, E. & PAZOS, A. 2010. Agonist-dependent modulation of G-protein coupling and transduction of 5-HT1A receptors in rat dorsal raphe nucleus. *Int J Neuropsychopharmacol,* 13, 835-43.

VAN GOETHEM, N. P., SCHREIBER, R., NEWMAN-TANCREDI, A., VARNEY, M. & PRICKAERTS, J. 2015. Divergent effects of the 'biased' 5-HT1 A receptor agonists F15599 and F13714 in a novel object pattern separation task. *Br J Pharmacol,* 172, 2532-43.

VAN HAGEN, B. T., VAN GOETHEM, N. P., LAGATTA, D. C. & PRICKAERTS, J. 2015. The object pattern separation (OPS) task: a behavioral paradigm derived from the object recognition task. *Behav Brain Res,* 285, 44-52.

VON BOHLEN UND HALBACH, O. 2007. Immunohistological markers for staging neurogenesis in adult hippocampus. *Cell Tissue Res,* 329, 409-20.

YAGI, S., CHOW, C., LIEBLICH, S. E. & GALEA, L. A. 2016. Sex and strategy use matters for pattern separation, adult neurogenesis, and immediate early gene expression in the hippocampus. *Hippocampus,* 26, 87-101.

YASSA, M. A. & STARK, C. E. 2011. Pattern separation in the hippocampus. *Trends Neurosci,* 34, 515-25.

YOKOYAMA C., MAWATARI A., KAWASAKI A., TAKEDA C., ONOE K., DOI H., NEWMAN-TANCREDI A., ZIMMER L., ONOE H. 2016. Marmoset Serotonin 5-HT1A Receptor Mapping with a Biased Agonist PET Probe 18F-F13714: Comparison with an Antagonist Tracer 18F-MPPF in Awake and Anesthetized States. *Int J Neuropsychopharmacol, 19(12).*

ZHANG, Y., D'SOUZA, D., RAAP, D. K., GARCIA, F., BATTAGLIA, G., MUMA, N. A. & VAN DE KAR, L. D. 2001. Characterization of the Functional Heterologous Desensitization of Hypothalamic 5-HT1A Receptors after 5-HT2AReceptor Activation. *Journal of Neuroscience,* 21, 7919-7927.

ZHANG, Y., GRAY, T. S., D'SOUZA, D. N., CARRASCO, G. A., DAMJANOSKA, K. J., DUDAS, B., GARCIA, F., ZAINELLI, G. M., SULLIVAN HANLEY, N. R., BATTAGLIA, G., MUMA, N. A. & VAN DE KAR, L. D. 2004. Desensitization of 5-HT1A receptors by 5-HT2A receptors in neuroendocrine neurons in vivo. *J Pharmacol Exp Ther,* 310, 59-66.

ZUIDEVELD, K. P., VAN DER GRAAF, P. H., NEWGREEN, D., THURLOW, R., PETTY, N., JORDAN, P., PELETIER, L. A. & DANHOF, M. 2004. Mechanism-based pharmacokinetic-pharmacodynamic modeling of 5-HT1A receptor agonists: estimation of in vivo affinity and intrinsic efficacy on body temperature in rats. *J Pharmacol Exp Ther,* 308, 1012-20.

Chapter 4

Acute effects of selective 5-HT_{1A} heteroreceptor stimulation on schizophrenia-related cognitive impairments in a chronic ketamine rat model

Britt T.J. van Hagen, Rudy Schreiber, Adrian Newman-Tancredi, Mark Varney and Jos Prickaerts

Abstract

Cognitive deficits are a key feature of schizophrenia that can severely impact daily functioning. Stimulation of 5-HT_{1A} receptors to improve cognition has led to differential effects, dependent on their location. Conventional non-biased 5-HT_{1A} receptor agonists can elicit positive effects which are believed to be regulated through post-synaptic 5-HT_{1A} heteroreceptors, but these can be counteracted by simultaneous activation of the 5-HT_{1A} autoreceptors. Therefore, selective targeting of post-synaptic 5-HT_{1A} heteroreceptors with biased agonists such as F15599 could be more effective to alleviate cognitive deficits in schizophrenia.

Here, the NMDA glutamate receptor antagonist ketamine (30mg/kg, IP) was used to induce schizophrenia-like cognitive deficits in rats. Three weeks after sub-chronic (5-days) ketamine or vehicle treatment, cognitive performance was measured in the Object Pattern Separation (OPS) task, which relies on hippocampal processes. An attentional set-shifting task was used to assess medial prefrontal cortex-regulated behavioral flexibility. Acute treatment with F15599 (0.04mg/kg IP) was compared to vehicle treatment to evaluate the effects of selective 5-HT_{1A} heteroreceptor activation on cognitive performance in both control rats and the schizophrenia-like rats.

In both tasks the ketamine-treated rats showed an impaired performance compared to control animals. Acute treatment with F15599 rescued the pattern separation deficit up to the performance level of control animals. Administration of F15599 prior to learning a new response-strategy in the set-shifting task improved performance in the schizophrenia-like rats. These data show that selective activation of 5-HT_{1A} heteroreceptors can be a successful treatment to relieve schizophrenia-related deficits that originate from both hippocampal or prefrontal cortex centered processing.

Chapter 5

Behavioral and neurochemical effects of the soluble guanylate cyclase stimulator riociguat on memory processes in mice

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Abstract

Upregulation of cyclic guanosine monophosphate (cGMP) through the inhibition of specific phosphodiesterases (PDEs) has previously been shown to improve memory performance. The current study aimed to target cGMP upregulation differently, using the soluble guanylate cyclase (sGC) stimulator riociguat, and investigate the acute effects on memory in both healthy mice and a biperiden induced memory deficit mouse model. Biochemical measurements were performed on hippocampal tissue to further elucidate the role of the nitric oxide (NO) – sGC – cGMP signaling pathway in memory function. Acute administration with a low dose of riociguat was able to enhance working-, short- and long-term spatial memory as measured with the object location task or Y-maze continuous alternation task. Pharmacokinetic measurements within brain tissue of acutely treated mice showed very poor or no brain penetration of riociguat. Western blots revealed an increase in activation of vasodilator stimulated phosphoprotein (VASP) at the behaviorally active dose of riociguat. No other effects were found on memory-related hippocampal plasticity measures including activation of CREB, AMPA receptor trafficking and PSD95. These findings support the assumption that the memory enhancing effects are due to a non-central effect. In this respect, further research is needed to investigate the possible contribution of hemodynamic or metabolic effects which are known to be regulated by sGC-cGMP signaling.

1. Introduction

Targeting intracellular signaling cascades can be a potential strategy to enhance multiple aspects of memory processes, without being limited to a single neurotransmitter system. Evidence is accumulating that second messenger molecules, cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP), are important in memory processes in general, and for long-term potentiation (LTP) in particular (Boess et al., 2004; Heckman et al., 2015). An approach to increase intracellular cGMP is through activation or stimulation of the heme-protein soluble guanylate cyclase (sGC). Normally, sGC is activated by nitric oxide (NO), which stimulates guanosine triphosphate (GTP) to be converted into cGMP. Subsequently, cGMP activates cGMP-dependent protein kinase (PKG) which triggers further downstream signaling (Mittendorf et al., 2009; Pfeifer et al., 2013). This specific pathway is believed to be involved in acquisition and early consolidation of memory through neurotransmitter release, protein synthesis and synaptic plasticity (Bollen et al., 2014; Devan et al., 2005; Kleppisch & Feil, 2009; Ota et al., 2008; Reneerkens et al., 2009; Rutten et al., 2007). In vitro studies have shown that NO-cGMP signaling is inhibited by amyloid-β (Aβ), one of the hallmark pathologies of Alzheimer's disease (AD), causing impairments in LTP, which could be reversed by stimulating cGMP activity (Puzzo et al., 2005). This led to the hypothesis that increased in NO-cGMP signaling could be beneficial in ameliorating AD-related memory impairments. Previous pre-clinical studies that have been successful in showing memory enhancement by stimulating this pathway targeted specific phosphodiesterases (PDEs), which are responsible for the degradation of cGMP. Through administration of PDE inhibitors cellular levels of cGMP increase, which leads to increased activation of PKG and cAMP response element binding (CREB), resulting in more protein synthesis. Stimulation of sGC-cGMP signaling leads to enhanced brain plasticity and memory function in both healthy animals (Bollen et al., 2014; Devan et al., 2005; Rutten et al., 2007; Rutten et al., 2005) and animal models for memory impairment and AD (Devan et al., 2005; Jin et al., 2014). Inhibition of cGMP specific PDE5 has even been shown to reduce Aβ -plaque load in a mouse model for AD (Puzzo et al., 2009), illustrating the pathways' potential as a target to improve the functioning of AD patients. Despite these findings, to this date none of the existing PDE inhibitors specific to cGMP have been successful in ameliorating cognitive impairments in humans. Therefore, it might be beneficial to investigate a different approach to increasing sGC-cGMP-PKG signaling for its memory enhancing potential.

In contrast to ameliorating memory impairments through inhibition of cGMP degradation, the current study aimed to investigate if directly stimulating cGMP synthesis might also be an effective strategy to enhance memory. A relatively new class of drugs targeting the NO-cGMP pathway are sGC stimulators and activators, which both increase intracellular cGMP after

administration. Activators and stimulators for sGC are clinically investigated for pulmonary hypertension (Evgenov et al., 2006; Ghofrani & Grimminger, 2009). The sGC stimulator riociguat (BAY 63-2521) has been clinically approved for the treatment of pulmonary arterial hypertension (Conole & Scott, 2013; Ghofrani et al., 2013), making this a well-suited candidate to investigate its effects on cognition in a translational manner. Interestingly, it has been shown that an increased activity of sGC, after administration of the sGC activator YC-1, significantly improved cognitive function in aged rats (Celikyurt et al., 2014). The natural activator of sGC is the molecule NO. YC-1, which is a relatively aspecific drug, directly mimics the action of NO. In contrast, sGC stimulators increase cGMP syntheses by binding to sGC and increasing its sensitivity to endogenous NO (Mittendorf et al., 2009). Directly upregulating the synthesis of cGMP when needed, i.e. at non-basal levels, using endogenous available NO, might be a subtle strategy for cognition enhancement with fewer unwanted effects on other adverse processes that might be regulated by this pathway. Based on studies with PDE inhibitors, it is already evident that enzymes involved in cGMP production or degradation only require subtle stimulation in order to have an optimal biological effect in the brain (Heckman et al., 2015).

The present study was designed to evaluate the cognition enhancing potential of the sGC stimulator riociguat in both healthy mice and in mice subjected to a pharmacological model for memory impairment (see below), in the object location task (OLT) and the y-maze alternation task. The OLT is a one-trial learning task for episodic-like spatial short-term and long-term memory in rodents (Murai et al, 2007). The specific timing of the treatment in relation to the learning or test trial of the behavioral task allows one to investigate acquisition, consolidation or retention memory processes (Prickaerts et al., 2005; van Goethem et al., 2012). The NO-cGMP pathway is believed to be mainly involved in memory acquisition and early consolidation (Bollen et al., 2014; Prickaerts et al., 2002; Rutten et al., 2007). The y-maze continuous alternation task is used to investigate spatial working memory, by assessing the amount of alternations made between the different arms of the y-maze, during a single exploration trial (Yamada et al., 1996).

In addition to investigating riociguat alone as a potential cognition enhancer, we want to investigate the potential of combining two drugs which target differential neurochemical cascades, in sub-optimal dosages. A combination of sub-optimal doses of the acetylcholinesterase inhibitor (AChEI) donepezil and riociguat will be evaluated on its potential to enhance memory performance in the OLT in mice. Donepezil is currently the most widely used drug on the market to reduce cognitive decline in AD patients (Melnikova, 2007).

To evaluate the effect of riociguat in a mice model for memory impairment, the muscarinic M1 receptor antagonist biperiden was used as a pharmacological model for memory deficits

(Klinkenberg & Blokland, 2011). This model was chosen due to its more selective effects on central cholinergic neurotransmission when compared to other cholinergic deficit models (e.g., scopolamine). Acute biperiden administration has been shown to induce cognitive impairments in rodents with fewer side-effects in other domains, like locomotion and attention (Blokland et al., 2016). Secondly, this pharmacological model has been shown to cause similar memory deficits in healthy humans when given acutely (Sambeth et al., 2015), enabling us to directly translate the pre-clinical studies to a parallel human study with riociguat (Borghans et al., this issue).

Apart from the acute effects of riociguat on cognition, we were also interested in its neurochemical effects. When considering the possible mechanism of action, it can be hypothesized that upregulation of cGMP through stimulation of sGC, will have similar biochemical changes as shown after administration of cGMP-specific PDE inhibitors. Following this rationale, it can be expected that riociguat is able to enhance memory performance through an upregulation of cGMP-PKG signaling and its regulation of pre-synaptic neurotransmitter release and post-synaptic plasticity changes (Francis et al., 2010), e.g., AMPA receptor trafficking (Kleppisch & Feil, 2009). The latter might be related to direct phosphorylation of its GluA1 unit and AMPA receptor membrane insertion or to phosphorylation of transcription factors including CREB, resulting in more AMPA receptor production (Bollen et al., 2014; Serulle et al., 2007). Another interesting transcription factor associated with sGC-cGMP activation is vasodilator-stimulated phosphoprotein (VASP), which is known as an important regulator of vascular processes (Giralt et al., 2013). The phosphorylation of VASP is mediated by NO-cGMP-PKG activity (Francis et al., 2010), and is therefore used as a biomarker for the effectiveness of NO-cGMP signaling in pulmonary and cardiovascular research (Kirsch et al., 2008; Schäfer et al., 2010). Phosphorylation of VASP has not yet been widely studied in relation to cognition, but has been shown to promote actin assembly and modulate dendritic spines and synapse formation (Lin, 2011), and found to induce LTP (Wang et al., 2005). Linked to this, VASP increases post-synaptic density protein (PSD)-95 and surface AMPA receptors, which are both important for synaptic plasticity. PSD-95 is known to bind to the AMPA receptor (Kennedy et al., 2008) and found to directly interact with sGC (Russwurm et al., 2001).

In summary, the mechanism of action leading to the pro-cognitive effects of sGC-cGMP stimulation is hypothesized to be mediated by CREB and VASP activation, thus increasing AMPA receptor trafficking and dendritic formation, which altogether results in improved glutamate signaling responsible for enhanced memory performance. To evaluate the effects of direct sGC stimulation on memory processes, experiments were performed in which the activity of the described biochemical markers were assessed after riociguat treatment and memory acquisition/learning. Taken together, the behavioral and biochemical measurements will provide

more insight into the effects of acute sGC stimulation on memory performance and further elucidate the role of the sGC-cGMP-PKG signaling pathway in memory processes.

2. Materials & methods

2.1 Animals

All procedures involving animals were carried out under the Dutch Experiments on Animals Act (EAA, amended 1996), in accordance with the U.K. Animals (Scientific Procedures) Act, 1986, and the European Directive (2010/63/EU) for animal experiments. The experimental protocol was approved after evaluation by the animal ethical committee of Maastricht University (licensed animal ethical committee: Min.VWS, GZBIVVB981845). The official protocol number for these studies was DEC 2013-059. All studies involving animals are reported in accordance with ARRIVE guidelines (Kilkenny et al., 2010).

In total 40 male C57BL/6 mice were supplied by Charles River (Sulzfeld, Germany) and tested between 4-5 months of age. The average body weight at the beginning of the study was 28.0 g. An additional cohort of 40 mice of the same age was used for the combination study, with an average body weight of 28.8 g at the start of the study. A total of 36, 4-months-old male mice (average weight of 23.2 g) were used for the biochemical studies. All animals were housed individually in standard green line Tecniplast IVC cages on sawdust bedding. The animals were housed on a reversed 12/12-h light/dark cycle (lights on from 19:00 h to 07:00 h) and received food and water ad libitum. The mice were housed and tested in the same room. A radio, playing softly, provided background noise in the room. Testing was performed between 09:00 h and 18:00 h, with a small desk lamp providing light for the experimenter.

2.2 Treatments

Both riociguat and biperiden were dissolved in a 0.5% tylose solution (98% of the total end volume) with 2% tween80, at an injection volume of 5 ml/kg. Doses of 0 (vehicle), 0.01, 0.03, 0.1 and 0.3 mg/kg of riociguat were administrated orally (P.O.) 30 min in advance, for both the OLT and the y-maze alternation task. Biperiden was given I.P. 30 min before the task at a dose of 0 (vehicle) or 3 mg/kg, as the latter is the optimal dose for cognitive impairment in the OLT as found in a pilot study previously performed in our lab (data not shown). Riociguat was used as the free base (MW: 422.42 g/mol) and biperiden was used as an HCl salt (MW: 311.46 g/mol). Both compounds were supplied by Beijing Mesochem Technologies CO (Beijing, China). Donepezil was given P.O. 30 min before the learning trial in the OLT at doses of 0 (vehicle), 0.03, 0.1 and 0.3 mg/kg to find the sub-optimal dose. Donepezil was dissolved in saline and was administered at an injection volume of 5 ml/kg. Donepezil

was a generous gift from Solvay Pharmaceuticals/ Abbvie Pharmaceuticals, provided as an HCl salt (MW: 379.492 g/mol). The combination treatment groups all received two P.O. injections 30 min before testing, with vehicle (0.5% tylose solution or saline), riociguat or donepezil. All solutions were prepared freshly at the day of the experiment.

2.3.1 The Object Location Task

The OLT has been derived from, and is very similar to, the ORT (Ennaceur & Delacour, 1988). The OLT has been performed as previously described elsewhere (Sierksma et al., 2013; Vanmierlo et al., 2011). The apparatus consisted of a circular arena, 40 cm in diameter. The back half of the 40 cm high wall was made of white polyvinyl chloride (PVC) and the front was made of transparent PVC. Fluorescent red tubes and a light bulb provided a constant illumination of about 20 lux on the floor of the apparatus. We used 4 different sets of two identical objects, which were divided in a semi-random manner between animals and over all treatment conditions. The objects consisted of (1) a cylinder with a pointed top, made of brass (diameter 2 cm and total height 9 cm), (2) a massive metal rectangular prism (2.5 cm × 5 cm × 7.5 cm) containing two holes (diameter 1.5 cm) (3) a white plastic cube with the corners rounded-off and a flat cylinder on top (5 cm x 5 cm x 6.5 cm) (4) a massive aluminum cube with a tapering top (4.5 cm × 4.5 cm × 8.5 cm). The mice were unable to displace the objects. Prior to compound testing, the animals were handled by the experimenter for 5 min on 3 days and allowed to explore the OLT arena, also for 5 min. Secondly, the animals were habituated to each object set for 5 min on 4 separate days. Subsequently, animals were accustomed to the complete OLT testing procedure including injections prior to testing. During habituation and repeated testing, object sets were presented to the animals in a balanced manner to avoid learning or place biases.

A test session comprised two trials, each with a duration of 4 min. Prior to the experimental trials the mice were put in an empty cage for 4 min to increase arousal during testing. In order to avoid the presence of olfactory cues the objects were thoroughly cleaned with a 70% ethanol solution after each trial. The time spent exploring each object during T1 and T2 was recorded manually on a personal computer. Exploration was defined in the following manner: directing the nose to the object at a distance of no more than 2 cm and/or touching the object with the nose. Sitting on the object was not considered to be exploratory behavior. Of note, treatment conditions were divided in a semi-random balanced manner over animals and testing sessions. In case of repeated testing, individual animals never received the same condition twice. The experimenter was always blind to the conditions that were being tested.

2.3.2 OLT statistical analysis

The readout parameters of the OLT are the times that mice spent exploring each object during T1 and T2. The same measurements and analysis are performed as for the ORT (Akkerman et al., 2012; Akkerman et al., 2012) The exploration time (in seconds) of each object during T1 are presented as 'a1' and 'a2'. The time spent in exploring the familiar and the moved object in T2 are represented as 'a3' and 'b', respectively. Using these read-outs, the following variables were calculated: e1 (=*a1+a2*), e2 (=*a3+b*) and d2 (=*b-a3/e2*). The d2 index is a relative measure of discrimination performance because it is corrected for exploratory activity and shows no correlation with e1 or e2 (Akkerman, Blokland et al., 2012). The d2 index can range from -1 to 1. A significant difference from zero, i.e. chance level, indicates that the mice remembered the object locations from T1, and a difference from the vehicle condition signifies an actual memory improvement. Of note, mice require a minimum amount of exploration in order to show reliable memory performance (Akkerman et al., 2012). Therefore, animals were removed from the analysis if they spent less than 6 s exploring the objects during T1 or T2.

One sample t-tests were used to compare the d2 index of the conditions to zero (i.e. chance level). To evaluate group differences, a one-way ANOVA was performed between the d2 values of the conditions. In case of a significant ANOVA effect, post-hoc Dunnett's t-tests were performed to compare the experimental conditions to the vehicle (24 h OLT) or biperiden + vehicle condition (1 h OLT).

2.4.1 The y-maze continuous alternation task

The y-maze continuous alternation test is a single trial task that measures spatial working memory and was performed as described previously (Anisman & Kokkinidis, 1975; Sierksma et al., 2014). The apparatus was made of grey PVC with three arms symmetrically placed together at a 120^{o} angle. Each arm was 40 cm long, 17 cm high, 4 cm wide at the bottom and 13 cm wide at the top. At the beginning of the trial each mouse was placed in one of three arms (randomly divided and balanced), and was then allowed to freely explore the apparatus for 6 min. The number and order of arm entries were taken as measurements. An arm entry was only counted if all four paws of the animal were placed completely inside the arm. When a mouse visited all 3 different arms consecutively it made a triad. In between trials the apparatus was cleaned with a 70% ethanol solution to avoid olfactory cues.

2.4.2 Y-maze continuous alternation task analysis

To measure spatial (working) memory, the percentage of alternations was calculated by dividing the number of triads made by the maximum possible alternations (i.e. total entries minus 2) multiplied by 100. A score of 50% alternations is considered chance level. Therefore, a significantly higher percentage than 50% is indicative of functional working memory. A minimal of 10 arm entries per mouse was considered sufficient to provide a reliable score. Therefore, mice with less than 10 arm entries were excluded from the analysis. One-sample t-tests were used to compare each condition to a score of 50%. A one-way ANOVA was performed to evaluate differences between the conditions. When the overall ANOVA was significant, post-hoc Dunnett's t-test were performed to compare the conditions to the biperiden + vehicle treatment.

2.5 Determination of drug plasma and brain concentrations

After behavioral analysis in the OLT and y-maze 16 mice were used for pharmacokinetic measurements of both riociguat and its main circulating active metabolite M-1 (BAY 60-4552), of which the pharmacological activity is 1/3 to 1/10 of that of riociguat (Conole & Scott, 2013). The mice were injected P.O. with 0.03 mg/kg (behaviorally active dose) or 0.3 mg/kg riociguat and sacrificed at either 30 min or 1.5h after administration, reflecting the time points of the behavioral OLT studies' initial learning (T1) or retrieval (T2), respectively. This results in 4 different treatment conditions of n=4 each; (0.03 mg/kg riociguat sacrificed at 30 min - 0.03 mg/kg riociguat sacrificed at 1.5 h - 0.3 mg/kg riociguat sacrificed at 30 min - 0.3 mg/kg riociguat sacrificed at 1.5 h). Prior to sacrifice blood samples were collected from the saphenous vein in heparin-coated blood collection tubes, which were temporarily stored on ice. Plasma was isolated using centrifugation (1500g for 10 min at 4˚C) and subsequently pipetted into micro tubes. Directly after sacrificing the animals through cervical dislocation, the brains were taken out and snap frozen in liquid nitrogen. Both plasma and brain samples were stored at -80˚C until further analysis.

All samples were shipped to Agilux laboratories, inc. (Worchester, MA, USA), who performed pharmacokinetic measurements on riociguat and M-1 levels in the brain and plasma samples. Individual standard curves were prepared in respective control matrices, appropriate dynamic ranges were achieved for both analytes and instrument settings were adjusted to optimize the MS signal. Quantification was performed using liquid– liquid extraction followed by a characterized liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay. Data were processed by Agilux Laboratories using the Masslynx software with the Quanlynx application manager (Waters Ltd.).

Data of riociguat and M-1 concentrations in each sample were provided to us in ng/ml. The lower limit of quantification (LLOQ) of riociguat was 1.25 ng/mL for plasma and 0.25 ng/mL for brain, for M-1 the LLOQ was 2.0 ng/mL for plasma and 0.25 ng/mL for brain. If the plasma or brain sample of riociguat was below the quantification limit (BQL), but one or more of the other samples in the same group had measurable values, the BQL was treated as zero.

2.6.1 Surface protein biotinylation and sample preparation

For the biochemical study a total of 36 mice were used, who were habituated to the OLT according to the procedure described above. To evaluate brain plasticity markers, mice first received acute treatment with the behaviorally active dose of riociguat or vehicle. 30 min after injection the mice underwent the first trial of the OLT (T1) as a learning paradigm and subsequently were sacrificed either 1.5 h or 24 h after T1. Animals were sacrificed by means of cervical dislocation, the brains were excised and both hippocampi were isolated. Coronal hippocampal slices of 350 µm thickness were obtained using a McIlwain tissue chopper. The slices were transferred in ice cold ACSF (124 mM NaCl, 4.4 mM KCl, 1 mM Na2HPO4, 25 mM NaHCO3, 2 mM CaCl, 2 mM MgSO4 and 10 mM glucose) and incubated with 1mM sulfo-NHS-SS-biotin (#21328, Thermo Scientific, Bleiswijk, The Netherlands) for 60 min on ice. Following biotin incubation, slices were washed with cold 100 mM glycine to remove the excess of biotin and flash-frozen in liquid nitrogen. Frozen hippocampal slices were mechanically dissociated in lysis buffer (1 mM EDTA, 1 mM EGTA, 1% glycerol, 0.1% triton and 1% IGEPAL CA-630 in PBS), containing protease and phosphatase inhibitors. Protein concentration was determined with Lowry protein assay (Bio-Rad Laboratories, Veenendaal, The Netherlands). For the membrane fractions, protein lysates (60 µg) were incubated overnight with streptavidin-coated Dynabeads (#65601, Thermo Scientific, Bleiswijk, The Netherlands) at 4 °C under constant rotation. Dynabeads containing surface biotinylated proteins were separated by cytosolic proteins by magnetic precipitation. Biotinylated proteins were eluted from streptavidin beads with 1xSDS loading buffer (1 M Tris HCL, 75% glycerol, 6% SDS, 15% -β-mercaptoethanol and 0.025% brome phenol blue in milliQ) at 95 °C for 5 min.

2.6.2 Western Blotting

Surface protein fractions (60 µg) and their corresponding total protein samples (8 µg) were resolved in 10% SDS-PAGE and then transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Veenendaal, The Netherlands). The membranes were blocked (50% Odyssey blocking buffer in PBS, Li-Cor, Lincoln, NE, USA) for 1 h at room temperature, followed by overnight incubation with the primary antibodies at 4 °C. The primary antibodies consisted of mouse anti-glutamate receptor 1 N-terminus (1:1000, MAB2263, Merck Millipore, Burlington, MA, USA), mouse

anti-PSD95 (1:2000, #56452, QED Bioscience inc., San Diego, CA, US), rabbit anti-pCREB (1:500, #9198S, Cell signaling technology, Danvers, MA, US), mouse anti-CREB (1:500, #9104S, Cell signaling technology, Danvers, MA, US), rabbit anti-pVASP Ser 329 (1:500, sc-23507, Santa Cruz Biotechnology, Dallas, TX, US), mouse anti-VASP (1:200, sc-46668, Santa Cruz Biotechnology, Dallas, TX, US) and mouse anti-GAPDH (1:1.000.000, 10R-G109A, Fitzgerald Industries, Acton, MA, US), as loading control. Membranes were subsequently incubated by secondary antibodies for 1 h at room temperature: goat anti-rabbit IRDye 800 (1:10.000, Li-Cor, Lincoln, NE, US) and donkey anti-mouse IRDye 680 (1:10.000, Li-Cor, Lincoln, NE, US). Membranes were visualized using the Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE, US) and protein bands were quantified using ImageJ (https://imagej.nih.gov/ij/). Raw intensity measures were normalized to GAPDH to control for loading differences. Outliers were excluded based on a Dixon Q-test for outliers. The group means of riociguat treatment and vehicle treatment were compared at each timepoint by a t-test for equal means.

3. Results

3.1 The effects of riociguat on natural forgetting in the OLT

Table 1 summarizes the results of the 24 h inter-trial interval OLT per treatment condition. There were no differences in exploration time between treatment conditions for both T1 (e1: $F_{4,68}$=0.71, n.s.) and T2 (e2: $F_{4,68}$=1.67, n.s.). If given vehicle treatment, the mice were not able to remember the object locations after 24h as is indicated with a d2 value that is not different from chance level (zero) as measured with a one-sample t-test. When given a dose of 0.03 mg/kg riociguat, the OLT performance was significantly higher than chance level ($p<0.001$), indicating well-functioning spatial memory. None of the other tested doses were able to increase the performance significantly higher than chance level. In Figure 1 a visual representation of the OLT performance in healthy mice is depicted.

A one-way ANOVA comparing the d2 values of each condition showed a significant difference between the OLT performance of the experimental conditions ($F_{4,68}$=4.19; $p<0.01$). Post-hoc Dunnett's t-tests comparing every condition to the vehicle treatment condition showed that a dose of 0.03 mg/kg riociguat significantly enhanced mice OLT performance ($p<0.01$).

Table 1 - Mean values (± SEM) of the different 24h interval OLT measures

Dose riociguat	e1 (s)	e2 (s)	d2	N
vehicle	25.73 (3.17)	17.76 (2.52)	-0.01 (0.07)	14
0.01 mg/kg	27.65 (3.17)	15.23 (1.85)	0.05 (0.07)	15
0.03 mg/kg	30.04 (4.44)	22.80 (2.49)	0.38 (0.05) ###	14
0.1 mg/kg	21.77 (2.66)	17.09 (1.67)	0.15 (0.07)	15
0.3 mg/kg	26.20 (4.25)	21.52 (3.35)	0.09 (0.09)	15

Displayed are the mean exploration times in T1 (e1) and T2 (e2) and discrimination performance (d2) of the different treatment conditions. The SEM is presented between brackets. One sample t-tests were performed on the d2 measures. A significant difference from zero (one-sample t-test; indicated by hashes; ###: $p<0.001$) indicates that the animals remembered the location of the object from T1.

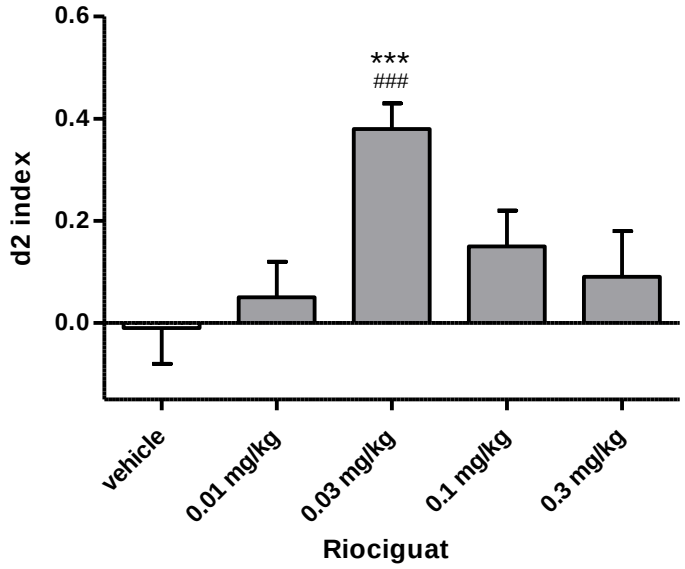

Figure 1 - Average d2 value and SEM of each treatment condition in a 24 h interval OLT. The discrimination index (d2) is indicated on the y-axis and the different doses of riociguat are shown on the x-axis. Hashes indicate a difference from zero (one-sample t-test: ###: $p < 0.001$). A difference from the vehicle condition is indicated with asterisks (post-hoc Dunnett's t-test: ***: $p<0.001$).

3.2 The effects of riociguat on a biperiden-induced memory deficit in the OLT

In this experiment the cognitive effects of riociguat were evaluated in combination with a biperiden-induced memory deficit with a 1 h inter-trial interval OLT. The results are summarized in Table 2. No significant differences were found between the exploration times of the different conditions for T1 (e1: $F_{5,76}=0.55$, n.s.) and T2 (e2: $F_{5,76}=1.78$, n.s.). After a 1 h interval, vehicle + vehicle treated mice were able to remember the location of the objects, indicated by the significant difference of the d2 value from chance level measured with a one-sample t-test ($p<0.001$). When given biperiden + vehicle the performance of the mice was not different from chance level indicating a spatial memory impairment after 3 mg/kg biperiden administration. One-sample t-tests comparing every biperiden + riociguat treatment to chance level showed that both biperiden + 0.03 mg/kg riociguat and biperiden + 0.1 mg/kg riociguat treated mice recognized the locations of the object after 1 h ($p<0.001$ and $p<0.01$, respectively).

Table 2 - Mean values (± SEM) of the different 1h interval OLT measures

Condition	e1 (s)	e2 (s)	d2	N
vehicle + vehicle	24.06 (3.1)	21.69 (3.33)	0.39 (0.07) ###	14
Biperiden (3 mg/kg) + vehicle	24.28 (2.68)	17.19 (2.11)	-0.04 (0.07)	14
biperiden + 0.01 mg/kg riociguat	23.68 (5.23)	20.34 (2.17)	0.08 (0.09)	13
biperiden + 0.03 mg/kg riociguat	18.00 (2.78)	19.03 (2.35)	0.37 (0.07) ###	14
biperiden + 0.1 mg/kg riociguat	26.05 (4.25)	18.16 (2.64)	0.25 (0.07) ##	13
biperiden + 0.3 mg/kg	22.99 (4.09)	29.30 (5.64)	0.06 (0.1)	14

Displayed are the mean exploration times in T1 (e1) and T2 (e2) and discrimination performance (d2) of the different treatment conditions. The SEM is presented between brackets. One sample *t*-tests were performed on the d2 measures. A significant difference from zero (one-sample t-test; indicated by hashes; ##: $p<0.01$; ###: $p<0.001$) indicates that the animals remembered the location of the object from T1.

To evaluate the performance differences between all treatment conditions, a one-way ANOVA was performed over the d2 values of every condition. The one-way ANOVA revealed a significant difference between conditions ($F_{5,76}=5.213$; $p<0.001$). Next, every treatment group was compared to the biperiden + vehicle group with a post-hoc Dunnett's t-test, and the latter showed a significant difference for the vehicle + vehicle condition ($p<0.01$) confirming that when treated with biperiden mice performed significantly lower than vehicle treated animals. Furthermore, Dunnett's t-tests showed that biperiden + 0.03 mg/kg riociguat ($p<0.01$) and biperiden + 0.1 mg/kg riociguat ($p<0.05$) treated mice performed significantly higher than biperiden + vehicle treated animals. This indicates that 0.03 and 0.1 mg/kg riociguat completely attenuated the biperiden-induced memory deficit. These results are graphically illustrated in Figure 2.

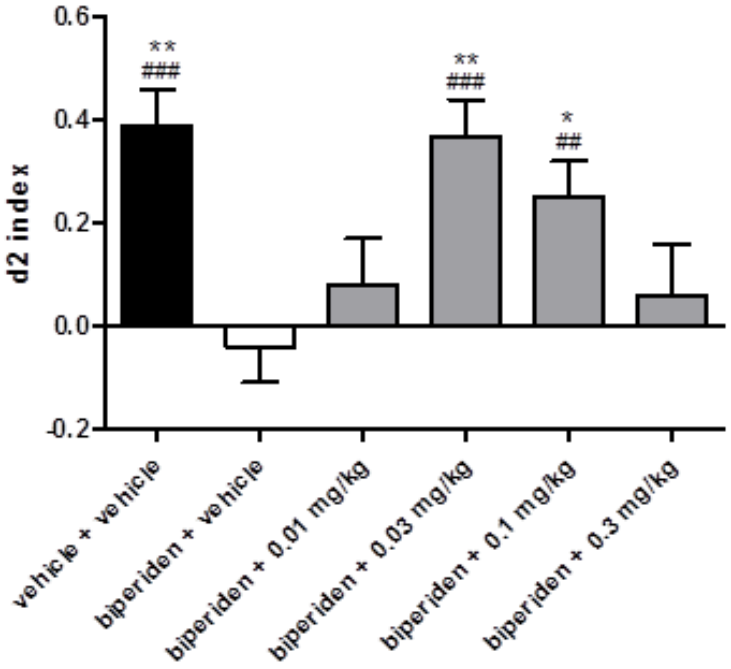

Figure 2 - Average d2 value and SEM of each treatment condition measured in a 1h OLT. Biperiden was always administered at a dose of 3 mg/kg. The discrimination index (d2) is indicated on the y-axis and the different treatment conditions are shown on the x-axis. Hashes indicate a difference from zero (one sample t-test: ##: p<0.01; ###: p<0.001). A difference from the biperiden + vehicle condition is indicated with asterisks (post-hoc Dunnett's t-test**:** *: p<0.05; **: p<0.01).

3.3 Effects of riociguat in the y-maze continuous alternation task after a biperiden-induced memory deficit

The y-maze continuous alternation task was performed to evaluate the acute effects of riociguat on spatial working memory after a biperiden-induced memory deficit. Table 3 summarizes the results of this experiment per treatment group. The mean alternation percentages were compared to 50% alternations (chance level) by one sample t-tests. Vehicle + vehicle (p<0.001), biperiden + 0.01 (p=0.001), biperiden + 0.03 (p<0.001) and biperiden + 0.1 mg/kg riociguat (p<0.05) treated animals showed significantly higher performances than chance level, indicating a well-functioning working memory. Biperiden + vehicle and biperiden + 0.3 mg/kg riociguat did not show a difference from chance level which indicates impaired working memory in these treatment conditions.

Table 3 - Mean values and SEM of the y-maze alternation task measures

Condition	Alternations %	SEM	n
vehicle + vehicle	66.12 ###	2.61	9
Biperiden (3 mg/kg) + vehicle	54.23	2.15	9
biperiden + 0.01 mg/kg riociguat	56.82 ##	1.35	9
biperiden + 0.03 mg/kg riociguat	65.04 ###	2.62	9
biperiden + 0.1 mg/kg riociguat	57.58 #	2.57	9
biperiden + 0.3 mg/kg riociguat	53.96	3.42	9

Displayed are the mean percentages of alternations and their SEM for every treatment condition. One sample t-tests were performed on the alternation percentages. A significant difference from 50% (one sample t-test; indicated by hashes; #: $p<0.05$; ##: $p<0.01$; ###: $p<0.001$) indicates that the animals have well-functioning spatial working memory.

A one-way ANOVA comparing the performance between the different treatment groups showed a significant effect ($F_{5,48}=4.441$; $p<0.01$). Dunnett's t-tests comparing the groups to the biperiden + vehicle treatment showed that both the vehicle + vehicle ($p=0.008$) and biperiden + 0.03 mg/kg riociguat ($p<0.05$) treated mice performed significantly higher. This indicates that a dose of 0.03 mg/kg riociguat can fully restore a biperiden-induced (working) memory deficit up to the level of performance of vehicle treated animals. Doses of 0.01 mg/kg and 0.1 mg/kg riociguat were able to show a functioning working-memory but did not show a significant difference from the biperiden + vehicle condition, therefore these doses are regarded to have an intermediate effect on spatial working memory performance after a biperiden-induced memory deficit. These results are graphically depicted in Figure 3.

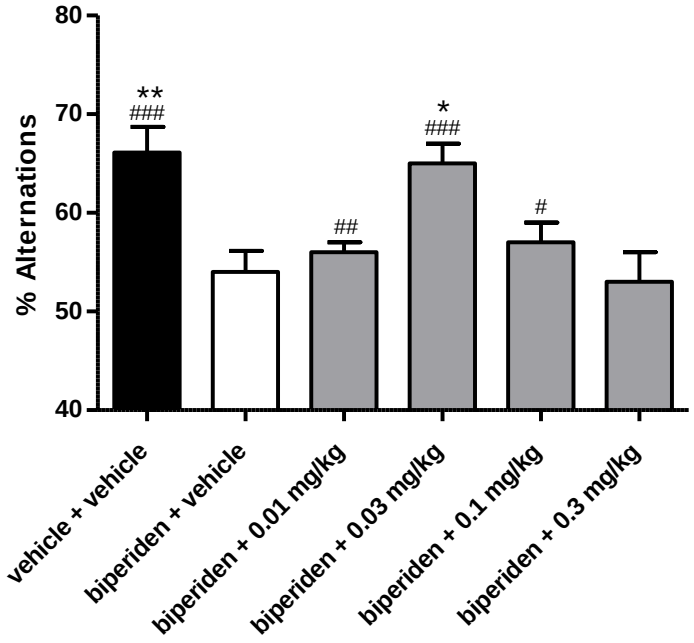

Figure 3 - Y-maze alternation task average percentages of alternations and SEM of each treatment condition. The mean alternation % is indicated on the y-axis and the different treatment conditions are shown on the x-axis. Biperiden was always given at a dose of 3 mg/kg. Hashes indicate a difference from chance level, i.e. 50% (one-sample t-test: #: $p<0.05$; ##: $p<0.01$; ###: $p<0.001$). A difference from the biperiden + vehicle condition is indicated with asterisks (post-hoc Dunnett's t-test: *: $p<0.05$; **: $p<0.01$).

3.4 Effects of combined sub-optimal doses of riociguat and donepezil in a 24 h inter-trial interval OLT

To evaluate the effects of combined drug treatment of riociguat with donepezil, a natural forgetting OLT was performed with the sub-optimal doses of these drugs given in combination. Firstly, donepezil was administered 30 min before the first trial (P.O.) at doses of 0.03, 0.1 and 0.3 mg/kg from which 0.1 mg/kg was found to be the sub-optimal dose (data not shown). In our previous experiment 0.01 mg/kg riociguat was found to be the sub-optimal dose at a 24 h inter-trial interval OLT.

A total of 4 treatments were tested with all groups receiving two injections 30 min in before T1 (P.O.; 5 ml/kg). Saline was used as a vehicle for donepezil while a 0.5% tylose solution was used as a vehicle for riociguat. The treatments tested were: vehicle (donepezil) + vehicle (riociguat), vehicle (donepezil) + 0.01 mg/kg riociguat, 0.1 mg/kg donepezil + vehicle (riociguat) and 0.1 mg/kg donepezil + 0.01 mg/kg riociguat. The results are summarized in Table 4.

Table 4 - Mean values (± SEM) of the different 24h interval OLT measures

Dose	e1 (s)	e2 (s)	d2	N
vehicle (saline) + vehicle (tylose)	24.65 (6.42)	21.16 (3.14)	0.02 (0.11)	9
vehicle (saline) + 0.01 mg/kg riociguat	26.06 (2.88)	21.21 (2.44)	0.11 (0.11)	10
0.1 mg/kg donepezil + vehicle (tylose)	34.93 (6.4)	18.34 (2.33)	0.01 (0.06)	9
0.1 mg/kg donepezil + 0.01 mg/kg riociguat	23.15 (3.66)	19.28 (2.41)	0.39 (0.11) ##	10

Displayed are the mean exploration times in T1 (e1) and T2 (e2) and discrimination performance (d2) of the different treatment conditions. The SEM is presented between brackets. One sample t-tests were performed on the d2 measures. A significant difference from zero (one-sample t-test; indicated by hashes; ##: $p<0.01$) indicates that the animals remembered the location of the object from T1.

When given a combination of riociguat and donepezil the mice were able to remember the object locations after 24h, as shown by a one-sample t-test comparing the d2 value to chance level ($p<0.01$), while the mice treated with sub-optimal doses of riociguat and donepezil alone did not show any object location memory. A one-way ANOVA comparing the mean performance of the different treatment conditions showed a significant treatment effect ($F_{3,34}=3.116$; $p<0.05$) and the post-hoc Dunnett's t-test revealed that the donepezil + riociguat treatment differed significantly from vehicle + vehicle treatment ($p<0.05$). Figure 4 shows a graphical representation of these findings. These results indicate that while these sub-optimal doses of donepezil and riociguat alone do not have an effect on memory performance in healthy mice, when given in combination they are equally effective in prolonging natural forgetting as a higher dose of either of these compounds alone.

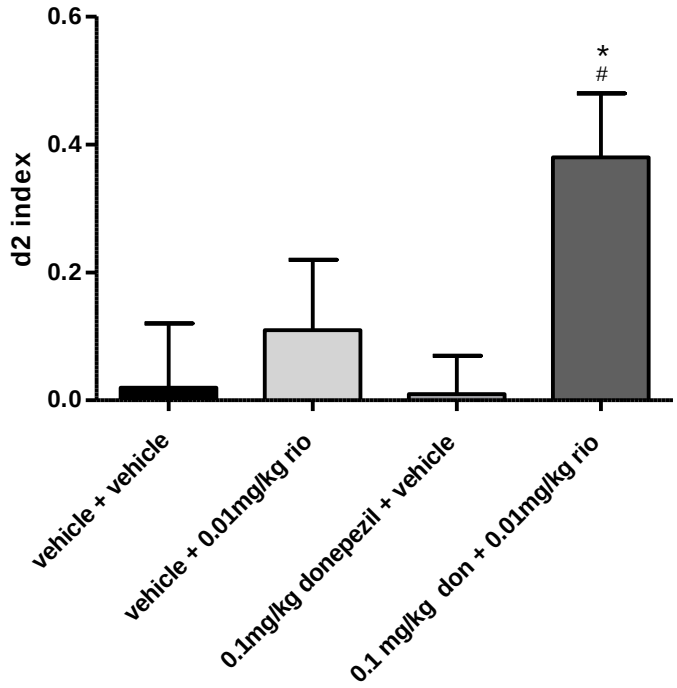

Figure 4 - 24 h interval OLT performance combination study riociguat and donepezil. Shown are the average d2 values and SEM of each treatment condition. The discrimination index (d2) is indicated on the y-axis and the different treatment conditions are shown on the x-axis. Hashess indicate a difference from zero (one-sample t-test: ##: $p < 0.01$). A difference from the vehicle + vehicle condition is indicated by an asterisk (post-hoc Dunnett's t-test: *: $p<0.05$).

3.5 Plasma and brain concentration of riociguat and M-1

Total plasma (C_P) and total brain (C_b) concentration, and the brain-to-blood ratios (C_B:C_P) for both riociguat and its metabolite M1 are listed in Table 6. Concentration levels were measured at 0.5 h and 1.5 h after compound administration, reflecting the timing of the learning trial of the behavioral tasks and the test trial of the 1 h OLT, respectively. Riociguat concentration was measured at two dosages, the behaviorally active and a 10-fold higher dose, while M1 was only measured in the highest dose. The behaviorally active dose of 0.03 mg/kg riociguat appears detectable in the brain only 0.5 h after administration. However, the ratio between plasma and brain concentration of riociguat is equally low for every condition, indicting no or poor brain penetration of riociguat. The brain penetration of the metabolite might be better, yet this was not tested at the memory enhancing dose.

Table 5 - Pharmacokinetics of riociguat and its metabolite M1

interval	**0.5 h**	**1.5 h**
riociguat (0.03 mg/kg)		
C_p (ng/ml)	2.87 (0.39)	1.32 (0.79)
C_b (ng/g)	0.15 (0.15)	BQL
C_b: Cp	0.07 (0.07)	n.a.
riociguat (0.3 mg/kg)		
C_p (ng/ml)	30.03 (3.04)	15.00 (1.85)
C_b (ng/g)	0.84 (0.10)	0.64 (0.07)
C_b:C_p	0.03 (0.00)	0.04 (0.00)
M1 (0.03 mg/kg)		
C_p (ng/ml)	N.D.	N.D.
C_b (ng/g)	N.D.	N.D.
C_b:C_p	N.D.	N.D.
M1 (0.3 mg/kg)		
C_p (ng/ml)	1.79 (0.27)	1.91 (0.24)
C_b (ng/g)	0.14 (0.14)	BQL
C_b:C_p	0.14 (0.14)	n.a.

Mean (±SEM) plasma and brain concentrations of riociguat and M1 (0.3 and 0.03 mg/kg, p.o.) 0.5 and 1.5 h after administration. The LLOQ for plasma and brain were 0.25 ng/ml and 0.4 ng/g, respectively. n.a.: not applicable; N.D.: not determined; BQL: below quantification limit.

3.6 Effect of riociguat on plasticity markers and VASP after 1.5 and 24h

In order to investigate the underlying molecular mechanisms responsible for the efficacy of riociguat on memory, mice received 0.03 mg/kg (P.O.) riociguat that was shown to be the optimal dose for reversing natural forgetting (Figure 1). Riociguat was administered 30 min prior to T1 and mice were sacrificed 2 h and 24 h after the injection, without undergoing T2. Treatment with riociguat did not affect membrane, total or the ratio of GluA1-containing AMPA receptors, either after 2 h (Figure 5: A1, B1, C1) or 24 h (Figure 5: A2, B2, C2). Similarly, no difference was observed for PSD95 (E1, E2) levels and CREB activation (F1, F2) at the two time points tested. In contrast to these plasticity markers, riociguat did increase VASP activation. This effect was observed only for the animals sacrificed 2 h after riociguat administration ($t=3.087$; $p=0.009$), while VASP activation was similar to that of the vehicle-treated animals 24 h post-injection.

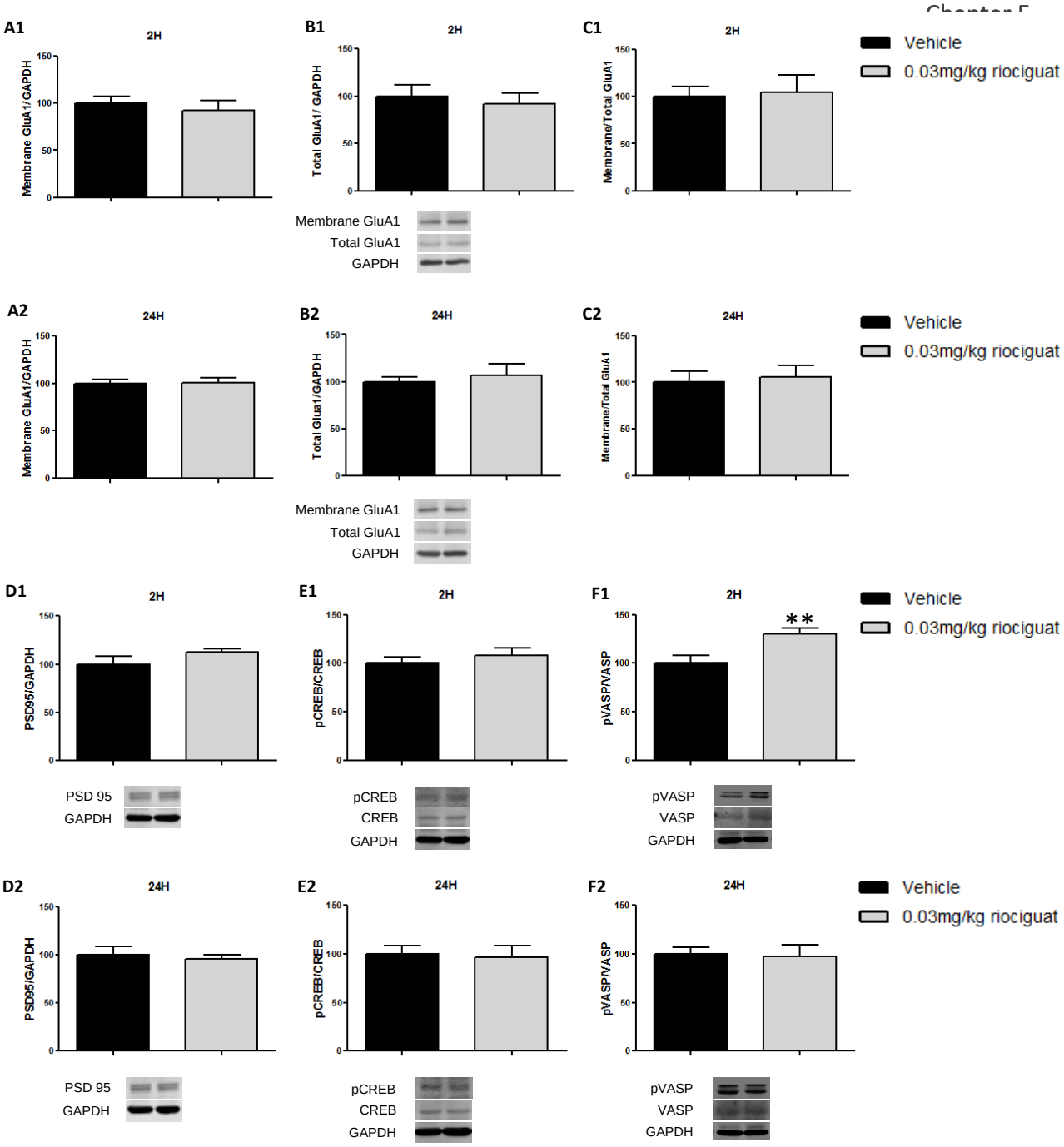

Figure 5- Protein levels of plasticity markers and VASP measured in mouse hippocampus of vehicle- and riociguat-treated animals. Treatment with riociguat (0.03 mg/kg) did not affect protein levels of membrane GluA1, total GluA1, the ratio membrane/total GluA1, PSD95 or CREB phosphorylation in the hippocampus of mice sacrificed 2 h (panels A1, B1, C1, D1, E1) and 24 h (panels A2, B2, C2, D2, E2) after the injection. VASP phosphorylation was significantly increased for the riociguat-treated mice compared to vehicle treated mice, sacrificed 2 h after the injection. Whereas 24 h after the injection no difference was observed between the two conditions. Asterisks indicate a significant difference from vehicle-treated mice (t-test: **: $p < 0.01$).

4. Discussion

4.1 Riociguat is able to enhance spatial memory performance in both healthy mice and a mouse model for memory impairment

In the first experiment we investigated the effects of riociguat on long-term spatial memory in healthy mice, by means of a 24 h interval OLT. It was found that a dose of 0.03 mg/kg riociguat (P.O.) was able to significantly counteract natural forgetting. None of the other doses tested were able to show a performance different from chance level, which indicates that riociguat has a very narrow efficacy dose-range window for improving memory in healthy mice. This window was slightly larger in a mouse model for memory impairment, as was shown by the 1 h OLT data with a biperiden-induced memory deficit. The finding that a dose of 0.1 mg/kg is also effective in impaired animals, but not in healthy animals, might be due to decreased NO-cGMP-PKG signaling in the deficit model. Due to lower levels of cGMP, riociguat can be given in a higher dose, since the threshold of overstimulation of the pathway is less easily reached. The dose of 0.03 mg/kg seems to be most efficacious in restoring both impaired spatial memory, as well as counteracting natural forgetting.

A combination of sub-optimal doses of riociguat and donepezil was also shown to be an effective enhancer of long-term spatial memory in the 24 h OLT. By combining two drugs that target different facets of memory-related neurotransmission, effects could combine to be additive or synergistically effective (Geerts & Grossberg, 2006). A treatment strategy with sub-optimal doses might also be more beneficial because of potentially lower side-effects (e.g., cardiovascular, gastrointestinal), due to the low dosing concentrations. Further investigations are needed to substantiate this claim.

In addition to the OLT experiments, a y-maze alternation task was performed to investigate spatial working memory. The previously shown most efficacious dose of riociguat in short- and long-term spatial memory (0.03 mg/kg), also proved to be most effective in restoring a biperiden-induced deficit of spatial working memory in mice. Interestingly, in contrast to the findings in the OLT experiments, two other dosages showed intermediate effects on working memory. Although the performance was not significantly higher from the biperiden + vehicle treated animals, mice treated with 0.01 and 0.1 mg/kg riociguat performed significantly above chance level. This implies that the working memory deficit induced by biperiden was, at least partly, restored by riociguat treatment at these doses.

Altogether, these results show that only a subtle stimulation of the NO-cGMP-PKG pathway was needed to improve memory functioning in mice, and overstimulation diminished the possible positive effects of the drug. The finding that the optimal dosage of riociguat was similar across different forms of memory in mice, could be beneficial for diseases showing general memory decline

(e.g. AD). Thus, a single dose would effectively enhance memory performance across working, short-term and long-term memory. Of note, all paradigms used in this study were measuring spatial forms of memory, therefore more research is needed to investigate if this holds also for non-spatial memory tests. Another consequence of the very specific range of effectiveness after riociguat administration is that when giving a standard dose/pill to human subjects, regardless of their body mass and metabolism, the level of cGMP-PKG stimulation needed to obtain optimal memory enhancing effects might be missed relatively easily.

4.2 Riociguat or its metabolite M-1 do not seem to enter the brain

The T_{max} and plasma $T_{1/2}$ of riociguat in rodents are approximately 1 and 2 h, respectively (Auspar Report, 2014; CHMP report, 2014). The $T_{1/2}$ of M1 is not known in rodents, but based on other species it is significantly longer, approximately 5 h. Riociguat was always given 30 min before y-maze testing or OLT learning. Thus, it should be optimally available during y-maze testing and OLT acquisition as well as OLT consolidation. In the plasma, riociguat was present at an average concentration of 2.82 ng/ml 30 min after oral administration, and this was roughly reduced by half (1.32 ng/ml) 1.5 h after administration, i.e. during OLT consolidation. The 10-fold dosage of riociguat shows a 10-fold increase in plasma concentration, which was also reduced by half 1.5 h after treatment. The brain concentration of riociguat was very low at a dose of 0.03 mg/kg 30 min after administration, and below the quantification limit 1.5 h after administration. A 10-fold increase of the dose does not clearly increase brain concentrations by 10-fold at the two time points. Due to the low brain concentrations of riociguat, the brain to blood concentration ratio of all measured groups are low and close to 0.04 which is the ratio of the approximate cerebral blood volume relative to total unperfused brain volume (Hitchcock & Pennington, 2006). This could indicate that the riociguat quantity measured in the brain samples was originating from the arteries within the brain tissue, but not the brain tissue itself. We can conclude from these data that riociguat has very poor or no brain penetration.

In order to exclude that riociguats' activity could be due to its main metabolite M1 being active in the brain, the concentrations of M1 were also evaluated. Interestingly, after administration of the high dose of 0.3 mg/kg, M1 showed a sufficient brain/plasma ratio of 0.14 at 30 min after riociguat treatment. However, the plasma concentration of M1 does not substantially change at 1.5 h after treatment, while the brain concentration becomes undetectable. This indicates that M1 also has poor brain penetration, which is even more likely at the lower memory-enhancing dose.

Taken together, since riociguat showed memory-enhancing effects in both spatial memory tasks, the pharmacokinetic data question the possibility that these effects were directly mediated by a central effect.

4.3 Underlying mechanism of memory enhancement after acute riociguat administration

The hippocampus is important for spatial memory (Brown & Aggleton, 2001). To investigate if drug-induced changes in neuronal plasticity underlie the acute effects of riociguat on memory performance, specific cGMP-PKG signaling-related biochemical markers were analyzed in hippocampal tissue. Hippocampal slices were dissected from mice which were treated with 0.03 mg/kg riociguat 30 min before the learning trial of the OLT and subsequently sacrificed 1.5 and 24 h after learning. Membrane AMPA receptors, total level of AMPA receptors, CREB activation and PSD-95 failed to show any difference between vehicle-treated animals and riociguat-treated animals on any timepoints after learning. Therefore, we can conclude that the memory-enhancing effects of riociguat were not mediated through these neuronal plasticity mechanisms, which seems to fit with the apparent lack in brain penetration of riociguat. Therefore, we must consider the possibility of an alternative (peripheral) mechanism of action.

Evidence supporting the existence of alternative mechanisms of memory enhancement have been found in prior experiments in which cGMP-specific drug targets were unable to show plasticity changes (Sierksma et al., 2013). Studies that reported chances on plasticity markers often used a deficit model in which these markers were decreased compared to baseline levels (Giralt et al., 2013; Puzzo et al., 2009). It can be argued that by using healthy mice for the biochemical analysis, an effect on PSD-95, pCREB and AMPA receptors could not be detected since these markers were already present at optimal levels, i.e. a ceiling effect. In other words, if we had used the biperiden-induced deficit model for the brain plasticity measurements after treatment + learning, effects could have been different. Furthermore, it has been demonstrated that a PDE5 inhibitor did not necessarily have to enter the brain to reverse a scopolamine-induced memory impairment in the ORT (Reneerkens et al., 2012). Altogether, these prior findings support the notion that riociguat might enhance memory through mechanisms other than hippocampal plasticity related to these specific markers.

Interestingly, analysis of pVASP and VASP in the hippocampus showed that acute riociguat treatment was able to significantly increase the pVASP/VASP ratio, which is indicative of pVASP activation, compared to vehicle 1.5 h after the learning trial of the OLT. PKG preferentially phosphorylates VASP at Ser 239, while PKA has a preference for Ser 157 (Begonja et al., 2013). Thus, pVASP Ser 239 was a measure for PKG activity within the hippocampus. Acute administration of 0.03

mg/kg riociguat was able to increase cGMP-PKG-VASP signaling within the mouse hippocampus. Thus, VASP might be involved in mediating effects that improve acquisition and/or early consolidation processes. 24 h after the learning trial, no difference was found anymore in pVASP/VASP ratio between riociguat and vehicle treated animals. The 24 h time point is similar to the test trial of the natural forgetting OLT, showing that VASP phosphorylation was not at hand anymore to possibly affect retrieval processes. VASP has been connected to plasticity changes because of its effect on synapses (Lin, 2011), AMPA receptors and LTP (Francis et al., 2010; Wang et al., 2005). If memory-related plasticity would be regulated through VASP activation, this contradicts the notion that riociguat appears not to be able to enter the brain. Alternatively, it could also be possible that the VASP increase is measured in arteries innervating the hippocampus. VASP activation also has been shown to increase blood flow and might thus have an effect on hippocampal function (Francis et al., 2010).

Riociguat is on the market for pulmonary hypertension, and its main known effects are vasodilation, vascular smooth muscle cell relaxation and increased blood oxygenation (Mittendorf et al., 2009). Since cognitive functioning requires increased levels of cerebral blood oxygenation to support neuronal capacity, the memory enhancing effect of riociguat could be explained by increased blood oxygenation supporting activation of brain structures important to memory function. The measured increase in VASP phosphorylation supports this notion, since this illustrates vascular changes within the hippocampus, during the acquisition and/or consolidation of spatial information. Coincidentally, the optimally active oral dose of 0.03 mg/kg riociguat in our mice is similar to the lowest oral dose that could lower arterial blood pressure in conscious spontaneous hypertensive rats (Mittendorf et al., 2009). Yet, only oral doses higher than 0.3 mg/kg have been found to decrease the mean arterial blood pressure (MAP) in anaesthetized healthy rats and no significant effects on heart rate (HR) are to be expected at such doses (Auspar Report, 2014). This implies that in our study, memory-enhancing doses of maximally 0.1 mg/kg are probably still too low to have an effect on MAP and HR. This would rule out any cardio- and cerebrovascular effects to improve the memory performance as found in the present study. More research is needed into the hemodynamic effects of riociguat at doses that improve memory performance.

Another interesting point is that it is known that sGC influences energy metabolism through activation of natriuretic peptides in the cardiovascular system (Moro & Lafontan, 2013; Pfeifer et al., 2013). The stimulation of sGC increases glucose metabolism and secondly, cGMP has been found to regulate mitochondrial biogenesis within muscle tissue (Nisoli et al., 2004; Pfeifer et al., 2013). Stimulation of sGC by riociguat could lead to an overall increase of energy metabolism, creating a higher overall availability of cellular energy supply. Effects on memory performance can be due to

the availability of high and rapidly metabolized energy supplies to both brain and muscle tissue during the learning task, leading to optimal homeostasis and simultaneous function of memory and motor processes, resulting in enhanced task performance.

Clearly, additional experiments are necessary to elucidate possible mechanisms of sGC-mediated memory enhancement, and answer the question if this effect is regulated centrally or peripherally. Since this is not the first study to show the contra-indicative result that a centrally regulated process appears to be enhanced by (additional) peripheral effects (Reneerkens et al., 2012). Therefore, it is obvious that central and peripheral pathways of cGMP-signaling still need to be unraveled in relation to memory function. Interestingly, Reneerkens et al. (2012), found that a PDE5 inhibitor that did not enter the brain was able to reverse a scopolamine, i.e. muscarinic receptor-induced object memory deficit, yet not a MK-801, i.e. NMDA receptor-induced object memory deficit. This implies an interaction between peripheral cGMP signaling and central muscarinic receptors to be beneficial for memory function. The biperiden model for memory impairment is also specific for muscarinic receptors. Therefore, it would be of interest to evaluate riociguats' effect on a memory impairment induced by MK-801 or another NMDA receptor antagonist. If sGC stimulation would not be able to reverse a dysfunction in NMDA signaling, this would further support a peripheral sGC-cGMP pathway which can counteract muscarinic receptor-induced central deficits on spatial and object memory function.

In conclusion, sGC stimulation through riociguat treatment was able to enhance spatial memory in healthy mice and restore spatial memory performance to the level of vehicle treatment in a cholinergic model of memory impairment. Furthermore, it was shown that when using a combination of suboptimal doses of the AChEI donepezil and riociguat in healthy mice, OLT memory enhancement was equal to the optimal dose of riociguat when given separately. Pharmacokinetic data showed that riociguat was not or poorly able to enter the brain, therefore it seems most likely that the enhanced memory performance is a result of better blood-oxygen supply reaching the brain through peripheral effects on hemodynamics. The exact mechanism through which riociguat was able to enhance memory function remains unclear until further investigation.

5. References

Akkerman, S., Blokland, A., Reneerkens, O., van Goethem, N. P., Bollen, E., Gijselaers, H. J., . . . Prickaerts, J. (2012). Object recognition testing: methodological considerations on exploration and discrimination measures. *Behavioural Brain Research, 232*(2), 335-347.

Akkerman, S., Prickaerts, J., Steinbusch, H. W., & Blokland, A. (2012). Object recognition testing: statistical considerations. *Behavioural Brain Research, 232*(2), 317-322.

Anisman, H., & Kokkinidis, L. (1975). Effects of scopolamine, d-amphetamine and other drugs affecting catecholamines on spontaneous alternation and locomotor activity in mice. *Psychopharmacology, 45*(1), 55-63.

Auspar Report (2014). Adempas, Australian Public Assessment Report for Riociguat. *available from: https://*www.tga.gov.au/sites/default/files/auspar-riociguat-140612.pdf.

Begonja, A. J., Gambaryan, S., Schulze, H., Patel-Hett, S., Italiano, J. E., Jr., Hartwig, J. H., & Walter, U. (2013). Differential roles of cAMP and cGMP in megakaryocyte maturation and platelet biogenesis. *Exp Hematol, 41*(1), 91-101 e104. doi: 10.1016/j.exphem.2012.09.001

Blokland, A., Sambeth, A., Prickaerts, J., & Riedel, W. J. (2016). Why an M1 Antagonist Could Be a More Selective Model for Memory Impairment than Scopolamine. *Frontiers in Neurology, 7*, 167. doi: 10.3389/fneur.2016.00167

Boess, F. G., Hendrix, M., van der Staay, F.-J., Erb, C., Schreiber, R., van Staveren, W., . . . Koenig, G. (2004). Inhibition of phosphodiesterase 2 increases neuronal cGMP, synaptic plasticity and memory performance. *Neuropharmacology, 47*(7), 1081-1092. doi: https://doi.org/10.1016/j.neuropharm.2004.07.040

Bollen, E., Puzzo, D., Rutten, K., Privitera, L., De Vry, J., Vanmierlo, T., . . . Prickaerts, J. (2014). Improved Long-Term Memory via Enhancing cGMP-PKG Signaling Requires cAMP-PKA Signaling. *Neuropsychopharmacology, 39*(11), 2497-2505. doi: 10.1038/npp.2014.106

Brown, M. W., & Aggleton, J. P. (2001). Recognition memory: what are the roles of the perirhinal cortex and hippocampus? *Nature Reviews Neuroscience, 2*(1), 51-61.

Celikyurt, I. K., Utkan, T., Ozer, C., Gacar, N., & Aricioglu, F. (2014). Effects of YC-1 on Learning and Memory Functions of Aged Rats. *Medical Science Monitor Basic Research, 20*, 130-137. doi: 10.12659/MSMBR.891064

CHMP report. (2014). Adempas, Committee for Medicinal Products for Human Use (CHMP) assessment report. *available from:* http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Public_assessment_report/human/002737/WC500165036.pdf.

Conole, D., & Scott, L. J. (2013). Riociguat: First Global Approval. *Drugs, 73*(17), 1967-1975. doi: 10.1007/s40265-013-0149-5

Devan, B. D., Duffy, K. B., Bowker, J. L., Bharati, I. S., Nelson, C. M., Daffin, L. W., Jr., . . . Ingram, D. K. (2005). Phosphodiesterase type 5 (PDE5) inhibition and cognitive enhancement. *Drugs of the Future, 30*(7), 725. doi: 10.1358/dof.2005.030.07.929862

Ennaceur, A., & Delacour, J. (1988). A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data. *Behavioural Brain Research, 31*(1), 47-59. doi: https://doi.org/10.1016/0166-4328(88)90157-X

Evgenov, O. V., Pacher, P., Schmidt, P. M., Haskó, G., Schmidt, H. H. H. W., & Stasch, J.-P. (2006). NO-independent stimulators and activators of soluble guanylate cyclase: discovery and therapeutic potential. *5*, 755. doi: 10.1038/nrd2038

Francis, S. H., Busch, J. L., Corbin, J. D., & Sibley, D. (2010). cGMP-dependent protein kinases and cGMP phosphodiesterases in nitric oxide and cGMP action. *Pharmacol Rev, 62*(3), 525-563. doi: 10.1124/pr.110.002907

Geerts, H., & Grossberg, G. T. (2006). Pharmacology of acetylcholinesterase inhibitors and N-methyl-D-aspartate receptors for combination therapy in the treatment of Alzheimer's disease. *J Clin Pharmacol, 46*(7 Suppl 1), 8S-16S. doi: 10.1177/0091270006288734

Ghofrani, H.-A., Galiè, N., Grimminger, F., Grünig, E., Humbert, M., Jing, Z.-C., . . . Rubin, L. J. (2013). Riociguat for the Treatment of Pulmonary Arterial Hypertension. *New England Journal of Medicine, 369*(4), 330-340. doi: 10.1056/NEJMoa1209655

Ghofrani, H. A., & Grimminger, F. (2009). Soluble guanylate cyclase stimulation: an emerging option in pulmonary hypertension therapy. *European Respiratory Review, 18*(111), 35.

Giralt, A., Saavedra, A., Carreton, O., Arumi, H., Tyebji, S., Alberch, J., & Perez-Navarro, E. (2013). PDE10 inhibition increases GluA1 and CREB phosphorylation and improves spatial and recognition memories in a Huntington's disease mouse model. *Hippocampus, 23*(8), 684-695. doi: 10.1002/hipo.22128

Heckman, P. R. A., Wouters, C., & Prickaerts, J. (2015). Phosphodiesterase Inhibitors as a Target for Cognition Enhancement in Aging and Alzheimer’s Disease: A Translational Overview. *Current Pharmaceutical Design, 21*(3), 317-331.

Hitchcock, S. A., & Pennington, L. D. (2006). Structure− brain exposure relationships. *Journal of medicinal chemistry, 49*(26), 7559-7583.

Jin, F., Gong, Q.-H., Xu, Y.-S., Wang, L.-N., Jin, H., Li, F., . . . Shi, J.-S. (2014). Icariin, a phoshphodiesterase-5 inhibitor, improves learning and memory in APP/PS1 transgenic mice by stimulation of NO/cGMP signalling. *International Journal of Neuropsychopharmacology, 17*(6), 871-881. doi: 10.1017/S1461145713001533

Kennedy, M. B., Marcora, E., & Carlisle, H. J. (2008). Scaffold Proteins in the Postsynaptic Density. In J. W. Hell & M. D. Ehlers (Eds.), *Structural And Functional Organization Of The Synapse* (pp. 407-440). Boston, MA: Springer US.

Kilkenny, C., Browne, W. J., Cuthill, I. C., Emerson, M., & Altman, D. G. (2010). Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biol, 8*(6), e1000412. doi: 10.1371/journal.pbio.1000412

Kirsch, M., Kemp-Harper, B., Weissmann, N., Grimminger, F., & Schmidt, H. H. (2008). Sildenafil in hypoxic pulmonary hypertension potentiates a compensatory up-regulation of NO-cGMP signaling. *FASEB J, 22*(1), 30-40. doi: 10.1096/fj.06-7526com

Kleppisch, T., & Feil, R. (2009). cGMP Signalling in the Mammalian Brain: Role in Synaptic Plasticity and Behaviour. In H. H. H. W. Schmidt, F. Hofmann & J.-P. Stasch (Eds.), *cGMP: Generators, Effectors and Therapeutic Implications* (pp. 549-579). Berlin, Heidelberg: Springer Berlin Heidelberg.

Klinkenberg, I., & Blokland, A. (2011). A comparison of scopolamine and biperiden as a rodent model for cholinergic cognitive impairment. *Psychopharmacology, 215*(3), 549-566. doi: 10.1007/s00213-011-2171-1

Lin, W. (2011). Vasodilator-stimulated phosphoprotein (VASP) promotes actin assembly in dendritic spines to regulate synaptic strength. *PhD Dissertation*.

Melnikova, I. (2007). Therapies for Alzheimer's disease. *Nat Rev Drug Discov, 6*(5), 341-342. doi: 10.1038/nrd2314

Mittendorf, J., Weigand, S., Alonso-Alija, C., Bischoff, E., Feurer, A., Gerisch, M., . . . Stasch, J. P. (2009). Discovery of riociguat (BAY 63-2521): a potent, oral stimulator of soluble guanylate cyclase for the treatment of pulmonary hypertension. *ChemMedChem, 4*(5), 853-865. doi: 10.1002/cmdc.200900014

Moro, C., & Lafontan, M. (2013). Natriuretic peptides and cGMP signaling control of energy homeostasis. *American Journal of Physiology - Heart and Circulatory Physiology, 304*(3), H358-H368. doi: 10.1152/ajpheart.00704.2012

Murai, T., Okuda, S., Tanaka, T., & Ohta, H. (2007). Characteristics of object location memory in mice: Behavioral and pharmacological studies. *Physiology & Behavior, 90*(1), 116-124. doi: https://doi.org/10.1016/j.physbeh.2006.09.013

Nisoli, E., Falcone, S., Tonello, C., Cozzi, V., Palomba, L., Fiorani, M., . . . Clementi, E. (2004). Mitochondrial biogenesis by NO yields functionally active mitochondria in mammals. *Proc Natl Acad Sci U S A, 101*(47), 16507-16512.

Ota, K. T., Pierre, V. J., Ploski, J. E., Queen, K., & Schafe, G. E. (2008). The NO-cGMP-PKG signaling pathway regulates synaptic plasticity and fear memory consolidation in the lateral amygdala via activation of ERK/MAP kinase. *Learn Mem, 15*(10), 792-805. doi: 10.1101/lm.1114808

Pfeifer, A., Kilic, A., & Hoffmann, L. S. (2013). Regulation of metabolism by cGMP. *Pharmacol Ther, 140*(1), 81-91. doi: 10.1016/j.pharmthera.2013.06.001

Prickaerts, J., de Vente, J., Honig, W., Steinbusch, H. W. M., & Blokland, A. (2002). cGMP, but not cAMP, in rat hippocampus is involved in early stages of object memory consolidation. *European Journal of Pharmacology, 436*(1), 83-87. doi: https://doi.org/10.1016/S0014-2999(01)01614-4

Prickaerts, J., Şık, A., van der Staay, F. J., de Vente, J., & Blokland, A. (2005). Dissociable effects of acetylcholinesterase inhibitors and phosphodiesterase type 5 inhibitors on object recognition memory: acquisition versus consolidation. *Psychopharmacology, 177*(4), 381-390. doi: 10.1007/s00213-004-1967-7

Puzzo, D., Staniszewski, A., Deng, S. X., Privitera, L., Leznik, E., Liu, S., . . . Arancio, O. (2009). Phosphodiesterase 5 Inhibition Improves Synaptic Function, Memory, and Amyloid-β Load in an

Alzheimer's Disease Mouse Model. *The Journal of Neuroscience, 29*(25), 8075-8086. doi: 10.1523/jneurosci.0864-09.2009

Puzzo, D., Vitolo, O., Trinchese, F., Jacob, J. P., Palmeri, A., & Arancio, O. (2005). Amyloid-β Peptide Inhibits Activation of the Nitric Oxide/cGMP/cAMP-Responsive Element-Binding Protein Pathway during Hippocampal Synaptic Plasticity. *The Journal of Neuroscience, 25*(29), 6887-6897. doi: 10.1523/jneurosci.5291-04.2005

Reneerkens, O. A., Rutten, K., Akkerman, S., Blokland, A., Shaffer, C. L., Menniti, F. S., . . . Prickaerts, J. (2012). Phosphodiesterase type 5 (PDE5) inhibition improves object recognition memory: indications for central and peripheral mechanisms. *Neurobiol Learn Mem, 97*(4), 370-379. doi: 10.1016/j.nlm.2012.02.008

Reneerkens, O. A. H., Rutten, K., Steinbusch, H. W. M., Blokland, A., & Prickaerts, J. (2009). Selective phosphodiesterase inhibitors: a promising target for cognition enhancement. *Psychopharmacology, 202*(1), 419-443. doi: 10.1007/s00213-008-1273-x

Russwurm, M., Wittau, N., & Koesling, D. (2001). Guanylyl cyclase/PSD-95 interaction: targeting of the nitric oxide-sensitive alpha2beta1 guanylyl cyclase to synaptic membranes. *J Biol Chem, 276*(48), 44647-44652. doi: 10.1074/jbc.M105587200

Rutten, K., Prickaerts, J., Hendrix, M., van der Staay, F. J., Şik, A., & Blokland, A. (2007). Time-dependent involvement of cAMP and cGMP in consolidation of object memory: Studies using selective phosphodiesterase type 2, 4 and 5 inhibitors. *European Journal of Pharmacology, 558*(1), 107-112. doi: https://doi.org/10.1016/j.ejphar.2006.11.041

Rutten, K., Vente, J. D., Şik, A., Ittersum, M. M.-V., Prickaerts, J., & Blokland, A. (2005). The selective PDE5 inhibitor, sildenafil, improves object memory in Swiss mice and increases cGMP Levels in hippocampal slices. *Behavioural Brain Research, 164*(1), 11-16. doi: https://doi.org/10.1016/j.bbr.2005.04.021

Sambeth, A., Riedel, W. J., Klinkenberg, I., Kähkönen, S., & Blokland, A. (2015). Biperiden selectively induces memory impairment in healthy volunteers: no interaction with citalopram. *Psychopharmacology, 232*(11), 1887-1897. doi: 10.1007/s00213-014-3822-9

Schäfer, A., Fraccarollo, D., Werner, L., & Bauersachs, J. (2010). Guanylyl cyclase activator ataciguat improves vascular function and reduces platelet activation in heart failure. *Pharmacological Research, 62*(5), 432-438. doi: https://doi.org/10.1016/j.phrs.2010.06.008

Serulle, Y., Zhang, S., Ninan, I., Puzzo, D., McCarthy, M., Khatri, L., . . . Ziff, E. B. (2007). A GluR1-cGKII Interaction Regulates AMPA Receptor Trafficking. *Neuron, 56*(4), 670-688. doi: https://doi.org/10.1016/j.neuron.2007.09.016

Sierksma, A. S. R., Rutten, K., Sydlik, S., Rostamian, S., Steinbusch, H. W. M., van den Hove, D. L. A., & Prickaerts, J. (2013). Chronic phosphodiesterase type 2 inhibition improves memory in the APPswe/PS1dE9 mouse model of Alzheimer's disease. *Neuropharmacology, 64*(Supplement C), 124-136. doi: https://doi.org/10.1016/j.neuropharm.2012.06.048

Sierksma, A. S. R., van den Hove, D. L. A., Pfau, F., Philippens, M., Bruno, O., Fedele, E., . . . Prickaerts, J. (2014). Improvement of spatial memory function in APPswe/PS1dE9 mice after chronic inhibition of phosphodiesterase type 4D. *Neuropharmacology, 77*(Supplement C), 120-130. doi: https://doi.org/10.1016/j.neuropharm.2013.09.015

van Goethem, N. P., Rutten, K., van der Staay, F. J., Jans, L. A. W., Akkerman, S., Steinbusch, H. W. M., . . . Prickaerts, J. (2012). Object recognition testing: Rodent species, strains, housing conditions, and estrous cycle. *Behavioural Brain Research, 232*(2), 323-334. doi: https://doi.org/10.1016/j.bbr.2012.03.023

Vanmierlo, T., Rutten, K., Dederen, J., Bloks, V. W., van Vark-van der Zee, L. C., Kuipers, F., . . . Mulder, M. (2011). Liver X receptor activation restores memory in aged AD mice without reducing amyloid. *Neurobiology of Aging, 32*(7), 1262-1272. doi: https://doi.org/10.1016/j.neurobiolaging.2009.07.005

Wang, H.-G., Lu, F.-M., Jin, I., Udo, H., Kandel, E. R., de Vente, J., . . . Antonova, I. (2005). Presynaptic and Postsynaptic Roles of NO, cGK, and RhoA in Long-Lasting Potentiation and Aggregation of Synaptic Proteins. *Neuron, 45*(3), 389-403. doi: https://doi.org/10.1016/j.neuron.2005.01.011

Yamada, K., Hiramatsu, M., Noda, Y., Mamiya, T., Murai, M., Kameyama, T., . . . Nabeshima, T. (1996). Role of nitric oxide and cyclic GMP in the dizocilpine-induced impairment of spontaneous alternation behavior in mice. *Neuroscience, 74*(2), 365-374. doi: https://doi.org/10.1016/0306-4522(96)00161-3

Chapter 6

Assessing spatial pattern separation in rodents using the object pattern separation (OPS) task

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Abstract

Pattern separation is the process of transforming highly similar sensory inputs into distinct, dissimilar representations. It takes place in the hippocampus and is thought to be used in episodic memory. Impaired pattern separation performance has been recognised to be a predictor for the development of cognitive impairments in humans such as dementia and to be present in patients with schizophrenia and post-traumatic stress disorder (PTSD). In this protocol we describe how to implement a simple and robust object pattern separation (OPS) task in mice and rats that we have previously established and validated. This two-trial memory task utilizes specific object locations so differences in performance can be calibrated with the extent of object movement. Changes in performance are indicative of spatial pattern separation. In contrast to other pattern separation tasks, the OPS task allows detection of spatial pattern separation performance bi-directionally. Furthermore, the OPS task is cheaper and easier to use and interpret than other tasks which use more than two objects or which are touch-screen based. The entire protocol, from vivarium acclimatization to training of the animals, takes approximately 30 days. After successful training, the animals can be tested repeatedly and three OPS experiments (n = 20-24 per experimental day) can be performed per week. A standard level of expertise undertaking behavioral studies in rodents is sufficient to successfully integrate this paradigm into an existing rodent test battery.

Introduction

Pattern separation is a memory process which is commonly defined as the ability to form separate representations from highly similar, yet slightly different events or stimuli. Originating from computational models of hippocampal functioning, pattern separation processes have only recently been shown to be essential for normal memory functioning in both human and animal studies[1,2]. This has led to increased interest in pattern separation as a research topic and subsequently a growing body of evidence emerged supporting the importance of pattern separation processes to memory function. Current literature indicates pattern separation performance as a predictor for the development of cognitive impairments as seen in mild-cognitive impairment (MCI, pre-dementia) and dementia[3]. Furthermore, pattern separation processes are hypothesized to be impaired in anxiety disorders like post-traumatic stress disorder (PTSD)[2]. Pattern separation deficits were also reported in schizophrenia patients and have been linked to hippocampal dentate gyrus (DG) dysfunction[4,5]. It is suggested that impaired pattern separation functioning results in over-generalization of every-day stimuli and situations, leading to psychotic associations which are likely to underlie the hallmark positive symptomatology of this disorder[6].

Since pattern separation processes have been linked to fundamental hippocampal functioning and different disease models, translatable tests to reliably measure pattern separation performance in rodents are of great interest to both pharmaceutical and fundamental neuroscience research. Different tests to measure pattern separation performance in rodents have been described, for example fear conditioning paradigms that gradually change context cues[2], response to three objects in a testing arena[7], spatial orientation in a radial arm maze[1], or response to visual stimuli measured using a touch screen[8].

Contrasting results have been presented regarding the underlying mechanisms of pattern separation performance. This could be due in part to the lack of consistency between the tasks used in different behavioral *in vivo* investigations. Because pattern separation takes place on different levels of mnemonic or even sensory processes[9,10], different paradigms probably measure different underlying neuronal mechanisms. Thus the fundamental mechanisms of pattern separation and the implications for psychiatric disorders are not fully understood.

In this protocol we describe a standard protocol for a standardized and simple object pattern separation (OPS) task. We developed our protocol to be easily implementable in any typical rodent facility and usable by personnel with standard animal behavioral training. Therefore its use should facilitate a more consistent investigation of spatial pattern separation processes in animal models and treatment intervention studies. Previously we have demonstrated that the OPS can assess the effects of putative pattern separation enhancing and impairing drugs on OPS task performance[11].

Development of the OPS task

We derived our OPS protocol from the popular and straightforward object recognition memory task (ORT) and its spatial variant, the object location task (OLT)[12-14]. Based on the same paradigm as the ORT and OLT, the OPS task exploits the naturally occurring exploratory behavior of both rats and mice which have an innate preference to explore novel stimuli. In the OPS, ORT and OLT tests the time an animal takes to explore two individual objects in a testing arena is used as a readout parameter (see **Fig. 1**). First a rodent learns the spatial arrangement of the two objects in a learning trial which comprises 3 or 4 min of free exploration time. After a 1 h inter-trial interval, the animal is again confronted with the object-set, but this time one of the objects has been moved to a new location which can vary in distance from the location it was in previously during in the learning trial. Subsequently, it can be determined whether the animals spent a different amount of time exploring the moved or the stationary object. Pattern separation performance is indicated by the relative time spent exploring the moved object compared to the stationary object. A greater distance between the starting location and the new location should lead to a better pattern separation performance for each rodent as it is easier to discriminate or recognize the new position as novel when the change is more apparent.

The difference between the OPS task and the OLT is that instead of moving one of the two objects to one new location that is far away from that initially used (which assesses spatial memory performance), the OPS task uses four possible new locations along a vertical axis. These locations gradually increase in separation and range from no displacement (i.e. the same position as in the learning trial), to a maximal displacement similar to that used in the test trial of an OLT[15] (see also Step 10 – 24 of the PROCEDURE, **Fig. 1 and 3**). Utilizing these subtle differences in locations enables experimenters to find the exact cutoff where rodents are able to differentiate a new spatial arrangement from an earlier encountered one. This refined measuring of spatial memory depends on pattern separation processes[3]. When using the smallest possible displacement at which the animals are able to discriminate between the 'old' (learning trial) and 'new' (test trial) situation, it is assured that the presented locations are highly similar to the rodents, and thus pattern separation processes have to be utilized in order to make this discrimination. Another difference and advantage is that the OPS task allows the detection of spatial pattern separation bi-directionally at small spatial separations when using a 1 h inter-trial interval. Thus in the OPS paradigm, unlike in the OLT, there is a spatial arrangement of the objects that allows detection of improvement or impairment of discrimination performance within the same experiment.

The difference between the OPS task and the ORT is more apparent. The ORT is also a one-trial learning task, but allows the assessment of consolidation of specifically object information (instead of spatial information) into memory. In the first (learning) trial an animal is put into an arena

in which two identical objects are placed. After a certain delay, the animal is given a second (test) trial, in which it is again placed in the same arena but now one of the objects has been replaced by a novel object. It is scored how much time the animal inspects the two objects. Subsequently it can be determined whether the animal spent a different amount of time on the novel or the familiar object[12-14].

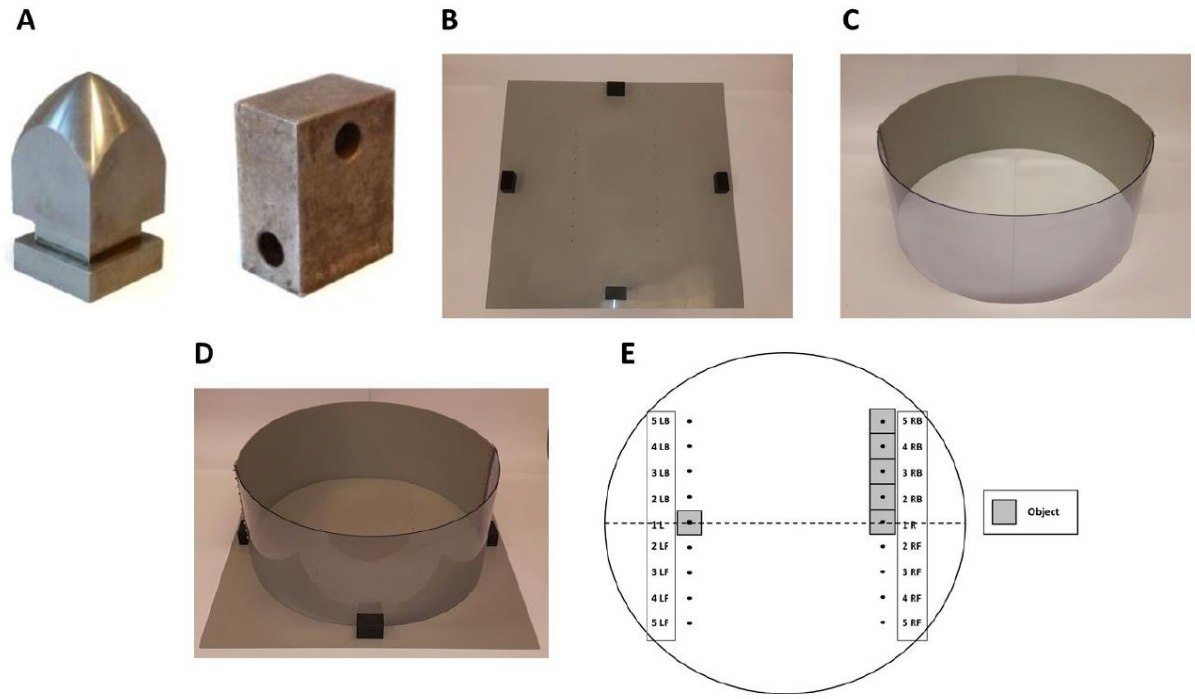

Figure 1 | *Objects, base plate and testing arena used for rats in the OPS paradigm.* ***(A)****: The two different objects used in the OPS task. Both objects are available in duplicate to form the object-sets. Usage of the two object-sets should alternate between OPS trials.* ***(B)****: The base plate of the OPS apparatus. On this base plate the testing arena can be placed between the black cubes (which are attached to the base plate), this ensures that the testing arena is always placed on the same location on the base plate (see also* ***D****). On the base plate all possible locations for the objects are indicated with black dots. These dots are 60 mm (rat base plate) or 45 mm (mouse base plate) apart and are indicated on both sides (left and right) and directions (forward and backward) of the testing arena (see also Fig 1E).* ***(C)****: The testing arena, the walls are half transparent and half grey or black.* ***(D)****: The testing arena put on the base plate, for scoring make sure the experimenter is located before the transparent part of the arena wall at an appropriate distance in order to reliably score the exploratory behavior of the animal while not disturbing the animal. For all exact dimensions of the apparatus and objects see the Materials section. For mice, similar equipment (although smaller) is used (see MATERIALS section).* ***(E)*** *Schematic representation of the top-view of the arena with the possible new locations for an object in T2 (indicated on the right). In the diagram, L and R refer respectively to 'left' and 'right'. Furthermore, B and F refer to 'backward' and 'forward' respectively, indicative of the general direction of the displacement of an object. The number indications (1-5) represent the 5 possible locations to where an object can be displaced. In T1 the placement of objects is always '1L' and '1R', in T2 (after a 1 h interval); one of the objects could get displaced to one of the new locations along the straight line (Position 1-5, see also Box 1). (Figure was adapted from: (van Hagen et al., 2014)[11,15])*

Comparison with other pattern separation tasks

Spatial pattern separation utilizes memory processes centered in the DG and CA3 region of the hippocampus[1,4]. As such, the OPS task is thought to rely on the capacity to perform hippocampal-mediated pattern separation. A comparable spatial task can be easily applied for humans and therefore the OPS task has significant translational value. In fact, a highly similar spatial pattern

separation task for humans has been described and has shown to be correlated with performance on the Rey Auditory Verbal Learning Test (RAVLT), which is sensitive to hippocampal dysfunction[3].

Alternative available pattern separation tasks are often based on fear conditioning[2] or radial arm maze[1] paradigms. Because the OPS task relies on the natural curiosity of rodents, especially for novel stimuli or places, it is not necessary to use positive or negative reinforcement in the form of reward or punishment incentives when using this paradigm. Thus the OPS task permits measurement of hippocampal driven pattern separation performance in the absence of any effect on the brain resulting from the punishment and reward incentives. Performance assessment in the OPS task relies on the ability of rodents to discriminate between small spatial changes in object locations within a familiar environment (**Fig. 1** and **Fig. 3**). When an object location is recognized by an animal as being different from one previously encountered, it will naturally spend more time exploring this new object location. Therefore, a difference in exploration times between the displaced and stationary object in the test-trial reflects pattern separation functioning. This is in contrast to pattern separation tasks that use emotional/fear components which will inherently utilize a broader range of (pattern separation) processes as opposed to solely cognitive spatial processes[9,10].

However because the spatial nature of the OPS task measures hippocampal driven pattern separation performance, this can alternatively be interpreted as a limitation of the paradigm when the research question demands measuring pattern separation on a broader level (i.e. when also wishing to assess emotional/fear components). Furthermore, OPS testing requires sufficient arousal, motivation and intact locomotor behavior of the animals in order to have sufficient exploration times of the objects and locations. When assessing the effects of drugs, genetic manipulations, lesions/ablations or other interventions on pattern separation performance, these aspects should be kept in mind. Interventions that alter the arousal, motivation or locomotor activity of the animals might bias the test results. Moreover, since ORT performance has been shown to be influenced by hormonal regulation in female rats[16], it is reasonable to suggest that hormonal regulation could affect performance in the OPS paradigm as well. We have not yet examined the applicability of female rodents in the OPS task.

Other alternative paradigms have been described that use three or more objects in a testing arena[7] or which are touch screen based[8]. Due to the simple nature and requirements of the set-up, the OPS task is less expensive and easier to use and interpret. The simplicity of the task has the additional advantage that no lengthy training procedures are needed in order for the assessor to learn how to interpret the rodent strategies or the rules that should be followed when performing a task. The use of different methods for rule- and strategy learning are a potential source for inter-study variability.

Experimental design

To allow for reliable OPS performance and measurements, important parameters for experimenters to consider before and during a study are listed below. Virtually anyone who is licensed to perform animal research and has experience with rodent handling and behavioral tasks can perform the OPS task. No specific level of research expertise or scientific background knowledge is required to follow the OPS protocol. Researchers thinking of implementing the OPS task are recommended to adequately teach themselves or technicians how to score exploratory behavior of rodents (see Step 16 of the PROCEDURE), as well-trained experimenters tend to need smaller group sizes and show more consistent results. Behavioral laboratories that already have adequate experience with ORT and/or OLT testing should have no problem implementing the OPS task within their rodent behavioral test battery. The OPS task, OLT and ORT can be performed in the same apparatus, which requires similar habituation and training. This makes the OPS paradigm useful for quick and easy assessment of spatial pattern separation performance which could potentially lead to more consistent and comparable spatial pattern separation studies across different laboratories.

Overview of protocol

Prior to embarking on a study, users need to choose and obtain appropriate animals to use as a model plus set up the required apparatus. Appropriate control groups must be chosen and used in order to minimize the risk of false positives or negatives. Figure 2 provides an overview of the whole procedure. The procedure itself starts with 3 preparatory sections: acclimatization (Steps 1-3); habituation (Steps 4-9); and training in the OPS task (Steps 10-31). Once these sections are completed the animals can be tested repeatedly. Three OPS task experiments can be performed per week, as described in Steps 32-36 of the PROCEDURE. Further OPS experiments can be undertaken on the same animals. If testing is combined with the use of pharmaceutical interventions, the animals can be tested repeatedly providing sufficient time to allow complete wash-out of the drug has elapsed between subsequent OPS testing sessions. For some studies, such as lesion/ablation studies, pre- and post-surgery studies can be performed. For studies such as these, post-experimental verification of the correct localization of the brain lesions/ablations should be carried out. Proper memory function of the animals used can be assessed during the OPS training steps (see Step 25 – 31 of the PROCEDURE). A characteristic linear OPS task performance (see **Fig. 4**) should result.

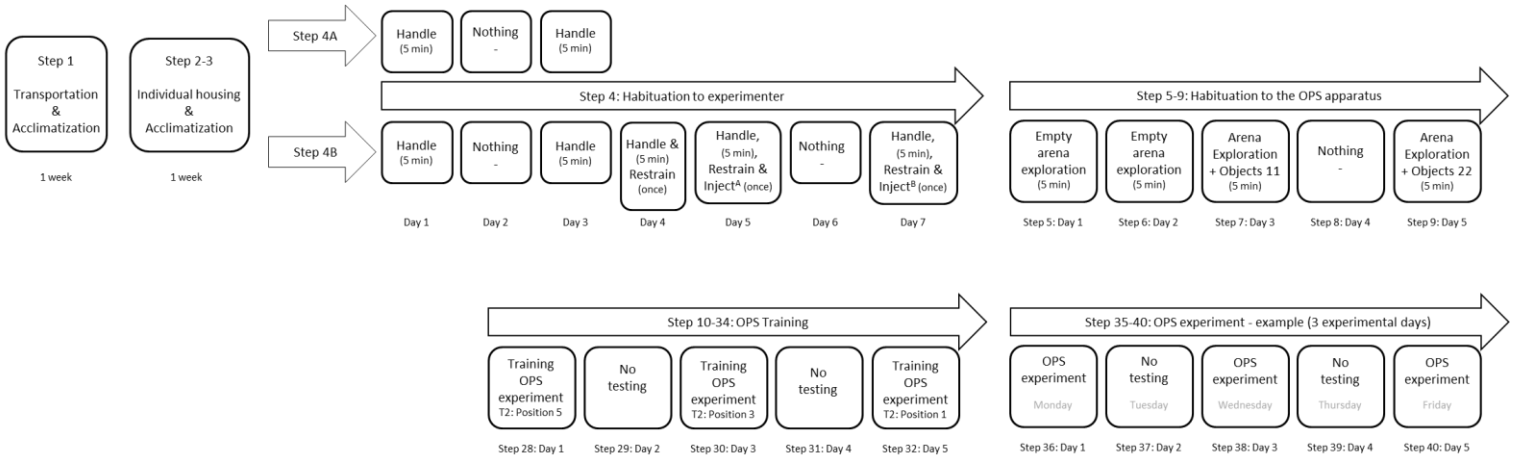

Figure 2 | *Flowchart of the OPS task procedure. This schematic overview shows all steps and timing for OPS training and testing as described in the Procedure section.*

Equipment

Setting up an OPS paradigm within a rodent testing facility requires a limited amount of equipment and expertise. Needed are a testing arena, a base plate and objects (see **Fig. 1**). Selecting appropriate objects for the rodents to explore is very important. When different object-sets are used in the OPS task, it is imperative that both object-sets are of equal interest to the rodents. The preference of one object-set over another will lead to confounding results in the experiment. In order to make sure all used object-sets are of equal interest to the rodents, pilot tests in which the exploration behavior towards all objects is meticulously assessed should be performed. Equal exploratory behavior/interest in the different objects is important. It has been shown that not all objects are of equal interest to rodents[17]. Furthermore, it is best to use objects that have a square/rectangular base in order to properly control the (dis)placement of the objects in the testing arena. Since the OPS task is used to assess recognition performance of very small changes in spatial rearrangements, it is imperative that the displacement of the objects is very precise (see **Fig 1** and **3**). This precise object displacement becomes obscured when objects with a big round base are used, because these are more difficult to precisely displace to the next location in the testing arena when using minimal floor markings.

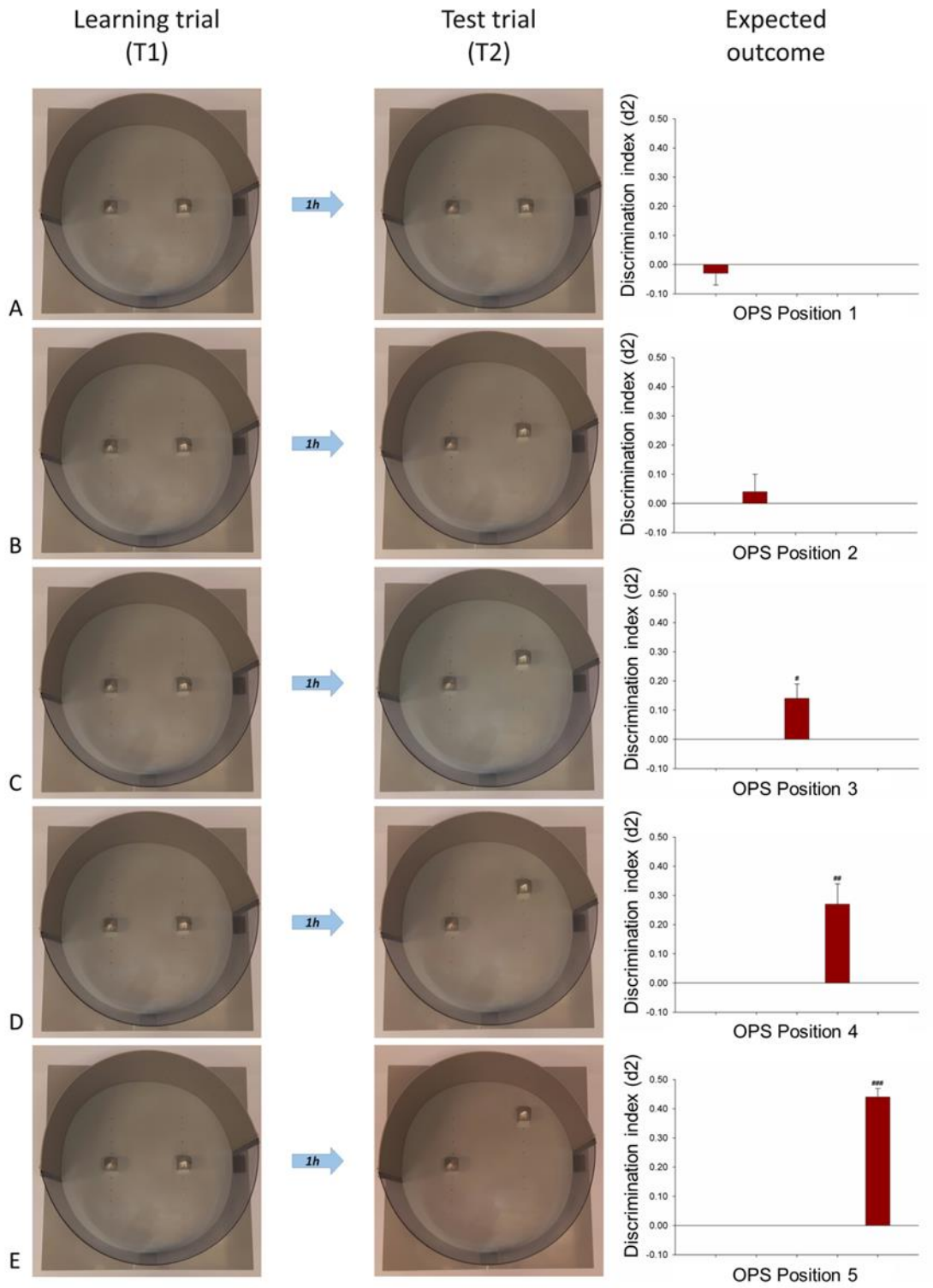

Figure 3 | *An example of object placement in both the learning trail (T1) and the test trial (T2). In this example one of the similar objects of the object-set is moved backward on the right side of the testing arena. The expected outcome of the discrimination index (d2, see also Table 6) is shown in the graphs in the right column under 'Expected outcome' (means + SEM). These graphs have been adapted from van Goethem et al. (2015)[11]. For this example, naïve Wistar rats were tested on position 1 (n = 13), 2 (n = 10), 3 (n = 14), 4 (n = 10) and 5 (n = 13). The data is similar as the data presented in Figure 4. The inter-trial interval is 1 h. Trial duration is 3 min for rats and 4 min for mice.* ***(A)****: In T1 both objects are placed symmetrically in the middle of the testing arena on the starting positions (position 1). In T2 position 1 is tested. No displacement, this means both objects should be placed symmetrically in de middle of the testing arena again, like in T1. Expected outcome, d2 ≈ 0.00.* ***(B)****: For T1 both objects are again placed on position 1. In T2 position 2 is tested. Minimal displacement, expected outcome, d2 ≈ 0.05.* ***(C)****: For T1 both objects are again placed on position 1. In T2 position 3 is tested. Displacement is half way, expected outcome, d2 ≈ 0.15.* ***(D)****: For T1 both objects are again placed on position 1. In T2 position 4 is tested, expected outcome, d2 ≈ 0.20 – 0.30.* ***(E)****: For T1 both objects are again placed on position 1. In T2 position 5 is tested. Displacement is maximal, expected outcome, d2 ≈ 0.30 – 0.45. Significant object location discrimination performance is found for position 3, 4 and 5 (or C, D and E; t-test compared to zero/chance level: #P < 0.05; ##P < 0.01; ###P < 0.001). Error bars show standard error of means.*

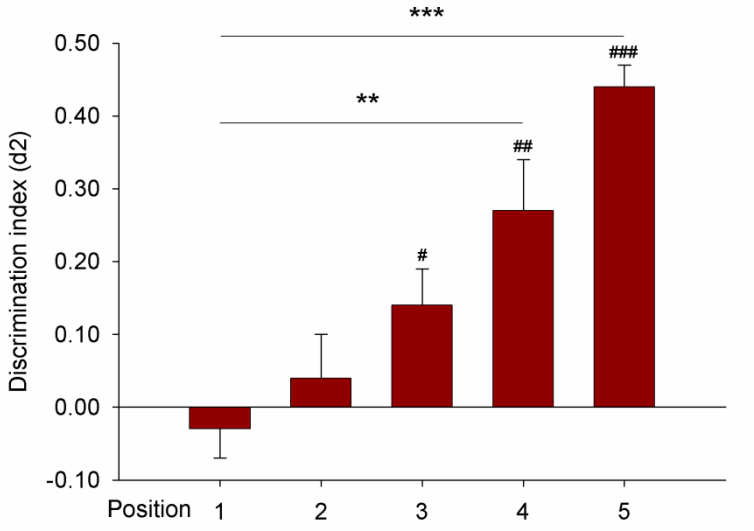

Figure 4 | *Characteristic OPS task results showing the d2 indices (means + SEM) for all five positions of untreated 4-month-old male Wistar rats (average weight 393 g). Naïve Wistar rats were tested on position 1 (n = 13), 2 (n = 10), 3 (n = 14), 4 (n = 10) and 5 (n = 13). Significant object location discrimination performance was found for position 3, 4 and 5 (t-test compared to zero/chance level: $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$), which increased with increasing distance from position 1 along the vertical axis (Position 3 < 4 < 5), which is indicative of spatial pattern separation. When compared with performance on position 1, position 4 and 5 differed significantly with respect to pattern separation performance, position 3 showed a trend (one-way ANOVA: $F_{4,55} = 13.89$; $P < 0.001$, Post-hoc Bonferroni t-tests: position 1 and 3, $P = 0.1$; position 1 and 4, $^{**}P < 0.01$; position 1 and 5, $^{***}P < 0.001$). On position 3, an increase or a decrease in pattern separation performance can be measured, i.e. performance after an experimental manipulation could resemble the performance on Position 5 or 1, respectively. Error bars show standard error of means. This figure was adapted from van Goethem et al. (2015)[11].*

Choice of Animals

So far, we have validated the OPS task with adult young (2-3 months old) male C57BL/6 mice and Wistar rats. We have found that Wistar rats perform well in this task when using a 1 h inter-trial interval and trial durations of 3 min[11]. Optimal inter-trial intervals and trial duration for the C57BL/6 mice were found to be 1 or 2 h and 4 min, respectively. A 30 min inter-trial interval was found to be too short for these mice, i.e. the performance was too high, leading to statistical ceiling effects and an inability to measure the OPS performance bi-directionally[15]. So far, we have not validated the 2 h inter-trial interval for rats, but we assume this interval could be used as well. Since we have not found differences in OPS performance between the 1 and 2 h inter-trial intervals in mice (on Position 3), we use the 1 h inter-trial interval in our OPS experiments. In addition, we have examined the potential of aged rats (2 year-old male Wistar rats) to be used in our OPS paradigm. Our preliminary findings indicate full applicability of aged male Wistar rats in the OPS task using a 1 h inter-trial interval and 3 min trial duration. We have not yet examined the applicability of other rat and mouse strains or rodent species (e.g., hamsters). If variations from expected results occur when testing aged animals, other strains or species, we suggest performing different inter-trial intervals (e.g., 30 min when the performance is too low, or 3 h when the performance is too high) or longer trial durations (e.g., 5 min or more when the exploration times are too low) to assess OPS performance. Since the

OPS task does rely on visual cues, the use of albino animals could be a matter of debate because of the well-known compromised visual acuity of albino animals as opposed to pigmented animals[16,18]. The use of an albino rodent strain might therefore affect certain parameters of behavioral tasks (e.g., the speed of training or discrimination performance). Despite these visual disabilities, the albino Wistar rat strain is often used in (spatial) behavioral paradigms which have led to reproducible results. New animal models should always carefully be assessed before they are used in the actual OPS experiments.

It is important to consider the sex of the animals when designing your experiment. It has been shown that the cognitive performance of female rodents fluctuates during the different stages of their estrous cycle[16]. In our experiments so far, we have only used male rats and mice. If female rodents are used in the OPS task we recommend, considering controlling for estrous cycle (i.e. estradiol and progesterone levels). Whilst we have validated the OPS task with adult male C57BL/6 mice and Wistar rats[11,15]. Other rodents strains (e.g., for rats: Long-Evans, Hooded Lister, Sprague Dawley, etc.; and for mice: OF1, NMRI, etc.) should also be useable in the OPS paradigm because they exhibit sufficient exploration of the objects[16]. Sufficient object exploration requires adequate energy levels, attention and interest in the objects. These traits may vary between rodent strains[16]. Therefore, we recommend to always carefully consider which rodent strain to use in any behavioral paradigm and always control for factors that could possibly confound the experimental results. Ways of controlling for these are for example performing pilot studies to assess proper performance (see the Expected results section in the ANTICIPATED RESULTS), but also the use different paradigms in order to make sure the chosen strain is able to perform as anticipated by the experimental design. To elaborate, apart from visual abilities, other possible confounders within an OPS task are locomotor, arousal and motivational aspects of the animals used. When the OPS task is applied to assess the pattern separation enhancing or impairing effects of experimental interventions, it should be verified that the seen improvement or impairment in OPS performance is not the result of possible confounding factors related to locomotor, arousal and/or motivational effects of the interventions. Additional tests/separate experiments which specifically assess these traits, before the actual OPS experiments start, are recommended. If possible, a within design (repeated measures) is recommended so every animal can serve as its own control. To obtain statistically reliable data, on average 12 to 16 animals per group are needed, more animals will strengthen statistical power. Novice experimenters tend score with higher inter-individual variability and therefore might need bigger group sizes when compared to well-trained experimenters. We thus recommend unexperienced experimenters to practice scoring during the habituation phase (Step 8 and 9).

Animal housing

Animal housing can be a source of stress for rodents. Care should be taken in regard to these procedures to warrant results that are reproducible and decrease variation in behavioral data/performance as much as possible. Upon arrival, reverse the day-night cycle of the animals in the vivarium (e.g., lights on from 7:00 a.m. until 7:00 p.m.). This way, the animals can be tested later on during the day in their active (dark) phase[19], under low illumination conditions (i.e. 20 lux provided by fluorescent red tubes and an indirect background light) (see Step 1 of the PROCEDURE). Individual housing is preferred (see Step 2 of the PROCEDURE) to ensure that the animals do not have too much interference between the learning and the test trial (see Step 10 – 24 of the PROCEDURE). Social housing leads to too much interference during the inter-trial interval and subsequently leads to decreased performance of the animals[16,20]. Individual housing can be a source of stress which in turn may affect the learning abilities, behavioral outcome and biochemical processes of rodents. These could potentially confound the OPS results. Stress and compromised well-being of the animals due to individual housing, could be assessed by corticosteroid measurements and careful observation of the animals. Before the actual OPS experiments start (see Step 32 – 36 of the PROCEDURE), proper cognitive function of the animals has to be assessed during the OPS training steps (see Step 25 – 31 of the PROCEDURE). With respect to individual housing and isolation stress, it has also been shown that individual housing leads to increased behavioral arousal in mice[21] and has no effect on stress signs[22]. Nevertheless, to minimize potential bias in the experimental results, acclimatization and habituation to the individual housing is imperative (see Step 2 of the PROCEDURE). Make sure to provide enough cage enrichment and keep this enrichment constant over the entire OPS procedure to avoid other possible interfering factors during the inter-trial intervals. It is important to keep the housing conditions of the animals constant during an OPS experiment. Always place the home-cage of an animal back at the same spot in the rack and never change the cage enrichment during an experiment. Novel objects in the home-cage can lead to interference during the inter-trial interval which can bias the results. The same applies for cleaning the home-cages; avoid cleaning or switching the cages on an experimental day (before the OPS experiment), as this could lead to stress and interference during the inter-trial interval[23]. This inter-trial interference also occurs when the animals are social- or group housed[20] and results in increased variance in the dataset, which can only be solved by increasing the animal group sizes.

Animal handling

Experimenter handling is always a source of stress for rodents in experimental studies[23]. Extensive habituation to the handling procedures are therefore imperative to minimize these stress levels of the animals (see also Step 4 of the PROCEDURE). Experimenter intervention during the OPS

testing can therefore be considered as a limitation to the paradigm. To minimize experimenter handling, clever designs can be found in the literature were a behavioral apparatus is used that requires only minimal animal handling due to utilization of holding areas within the testing apparatus. During the inter-trial interval, an animal can stay within this holding area until the next trial starts. This minimizes experimenter handling and also offers the possibility to make use of a continuous running protocol[24-27]. However, this does require an animal to stay within this holding area for the total duration of the inter-trial interval (which is in the case of the OPS task 1 h). Hence, to adapt such a behavioral apparatus to the OPS task would require modifications to keep the testing schedules workable (e.g., making use of multiple holding areas/chambers so multiple animals can be tested in sequence, or use multiple apparatuses). Habituation sessions to familiarize the animals to the experimenter and the handling procedures should be performed carefully (see Step 4 of the PROCEDURE). Not habituating the animals properly could lead to stressed animals and biased results. The whole idea of this procedure is to let the rodents get accustomed the smell and handling of the experimenter. Proper habituation to handling will greatly benefit the experimenter later on when the animals need to be handled regularly during testing and/or possible administration of substances. Ideally only one experimenter should be assigned to an animal cohort. This person should habituate the animals to the handling procedure and also perform the OPS experiment. Switching between experimenters is a cause of stress for rodents and will bias your experimental results[28]. Be gently with the rodents and avoid restraining them too much. Restraining an animal will lead to animals that are even more difficult to handle later on. Animals that do not habituate to handling well should be given a little more time to get accustomed to the handling procedure. Never pick up a rodent at the tip of the tail as this may cause severe skin injury and stress (see Step 4 of the PROCEDURE). Handling on additional days without restraining and/or injecting the animals will attenuate their stress levels during subsequent handling. Consider weighing your animals on separate days from the OPS protocol to increase low stress contact moments.

Timing of experiments

Since rodents are nocturnal species, it is important to carefully consider the timing of the OPS experiments. Nocturnal species are more active during the dark phase, so testing during the light (resting) phase is likely to result in behavioral differences, behavioral inhibition and/or cognitive disruption[19]. For this reason, we typically test our rodents in all OPS task experiments during the dark/active phase. The OPS task does rely on visual cues. Therefore the task should be performed in low light conditions. The lighting conditions should be about 20 lux which is equal in the different parts of the testing arena. After habituation to the reversed day-night cycle, we can test our animals in the daytime during their active (dark) phase (e.g., between 7:00 a.m. and 7:00 p.m.). Make sure to

allow enough time for the animals to get acclimated to the reversed day-night cycle (at least 2 weeks, see also Step 1 – 4 of the PROCEDURE) in order for their circadian rhythm to adjust. In addition to the impact of light, the behavioral outcome of rodents is also dependent on their circadian rhythm[19]. Consequently, when testing repeatedly, it is advised to conduct the OPS experiments on the same time on every experimental test day. Finally, in regard to rodent caretaking, it is important to consider when the cages of the animals are cleaned. Cage cleaning is quite stressful for rodents[23] and should therefore never occur right before an experiment. Plan the cleaning/changing of the cages either after the experiment or on a day when no testing occurs.

Testing environment

The housing and testing conditions of the rodents depend largely on the availability of space in the animal facility. Ideally, the animals should be housed in the same room that is dedicated to OPS testing. Since this is not always an option, careful considerations will have to be kept in mind when the animals are housed in a different room as where the testing takes place (see Step 3 of the PROCEDURE). To warrant reliable and reproducible results, the following parameters should be considered.

When housing and testing occurs within the same room, make sure the placement of the cages will not interfere with the behavior of the animals within the testing arena. Ideally, the animal cages should be placed on the right (50% of animals) and left (50% of animals) side of the testing arena at a sufficient distance (as far away from the testing arena as possible). This way, potential side preferences of the animals within the testing arena should be eliminated. To elaborate on this; because of the unavoidable noises (and odors) coming from the side on which the home-cages are placed, the animal which is being tested can get distracted and prefer this side of the arena. Since the OPS task is based on spatial arrangement of objects within the testing arena, this may confound the results. If the animals are housed on both the left and right side of the testing arena, this potential problem will be balanced out. Another possibility is to house the animals on the front and back of the testing arena. If your room is big enough this problem will less likely occur. It is always wise to check for side preferences (left and right / front and back) to assess if everything in your behavioral room is placed correctly (see also ANTICIPATED RESULTS).

When the rodents are housed in a different room from where they are tested, they will have to be transported to the testing room for the actual experiment. Ideally, the cages of the rodents are permanently placed in movable racks so the animals can be transported easily, with the least amount of stress. Make sure to habituate the animals to this transportation procedure as it may cause stress the first few times they are moved and placed in another room (e.g., perform this procedures twice before actual handling). Before handling (see Step 4 of the PROCEDURE) or testing (see Step 10 – 36

of the PROCEDURE) the animals, leave them undisturbed for at least 30 min in order for them to become acclimatized to the environment.

In case a dedicated room for OPS testing is available, the base plate, testing arena and OPS objects can stay untouched until the next testing session. Also, all other things in the room can stay similar (e.g., desk, PC, etc.). This way the same spatial arrangement of the room is guaranteed over subsequent test days, which is preferable when performing any behavioral rodent task related to spatial learning. Store the OPS objects that are not being used during the learning/test trial out of sight of the animals in the testing arena (e.g., under the table on which the testing arena is placed or behind the testing arena on the side of the grey/black half of the arena wall). If the testing room is used for different behavioral tests by multiple persons, the experimenter has to make sure that the testing conditions and lay-out of the room are exactly replicated over subsequent experimental days. Changes in the environment may cause variability in the behavioral outcomes.

Habituation to the OPS procedure

After the rodents are habituated to the handling by the experimenter (see Step 4 of the PROCEDURE), they have to get habituated to the OPS testing apparatus (see Steps 5 – 9 of the PROCEDURE). First the animals can get familiarized with the testing arena on two separate days. Subsequently the animals can get familiarized with the OPS objects within the testing arena again on two separate days. It is important to habituate/familiarize the animals with the sets of objects that are going to be used. Using novel objects on each experimental day is difficult to achieve if multiple testing sessions are contemplated (i.e. a lot of equally interesting objects will have to be found and used). The OPS task is designed for repeated testing and therefore the animals should get familiarized with the used objects so the pattern separation performance resembles an 'update' of the memory trace[29] (in contrast with reflecting absolute novelty[30]). If properly habituated, the animals can be tested for at least 10 times given that the two sets of objects are alternated between testing days.

When using mice, in order to increase their arousal at testing, mice are placed in an empty cage (only bedding, no enrichment) for 4 min immediately preceding the trials in OPS experiments. This will increase the exploratory behavior of the mice during testing[15,31]. In our lab, we only add this extra procedure when using mice, since rats show sufficient exploratory behavior when placed immediately from the home-cage into the testing arena. Therefore, when using mice, the extra procedure of making such an 'incubation cage' should be taken into account when preparing the testing room. Mice should get accustomed to the empty cage and the procedure (during Steps 8 and 9 of the PROCEDURE) 4 min before they go into the testing arena with the two similar objects. The bedding material should be changed after testing every cohort of animals. This allows the bedding to

be soiled in a similar fashion when the animals are tested in the same order on each habituation day. Alternatively, an empty cage without bedding could be used, but make sure to clean this cage after every animal. This will be more time-consuming as the cage needs to be cleaned with an ethanol solution and the ethanol needs to be evaporated completely before a new mouse can be put in the empty cage.

Planning for OPS testing

The animals are familiarized with the OPS objects during habituation to OPS testing (Steps 8 and 9 of the PROCEDURE). During the actual OPS testing the rodents' reaction to changes in the position of the OPS objects is monitored. Thus, the precise positioning of the objects for each rodent and each test needs to be determined prior to starting the OPS task using a randomization schedule (discussed further in the OPS testing section, see also **Table 4-5**). Objects are displaced to one of five different positions numbered 1-5 along a vertical axis. These positions are indicated on the base plate of the OPS apparatus (**Fig. 1**). The most straightforward way of measuring pattern separation is by first establishing performance on position 1, 3 and 5. **Figure 1E and 3** show the various possible positions in the arena visually and **Box 1** lists what use of each position means.

Box 1 | Positioning of the OPS objects

Position 1: No displacement. The placement of the objects is exactly the same as during T1. Therefore, an improvement on position 1 is impossible, as the conditions in T1 and T2 are exactly the same. The d2 value should be ~0.00 (i.e., chance level) or should at least not significantly differ from zero ($P > 0.05$). Position 1 serves as a control, as no pattern separation can or should be detected here.

Position 2: At position 2, the displacement of one of the objects is very minimal, and rodents normally show no to only very slight pattern separation performance at this position. When an improvement is found at position 3 within a certain experimental condition, the experimenter could consider testing the experimental condition at position 2 as well. By experimentally increasing pattern separation performance at position 3, this performance might increase at position 2 as well. Therefore, position 2 can be used to further investigate or refine the improved performance within an experimental condition. If no effect or even an impairing effect is found at position 3, the experimenter should consider increasing the displacement to position 4 (i.e., when an impairment in performance is found at position 3, it should also be present at position 2). The d2 value should be ~0.05 or slightly above chance level. The d2 value will not significantly differ from zero/chance level ($P > 0.05$) in naive/ untreated animals.

Position 3: At position 3, the displacement of one object is exactly halfway between the displacements at positions 1 and 5. This is the most relevant position for testing experimental conditions. The d2 value should be ~0.15 and significantly differ from zero/chance level ($P < 0.05$) and could show a trend or a significant difference ($P < 0.05$) from performance at position 1 (ref. 15). A significant difference from chance level ($P < 0.05$) indicates that the rodents perceive the displaced object as being in a 'novel' location, meaning they show functioning spatial pattern separation. At position 3, performance can vary bidirectionally, i.e., the d2 value can either decrease or increase (as compared with baseline, d2 ≈ 0.15), depending on the experimental condition that is tested. For example, in drug testing, a pattern separation–impairing drug will decrease the d2 value (e.g., from 0.15 to 0.00), whereas a pattern separation–improving drug will increase the d2 value (e.g., from 0.15 to 0.40). This way, a pattern separation–improving drug can show 'position 5 performance' (d2 ≈ 0.40) at position 3, whereas a pattern separation–impairing drug can show 'position 1 performance' (d2 ≈ 0.00) at position 3 (ref. 11). The same holds for, e.g., lesion studies, food-deprivation studies and genetic manipulations.

Position 4: Position 4 is located between position 3 and position 5. Similar to position 2, position 4 is considered to be a measure that can be used to further investigate or refine the performance found at position 3. When testing an experimental condition and no or only a slight effect is found at position 3, the experimenter could consider testing the experimental condition at position 4 to see whether performance there is enhanced as compared with baseline (i.e., the control group). Testing at position 4 when an improvement has already been found at position 3 would not be very informative, because of ceiling effects (i.e., when an improvement in performance has already been found at position 3, it should also be present at position 4). The d2 value should be between 0.20 and 0.30 and should significantly differ from zero/chance level and performance at position 1 ($P < 0.05$).

Position 5: This position represents maximal displacement. At position 5, the performance cannot go higher, as the displacement is maximal here. Therefore, at position 5 a ceiling effect is encountered (i.e., OPS performance cannot improve any further). The only pattern separation effect (or spatial memory effect) that can be measured at position 5, with a 1-h inter-trial interval, is performance impairment (similar to the OLT[32]). The d2 value should be between 0.30 and 0.45 in naive/untreated animals and should significantly differ from zero/chance level and performance at position 1 ($P < 0.05$).

OPS testing

Animals undertake the OPS task during training in the OPS task (Steps 10-24 of the PROCEDURE), performance measurement (Steps 25-31 of the PROCEDURE) and the actual tests (an example is described in Steps 32-36 of the PROCEDURE). The OPS task is a two trial spatial learning paradigm. The task starts with the first trial, which is called the learning trial or T1. After T1, there is a second trial (1 h after the end of T1), which is called the test trial or T2. The object exploration times of this trial are used to calculate the discrimination index (d2) to assess the pattern separation performance of the animals (see ANTICIPATED RESULTS, **Table 6**).

Only relative short inter-trial intervals (i.e. 1 or 2 h) can be utilized in the OPS task in order to reliably measure spatial pattern separation bi-directionally[15]. Using longer inter-trial intervals within the OPS paradigm leads to statistical floor effects. At longer inter-trial intervals, small spatial

separations are not remembered by rodents, leading to a need to increase the spatial separation to a level which could arguably require (normal) long term spatial memory instead of pattern separation processes. Having to use a short inter-trial interval (i.e. 1 or 2 h) results in a potential limitation of the OPS paradigm when wanting to differentiate between the different phases of memory functioning (i.e. the memory acquisition, consolidation and retrieval processes). The action of a certain intervention is likely to overlap the different phases of memory functioning when utilizing a short inter-trial interval. Therefore, we utilize the OPS task as a paradigm to measure spatial pattern separation per se, not specified to specially an acquisition, consolidation or retrieval memory process.

The duration of both T1 and T2 is 3 min for rats[11] and 4 min for mice[15]. This trial duration is in line with earlier studies using object recognition or object location as a primary readout[16,32]. Although short, we have found robust pattern separation performance when using this trial duration [11,15] (**Fig. 4**). Having a longer trial duration for mice (4 min) helps to increase their exploration time and discrimination performance. Therefore, when planning an OPS experiment, it's feasible to use a 5 min planning for rats (5 min per rat), and a 6 min planning for mice (6 min per mouse) (see **Table 2-3**). With a 3 min (rat) and a 4 min (mouse) trial duration, 2 min will remain which can be used for cleaning, changing objects and handling animals (and optional administration of drugs).

Careful analysis of a large dataset of ORT results has shown reliable object discrimination performance (i.e. a stabilized discrimination index (d2), see also Statistical analysis of the ANTICIPATED RESULTS) when familiarized rats manifested a total exploration time of minimally 7 sec in T1 and 10 sec in T2. Although these minimal exploration times for both objects seem low, apparently rats are able to learn object features within this relative short timeframe[30]. Even though the ORT is not a spatial task, this cutoff for total exploration time has been applied for the OLT in familiarized animals as well (both for rats and mice)[32,33]. Our results indicated that a similar cutoff can be applied to the OPS task. Hence we believe that in order for the animals to properly learn the spatial arrangement of the objects in the testing arena, a similar cutoff in minimal exploration time as found for the ORT can be used (i.e. T1/e1 ≥ 7 sec and T2/e2 ≥ 10 sec for familiarized animals[30]) (see Step 10 – 24 of the PROCEDURE). We rarely encounter non-performers in the OPS task when using either male Wistar rats or C57BL/6 mice. When encountered, this probably relates to elevated anxiety and/or stress levels which could be counteracted by proper habituation of the animals to the test and handling procedures. Alternatively, using different inter-trial intervals (e.g., 30 min when the performance is too low, or 3 h when the performance is too high) or longer trial durations (e.g., 5 min or more when the exploration times are too low) could help minimizing the amount of non-performers. Animals that do show a lower exploration time in one or both of the OPS trials should be removed from the dataset.

The randomization scheme should be designed in which both the positions to be tested in T2 and the objects used are assigned in a balanced manner to prevent potential biases due to preferences for particular locations or objects. An example is given for 12 animals in the appended randomization scheme in which position 1, 3, and 5 (**Table 4**) or position 3 only (**Table 5**) are tested in a balanced way with two objects. Trained animals have to be assigned (pseudo-) randomly to experimental groups to warrant reliable results (for examples see **Table 4-5**). Moreover, if applicable the experimenter should always be blinded to the treatment condition of the animals.

Table 1| TROUBLESHOOTING

Step	Problem	Possible reason	Solution
3, 26, 28, 30, 32, 34, and 36	The rodent shows side preference in the testing arena	Home cages are located on the same side of the testing arena that the animal prefers	House (or place) the animals on both sides (left and right OR front and back) of the testing arena
		Stressors or noises in the testing room	Check the testing room for possible stressors (e.g., temperature, humidity, lighting conditions, noises and airflow)
		The testing arena is unequally lit	Make sure the testing apparatus is equally lit across the base plate (~20 lux)
		Home cage of the animal is too close to one side of the testing arena	Place the home cage further away from the testing arena
4A(iii)	After handling the rodent twice for 5 min, the animal still appears uncomfortable	The animal is stressed or anxious	More handling is necessary before starting the actual OPS procedure. Handle this animal twice more for 5 min each on separate days (Step 4)
			Check the testing room for possible stressors When transporting animals to a testing room, prolong the time to acclimatize before the start of the experiment
16	Automated nose-tracking with a video-tracking system and software leads to false-positive object exploration	Unreliable/inconsistent nose-tracking of the animal—e.g., when an animal is sitting on top of an object and is looking up, its nose will be in the indicated region of interest. Although this is not considered to be exploratory behavior, a video-tracking system might not be able to tell the difference	Filming from multiple sides of the testing arena might solve this issue. The video-tracking system and software must be compatible with this. Consider manually scoring the trials from recorded video files

Table continued

Step	Problem	Possible reason	Solution
16, 19, 21, 22, 24, 26, 28, 32, 34, and 36	The rodent does not explore the objects sufficiently (i.e., <7 s during T1 and/or <10 s during T2)	The animal's arousal is too low during testing	For mice: place the mouse in an empty cage (only bedding, no enrichment) for 4 min immediately preceding the learning and test trials (both T1 and T2) to increase the exploratory behavior of the mouse (Step 12)
		The animal is stressed or anxious	Check the testing room for possible stressors (e.g., temperature, humidity, lighting conditions, noises and airflow); make sure the home cage has not been cleaned on the experimental day; when transporting animals to a testing room, prolong the time to acclimatize before the start of the experiment; increase the trial duration (e.g., by 1 min)
		The animal is not interested in the objects	Make sure to have interesting objects for the animals to explore (such as those shown in Fig. 1). Objects can be made more attractive by adding holes to them (always habituate/ familiarize the animals with the objects before testing)
		When drugs have been administrated to the animal, arousal and/or locomotor activity of the animal may be altered (side-effects)	If possible, administer the drug earlier; check whether the effects hold after repeated administration; increase the trial duration (e.g., by 1 min)
21, 22, 23, 24, 26, 28, 30, 32, 34, and 36	d2 values at positions 1, 3 and 5 are not similar to those of the examples given in Fig. 4	Objects are still too novel (leads to higher performance than anticipated)	Repeat Steps 8 and 9, i.e., show both object sets again While in the experiment (Steps 32–36), repeat Steps 25–31 until stable OPS performance is shown
		The animal is stressed or anxious	Check the testing room for possible stressors (e.g., temperature, humidity, lighting conditions, noises and airflow)
			Make sure the home cage has not been cleaned on the experimental day
	The d2 value does significantly differ from zero/chance level while using position 1 and/or 2 in T2 ($P < 0.05$) The baseline (i.e., no experimental condition) d2 value does not significantly differ from zero/chance level ($P > 0.05$) while using position 3, 4 or 5 in T2 (i.e., OPS performance is too low)	Side preference in the testing arena Objects are incorrectly placed in the testing arena during T1, T2, or both	Check which side is preferred or avoided by the animal and subsequently check the behavioral room to see whether there are factors that could explain this behavior. Known causes of side preferences are unequal light conditions on the floor of the testing arena, home cage that is too close to the testing arena, housing of conspecifics on one side of the testing arena, and uneven airflow or noises in adjacent rooms (stressors). Exclude the animal
			Carefully place the objects at the correct locations (both T1 and T2)
		Animal is stressed or anxious	Check the testing room for possible stressors (e.g., temperature, humidity, lighting conditions, noises and airflow); make sure the home cage has not been cleaned on the experimental day
	The baseline (i.e., no experimental condition) d2 value does not significantly differ ($P > 0.05$) from that of position 1 while using position 3, 4 or 5 in T2	Objects are incorrectly placed in the testing arena during T1, T2, or both	Carefully place the objects on the correct locations (both T1 and T2)
	Pattern separation performance varies within the same position	Animal is stressed or anxious	Check the testing room for possible stressors (e.g., temperature, humidity, lighting conditions,

Table continued

Step	Problem	Possible reason	Solution
	between similar groups on different experimental days		noises and airflow); make sure the home cage has not been cleaned on the experimental day
		Objects are incorrectly placed in the testing arena during T1, T2, or both	Carefully place the objects on the correct locations (both T1 and T2)
		Testing conditions differ between experimental days	Always start testing at the same time on an experimental day (effects of circadian rhythm[19])
		Animals are stressed or anxious during an experimental day	Check the testing room for possible stressors (e.g., temperature, humidity, lighting conditions, noises and airflow); make sure the home cage has not been cleaned on the experimental day; when transporting animals to a testing room, prolong the time to acclimatize before the start of the experiment; shared testing room—mark the position of the setup and place it back in precisely the same location
	d2 index is 1 or −1	The animal hides behind one of the objects, resulting in observed exploration of only one object	Animal is stressed; exclude the animal from the dataset
		Side preference (lateralization)	Resolve side preferences as indicated elsewhere in this table
		Objects are still too novel (leads to higher performance than anticipated)	Repeat Steps 8 and 9, i.e., show both object sets again. While in the experiment (Steps 32–36), repeat Steps 25–31 until stable OPS performance is acquired
		Animal is stressed or anxious	Check the testing room for possible stressors (e.g., temperature, humidity, lighting conditions, noises and airflow); make sure the home cage has not been cleaned on the experimental day

Table 2| Testing schedule for two OPS experiments using mice in the presence or absence of injection.

Time	Exp 1 T1	Exp 1 T2	Exp 2 Inj.	Exp 2 T1	Exp 2 T2
0:00	1		1		
0:06	2		2		
0:12	3		3		
0:18	4		4		
0:24	5		5		
0:30	6		6	1	
0:36	7		7	2	
0:42	8		8	3	
0:48	9		9	4	
0:54	10		10	5	
1:00		1		6	
1:06		2		7	
1:12		3		8	
1:18		4		9	
1:24		5		10	
1:30		6			1
1:36		7			2
1:42		8			3
1:48		9			4
1:54		10			5
2:00					6
2:06					7
2:12					8
2:18					9
2:24					10

The timing information is in the format hour:minute. The numbers in the tables correspond to the individual animal numbers. T1 and T2 are respectively, the learning trial and the testing trial of the OPS task. The number of the animal to be injected at each time point is indicated in the 'Inj.' column. This OPS test schedule for mice that have to be injected with a drug that has a T_{max} of 30 min and has to be maximally active during the memory acquisition phase (i.e. the animal has to be injected 30 min before the learning trial T1).

Table 3| Testing schedule for two OPS experiments using rats in the presence or absence of injection.

Time	Exp 1 T1	Exp 1 T2	Exp 1 Inj.	Exp 1 T1	Exp 1 T2
0:00	1		1		
0:05	2		2		
0:10	3		3		
0:15	4		4		
0:20	5		5		
0:25	6		6		
0:30	7		7	1	
0:35	8		8	2	
0:40	9		9	3	
0:45	10		10	4	
0:50	11		11	5	
0:55	12		12	6	
1:00		1		7	
1:05		2		8	
1:10		3		9	
1:15		4		10	
1:20		5		11	
1:25		6		12	
1:30		7			1
1:35		8			2
1:40		9			3
1:45		10			4
1:50		11			5
1:55		12			6
2:00					7
2:05					8

The timing information is in the format hour:minute. The numbers in the tables correspond to the individual animal numbers. T1 and T2 are respectively, the learning trial and the testing trial of the OPS task. The number of the animal to be injected at each time point is indicated in the 'Inj.' column. This OPS test schedule is for rats that have to be injected with a drug that has a T_{max} of 30 min and has to be maximally active during the memory acquisition phase (i.e. the animal has to be injected 30 min before the learning trial T1).

MATERIALS

Reagents

- Rodents: for the assessment of spatial pattern separation performance using the OPS task, both commercially available or in-house laboratory-bred rodents can be used. In our experiments, we normally use adult male Wistar rats (2-3 months old) or adult male C57BL/6 mice (2-3 months). Both the Wistar rats and C57BL/6 mice we use are ordered from Charles River (Sulzfeld, Germany; Wistar rat: Crl:WI, 003 and C57BL/6 mouse: C57BL/6NCrl, 027). These rodent strains are known to show robust performance in memory tasks regarding object recognition or location performance[16,32].

!CAUTION: All experiments performed on rodents must be performed in accordance with relevant governmental guidelines and regulations after being approved by a local ethical committee for the care and use of laboratory animals. All procedures should be designed and performed to minimize the potential discomfort of the animals during the behavioral experiments. Our experimental procedures are in strict accordance with the Dutch Experiments on Animals Act (EAA, amended 1996) and the European Directive (2010/63/EU) of the European Parliament and of the Council of the European Union (86/609EEC) on the protection of animals used for scientific purposes (22 September 2010) and were approved by the local ethical committee for animal experiments of Maastricht University. Maastricht University has a license to perform animal experiments (license ex art 2, 1 and art6 EAA: DGVGZ/VVP-83267). The detailed project proposal of the studies conducted to validate the herein described OPS protocol was approved after careful evaluation of an ethical committee of Maastricht University (license animal ethical committee: Min.VWS, GZBIVVB 981845, 16 April 1998). The assigned official protocol numbers provided to these studies were: DEC 2012-062 and DEC 2013-013 for the rat studies and DEC 2013-059 for the mouse studies.

- Ethanol (70% (vol/vol), Boom B.V. The Netherlands, cat. no. 84010059.2500, or similar)
- Laboratory animal bedding (Tecnilab-BMI, Rehofmix corncob bedding for rodents MK1500, cat. no. 97150 or other approved bedding for rodents)
- Rodent chow in pellet form (SSNIFF rat/mouse maintenance, 10mm sterilized 25kGy, bag of 10 kg cat. no. V1534-703 or other approved rat/mouse chow). Store appropriately, particularly hygienic, cool, and dry. Optimum storage condition ≤18°C. Closed bags can be stored until expiration date. Open bags should be used within 6 weeks provided that they were properly stored.

Equipment

- Animal housing (see EQUIPMENT SETUP)
- Water bottles that are compatible with both the IVC rat and mouse cages (transparent polycarbonate bottles 300 ml, Tecniplast cat. no. ACBT0262). Our water bottles are equipped with polished AISI 316 stainless steel bottle caps with a 25 mm drinking nipple with a 1.8 mm diameter hole for rats (Tecniplast cat. no. ACCP2521) or a 65 mm drinking nipple with a 2.2 mm diameter hole for mice (Tecniplast cat. no. ACCP6521).
- OPS objects (see Equipment setup)
- Worktable on which the testing apparatus is placed (base plate, testing arena and objects). We use a normal white work table/office desk (1600 mm x 800 mm x 750 mm) (Gispen IC).
- Viscose cleaning cloths (Sorbo cat. no. 09660604) or other cleaning cloths (e.g. paper tissues).
- Computer (see Equipment setup).
- Table to put the PC on. We use a normal white working table/office desk (1600 mm x 800 mm x 750 mm) (Gispen IC) and a chair for the experimenter to sit on.
- Fluorescent red tubes and a small background light (e.g. a desk lamp, 25 W) which can be dimmed with white filter paper if the light intensity is too bright (> 20 lux).

  !CRITICAL: Test the animals preferably in their active phase (i.e. night phase). The OPS task does rely on visual cues. Therefore the task should be performed in low light conditions. The lighting conditions should be about 20 lux which is equal in the different parts of the testing arena and the inside of the home-cages of the animals during testing. Higher light intensities could lead to distress, behavioral disturbances and circadian rhythm suppression of the animals[23]. These might affect the behavioral outcome of the animals, and hence confound the OPS results. Housing conditions during the dark phase, when no testing occurs, should be approximately 1 lux.
- Radio to provide background noise in the housing room/vivarium 24 h per day. This way the animals get accustomed to noises in the housing room/vivarium.
- Permanent marker (Edding 400 round-tip, black (col. 001) or equivalent).
- Electronic timer (e.g. stopwatch or timer).
- Squeeze bottles (Nalgene Right-To-Know Wash Bottle for Ethyl Alcohol, LDPE, 500 mL; Thermo Scientific, cat. no. 20-2425-0502 or equivalent).
- Laboratory gloves; use powder-free nitrile or vinyl gloves as rodents dislike (the texture and/or odor of) latex gloves (Kimtech science, purple nitrile gloves; cat. no. 90625, or equivalent).
- (Optional) Digital camcorder with stand and mount to record OPS task trials from above (to do re-scoring or scoring of the exploratory behavior later on).

- (Optional) Video-tracking system with adequate nose tracking software, for example, Noldus Information Technology - EthoVision XT software, or other commercially available software.

Reagent setup

Rodents: Rats and mice should be maintained on a reversed 12-h light/dark cycle with lights off between 7:00 a.m. and 7:00 p.m so the animals can be tested during the day in their active (dark) phase. Keep rodents in a vivarium that is both temperature- (22 ± 1 °C) and humidity-controlled (55% ± 10%) with free access to food and water. Appropriate requirements for rodent husbandry and environment can be found in Appendix A of the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (ETS No. 123).

All OPS task experiments should be performed during the dark/active phase of the rodents (e.g., between 7:00 a.m. and 7:00 p.m.) and conducted on the same time on every experimental test day when testing repeatedly. The experimenter should not change his or her 'olfactory signature' (and avoid volatile odors, see also Step 4 - !CAUTION of the PROCEDURE) while interacting with the animals throughout the entire experiment. Moreover, the experimenter should have sufficient experience in scoring the exploratory behavior of the animals (see also Step 16 of the PROCEDURE), and if applicable, always be blinded to the experimental condition of the animals, both during testing/scoring and during the analyses of the results.

Equipment setup

Rodent home-cages and test rooms in the vivarium: A sufficient number of home-cages must be available for proper housing of the rodents (including bedding, food, water and cage enrichment). In our animal facility we use an individually ventilated cage (IVC) system. Conventional rodent cages will also suffice as long as they meet the international guidelines for the proper care and use of laboratory animals. For our IVC system for rats we use: transparent polysulfone plastic (Sealsafe plus) Green Line cages with a floor area of 904 cm^2, with a standard stainless-steel wire food lid and a standard polysulfone plastic top that can hold two water bottles (Tecniplast, cat. no. GR900). For mice we use: transparent polysulfone plastic (Sealsafe next) Blue Line Next cages with a floor area of 435 cm^2, with a standard stainless-steel wire food lid and a standard polysulfone plastic top that can hold one water bottle (Tecniplast, cat. no. 1145T). Place the cages in wheel racks. We use stainless-steel racks on wheels, for holding the IVC cages. Our rack configuration accommodates 28 IVC Green Line cages for rats (Tecniplast, cat. no. GR28, 28 cages 4w x 7h, single sided), or 72 IVC Blue Line Next cages for mice (Tecniplast, cat. no. TN72, 72 cages 8w x 9h, single sided). All IVC racks are coupled to an air handling unit (Tecniplast, Smart Flow). House the rodents in a dedicated room in the vivarium. The rodents can be housed in the room in which testing will take place, or in a

separate room dedicated to housing only (see Step 3 of the PROCEDURE). The home-cages should contain adequate cage enrichment. Examples of suitable items for rats include cardboard tubes, 90 mm in diameter x 125 mm long, thickness cardboard 5 mm (Bioservice/Datesand, play tunnels rat, cat. no. CS3B02A); irradiated Aspen bricks, 100 x 20 x 20 mm (Bioservice/Datesand/Tapvei, cat. no. CS3C09); carton nesting material (Datesand, Sizzlenest suitable for all species, cat. no. CS1a09), and additional twisted paper nesting material (Harlan, Teklad, cat. no. 7979C.CS). Suitable cage enrichment for mice includes Cardboard tubes, 75 mm in diameter x 38 mm long/high, thickness cardboard 1.25 mm (Bioservice/Datesand, play tunnels mouse, cat. no. CS3B01); red transparent polycarbonate hiding house with 2 holes/entrees, 100 x 90 mm at the base and 75 x 65 mm at the top (Bioservice/Zoonlab, mouse house, cat. no. 3084010); carton nesting material (Datesand, Sizzlenest suitable for all species, cat. no. CS1a09); and additional twisted paper nesting material (Harlan, Teklad, cat. no. 7979C.CS).

Circular testing arena: An arena with walls that are 400mm high is required. Half of the 400 mm high wall should comprise grey (RAL7035) or black (RAL9005) polyvinyl chloride, the other half of transparent polyvinyl chloride. For rats the diameter should be 830 mm and for mice the diameter should be 400 mm. Ours was made by our local technical department IDEE, Maastricht University; see also **Fig. 1**).

!CRITICAL: In our experiments we always use a circular testing arena to prevent a rodent from spending too much time in the corners (as is often the case when using square testing areas). Especially when placing objects in a square arena, the tendency to hide between the corners and objects becomes higher. Always habituate the animals extensively to the testing arena and procedures before actual testing (see also Step 5 – 9 of the PROCEDURE).

!CRITICAL: One half of the circular testing arena has to be made out of transparent material in order for the observer to do live visual scoring (if live visual scoring is not performed the testing arena can be entirely made out of grey or black material and trials are subsequently filmed from the top, see also Step 16 of the PROCEDURE).

Arena floor or base plate: A square 1000 mm x 1000 mm (rats) or 600 mm x 600 mm (mice) floor is required and should be made out of either grey (RAL7035) or black (RAL9005) polyvinyl chloride. Indicate all possible locations for objects in the arena using a permanent marker (see **Fig. 1**), starting with a mark in the middle and making 4 further marks so that they are 60 mm apart for rats and 45 mm apart for mice. When faded, re-draw again with a permanent marker. (Our arena was made by our local technical department IDEE, Maastricht University, see also **Fig. 1**).

!CRITICAL: The advantage of using a grey colored base plate is that animals with both white or dark coat colors show sufficient contrast to the floor for clear video recording and subsequently for reliable video scoring or automated tracking.

Cleaning of the testing arena, base plate and objects: The testing arena, base plate and objects must be cleaned and dried thoroughly before testing. Cleaning can be performed with a 70% ethanol solution and a viscose cleaning cloth. It is important to clean the base plate and the objects after every animal/trial (see Step 5 – 9, of the PROCEDURE - CRITICAL STEP).

OPS objects: Two different sets of objects are required. Those we use for rats and mice are similar in shape and material and only differ in size. We use two different kinds of objects. These are: a massive metal cube (rats: 100 mm high x 50 mm width x 75 mm long; mice: 75 mm high x 25 mm width x 50 mm long) with two holes in it (diameter: 19 mm and 15 mm for rats and mice, respectively), and a massive aluminium cube with a square base and a tapering top (rats: 120 mm high x 60 mm x 60 mm (base); mice: 85 mm high x 45 mm x 45 mm (base)) and a milled slot with a small hole (diameter 5 mm) drilled halfway through (all objects are made by our local technical department IDEE, Maastricht University) (see also **Fig. 1**).

!CRITICAL: It is best to use objects that have a square/rectangular base in order to properly control the (dis)placement of the objects in the testing arena.

Personal computer: A personal computer is required to manually live score the exploratory behavior of the rodents and/or for video-recording of the OPS trials. We use our own in-house developed software (ORT scoring v2.1, developed by Manon Schaap with Delphi application, Object Pascal programming, Maastricht University 2008). In this program, exploration of either object is indicated by the experimenter with simple key presses and its basic output is the total amount of time (in ms) an animal explores the left and right object individually. Other software tools or timers/stopwatches could be used as well.

!CRITICAL: In our experiments we prefer live visual scoring (and sequent re-scoring of videotaped trials by another observer) over automated scoring/tracking programs. Since exploratory behavior of rodents is a rather complex behavior to be detected by automated tracking software, many false positives tend to occur with automated scoring[34] (see also Step 16 of the PROCEDURE).

(Optional) Video-tracking system: Install a digital camcorder above the testing arena on a stand and mount it in such a way that the entire floor within the testing arena is visible and the exploration behavior of the animals can be recorded from above. If using nose-tracking software, connect the camera to the video-tracking system being used. Ensure all the tracking parameters of your video-tracking system are optimized to perform proper nose-tracking of your rodents within your vivarium environment and for your OPS apparatus (testing arena, base plate and objects).

Table 4| Randomization schemes of objects and locations for OPS testing on Position 1, 3 and 5

		Learning trial (T1)	Test trial (T2)				
Animal number	Objects	Position of stationary objects	Object moved left backward	Object moved left forward	Object moved right backward	Object moved right forward	New position of the object
1	22	1	NA				1
2	11	1			X		3
3	22	1	X				5
4	11	1	NA				1
5	22	1		X			3
6	11	1				X	5
7	11	1	NA				1
8	22	1			X		3
9	11	1	X				5
10	22	1	NA				1
11	11	1		X			3
12	22	1				X	5

Randomization scheme for 12 animals to be tested on position 1, 3 and 5 in the OPS task. From left to right the columns show: 'Animal nr.', this corresponds to the ID numbers of the animals; 'Objects', refers to the object-set, here the sets of two objects are called '11' (object-set 1), and '22' (object-set 2); 'Learning trial (T1)', shows the position the objects should be on during the first trial (T1), this is always position 1; the column 'Test trial (T2)', indicates the new location (side, direction and position) to which one object should be moved to. The sub-columns show whether the right or the left object has to be moved, to which position and in what direction (backward or forward) this object has to move. 'Position' indicates the position to where the object has to get displaced to. The 'X' indicates where the object needs to be in T2, e.g., Rat 2 gets object-set 11, in T1 both objects are placed symmetrically on position 1 on both the left and the right side of the testing arena, in T2 the right object has to be moved backward to position 3. Since there is no displacement when testing position 1 in T2, the 'Moved object' is indicated as 'n.a.' when position 1 is utilized in T2.

Table 5| Randomization schemes of objects and locations for OPS testing on Position 3

		Learning trial (T1)	Test trial (T2)				
Animal number	Objects	Position of stationary objects	Object moved left backward	Object moved left forward	Object moved right backward	Object moved right forward	New position of the object
1	11	1		X			3
2	22	1			X		3
3	11	1	X				3
4	22	1				X	3
5	11	1		X			3
6	22	1			X		3
7	22	1	X				3
8	11	1				X	3
9	22	1		X			3
10	11	1			X		3
11	22	1	X				3
12	11	1				X	3

Randomization scheme for 12 animals to be tested on only position 3 in the OPS task. For additional explanation see footnote underneath Table 4. Object location discrimination performance on position 3 can be used to assess pattern separation bi-directionally, i.e. improved or impaired performance (e.g., for respectively drug testing or model validation).

PROCEDURE

Transportation and acclimatization • TIMING 7 d

CRITICAL Group housing for a week of acclimatization is required if animals have arrived from a commercial registered breeder or obtained from another animal facility and they have been group housed previously in transportation boxes.

1| Group house mice and/or rats in home-cages (groups of 2-3 per cage) and place them on a reversed day-night cycle. Allow the animals to get acclimatized to their new cage and environment for 7 days. During these 7 days the animals should be left undisturbed. Water and food should be available *ad libitum*.

CRITICAL STEP: Transportation of the animals and housing them in a new cage and environment is a source of stress[23] which in turn may affect the learning abilities and behavioral outcome of rodents. This can bias your experimental results. Acclimatization and habituation to the new environment is necessary to attenuate this stress.

Individual housing, housing conditions and acclimatization • TIMING 7 d

2| After acclimatization to the new environment, day-night cycle and cages, transfer the animals to individual housing with *ad libitum* access to food and water and allow them to get acclimatized to their new cage and individual housing for 7 days. For easier transportation, all home-cages of the animals should ideally be placed in/on movable wheel/rolling racks in case the animals need to be transported to the testing room (Step 3). Upon individual housing, number the animals on the ID tags or cages with a pen or a marker for easier identification. If the animals need to be housed in a different room to where the OPS task is to be performed they will need to also be habituated to the test room and transportation so proceed to the next step. Otherwise proceed to Step 4.

CRITICAL STEP: Individual housing throughout the OPS procedure is preferred to ensure that the animals do not have too much interference between the learning and the test trial (Step 10 – 24). Ensure sufficient cage enrichment is provided and keep this enrichment constant over the entire OPS procedure to avoid other possible interfering factors during the inter-trial intervals.

CRITICAL STEP: Ideally, the animals should be housed in a room that is dedicated to OPS testing. If this is possible the animal cages should be placed on the right (50% of animals) and left (50% of animals) side of the testing arena as far away from the testing arena as possible. It is always wise to check for side preferences to assess if everything in your behavioral room is placed correctly (see also ANTICIPATED RESULTS). Ensure the spatial arrangement of the base plate, testing arena, OPS objects and all other things in the room (e.g., desk, PC, etc.) stays similar throughout the OPS task. Store the OPS objects that are not used during the learning/test trial out of sight of the animals in the testing

arena (e.g., under the table on which the testing arena is placed or behind the testing arena on the side of the grey/black half of the arena wall).

3| **Optional:** If the animals cannot be housed in the room in which OPS testing will take place, habituate the animals on two separate days to the transportation procedure and the test room by moving them to the test room and leaving them undisturbed in the test room for at least 30 min. The test room should be set up as if testing is to take place and the animal cages should be placed on the right (50% of animals) and left (50% of animals) side of the testing arena at a sufficient distance (as far away from the testing arena as possible).

CRITICAL STEP: Moving rooms may cause stress the first few times the animals are moved.

?TROUBLESHOOTING

Habituation to experimenter • TIMING 8-12 d; 5 min of habituation per rodent per day

CRITICAL Animals must become habituated to the experimenter.

!CAUTION: Experimenters should keep everything consistent over the entire OPS procedure. Since rodents have a highly developed olfactory system, they rely heavily upon smell. Therefore, it is important for the experimenter to keep his/her smell as similar as possible. Keep a personal lab coat within the testing room that is not worn by someone else. Furthermore, this also implies that the experimenter has to use the same deodorant, shampoo, shower gel, hair gel, etc. throughout the entire OPS procedure. Avoid the use of volatile odors or perfume. Changing the 'olfactory signature' of the experimenter is a cause of stress that will lead to inconsistent results.

!CAUTION: If using gloves when handling animals, always use powder-free nitrile or vinyl gloves as rodents dislike (the texture and/or odor of) latex gloves.

4| Habituate the animals to the experimenter. If the planned experiment requires the testing of naïve animals or animals in which restraining procedures and/or injections are not required (e.g., transfected animals, animals with a minipump, etc.), follow option A at least twice for 5 min on separate days (Day 1 and 3), leaving one day in between (Day 2) during which the animals are left undisturbed. If the planned experiment does require restraining and/or injection of the animals, follow option B (see also the flowchart in **Fig. 2**).

(A) Habituation in the absence of additional procedures.

(i) Gently open the lid of the home-cage and put your hand in the cage, let the rodent smell and explore your hand for a short period of time. The animals should be allowed sufficient time to become aware of your presence before moving to the next step.

(ii) Pick up the rodent gently and after lifting it out of its cage place on the arm of your lab coat. Pick up a rat by the thorax if possible. If the rat keeps fleeing or is behaving

unpredictable, pick it up by the base of the tail. Pick up mice by the base of the tail. During the first handling sessions, rats will typically move towards your cubital fossa (elbow pit) and tend to hide their head there. Just let the rat do this for a while and gently pet the animal so that it becomes accustomed to being touched/approached from above. Mice tend to get less accustomed to handling then rats. Another, less stressful option for mice is to let the mouse climb onto an object (e.g., a small house or cup) after which it can be placed on your arm.

(iii) Leave the rodent to explore or rest on your arm for 5 min, after this, gently return it to the home-cage. During later sessions if using a rat , consider picking it up and putting it back in a different position on your arm a couple of times, to get the animal accustomed to being handled.

CRITICAL STEP: If rodents are not properly habituated to the handling procedures, they will not be comfortable in the testing apparatus which can bias experimental results. We advise to handle the animals at least twice on separate days, but if the animals do not seem comfortable after two days this step can be repeated until the animals look calm and comfortable.

?TROUBLESHOOTING

(B) Habituation if additional procedures will be carried out.

CRITICAL If the planned experiment involves drug testing that requires administration of injections, it is important to habituate the animals to this procedure as well as handling. Habituation sessions should be performed for both the restraining of an animal and the actual injection(s). Restraining and injecting an animal for the first time right before the actual test leads to excessive stress which can bias the experiment. We recommend to give the animals at least one saline injection during habituation, using the route of administration planned for the experiment. If repeated injections are necessary, consider alternatives such as the use of mini-pumps to reduce the discomfort of the animals

(i) Habituate animals to the experimenter first by carrying out Step 4 option A at least twice for 5 min on separate days (Days 1 and 3; leave one day in between where the animals are left undisturbed) (Step 4A, **Fig. 2**).

(ii) On the subsequent day (Day 4) handle the animals as described in option A and also restrain the animal once for a few sec during the 5 min habituation without injecting it. Gently pet or leave the animal on your arm for the rest of this habituation phase. To keep stress levels as low as possible, restrain the animal smoothly and without hesitation.

(iii) On the following day (Day 5), again take out and restrain the animal as described in the previous step and also administer a saline injection via the route that will be used during the OPS task (e.g., i.p., p.o., s.c., i.m.).

CRITICAL STEP: If multiple injection routes are required just carry out one injection.

(iv) If multiple injection routes will be required, leave the animals undisturbed for one day (Day 6) and then repeat the previous step on Day 7, administering a saline injection via the second required route of administration.

Habituation to OPS apparatus • TIMING Typically, this habituation procedure can be performed in 5 days.

CRITICAL After the rodents are habituated to the handling by the experimenter, they must be habituated to the testing apparatus.

CRITICAL The testing arena and the objects must be cleaned thoroughly after every animal with a cleaning cloth and a 70% ethanol solution in order to remove all olfactory traces that could attract or repel an animal towards, or away from an object or location. Make sure that the ethanol is completely evaporated when the next animal is introduced into the testing arena (not doing this will influence the results of the experiment).

5| Handle the animal for 5 min as described in Step 4 option A. If using mice, in order to increase their arousal at testing, next place the mouse in an empty cage (only bedding, no enrichment) for 4 min before moving to the next step. If using rats instead proceed instead to the next step.

6| Place the animal in an empty testing arena (facing the middle of the transparent segment of the arena or facing the middle of the grey/black half of the arena). Let the animal freely explore the arena for 5 min (during this time the second animal can be handled as described in Step 5). After 5 min, gently remove the animal from the arena and place it back in the home-cage and the holding rack.

7| Repeat Steps 5 and 6 on the next day (day 2 of OPS habituation).

8| On the next day (day 3 of OPS habituation) place the first set of similar objects in the testing arena randomly on the indicated possible locations. Make sure one object is located on the left vertical axes and the other one is located on the right vertical axes (e.g., see **Fig. 3**). Repeat steps 5 and 6 to give the animals the opportunity to be familiarized with the objects. Let the animal freely explore the testing arena with both objects for 5 min.

CRITICAL STEP: For new experimenters, it helps to practice scoring during the habituation phase. This can be done by carrying out step 16 option A whilst the animal is exploring the testing arena.

9| Leave the animals to rest for one day (day 4 of OPS habituation) and on the following day (day 5 of OPS habituation) repeat Step 8, but using the second set of similar objects and different locations along the left and right vertical axes.

OPS task procedure and training – 1 h inter-trial interval • TIMING 6 d; 2 h per day for a cohort of 12 rats or 10 mice

CRITICAL The following steps should be carried out carefully in order to obtain reliable task performance of the animals. The procedure for T1 (Steps 10 - 20) and T2 (Steps 21 - 24) is similar except that in T2 one of the two similar objects is displaced along a vertical axis. Placement should be determined by a randomised scheme (**Table 4-5**) and be to one of five different positions along a vertical axis (ranging from the same position as in T1, to a maximal displacement similar to the new location in the OLT[15], see also **Fig. 3** and **Box 1**).

!CAUTION: OPS testing should always be performed in a quiet room and area. Even soft noises could distract the animals and influence and/or bias the results.

10| OPS task procedure – Learning Trial (T1): Thoroughly clean the testing arena, base plate and OPS objects with a cleaning cloth and a 70% ethanol solution. When testing mice: prepare an empty cage with only bedding and put this cage somewhere in the room away from the testing arena.

CRITICAL STEP: Ensure the testing arena is on the base plate at the same position it has been previously as it must be in the same place throughout the entire experiment. Make sure that all possible object positions in the testing arena are clearly visible (**Fig. 1**).

CRITICAL STEP: Store the OPS objects that are not used during the trial out of sight of the animals in the testing arena (e.g., under the table the testing arena is on or behind the testing arena behind the grey- or black colored half of the arena wall).

11| Place two similar objects in the testing arena on the starting positions. The starting positions are 2 horizontally symmetrical locations on the middle line of the testing arena approximately 25 cm (rat arena) or 12 cm (mouse arena) from the wall on each side ('Position 1', **Fig. 3** and **Table 4-5**).

CRITICAL STEP: The experimenter has to choose with which object-set (1 or 2, **Fig. 1**) to start with. It is important to alternate between the two object-sets on subsequent testing days (e.g., rat 1 gets to explore object-set 1 on test-day 1, object-set 2 on test-day 2, object-set 1 again on test-day 3, etc.). A randomization scheme should be designed in which both the positions to be tested in T2 (Step 21 - 24) and the objects are used in a balanced manner to prevent potential biases due to preferences for particular locations or objects (**Table 4**).

CRITICAL STEP: Although they are similar, allocate each individual object to one side of the testing arena and keep it like this for this test day. Small differences in the texture of the objects might be more salient to rodents than they are to us and might subsequently lead to inconsistent performance

of the animals. Also, make sure that the objects are always oriented the same way (i.e. holes in the objects should always face the same direction).

12| When using mice, place the mouse in an empty cage (only bedding, no enrichment) for 4 min immediately preceding T1 to increase the arousal of the mouse at testing.

13| Start the timer or stopwatch to time the total time required to carry out T1 for the animal (5 min per rat; 6 min per mouse).

CRITICAL STEP: Tracking the entire time with each animal is necessary to exactly time the appropriate inter-trial interval (1 h) for each individual animal.

14| Gently get the home-cage of the animal out of the rack and place it on a table in the room.

CRITICAL STEP: Do not place the home-cage of the animal in close proximity of the testing arena, as this could potentially attract the animal towards its cage while it is in the OPS task. In our laboratory, we place the home-cage of the animal on the same table where the PC is located.

15| Take the animal out of its home-cage and place it in the testing area containing the two symmetrically placed objects facing the middle of the front transparent half of the arena or facing the middle of the grey/black half of the arena (i.e. facing away from the objects).

CRITICAL STEP: Ensure the starting point for the animals is kept identical during the entire OPS habituation, training and experimental procedures. Changing the starting position can lead to biased results. Since this is a spatial task, the animals will always be placed closer to one object than the other during the test trial (T2, Step 21 – 24). As long as the positions of the objects and the experimental conditions are tested in a balanced manner, the possible confounding effect of animals being placed in closer proximity to one object as opposed to the other is minimized.

CRITICAL STEP: Make sure that the cleaning ethanol is completely evaporated when the animal is introduced to the testing arena (not doing this can influence the results of the experiment).

16| Start a second timer or stopwatch to exactly time the time in the arena (3 min per rat; 4 min per mouse, see **Table 2-3**) and ensure the correct inter-trial interval (1 h) for each individual animal. Also, start scoring and/or recording the behavior of the animals. For live visual scoring of the exploratory behavior of the rodents follow option A. Video-recording of the trial is also advisable if possible as described in option B to (manually or automatically) score or enable rescoring of the exploratory behavior. Option C describes how to score exploratory behavior automatically using a video-tracking system using nose-tracking software. Option B offers some advantages, for instance because observation is from above it is always possible to see what an animal is doing and it does not get hidden behind an object as can happen during live scoring. Also the trials can be scored by different observers on different moments which allows for cross-comparisons (and measuring inter-observer reliability) and a permanent archive can be created which allows later access for rescoring

or meta-analyses. An example of an OPS experimental trial is given in the Supplementary Information (SI) in which a video of T1 and T2 is given. The exploration and discrimination performance of one mouse in given in a separate SI file as well (see: SI video files and SI Excel file: Scoring of SI video files; output and calculations of the parameters outlined in Table 6).

CRITICAL STEP: Total exploration times should always minimally be 7 sec in T1 for familiarized animals to properly learn the spatial arrangement of the objects in the testing arena[30]. Total exploration times should always minimally be 10 sec in T2 for familiarized animals in order to draw reliable conclusions with regard to the discrimination performance[30].

(A) Live visual scoring

(i) Carefully monitor the animals while in the testing arena. The experimenter should be placed at a position within the room where he/she can see as much of the testing arena as possible and should look through the transparent part of the testing arena in order to monitor the animal. A disadvantage of this way of scoring behavior is that the experimenter cannot see what an animal is doing while it is behind one of the objects exploring the lower part of the object. Only exploratory behavior that can properly be seen should be scored. Score the cumulative exploration behavior of the animals with appropriate software or stopwatches/timers. The observer should remain as quiet as possible while the animal is interacting with the objects. Even soft sudden noises (e.g., cracking of a chair) could distract the animals and influence the exploratory behavior. Exploration is defined as follows: directing the nose to the object at a distance of no more than 2 cm and/or touching the object with the nose. Sitting on, or leaning to, an object is not considered as exploratory behavior; the animals should only be scored as exploring when actively investigating an object. We recommend using a personal computer to manually score (live visual scoring) the exploratory behavior of the rodents. Alternatively, separate stopwatches can be used to measure the amount of exploratory behavior per object but this is more time-consuming and demanding than using simple software that accumulates exploration time.

CRITICAL STEP: Well-trained experimenters are required; new experimenters should already have practised scoring during Steps 8 and 9 if necessary.

(B) Video-recording for scoring

(i) Record the animals behavior using the preinstalled a digital camcorder.

(C) Automated scoring by video-tracking system

(i) Video-track the OPS trials for object exploration. The regions of interest where the software has to score are the areas around the objects (no more than 2 cm away from the

object, see Step 16A) and the objects themselves. The software should continuously track the nose of the animal and only score behavior as exploratory if the nose of the animal is within the indicated region of interest. An advantage of automated scoring by a video-tracking system is that the total distance moved as a measure for locomotor behavior of the animals can also be analyzed. To accomplish this, the center point of the animal can be tracked (so nose tracking is not necessary). This way the potential confounding effect of locomotor deficiencies of the animals can be assessed.

CRITICAL STEP: Before analyzing the OPS dataset generated by the video-tracking system, check whether manual scoring from a few video files correlate to the data generated by the video-tracking system. All parameters need to be set right in order to get the most reliable results.

?TROUBLESHOOTING

17| After the trial duration expires (3 or 4 min) take the animal out of the arena and place it back in the home-cage and the holding rack to be kept ready for its learning trial (T2) which must start in 1 h. Store the acquired data.

18| Clean the testing arena, base plate and OPS objects with a cleaning cloth and a 70% ethanol solution. Again, store the cleaned objects out of sight of the animals.

CRITICAL STEP: it is recommended to clean the used objects after each animal instead of cleaning the to-be-used objects before each animal. This ensures all the ethanol to be evaporated from the arena and objects upon using them again.

CRITICAL STEP: Steps 11- 18 need to be completed within 5 min for a rat or 6 min for a mouse to keep on schedule for time-planning.

19| Repeat Steps 11-18, using the objects and position determined by the randomization schedule on each animal until the remaining 11 rats (12 rats in total) or 9 mice (10 mice in total) have been through T1.

20| When an hour has elapsed since the first animal completed testing in T1, proceed to the next step for the second trial (T2) (Step 21 – 24). If using 12 rats or 10 mice, with 5 min or 6 min having been used for each rat or mouse trial, 1 h will have elapsed since the first animal was completed testing in T1 ((12 rats * 5 min) or (10 mice * 6 min)) so you should immediately proceed to the next step.

21| OPS task procedure – Test Trial (T2): Repeat Steps 11-18 with the same set of objects that was used for the animal in T1 but with one of the two objects displaced to one of 5 positions along the vertical axis indicated on the base plate of the OPS apparatus. The position to place the object on should be as determined by the randomisation scheme (**Table 4-5**).

CRITICAL STEP: Ensure the object that was presented on the left side of the testing arena in T1 is again on the left side in T2 and that the object presented on the right side in T1 is now again on the right side of the testing arena in T2.

CRITICAL STEP: Use a balanced randomization scheme for both the objects and the object positions in T2 to prevent potential biases due to preferences for particular locations or objects. An example is given for 12 animals in the appended randomization scheme in which position 1, 3, and 5 or only position 3, are tested in a balanced way with two objects (**Table 4 and 5, respectively**).

22| Repeat Step 21 for the remaining 11 rats (12 rats in total) or 9 mice (10 mice in total) tested in T1, with objects used and placed as determined by the predetermined randomization scheme.

23| If 5 min in total is used for rats and 6 min in total used for mice, 2 h will have elapsed since the first animal was tested in T1 ((12 rats * 5 min * 2 trials) or (10 mice * 6 min * 2 trials)). If desired, repeat steps 10-22 with a second cohort of 12 rats or 10 mice.

24| After finishing the OPS experiment, score any videotaped material that has not been automatically analyzed by software as described in step 16 option A and analyze the stored data using spreadsheet software or a statistical program (ANTICIPATED RESULTS).

?TROUBLESHOOTING

Further OPS training

CRITICAL After the rodents are habituated to the experimenter, the testing arena, the OPS objects and the OPS task the actual training in the OPS task can start. This measures the performance of the animals in the task and assesses whether they show reliable pattern separation performance. The most straightforward way of measuring pattern separation is by first establishing performance on position 1, 3 and 5 in the test trial, or T2 (Step 10 – 24, see also **Box 1**). When the linear relationship (**Fig. 4**) is established the actual OPS experiment (Step 32 – 36) can start. Total exploration times should always minimally be 7 sec in T1 and 10 sec in T2, in order to draw reliable conclusions with regard to the discrimination performance of familiarized animals[30].

25| Leave the animals for a day before further testing.

26| On the next day repeat Steps 10-24 using the same objects for T1 and T2 but displacing the object in the T2 to 'position 5' (where the displacement of the object is maximal). The expected outcome is that the d2 value should be approximately 0.30 – 0.45 (see ANTICIPATED RESULTS and **Fig. 3**).

CRITICAL STEP: Performance on position 5 is most informative in this first stage of training because it shows whether the animals have the capacity to learn. Displacement to position 5 enables the highest performance to be achieved (a d2 of approximately 0.30 – 0.45). Starting with other positions will be less informative at this stage (e.g., starting with position 1, where a d2 of approximately 0.00

is anticipated, will not be informative with regard to the learning abilities of the rodents, since there is no novelty for the animals to explore and hence no way of assessing whether the animals are able to learn spatial information properly). A low discrimination performance on position 5 indicates deviant behavior that might relate to anxious or stressed behavior or erroneous displacement of the object(s) in T2 (see also the **TROUBLESHOOTING** table).

27| Leave the animals for a day before further testing.

28| On the next day repeat steps 10-24 starting with the second set of similar objects for T1 and T2 but displacing the object in T2 to 'position 3' (where the displacement of the object in T2 is half way between the starting position, 'position 1', and the maximum position, 'position 5'). The expected outcome is that the d2 value should be approximately 0.15 (ANTICIPATED RESULTS and **Fig. 3**).

29| Leave the animals for a day before further testing

30| On the next day repeat Steps 10-24 starting with the first set of similar objects for T1 and T2 and not displacing the object in T2 but leaving it on 'position 1'. The expected outcome is that the d2 value should be approximately 0.00 (ANTICIPATED RESULTS and **Fig. 3**).

31| Leave the animals for a day before further testing.

- **PAUSE POINT:** this rest period might also extend over the weekend if desired.

?TROUBLESHOOTING

OPS experiment • TIMING 2 h per day for a cohort of 12 rats or 10 mice

CRITICAL After the rodents have been habituated and trained for the OPS procedure and show reliable OPS performance (**Fig. 3** and **Fig. 4**), the actual experiment can start. We give an example below for a an experimental week (3 testing days) where 3 OPS experiments are performed in which different experimental conditions are tested on OPS position 3 in order to assess whether the experimental conditions impair or improve OPS performance.

!CRITICAL: When the testing involves the administration of drugs, timing of the testing should be customized according to the T_{max} of the drug. An example for a drug with a T_{max} of 30 min that has to be maximally active during the memory acquisition phase is shown in **Table 3** (rats) and **Table 2** (mice).

!CRITICAL: During drug testing, the half-life of the drug should always be taken into account in order to prevent accumulation of the drug in the system. In general, an interval of at least 6 times the half-life of a drug (98.4% drug eliminated) should be interposed between subsequent drug testing/experiments.

CRITICAL STEP: When testing different experimental groups, experimenters should always be blinded to the treatments or conditions of the animals.

CRITICAL STEP: A randomization scheme should be designed in which the objects, the locations to be tested in T2 (i.e. left and right) and the experimental conditions are used in a balanced manner to prevent confounding results (see **Table 5**).

?TROUBLESHOOTING

32| **Monday**: Repeat Steps 10-24 starting with the first set of familiar objects, placing the objects on position 3 during T1. In T2 displace the object to 'position 3'. The expected outcome is that the d2 value should be approximately 0.15 in the control or vehicle condition (ANTICIPATED RESULTS and **Fig. 3**). Experimental conditions that improve or impair pattern separation performance will show an increase (e.g., a d2 value of 0.30) or a decrease (e.g., a d2 value of 0.00) of the discrimination index (d2), respectively.

33| **Tuesday:** Leave the animals for a day with no testing.

34| **Wednesday:** Repeat Step 32 but with using the second set of similar objects.

35| **Thursday:** Leave the animals for a day with no testing.

36| **Friday:** Repeat Step 32

Ongoing experiments TIMING Variable

37| Animals can be tested repeatedly in the OPS task so if desired further studies can be undertaken with the same sets of object. In order to prevent recent memory effects of the objects, at least 48 h should be interposed between subsequent experiments. This means that during one week (Monday – Friday), three OPS experiments can be performed in one cohort of animals (Monday, Wednesday and Friday) (**Fig. 2**).

Additional endpoints TIMING Variable

38| After the necessary experimental conditions are tested in the OPS experiment and if required by the experimental design, anesthetize the animals, euthanize and dissect for tissue collection according to protocol requirements. In our laboratory anesthesia and euthanasia gets performed conform Annex IV of Directive 2010/63/EU.

CRITICAL STEP: All personnel involved with anesthesia or euthanasia of animals should have all required licenses and be adequately trained to perform these procedures.

• TIMING

Step 1, Transportation and acclimatization: 7 d

Steps 2-3, Individual housing, housing conditions and acclimatization: 7 d

Steps 4-9, Habituation to experimenter and OPS apparatus: 8-12 d

Steps 10-31, OPS task procedure and training – 1 h inter-trial interval: 6 d

Steps 32-36, OPS experiment – 1 h Inter-trial-interval - OPS experiment: 2 h per day for a cohort of 12 rats or 10 mice

Step 37, On-going experiments: variable

Below is an example of what is required on a daily basis for a typical experiment:

Days 1-7: Acclimatization of newly arrived animals (Step 1)

Days 8-14: Acclimatization to individual housing (Step 2)

Day 15-19: Acclimatization to handling (Step 4)

Day 20-24: Acclimatization to the OPS apparatus (Step 5)

Day 25-30: OPS task training (Steps 10-31)

Day 31-35: 3 OPS experiments (in 5 days, N=20-24) (Steps 32-36)

Day 36-X: Ongoing experiments (3 OPS experiments per week, variable) (Step 37)

?TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

ANTICIPATED RESULTS

Statistical analysis

The OPS task provides measures for exploration time and discrimination performance. The outcome measures of the task are the times spent by rodents exploring each object location during the learning trial (T1) and the test trial (T2). The time spent in exploring the two symmetrically placed objects in T1 is represented by 'a1' and 'a2', respectively. The time spent in exploring the stationary and the moved object in T2 are represented by 'a3' and 'b', respectively. From these exploration times the following variables are calculated: e1, e2, and d2 (**Table 6**). Total exploration times in both trials should be sufficient (i.e. T1/e1 ≥ 7 sec and T2/e2 ≥ 10 sec for familiarized animals) in order to reliably assess discrimination performance[30]. If one or both of these criteria are not met, exclusion of the animal is warranted. The d2 index is a relative measure of discrimination performance since it is corrected for total exploratory activity in the test trial (e2). The d2 index can range from -1 to 1; with -1 indicating complete preference for the familiar/stationary object location, 1 indicating complete preference for the novel object location, and 0 signifying no preference for either object location (chance performance). A d2 index should never be 1 or -1, since this means an animal completely ignored one of the objects or sides of the testing arena (Steps 21 – 24 - TROUBLESHOOTING). Ignoring one side or object is deviant behavior and often emerges when an animal is anxious. Animals should always explore both objects in order to draw reliable conclusions with regard to their pattern separation performance[15]. Animals having a d2 index of -1 or 1 should be excluded from the dataset since this performance and outcome will bias the results. Additional measures such as total distance moved during both trials can serve as an assessment of locomotor activity which can be a valuable readout in for example; pharmacological studies or when using deficit models (e.g., transgenic or pharmacological models). Total distance moved can be easily assessed when using an automated scoring program (see also Step 16 option C).

Spreadsheet software (e.g., Microsoft Excel) should be used to enter and organize the different OPS parameters. For faster OPS data analysis reusable templates can be made using spreadsheet software (see also the SI video files and SI Excel file: Scoring of SI video files; output and calculations of the parameters outlined in Table 6). The OPS parameters can be analyzed using a statistical program (e.g., IBM SPSS Statistics). One-sample *t*-statistics can be performed in order to assess whether the d2 index significantly differs from zero (i.e. chance level performance). However, comparison of the value of d2 with the value zero with no variance may not be the most suitable way for analyzing performance because of an increased chance of making a type I error. Another possibility is to perform a *t*-test that compares to a fictive group that shows no discrimination. The fictive group is a standard data set with a mean d2 of 0.00 (no discrimination, i.e. chance

performance) and a standard error of the mean (SEM) that is usually observed in the same type of experiments (i.e. 0.07). To obtain an average SEM, an earlier study performed in our lab calculated the mean value of 10 previous random independent ORT experiments, the SEM was 0.07. On basis of these data, a fictive group was constructed for the ORT. Since the d2 index is also used in the OPS task, similar variance in the data can be expected[35]. Experimental conditions, including the different positions, should also be compared using; one-way ANOVAs, repeated measures ANOVAs, or paired-samples *t*-statistics, depending on the experimental design (e.g., performance difference between different positions, groups of animals within positions, drug treatments, etc.). When the overall ANOVA is significant, post-hoc *t*-tests can be performed. Normally, an α level of 0.05 is considered significant. A d2 value that is significantly different from zero (as indicated by one-sample *t*-statistics) signifies an intermediate effect, and as such, already indicates recognition of the familiar object location implying functioning pattern separation. A significant difference from a fictive group that shows no discrimination (i.e. with a mean of 0.00 and a SEM of 0.07) indicates an even stronger effect. When a d2 index shows both a difference from zero and a between group effect (as indicated by ANOVA or paired-samples *t*-statistics), it is called a full effect[35].

Table 6| Derived Measures in the Object Pattern Separation (OPS) Task

Trial number	Exploration time (s)	Discrimination index
T1	$e1 = a1 + a2$	Not applicable
T2	$e2 = a3 + b$	$d2 = (b - a3) / e2$

e1 is the time spent in exploring both identical object locations (a1 and a2) during the learning trial (T1). e2 is the time spent in exploring both the familiar (a3) and new object location (b) in the test trial (T2). d2 corresponds to the ability to discriminate between the old and new object location during the test trial (T2). The d2 index is corrected for exploration time during T2 and is therefore independent of the animals' exploratory behavior during this trial.

Side preference (or lateralization) of a cohort of animals can be checked for by comparing the amount of time an animal spent exploring the left and right object during T1. Similarly, it can be checked whether there is side preference with regard to forward and backward displacement of the moved object in T2 (so a preference for forward or backward moved objects). These separate exploration times should not differ too much. One approach to check for side preference is to calculate the difference between the exploration times of the left and right (or forward and backward displaced) objects during T1 (i.e. (a1 – a2)) or T2, respectively. Subsequently, a *t*-test can be performed where the difference scores between a1 and a2 are compared to the value zero. In addition, when using an automated tracking program, the amount of time an animal spends in each half (or even each quarter) of the testing arena can be assessed to check for possible area preferences/avoidances or lateralization, both during T1 and T2. There should be no preference for

any specific part of the testing arena during T1. During T2, it is to be expected that an animal shows preference for the specific part of the testing arena to where an object is moved (and identified as moved) hence checking for side preference in T2 is less informative.

Expected results

When the herein described procedures for OPS testing (**Fig. 2**) are applied to naïve adult male Wistar rats or C57BL/6 mice, gradual pattern separation performance effects (spatial discrimination) over the different positions should occur (**Fig. 3**). When all procedural steps are carried out successfully, a graph as shown in **Fig. 4** should follow. **Fig. 4** shows a linear relationship of the discrimination index (d2) between the different positions. As the distance of the moved object compared to the stationary object increases, the pattern separation performance (i.e. the d2 index) increases as well. Usually, rodents show a good pattern separation performance when the displacement is maximal (i.e. position 5) and a 1 h retention interval is interposed between the trials. However, when the displacement of the object along a vertical axis is in-between the starting position (position 1) and the maximum position (position 5), the pattern separation performance is attenuated. This indicates that it is more challenging for rodents to discriminate between the locations of the objects when the displaced object is closer to its starting position. In other words, a greater distance between the starting location and the new location leads to a better pattern separation performance of the rodents. It is easier to discriminate or perceive the new position as novel when the change is more apparent.

In our laboratory, we mainly use the 1 h inter-trial interval between T1 and T2 in the OPS task. We have found that an inter-trial interval of 2 h leads to similar results on position 3 in untreated mice[15]. Time dependent forgetting can be manipulated with experimental conditions if desired. Although a linear relationship (indicating gradual pattern separation performance effects) has always been observed when performing the OPS task, we have found small sporadic differences in results for mice and rats on position 3 with regard to the statistical outcome of the post-hoc *t*-tests performed after an ANOVA effect. To elaborate, OPS performance on position 3 compared to the performance on position 1, has led to a significant effect for rats, but not for mice in the studies described in van Hagen et al. (2015)[15]. Both rats and mice showed a significant effect from zero (i.e. chance level), indicating an intermediate effect, and as such, recognition of the familiar object location implying functioning pattern separation. Both in our rats and mice studies we have encountered this phenomenon (i.e. position 3 not always reaching statistical significance when compared to position 1) and as such we do not assign this performance to be a specific species characteristic. The reason for this discrepancy is evident; it results from the fact that position 3 is the

cutoff distance where rodents are just able to differentiate a new spatial arrangement from an earlier encountered one. When using this smallest possible displacement at which the animals are able to discriminate between the 'old' (learning trial) and 'new' (test trial) situation, it is assured that the presented locations are highly similar to the rodents, and thus pattern separation processes have to be utilized in order to make this discrimination. Inherent to this, the discrimination performance is still low so it gives the opportunity to measure spatial pattern separation bi-directionally (i.e. have the ability to measure an improvement or an impairment of the OPS performance to resemble the performance on position 5 or 1, respectively). Because the SEM can fluctuate between animal cohorts, and such a refined displacement is used, these slight differences between animal cohorts can occur. Using post-hoc *t*-tests that do not perform all pairwise comparisons but instead compare only to the control condition (i.e. position 1, using for example Dunnett's tests) could help to prevent this. Position 3 should at least always be statistically different from chance level (i.e. zero) to be able to measure improvement or impairment after an experimental intervention.

We have successfully utilized the OPS task for assessing drug effects on pattern separation performance in male Wistar rats[11]. As intact hippocampal DG functioning is important for proper pattern separation performance[4], currently, we are investigating the role of adult neurogenesis in the DG in OPS performance. Preliminary results indicate that an increase in adult neurogenesis in the DG, due to a pharmacological manipulation, leads to improved performance in the OPS paradigm. This is in accordance with current hypotheses that adult neurogenesis is important in spatial pattern separation processes. In addition, we are examining the potential of our OPS paradigm for evaluating pattern separation performance in aged rats. For these studies we used 2 year-old male Wistar rats. Our preliminary findings indicate full applicability of aged rats in the OPS task using a 1 h inter-trial interval and 3 min trial duration. The group of aged animals shows deficits in OPS performance which can be attenuated with cognition enhancing drugs.

In conclusion, the procedures reported in this protocol should provide a framework for assessing spatial pattern separation performance using the OPS task in different experimental conditions. The OPS task solely relies on the natural tendency of rodents to explore novelty and as such does not require punishment or reward incentives or food restriction. The OPS paradigm can be easily implemented in every standard rodent behavioral testing facility by personnel with regular rodent behavioral expertise. The OPS task is not expensive to incorporate in the behavioral test battery of any animal facility already performing rodent behavioral tasks. Especially when ORT or OLT apparatus and experience are present, the OPS task is relatively easy to implement and perform. The OPS task can be a valuable add-on in test-batteries when validating new animal models of disease or when investigating novel treatment interventions. Moreover, pattern separation has been linked to neuronal plasticity including adult born neurogenesis[1], opening possibilities to be utilized in this field

of research as well. The OPS task could allow for quick, easy and more comparable research in the fields of psychopathology including MCI, dementia, PTSD and schizophrenia, but also more fundamental neurosciences investigating plasticity or memory formation.

References

1. Clelland, C. et al. A functional role for adult hippocampal neurogenesis in spatial pattern separation. *Science* **325**, 210-213 (2009).
2. Kheirbek, M.A., Klemenhagen, K.C., Sahay, A. & Hen, R. Neurogenesis and generalization: a new approach to stratify and treat anxiety disorders. *Nat. Neurosci.* **15**, 1613-1620 (2012).
3. Reagh, Z.M. et al. Spatial discrimination deficits as a function of mnemonic interference in aged adults with and without memory impairment. *Hippocampus* **24**, 303-314 (2013).
4. Schreiber, R. & Newman-Tancredi, A. Improving cognition in schizophrenia with antipsychotics that elicit neurogenesis through 5-HT_{1A} receptor activation. *Neurobiol. Learn. Mem.* **110**, 72-80 (2014).
5. Das, T., Ivleva, E.L., Wagner, A.D., Stark, C.E.L., Tamminga, C.A. Loss of pattern separation performance in schizophrenia suggests dentate gyrus dysfunction. *Schizophr. Res.* **159**, 193-197 (2014).
6. Tamminga, C. A., Stan, A. D. & Wagner, A. D. The hippocampal formation in schizophrenia. *Am. J. Psychiatry* **167**, 1178–1193 (2010).
7. Bekinschtein, P. et al. BDNF in the dentate gyrus is required for consolidation of 'pattern-separated' memories. *Cell Rep.* **5**, 759-768 (2013).
8. Oomen, C.A. et al. The touchscreen operant platform for testing working memory and pattern separation in rats and mice. *Nat. Protoc.* **8**, 2006-2021 (2013).
9. Sahay, A., Wilson, D.A. & Hen, R. Pattern separation: a common function for new neurons in hippocampus and olfactory bulb. *Neuron* **70**, 582-588 (2011).
10. Lacy, J.W., Yassa, M.A., Stark, S.M., Muftuler, L.T. & Stark, C.E.L. Distinct pattern separation related transfer functions in human CA3/dentate and CA1 revealed using high-resolution fMRI and variable mnemonic similarity. *Learn. Mem.* **18**, 15-18 (2011).
11. van Goethem, N.P., Schreiber, R., Newman-Tancredi, A., Varney, M. & Prickaerts, J. Divergent effects of the "biased", 5-HT_{1A} receptor agonists F15599 and F13714 in a novel object pattern separation task. *Br. J. Pharmacol.* **172**, 2532-2543 (2015).
12. Ennaceur, A. & Delacour, J. A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data. *Behav. Brain Res.* **31**, 47-59 (1988).
13. Leger, M. et al. Object recognition test in mice. *Nat. Protoc.* **8**, 2531-2537 (2013).
14. Antunes, M. & Biala, G. The novel object recognition memory: neurobiology, test procedure, and its modifications. *Cog. Process* **13**, 93-110 (2012).
15. van Hagen, B.T.J., van Goethem, N.P., Lagatta, D.C. & Prickaerts, J. The object pattern separation (OPS) task; a behavioral paradigm derived from the object recognition task. *Behav. Brain Res.* **285**, 44-52 (2015).
16. van Goethem, N.P. et al. Object recognition testing: rodent species, strains, housing conditions, and estrous cycle. *Behav. Brain Res.* **232**, 323-334 (2012).
17. Heyser, .J. & Chemero, A. Novel object exploration in mice: Not all objects are created equal. *Behav. Proces.* **89**, 232-238 (2012).
18. Prusky, G.T., Harker, K.T., Douglas, R.M. & Whishaw, I.Q. Variation in visual acuity within pigmented, and between pigmented and albino rat strains. *Behav. Brain Res.* **136**, 339-348 (2002).
19. Roedel, A., Storch, C., Holsboer, F. & Ohl, F. Effects of light or dark phase testing on behavioural and cognitive performance in DBA mice. *Lab. Anim.* **40**, 371–381 (2006).
20. Akkerman, S. et al. PDE5 inhibition improves object memory in standard housed rats but not in rats housed in an enriched environment: implications for memory models? *PLoS ONE* **9**, e111692 (2014).
21. Bartolomucci, A. et al. Individual housing induces altered immuno-endocrine responses to psychological stress in male mice. *Pyschoneuroendocrin.* **28**, 540-558 (2003).
22. Arndt, S.S. et al. Individual housing of mice – impact on behaviour and stress responses. *Physiol. Behav.* **97**, 385-393 (2009).
23. Castelhano-Carlos, M.J. & Baumans, V. The impact of light, noise, cage cleaning and in-house transport on welfare and stress of laboratory rats. *Lab. Anim.* **43**, 311-327 (2009).

24. Ameen-Ali, K.E., Eacott, M.J. & Easton, A. A new behavioural apparatus to reduce animal numbers in multiple types of spontaneous object recognition paradigms in rats. *J. Neurosci. Met.* **211**, 66-76 (2012).
25. Ameen-Ali, K.E., Easton, A. & Eacott, M.J. Moving beyond standard procedures to assess spontaneous recognition memory. *Neurosci Biobehav. Rev.* **53**, 37-51 (2015).
26. Albasser, M.M., Chapman, R.J., Amin, E., Iordanova, M.D., Vann, S.D. & Aggleton, J.P. New Behavioral protocols to extend our knowledge of rodent object recognition memory. *Learn. & Mem.* **17**, 407-419 (2010).
27. Kinnavane, L., Albasser, M.M. & Aggleton, J.P. Advances in the behavioural testing and network imaging of rodent recognition memory. *Behav. Brain Res.* **285**, 67-78 (2015).
28. Sorge, R.E. et al. Olfactory exposure to males, including men, causes stress and related analgesia in rodents. *Nat. Met.* **11**, 629-632 (2014).
29. Ennaceur, A. One-trial object recognition in rats and mice: methodological and theoretical issues. *Behav. Brain Res.* **215**, 244-254 (2010).
30. Akkerman, S. et al. Methodological considerations on discrimination and exploration measures in object recognition. *Behav. Brain Res.* **232**, 335-347 (2012).
31. Bruno, O. et al. GEBR-7b, a novel PDE4D selective inhibitor that improves memory in rodents at non-emetic doses. *Br. J. Pharmacol.* **164,** 2054-2063 (2011).
32. Vanmierlo, T. et al. Liver X receptor activation restores memory in aged AD mice without reducing amyloid, *Neurobiol. Aging* **32,** 1262-1272 (2009).
33. Zeef, D.H., van Goethem, N.P., Vlamings, R., Jahanshahi, A., Hescham, S.A., Prickaerts, J., & Temel, Y. Memory deficits in the transgenic rat model of Huntington's disease. *Behav. Brain Res.* **227**(1), 194-198 (2012).
34. Rutten, K. et al. Automated scoring of novel object recognition in rats. *J. Neurosci. Met.* **171**, 72-77 (2008).
35. Akkerman, S., Prickaerts, J., Steinbusch, H.W.M. & Blokland, A. Object recognition testing: statistical considerations. *Behav. Brain Res.* **232**, 317-322 (2012).

Chapter 7

General Discussion & Conclusions

Summary

In this dissertation we studied cognitive processes with emphasis on pattern separation and spatial memory. Pattern separation is the process of transforming highly similar sensory inputs into distinct, dissimilar representations. It takes place in the hippocampus and is thought to be used in memory formation and retrieval. Impaired pattern separation performance has been recognised to be a predictor for the development of cognitive impairments in humans such as dementia and is present in patients with schizophrenia and post-traumatic stress disorder (PTSD). Moreover, the role of the serotonin 1A receptor (5-$HT1_AR$) in pattern separation processes was studied. The functioning of different 5-$HT1_AR$ subpopulations was assessed on a behavioral and biochemical level in order to evaluate different possibilities to utilize this receptor subtype (and subpopulations) for treatment possibilities in different psychiatric and neurological disorders. This is described in more detail in **Chapter 1**, which gives a general introduction on cognitive enhancement, pattern separation & spatial memory, and the the 5-$HT1_AR$. Furthermore, this chapter contains the aim of this dissertation; "The aim of this dissertation was to gain more insight in the neuronal signaling pathways involved in behavioral pattern separation processing and spatial memory, through investigation of the potential of different pharmacological agents to either impair or enhance these processes."

Chapter 2 elaborates more on the pattern separation proces and describes different paradigms to assess memory processes in rodents. The object recognition task (ORT) is a widely used paradigm to measure object memory processes in rodents. Recently, the memory process known as pattern separation has received increasing attention, as impaired pattern separation can be one of the cognitive symptoms of multiple neurological and psychiatric disorders. In the search for an easily implemented task for rodents that can be used to measure pattern separation, we developed a task derived from the ORT and the object location task (OLT), which we called the object pattern separation (OPS) task. This task aims to measure spatial pattern separation per se, which utilizes memory processes centered in the DG and CA3 region of the hippocampus. Adult male C57BL/6 mice and adult male Wistar rats were used to validate different object locations which can be used to measure spatial pattern separation. Furthermore, different inter-trial time intervals were tested with the most optimal object location, to further evaluate pattern separation-related memory in mice. We found that specific object locations show gradual effects, which is indicative of pattern separation, and that the OPS task allows the detection of spatial pattern separation bi-directionally at intermediate spatial separations. Thus, object locations and time intervals can be specifically adjusted as needed, in order to investigate an expected improvement or impairment. We conclude

that the current spatial OPS task can be best described as a specific version of the ORT, which can be used to investigate pattern separation processes.

In **Chapter 3** the focus is on cognition enhancement and the role of 5HT1$_A$Rs on the pattern separation process and spatial memory in rodents. Several studies were performed to increase our understanding of the role this receptor, its different subpopulations and their different ligands, plays in enhancing pattern separation processes and spatial memory.

The 5-HT$_{1A}$R can exert differential effects on cognition-related neurotransmission depending on the location (or subpopulation) of the receptors. A subpopulation of 5-HT$_{1A}$ autoreceptors are located in the raphe nuclei, such as the dorsal raphe nucleus (DRN), and exert inhibitory effects on serotonergic transmission, whereas 5-HT$_{1A}$ heteroreceptors are mainly located in limbic regions, such as the hippocampus and cortical regions, and can exert indirect stimulatory effects. The study outlined in this Chapter aimed to identify how different 5-HT$_{1A}$R subpopulation activity mediates cognition, specifically spatial pattern separation performance, following acute and chronic stimulation.

Male Wistar rats were treated with either F13714, a biased agonist which preferentially activates 5-HT$_{1A}$ autoreceptors, or F15599, a biased agonist that preferentially activates 5-HT$_{1A}$ heteroreceptors, both acutely and chronically for 14 days. Body temperature measurements were taken daily. Object pattern separation (OPS) performance was measured directly after acute treatment and at day 15 of chronic treatment. Animals were sacrificed after behavioral testing to measure 5-HT$_{1A}$R density and cognition-related markers in the hippocampus and DRN.
Acute treatment with F13714 impaired OPS performance, whereas chronic treatment increased performance to vehicle levels. Body temperature was measured as a functional correlate of 5-HT$_{1A}$ receptor stimulation. It dropped from day 4 onwards and in parallel the number of 5-HT$_{1A}$ receptors decreased in the DRN. F15599 enhanced OPS performance both acutely and chronically and caused an acute drop in body temperature, which rose again during chronic treatment. Furthermore, BDNF levels and doublecortin positive newborn neurons increased in the dorsal hippocampus after chronic F15599 treatment.
In conclusion, these studies showed the divergent effects of two 5-HT1$_A$R biased agonists. Subpopulation specific targetting of 5-HT1$_A$Rs might prove to be a valuable tool for pattern separation impairments. The studies outlined in this Chapter indicate that chronic treatment with F13714 may result in desensitization of 5-HT$_{1A}$ autoreceptors,which causes a reversal of the initial impairment measured in OPS performance. Chronic post-synaptic stimulation of 5-HT$_{1A}$ heteroreceptors with F15599 may have therapeutic potential to treat pattern separation impairments.

Assessment of the potential of 5-HT_{1A} heteroreceptor stimulation on a rodent cognitive-deficit model would be the next logical step. **Chapter 4** introduces a preclinical pharmacological model for cognitive impairments associated with schizophrenia (CIAS). Cognitive deficits are a key feature of schizophrenia that can severely impact daily functioning. Stimulation of 5-$HT1_A$ receptors to improve cognition has led to differential effects, dependent on their location. Conventional non-biased 5-$HT1_A$ receptor agonists can elicit positive effects which are believed to be regulated through post-synaptic 5-$HT1_A$ heteroreceptors, but these can be counteracted by simultaneous activation of the 5-$HT1_A$ autoreceptors. Therefore, selective targeting of post-synaptic 5-$HT1_A$ heteroreceptors with biased agonists such as F15599 could be more effective to alleviate cognitive deficits in schizophrenia.

Here, the NMDA glutamate receptor antagonist ketamine (30mg/kg, IP) was used to induce schizophrenia-like cognitive deficits in rats. Three weeks after sub-chronic (5-days) ketamine or vehicle treatment, cognitive performance was measured in the OPS task, which relies on hippocampal processes. An attentional set-shifting task was used to assess medial prefrontal cortex-regulated behavioral flexibility. Acute treatment with F15599 (0.04mg/kg IP) was compared to vehicle treatment to evaluate the effects of selective 5-$HT1_A$ heteroreceptor activation on cognitive performance in both control rats and the schizophrenia-like rats.

In both tasks the ketamine-treated rats showed an impaired performance compared to control animals. Acute treatment with F15599 reversed the pattern separation deficit up to the performance level of control animals. Administration of F15599 prior to learning a new response-strategy in the set-shifting task, improved performance in the schizophrenia-like rats. These data show that selective activation of 5-$HT1_A$ heteroreceptors can be a successful treatment to relieve schizophrenia-related deficits that originate from both hippocampal and/or prefrontal cortex centered processing.

Chapter 5 describes another mechanism to enhance spatial memory processes in preclinical pharmacological rodent models for cognitive impairment. Upregulation of cyclic guanosine monophosphate (cGMP) through the inhibition of specific phosphodiesterases (PDEs) has previously been shown to improve memory performance. The current study aimed to target cGMP upregulation differently, using the soluble guanylate cyclase (sGC) stimulator riociguat, and investigate the acute effects on memory in both healthy mice and a biperiden induced memory deficit mouse model. Biochemical measurements were performed on hippocampal tissue to further elucidate the role of the nitric oxide (NO) – sGC – cGMP signaling pathway in memory function. Acute administration with a low dose of riociguat was able to enhance working-, short- and long-term spatial memory as measured with the object location task or Y-maze continuous alternation task. Pharmacokinetic measurements within brain tissue of acutely treated mice showed very poor or no brain penetration

of riociguat. Western blots revealed an increase in activation of vasodilator stimulated phosphoprotein (VASP) at the behaviorally active dose of riociguat. No other effects were found on memory-related hippocampal plasticity measures including activation of CREB, AMPA receptor trafficking and PSD95. These findings support the assumption that the memory enhancing effects are due to a non-central effect. In this respect, further research is needed to investigate the possible contribution of hemodynamic or metabolic effects which are known to be regulated by sGC-cGMP signaling.

After validating the OPS paradigm, it was decided to share a highly detailed protocol of this novel task with the scientific community. This protocol was published and is outlined in **Chapter 6**. In this protocol we describe how to implement a simple and robust OPS task in mice and rats that we have previously established and validated. This two-trial memory task utilizes specific object locations so differences in performance can be calibrated with the extent of object movement. Changes in performance are indicative of spatial pattern separation. In contrast to other pattern separation tasks, the OPS task allows detection of spatial pattern separation performance bi-directionally. Furthermore, the OPS task is cheaper and easier to use and interpret than other tasks which use more than two objects or which are touch-screen based. The entire protocol, from vivarium acclimatization to training of the animals, takes approximately 30 days. After successful training, the animals can be tested repeatedly and three OPS experiments (n = 20-24 per experimental day) can be performed per week. A standard level of expertise undertaking behavioral studies in rodents is sufficient to successfully integrate this paradigm into an existing rodent test battery.

To summarize, this dissertation showed that spatial pattern separation can be successfully measured using the OPS task in both healthy rodents and a rat model for CIAS. The task lends itself for easy application and adaptation to study both the enhancing and impairing effects of different drug treatments. The experiments in this dissertation provide evidence that 5-HT1_ARs mediate pattern separation performance and that selective targeting of post-synaptic heteroreceptors could potentially be a successful strategy to enhance pattern separation performance and schizophrenia-related cognitive impairments. We provided evidence that chronic stimulation of 5-HT1_A ¬heteroreceptors with F15599 increases hippocampal plasticity which possibly mediates the beneficial effects on pattern separation. Acute treatment with F15599 can alleviate ketamine-induced schizophrenia-like cognitive impairments, possibly through enhanced cortico-hippocampal signaling resulting in restoral of glutamatergic and GABAergic levels. However, it must be noted that further experiments are needed to elucidate the exact role of 5-HT1_ARs in cognitive functioning. The studies truly highlight the complexity of 5-HT1_AR signaling which appears to be highly dependent on the localization of the receptors within different brain structures.

Valorisation Addendum

Relevance & Audiences

Cognitive impairment is a common feature in many neurological and psychiatric conditions, like Alzheimer's disease (AD), dementia, schizophrenia, post-traumatic stress disorder (PTSD) and aging. Cognitive deficits include a wide variety of different symptoms which can affect multiple types of mental functions in different levels of severity depending on the nature of the underlying condition. The intricate nature of cognitive functions and inter-personal differences between patients makes it difficult to study cognitive impairments. Therefore, many of the mechanisms serving cognitive function are not fully understood yet. Our cognitive abilities enable us to perform many of the tasks important in day-to-day living. Therefore, cognitive impairments can have a great impact on everyday functioning of the patient and their quality of life. Hence, it is important to study different types of cognitive impairments to gain a better understanding of its underlying mechanisms and ultimately develop targeted treatments which can ameliorate cognitive impairments.

The studies in this dissertation have mainly focused on pattern separation, a specific type of cognitive function that entails the ability to discriminate between two highly similar but slightly different memory formations. Given that most people constantly move around in the same environments, seeing the same surroundings, the same people and the same objects on a very regular basis. One can imagine that pattern separation is of high importance to even the simplest aspects of day-to-day living. Impaired pattern separation has a severe impact on memory function, leading for example to difficulties in making a distinction between situations. Next to a decreased memory function, this can affect judgment and decision making which has a negative influence on the long-term outcome and chances of recovery for the patient.

Pattern separation has been found to be impaired in diseases like schizophrenia, PTSD and AD. Schizophrenia is a disabling psychiatric disorder that affects about 1% of the population worldwide. While schizophrenia is most known for its positive and negative symptomatology, cognitive deficits are also an important symptom category. In recent history, the importance of cognition in schizophrenia patients has been under-acknowledged and all available treatment strategies focus on the treatment of positive and negative symptoms. However, it was found that the extent of cognitive deficiencies is the best predictor of functional outcome when compared to the positive and negative symptomatology. Therefore, the development of treatment for cognitive impairments will have a substantial beneficial effect on the functional outcome and quality of life of patients. Furthermore, it will also lessen the significant and long-lasting health, social and financial burden for

the patients, their families, caregivers and the wider society. In addition to the social and economic benefits, amelioration of cognitive impairment would re-establish a patient's ability to function independently which has a major positive influence on recovery and chances of re-lapse.

Pattern separation impairment is of specific interest in the treatment of cognitive deficits in schizophrenia. It has been proposed that this particular impairment leads to the exacerbation of some of the more pronounced positive and negative symptoms, or might even be the underlying cause of the disease. The disability to judge situations and overgeneralization of potential threats increases anxiety and paranoia. This could lead to the development and sustenance of the commonly seen psychotic beliefs, which make schizophrenia such a debilitating disorder. Development of a treatment which could tackle all these symptoms at once would be of high benefit to both patients and society.

In addition, pattern separation impairments have been found to occur in a wide variety of psychiatric diseases. In AD for instance, it has been found to be one of the first cognitive abilities to be impaired in the early stages of the disease. This highlights the fundamental nature of pattern separation processing and its importance to support overall cognitive function. A cognitive enhancer that could ameliorate pattern separation dysfunction could not only benefit schizophrenia or AD patients, but improve the outcome of a broad spectrum of patients suffering from different psychiatric diseases. The 5-HT1AR sub-population has been studied widely in this respect, due to its distribution through the brain and wide involvement in many mechanisms important to cognition. However, most of the drugs that (partially) target 5-HT1AR's only show moderate results in their memory and cognition enhancing effects, due to the differentiating effects that stimulation of 5-HT1AR's can have based on their location within the brain. In this dissertation a novel type of 5-HT1AR agonists was studied in their ability to enhance cognition and specifically pattern separation processing. These 'biased' agonists have the ability to specifically target either the pre-synaptic autoreceptors or post-synaptic heteroreceptors, enabling the ability to specifically target the receptors that show beneficial effect without the opposing effect of the other sub-group.

In this dissertation it was shown that the cognitive performance of rodents can be improved by specifically targeting post-synaptic 5-HT1A heteroreceptors. In summary, it was shown that:

1. A biased post-synaptic 5-HT1AR agonist (F15599) can improve pattern separation performance in healthy rats when given both acutely and chronically;
2. Chronic biased post-synaptic 5-HT1A heteroreceptor stimulation enhances hippocampal plasticity;
3. That chronic exposure to a pre-synaptic 5-HT1AR agonist (F13714) will desensitize the pre-synaptic autoreceptors and restore the initial acute impairment.
4. That acute administration of a post-synaptic 5-HT1AR agonist ameliorates a pattern separation deficit in a pharmacological rat model for schizophrenia
5. That acute administration of a post-synaptic 5-HT1AR agonist ameliorates a behavioral flexibility deficit in a pharmacological rat model for schizophrenia

The possibility to study the behavioral effects of differently located 5-HT1ARs separately is a major benefit of these biased agonists. This dissertation demonstrates that there can be benefits in separately targeting these sub-populations and that it will provide us with new insights into the mechanisms of these receptors. This brings us one step closer to unravelling the 5-HT1AR system mechanisms and diseases associated with a dysfunction in this receptor population. Increasing our knowledge on the exact mechanisms will contribute to the development of successful treatments of psychiatric disease.

Post synaptic 5-HT1A heteroreceptors are expressed on many types of non-serotonergic neurons with a high distribution in area's important to memory and cognition, activation has many (indirect) effects on different signaling cascades. It was shown that specifically targeting this sub-population of receptors is potentially more effective than using a non-biased 5-HT1AR agonist in the treatment of pattern separation, because it circumvents the impairing effects of autoreceptor stimulation. It was shown that chronic stimulation of post-synaptic 5-HT1ARs with F15599 did not lead to desensitization to the treatment. Implying that this drug potentially can be given as a long-term treatment, without a decrease in its behavioral benefits.

Furthermore, it was shown that chronic treatment with F15599 enhances hippocampal plasticity measures, illustrating that chronic post-synaptic 5-HT1AR stimulation also has structural effects which support its beneficial behavioral effects. As enhanced hippocampal plasticity has been linked strongly to enhanced memory function, the beneficial effects might not be limited to pattern separation. These findings are also of interest to the investigation of other cognitive modalities which are influenced by 5-HT1AR function and the symptomatic treatment of cognitive dysfunctions.

Therefore, the results from these studies are of interest for the pharmaceutical industry. Developing new symptomatic treatments for schizophrenia, AD or other neurological or psychiatric disorders that show impaired cognition, is commercially interesting and has great societal impact. The industry has appropriate financial resources and infrastructure organization to bring a new drug to the market. Of note, research on the biased 5-HT1AR agonists is a collaboration with Neurolixis (Dana Point, USA).

In summary, the results of the studies described in this dissertation are of importance to patients, their family and caretakers, governments (reduction in medical costs) and pharmaceutical industries.

Activities/Products & Innovation

An innovation derived from this dissertation is the development of the Object Pattern Separation task. This novel task allows for easy, effective and efficient pre-clinical screening of spatial pattern separation-enhancing drugs, which can be of interest to the pharmaceutical industry.

An actual product that can be further developed based on these studies is the use of the biased 5-HT_{1A}R agonist F15599 in the treatment of schizophrenia related cognitive impairment. This dissertation showed there is an implication that treatment with F15599 might ameliorate both pattern separation and set-shifting impairments in schizophrenia patients. Since F15599 has already been approved for safe administration in humans, a next logical step would be to administer the treatment in a clinical trial to schizophrenia patients. If this treatment would prove to be effective in humans, this would be the first treatment available specifically for cognitive impairment in schizophrenia. This would not only be of high benefit to patients suffering from the disease but would also have high financial benefits for to the company being first-to-market with this type of treatment. The pharmaceutical industry would also have the financial resources and infrastructure to set-up the required clinical studies and bring this treatment to market. Furthermore, follow-up studies with these biased compounds could lead to new indications to be patented. For a whole spectrum of psychiatric diseases that show pattern separation dysfunction or other types of cognitive dysfunction.

In conclusion, the findings that the biased agonist F15599 can enhance spatial pattern separation processes and can be given chronically has shown the potential of biased 5-HT_{1A}R agonists as a treatment for cognitive deficits in schizophrenia patients. Furthermore, the application of these biased agonists is also a promising strategy for the treatment of other cognitive impairments in psychiatric diseases and a way to enhance the knowledge of the specific mechanisms of receptor sub-populations.

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