Universitat Autònoma de Barcelona

Effects of orexin-A blockade on memory facilitation by intracranial self-stimulation

Soleil García Brito

Ph.D. Thesis

Neuroscience

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I per tal que consti als efectes oportuns, signen el present certificat a Bellaterra, a 16 de Març de 2018

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CONTENTS

5-HT	Serotonin (5-hydroxytriptamine)
ACh	Acetylcholine
AD	Anterodorsal thalamic nucleus
AM	Anteromedial nucleus
Arc	Activity-regulated cytoskeletal-associated protein
AV	Anteroventral nucleus
BDNF	Brain-derived neurotrophic factor
BF	Basal Forebrain
CA1	Cornu ammonis 1
CA2	Cornu ammonis 2
CA3	Cornu ammonis 3
Cg	Cingulate Cortex
CL	Centrolateral nucleus of the thalamus
CM	Centromedial nucleus of the thalamus
Cn	Caudate nucleus
CPu	Caudate-putamen
CSF	Cerebrospinal fluid
DA	Dopamine
DG	Dentate gyrus
DMSO	Dimethyl sulfoxide
dRSC	Dysgranular retrosplenial cortex
E	East
ERK	Extracellular signal-regulated kinase
GABA	gamma-Aminobutyric acid
Glu	Glutamate
GPCR	G protein-coupled receptor
gRSC	Granular retrosplenial cortex
HDB	Horizontal limb of the Diagonal band of Broca
HPC	Hipoccampus
ICCS	Intracranial self-stimulation
icv	Intracerebroventricular
IL	Infralimbic cortex
ip	Intraperitoneal
ITI	Inter-trial Interval
LC	Locus coreuleus

ABBREVIATIONS

LD	Laterodorsal nucleus of the thalamus
LH	Lateral hypothalamus
LTDtg	Laterodorsal tegmental area
MD	Mediodorsal nucleus
MFB	Medial forebrain bundle
MLDA	Mesolimbic dopaminergic pathway
mPFC	Medial prefrontal cortex
MS	Medial septum
N	North
NA	Noradrenaline
NAc	Nucleus accumbens
NAcS	Nucleus accumbens shell
NMDA	N-methyl-d-aspartate
NMDAR	N-methyl-d-aspartate receptor
OFC	Orbitofrontal cortex
OX1R	Orexin-A receptor
PPTg	Pedunculopontine tegmental nucleus
PV	Paraventricular nucleus of the thalamus
Re	Reunien nucleus
Rh	Rhomboid nucleus
RN	Raphe Nuclei
RSC	Retrosplenial cortex
S	South
SA	Septal area
SI	Substantia innominata
Sn	Substantia nigra
Snc	Substantia nigra pars compacta
SVD	Simultaneous visual discrimination
VDB	Vertical limb of the Diagonal band of Broca
VTA	Ventral tegmental area
W	West

Learning and memory are the main tools for adaptation for both animals and humans. The integration of relevant acquired information into an existing network helps us navigate through in ever-changing contexts. While explicit memory allows us to remember “what” we know, implicit memory confers us the ability to remembering “how” to perform actions we have learnt in the past. Thus, research into both memory-enhancing treatments and those that can aid the recovery of memory is important in order take steps research towards an improvement in adaptive function and, consequently, quality of life.

Intracranial self-stimulation (ICSS) is a rewarding operant response that arises from stimulation of several areas belonging to the reward neural system, such as the medial forebrain bundle (MFB). It has been used as a memory-enhancement treatment for over 30 years. Studies in animals have consistently shown that this treatment can facilitate the acquisition and retention of both explicit and implicit memory tasks. ICSS has also been shown to recover loss of memory function due to neuroanatomical lesions and normal aging. Thus, understanding the underlying mechanisms through which ICSS treatment facilitates memory is of paramount importance, not only to advance research into a more effective administration of the procedure, but also in regards to the possible significant translational relevance. In order to explain its facilitating effects on learning and memory, several mechanisms have been proposed. Firstly, ICSS has shown to promote structural plasticity and the expression of plasticity-related genes in the hippocampus and other memory-related structures. In addition, ICSS boosts general arousal states through the activation of excitatory neurotransmission systems, such as the dopaminergic, cholinergic, noradrenergic and glutamatergic systems. The involvement of these neurotransmitters in learning and memory has been well documented. However, research exploring the implication of other neurochemical systems in the facilitative effects of ICSS on memory has yet to be carried out.

One such system is the orexinergic system. Orexins are excitatory neuropeptides that are thought to play a role in homeostatic regulation and food intake. Orexin-A is one of the molecular forms of orexin, which binds to both orexin receptors, the orexin-A selective receptor (OX1R) and the orexin-A and orexin-B receptor (OX2R). However, it is mostly through the binding with OX1R that this neuropeptide is involved in processes that overlap with those affected by ICSS. For instance, orexin-A participates in reward processing through interactions with the dopaminergic system in several reward-related regions in the brain. Furthermore, its involvement in reward processing ranges from food consumption, to drug-seeking behavior, to development of preference for objects or contexts associated to reinforcers. It has also been linked to ICSS, as orexin-A administration can affect the threshold of the electrical stimulation rewarding effect. Moreover, orexin-A plays a part in the regulation of arousal states mainly through the stimulation of the noradrenergic and cholinergic systems. In addition, OX1R action within the cholinergic system also seems to modulate attentional processing, especially when it comes to tasks requiring heightened attentional demand. Perhaps more importantly, orexin-A and OX1R have been gaining relevance with regard to learning and memory processes, in that it has been shown to be capable of facilitating the acquisition of several types of memory. Interestingly, one of the hypothalamic regions where orexinergic neurons are located is the lateral hypothalamus, which is also where the highest rates of ICSS behavior are recorded.

In order to study the relationship between the orexinergic system and the facilitating effect of ICSS on learning and memory, this dissertation presents four studies which set out to explore whether or not blocking OX1R, using intracerebroventricular microinfusions of OX1R antagonist SB-334867, affects the facilitating effects of ICSS on two different memory tasks. Specifically, the effects of ICSS and OX1R blockade were studied in a standard spatial version of the Morris Water Maze (MWM) and an adapted version of a simultaneous visual discrimination (SVD) implicit task in the same apparatus. This has allowed us to compare the effects of the treatments on different types of cognitive strategies and memory processes in a similar experimental condition.

In **Study I** we aimed to determine the effects of ICSS on a SVD in the MWM. We found that ICSS facilitates the acquisition and retention of this task, while promoting an inflexible expression of the discrimination memory. The results of this study also provided the basis for further studies into the implications of orexins and ICSS on an SVD task. In **Study II** we characterized the condition of training intensity – weak, 2 trials or strong, 6 trials – and found that the blockade of OX1R caused learning impairment of a spatial memory task in the MWM, as well as a decrease in c-Fos expression in memory-related areas that also express OX1R. The administration of SB-334867 only impaired the performance and lessened the activation of memory-related areas in animals that had undergone weak training, which suggests that stronger training has a compensatory effect. These results allowed us to determine the appropriate training condition to apply in the following experiment. In **Study III**, we used the previously characterized training protocol in order to assess the effects of OX1R blockade on the facilitating effects of ICSS on a spatial memory task in the MWM, as well as the activation of memory-related areas.

ICSS treatment compensated for the deficits caused by SB-334867 administration, but its facilitative effects on the consolidation and retention of the spatial memory task were partially negated by the OX1R blockade. In addition, ICSS facilitation of the reversal task was completely negated in animals that received SB-334867 microinfusions. At the same time, ICSS-treated animals showed a higher activation of memory-related areas, while the affectation by OX1R blockade resulted in a general reduction in activity. Interestingly, SB-334867 infusions partially or totally negate the increase in activation observed in ICSS-treated animals depending on the brain region. Taken as a whole, these results suggest that the orexinergic system is involved in the facilitative effect of ICSS, especially on the cognitive flexibility aspect of a spatial memory task. In **Study IV**, we evaluated the effects of OX1R blockade on the facilitation of an SVD task in the MWM by ICSS, as well as the acetycholinesterase (AChE) activity in some important cholinergic areas. Our results show that SB-334867 administration impairs discrimination learning, especially regarding the number of errors committed by the animals. The deficit observed during the training phase and retention was compensated by ICSS. Animals that received ICSS showed a rigid and inflexible discrimination memory when tested in a reversal task, in a similar way to Study I. This effect was annulled by the administration of SB-334867. In addition, the levels of AChE activity were reduced by the inactivation of OX1R, showing that animals with lower AChE activity performed worse in several aspects of the task. ICSS reverted SB-334867 effects, but had no clear effects on AChE levels of animals that did not receive infusions of the antagonist. These results suggest that the orexinergic system also plays a role in the effect of ICSS on an SVD task. Moreover, present results indicate that multiple neurochemical systems participate in the facilitating effects of ICSS on implicit memory.

This dissertation sheds further light on the implication of the orexinergic system in the facilitation of memory by ICSS. More specifically, it highlights the extensive involvement of orexin-A and its selective receptor OX1R in aspects such as consolidation, long-term retention and cognitive flexibility in both implicit and explicit memory. In addition, this dissertation provides insight into the differential participation of OX1R in the reversal of each task, depending on how flexible that type of memory is. Overall, we concluded that blocking OX1R partially impedes the facilitation of memory by ICSS, but that this effect varies depending on the type of memory task and specific aspects of memory.

El aprendizaje y la memoria son procesos cognitivos claves para la adaptación. La integración de la nueva información adquirida a las redes neuronales existentes ayuda a los individuos a desenvolverse en contextos constantemente cambiantes. Mientras que la memoria explícita nos permite recordar “lo que sabemos”, la memoria implícita nos confiere la capacidad de recordar “como hacer lo que sabemos”. Así, investigar tratamientos que puedan potenciar las capacidades de memoria, o incluso paliar sus posibles déficits, constituye un hito importante para la mejora de esta capacidad cognitiva y, por lo tanto, de la calidad de vida.

La autoestimulación eléctrica intracraneal (AEIC) es una respuesta operante que surge a partir de la estimulación del sistema neural del refuerzo como, por ejemplo, del haz prosencefálico medial. Desde hace más de 30 años, los datos experimentales indican que este procedimiento constituye un potente tratamiento para la facilitación de la memoria. Estudios en animales han confirmado de forma consistente que este tratamiento puede facilitar la adquisición y la retención de tareas explícitas e implícitas. La AEIC también ha demostrado ser capaz de recuperar la pérdida de memoria producida por lesiones cerebrales o por el envejecimiento. Así, dilucidar los mecanismos que subyacen a la acción facilitadora de la AEIC sería de suma importancia, no sólo para optimizar las condiciones de administración, sino también en cuanto a su potencial relevancia translacional. Los posibles mecanismos implicados en el efecto facilitador de la AEIC podrían ser múltiples. Así, la AEIC promueve mecanismos de plasticidad en el cerebro relacionados con los procesos de aprendizaje y memoria, como cambios estructurales o la expresión de genes relacionados, en el hipocampo y otras estructuras de diferentes sistemas de memoria. Además, la AEIC produce un arousal generalizado en el cerebro a través de la activación de sistemas de neurotransmisión excitatorios, como el dopaminérgico, el colinérgico, el noradrenérgico o el glutamatérgico. La participación de estos neurotransmisores en el aprendizaje y la memoria ha sido ampliamente documentada. Sin embargo, la implicación de otros sistemas neuroquímicos en el efecto de la AEIC sobre la memoria no ha sido estudiada.

Uno de los candidatos es el sistema orexinérgico. Las orexinas son neuropéptidos excitatorios que participan en procesos homeostáticos. La orexina-A se une a los dos receptores de orexinas, el OX1R y el OX2R. Sin embargo, es a través de su acción sobre el OX1R que la orexina-A participa en procesos que también son modulados por la AEIC. Por ejemplo, la orexina-A participa en los procesos de refuerzo a partir de sus interacciones principalmente con el sistema dopaminérgico mesolímbico. Así, está

implicado en el efecto reforzante de la ingesta de alimentos, del consumo de drogas y en el desarrollo del condicionamiento de preferencia por objetos o contextos asociados a un estímulo reforzante. Este sistema neuroquímico también se ha relacionado con la conducta de AEIC, ya que la administración de orexina-A puede influir sobre la intensidad umbral de estimulación. Además, la orexina-A regula los estados de arousal probablemente a través de la modulación de vías noradrenérgicas y colinérgicas. La activación de los receptores OX1R en neuronas colinérgicas parece también modular procesos atencionales, especialmente en aquellas tareas con alto requerimiento atencional. El estudio de la participación de las orexinas, y de los receptores OX1R en particular, en la modulación de los procesos de aprendizaje y memoria está ganando relevancia; ya que se ha demostrado que la activación de estos receptores puede facilitar diferentes tipos de memoria. Por último, las neuronas que sintetizan orexinas se encuentran principalmente en el hipotálamo lateral, precisamente el área cerebral donde la AEIC causa una mayor tasa de respuesta.

Con el objetivo de estudiar la relación entre el sistema orexinérgico y el efecto facilitador de la AEIC sobre el aprendizaje y la memoria, se han llevado a cabo cuatro experimentos orientados a evaluar si el bloqueo de los OX1R, mediante la administración intracerebroventricular de SB-334867, afecta a la facilitación de la memoria producida por la AEIC en dos tareas distintas de aprendizaje, una explícita y otra implícita. Específicamente, los efectos de la AEIC y del bloqueo de los OX1R fueron estudiados en una versión espacial estándar del laberinto Acuático de Morris (LAM), y en una tarea de discriminación visual simultánea (DVS), una versión implícita adaptada para el mismo laberinto. Esto nos permitía comparar los efectos de los tratamientos sobre estrategias cognitivas y procesos mnemónicos diferentes en condiciones experimentales similares.

En el Estudio I, nos propusimos determinar los efectos de la AEIC sobre la DVS en el LAM. Observamos que la AEIC facilita la adquisición y la retención de esta tarea, pero que promueve una expresión inflexible de la memoria. Estos resultados aportaron el modelo experimental para el estudio posterior sobre la implicación de las orexinas en una tarea de DVS. En el Estudio II, se evaluó la influencia de la intensidad de entrenamiento – bajo, 2 ensayos o alto, 6 ensayos – y los efectos del bloqueo de los OX1R en una tarea espacial en el LAM. Los resultados demostraron que la administración de SB-334867 ocasiona un deterioro de la memoria espacial, pero únicamente en la condición de bajo entrenamiento. Además, los sujetos que recibieron el bloqueador mostraron una disminución de la expresión de c-Fos en diversas áreas cerebrales relacionadas con la memoria espacial y que expresan OX1R. Todo ello parece indicar que un alto entrenamiento podría compensar el deterioro producido por el bloqueador. Estos resultados nos permitieron determinar la condición de entrenamiento que debíamos aplicar en el siguiente experimento. En el Estudio III, usamos el protocolo de bajo entrenamiento del Estudio II para evaluar los efectos del bloqueo de OX1R sobre la facilitación de la AEIC en la memoria espacial y en la activación de regiones relacionadas con la memoria. El tratamiento de AEIC compensó los déficits causados por el SB-334867, pero perdió parcialmente su efecto facilitador sobre la consolidación y la retención. Además, en la fase de reversal, el SB-334867 anuló por completo el efecto de la AEIC. Los resultados de c-Fos pusieron de manifiesto una mayor activación de las àreas de memoria en los animales tratados con AEIC y reducción en los que fueron infundidos con SB-334867. La infusión de SB-334867 ocasionó la anulación parcial o total del efecto potenciador de la AEIC sobre la actividad, dependiendo de la región. En conjunto, estos resultados sugieren que el sistema orexinérgico está involucrado en el efecto facilitador de la AEIC sobre la memoria espacial, especialmente en la flexibilidad cognitiva. En el Estudio IV, evaluamos los efectos del bloqueo del OX1R sobre la facilitación de una tarea de DVS en el LAM por la AEIC, así como sus efectos sobre los niveles de acetilcolinesterasa (AChE) en dos regiones colinérgicas relevantes. Nuestros resultados muestran que el SB-334867 deteriora el aprendizaje de DVS. El déficit observado durante las fases de entrenamiento y retención fue contrarrestado por la AEIC. Y, al igual que en el Estudio I, los animales que recibieron este tratamiento mostraron una memoria de discriminación mas rígida e inflexible a la hora de realizar el reversal. Este efecto fue anulado por la administración de SB-334867. Además, la inactivación del OX1R redujo los niveles de AChE, mostrando una correlación significativa entre estos niveles y la ejecución de la tarea. La AEIC revierte estos efectos del SB-334867, aunque en ratas sin el inhibidor no parece tener efectos sobre los niveles de este enzima. En conjunto, estos resultados sugieren que el sistema orexinérgico participa en el efecto de la AEIC sobre una tarea de DVS, pero no excluyen la participación de otros sistemas neuroquímicos en la facilitación de la AEIC sobre la memoria implícita.

En resumen, esta tesis aporta nuevos datos acerca de la implicación del sistema orexinérgico en la facilitación de la memoria por la AEIC. Más específicamente, destaca la participación de la orexina-A y su receptor OX1R en la consolidación, la retención a largo plazo y la flexibilidad cognitiva en dos tareas, de memoria explícita y de memoria implícita respectivamente. Además, dedica una especial atención a la participación diferencial del OX1R en un aprendizaje de reversal, dependiendo de las características

de cada tarea en términos de flexibilidad. En general, el presente trabajo sugiere que el bloqueo de los receptores OX1R impide la facilitación de la memoria por AEIC de forma parcial, pero que este efecto depende del tipo y del proceso de memoria que se evalúe.

CHAPTER I

GENERAL INTRODUCTION

1 INTRACRANIAL SELF-STIMULATION (ICSS)

1.1 Introduction

Intracranial self-stimulation (ICSS) is an operant response, provoked by the stimulation of reward pathways in the brain. James Olds and Peter Milner inadvertently discovered this phenomenon in 1953, while studying the relationship between the stimulation of the reticular activating system (RAS) and motivational processes (Olds & Milner, 1954). The electrode implantation targeted the RAS to evaluate whether the animals would develop a preference for the maze location where the stimulation was given. The researchers did not expect to find that only one of the animals would develop this preference; due to an unsuccessful implantation, the electrode had found its way into the septal area (SA) of the basal forebrain (BF). The researchers subsequently carried out a variety of experiments that shed light on some of the characteristics of this phenomenon. The stimulation led to an increase walking speed and exploration, as well as allowing the researchers to easily shape “a wide range of behaviors” (Milner, 1989). Later on, the animal was trained to press a lever that would deliver the stimulation train. According to Milner (1989), this was done in order to confirm that the observations were not a result of increased motor excitability as opposed to a reinforcing event. It also allowed the researchers to show that the behavior was easily extinguished when the contingency was eliminated (Olds, 1958).

One of the most evident characteristics of ICSS is that it is highly rewarding. This has been corroborated by homeostatic studies, which found that food-deprived animals (Routtenberg & Lindy, 1965; McMurray et al., 2017) and humans (Bishop et al., 1963) would choose brain stimulation over food. Similarly, thirsty animals (Falk, 1961) and hypothermic (Carlisle & Snyder, 1970) animals preferred ICSS to water or heat, respectively. Perhaps more interestingly, the animals trained in an ICSS paradigm would even endure electric shocks in order to reach the ICSS lever (Olds, 1958). Nevertheless, the studies that looked to compare ICSS to food (Olds, 1958; (Hoebel, 1969), and sex (Olds, 1958; Olds & Fobes, 1981) concluded that animals would respond in a very similar manner to both. Thus, it can be argued that ICSS leads to behavioral responses similar to those established by natural rewards. However, ICSS as a reinforcement paradigm has several advantages over food, water or sex. Firstly, the motivation for the behavior is not a result of a deficit state – food and water deprivation – and it presents no satiety (Olds, 1958). Secondly, the direct activation of the reward pathways allows for the stimulus to bypass sensorial inputs (Wise, 2002); this presents the opportunity to study the reward pathway with some neuroanatomical and neurochemical specificity. Finally, the parameters of the stimulation that the animals receive are adjustable, which enables the researchers to achieve optimal stimulus-response ratio, and the output is easily operationalized into objective data (Carlezon & Chartoff, 2007).

Amongst the species that have been studied for ICSS behavior are dogs (Stark et al., 1962; Kassil’ et al., 1982), cats (Roberts, 1958; Kassil’ et al., 1982; Ángyán, 1975) pigeons (Goodman & Brown, 1966; Zeigler et al., 1978), goldfish (Boyd & Gardner, 1962), non-human primates (Campbell, 1972; German & Fetz, 1976) and humans (Bishop et al., 1963; Heath, 1963). In addition, peripheral self-stimulation has also been documented for fish and reptiles (Campbell, 1972). However, it is mostly studied in rodents for assessing activation of the central nervous system (CNS) in relation to dysregulations of motivational processes (Olds & Fobes, 1981; Carlezon & Chartoff, 2007; Stoker & Markou, 2011), and for evaluating characteristics of drug addiction (Negus & Miller, 2014; Tedford et al., 2014; Harris et al., 2015; Miller et al., 2015; Radke et al., 2016) and withdrawal (Holtz et al., 2015; Muelken et al., 2015; Harris et al., 2017). Nevertheless, the study of ICSS is not limited to reward-related research. In 1975, Aryeh Routtenberg became interested in the mesolimbic dopaminergic pathway (MLDA) as a substrate through which stimulation would affect memory consolidation (Routtenberg, 1975). Since then, several researchers have studied ICSS in relation to its influence upon cognitive and memory-related processes (Destrade & Jaffar, 1978; Huston & Mueller, 1978; Velley et al., 1978; Redolar-Ripoll et al., 2002; Ramkumar et al., 2008; Segura-Torres et al., 2010; Ilango et al., 2011; Kádár et al., 2011; Chamorro-López et al., 2015; Kádár et al., 2016) in which ICSS is used as a facilitative or recovery treatment for different types of memory.

1.2 Neural basis of ICSS

The SA was the first site where an electrode resulted in reports of ICSS behavior. Olds and Milner (1954) also attempted to generate the operant response from implantations in different brain areas: the corpus callosum (CC), caudate nucleus (Cn), cingulate cortex (Cg), hippocampus (HPC), medial lemniscus (ml), mamillothalamic tract (mt), medial geniculate (mg) and the tegmentum (Tg). Their results have been supported by later studies, in which it became evident that the self-stimulation rates and behavioral outcome would vary depending on the site (Table 1). Amongst other areas of the brain that have been targeted under an ICSS paradigm in rodents are the olfactory bulb (OB) and HPC (Phillips et al., 1977), medial prefrontal cortex (mPFC) (Mora et al., 1976), nucleus accumbens (NAc) (Zacharko et al., 1983), medial forebrain bundle (MFB) of the lateral hypothalamus (LH) (for review see Wise & McDevitt, 2017), ventral tegmental area (VTA) (Druhan et al., 1989), locus coeruleus (LC) (Crow et al., 1972) brainstem (Rompre & Miliaressis, 1985) and cerebellum (Corbett et al., 1982). A summary of the sites where ICSS can be obtained is depicted in Figure 1.

Table 1. Rate of self-stimulation and behavioral output.

ICSS site	Rate	Behaviour	Reference
MFB	↑↑↑↑	Arousal Focal Exploration Approach Ejaculations Seizures Grooming	Hodos & Valenstein, 1962 Steiner et al., 1978 St-Laurent, 1988 Olds & Olds, 1963 Ellman & et al, 1975 Singh et al., 1996
Septal Area	↑↑	Arrest Ambivalence	Hodos & Valenstein, 1962 St-Laurent, 1988
Prefrontal Cortex	↑	Arrest	St-Laurent, 1988 Bogacz et al., 1965
Hippocampal formation	↑	Diffuse exploration Flight behaviour Seizures	St-Laurent, 1988 Olds & Olds, 1963 Campbell et al., 1978
Ventral Tegmental Area	↑↑↑	Focal exploration Approach Stereotypy	St-Laurent, 1988 Olds & Olds, 1963 Ilango et al., 2014a Singh et al., 1996
Preoptic area	↑↑	Focal exploration	St-Laurent, 1988
Raphe nuclei	↑	Diffuse exploration	St-Laurent, 1988 Sheard & Aghjanian, 1968
Locus Coeruleus	↑↑↑	Non-systematised exploration	St-Laurent, 1988 Crow et al., 1972 Steiner et al., 1978 Ellman et al, 1975
Reticular formation	↑↑	Ambivalent	St-Laurent, 1988 Olds & Olds, 1963

Table 1. Rate of self-stimulation and behavioral output in studies that compared different ICSS sites. Number of arrows indicate lower or higher rate of stimulation.

The MFB of the LH has been the most frequently targeted site within the paradigm of ICSS and reward. This is probably because the rate of self-stimulation is the highest and most easily sustained when applied to this site (Olds & Olds, 1963). Notwithstanding, as many other sites are responsive to the activity of the fibers of the MFB, researchers have long looked for the common thread among them. Hence, the study of reward pathways and the relevant neurotransmission systems requires the operationalization of the variables involved. As described by Wise and Bozart (1984), the phenomena of reward could be studied by looking at four different but related events, which involve many of the areas mentioned above. Therefore, these areas could be seen as elements belonging to a wider circuit that interconnects in series and responds to the activation of four different fundamental components, assessed by specific methods: 1) the MFB – by electrical stimulation; 2) the VTA – by morphine injections; 3) connections between VTA and the NAc – by electrical stimulation; and 4) the NAc – by amphetamine injections.

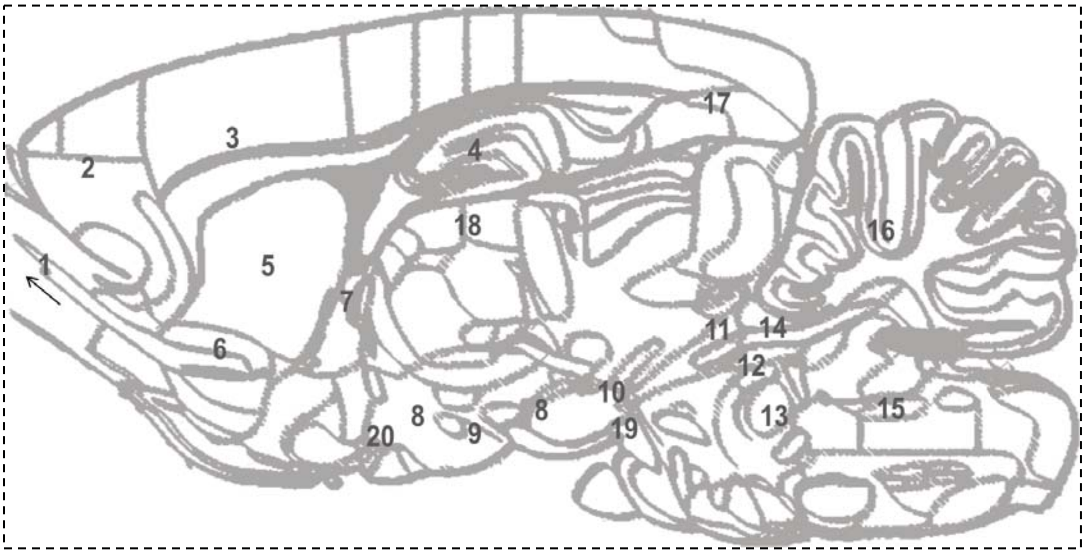


Figure 1. Summary of main sites that produce ICSS behavior. Some structures do not appear in brain section due to laterality: 1. Olfactory bulb; 2. Medial prefrontal cortex; **3.** Cingulate cortex; **4.** Hippocampus; **5.** Caudate nucleus; **6.** Nucleus accumbens; **7.** Septum; **8.** Medial forebrain bundle; **9.** Ventromedial hypothalamus; **10.** Ventral tegmental area; **11.** Raphe nucleus; **12.** Locus coeruleus; **13.** Motor nuclei trigeminal nerve; **14.** Mesencephalic nucleus trigeminal nerve; **15.** Nucleus of the solitary tract; **16.** Cerebellum; **17.** Entorhinal cortex; **18.** Mediodorsal thalamus; **19.** Substantia nigra; **20.** Amygdala. Adapted from Phillips & Fibiger, 1989; and Wise, 1996.

1.2.1 *Medial forebrain bundle*

The MFB is a neural pathway, which is broadly accepted to form part of the reward system. It is comprised of a set of long ascending and descending axons, arranged in a rostrocaudal axis, passing through the LH and extending from the OB and mPFC to structures in the brainstem. Short interconnecting axons also project to and from lateral and adjacent regions of the hypothalamus, adding to the complexity of the pathway. While the descending projections activate areas involved in the reinforcing aspect of ICSS (Bozarth et al., 1980; Wise, 1996), the ascending fibers also participate in the general activation of the brain (Takagi et al., 1980).

Many structures have been anatomically and functionally linked to the MFB (see Figure 2). There has long been a debate regarding this extensive network and about whether these sites are a single system or multiple circuits linked in parallel. In fact, several researchers have questioned the notion of a unitary system located inside the MFB, as well as its indispensability to ICSS. Valenstein and Campbell (Valenstein & Campbell, 1966) originally found that self-stimulation behavior could still be obtained despite a 90 percent degradation of the fibers of the MFB. Results obtained after extensive anterior and posterior lesions of the MFB supported this notion (Lorens, 1966). Later, anatomically specific ablation studies showed that hypothalamic ICSS was still maintained after disconnection with ipsilateral forebrain and caudal structures (Huston & Borbély, 1973; Huston et al., 1982; Stellar, Illes, & Mills, 1982; Sprick et al., 1985; Fakhoury et al., 2016), and that causing a lesion in an ascending or descending fiber would differentially affect the ICSS rewarding efficacy (Gallistel et al., 1996). A key example of the non-exclusive nature of ICSS behavior is the MLDA, one of the most important components of the MBF. This system is activated by the myelinated descending fibers (Bielajew & Shizgal, 1986) from the LH to the VTA and contains the ascending dopaminergic fibers from the VTA in the midbrain to the NAc in the BF (You et al., 2001), and is commonly referred to as the reward pathway. However, lesion studies have also shown that the maintenance of ICSS behavior cannot be attributed to this system alone (Hand & Franklin, 1985; Johnson & Stellar, 1994).

Taken together, the evidence coming from lesion and ablation studies suggests the existence of fibers that connect with collateral neurons and alternative systems capable of maintaining the very complex ICSS behavior.

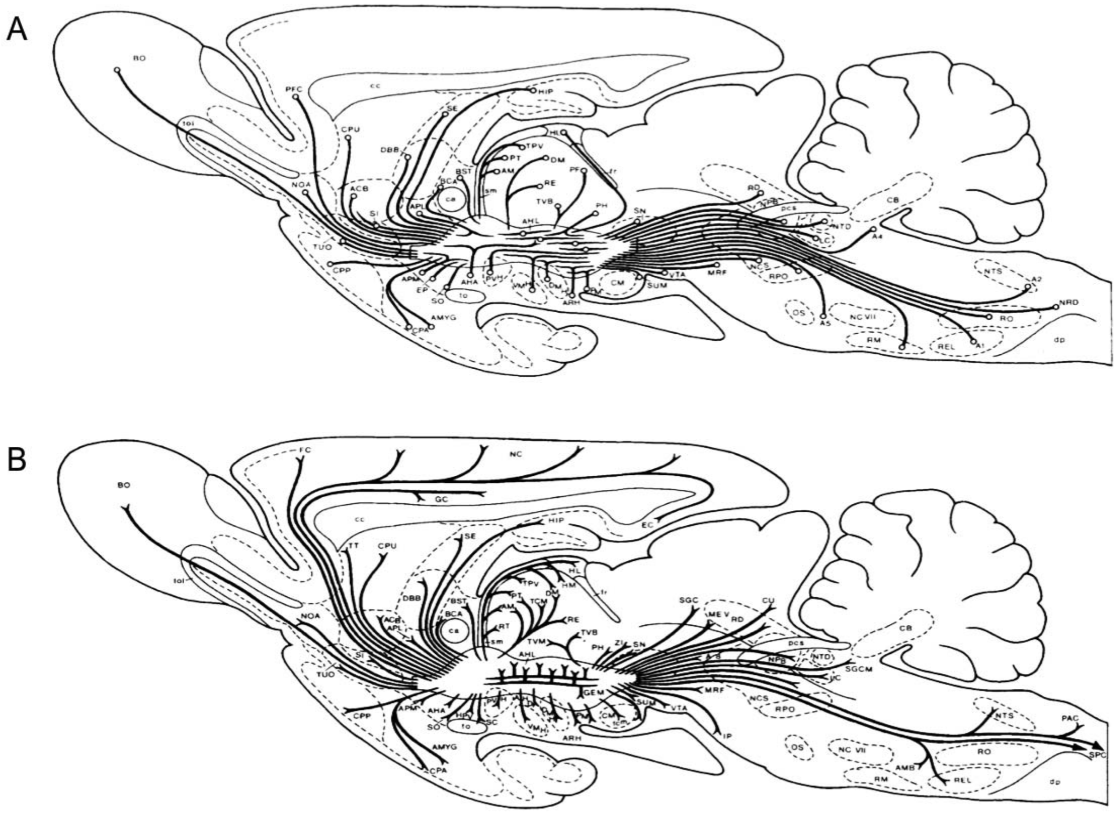


Figure 2. Diagram of fiber projections comprising the reward system. Figure 2A depicts input to the MFB. Figure 2B depicts output of the MFB. Adapted from Nieuwenhuys et al., 1982.

1.2.2 Neurotransmission of the ICSS

Perhaps it does not come as a surprise that researchers interested in studying reward saw in this novel method a way to elucidate the pharmacological aspects of addiction, food intake, and reward-seeking behavior in general. The catecholamine system, specifically noradrenaline (NA), was the first one to be functionally linked to reward and self-stimulation in the early 60s. Since then, additional systems that interact with the MFB and its components have been identified. Among them are the dompaminergic, glutamatergic, cholinergic, GABAergic, serotoninergic and opioid systems. The first indications of a possible role for neurotransmitters in the ICSS rewarding effect came from an overlap in mapping studies, which entailed a correlation between ICSS loci, and histochemical studies of neurons and pathways.

As mentioned above, NA was the first neurotransmitter hypothesized to mediate the brain stimulation reward and supporting evidence rapidly came from dose-dependent pharmacological studies where the enhancement (Stein, 1964) or reduction (Stein, 1964; Poschel & Ninteman, 1963) of catecholaminergic synaptic action would in turn increase or decrease ICSS behavior, respectively. It is important to remember that at this time, distinctions between NA and DA had not yet been established. For that reason, when lesion studies on cortical NA (Cooper et al., 1974) and in the LC (Corbett et al., 1977) failed to disrupt ICSS in the same nucleus or in the LH, DA came to be considered as the most likely substrate for ICSS behavior.

Early evidence for dopaminergic involvement in brain stimulation reward came from studying the catecholamine (German & Bowden, 1974) and neuroleptic (Edmonds & Gallistel, 1974; Franklin, 1978; Fouriezos et al., 1978) systems. Additional supporting evidence arrived from extensive mapping studies using moveable electrodes and fluorescence histochemistry (Corbett & Wise, 1980), which confirmed that ICSS rate was proportional to the density of DA neurons around the tip of the electrode as well as the confinement of ICSS positive sites to zones with DA containing bodies. ICSS in the MFB has also been directly linked to an increase in DA levels in the NAc (Blaha & Phillips, 1990; Phillips et al., 1992; Taber et al., 1995; Rada et al., 1998; Owesson-White et al., 2008; Hernandez et al., 2012; Klanker et al., 2017). Moreover, the blockade of DA transporter increases ICSS behavior (Hernandez et al., 2012), whereas causing lesions in DA neurons (Fibiger et al., 1987) and blocking DA receptors (Lippa etal., 1973; Fouriezos & Wise, 1976) impairs it. More recently, studies using optogenetic techniques have helped confirm the reinforcement caused by the stimulation of DA neurons in the VTA or substantia nigra (Sn) of rodents (Witten et al., 2011; Kim et al., 2012; Ilango et al., 2014b; Wang et al., 2017) and non-human primates (Stauffer et al., 2016).

However, the notion of DA as a crucial and central neurotransmitter to the ICSS behavior has also been questioned. Although some studies have found that both, D1 and D2, receptors in the NAc are necessary for the positive reinforcement from ICSS in the VTA (Steinberg et al., 2014; Steidl et al., 2017), other have argued that each receptor has distinct roles in the facilitation/suppression of ICSS in VTA and MFB. In that sense, the coactivation of both receptors leads to the suppression of ICSS; more specifically, the direct administration of D2 agonists into the NAc suppresses ICSS (Singh et al., 1997), while a D1 agonist increases the rewarding effects of the stimulation (Nakajima & McKenzie, 1986). Likewise, MFB-LH stimulation alters D1, but not D2, receptor expression in the NAc or ventral pallidum (VP) (Simon et al., 2016). In general, it has been reported that animals are capable of maintaining self-stimulation when DA has been depleted within the reward system (Cooper et al., 1974; Phillips & Fibiger, 1978). Moreover, collision studies have shown that DA fibers are not directly depolarized by the electrode tip in the LH, but by descending unmyelinated axons, which activate DA neurons in the VTA (Yeomans et al., 1988; Gratton & Wise, 1988). Additionally, the DA neurons projecting to the NAc do not seem to be activated in series by stimulation in the LH of the MFB (Cossette et al., 2016). This suggests the existence of additional neurotransmitters that form part of the reward substrate.

The ultrafast population of acetylcholine (ACh) fibers was found to contribute to the rewarding effects of MFB stimulation by activating dopaminergic neurons in the VTA (Gratton & Wise, 1985; Yeomans et al., 1985; Rada et al., 2000). In addition, dopaminergic neurons in the Sn are innervated by ipsilateral cholinergic afferents from the pedunculopontine tegmental nucleus (PPtg) (Clarke et al., 1987), and stimulation of the MFB-LH increases c-Fos immunoreactivity in this nucleus (Arvanitogiannis et al., 1997). This anatomical and functional relationship has been further explored by blocking muscarinic (Yeomans et al., 1993) or enhancing nicotinic (Clarke & Kumar, 1984) receptors in this area, thus provoking a disruption of and increasing the rewarding effects of hypothalamic stimulation, respectively. Moreover, VTA efferent glutamate-dopamine neurons target cholinergic interneurons in the NAc (Chuhma et al., 2014), where they increase DA release (Threlfell et al., 2012; Cachope et al., 2012; Surmeier & Graybiel, 2012, Warner-Schmidt et al., 2012).

Additionally, glutamate (Glu) had been found to activate the VTA in a MFB stimulation paradigm (You et al., 2001) by making contact with neighboring neurons (Dobi et al., 2010). In fact, the stimulation of Glu neurons in the VTA (Wang et al., 2015) and PPTg (Yoo et al., 2017) has reinforcing effects. Furthermore, stimulation of the dorsal raphe nucleus supports the reinforcement of ICSS through its Glu projections to the VTA and NAc (Qi et al., 2014; Luo et al., 2015; Hernandez et al., 2016), despite serotonin (5-HT) not contributing to this effect (McDevitt et al., 2014). However, long and constant stimulation of Glu neurons in the VTA is aversive to mice (Yoo et al., 2016; Qi et al., 2016). Moreover, Glu neurons in the VTA co-release GABA after stimulation, which promotes positive reinforcement (Yoo et al., 2016). This rewarding effect has also been observed after stimulation of GABA LH-VTA projections (Barbano et al., 2016). What initially appeared to be a counterintuitive premise had been addressed previously by researchers who observed that GABA activity in the VTA increases prior to MFB ICSS and decreases immediately after (Steffensen et al., 2001), behaving like an integrator of the brain stimulation reward (Lassen et al., 2007). Hence, the inhibition of local GABAergic neurons in the VTA could be responsible for the increase in DA release in the NAc that has been observed after direct photostimulation of LH-VTA GABAergic neurons (Nieh et al., 2016), even in a predictive manner (Sharpe et al., 2017). The opioid system is another group of inhibitory transmitters that have been linked to ICSS behavior in rodents, since injecting them in the VTA increases ICSS rate (Broekkamp & Phillips, 1979; Ide et al., 2017), and decreases ICSS threshold (van Wolfswinkel et al., 1985).

Importantly, the orexinergic system has also been studied in relation to its involvement in reward processing. Orexin fibers project from the LH to the VTA (Peyron et al., 1998; Nambu et al., 1999), where they innervate and excite both DA and non-DA neurons (Korotkova et al., 2003; Borgland et al., 2006) through N-metyl-D-asparte (NMDA) potentiation (Narita et al., 2006; Borgland et al., 2009). Furthermore, the administration of orexin-A in the VTA facilitates LH stimulation by reducing the aversive effects of other neurotransmitters (Muschamp et al., 2014). Notwithstanding, intracrebroventricular (icv) or intra-VTA orexin-A administration has also been shown to increase the ICSS threshold (Boutrel et al., 2005; Hata et al., 2011).

Taken together, the roles of each neurotransmitter and the interaction between them portray a complex system that requires further elucidation. Although the DA system is the most widely implicated in the rewarding effects of ICSS, other neurotransmitters interact with it and support its role. A representation of the systems involved in MFB ICSS can be seen in Figure 3.

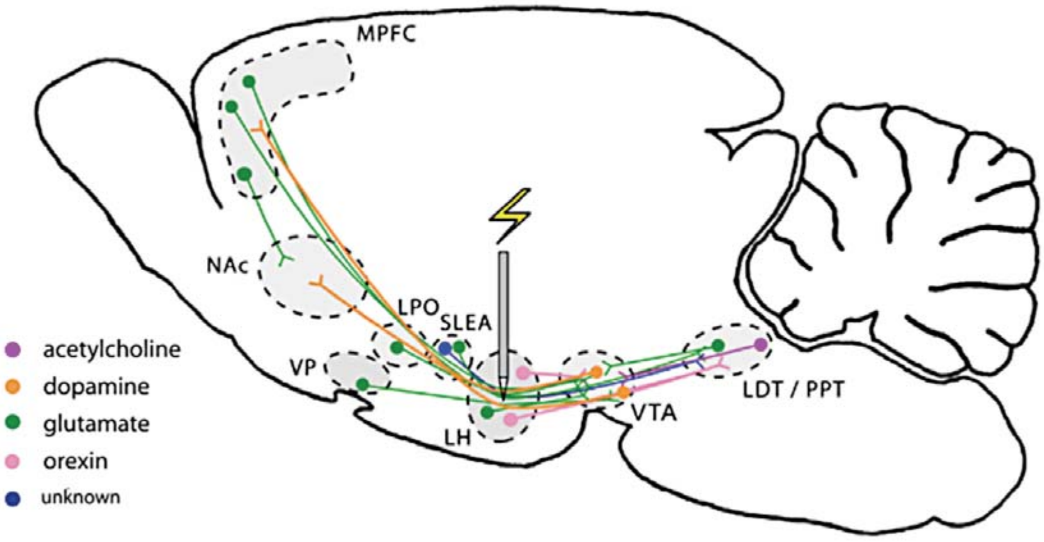


Figure 3. Sagital representation of pathways involved in MFB-LH ICSS rewarding effect. From antero-posterior representation: MPFC, medial prefrontal cortex; NAc, nucleus accumbens; VP, ventral pallidum; LPO, lateral preoptic area; SLEA, sub-lenticular extended amygdala; LH, lateral hypothalamus; VTA, ventral tegmental area; LDT, laterodorsal tegmental area; PPT, pedunculopontine tegmental area. Adapted from Shizgal, 2012.

1.3 Learning and Memory facilitation by ICSS

Since its discovery, several researchers have studied ICSS regarding its influence upon cognitive and memory-related processes in which ICSS is used as a facilitative or even recovery treatment for different types of memory. Not only has it been proven effective as a memory-enhancing treatment in animals, but also in humans. A study performing hypothalamic stimulation in the hope of treating a patient's morbid obesity threw up an unexpected result: they found that the patient experienced heightened memory functions due to direct increased activation of the HPC (Hamani et al., 2008). In addition, the stimulation of areas belonging to the extensive network connected to MFB-LH, such as the entorhinal cortex (Mann et al., 2017) and fornix (Hescham et al., 2013), has provided treatment for cognitive deficits of neural pathologies like Alzheimer's disease. Considering the wide range of areas and systems implicated in the ICSS rewarding effects, it goes without saying that the study of the mechanisms through which this phenomenon exerts its action on additional neural processes is extremely complex. Subsequently, the mechanisms through which ICSS enhances or facilitates learning and memory are not entirely known.

A central theory of reinforcement by Huston et al. (1977) proposed that ICSS would improve the learning of various tasks by acting directly on short-term memory processes. Coulombe and White (1980, 1982) also argued that administering an ICSS treatment after training to learn a task could promote the strengthening of the association that had been created. They based their premise on results obtained in an aversive conditioning task, where animals that underwent ICSS treatment needed 80% less of stimuli pairings in order to learn the task compared to the controls. This was also observed in a two-way active avoidance (TWAA) task, where ICSS seemed to accelerate the acquisition to a point at which the treated animals learned in 24h what control animals took 7 days to learn (Aldavert-Vera et al., 1996).

One of the questions that arise from the facilitative effect of ICSS on memory is whether or not it is a result of a pairing between the learning process and the rewarding event of ICSS. For example, if the rewarding stimulation were associated to the lack of aversive stimuli or the operative completion of an appetitive task, then it would potentiate the learning process and formation of the memory (Huston & Oitzl, 1989). However, the stimulation of the MFB can facilitate different types of memory conditioning even when it is administered at levels below the rewarding threshold (Destrade & Jaffar, 1978). Furthermore, the stimulation of areas associated to reward, such as the NAc does not facilitate memory, whereas amphetamine administration into the caudate, a non-rewarding site, does (White & Milner, 1992). This has also been observed in spatial memory tasks, with the stimulation of the entorhinal cortex (Stone et al., 2011; Mann et al., 2017).

Considering that the facilitating effects of ICSS are not directly dependent on the rewarding event, an additional phenomenon must be taking place. One of the characteristics of ICSS in the MFB-LH is the propagation of a generalized arousal state (Wright & Craggs, 1977; Destrade & Jaffar, 1978; Wright & Craggs, 1979; Stephens & Herberg, 1979; Nieh et al., 2016). It has been reported that a modulation of the arousal states could result in the positive enhancement of memory acquisition and consolidation (Nielson et al., 1996; Cahill & McGaugh, 1998), as well as the retrieval of memories (Przybyslawski & Sara, 1997; Tang et al., 2017), via the modulation of short-term thalamo-cortical plasticity (Castro-Alamancos & Connors, 1996). Nevertheless, states of high arousal can also result in the inability to engage the cognitive systems that would process information in a more flexible manner, thus failing to grasp the contextual details of a spatial learning situation (Maran et al., 2017). Therefore, the modulation of arousal states could stand as a possible general mechanism, among others, through which the ICSS treatment is capable of facilitating very diverse memory processes.

1.3.1 *Implicit and explicit learning and memory facilitation*

Early evidence of MFB-LH ICSS facilitation of learning and memory comes mainly from implicit memory paradigms. This treatment was originally tested to assess whether it could facilitate passive avoidance learning, with considerable success (Mondadori et al., 1976; Mondadori et al., 1977; Huston & Mueller, 1978). Additionally, more extensive work in our laboratory has corroborated the ability of ICSS to facilitate the acquisition of a TWAA task regardless of whether it is administered pre-or post-training (Segura-Torres et al., 1988). However, only a post-training ICSS treatment was capable of sustaining the facilitation (Redolar-Ripoll et al., 2002). Furthermore, as reported by one of the studies presented in this dissertation, ICSS can also facilitate robust and reversal resistant memory consolidation in a visual discrimination task in an implicit memory paradigm in the Morris Water Maze (MWM) (García-Brito et al., 2017). This treatment has even been proved capable of recovering the loss of memory function induced by lesions in the parafascicular nucleus (Redolar-Ripoll et al., 2003), the basolateral (Segura-Torres et al., 2010a) and lateral amygdala (AMG) (Kádár et al., 2014), as well as normal aging (Aldavert-Vera et al., 1997).

In addition, brain stimulation in the MFB of the LH has also been used in explicit memory paradigms, which have revealed a similar outcome. The ICSS treatment has been found able to facilitate spatial memory in rodents in the MWM (Soriano-Mas et al., 2005; Ruiz-Medina et al., 2008a; Takahashi et al., 2009; Chamorro-López et al., 2015). Other research groups have also observed that it can reverse stress-induced behavioral deficit performance in the T-maze (Ramkumar et al., 2008). While contingent ICSS promotes a rigid type of consolidation in implicit tasks, such as the visual discrimination paradigm we mentioned above, when it comes to spatial memory formation the organization and subsequent consolidation of information is more flexible. Accordingly, it has been reported that stimulated animals that had learned to hide/retrieve their food from one place in their home cage, quickly learnt the new location of the food, showing high cognitive flexibility in spatial memory (Hermer-Vazquez et al., 2005); likewise, animals that were stimulated in the MFB-LH achieved a more flexible memory expression in a delayed spatial alternation T-maze task (Soriano-Mas et al., 2005).

1.3.2 *Neural mechanisms of ICSS facilitation*

The mechanisms through which the ICSS is able to facilitate different types of learning and memory tasks have become somewhat clearer in the last few years. Several studies point at a possible modulation of ICSS upon memory-related elements in different areas of the brain as a mechanism for this facilitation. These mechanisms range from neurochemical, to molecular and morphological.

Regarding the modulation of neurotransmitter levels as a result of ICSS, brain stimulation in the MFB-LH increases levels of NA, DA, Glu, and acetylcholinesterase (AChE) activity in the HPC (Shankaranarayana Rao et al. 1998a), despite a stress-induced atrophy in the HPC (Ramkumar et al., 2008) the PFC (Ramkumar et al., 2012), and lesions to the AMG (Kádár et al., 2014). At a more molecular level, ICSS has been shown to increase c-Fos expression in CA3 and dentate gyrus (DG), and modulate the expression of over 60 hippocampal genes, some of which are related to neural plasticity, learning and memory (Huguet et al., 2009). Moreover, Kádár et al. (Kádár et al., 2011) found that the administration of MFB-LH ICSS results in the induction of the expression of several genes in AMG that are related to synaptic plasticity; among these genes are Fos, BDNF, Arc, ICER and CART; the majority of these genes were regulated in the HPC as well (Kádár et al., 2013), where an increased expression of the activity-regulated cytoskeletal-associated (Arc) protein was also found. Moreover, ICSS administration after a TWAA task increases levels of c-Fos in the AMG, HPC, LH and dorsal striatum, while

Nurr1 is only elevated in the HPC, AMG and dorsal striatum (Aldavert-Vera et al., 2013). In addition, it has also been found to increase c-Fos expression in the retrosplenial cortex (RSC) (Kádár et al., 2016). Furthermore, ICSS boosts neurite extensions in a PC12 mutant cell line through P38 MAPK enhancement, while an aversive event (immobilization) has the opposite effect (Gomita et al., 2017).

Ex vivo experiments further corroborate the morphological changes that appear in the HPC after ICSS treatment, suggestive of long-term potentiation (LTP) capabilities. Several studies have reported that ICSS in the MFB generates structural plasticity changes in the dendrites of CA3 hippocampal neurons (Shankaranarayana Rao et al., 1993; Rao et al., 1994). Said changes have proved sustainable over long periods of time (at 30 and 60 days after ICSS) (Shankaranarayana Rao et al., 1998b). The authors hypothesized that the alterations in the length and complexity of the dendritic branching might be due to an ICSS-induced increase in synaptic plasticity in CA3 (Shankaranarayana Rao et al., 1998c; Shankaranarayana Rao et al., 1999). Moreover, the enhancing effects of ICSS are such that it is capable of reverting stress-induced dendritic atrophy in both CA3 (Ramkumar et al., 2008) and PFC (Ramkumar et al., 2012) neurons. A more recent study has noted that CA1 pyramidal cells also experience morphological changes involving dendritic branching density and complexity, which seem to be related to an improvement in learning as a result of ICSS (Chamorro-López et al., 2015).

Finally, additional reports describe that ICSS in the MFB-LH of rodents significantly increases neurogenesis in the DG (Takahashi et al., 2009). These new neurons then matured and managed to integrate within hippocampal functional circuits, somehow reflecting the increased ability of the treated animals to acquire and consolidate a spatial memory task in the MWM. Brain stimulation in other areas, such as the anterior thalamic nucleus (Toda et al., 2008), the medial septum (Jeong et al., 2017) and the entorhinal cortex (Stone et al., 2011) has also reported neurogenesis in the DG. Stone et al., (2011) also noted that ICSS-derived neurogenesis and spatial performance decreased with the administration of a neurogenesis inhibitor, indicating that the enhanced cognitive ability originated from the ICSS-induced neurogenesis.

As we have laid out above, the mechanisms that allow for ICSS facilitation of learning and memory of diverse memory tasks are not known. Although some of the resulting effects of ICSS treatment on memory-related areas have been identified, there seems to be a missing link missing capable of bridging the gap between the rewarding experience, the arousal events in the brain, and the facilitation of learning and memory. Some of the neurochemical correlates of ICSS indicate that a neurotransmission system could be involved in many of the functional and cognitive aspects we find ICSS to affect. One of the systems that could be mediating the facilitative effect of ICSS will be explored in the next section.

2 Orexins

2.1 Introduction

Orexins, also known as hypocretins, are two neuromodulator peptides discovered by independent researchers Luis de Lecea and Takeshi Sakurai at practically the same time (de Lecea et al., 1998; Sakurai et al., 1998). While de Lecea named them after their location (hypothalamus, hypocretins), Sakurai focused on the primary associated function – stimulation of food intake (from Greek “orexis”, meaning appetite). They are exclusively produced in the perifornical area, lateral hypothalamus (LH) and posterior hypothalamus of rats (Peyron et al., 1998; Nambu et al., 1999) and humans (Elias et al., 1998) (Figure 4).

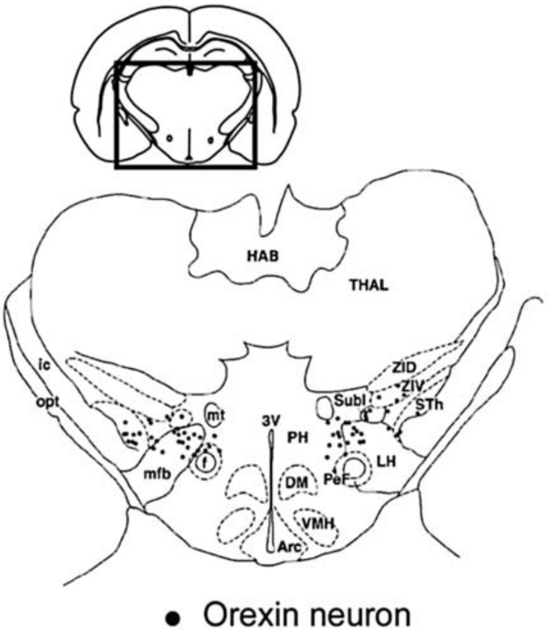


Figure 4. Coronal section depicting prepro-orexin mRNA-containing neurons, shown in black superimposed upon anatomical structures of the hypothalamus. Abbreviations: LH, lateral hypothalamus; PeF, perifornical nucleus; PH, posterior hypothalamus; STh, subthalamic nucleus; SubI, subincertal nucleus; ZIV, ventral zona incerta; THAL, thalamus; HAB, habenular complex; ic, internal capsule; opt, optic tract; mt, mammillothalamic tract; f, fornix; mfb, medial forebrain bundle; 3V, third ventricle; Arcn, arcuate hypothalamic nucleus; DM, dorsomedial hypothalamic nucleus; and VMH, ventromedial hypothalamic nucleus. Adapted from Tsujino & Sakurai, 2013.

Orexin neurons exist in two molecular forms, orexin-A and orexin-B. These excitatory neuropeptides share approximately 50% sequence identity and derive from the same precursor protein – prepro-orexin. Orexin-A is 33-aa residue peptide with two interchain disulfide bonds, whereas orexin B is a linear 28-aa residue peptide (Nakamachi, 2016). A representation of the peptides is depicted in Figure 5.

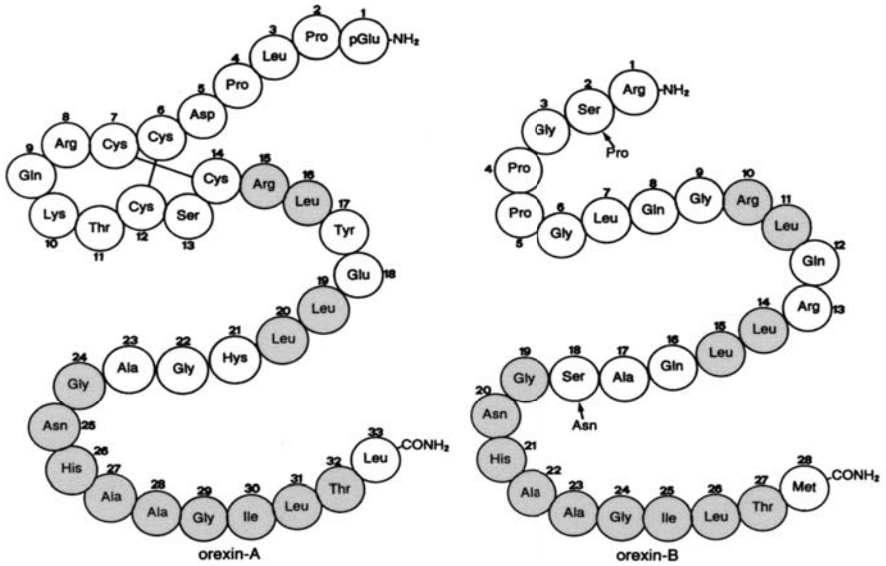


Figure 5. Orexin-A and orexin-B amino acid sequence. The orexin-A amino acid sequence is identical in humans, sheep, pigs, rats, and mice. Human orexin-B differs from that of pigs, rats, and mice in two amino acid substitutions (arrows). Identical amino acids between orexin-A and orexin-B are shadowed. Adapted from Spinazzi et al., 2006.

Both the site of origin and the sequences of the peptides are strongly preserved across vertebrate species (Wong et al., 2011), which suggest that they play a role in processes related to food intake and energy homeostasis. In order to survive lengthy periods of irregular food consumption, an animal must be able to carefully monitor its levels of hunger and energy, while modulating its wakefulness state in response to that previous assessment. To do so, the orexinergic system interacts with feeding cues, such as glucose/leptin/ghrelin, as well as monoaminergic/cholinergic centers (Yamanaka et al., 2003a), in order to coordinate the regulation of arousal and energy consumption in relation to food seeking behavior. As such, orexin-A administration consistently stimulates food intake in rodents (Sakurai et al., 1998; Edwards et al., 1999), and zebrafish (Yokobori et al., 2011), while the orexinergic system works to detect hunger/satiation states. Specifically, this population of peptides is inhibited in the presence of high concentrations of extracellular glucose and leptin, and excited by decreased levels of glucose and high levels of ghrelin (Yamanaka et al., 2003b; Burdakov et al., 2005; González et al., 2009; Schöne et al., 2011). This suggests that orexin neurons are integrators of “need-to-feed” signals. Furthermore, these neuropeptides also participate in the regulation of energy levels in response to food availability. For example, animals that face reduced food availability modify the rate of energy consumption, which allows for higher locomotor activity and longer periods of wakefulness (Itoh et al., 1990; Challet, Pévet, & Malan, 1997). This adaptative response is lacking in orexin-ablated transgenic mice, which are not capable of responding to fasting periods with an increased level of activity and alertness (Yamanaka et al., 2003b; Akiyama et al., 2004).

Since their discovery, it became clear that the orexin neurons are affected by a great number of brain areas and neurotransmission systems. Two groups of researchers carried out mapping studies using a genetically encoded retrograde tracer (Sakurai et al., 2005 ; Yoshida et al., 2006), which allowed them to identify the afferent innervations to orexin neurons. These included neurons coming from the infralimbic cortex (IL), basal forebrain (BF), nucleus accumbens shell (NAcS), lateral septum, bed nucleus of the stria terminalis, ventrolateral preoptic nucleus, posterior and dorsomedial hypothalamus, and raphe nuclei (RN). Several studies have shed further light on the way in which the neurotransmitters, neuropeptides, hormones and other factors coming from these areas modulate orexin neuron activity (Table 2).

Previously, it had been reported that the projections from orexinergic LH neurons reach the entire brain (Peyron et al., 1998; Nambu et al., 1999). These studies consistently found that the most densely innervated areas were monoaminergic nuclei locus coeruleus (LC) and RN, as well as the paraventicular nucleus of the thalamus (PV), the tuberomammillary nucleus, the arcuate nucleus in the hypothalamus (Arcn) and the ventral tegmental area (VTA). However, the scope of the projections is extensive, including cortical and subcortical regions, all across the neuroaxis (Figure 6).

The orexinergic system as a whole seems to influence multiple brain areas and therefore, is involved in a wide range of processes, with some researchers taking the extraordinary step of dubbing it as the “key to a healthy life” (Chieffi et al., 2017). In addition to their role in food intake and energy homeostasis, orexins have been thoroughly studied in order to determine their role in stress response [for review see Kukkonen et al., 2002), reward (Borgland et al., 2009; Aston-Jones et al., 2010; Muschamp et al., 2014), as well as their participation in arousal (for reviews see Sakurai, 2007; Li, Hu, & De Lecea, 2014), attention (Fadel & Burk, 2010; Zajo, Fadel, & Joshua, 2016) and memory processes (Telegdy & Adamik, 2002; Mavanji et al., 2017). Therefore, and given the objectives of this compilation, we will expand on the description of the orexinergic system in relation to reward and cognitive processes such as arousal, attention and memory.

Table 2. Factors that modulate orexin neurons' activity.

Factor	Receptor in orexin neuron	Reference
Excitatory		
Glutamate	AMPAR, NMDAR, mGluRs	Yamanaka et al., 2003b Li et al., 2002
Ghrelin	GHSR	Yamanaka et al., 2003b
Cholecystokinin	CCK-A	Tsujino et al., 2005
Neurotensin	ND	Tsujino et al., 2005
Vasopresin	V1a	Tsujino et al., 2005 Tsunematsu et al., 2008
Oxytocin	V1a	Tsujino et al., 2005
Glucagon-like peptide 1	ND	Acuna-Goycolea & van den Pol, 2004
CFR	CFR1	Winsky-Sommerer et al., 2004
mACh	M3 (27% of orexin neurons)	Sakurai et al., 2005
ATP	P2X	Wollmann et al., 2005
Inhibitory		
Glucose	Unknown	Yamanaka et al., 2003b
GABA	$GABA_A$, $GABA_B$	Li et al., 2002 Yamanaka et al., 2003b Xie et al., 2006
Serotonin	$5\text{-}HT_{1A}$	Yamanaka et al., 2003a Muraki et al., 2004 Sakurai et al., 2005
Noradrenaline	α_2	Yamanaka et al., 2003a Yamanaka et al., 2006 Sakurai et al., 2005
Dopamine	α_2	Yamanaka et al., 2003a Yamanaka et al., 2006 Sakurai et al., 2005
Neuropeptide Y	Y_1	Broberger et al., 1998 Fu et al., 2004
Leptin	OB-R	Yamanaka et al., 2003b
mACh	ND (6% of orexin neurons)	Yamanaka et al., 2003a Sakurai et al., 2005
Adenosine	A_1	Liu & Gao, 2007

Table 2. Factors that modulate orexin neurons' activity. α2, α2 adrenergic receptor; 5-HT1A, 5-hydroxytryptamine receptor 1A; AMPAR, α-amino-3- hydroxy-5-methyl-4-isoxazole propionic acid receptor; CCK-A, cholecystokinin receptor A; CRFR1, corticotropin-releasing factor receptor 1; GABA, γ-aminobutyric acid; GHSR, growth- hormone secretagogue receptor; mACh, muscarinic acetylcholine; mGluRs, metabotropic glutamate receptors; ND, not determined; NMDAR, N-methyl-d-aspartate receptor; OB-R, leptin receptor; P2X, purinocepter adenosine. Adapted and modified from Sakurai, 2007.

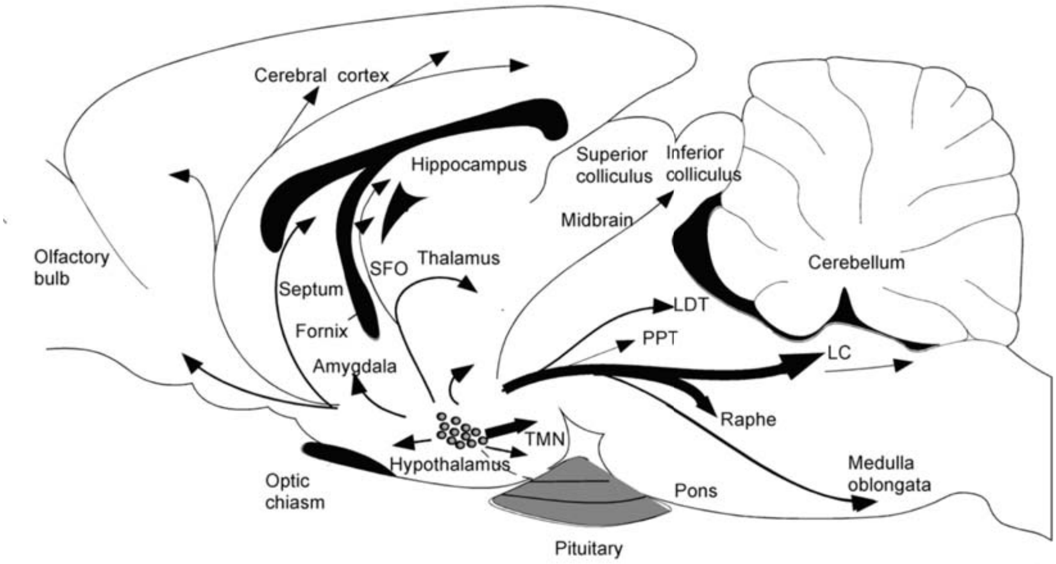


Figure 6. Sagittal section depicting prepro-orexin mRNA-containing neurons, shown in black superimposed upon anatomical structures of the hypothalamus. Abbreviations: PPT, pedunculopotine tegmental nucleus; LDT, laterodorsal tegmental nucleus; LC, locus coeruleus. Width of arrow signals density of fibers. Adapted from Tsujino & Sakurai, 2013.

2.2 Orexin receptors

Orexin-A and orexin-B peptides bind selectively to OX1 and OX2 receptors (OX1R and OX2R, respectively). They share 64% amino acid homology, but OX1R is more selective than OX2R. While OX1R binds exclusively to orexin-A (IC50 20nM), OX2R is less selective and binds to both peptides with high affinity (IC50: orexin-A, 38nM; and orexin B, 36 nM, respectively) (Sakurai et al., 1998). These receptors, like their peptides, are preserved across species with some studies observing a 94% identity in amino acids sequence between rats and humans (Sakurai et al., 1998). However, OX2R is probably older than OX1R, which is specific to mammalian species (Wong et al., 2011).

Orexin receptors spread widely across the neural axis. We have already mentioned studies that mapped the distribution of orexin neurons and fibers in the rat brain (Peyron et al., 1998; Nambu et al., 1999). In addition, the differential localization of OX1R and OX2R has been specified by *in situ hybridazation* studies (Hervieu et al., 2001; Marcus et al., 2001; Trivedi et al., 1998). The reports are in agreement regarding the widespread strong expression of OX1R in the retrosplenial cortex (RSC), the CA2 and DG of the hippocampus (HPC), prefrontal cortex (PFC), cingulate cortex (Cg) medial septum (MS), anterodorsal thalamic nucleus (AD), the midline thalamic nuclei, anterior and ventromedial hypothalamic area, LC and pedunlopontine and laterodorsal tergmental nuclei

(PPTg/LTDtg). Moreover, the strongest expression of OX2R is localized in the olfactory tubercule, CA3 of the HPC, Arcn, tuberomammillary nucleus, LH, medial portion of the PV nucleus of the hypothalamus, habenula, and the reticular formation. Furthermore, both receptors are markedly expressed in the PV of the thalamus, substantia nigra pars compacta (Snc), VTA, RN and the zona incerta. A schematic summary of the expression of these receptors is depicted in Figure 7.

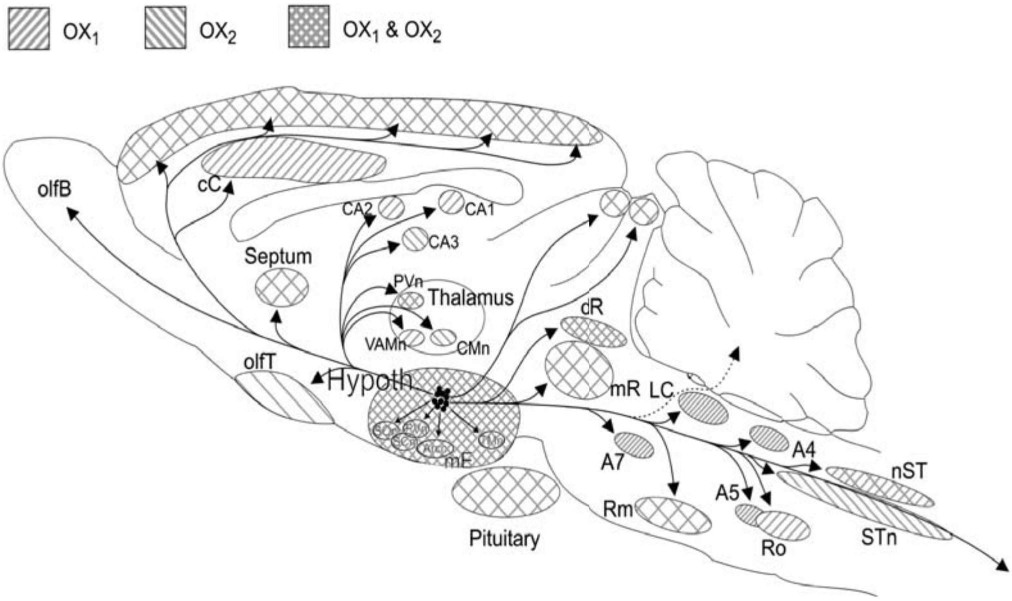


Figure 7. Orexinergic pathways and orexin receptor mRNA distribution in the rat according to Hervieu et al., 2001 (OX1R), Marcus et al., 2001 and Trivedi et al., 2001. Only a selection of the sites is shown. The receptor density is indicated by levels of hatching (3 levels). Abbreviations: Arcn, arcuate nucleus; CA1–3, areas of hippocampus; cC, cingulate cortex; CMn, centromedial nucleus; dR, dorsal raphe nucleus; LC, locus ceruleus; mE, median eminence; mR, median raphe nucleus; nST, nucleus of solitary tract; olfB, olfactory bulb; olfT, olfactory tubercle; PVn, paraventricular nucleus (in the thalamus and hypothalamus); Rm, nucleus raphe magnus; Ro, nucleus raphe obscurus; SCn, suprachiasmatic nucleus; SOn, supraoptic nucleus; STn, spinal trigeminal nucleus; TMn, tuberomamillary nucleus; VAMn, ventral anteromedial nucleus. Adapted from Kukkonen et al., 2002.

Furthermore, studies have expanded on the pharmacology of orexin receptors by transfecting Chinese hamster ovary (CHO). It is known that they are both heterotrimeric G protein-coupled receptors (GPCRs); OX1R couples to G_q, whereas OX2R can signal to G_q or G_i/G_o. The activation of the receptors coupled to G_q depolarize neurons and elevates intracellular Ca^{2+} through voltage-gated calcium channels (Peltonen et al., 2009) or intracellular release (Sakurai et al., 1998; Smart et al., 1999), mediated by an increase in phospholipase C activity (Lund et al., 2000; Holmqvist et al., 2002). This causes release of inositol triphosphate (IP_3) and diacylglycerol (DAG), which enhances the intracellular Ca^{2+}

release from the endoplasmic reticulum, increasing protein kinase C (PKC) activity (Spinazzi et al., 2006; Scammell & Winrow, 2011; Kukkonen & Leonard, 2014) (Figure 8). There is also evidence that OX2R coupling to $G_{i/o}$ could hyperpolarize the cell by activating the G-protein-coupled inward rectifier (GIRK) K^+ channels (Hoang et al., 2003).

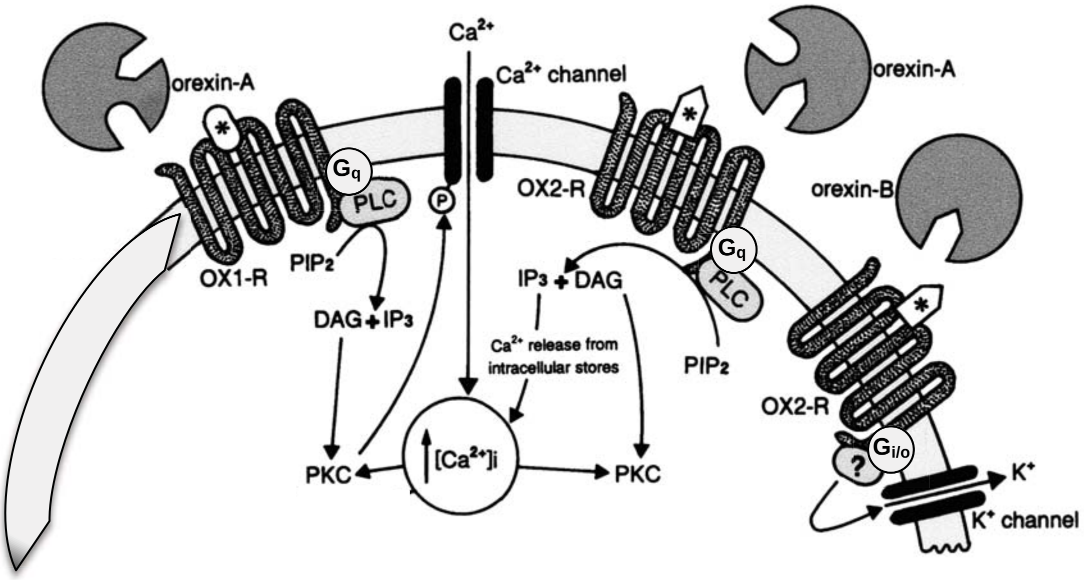


Figure 8. Schematic drawing of the main signaling pathways of OX1R and OX2R upon activation by orexin-A or orexin-B, respectively. Asterisks denote the binding region. Abbreviations: G, G protein; P, phosphorylation site; PIP_2, phosphatidylinositol; other abbreviations are indicated in the text. Adapted from Spinazzi et al., 2006.

The postsynaptic excitation of target neurons takes place through the activation of Na^+/Ca^{2+} exchange (Burdakov et al., 2003; Wu et al., 2004; Acuna-Goycolea & van den Pol, 2004), but orexin neurons can also stimulate presynaptic GABA or glutamate (Glu) release (van den Pol et al., 1998; Li et al., 2002). Furthermore, GPCRs are involved in the modulation of ERK and other MAPK pathways (for review see Rozengurt, 2007), which are related to neural plasticity (Giese & Mizuno, 2013). In that sense, one of the ways in which orexin-A could modulate plasticity changes is by the activation of OX1R, which results in the precipitation of AMPAR trafficking to the membrane in cultures of rat prefrontal cortex and striatum (Shin et al., 2009). It is important to note that this plasticity change was repressed by the removal of extracellular Ca^{2+} or by an inhibitor of the MAPK/ERK kinase 1. Similarly, orexin-A induces NMDAR trafficking to the membrane in the rat VTA through PKC activity (Borgland et al., 2006), suggesting that orexin is critical for synaptic plasticity induction in this region.

2.2.1 *Orexin-A selective antagonist: SB-334865*

Given the involvement of the orexinergic system in the aforementioned number of processes (see 2.1), there has been an increasing interest in developing pharmacological treatments for the most frequent orexin-related disorders, such as insomnia, drug addiction, diabetes and obesity (Boss et al., 2009).

The first compound developed with this purpose was the SB-334867 (Smart et al., 2001). This non-peptide has a high level of central nervous system penetration (Porter et al., 2001) and is an OX1R selective antagonist (50× over OX2R). Its administration inhibits Ca^{2+} elevation (Smart et al., 2001) and carries *in vivo* activity following intraperitoneal (ip) dosing. Moreover, behavioral observations report that the administration of SB-334867 induces rapid satiety, while also reducing active behaviors, such as grooming, sniffing, rearing and locomotion (Rodgers et al., 2001). It is important to note that this compound has so far been tested exclusively in animals. However, there have been developments in the pharmaceutical industry towards orexinergic antagonism in humans. Almorexant (ACT-078573) is an orally available dual orexin receptor antagonist introduced by Actelion in 2007. It has been shown to induce sleep in rats, dogs and humans (Brisbare-Roch et al., 2007), and to even increase rapid eye movement (REM) (Roecker & Coleman, 2008). Other compounds selective for OX1R, OX2R or of dual affinity are presented in Table 3.

Table 3. Main orexin antagonists and their affinity for OX1R and OX2R.

Compound	**Selectivity**	**Potency**		**Reference**
		OX1R	OX2R	
SB-334867	OX1R (~50×)	K_b = 40 nM	K_b = 1995 nM	Smart et al., 2001 Porter et al., 2001
SB-674042	OX1R (~130×)	K_b = 1 nM	K_b = 129 nM	Langmead et al., 2004
SB-408124	OX1R (~60×)	K_b = 22 nM	K_b = 1405 nM	Langmead et al., 2004
JNJ-10394049	OX2R (~630×)	K_i = 3162 nM	K_i = 5 nM	Dugovic et al., 2009
EMPA	OX2R (>900×)	K_i > 900 nM	K_i = 1 nM	Malherbe et al., 2009
Almorexant	Dual	IC_{50} = 13 nM	IC_{50} = 8 nM	Brisbare-Roch et al., 2007
MK-4305	Dual	K_i = 0.55 nM IC_{50} = 50 nM	K_i = 0.35 nM IC_{50} = 56 nM	Cox et al., 2010
Merek DORA-5	Dual	K_i = 0.6 nM IC_{50} = 29 nM	K_i = 1.2 nM IC_{50} = 27 nM	Whitman et al., 2009

Table 3. Main orexin antagonists and their affinity for OX1R and OX2R. Abbreviations: K_b, blocking constant; K_i, inhibitory constant; IC_{50}, half maximal inhibitory concentration; nM, nanomolar. Adapted from Scammell & Winrow, 2011.

2.3 Orexin-A and Reward

As mentioned above (see 2.2), the ubiquitous distribution of OX1R across the brain includes expression in brain regions associated with reward, such as the VTA and NAc. As previously stated (see *1.2.2*), the orexin-A fibers increase the activation of dopamine (DA), as well as non-DA neurons of these two areas (Korotkova et al., 2003; Borgland et al., 2006), and participate in reward processing. The studies that attempt to explore the involvement of orexin-A in reward are mainly focused on the effects of orexin-A administration or OX1R blockade on food and drug consumption, reinstatement of drug-seeking behavior, conditioned place preference (CPP) and intracranial self-stimulation (ICSS) thresholds.

The activation of OX1R seems necessary for food-reinforcement in mice (Sharf et al., 2010) and blocking this receptor using SB-334867 in rats results in reduced attempts to self-administer high-fat food pellets (Nair et al., 2008; Borgland et al., 2009). This system also participates in the reinforcing effects of addictive drugs, such as cocaine or morphine. In fact, orexin-A administration increases the cocaine self-administration rate in mice (Muschamp et al., 2014) and rats (España et al., 2011), whereas the blockade of OX1R using SB-334867 results in reduced attempts to self-administer cocaine (Borgland et al., 2009; España et al., 2010) and morphine (Farahimanesh et al., 2017). It has been suggested that the role of orexin-A may lie in the link to the cue to administer a substance. Thus, it has been shown that administering orexin-A into the VTA reinstates previously extinguished drug-seeking behavior for different substances, including cocaine (Boutrel et al., 2005; Harris et al., 2005), morphine (Aston-Jones et al., 2009, Aston-Jones et al., 2010) and ethanol (Brown et al., 2016).

The involvement of this system in establishing a link between reinforcing effects of drugs and food and a cue or particular context is further explored using the CPP paradigm. It has been observed that orexin neurons are activated when rats prefer contexts associated with addictive drugs or natural reinforcers (Boutrel et al., 2005; Harris et al., 2005; Lawrence et al., 2006; Smith et al., 2010). Moreover, OX1R has been shown to be more involved than OX2R in CPP (Rashidy-Pour et al., 2015). Specifically, orexin-A injections into the VTA promote CPP, even showing a positive correlation between the intensity of reward seeking and the percentage of c-Fos positive cells in the LH (Harris et al., 2005; Aston-Jones et al., 2009) and in the septum (Aston-Jones et al., 2010). Furthermore, OX1R antagonist in the NAc has been shown to weaken a cocaine-induced CPP (Gozzi et al., 2011); this study also revealed that the inactivation of OX1R in the NAc led to an attenuation of the excitatory effects of D-amphetamine in the nucleus accumbens shell (NAcS) portion of the striatum. Conversely, the injection of orexin-A in NAcS induced DA phasic release, which was in turn inhibited by SB-334867 administration (Quarta et al., 2010; Patyal et al., 2012). An increased signaling of DA has also been observed after local injections of orexin-A into the VTA (Aston-Jones et al., 2010), which had previously been associated to Glu-dependent long term potentiation (LTP) in VTA DA neurons (Aston-Jones et al., 2009). Incidentally, orexin-A-induced cocaine self-administration has been found to translate into stronger excitatory presynaptic Glu inputs to the VTA (Borgland et al., 2009).

Intracranial self-stimulation (ICSS) has also been used to assess the nature of orexin-A participation in reward, specifically by observing the effect of its administration or blockade on the ICSS threshold. For instance, if a drug or substance potentiates the rewarding effect of ICSS, its administration would lower the ICSS threshold. However, data from ICSS studies into orexin is sparse and contradictory. Thus, while both icv and intra-VTA orexin-A administration have been shown to increase ICSS threshold (Boutrel et al., 2005; Hata et al., 2011), it seems that intra-insular or ip administration does not affect the threshold or rate of ICSS (Hollander et al., 2008; Riday et al., 2012). Nevertheless, ICSS studies also allow for the exploration of additional systems that might affect orexinergic participation in reward. For instance, the threshold increase caused by orexin-A infusion can be inhibited by administering a corticotropin-releasing factor (CRF) antagonist (Hata et al., 2011), which suggests that the effects of orexin-A on ICSS threshold is related to stress processing. Moreover, orexin-A can also counteract the anti-reward effects on LH self-stimulation of dynorphin, a neuropeptide that is coreleased with orexin-A in the VTA (Muschamp et al., 2014).

2.4 Orexin-A and Arousal

Orexinergic neurons have been referred to as a link between energy balance (food availability) and arousal states (for review see Berthoud & Münzberg, 2011). In that sense, LH orexinergic neurons monitor hunger/satiety cues (glucose, leptin and ghrelin) and mediate the augmentation of arousal when animals are in prolonged fasting conditions (Yamanaka et al., 2003b). This is presumably an adaptative response that would increase locomotor activity and the chances of finding food.

According to experiments on the narcoleptic syndromes of orexin null mice, OX2R plays a more critical role in the control of sleep/wakefulness than OX1R (Willie et al., 2001; Willie et al., 2003). However, OX1R and orexin-A's involvement in arousal should not be disregarded, as OX1R is widely expressed in regions involved in sleep/wakefulness regulation, where orexin-A depolarizes neurons, such as the noradrenergic LC (Hagan et al., 1999), the cholinergic LDTg/PPTg (Burlet et al., 2002), and in the basal forebrain (BF) (Arrigoni et al., 2010).

Importantly, orexin-A levels in cerebrospinal fluid (CSF) is practically undetectable in narcoleptic patients (Nishino et al., 2000; Mignot et al., 2002). In fact, low concentrations of orexin-A in the CSF is now one of the criteria for narcolepsy diagnosis (American Academy of Sleep Medicine., 2014). Moreover, disorders in the sleep-wake cycle are also found in Alzheimer's disease (Peter-Derex et al., 2015), while a significant loss of orexin-A neurons (Fronczek et al., 2012) and a decrease in orexin-A levels in CSF (Slats et al., 2012) is also observed in these patients. In addition, extracellular levels of orexin-A in humans are higher during positive emotions, social interactions and anger in contrast to quiet wakefulness states, sleep or post-operative pain, when levels are lower (Blouin et al., 2013). This suggests that this peptide could also be involved in other activities that require a state of arousal, beyond just being awake. Observations in animals are consistent with this notion, showing that icv administration of orexin-A increases locomotor activity and grooming response (Hagan et al., 1999).

Furthermore, *in vivo* studies have also confirmed that rats' normal sleep/wake period is altered by orexin-A. The normal awaken period of rats increase after icv injections of orexin-A in a dose dependent manner (Piper et al., 2000). This study revealed that the electroencephalogram (EEG) readings of the animals during the sleep period are also altered, showing a sharp decrease in paradoxical sleep. Further studies into alterations in electrical activity as a result of orexin-A administration confirm that it promotes a gamma band in the EEG (Modi et al., 2017), which is a measure of arousal from coma states. It had previously been reported that the microinjection of orexin-A into the BF increased cortical release of ACh while also stimulating an arousal state, as read by the EEG, in both freely behaving rats (Thakkar et al., 2001) and in animals under isoflurane anesthesia (Fukuda et al., 2007). Fukuda et al., (2007) also showed that the OX1R selective antagonist SB-334867 was capable of reducing these measures of arousal – EEG and cortical ACh release – induced by PPTg electrical stimulation. A similar result was found in cats, as administering this neuropeptide into the LDTg increased the duration of wakefulness episodes (Xi et al., 2001), mediated by GABAergic activity in relation to the orexinergic input (Xi et al., 2015). In addition, the induction of an orexinergic current to PTTg/LDTg provokes a Ca^{2+}-dependent resonance that promotes arousal and cortical gamma frequencies (Kohlmeier et al., 2013; Ishibashi et al., 2015). Furthermore, the wakefulness-promoting effects of orexin-A are possible thanks to its innervation of cholinergic neurons in the LDTg that project to the mPFC (Cid-Pellitero & Garzón, 2011). Taken as a whole, the evidence suggests that the generation of cortical arousal through the stimulation of cholinergic pathways is at least mediated by orexin-A.

Finally, the LC is known for being a key modulator of arousal states (for review see Berridge & Waterhouse, 2003), and it is also one of the brain regions with the densest expression of OX1R (Hervieu et al., 2001; Marcus et al., 2001). Accordingly, the application of orexin-A into the LC has been found to increase the firing rate of NA neurons, accompanied by an observable increased state of arousal (Hagan et al., 1999). The symbiosis between these two systems might even go beyond increasing levels of arousal, as optogenetic studies have observed that the LC is the main effector of orexin-A neurons signaling with regard to sleep-to-wake transitions (Carter et al., 2012). Moreover, a deficiency of OX1R in the LC seems to result in a failure to maintain wakefulness, as seen in a narcolepsy/cataplexy mouse model ($OX1R^{-/-}OX2R^{-/-}$), where the animals significantly improved the consolidation of wakefulness episodes when the OX1R was restored in the LC (Hasegawa et al., 2014).

A representation of the pathways through which the orexinergic system exerts its action upon arousal, attention and memory can be seen in Figure 9.

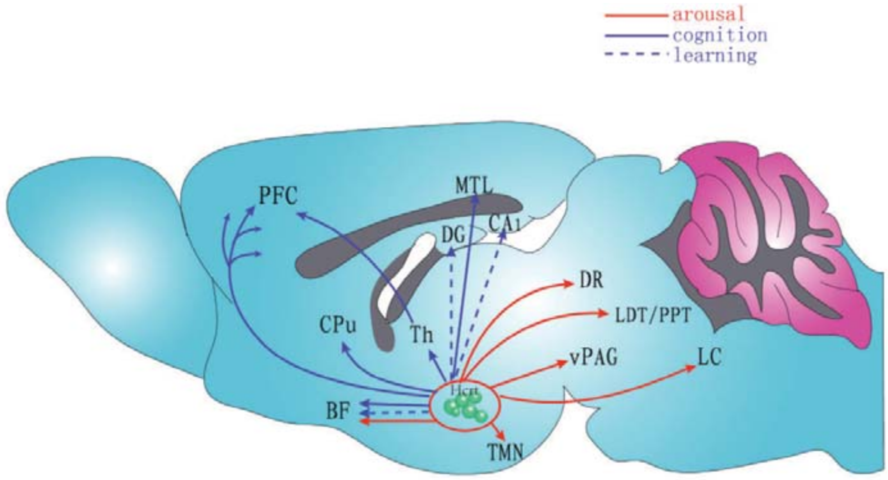


Figure 9. Orexin pathways to arousal and cognition areas. Abbreviations of main areas: PFC, prefrontal cortex; BF, basal forebrain; CA1, cornus ammonis 1; DG, dentate gyrus; LDT/PPT, laterodorsal and pedunculopontine tegmental nucleus; LC, locus Coeruleus. Adapted from Li et al., 2014.

2.5 Orexin-A and Attention

Fadel and Burk (2010) suggested that a dysfunction in the orexin – ACh interactions in the BF and PFC could help explain the attentional deficits that are observed in age-related cognitive decline and in many neurodegenerative diseases. In fact, many reports have supported this latter idea, particularly regarding Alzheimer's disease (Fronczek et al., 2012; Slats et al., 2012; Wennström et al., 2012; Fadel et al., 2013). Both the BF and the PFC have been highlighted for their importance to attentional processing (for reviews see Sarter et al., 2001, Sarter et al., 2005 and Villano et al., 2017). In short, while the cholinergic system in the BF is selectively activated during attentional performance, the PFC undertakes the optimization of the attention processing.

The orexinergic system appears to be a suitable candidate for the exploration of this functional relationship between the regions, since the administration of orexin-A into the BF has been shown to increase cortical ACh release in the PFC (Fadel et al., 2005; Arrigoni et al., 2010). Fadel (2005) also found a widespread distribution of orexin fibers in cholinergic regions on the basal forebrain, suggesting that the orexin projections to the basal forebrain form an important link between hypothalamic arousal and forebrain attentional systems. A later study performed by the same group found that administering the OX1R selective antagonist – SB-334867 – into the BF results in observable disturbances in the performance of rats in a signal detection-two lever sustained attention task (Boschen et al., 2009). Specifically, the authors noticed that the disruption in the performance was mainly due to a decrease in the level of accuracy regardless of signal presentation. This is consistent with previous findings from a study applying lesions to the cholinergic fibers in the BF, which found a decrease in accuracy when no signal was presented (Burk & Sarter, 2001). Moreover, in an attempt to determine whether the administration of orexin-A could enhance attention in rats, the same group administered orexin-A into the BF. They concluded that although orexin-A does not appear to enhance performance when the animals are well trained in the task, it improves the animals' performance when a visual distracter increases the attentional demands (Zajo et al., 2016). The same study also found that orexin-A injections can reverse performance impairments in animals that have suffered a 192 IgG-saporin-induced cholinergic lesion in the mPFC. All the results obtained by this group are consistent with an additional study that reported that while orexin-A in the BF facilitated olfactory discrimination acquisition and reversal, the OX1R antagonist impaired both portions of the task (Piantadosi et al., 2015). They also added that orexinergic action could be carried out through an increased level of cortical ACh activity.

Furthermore, the specific nature of the activation of the PFC by the orexinergic inputs has also been explored in *in vitro* studies, which have found that the excitation of orexin-A fibers that innervate mPFC results in a depolarization of its pyramidal neurons (Song et al., 2005; Xia et al., 2009; Yan et al., 2012). *In vivo* studies in rats have shown that intranasal administration of orexin-A increases ACh and Glu input to the PFC (Calva et al., 2018). In addition, local mPFC blockade of OX1R inhibits pyramidal neuron activity, which is reflected by a reduction in the power of the gamma oscillations in the area (He et al., 2015). Importantly, reduced gamma oscillations in the PFC has been shown to be a hallmark of schizophrenic patients with impaired cognitive flexibility (Cho et al., 2006). This suggest that orexin-A action of PFC excitation does not come exclusively from its effects of cholinergic inputs, but that it can, in its own right, promote a states of activity that would be associated to heightened attentional requirement.

2.6 Orexin-A and Learning and Memory

It should not be forgotten that OX1R is widely expressed in memory-related areas, such as the DG and the CA2 in the HPC as well as the RSC and the mPFC. Additionally, this receptor can also be found in CA1 to a lesser extent. Subsequently, the administration of orexin-A or its main receptor’s selective antagonist, SB-334867, affect the functionality of these areas and the learning and memory processes in which they are involved. Several behavioral studies have reported that the administration of orexin-A generally improves memory in implicit (Jaeger et al., 2002; Telegdy & Adamik, 2002; Akbari et al., 2008; Mavanji et al., 2017) and explicit (Akbari et al., 2006; Akbari et al., 2007; Yang et al., 2013; Zhao et al., 2014) memory tasks. A summary of the specific effects of orexin-A or its blockade on different aspects of learning in memory can be seen in Table 4.

Akbari et al. have undertaken an extensive study of the effects of orexin-A on memory, as reported in several publications. They first found that blocking the OX1R in the CA1 using SB-334867 resulted in an impaired acquisition (pre-training administration), consolidation (post-training administration) and retrieval (pre-probe administration) of a MWM task (Akbari et al., 2006). Their next study concluded that blocking the OX1R, this time in the DG, also results in impairment of acquisition and consolidation, but not retrieval of the spatial task (Akbari et al., 2007). Mixed results were obtained when exploring the effects of orexin-A blockade in CA1 and DG for a passive avoidance task; SB-33487 administration in the CA1 impairs the retrieval, whereas blocking OX1R in DG impaired acquisition and consolidation, but not retrieval (Akbari et al., 2008). Overall, they concluded that orexins in the HPC play an important part in the learning and memory formation of different tasks, possibly through the modulation of the activity of CA1 and DG.

There is mounting evidence of orexin-A positively modulating synaptic plasticity in the HPC. Microinfusion of orexin-A into remote areas connecting to the HPC, such as the LC, is capable of producing long-term potentiation (LTP) in the perforant path of the DG (Walling et al., 2004). Locally, an *in vitro* study looking at mice brain slice preparations provided evidence of orexin-A neuropeptide inducing and modulating the LTP of synaptic transmission in the CA1 Schaeffer collaterals in an age-dependent manner (Selbach et al., 2004; Selbach et al., 2010). The same study reported that the LTP in the HPC was associated with sharp-wave and theta rhythm, in addition to providing pharmacological evidence of coactivation of Glu, GABA, NA and ACh. An additional study seeking to uncover the underlying neurotransmission systems participating in this effect, found that orexin-A enhanced the facilitation and consolidation of a passive avoidance task, and despite DA, ACh and GABAergic systems being involved in this action, they could not determine a critical relevance of any of them on their own (Telegdy & Adamik, 2002).

However, some conflicting data has been reported regarding the affectation of memory processes due to orexin-A, suggesting that its role in this area of study has not been yet fully characterized. Two studies using a dual orexin receptor antagonist (Almorexant) found that blocking the action of orexin-A did not result in impairment of spatial memory (Dietrich & Jenck, 2010; Morairty et al., 2014) or passive avoidance learning (Dietrich & Jenck, 2010). An even more dramatic result that had been previously reported examined the extent of the effect of orexin-A, finding that it impairs MWM performance and LTP of CA1 Schaeffer collaterals (Aou et al., 2003). Here, the icv administration of the orexin-A was performed 2 h previous to the behavioral testing, at doses of 1µ at 1nM and 10nM. Nevertheless, a later study (Wayner et al., 2004) found that orexin-A enhanced LTP in the medial perforant path of the dentate granule cells in the dentate gyrus (DG), an effect which is blocked by SB-334867. This latter study found that the effective LTP-inducing dose of orexin-A was 1µ at 90nM. They went on to conclude that the same peptide could have different effects on the modulation of hippocampal synapses depending on the dosage and the post-synaptic receptors, which could explain differing results in the DG and the CA1. Further supporting evidence of OX1R mediating activity in the HPC comes from *in vivo* single unit recordings, showing that icv injections of orexin-A generally promotes excitatory responses of pyramidal cells in the CA1, while SB-334867 inhibits them (Riahi et al., 2015; Chen et al., 2017), as well

Table 4. Effects of orexin-A administration and OX1R blockade on memory tasks and neural markers of memory.

Procedure	Admin.	Time	Effect	Reference
Behavioral results				
Passive avoidance	**OrxA** icv	pre-post	↑ Learn., cons., retr.	Telegdy & Adamik, 2002
	OrxA icv	post	↑ Retention	Jaeger et al., 2002
	SB CA1	pre-post	↓ Retr.	Akbari et al., 2008
	SB DG	pre-post	↓ Acq. and cons.	
	Almorex po	pre	– No effect	Dietrich & Jeck, 2010
Morris Water Maze	**OrxA** icv	pre	↓ Acquisition	Aou et al., 2003
	SB CA1	pre-post	↓ Acq., cons. and retr.	Akbari et al., 2006
	SB DG	pre-post	↓ Acq. and cons.	Akbari et al., 2007
	Almorex po	pre	– No effect	Dietrich et al., 2010
	Almorex po	pre	– No effect	Morairty et al., 2014
	OrxA icv	pre	↓↑ Retention	Zhao et al., 2014
Social memory	**OrxA** na	pre	↓↑ Consolidation	Yang et al., 2013
Two-way active avoidance	**OrxA** CA1	post	↓↑ Response/Acq.	Mavanji et al., 2017
Physiological, Pharmacological and Neurogenesis results				
LTP	**OrxA** icv	---	↓ LTP in CA1	Aou et al., 2003
	OrxA CA1	60min pre	↑ LTP in CA1	Selbach et al., 2004, 2010
	OrxA DG	30min pre	↑ LTP in DG	Wayner et al., 2004
	SB ip	30min pre	↓ LTP in DG	
	OrxA LC	60min pre	↑ LTP n DG	Walling et al., 2004
	SB DG	5m-24h pre	↓ LTP in DG	Akbari et al., 2011
	OrxA na	---	↓↑ LTP (+pCREB)	Yang et al., 2013
Activation	**OrxA** icv	---	CA1 excitation	Riahi et al., 2015
	SB icv		CA1 inhibition	
	OrxA CA1	imm. pre	↑ CA1 firing rate	Chen et al., 2017
	SB CA1		↓ CA1 firing rate	
Neurotrans-mitters	**OrxA** icv	---	◎ DA, ACh, GABA	Telegdy & Adamik, 2002
	OrxA CA1	60min pre	◎ Glu, GABA, ACh, NA	Selbach et al., 2004
Neurogenesis	**OrxA** icv	---	↑ BrdU positive	Ito et al., 2008
	SB ip		↓ BrdU positive	
	OrxA icv	---	↓↑ BrdU/DCX+ERK	Zhao et al., 2014

Table 4. Effects of orexin-A and OX1R blockade on memory tasks and neural markers of memory. Abbreviations: OrxA, orexin-A; SB, SB-334867; Almorex, almorexant; icv, intracerebroventricular; ip, intraperitoneal; po, oral; na, nasal; BrdU, bromodeoxyuridine; Learn, learning; cons, consolidation; retr, retrieval; Acqu, acquisition. ↑ improves; ↓ impairs; ↓↑ restores; ◎ necessary for orexin-A action.

as from a physiological study in which OX1R was blocked using SB-334867 causing an impairment of the high frequency-induced LTP in the DG (Akbari et al., 2011).

Furthermore, the capability of orexin-A to restore lost ability to learn a memory task in models that involve memory impairment has been explored in several types of paradigms. For instance, orexin-A improved retention of a passive avoidance task even in a mouse model of overproduction of Aβ (Jaeger et al., 2002). Moreover, in an orexin deficiency model in which orexin neurons degenerate at 3 months of age (orexin/ataxin-3-trangenic mouse model), orexin-A administration restored the animals' ability to acquire social memory (Yang et al., 2013) and a two-way active avoidance (TWAA) task (Mavanji et al., 2017). Additionally, Yang et al. (2013) found that orexin-A enhanced LTP in the HPC. Importantly, they noticed that the impaired LTP in the HPC was accompanied by a decrease in the phosphorylation of CREB (pCREB), a necessary process for the formation of long-term memory in a number of HPC-dependent memory tasks (Suzuki et al., 2011; Giese & Mizuno, 2013). Moreover, icv orexin-A administration is not only capable of attenuating the impairing effects of a Pentylenetetrazol (PTZ)-induced hippocampal atrophy on a MWM task, but it also induces neurogenesis in rats' DG through ERK1/2 activation (Zhao et al., 2014). Other reports of cell proliferation in the DG come from depression studies, which found that the icv administration of orexin-A also increases the number of BrdU-positive cells in the DG (Ito et al., 2008).

The findings of the aforementioned studies implicate orexin-A in the facilitation of the acquisition of diverse types of memory, while also shedding light on the mechanism that could allow for this effect to take place.

3 Morris Water Maze

3.1 Introduction

Richard Morris first developed the Morris Water Maze (MWM) in 1981 in St Andrews University, Scotland (Morris, 1981). Since then, this task has been extensively used to assess learning and memory in rodents (for review see D’Hooge & De Deyn, 2001). A comprehensive literature search revealed that there are currently over 6800 published studies, which have used this model or an adaptation of it to evaluate the effects that aging, lesions, drugs and experimental treatments could have on learning and memory in rodents.

The basic principle of the task is that water, a natural aversive stimulus to rodents, would motivate the animal to escape; in order to so, they must consistently find a submerged platform with the help of the presence and configuration of environmental cues, which they must remember. Although it seems simple, carrying out this task involves several sophisticated mnemonic processes. To begin with, the completion of this task requires the identification of relevant cues to aid navigation as well as the association between the platform and escape from the aversive stimulus (water). The animals then need to locate the distal cues in reference to the escape platform, which must be acquired, processed, consolidated, retained and retrieved in order to accomplish the objective of escaping. This procedure offers a series of advantages over other spatial memory tests when it comes to assessing cognitive function in rodents (see Table 5).

Despite the fact that its original purpose was to assess spatial navigation and memory, it has also been adapted to allow for the evaluation of non-spatial learning, specifically visual discrimination paradigms (Morris, 1984; Hagan et al., 1986; Whishaw & Petrie, 1988; Selden et al., 1990; Packard & McGaugh, 1992; Robinson et al., 2001; Hyde et al., 2002; Moreau et al., 2013; García-Brito et al., 2017). However, the apparatus remains the same, regardless of the paradigm. It consists of a circular tank, measuring 180-200cm in diameter and 60-80cm in height, filled with water at a temperature between 19ºC and 24ºC (Vorhees & Williams, 2006). The maintenance of the temperature is of great importance in order to ensure that the animals want to escape while avoiding impaired learning due to hypothermic states (Rauch et al., 1989). The specific characteristics of the configuration, measured variables, strategies used and neural basis for memory formation in each version of the MWM will be described in the following subsections.

Table 5. **Comparison between main spatial memory tests.**

Item	MWM	T-maze	OLT	RAM	Barnes maze	Other water mazes
No deprivation (food/water)	✓	✗	✓	✗	✓	✓
No pre-training	✓	✓	✗	✗	✓	✗
Non-painful aversive stimulus	✓	✓	✓	✓	✓	✓
No olfactory trails	✓	✗	✗	✗	✗	✓
Check non-mnemonic strategies	✓	✗	✗	✗	✗	✓
Check anxiety	✓	✗	✗	✗	✗	✗
Short learning period	✓	✓	✓	✗	✓	✗
Reversal	✓	✓	✗	✓	✓	✓

Table 5. Comparison between memory tests. Shows adequacy of each test to assess useful items, or avoiding potentially negative items associated to memory evaluation procedures. Abbreviations: MWM, Morris water maze; OLT, object location test; RAM, radial arm maze.

3.2 Morris Water Maze and Spatial Learning and Memory

3.2.1 *Standard configuration and procedure of the spatial MWM*

While the MWM is not a labyrinth, it is considered to be a maze because the animals must search the interior of the apparatus for the escape platform. This platform is submerged under water and located in the middle of one of the four quadrants of the swimming pool. Two virtual perpendicular divisions of the circular tank delineate the quadrants, and define the four cardinal points which will serve as the entry in each trial: North (N), South (S), East (E) and West (W). A dark curtain surrounds the tank serving as a cover from outside stimuli, as well as a surface to hang the spatial cues. These cues are generally distinctive objects (lights, boxes, toys, balls, etc.) that provide distal visual aid for the animals to locate themselves within the tank and in reference to the platform, regardless of the entry point. A closed-circuit video camera is mounted above the center of the tank, which allows for the relay of the recorded video to the analysis software (Morris, 1981; Morris, 1984; Vorhees & Williams, 2006). A possible configuration of the spatial MWM can be seen in Figure 10.

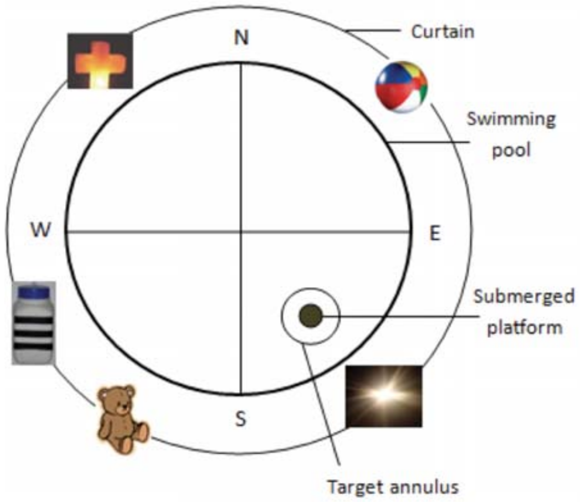


Figure 10. Configuration of a Morris Water Maze apparatus for a spatial memory task. Shown in picture: light box depicting cross, beach ball, light, teddy bear, striped container.

The standard training procedure consists of three phases: the habituation phase, the acquisition phase, and the probe trial (for review see Vorhees & Williams, 2014). The habituation phase is performed with no cues or platforms present in the tank, in which animals swim freely for 60-120s. The acquisition phase is also known as the training period; here, the animals are repeatedly introduced into the tank from a different entry point (pseudorandomized) in each of the trials, and guided to the platform after 60-120s. If the animals find the platform before the completion of the trial, they are left on it for 15-30s and then removed by the experimenter. There is usually an intertrial interval (ITI) in which animals can rest for 90-120s. As the trials progress, the animal learns to locate the platform by using the distal cues as spatial guides. To assess the spatial memory at the end of the learning, a probe trial is given. This is also known as *retention test*. It can take place anytime between 24h to 72h later (Chamorro-López et al., 2015) and consists of removing the platform before introducing the animal into the tank. If the animal has learned to locate the platform based on the spatial cues, it will be reflected in a more accurate navigation trajectory. An additional learning phase, reversal learning attempts to assess cognitive flexibility. This added difficulty tests the animals' ability to learn a new platform position, usually mirroring the initial location, in reference to the same contextual cues.

3.2.2 *Measured variables in spatial MWM*

The most frequently measured variable during the acquisition phase of the spatial MWM is the *latency to target*. It is easily quantified and it is also somewhat resistant to environmental changes (Crabbe et al., 1999). Additional variables are considered and recorded in order to minimize the impact of strange factors that may influence the animals' performance. Thus, the control variables of *speed*, *time near walls* and *total length* are analyzed for the acquisition session. Each allows for the screening of problems of significant differences between animals and experimental groups. Specifically, the variable *speed* screens for either outstanding or deficient locomotor skills that might be altering the escape latency; the variable *time near walls* equated to a measurement of thigmotaxis assesses anxiety levels (Treit & Fundytus, 1988; Korz, 2006; Harris et al., 2009); lastly, the variable *total length* is treated as a correlate of escape latency and speed which should covary accordingly. The same control variables are maintained across each trial and session, including the probe trial and the reversal learning.

However, given that the platform is removed during the probe trial, an additional set of variables is used to further assess the spatial memory of the animals. Among the variables usually recorded and analyzed for the retention test, are: time spent in target quadrant, time spent in target annulus, mean proximity to target, target crossing and the Whishaw's error. Maei et al. (2009) reviewed the sensitivity of all these variables and concluded that the mean proximity to target was the most consistent in detecting group differences, regardless of the sample or effect size and using both parametrical and non-parametrical statistical analyses. Gallagher et al. (1993) originally developed this measure in order to optimize the use of computer tracking software to identify the position of the animal with respect to the target location. In the original article, the proximity to the target was obtained every 10 seconds and averaged every second. Since it was established, other researchers have suggested this measure should become a standard variable in all spatial memory procedures in the MWM (Pereira & Burwell, 2015). Furthermore, the analysis of the accuracy of the navigation path to the platform, otherwise known as the Whishaw's error or corridor, provides an additional index to assess the animals' search precision by quantifying the amount of time spent inside a virtual corridor of pre-established width (Whishaw, 1995).

3.2.3 *Navigation strategies in spatial MWM*

According to Brandeis et al. (1989), animals can apply 3 different navigation strategies to reach the escape platform. They could be using a *praxis* strategy, which involves a sequence of moves and turns that will lead them to the platform; they could use a *taxis* strategy, in which animals approach the platform using proximal cues; or they could apply a *mapping* or *spatial strategy*, which consists of locating the platform in reference to the distal cues. A more recent study has identified different search strategies and grouped them into *spatial (allocentric)* or *non-spatial (egocentric)* (Rogers et al., 2017) (Figure 11). An allocentric strategy would include swimming in directly to the platform (direct swim), focally searching for the platform (focal swim) or having a rough estimate of goal direction (directed search). Conversely, an egocentric strategy would include swimming in concentric circles (chaining), swimming in a scattered focal search (scanning), swimming near the walls (thigmotaxis) or randomly searching for the platform. Ideally, as the training progresses in healthy subjects, we would observe a transition from egocentric to allocentric strategies.

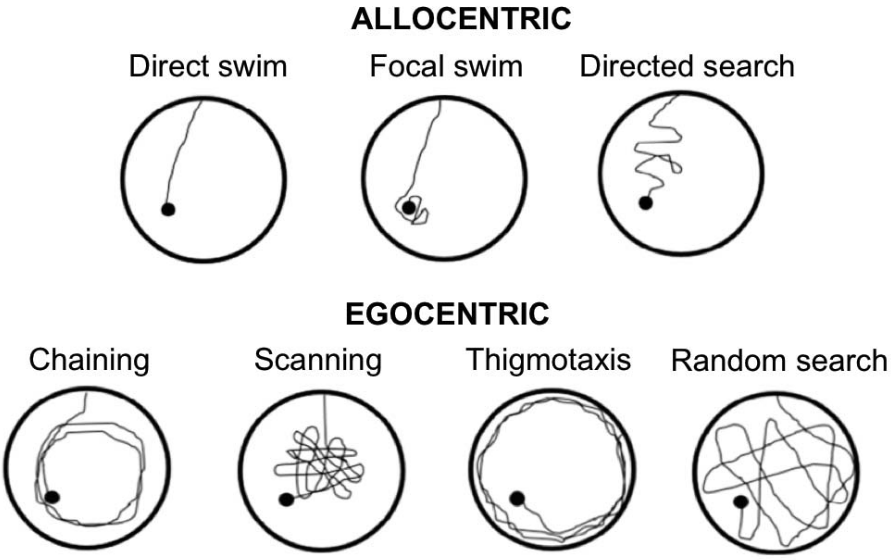


Figure 11. Strategies grouped by type of learning. Adapted from Rogers et al., 2017.

When applying the MWM to the study of spatial memory processing, the addition of a probe trial and the analysis of the aforementioned measures can prove to be an excellent way of ruling out the animals learning to find the platform by applying non-spatial strategies.

3.3 Morris Water Maze and Simultaneous Visual Discrimination

3.3.1 *Configuration and procedure of the SVD MWM*

It is important to note that most of the advantages that the spatial MWM holds over other memory tests would be translatable to a visual discrimination task performed with the same apparatus. For instance, there is no need for deprivation procedures as the main motivation to achieve the goal is to escape the aversive condition that water naturally represents to rodents. It also provides an opportunity to analyze more than one aspect of the decision-making process, such as levels of anxiety and strategy selection. Finally, it does away with any influence olfactory tracks may have on the navigation path.

Morris originally developed a visual discrimination task to be performed by rats in the MWM in order to assess the visual acuity of the animals (Morris, 1984). It consisted of two cues, one of which would signal the location of the escape platform. The location of the correct and incorrect visual cues would be rotated in each trial, meaning that the animal must learn to approach the platform on the basis of the visual pattern associated with it and not on the basis of its spatial location. Since the presentation of the cues was simultaneous and not sequential, we will refer to the task as Simultaneous Visual Discrimination (SVD) from here on.

Several studies have used Morris' setup or a modified version of this configuration to test SVD in the MWM. The different configurations range from using two patterned balls, one anchored to the platform and a second one used as a decoy located in a different quadrant (Whishaw & Petrie, 1988; Selden et al., 1990; Packard & McGaugh, 1992; Moreau et al., 2013), to using two different platforms with no attachments at all (Hagan et al., 1986). Additionally, some studies have made use of externally placed cards displaying a variety of patterns, one of which signals the location of the hidden escape platform (Robinson et al., 2001; Hyde et al., 2002; García-Brito et al., 2017). For this configuration it is of outmost importance to eliminate the possibility of the animal seeing any other external cues, such as doors, windows, furniture, or the experimenter. In order to do so, a curtain may be used as a barrier between the tank and the exterior, as well as serving as a place from which to hang the cues. Figure 12 illustrates a possible configuration of the SVD task using cards patterned with vertical or horizontal black and white stripes.

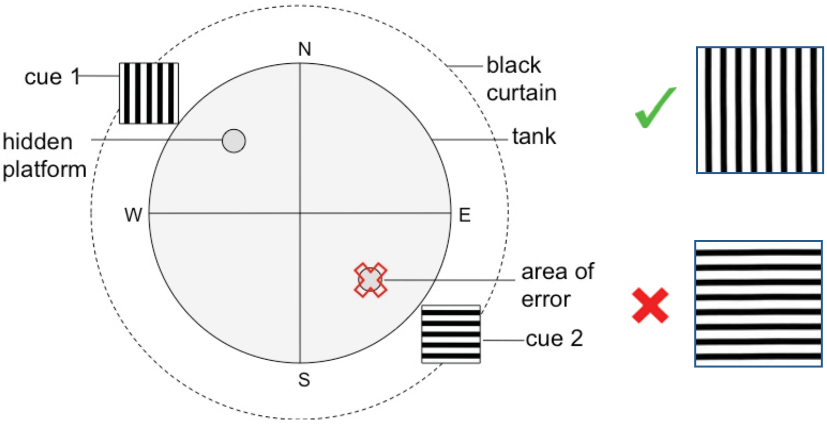


Figure 12. Configuration a Morris Water Maze apparatus for a Simultaneous Visual Discrimination task. Green tick indicates correct cue associated to escape platform. Red cross indicates wrong cue associated to the area of error.

It is important to note that removing the spatial contiguity between the correct cue and the escape platform by placing the cues outside of the perimeter of the tank would increase the difficulty of the task (Whishaw & Petrie, 1988), which could result in the animals struggling to incorporate the strategies needed to accomplish the goal of mounting the platform. We will explore the possible implications that an increase in difficulty could have on the neural involvement required to succeed at a visual discrimination learning task in the next section (see *4.1.3*, and *4.1.5*).

Finally, the procedure for this task is reminiscent of the spatial task in almost every way, with a few variations. One additional consideration is the need to alter the location of the cues in each trial in relation to each other, in order to avoid any associations between the escape platform and a particular configuration of both cues, instead of the association of one specific cue to the platform. An example of a pseudo randomized rotation protocol can be seen in Annex 1. Similarly, the test procedure would consist of introducing the animal into the tank having previously removed the escape platform, while the reversal of the learning would involve exchanging the cues in relation to their previous association to the platform.

3.3.2 Measured variables in SVD MWM

For this version of the MWM, the *latency to target* continues to be a valuable variable to measure and analyze in acquisition and reversal trials; it provides easily quantifiable and interpreted information just as it does in the spatial version of the apparatus. However, this task implies a 50% chance of achieving the goal of finding and mounting the escape platform, and contrary to the spatial memory task, there is only one possible wrong decision. Therefore, an additional variable that must be quantified in order to assess whether the animal is correctly discriminating between cues in the SVD MWM is choice accuracy, computed into *errors*. This is done by assigning an area of error in front of the incorrect cue, and counting every time the animal enters said area. This particular notion was first encountered by Hagan et al., (1986), who found that in addition to latency, choice accuracy was affected in a group of animals with cholinergic deficits. Additionally, Whishaw and Petrie (1988) reported that this tendency to commit errors depended on the characteristics of the discrimination objects; they found that animals committed a significant amount of errors when they attempted to discriminate between vertical/horizontal lines but not different colors (black and white) in an SVD task in the MWM. Similarly, Packard and McGaugh (1992), who used vertical and horizontal patterns of their SVD cues, discovered that latency alone was not enough to determine the learning deficit of animals that had suffered a striatum lesion. Thus, the biggest indication of impairment came from a lack of choice accuracy – higher number of errors – in the later trials. Further supporting the importance of measuring errors in an SVD task in the MWM, errors have also been found to be a more reliable measure of the effects of an enhancing intracranial self-stimulation (ICSS) treatment in an SVD task in the MWM than latency to target (García-Brito et al., 2017).

Furthermore, the probe trial also benefits from using other accuracy measures, such as Whishaw’s error, and the average proximity to target (see *3.2.2*), as they provide further information regarding the animals established associations between the escape platform and either cue.

3.3.3 *Navigation strategies in the SVD MWM*

In order to find and mount the platform, the animals must have established an association between the goal (platform) and the positive stimulus (correct cue). While studying the effects of muscarinic receptor antagonism, Whishaw and Petrie (1988) identified two sets of swimming strategies their animals were using upon coming into contact with the wrong cue: *error and return* and *error and swim*. The former would describes the trajectory of an animal that initiated an incorrect swimming path but stopped before reaching the incorrect cue, redirected its swimming path and managed to find and mount the platform by identifying the correct cue. The latter refers to animals that, having tried and failed to mount the platform due to the incorrect choice, proceeded to swim in a seemingly random manner, instead of redirecting their swimming trajectory towards the correct cue.

A second study, presented as one of the experimental studies in this compilation (García-Brito et al., 2017), identified two types of swimming trajectories specifically regarding the errors committed by the animals. When the animals manage to successfully discriminate between the cues and associate the correct cue with the escape platform, they would engage in a *direct* strategy regardless of the entry point of the contiguity between the two cues (for different contiguity relationships between the two cues throughout acquisition sessions, see Annex 1). In contrast, animals that failed to incorporate the association or had trouble discriminating between the cues would employ a *trial and error* strategy. In this case, the rat has a 50% chance of finding the escape platform in every single trial, but it will result in a higher number of errors.

4 Neuroanatomical basis of Learning and Memory

Learning and memory are the main tools for adaptation. Every living being's memory-related experiences result in observable changes to the brain structures involved in the process of acquiring information and incorporating it into a pre-existing network, allowing them to react to an ever-changing context and behave accordingly. While learning is the process that produces neural modifications, memory is the inferred observable or quantifiable outcome (Morgado-Bernal, 2011). Thus, in order to learn and form memories, these changes must occur in the functionality and morphology of structures in the one organ that processes the information: the brain.

The study of the differential involvement of a number of brain structures in learning and memory has revealed that some areas may specialize in the processing of different types of memories, albeit not in an exclusive manner. While the hippocampus (HPC) has been deemed the most important brain structure for spatial memory (Eichenbaum, 2017a; Eichenbaum, 2017b) the caudate putamen (CPu) in the striatum has been found to be critical to the completion of visual discrimination tasks (Packard & McGaugh, 1992). However, there seem to be functional interactions between these two systems when it comes to learning a simultaneous visual discrimination (SVD) task in a water maze (Fidalgo et al., 2012).

Several other structures have been studied in order to ascertain their role in either type of learning. For instance, the retrosplenial cortex (RSC) is involved in spatial learning and in long-lasting memory storage (Pothuizen et al., 2008; Pothuizen, Davies, Albasser et al., 2009; Sugar et al., 2011; Miller et al., 2014), and the thalamic nuclei are thought to be of great importance for the acquisition and retention of spatial navigation tasks (Kealy et al., 2008; Lopez et al., 2009; Aggleton & Nelson, 2015). On the other hand, several components of the basal forebrain (BF) have been found to be critical for the acquisition of a simultaneous visual discrimination (SVD) task (Marston et al., 1993; Kornecook et al., 1998). At the same time, the functional integrity of the BF is essential for efficient performance in a spatial Morris Water Maze (MWM) task (Waite et al., 1994; Brandner & Schenk, 1998). Moreover, regions like the prefrontal cortex (PFC) participate in a number of aspects of the learning process in both spatial learning (Cholvin et al., 2016; Farina & Commins, 2016) and visual discrimination (Dillon et al., 2009; Fidalgo et al., 2014).

4.1 Learning and Memory neural structures

4.1.1 Hippocampus

In the rat, the HPC is located in the medial temporal lobe of both hemispheres, ventral to the corpus callosum. It is entrapped between the cortex and the dorsal thalamus, and belongs to the hippocampal formation (Figure 13), which is comprised of the three subfields of the cornus ammonis three subfields, CA1, CA2 and CA3, the dentate gyrus (DG), the pre-subiculum, subiculum and parasubiculum, the entorhinal cortex and the fasciola cinereum.

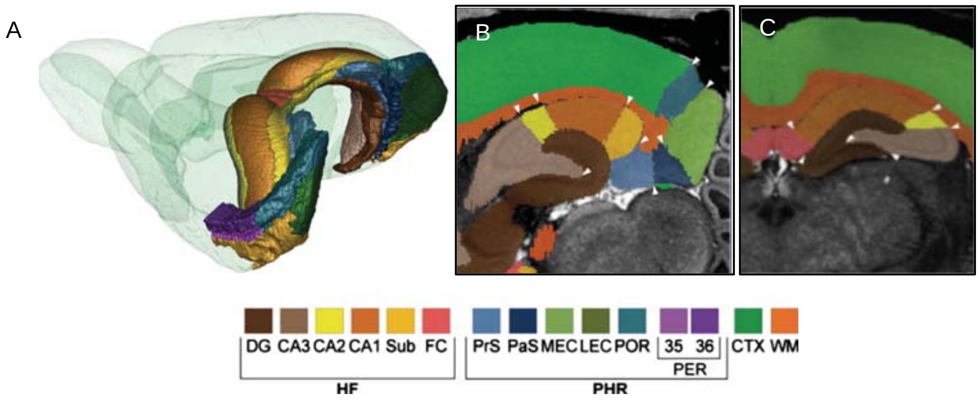


Figure 13. Representation of the rat hippocampal formation. (A) 3D representation of the dorsal and ventral rat hippocampus. (B) Anatomical delineations of the rat hippocampus in a horizontal section. (C) Anatomical delineations of the rat hippocampus in coronal section. Abbreviations: CA1, cornus ammonis area 1; CA2, cornus ammonis area 2; CA3, cornus ammonis area 3; DG, dentate gyrus; CTX, cerebral cortex; FC, fasciola cinereum; LEC, lateral entorhinal cortex; MEC, medial entorhinal cortex; PaS, parasubiculum; PER 35/36, perirhinal area 35/36; POR, postrhinal cortex; PrS, presubiculum; Sub, subiculum; WM, white matter. Panel below shows color-coding of each area. Adapted from Kjonigsen et al., 2015.

While most of the neurons in the DG are small glutamatergic granule cells, the CA1, CA2 and CA3 defined layer is comprised of large pyramidal neurons. Additionally, the local circuit is comprised of GABAergic interneurons morphologically classified as basket cells (Andersen et al., 2007). The trisynaptic circuit is the main pathway that connects the neurons in the hippocampus; (1) the perforant path projects to the granular cells in the DG (2) which send their axons, otherwise known as mossy fibers, to the apical dendrites of pyramidal neuron of CA3. Finally, (3) the collateral fibers from the CA3 pyramidal neurons, also called Schaffer collaterals are sent to the apical dendrites of pyramidal cells in CA1 (Brodal, 2010) (Figure 14).

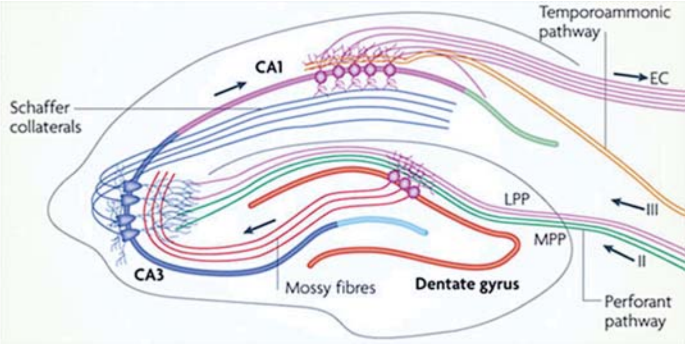


Figure 14. Trisynaptic circuit in the Hippocampus. Adapted from Deng et al., 2010.

CA1 long-term potentiation (LTP) and long-term depression (LDT) in the N-methyl-D-Aspartate receptors (NMDAR) are the most emblematic forms of synaptic plasticity (Morgado-Bernal, 2011). However, the mossy fibers in the DG have received increased attention as an alternative form of LTP, since this particular event can be triggered by increases in Ca^{2+} and does not require the activation of NMDAR (Malenka & Bear, 2004). Additionally, although CA2 has been found to be an “LTP-resistant” structure within the HPC, its activity can potentiate synaptic plasticity in atypical ways (Caruana et al., 2012).

The HPC is critical for the processing and the formation of declarative or relational memories. In Alzheimer’s disease patients, there is a clear atrophy of this structure (for review see Weiner et al., 2017) and a severe impairment of declarative memories, such as spatial memory (Vaughan et al., 2006; Pengas et al., 2012). Animal studies have also shed light on the significant role of the hippocampus in the learning of a spatial task in the MWM in rodents (Packard & McGaugh, 1992), especially when it comes to the use of allocentric search strategies (Eichenbaum et al., 1990). In addition, this structure is involved in choice accuracy during a spatial memory task, but not during a visual discrimination task (Bett et al., 2012). Furthermore, several studies have reported a correlation between HPC inactivation and a disruption in spatial memory retrieval in the MWM, either by agonism of GABA (Cholvin et al., 2016) or antagonism of NMDAR (Farina & Commins, 2016). Some studies have also shown that the activation of hippocampal specific structures is necessary for spatial memory acquisition and retrieval, namely CA3 (He et al., 2002), CA1 (Teather et al., 2005) and DG newly born granule neurons (Tronel et al., 2015). Although most of the evidence points to the HPC being critical for spatial memory, the dorsal portions of CA1 and CA3 have been shown to activate for both spatial and non-spatial recognition memory tasks (Beer et al., 2014).

4.1.2 *Retrosplenial cortex*

The retrosplenial cortex (RSC) is a posterior cingulate region in the medial portion of the brain, located in both hemispheres and dorsal to the corpus callosum. It is heavily involved in spatial learning and long-lasting memory storage (Pothuizen et al., 2008; Pothuizen et al., 2009; Miller et al., 2014; Powell et al., 2017), especially when there is a need to use distal visual cues to solve spatial problems (Hindley et al., 2014). The RSC is divided into two parts (Figure 15), the granular (gRSC) and dysgranular retrosplenial cortex (dRSC) and it consists of 6 cortical layers of neurons (Wyss & Van Groen, 1992).

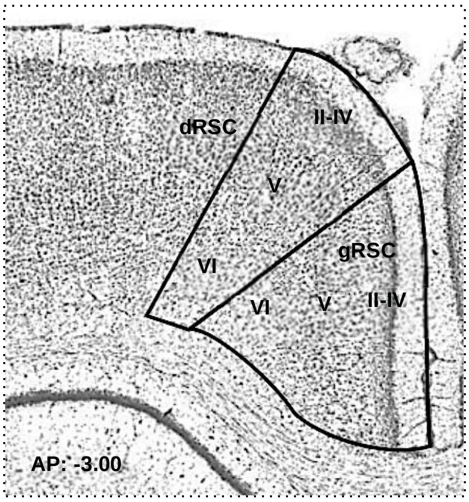


Figure 15. Diagram of a coronal section of the retrosplenial cortex. Adapted from Paxinos & Watson, 2007.

The RSC contains head direction cells (Cho & Sharp, 2001) and place cells (Alexander & Nitz, 2015), which could help provide key information for goal-directed navigation in a spatial task (Vedder et al., 2017). In aging studies in rats, the ability of the RSC to reorganize the functional connectivity of its neural network has proven to be a predictor of successful cognitive aging (Ash et al., 2016). This is especially interesting when considering that the RSC may be the place where compensatory processes take place in prodromal Alzheimer’s disease (Dillen et al., 2016). Moreover, the gRSC seems to be active both during acquisition and long-term retention of a spatial memory task (Malinowska et al., 2016). Although the whole of the RSC is required to use visual cues to determine location in a spatial task (Hindley et al., 2014), there is a functional dissociation between the gRSC and dRSC when it comes to navigation; the former activates for both internal and external cues, and the latter is selectively active when distal cues are present (Pothuizen et al., 2009). Furthermore, the RSC has also been shown to be active during paradoxical sleep (Koike et al., 2017), showing one of the possible processes through which it contributes to memory consolidation.

4.1.3 *Prefrontal Cortex*

The prefrontal cortex (PFC) has been traditionally associated with executive functions in humans, and this notion has been extended to the role of the PFC in rule learning at high levels of complexity (Badre et al., 2010). It has also been associated with behavioral and cognitive flexibility (for review see Marton et al., 2018) and attentional processes (Sarter et al., 2005). Approximately 80-90% of neurons in the PFC are excitatory glutamatergic pyramidal neurons, with the remaining 10-20% being inhibitory GABAergic interneurons (Kolb & Gibb, 2015). The PFC is anatomically divided into 3 distinct areas: (1) the medial prefrontal cortex (mPFC), which comprises the anterior cingulate cortex (ACC), the infralimbic cortex (IL) and the prelimbic cortex (PL); (2) the orbitofrontal cortex (OFC), encompasses the lateral orbital cortex (LO), ventral orbital cortex (VO) and insular regions; (3) the lateral prefrontal cortex, divided in the ventrolateral and dorsolateral prefrontal cortex (Murray et al., 2016). Figure 16 depicts mPFC and OFC subregions.

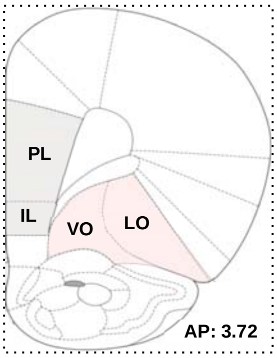


Figure 16. Diagram of the prefrontal cortex. Highlighted in grey is the mPFC; highlighted in pink is the OFC. Adapated from Paxinos & Watson, 2007.

Several studies have shown that the rodent PFC is critical to rule-guided switching between memory strategies (Hyde et al., 2002; Ragozzino et al., 2003; Rich & Shapiro, 2009; Durstewitz et al., 2010). In this sense, the neuronal activity patterns in the mPFC appear to predict the switching between a spatial or place strategy and a discrimination or egocentric strategy (Rich & Shapiro, 2009). Incidentally, one of the latest cognitive deficits to be reported in Alzheimer's patients is the impairment in the ability to switch from allocentric to egocentric strategies in spatial representations, which could implicate the PFC (Ruggiero et al., 2018).

The role of the PFC in behavioral and cognitive flexibility has been tested extensively by administering a reversal test on an already learnt task. Human neuroimaging studies report an increase in PFC activity while the subjects are performing reversals (for review see Izquierdo et al., 2017). Both the mPFC and the OFC seem to be involved in this effect, but there is a differentiation between the specific aspects of flexibility they participate in. While the IL sorts through known strategies, the PL selects and maintains the strategy in order to respond to the conflict elicited by the altered task (Oualian & Gisquet-Verrier, 2010), allowing for the strategy switching aspect of a reversal task to take place (Young & Shapiro, 2009). The role of the OFC seems to be more extensive, since its inactivation impairs performance of reversal phases of visual discrimination (Izquierdo et al., 2013), instrumental spatial (Boulougouris et al., 2007), spatial (Young & Shapiro, 2009) and probabilistic discrimination tasks (Dalton et al., 2016), while its activation positively correlates with an improved reversal in a spatial task in a water maze (Sampedro-Piquero et al., 2015). Moreover, the PFC seems to have a specific relation to perseverative errors during a reversal (Méndez et al., 2010), which occur when there are deficits in the function of the mPFC (Kolb et al., 1974; (Ferry et al., 2000) and the OFC (Chudasama & Robbins, 2003); Boulougouris et al., 2007).

It is important to note that cortical cholinergic inputs have been hypothesized to contribute to learning and memory, particularly in tasks that place high demand on attentional processing (Sarter et al., 2003); Dalley et al., 2004). Since the PFC has been extensively linked to attentional processing, the deficits observed in cognitive flexibility aspects, such as rule shifting, perseverative errors and reversal learning could be due to inadequate cholinergic function in its components. In that sense, the participation of the PFC in the increasing attentional demands of a task may result in an elevated release of acetylcholine (ACh) in the mPFC (Kozak et al., 2006; (Berry et al., 2015). Moreover, Hagan et al. (1986) have reported that muscarinic cholinergic blockade impairs performance of a visual discrimination in the MWM especially in terms of choice accuracy, resulting in more perseverative errors.

4.1.4 *Thalamic nuclei*

The thalamus is the largest component of the diencephalon, and it is the main relay site for all sensory pathways (except olfaction) to the cerebral cortex. In functional magnetic resonance imaging (fMRI) studies in humans, the thalamus has been found to participate in memory and attention processes (De Bourbon-Teles et al., 2014). From a functional point of view, the thalamus can be broadly divided in three areas: the associative nuclei, the intralaminar nuclei and the midline nuclei (see Figure 17). Anatomically, we could define the main nuclei involved in learning and memory processes into the anterior thalamic nuclei (ATN) – anterodorsal (AD), anteroventral (AV) and anteromedial (AM) –, mediodorsal nucleus (MD), laterodorsal (LD), centromedial (CM), centrolateral (CL), reuniens (Re), rhomboid (Rh) and paraventricular (PV) nucleus. The thalamus is mainly comprised of excitatory relay neurons which project to the cortex, but there is also a local circuit of GABAergic neurons (Arcelli et al., 1996).

Associative			Intralaminar		Midline		
ATN	MD	LD	CM	CL	Re	Rh	PV

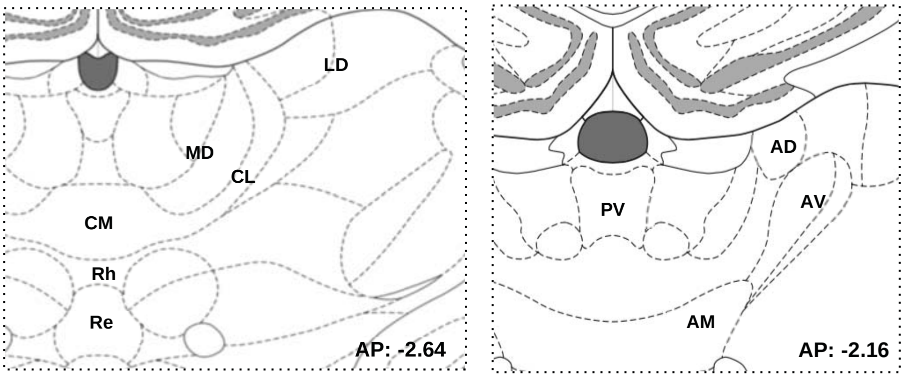


Figure 17. Representation of Thalamic nuclei. Coronal section of rat thalamus. Lateral structures only shown on the right hemisphere. Abbreviations: CM, centromedial; Rh, rhomboid; Re, reuniens; MD, mediodorsal; CL, centrolateral; LD, laterodorsal; PV, paraventricular; AD, anterodorsal; AV, anteroventral; AM, anteromedial. Adapted from Paxinos & Watson, 2007.

Each nucleus participates in different aspects of learning and memory, depending on their connections to different cortical areas (see 4.2). The ATN support allocentric spatial memory (Wolff et al., 2008; Jankowski et al., 2013; Dumont et al., 2014a), and a lesion to these nuclei can lead to a deficit in spatial memory tasks (Harland et al., 2014; Dumont et al., 2014b; Perry et al., 2018). Furthermore, there seems to be a dissociation regarding the types of memory that the thalamic nuclei support. Thus, structures that participate in spatial learning, such as the anterior thalamic nuclei (ATN) do not seem to be necessary for the acquisition of an SVD task in the MWM (Moreau et al., 2013). The AD is one of the brain regions that contain head direction cells (Clark et al., 2012), which could explain its role in orientation during a spatial navigation task. Moreover, the MD has been found to be of great importance to discriminative learning (Cross et al., 2012) and critical to rule switching in the expression of behavioral flexibility (Marton et al., 2018). Furthermore, the intralaminar nuclei may contribute to consolidation processes in remote spatial memory (Lopez et al., 2009; Pereira de Vasconcelos & Cassel, 2015), whereas the midline thalamic nuclei, Re and Rh, appear to be essential for learning the behavioral strategies that require the use of spatial mappings (Cain et al., 2006).

4.1.5 *Basal Forebrain*

The basal forebrain is a collection of cholinergic, GABAergic, and glutamatergic projection neurons and local GABAergic interneurons (Gritti et al., 2003). Activity in this system has been linked to control of arousal (Xu et al., 2015), attention (Klinkenberg et al., 2011) and learning and memory (Okada et al., 2015). The BF is divided into the medial septum (MS), the vertical and horizontal limbs of the diagonal band of Broca (VDB/HDB), the nucleus basalis magnocellularis (NBM) and the substantia innominata (SI) (Figure 18).

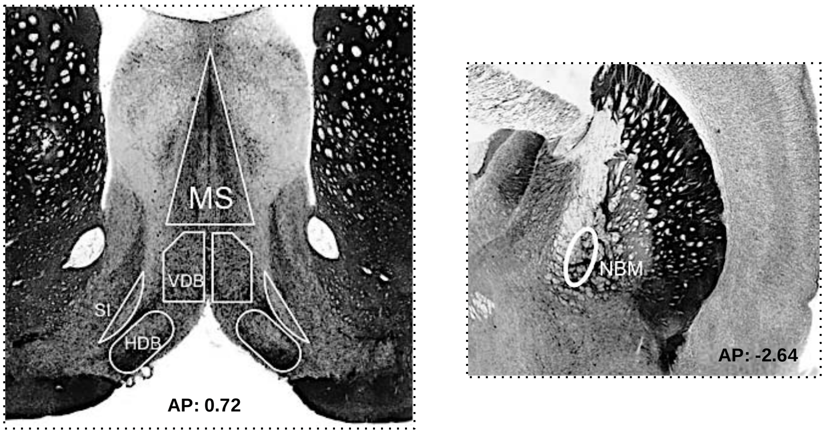


Figure 18. Basal forebrain system. Coronal sections of a rat brain. Abbreviations: MS, medial septum; SI, substantia innominate; VDB/HDB, diagonal band; NBM, nucleus basalis magnocellularis. Adapted from Paxinos & Watson, 2007.

Early studies suggested that this complex did not play a relevant role in spatial learning and memory, since the removal of cholinergic fibres to the HPC had not effect, regardless of the animal’s age (Torres et al., 1994; Baxter & Gallagher, 1996). Instead, this system seemed to be specifically related to visual discrimination tasks, as shown by an impairment to the acquisition of learning in an operant chamber after lesions to the MS and VDB (Marston et al., 1993). However, since more recent studies have found that abnormal cholinergic neurotransmission of the BF contributes to memory deficits in advanced age and Alzheimer’s disease (Haense et al., 2012; (Grothe et al., 2013), the notion of its role in spatial memory has been rekindled. Additional contrasting results have shown that cholinergic depletions in an Alzheimer’s model in rats impair the acquisition of a spatial MWM, but not that of a simple discrimination task in the radial maze (Deibel et al., 2016). A more localized lesion of the BF, specifically the ablation of MS and VDB neurons, has also been shown to impair spatial object location memory (Okada et al., 2015).

One of the most relevant roles of the cholinergic system in the BF is in relation to attention. This region is selectively activated during tasks that demand an attentional performance (Arnold et al., 2002), and has been established as a necessary component of the neuronal circuits mediating sustained attention (for review see Sarter et al., 2001 and Klinkenberg et al., 2011). In addition, the attentional deficiencies that have been deemed a core feature of Alzheimer disease, may be due, at least in part, to damage to the cholinergic system in the BF (for review see Lawrence & Sahakian, 1995). Furthermore, insufficient involvement of the cholinergic system of the BF has resulted in impairment of an SVD task in the MWM (Dotigny et al., 2008), possibly because it did not support the attentional demands involved in this function. Nevertheless, other neurotransmission systems, such as the GABAergic projections to the cortex, may be mediating the executive aspects of the attentional function of the BF (Burk & Sarter, 2001; Dotigny et al., 2008).

4.2 Anatomical and functional relationship between neural structures

In humans, functional imaging studies have demonstrated correlations between hippocampal and prefrontal activity in a variety of memory tasks (for review see (Ritchey et al., 2015). This has led to the notion that the HPC organizes memories, whereas the PFC extracts the rules that govern a set of those memories. Furthermore, the relationship between HPC and PFC supports context-guided memory. For example, when rats enter a spatial context, hippocampal networks send information about the context to the PFC, which evaluates the possible choices and sends the outcome information to the HPC, guiding the retrieval of the appropriate memories (Place et al., 2016). It has also been reported that a coordinated release of ACh in both the PFC and HPC is associated with reward and arousal, which offers dual mechanisms to support the acquisition of a cognitive task (Teles-Grilo Ruivo et al., 2017). However, the activation of any one isolated structure cannot be taken as a direct predictor of spatial learning and memory; instead, the activity of a set of areas usually associated to spatial learning, like the PFC and the RSC, offers a better correlate for MWM learning (Shires & Aggleton, 2008). Specifically, the CA1 in the HPC distributes its fibers to the mPFC (Hoover & Vertes, 2007) and receives indirect projections back through thalamic relays (Vertes, 2006). In the processing of spatial learning and memory, the HPC and RSC not only share extensive connections between them (Wright et al., 2010), but they also seem to work in synchrony by coordinating theta waves during paradoxical sleep in order to consolidate memory (Koike et al., 2017). One other cortical area that is interconnected with the PFC is the RSC. The RSC receives projections from the OFC (Shibata & Naito, 2008), and sends its afferents to the VO of the PFC (Shibata et al., 2004), suggesting that PFC differentially supports the functions of RSC in behavior and spatial memory, depending on the interconnected targets.

The three cortical structures mentioned above are interconnected by thalamic nuclei that serve as relays (Vertes, 2006; Aggleton et al., 2010; Prasad & Chudasama, 2013). However, there is dissociation in the specific connections between them. While lesions in the AD impede c-Fos expression in the granular retrosplenial cortex (gRSC) (Jenkins et al., 2004), lesions in the RSC impair the representation of head direction cells in the AD (Clark et al., 2010). Thus, the role of these cells in providing orientation in a spatial navigation task through its projections to the HPC (Arleo et al., 2013) may also depend on the integrity of the RSC. To further support the notion of a reciprocal functional network, ATN lesions also produce atrophy in the spine density of CA1 and RSC (Harland et al., 2014). The intricate interconnection between these structures also provides the basis for the retrieval of recent memories (Lopez et al., 2017). Furthermore, the main mPFC input from the thalamus projects from the MD nucleus in the thalamus; this connection could help to support the PFC in the integration of associative information (Mitchell, 2015) and seems to be especially critical for behavioral flexibility (Marton et al., 2018). Another important projection to the mPFC comes from the midline nuclei, that is, the Re and Rh (Hoover & Vertes, 2007), which lends relevance to the role of these nuclei in the learning of behavioral strategies applied in spatial mappings (Cain et al., 2006) and as mentioned above, behaves as a relay for limbic information between the mPFC and HPC, closing the information loop (Vertes, 2006; Cassel et al., 2013).

Furthermore, one of the key components of the neuronal circuitry necessary for memory formation is the innervation of the HPC by BF cholinergic, glutamatergic and GABAergic neurons, also called septohippocampal projections, which modulate the cell excitation, synaptic plasticity and the rhythm oscillations of glutamatergic pyramidal neurons in the HPC (Teles-Grilo Ruivo & Mellor, 2013). Specifically, Glu, ACh and GABA neurons in the MS and the VDB/HDB project to HPC and promote theta waves (Andersen et al., 2007; Huh et al., 2010; Zhang et al., 2010). In addition, GABAergic fibers from the MS and VDB/HDB that target interneurons in CA1 of the HPC and in the RSC are believed to contribute to the temporal coordination of neural activity (Unal et al., 2015).

Moreover, the anatomical and functional connections between the BF and the PFC have also been of great interest in regard to the role of ACh in attentional processes. Sarter et al. (2005) argue that the role of the cortical cholinergic input is to optimize the processing of different signals in situations that require attention. The NBM and SI project uniformly to the PFC (Mesulam, 2004) releasing ACh widely. The BF also sends glutamatergic and GABAergic afferent projections to the PFC (Henny & Jones, 2008). At the same time, the activity in the BF can be modulated by glutamatergic projections from the PFC (Fadel et al., 2001); Turchi & Sarter, 2001), thus providing a symbiotic mechanism for the regulation of attentional processing.

CHAPTER II

HYPOTHESIS AND OBJECTIVES

1 Hypothesis

Intracranial self-stimulation (ICSS) in the lateral hypothalamus (LH) can facilitate different types of memory through the promotion of a generalized arousal, characterized by increased activity in some excitatory neurotransmission systems in multiple brain regions, specifically in those related to memory. ICSS promotes plastic changes in memory-related structures similar to those resulting from training in a memory task. The orexinergic system, especially orexin-A, is implicated in several processes that are also affected by ICSS, such as reward, arousal, attention and memory. Additionally, orexin-producing neurons are located in the LH, where ICSS produces the highest rates of stimulation.

Therefore, we hypothesize that the orexin-A selective receptor (OX1R) is involved in the facilitating effects of ICSS upon different types of memory and that said facilitating effects will be impaired by the administration of OX1R selective antagonist SB-334867.

2 Objectives

2.1 General objective

The general objective of this thesis is to study the implication of the orexinergic system in the facilitating effect of ICSS on different aspects of two types of memory tasks, a spatial task and a simultaneous visual discrimination task (SVD) in the Morris Water Maze (MWM).

2.2 Specific objectives

- To study the effects of ICSS on an SVD in the MWM.
- To characterize the condition of training intensity in which the blockade of OX1R using SB-334867 causes learning impairments.
- To determine the effects of OX1R blockade on the facilitation of a spatial memory task in the MWM by ICSS.
- To characterize the activation of memory-related neuroanatomical areas affected by ICSS and SB-334867 in a spatial memory task.
- To study the effects of OX1R blockade on the facilitation of an SVD in the MWM.
- To evaluate the effects of ICSS and SB-334867 on the activity of AChE in cholinergic areas related to attentional processing in SVD learning in the MWM.

CHAPTER III

EXPERIMENTAL STUDIES

STUDY I

1 Facilitation of a simultaneous visual discrimination task by intracranial self-stimulation

Behavioural Brain Research 2017; 317, 360-366 (Annex 2)

Intracranial self-stimulation also facilitates learning in a visual discrimination task in the Morris water maze in rats.

Soleil García-Brito, Ignacio Morgado-Bernal, Neus Biosca-Simon; Pilar Segura-Torres.

1.1 Introduction

The electrical activation of the medial forebrain bundle (MFB) via Intracranial self-stimulation (ICSS) has been confirmed in our and other laboratories as a treatment capable of consistently facilitating the acquisition and retention in a wide array of experimental paradigms, for both implicit (Huston et al., 1977; Huston & Mueller, 1978; Redolar-Ripoll et al., 2003; Ruiz-Medina et al., 2008b) and explicit memory (Soriano-Mas et al., 2005; Chamorro-López et al., 2015) in rats. Several mechanisms of action have been proposed to explain these facilitating effects of ICSS on learning and memory. Stimulation of the MFB has been linked to activation of general arousal systems (Wise, 2005; Newman & Feldman, 1964) due to activity of dopaminergic, noradrenergic and serotoninergic ascendant fibers (Nieuwenhuys et al., 1982; Shankaranarayana Rao et al., 1998a). Learning and memory facilitation has also been linked to structural plasticity induced by ICSS (Shankaranarayana Rao et al., 1993). Recent work undertaken in our laboratory has shown an increase in the density of dendrite spines in the CA1 neurons of the hippocampus in rats that received ICSS after training in a spatial task (Chamorro-López et al., 2015). These morphological modifications could be related to changes in the expression of several plasticity-related genes caused by the post-training ICSS treatment, with increased levels of Nurr1, c-Fos and Arc protein consistently being found in hippocampus, amygdala, dorsal striatum, lateral hypothalamus or retrosplenial cortex (Huguet et al., 2009; Aldavert-Vera et al., 2013; Kádár et al., 2014; Kádár et al., 2016).

While most evidence supporting the facilitating effect of the post-training ICSS on explicit memory comes mainly from spatial learning tasks in T-mazes and the Morris Water Maze (MWM), the type of implicit memory that has been subjected to ICSS treatment effects is an amygdala-dependent emotional memory. Thus, the most commonly used tasks have been aversive classical conditioning and avoidance learning. While some pioneering studies have looked into the effects of ICSS on other types of implicit tasks, such as appetitive classical conditioning (Coulombe & White, 1980), there are none that focus on tasks more related to perceptual learning and memory. Perceptual abilities of recognition and discrimination between stimuli are the foundation of most of the learning processes both in animals and humans and, therefore, if ICSS were able to facilitate visual discrimination conditioning and memory it would extend the range of cognitive processes – involving stimuli perception – that are improved by ICSS or the stimulation of reward pathways. Furthermore, since a deficit in implicit learning and memory related to visual discrimination are observed in both Parkinson's disease (Appollonio et al., 1994) and the later stages of Alzheimer's disease (Manzanero, 2007), the possibility of positively affecting this type of memory could also be interesting in the field of neurodegenerative diseases.

In order to study the possible effect of post-training ICSS on a simultaneous visual discrimination task in the MWM (SVD), we modified the configuration of the MWM based on the model presented by Packard and McGaugh (Packard & McGaugh, 1992) of a two-platform task, in a non-spatial version of the MWM task, in which two visible white rubber balls were painted with black horizontal and vertical stripes and used as cues attached to the escape/non-escape platforms. As ICSS treatment demonstrates a higher effectiveness on high difficulty conditions (Aldavert-Vera et al., 1996; Redolar-Ripoll et al., 2002; Redolar-Ripoll et al., 2003) an SVD task would present the appropriate setup, given that the task involves the need to identify and compare two similar stimuli in order to solve it. Moreover, this task in the MWM does not require caloric restriction in order for the animal to learn to find the platform, thus reducing the possible interference of the motivational states on learning (Kennedy & Shapiro, 2009). This task is considered to be a non-declarative memory task (Bussey et al., 1997), which also requires the animal to establish an association between a specific stimulus and the location of the platform, generating an instrumental escape response; this associative nature would also involve the use of relatively inflexible memory processes (Hirsh, 1974) which could mean that reversing or changing a well-consolidated memory would be extremely challenging. This suggests that, should the acquisition of the SVD task be facilitated by the ICSS treatment, the retention of the memory will be stronger while the reversal learning will be challenged.

1.2 Materials and Methods

1.2.1 *Subjects*

A total of Forty-two Wistar male rats with mean age 90.35 days (SD=2.20), and a mean weight of 390.57g (SD=20.83) from our laboratory's breeding stock were used. Three days before the stereotaxic procedure they were isolated and kept in individual cages (50x22x14cm, plastic bottomed and sawdust-bedded). The animals were kept under conditions of controlled temperature and humidity, and subjected to an artificial 12h light/dark cycle (light on at 08:00). The experimental work was carried out during the first half of the light cycle. All subjects were in an ad libitum regime of food and water. All procedures were carried out in compliance with the European Community Council directives for care and use of laboratory animals and were approved by the institutional animal care committee.

1.2.2 *Surgery*

Previous to the surgery, two sessions of handling took place in order to diminish emotional reactivity of the animals towards experimental manipulation. Under general anesthesia (150mg/kg Imalgène® ketamine chlorhydrate (Merial, Lyon, France) and 0.08mg/kg Rompun® xylazine (Bayer, Barcelona, Spain); i.p.), all rats were chronically implanted with a monopolar stainless steel electrode (150µm in diameter) aimed at the right lateral hypothalamus (LH) into the fibers of the MFB, according to coordinates from the stereotaxic atlas of Paxinos & Watson (2007), anterior: -1.8mm from bregma, lateral: 2.0mm (right hemisphere) and ventral: -8.5mm with the cranium surface as the dorsal reference. In the post-surgery recovery period (7 days), the animals were weighed and handled daily.

1.2.3 *Group designation and ICSS behavior shaping*

The rats were randomly distributed into two groups, Sham and ICSS, according to the independent variable “ICSS-treatment”. Subjects in the ICSS group were taught to self-stimulate by pressing a lever in a Skinner box (25x20x20cm). Electrical brain stimulation consisted of 0.3s trains of 50Hz sinusoidal waves at intensities ranging from 20 to 250µA. The optimum intensity (OI), defined by the lowest intensity that led to a stable rate of about 250 responses in 5min, was established.

1.2.4 *Morris Water Maze Apparatus.*

The MWM consisted of an elevated circular pool (2m diameter; 60cm above the pool floor) filled with water (45cm height) maintained at 22±2°C. The pool was in the middle of a semi-dark room and surrounded by black curtains reaching from a false ceiling to the base of the pool forming a circular enclosure 2.4m in diameter. In an adapted version of the two-platform task of Packard and McGaugh (Packard & McGaugh, 1992), four imperceptible nylon threads hung from the false ceiling at equal distances from one another to provide suspension for the two mobile cues throughout the training. These cues rested in the middle of the virtual quadrant in the tank, 45cm above the water level, and consisted of identical squares (40cm^2) with a vertical or horizontal black and white stripes pattern of 1cm wide stripes, as represented in Figure 19. For the escape task, a clear Plexiglas platform (11cm diameter) was placed centrally in one of the four equal quadrants in which the tank was virtually divided, with its top 2cm below the surface of the water. All swim paths were recorded using a closed-circuit video camera (Smart Video Tracking System, Version 2.5, Panlab) with a wide-angle lens was mounted 1.75m above the center of the pool inside the false ceiling.

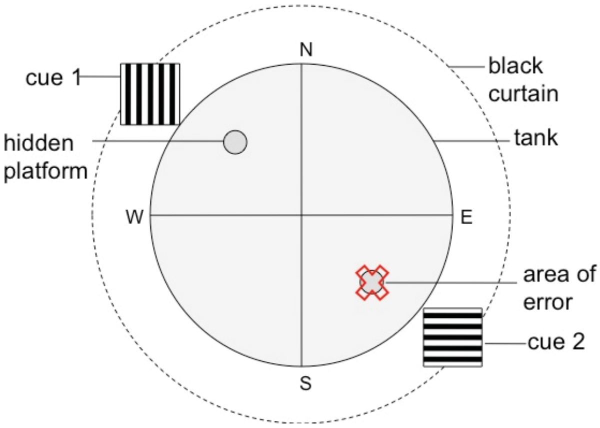


Figure 19. Representation of one of the configurations for MWM in the simultaneous visual discrimination task. *Escape area* is associated to cue 1 and illustrated with a clear platform. *Area of error* represents the “no escape” associated to cue 2 and is signaled with a red X.

1.2.5 Behavioral procedure

Acquisition sessions

Seventy-two hours after the ICSS shaping, all subjects were given six daily trials for five days (acquisition sessions). The average intertrial interval (ITI) was 120s. Starting from one of four different cardinal points (N, E, S and W) in a pseudorandom schedule each water-maze trial consisted of one swim from the edge of the pool to the platform. The correct cue (1) was associated with the escape platform (*escape area*), while the incorrect cue (2) was associated with the area of no escape (*area of error*). When a rat failed to find the platform within 90s, it was manually guided to the platform for 15s and then removed from the tank. When a rat found the platform it was left on it for 15s and then removed from the tank. The position of the two cues was manipulated so that every ten trials the correct cue was closer, farther or at the same distance than the incorrect cue in relation to the starting point. Thus, the correct cue changed quadrants every three trials (½ of each session), while the incorrect cue changed position after each trial among the remaining quadrants (see Annex 1).

Probe test and reversal

The probe test took place seventy-two hours after the last acquisition session and consisted of removing the platform and placing the animal in the pool from the East (E) starting position. Immediately after the 60s, the reversal trials were initiated. The platform was placed in the quadrant associated to the incorrect cue and the animal was directed to mount the platform for 15s and then removed. After 120s ITI three reversal trials took place, which consisted of the exchange of the cues’ associations. Animals were again placed in the tank from the three remaining starting points (N, W, S), and the cues changed quadrants anticlockwise for each trial, which had duration of 90s and an ITI of 120s.

1.2.6 *ICSS treatment*

Immediately after each SVD acquisition session, the ICSS rats were placed in the self-stimulation box and received the ICSS treatment, consisting of 2500 trains of stimulation at the OI established during the shaping phase for each rat. Rats in the Sham group were instead placed in the self-stimulation box for 45min after each training session without receiving any stimulation. Figure 20 depicts the experimental procedure.

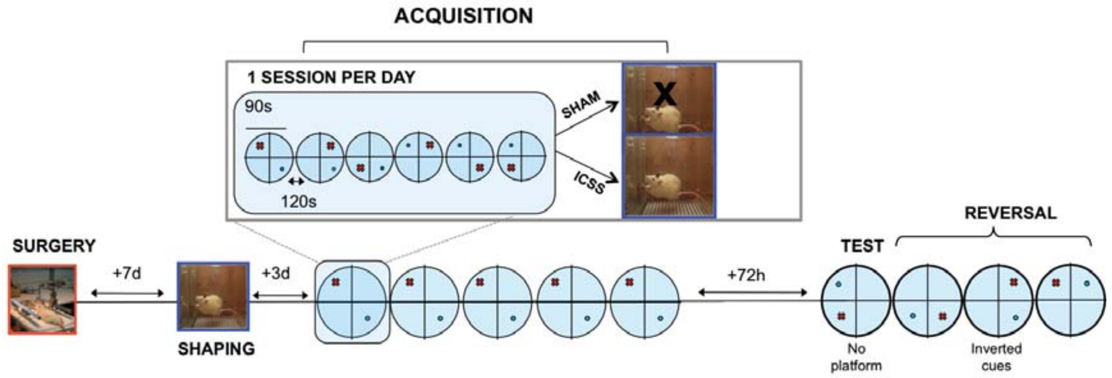


Figure 20. Diagram depicting experimental procedure in Study I.

1.2.7 *Histology*

The animals were transcardially perfused with 4% paraformaldehyde in phosphate buffer 0.1M (PB; pH=7.4). The brains were removed and post fixed overnight in the same solution. They were then placed in a 30% sucrose solution before being cut into 40µm sections on a freezing stage microtome (Cryocut 1800 with microtome 2020, Jung). The tissue was stained with Cresyl Violet and examined for electrode tip placement under a microscope for histological determination of the electrode location.

1.2.8 *Statistical analysis*

All statistical analysis was carried out with SPSS statistical package v. 23 (SPSS Inc., Chicago, IL, USA). Analysis was conducted with a 2×5 mixed ANOVA (GROUP×SESSIONS) for the acquisition phase, and independent samples *t*-test analysis for the probe test and reversal (the average score of the three trials was analyzed). The main outcome variables for acquisition and reversal in the SVD were: 1) *Escape latency*: time (s) needed to find and climb onto the platform (the maximum value was 90 s), and 2) *Number of errors*: number of contacts with area associated to incorrect cue (no escape). In the probe test *Number of target crossings*, *Percentage of time spent in the target quadrant*, *Number of errors*, *Percentage of time spent in the error quadrant* were compared between groups. In addition, a one-sample *t*-test against a constant (50) was used for each group to determine whether the *Percentage of time spent in the target quadrant* was different from chance level (50%). Moreover, the control variables *Percentage of time spent near the walls* (measure of thigmotaxis), *Length* (total distance in cm) and *Speed* were also analyzed. When the effect of SESSIONS factor was statistically significant, polynomial contrasts explored the presence of linear and/or quadratic trends in the performance. A Chi-square test for independence was performed to determine the relation between the group and the strategy used. In addition, a regression analysis was performed to examine the relationship between ICSS parameters and SVD performance. The α level for all tests was set at 0.05.

1.3 Results

A total of five subjects were excluded from the analysis (two subjects lost the electrode in the middle of the treatment, and three did not continue to respond to the ICSS treatment). The final sample consisted of 37 subjects (Sham: n=19, ICSS: n=18). There was no statistical difference between groups in weight change.

1.3.1 *Acquisition sessions*

Mean *Escape latencies* are depicted in Figure 21A. The SESSIONS factor was significant [$F_{4,140}$=22.626, P<0.001], but since interaction GROUP×SESSIONS does not reach significance [$F_{4,140}$=2.034, P=0.09], a similar evolution between groups can be assumed. In addition, the GROUP factor was not significant [$F_{1,35}$=0.181, P=0.67]. Furthermore, both groups learnt the task in terms of the decrease of the *Escape latencies*, revealing a significant downward linear function (Polynomial contrast, Sham P<0.001; ICSS P<0.001).

Means of the *Number of errors* made are depicted in Figure 21B. The main effects of GROUP and SESSIONS are significant [$F_{1,35}$=18.024, P<0.001 and $F_{4,140}$=4.072, P=0.004, respectively], but there is no interaction GROUP×SESSIONS [$F_{4,140}$=1.224, P=0.30]. It is important to point out that no differences between groups were found in session 1 (P=0.267), before the ICSS administration, and the analysis of the sessions 2 to 5 confirms the lower *Number of errors* from the ICSS group [$F_{1,35}$=17.64, P<0.001]. Additionally, the decrease in the *Number of errors* in the ICSS group followed a linear evolution (P<0.001), while the Sham group did not (P=0.268). Furthermore, a simple effects analysis found that the differences between groups appeared in the third session ($P=0.025$), were maintained in the fourth session ($P=0.018$) and were stronger in the last acquisition session ($P<0.001$).

No differences were found for GROUP×SESSIONS for control variables: *Percentage of time spent near the walls*, *Length* or *Speed*.

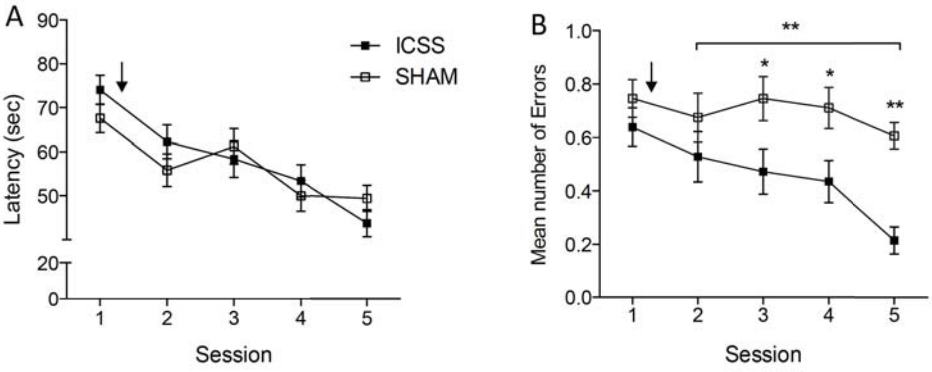


Figure 21. Facilitative effects of ICSS on acquisition. (A) Mean *Escape latencies* (±SE) for the five acquisition sessions. Arrow shows start of ICSS treatment; (B) Mean *Number of errors* (±SE) committed during the acquisition sessions by each group. Group factor significance is depicted with a horizontal bracket. * P<0.05; **P<0.001

1.3.2 Probe test

ICSS group had a higher *Number of target crossings* than the Sham group [*Welch's* $F_{1,23.95}=4.974$, $P=0.035$] (Figure 22A). Moreover, the ICSS group also showed a higher preference for the *target quadrant* [$t_{35}=17.848$, $P<0.001$] (Figure 22B), although neither group performed above chance level (Sham: $t_{18}=-2.744$, $P=0.86$; ICSS: $t_{17}=-1.19$, $P=0.45$).

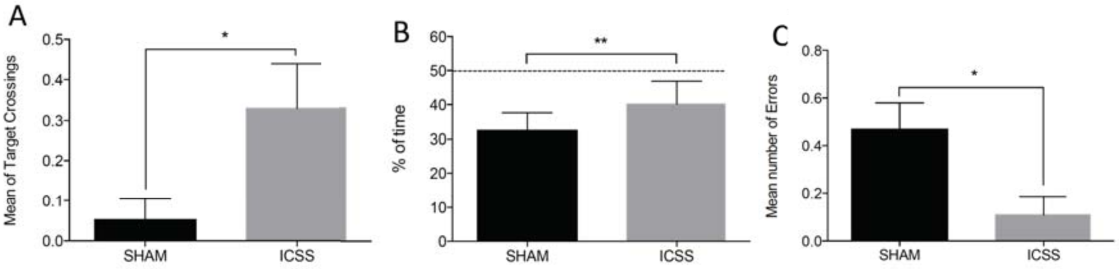


Figure 22. Facilitative effects of ICSS on the probe test. (A) Mean *Target crossings* (±SE); (B) Mean *Percentage of time spent in target quadrant* (±SE). The dotted line represents chance level; (C) Mean *Number of Errors* (±SE). *P<0.05; **P<0.001

Concerning the *Number of Errors* (Figure 22C), the ICSS group committed fewer errors than the Sham group during the first 30 seconds of the probe test [*Welch's*

$F_{1,30.572}$=6.687, P=0.015]. Finally, means of *Percentage of time spent in the error quadrant* were not statistically different between groups, although Sham animals showed a tendency for higher preference [t_{35}=3.767, P=0.06].

1.3.3 *Reversal trial*

The ICSS group had significantly higher *Escape latencies* than the Sham group [t_{35}=4.532, P=0.04] (Figure 23A). Moreover, the ICSS group made significantly more *errors* than the Sham group [*Welch's* $F_{1,24.615}$=5.113, P=0.03] (Figure 23B).

No differences were found between groups for control variables (*Percentage of time spent near the walls*, *Length* or *Speed*) either in the probe test or in the reversal session.

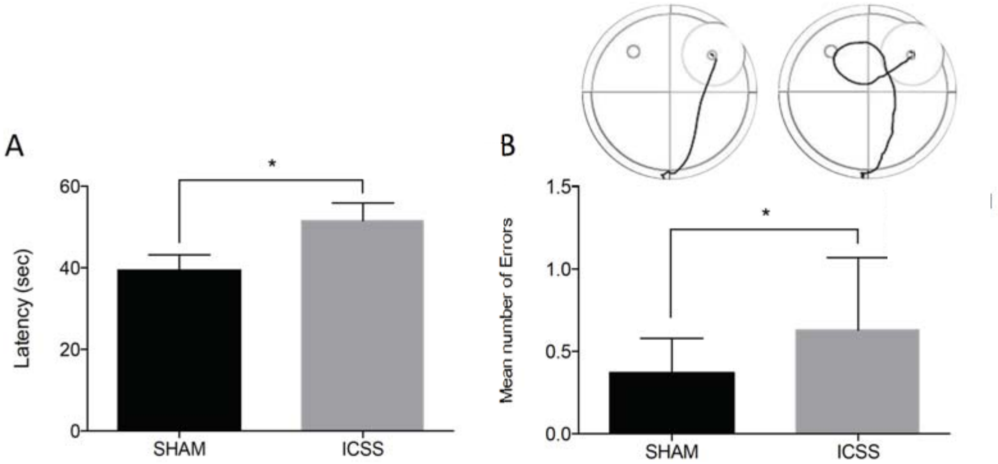
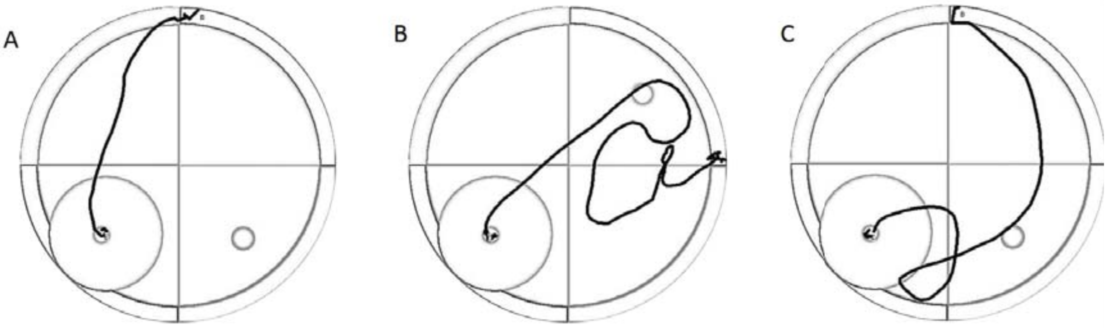


Figure 23. Impairing effects of ICSS on reversal. (A) Mean *Escape latencies* (±SE); (B) Mean *Number of errors* (±SE). An example of the trajectory of Sham and ICSS animals is presented above corresponding bars. *P<0.05

1.3.4 *Swimming trajectories*

The qualitative analysis of swimming trajectories revealed that rats followed two defined strategies. While some animals performed the task in a "direct" manner (Figure 24A), others appeared to have applied a "trial and error" strategy (Figures 24B and 24C), which is exemplified by a frequent visit to the incorrect cue's associated area (error area) before choosing to approach the correct one. A chi-square test confirmed that the Sham group significantly preferred the "trial and error" strategy, while the ICSS-treated animals adopted a "direct" strategy ($\chi^2_{1,37}$ = 10.078, P = 0.006). A contingency table (Figure 24D) displays the frequency and percentages for each group and strategy.

At the same time, some animals that appeared to have chosen the correct cue failed to mount the platform due to their trajectory missing the target by a few millimeters. Figure 25 shows two examples (Figures 25A and 25B) of this behavior.



D

		STRATEGY			TOTAL
		Trial and Error	Direct	N/a	
GROUP	Sham	**68.4%** (13)	10.5% (2)	21.1% (4)	100% (19)
	ICSS	22.2% (4)	**55.6%** (10)	22.2% (4)	100% (18)

Figure 24. Swimming trajectories in the acquisition. Images of direct vs trial and error swimming trajectories that the rats used to solve the SVD task. In the lower-left quadrant, the inner concentric circle corresponds to the platform; outer concentric circle comprises the target zone; and in the lower-right quadrant, the circle represents the error area. (A) ICCS animal; (B) and (C) Sham animals; (D) Percentage of animals preferring “trial and error” or “direct” strategies, by group. N/a: non-applicable. Frequency is shown in brackets after the percentage.

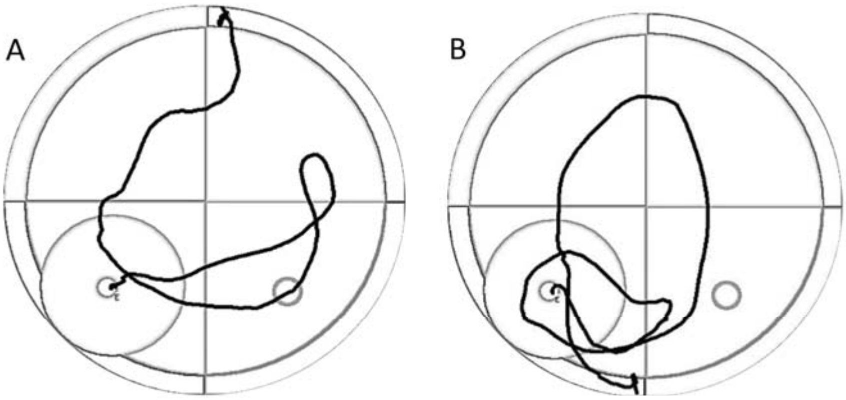


Figure 25. Swimming trajectories of rats missing the platform. The trajectory of some animals that approached the platform but failed to located it. This behavior could explain the lack of differences in latency between groups. Both figures belong to ICSS subjects.

1.3.5 *Histology*

Evaluation of the location of the electrode tip under the microscope revealed that they were all between AP -1.80mm and -3.14mm from Bregma according to the stereotaxic atlas (Paxinos & Watson, 2007). Additionally, a regression analysis showed that there is no relationship between the histological location of the electrode tip and the ICSS parameters or the performance in the SVD sessions.

1.4 Discussion

The present results indicate that post-training ICSS treatment facilitates the acquisition and retention of a visual discrimination task in the MWM. Although the escape latencies were equal for both groups, the ICSS subjects committed fewer errors than the Sham animals during the acquisition and the probe test. During the probe test, which assessed the memory retention after 72h, the ICSS group spent more time in the target quadrant and achieved more target crossings than the Sham subjects, indicative of a higher level of memory in the ICSS subjects. Present results are in agreement with those obtained in our laboratory regarding ICSS facilitation of the acquisition and retention of implicit avoidance memory tasks (Redolar-Ripoll et al., 2003; Ruiz-Medina et al., 2008b).

Furthermore, during the reversal phase ICSS treatment caused higher escape latencies and number of errors than the non-treated animals. Our results in the reversal test are also consistent with Hirsh's (1974) consideration of a visual discrimination task as an inflexible and associative memory process and with previous work on the difficulty of achieving the reversal of a well-consolidated visual discrimination task (Wilson & Cook, 1995). One aspect we believe to be of paramount importance is the fact that throughout the three phases of the experimental design, results consistently showed that the mean number of errors of each group was indicative of the facilitative effect of the ICSS treatment upon the visual discrimination task. Altogether, quantitative and qualitative results revealed that the ICSS rats applied a more efficient and direct strategy than Sham animals to learn the task and that ICSS treatment promotes a stronger and better-consolidated perceptual memory. As far as we know, this is the first time that the facilitation of the acquisition and retention of a visual discrimination task by post-training ICSS has been demonstrated.

On the other hand, and contrary to what we had expected, there were no differences between groups for the escape latencies in the acquisition sessions. This may seem contradictory with the idea of ICSS facilitation of the task, but these findings are similar to those of Packard and McGaugh (Packard & McGaugh, 1992), where escape latencies to mount the platform did not completely reflect the behavioral deficit observed in rats with lesion of the caudate nucleus. There are several possible explanations for this lack of differences. Looking at the trajectories of ICSS and Sham animals, some of them seem to experience difficulty finding the exact location of the escape platform as it is illustrated in Figures 25A and 25B, probably because the cue was too far from the platform. Therefore, even if rats accurately identify the correct cue, failing to find and mount the platform could cause the animal to retreat from the area in search of the alternative cue. This translates into valuable lost time, which would directly affect the escape latency values in the overall results. Thus, reducing the distance between the cues and the areas of *Escape* and *Error* would help avoid this type of error. In fact, a complementary experiment performed in our laboratory confirmed this hypothesis. As it is shown in Figure 26, when the distance between the edge of the tank and the cue was reduced from 35cm to 25cm, the ICSS group solved the task showing significantly lower latencies than the Sham animals in the last acquisition session. Other studies performed in the MWM where the visual discrimination cues were separated from the platforms have prevented this effect by concomitantly using contextual cues, providing spatial information that facilitate the task (Hyde et al., 2002).

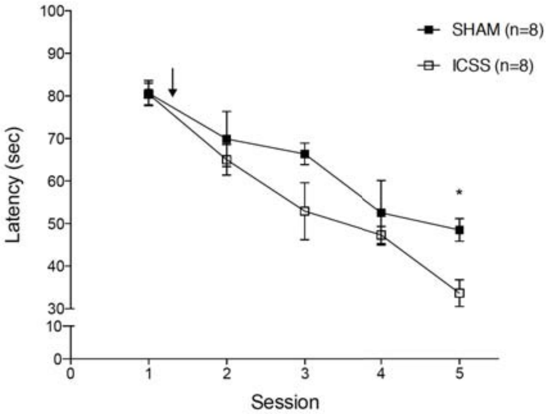


Figure 26. Significant effects of ICSS on acquisition latencies when cues were closer to the MWM. This figure depicts the Mean *Escape latencies* (±SE) for the acquisitions sessions of a complementary experiment where distance between cues and *Area of escape* and *Area of error* was reduced. Arrow shows start of ICSS treatment sessions. *P<0.05

Furthermore, the different strategies followed by the animals and the lower number of errors committed by ICSS group, suggests that ICSS treatment promotes a discriminatory perceptive learning process instead of a “trial and error” strategy. As in the aforementioned study by Packard and McGaugh (1992), our present results also show that the *number of errors* could be a more sensitive measure than latencies to detect the degree of facilitation in the acquisition of visual discrimination tasks.

Moreover, this improvement could be related to increases in some excitatory neurotransmitters levels, such as dopamine (DA), acetylcholine (ACh) or glutamate (GLU), in the hippocampus and cortical regions (Shankaranarayana Rao et al., 1998a; Murakami et al., 2015). In that sense, it has been observed that the blockade of DA and ACh (Turchi et al., 2008) or GLU (Gargiulo et al., 1998) results in a higher number of errors to criterion in this kind of tasks, while an ACh blockade also impairs the strategy selection (Whishaw & Petrie, 1988). Additionally, lesions of the cholinergic nucleus basalis magnocellular have been shown to increase perseverative errors in a simple-stimulus response visual perceptual task (Wilson & Cook, 1995), which could be linked to the modulatory effects of ACh on the attentional aspects of the task (Tsui, 2011). Furthermore, evidence from human studies shows that an enhanced efficacy of the cholinergic system's function facilitates the consolidation processes in a visual discrimination task (Beer et al., 2013) and promotes long-lasting improvements in perceptual learning (Rokem & Silver, 2013). All in all, an increased function of some excitatory neurotransmission systems activated by ICSS in memory-related regions, such as the hippocampus and cortex, could explain the lower number of errors and the accurate trajectory portrayed by ICSS-treated subjects in the present experiment. The specific mechanisms through which the ICSS could promote the activity of these memory-modulatory neurotransmitters are yet to be defined. However, descendent fibers from the MFB could explain such an increase, since ICSS functionally activates monoaminergic and cholinergic brainstem regions, such as the locus coeruleus, the ventral tegmental area and the pedunculopontine area (Arvanitogiannis et al., 1997).

In summary, the main goal of the present research was to determine whether a visual discrimination task, in which the perceptive component is critical, could be facilitated by post-training administration of ICSS in the MFB. ICSS led to the animals not only committing fewer errors, but also to them using a more accurate strategy to solve the task. Moreover, cognitive flexibility assessed by a reversal test was compromised by a strengthened memory consolidation. An implication of some excitatory ICSS-related transmitters is suggested. As far as we know, this is the first time that ICSS has been able to facilitate this type of implicit-perceptual learning and its retention. These findings, together with previous research in our laboratory, contribute to the establishment of post-training ICSS in the MFB as a generic treatment useful for facilitating a wide range of learning tasks and procedures.

STUDY II

2 Effects of Orexin-A receptor (OX1R) blockade on a spatial memory and activation of memory-related areas

Under revision at Behavioural Brain Research

Increased training compensates for OX1R blockage-impairment of spatial memory and c-Fos expression in hippocampal-thalamic-cortical circuit areas.

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Pilar Segura-Torres.

2.1 Introduction

Orexin neurons are considered “multi-tasking” neurons which regulate a set of vital body functions, including sleep-wake states, feeding behavior, energy homeostasis and reward systems (Chieffi et al., 2017). More recent studies have also linked this system to cognitive processes such as attention, learning and memory, the latter being the focus of the present study. The orexin system consists of two excitatory neuropeptides, orexin-A and orexin-B, which are produced by a few thousand neurons localized in the lateral hypothalamus (LH) and the adjacent perifornical area (de Lecea et al., 1998; Sakurai et al., 1998). The orexin-A selective receptor (OX1R) has been more thoroughly studied for its implication in memory (Li et al., 2014), while the OX2R, with a similar affinity for both orexin-A and orexin-B (Sakurai et al., 1998), is more involved in sleep and wakefulness (Sakurai, 2007). Orexin producing neurons project locally within the hypothalamus and widely throughout the brain, with prominent input in areas involved in arousal, reward and learning and memory processes.

In support of it playing a general modulatory role on memory, several morphological studies have shown that these projections reach regions related to different memory systems, including (1) the hippocampus (HPC) and other related structures such as the retrosplenial cortex (RSC) and the medial prefrontal cortex (mPFC); (2) several thalamic nuclei; (3) the amygdala and the striatum (Hervieu et al, 2001; Li et al., 2014; Marcus et al., 2001; Nambu et al., 1999; Peyron et al., 1998).

Interestingly, the electrophysiological effects of orexin-A in the HPC are similar to those that take place during learning. Thus, the administration of orexin-A induces long-term potentiation (LTP) in Schaffer collateral CA1 synapses (Selbach et al., 2010) and in the DG (Walling et al., 2004; Wayner et al. 2004). Moreover, the excitatory effects on CA1 hippocampal neurons have recently been shown to be mediated via OX1R (Chen et al., 2017). Together with these results, it should be noted that the manipulation of the orexin system functionally affects the animal’s performance in different memory tasks. Initial studies showed that the intracerebroventricular (icv) administration of orexin-A improves the animals’ performance in a passive-avoidance task (Telegdy & Adamik, 2002) and attenuates the retention deficits shown by a senescence-accelerated mouse strain in a T-maze and active avoidance task (Jaeger et al., 2002).

More recently, the administration of orexin-A has been shown to have ameliorating effects on a spatial memory in the Morris Water Maze (MWM) (Zhao et al., 2014).

Perhaps the most conclusive results of the involvement of orexin-A in hippocampal-dependent learning come from studies using the selective OX1R antagonist SB-334867 and the MWM. Specifically, administering SB-334867 in the CA1 impairs the acquisition, consolidation and recall of the task (Akbari et al., 2006), while DG SB-334867 microinfusion has also been found to impair acquisition and consolidation, but not retrieval (Akbari et al., 2007). In these studies, the administration of the antagonist is performed in specific hippocampal regions. However, the effects of the blockade of OX1R in additional brain structures that are also involved in spatial memory have been scarcely studied.

Furthermore, considering that increased training could at least partially compensate for cognitive deficits after lesions to the dorsal HPC in a spatial reference memory task (Ramos, 2009) or amygdala lesions in an emotional memory task (Segura-Torres et al., 2010), the present study aims to assess whether the expected negative effect of OX1R blockade on the MWM task could also be compensated for or alleviated by increased training.

The aim of the study is to test the effects of post training icv infusions of OX1R antagonists on spatial memory in a weak and strong MWM training. To test this we studied how post-training icv administration of SB-334867 affected the consolidation of a spatial learning and memory task in the MWM under two quantitatively different training conditions: 2 or 6 daily trials. A retention test was also performed to evaluate whether the effects of SB-334867 are still present after 72h, after which a reversal test was carried out to assess cognitive flexibility. We used icv microinfusions because we wanted to block OX1R in several areas given their relevance regarding memory-related processes. Finally, we tested the level of activation in the different brain structures involved in spatial learning and memory, and which have also been shown to express OX1R: HPC, RSC, infralimbic (IL) and prelimbic (PL) regions of the mPFC, and some thalamic nuclei such as Reuniens (Re), Rhomboid (Rh), Anterodorsal (AD), Centrolateral (CL), Centromedial (CM), Mediodorsal (MD), Laterodorsal (LD) and Paraventricular (PV) by using c-Fos as a marker of cellular activation.

2.2 Materials and Methods

2.2.1 *Experimental subjects*

A total of 38 male Wistar rats with a mean age 94.57±2 days and a mean weight of 415±17.2g from our laboratory's breeding stock were used. Three days before the stereotaxic procedure they were isolated and kept in individual cages (50x22x14cm, plastic bottomed and sawdust-bedded). The animals were kept under conditions of controlled temperature and humidity, and subjected to an artificial 12h light/dark cycle (light on at 08:00). All behavioral tests took place during the first 6h of light. All subjects were kept in an ad libitum regime of food and water. All procedures were carried out in compliance with the Directive 2010/63/EU and were approved by the institutional animal care committee.

2.2.2 *Stereotaxic surgery*

Previous to the surgery, two sessions of handling took place in order to diminish emotional reactivity of the animals towards experimental manipulation. Under general anesthesia (150 mg/kg Imalgène® ketamine chlorhydrate (Merial, Lyon, France) and 0.08 mg/kg Rompun® xylazine (Bayer, Barcelona, Spain); i.p.) all rats were chronically implanted with a 7.5mm infusion guide cannula (Plastics One®, Raonoke, VA, US, purchased through Bilaney consultants, Düsseldorf, Germany; ref: C315G/PK/Spc) into the left lateral ventricle (LV), according to coordinates from the stereotaxic atlas of Paxinos & Watson (2007) at anterior: -0.7mm from bregma, lateral: 1.6mm and ventral: -4.0mm. A dummy cannula (Plastics One®; ref: C315DCN/Spc), filled the infusion guide cannula and expanded 0.5mm into the parenchyma. The cannula was fixed in position using an auto-polymerizing acrylic resin (Vertex self-curing, Dentimex, Netherland). The animals were weighed and handled daily during the post-surgery recovery period (7 days).

2.2.3 *Morris Water Maze Apparatus.*

The MWM consisted of an elevated circular pool (2m diameter; 60cm above the pool floor) filled with water (45cm height) maintained at 22 ± 2ºC. The pool was in the middle of a semi-dark room and surrounded by black curtains reaching from a false ceiling to the base of the pool forming a circular enclosure 2.4m in diameter. A clear Plexiglas platform (11cm diameter) was placed centrally in one of the four equal quadrants in which the tank was virtually divided, with its top 2 cm below the surface of the water. All swim paths were recorded using a closed-circuit video camera (Smart Video Tracking System, Version 2.5, Panlab) with a wide-angle lens was mounted 1.75m above the center of the pool embedded in the false ceiling.

2.2.4 *Behavioral procedure: Training, retention test and reversal.*

All animals underwent one habituation session to the MWM in order to reduce emotional reactivity. During the training phase, animals were given either 2 or 6 trials for 5 days. The duration of the trial varied according to the number of trials: 120s (2 trials) and 90s (6 trials). The average intertrial interval (ITI) was 120s. Starting from one of four different cardinal points (N, E, S and W) in a pseudorandom schedule each trial consisted of one swim from the edge of the pool to the platform. When an animal failed to find the platform after 90s or 120s, it was manually directed to mount it for 15s and then removed from the tank.

Seventy-two hours after the last training session each animal completed a retention test, which consisted of removing the platform and placing the animal in the pool from the E starting position for 60s. Immediately after the 60s, subjects underwent reversal learning, in which the platform was placed in the opposite quadrant of the pool and the animals were directed to mount the platform and left there for 15s. After 120s ITI, animals were again placed in the pool from the remaining starting points (N, W, S) and left to find the platform for 90s or 120s, depending on the group.

2.2.5 Post-training microinfusion procedure.

All animals underwent two habituation sessions to the microinfusion apparatus in order to reduce initial emotional and physical reactivity. Immediately after each training session, the animals were gently restrained while its dummy was removed and replaced with a 26-gauge injector (PlasticOne®, Roanoke, VA, USA; ref.: C313CT) extending 0.5mm below the cannula tip. The injectors were connected by polyethylene tubing (PlasticOne®, Roanoke, VA, USA; ref.: C315I/PK/Spc) to two 10µL syringes (SGE Analytical Science, Cromlab S.L. Barcelona, Spain) that were placed in the infusion pump (11 Plus Syringe Pump, Harvard Apparatus Inc., Holliston, Massachusetts, USA). The selective OX1R antagonist SB-334867 (Tocris Bioscience, Bristol, UK) was dissolved in dimethyl sulfoxide (DMSO) (Tocris Bioscience, Bristol, UK), aliquoted in tightly sealed vials and stored at -20ºC for up to 5 days. The animals were administered a total volume of 5µL of DMSO or SB-334867 (5µg/5µl) into the left LV at a rate of 2.5 µL/minute. The injectors were left in place for an additional 60s to allow for the diffusion of the solution away from the tip. Figure 27 depicts the experimental procedure.

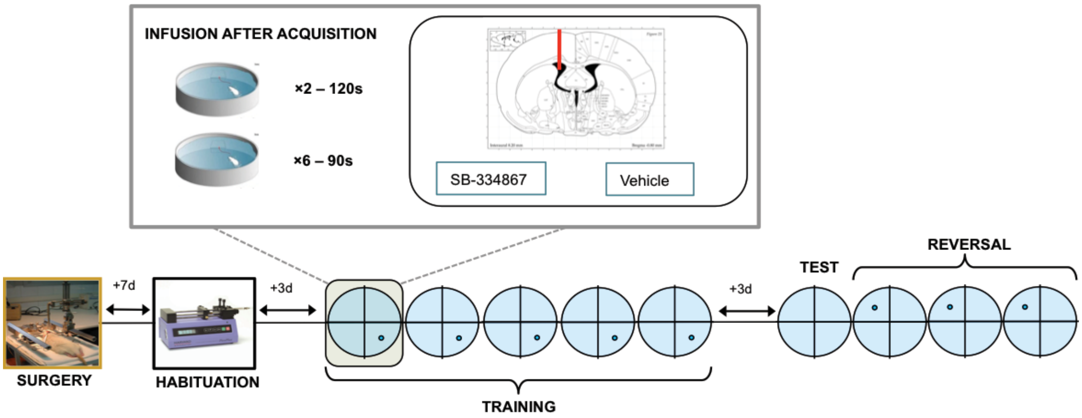

Figure 27. Diagram depicting experimental procedure in Study II.

2.2.6 *c-Fos inmunolocalization*

2.2.6.1 Tissue collection.

Ninety minutes after the end of the retention/reversal session, animals received a pentobarbital overdose (150mg/Kg, i.p.) and were transcardially perfused with a solution of 0.1M of phosphate buffer saline (PBS), pH 7.4, followed by a solution of 4% paraformaldehyde in PBS. Brains were removed and post-fixed in 4% paraformaldehyde in PBS, then cryoprotected in 15% and 30% sucrose in PBS and stored at -80ºC. Serial coronal sections (30µm) were obtained in a cryostat (Cryocut 1800, with 2020 JUNG microtome) at -20ºC between the coordinates 4.68 and 2.16, and -1.56 and -3.36 AP of Bregma (Paxinos & Watson, 2007) and stored at -80ºC until immunostaining. From the same subject, localized coronal sections (40µm), between the coordinates -0.6 and -1.20 of Bregma, were mounted onto a gelatin-coated slide, stained with cresyl violet and examined for cannula placement.

2.2.6.2 c-Fos immunochemistry.

Free-floating coronal sections were incubated with 0.3% H_2O_2 in Tris-buffered saline (TBS) for 30m and in 0.1% Bovine Serum Albumin (BSA) in 0.3% Triton X-100 in TBS for 30m. Sections were incubated in primary antibody rabbit anti-cFos (Santa Cruz Biotechnology Inc. Santa Cruz, CA, US; ref.: sc-166940) at 1:2000 in 0.1% BSA in 0.3% Triton X-100 in TBS for 60m at room temperature and then overnight at 4ºC. The next day samples were incubated with biotinylated goat anti-Rabbit-IgG secondary antibody (Jackson Immunoresearch Inc., USA; ref.: 111-066-144) at 1:5000 in 0.1% BSA in 0.3% Triton X-100 in TBS for 60m and subsequently treated with Streptavidin-HRP (Pelkin Elmer ® Life Science Inc., USA) at 1:3600 in 0.3% Triton X-100 in TBS, followed by treatment with 3,3'-Diaminobenzidine (DAB) solution (Vector ®) for 7m. Negative controls without primary or secondary antibodies were included.

2.2.6.3 Image acquisition and analysis.

Using a BX-41 Olympus microscope attached to an Olympus DP-70 digital camera (Japan), microphotographs of the following brain regions were captured: HPC, RSC, IL, PL, Re, Rh, AD, CL, CM, MD, LD and PV. Appropriate grey threshold and particle size were set for each area and maintained for all subjects. The image analysis software Image-J 1.43 (http://rsb.info.nih.gov/ij/) was employed to bilaterally count the number of c-Fos immunostained nuclei using regions of interest (ROIs) (see Figure 28A - 28D in Results section). Three histological sections were counted and averaged for all areas, except for AD where only 2 sections of each animal were suitable for the analysis. The coordinates for each area were matched for each group in order to reduce variability between samples (AP: PFC, 3.00mm to 2.52mm; AD, -2.04mm to -2.28mm; HPC-RSC-Thalamic nuclei, -2.64mm to -3.00mm). In order to remove background noise, each image was digitally smoothed and subtracted from the original.

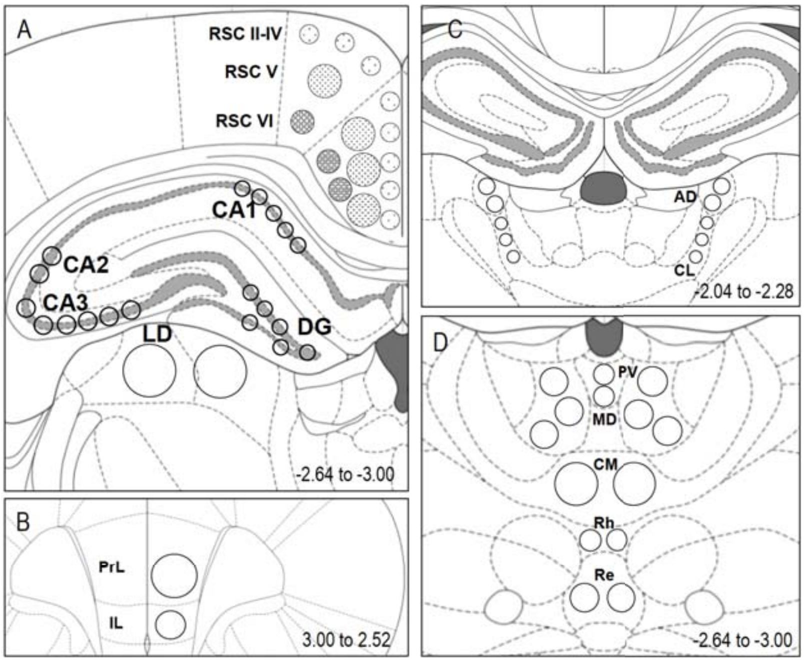

Figure 28. Regions of interest (ROIs) for the quantification of c-Fos immunopositive nuclei in the (A) HPC (CA1, CA2, CA3, DG), RSC (layers 2-4, 5 and 6) and LD. (B) IL and PL. (C) AD and CL. (D) PV, MD, CM, Rh and Re are shown superimposed on coronal sections adapted from Paxinos & Watson’s atlas (2007). Abbreviations: CA1- CA2-CA3, cornu ammonis 1, 2 and 3; DG, dentate gyrus; RSC, retrosplenial cortex; LD, laterodorsal nucleus; IL, infralimbic cortex; PL, prelimbic cortex; AD, anterodorsal nucleus; CL, centrolateral nucleus; PV, paraventricular nucleus; MD, mediodorsal nucleus; CM, centromedial nucleus; Rh, rhomboid nucleus; and Re, reuniens.

2.2.7 *Statistical analysis*

Statistical analysis was performed using SPSS v23 (SPSS Inc. Chicago, IL, USA). Analysis of the memory task in the MWM was conducted independently for each training condition (2 or 6 trials) with a 2×5 mixed ANOVA (SESSIONS×INFUSION) for the training phase and independent sample *t*-test analysis for the retention test and reversal (the average score of the three trials was analyzed). When the effect of the SESSIONS factor was statistically significant, polynomial contrasts explored the presence of linear and/or quadratic trends in the performance. The main outcome variable for training and reversal sessions in the MWM was the *Escape latency* or time (s) needed to find and climb onto the platform. Results for the retention test were analyzed for the totality of the trial (60s) and, in addition, for the first half of the trial (30 s). In the retention test, the following variables were analyzed: (1) Percentage of time spent in the target quadrant in the first half of the trial [TQ30] and its totality [TQ60]; (2) Average Distance to target [DT30 and DT60]; (3) Percentage of time spent in target annulus [Ann30 and Ann60]; and (4) Whishaw's Error [WE]. In addition, a one-sample *t*-test against a constant (25) was used for each group to determine whether TQ30 and TQ60 were different from chance level (25%) both at the first 30s (Chance30) and at 60s (Chance60). Moreover, the *percentage of time spent near the walls* (measure of thigmotaxis – anxiety), *length* (total distance in cm) and *speed* (measure of motor activity) were analyzed and considered as control variables for each group. To analyze c-Fos quantitative data we used a 2×2 mixed analysis of variance with INFUSION (SB or DMSO) and TRAINING (2 or 6 trials per day) as independent factors between subjects, and the within group factors HEMISPHERE, REGION and LAYER, depending on the brain structure. Greenhouse-Geisser correction and Welch's *t*-test were used when sphericity was violated and homogeneity of variables was not reached, respectively. Additionally, in order to study the underlying functional relationship between the regions in which significant main factors effects on c-Fos expression were observed, a principal components analysis (PCA) using the *oblimin* method of rotation was conducted. Statistical significance for all tests was set at α=0.05.

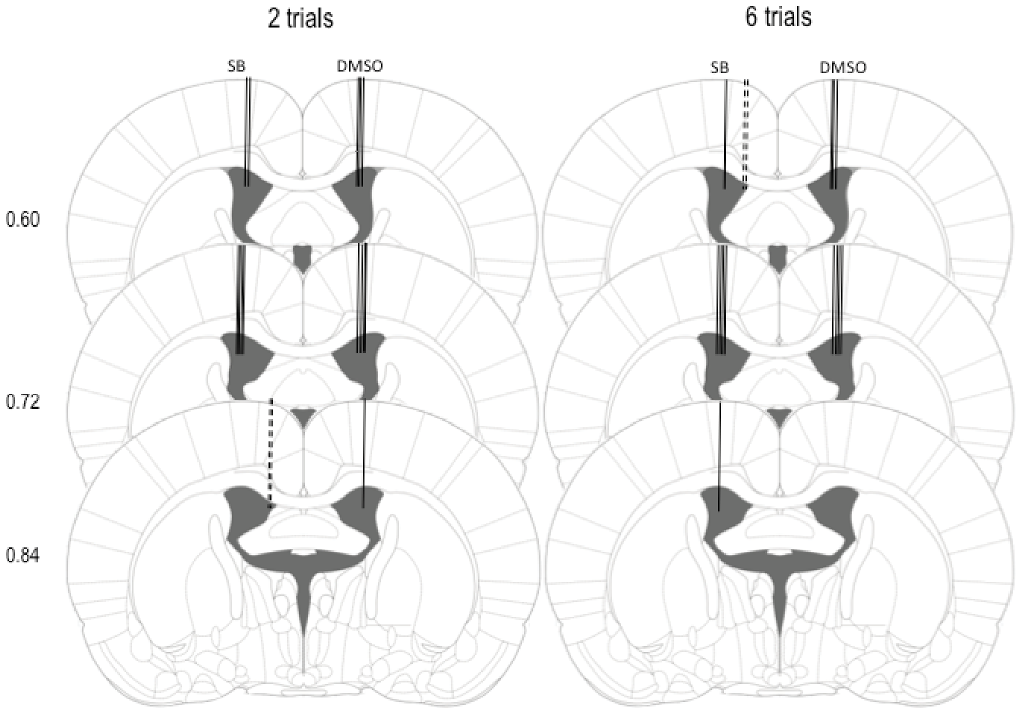

Figure 29. Representation of cannula placement throughout rostro-caudal locations in the left lateral ventricle (Paxinos & Watson, 2007) for animals in 2 and 6 trials. Discontinuous lines depict misplacements that resulted in subject removal from experiment.

2.3 Results

A total of seven subjects were excluded from the analysis of the behavioral data for two reasons: (1) misplaced cannula (N=4); and (2) outliers for control variables (N=3). The final sample consisted of 31 subjects (DMSO2: N=8; SB2: N=7; DMSO6: N=8; SB6: N=8). There was no statistical difference between groups in age at the start of the experiment or in weight evolution throughout the experiment. A representation of the cannula placement for all animals can be seen in Figure 29.

2.3.1 *Effects of SB-334867 infusion on Morris Water Maze*

2.3.1.1 Training

2-trial condition. A mixed ANOVA of the escape latencies for the training phase showed an interaction for SESSIONS×INFUSION [$F_{4,52}$=7.577, P<0.001], indicating a difference between the groups depending on the session (Figure 30A). The simple effects analysis detected differences between groups in all sessions except for session 1, indicating a similar starting point for both groups (P=0.83), as well as higher latencies for the SB animals in every session after the first administration (session 2, P=0.045; session 3, P=0.002; session 4, P=0.002; and session 5, P=0.001). The intragroup analysis of latencies throughout training sessions showed that both groups adjust to a significant downward linear function, but the performance of the DMSO group had a steeper slope [DMSO: $F_{1, 13}$=90.32, P<0.001; SB: $F_{1, 13}$=5.12, P=0.041].

6-trial condition. An ANOVA showed a significant effect of the factor SESSION [$F_{4,56}$=51.322, P<0.000], but no significance for the factor INFUSION [$F_{1, 14}$=3.997, P=0.065] or interaction effect SESSIONS×INFUSION [Greenhouse-Geisser $F_{4,56}$= 2.586, P=0.084]. The analysis of simple effects for each training session showed statistical significance between groups in session 2 (P=0.02) and session 4 (P=0.03) (Figure 30B). The polynomial contrast for the evolution of latencies showed that both groups adjusted to similar linear functions [DMSO: $F_{1, 14}$=88.04, P<0.001; SB: $F_{1, 14}$=48.50, P<0.001].

Finally, no differences in control variables for *percentage of time spent near the walls (thigmotaxis)*, *length* and *speed* were found between groups in any training condition (see Table 6).

2.3.1.2 Retention test

2-trial condition. A general disruptive effect of SB-334867 on the retention test was observed. Thus, SB group had a lower preference for target quadrant both in the first 30s and the total trial (TQ30: $t_{1,13}$=2.181, P=0.048; TQ60: $t_{1,13}$=3.369, P=0.005) when compared to the DMSO controls (Figure 30C and 30D). These figures also show that DMSO animals performed above chance level (25%) for both the first 30s and the total trial (Chance30: $t_{1,13}=3.274$, $P=0.011$; Chance60: $t_{1,13}=3.549$, $P=0.008$) while the SB animals did not (Chance30: $t_{1,13}=0.56$, $P=0.958$; Chance60: $t_{1,13}=0.775$, $P=0.473$). Furthermore, SB animals' average distance to the platform was significantly higher than DMSO animals also for both in the first 30s and the total trial (DT30: $t_{1,13}=2.323$, $P=0.037$; DT60: $t_{1,13}=3.286$, $P=0.007$) (Figure 30F and 30G, respectively). Likewise, SB subjects showed a lower preference for Ann30 ($t_{1,13}=2.420$, $P=0.033$) and Ann60 ($t_{1,13}=3.089$, $P=0.011$) (Figure 30H and 30I). Similarly, they had a worse performance regarding WE ($t_{1,13}=2.371$, $P=0.038$) (Figure 30J).

6-trial condition. Both SB and DMSO animals performed above chance level (25%) for both the first 30s (SB: $t_{1,14}=3.180$, $P=0.015$; DMSO: $t_{1,14}=3.918$, $P=0.006$) and the total trial (SB: $t_{1,14}=3.966$, $P=0.005$; DMSO: $t_{1,14}=5.669$, $P=0.001$) (Figure 30E). No differences between groups were found for any other analyzed variable.

Table 6. **Values of latency and control variables.**

Variable	Session	SB2 (N=7)	DMSO2 (N=8)	SB6 (N=8)	DMSO6 (N=8)
Latency	1	111.29±5.72	113.06±5.60	74.89±4.98	78.75±4.25
	2	113.31±5.20	86.20±9.28	64.79±3.55	52.16±3.21
	3	103.31±7.14	50.42±10.05	48.85±9.28	31.67±5.41
	4	96.50±9.44	47.08±8.32	44.78±6.64	25.36±5.11
	5	90.19±14.16	31.45±5.35	31.63±5.09	20.40±3.31
	Reversal	91.92±11.22	55.28±9.04	50.40±8.75	31.88±5.72
Length	1	6059.9±1052.3	6885.0±1603.7	4534.7±813.9	4442.6±576.4
	2	8640.9±1256.2	6607.3±2406.1	4611.1±1016.9	3562.7±605.7
	3	7708.4±1296.1	4155.6±2789.5	3125.8±1972.3	2039.9±1083.4
	4	6925.8±1479.8	3727.4±2710.8	2872.5±1373.7	1624.7±1103.2
	5	6531.6±3056.4	3196.2±2377.4	1863.4±1084.9	1322.8±787.9
	Reversal	5579.6±1708.1	3450.2±2056.0	2942.6±1654.4	1768.2±1018.7
Speed	1	57.43±6.75	61.21±10.45	62.30±7.05	57.79±4.43
	2	76.42±7.83	77.20±10.26	69.74±5.54	68.51±12.11
	3	77.61±14.98	76.36±15.73	64.40±5.25	65.00±14.68
	4	75.96±13.76	78.40±16.82	63.38±6.87	61.62±10.51
	5	78.11±19.69	78.18±16.49	61.08±10.56	64.89±9.88
	Reversal	63.14±19.47	64.33±14.32	62.28±7.79	60.22±10.62
Walls	1	53.90±12.38	52.38±14.18	44.73±11.49	41.57±13.71
	2	48.65±15.79	48.75±23.38	39.75±16.33	40.43±12.22
	3	40.17±13.01	38.07±16.55	38.09±19.75	29.70±13.17
	4	43.71±18.12	36.06±23.45	27.97±16.48	27.20±14.79
	5	35.49±22.58	36.26±15.31	28.97±10.84	26.98±7.38
	Reversal	45.05±12.01	31.00±9.74	23.89±8.45	17.26±9.29

Table 6. Values of latency and control variables for the training phase (1 to 5 sessions) and the reversal session (average of 3 trials). Latency (sec, mean±SEM) Length (cm, mean±SEM); Speed (cm/s, mean±SEM) and Thigmotaxis (percentage, mean±SEM).

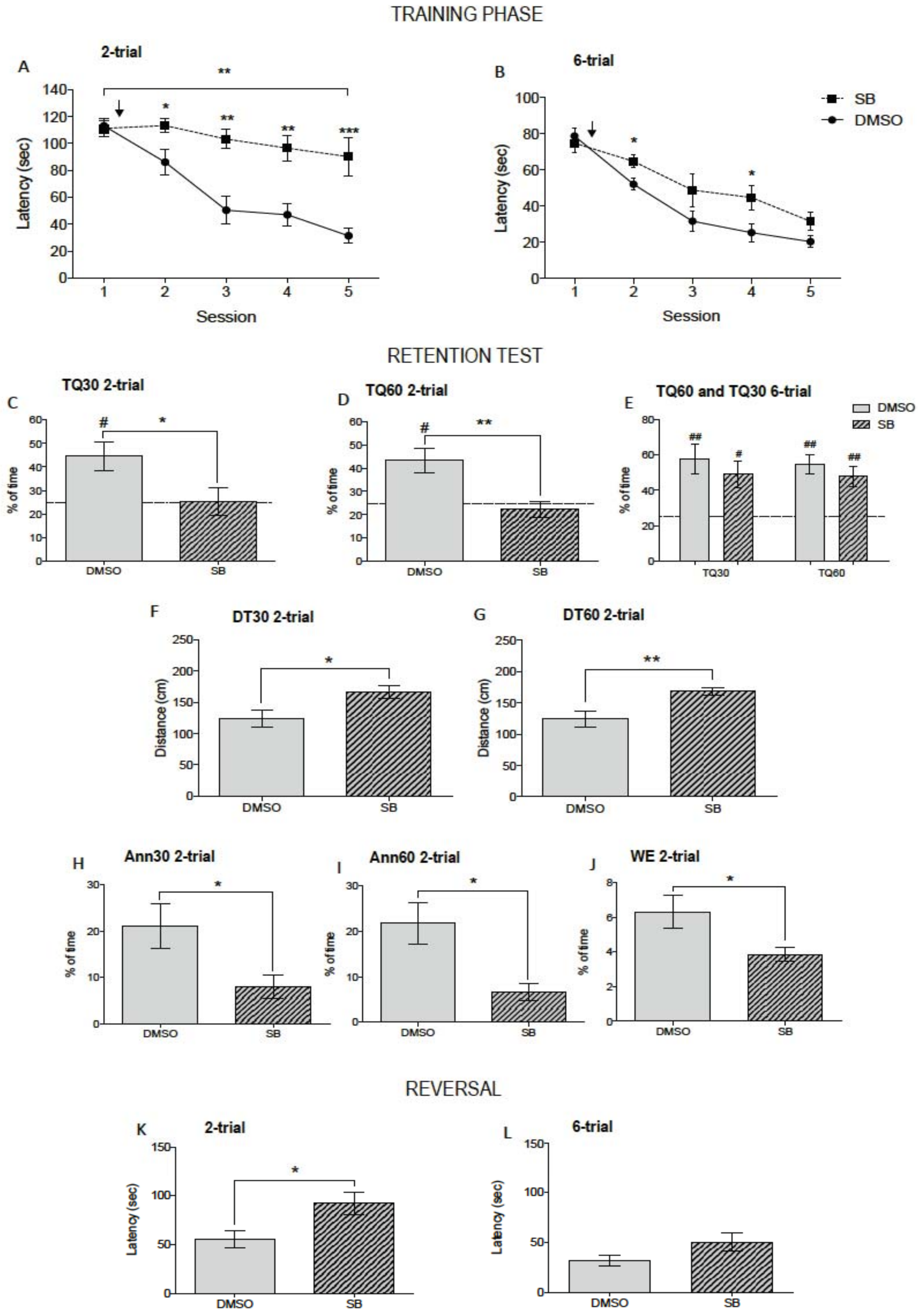

Figure 30. Differential effects of SB-334867 on MWM task depending on the number of trials. Mean *Escape latencies* (±SEM) for the five sessions of the training phase in the 2-trial (A) and 6-trial (B) training conditions. Arrow shows start of infusions. Retention test variables for 2-trial condition: TQ30 and TQ60 (C and D), DT30 and DT60 (F and G), in the Ann30 and Ann60 (H and I) and the WE (J); and 6-trial condition: TQ30 and TQ60 (E). Reversal variables: *Escape latencies* (±SEM) in 2-trial (K) and 6-trial (L) training condition. Abbreviations: TQ, percentage of time spent in target quadrant; DT, average distance to target; Ann, percentage of time spent in target annulus; WE, Whishaw's error. Group factor significance is depicted with *P<0.05; **P<0.01; ***P=0.001. The dotted line in retention test represents chance level # P<0.05.

2.3.1.3 Reversal

2-trial condition. Analysis of the mean latencies for the three reversal trials showed a significant effect of INFUSION [$F_{1,\,13}$=6.496, P=0.024], with SB animals taking longer to find the platform than DMSO animals (Figure 30K). Moreover, the SB group spent significantly more time near the walls ($t_{1,13}$=2.330 P=0.027).

6-trial condition. Analysis of the mean latencies for the three reversal trials showed no effect for the factor INFUSION [$F_{1,\,14}$=3.136, P=0.098] (Figure 30L).

2.3.2 *Effects of SB-334867 infusion and/or amount of training on c-Fos expression*

The final sample varied from 23 to 25 subjects depending on the area of analysis (DMSO2: N=8; SB2: N=5; DMSO6: N=6; SB6: N=6). The general linear model of variance analysis of the c-Fos expression showed that there were no effects of the HEMISPHERE factor or its interactions with TRAINING or INFUSION in any of the analyzed areas. Thus, we proceeded to adjust the model by removing this factor and the INFUSION and TRAINING factors were analyzed bilaterally in all the regions with the exception of the medial nuclei of the thalamus. Figure 31 shows c-Fos quantification (top) and representative photographs (bottom) of one subject in each group for the HPC, RSC and PFC. Figure 32 depicts quantification (left) and representative photographs (right) of one subject in each group for the associative nuclei, intralaminar nuclei and midline nuclei of the thalamus. Table 7 summarizes the *p*-values of the statistical results for all factors, their interaction and, when appropriate, simple effects for each of the regions studied.

2.3.2.1 Hippocampus

No interaction of INFUSION×TRAINING factors was found in any of the hippocampal areas. A TRAINING effect was observed in the HPC [CA2: $F_{1,19}$=10.241, P=0.005; CA3: $F_{1,21}$=14.767, P=0.001; DG: $F_{1,21}$=10.424, P=0.004], with the exception of CA1 [$F_{1,21}$=3.344, P=0.08]. In general, higher c-Fos expression was observed in the 6-trial compared to the 2-trial condition. The study of simple effects detected differences between the DMSO6 and the DMSO2 groups in CA2 (P=0.028) and CA3 (P=0.053); and between the SB6 and the SB2 groups in DG (P=0.01) (Figure 31A).

The INFUSION factor was significant in CA2 [$F_{1,19}$=12.969, P=0.001] and DG [$F_{1,21}$=8.068, P=0.01], in the sense that SB groups showed lower c-Fos expression. Particularly, differences between the DMSO6 and the SB6 groups were found in CA2 (P=0.008) and between the DMSO2 and the SB2 groups in DG (P=0.036).

2.3.2.2 Restrosplenial Cortex

In the gRSC (Figure 31B), since the LAYER interacted significantly INFUSION factor [$F_{2,42}$=3.595, P=0.036], we proceeded to study each layer. A general effect of INFUSION was observed; being significant for layers II-IV [$F_{1,21}$=6.829, P=0.016] and VI [$F_{1,21}$=7.317, P=0.013], and tending to significance for layer V [$F_{1,21}$=3.253, P=0.06]. However, the analysis of simple effects showed a lower c-Fos expression in the SB2 group compared to its control, in each of the layers (II-IV: P=0.05; V: P=0.02; VI: P=0.02). TRAINING was not significant in any of the gRSC layers.

The differences in the dRSC were independent of the layers. There is a main effect of the TRAINING [$F_{1,21}$=6.920, P=0.016], with the DMSO6 group showing a higher expression of c-Fos than DMSO2 group (P=0.035). The INFUSION was also significant in dRSG [$F_{1,21}$=10.430, P=0.004], and simple effects pointed out significant differences between the DMSO6 and the SB6 groups (P=0.019).

2.3.2.3 Prefrontal cortex

A main TRAINING effect was observed in PL [$F_{1,20}$=6.180, P=0.022], independent on the infusion condition (Figure 31C). However, simple effects seemed to show that training had a greater effect on the infused animals (SB6 vs. SB2: P=0.016). Moreover, INFUSION was also significant in PL [$F_{1,20}$=13.405, P=0.002], especially for the groups in the 2-trial condition (DMSO2 vs. SB2: P=0.019). Although no differences were found in the IL, both factors showed a tendency towards significance [TRAINING: $F_{1,20}$=3.222, P=0.08; INFUSION: $F_{1,20}$=3.737, P=0.06] (Figure 31C), and results seemed to mirror the ones observed in PL.

2.3.2.4 Thalamus

The thalamic nuclei that were analyzed are (1) the specific associative regions AD, MD and LD (Figure 32A), (2) rostral intralaminar CM and CL nuclei (Figure 32B), and (3) unspecific nuclei of the midline, the ventral Re and Rh and the dorsal PV (Figure 32C). Of these nuclei, the only ones that did not show any significant result were the PV and the LD; the latter probably due to the reduced amount of expression observed in all the groups. The factor TRAINING was significant for the remaining nuclei: AD [$F_{1,19}$=14.197, P=0.001], MD [$F_{1,20}$=5.571, P=0.029], CM [$F_{1,21}$=12.523, P=0.002], CL [$F_{1,20}$=7.924, P=0.011], Re [$F_{1,20}$=5.712, P=0.027], and Rh [$F_{1,20}$=4.778, P=0.041], independent of the INFUSION factor. Simple effects showed a greater activation for the 6-trial groups of both conditions in CM (DMSO6 vs DMSO2: P= 0.014; SB6 vs SB2: P=0.028), while in CL and Rh this effect was only seen for the SB groups (CL: SB6 vs SB2, P=0.020; Rh: SB6 vs SB2, P=0.032). Moreover, differences in AD were observed only in the control

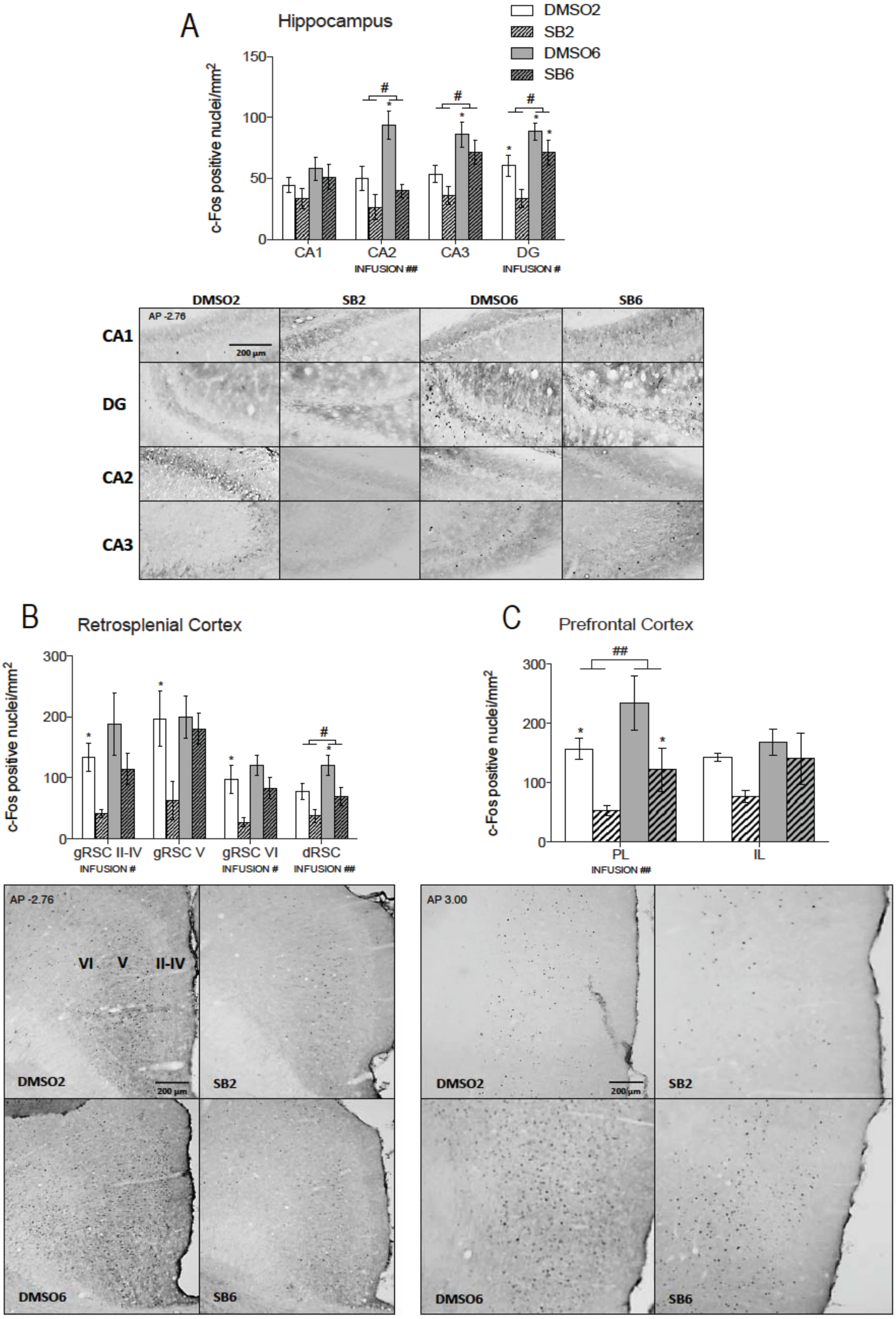

Figure 31. Effects of SB-334867 and training on c-Fos expression in (A) Hippocampus, (B) Retrosplenial Cortex (with Layers II-IV, V and VI), and (C) Prefrontal Cortex for animals receiving a vehicle (DMSO) or OX1R antagonist (SB-334867) infusion, and weak (-2) or strong (-6) training in the MWM. Training factor significance is depicted above bars, and infusion factor significance is shown below the X axis with # P<0.05; ## P<0.01. Group contrasts are depicted with *P<0.05. Microphotographs of each area are found below the corresponding graphs. Scale bar: 200µm.

condition (DMSO6 vs DMSO2: *P*=0.017).

The INFUSION factor was only significant in CM ($F_{1,21}$=8.065, *P*=0.01), CL [$F_{1,20}$=4.934, *P*=0.038], and in AD [$F_{1,19}$=21.820, *P*<0.001]. The simple effects analysis showed that the SB2 group under-expressed c-Fos when compared to DMSO2 (*P*=0.035) in CL, while it affected both conditions of training, the 6-trial (DMSO6 vs SB6: *P*=0.005) and 2-trial (DMSO2 vs SB2: *P*=0.042) in the AD.

Microphotographs of the areas that showed significant differences between groups can be seen in Figure 32D.

Table 7. ANOVA and simple effects analysis *p*-values of c-Fos expression for all areas analyzed.

			ANOVA analysis (all *p*-values)			**Simple effects analysis (all *p*-values)**			
			FACTORS			**TRAINING**		**INFUSION**	
			TRAINING	*INFUSION*	*INT*	*DMSO6 vs DMSO2*	*SB6 vs SB2*	*DMSO2 vs SB2*	*DMSO6 vs SB6*
RSC	gRSC	Layers II-IV	0.058	0.016 *	0.77	*ns*	*ns*	0.05 *	*ns*
		Layer V	0.129	0.06	0.67	*ns*	*ns*	0.02 *	*ns*
		Layer VI	0.062	0.013 *	0.43	*ns*	*ns*	0.02 *	*ns*
	dRSC	-	0.016 *	0.004 **	0.71	0.035 *	*ns*	*ns*	0.019 *
Hippocampus		CA1	0.08	0.3	0.8	*ns*	*ns*	*ns*	*ns*
		CA2	0.005 **	0.002 **	0.24	0.022 *	*ns*	*ns*	0.012 *
		CA3	0.001 **	0.08	0.86	*ns*	*ns*	*ns*	*ns*
		DG	0.004 **	0.01 *	0.89	0.03 *	0.035 *	0.046 *	*ns*
Prefrontal cortex		PL	0.022 *	0.002 **	0.87	*ns*	0.016 *	0.019 *	*ns*
		IL	0.08	0.06	0.44	*ns*	*ns*	*ns*	*ns*
Thalamus		Re	0.027 *	0.284	0.757	*ns*	*ns*	*ns*	*ns*
		Rh	0.041 *	0.137	0.251	*ns*	0.032 *	*ns*	*ns*
		CM	0.002 **	0.01 *	0.98	0.014 *	0.028 *	*ns*	*ns*
		CL	0.011 *	0.038 *	0.39	*ns*	0.020 *	0.035 *	*ns*
		MD	0.029 *	0.227	0.721	*ns*	*ns*	*ns*	*ns*
		LD	0.089	0.425	0.51	*ns*	*ns*	*ns*	*ns*
		AD	0.001 **	<0.001 ***	0.57	0.017 *	*ns*	0.042 *	0.005 **
		PV	0.371	0.813	0.606	*ns*	*ns*	*ns*	*ns*

Table 7. ANOVA and simple effects analysis *p*-values of c-Fos expression for all areas analyzed. Abbreviations: RSC, retrosplenial cortex (g, granular; d, dysgranular); CA1- CA2-CA3, cornu ammonis 1, 2 and 3; DG, dentate gyrus; PL, prelimbic; IL, infralimbic; Re, reuniens; Rh, rhomboid; CM, centronedial; MD, mediodorsal; LD, laterodorsal; AD, anterodorsal; PV, paraventricular; INT, interaction; *ns*, no significance. Factor significance is depicted with * P < 0.05; ** P < 0.01; *** P < 0.001.

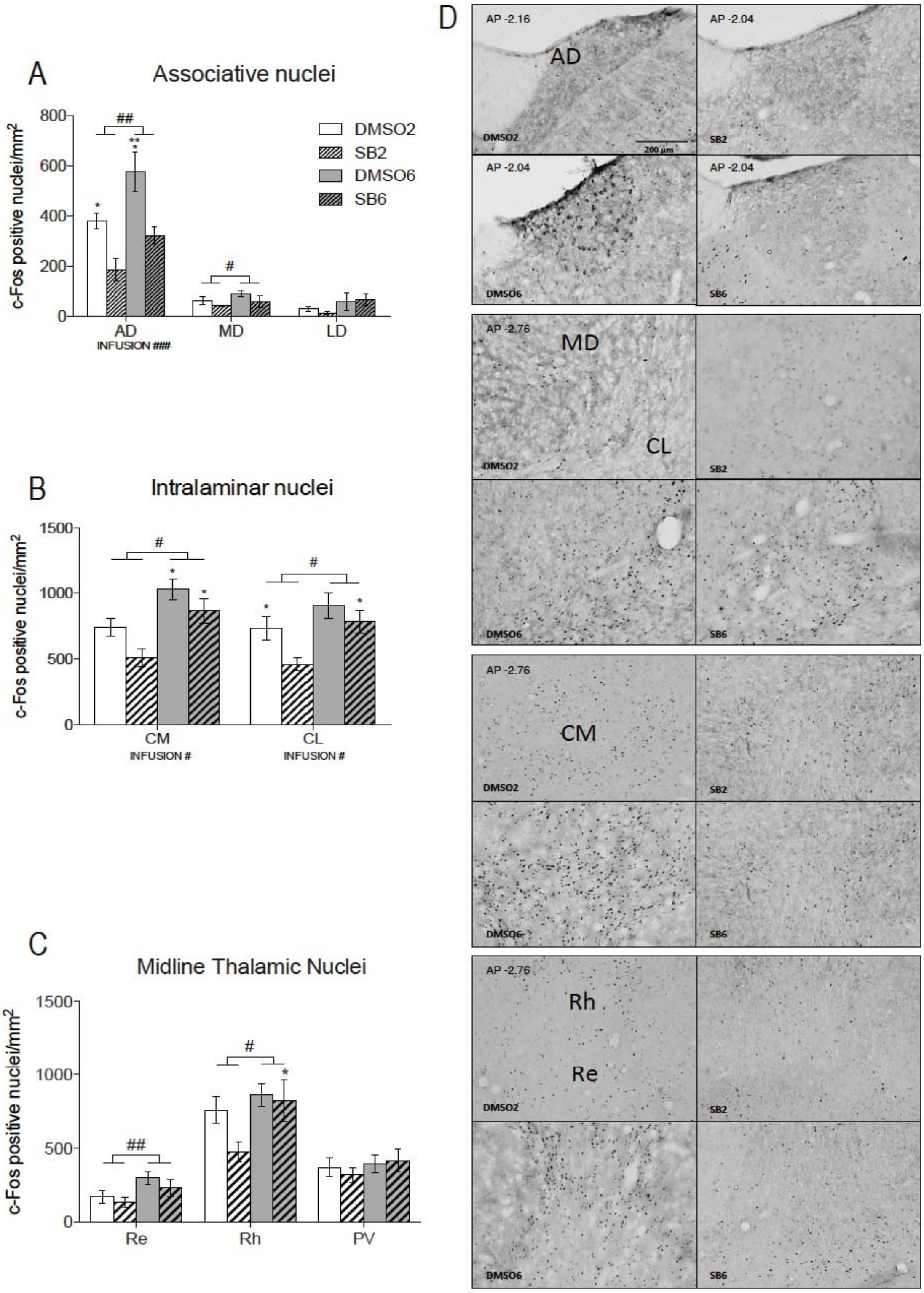

Figure 32. Effects of SB-334867 and training on c-Fos expression in the Thalamus. (A) Associative nuclei, (B) Intralaminar nuclei, and (C) Midline nuclei for animals receiving a vehicle (DMSO) or OX1R antagonist (SB-334867) infusion, and weak or strong training in the MWM. Abbreviations: AD, anterodorsal nucleus; MD, mediodorsal nucleus: LD, laterodorsal nucleus; CM, centromedial nucleus; CL, centrolateral nucleus; Re, reuniens nucleus; Rh, rhomboid nucleus; and PV, paraventricular nucleus. Training factor significance is depicted above bars, and Infusion factor significance is shown below the X axis with #P<0.05; ##P<0.01; ###P≤0.001. Group contrasts are depicted with *P<0.05; **P<0.01. Microphotographs of each area are found next to the corresponding graphs. Scale bar: 200µm.

2.3.2.5 Principal component analysis (PCA)

A PCA was performed in order to identify subsets of functionally related areas regarding c-Fos expression. This was based on the regions where INFUSION factor was significant. The bilateral areas were included in the PCA as the average of both hemispheres, given that no hemispheric differences were detected. The PCA included the following areas: PL, aRSG, gRSC II-IV, gRSC VI, CA2, GD, CM, CL, and AD.

Bartlett's sphericity test was statistically significant (KMO=0.707; *P*<0.001). A 3-component model explained 80.13 percent of variance. Table 8 shows a rotated factor loading. Factor 1 included seven items (all the cortical and thalamic areas included). Factor 2 consisted of the CA2, while Factor 3 was loaded by the DG. Factor correlations were low for the three factors (*r* ≤ /.055/).

Table 8. Structure matrix for subsystems of c-Fos immunoreactivity as shown by principal components analysis

BRAIN REGION	NUCLEUS/ SUBREGION	FACTOR 1	FACTOR 2	FACTOR 3
Cortex	gRSC II-IV	**0.910**		
	gRSC VI	**0.875**		
	dRSC	**0.904**	0.298	
	PL	**0.732**		
Thalamus	CL	**0.864**		
	CM	**0.740**		
	AD	**0.861**		
Hippocampus	CA2		**0.966**	
	DG			**0.976**
Percent variance explained		*57.4%*	*13.1%*	*9.7%*

Table 8. Structure matrix for subsystems of c-Fos immunoreactivity as shown by principal components analysis. The table depicts *Oblimin* rotated values of factor loadings for SB-334867 infused groups. Values in bold correspond to component loadings higher than 0.70; empty cells are residual scores between 0.20 and -0.20. The percentage of the variance explained by each factor is depicted at the bottom. Abbreviations: gRSC II-IV, layer 2-4 of the granular retrosplenial cortex; gRSC VI, layer 6 of granular retrosplenial cortex; dRSC, dysgranular retrosplenial cortex; PL, prelimbic cortex; CL, centrolateral nucleus; CM, centromedial nucleus; AD, anterodorsal nucleus; CA2, cornu ammonis 2; DG, dentate gyrus.

2.4 Discussion

We found that post training-administration of SB-334867 impaired the memory consolidation of the MWM task, especially in the weak training condition. Furthermore, we observed that animals from the strong training condition showed higher c-Fos protein expression in most of the areas analyzed. In contrast, reduced activation was detected in the SB-334867 infused animals, especially in those areas with a higher density of OXR1.

2.4.1 *Effects of SB-334867 on the MWM task in two training conditions*

In the 2-trial condition, SB-334867 infused animals showed a higher latency to the target, suggesting an increase in the difficulty of learning and consolidating the task. SB-334867 effects also show up in the retention test and in the reversal indicating that the animals that received this treatment did not remember the platform location and their cognitive flexibility was compromised. These differences were not found in the 6-trial condition groups.

An important contribution of this study is that the impairing effect of SB-334867 on the MWM depends on the level of training, since a higher level of training seems to counteract the negative effects produced by the blockade of OX1R. To further support this idea, it has been reported that rats treated orally with a dual orexin receptor antagonist (almorexant) are capable of acquiring a spatial memory task in the MWM when the total number of trials is high (Dietrich & Jenck, 2010; Morairty et al., 2014). In addition, the detrimental effect blocking OX1R observed in the 2-trial condition is consistent with studies that use intra-hippocampal SB-334867 infusions together with a weak training paradigm in the MWM (Akbari et al., 2006; Akbari et al., 2007).

Our results also show that SB-334867 negatively affected the 2-trial group's ability to remember the location of the platform during the retention test. The lack of differences in variables assessing anxiety or motor activity support the notion that the differences observed are not due to other orexin-related functions that could have affected the execution of the response. Thus, given that the administration was performed post-training, the effect on retention can mainly be attributed to memory consolidation impairment. This result does not exclude the more general role of OXR1 in memory, since the data shows that SB-334867 can also impair memory recovery in the MWM when administered pre-test (Akbari et al., 2006).

The cognitive flexibility assessed in the reversal test was impaired for the SB-334867 infused animals only in the 2-trial condition. This result could be attributed to a direct effect of OXR1 blockade on the reversal phase. But, since no impairment was observed in the SB6 group and taking the time since the last blocker administration into account, it is difficult to argue in favor of a deteriorating anterograde effect. Instead, based on the poor performance showed by the SB2 group in the training and retention sessions, the animals failed to incorporate the rules needed to solve the task and they were not able to use them flexibly in the reversal session. However, the SB-334867 could also have a direct effect on cognitive flexibility, as shown in an olfactory discrimination task when administered before the reversal trial (Piantadosi et al., 2015).

Overall, our results confirm that post-training OX1R blockade clearly has a detrimental effect on the spatial MWM task in a training-dependent manner. This effect is maintained over time, affecting long-term memory at 72h and subsequent reversal learning. It is important to point out that the administration of SB-334867 was performed in the lateral ventricles; thus, in contrast to preceding works in spatial memory where infusions were localized in the HPC (Akbari et al., 2006; Akbari et al., 2007), the impairing effects observed here could involve other regions.

2.4.2 *Study of c-Fos expression*

Animals in the 6-trial condition showed a higher number of c-Fos immunoreactive cells than those in the 2-trial condition for most of the areas analyzed, the DG, CA2 and CA3 of the hippocampus, the dRSC and PL cortices, and the Re, Rh, Cm, CL, MD and AD thalamic nuclei. In gRSC, CA1, IL, and LD there was only a tendency to higher c-Fos expression for the strong training groups, while no effects were observed in the PV. Thus, the amount of training seems to cause a widespread activation in the hippocampal-thalamic-cortical circuit, supporting the notion of its involvement in spatial memory.

The higher c-Fos expression observed in the different hippocampal regions in the 6-trial condition is consistent with the fact that the HPC is the most important area for spatial memory (Eichenbaum, 2017a). Furthermore, an overexpression of c-Fos was also observed in RSC, which connects with the HPC (Wright et al., 2010). This brain region is also involved in spatial learning and in long-lasting memory storage (Miller et al., 2014; Pothuizen et al., 2008; Pothuizen et al., 2009; Sugar et al., 2011). The fact that significant differences were found in the dRSC, more interconnected with visual areas than the gRSC (van Groen & Wyss, 1990; van Groen & Wyss, 1992), could be related to the higher reliance on visual cues from the environment required in a spatial MWM task.

Moreover, it has also been suggested that the PFC and thalamic nuclei form part of that circuit (Vertes, 2006; Jo et al., 2007; Preston et al., 2013; Miller et al., 2014;

Eichenbaum, 2017b; Jadhav et al., 2016). Our results showed that the groups subjected to a stronger training have higher levels of expression of c-Fos in PL. This supports the implication of the PL not only in the spatial memory system, but also in the cognitive flexibility aspect of it (Becker et al., 1981; de Bruin et al., 1994), especially regarding visuospatial components (Shaw et al., 2013) required to solve MWM reversal learning.

Furthermore, our results show that more training generally increased the level c-Fos in the thalamus. The associative nuclei AD and MD are a relay between HPC, RSC and PFC (Aggleton et al., 2010; Prasad & Chudasama, 2013). While the role of the MD in memory processes comes from its involvement in executive functions through its connections to mPFC (Mitchell & Chakraborty, 2013), the AD is critical for the processing of allocentric references in spatial memory (Wolff et al., 2008; Jankowski et al., 2013; Dumont et al., 2014a), thus being of great importance for the acquisition of a spatial navigation task (van Groen et al., 2002a; Kealy et al., 2008; Aggleton & Nelson, 2015). In fact, AD is thought to be part of the “dorsal hippocampal pathway” involved in spatial cognition (Prasad & Chudasama, 2013). Furthermore, LD does not show significant alterations in c-Fos expression related to the amount of training. This result, together with the low number of c-Fos immunoreactive cells observed in this area in all the groups, suggests a dissociative role for LD and AD in this task. Even though they are connected and functionally related (Clark & Harvey, 2016), the LD is involved in the somatosensory aspect of spatial navigation through head direction cells (Taube, 2007), and its selective lesion is not enough to cause a prominent impairment in a spatial task (van Groen et al., 2002b).

The relevant role of the unspecific nuclei of the thalamus in different aspects of arousal has been well established. Thus, the rostral intralaminar nuclei (CL and CM) could contribute to cognitive functions, such as attention, while the ventral midline nuclei (Re and Rh) are more related to multimodal sensory processing (Pereira de Vasconcelos & Cassel, 2015). All of them have dense reciprocal connections with the mPFC (Saalmann, 2014), with the Re and Rh also presenting neural links to the HPC (Vertes et al., 2007; Cassel et al., 2013) that would support their functions. Moreover, Re and Rh are also thought to be part of the “ventral hippocampal pathway” involved in executive control (Prasad & Chudasama, 2013). In contrast, the PV has neither mPFC nor hippocampal connections, and has been mainly linked to anxiety-related responses (Li et al., 2010), viscerolimbic and homeostatic functions (Kirouac, 2015; Pereira de Vasconcelos & Cassel, 2015). Accordingly, our results showed a lack of training effects on c-Fos expression in the PV.

A reduced c-Fos expression was observed in SB-334867 infused groups, mainly in areas that have been previously reported to have a high density of OX1R (Hervieu et al., 2001; Marcus et al., 2001). In some of these areas, significant differences were only observed in the weak training, between SB2 and DMSO2 groups, but not in the strong training condition (SB6 vs DMSO6). These results suggest that increased training can counteract the decrease in brain activity observed in the SB-334867 infused subjects.

In relation to the HPC, SB-334867 infused animals showed less c-Fos expression in CA2 and DG, where there is a higher density of OX1R, but not in CA1 or CA3. Results in the DG agree with a modulatory role of the orexinergic system on synaptic plasticity (LTP) (Akbari et al., 2011) and neurogenesis (Zhao et al., 2014) in this region. DG is one of the regions in which the level of c-Fos expression does not differ between 6-trial groups, thus reproducing the memory performance in the MWM. It follows that DG could be a region which is sensitive to the observed compensatory effect of training. Although there are no experimental antecedents regarding the role of orexins in CA2 in relation to learning, our results suggest that CA2 does not seem to mediate the compensatory effect observed after the increase in training. This functional differentiation between DG and CA2 is also reinforced by the PCA analysis, which shows that these regions belong to two distinct SB-334867 action components.

The data obtained in the RSC are consistent with a significant response attenuation in the RSC after the blockade of OX1R (Gozzi et al., 2013). In our case, differential effects were observed for the gRSC and dRSC. In the dRSC, despite it being highly sensitive to the amount of training, it is not enough to compensate for the global reduction of c-Fos expression associated with the blockade of OX1R. Conversely, the c-Fos activity reduction in the gRSC, similar to that in DG, was only observed in the weak training condition, and was more pronounced in layers II-IV, where OX1R expression is higher, and in layer VI, which has been previously found to be reactive to orexin fibers activation (Hay et al., 2014). The different impact of the level of training in the compensatory effects in dRSC and gRSC activity could be related to the fact that animals are more prone to using a response strategy to solve the task when the number of trials is low (Martel et al., 2007). This type of strategy consists of proprioceptive and non-visual inputs, thus amplifying the importance of the gRSC, given that it is more connected to head-direction areas (van Groen & Wyss, 1992).

With regard to the PL, SB-334867 infused groups showed a lower c-Fos expression, which is consistent with results in previous studies showing that the blockade of OX1R reduced activity of the mPFC (Gozzi et al., 2011; He et al., 2015). The c-Fos expression level in the PL seems to mirror performances in the reversal test, where the infusion of SB-334867 resulted in cognitive flexibility impairment of the weak training group, effect that was cancelled in the condition of strong training.

In the thalamus, OX1R blocked is associated to a reduction of the expression of c-Fos in CL and AD, which have been reported to express the most OX1R. The decrease of c-Fos protein in the CL is also consistent with previous reports of orexin-A depolarizing neurons in this nucleus (Govindaiah & Cox, 2006). While c-Fos expression in the CL reproduces behavioral outcome, AD could be considered to be the region where the ORX1 blockade had stronger effects since it affected both training conditions. A considerable amount of data support a dense expression of OX1R in AD in different mammals (Gravett et al., 2011; Lee, 2005). Although the orexinergic system in this nucleus has been traditionally associated to motivation, arousal, and autonomic regulation, our results suggest a possible involvement of AD in spatial memory. The significant reduction of c-Fos in CM observed in the SB-334867 infused groups seems to be inconsistent with preceding studies reporting the low expression of OX1R in this nucleus (Hervieu et al., 2001) and the lack of the effects of orexin-A on the depolarization of CM neurons (Govindaiah & Cox, 2006). However, the lack of difference between SB-334867 infused animals and its respective controls lends weight to the idea that the orexinergic system is considerably less relevant in the CM. When taken as a whole, the autocorrelation observed among these thalamic nuclei, the RSC and the mPFC, suggest a common subsystem of SB-334867 sensitive regions. Nevertheless, it is important to consider that the effect of OX1R blockade on the expression of c-Fos in each of the areas could be either direct or indirect, due to the high connectivity among these areas.

Overall, the results showed a deteriorating effect of OXR1 blockade on spatial memory consolidation in the MWM, and that such an effect can be counteracted by increased training. Additionally, c-Fos expression levels seem to reproduce the behavioral results in a set of regions. Thus, an increase in the amount of training counters the decrease in the expression of c-Fos, observed in the SB-334867 infused and low-trained animals, in DG, gRSC, PL and CL. The activation of these areas could constitute a neuroanatomical substrate involved in the compensatory mechanisms of training upon SB-334867 effects on spatial learning.

STUDY III

3 Effects of Orexin-A receptor blockade on spatial memory facilitation by intracranial self-stimulation

Manuscript in preparation

ICSS compensates for OX1R blockade-induced impairment of acquisition and retention but not reversal deficits.

Soleil García-Brito, Laura Aldavert-Vera, Gemma Huguet-Blanco, Pilar Segura-Torres

3.1 Introduction

Intracranial self-stimulation (ICSS) is an operant response in which subjects self-administer small electric shocks to the medial forebrain bundle (MFB) in the lateral hypothalamus (LH), resulting in a highly rewarding pattern of activation. As a treatment, it has been reported to facilitate the acquisition and retention of implicit (Huston et al., 1977; Huston & Mueller, 1978; Redolar-Ripoll et al., 2002; Ruiz-Medina et al., 2008b; García-Brito et al., 2017) and explicit (Soriano-Mas et al., 2005; Chamorro-López et al., 2015) memory tasks in rats, even managing to recover lost memory function caused by lesions (Segura-Torres et al., 2010; Kádár et al., 2014) and old age (Aldavert-Vera et al., 1997).

Several mechanisms to explain its facilitative effects on learning and memory have been proposed. For instance, ICSS has been linked to the structural plasticity of very specific memory-related areas, such as the pyramidal dendrites in CA3 (Shankaranarayana Rao et al., 1993) and CA1 after training in a spatial memory task (Chamorro-López et al., 2015). Additionally, ICSS treatment has been found to be capable of activating general arousal systems (Newman & Feldman, 1964; Wise, 2005) through dopaminergic, noradrenergic and serotoninergic ascendant fibers (Nieuwenhuys et al., 1982; Shankaranarayana Rao et al., 1998a). These as well as other neurotransmission systems have been extensively studied in order to ascertain the effects of ICCS on reward (Rolls, 1974; Fibiger et al., 1987; Negus & Miller, 2014; Murakami et al., 2015) and memory (Owesson-White et al., 2008; Ramkumar et al., 2008; Vega-Flores et al., 2014). However, research exploring the involvement of the orexinergic system, more specifically orexin-A, has yet to be carried out. Since its discovery, this neuropeptide has been studied due to its implication in homeostatic functions and food intake (Sakurai et al., 1998; for review see Kukkonen et al., 2002) and arousal (Sakurai, 2007; Li et al., 2014). Additionally, researchers have looked into the key role it plays in reward processes (Borgland et al., 2009; Aston-Jones et al., 2010; Arias-Carrión et al., 2014; Muschamp et al., 2014). Both arousal and reward are of paramount importance when considering ICSS effects.

This neurochemical system has recently gained relevance in relation to learning and memory processes. The orexin-A receptor (OX1R) is expressed widely throughout memory-related areas in the brain (Hervieu et al., 2001; Marcus et al., 2001), including CA1, DG and CA2 of the hippocampus (HPC), the prefrontal cortex (PFC) and retrosplenial cortex (RSC). Several studies have described how the selective blockade of OX1R can impair a spatial task in the Morris Water Maze (MWM) (Akbari et al., 2006; Akbari et al., 2007), while orexin-A administration to rodents has been reported to have facilitative effects on passive avoidance (Jaeger et al., 2002; Telegdy & Adamik, 2002) and spatial (Zhao et al., 2014) memory tasks.

The mechanisms through which orexin-A seems to affect spatial memory processes are somewhat comparable to those of ICSS. An increase in levels of orexin-A has been linked with a rise in phosphorylation of MAPK proteins *in vitro* (Ammoun et al., 2006; Kukkonen & Leonard, 2014) and more specifically in hippocampal cells in rodents *in vivo* (Selbach et al., 2010; Yang et al., 2013; Zhao et al., 2014); these proteins are closely related to plasticity signaling (Thomas & Huganir, 2004; Giese & Mizuno, 2013). Similarly, ICSS has been reported to increase the expression of learning and memory-related genes in the HPC (Kádár et al., 2013), while consistently increasing the expression of Nurr1, c-Fos and Arc protein in HPC, LH and RSC (Huguet et al., 2009; Aldavert-Vera et al., 2013; Kádár et al., 2014; Kádár et al., 2016). Another important correlate is neurogenesis in the DG, which has been reported for both orexin-A (Ito et al., 2008) and ICSS (Takahashi et al., 2009) administration in rats. Perhaps more interestingly, the orexin-A-producing neurons originate in the LH (de Lecea et al., 1998; Sakurai et al., 1998), which is, incidentally, the place in which ICSS electrodes are implantated. Thus, the stimulation of the neural substrate of ICSS could also be activating orexinergic pathways that give rise to the events laid out above.

Therefore, we set out to study the relationship between the orexin-A system and the facilitative effect of ICSS on spatial memory. In order to achieve this objective, we evaluated how post-training intracerebroventricular (icv) microinfusions of OX1R selective antagonist SB-334867 affects the facilitative effect of ICCS on the acquisition, retention and reversal of a spatial memory task in the Morris Water Maze (MWM). Moreover, we attempted to assess the effects of both of these variables on the activation of memory-related areas, using c-Fos as a cellular activation marker.

3.2 Materials and Methods

3.2.1 *Experimental subjects*

A total of 48 male Wistar rats with a mean age 94.79±3.17 days and a mean weight of 408.50±6.14g from our laboratory's breeding stock were used. Three days before the stereotaxic procedure they were isolated and kept in individual cages (50×22×14cm, plastic bottomed and sawdust-bedded). The animals were kept under conditions of controlled temperature and humidity, and subjected to an artificial 12-hour light/dark cycle (light on at 08:00). All behavioral tests took place during the first 6 hours of light. All subjects were kept in an ad libitum regime of food and water. All procedures were carried out in compliance with the Directive 2010/63/EU and were approved by the institutional animal care committee.

3.2.2 *Stereotaxic surgery*

Before undergoing the surgery, two sessions of handling took place in order to diminish the animals' emotional reactivity towards experimental manipulation. Under general anesthesia (150 mg/kg Imalgène® ketamine chlorhydrate (Merial, Lyon, France) and 0.08 mg/kg Rompun® xylazine (Bayer, Barcelona, Spain); i.p.) all rats were chronically implanted with a 7.5mm infusion guide cannula (Plastics One®, Raonoke, VA, US, purchased through Bilaney consultants, Düsseldorf, Germany; ref: C315G/PK/Spc) into the left lateral ventricle (LV), according to coordinates from the stereotaxic atlas of Paxinos and Watson (2007) at anterior: -0.7mm from bregma, lateral: 1.6mm and ventral: -4.0mm. A dummy cannula (Plastics One®; ref: C315DCN/Spc), filled the infusion guide cannula and expanded 0.5mm into the parenchyma. The cannula was fixed in position using an auto-polymerizing acrylic resin (Vertex self-curing, Dentimex, Netherland). The animals were weighed and handled daily during the post-surgery recovery period (7 days).

3.2.3 *Morris Water Maze Apparatus.*

The MWM consisted of an elevated circular pool (2m diameter; 60cm above the pool floor) filled with water (45cm height) maintained at 22 ± 2ºC. The pool was in the middle of a semi-dark room and surrounded by black curtains hanging from a false ceiling to the base of the pool forming a circular enclosure 2.4m in diameter. A clear Plexiglas platform (11cm diameter) was placed centrally in one of the four equal quadrants in which the tank was virtually divided, with its top 2cm below the surface of the water. All swim paths were recorded using a closed-circuit video camera (Smart Video Tracking System, Version 2.5, Panlab) with a wide-angle lens mounted 1.75m above the center of the pool embedded in the false ceiling.

3.2.4 *Intracranial self-stimulation shaping procedure.*

Once rats had recovered from surgery (7 days), they were randomly distributed into four groups, following a 2×2 experimental design: SB, Control, ICSS and SB+ICSS. Subjects in the ICSS and SB+ICSS groups were trained to self-stimulate by pressing a lever in a conventional Skinner box (25×20×20 cm). Electrical brain stimulation consisted of 0.3-s trains of 50 Hz sinusoidal waves at intensities ranging from 5 to 250 µA. The ICSS behavior was shaped to establish the optimal intensity (OI), or lowest intensity that would lead to a stable rate of about 250 responses in 5min.

3.2.5 *Behavioral procedure.*

3.2.5.1 Acquisition, Probe test and Reversal.

All animals underwent one habituation session in the MWM in order to reduce emotional reactivity. During the acquisition phase, animals underwent 2 trials for 5 consecutive days. Each trial lasted for 120s and the average intertrial interval (ITI) was 120s. At the beginning of each trial, the animal was placed into the pool at one of four different cardinal points (N, E, S and W) in a pseudorandom schedule. When an animal failed to find the platform after 120s, it was manually directed to mount it for 15s and then removed from the tank.

Seventy-two hours after the last acquisition session each animal performed a probe test, which consisted of removing the platform and placing the animal in the pool from the E starting position for 60s. Immediately after the 60s, the reversal phase was initiated, in which the platform was placed in the opposite quadrant of the pool and the animals were directed to mount the platform and then left there for 15s. After 12 s ITI, animals were again placed in the pool from the remaining starting points (N, W, S) and left to find the platform for a maximum of 120s.

3.2.5.2 Post-training microinfusion procedure.

All animals underwent two habituation sessions with the microinfusion apparatus in order to reduce initial emotional and physical reactivity. Immediately after each acquisition session, the animals were gently restrained while its dummy was removed and replaced with a 26-gauge injector (PlasticOne®, Roanoke, VA, USA; ref.: C313CT) extending 0.5mm below the cannula tip. The injectors were connected by polyethylene tubing (PlasticOne®, Roanoke, VA, USA; ref.: C315I/PK/Spc) to two 10µL syringes (SGE Analytical Science, Cromlab S.L. Barcelona, Spain) that were placed in the infusion pump (11 Plus Syringe Pump, Harvard Apparatus Inc., Holliston, Massachusetts, USA). The selective OX1R antagonist SB-334867 (Tocris Bioscience, Bristol, UK) was dissolved in dimethyl sulfoxide (DMSO) (Tocris Bioscience, Bristol, UK), aliquoted in tightly sealed vials and stored at -20ºC for up to 5 days. The animals in Control and ICSS groups were administered a total volume of 2µL of DMSO, and the animals in SB and SB+ICSS groups received SB-334867 (5µg/2µl). Microinfusions were administered into the left LV at a rate of 1µL/min. The injectors were left in place for an additional 60s to allow for the diffusion of the solution away from the tip.

3.2.5.3 Intracranial self-stimulation procedure.

Immediately after the microinfusion procedure, the ICSS and SB+ICSS rats were placed in the self-stimulation box and received the ICSS treatment, consisting of 2500 trains of stimulation at the OI established during the shaping phase for each rat. Rats in the Control and SB groups underwent sham treatment, by being placed in the self-stimulation box for 45min after each training session without receiving any stimulation. Figure 33 depicts the experimental procedure.

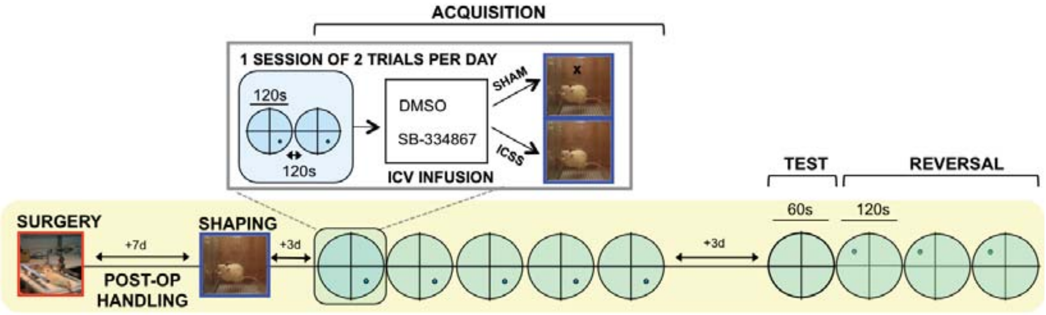

Figure 33. Diagram of experimental procedure in Study III.

3.2.6 *c-Fos inmunolocalization*

3.2.6.1 Tissue collection.

Ninety minutes after the last reversal test animals received a pentobarbital overdose (150mg/Kg, i.p.) and were transcardially perfused with a solution of 0.1M of phosphate buffer saline (PBS), pH 7.4, followed by a solution of 4% paraformaldehyde in PBS. Brains were removed and post-fixed in 4% paraformaldehyde in PBS, then cryoprotected in 15% and 30% sucrose in PBS and stored at -80ºC. Serial coronal sections (30µm) were obtained in a cryostat (Cryocut 1800, with 2020 JUNG microtome) at -20ºC between the coordinates 4.68mm and 2.16mm, and -1.56mm and -3.36mm AP of Bregma (Paxinos & Watson, 2007) and stored at -80º C until immunostaining. From the same subject, localized coronal sections (30µm), between the coordinates -0.6mm and -1.20mm of Bregma, were mounted onto a gelatin-coated slide, stained with cresyl violet and examined for cannula placement.

3.2.6.2 c-Fos immunochemistry.

Free-floating coronal sections were incubated with 0.3% H_2O_2 in Tris-buffered saline (TBS) for 30min and in 0.1% Bovine Serum Albumin (BSA) in 0.3% Triton X-100 in TBS for 30min. Sections were incubated in primary antibody rabbit anti-cFos (Santa Cruz

Biotechnology Inc. Santa Cruz, CA, US; ref.: sc-166940) at 1:2000 in 0.1% BSA in 0.3% Triton X-100 in TBS for 60min at room temperature and then overnight at 4ºC. The next day samples were incubated with biotinylated goat anti-Rabbit-IgG secondary antibody (Jackson Immunoresearch Inc., USA; ref.: 111-066-144) at 1:5000 in 0.1% BSA in 0.3% Triton X-100 in TBS for 60min and subsequently treated with Streptavidin-HRP (Pelkin Elmer ® Life Science Inc., USA) at 1:3600 in 0.3% Triton X-100 in TBS, followed by treatment with 3, 3'-Diaminobenzidine (DAB) solution (Vector ®) for 7min. Negative controls without primary or secondary antibodies were included.

3.2.6.3 Image acquisition and analysis.

Using a BX-41 Olympus microscope attached to an Olympus DP-70 digital camera (Japan), microphotographs of the following brain regions were captured: Cornu ammonis 1 (CA1), Dentate gyrus (DG), granular retrosplenial cortex (gRSC), Prelimbic cortex (PL) and Orbitofrontal cortex (OFC). Appropriate grey threshold and particle size were set for each area and maintained for all subjects. The image analysis software Image-J 1.43 (http://rsb.info.nih.gov/ij/) was used to bilaterally count the number of c-Fos immunostained nuclei using regions of interest (ROIs) (Figure 34). Two or three histological sections for each animal were counted and averaged. The coordinates for each area were matched for each group in order to reduce variability between samples (AP: PFC, 3.00mm to 2.52mm; HPC-RSC, -2.64mm to -3.00mm). In order to remove background noise, each image was digitally smoothed and subtracted from the original.

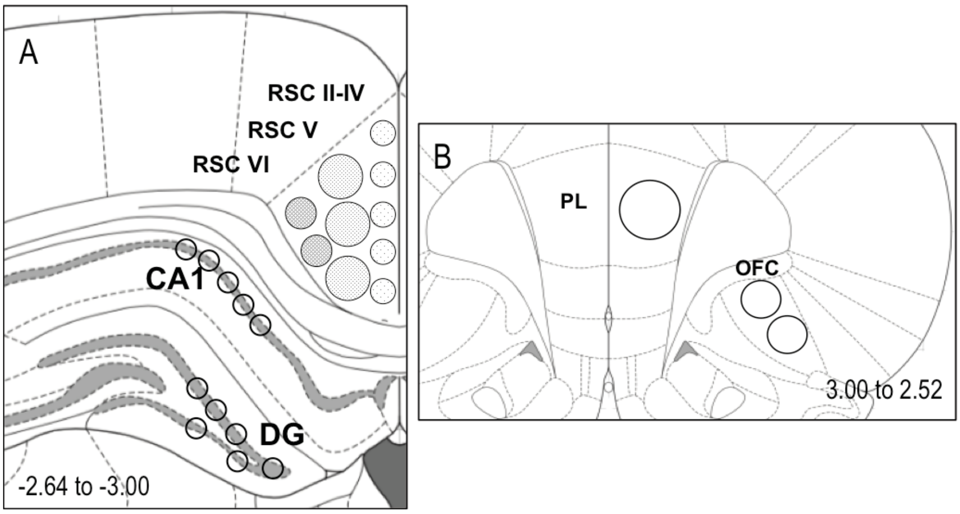

Figure 34. Regions of interest (ROIs) for the quantification of c-Fos immunopositive nuclei in the (A) HPC (CA1 and DG), gRSC (layers 2-4, 5 and 6). (B) PL and OFC are shown superimposed on coronal sections adapted from Paxinos & Watson, 2007. Abbreviations: CA1, cornu ammonis 1; DG, dentate gyrus; RSC, retrosplenial cortex; PL, prelimbic cortex; OFC, orbitofrontal cortex.

3.2.7 *Statistical analysis*

Statistical analysis was performed using SPSS v23 (SPSS Inc. Chicago, IL, USA). Analysis of the acquisition phase of the memory task in the MWM was conducted with a 4×5 mixed ANOVA (GROUP×SESSION). When the effect of the interaction factor was statistically significant, polynomial contrasts were applied to explore the presence of linear and/or quadratic trends in performance. A multiple comparison analysis (Tukey HSD) was performed to assess differences between groups across each session. The main outcome variable for acquisition and reversal sessions in the MWM was the *Escape latency* or time (s) needed to find and climb onto the platform. Results for the retention test were analyzed with a one-way ANOVA for the totality of the trial (60s) and also for the first half of the trial (30s). In the probe test, the following variables were analyzed: (1) Percentage of time spent in the target quadrant in the first half of the trial [TQ30] and its totality [TQ60]; (2) Gallagher's measure or Proximity to target [PT30 and PT60]; (3) Percentage of time spent in target annulus [Ann30 and Ann60]; and (4) Wishaw's Error [WE]. In addition, a one-sample *t*-test against a constant (25) was used for each group to determine whether TQ30 and TQ60 differed from chance level (25%) (Chance30 and Chance60, respectively). Moreover, the *percentage of time spent near the walls* (measure of thigmotaxis - anxiety), *length* (total distance in cm) and *speed* (motor activity measure) were analyzed and considered as control variables for each group. We also used a one-way ANOVA to analyze the average score of the three reversal trials. To analyze c-Fos quantitative data we used a 4×2 mixed analysis of variance with GROUP as an independent factor between subjects, and the HEMISPHERE within group factors, according to each brain structure. A Post Hoc (Tukey HSD) test was performed to detect differences among groups. A Greenhouse-Geisser correction was used when sphericity was violated and a Welch's *t*-test and Post Hoc Games-Howell correction was applied when homogeneity of variables was not reached.

3.3 Results

One subject was excluded from the analysis due to a misplaced cannula. The final sample consisted of 47 subjects (SB: N=11; Control: N=12; ICSS: N=12; SB+ICSS: N=12). There was no statistical difference between groups in age at the start of the experiment or in weight evolution throughout the experiment. Similarly, the infusion of SB-334867 had no significant effect on the average OI of stimulation or the time of the ICSS treatment between groups.

3.3.1 *Effects of SB-334867 infusion and ICSS on Morris Water Maze*

3.3.1.1 Acquisition phase

A mixed ANOVA of the escape latencies for the acquisition showed an interaction GROUP×SESSION [Greenhouse-Geisser: $F_{12,172}$=3.376, P<0.001], indicating a difference between the groups depending on the session (Figure 35). The simple effects analysis detected differences between groups in all sessions except for session 1, previous to receiving any treatment, indicating a similar starting point for all groups (session 1, P=0.778; session 2, P=0.019; session 3, P=0.003; session 4, P<0.001; and session 5, P<0.001). A multiple comparison (Tukey HSD) into each session showed that the SB group's latencies were higher than the ICSS group (session 2: P=0.015; session 3: P=0.005; session 4: P<0.001; session 5: P<0.001), the Control group (session 3: P=0.006; session 4: P=0.001; session 5: P<0.001) and the SB+ICSS group (session 4: P<0.001; session 5: P<0.001).

The intragroup analysis of latencies throughout acquisition sessions showed that although all groups adjust to a significant downward linear function [SB: $F_{1,43}$=12.47, P=0.001; Control: $F_{1,43}$=89.85, P<0.001; ICSS: $F_{1,43}$=95.93, P<0.001; SB+ICSS: $F_{1,43}$=88.13, P<0.001], the SB group's slope was significantly less pronounced when compared to each of the other groups [Control: $F_{1,43}$=16.84, P<0.001; ICSS: $F_{1,43}$=22.15, P<0.001; SB+ICSS: $F_{1,43}$=14.44, P<0.001]. In this sense, the SB group was the only one to show no significant decrease in the to target across the first three sessions of acquisition [SB: $F_{1,43}$=0.01, P=0.925; Control: $F_{1,43}$=6.69, P=0.013; ICSS: $F_{1,43}$=19.82, P<0.001; SB+ICSS: $F_{1,43}$=5.71, P=0.021]. In addition, the ICSS group was the only group to also adjust to a quadratic function [$F_{1,43}$=6.12, P=0.018], indicating a sharper decline in the latencies after the first ICSS treatment.

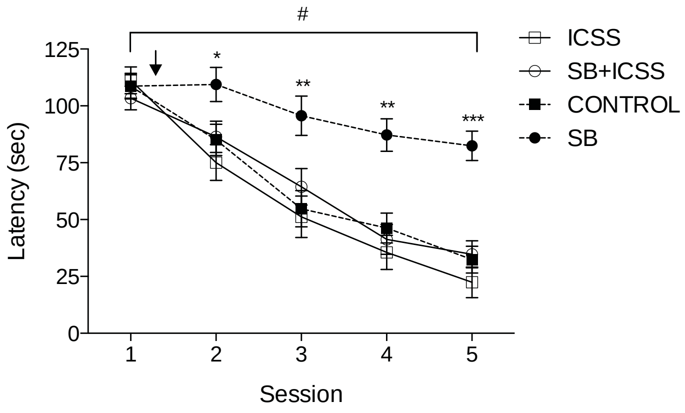

Figure 35. Effects of ICSS and SB-334867 on the acquisition of a MWM task. Mean *Escape latencies* (±SEM) for the five sessions of the training phase. Arrow shows start of ICSS and microinfusions. Interaction factor significance is depicted with *P<0.05; **P<0.01; ***P=0.001. The dotted line in retention test represents chance level #P<0.05.

3.3.1.2 Retention test

All groups performed above chance level (25%) except for the SB group in both Chance30 [SB: $t_{1,11}$=1.377, P=0.198; Control: $t_{1,12}$=3.822, P=0.002; ICSS: $t_{1,12}$=2.968, P=0.016; SB+ICSS: $t_{1,12}$= 4.096, P=0.001] and Chance60 [SB: $t_{1,11}$=0.775, P=0.456; Control: $t_{1,12}$=3.607, P=0.004; ICSS: $t_{1,12}$=3.113, P=0.012; SB+ICSS: $t_{1,12}$=3.107, P=0.009] (Figure 36A). Although the GROUP factor did not reach significance for TQ60 variable [$F_{3,43}$=2.307, P=0.090], the SB group performed worse than the other groups (Control: P=0.043; ICSS: P=0.029; SB+ICSS: P=0.038).

The proximity to target was significantly different between groups for PT30 [$F_{3,43}$=2.945, P=0.043] and PT60 [$F_{3,43}$=3.249, P=0.031]. A Post Hoc analysis for PT30 showed a lower distance to target for the ICSS group compared to the Control (P=0.038) and SB (P<0.001) groups. The SB+ICSS group also achieved a lower distance than the SB group for both the first half (P=0.009) and the totality of the test (P=0.012) (Figure 36B).

Differences between groups were also observed for WE in the first 30s of the trial [Welch: $F_{3,21.969}$=4.092, P=0.019], for which a Post Hoc showed that ICSS had higher values than the Control group (P=0.003) and the SB group (P=0.006) (Figure 36C).

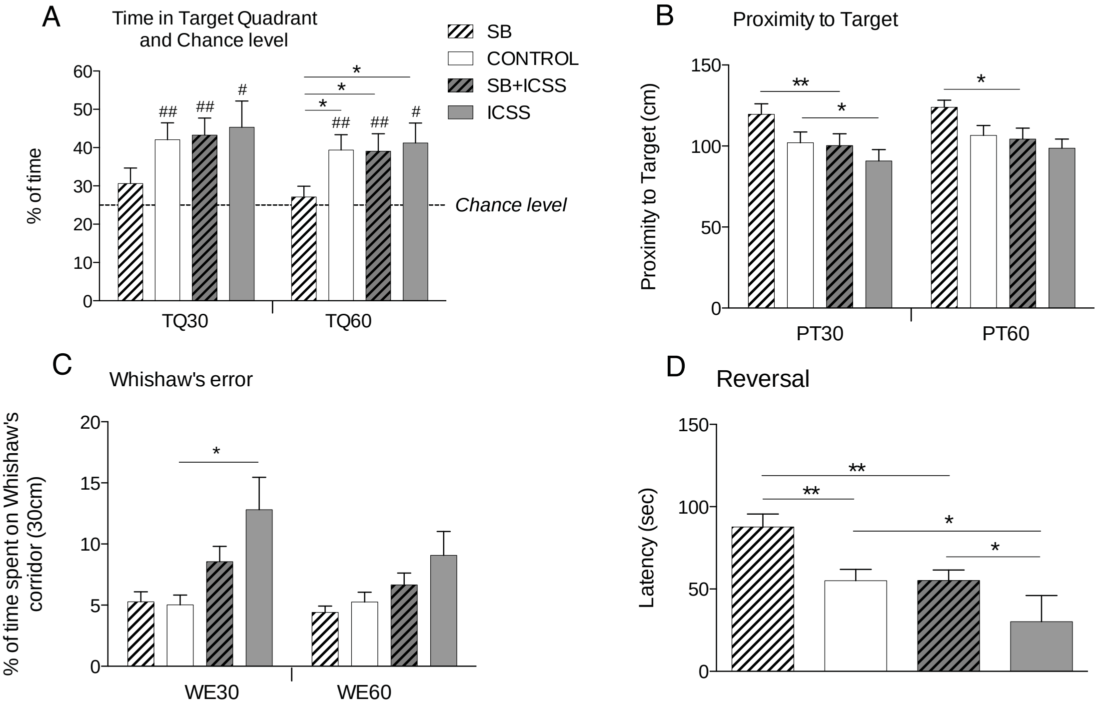

Figure 36. Effects of ICSS and SB-334867 on the retention and reversal of a MWM task. Retention test variables: (A) TQ30 and TQ60, (B) PT30 and PT60 and the (C) WE; (D) Reversal variables, *Escape latencies* (±SEM). Abbreviations: TQ, percentage of time spent in target quadrant; PT, proximity to target; WE, Whishaw's error. Group contrasts are depicted with *P<0.05; **P<0.01. The dotted line in retention test represents chance level #P<0.05; ##P<0.01.

3.3.1.3 Reversal

Analysis of the mean latencies for the three reversal sessions showed a significant effect of GROUP [$F_{3,43}$=11.135, $P<0.001$], for which a Post Hoc showed higher latencies for SB compared to Control (P=0.001) and SB+ICSS (P=0.001), while ICSS showed lower latencies than Control (P=0.014) and SB+ICSS (P=0.013) groups (Figure 36D).

Differences among groups were observed in thigmotaxis values [$F_{3,43}$=9.314, $P<0.001$]. Specifically, the SB group spent more time near the walls than SB+ICSS group ($P<0.001$).

3.3.2 *Effects of SB-334867 infusion and ICSS on c-Fos expression*

The final sample was 38 (SB: N=9; Control: N=10; ICSS: N=9; SB+ICSS: N=10). The excluded subjects were outliers for c-Fos expression in one or more brain areas (N=4) or their samples were insufficient in number and/or quality (N=5). The general linear model of variance analysis of c-Fos expression showed that there were no effects of the HEMISPHERE factor or its interactions with GROUP in any of the analyzed regions. Thus, all areas were analyzed bilaterally. Figures 37-39 show c-Fos quantification and representative photographs of one subject in each group for the HPC, RSC and PFC.

3.3.2.1 Hippocampus

The analysis of the means of the c-Fos cell count showed differences between groups in CA1 [$F_{3,34}$=21.114, $P<0.001$]. The Post Hoc (Tukey HSD) analysis showed that c-Fos expression was increased in the SB+ICSS and ICSS groups, in both infusion conditions, compared to Control and SB groups (SB+ICSS vs Control: $P<0.001$; ICSS vs Control: P=0.001; SB+ICSS vs SB: $P<0.001$; ICSS vs SB: $P<0.001$) (Figure 37A).

There were also group differences in the DG [$F_{3,34}$=26.747, $P<0.001$] and, similarly to CA1, the ICSS treatment increased c-Fos expression in animals that received SB-334867 infusions (SB+ICSS vs Control: P=0.013; SB+ICSS vs SB: $P<0.001$) and in those that did not (ICSS vs Control: $P<0.001$; ICSS vs SB: $P<0.001$). Additionally, lower c-Fos expression was detected in groups that received SB-334867 infusion (SB vs Control: P=0.048), even when the ICSS treatment was administered (SB+ICSS vs ICSS: P=0.006) (Figure 37B).

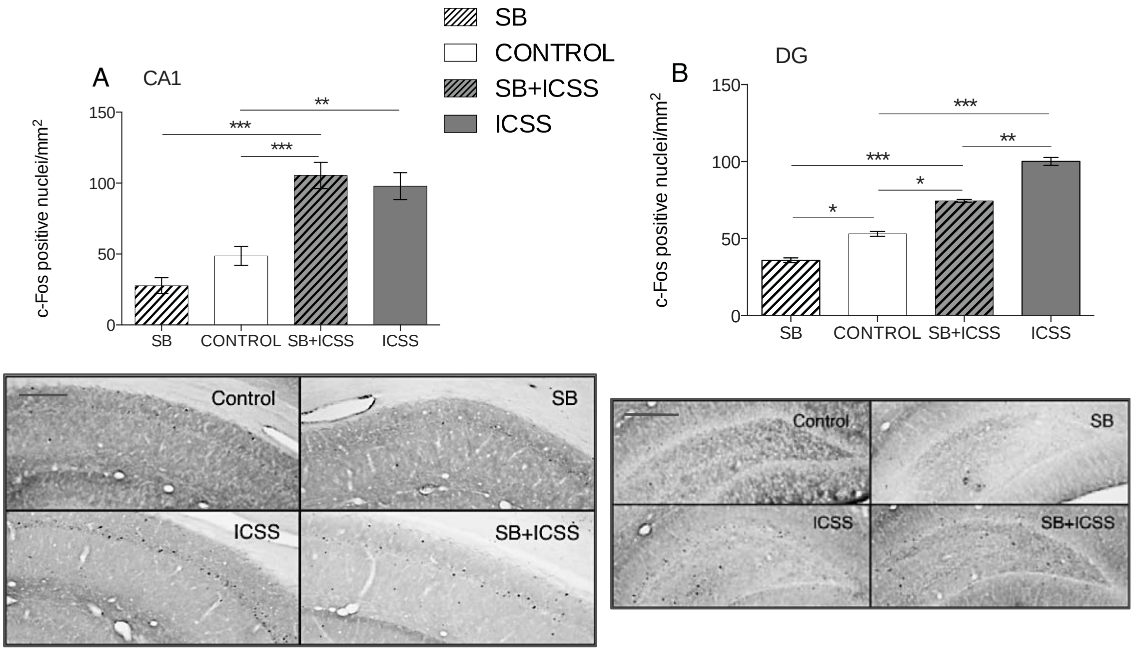

Figure 37. Effects of SB-334867 and ICSS on c-Fos expression in the HPC. (A) Cornus ammonis 1, (B) Dentate gyrus. Group contrasts are depicted with *P<0.05 **P<0.001 ***P<0.001. Microphotographs of each area are found below the corresponding graphs. Scale bar: 200µm.

3.3.2.2 Restrosplenial Cortex (granular)

The analysis showed differences between groups in gRSC II-IV [Welch: $F_{3,15.591}$=32.821, P<0.001], and gRSC VI [$F_{3,15.697}$=23.507, P<0.001], while there was a tendency to significance in gRSC V [$F_{3,17.216}$=2.446, P=0.099] (Figure 38). The Post Hoc analysis for gRSC II-IV showed that SB-334867-infused animals had a lower c-Fos expression (SB vs Control: P=0.033), while those that received ICSS treatment

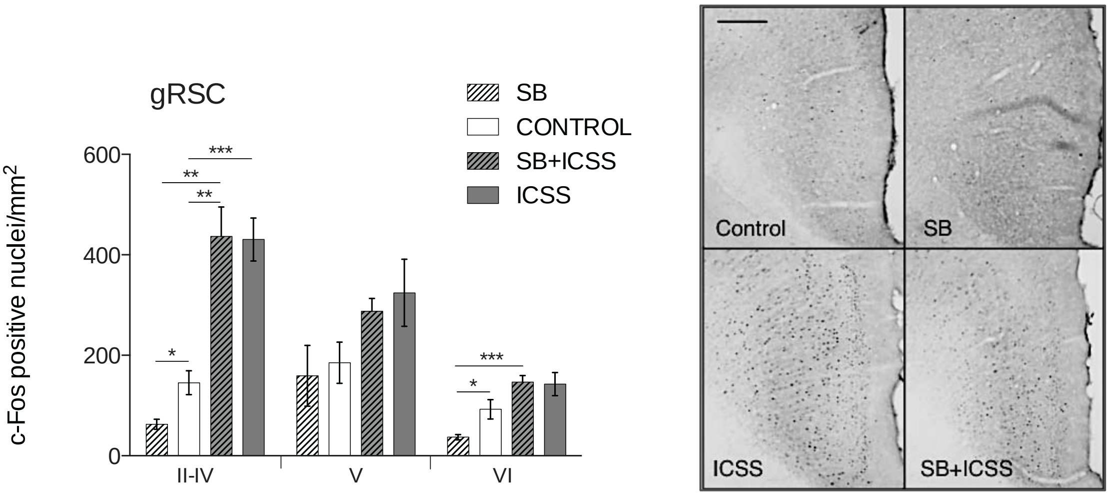

Figure 38. Effects of SB-334867 and ICSS on c-Fos expression in the RSC. Group contrasts are depicted with *P<0.05 **P<0.01 ***P<0.001. Microphotographs of each area are found next to the corresponding graphs. Scale bar: 200µm.

had an increased number of c-Fos labeled cells (SB+ICSS vs SB: *P*=0.001; ICSS vs Control: *P*<0.001; ICSS vs SB: *P*=0.007), even in the presence of OX1R antagonist (SB+ICSS vs Control: *P*=0.006). In gRSC VI, the number c-Fos labeled cells also decreased with the OX1R blockade (SB vs Control: *P*=0.045), while ICSS treatment increased the c-Fos count (SB+ICSS vs SB: *P*<0.001).

3.3.2.3 Prefrontal cortex

Differences between groups were also observed for the PL [Welch: $F_{3,17.300}$=15.405, *P*<0.001]. In PL, unlike the other regions, ICSS did not significantly increase the expression of c-Fos compared to the Control group (ICSS vs Control: *P*=0.150), although ICSS animals had a significantly higher number of c-Fos labelled cells than the SB group (ICSS vs SB: *P*=0.004). The OX1R blockade significantly reduced c-Fos expression in both stimulated (SB+ICSS vs ICSS: *P*=0.016) and non-stimulated animals (SB vs Control: *P*=0.003), while the ICSS treatment increased the c-Fos count in SB-infused animals (SB+ICSS vs SB: *P*=0.008) (Figure 39A).

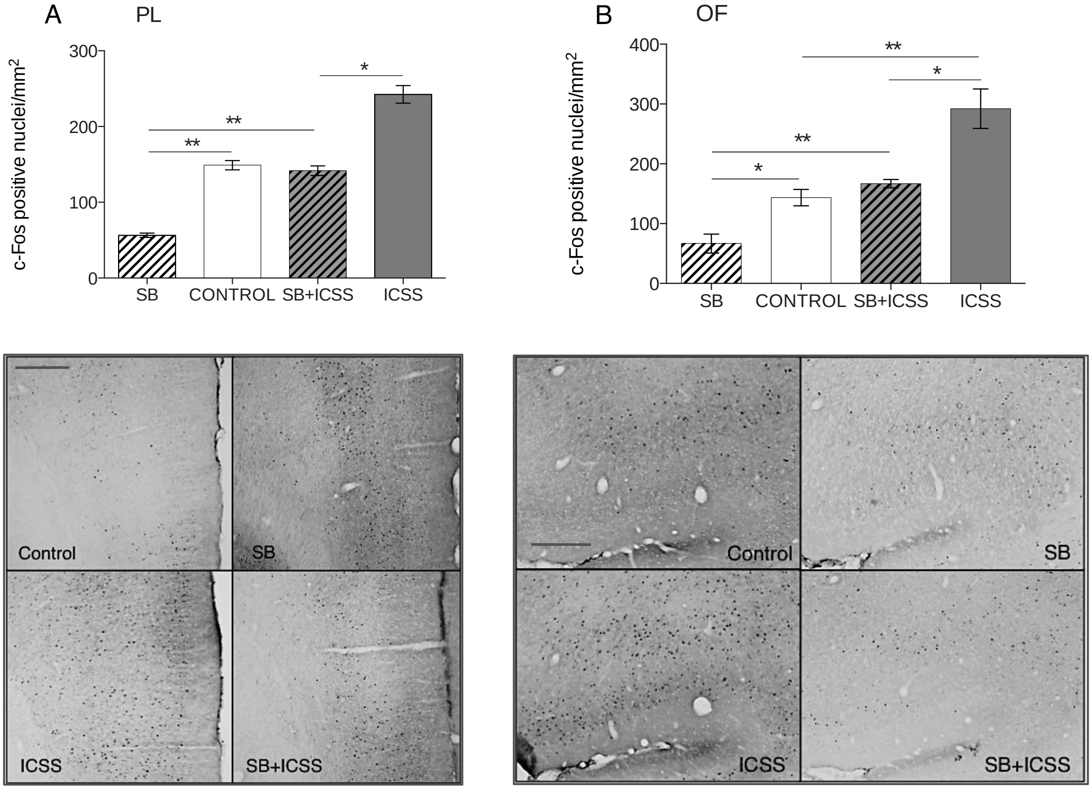

Figure 39. Effects of SB-334867 and ICSS on c-Fos expression in the PFC. (A) Prelimbic cortex, (B) Orbitofrontal cortex. Group contrasts are depicted with *P<0.05 **P<0.01. Microphotographs of each area are found below the corresponding graphs. Scale bar: 200µm.

Differences between groups were also found for the OFC [$F_{3,18.279}$=15.976, *P*<0.001]. Similarly to PL, a general decrease of c-Fos expression was observed after SB-334867 infusion (SB vs Control: *P*=0.010), even after receiving ICSS treatment (SB+ICSS vs ICSS: *P*=0.021). In the subregion, the ICSS treatment increased c-Fos expression in SB-infused animals (SB+ICSS vs SB: *P*=0.001), and, unlike PL, it significantly increased the number of c-Fos cells in this area compared to the Control and SB groups (ICSS vs Control: *P*=0.008; ICSS vs Control: *P*<0.001) (Figure 39B).

3.4 Discussion

We found that the blockade of OX1R impaired the consolidation, retention and reversal of a spatial memory task in the MWM, and replicated our previous results from the same experimental design. Present results also confirm the post-training ICSS as a memory-enhancing treatment, and demonstrate that ICSS is also able to facilitate the reversal of a MWM. It is worth noting that the capability of ICSS to ameliorate detrimental effect of SB-334867 was observed for the first time. Furthermore, SB-334867-infused animals showed a reduction in c-Fos expression in most of the brain areas analyzed, but only when they did not receive ICSS treatment.

The blockade of OX1R deeply affected the ability of animals to learn the task. This difficulty is evident from the fact that the SB group showed no sign of learning in the first three sessions of the training phase. This result is consistent with our previous study (Study II) as well as others in which the infusion was administered post-training but in specific hippocampal areas (Akbari et al., 2006; Akbari et al., 2007). The SB's group poor performance during the training phase is also evident in the retention test, in which they showed no sign of remembering the location of the platform. In addition, this group not only had a worse performance compared to the other groups, but also failed to perform above chance levels. Given that the last administration of the OX1R antagonist took place 72h before the retention test, the impaired retrieval shown by these animals cannot be attributed to an acute effect of the blockade of OX1R, but to failed consolidation. Nevertheless, the repeated administration of SB-334867 after each acquisition session could have caused long-term neural changes that could also partially explain the null performance observed in the retention test 72h after the last infusion. In fact, we have also found that activation of both the DG of the HPC and layers II-IV and VI of the gRSC is significantly lower in animals that have received the SB-334867 infusion. To further support this notion, the inactivation of OX1R has been reported to result in an impaired long-term potentiation (LTP) induction in the DG which may last beyond 24h, even when applying a single administration of SB-334867 (Akbari et al., 2011).

Importantly, the reversal test results, which indicate a deteriorating effect of SB-334867 on cognitive flexibility, also replicated previous results reported in Study II for the weak training condition. Accordingly, we also found a reduced c-Fos expression in areas involved in behavioral and cognitive flexibility in spatial reversal tasks, such as the PL (Delatour et al., 2000) and the OFC (Young and Shapiro, 2009), for animals that received the SB-334867 infusion. This suggests that the antagonism of OX1R affects the execution of the function of these regions regarding both the cognitive flexibility and attentional processing necessary to carry out a reversal task. Taken as a whole, the results reported above suggest that the blockade of OXR1 significantly impairs the consolidation and reversal of a spatial memory task, which replicates the results obtained in Study II.

Furthermore, present results demonstrate once again that post-training ICSS facilitates spatial memory in the MWM (Ruiz-Medina et al., 2008a; Chamorro-López et al., 2015). Although this effect is not evident in the training phase, it is clearly seen in the retention test. Taking a closer look at the consecutive training sessions, however, we see that we cannot rule out that the ICSS treatment has an effect on the acquisition of the task. More specifically, the ICSS group is the first to outperform the SB group in the session following the first treatment administration during the training phase, suggesting a certain accelerating effect of ICSS on memory consolidation. It is important to consider that, as observed in a previous study (Ruiz-Medina et al., 2008a), the progressive increase in training can eliminate differences between the ICSS group and its control, due to a “floor effect” in our data. Moreover, in the retention test, the analysis of the proximity to target and the Whishaw’s error index reveals that the animals that receive the ICSS treatment were able to locate the platform more accurately. Out of all the variables analyzed for this portion of memory evidence, proximity to target has been reported to be the most sensitive measure when it comes to the assessment of spatial memory in the MWM, especially in probe trials (Gallagher et al., 1993; Maei et al., 2009; Pereira & Burwell, 2015).

To further support the notion of the long-term facilitating effect of ICSS on a spatial task, the ICSS-treated animals displayed potentiated activation of the CA1 and DG of the HPC and layer II-IV of the gRSC 72h after 72h the last ICSS administration. Previous reports of ICSS increasing c-Fos expression in the HPC only support short-term (90min) effects of the treatment (Huguet et al., 2009; Aldavert-Vera et al., 2013). This apparently conflicting outcome may be due to the greater number of ICSS sessions administered in the present study and/or to the fact that, unlike in previous research, animals were trained in a hippocampal-dependent task. In support of the latter idea, ICSS has been reported to not only improve a MWM performance but also induce long-term enhancement of dendritic branching density, observed after 72h in CA1 (Chamorro-López et al., 2015) and after 10 (Shankaranarayana Rao et al., 1999) and 60 days in CA3 (Shankaranarayana Rao et al., 1998b). In addition, a single session of ICSS is enough to potentiate RSC activation for 48h, even when the task involved was a non-declarative emotional task (Kádár et al., 2016). This differential delayed responsiveness of RSC could be related to its role in long-lasting memory consolidation and/or memory retrieval (Todd & Bucci, 2015), especially regarding spatial memory (Miller et al., 2014).

Moreover, we have found that ICSS also facilitates the ability of subjects to achieve reversal learning of a spatial task in the MWM for the first time. This enhancing effect on cognitive flexibility has previously been demonstrated in the reversal of a delayed alternation task in a T-maze (Soriano-Mas et al., 2005). One possible explanation for this facilitation is that ICSS increases the levels of excitatory neurotransmitters, such as dopamine (DA), noradrenaline (NA), acetylcholine (ACh) and glutamate (Glu) in the HPC and cortical regions (Shankaranarayana Rao et al., 1998a). As we have mentioned above, the ICSS treatment increases activity in several regions of the HPC, but it was also found to increase c-Fos expression in the OFC of the PFC. The enhanced activation of these areas, as well as the effect of ICSS on long-term neural plasticity, could go some way to explaining this treatment's facilitation of hippocampal-dependent memories as well as its flexible expression. The fact that ICSS treatment did not increase activation in the PL 90min after the reversal test is consistent with findings at shorter intervals (Flores et al., 1997; Hunt & McGregor, 2002), and with the OFC being found to be of greater relevance for the processing of a spatial reversal task (Boulougouris et al., 2007; Izquierdo et al., 2017). It is important to mention that MWM control variables related to anxiety (thigmotaxis) and locomotor activity (speed) did not differ from group to group. Consequently, the influence of any secondary effects of the ICSS treatment or other orexin-related functions on the execution of the response can be safely ruled out.

After reviewing the effects of each of the treatments – SB-334867 infusion and ICSS – on the memory task and the activation of the different brain regions, we must address the question of whether or not the orexinergic system blockade affects the ability of ICSS to facilitate a spatial memory task. ICSS treatment was found to be capable of counteracting the impairing effect of the OX1R blockade in the training phase and the retention test, as reflected by the equal values of SB+ICSS and Control groups. However, the facilitative effect of ICSS is partially annulled by the administration of SB-33486. Thus, although the execution of the SB+ICSS group did not differ from the Control or ICSS groups in the training phase, the inability of the SB+ICSS group to match the performance of the ICSS group becomes apparent. Our laboratory has previously reported that ICSS is capable of maintaining its facilitative effects on an emotional memory task after localized lesions of the parafascicular nucleus (Redolar-Ripoll et al., 2003) and amygdala (Segura-Torres et al., 2010; Kádár et al., 2014). However, the facilitating effects of ICSS on a spatial memory task seem to be negated in animals exposed to prolonged conditions of stress, which could affect several brain regions (Ramkumar et al., 2008). Therefore, our results could be related to the extensive effect an icv administration of an OX1R antagonist would have on neural systems supporting spatial memory. The activation of several areas belonging to the neuroanatomical substrate of spatial memory offer a wider picture of the implication of OX1R activity in the facilitation promoted by ICSS. In layer II-IV of the RSC, the stimulation treatment manages to not only to compensate for the SB-334867-induced decrease in c-Fos expression, but also potentiate the expression to the same level as the ICSS group. Taken as a whole, this indicates that the neurochemical mechanisms through which ICSS activates the RSC include other systems apart from the orexinergic system. However, the inability of the ICSS to equate the SB+ICSS to ICSS animals' performance in the MWM suggests that OX1R is at least participating in the facilitation of spatial memory by ICSS. Similarly, the lack of potentiation of DG activation, suggests a greater participation of the orexin system in this area regarding the facilitation exerted by ICSS. This is consistent with the fact that DG has a denser expression of OX1R receptors than the RSC (Hervieu et al., 2001; Marcus et al., 2001) and, therefore could be more sensitive to the detrimental effects of SB-334867.

Similar results regarding the compensatory effects of ICSS were observed in the reversal, but in this phase the OX1R blockade completely negated the ICSS facilitation since the SB+ICSS group performed significantly worse than the ICSS group. In addition, the ICSS treatment did not potentiate the activation of the OFC in animals that received the SB-334867 infusion, resulting in similar levels of activation in the SB+ICSS and Control groups. As mentioned above, one of the mechanisms through which ICSS could be exerting its facilitating effects on the reversal task is by increasing the levels of hippocampal and cortical excitatory neurotransmitters. The blockade of OX1R in the basal forebrain interrupts attentional processes (Boschen et al., 2009), probably through its cholinergic projections to the PFC (Fadel et al., 2005; Arrigoni et al., 2010), where SB-334867 has also been shown to reduce the power of the gamma oscillations (He et al.,

2015) which in turn can affect cognitive flexibility (Cho et al., 2006). Therefore, the proposed pathway of ICSS facilitation would be interrupted by the icv administration of the OX1R antagonist, resulting not only in the loss of facilitation of a reversal task in the MWM, but also the potentiation of the activation of prefrontal areas.

Our results suggest that the facilitating effect of the ICSS is partly mediated by the orexin system, due to the fact that blocking OX1R leads to the loss of said effect. This is especially evident when it comes to the effects of ICSS on the cognitive flexibility required to perform a successful reversal trial. Another important finding is the ability of ICSS to compensate for the detrimental effect of SB-334867 administration on the consolidation of a spatial memory task in the MWM. Taken as a whole, these results indicate that multiple neurochemical systems, in addition to the orexinergic system, participate in the memory enhancement attributed to ICSS.

STUDY IV

4 *Effects of Orexin-A receptor blockade on simultaneous visual discrimination facilitation by intracranial self-stimulation*

Manuscript in preparation

Soleil García-Brito, Laura Aldavert-Vera, Gemma Huguet-Blanco, Pilar Segura-Torres.

4.1 Introduction

Intracranial self-stimulation (ICSS) of the medial forebrain bundle (MFB) in the lateral hypothalamus (LH) has been proven to be an effective treatment to improve memory acquisition and retention of both implicit (Huston et al., 1977; Huston & Mueller, 1978; Redolar-Ripoll et al., 2002; Ruiz-Medina et al., 2008b; García-Brito et al., 2017) and explicit (Soriano-Mas et al., 2005; Chamorro-López et al., 2015) memory tasks in rats. As seen in the previous studies presented in this dissertation, ICSS exerts its facilitative effects through several mechanisms of action ranging from activation of arousal neurotransmission systems (Newman & Feldman, 1964; (Nieuwenhuys et al., 1982; Shankaranarayana Rao et al., 1998a; Wise, 2005) to dendritic structural changes (Shankaranarayana Rao et al., 1993, Shankaranarayana Rao et al., 1999; Chamorro-López et al., 2015) to an increased expression of plasticity-related genes in memory-related areas (Huguet et al., 2009; Aldavert-Vera et al., 2013; Kádár et al., 2014; Kádár et al., 2016).

One of the neurotransmission systems that could participate in the pathways through which ICSS exerts its effects on learning and memory is the orexinergic system. The orexinergic neurons are located in the lateral hypothalamus (LH) (de Lecea et al., 1998; Sakurai et al., 1998), where the ICSS treatment is delivered. Moreover, orexin-A has been linked to an enhanced reward (Muschamp et al., 2014), increased arousal (Modi et al., 2017), heightened attention (Fadel & Burk, 2010) and improved learning of both implicit (Akbari et al., 2008) and explicit (Zhao et al., 2014) memory tasks. Furthermore, blocking the orexin-A selective receptor (OX1R) results in the impairment of these functions.

In Study I, we reported that ICSS was capable of facilitating the acquisition of a simultaneous visual discrimination (SVD) task, especially when in comes to the number of errors committed, which could be related to an enhanced cholinergic function (Hagan et al., 1986; Dotigny et al., 2008). We also found that the enhancing effect on memory was maintained for at least 72h and that the nature of the inflexible learning was reflected in the impairment of the ICSS-treated animals' ability to reverse the learning. Moreover, orexin-A is especially involved in attentional processes through enhancement of acetylcholine (ACh) in the basal forebrain (BF) (Villano et al., 2017), ACh and glutamate (Glu) in the prefrontal cortex (PFC) (Calva et al., 2017), which could play a part in the increase in perseverative errors observed in discrimination tasks after the blockade of

OX1R in the BF (Piantadosi et al., 2015). In addition, orexin-A seems to be implicated in memory formation through modulation of the Glu, ACh and GABA systems in the hippocampus (HPC) (Zhang et al., 2010; Teles-Grilo Ruivo & Mellor, 2013; Teles-Grilo Ruivo et al., 2017). It is important to mention that the OX1R is widely expressed across the aforementioned areas (Hervieu et al., 2001; Marcus et al., 2001).

Therefore, in order to evaluate whether the orexinergic system could participate in the facilitative effects of ICSS on an SVD memory task, animals' performance in such tasks were tested in the Morris Water Maze (MWM) for 5 sessions (5 days) of training as well as a retention/reversal test 72h after the last day training. Each training session was followed by the administration of orexin-A receptor (OX1R) selective antagonist SB-334867 or a vehicle, followed by the ICSS treatment. Additionally, we measured the levels of acetylcholinesterase (AChE) present in the medial septum (MS) of the BF and the laterodorsal tegmental nucleus (LDTg) in the brainstem in order to assess whether cholinergic activity was affected by blocking OX1R and the administration of ICSS.

4.2 Materials and Methods

A total of 28 male Wistar rats with a mean age 92.65±3.27 days and a mean weight of 412.72±6.23g from our laboratory's breeding stock were used. The configuration and procedure is almost identical to the one used in Study I, with the exception of the distance between the visual cues and the water level, which was reduced from 35 cm to 25 cm. All procedure regarding ethical statements, stereotactic implantation of electrodes and cannulae, ICSS shaping and treatment, and drug microinfusion have previously been described in Study III. All procedures regarding memory training and testing in the water maze, and statistical analysis has been described in Study I. A schematic representation of the experimental procedure can be seen in Figure 40.

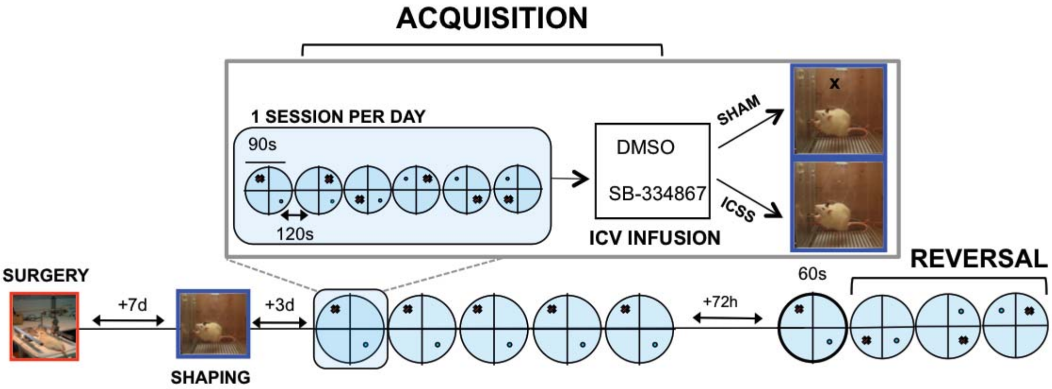

Figure 40. Diagram of experimental procedure in Study IV.

4.2.1 *Tissue collection.*

The euthanasia procedure, perfusion, brain post-fixation, crioprotection and freezing has already been described in Studies I, II and III. Serial coronal sections of the brain (30µm) were obtained in a cryostat (Cryocut 1800, with 2020 JUNG microtome) at -20º C between the coordinates 4.68 and 2.16, and -1.56 and -3.36 AP of Bregma (Paxinos & Watson, 2007) and stored at -80º C until staining. From the same subject, localized coronal sections (40µm), between the coordinates -0.6 and -1.20, and -2.28 and -3.12 of Bregma, were mounted onto a gelatin-coated slide, stained with cresyl violet and examined for cannula and electrode placement, respectively.

4.2.2 *AChE enzymatic staining and quantitative analysis.*

A free-floating AChE method was used to quantify AChE activity in the the MS and LDTg.The sections were collected in 0.1M sodium acetate buffer (pH 7.4) and incubated in 0.1M sodium acetate buffer (pH 5.0) containing 1.2 mg/ml of glycine, 1mg/ml of $CuSO_4$, 0.03mg/ml of ethopropazine hydrochloride and 1.16mg/ml of acetylcholine iodide for 4h30min in gentle agitation and protected from light. The enzymatic reaction was performed using 1% Na_2S in 0.1M sodium acetate buffer for 15min in the dark, and then rinsed with PBS. Sections were then dehydrated and mounted onto slides for microscopy analysis. Microphotographs from one out of three sections taken from every rat in each group were captured with a 10× objective lens using a BX-41 Olympus microscope attached to an Olympus DP-70 digital camera (Japan). Lighting parameters were identical for all microphotographs. The image analysis software Image-J 1.43 (http://rsb.info.nih.gov/ij/) was employed to assess the intensity levels of AChE staining. Intensity measures were obtained from a region of interest (ROI) drawn in MS and LDTg areas and counted in each section (AP: MS, 1.20mm to 0.60mm; LDTg, -8.88mm to -9.48mm). ROIs were composed of 5 or 3 circular areas in the MS and LDTg, respectively. For every section, each component of the ROI was individually situated in order to obtain the complete set of equidistant circular areas inside each specific area. Quantification means from three sections of each animal were then used for the corresponding statistical analysis.

4.2.3 *Statistical analysis*

All statistical analysis was carried out with SPSS statistical package v. 23 (SPSS Inc., Chicago, IL, USA). Analysis was conducted with a 4×5 mixed ANOVA (GROUP×SESSIONS) for the acquisition phase, and independent samples *t*-test analysis for the probe test and reversal (the average score of the three trials was analyzed). The main outcome variables for acquisition and reversal in the SVD were: 1) *Escape latency*:

time (s) needed to find and climb onto the platform, and 2) *Number of errors*: number of contacts with the area associated with the incorrect cue (no escape). In the probe test, the first part of the trial and the totality of the trial were analyzed for the following variables: *Percentage of time spent in the target quadrant*, *Percentage of time spent in the target annulus*, *Number of target crossings*, *Proximity to target*, *Whishaw's error* and *Number of errors*. In addition, a one-sample *t*-test against a constant (33) was used for each group to determine whether the *Percentage of time spent in the target quadrant* was different from chance level (33%, compared to the three options of navigation: target quadrant, error quadrant or the remaining two quadrants). Moreover, the control variables *Percentage of time spent near the walls* (measure of thigmotaxis - anxiety), *Length* (total distance in cm) and *Speed* (motor activity measure) were also analyzed. A one-way ANOVA was used to analyze AChE quantitative data. Polynomial contrasts, Post Hoc tests and adjustments in the case of unequal variances were carried out as described in Study III. A Spearman correlation analysis was performed to ascertain the relationship between AChE and the behavioral data. The α level for all tests was set at 0.05.

4.3 Results

The final sample consisted of 28 subjects (SB: n=7; Control: n=7; ICSS: n=7; SB+ICSS: n=7). There was no statistical difference between groups in weight change across the experimental procedure. Furthermore, no differences in rate or OI of ICSS were found among groups.

4.3.1 *Training phase*

There is a significant effect of interaction GROUP×SESSIONS [$F_{12,96}=5.622$, $P<0.001$] for the *Escape latency* (Figure 41A). All groups had equal escape latencies in the first session ($P=0.477$), but they started to differ after the first infusion/ICSS session (session 2: $P=0.002$; session 3: $P<0.001$; session 4: $P<0.001$; session 5: $P<0.001$). There was a decrease in the *Escape latency* across all groups, revealed by a significant downward linear function [SB: $F_{1,24}=88.86$, $P<0.001$; Control: $F_{1,24}=170.63$, $P<0.001$; ICSS: $F_{1,24}=399.74$, $P<0.001$; SB+ICSS: $F_{1,24}=149.06$, $P<0.001$], but only the ICSS group adjusted to a quadratic function [$F_{1,24}=27.36$, $P<0.001$], showing a sharper decline in the first three sessions compared to the rest of the groups [SB: $F_{1,24}=0.01$, $P=0.925$; Control: $F_{1,24}=6.69$, $P=0.013$; ICSS: $F_{1,24}=19.82$, $P<0.001$; SB+ICSS: $F_{1,24}=5.71$, $P=0.021$]. A Post-Hoc (Tukey HSD) into each session showed that the SB group latencies were higher than ICSS group (session 2: $P=0.001$; session 3: $P<0.001$; session 4: $P<0.001$; session 5: $P<0.001$), the Control group (session 2: $P=0.046$; session 3: $P=0.009$; session 4:

P=0.039; session 5: P<0.001) and the SB+ICSS group (session 3: P=0.008; session 4: P=0.044). In addition, latencies for the ICSS group were lower than the Control and SB+ICSS groups in the third (Control: P=0.004; SB+ICSS: P=0.010) and fifth (Control: P=0.034; SB+ICSS: P=0.050) sessions of the training phase.

Regarding the *number of errors*, there is no interaction GROUP×SESSIONS [$F_{12,96}$=0.765, P=0.685], but the main effects of GROUP and SESSIONS are significant [$F_{3,24}$=3.680, P=0.049 and $F_{12,96}$=4.458, P=0.002, respectively] (Figure 41B). Although there are no differences between each group in any individual session, an analysis of simple effects within each session showed that the ICSS group significantly and consistently committed fewer errors in later sessions compared to the first one (S3: P=0.026, S4: P=0.44, S5: P=0.018), while the Control group only showed differences between the first and fifth sessions (P=0.045) and the SB+ICSS group between the second and fourth sessions (P=0.047). The SB group is the only one that did not commit fewer errors across sessions (S1 vs all sessions: P>0.05).

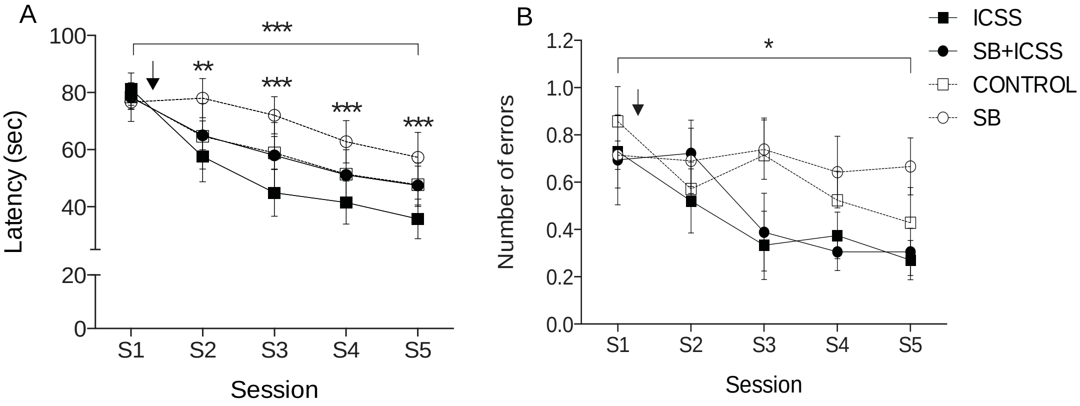

Figure 41. Effects of ICSS and SB-334867 on the acquisition of an SVD task in the MWM. (A) Mean *Escape latencies* (±SE) for the five acquisition sessions. Interaction significance depicted with a horizontal bracket; (B) Mean *Number of errors* (±SE) committed during the acquisition sessions by each group. Group factor significance is depicted with a horizontal bracket. Arrow shows start of ICSS treatment and SB-334867 infusion. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

4.3.2 *Probe test*

The time the animals spent in the *target annulus* was different between groups in the first 30 seconds [$F_{3,24}$=3.418, P=0.033] and in the totality of the trial [Welch: $F_{3,12.438}$=15.210, P<0.001] (Figure 42A). For the first 30 seconds of the trial, the ICSS group outperformed the rest of the groups (SB: P=0.014; Control: P=0.018; SB+ICSS: P=0.022). ICSS group also outscored the Control and SB groups (P=0.017 and P<0.001, respectively) for the totality of the trial, whereas the SB+ICSS group recovered and had a higher percentage of time in the target annulus than the SB group (P=0.045). Group differences were also found for the *proximity to target* [Welch: $F_{3,11.528}$=32.430, P<0.001] (Figure 42B). A Post Hoc revealed that the ICSS group swam closer to the platform (correct cue) than the Control (P=0.028), while the SB group swam farther from the platform than the rest of the groups (Control: P=0.006; SB+ICSS: P=0.020; ICSS: P<0.001).

Whishaw's error was also different between groups for the first 30 seconds [$F_{3,24}$=6.810, P=0.002] and the totality of the trial [$F_{3,24}$=15.072, P<0.001] (Figure 42C). During the first half of the trial, the ICSS group's navigation was more accurate than the Control, SB+ICSS and SB groups' (P=0.025 and P<0.001, respectively), while the Control group performed better than the SB group (P=0.026). For the totality of the trial, the ICSS group's performance was better than the rest (Control: P<0.001; SB+ICSS: P<0.001; ICSS: P<0.001). The *number of target crossings* showed a significant difference between groups [$F_{3,24}$=5.551, P=0.005] (Figure 42D), where once again the ICSS performed better than the other groups (SB: P=0.007; Control: P=0.023; SB+ICSS: P=0.044). Regarding the *number of errors*, there is a tendency towards a significant difference between groups [$F_{3,24}$=2.081, P=0.064] (Figure 42E), where the ICSS group committed less errors than the SB group (P=0.045). No differences regarding *percentage of time spent in the target quadrant* were found between groups. Only the ICSS and Control groups spent enough time in the target quadrant to differ from *chance* level (ICSS: P=0.002; Control: P=0.018) (Figure 42F).

4.3.3 *Reversal trial*

Group differences in the *Escape latencies* were found [$F_{3,24}$=3.316, P=0.037] (Figure 42G). The ICSS group had significantly higher *Escape latencies* than the Control group (P=0.045). Differences in the *number of errors* were also found [$F_{3,24}$=6.548, P=0.002] (Figure 42H), with the ICSS group making more errors than the SB (P=0.010), Control (P=0.010) and the SB+ICSS groups (P=0.007).

No differences in control variables (*Percentage of time spent near the walls*, *Length* or *Speed*) were found between groups either in the acquisition, probe test or in the reversal session.

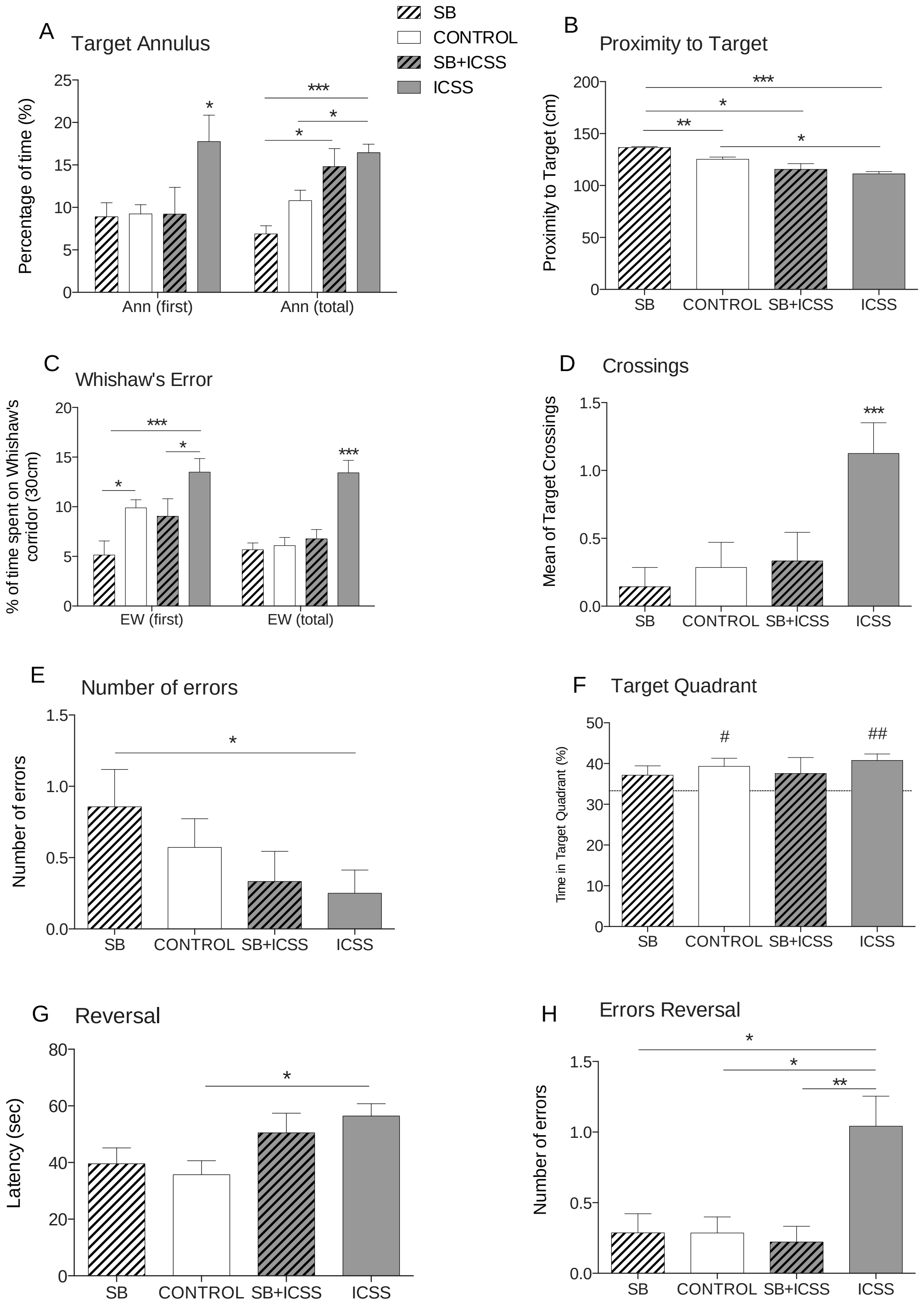

Figure 42. Effects of ICSS and SB-334867 on the retention of an SVD task in the MWM. (A) Target Annulus, first 30 seconds and totality of the test; (B) Proximity to target (cm); (C) Whishaw's error; (D) Number of crossings over the target; (E) Number of errors; (F) Percentage of time spent in the target quadrant (G) Latency in reversal; (H) Number of errors in reversal. Abbreviations: Ann, percentage of time spent in the target annulus; WE, Whishaw's error. Group differences are depicted with *P<0.05; **P<0.01; *** P<0.001. The dotted line in retention test represents chance level # P<0.05; ## P<0.01.

4.3.4 AChE activity

There was a significant difference between groups in AChE activity in the LDTg [$F_{3,24}$=14.290, *P*<0.001] (Figure 43A) and a tendency to significance in the MS [Welch: $F_{3,12.963}$=2.555, *P*=0.061] (Figure 43B). Generally, SB-infused groups had lower AChE activity than vehicle groups in both the LDTg (Control vs SB, *P*=0.019; Control vs SB+ICSS, *P*=0.012; ICSS vs SB, *P*<0.001; ICSS vs SB+ICSS, *P*<0.001) and in the MS (Control vs SB, *P*=0.045; ICSS vs SB, *P*=0.036; ICSS vs SB+ICSS, *P*=0.017), with the exception of the Control vs SB+ICSS (*P*=0.055).

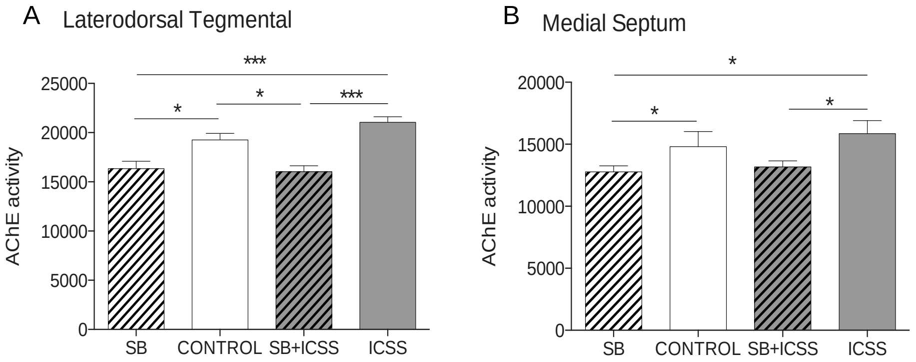

Figure 43. Effects of ICSS and SB-334867 on AChE expression. (A) Laterodorsal tegmental nucleus; (B) Medial septum. Group contrasts are depicted with *P<0.05 **P<0.01 ***P<0.001.

4.3.5 Correlation analysis

All Spearman correlation values are shown in table 9. There was moderate positive correlation between AChE levels in LDTg and number of errors in the reversal test (Figure 45A). In addition, there were negative correlations between the LDTg and mean latencies in sessions 2, 3 and 5 of the training phase (Figures 45B-45D). Finally, AChE levels in the MS negatively correlated with number of errors in session 2 of the training phase (Figure 45E), immediately after the first infusion/ICSS session.

Table 9. Correlations between AChE activity and behavioral correlates.

AChE levels	Number of errors reversal	Mean latency session 2	Mean latency session 3	Mean latency session 5	Number of errors session 2
LDTg	r_s = 0.448*, *P*=0.017	r_s = -0.582**, *P*=0.001	r_s = -0.489**, *P*=0.008	r_s = -0.476*, *P*=0.010	
MS					r_s = -0.502**, *P*=0.006

Table 9. Correlations between AChE activity and behavioral correlates. Abbreviations: LDTg, laterodorsal tegmental nucleus; MS, medial septum. *Correlation is significant at the 0.05 level, **Correlation is significant at the 0.01 level.

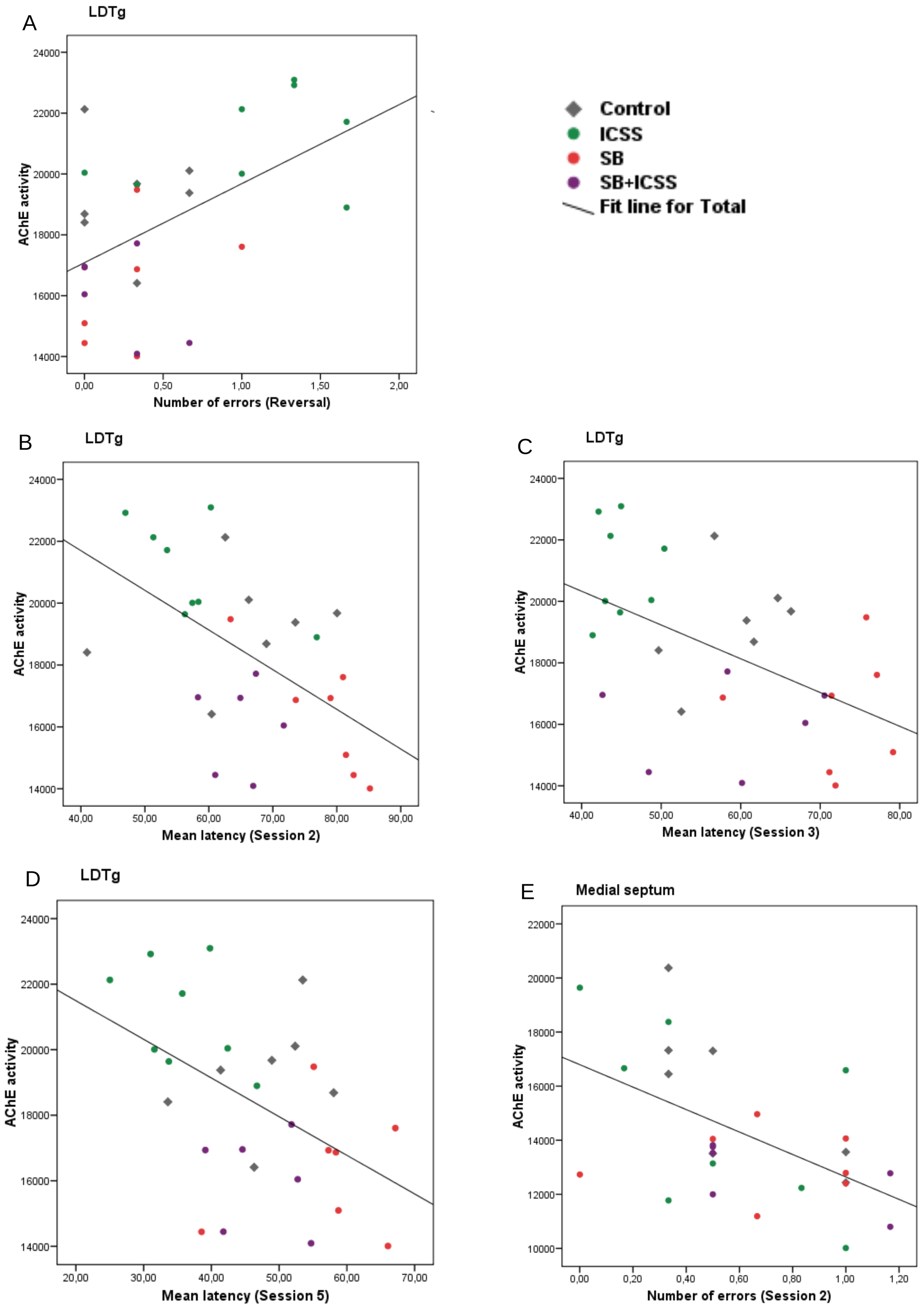

Figure 44. Scatterplots depicting correlations between AChE levels and behavioral correlates. (A) LDTg and number of errors in the reversal session; (B) LDTg and mean latency in session 2; (C) LDTg and mean latency in session 3; (D) LDTg and mean latency in session 5; (E) MS and number of errors in session 2. Abbreviations: LDTg, laterodorsal tegmental nucleus; MS, medial septum.

4.4 Discussion

The results of this study confirm the ICSS facilitative effect on SVD memory consolidation observed in Study I. Moreover, they provide additional data in favor of the general negative impact of OX1R blockade on implicit memory, as previously seen in Study III for explicit memory. Furthermore, the facilitative effects of ICSS on an SVD task in the MWM seem to have been partially annulled by the administration of SB-334867. An especially interesting differential effect in the reversal portion was observed in this study, in contrast to the Study III.

The detrimental effects of the blockade of OX1R are evident in the training phase, as the SB group’s escape latencies and number of errors committed remain higher than the rest of the groups throughout the 4 sessions that followed the first SB-334867 administration. Furthermore, these impairing effects also seemed to be evident in the retention test, performed 72h after the last administration of SB-334867, where both proximity to target and navigation accuracy were considerably affected by the administration of the OX1R antagonist. Although the detrimental effect of SB-334867 has been reported in other implicit memory tasks (Jaeger et al., 2002; Telegdy & Adamik, 2002; Akbari et al., 2008; Mavanji et al., 2017), this is the first time, to our knowledge, that the blockade of OX1R has been shown to impair the performance of an SVD task in the MWM.

In contrast to our results, this OX1R antagonist seems to have no effect on escape latency in a non-spatial visual discrimination task in the MWM (Akbari et al., 2006). However, unlike our design, the procedure used by these researchers cannot be considered a simultaneous discrimination of two visual stimuli, but more of a visual acuity test in which a visible platform is used. On the other hand, in an olfactory discrimination task, subjects that received an infusion of SB-334867 into the BF needed a higher number of trials to criteria during the acquisition of the task (Piantadosi et al., 2015). This outcome prompted the authors to suggest that this cholinergic center could be especially relevant to the attentional requirements of discrimination memory. In agreement with this idea, the administration of orexin-A directly into the BF seems to attenuate attentional deficits, caused by lesions of cortical cholinergic projections from the BF, in the discrimination of the temporal presentation of visual signals (Zajo et al., 2016). This notion seems to be supported by present results showing that AChE intensity in both the MS and LDTg diminished in the SB group compared to the Control. Incidentally, the SB group had the worst performance. Together with these results, the positive correlation between performance – based on latency analysis – and AChE levels, led us to interpret enzymatic activity as a result of cholinergic activity (Sergutina & Rakhmanova, 2014; Kaizer et al., 2018). Additionally, in agreement with present results, Piantadosi et al. (2015) also found that animals suffering from OX1R blockade in the BF committed a higher number of errors to criteria than other groups. This is supported by the fact that animals with the highest number of errors in session 2 of the training phase also displayed lower AChE activity in the MS region. This suggests that the inactivation of orexinergic activity leads to diminished cholinergic activity, which in turn could result in an impairment of the ability to perceive differences between two stimuli.

Regarding the reversal phase, the SB group's performance was unexpectedly equal to the Control group. Although these results seem contrary to preceding reports that state that the blockade of OX1R give rise to a higher number of trials and errors to criteria (Piantadosi et al., 2015), the difference in the time between administration and reversal could explain the differing results. In our study, the gap of 72h between the last SB-334867 infusion and the reversal could be behind the dissipation of the detrimental effects. Thus, animals with OX1R blockade probably acquire the reversal task as if they were in the initial stage of training.

Moreover, the effects of ICSS on an SVD task in the MWM replicate results in Study I, in that the ICSS group performed better than the Control throughout the training sessions and the retention test. Other implicit memory paradigms have also been facilitated by ICSS treatment (Redolar-Ripoll et al., 2002; Aldavert-Vera et al., 2013) and one study that explored perceptive associations between stimuli found that ICSS strengthened this association (Coulombe & White, 1982). However, there is not much precedent for the facilitation of a memory task of highly perceptive character by ICSS, with the obvious exception of the results presented in the published work of Study I (García-Brito et al., 2017). Specifically, ICSS improves performance by reducing both escape latency and number of errors committed across the training phase. In addition, said improvement in the performance was maintained for 72h, as revealed by the close proximity to target and number of crossings in the probe test. Proximity to target has been identified as the most sensitive probe test variable for spatial configurations in the MWM (Maei et al., 2009), and it was one of the two variables that demonstrated the facilitating effects of ICSS treatment on the retrieval of visual discrimination memory. However, the enhancing effects of ICSS we have observed in the behavioral performance are not reflected in the AChE activity in either cholinergic centers, LDTg or the MS. This is in contrast with previous reports of the capability of ICSS to increase levels of AChE in the

HPC and motor cortex (Shankaranarayana Rao et al. 1998a). A plausible explanation for this conflicting data is that the facilitation of an SVD task by ICSS is not mediated by AChE activity in the areas analyzed. However, considering that AChE correlations with performance show that subjects in the ICSS group are primarily located in the in the high-AChE-activity-better-performance area, we cannot rule some form of relationship. Moreover, ICSS facilitation of the SVD resulted in a characteristically rigid memory of the association between the correct cue and escape platform, as shown by a higher escape latency and number of errors in the reversal learning of the SVD task. Additionally, the data presented in this study replicated the results obtained in Study I, where the ICSS group's perseverative errors during the reversal of the discrimination task were higher than the Control group.

Furthermore, the facilitative effect of ICSS upon an SVD task seems to be completely negated by the OX1R blockade. It is noteworthy that previous studies carried out in our lab reported that the facilitation of implicit memory by ICSS was not impeded by lesions of either the parafascicular nucleus of the thalamus (Redolar-Ripoll et al., 2003) or the amygdala (Segura-Torres et al., 2010; Kádár et al., 2014), suggesting that the affectation of and extended number of brain regions by the icv infusion of SB-334867 is behind the inability of ICSS to exert its facilitative effect. On the other hand, in keeping with results obtained on explicit memory in Study III, ICSS compensates for the detrimental effect of SB-334867 infusion by equating SB+ICSS group to Control subjects. This is revealed by the SB+ICSS group's recovery in performance regarding escape latencies and number of errors during the training phase, as well as the percentage of time spent near the platform and proximity to target in the retention test. This compensation could result from the ICSS-induced activation of critical brain regions for discrimination memory, such as the caudate-putamen (Packard & McGaugh, 1992). Similarly, previous studies on hippocampal-independent learning have demonstrated that ICSS potentiates immediate expression genes – c-Fos and Nurr1 – in this region (Aldavert-Vera et al., 2013).

In summary, the inability of ICSS to facilitate an SVD task after the infusion of an OX1R antagonist suggests that the orexin system is involved in facilitative effect of the stimulation treatment. In addition, present results provide data that supports the compensating capability of ICSS treatment in several types of memory.

CHAPTER IV

GENERAL DISCUSSION

The main objective of this thesis is to evaluate the involvement of orexin-A and its receptor OX1R in the facilitating effect of intracranial self-stimulation (ICSS) on two types of memory, tested in a standard spatial version of the Morris Water Maze (MWM) and a simultaneous visual discrimination task (SVD) in the same maze. ICSS in the medial forebrain bundle (MFB) of the lateral hypothalamus (LH) has been found to be capable of facilitating both explicit (Soriano-Mas et al., 2005; Ruiz-Medina et al., 2008a; Ramkumar et al., 2008; Takahashi et al., 2009; Chamorro-López et al., 2015) and implicit (Huston & Mueller, 1978; Redolar-Ripoll et al., 2002; Ruiz-Medina et al., 2008b) memory tasks. The enhancing effects of this treatment have been linked to several routes of action, which probably involve complex dynamics and the affectation of various systems and mechanisms. For instance, ICSS promotes structural plasticity changes (Shankaranarayana Rao et al., 1998b; Chamorro-López et al., 2015), as well as the expression of plasticity-related genes in the hippocampus (HPC) and other memory-related structures (Huguet et al., 2009; Aldavert-Vera et al., 2013; Kádár et al., 2014; Kádár et al., 2016). ICSS treatment also boosts the activation of numerous excitatory neurotransmission systems throughout the brain (Newman & Feldman, 1964; (Nieuwenhuys et al., 1982; Shankaranarayana Rao et al., 1998a; Wise, 2005).

The orexinergic system is implicated in several processes that are also affected by ICSS, such as reward, arousal, attention and memory (Akbari et al., 2006; Fadel & Burk, 2010; Berthoud & Münzberg, 2011; Arias-Carrión et al., 2014). Additionally, orexin-producing neurons are located in the LH (de Lecea et al., 1998; Sakurai et al., 1998), where ICSS produces the highest rates of stimulation (St-Laurent, 1988). Therefore, we set out to explore whether or not the orexinergic system, specifically orexin-A, through its selective receptor OX1R, plays a role in the facilitating effect of ICSS on two types of memory. In order to study this relationship, two preparatory experiments were carried out.

Although previous work in our laboratory had concluded that ICSS had a facilitative effect on implicit memory tasks of emotional character (Redolar-Ripoll et al., 2002; Ruiz-Medina et al., 2008b; Aldavert-Vera et al., 2013), the effect of ICSS had never been tested in a task requiring high levels of perceptual visual discrimination. In addition, we were looking to compare the orexin-ICSS relationship in two different memory tasks, which meant that we needed to establish a common and comparable set of parameters governing the two tasks. Therefore, we adapted the configuration and procedure for a visual discrimination task in the MWM from Packard and McGaugh (1992) not only to elucidate the effects of ICSS on the implicit task, as shown in Study I, but also to obtain comparable parameters in the study the effect of orexin blockade on ICSS memory facilitation.

Moreover, conflicting data regarding the involvement of the orexinergic system in spatial memory was found, which indicated that the effects of orexin antagonism on a spatial memory task differed depending on the intensity of training the animals underwent. Some studies found that intra-HPC administration of SB-334867, an OX1R selective antagonist, impaired spatial memory in a weak training protocol (Akbari et al., 2006, 2007). However, this type of memory did not seem to be affected by the administration of Almorexant, a dual antagonist of OX1R and OX2R, when animals endured stronger training (Dietrich & Jenck, 2010; Morairty et al., 2014). One of the critical differences between these studies was the number of trials that animals underwent in the training portion of the task. Therefore, the design used in Study II attempted to resolve whether or not the detrimental effects of OX1R blockade on memory was contingent on the intensity of training. Consequently, these results allowed for the characterization of a model in which OX1R blockade impaired a spatial memory task. In the aforementioned studies the administration route for the orexin receptor antagonists, intra-HPC, intraperitoneal or oral administrations (Akbari et al., 2006, 2007; Dietrich & Jenck, 2010; Morairty et al., 2014), respectively) also varied. In the interest of blocking OX1R in several brain regions that express OX1R and participate in both types of memory, we used an intracerebroventricular (icv) administration. This route of administration is also preferable to oral or intraperitoneal alternatives not only in order to avoid metabolization differences between subjects (Turner et al., 2011), but also due to the fact that the solvent for SB-334867 – dimethyl sulfoxide (DMSO) – can cause irritation to the skin.

Once we had characterized the effects of post-training ICSS on an SVD task, as well as the training protocol that would show memory impairment after SB-334867 administration, we proceeded to evaluate rats' performance in a spatial memory task, as well as in an SVD task, in the presence of ICSS and OX1R antagonist administration. Additionally, we looked into the effects of both treatments on the activation of several memory-related regions implicated in a spatial memory task, in addition to the levels of acetylcholinesterase (AChE) activity in relevant cholinergic centers after an SVD task in the MWM.

Intracranial self-stimulation facilitates implicit and explicit memory

Results obtained in this dissertation confirm that ICSS is a treatment capable of facilitating both explicit and implicit memory tasks, as shown by Study III, and studies I and IV, respectively. Additionally, it annexes SVD tasks performed in the MWM to the array of memory paradigms that can be potentiated by this treatment. The strengthening of perceptual associations by ICSS has been reported previously for light and tone stimuli (Coulombe & White, 1982), but it had not been tested in a simultaneous discrimination paradigm that required differentiating between visual patterns in order to achieve the goal of escaping from the pool. Expanding our knowledge into the memory processes that can be enhanced by ICSS is a valuable step towards potential translational applications. Although explicit memory deficits are broadly accepted to be part of early onset Alzheimer’s disease, damage to areas related to perceptual learning is evident in the later stages (Manzanero, 2007). More specifically, patients with Alzheimer’s disease show deficiencies in visuo-perceptive priming (Boccia et al., 2014) as well as visual discrimination (Harnish et al., 2010).

Moreover, ICSS treatment has consistently improved memory retention in both tasks. This is especially interesting when considering that the outcome regarding comparable criteria between memory tasks was somewhat similar. For example, the value of the variable “proximity to target” was consistently improved by ICSS across both memory tasks. Although this measurement had already been identified as the most sensitive for detecting differences between groups in the probe test of a spatial memory task (Maei et al., 2009), this is the first time, to our knowledge, that this measurement has been assessed in an SVD task.

Furthermore, the comparative analysis of the effects of ICSS on the reversal session between tasks reveal that the strengthening of the learning might result in different outcomes when it comes to cognitive flexibility. Implicit memory has been described as intrinsically inflexible (Hirsh, 1974), whereas hippocampus-dependent memory allows for the flexible use of the information that could guide spatial navigation in diverse situations (Eichenbaum, 2017a). ICSS has been shown to improve cognitive flexibility in a spatial memory task where animals were required to use the contextual configuration to locate their food (Hermer-Vazquez et al., 2005). Likewise, stimulation of the MFB in the LH helped animals achieve a more flexible expression of spatial memory in an alternation T-maze task (Soriano-Mas et al., 2005). Accordingly, present results confirm that ICSS promotes cognitive and behavioral flexibility in a spatial memory task. In contrast, ICSS facilitative effects on an SVD task seem to have the opposite effect on the reversal portion of this paradigm, suggesting that stronger SVD consolidation would result in a more rigid and inflexible memory, as shown by higher escape latencies and number of errors. Previous experiments carried out in our laboratory obtained similar findings in an active avoidance conditioning, in which ICSS increased the resistance to the extinction of a well-acquired emotional memory (unpublished data).

Finally, present results support previous research showing the ability of ICSS treatment to cause significant activation of brain regions related to the hippocampal system of explicit memory (Aldavert-Vera et al., 2013; Kádár et al., 2016). Although the work carried out in this thesis did not analyze the activation of structures related to the SVD task, we have previously been able to verify that ICSS also activates regions critical for this kind of memory, as is the case with the dorsal striatum (Aldavert-Vera et al., 2013).

OX1R blockade impairs memory

The infusion of SB-334867 into the lateral ventricle resulted in a marked impairment to memory consolidation and retention in both the spatial and SVD memory paradigms. Present results corroborate what previous studies have also reported regarding the detrimental effect of OX1R blockade on explicit (Akbari et al., 2006; Akbari et al., 2007; Yang et al., 2013; Zhao et al., 2014) and implicit (Jaeger et al., 2002; Telegdy & Adamik, 2002; Akbari et al., 2008; Mavanji et al., 2017) memory tasks. In addition, present results broaden our knowledge regarding the relevant factors to consider when using SB-334867 as a memory-impairing drug. Specifically, the intensity of training is a procedural criterion that could determine whether or not the administration of this drug results in learning deficits in a spatial memory task. Moreover, the detrimental effects resulting from the blockade of the orexin-A receptor are in agreement with animal models of memory impairment both implicit (Jaeger et al., 2002; Mavanji et al., 2017) and explicit (Yang et al., 2013) tasks, which show that orexin-A administration can aid the recovery of memory in animals with orexin neuron degeneration, and with Aβ overproduction. This notion is also consistent with clinical observations of patients with Alzheimer's disease suffering from significant losses of orexin-A neurons (Fronczek et al., 2012) and low levels of orexin-A in cerebrospinal fluid (Slats et al., 2012).

Interestingly, the impaired performance in animals infused with SB-334867 was reflected in the lack of accuracy and proximity to target during memory retrieval of the SVD task. The weight that these variables carry towards the detection of memory deficits has been reported previously with regard to a spatial memory task (Maei et al., 2009; Pereira & Burwell, 2015). However, this is the first time, to our knowledge, that proximity to target variable has detected deficits in an SVD task in the MWM. In addition, we observed the differential effects of the OX1R blockade on the reversal phase of both tasks. The administration of SB-334867 impaired cognitive flexibility in the spatial version of the memory task, whereas it did not seem to affect the reversal of the SVD task. Although there are no previous reports of the effects of OX1R blockade on the reversal of a spatial memory task, an impairment of cognitive flexibility has been observed in a discrimination task when the administration of SB-334867 takes place immediately before the execution of the reversal session (Piantadosi et al., 2015). However, it is important to note that the contingency in time between OX1R blockade and the execution of the reversal may play a big part in its detrimental effects on a discrimination task. This could help explain the lack of effect on the reversal detected in Study IV, where animals received the last SB-334867 infusion 72h before the reversal test took place.

Our results also showed that c-Fos expression diminished in several memory-related areas in animals with OX1R blockade. This is consistent with the fact that all the areas that were affected by the SB-334867 administration, also express OX1R (Hervieu et al., 2001; Marcus et al., 2001). In addition, our observations suggest that the orexinergic system could participate in different memory processes through the regulation of the activation of areas associated with the specific types of memory. For instance, the opposite effect on c-Fos expression was reported in a study exploring the effects of SB-334867 on fear memories (Flores et al., 2014). Here, the researchers found that blocking OX1R not only facilitated the extinction of the conditioned fear response, but it also increased activity in the infralimbic cortex, one of the critical structures for this function.

Overall, present results corroborate the impairing effects of OX1R blockade on different types of memory. This detrimental effect could take place through several mechanisms, including the regulation of activity in specific memory-related areas.

Effects of OX1R blockade on memory facilitation by ICSS

Study III and Study IV aimed to address the hypothesis of this dissertation. Our results suggest that the orexinergic system is involved in the facilitating effects of ICSS on memory. Yet, the extent to which blocking OX1R interrupts memory facilitation by ICSS seems to differ between the spatial and SVD tasks. Despite obtaining similar results in both tasks, OX1R blockade appears to partially negate the facilitating effect of ICSS on spatial memory, whereas it completely hinders ICSS facilitation of an SVD task. This is especially noticeable when we look at specific variables analyzed for both paradigms in the retention test. The analysis of Whishaw's error or navigation accuracy, which assesses the precision of animals in their approach to the goal (Whishaw, 1995), reveals that despite the blockade of OX1R, the facilitative effect of ICSS can be maintained in the spatial memory task, but not in the SVD task.

A valuable consideration regarding the differential effects of OX1R blockade on the facilitating effects of ICSS lies in the importance of object saliency for the memory tasks. Orexins contribute to the perception of relevant and salient contextual cues (Mileykovskiy et al., 2005, Petrovich et al., 2012), which indicates that they play a big part in the control of the selective attention needed to perform simultaneous discrimination between visual stimuli (Teng et al., 2015). Perhaps the facilitating effect of ICSS on an SVD task is dependent on the detection of salient individual stimuli, while a greater reliance on richer and more complex configurations may be required in spatial tasks (Lopez et al., 2008). Thus, the richness of the context could help compensate for the loss of orexinergic function and still allow ICSS to improve spatial learning and memory.

It is important to note that our experimental procedure included numerous registers designed to control for any side effect that the OX1R blockade may have on the execution of the memory task, especially considering the wide implication of orexins in physiological functions (Li et al., 2014), such as food intake (Sakurai et al., 1998), locomotor activity (Hagan et al., 1999) and reward (Hata et al., 2011; Patyal et al., 2012). No effects of SB-334867 infusion were observed in the weight of the animals, motor activity in the MWM, or in the ICSS behavior.

An unexpected outcome of the studies carried out for this dissertation was that ICSS treatment was capable of compensating for the detrimental effects of OX1R blockade on both memory paradigms. The compensatory effects of ICSS could be to some extent similar to those observed as a result of an increase in training observed in Study II, where increased training recovered the performance to the point of equating SB-334867-infused group to the control group. In any case, a qualitative comparison of the performance of the animals that received SB-334867 and ICSS (SB+ICSS group in Study III), and the performance of those that underwent stronger training (SB6 in Study II), indicates that the effect of ICSS is more powerful. This idea is in agreement with previous research from our laboratory in an implicit memory task (Redolar-Ripoll et al., 2002; Segura-Torres et al., 2010).

The capability of ICSS to recover loss of memory function has already been reported. Specifically, ICSS has compensated for hippocampal atrophies as a result of chronic stress exposure (Ramkumar et al., 2008). Similarly, ICSS has also been shown to ameliorate implicit memory impairments due to localized lesions in the parafascicular nucleus of the thalamus (Redolar-Ripoll et al., 2003) or amygdala (Segura-Torres et al., 2010; Kádár et al., 2014), as well as normal aging (Aldavert-Vera et al., 1997).

The study of the mechanisms for ICSS memory facilitation is a highly complex one, particularly due to the variety of neurochemical systems and functional connections between areas involved in memory processing which are also affected by ICSS. In order to further explore the role of the orexinergic system in the facilitating effect of ICSS on different types of memory, future studies should evaluate how ICSS impacts the function of the orexinergic system, by examining whether or not the expression of OX1R is altered by ICSS. The repercussions of such interventions on memory processing could also be examined. In addition, establishing a clear contingency between the administration of SB-334867 and/or ICSS, and specific cognitive processes, while evaluating the activation in brain regions related to such processes, would help to shed more light on the involvement of the orexinergic system within the neural network implicated in the facilitating effects of ICSS on memory.

CHAPTER V

CONCLUSIONS

Results obtained in this thesis support our group’s hypothesis regarding the relationship between ICSS and the modulation of learning and memory. The results also shed further light on the neurochemical systems that may be implicated in such effects.

ICSS treatment has once again been shown to be capable of facilitating learning and memory in a wide variety of tasks involving different memory systems. It has been suggested that ICSS could exert its effects due to its capacity to activate multiple brain regions, including those related to memory. ICSS seems to promote plasticity mechanisms in these regions that could support both learning and memory.

The potentiating effect of ICSS on memory seems to be partially mediated by orexin-A receptor (OX1R), since blocking these receptors reduces the facilitative effect of ICSS to different extents depending on the memory system and cognitive process evaluated. These results indicate that the facilitation of memory by ICSS involves multiple neurochemical systems, which could explain the impressive capacity of ICSS treatment to compensate for memory deficits, such as those caused by OX1R blockade or by specific cerebral lesions.

ICSS, and therefore, the activation of the reward brain system offer us a fascinating opportunity to explore general and effective ways of improving learning and memory.

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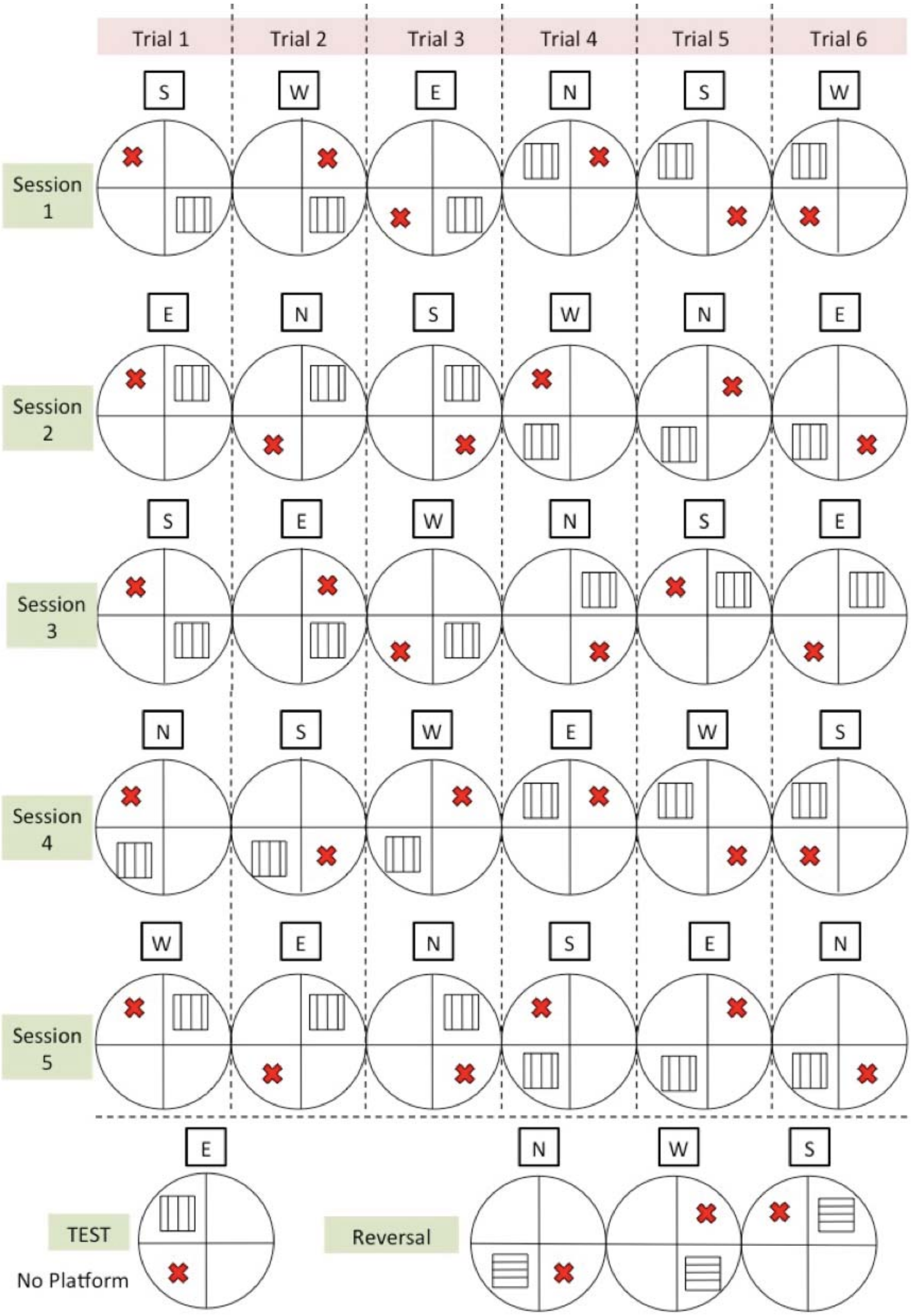
ANNEX

Annex 1. SVD protocol in the MWM.



Annex 1. SVD protocol in the MWM. Depicts all the configurations applied in the acquisition, retention (test) and reversal sessions. Patterned square represents correct cue/presence of platform while X represents incorrect cue/absence of platform. Starting points are shown for each trial (N, North; E, East; S, South; O, West).

Annex 2. Copy of published Manuscript for Study I.

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Research report

Intracranial self-stimulation also facilitates learning in a visual discrimination task in the Morris water maze in rats

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HIGHLIGHTS

- Self-stimulation (ICSS) facilitates the learning of a visual discrimination task.
- A direct, instead of a trial and error strategy is preferred by ICSS animals.
- *Number of errors* is a more sensitive measure than *latency* in visual discrimination.
- A strengthened implicit memory caused by ICSS, challenges reversal learning.

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ABSTRACT

Intracranial self-Stimulation (ICSS) of the medial forebrain bundle is a treatment capable of consistently facilitating acquisition of learning and memory in a wide array of experimental paradigms in rats. However, the evidence supporting this effect on implicit memory comes mainly from classical conditioning and avoidance tasks. The present work aims to determine whether ICSS would also improve the performance of rats in another type of implicit task such as cued simultaneous visual discrimination in the Morris Water Maze. The ICSS treatment was administered immediately after each of the five acquisition sessions and its effects on retention and reversal were evaluated 72 h later. Results showed that ICSS subjects committed fewer errors than Sham subjects and adopted more accurate trajectories during the acquisition of the task. This improvement was maintained until the probe test at 72 h. However, ICSS animals experienced more difficulties than the Sham group during the reversal of the same learning, reflecting an impairment in cognitive flexibility. We conclude that post-training ICSS could also be an effective treatment for improving implicit visual discrimination learning and memory.

1. Introduction

The electrical activation of the medial forebrain bundle (MFB) via Intracranial self-stimulation (ICSS) has been confirmed in our and other laboratories as a treatment capable of consistently facilitating the acquisition and retention in a wide array of experimental paradigms, for both implicit [1–4] and explicit memory [5,6], in rats. Several mechanisms of action have been proposed to explain these facilitating effects of ICSS on learning and memory. Stimulation of the MFB has been linked to activation of general arousal systems [7,8], due to activity of dopaminergic, noradrenergic and serotoninergic ascendant fibers [9,10]. Learning and memory facilitation has also been linked to structural plasticity induced by ICSS [11]. Recent work undertaken in our laboratory has shown an increase in the density of dendrite spines in the CA1 neurons of the hippocampus in rats that received ICSS after training in a spatial task [6]. These morphological modifications could be related to changes in the expression of several plasticity-related genes caused by the post-training ICSS treatment, with increased levels of Nurr1, c-Fos and Arc protein consistently being found in hippocampus, amygdala, dorsal striatum, lateral hypothalamus or retrosplenial cortex [12–15].

While most evidence supporting the facilitating effect of the post-training ICSS on explicit memory comes mainly from spatial learning tasks in T-mazes and the Morris Water Maze (MWM), the type of implicit memory that has been subjected to ICSS treatment effects is an amygdala-dependent emotional memory. Thus, the most commonly used tasks have been aversive classical condition-

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ing and avoidance learning. While some pioneering studies have looked into the effects of ICSS on other types of implicit tasks, such as appetitive classical conditioning [16], there are none that focus on tasks more related to perceptual learning and memory. Perceptual abilities of recognition and discrimination between stimuli are the foundation of most of the learning processes both in animals and humans and, therefore, if ICSS were able to facilitate visual discrimination conditioning and memory it would extend the range of cognitive processes – involving stimuli perception – that are improved by ICSS or the stimulation of reward pathways. Furthermore, since a deficit in implicit learning and memory related to visual discrimination are observed in both Parkinson's disease [17] and the later stages of Alzheimer's disease [18], the possibility of positively affecting this type of memory could also be interesting in the field of neurodegenerative diseases.

In order to study the possible effect of post-training ICSS on a simultaneous visual discrimination task in the MWM (SVD), we modified the configuration of the MWM based on the model presented by Packard and McGaugh [19] of a two-platform task, in a non-spatial version of the MWM task, in which two visible white rubber balls were painted with black horizontal and vertical stripes and used as cues attached to the escape/non-escape platforms. As ICSS treatment demonstrates a higher effectiveness on high difficulty conditions [20–22] a SVD task would present the appropriate setup, given that the task involves the need to identify and compare two similar stimuli in order to solve it. Moreover, this task in the MWM does not require caloric restriction in order for the animal to learn to find the platform, thus reducing the possible interference of the motivational states on learning [23]. This task is considered to be a non-declarative memory task [24], which also requires the animal to establish an association between a specific stimulus and the location of the platform, generating an instrumental escape response; this associative nature would also involve the use of relatively inflexible memory processes [25] which could mean that reversing or changing a well-consolidated memory would be extremely challenging. This suggests that, should the acquisition of the SVD task be facilitated by the ICSS treatment, the retention of the memory will be stronger while the reversal learning will be challenged.

2. Materials and methods

2.1. Subjects

A total of Forty-two Wistar male rats with mean age 90.35 days (SD = 2.20), and a mean weight of 390.57 g (SD = 20.83) from our laboratory's breeding stock were used. Three days before the stereotaxic procedure they were isolated and kept in individual cages (50 × 22 × 14-cm, plastic bottomed and sawdust-bedded). The animals were kept under conditions of controlled temperature and humidity, and subjected to an artificial 12-h light/dark cycle (light on at 08:00). The experimental work was carried out during the first half of the light cycle. All subjects were in an ad libitum regime of food and water. All procedures were carried out in compliance with the European Community Council directives for care and use of laboratory animals and were approved by the institutional animal care committee.

2.2. Surgery

Previous to the surgery, two sessions of handling took place in order to diminish emotional reactivity of the animals towards experimental manipulation. Under general anesthesia (150 mg/kg Imalgène® ketamine chlorhydrate (Merial, Lyon, France) and 0.08 mg/kg Rompun® xylazine (Bayer, Barcelona, Spain); i.p.), all rats were chronically implanted with a monopolar stainless steel electrode (150 μm in diameter) aimed at the right lateral hypothalamus (LH) into the fibers of the MFB, according to coordinates from the stereotaxic atlas of Paxinos and Watson [26], anterior: −1.8 mm from bregma, lateral: 2.0 mm (right hemisphere) and ventral: −8.5 mm with the cranium surface as the dorsal reference. In the post-surgery recovery period (7 days), the animals were weighed and handled daily.

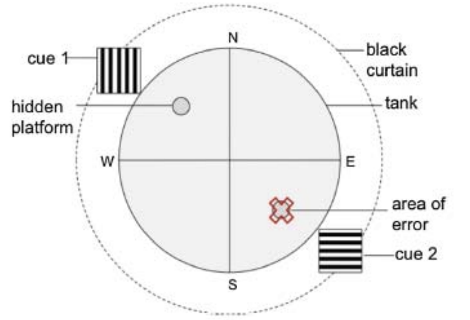


Fig. 1. Representation of one of the configurations for MWM in the simultaneous visual discrimination task. *Escape area* is associated to cue 1 and illustrated with a clear platform. *Area of error* represents the "no escape" associated to cue 2 and is signaled with a black X.

2.3. Group designation and ICSS behavior shaping

The rats were randomly distributed into two groups, Sham and ICSS, according to the independent variable "ICSS-treatment". Subjects in the ICSS group were taught to self-stimulate by pressing a lever in a Skinner box (25 × 20 × 20 cm). Electrical brain stimulation consisted of 0.3 s trains of 50 Hz sinusoidal waves at intensities ranging from 20 to 250 μA. The optimum intensity (OI), defined by the lowest intensity that led to a stable rate of about 250 responses in five minutes, was established.

2.4. Morris water maze apparatus

The MWM consisted of an elevated circular pool (2 m diameter; 60 cm above the pool floor) filled with water (45 cm height) maintained at 22 ± 2 °C. The pool was in the middle of a semi-dark room and surrounded by black curtains reaching from a false ceiling to the base of the pool forming a circular enclosure 2.4 m in diameter. In an adapted version of the two-platform task of Packard and McGaugh [19], four imperceptible nylon threads hung from the false ceiling at equal distances from one another to provide suspension for the two mobile cues throughout the training. These cues rested in the middle of the virtual quadrant in the tank, 45 cm above the water level, and consisted of identical squares (40 cm^2) with a vertical or horizontal black and white stripes pattern of 1 cm wide stripes, as represented in Fig. 1. For the escape task, a clear Plexiglas platform (11 cm diameter) was placed centrally in one of the four equal quadrants in which the tank was virtually divided, with its top 2 cm below the surface of the water. All swim paths were recorded using a closed-circuit video camera (Smart Video Tracking System, Version 2.5, Panlab) with a wide-angle lens was mounted 1.75 m above the center of the pool inside the false ceiling.

2.5. Behavioral procedure

2.5.1. Acquisition sessions

Seventy-two hours after the ICSS shaping, all subjects were given six daily trials for five days (acquisition sessions). The average intertrial interval (ITI) was 120 s. Starting from one of four different cardinal points (N, E, S and W) in a pseudorandom schedule each water-maze trial consisted of one swim from the edge of the pool to the platform. The correct cue (1) was associated with the escape platform (*escape area*), while the incorrect cue (2) was associated with the area of no escape (*area of error*). When a rat failed to find the platform within 90 s, it was manually guided to the platform for 15 s and then removed from the tank. When a rat found the platform it was left on it for 15 s and then removed from the tank. The position of the two cues was manipulated so that every ten trials the correct cue was closer, farther or at the same distance than the incorrect cue in relation to the starting point. Thus, the correct cue changed quadrants every three trials (½ of each session), while the incorrect cue changed position after each trial among the remaining quadrants (see Fig. S1 in the Supplementary material in the online version at DOI: http://dx.doi.org/10.1016/j.bbr.2016.09.069).

2.5.2. ICSS treatment

Immediately after each SVD acquisition session, the ICSS rats were placed in the self-stimulation box and received the ICSS treatment, consisting of 2500 trains of stimulation at the OI established during the shaping phase for each rat. Rats in the Sham group were instead placed in the self-stimulation box for 45 min after each training session without receiving any stimulation.

2.5.3. Probe test and reversal

The probe test took place seventy-two hours after the last acquisition session and consisted of removing the platform and placing the animal in the pool from the East (E) starting position.

Immediately after the 60 s, the reversal trials were initiated. The platform was placed in the quadrant associated to the incorrect cue and the animal was directed to mount the platform for 15 s and then removed. After 120 s ITI three reversal trials took place, which consisted of the exchange of the cues' associations. Animals were again placed in the tank from the three remaining starting points (N, W, S), and the cues changed quadrants anticlockwise for each trial, which had duration of 90 s and an ITI of 120 s.

2.6. Histology

The animals were transcardially perfused with 4% paraformaldehyde in phosphate buffer 0.1 M (PB; pH = 7.4). The brains were removed and post fixed overnight in the same solution. They were then placed in a 30% sucrose solution before being cut into 40 μm sections on a freezing stage microtome (Cryocut 1800 with microtome 2020, Jung). The tissue was stained with Cresyl Violet and examined for electrode tip placement under a microscope for histological determination of the electrode location.

2.7. Statistical analysis

All statistical analysis was carried out with SPSS statistical package v. 23 (SPSS Inc., Chicago, IL, USA). Analysis was conducted with a 2 × 5 mixed ANOVA (GROUP × SESSIONS) for the acquisition phase, and independent samples *t*-test analysis for the probe test and reversal (the average score of the three trials was analyzed). The main outcome variables for acquisition and reversal in the SVD were: (1) *Escape latency*: time (s) needed to find and climb onto the platform (the maximum value was 90 s), and (2) *Number of errors*: number of contacts with area associated to incorrect cue (no escape). In the probe test *Number of target crossings*, *Percentage of time spent in the target quadrant*, *Number of errors*, *Percentage of time spent in the error quadrant* were compared between groups. In addition, a one-sample *t*-test against a constant (50) was used for each group to determine whether the *Percentage of time spent in the target quadrant* was different from chance level (50%). Moreover, the control variables *Percentage of time spent near the walls* (measure of thigmotaxis), *Length* (total distance in cm) and *Speed* were also analysed. When the effect of SESSIONS factor was statistically significant, polynomial contrasts explored the presence of linear and/or quadratic trends in the performance. A Chi-square test for independence was performed to determine the relation between the group and the strategy used. In addition, a regression analysis was performed to examine the relationship between ICSS parameters and SVD performance. The α level for all tests was set at 0.05.

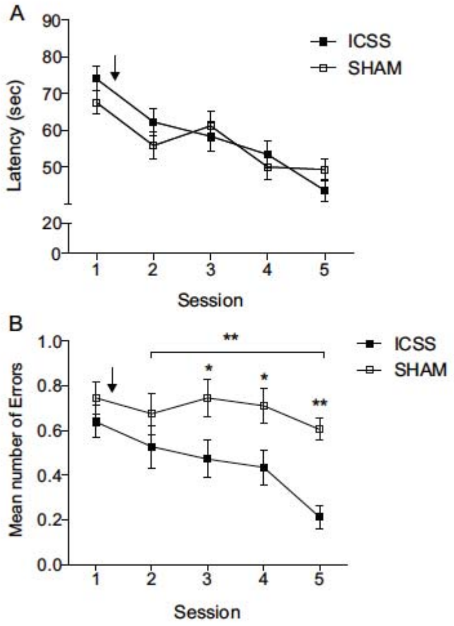


Fig. 2. Facilitative effects of ICSS on acquisition. (A) Mean *Escape latencies* (±SE) for the five acquisition sessions. Arrow shows start of ICSS treatment; (B) Mean *Number of errors* (±SE) committed during the acquisition sessions by each group. Group factor significance is depicted with a horizontal bracket. *P < 0.05; **P < 0.001.

3. Results

A total of five subjects were excluded from the analysis (two subjects lost the electrode in the middle of the treatment, and three did not continue to respond to the ICSS treatment). The final sample consisted of 37 subjects (Sham: n = 19, ICSS: n = 18). There was no statistical difference between groups in weight change.

3.1. Acquisition sessions

Mean *Escape latencies* are depicted in Fig. 2A. The SESSIONS factor was significant [$F_{4,140} = 22.626$, $P < 0.001$], but since interaction GROUP × SESSIONS does not reach significance [$F_{4,140} = 2.034$, $P = 0.09$], a similar evolution between groups can be assumed. In addition, the GROUP factor was not significant [$F_{1,35} = 0.181$, $P = 0.67$]. Furthermore, both groups learnt the task in terms of the decrease of the *Escape latencies*, revealing a significant downward linear function (Polynomial contrast, Sham $P < 0.001$; ICSS $P < 0.001$).

Means of the *Number of errors* made are depicted in Fig. 2B. The main effects of GROUP and SESSIONS are significant [$F_{1,35} = 18.024$, $P < 0.001$ and $F_{4,140} = 4.072$, $P = 0.004$, respectively], but there is no interaction GROUP × SESSIONS [$F_{4,140} = 1.224$, $P = 0.30$]. It is important to point out that no differences between groups were found in session 1 ($P = 0.267$), before the ICSS administration, and the analysis of the sessions 2 to 5 confirms the lower *Number of errors* from the ICSS group [$F_{1,35} = 17.64$, $P < 0.001$]. Additionally, the decrease in the *Number of errors* in the ICSS group followed a linear evolution ($P < 0.001$), while the Sham group did not ($P = 0.268$). Furthermore, a simple effects analysis found that the differences between groups appeared in the third session ($P = 0.025$), were maintained in the fourth session ($P = 0.018$) and were stronger in the last acquisition session ($P < 0.001$).

No differences were found for GROUP × SESSIONS for control variables: *Percentage of time spent near the walls*, *Length* or *Speed*.

3.2. Probe test

ICSS group had a higher *Number of target crossings* than the Sham group [*Welch's* $F_{1,23.95} = 4.974$, $P = 0.035$] (Fig. 3A). Moreover, the ICSS group also showed a higher preference for the *target quadrant* [$t_{35} = 17.848$, $P < 0.001$] (Fig. 3B), although neither group performed above chance level (Sham: $t_{18} = -2.744$, $P = 0.86$; ICSS: $t_{17} = -1.19$, $P = 0.45$).

Concerning the *Number of Errors* (Fig. 3C), the ICSS group committed fewer errors than the Sham group during the first 30 s of the probe test [*Welch's* $F_{1,30.572} = 6.687$, $P = 0.015$]. Finally, means of *Percentage of time spent in the error quadrant* were not statistically different between groups, although Sham animals showed a tendency for higher preference [$t_{35} = 3.767$, $P = 0.06$].

3.3. Reversal trial

The ICSS group had significantly higher *Escape latencies* than the Sham group [$t_{35} = 4.532$, $P = 0.04$] (Fig. 4A). Moreover, the ICSS group made significantly more *errors* than the Sham group [*Welch's* $F_{1,24.615} = 5.113$, $P = 0.03$] (Fig. 4B).

No differences were found between groups for control variables (*Percentage of time spent near the walls*, *Length* or *Speed*) either in the probe test or in the reversal session.

3.4. Swimming trajectories

The qualitative analysis of swimming trajectories revealed that rats followed two defined strategies. While some animals performed the task in a "direct" manner (Fig. 5A), others appeared to have applied a "trial and error" strategy (Figs. 5B and C), which is exemplified by a frequent visit to the incorrect cue's associated area (error area) before choosing to approach the correct one. A chi-square test confirmed that the "trial and error" strategy was preferred significantly by the Sham group, while the ICSS-treated animals adopted a "direct" strategy ($\chi^2_{1,37} = 10.078$, $P = 0.006$). A contingency table (Fig. 5D) displays the frequency and percentages for each group and strategy.

At the same time, some animals that appeared to have chosen the correct cue failed to mount the platform due to their trajectory missing the target by a few millimeters. Fig. 6 shows two examples (Figs. 6A and B) of this behavior.

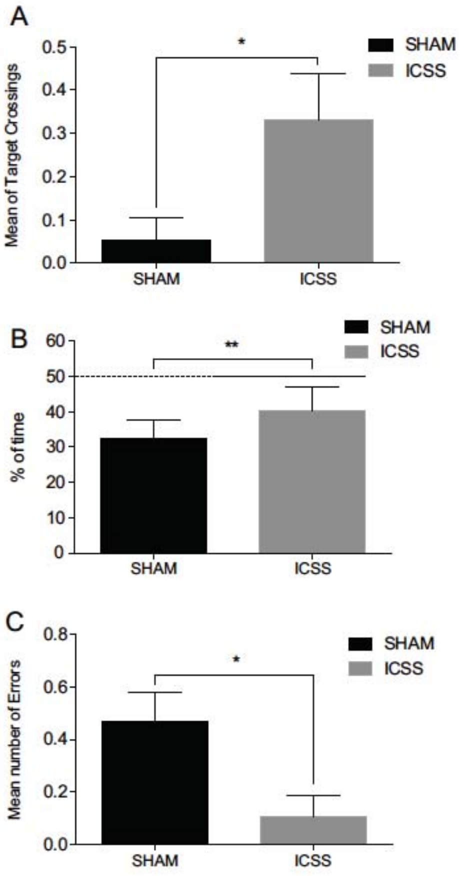


Fig. 3. Facilitative effects of ICSS on the probe test. (A) Mean Target crossings (±SE); (B) *Mean Percentage of time spent in target quadrant* (±SE). The dotted line represents chance level; (C) Mean *Number of Errors* (±SE). * $P < 0.05$; ** $P < 0.001$.

3.5. Histology

Evaluation of the location of the electrode tip under the microscope revealed that they were all between AP −1.80 mm and −3.14 mm from Bregma according to the stereotaxic atlas [26]. Additionally, a regression analysis showed that there is no relationship between the histological location of the electrode tip and the ICSS parameters or the performance in the SVD sessions.

4. Discussion

The present results indicate that post-training ICSS treatment facilitates the acquisition and retention of a visual discrimination task in the MWM. Although the escape latencies were equal for both groups, the ICSS subjects committed fewer errors than the Sham animals during the acquisition and the probe test. During the probe test, which assessed the memory retention after 72 h, the ICSS group spent more time in the target quadrant and achieved more target crossings than the Sham subjects, indicative of a higher level of memory in the ICSS subjects. Present results are in agreement with those obtained in our laboratory regarding ICSS facilitation of the acquisition and retention of implicit avoidance memory tasks [3,4].

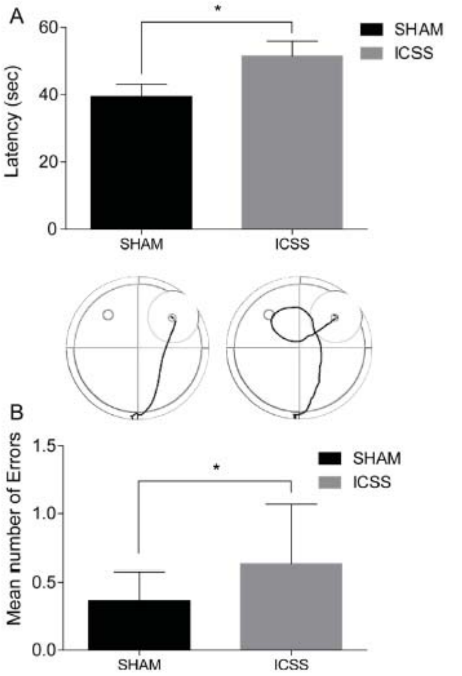


Fig. 4. Impairing effects of ICSS on reversal. (A) Mean *Escape latencies* (±SE); (B) Mean *Number of errors* (±SE). An example of the trajectory of Sham and ICSS animals is presented above corresponding bars. * P < 0.05.

Furthermore, during the reversal phase ICSS treatment caused higher escape latencies and number of errors than the non-treated animals. Our results in the reversal test are also consistent with Hirsh's [25] consideration of a visual discrimination task as an inflexible and associative memory process and with previous work on the difficulty of achieving the reversal of a well-consolidated visual discrimination task [27]. One aspect we believe to be of paramount importance is the fact that throughout the three phases of the experimental design, results consistently showed that the

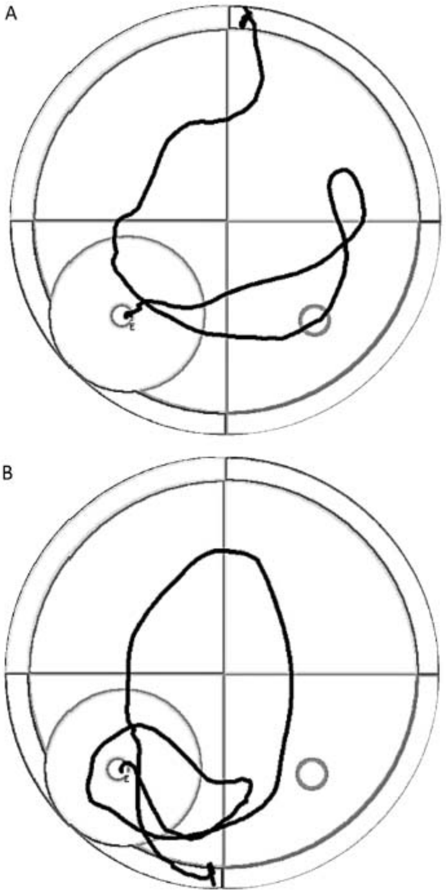
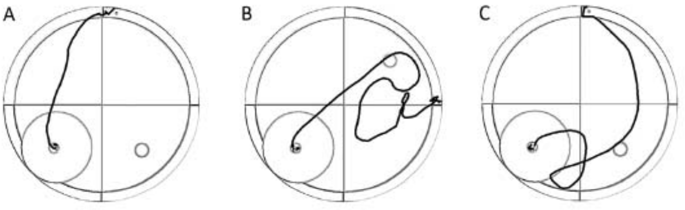


Fig. 6. Swimming trajectories of rats missing the platform. The trajectory of some animals that approached the platform but failed to located it. This behavior could explain the lack of differences in latency between groups. Both figures belong to ICSS subjects.



D

		STRATEGY			TOTAL
		Trial and Error	Direct	N/a	
GROUP	Sham	**68.4%** (13)	10.5% (2)	21.1% (4)	100% (19)
	ICSS	22.2% (4)	**55.6%** (10)	22.2% (4)	100% (18)

Fig. 5. Swimming trajectories in the acquisition. Images of direct vs trial and error swimming trajectories that the rats used to solve the SVD task. In the lower-left quadrant, the inner concentric circle corresponds to the platform; outer concentric circle comprises the target zone; and in the lower-right quadrant, the circle represents the error area. (A) ICCS animal; (B) and (C) Sham animals; (D) Percentage of animals preferring "trial and error" or "direct" strategies, by group. N/a: non-applicable. Frequency is shown in brackets after the percentage.

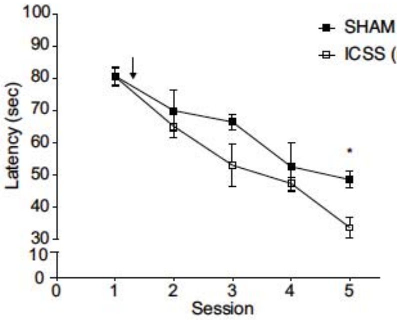


Fig. 7. Significant effects of ICSS on acquisition latencies when cues were closer to the MWM. This figure depicts the Mean *Escape latencies* (±SE) for the acquisitions sessions of a complementary experiment where distance between cues and *Area of escape* and *Area of error* was reduced. Arrow shows start of treatment sessions. * P < 0.05.

mean number of errors of each group was indicative of the facilitative effect of the ICSS treatment upon the visual discrimination task. Altogether, quantitative and qualitative results revealed that the ICSS rats applied a more efficient and direct strategy than Sham animals to learn the task and that ICSS treatment promotes a stronger and better-consolidated perceptual memory. As far as we know, this is the first time that the facilitation of the acquisition and retention of a visual discrimination task by post-training ICSS has been demonstrated.

On the other hand, and contrary to what we had expected, there were no differences between groups for the escape latencies in the acquisition sessions. This may seem contradictory with the idea of ICSS facilitation of the task, but these findings are similar to those of Packard and McGaugh [19], where escape latencies to mount the platform did not completely reflect the behavioral deficit observed in rats with lesion of the caudate nucleus. There are several possible explanations for this lack of differences. Looking at the trajectories of ICSS and Sham animals, some of them seem to experience difficulty finding the exact location of the escape platform as it is illustrated in Figs. 6A and B, probably because the cue was too far from the platform. Therefore, even if rats accurately identify the correct cue, failing to find and mount the platform could cause the animal to retreat from the area in search of the alternative cue. This translates into valuable lost time, which would directly affect the escape latency values in the overall results. Thus, reducing the distance between the cues and the areas of *Escape* and *Error* would help avoid this type of error. In fact, a complementary experiment performed in our laboratory confirmed this hypothesis. As it is shown in Fig. 7, when the distance between the edge of the tank and the cue was reduced from 35 cm to 25 cm, the ICSS group solved the task showing significantly lower latencies than the Sham animals in the last acquisition session. Other studies performed in the MWM where the visual discrimination cues were separated from the platforms have prevented this effect by concomitantly using contextual cues, providing spatial information that facilitate the task [28].

Furthermore, the different strategies followed by the animals and the lower number of errors committed by ICSS group, suggests that ICSS treatment promotes a discriminatory perceptive learning process instead of a "trial and error" strategy. As in the aforementioned study by Packard and McGaugh [19], our present results also show that the *number of errors* could be a more sensitive measure than latencies to detect the degree of facilitation in the acquisition of visual discrimination tasks.

Moreover, this improvement could be related to increases in some excitatory neurotransmitters levels, such as dopamine (DA), acetylcholine (ACh) or glutamate (GLU), in the hippocampus and cortical regions [10,29]. In that sense, it has been observed that the blockade of DA and ACh [30], or GLU [31] results in a higher number of errors to criterion in this kind of tasks, while an ACh blockade also impairs the strategy selection [32]. Additionally, lesions of the cholinergic nucleus basalis magnocellular have been shown to increase perseverative errors in a simple-stimulus response visual perceptual task [33], which could be linked to the modulatory effects of ACh on the attentional aspects of the task [34]. Furthermore, evidence from human studies shows that an enhanced efficacy of the cholinergic system's function facilitates the consolidation processes in a visual discrimination task [35] and promotes long-lasting improvements in perceptual learning [36]. All in all, an increased function of some excitatory neurotransmission systems activated by ICSS in memory-related regions, such as the hippocampus and cortex, could explain the lower number of errors and the accurate trajectory portrayed by ICSS-treated subjects in the present experiment. The specific mechanisms through which the ICSS could promote the activity of these memory-modulatory neurotransmitters are yet to be defined. However, descendent fibers from the MFB could explain such an increase, since ICSS functionally activates monoaminergic and cholinergic brainstem regions, such as the locus coeruleus, the ventral tegmental area and the pedunculopontine area [37].

In summary, the main goal of the present research was to determine whether a visual discrimination task, in which the perceptive component is critical, could be facilitated by post-training administration of ICSS in the MFB. ICSS led to the animals not only committing fewer errors, but also to them using a more accurate strategy to solve the task. Moreover, cognitive flexibility assessed by a reversal test was compromised by a strengthened memory consolidation. An implication of some excitatory ICSS-related transmitters is suggested. As far as we know, this is the first time that ICSS has been able to facilitate this type of implicit-perceptual learning and its retention. These findings, together with previous research in our laboratory, contribute to the establishment of post-training ICSS in the MFB as a generic treatment useful for facilitating a wide range of learning tasks and procedures.

Acknowledgments

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