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**THE ROLE OF THE HIPPOCAMPUS AND POST-LEARNING HIPPOCAMPAL
ACTIVITY IN LONG-TERM CONSOLIDATION OF CONTEXT MEMORY**

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B. Sc., University of Lethbridge, 2009

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ABSTRACT

Sutherland, Sparks and Lehmann (2010) proposed a new theory of memory consolidation, termed Distributed Reinstatement Theory (DRT), where the hippocampus (HPC) is needed for initial encoding but some types of memories are established in non-HPC systems through post-learning HPC activity. An evaluation of the current methodology of temporary inactivation was conducted experimentally. By permanently implanting two bilateral guide cannulae in the HPC and infusing ropivacaine cellular activity could be reduced by 97%. Rats were trained in a context-fear paradigm. Six learning episodes distributed across three days made the memory resistant to HPC inactivation while three episodes did not. Blocking post-learning HPC activity following three of six training sessions failed to reduce the rat's memory of the fearful context. These results fail to support DRT and indicate that one or more memory systems outside the HPC can acquire context memory without HPC post-event activity.

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LIST OF ABBREVIATIONS

aCSF	Artificial Cerebrospinal Fluid
ANOVA	Analysis of variance
AP	Action Potential
AM	Morning (12-midnight to 12-noon)
Ca ⁺	Calcium ion
CA1	Cornu Ammonis 1
CA3	Cornu Ammonis 2
Cl ⁻	Chloride ion
CR	Conditioned Response
CS	Conditioned Stimulus
DAB	3,3'-Diaminobenzidine Tetrahydro-Chloride
DG	Dentate Gyrus
GABA-r	Gamma Aminobutyric Acid receptor
dHPC	dorsal Hippocampus
DRT	Distributed Reinstatement Theory
EC	Entorhinal Cortex
HPC	Hippocampus
IEG	Immediate-Early Gene
KCl	Potassium Chloride
LTP	Long-Term Potentiation
MECS	Maximal Electric Convulsive Shock
MRN	Median Raphe Nucleus
MTL	Medial Temporal Lobe
MTT	Multiple Trace Theory
Na ⁺	Sodium ion
NMDA	N-Methyl-D-Aspartic acid
PAG	Periaqueductal Grey
PFA	Paraformaldehyde
PM	Afternoon/Evening (12-noon to 12-midnight)
RA	Retrograde Amnesia
RNA	Ribonucleic Acid
ROP	Ropivacaine
SMSC	Standard Model of Systems Consolidation
SWP	Sharp-wave Ripple events
TTX	Tetrodotoxin
US	Unconditioned Stimulus
vHPC	ventral Hippocampus
vlPAG	ventro-lateral Periaqueductal Grey

CHAPTER 1: GENERAL INTRODUCTION

Every day we experience many different episodes. Some may be similar to episodes you have experienced previously, and some may be completely unique. Some experiences are forgotten after a few hours, days, or years, while others may be remembered for the rest of our lives. Information about place, time, people you are with, sounds, smells and emotions can all be integrated to form a vivid memory that can be recollected at a later point in time. Almost all of us take memory abilities for granted, but how the brain accomplishes memory skills is still largely a mystery. How is memory stored, how is it consolidated from a short-term state to a long-term state, where in the brain is it stored and how come some things are forgotten while others are not, are still uncertain. In spite of many decades of sustained scientific investigations, we are still far from having a complete understanding of how memory works. There are however, some prevalent theories as to how the brain organizes and stores our everyday experiences.

Memory can be divided into several subdivisions. These subdivisions were neatly mapped out by Larry Squire and Stuart Zola in 1996, and include skills and habits, motor-memory, memory of facts and memory of personal experiences. According to them conscious or declarative memories are memories of world facts and autobiographical events. These memories can be reported if someone were to ask you about them and they depend on structures located within the medial temporal lobe (MTL; Squire & Zola, 1996). These are also the type of memories most commonly referred to when memory is being discussed.

In 1957 Scoville and Milner published the first article describing the world-famous amnesic patient H.M. In 1953 H.M. had parts of his brain removed as an attempt to relieve him of his epilepsy. The tissue excised was in the MTL and included structures such as the hippocampus (HPC), the amygdala, and adjacent cortical regions. After recovering from the surgery, H.M. had seemingly lost his ability to form new declarative memories, however, his non-declarative memories, such as motor learning, were still intact (Corkin, 1968). The initial observation that H.M. had only lost his most recent autobiographical memories (Scoville & Milner, 1957) led to speculations as to how memory is stored in the brain. Marr (1971) suggested that memories were stored in the neocortex, and that they only relied on the HPC for a short period of time after initial learning. He proposed that the HPC was important for rapidly acquiring information and it was important in establishing a permanent neocortical trace that contained information about the constancies in information across episodes. The neocortical memory became HPC-independent after a few episodes of sleep. This theory was elaborated on by Squire, Cohen and Nadel (1984) who first described the Standard Model of Systems Consolidation (SMSC; Rudy, 2008). This theory elaborates on Marr's idea of the memory relying on the HPC for only a short period of time. The SMSC suggests that new memories rely on the MTL and the HPC in that it stores an index which connects multiple traces between multiple cortical areas and the HPC. Recalling the memory activates the HPC trace which in turn activates multiple regions in the neocortex. As the memory strengthens, the neocortical areas form their own interconnections until they are strong enough to be activated without the input from the HPC. The memory has now consolidated in the neocortex and does not depend on the MTL or HPC for retrieval. The

time interval for this form of consolidation to occur is thought to vary from 3 days to decades (Sutherland & Lehmann, 2011).

Even though the SMSC seems to explain many of the observed phenomena related to amnesia, it does not explain them all. Nadel and Moscovitch (1997) conducted a review of the available literature from human studies and found a difference between autobiographical memories and factual memories (the difference between knowing last time you ate an apple and knowing that an apple is round). They concluded that if the entire HPC was gone, there were no autobiographical memories spared at all. A re-evaluation of patient H.M. (Steinvorth, Levine, & Corkin, 2005) confirmed that all his autobiographical memories were lost. Based on these more recent findings, a modification of the SMSC was suggested, referred to as the Multiple Trace Theory (MTT; Nadel & Moscovitch, 1997). This theory modified the existing SMSC by dividing semantic memory into autobiographical and factual memory claiming that autobiographical memory is always dependent on the HPC, while factual memory only temporarily depends on the HPC. Each re-activation of an autobiographical memory leads to factual information about the event being extracted and stored elsewhere in the cortex where it is then integrated with pre-existing semantic memory. Every time a memory is being recalled, a new trace is formed in the HPC with most of or all of the same neocortical connections as the previous trace. Older memories would have been re-activated more frequently and therefore have more traces in the HPC. This is why these memories are most likely spared when only parts of the HPC are removed.

There are still controversies about memory storage left to be explained. Both SMSC and MTT give the HPC a role where it is always needed for new complex

memories to be formed. This does not explain the data presented by Wiltgen, Sanders, Anagnostaras, Sage and Fanselow (2006) where rats given dorsal HPC (dHPC) damage following training in context fear conditioning showed complete retrograde amnesia (RA) while rats given dHPC damage before training performed as well as controls. These data suggests that there is a memory system capable of learning new tasks independent of the HPC. More data to support this were presented by Lehmann et al. (2010) who showed that rats exposed to 12 pairings of context-shock followed 7-10 days later by complete HPC lesion showed RA. On the other hand, exposing rats to 10 pairings distributed over five days made the memory HPC-independent and no RA were seen. This indicates that while the HPC is undamaged other memory systems can acquire the memory as long as the pairings are multiple and distributed, and with a damaged HPC these systems enable the animal to learn as fast as controls. These results cannot be explained by SMSC as time was not a factor which could explain the different results. In both experiments testing was done 17-20 days after the first pairing. MTT favors the multiple exposures over the temporal aspect when explaining why some memories are spared, but it does not explain why there is a difference if the pairings are distributed or not. Even though these two theories explain many of the observed phenomena in the memory literature, they both fail to explain it all.

The most recent alternative to the SMSC and MTT came from Sutherland, Sparks, and Lehmann (2010) and Sutherland and Lehmann (2011). After careful reviews of the rat literature on HPC lesion and retrograde amnesia the authors suggested two variations of long-term memory consolidation. The first idea is a dual-storage model in which the HPC as well as non-HPC memory systems independently acquire information.

Importantly, normally when the HPC is intact non-HPC systems are suppressed. This idea helps explain results such as the ones first presented by Maren, Aharonov, and Fanselow (1997) where there was no effect of dHPC lesions on context fear when training and testing both occur after surgery, despite profound RA from the same damage with training taking place before surgery. However, if multiple, distributed learning episodes are experienced, then memories can be established in non-HPC systems (Lehmann et al., 2009). Lehmann et al. (2009) showed that memories established by context-shock pairings occurring multiple times over several days the memory withstood extensive HPC damage. These results clearly demonstrate that a robust long-term context fear memory can be established in non-HPC networks that do not require an intact HPC to be retrieved. On the simplest view, memories can be established rapidly and are dependent on the HPC for maintenance and with repeated spaced learning episodes memories can be established in parallel in non-HPC networks. A less parsimonious explanation of the phenomena observed in the two last-mentioned studies is outlined in the Distributed Reinstatement Theory (DRT) (Sutherland et al., 2010). Non-HPC networks acquire only weak memories that are strengthened by bouts of HPC activity (so-called *replay*) triggered by successive learning episodes. When very similar information is reiterated, for example if there are multiple, spaced context-shock pairings, the post-event bouts of HPC replay lasting perhaps hours, incrementally strengthens the non-HPC memory which eventually reaches a threshold enabling activation by relevant cues in the absence of the HPC.

Even though all the theories presented to date are different, and offer different explanations as to how the brain stores memories, they all have certain similarities. There

is little debate on whether or not the HPC is important for memory, and in the more recent years – following the presentation of the MTT – it is a general agreement that autobiographical memories always depend on the HPC. However, there is far less certainty in how the HPC communicate with the rest of the cortex and how some memories seem to be still remembered after HPC damage as well as some information can be learned and remembered following HPC damage. The aim of this thesis is to experimentally distinguish between the two alternative hypotheses presented by Sutherland, Sparks, and Lehmann (2010), namely parallel memory encoding in HPC and non-HPC networks vs. establishing non-HPC memory in means of HPC replay activity. Here I ask if post-event HPC activity is necessary for long-term context memories to be established elsewhere.

Hippocampal Replay

In 1973 Bliss and Lømo discovered that by electrically stimulating HPC neurons at a specific frequency they would get long term changes in the synapses and how the neurons communicated. This process became known as long-term potentiation (LTP) which is defined as a persistent strengthening of synapses produced by a low-frequency, intense electrical stimulation (Rudy, 2008). LTP became the description of how memories are stored at the cellular level as the changes observed with LTP were long-lasting. Marr (1970) hypothesized that in order for these synaptic changes to happen within the HPC, there must be as little sensory stimulation as possible. The only time when this event could take place would therefore be during sleep. In 1989, Buzsaki described the phenomenon of sharp-wave ripple events (SWP) happening within the HPC

during sleep. SWP is a specific characteristic of neuron electrical activity. These SWP arose in the HPC and was propagated out to the output layers of the entorhinal cortex (EC), and from where they could propagate out to the neocortex. Buszaki also claimed that the SWP provided optimal conditions for synaptic plasticity in afferent neurons. Palvides and Winson (1989) showed that hippocampal neurons that have been selectively activated during a prior episode when the rat was awake are selectively more active during subsequent sleep, especially the part of the sleep cycle known as slow-wave sleep (SWS). McClelland et al. (1995) concluded this as evidence of the HPC *replaying* memories from recent experiences. Replay is present not only during sleep, but also during quiet wakefulness. It is seen after a task is completed, and decline substantially over about 30 min. However, a small degree of replay can still be seen in the HPC 24 hrs following task completion (Hoffman & McNaughton, 2002). Neuronal firing patterns related exclusively to SWS has also been found in the outside of the HPC, in the neocortex. Siapas and Wilson (1998) demonstrated that there is a temporal correlation between the neocortical firing patterns and the SWP found in the HPC. These events are not observed following tasks that do not depend on the HPC (Kali & Dayan, 2004). The replay co-occurring in the neocortex and HPC has been thought to support a transfer of memory representations, which eventually leads to memories being stored in the neocortex.

The hypothesis of the co-activation of neocortical and HPC replay being important in the process of memory consolidation is clearly compatible with SMSC in which memories only temporarily depend on the HPC before they are consolidated in the neocortex. There might be some difficulty with this relationship if the probability of

replay is very low after 1 day, since most SMSC theorists posit the systems consolidations to occur over weeks to months. Also the DRT describes post-event activity, such as replay, as being an important feature of making memories independent of the HPC. The evidence of the role of replay is, however, still only largely correlational. To shine further light on the role of HPC, we aim to disrupt this activity following training sessions of context fear to evaluate whether or not non-HPC memory systems can still acquire the memory.

Context Fear Memory

Classical conditioning is a learning paradigm where a neutral stimulus elicits a response because of its predictive relationship with a specific event (Kolb & Whishaw, 2006). Pavlov first described this process in 1927 when testing dogs; the presentation of food (conditioned stimulus; CS) elicited salivation (conditioned response; CR). By pairing a tone (unconditioned stimulus; US) and food together multiple times, the tone was eventually enough to produce the salivation alone – without the presence of food. The pairing of a US and a CS to elicit a CR has been used in multiple behavioural paradigms. In the rat, a common version of this is contextual fear conditioning, where the acquisition and retention of an aversive stimuli (such as a foot-shock) is connected to a specific context (Antoniadis & McDonald, 1999). Memory is usually measured by the presence of an involuntary response (such as freezing) when the rat is placed back into the context. Fear conditioning to context is a popular measure of learning as it is rapidly acquired and the memory last for several months (LeDoux, 1994).

Context fear conditioning is a frequently used behavioural paradigm and there is a general agreement that the anatomical circuitry involved in eliciting a freezing response involves mainly the HPC and the amygdala. Antoniadis and McDonald (1999) outlined a pathway where the freezing response commonly measured as an indication of learning and memory is mediated by a pathway that relies on both of the structures as well as the periaqueductal grey (PAG) and the median raphe nucleus (MRN). This pathway can produce a freezing-response after only one pairing of context and shock, and damage to the amygdala or the HPC after training blocks the learned response (Antoniadis & McDonald, 1999).

O'Keefe and Nadel (1978) proposed that the HPC was involved in creating and storing spatial maps of environments. Considerable experimental support emerged thereafter, and in 1992 Kim and Fanselow showed that contextual fear memory is lost following a HPC lesion. Bannerman et al. (2004) divided the hippocampus in to two functional regions, where the dHPC had a preferential role in learning context memories, and the ventral HPC (vHPC) was important in anxiety-related behaviours. Because the vHPC has strong connections with the medial baso-lateral amygdala (Alvarez & Ruarte, 2002) and the dHPC get its input from the EC, (Corbit, Ostlund, & Balleine, 2002) there is anatomical evidence to support the regional differences in behaviour. The amygdala has been shown to be a key structure in emotional memory (LeDoux, 1994; Kolb & Whishaw, 2006), specifically in anxiety and fear memory. Set in the perspective of fear conditioning, the amygdala seem to be responsible for the feelings of anxiety towards a context that by itself would not normally produce freezing (Kolb & Whishaw, 2006). Even though both the amygdala and the HPC play important roles in the expression of

freezing in response to remembering an aversive context, it is based on the current literature safe to say that the HPC plays a critical role in this task.

There are also clear anatomical connections between the amygdala and the other structures implemented in playing a part in contextual fear conditioning and the freezing response. The ventro-lateral PAG (vlPAG) seem to be most involved in context fear as temporary inactivating this region reduces conditional freezing (Carrive, Lee, & Su, 2000). The amygdala and the vlPAG share direct and reciprocal connections through the central nucleus of the amygdala as well as with the MRN (Vivanna & Brandao, 2003). Rats that have had lesions to the MRN show decreased freezing in a contextual fear paradigm (Avanzi & Brandao, 2001).

We have chosen to use contextual fear conditioning in the main experiment because other studies have shown that a rat can learn context fear in the absence of the HPC (Maren et al. 1997). Furthermore, multiple exposures spread out over several days create a fear memory resistant to HPC damage, whereas the memory created by the same number of context-shock pairings in the same session is not (Lehmann et al., 2009). Based on these results it is clear that context fear often is dependent on the HPC, but that other memory systems are capable of learning the association in the absence of the HPC. These findings make it a good paradigm to test if HPC replay is the mechanism responsible for making the memory HPC-independent.

Temporary Inactivation

For decades psychologists and neuroscientists have studied patients with permanent lesions in order to understand brain function (Lomber, 1999). This kind of

research has led to some incredible and very important findings within our field (Scoville & Milner, 1957 as an example) but it does present some problems. Naturally occurring lesions or lesions from surgery are usually not restricted to one structure within the brain. It is also hard to find two patients with exactly the same lesion. And because a patient usually do not subject themselves to testing prior to having a lesion, it can be hard to establish what effect the lesion had on the individual. The lesion method has been applied to animal experimentation where these kinds of factors can be better controlled.

However, there are still some caveats to consider when using permanent lesions. When performing surgery on an animal a significant amount of time is required for postoperative recovery. The timeframe of recovery can cause some problems. If the testing starts too soon after surgery there is little control over whether or not the changes seen are caused by the general trauma of surgery or by the removal of brain tissue (Lomber, 1999). However, if testing starts too long after surgery, there might have been plastic changes occurring within the brain which compensate for the loss of tissue, allowing the animal to carry out a task but relying on cortical structures other than the ones used by an undamaged brain (Lomber, 1999). This kind of recovery would give rise to false results and false interpretations of the data. Other issues to consider with permanent lesions are destruction of fibers of passage which could cause damage to brain structures distant to the lesion site, destruction of blood supply, and the chance of causing permanent seizures (Lomber, 1999). Because of the obvious large drawback of permanent lesions – that they are indeed permanent – another method of studying functions of specific brain regions is becoming more popular. This technique is known as

temporary inactivation, where targeted regions of the brain are shut down for a specific time interval.

In 1968, Avis and Carlton described a method of inactivating electrical activity in the HPC by injecting potassium chloride (KCl). The disruption was enough to produce RA for events learned 24 hrs prior to the injection. This was the first paper that recognized that by suppressing HPC electrical activity one could achieve amnesia. Following this event many methods have been described for temporarily inactivating parts of the brain. Techniques are either chemical or cryogenic and consist of either injecting an agent in to the target region or freezing parts of the cortex (Lomber, 1999). The benefits of these techniques are great as it eliminates many of the problems observed using permanent lesions. Because chemicals can be injected in to the brain without any extensive surgical intervention and has almost immediate function, the effect of surgery trauma or “recovery of function” is avoided. The technique had provided researchers with much greater flexibility when it comes to research design, and a greater number of new studies can now be pursued (Lomber, 1999). Different chemicals inactivate the cortex at different time intervals, and it is possible to regulate wither or not fibers of passage are affected (for review, see Lomber, 1999). One disadvantage in using temporary inactivation in replacement of permanent lesions is that it is hard to compare the results to lesion data from the human literature (Lomber, 1999). However, for research whose aim is not to compare results across fields the method is often preferable.

Even though temporary inactivation has been employed for decades, there are still great inconsistencies within the literature as to what methods are used, and most papers published does little to establish the extent of HPC inactivation or the accurate temporal

properties of their techniques (Sutherland, Sparks, Lehmann, 2010). It is therefore the aim of the first three experiments to establish optimal placement of infusion sites, proper infusion technique, and the temporal properties of the non-toxic local anesthetic ropivacaine. For the purpose of this thesis the dHPC will be defined as the septal half of the structure while the vHPC will be defined as the temporal half.

Immediate Early Genes

Immediate-early gene (IEG) is a term that describes a group of genes which all responds rapidly after cellular stimulation (Sweatt, 2003). IEGs code for proteins with a wide variety of functions. One major category is transcriptional factors, which includes cFos, cJun, Jun-B, and zif268 (Sweatt, 2003). These genes are believed to function in coupling short-term signals to long-term changes within the neuron by altering the target gene expression (Curran & Morgan, 1994). cFos is considered to be an IEG because its synthesis is directly triggered by stimulating the target cell and is always present in resting neurons at very low concentrations (Purves et al. 2004). When the neuron is stimulated, IEG RNA is synthesized within 5-15 min (Sweatt, 2003) and the target protein is visible within the cytoplasm after 30 min and the amount rises dramatically until 60 min after stimulation (Purves et al. 2004). The cascade of events related to the neuronal action potential (AP) leads to elevated intracellular Ca⁺ levels which in turn results in cFos expression (Morgan & Currant, 1986). Due to the connections between APs and cFos the largely synchronized and extensive activation of neurons that occurs during seizure can provoke cFos expression in nearly all cFos producing cells (Curran & Morgan, 1994). The production of cFos plays a role in stimulus-response coupling that is

common to most cell types (Morgan & Curran, 1991); in this sense, cFos can be considered as a marker of gene activation (Pompeiano, Cirelli, Arrighi, & Tononi, 1995) during both seizures and immediately following behaviour. The visualization of cFos can be used to map brain areas which were active during a specific task (Pompeiano et al. 1995).

Accordingly, cFos serves as an appropriate tool for the present experiments allowing measurement of spatial and temporal extent of HPC inactivation. We will take advantage of cFos expression generated by electrically induced seizures during the first three experiments where we aim to establish a reliable method of temporarily inactivating the HPC.

Our fourth experiment consists of training rats in a context fear paradigm similar to that used by Lehmann et al. (2009) and consequently blocking the replay activity following the training events in order to evaluate the role of HPC replay activity in memory consolidation. When testing for consolidation, temporary inactivation will be used in place of a permanent lesion, and cFos expression will be used to confirm the effectiveness of HPC inactivation during testing.

CHAPTER 2 – ONE VS. TWO INFUSION SITES

Introduction

Following Avis and Carlton's (1968) temporary inactivation of electrical currents in the HPC there have been numerous studies conducted that include reversible inactivation targeted at different brain structures. In spite of the increase seen in these kinds of studies the past decades, there are still great inconsistencies found when evaluating the methodology used by the different researchers. Normal irregularities when evaluating the HPC literature include the number of infusion sites and where within the HPC these sites are placed relative to the study's findings and conclusions. Single bilateral infusions sites are often used to inactivate the entire HPC. For a long time small HPC permanent lesions were used and the behavioural results of these studies were accredited to the HPC structure as a whole. Problems with this and the interpretation of these data are discussed in Sutherland et al. (2010). Small HPC lesions leave a part of the structure still functional, making the conclusion that any behavioural finding being accredited to non-HPC structures somewhat problematic. By using only one single bilateral infusion site to temporarily inactivate the HPC there is a chance that some HPC tissue remains active which can cause a researcher to draw incorrect conclusions from any experimental findings. There has not yet been a study published which would indicate the extent of inactivation one infusion site allows. Therefore, the possibility that a researcher takes experimental findings where only parts of the HPC were inactivated and generalize to the entire HPC remains a problem.

Another major problem that becomes evident when evaluating the recent studies published is that very few evaluate whether or not the targeted structure was successfully inactivated at the time of the experimental testing. In the permanent lesion literature histological data is commonly used to evaluate the success of the surgery. This has not translated in to the temporary inactivation studies where indirect methods or no controls at all are used to evaluate the extent of inactivation. No study so far has evaluated the extent of inactivation at the time of testing. Consideration of these observations provides reason to believe that a careful evaluation of the current methodology is warranted.

The aim of the present experiment is to evaluate the extent of inactivation when infusing a local anesthetic drug in to a single bilateral site aimed at the dHPC using the IEG cFos. cFos is produced within most neuron types in response to APs (Curran & Morgan, 1991) and can therefore be used as a marker for neuronal activation (Pompeiano et al., 1995). cFos can be seen in a neuron soon after relevant behavioural activation or in response to seizures. By using a seizure-inducing method together with infusion of temporary inactivating drugs we can maximize the expression of cFos in large parts of the brain. This will create a reliable control for the inactivation technique and make it possible to evaluate which parts of the brain are inactivated and which parts remain active.

Methods

Subjects: Two adult male Long-Evans rats weighing 600-650 g were included in this study. The rats were housed in pairs in a 12 hr-12 hr light-dark cycle room with access to rat chow and water *ad libitum*.

Surgery: The rats were anesthetized using 4 % isoflurane gas in oxygen flowing at 1.5 l/min. When deeply anesthetized the isoflurane gas was lowered to 2 % and kept between 1-2 % throughout the length of the surgery. The rats were injected with 0.03 mg/kg buprenorphine prior to making the first incision. The rats were installed with 23-gauge stainless steel guide cannulae bilateral in to the dorsal HPC (-3,5 mm in the anterior/posterior direction, ± 2 mm in the lateral/medial direction and -3.5 mm in the ventral direction based on bregma) measuring 10 mm long. Three anchoring screws were tapped into the skull and the cannulae were held in place using dental acrylic. The guide cannulae were occluded using 30 g dummy cannulae which stayed in place until infusion. Following surgery the rats were injected with 0.1 cc/500 g Metacam and kept in the surgical suite for 24 hrs for inspection before being placed back in their home cages for an additional six days.

Infusion/drug: On the test day the rats were brought back in to the surgical room. The dummy cannulae were removed and the rats were restrained by hand and the infusion needles put in place. The rats were infused with 10 mg/ml ropivacaine (ROP; Naropin®, AstraZeneca) through both guide cannulae using a 30 g stainless steel infusion needle extending the same length as the infusion cannulae. A total of 0.7 μ l of drug was infused at 0.29 μ l/min using a 10 ml Hamilton syringe connected to a Harvard infusion apparatus. The needles were left in the guide cannulae for an additional 4.5 min to allow the drug to properly diffuse. The infusion needles were carefully removed and the dummy cannulae were placed back in to the guide cannulae. The rats were then transported back to their home cage for 45 min.

Shock: 45 min after the drug infusion the rats were brought in to a novel testing room in a clear plastic cage with a high plastic lid and a soft towel covering. The rats were then connected to a UGO Basite Maximal Electric Convulsive Shock (MECS) machine using two ear-clamps which had been dipped in saline. A shock was delivered at 100 pulses/sec for 1.1 sec with a pulse width of 5 ms and a current of 85 mA. After the shock administration and the seizure activity had stopped, the animals were transported back to their home cage. Administration of electroconvulsive shock leads to a rapid increase of cFos expression in several regions of the brain, including the HPC. The cFos expression can be blocked with the use of sodium channel blockers (Cole, Abu-Shakra, Saffen, Baraban, & Worley, 1990).

Perfusion: 45 min following the shock the rats were given an overdose of a sodium pentobarbital (Euthansol) and perfused with 1 % buffer and 1% paraformaldehyde (PFA). The brain were extracted and stored in 1 % PFA for 24 hrs before being switched to 30 % sucrose with 0.02 % sodium azide until slicing.

Immunocytochemistry: The brains were sectioned on a sliding microtome at 40 μ m thick and divided into 12 series. One series was labeled immunohistochemically for the IEG cFos using cFos rabbit polyclonal IgG (SantaCruz Biotechnology) against Biotin-SP-conjugated AffiniPure Donkey Anti-Rabbit against peroxidase-conjugated streptavidin (both from Jackson ImmunoResearch laboratories Inc.). To view the labeling we used 3.3'-Diaminobenzidine tetrahydro-chloride (DAB).

Stereology: Unbiased counts of the cFos protein were made using optical fractionator in StereoInvestigator 9.03 32-bit from MBT Bioscience-MicroBrightfield, Inc. HPC subregions CA1, CA2, CA3 and DG were traced and counted together, starting

at the first section showing both DG and CA3, and ending on the last section showing both DG and CA1. The dorsal and ventral HPC was counted separately and the distinction was made so that the superior parts of the HPC were counted as dHPC, while inferior and rostral parts of the HPC were counted as vHPC. Every 12th section through the whole HPC was counted. Section thickness was measured at every 3rd counting site. Top and bottom guard-zone was set at 5 μm to decrease any chance of double-counting. The use of stereology and optical fractionator has been shown to be less biased and more accurate than other more direct counting methods when evaluating cFos expression (Mura, Murphy, Feldon, & Jongen-Relo, 2004).

Statistical analysis: The cFos cell counts for the dHPC and vHPC were calculated as a percentage of total amounts of cells expressing cFos. Standard error bars indicate standard error of the mean (SEM) (sample standard deviation $/\sqrt{n}$). Ratios in fig. 2.3, were calculated based on the number of cFos expression cells counted in the dorsal (septal) or ventral (temporal) part of the HPC vs. the total number of cFos-positive cells in the whole HPC (dHPC cFos / total cFos counted within the whole HPC * 100).

Results

Infusion sites during temporary inactivation of the HPC 2001-2011

Author	Target structure	Extent of inactivation	Number of sites
Holahan & Aryeh, 2011	CA3 of dorsal HPC	not mentioned	Bilateral
Telenesky et al., 2011	HPC	not mentioned	Bilateral
Cimadevilla et al., 2011	HPC	not mentioned	Bilateral dHPC
Lasster et al. 2010	vHPC	not mentioned	Bilateral vHPC, DG, dHPC
Parsons & Otto, 2010	dHPC	not mentioned	Bilateral dHPC
McEown & Treit, 2010	dHPC or vHPC	Estimated	Bilateral vHPC or dHPC
McDonald et al., 2010	dHPC	not mentioned	Bilateral dHPC
Jo & Lee, 2010	HPC	not mentioned	Bilateral dHPC
Kelemen, & Fenton, 2010	left or right HPC	not mentioned	Bilateral dHPC

Gomes et al. 2010	CA1	not mentioned	Bilaterally above CA1
Cohen et al. 2009	dHPC	not mentioned	Bilaterally
Iordanova et al., 2009	HPC	not mentioned	Bilateral dHPC
Cimadevilla et al., 2009	unilateral HPC	not mentioned	Bilateral right dHPC
McEown & Treit, 2009	dHPC or vHPC	not mentioned	Bilaterally dHPC or vHPC
Esclassan et al., 2009	dHPC or vHPC	not mentioned	Bilaterally dHPC or vHPC
Czerniawski et al., 2009	dHPC or vHPC	not mentioned	Bilaterally dHPC or vHPC
Klur et al., 2009	right or left HPC	not mentioned	Bilaterally dHPC
Tan, 2008	dHPC or vHPC	not mentioned	Bilaterally dHPC or vHPC
Atkins et al., 2008	vHPC	estimated	Bilateral vHPC
Cimadevilla & Aria, 2008	right dHPC	estimated	Unilateral right dHPC
Parsons & Otto, 2008	dHPC	estimated	Bilateral dHPC
Hafting et al. 2008	HPC	Estimated	Bilateral dHPC
Atallah et al. 2008	dHPC	not mentioned	Bilateral dHPC
Shahidi et al. 2008	DG	not mentioned	Bilateral DG
Yoon et al. 2008	dHPC	not mentioned	Bilateral dHPC
Chang et al. 2008	dHPC	not mentioned	Bilateral dHPC
Luft et al. 2008	dHPC CA1	infusion with dye	Bilateral CA1 of dHPC
McHugh et al. 2008	dHPC or vHPC	not mentioned	Bilateral dHPC or vHPC
Howland et al. 2008	dHPC or vHPC	not mentioned	Bilateral dHPC or vHPC
Cimadevilla et al. 2008	unilateral or bilateral HPC	not mentioned	Bilateral dHPC
Calfa et al. 2007	dHPC or vHPC	not mentioned	Bilateral dHPC and vHPC
Amaral et al. 2007	HPC	not mentioned	Bilateral CA1 of dHPC
Rogers & See, 2007	vHPC	not mentioned	Bilateral vHPC
Maren & Hobin, 2007	dHPC	not mentioned	Bilateral dHPC
Burman & Gewirtz, 2007	dHPC	not mentioned	Not specified
Cimadevilla et al. 2007	unilateral HPC	not mentioned	Bilateral dHPC
Akbari et al. 2007	DG	not mentioned	Bilateral DG
Stouffer & White, 2007	dHPC or vHPC	not mentioned	Bilateral dHPC or vHPC
Bhatti et al, 2007	HPC (mossy fibers)	Injected with dye	Bilateral hilus of dHPC
Bertoglio et al, 2006	dHPC or vHPC	Injected with dye	Bilateral dHPC or vHPC
Akbari et al. 2006	CA1	not mentioned	Bilateral CA1
de Lima et al, 2006	dHPC	Injected with dye	Bilateral CA1 of dHPC
Prado-Alcala et al, 2006	HPC	not mentioned	Bilateral dHPC
Igaz et al 2006	HPC	methylene blue dye	Bilateral CA1 of dHPC
White & Gaskin, 2006	dHPC	not mentioned	Bilateral dHPC
Hobin et al, 2006	vHPC	not mentioned	Bilateral vHPC
Gaskin & White, 2006	dHPC	methylene blue dye	Bilateral dHPC
Kubik et al. 2006	HPC	not mentioned	Bilateral dHPC
Cimadevilla et al. 2005	unilateral dHPC	not mentioned	Unilateral right dHPC
Stone et al. 2005	HPC	not mentioned	Bilateral dHPC
Holahan, 2005	HPC	not mentioned	Bilateral dHPC
Gaskin et al. 2005	dHPC	methylene blue dye	Bilateral dHPC
Klement et al. 2005	dHPC	not mentioned	Bilateral dHPC
Micheau et al. 2004	HPC	not mentioned	Bilateral dHPC
Corcoran & Maren, 2004	dHPC	not mentioned	Bilateral dHPC
Black et al. 2004	dSUB or vSUB (HPC)	mathematical formula	Bilateral dSUB or vSUB
Maren & Holt, 2004	dHPC or vHPC	not mentioned	Bilateral dHPC or vHPC
Quiroz et al., 2003	HPC	unilateral cFos expression	Bilateral dHPC
Chang & Gold, 2003	HPC	not mentioned	Bilateral HPC
Zhang et al. 2002	dHPC or vHPC	not mentioned	Bilateral dHPC and vHPC
Schroeder et al. 2002	HPC	Estimated	Bilateral dHPC
Jezek et al. 2002	HPC	not mentioned	Bilateral dHPC
Bast et al. 2001	vHPC	not mentioned	Bilateral vHPC
Maruki et al. 2001	dHPC or vHPC	not mentioned	Bilateral dHPC or vHPC

Cimadevilla et al. 2001	Unilateral HPC	not mentioned	Bilateral HPC
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Table 2.1. Studies using temporary inactivation of the HPC in the period of 2001-2011.

Studies were found when searching for “Hippocampus AND inactivation” in Web of Science, June 6th 2011. Only studies in rats that were targeting the HPC were included in the methodological evaluation.

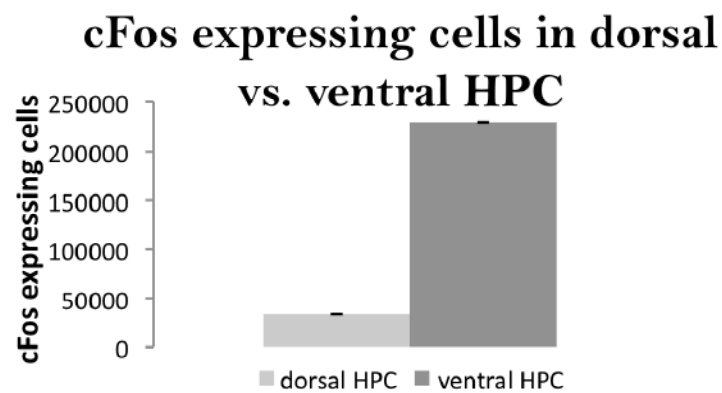


Figure 2.1. The absolute number of cFos expressing cells (\pm SEM) in dorsal versus ventral HPC 45 minutes following infusion of ROP into one single bilateral HPC infusion site as estimated using unbiased stereology. The dHPC had an average of 33'765 cFos expressing cells while the vHPC had an average of 229'694 cells.

Dorsal and Ventral HPC cFos Expression

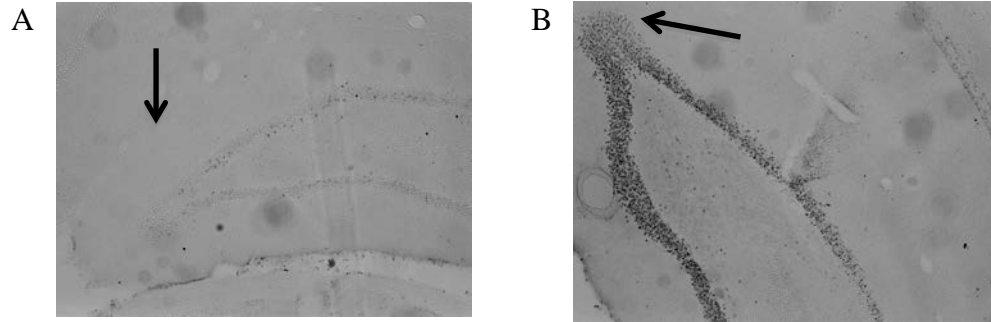


Figure 2.2. Images taken with a 10x0.3NA magnifying lens of the cFos in both the dorsal (A) and ventral (B) HPC of the same subject. The small black dots each indicate a cell expressing the cFos protein. The arrows are pointing at the tip of the DG.

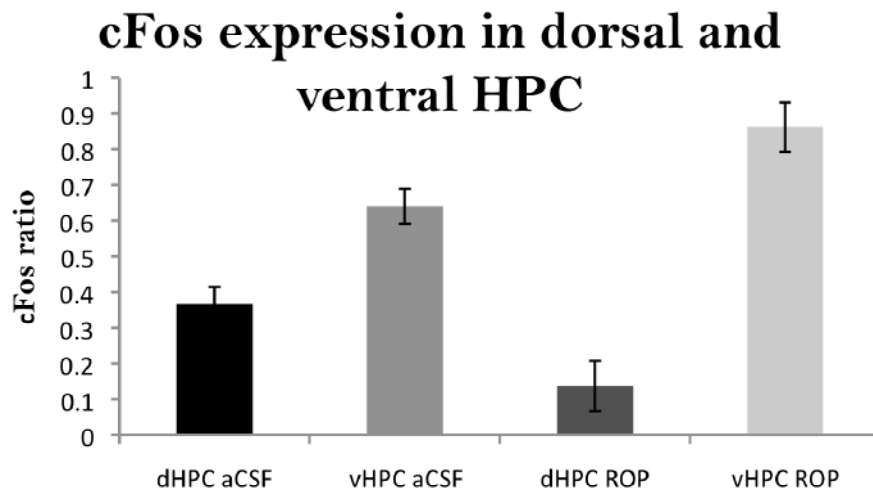


Figure 2.3. Ratio of number of cFos-positive cells (\pm SEM) in dorsal and ventral HPC compared to total number of cFos expressing cells. aCSF groups are unilateral control hemispheres from all subjects included in the study described in Chapter 3. ROP brains are full brains as described in the methods section in this chapter.

The findings described in the results section clearly show that one infusion site in the dHPC leaves a large part of the HPC still active. The literature review described in

table 2.1 shows that 26% of all studies evaluated were infusing a drug in to a single bilateral HPC site while ascribing effects to inactivation of the entire HPC. 20% of the studies inactivated either the dorsal or the ventral part of the HPC to compare the properties of the two regions, while only one study inactivated both the dHPC and the vHPC when evaluating the properties of the entire structure. 90% of the studies did only one bilateral infusion site; 26% of these related their effects to the entire HPC. None of the papers in Table 2.1 showed that the targeted structure was inactivated at the time of behavioural testing. One paper used seizure activity and the IEG cFos as a measure of extent of inactivation; however, the test was done on a later injection of the drug, and not on the drug infusion associated with behaviour (Quiroz, Quirarte, Morales, Diaz-Cintra, & Prado-Alcala, 2003). None of the evaluated articles evaluated extent of inactivation at the time of testing. 9% of the studies inferred the diffusion of the infused drug on previous research reports and mathematical calculations. 11% evaluated the extent of inactivation by infusing a dye through the guide cannulae at the time of perfusion and then used histological data to estimate the extent of inactivation. Only one paper used any form of function to physiological measure, in this case IEG expression, to accurately evaluate the extent of inactivation. However, this was done at a time point after the testing, and did not test the inactivation during the experiment itself. The remainder of the studies did not address the issue at all or used histology to confirm proper placement of the cannulae tip, sometimes only in a smaller group of the animals used in the study.

In this experiment, active cFos expressing cells following ROP infusion and MECS were counted and analyzed. The HPC subregions CA1, CA2, CA3 and DG were counted together but there was a separation between dorsal and ventral HPC. Superior

and caudal parts of the HPC were counted as dHPC while the inferior and rostral parts were counted as vHPC. The distribution of cFos is shown in fig. 2.1. The dHPC showed very little cFos expression (mean = 33,765 cells, as indicated by the stereology technique described in the methods section) while the vHPC showed far more cFos expression (mean = 229,694 cells). Of the total amount of cells expressed, 83 % of the cFos was found in the vHPC. In control hemisphere taken from the subjects in chapter 3 the distribution of cFos expressing cells were 36% in the dHPC and 64% in the vHPC (fig. 2.3).

Discussion

Several studies have shown behavioural differences when evaluating dorsal and ventral HPC. Bannerman et al. (2004) concluded from a literature review that the dHPC plays a large role in spatial memory while the vHPC is preferentially involved in anxiety. This explains the findings presented by Kim and Fanselow (1992) where dHPC lesions reduced context fear memory at multiple time points following surgery; however, they did not find any effect on tone-shock pairing memory. The opposite was reported by Yoon and Otto (2007) where rats with vHPC lesion show impaired acquisition and expression of auditory trace fear conditioning. Similar differences between the HPC regions were shown by Kjelstrup et al. (2002) where rats with lesions to the vHPC performed just as well as controls in the Morris water task, indicating that their spatial memory was intact. These studies and more indicate that there is at least functional specialization within the HPC and that the different parts of the structure contribute

differently to memory. The present experiment clearly shows that when injecting only one site bilaterally in to the HPC a large portion of the structure is still active. Here, 17 % of the total cFos expressing cells counted were found in the dHPC. Most of these were found towards the caudal end of the dHPC indicating that the drug did not spread all the way through this part of the structure. When comparing these results to the artificial cerebrospinal fluid (aCSF) and non-infusion control hemispheres in all subjects of Chapter 3, 36 % of the cFos expressing neurons were found in the dHPC (Fig. 2.3). Only having two subjects in this experiment does not give high enough power to conduct reliable statistical analysis on these data sets, however, the difference in cFos expression between the dorsal and ventral sub-regions and the different conditions are clearly illustrated in fig. 2.3. This difference emphasizes the importance of using more than one infusion site as well as proper measures of extent of inactivation if the aim of the study is to clarify HPC function.

CHAPTER 3 – LENGTH OF THE INFUSION NEEDLE

Introduction

Infusion of local anesthetics, receptor blockers, and other agents directly in to the rodent brain has become increasingly popular the last decades. Injections can be made during a surgical procedure while the animal is under anesthetics, or the agent can be injected in to the brain in an awake animal through permanently installed cannulae. The last option is popular in studies where parts of the brain are inactivated using local anesthetics and where the goal is for the function of the targeted region to be restored following some time interval. While restraining the animal, needles are lowered in to the permanent guide cannulae and left in place for the duration of the drug infusion and some variable amount of extra time to allow for the drug to diffuse away from the infusion tip before the needle is removed.

Even though this is a common form of behavioural manipulation there are great inconsistencies in the literature as to how the infusions are conducted. One of these inconsistencies is the length of the infusion needles used when infusing the drug. The lengths of the needles vary from being flush with the permanent guide cannulae to protruding 2 mm lower than the guide cannulae. Presumably protrusion could disrupt neuronal activity. There has not been any studies published investigating the effect of needle protrusion into healthy brain tissue. It is therefore the aim of this study to evaluate the effect of using protruding infusion needles on tissue when nothing is infused or while injecting a local anesthetic or a control vehicle.

Methods

Subjects: Four adult male Long-Evans rats weighing 520-580 g at the time of the surgery were included in this study. The rats were housed in pairs or trios in a room where the light cycle was set to 12 hr light -12 hrs dark (lights on at 7.30 am) and given access to water and rat chow *ad libitum*.

Surgery: The surgical procedure was mainly the same as described in Chapter 2. Each rat was implanted with 2 bilateral stainless steel cannulae targeting the dorsal and ventral HPC and where 10 and 13 mm long, respectively. The coordinates for the dHPC was the same as described in Chapter 2, the coordinates for the vHPC was 5.6 mm anterior/posterior direction, 5.2 mm in the lateral/medial direction and 6 mm in the ventral/dorsal direction.

Drug infusion: The procedures for the drug infusion were the same as described in Chapter 2. All four infusion sites were used simultaneously. ROP was infused to inactivate HPC cellular activity while aCSF were infused as a control vehicle.

Groups: The rats were randomly assigned to four different groups. Group 1 had infusion needles lowered in to all four guide cannulae with no drug infusion. The infusion needles were flush with the guide cannulae in the right hemisphere and protruding 1mm below the guide cannulae in the left hemisphere. The infusion needles were kept in place for the same amount of time as a regular infusion and diffusion would take. Group 2 had infusion needles protruding 1 mm below guide cannulae in the left hemisphere and flush infusion needles in the right hemisphere. Both hemispheres had ROP infused. Group 3 had 1 mm protruding needles in both hemispheres. Left hemisphere had aCSF infused

and right hemisphere had ROP. Group 4 had flush needles in both hemispheres and both hemispheres were infused with aCSF.

Procedure: 45 min following infusion all animals with the exception of group 1 where given MECS following the same protocol as described in Chapter 2. 45 min following MECS the rats were perfused and their brains harvested and stored in the same manner as described in Chapter 2.

Perfusion/Immunocytochemistry/stereology: All brains were cut, labeled and analyzed in the same way as described in Chapter 2. Prior to sectioning a cut was made in to the cortex of the right hemisphere in each brain for accurate identification of left and right hemisphere during stereological analysis. The brains were counted using StereoInvestigator in the same manner as described in Chapter 2, but keeping only left and right hemisphere separate.

Results

Length of infusion needles used between 2001 and 2011

Author	Infusion Needle	Author	Infusion Needle
Holahan & Aryeh, 2011	extended 1 mm	Maren & Hobin, 2007	not mentioned
Telenesky et al., 2011	prodruding 1 mm	Burman & Gewirtz, 2007	protruding 1 mm
Cimadevilla et al., 2011	prodruding 2 mm	Cimadevilla et al. 2007	protruding 2 mm
Lasster et al. 2010	prodruding 1 mm	Akbari et al. 2007	protruding 0.5 mm
Parsons & Otto, 2010	prodruding 1 mm	Stouffer & White, 2007	protruding 0.5 mm
McEwon & Treit, 2010	not mentioned	Bhatti et al, 2007	not mentioned
McDonald et al., 2010	protruding 1 mm	Bertoglio et al, 2006	protruding 1.5 or 3 mm
Jo & Lee, 2010	protruding 1 mm	Akbari et al. 2006	protruding 0.5 mm
Kelemen, & Fenton, 2010	reference other papers	de Lima et al, 2006	protruding 1 mm
Gomes et al. 2010	protruding 1 mm	Prado-Alcala et al, 2006	not mentioned
Cohen et al. 2009	protruding 1 mm	Igaz et al 2006	not mentioned
Iordanova et al., 2009	protruding 1 mm	White & Gaskin, 2006	protruding 1 mm
Cimadevilla et al., 2009	protruding 2 mm	Hobin et al, 2006	not mentioned
McEwon & Treit, 2009	not mentioned	Gaskin & White, 2006	protruding 1 mm

Esclassan et al., 2009	protruding 1 mm	Kubik et al. 2006	not mentioned
Czerniawski et al., 2009	protruding 1 mm	Cimadevilla et al. 2005	protruding 1 mm
Klur et al., 2009	protruding 1 mm	Stone et al. 2005	Flush
Tan, 2008	not mentioned	Holahan, 2005	protruding 1 mm
Atkins et al., 2008	not mentioned	Gaskin et al. 2005	not mentioned
Cimadevilla & Aria, 2008	protruding 2 mm	Klement et al. 2005	protruding 1.4 mm
Parsons & Otto, 2008	not mentioned	Micheau et al. 2004	not mentioned
Hafting et al. 2008	protruding 0.9 mm	Corcoran & Maren, 2004	protruding 1 mm
Atallah et al. 2008	protruding 0.5 mm	Black et al. 2004	protruding 1 mm
Shahidi et al. 2008	protruding 1 mm	Maren & Holt, 2004	protruding 1 mm
Yoon et al. 2008	protruding 1 mm	Quiroz et al., 2003	not mentioned
Chang et al. 2008	protruding 1 mm	Chang & Gold, 2003	protruding 1 mm
Luft et al. 2008	protruding 1 mm	Zhang et al. 2002	protruding 1.5 mm
McHugh et al. 2008	protruding 2 mm	Schroeder et al. 2002	not mentioned
Howland et al. 2008	protruding 1 mm	Jezek et al. 2002	protruding 2 mm
Cimadevilla et al. 2008	protruding 2 mm	Bast et al. 2001	protruding 1.6 mm
Calfa et al. 2007	protruding 1-2 mm	Maruki et al. 2001	protruding 0.5 mm
Amaral et al. 2007	protruding 1 mm	Cimadevilla et al. 2001	protruding 2 mm
Rogers & See, 2007	not mentioned		

Table 3.1 A list over studies published between 2001 and 2011 which can be accessed through the University of Lethbridge Web of Knowledge as of June 6th 2011. Keyword criterion was “hippocampus AND inactivation”. Only studies targeting the rat HPC was included in the analysis. The table highlights the length of the infusion needles used in the individual studies.

Effect of protruding infusion needle on cFos

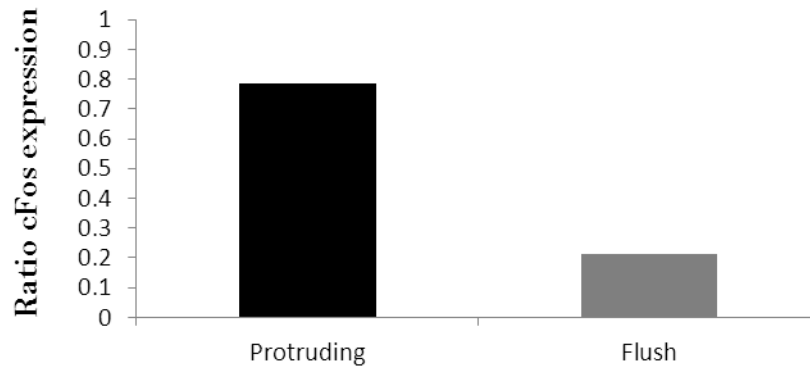


Figure 3.1. The difference in cFos expression by lowering a flush infusion needle compared to a protruding needle. No infusions were made. 79% of the total cFos expressed within the HPC was found in the hemisphere with a protruding infusion needle.

cFos expression in each condition

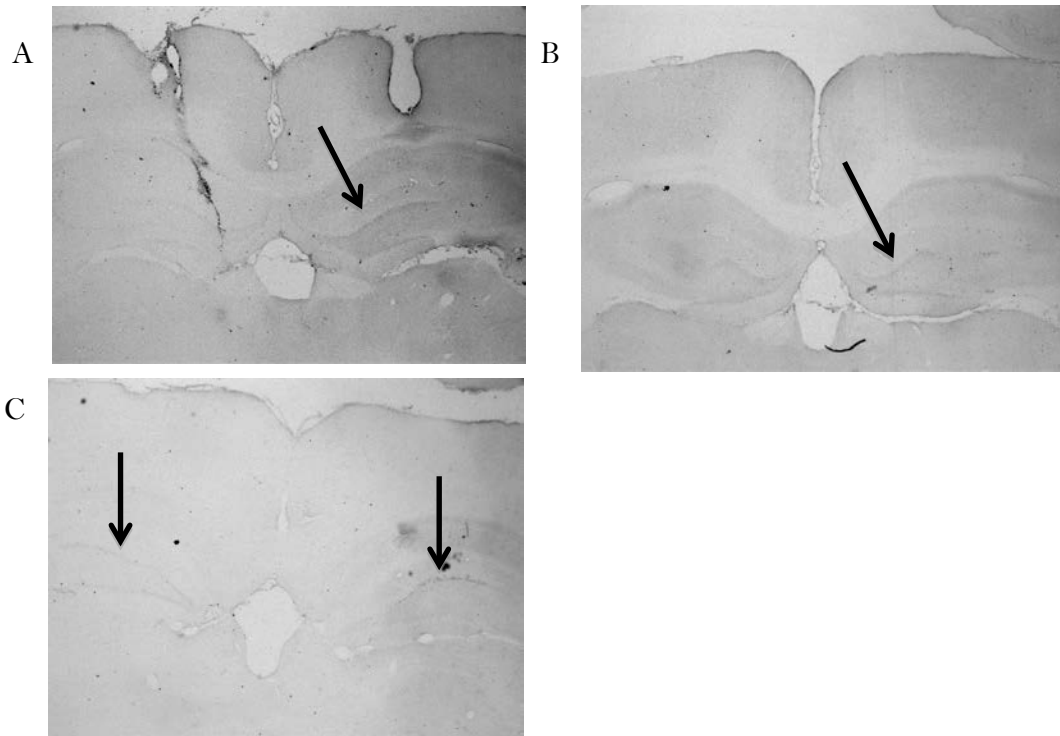


Figure 3.2. cFos expression in Group 1 (A), Group 2 (B) and Group 3(C). All pictures were taken on 2.5x magnification. A) The amount of cFos expressed following lowering of flush (right) or protruding (left) infusion needles. B) The amount of cFos expressed following ROP infusion through a flush (right) and protruding (left) needle. C) The amount of cFos expressed after infusion aCSF (right) or ROP (left) through protruding infusion needles. The arrows indicate region with the greatest difference.

Effect of protruding infusion needle on ROP inactivation

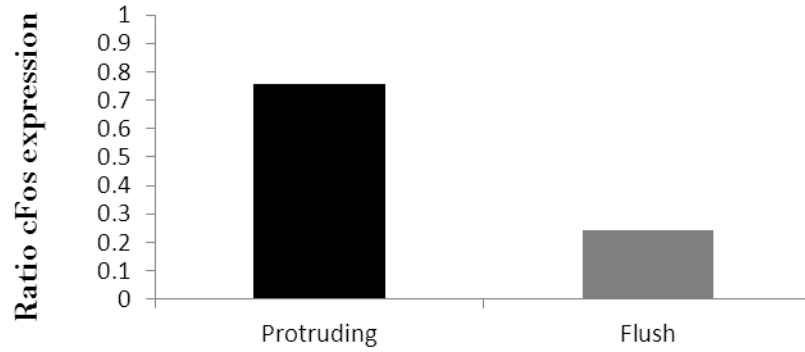


Figure 3.3. Ropivacaine infused in to the hippocampus through a flush infusion needle compared to a protruding needle. When comparing the ratio of cFos expressing neurons seen in the whole brain to the two hemispheres, 76% of the cFos is found in the hemisphere with the protruding infusion needle.

Effect of protruding infusion needle on ROP vs. aCSF

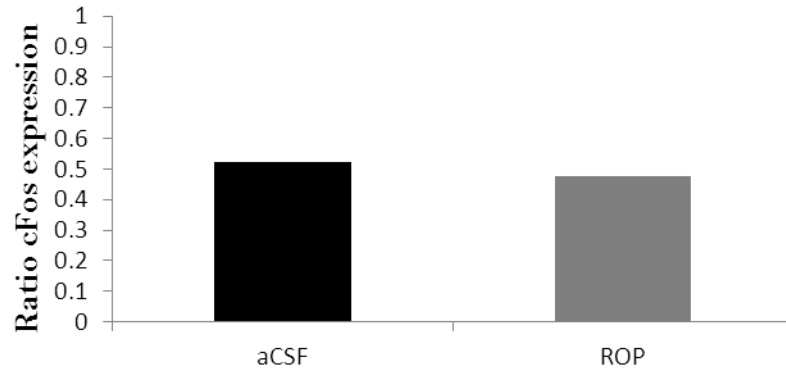


Figure 3.4. The effect of using a protruding infusion needle while infusing aCSF and ROP. The ratio of cFos expression was 52% in the aCSF hemisphere and 48% in the ROP hemisphere.

Hemispheric difference in cFos expression

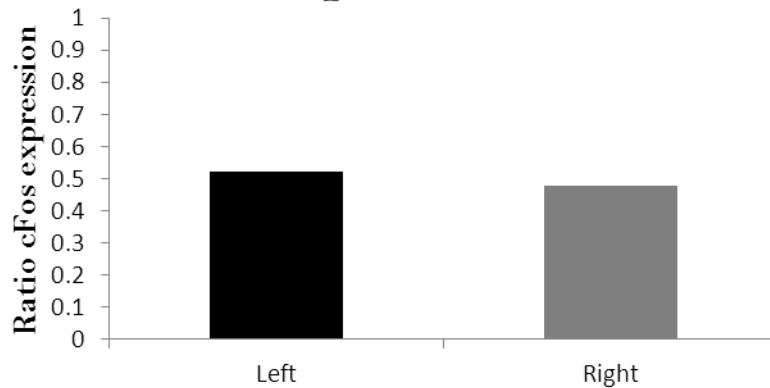


Figure 3.5. The difference in cFos expression following flush infusion needles between the hemispheres. 52% of the cFos was found in the left hemisphere and 48% was found in the right hemisphere.

The results are expressed as a ratio between the two hemispheres of each subject. No SEM or SD was conducted as each group only had one subject. Group 1 had unilateral flush infusion needles and unilateral protruding infusion needles. No infusions were made through the needles and the group did not receive MECS. There were more cFos expressed in the hemisphere where the needle was lower than the permanent guide cannulae (Fig. 3.2). This indicates that there was an effect of disrupting healthy brain tissue which is accredited the protruding needle and not infusions or MECS. The higher expression of cFos associated with a protruding infusion needle was also visible when ROP was infused (Fig. 3.3). Group 3 had ROP infused in one hemisphere and aCSF in the other hemisphere. All infusion needles were protruding. When comparing cFos expression following protruding needle infusion of ROP and aCSF, the aCSF hemisphere and ROP hemisphere are almost identical (52% in the aCSF hemisphere vs. 48% in the ROP hemisphere) (fig. 3.4). Fig. 3.5 shows that with flush infusion tips there are only slight differences in cFos expression between the hemispheres, where the left hemisphere has a slightly higher percentage of cFos expression (52 vs. 48%). Interestingly, this is the same hemispheric difference as seen in the group which had aCSF and ROP infusions both with protruding needles, indicating that there were no effect of infusion ROP when using a protruding needle as compared with a control condition.

Discussion

Infusion needles protruding up to 3 mm below a permanently installed infusion cannulae seem to be the current standard in the methodology of temporary inactivating the HPC. In fig 3.2 it is clear that by only lowering an infusion needle protruding 1 mm below the guide cannulae there is greater cFos activation than the baseline expressed in the contralateral hemisphere where a flush needle was used. This activity can only be accredited to the protruding infusion needle as no drugs or vehicles were infused and the group was not given MECS. The cFos expression is related to excitation of neurons, possibly even seizure activity. It is therefore not unreasonable to conclude that lowering a needle into healthy tissue causes temporary strong discharge of neurons nearby. When combining these discharges with behavioural experiments and data it becomes possible to misinterpret the experimental findings. The cFos expressed in correlation with the protruding needle is visible even when the lowering of the needle is combined with infusion of a Na⁺ channel blocker, ROP. ROP would usually block APs from taking place but the discharges have already taken place when the drug effect begins somewhere around 20 min following infusion (see Chapter 4). Because of cFos expression tapering off 60 min after stimulation (Purves et al., 2004) and the cFos measured in fig. 3.2 was measured almost 2 hrs after the needles were lowered, it is reasonable to assume that the seizure activity continued after the ROP had taken full effect. Fig. 3.4 shows that the cFos expression correlated with protruding infusion needles makes comparing inactivation to control hemispheres complicated, as they display nearly the same level of cFos. The

differences seen between the hemispheres are due to the experimental manipulations as there are no difference in cFos expression following aCSF infusion and MECS (fig. 3.5).

Based on these findings it is clear that using a flush infusion needle is preferable to a protruding one. Depending on the experimental design, there is reason to believe that the use of a protruding infusion needle may interfere with the behavioural data confounding simple interpretations.

Chapter 4 – Ropivacaine Timeline

Introduction

In the previous two chapters we have underlined several issues with the current state of temporary inactivation methodology. There are little to no measure of how much of the brain is actually inactivated following drug infusion. In the case of the HPC infusion sites are too few and may not be enough for the drug to cover the extent of tissue that the researchers wish. Finally, if infusion needles are longer than permanent cannulae confounding neuronal activation may occur in the surrounding tissue. One last inconsistency is the type of drug that is being infused. Some drugs are injected because they target specific receptors, whose function is of interest to the researcher (Glu-r antagonist, Burman & Gewirtz, 2007, Micheau, Riedel, Roloff, Inglis, & Morris, 2004; OX1-r antagonist, Akbari, Naghdi, & Motamedi, 2006, Akbari, Nghdi & Motamedi, 2007; MEK ½ inhibitor, Igaz et al., 2006; NMDA-r antagonist, Gomes et al., 2010, Tan, 2008, Luft, Amaral, Schwartzmann, & Roesler, 2008; GABA_A-r antagonist, Shahidi, Komaki, Mahmoodi, & Lashgri, 2008; ZIP, Cohen, Kozlovsky, Matar, Kapla & Zohar, 2009) while others are infused with the aim of shutting down electrical activity and neuronal signaling in the targeted region. To block APs within the brain, the GABA_A-receptor agonist muscimol has been a common drug for a long time. The benefits of using this drug is that it targets only specific receptors which will increase the influx of Cl⁻ ions making the neuron less likely to fire (Kolb & Whishaw, 2006) and it does not disrupt fibers of passage (McEown & Treit, 2010). Other popular drugs like lidocaine,

tetradotoxin (TTX) and bupivacaine are sodium (Na⁺) channel blockers, and infusion these drugs means that all APs in the region – including in fibers of passage – will be blocked (McEown & Treit, 2010). Setting aside the specific function of these local anesthetics, there has also been concerns regarding the toxicity of these drugs and what effect they may have on the brain and behaviour when infused one or multiple times. Bast, Zhang and Feldon (2001) reported that infusion muscimol or TTX in to the vHPC resulted in decreased locomotion during exploration of a novel arena. As a result of these types of findings, finding a drug with low toxicity is important. Ohmura, Kawada, Ohta, Yamamoto and Kobayashi (2001) compared bupivacaine, levobupivacaine and ROP – all Na⁺ channel blockers - and found that ROP had the least toxic effect on the central nervous system. There are no papers published using ROP as the local anesthetic infused in to the awake brain. It is therefore the aim of this study to establish the timeline as to when the drug takes effect and how long this effect is present when infusion 0.7 µl 10 mg/ml ROP in to two bilateral HPC sites, as well as to show that this dose is enough to completely inactivate the entire HPC.

Methods

Subjects: Ten adult male Long-Evans rats weighing 530-650 g at the time of the surgery were included in this study. The rats were housed in pairs or trios in a room where the light cycle was set to 12 hr light -12 hrs dark (lights on at 7.30 am) and given access to water and rat chow ad libitum.

Surgery: The surgical procedure was similar to that described in Chapter 3. Each rat was implanted with 2 bilateral stainless steel cannulae targeting the dorsal and ventral HPC and where 10 and 13 mm long, respectively. The coordinates were the same as described in Chapter 3.

Drug infusion: The procedures for the drug infusion were the same as described in Chapter 3. 10 mg/ml ROP was infused unilaterally. Three animals had aCSF infused in the control hemisphere while 6 animals had no control infusion.

Groups: The rats were randomly assigned to five different groups. Group 1 ($n=2$) were infused with were infused with ROP and aCSF unilaterally and received MECS 20 min after infusion; group 2 ($n=2$) received MECS 45 min after infusion (one animal had aCSF); group 3 ($n=2$) received MECS 1.5 hrs after infusion; group 4 ($n=2$) received MECS 3 hrs after infusion and group 6 ($n=2$) received MECS 6 hrs after infusion.

Procedure: MECS were administered following the same protocol as described in Chapter 2. 45 min following MECS the rats were perfused and their brains harvested and stored in the same manner as described in Chapter 2.

Perfusion/Immunocytochemistry/Stereology: All brains were cut, labeled and analyzed in the same way as described in Chapter 3.

Results

The drugs used to temporarily inactive the hippocampus in the period of 2001–2011

Author	Drug	Author	Drug
Holahan & Aryeh, 2011	Lidocaine	Maren & Hobin, 2007	Muscimol
Telnesky et al., 2011	TTX	Burman & Gewirtz, 2007	GluR antagonist/muscimol
Cimadevilla et al., 2011	TTX	Cimadevilla et al. 2007	TTX

Lasster et al. 2010	Baclofen/muscimol	Akbari et al. 2007	OX1R antagonist
Parsons & Otto, 2010	Muscimol	Stouffer & White, 2007	Muscimol
McEwon & Treit, 2010	Muscimol	Bhatti et al, 2007	Lidocaine
McDonald et al., 2010	Muscimol	Bertoglio et al, 2006	Lidocaine
Jo & Lee, 2010	Muscimol	Akbari et al. 2006	OX1R antagonist
Kelemen, & Fenton, 2010	TTX	de Lima et al, 2006	Muscimol
Gomes et al. 2010	NMDr antagonists	Prado-Alcala et al, 2006	TTX
Cohen et al. 2009	ZIP	Igaz et al 2006	MEK 1/2 inhibitor
Iordanova et al., 2009	Muscimol	White & Gaskin, 2006	Muscimol
Cimadevilla et al., 2009	TTX and lidocaine	Hobin et al, 2006	Muscimol
McEwon & Treit, 2009	Lidocaine	Gaskin & White, 2006	Muscimol
Esclassan et al., 2009	Muscimol	Kubik et al. 2006	TTX
Czerniawski et al., 2009	Muscimol	Cimadevilla et al. 2005	TTX
Klur et al., 2009	Lidocaine	Stone et al. 2005	Muscimol
Tan, 2008	NMDAr antagonist	Holahan, 2005	Muscimol
Atkins et al., 2008	Lidocaine	Gaskin et al. 2005	Muscimol
Cimadevilla & Aria, 2008	TTX	Klement et al. 2005	TTX
Parsons & Otto, 2008	Muscimol	Micheau et al. 2004	GluR antagonist
Hafting et al. 2008	Muscimol	Corcoran & Maren, 2004	Muscimol
Atallah et al. 2008	Muscimol	Black et al. 2004	Lidocaine
Shahidi et al. 2008	PTX	Maren & Holt, 2004	Muscimol
Yoon et al. 2008	Muscimol	Quiroz et al., 2003	TTX
Chang et al. 2008	Lidocaine	Chang & Gold, 2003	Lidocaine
Luft et al. 2008	AP5	Zhang et al. 2002	Muscimol/TTX
McHugh et al. 2008	muscimol and AP5	Schroeder et al. 2002	Bupivacaine
Howland et al. 2008	Lidocaine	Jezek et al. 2002	TTX
Cimadevilla et al. 2008	TTX	Bast et al. 2001	Muscimol/TTX
Calfa et al. 2007	Lidocaine	Maruki et al. 2001	Muscimol
Amaral et al. 2007	Muscimol	Cimadevilla et al. 2001	TTX
Rogers & See, 2007	Muscimol		

Table 4.1. A list over studies published between 2001 and 2011 which can be accessed through the University of Lethbridge Web of Knowledge as of June 6th 2011. Keyword criterion was “hippocampus AND inactivation”. Only studies targeting the rat HPC was included in the analysis. The table highlights the different drugs used in the individual studies.

Ropivacaine Timeline

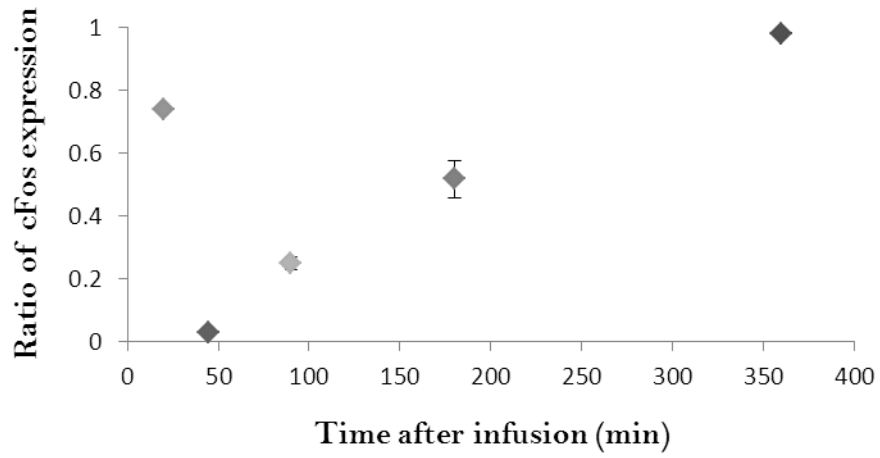


Figure 4.1. The ratio of number of cFos positive cells in HPC with MECS between 0.7 μ l of 10 mg/ml Ropivacaine into one HPC and no infusion in the HPC in the other hemisphere.

ROP inactivated and aCSF infused hemispheres 45 min after infusion

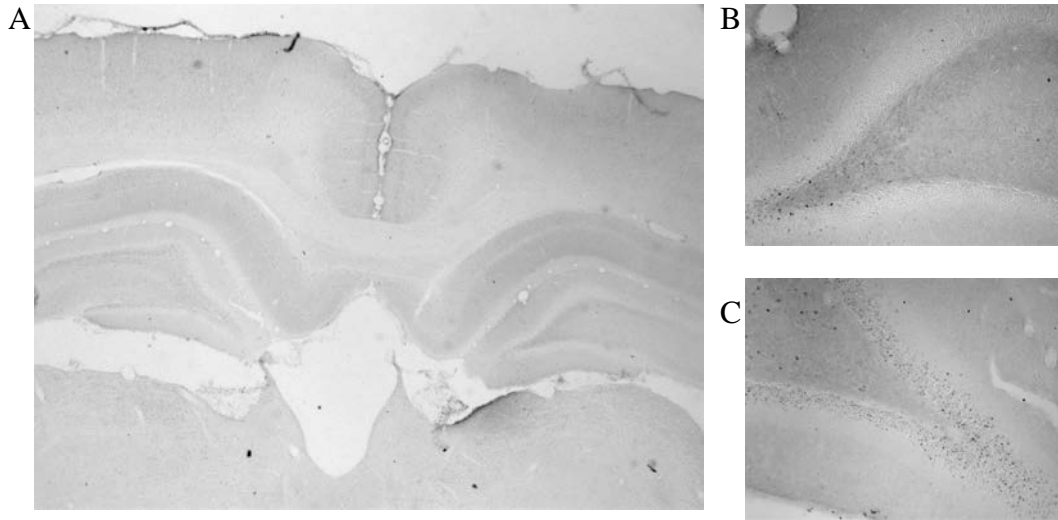


Figure 4.2. The inactivation of unilateral HPC. A) Bilateral dHPC 45 min following infusion of ROP unilaterally. cFos cells are seen as small black dots and are visible on the left side of the hippocampus and in the surrounding cortex. Image is taken at 2.5x magnification. B) 20x magnification of the same section as in A). This is the inactivated hemisphere and few cFos expressing cells are seen. C) The aCSF infused control hemisphere magnified 20x. cFos cells are expressed in all subregions of the HPC.

Two rats were removed from the study due to problems with surgery or infusion. Eight rats were included in the analysis. The number of cFos expressing cells was calculated in each hemisphere individually and then the hemispheres within each animal were compared against each other. The results are presented as ratio of cFos expression between the two hemispheres. 20 min after infusion approximately 25 % of the neurons are inactivated and 45 min after infusion 97 % of the neurons are no longer expressing cFos. Over the next 5 hrs there is a gradual increase in cell firing, where at 1.5 hrs after infusion 25 % of the neurons express cFos, at 3 hrs 52 % of the neurons express cFos, and at 6 hrs there are no longer any inactivation observed (fig. 4.1).

Discussion

This experiment has clearly outlined the timeline of effect of the non-toxic Na⁺ channel blocker ROP. 45 min after infusion 97 % of the cFos expressing neurons were turned off, making this the ideal time-point for behavioural testing. Six hrs following infusion the inactivated hemisphere the infused hemisphere expressed 97 % of cFos when compared to the control infused hemisphere. These results are important to take in to consideration when designing an experiment. Testing the rat prior to 45 min may lead to false behavioural results as most of the HPC is still active, and testing after 3 hrs may also lead to false data as much of the HPC has restored function, at least as indexed by cFos, by this time. Another important finding is that using two bilateral infusion sites led to the inactivation of 97 % of the entire HPC, indicating that this method is much more preferred when testing HPC function than one bilateral dorsal infusion sites which leaves the ventral HPC fully functional (see Chapter 2). In conclusion, we have now established a method of inactivating 97 % of the entire HPC without causing seizure/excitation to the surrounding tissue as well as having an established timeline as to the effect of a non-toxic drug.

Chapter 5 – The role of hippocampal replay in making context memories

hippocampus-independent

Introduction

How memories are stored as long-term memories in our brains is still largely uncertain at the systems level and a topic of great scientific research. The first major theory of how long-term memories are consolidated described a system where the HPC played only a temporary role. Following some period of time, weeks or months or even years, memories were consolidated outside the HPC (Squire et al., 1984). This theory, known as the SMSC, explains findings such as the initial description of H.M. only having lost his most recent memories, as well as the experimental results presented by Kim and Fanselow (1992) where context-fear memory was impaired if the HPC was damaged 1 day after learning but was spared if the HPC was damaged 28 days after learning. However, it does not explain some findings from the human literature where recent and remote autobiographical memories seemed to be completely disrupted (for review, see Nadel & Moscovitch, 1997). A new modified theory was then formulated. This theory distinguishes between autobiographical memories and factual memories and states that only factual memories can become HPC independent. Autobiographical memories are always dependent on the HPC. This became known as the MTT (Nadel & Moscovitch, 1997). Still, there are some findings that cannot be explained by the SMSC nor the MTT. Studies such as the one by Wiltgen et al. (2006) show that rats can learn context-fear associations without the HPC being available. This indicates that there are other memory systems within the brain that are capable of acquiring new information without the influence of the HPC. Sutherland et al. (2010) suggest two possible mechanisms as to how memories come to be stored outside of the HPC. One possibility is a dual-storage

model where the HPC and non-HPC memory systems independently acquire memory representations. The HPC acquires the memory fast; needing only one or a few iterations, while non-HPC systems require multiple distributed learning episodes in order to build a representation of the event. The other suggestion is the DRT where post-event HPC activity such as replay aids in consolidating the memory somewhere outside the HPC (Sutherland et al., 2010, Sutherland & Lehmann, 2011). Each time a similar event takes place replay aids in making the non-HPC trace stronger, eventually rendering it resilient to HPC damage. If the DRT is correct, then blocking post-learning HPC activity should obstruct the memory of the task and it will not be consolidated outside the HPC.

Lehmann et al. (2010) described a behavioural paradigm using contextual fear conditioning whereby multiple distributed learning episodes establish a memory that is HPC independent, able to survive permanent HPC damage. It is our aim to use this behavioural paradigm and to block replay activity after the training sessions using the local anesthetic ROP. During retention testing the HPC will be inactivated in the same manner. If the DRT is correct, blocking the replay session should disrupt establishment of the memory in non-HPC networks and impair performance during retention testing, the animals should freeze less than controls that did not have the replay episodes blocked. The results of this study should provide a good test of the importance of HPC replay in establishing memories outside the HPC.

Methods

Subjects: 66 adult Long-Evans rats weighing 330-560 g at the time of surgery.

The rats were housed in pairs in an animal housing room maintained at a 12:12 hr light:dark cycle (lights off at 7.30 pm) and given access to water and rat chow *ad libitum*.

Surgery: Prior to testing all animals were implanted with four stainless steel guide cannulae following the same procedure as described in Chapter 3.

Apparatus: Two MedAssociates, inc. Modular Test Chambers placed inside a MedAssociates, inc. Melamine box with one wall removed in a dimly light room were used. The boxes were placed on top of each other. The apparatus were connected to a computer installed with FreezeFrame TM version 1.6e (ActiMetrics Software). The sessions were recorded using a Sony Hyper HAD B&W video camera connected to a PC computer. A back-up Sony Hanycam HDD camcorder was placed on a tripod immediately behind the main video camera in case of any failure in recording the sessions. The cameras were positioned so that they could film both boxes simultaneously without adjustment. Outside of the two tripods with cameras there were no external cues in the room visible from the testing chamber.

Procedure: All acquisition sessions were conducted the same way. The animals were transported in pairs in separate clear plastic cages in to the testing room and the pairs were tested simultaneously. The rats were carefully placed within the testing chamber at the same time. After 45 sec within the chamber a 0.9 mA foot-shock came on for 2 sec. After an additional 13 sec the rats were removed from the chamber and transported back to their home cages. Following each run the chambers were cleaned out using Quatsyl-D Plus animal care disinfectant to prevent any odor transfer. The morning

(AM) sessions were run between 8.30 am and noon and the afternoon (PM) sessions were run between 4.00 pm and 7.30 pm.

5.1 Normal acquisition: Group 1 ($n=11$) received one acquisition session in the AM on three consecutive days. Group 2 ($n=10$) were given one acquisition session in the AM and PM (total of six sessions) on three consecutive days. On day 4 both groups were placed back in to the chamber for 5 min without presentation of any shock. The level of freezing was measured as an index of memory of the shock-context pairing.

5.2 Making context memory HPC-independent: Group 3 ($n=16$) received one acquisition session in the AM on three consecutive days. Group 4 ($n=13$) received two acquisition sessions per day; one in the AM and one in the PM. Following the PM session the animals were infused with a control vehicle (aCSF). On day four animals in both groups 3 and 4 were given infusions of ROP through all guide cannulae 45 min prior to testing. During retention testing the animals were placed in the chamber for 5 min without the shock being presented and the level of freezing was measured as an index of memory.

5.3 The role of HPC replay: Group 5 ($n=16$) received two acquisition sessions per day; one in the AM and one in the PM. Immediately before entering the chamber for the PM session the animals were infused with ROP in order to turn off any post-event HPC activity. Maximum 15 min went by between start of infusion and the animals being one acquisition and back in their home cages. On day 4 the animals were infused with ROP and paced back in their home cages. 45 min after infusion the animals were placed back in the chamber for 5 min without any presentation of shock. The level of freezing was measured as an index of memory.

Drug/infusion: The groups receiving ROP infusions were infused with 0.7 μ l 10 mg/ml ROP at the rate of 0.29 μ l/min with an additional 4.5 min diffusion time. The procedures were the same as described in Chapter 2. On day 4 after infusion the rats were placed back in their home cages for 45 min before being tested for retention.

Perfusion/immunohistochemistry/stereology: The animals were perfused 45 min after retention testing in the same way as described in Chapter 2. The brains were cut using a sliding microtome and labeled for the cFos protein. The whole brain was counted at once using StereoInvestgator as described in Chapter 2. Animals which received ROP infusions on test day and failed to have a minimum of 80 % of the cFos expression reduced compared to non-infused controls were eliminated from the study.

Statistical Analysis: The data were collected using FreezeView TM version 2.1 (ActiMetrics Software). Freezing threshold was set individually for each animal to eliminate errors related to the animal's position and defecation by a researched blind to the experimental conditions. The data sets were analyzed using IBM SPSS Statistics version 19. The data sets were subjected to a one-way ANOVA with *post hoc* Scheffe comparisons.

Results

Normal Acquisition

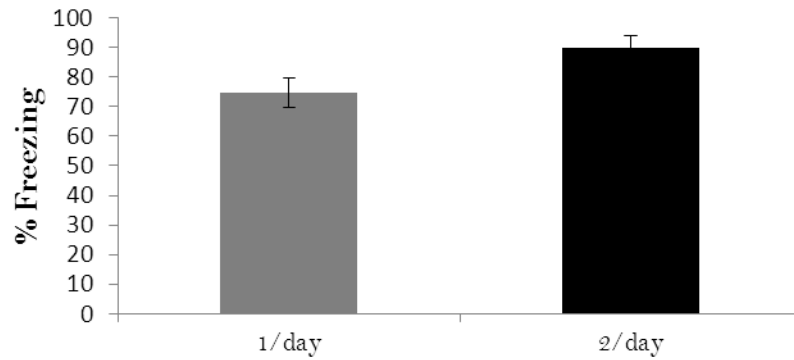


Fig 5.1. Mean (\pm SEM) percent time freezing by Group 1 (1/day) and Group 2 (2/day) during retention testing in the conditioned context.

Making Context Memories HPC-independent

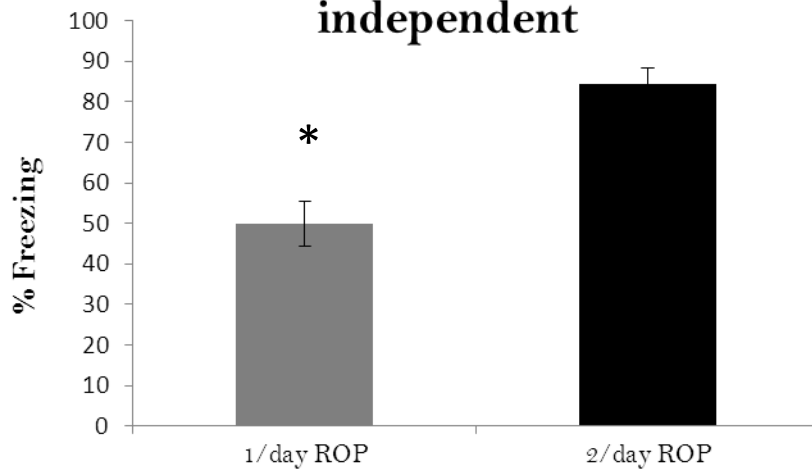


Figure 5.2. Mean (\pm SEM) percent time spent freezing by Group 3(1/day ROP) and Group 4 (2/day ROP).

Effect of inactivating HPC

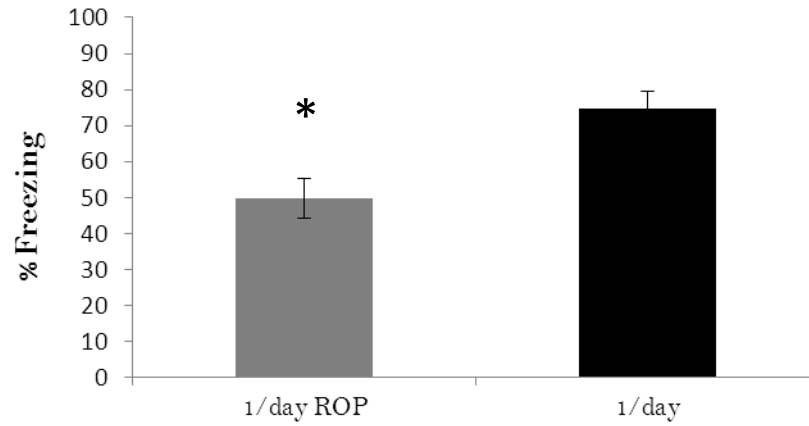


Figure 5.3. Mean (\pm SEM) percent time spent freezing by Group 3 (1/day ROP) and Group 1 (1/day).

Effect of HPC replay inactivation

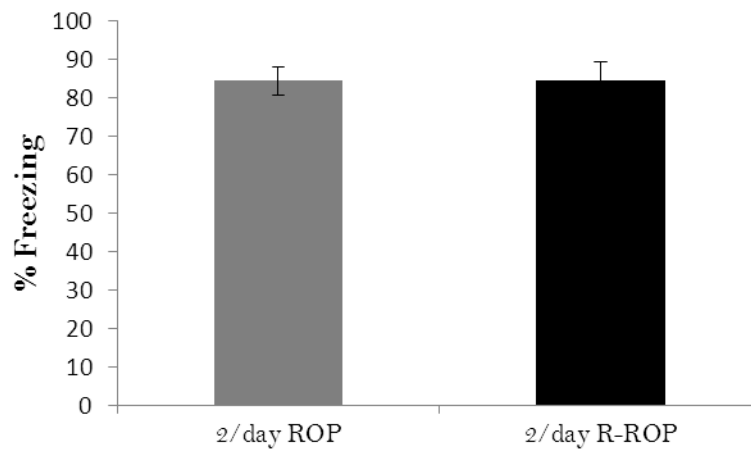


Figure 5.4. Mean (\pm SEM) percent time spent freezing by 2/day ROP and 2/day R-ROP. Group 5 (2/day R-ROP) had post-event HPC replay activity turned off by the means of ROP infusion.

Test day cFos levels

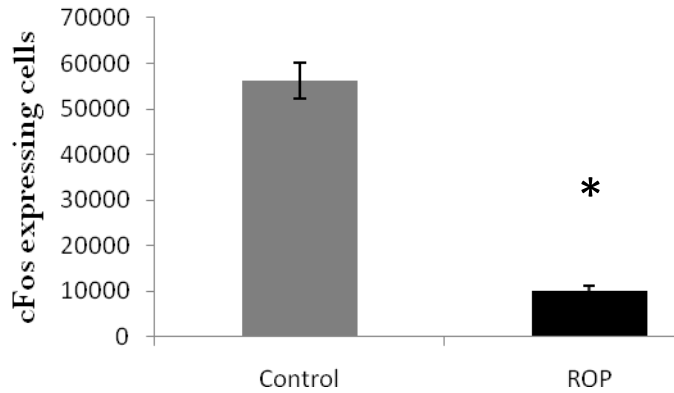


Figure 5.5. Number cFos positive cells (\pm SEM) at the time of retention testing.

Effect of HPC inactivation and replay blockage on 2/day freezing levels

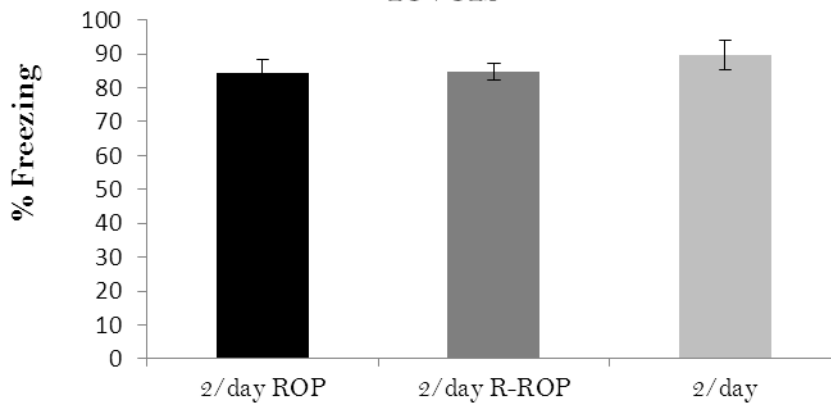


Figure 5.6. Mean (\pm SEM) percent time spent freezing by Group 4 (2/day ROP), Group 5 (2/day R-ROP) and Group 2 (2/day).

10 rats had problems connected to their infusions and showed cFos expression which exceeded 20% of control levels. On the basis of insufficient inactivation these

animals were therefore eliminated from the study. Further, one animal was eliminated due to freezing levels only slightly increasing from day 1 to retention testing. The back-up system for behavioural measurements was not needed in any analysis of the experimental data. A between groups one-way ANOVA showed significant difference between groups ($F(4,53)=12.481, p<0.001$).

5.1 Normal acquisition: Both control groups were able to learn the association between context and shock. Post hoc analysis revealed that there were no difference between the group that was trained 1/day (AM) group that were exposed to the context both in the AM and the PM ($p=0.123$).

5.2 Making context memory HPC-independent: Post hoc analysis of Group 3 who was exposed to the context-shock pairing in the AM and then had the HPC inactivated during retention testing (1/day ROP) and Group 4 who were exposed to the pairing in the AM and PM and had the HPC inactivated during retention (1/day ROP) showed that Group 3 froze significantly less than Group 4 on retention day ($p<0.001$). Group 3 (1/day ROP) also froze significantly less than Group 1 (1/day) ($p=0.036$) (fig.5.3).

5.3 The role of HPC replay: Post hoc comparison of group 4 (2/day ROP) and group 5 (2/day R-ROP) revealed no difference between groups ($p=1$) (fig. 5.4).

Histology: cFos expressing cells were counted in all HPC sub-regions as one. Results are displayed in fig. 5.5. There were no statistical significance between the cFos expressed in

the two control groups ($p>0.001$) and their results were grouped together. Animals in the inactivated group that failed to have more than 80 % of total cFos expression inactivated were eliminated from the study on the basis of failed inactivation. The average suppression of cFos expression was 82.3 % compared to the two non-infused control groups. A one-way ANOVA with between group factor showed a significant difference in cFos expression between the two groups ($F(1,53)=147.805, p<0.001$).

Effect of infusion: Fig. 5.6 shows groups 2, 4, and 5. All of these groups were trained twice per day. Group 2 did not have any infusions; Group 4 had aCSF infusions associated with PM training session and ROP on test day while group 5 had ROP infusions associated with PM training session as well as on retention day. A one-way ANOVA with between-groups factor show that there is no significant difference between the freezing behavior across these groups indicating no effect of infusion ($F(2,350)=0.718, p=0.495$).

Discussion

Sutherland et al. (2010) and Sutherland and Lehmann (2011) have recently suggested two models of memory consolidation as an alternative to the SMSC and MTT. The first suggestion was a dual-storage model (Sutherland et al. 2010). This model is based on different memory systems acquiring information independently with each system have its own learning rate and decay parameters. The other suggestion was the DRT (Sutherland et al. 2010, Sutherland and Lehmann, 2011) which is similar to the dual-storage model in the way that different memory systems can acquire information.

However, according to the DRT the acquisition by some non-HPC systems is dependent on HPC replay which occurs after an experience. In this experiment we aimed at dissociating the two models by inactivating HPC replay and then testing retention for context memory.

In this paradigm animals received context-shock pairings once or twice per day for three days. Normal animals that had no infusions showed good retention of the context-shock pairing displayed by high levels of freezing during 5 min of retention testing. There was a trend for the 2/day group to perform better than the 1/day group but this was not statistically significant. Using these parameters of 3 vs. 6 sessions we observed a significant decrease in performance when the animals had only 3 sessions before inactivating the HPC, indicating that this was not enough for any extra-HPC memory systems to fully acquire the memory. There was no effect of ROP at the time of retention if rats had received six context-shock pairings. This indicates that six but not three pairings was sufficient to establish a memory outside the HPC.

Group 5 (2/day R-ROP) had HPC replay turned off by the means of ROP infusion following the PM acquisition session each day as well as during retention testing. According to the DRT this would disrupt the process of consolidating the memory outside of the HPC, and we would expect to see freezing levels similar to that of Group 3 (1/day ROP) that only had half of the acquisition sessions – and consequently also half of the replay episodes – of Group 4 (2/day ROP). If replay activity is the mechanism by which memories are established in other cortical regions then blocking half of the replay sessions should also block consolidation. According to these results this is not the case. When blocking replay after half of the acquisition sessions there were no effect on

freezing. Group 5 (2/day R-ROP) froze slightly more than Group 2 (2/day) and Group 4 (2/day ROP) showing that the animals still remembered the context-shock pairing. These results therefore favor the dual-storage model, where different memory systems acquire representations independent of the HPC but at longer learning rates.

Based on the current results the SCMS, MTT, and DRT all fall short of describing the process of consolidating short-term memory into long-term memory. The SCMS claims that memories are only temporarily dependent on the HPC, and by reiteration of memories between HPC and non-HPC networks the memory eventually, over a period of days to months, become independent of the HPC and stored elsewhere (Sutherland & Lehmann, 2011). If this were true, one could predict a RA pattern similar to Ribot's law, where new memories are lost while older memories are spared in a linear fashion following HPC damage (Rudy, 2008). However, this is not the case, as literature using complete HPC damage fails to find any temporal gradients of RA (Sutherland et al., 2010, Sutherland & Lehmann, 2011). MTT differentiates between different types of memories and claim that spatial information always depends on the HPC (Rudy, 2008). As there were no other form of cues associated with the shock in this paradigm, the context must have been what triggered the memory-associated freezing behaviour. According to MTT the spatial information should always depend on the HPC, and even after multiple, distributed learning episodes the memory would be lost after HPC damage. As this was not the case, information about context can be stored somewhere outside of the HPC and MTT fails to explain why. The DRT supports the findings that memories can be stored outside the HPC, and that this happens by means of post-event HPC replay. Re-exposing the animal to the same situation multiple times leads to multiple replay

episodes and the memory is eventually stored in cortical networks other than the HPC. This does not fit our finding of the memory still being expressed after blocking HPC replay activity. These considerations lead to better support for a dual-storage model of memory consolidation. Both HPC and non-HPC networks acquire independent memory traces simultaneously. The HPC has a faster rate of learning and can express the memory after few exposures while non-HPC networks require more time as well as several re-exposures in order to firmly consolidate the memory.

Chapter 6 – General Discussion

Many theories of memory long-term consolidation have been developed. As outlined in Chapter 1 and Chapter 5 they all have features that help explain the available literature and they also have shortcomings in explaining some of the more recent research published. The two most recent suggestions, the dual-storage model and the DRT are the most encompassing theories, but they also have deficiencies. The dual-storage model does not explain all behavioral data available. In the literature review done by Sutherland et al. (2010) dual memory traces and different learning rates can explain all behavioural findings with the exception of tasks using odour and/or flavours as the key ingredient in their tasks as RA is observed when animals undergo surgery 1-2 days following training but not 3-9 days after.. One explanation of these results was presented by Rudy and Sutherland (2008). The authors claim that HPC lesions made within the timeframe on cellular consolidation cascades outside the HPC and affect connecting neocortical structures through synchronous discharge and APs. If this is true one can assume that odour/flavour memory never depended on the HPC but is disrupted by the side-effects of permanent lesions if these lesions are made within the timeframe of cellular consolidation.

The other more complex, but more applicable theory may be DRT. According to the DRT post-event HPC activity, such as replay, is necessary for the memory representation to be established outside the HPC in other cortical networks (Sutherland et al., 2010). Because replay activity diminishes rapidly after the first 30 min of rest and is almost gone after 24 hrs (Hoffman & McNaughton, 2002), multiple and distributed

learning episodes are thought to be needed to establish a sufficiently strong memory trace outside the HPC, on that can be used to recall the memory if the HPC is inactivated. This theory explains findings like the one made by Lehmann et al. (2010), (replicated here in section 5.2) where few learning episodes or multiple learning episodes in close temporal proximity are not sufficient to make the context memory HPC independent. However, multiple and distributed learning episodes creates a memory that appears to be unaffected by HPC damage. The DRT can be used to explain the flavor/odour findings by only one session of replay activity being needed in order for the memory to consolidate outside the HPC. If the DRT is correct, one would assume that there would be marked constancies in the replay activity after each training session over a period of days.

There are still many questions unanswered about replay, such as does it facilitate consolidation, does it play a role in the consolidation of all types of memories, and is replay even a part of the consolidation process at all or does it simply represent already acquired memories (O'Neill, Pleydell-Bouverie, Dupret, & Csicsvari, 2010). In Chapter 5 we block post-learning replay activity following half of the learning sessions. This did not affect retention day performance, indicating that the replay was not necessary for the memory to consolidate outside the HPC. Considering the arguments presented by O'Neill et al., (2010) there is still a chance that replay is necessary for consolidation of other types of memories more complex than context-fear. There is also a chance that blocking replay for a few hrs following only half of the learning sessions in Chapter 5 may have been insufficient and that longer-lasting blockage or blockage after all six sessions is needed in order to see a behavioural effect. On the other hand, because of the significant uncertainty about the specific processes involving replay, it is hard to adapt the DRT as a

reliable model for memory consolidation. More work on the effect of blocking replay on behavior and learning is needed in order to draw firmer conclusions.

Summary of results

Single vs. multiple bilateral infusion sites: Through a series of experiments we set out to cast more light on the current situation of theories of memory consolidation. By conducting a literature review of the current research where temporary inactivation of the HPC in rats are used it became clear that there is great inconsistencies in the methodology as well as few controls to evaluate the methods used. We therefore started this thesis by evaluating the extent of inactivation by a single bilateral infusion site. Because 90% of the reviewed articles only used single bilateral infusions sites, most commonly aimed at the dHPC, it was important to establish if any and how much of the HPC remained active. The results were consistent with the hypothesis that by injecting a local anesthetic only into the dHPC, most of the neurons in the vHPC would still remain active. This becomes important when considering anatomical connections and regional specifications within the HPC. It also helps explain findings such as the ones by Kim and Fanselow (1992) who reported that a dHPC lesion decreased performance in the context fear paradigm but not in the cued fear. According to Bannerman et al. (2004) the dHPC is important in the context part of memory while the vHPC has more close connections with the amygdala and therefore is more important in the expression of anxiety and fear. Kim and Fanselow (1992) used small dHPC lesions when evaluating the extent of RA on context fear and auditory cued fear. They found no effect of lesion on auditory fear, concluding that this was a HPC-independent task. This was not the findings of Lehmann,

Sparks, O'Brien, McDonald and Sutherland (2010). Comparing different HPC lesion sizes, the authors found that only complete HPC damage showed an effect of fear-potentiated startle, indicating that auditory fear is based on the HPC but that there might be a functional segregation within the HPC.

Flush vs. extended infusion needles: From the 65 articles with HPC infusions reviewed only one article used infusion needles that were the same length as the permanent guide cannula when temporarily inactivating the HPC. The remainder of the needles used were protruding somewhere between 0.5 mm and 3 mm below the end of the guide cannulae. Disrupting healthy brain tissue can lead to complications and we therefore hypothesized that using protruding infusion needles would lead to pronounced discharge by cells surrounding the infusion needle. By using a 1 mm protruding infusion needle we did find an increased number of cFos expressing cells that could be associated with pronounced discharge of cells. The expression of cFos was present both when ROP was infused and when there were no infusions, indicating that it was the infusion needle that caused the cFos activation and that this activation was persistent enough to withstand a Na⁺ channel blocker. When using infusion needles that were flush with the guide cannulae the cFos activity was not present, neither when there were no infusions nor when ROP was infused. It is therefore clear that it is advantageous to use a flush infusion needle to prevent any confounds in the behavioural results which could be attributed to seizures.

Ropivacaine timeline: Because of observed side effects, such as decreased locomotion, associated with the toxicity of some of the most commonly used temporary inactivating drugs, we wanted to use a local anesthetic not currently used in rat behaviour

but that have a lower toxicity level than its derivatives. Ropivacaine is currently used in hospitals but have not yet been adapted in to animal research. The results reported in the experiment described in Chapter 4 showed that ROP infusion into two bilateral sites reduced cFos expression by 97%. The effect of ROP is maximized 45 min after infusion and there was complete recovery of function indexed by cFos after 6 hrs. Because the main experiment of this thesis, described in Chapter 5, required multiple infusions of the drug in to the same animal, it was important to use a drug that has low toxicity levels. As we were testing the animals every day, it was also important that we knew the timeline of the effect of the drug. Based on reports of the timeline of a similar drug, bupivacaine, we had hypothesized a function quite similar to the one presented in the Chapter 4 result section. The main difference is that bupivacaine seem to be faster acting and its effectiveness declines faster than with ROP (Schroeder, Wingard, & Packard, 2002).

This study also show that by using two bilateral infusion sites 97% of the entire HPC is inactivated which makes this method more efficient than using one single bilateral infusion site when testing the function of the HPC as a whole.

The role of hippocampal replay in making context memories hippocampus-independent: In the final experiment we wanted to distinguish among predictions of the current theories of memory consolidation. According to the DRT model, post-event HPC replay activity is necessary to facilitate memory consolidation outside of the HPC. In these experiments the results reported by Lehmann et al. (2010) were systematically replicated, by showing that three learning episodes distributed over three days were insufficient to make the memory HPC-independent. However, six learning episodes distributed over the same amount of time made the memory survive HPC inactivation.

We then blocked the replay activity associated with half of six acquisition sessions. According to DRT, this group should perform at the same level as the three session group on retention day. This was not the case. We found that there were no effect of blocking replay as the animals froze during the retention just as much as controls and more than the group that had experienced only three sessions. These results fail to support the DRT as a satisfactory model for memory consolidation. An alternative view should be considered.

Interpretation

It is clear that temporary inactivation is a powerful method in studying brain function. However, great inconsistencies in methodology and weak control measures on extent and successful inactivation makes many of the results presented so far questionable. There is need for a literature standard which does not cause behavioural confounds. Using flush infusion needles together with proper measurements of inactivation at the time of testing would help improve the standard of studies that adapt this methodology.

The results from Chapter 5 fail to support the DRT as a satisfactory model for memory consolidation. A non-HPC context-fear memory was established even though post-event HPC activity was blocked. This means that non-HPC context memory systems do not require post-event HPC input to effectively establish a memory. A consolidation model like the dual-storage model where memory traces are established independently of one another is most likely correct. The non-HPC systems require more reiterations of the

learning episode in order to form a strong memory representation. This is why RA is observed with HPC inactivation or damage if only few sessions are used.

Strengths/short-comings of the study

In the control experiments (Chapters 2-4) the biggest weakness was the number of rats per group. Many of the groups had an $n = 1$. When doing behavioural research this is less than ideal as the results should represent the majority of a population and not just a single few. On the other hand, these experiments were meant as simple controls of methodology using cFos and MECS. Inactivating only the dHPC in two animals showed that in both animals all of the vHPC is left functional. It is unlikely that it is necessary to have a higher n than the one used in this experiment as if there even is a chance that some of the HPC is left functional the hypothesis would be supported. The aim of the three studies was identify key variables in the current literature that introduce interpretive difficulties involving behavioural effects of HPC inactivation and to find a method that can avoid interpretive problems. Chapter 3 had group sizes of $n=1$ in all four groups. There could be a benefit of adding one more rat to each group; however, if there is any chance that there is seizure-like activity observed using a protruding infusion needle and this activity is not observed with a flush needle than the choice of which needle length to use is simple. Even though each group only had $n=1$ there were several infusion sites that had protruding needles and they all showed the same increased level of cFos expression, a level that was not associated with the flush needle tip. Because of these findings, we think we there is a strong case when arguing that in order to eliminate any possible

cofounds and false behavioural results, flush infusion needles are recommended, as are two infusion sites in each HPC.

In Chapter 4 the timeline of the efficacy of ROP was calculated using unilateral infusions and cFos expression after MECS as a within-subject control. One animal was eliminated from the study which caused one group (6 hrs) to have a smaller n than the other four groups. Even though the n were small again, we do not believe that it creates significant problem because the experiment was to examine relatively low variability physiological data and not behavioural data. In the groups were that had $n=2$ the SEM was fairly small (between 0.001 and 0.17) indicating that the results are consistent and it is safe to assume that the values reported in fig 4.1 would not change even if more rats were added to the different groups.

Chapter 5 was the main experiment in the thesis. The behavioural paradigm was based upon Lehmann et al. (2010). They had already shown that multiple distributed context-shock pairings would successfully make a context memory independent of the HPC. The main difference in the study presented by Lehmann et al. and this study is the use of permanent lesions and temporary inactivation. In the study described by Lehmann et al. (2010) the rats underwent surgery and complete HPC damage using the neurotoxin N-methyl-D-aspartic acid (NMDA). NMDA is an excitotoxin and the immediate effect of injecting NMDA is seizures (Zaczek, Collins, & Coyle, 1981). The freezing levels observed in the groups that did not successfully make the context memory HPC-independent were lower than 20%. This is substantially lower than the freezing levels seen in the same groups in this study. We chose to use temporary inactivation as a replacement for permanent lesions to make the study more temporally compacted and to

eliminate some confounds of using permanent lesions (see Chapter 1). The fact that the groups in this study froze more than the corresponding groups in the Lehmann et al. study is likely to be a true difference between using permanent lesions and temporary inactivation. There might be an effect of injecting excitotoxic drugs in to a brain structure and causing subsequent seizure activity and permanent damage that is different than turning off the electrical currents temporarily. There is a possibility that the permanent lesions affect structures that are connected to the target structure and therefore affect regions other than the one targeted. There is also the possibility that simply turning off electrical currents is not enough to turn off all memory related processing within the HPC and this is why we still see some memory when ROP is used. Based on the results of section 5.3 we believe that the reason some freezing is observed in Group 3 (1/day ROP) is that another memory system has already started acquiring the memory and it is at this time not as strong as a HPC memory nor has the memory had a chance to fully consolidate outside of the HPC. Another methodological difference between the Lehmann et al. (2010) study and the present experiment is that they used multiple learning episodes on one day while we used few learning episodes distributed over time. It might be that the temporal component is more important than multiple exposures in making the memory HPC independent.

One of the major assumptions in Chapter 5 is that by inactivating the HPC immediately before an acquisition session replay activity associated with that session was eliminated. Unfortunately there was very little control over whether or not this actually happened. HPC replay is described as diminishing after only 30 min (Hoffman & McNaughton, 2002). Turning off the entire HPC for a period of approximately 3 hrs we

predict that the replay activity is turned off and not just delayed until the effect of the drug wears off. Girardeau, Benchenane, Wiener, Buzsaki and Zungo (2009) blocked replay activity using electrical currents for 1 hr immediately following an acquisition session in a radial-arm maze paradigm. This led to the delay in above-chance performance on the task as well as consistently performing below control rats, even though they did learn the task. Quiroz, Martinex, Quirarte, Morales, Diaz-Cintra and Prado-Alcala (2003) showed that by temporarily inactivating the dHPC using TTX infusions following a single session of context-fear conditioning rats that received mild foot-shock showed RA while rats with a higher intensity foot-shock showed did not. The authors concluded that by inactivating the HPC immediately after acquisition the consolidation process was disrupted and if the emotional aspect of the memory was not strong enough (mild foot-shock) the memory was lost. Sutherland et al. (2010) and Sutherland and Lehmann (2011) discuss the process of cellular consolidation which takes place from a few hrs to 24 hrs following a learning episode. Therefore, even if the replay activity *per se* and the sharp-wave ripple events characteristic of this activity is merely delayed by the ROP infusion in this experiment, the inactivation should disrupt any cascade of neural circuitry events eventually leading to cellular consolidation. Therefore, the conclusion that post-event HPC activity is redundant for the memory to be consolidated outside of the HPC still stands strong.

Because only 10 animals were eliminated due failed ROP infusion at the time of testing in one or more infusion sites, we are fairly confident that the post-training HPC activity was turned off satisfactory in all cases and that the data presented are an accurate reflection of the effects of replay blockage.

In Chapter 2 we described how there is a general lack of control on the extent on inactivation following infusion of some drug in the literature. To avoid this pitfall the brain tissue was labeled for the IEG cFos following testing in order to verify the extent of inactivation. The basal cFos expression seen in the control animals was lower than expected, making it difficult to establish the degree of inactivation at all four infusion sites. Quiroz et al. (2003) presented an alternative to simply using baseline cFos expression as a control and instead infused kainic acid to induce seizure activity and this way creating a more visible control for the inactivation. It would have been possible to give the rats MECS following retention testing to better establish the inactivation. Because there were still a significant difference between control cFos levels and cFos levels in inactivated brain, the method used was sufficient in showing the desired results, but could have been strengthened.

Conclusion and future directions

Wiltgen et al. (2006) showed that if the HPC was damaged prior to context fear conditioning the animal could learn the task as well control animals. However, if the damage happened after the training the memory was lost. Sutherland et al. (2010) explains this phenomenon with overshadowing, where the HPC overshadows other memory systems, slowing down their learning time when present. This interference must be related to HPC activity, as it disappears when the HPC is inactive. The HPC therefore appear to have some kind of interfering interaction with the other memory systems, however, this interaction must take place at the time of learning and not after.

The role of replay. Replay activity is a phenomenon which is becoming well established within the literature. The aim of Chapter 5 was to establish whether or not this activity is necessary for a memory to become HPC independent. The results failed to support this theory. However, replay is still a seemingly reliable phenomenon. The function of replay has been discussed previously (see O'Neill, 2010) and the options are many. There might still be a role for replay in memory consolidation, but it would be consolidation within the HPC, not involving non-HPC networks. It is also unknown if the memory representation is the same for a rat that has a HPC independent memory as it is for a rat that still has an intact HPC. There is a possibility that replay helps facilitate more complex representations and that we would see an effect of turning replay off if the task had greater complexity. Girardeau et al. (2009) blocked replay activity during sleep following training in a radial arm maze. The rats still learned the task, however it took them longer to do so and they never reached the same levels as the controls. If HPC replay is needed in order for the memory to consolidate within the HPC, the researchers would have blocked the possibility for the HPC to learn the task. Every time the rat enters the maze, the learning is new for the HPC but not for the non-HPC systems. Their findings are therefore consistent with the dual-storage view. It takes the non-HPC network more trials to learn the task compared to the HPC network (controls). Because the HPC-replay blocked rats never reach the performance level of the controls, it is possible that the HPC is capable of more detailed memory representations than other memory systems, and therefore a HPC dependent memory could be superior to a less detailed non-HPC memory.

A modified theory of memory consolidation. Based on the findings in Chapter 5 DRT was not supported as a model for memory consolidation for simple memory representations. Instead, a dual-storage model where HPC and non-HPC memory systems operate independently is favored. However, this is still a very simplified description of the literature available. The HPC interacts with other memory systems at the time of learning by at least one process, the one of overshadowing. The functions of this are unknown and not adequately constrained by current evidence. It is possible that the HPC is capable of more detailed memory representations than other memory systems, and therefore a HPC dependent memory could be superior to a less detailed non-HPC memory. Post-learning HPC replay activity may aid in facilitating storage of more complex representations. A great challenge for future research will be to find a task complex enough to see an effect of replay blockage, but still simple enough for non-HPC memory systems to learn. Such a task can become a powerful tool in further investigating the interactions between memory systems with the brain.

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