HIPPOCAMPAL OVERSHADOWING: EXPLORING THE UNDERLYING MECHANISMS

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ABSTRACT

The aim of the current thesis was to evaluate the underlying mechanisms of hippocampal (HPC) overshadowing. One theory to explain this phenomenon predicts that perceptual representation of an event is stored in non-HPC cortical areas. Following multiple distributed exposure to the same or similar event, this representation becomes stable and the cellular activation patterns can be recalled in the absence of HPC input. Through a series of experiments, the hypothesis that the dysgraunlar layer of the retrosplenial cortex (RSD) fulfill these requirements was tested. Using double *in-situ* hybridization for the mRNA of immediate-early genes Arc and Homer1a, the cellular activation patterns in HPC subregion CA1 and RSD were evaluated. In addition, temporary inactivation of HPC was used to evaluate cellular activation patterns of a HPC-independent memory in RSD. The results presented here support the idea that cellular activation patterns for may be stored – at least in part – in the RSD.

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ACh – acetylcholine
aCSF – artificial cerebrospinal fluid
ATN – anterior thalamic nucleus
AV – anteroventral anterior thalamic nuclei
BLA – basolateral amygdala
CaMKII – calmodulin-dependent kinase II
CAS – configural associative system
CR – conditioned response
CS – conditioned stimulus
DAPI – 4',6'-diaminidino-2-phenylindole
DEPC – diethylcarbonate
DG – dentate gyrus
EPSP – excitatory post-synaptic potential
dISH – double in-situ hybridization
GABA – ϒ-aminobutyric acid
$GABA_A$-R - ϒ-aminobutyric acid type A receptor
H1a – Homer1a
HPC – hippocampus
HV – high voltage
IEG – Immediate early gene
i.p. – intraperitoneal
ISH – in situ hybridization
LSD – least significant difference
LTP – Long-term potentiation
MECS – maximal electric convulsive shock
mGluR – metabotropic glutamate receptors
mRNA – messenger RNA
MTL – medial temporal lobe
PBS – phosphate buffered saline
PFA – paraformaldehyde
PHR – perirhinal cortex
RNA – ribonucleic acid
ROI – region of interest
RSC – retrosplenial cortex
RSD – retrosplenial cortex dysgranular layer
RSG – retrosplenial cortex granular area
RTF – regulatory transcription factors
SAS – simple associative system
SSC – saline-sodium-citrate
TBS – Tris Buffered Saline
TBST – Tris Buffered Saline, Tween
TSA – Tyramide signaling amplification
US – unconditioned stimulus

CHAPTER 1: GENERAL INTRODUCTION

1.1 General introduction

Since the scientific study of cognition began, multiple theories of learning and memory have been proposed. It is safe to say that the accumulated experimental evidence has not led to a consensus on any theory of memory. Most experimental approaches have relied on behavioural evidence and observing the behavioural outcomes of various manipulations. The emergence of electrophysiological recordings of awake, behaving animals (Hubel & Wiesel, 1959) widened the focus of observations from exclusively behavioural to include important physiological observations. Multiple studies were conducted with the aim of investigating the activation and role of specific brain systems and circuits in different learning and memory tasks, especially tasks involving spatial navigation and spatial memory (e.g. O'Keefe & Nadel, 1978; McNaughton, Barnes & O'Keefe, 1983; Chen, Lin, Green, Barnes & McNaughton, 1994; Chen, Lin, Barnes & McNaughton, 1994; Jung, Wiener, & McNaughton,1994; Gothard, Skaggs, Moore, & McNaughton, 1996; Moser & Moser, 1998; Kudrimoti, Barnes & McNaughton, 1999; Leutgeb, Leutgeb, Moser & Moser, 2007; Hafting, Fyhn, Bonnevie, Moser & Moser, 2008; Kjelstrup et al., 2008). Recording studies have led to an increased understanding of basic physiology of neural systems and circuits, including their relation to learning and memory. However, a significant gap between single neuron physiology and behaviour still exists, particularly at the level of understanding how large populations of neurons interact across systems. One favourable system for examining these issues is the hippocampus (HPC), a cortical region that has a very well established connection to spatial learning and memory, as well as to context-specific memory processes. A goal of this thesis is to find support for a theoretical approach that contains a neural systems level explanation of the behavioural outcomes of certain

recent learning and memory experiments, especially of the observations underlying the phenomenon termed *"hippocampal overshadowing".*

1.2 Theories of hippocampal overshadowing

Early theories of learning and memory were built on the idea that the repeated pairing of two events in time will result in the growth of a connection, or association, between their internal representations (Pavlov, 1927). If two independent events, such as A and B, occur in temporal proximity, a later presentation of A will retrieve a cerebral representation of B. The co-occurrence of A and B produces a connection or association between them (Pavlov, 1927; Rudy, 2008). The existence of this association will then allow the first event A to excite the representation of the second event B, which may then lead to the performance of a response. This type of learning has been termed *Pavlovian conditioning* and is a classic example of associative learning. Pavlov (1927) also outlined a possible scenario which included overshadowing. In the Pavlovian sense, overshadowing refers to a form of associative competition where a conditioned stimulus (CS) supports conditioning when paired on its own with an unconditioned stimulus (US), but fails to develop conditioned properties when it is paired with an US in conjunction with a more salient stimulus (Pavlov, 1927).

Overshadowing is not unique to conditioning of different exteroceptive cues. Maren, Aharonov and Fanselow (1997) provided what has become the hallmark experiment when considering overshadowing involving the HPC. They showed that if a context is paired with a shock, rats learn to fear the paired context after only one learning episode. If the HPC is damaged after the learning episode, the context fear memory is lost. On the other hand, if the HPC is damaged prior to the learning episode, rats learn the association just as quickly as if the HPC was still intact. This result indicates very clearly that there is more than one

memory system that can support learned associations. How is it that if a system outside of the HPC can efficiently acquire the memory, that it apparently does not do so when the HPC is intact? Overshadowing suggests that when two or more memory systems are active during an event, a competition occurs between them and the dominating system will acquire the memory and overshadow the weaker system (Sutherland, Sparks & Lehmann, 2010). When considering context memories, including context fear, the HPC is the default system that supports the learned association. Biedenkapp and Rudy (2009) argued that the HPC system and an extra-HPC system compete for control over the fear system during contextual fear conditioning, and that under normal conditions, the HPC win this competition. In both of these examples, there is a competition of the output systems. In a very similar view, building on Sutherland and Rudy's (1989) configural association theory, Fanselow (2010) suggested that a HPC configural or gestalt-like context representation overshadows any individual stimulus elements in the context. However, if there is no configural representation of the context then simple associations are formed. Maren et al. (1997) argued that in the case of contextual fear conditioning, rats use a HPC-dependent configural strategy to learn the association, which normally overshadows a non-HPC-dependent strategy. Rats with HPC lesions, however, learn associations between the fear and the most salient unimodal element in the context, such as odor (Maren et al., 1997). The idea that the HPC competes with other systems at the time of acquisition is a popular one and has been tested and described by many (Maren et al., 1997; Frankland, Cestari, Filipkowski, McDonald & Silva, 1998; Cho, Friedman, & Silva, 1999; Wiltgen, Sanders, Anagnostaras, Sage & Fanselow, 2006; Biedenkapp & Rudy, 2009, to mention some).

An alternative view to the theory that the competition occurs during memory acquisition, is that the competition occurs during retrieval and behavioural output. A classic example comes from McDonald and White (1993), who elegantly showed a triple-

dissociation between the HPC, amygdala and striatum in solving different versions of the radial-arm maze (win-shift, conditioned cue preference, and win-stay; respectively). A well-designed study done by Sparks, Lehmann and Sutherland (2011) used temporary inactivation to demonstrate that if the HPC is inactivated at the time of learning to fear a context, the association is learned, but the conditioned response is only observed if the HPC is inactivated at the time of recall, and not if the HPC is active. The authors suggested that the HPC was interfering with retrieval by the other system, indicating that competition may happen not only during acquisition, but also during retrieval or behavioural output. Whether competition happens at the time of learning or at the time of retrieval, no neural mechanisms that can explain these findings have been presented.

A last account of overshadowing states that the perceptual representation of a context is stored in regions of association neocortex. The perceptual representation is created by a combination of bottom-up sensory inputs, as well as, top-down inputs from systems that include HPC. Thus, HPC output to association cortex plays a critical role in creating a stable context representation. If the HPC is damaged or inactivated, the representation is significantly altered. As the cortical representation of the context is now incomplete, the target memory connected to the context is not retrieved (Sutherland et al., 2010; Lee, McDonald & Sutherland, in preparation). This last view suggests that at the time of learning, multiple systems contribute to forming an activation pattern unique to the event. When removing one or more of the systems involved in the initial encoding, the pattern is altered. If significantly altered, the behavioural outcome is retrograde amnesia. This view allows for specific predictions of neuronal patterns following learning, and can therefore be tested in detail. If unique representations are stored in neocortex, one can expect that the pattern of neuronal firing will be different between two different contexts. In addition, if this representation includes information coming from the HPC, the pattern will

change when the HPC is inactivated or damaged. Lastly, if the contextual representation is to survive HPC damage, the neuronal pattern will still be present even after the HPC is removed. Possible underlying mechanisms have already been proposed. Leutgeb and Leutgeb (2007) described a property of the cellular network within the HPC termed *pattern completion*. Pattern completion is the retrieval of a memory when only part of the original sensory information is available. When an animal explores an environment, different cells within the HPC fire at different locations. The firing pattern is stable within the same space even at different time-points, but they differ between different locations (Leutgeb et al., 2007). Similarly, when exposed to different contexts, cells in the HPC discriminate by means of different firing patterns. This observation has been termed *pattern separation* (Leutgeb & Leutgeb, 2007). The phenomena of pattern completion and pattern separation have been demonstrated using intra-cortical recordings of cell assemblies from both CA1 and CA3 of the HPC (Leutgeb et al., 2007). If an event such as pattern completion and pattern separation take place outside of the HPC, this could provide a mechanism for overshadowing. A non-HPC system that can acquire a contextual memory, should also be able to discriminate between contexts (Lehmann et al., 2009). When the HPC is removed, the neuronal firing pattern outside of the HPC will change. However, through the process of pattern completion the memory may still be recalled. Assuming that a phenomenon such as pattern completion requires multiple, distributed exposures to the same event, this could provide a prediction to what happens to the cellular firing patterns within a non-HPC system in the absence of the HPC, and this prediction can be tested.

1.3 The hippocampus and retrosplenial cortex

Since the discovery that removal of the HPC in an epileptic patient resulted in loss of any previously established episodic memories, as well as in the ability to form new ones

(Scoville & Milner, 1957), the HPC has served a central role in the study of learning and memory. The HPC is located in the medial temporal lobe (MTL) and consists of four principal subfields, DG, CA1, CA2, and CA3 (Lorente de Nó, 1934). An intricate network of reciprocal connections within the parahippocampal region allows for fine-tuned analysis of sensory information, and the integration of this information into rich autobiographical experiences and memories (Eichenbaum & Lipton, 2008). The organization of the MTL involves bidirectional pathways between the cerebral cortex and the HPC, which is largely conserved across mammalian species (Manns & Eichenbaum, 2006). Anatomical and behavioural findings regarding this region obtained from rodent, monkey, and human data are, in general, quite consistent.

The retrosplenial cortex (RSC) is highly interconnected, as well as functionally similar to the regions within the HPC system (Sugar, Witter, van Strien & Cappaert, 2011), where damage to the region results in impaired performance in spatial memory tasks (Sutherland et al., 1988), allocentric working memory tasks (Vann & Aggleton, 2004), egocentric memory tasks (Cooper & Mizumori, 1999; Whishaw et al., 2001), and in detecting the novelty of a spatial arrangement (Vann & Aggleton, 2002). The RSC is divided in to the granular area (RSG) and the dysgranular area (RSD). The rodent RSD is comprised of Brodmann's area 29d. RSD receives HPC formation projections from subiculum, postsubiculum, and entorhinal cortex, and share reciprocal connections with anterior thalamic nuclei (ANT), neocortex and HPC (van Groen and Wyss, 1992). In addition, RSD also receives visual information from visual cortex areas 17 and 18 (Wyss & van Groen, 1992).

Considerable evidence indicates a strong role for the HPC in combining object and spatial information (Eichenbaum & Lipton, 2008; Eichenbaum & Otto, 1993). Results from studies in rats, monkeys and humans suggest that HPC neurons encode associations

between specific stimuli in a unique location or behavioural context (Eichenbaum & Lipton, 2008). HPC firing patterns reflect unique conjunctions of stimuli with their significance, the animal's specific behaviours, and the places and contexts in which the stimuli occur (Eichenbaum & Lipton, 2008; Eichenbaum & Otto, 1993). This was further supported by the findings of HPC *place cells* which can be found in throughout the HPC, especially in CA1 and CA3. Place cells are neurons which fire when the animal is in a specific location (O'Keefe & Nadel, 1978). Place cells encode information through several means, such as firing rate, pattern of discharge and spike timing (Royer et al., 2012). It has been hypothesized that the place cells in CA1 have a central role in path integration, a process of continuous monitoring of position within an environment (Leutgeb et al., 2007; McNaughton, Battaglia, Jensen, Moser & Moser, 2006). Spatial processing is evident within most regions of the parahippocampal region, progressively becoming more spatially selective closer to the HPC, with the place cells being a very finely tuned system, especially in the septal portion of the CA fields. It seems clear that the information driving the place cell network originates in upstream cortical areas (Skaggs, McNaughton, Wilson, & Barnes, 1996). This prediction was confirmed for RSC by Cooper and Mizumori (2001) who showed that following temporary inactivation of the RSC, HPC place cells maintained their firing fields relative to each other, but lost their orientation to the external context. This finding indicates that the RSC may contribute to the integration of visual and self-motion cues during navigation (Cooper & Mizumori, 2001). Further evidence for this can be found in the presence of *head-direction* cells in RSC (Chen et al., 1994a, 1994b). These cells are similar to place-cells, but instead of firing in a specific place, they fire when an animals head is facing a specific location. In addition, a growing body of research is showing that damage to the RSC has significant impact on performance in several behavioural tasks, such as the fixed location (Sutherland, Whishaw, & Kolb, 1988; Sutherland & Hoesing, 1993; Warburton, Aggleton & Muir, 1998;

Vann & Aggleton, 2002; Harker & Whishaw, 2004) or daily-changing location of a hidden

platform in a water task (Sutherland & Hoesing, 1993; Sutherland et al., 1988; Harker &

Whishaw, 2004; Vann, Wilton, Muir, & Aggleton, 2003), objects-in-place discrimination

(Vann & Aggleton, 2002), and animals with RSD lesions are less reliant on distal cues to

control performance of a working-memory task in the radial-arm maze (Vann & Aggleton,

2002; Vann & Aggleton, 2005; St-Laurent, Petrides, Sziklas, 2009), as well as on tasks

requiring rats to use directional information (Pothuizen, Aggleton, & Vann, 2008). In

addition, Maviel, Durkin, Menzaghi and Bontempi (2004) demonstrated that when testing

for long-term memory storage, RSC was identified as a cortical region involved in memory

consolidation. Long-term memory storage was also accompanied by structural changes such

as synaptogenesis and laminar reorganization, and led the authors to suggest that the RSC is

involved in associative processing of complex visuospatial representations, including the

gradual establishment of extra-HPC spatial maps (Save, Nerad, Poucet, 2000; Maviel et al.,

2004). The complementary findings of behaviour and cellular activity in HPC and RSC makes

the investigation of the interaction of these two regions a particularly interesting one.

1.4 Immediate early genes

Immediate early genes (IEGs) have become more and more common when

evaluating neuronal activation within single systems or between multiple systems (e.g.,

Rosen, Fanselow, Young, Sitcoske, & Maren, 1998; Guzowski, McNaughton, Barnes & Worely,

1999; Huff et al., 2006; Kubik, Myashita & Guzowski, 2007). The use of IEG labeling

provides researchers with a method of evaluating neuronal activation during specific time

points during behavioural episodes, and these activations patterns can be compared across

multiple regions within the brain. In the current thesis, the use of IEGs becomes useful as

one of the goals is to evaluate cellular activation patterns in HPC and non-HPC cortical regions, as well as their interaction.

IEGs were first described as proto-oncogenes, a term which referred to a group of genes which were rapidly and transiently activated by neurotransmitters (Robertson, 1992). In the absence of stimulation, the transcription and translation of IEGs in the brain is generally low. However, following cellular stimulation, transcriptional activation of IEGs is rapid, and the mRNAs have a very short half-live (Robertson, 1992). The expression of certain specific IEGs enable a stable change in synaptic strength (Abraham et al., 1993; Worley et al., 1993), which has been related to learning (Hess, Lynch & Gall, 1995; Nagahara & Handa, 1995; Vann, Brown, Erichsen & Aggleton, 2000). Although there are many different IEGs, two are specifically interesting with respect to the current thesis. Arc and Homer1a (H1a) are effector IEGs and have a number of cellular functions capable of modifying synaptic function (Vazdarjanova, McNaughton, Barnes, Worley & Guzowski, 2002; Lanahan & Worley, 1998). Transcription of Arc and H1a is dramatically up-regulated in the same HPC and neocortical neurons of rats after exploration of a novel environment (Vazdarjanova et al., 2002). Importantly, nuclear Arc mRNA labeling is visible 5 minutes after cellular activation (Guzowski et al., 1999; Vazdarjanova et al., 2002), while intra-nuclear foci of H1a appear 25 minutes after novel context exploration (Vazdarhanova et al., 2002). In addition, 16 minutes after novel context exploration, intra nuclear foci number for Arc returns to control levels (Vazdarjanova et al., 2002). H1a foci are visible in the nucleus one hour after stimulation (Guzowski, McNaughton, Barnes, & Worley, 2001). Therefore, when designing an experiment in which neuronal activation during two different events are of interest, utilizing double fluorescent *in situ* hybridization (dISH) for Arc and H1a becomes a powerful tool. Such an experiment needs to take into consideration that experiences related to nuclear H1a labeling need to have taken place 20 minutes to 1 hour prior to

sacrificing the animal, and experiences related to Arc labeling need to have taken place

between 5 and 15 minutes prior.

1.5 The goal of the current thesis

Predictions related to HPC overshadowing have been outlined in section 1.2. One

hypothesis is that HPC and non-HPC memory systems compete for associative strength at

the time of acquisition (Maren et al., 1997), while other experiments suggest that the

competition occurs also during recall (Sparks et al., 2012). Although parsimonious, these

theories do not provide any clear mechanisms that can explain the behavioural outcomes of

experiments such as those by Maren et al. (1997) and Sparks et al. (2012). There is however

a view which outlines a hypothetical process that makes some clear predictions. The idea is

that perceptual representations of the context are created in association cortex. The

representations are created from convergence of bottom up (from primary sensory

cortices) and top-down (from HPC) sources. If either bottom-up or top-down inputs are

altered or inactive, the neuronal firing pattern changes, which in turn can lead to a failure of

perceptual representations of the context to retrieve the target memory.

Based on experimental and observational findings, it has been suggested that

different networks contribute to a holistic memory representation by providing different

aspects of an event. Each system is responsible for encoding, storing, and retrieving

information concerning the different types of characteristics they support (Milner, 1959;

Nadel & Moscovitch, 1997; Sherry & Schacter, 1987; McDonald & White, 1993; Squire &

Zola, 1996). The idea of different neuronal firing patterns for different experiences has also

previously been demonstrated. Leutgeb et al. (2007) showed that regions within the HPC

activated non-overlapping activity patterns when exposed to environments differing in

shape, providing experimental support that the HPC pattern-separates between similar

memories. Similarly, Guzowski et al. (1999) used Arc mRNA activation pattern to show that when rats were exposed to the same context twice, significantly more cells were activated in both exploration sessions compared to when two different contexts was explored. There are however no experiments published where both behaviour and neural activation within the frames of overshadowing have been investigated.

In the current thesis, it is predicted that HPC forms two different neuronal activation patterns when exposed to two different contexts, and that these differences can be detected using Arc and H1a dISH. There should be fewer cells expressing both IEGs if a rat visits two different context than if it visits the same context twice. In addition, it is hypothesized that a similar context-specific activation occurs in RSD. In order to shed light on how HPC may interact with cortical regions, the experiment will have rats explore either one or two contexts until exploratory behaviour reaches an asymptote. It will be demonstrated that when a rat enters a context that has become very familiar, the rat will explore the context significantly less than if the context was novel. It will also be demonstrated that recognition of a context that has only been encountered once is lost following HPC inactivation, while this is not the case if the context is familiar and has been explored multiple times over the course of days. Lastly, the cellular activation pattern for exploration in a novel compared to a familiar network will be evaluated in the presence and absence of HPC input. If a representation of different contexts is indeed stored in the cortex, these networks become stable after multiple, distributed training sessions. If at this time the HPC is inactivated, the cortical network should remain stable. However, if a context is explored only once, a subsequent exploration with the HPC inactivated will lead to a new unique neuronal firing pattern.

The first aim of this thesis is to establish a behavioural task suitable for testing of the hypothesis. In Chapter 2 rats will be trained in one or two different contexts over the course

of days in order to establish a behavioural pattern of exploration and habituation that can be used in later experiments. In Chapter 3, a method of temporarily inactivating the HPC within the time-frames presented by the use of Arc and H1a mRNA labeling will be established. HPC overshadowing phenomena suggest that the HPC and at least one non-HPC system acquire information about a context, and that they do so in parallel. This will be evaluated in Chapters 4 and 5, where cellular firing patterns in CA1 and RSD are evaluated following exploration of one context twice or two different contexts in succession. In addition, inactivation of the HPC will result in the disruption of the cortical context representation for a novel context, while the cortical context representation of a very familiar context will remain unaffected. In Chapter 6 this is tested using both behaviour and IEG labeling in RSD. It is hypothesized that when the HPC is inactivated following the exploration of a familiar context, but prior to re-entry to the same context, the proportion of cells that are active in both contexts will be significantly higher than if the same experiment was done using a novel context. If this is the case, it would support the idea that perceptual representations for a context are stored outside of the HPC, and following multiple, distributed exposures to the context this pattern becomes stable and can be re-activated in the absence of HPC input.

CHAPTER 2: EXPLORATORY BEHAVIOUR OF THE *RATTUS NORVEGICUS*

2.1 Introduction

Rattus norvegicus – or the Norway rat – has been used in behavioural, neural and physiological research for more than a century (Iwaniuk, 2005). Over this time-span, the different behavioural characteristics of the rat has been widely studied, and different aspects of the rat's natural behaviour, such as neophilia, neophobia, and social interactions has been utilized in several behavioural studies, such as novel object interaction (Ennaceur & Delacour, 1988), place preference (Rossier, Kaminsky, Schenk and Bures, 2000), and social food preference (Galef, Whiskin, & Bielavska, 1997). Most relevant to this thesis is the neophilic approach to unfamiliar places.

The Norway rat is highly exploratory and when in their home range/enclosure, the rat will move around in the search for food, water, nesting material or shelter (Barnett, 2005). The rat's natural tendency to explore provides an avenue for studying the neural basis of both spatial and motor behavior and can also be used as an assay of brain function (Gharbawie, Whishaw & Whishaw, 2004). One of the major functions of exploration is information gathering (Save & Poucet, 2005). When rats are exposed to a novel environment, there is a pronounced increase in behavioural activation, especially rearing (Cerbone & Sadile, 1994). Rearing is a natural behaviour for the rat which involves raising up on the hind legs in order to better look around and smell the surrounding area. Rats use this behaviour frequently, and it is considered a sign of curiosity, part of its exploratory repertoire. As the rat becomes more familiar with the environment, the number of rears decrease (Cerbone & Sadile, 1994; Thiel, Huston, & Schwarting, 1998). The decrease in rearing is attributed to habituation (Save & Poucet, 2005). Behavioral habituation provides one of the most elementary forms of learning, both in animals and humans (Thiel et al.,

1998), and is defined as a response decrement with repeated or continuous presentation of indifferent stimuli, which is not dependent on muscle fatigue or receptor adaptation (Cerbone & Sadile, 1993; Thompson & Spencer, 1966). As an environment loses its novelty, exploratory behaviour and rearing diminish, and behaviours such as grooming and sleep take over (Cerbone & Sadile, 1993). The diminished response due to familiarly requires learning-related processes and recognition or recall (Dai, Krost and Carey, 1995; Thiel et al., 1998).

The aim of the current experiment is to determine how many exploratory episodes are required for a Long-Evans male rat to become habituated to two contexts, as determined by rearing activity. HPC overshadowing has mainly been tested in behavioural paradigms depending on context. Here we seek to establish a new behavioural test which shows gradual habituation, and presumably the formation of a stable context representation in memory, across multiple, distributed exploration sessions. Scoring rearing behaviour over time provides a useful measuring frame to evaluate whether the animal has a stable context memory, as rearing occurs more frequently in a novel environment compared to a very familiar one (Barnett, 2005). The findings from this experiment will be an important foundation for subsequent experiments outlined in this thesis.

2.2 Materials and Methods

Animals

12 naïve male, hooded Long-Evans adult rats (Charles River, QC) weighing 330-450 g served as subjects. Animals were housed in pairs in standard Plexiglas cages in a vivarium maintained at a 12 hr light/dark cycle (lights off at 7.30 pm) with *ad libitum* access to food and water. All procedures were approved by the University of Lethbridge Animal Welfare Committee in accord with the Canadian Council of Animal Care (CCAC) guidelines.

Apparatus

Each day of testing, the rats were collected from their home-cage individually, and transported to one of two testing rooms in a clear Plexiglas cage with bedding. The two testing rooms were located adjacent to each other so the route was the same for each testing room.

Room 1: Room 1 contained a clear Plexiglas container on a table in the center of the room. The dimensions of the container was 48.3 cm long, 26.7 cm wide, and 20.3 cm deep. The container had a thick layer of bedding and a raised plastic lid. On the table 50 cm in front of the container was an electrical turning table with an LED lamp mounted to it. The LED lamp was covered with a tall cup with vertical slits in it. When the turning table was plugged in the lamp and cup would rotate, displaying large white vertical rotating stripes on the walls of the room. No other light was on. Between the container and the turning table was a small glass jar with a cotton ball in it. Prior to testing, 3 drops of tee tree oil (Life Brand®MD, 100% Pure Tea Tree Oil) was dropped on the cotton ball. During testing, a random mix of hit list songs from the 90's was playing at ~75 dB using a sound meter (Sound Meter Ver 1.6.0; Smart Tools co. for Android; Figure 2.1 A).

Room 2: The second testing room was identical to the first room in size and shape. However, the Plexiglas container did not have any bedding in it, and with the exception of a camcorder in one corner, there were no other visual or auditory cues in the room. There was no music playing the room, and background noise was measured at ~ 55 dB. The room was fully lit using traditional ceiling light (Figure 2.1 B).

Training

Each rat was randomly assigned to two different behavioural groups (see Table 2.1). Rats in group AA were trained in the same context twice per day, while rats in group AB where trained in two different contexts. Whether room 1 or room 2 served as Context A (and vice versa) was counterbalanced in each group, so that 3 animals in group AA were exposed to room 1 and 3 animals were exposed to room 2. Similarly, 3 animals in group AB were exposed to room 1 first, while the other 3 animals in this group was exposed to room 2 first. During exploration day, each rat spent 10 min in each context; during test day each rat spent 5 min in each context. The Plexiglas cages were cleaned using dilute Quatsyl-D Plus animal care disinfectant following each rat, and any feces were removed. Each training session was videotaped using Vixia HF S100 HD Camcorders mounted on a Velbon CX540 tripod. The video was subsequently scored by a trained, blind experimenter. The rats were habituated to the context until their rearing behaviour had plateaued for at least 2 days in a row, showing no more decrease in amount of rearing while being in the contexts.

Behavioural scoring

The behaviour of the rats during context exploration was scored by the number of instances of rearing. Rearing is defined as the rat raising up on his hind-legs with both front paws up in the air or touching the Plexiglas sides of the cage. Rearing behaviour was binned into 30 second bins. The data was entered in to SPSS© Statistics (version 21; IBM©, Armonk, NY, USA) for statistical analysis.

2.3 Results

There was a significant decrease in rearing behaviour from one day to the next, with the exception of from day 7 to day 8 (one-tailed t-test; p = 0.075) and from day 10 to 11 (p =

0.26) and from day 11 to 12 ($p = 0.37$), satisfying the habituation criterion for no change in rearing on two consecutive days. Training therefore stopped after 12 days. Figure 2.2 shows the gradual decrease of rearing over time.

The animals displayed the same amount of rearing in each room, with the exception of on days 10 and 11, when rats in the light room reared less than rats in the dark room ($p = 0.004$ and $p = 0.012$, respectively). Figure 2.3 depicts the different rearing behaviour in the two rooms across all training days.

Although rats in group AA received twice as many pairings to the context as rats in group AB, both groups followed the same pattern of decrease in rearing behaviour, with the exception of Day 1 ($p = 0.05$), Day 2 ($p = 0.002$) and Day 3 ($p = 0.31$) where rats in group AB reared more than rats in group AA (figure 2.4).

Rats in group AB showed the same amount of rearing in context A and context B on individual test days. However, rats in group AA displayed significantly less rearing when entering the same context for the second time on Day 1 ($p = 0.007$) and on Day 2 ($p = 0.003$). Figure 2.5 outlines this difference.

2.4 Discussion

It has long been known that the Norway rat shows neophilic behaviour when exposed to novel environments, meaning that they explore places that are new. Exploratory behaviour is characterized by activity such as rearing, and when an environment becomes familiar, the amount of rearing diminishes. In the current experiment I wanted to establish how long it took for the rat to become habituated to one or two novel contexts, as indicated by rearing behaviour. Rats were exposed to either two different contexts or one context twice on each test day. We found that the amount of rearing behaviour decreased significantly from day to day, until day 10. There were no significant decreases from Day 10

to Day 11, nor from day 11 to day 12, indicating that an asymptote had been reached. There was also no difference in rearing behaviour between the two different contexts, with the exception of days 10 and 11, where rats exposed to room 1 reared significantly more than rats exposed to room 2. This effect may be due to the fact that room 1 may have been more sensory rich and it could therefore take the rats slightly longer to become habituated to this context. A second explanation may be that the music in this room was a playlist of 5 songs played on a shuffle setting, and the rats may therefore be exposed to different songs on each trial. Although the sound level (dB) and style of music was the same, this could explain why the rats in room 1 took slightly longer to habituate.

Evaluating the data in more detail, we revealed that rats exposed to the same context twice decreased their rearing behaviour faster than rats exposed to two different contexts; however this effect was only visible on the first three days of testing. It still took the rats the same amount of time to reach behavioural plateau. In addition, rats in group AA reared significantly more in the first exposure on training days 1 and 2. There were no differences in rearing between exposures on individual days in group AB.

This experiment is not the first to show that rearing behaviour diminished over days (Cerbone & Sadile, 1994; Thiel et al., 1998). It is however the first experiment to measure this behaviour over a longer period of time. As the experiments outlined in chapter 6 include behavioural observations following 12 days of context exploration, the current experiement is important to the interpretation of the results presented later in this theses. The current findings are consistent with those made by Thiel et al. (1998) and Cerbone and Sadile (1994), who showed that when re-exposing the rat to the same environment on two and three consecutive days, respectively, rearing decreased.

Our current findings demonstrating the amount of time it takes for a rat to habituate to one or two contexts provides us with an important foundation for the remainder if this

thesis. When evaluating neuronal activation, different systems within the brain and how they interact, it is important, in the context of the goals of the present thesis, to know whether the context memory is relatively new or very stable/well established. We therefore now have a behavioural measure that we hypothesize distinguishes between novel and established context memories, this is important for the experiments outlined in Chapters 4-6.

CHAPTER 3: TEMPORARY INACTIVATION OF THE RODENT HIPPOCAMPUS USING MUSCIMOL

3.1 Introduction

For the purpose of this thesis, it was important to establish a method that could inactivate the rat hippocampus within the time frame of the difference in H1a and Arc mRNA transcription (5-10 min). The only drug that has been previously shown to suppress neuronal firing within that time-frame, is muscimol. Muscimol is a constituent and psychoactive ingredient from the mushroom *Amanita muscaria*, and is regarded as a compound that activates all ϒ-Aminobutyric acid (GABA) type A receptor (GABA $_A$-R) subtypes (Krogsgaard-Larsen, Hjeds, Curtis, Lodge, & Johnson, 1979). GABA is the major inhibitory neurotransmitter in the mammalian central nervous system, and it acts primarily through the GABA$_A$-R (Chandra et al., 2010). These are the receptors activated by muscimol.

According to a recording study conducted by Hafting, Fyhn, Bonnevie, Moser and Moser (2008), HPC pyramidal neurons stop firing around 7 min after intra-HPC infusion of muscimol. Based on this finding, muscimol was selected as the candidate drug to inactivate the HPC in the time-frame needed. The current study aimed at determining the efficacy of the drug at different time-points crucial for the experiment outlined in Chapter 6. Muscimol was infused unilaterally in to an awake rat HPC. At different time-points following infusion, rats sustained artificial brain-wide seizures in order to activate all excitable cells. IEG labeling within HPC was then measured using unbiased confocal stereology, comparing the muscimol-infused hemisphere with the control hemisphere. In this manner, a quantification of muscimol-induced cellular inactivation in the HPC, as well as a time-line for this inactivation, was established.

3.2 Materials and Methods

4 male Long-Evans rats served as subjects. Housing and handling guidelines were as described in Chapter 2.

Permanent cannulae implants

Rats were anesthetized using 4% isoflurane gas (Benson Medical Industries, Inc., ON, CA) in oxygen flowing at 1.5 l/min. surgical anesthetic plane was established and maintained using 1-2% isoflurane throughout the surgery. The rats were positioned in a stereotaxic apparatus (Kopf Instrumentals, Tujunga, CA, USA) and injected with buprenorphine (Temgesic®, 0.03 mg/kg, s.c.; Schering Plough, Hertfordshire, UK) prior to making the first incision. The scalp was retracted and seven burr holes, one for each cannular as well as three holes for the anchoring screws, were drilled into the skull. Two 23-gauge stainless steel guide cannulae were implanted bilaterally aimed at dorsal and ventral HPC (see Table 3.1 for surgical coordinates). Cannulae for the dorsal HPC were 11 mm in length, and cannulae for the ventral HPC were 14 mm. 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-gauge dummy cannulae which stayed in place until infusion. Following surgery the rats were injected with the anti-inflammatory drug meloxicam (Metacam®, 5 mg/ml, 0.02 mg/kg, s.c., Bueringer Integelheim, Burlington, ON, CA) and kept under close monitoring for 24 h before being returned to their home cage for an additional six days.

Intracranial infusion of muscimol

On the test day, the rats were brought in to a quiet room and gently restrained by hand as the dummy cannulae were removed and the infusion needles were inserted. The methods related to the infusion of the drug followed those outlined by Gulbrandsen and Sutherland (2014). The rats were immediately infused unilaterally in the right hemisphere with 0.4 µl muscimol (1 µg/µl, Sigma Life Science, Sigma-Aldrich, St. Louis, MO) per site at a rate of 0.25 µl/min. The left hemisphere were infused with the same volume of aCSF (pH ~ 7.4). Following infusion, the needles were left in place for an additional 5 minutes to allow for diffusion, after which the infusion needles were gently removed and the dummy cannulae re-inserted.

Electroconvulsive shock

Following infusion, the rats were brought to a novel testing room in a clear Plexiglas cage with a high plastic lid and soft towel covering. At 4 different time points (10 min, 15 min, 20 min, 25 min following end of infusion), each rat was connected to a Maximal Electric Convulsive Shock (MECS; UGO Basile, Collegeville, PA, USA) machine using two ear-clamps dipped in saline. An 1 s shock was delivered (100 pulses/sec; 0.5 ms pulse with; 85 mA current intensity). After the shock administration and after observable seizure activity had stopped, the rats were transported to a separate room and prepared for perfusion. Within 5 minutes of MECS, each rat received a lethal injection of sodium pentobarbital (Euthasol®, 0.3 ml/kg, 320 mg/mL, i.p.; Schering-Plough, Pointe-Claire, QU, CA) and were transcradially perfused with 1X phosphate-buffered saline (1xPBS) followed by 5% paraformaldehyde (PFA) in 1XPBS in DEPC water. Brains were drop-fixed in PFA for 2 hrs and were transferred to a solution of 30% sucrose 1xPBS for an additional 2 days before being frozen in -80°c until sectioning.

Arc in situ hybridization

All brains were sectioned at 40 μm using a Leica CM3050 S frozen cryostat (Leica Biosystems, Concord, ON, CA). The tissue was directly mounted on to Fisherbrand® Superforst® Plus microscope slides (Fisher Scientific, Ottawa, ON, CA) and stored at -80°c until labeling. Every 24th section was labeled for the immediate early gene using Arc *in situ* hybridization (ISH). Slides containing the sections were fixed in 4% paraformaldehyde before 1 ml of proteinase K buffer (3μl/1000μl) was added to the slides and left for 30 min in room temperature. The tissue was then again incubated in 4% PFA, before being treated with acetic anahydride, incubated in 1:1 acetone/methanol before prehybridization in buffer and 50% formamide for 60 min in a humid chamber at room temperature. 120 μL of hybridization buffer with denatured probes was then added to the slides which were left in a humid chamber at 56°C overnight. The following morning, the tissue was cooled down by being left in room temperature for 15 minutes followed by 2 post-hybridization washes in SSC before a third wash in SSC with RNase A (10 mg/ml) for 30 min in 37°C in order to break down any single-stranded RNA. Following this step, the tissue was again washed twice in SSC, before being washed in 0.5X SSC in 55°C for 30 min, followed by 5 min in 0.5X SSC in room temperature. After quenching endogenous peroxidases in 2% H_2O_2, slides were blocked with 150μL blocking buffer + 5% sheep serum per slide for 60 minutes, and then incubated with 150 μL anti-digoxigenin-POD (1:300; Roche Diagnostics, Basel, Switzerland) and stored at 4°C overnight. Slides were washed with TBST, and 120 μL of 1:100 Tyramide Signaling Amplification (TSA) Biotin Tyramide Reagent Pack (PerkinElmer, Waltham, MA) was added to the slides which were left for 60 min in a humid chamber at room temperature. Following an additional 3 washes in TBST, 120 μL of 1:200 Streptavidin-Texas red (PerkinElmer, Waltham, MA) was added to the slides for 30 min in a humid chamber in room temperature. Following this, the tissue went through an additional 3 washes in TBST

before being washed in TBS for 5 min. The sections were then counter-stained using 120 µL 4', 6'-diaminidino-2-phenylindole (DAPI; 1:2000; Sigma-Aldrich, St. Louise, MO) for 60 minutes before being cover-slipped with Vectashield© (Vector Laboratories, Burlingame, CA) and stored at 4°C. 1-2 days following cover-slipping, the slides were sealed using a commercially available nail-polish.

Confocal stereological analysis

Image stacks were taken using an Olympus BX61 FluoView FV1000 confocal microscope powered by an Olympus BX-UCB connected to an Olympus U-RFL-T mercury burner. The microscope was equipped with four lasers: a 405, 473, 559, and 635 (Olympus FV10-MCPSU, OLYMPUS America, Inc., Center Valley, PA, USA). A 60X oil-immersion objective was used to collect image stacks through the entire section (1 µm optical sections at 1µm step size). Exposure levels were set as high as possible without showing any over-exposure within any of the different laser-specific ranges. This means that when imaging for 2 channels (DAPI and Texas Red), each channel was set independently matching the intensity of the dISH label at the top, middle, and bottom of the section, creating a linear decrease in high-voltage (HV) when imaging from the top to the bottom of the section, controlling for any bias in the labelling process.

Unbiased stereology was conducted using the optical fractionator function in StereoInvestogator (10.54, 32-bit; MBF Bioscience – MicroBrightField, Inc.). The region of interest (ROI) was traced at a low magnification (10x). A grid was then placed on top of the ROI. The size and shape of the grid was determined through a pilot study, ensuring sufficient sampling sites to generate enough counts to have a coefficient of error of less than 0.05 without over-sampling. Once the grid size had been determined, it remained the same for each rat. When initiating a count at a new ROI (different hemisphere or new section) the

grid was placed in a random fashion on top to the ROI, and within each grid square one sampling site was established. 2 different counts were obtained within each sampling site; nucleus and Arc positive cells. Only cells that expressed 2 inter-nuclei foci was counted as IEG positive. Live view images were displayed through a Q Imaging Retiga Fast1394 2000R camera mounted above the stage and binoculars. The software was installed on a LG computer connected to the confocal microscope controlled by Olympus FluoView ver4.0b software. A DAPI filter (405 laser) and Texas red filter (559 laser) was used to visualize the ISH; DAPI visualizing the DAPI stain (cellular nuclei) and Texas red visualizing Arc mRNA.

This method allows for an unbiased selection of sampling sites, and provide an accurate estimate of total counts in CA1 as a hole, without having to count every single cell (Mura, Murphy, Feldon, & Jongen-Relo, 2004).

Statistical analysis

Statistical analysis was conducted using Microsoft Excel 2013 (Microsoft, Redmond, WA).

3.3 Results

The results of this study are outlined in Figure 3.1. In the control hemisphere, an average of 40.85% of cells counted (±0.82%) expressed Arc mRNA after being exposed to the MECS. The right, infused hemispheres showed 0.02%, 0.03%, 0.06% and 0.11% Arc mRNA labeling at 10, 15, 20, and 25 min following end of infusion, respectively. Figure 3.2 shows images from control and muscimol infused hemispheres 10 min after infusion.

3.4 Discussion

      The current study aimed at verifying that the muscimol inactivation method would be effective in suppressing HPC neuronal activity as measured by MECS and IEG labeling in the time-frame to be useful for the later dISH experiments. Rats were injected with muscimol unilaterally and at 10, 15, 20, or 25 min following the end of infusion of the drug, the rats were subjected to MECS before being sacrificed. MECS has previously been shown to be effective when evaluating neuronal activation and properties if temporary inactivation (Gulbrandsen & Sutherland, 2014), and is therefore a very useful tool when evaluating neuronal activation. The presence of mRNA for the IEG Arc indicated cellular activation, while the absence of Arc indicates successful inactivation. In the control hemisphere approximately 40% of DAPI labeled neurons expressed Arc. This is likely to be an underestimate of neuronal activation since we know that Arc is not expressed in glia cells (Guzowski et al., 1999); however glia are labeled by DAPI. According to Demchuk (2014), approximately 16% of the DAPI labeled cells in CA1 are classified as glia, as determined by double-labelling with NeuN and DAPI. In the studies yet to be described in this thesis, animals will be tested between 15 and 20 minutes post infusion. According to the current results, less than 1% of cells are expressing Arc, indicating that over 99% of the cells that can express Arc mRNA following MECS are inactivated during this time. These results therefore provide evidence that the HPC will be effectively inactivated by muscimol at the time of behavioural testing in the experiment described in Chapter 6.

CHAPTER 4: CONTEXT DISCRIMINATION IN CA1 AS INDICATED BY IMMEDIATE EARLY GENE ACTIVATION

4.1 Introduction

An understanding of the cellular basis of long-term memory and hippocampal overshadowing will require detailed knowledge of the specific circuits involved at a population level. A common learning theory states that when one is exposed to a novel setting, a unique pattern of neuronal activity across a wide neuronal population is activated in HPC and connected areas. Following a process of cellular consolidation, reactivation of the pattern is a basis for recall of the specific event or context. When re-visiting this context or event setting, parts of the network or the network in its entirety are reactivated, leading to a strengthening of the connections between the neurons that are activated. Previous research has shown that repeated learning episodes distributed over days can successfully make a context memory that was initially dependent on the HPC, become HPC independent (Lehmann et al., 2009; Gulbrandsen et al., 2013).

The HPC plays an important role in context learning and spatial navigation. Several recording studies have shown that HPC pyramidal and granule cells fire in a consistent relation to the location of the animal in an environment (O'Keefe & Dostrovsky; 1971; O'Keefe & Conway, 1978; McNaughton, Barnes, & O'Keefe, 1983; Muller, Kubie, & Ranck, 1987; Eichenbaum, Wiener, Shapiro & Cohen, 1989; Wiener, Paul, & Eichenbaum, 1989; Wilson & McNaughton, 1994). When manipulating cues within an environment, Gothard et al. (1996) found that HPC neurons fire in response to both the fixed environment and the movable objects, but that the two may be encoded by a different subset of neurons. Studies have shown that the HPC neurons fire in response to non-spatial variables, such as sampling of a cue, reward contingencies, etc. (Berger, Rinaldi, Weisz, & Thompson, 1983; Eichenbaum,

Kuperstein, Fagan & Nagode, 1987; Wiener et al., 1989; Otto & Eichebaum, 1992; Young, Fox & Eichenbaum, 1994). In more detail, previous studies using parallel ensemble recording indicate that a fraction of CA1 neurons are specifically activated during exploration of a given environment (typically, 30-50%, depending on environment size; Guzowski et al., 1999; Gothard et al., 1996; Wilson & McNaughton, 1994) and the cell groups demonstrating strong activity in each of two distinct environments are uncorrelated (Guzowski et al., 1999). Similar findings have been obtained using IEG labeling as an indication of cellular activation. The proportion of HPC neurons expressing Arc in the three major subfields of the HPC (CA1, CA3 and DG) are comparable to the proportions of neurons that are observed to express *place fields* within an environment of comparable size (Guzowski et al., 1999, Chawla et al., 2005; Vazdarjanova & Guzowski, 2004; Wilson & McNaughton, 1994). When two different environments are experienced, the proportion of overlap also corresponds to unit recording observations (Guzowski et al., 1999; Vazdarjanova & Guzowski, 2004).

The aim of the current study is to determine whether exploration of two contexts will activate different subpopulations of CA1 neurons. Labeling of cells for H1a and Arc mRNA with proper timing of context exposure will enable quantification of the extent of overlap in the CA1 representations of the two contexts. Vazdarjanova and Guzowski (2004) described all neurons that were activated in one context – as measured by IEG labeling – as being part of the same neuronal firing pattern. In this way, when comparing IEG labeling patterns after exposure to either the same context twice or two different contexts in succession, activation patterns were specific for the two exposures and could be used to reveal differences between cellular population responses within a specific rat. I therefore hypothesize that rats introduced to the same context twice should reactivate very similar ensembles, whereas two different ensembles should be activated if the rat is placed in two different contexts. This should be apparent by a difference in the number of Arc and H1a co-

labeled cells. There will be significantly more co-labeled cells if a rat enters the same context twice, compared with the case when the rat enters two different contexts. A comparison of left and right hemisphere and dorsal and ventral CA1 will also be conducted. The asymmetry of HPC function has been reported several times in humans (Squire et al., 1992; Motley & Kirwan, 2012) where the right HPC is more sensitive to spatial tasks compared to the left HPC. It is therefore possible that we see more IEG labeling in one hemisphere compared to the other. There is also a possibility that dorsal CA1 will have more cells labeled compared to ventral CA1. As cells in dorsal CA1 have smaller and more place fields (Kjelstrup et al., 2008) one could predict that more cells in dorsal CA1 will be active when exploring a context.

4.2 Materials and Methods

27 naïve male Long-Evans adult rats (Charles River, QC) weighing 330-450 g served as subjects. Housing and handling protocols were as described in Chapter 2. Testing rooms and behavioural procedures were as described in Chapter 2.

Behaviour

Rats were divided in to 4 behavioural groups (AA 3days n = 6; AB 3days n = 4; AA 12 days n = 5; AB 12 days n = 5) and one home-cage control group (n = 7). The details of the groups are outlined in table 4.1. Group 1 and 2 were trained for 3 days and tested on day 4; group 3 and 4 were trained for 12 days and tested on day 13. Group 5 served as home-cage controls and were not subjected to any behavioural training. Groups 1 and 3 were trained and tested in both contexts A and B; Groups 2 and 4 were trained and tested in only one context, either A or B. Which room (light or dark) served as Context A and Context B were counter-balanced in both groups. All rats were trained as described in Chapter 2.

Perfusion

5 minutes after exposure to the last context on test day, rats received a lethal injection of sodium pentobarbital (Euthasol®, 0.3 ml/kg, 320 mg/mL, ip.; Schering-Plough, Pointe-Claire, QU, CA) and were transcradially perfused with 1X phosphate-buffered saline (1xPBS) followed by 5% PFA in 1xPBS in DEPC water. Brains were drop-fixed in PFA for 2 hrs and were transferred to a solution of 30% sucrose 1XPBS for an additional 2 days before being frozen in -80°c until sectioning.

Double in-situ hybridization

Arc ISH labeling was conducted as described in Chapter 3. In addition, 150μL 1:1000 anti-Fluorescein-HPR antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove PA) was added to label for H1a, and the tissue was left over night. The following day 120μL 1:100 FITC-tyramide dye was added to the slides for 30 min before washes and cover-slipping.

Unbiased confocal stereology

Following dISH, every 12th section through the HPC was counted using unbiased stereology as outlined in Chapter 3. FITC filter was used to visualize H1a mRNA. Laser intensity remained constant across all analysis; however, the high-voltage (HV) function was set prior to each image stack. The levels was set at 3 depths throughout the tissue; -15 μm, - 25 μm and -35 μm. HV was set as high as possible without leading to any over-exposure within any of the three channels (DAPI, Texas Red and FITC). Counts for nucleus, H1a positive, Arc positive, and co-labeled cells were obtained as described in Chapter 3.

4.3 Results

Individual data points were extracted from StereoInvestigator and transferred to SPSS for statistical analysis. An average of 732,406.77 ($\pm$ 39,136.83) cells were counted and evaluated for IEG expression across all groups. All data shows labeled cells as a proportion of total CA1 cellular nuclei (DAPI) counted (all cells expressing IEG/nuclei counted), unless otherwise stated.

3-day exploration:

Average absolute number of cells expressing any IEG for the 3-day exploration group was 121,241.43 ($\pm$ 11,995.1), where 80,501.39 ($\pm$ 7,660.61) were expressing H1a, 67,276.85 ($\pm$ 5,996.75) Arc, and 26,536.81 ($\pm$ 3,210.54) were co-labeled.

When comparing groups AA, AB and home-cage controls after 3 days of exploration, a one-way ANOVA revealed significant between-group effects. Using the proportion of cells counted that were expressing IEGs, there was a difference in total labeled cells ($F_{(2,15)} = 4.17$; $p = 0.04$), H1a mRNA ($F_{(2,15)} = 22.93$; $p < 0.001$), and on co-labeled cells ($F_{(2,15)} = 21.52$; $p < 0.001$). There was no difference in number of cells expressing Arc mRNA ($F_{(2,15)} = 0.19$; $p = 0.83$).

Post-hoc Fisher LSD revealed that of the total cells counted, significantly fewer where expressing any IEG in home-cage control animals compared to group AB ($p = 0.019$) but not compared to group AA ($p = 0.06$). Home-caged animals also had significantly fewer H1a expressing cells compared to group AA ($p < 0.001$) and AB ($p < 0.001$), and fewer co-labeled cells compared to groups AA ($p < 0.001$) and AB ($p = 0.004$). There were no differences in Arc labeling between home-caged animals and group AA ($p = 0.55$) nor AB ($p = 0.79$). Importantly for the current hypothesis, there were significantly more co-labeled

cells in group AA compared to group AB (p = 0.009). These findings are summarized in Figure 4.1.

Paired-samples t-test showed no differences between left and right hemispheres in any of the aforementioned measures. However, a one-way ANOVA showed significant between-groups effects on cells expressing H1a mRNA ($F_{(2,15)}$ = 12.61; p < 0.001) and on co-labeled cells ($F_{(2,15)}$ = 7.71; p = 0.006) in the left hemisphere. *Post-hoc* Fisher LSD revealed that home-cage control animals had significantly fewer cells expressing H1a mRNA compared to both groups AA and AB (p's < 0.001), as well as fewer co-labeled cells compared to group AA (p = 0.002) and approaching significance compared to group AB (p = 0.067). In addition, group AA tended to have more co-labeled cells than group AB (non-significant trend, p = 0.081).

In the right hemisphere, there was a significant between-groups effect of co-labeled cells ($F_{(2,15)}$ = 13.04; p = 0.001) and H1a ($F_{(2,15)}$ = 15.18; p < 0.001). *Post-hoc* Fisher LSD revealed that home-caged animals had significantly fewer co-labeled cells compared to group AA (p < 0.001) and AB (p = 0.028). In addition, AA had significantly more co-labeled cells than AB (p = 0.023). Home-cage controls also had significantly fewer H1a mRNA expressing cells than AA (p = 0.001) and AB (p < 0.001). The results from left and right hemispheres are displayed in Figure 4.2.

CA1 can be divided in to dorsal and ventral portions. The separation was made where the rostral and caudal CA1 meet up in coronal sections, approximately -4.7 mm posterior to the bregma. Paired samples t-tests showed a significant difference between dorsal and ventral CA1 in group AB in total cells labeled (p = 0.011), H1a (p = 0.019) and Arc (p = 0.024) but not in co-labeled cells (p = 0.27), where the highest concentration was found in the dorsal CA1. There were no significant differences in IEG labeling in dorsal and ventral CA1 in group AA nor in home-cage control animals. A one-way ANOVA revealed significant

between-group differences in total labeled cells ($F_{(2,15)}$ = 6.34; p = 0.012), H1a ($F_{(2,15)}$ = 21.16; $p < 0.001$) and co-labeled cells ($F_{(2,15)}$ = 5.47; p = 0.019) in dorsal CA1. *Post-hoc* Fisher LSD revealed that home-cage control had significantly fewer cells expressing IEGs compared to group AB (p = 0.004), but not AA (p = 0.058). Home-cage controls also had significantly fewer H1a compared to AA ($p < 0.001$) and AB ($p < 0.001$) and fewer co-labeled cells (AA: p = 0.01; AB: p = 0.031). A one-way ANOVA also revealed significant between-groups effects in ventral CA1 for H1a ($F_{(2,15)}$ = 7.28; p = 0.008) and co-labeled cells ($F_{(2,15)}$ = 34.8; p < 0.001). *Post-hoc* Fisher LSD tests showed that home-cage control animals had significantly fewer H1a compared to both groups (AA: p = 0.003; AB: p = 0.023) as well as fewer co-labeled cells (AA: $p < 0.001$; AB: p = 0.008). In addition, group AA had significantly more co-labeled cells compared to group AB ($p < 0.001$). The results are consistent with the predictions based on the knowledge that dorsal CA1 have more place fields compared to ventral CA1 and more IEG expressing cells is therefore expected in dorsal CA1. These results are summarized in Figure 4.3.

12-day exploration:

The average absolute number of cells expressing any IEG for the 12 day exploration group was 177,067.0 ($\pm$ 25,836.66), where of 100,452.4 ($\pm$ 17,177.0) were expressing H1a, 116,629.54 ($\pm$ 16,535.23) Arc, and 40,014.94 ($\pm$ 7,791.22) were co-labeled.

A one-way ANOVA showed significant between-group effects on total cells labeled ($F_{(2,16)}$ = 7.78; p = 0.005), H1a mRNA labeling ($F_{(2,16)}$ = 17.05; $p < 0.001$), and co-labeled cells ($F_{(2,16)}$ = 0.79; p = 0.002). There were no significant groups effects on Arc mRNA labeling ($F_{(2,16)}$ = 3.24; p = 0.07).

Post-hoc Fisher LSD showed that home-cage control animals had significantly fewer cells labeled with IEGs compared to both group AA (p= 0.012) and AB (p = 0.003), fewer H1a

mRNA (AA: p = 0.002; AB: p < 0.001) and fewer co-labeled cells (p's = 0.002). Home-caged animals also had significantly fewer Arc mRNA expressing cells compared to group AA (p = 0.038) but not group AB (p = 0.07). There were no significant differences between group AA and BB. Contrary to the results obtained following 3 days of exploration, there was no longer any difference in the proportion of cells counted that were co-labeled in groups AA and AB. The statistical results are summarized in Figure 4.4, while confocal images from dorsal CA1 from groups AA and AB are shown in Figure 4.5 A and B.

Paired samples t-tests between right and left CA1 found no significant differences in IEG labeling between the two hemispheres.

A one-way ANOVA for left CA1 showed significant between-groups effects on proportion of cells labeled with IEGs ($F_{(2,16)}$ = 7.77; p = 0.001), H1a mRNA labeling ($F_{(2,16)}$ 15.97; p < 0.001), Arc mRNA labeling ($F_{(2,16)}$ = 3.81; p = 0.048) and on co-labeled cells ($F_{(2,16)}$ = 10.59; p = 0.002). *Post-hoc* Fisher LSD revealed that home-cage control animals had significantly fewer cells labeled with IEGs compared to groups AA and AB (p's = 0.005), fewer H1a mRNA labeled cells compared to group AA (p = 0.001) and group AB (p < 0.001). Home-cage animals also had significantly fewer cells labeled with Arc mRNA compared to group AA (p = 0.02) but not groups AB (p = 0.085), but they had fewer co-labeled cells compared to groups AA (p = 0.001) and group AB (p = 0.004).

As in right CA1, a one-way ANOVA for IEG labeling showed significant between-groups effects on proportion of cells labeled with IEGs ($F_{(2,16)}$ = 7.01; p = 0.008), cells labeld with H1a mRNA ($F_{(2,16)}$ = 15.32; p < 0.001) and that were co-labeled ($F_{(2,16)}$ = 8.1; p = 0.005) while there were no effect on Arc mRNA labeling ($F_{(2,16)}$ = 2.25; p = 0.143). *Post-hoc* Fisher LSD revealed that home-caged animals had significantly fewer cells labeled with IEGs compared to groups AA (p = 0.038) and AB (p = 0.003), fewer H1a mRNA compared to group AA (p = 0.009) and AB (p < 0.001) and fewer co-labeled cells compared to groups AA (p =

0.008) and AB (p = 0.003). In addition, group AA had significantly fewer H1a mRNA labeled cells compared to group AB (p = 0.042). The expression patterns in left and right CA1 is consistent with what was found when evaluating the structure as a whole. The results of left and right CA1 are summarized in Figure 4.6.

Group AB had significantly more Arc mRNA (p = 0.022) and total cells labeled with IEGs (p = 0.028) in dorsal compared to ventral CA1. There were no statistically significant differences in group AA nor home-cage controls. A one-way ANOVA showed significant between-groups effect of total cells labeled with IEGs ($F_{(2,16)}$ = 6.24; p = 0.012), H1a ($F_{(2,16)}$ = 11.47; p = 0.001) and co-labeled cells ($F_{(2,16)}$ = 5.35; p = 0.019) in dorsal CA1. *Post-hoc* Fisher LSD revealed that home-cage control animals had significantly fewer cells labeled with IEGs compared to group AA (p = 0.034) and AB (p = 0.005). Home-cage control animals also had significantly fewer cells labeled with H1a mRNA (AA: p = 0.009; AB: p < 0.001) and co-labeled cells (AA: p = 0.029; AB: p = 0.01). A one-way ANOVA showed significant between-groups differences in total cells labeled with IEGs ($F_{(2,16)}$ = 5.48; p = 0.017); H1a ($F_{(2,16)}$ = 12.4; p = 0.001) and co-labeled cells ($F_{(2,16)}$ = 11.03; p = 0.001) in ventral CA1. *Post-hoc* Fisher LSD revealed that home-cage control animals had significantly fewer total cells labeled compared to groups AA (p = 0.039) and AB (p= 0.007), as well as fewer H1a mRNA labeled cells (AA: p = 0.008; AB: p < 0.001) and fewer co-labeled cells (AA: p = 0.001; AB: p = 0.003). After 3 days of exploration, group AB had significantly more IEG labeling in dorsal compared to ventral CA1, while after 12 days of exploration this difference was gone. No other dorsal and ventral CA1 effects was found. The results for dorsal and ventral CA1 are summarized in Figure 4.7.

<u>3 vs. 12 days of exploration</u>

When comparing the proportion of cells expressing any IEG after 3 days of exploration and 12 days of exploration, a one-way ANOVA failed to find any between-group differences (AB: $F_{(1,9)}$ = 0.821; p = 0.39; AB: $F_{(1,8)}$ = 0.73; p = 0.42), nor was there any difference on total labeled cells after 3 days ($F_{(1,8)}$ = 0.158; p = 0.703) or after 12 days ($F_{(1,9)}$ = 0.48; p = 0.508). These results are summarized in Figure 4.8.

Similarly, there was no difference in proportion of all IEG expressing cells being co-labeled after 3 days compared to 12 days in group AB ($F_{(1,9)}$ = 0.77; p = 0.406). However, this change approached significance in group AA ($F_{(1,8)}$ = 5.43; p = 0.053). When considering between-groups effects on co-labeled cells, there was a significant difference between the groups after 3 days ($F_{(1,8)}$ = 18.62; p = 0.004) but not after 12 days ($F_{(1,9)}$ = 0.47; p = 0.513). These results indicate that although the proportion of co-labeled cells changed from 3 days of exploration to 12 days of exploration in group AA, this change was not due to a drop in IEG expressing cells overall. These results are summarized in Figure 4.9.

4.4 Discussion

The current study aimed at showing activation of two different cellular firing patterns when a rat is exposed to two different contexts. By taking advantage of the IEGs H1a and Arc and dISH labelling this study showed that when a rat explored one context on 3 consecutive days there was significantly more co-labeled cells in CA1 than if the rat explored two different contexts over the same time interval. This indicates that the CA1 has separate ensembles activated by different contexts, as has been previously described (Guzowski et al., 1999; Leutgeb et al., 2007). This effect was gone after 12 days of exploration. The proportion of cells expressing any IEG was not significant between 3 days of exploration and 12 days of exploration; however, the proportion of these cells being co-labeled dropped

from 3 days to 12 days in group AA, and this decrease was significant at a confidence level of 9%. These findings indicate that there is a shift in how the HPC responds based upon relative familiarity to contexts. The difference in co-labeled cells following exploration in two different context as compared to the same context twice indicates that CA1 represents the two contexts differently as indicated by the pattern of activation. Population of neurons can change their firing rate in response to changes in cues within the same location (Leutgeb et al., 2005). It is therefore possible that the representation for exploring the same context twice was still treated as two different events after 3 days of exploration, as changes in the electrophysiological properties of the same cell population cannot be detected using IEGs. However, a change in the pattern of activity did occur, and this change should be analyzed further.

Lever, Wills, Cacucci, Burgess and O'Keefe (2002) found that when recording place cells from CA1, the place fields in two contexts of the same shape and size diverged over the course of 6 days. The separation of place fields over the course of days can explain the findings of the current study at least in part, as there were fewer co-labeled cells after 12 days of exploration when compared to 3 days. However, as the proportion of cells expressing any IEG stayed the same after 12 days, there is an indication that information is being processed differently in very familiar contexts. A possible explanation lies in a theory presented by Davachi and DuBrow (2015) who suggested that when a sequence is repeated multiple times, the presentation of one item in the sequence triggers forward pattern completion of the full sequence in the HPC. In the current experiment, the sequence is the presentation of Context A, a break in the home-cage, followed by the presentation of Context B. Specific to the temporal organization of episodic memory, the HPC is essential to remembering unique sequences of events as well as the ability to disambiguate sequences that share common events (Fortin, Agster, & Eichenbaum, 2002; Kesner, Gilbert & Barua,

2002; Agster, Fortin, & Eichenbaum, 2002). Furthermore, studies have shown that the HPC is particularly involved in bridging temporal gaps that are devoid of specific external cues in order to bind discontinuous events that compose sequential memories (Meck, Church & Olton, 1984; Agster et al., 2002; Kesner, Hunsaker & Gilbert, 2005; Farovik, Dupont, & Eichenbaum, 2010; MacDonald, Lepage, Eden, & Eichenbaum, 2011). If it is the case that after 12 days of exploration there is an anticipation of the second context exploration, one can predict that as cells that would normally activate during the second context exploration activate earlier, resulting in an increase of H1a labeled cells. As these same cells may still activate during the second context exposure, there is no prediction of decreased number of Arc or co-labeled cells. Although there was a slight increase in H1a expression in group AB from 3 to 12 days of exploration, this increase was not significant (p = 0.266). There change in H1a expression in group AA was also non-significant (p = 0.666). Although a change in the representation of patterns of activity occurred, this change does not support the theory of anticipation and forward pattern completion.

There was no difference in Arc labeling between the behavioural groups (AA and AB) and home-cage control animals for any of the time-points. The increased levels of Arc mRNA labeling in the control subjects may be due to the experience of being removed from their home cage. As the ARC mRNA can be visible within the nucleus after as short of time as 2 min (Guzowski et al., 2005) this was a possibility due to the methods used. As the rats could not be euthanized within the vivarium, it took between 2 and5 minutes to inject the rats with euthansol after leaving the housing room (mean = 7.94; ± 0.64 min) this could account for the high level of ARC mRNA in the control population.

The current study failed to find any significant difference between CA1 in left and right hemisphere. According to a study by Shipton et al. (2014) rodents with the dorsal left HPC inactivated displayed impaired associative spatial long-term memory, while right HPC

inactivation had no effect on the same task. Recent work using healthy human subjects had found different activation in left and right HPC, where left HPC were more sensitive to semantic tasks, while right HPC was more sensitive to spatial processing (Motley & Kirwan, 2012). As the current behavioural task does not require the rat to solve any specific problems, this may be why no difference between the hemispheres was observed.

After 3 days of context exploration there were significantly more cells expressing H1a or Arc mRNA label in the dorsal HPC compared to the ventral HPC in group AB. At 12 days, only total IEG expressing cells and Arc were significantly greater in number in dorsal HPC. Kjelstrup et al. (2008) reported that the HPC place-fields change across the longitudinal axis where dorsal HPC has the smallest place fields of 0.98 ±0.03 m to the ventral HPC of 5.52 ± 0.54 m. When exposed to a larger environment, place cells in CA1 have more than one place field (Park, Dvorak & Fenton, 2011). As the place fields in dorsal CA1 are smaller than in the ventral, one could then predict that there might be more place fields in the dorsal CA1 compared to ventral CA1 which in turn explains the significantly greater IEG labeling in this region. After 12 days of exploration this overall effect is gone and only the number of Arc mRNA labeled cells was higher in dorsal compared to ventral HPC. This effect may be driven by the same phenomenon as previously explained in relation to home-caged control animals where the novel process of perfusion, and not to the exposure of the second context *per se*.

The current study was designed to evaluate the cellular firing pattern activated in CA1 following exposure to either one or two contexts. The results support the idea that CA1 has two distinct firing patterns that are activated when the rat enters two different contexts - as indexed by fewer CA1 cells co-labeled cells that observed in rats who enter the same context twice – during a time of learning where context exploration is still high (see Chapter 2). However, surprisingly, this effect was gone after 12 days of exploration; a time which is

associated with low levels of exploration and rearing. Although previous studies have

reported the so-called "A-B effect" (less overlap in the population of activated CA1 cell when

two contexts are visited compared to repeated visits to the same context; Guzowski et al.,

2005; Ramirez et al., 2013), this is the first study to quantify the extent of the A-B effect

across the entire septo-temporal axis of CA1, and the first to examine the effect after

repeated, distributed context exposures. Importantly the context exposures followed a

regimen consistent with studies that have created HPC-independent context memories

(Lehmann et al., 2009; Gulbrandsen et al., 2013). The change in HPC firing pattern from 3

days to 12 days is most interesting, as this is a novel finding and is contrary to any of the

predictions made regarding IEG activation. Possible mechanisms underlying this finding will

be discussed in more detail in Chapter 7.

CHAPTER 5: CONTEXT DISCRIMINATION IN RETROSPLENIAL CORTEX AS INDICATED BY IMMEDIATE EARLY GENE ACTIVATION

5.1 Introduction

The behavioural observations underlying the phenomenon of HPC overshadowing imply that at least one non-HPC cortical area is capable of discriminating between contexts in a manner similar to HPC. The retrosplenial cortex (RSC) is a nodal point for the transfer of information between the HPC formation, many neocortical regions, and the thalamus (Wyss & van Groen, 1992), and recent publications have shown that during spatial learning, experience-dependent memory traces are formed (Czajkowki et al., 2014). Other studies have demonstrated that the RSC makes an important contribution to the control of spatially guided behaviour (Sutherland et al., 1988). Functional studies demonstrate that lesions of the RSC impair place learning of rats in a water maze; however the animals are not impaired in learning *per se*. Thus, the RSC is essential to the ability to move accurately to a point in space using the relationship among distal cues (Sutherland et al., 1988). Similar results have been found using fMRI of human participants. Patients with RSC damage can recognize landmarks, but they have difficulty navigating effectively, even in familiar environments (Maguire, 2001).

The RSC can be divided into 4 distinct regions; RSGa, RSGb, RSGc, and RSD. In the current thesis, area RSD will be considered. The reason for this is the high interconnectivity with the thalamus, HPC and neocortex. RSD connects to the HPC via the postsubicular, parasubicular, entorhinal, and perirhinal cortices. RSD receives HPC input from the subiculum, postsubiculum and entorhinal cortex. In addition, RSD projects to orbitofrontal cortex and to anterior cingulate cortex. Both of these regions are important for complex behaviour, such as decision-making, reward anticipation and emotion. In addition, RSD

connects to primary and association visual cortex, and subcortical areas dorsal striatum, thalamus, superior colliculus, periaqueductal gray, and ventral pontine nuclei (Wyss and van Groen 1992), areas which are important for motor function, motivation and sensory integration. With connections to both neocortical and subcortical structures, the presence of head direction cells, and findings from previous behavioural studies, RSD is an excellent candidate for this study. It is hypothesized that RSD will show a larger overlap in cellular firing patterns when exposed to the same context twice as compared to when two different contexts are explored.

5.2 Materials and Methods

Analysis was conducted using the tissue collected during the experiment described in Chapter 4. The methods were therefore largely identical. dISH data was collected from the dysgranular layer of RSC as outlined by Paxinos and Watson (2007). A depiction of the specific region analyzed is found in Figure 5.1. Unbiased confocal stereology was done in the same manner as described in Chapter 4; however, the ROI was drawn out using 4x magnification, and the grid-size had been altered to fit the current experiment. Counts for total number of cells, as indicated by DAPI stain, cells expressing H1a mRNA, Arc mRNA or both were collected using unbiased stereology as described in Chapter 4. Individual data points were extracted from StereoInvestigator and transferred to SPSS for statistical analysis. All data is shown as a portion of total cells counted, unless otherwise stated.

5.3 Results

3-day exploration

The average absolute number of cells expressing IEGs in the 3-day exploration

groups was 177,154.77 ($\pm$ 20,575.42), where of 119,459.31 ($\pm$ 17,245.08) were expressing H1a, 115,470.78 ($\pm$ 16,044.94) Arc, and 57,775.33 ($\pm$ 12,897.78) were co-labeled.

A one-way ANOVA showed significant between-group difference on the total number of cells labeled ($F_{(2,15)}$ = 10.48; p = 0.002), number of cells expressing H1a ($F_{(2,15)}$ = 35.67; p < 0.001) and in co-labeled cells ($F_{(2,15)}$ = 35.36; p < 0.001). There was no significant between-groups effect on number of cells expressing Arc mRNA foci ($F_{(2,15)}$ = 2.93; p = 0.08).

Post-hoc Fisher LSD comparisons revealed that the home-cage control group had significantly fewer overall IEG labeled cells compared to both group AA (p = 0.003) and AB (p = 0.002). The home-cage control group also had significantly fewer H1a labeled cells compared to groups AA (p < 0.001) and AB (p < 0.001), and fewer co-labeled cells (AA: p < 0.001; AB: p =0.001). The home-cage control group had significantly fewer Arc labeled cells compared to group AA (p = 0.038) but not to group AB (p = 0.14). In addition, group AB also had significantly fewer co-labeled cells compared to group AA (p = 0.001). These results are consistent with the hypothesis that RSD creates two unique cellular firing patterns when exposed to two different contexts, as compared to when the same context is explored twice. A summary of these findings is outlined in Figure 5.2.

12-day exploration

The average absolute number of cells expressing IEGs in the 12-day exploration groups was 190,287.82 ($\pm$ 25,872.28) where of 108,536.86 ($\pm$ 16,904.56) were expressing H1a, 134,966.83 ($\pm$ 20,152.96) Arc, and 53,215.87 ($\pm$ 10,622.27) were co-labeled.

A one-way ANOVA revealed significant between-groups differences in total labeled cells ($F_{(2,16)}$ = 11.47; p = 0.001), H1a ($F_{(2,16)}$ = 32.32; p < 0.001), and co-labeled cells ($F_{(2,16)}$ = 10.21; p = 0.002). There was no difference on Arc mRNA labeling ($F_{(2,16)}$ = 3.07; p = 0.079).

Post-hoc Fisher LSD revealed that home caged control animals had significantly fewer IEG labeled cells compared to group AA ($p = 0.008$) and group AB ($p < 0.001$). Home-cage animals also had significantly fewer cells labeled for H1a mRNA (AA: $p < 0.001$; AB: $p < 0.001$) and fewer co-labeled cells compared to group AA ($p = 0.005$) and group AB ($p = 0.001$). Home-cage control animals also had significantly fewer Arc mRNA labeled cells than animals in group AB ($p = 0.044$) but not group AA ($p = 0.074$). In addition, group AA had significantly fewer H1a labeled cells compared to group AB ($p = 0.016$). These results are contradictory to the hypothesis that RSD creates two independent cellular firing patterns when exposed to two different familiar contexts. The results are however similar to what was reported in CA1 in Chapter 4. These results are summarized in figure 5.3.

3 vs. 12 days of exploration

A one-way ANOVA failed to find any difference in total cells expressing IEG between 3 days and 12 days of exploration in groups AA ($F_{(1,8)} = 1.01$; $p = 0.35$) nor in group AB ($F_{(1,9)} = 0.15$; $p = 0.71$; Figure 5.4), indicating that the same proportion of cells are active after 3 days of explorations as after 12 days of exploration. There was significant between-groups effect on the proportion of IEG expressing cells that were co-labeled in group AA ($F_{(1,8)} = 8.53$; $p = 0.022$) but not in group AB ($F_{(1,9)} = 0.89$; $p = 0.37$). Figure 5.5 show that the proportion of IEG expressing cells that were co-labeled in group AA was significantly higher after 3 days of exploration compared to after 12 days of exploration, while there was no change in group AB. The decrease in proportion of co-labeled cells in group AA was significant ($F_{(1,8)} = 14.95$; $p = 0.006$).

RSD and CA1 correlation

There was no significant correlation between total cells expressing IEGs between

CA1 and RSD after 3 days of exploration (Pearson's r = 0.137; p = 0.746; figure 5.6);

however, there was a significant positive correlation after 12 days of exploration (Pearson's

r = 0.799; p = 0.006; figure 5.7). This significant correlation also holds true for H1a mRNA

labeling (Pearson's r = 0.715; p = 0.02), Arc mRNA labeling (Pearson's r = 0.863; p = 0.001)

and for co-labeled cells (Pearson's r = 0.873; p = 0.001) after 12 days of exploration.

5.4 Discussion

The current experiment found support for the hypothesis that the RSD displays two

different cellular activation patterns when entering two different contexts, as compared to

when the same context was entered twice. This was true when the animals had 3 days of

exploration, however, the difference was not apparent after 12 days of exploration. The

change was not due to an overall drop in proportion of cells expressing IEGs; however,

when considering all cells labeled with IEGs, the proportion of co-labeled cells significantly

decreased from 3 days of exploration to 12 days of exploration in group AA, while the

proportion remained the same in group AB. The results obtained in this experiment show

that the RSD respond to context exploration in a similar manner to CA1. Interestingly, the

correlation between cellular activation in the two regions was not significant after 3 days of

exploration, while there was a strong positive correlation after 12 days. These results

indicate that when a context becomes increasingly familiar, the coherence between the two

regions also increases.

Previous research has shown that RSC is important in the acquisition of temporal

order (Bowers, Verfaellie, Valenstein, & Heilman, 1988). It is possible that when repeating

the sequence context exploration – home-cage – context exploration over many days, the

sequence is learned, and instead of the activation pattern coding for the individual contexts,

it is now differentiating between the two context exposures. Such a function would predict the cellular activation pattern observed in this experiment.

RSC receive input from layer V in the caudal portions of entorhinal cortex (Insausti, Herrero & Witter, 1998). This region receive strong input from the HPC (Insausti et al., 1998). As the entorhinal cortex contain neuronal codes for spatial maps and spatial processing (Hafting, Fyhn, Molden, Moser & Moser, 2005), it has been hypothesized that this part of the brain can support changes in coding schemes that could alter the firing pattern in other brain regions. These kinds of changes cannot be detected using IEGs. It is therefore possible that the change in activity pattern observed after 12 days of exploration is driven by input from entorhinal cortex and results in similar changes as those discussed in relationship with CA1.

As it has previously been shown that after 3 pairings, a context-fear memory is lost following HPC inactivation (Gulbrandsen et al., 2013), the current findings predict that following multiple, distributed sessions the RSD can support contextual memory in the absence of the HPC.

CHAPTER 6: CONTEXT SPECIFIC ACTIVATION IN RETROSPLENIAL CORTEX FOLLOWING HIPPOCAMPAL INACTIVATION AS MEASURED BY IMMEDIATE EARLY GENE ACTIVITY

6.1 Introduction

The RSC is closely connected with the HPC system in many ways. Anatomically, self-motion cues arrive in the RSC via connections with the ATN and/or posterior parietal cortex (Zilles & Wree, 1995). RSD also receives prominent input from visual areas 17 and 18 (Wyss & van Groen, 1992). RSC communicates with the HPC via reciprocal projections through the entorhinal cortex and the pre-, para-, and postsubiculum (van Groen, Vogt, & Wyss, 1993). Permanent lesion studies have demonstrated that the RSC is required for Morris water task acquisition and retention of a hidden, but not visible, platform location (Sutherland et al., 1988; Sutherland & Hoesing, 1993; Warburton et al., 1998). Electrophysiological studies have demonstrated that single cells in the RSC are controlled by multimodal sensory information (Musil & Olson, 1993). In rodents, a subpopulation of cells in RSC fire maximally when subjects face a particular direction in space (Chen et al., 1994a; 1994b). Taken together, the anatomical, lesion, and electrophysiological data suggest that the RSC may contribute multimodal sensory information for use during navigation (Cooper & Mizumori, 2001). As demonstrated in Chapter 5, the RSD is also able to pattern separate between two different contexts indexed by the activation of two different neuronal subpopulations when exposed to two different contexts after 3 days of exploration. It is clear from the studies described so far in this thesis that RSD and CA1 respond similarly to the same experience, however, it is unclear whether or not the two systems can operate independently or are intimately and functionally interconnected in their context specific activity.

The phenomenon of HPC overshadowing implies that at least one non-HPC system is able to recall a context memory in the absence of the HPC, if enough distributed learning has taken place (Lehmann et al., 2009; Sutherland et al., 2010; Gulbrandsen et al., 2013). In Chapter 5 it was demonstrated that after 12 days of exploration the pattern of neural activation, as indicated by IEG labeling, in CA1 and RSD was highly similar and significantly correlated. This is also the same amount of distributed training sessions that has been used in previous studies where context memories that initially depend on the HPC have become HPC independent (Lehmann et al., 2009; Gulbrandsen et al., 2013). It is therefore the aim of the current study to compare the neuronal activation pattern in RSD when the HPC is active to when the HPC is temporarily inactive.

In the current study, animals were trained and tested in a similar manner to animals described in Chapter 2. On test day (day 13) animals were introduced to either the familiar context(s) or to a novel context. In addition, the animals received an intra-HPC injection of muscimol using a method that leads to nearly complete HPC inactivation during the second context exposure (Chapter 3). Based on previous experiments and the results obtained in Chapter 5, one could expect that rats that are exposed to a novel context will show amnesia for this context when re-exposed to it following HPC inactivation. This could be observed in the analysis of rearing behaviour, as outlined in Chapter 2. When the rats were exposed to a novel environment, they reared significantly less the second time they entered the same environment, indicating a habitual response and memory for previous exploration. Therefore, if a memory is HPC dependent, there should be no drop in rearing behaviour when the rats re-enter the context. In addition, IEG labeling in RSD should show little overlap in H1a and Arc labeled cells. When rats are exposed to a familiar context which should have become HPC-independent, there will be no change in rearing behaviour, and one would expect to see a high number of co-labeled cells in RSD when the animals enter the

same context twice; however, if the RSD is not able to independently support such a context memory, one would expect a significantly lower level of co-labeled cells. As there were no difference in cellular activation patterns between groups AA and AB in RSD after 12 days of exploration (Chapter 5), no difference between these two groups are expected following HPC inactivation.

6.2 Materials and Methods

21 naive male Long-Evans rats served as subjects. Handling and housing methods were as described in Chapter 2. The rats were divided into 4 behavioural groups. Three of the groups, group AxA, AxB, and BxB all received permanent cannulae implants in the dorsal and ventral HPC as described in Chapter 3. The details of the groups and training are outlined in Table 6.1. All animals were exposed trained for 12 days and tested on day 13. Groups AxA, AxB and BxB were all injected with muscimol on test-day, while group BcB served as a novel-context control group. Groups BxB and BcB were trained and habituated to one context (Context A for 12 days) and on test day they were placed in a novel context, Context B.

All rats were allowed to explore the contexts on successive days as outlined in Chapter 2 with one slight difference. Each day following exposure to Context A the rats were transported to a separate room. Here the rats were subjected to sham intra-cranial infusions. The methods were as described in Chapter 3; however, the infusion cannulae were never inserted into the guide cannulae. Following the sham infusion the rats were returned to their home cage for 10 minutes before being brought into the second context, keeping the between context interval consistent at 20 minutes. On test day, the sham-infusion was replaced with actual infusion of muscimol, as described in Chapter 3.

Post-testing methods were as described in Chapter 4. Tissue was labeled for dISH and both HPC and RSD were analyzed using unbiased confocal stereology. HPC inactivation was quantified as described in Chapter 3.

6.3 Results

Behaviour

Test-day rearing behaviour was compared to the behaviour of the rats described in Chapter 2. A one-way ANOVA showed significant between-groups effect ($F_{(5,60)}$ = 51.77; $p <$ 0.001). *Post-hoc* Fisher LSD revealed that group BcB reared significantly more than all groups (all *p's* < 0.001) with the exception of group BxB (p = 0.977). Similarly, group BxB also reared significantly more than any other group (all p < 0.001) with the exception of group BcB. There was no difference in rearing between groups AB and AxB (p = 0.204) nor between groups AA and AxA (p = 0.865). These data are consistent with the hypothesis that rats that are exposed to a familiar context will show low levels of rearing, while rats that are exposed to a novel context will rear significantly more. These results are summarized in Figure 6.1.

A one-way ANOVA failed to reveal any significant difference on test-day rearing behaviour between first and second context exposure in group AxA ($F_{(1,11)}$ = 1.21; p = 0.296), group AxB ($F_{(1,9)}$ = 1.2; p = 0.305) or group BxB ($F_{(1,7)}$ = 0.87; p = 0.386). However, group BcB reared significantly more during the first exposure as compared to the second ($F_{(1,11)}$ = 10.66; p = 0.008). This data is again consistent with the hypothesis that animals in groups AxA and AxB do not change their rearing behaviour following HPC inactivation, while group BxB failed to show the typical drop in rearing when re-exposed to the same context (group BcB). These results are summarized in Figure 6.2.

Sutherland et al. (2010) showed that experiments where rats have less than 75% damage to the HPC show memory task behavioural results inconsistent with complete (>75%) HPC lesions. Therefore, rats that showed Arc mRNA labeling in above 25% of control levels were eliminated from further analyses. Animals from groups AA/AB and group BcB were analysed for full HPC Arc mRNA as described in Chapter 3. This led to the exclusion of 3 animals (1 from group AxB; 2 from group BxB). Following removal of these outliers, the average absolute number of cells expressing Arc in the control groups were 601,342.5 ($\pm$ 77,744.92) while the absolute number of Arc expressing cells in the inactivated groups were 109,975.48 ($\pm$ 9,749.31). The proportion of HPC cells labeled with Arc mRNA in groups AxA, AxB, and BxB 2.15%. A one-way ANOVA showed that HPC Arc mRNA labeling was significantly higher in groups AA and AB compared to groups AxA and AxB ($F_{(1,13)}$ = 0108,48; $p < 0.001$) and group BxB had significantly fewer HPC Arc mRNA labeled cells compared to group BcB ($F_{(1,6)}$ = 161.79; $p < 0.001$). All data related to this chapter is presented in the absence of these 3 animals. A comparison of control and HPC inactivated Arc mRNA labeling is shown in Figure 6.3.

Group AxA

The average absolute number of cells counted in this group was 5,346,133.67 ($\pm$ 386,723.0) where of 92,111.24 ($\pm$ 11,818.53) were expressing any IEG. Of these, 79,417.75 ($\pm$ 11,326.52) were expressing H1a, 32,010.31 ($\pm$ 5,063.9) Arc, and 19,316.82 ($\pm$ 3,408.31) were co-labeled.

Due to a strong *a priori* prediction about cellular activation patterns, IEG labeling in group AA (Chapter 4) and AxA was compared using independent samples t-tests with equal variance assumed. There was no between-groups difference in total cells labeled with IEG (*p*

= 0.527), H1a mRNA (p = 0.108), or in co-labeled cells (p = 0.206). However, there was a significant decrease in Arc mRNA labeled cells (p = 0.001). This indicates that the inactivation of the HPC significantly reduced IEG labeled in RSD. However, the lack of a difference in co-labeled cells indicate that there are still some activity in RSD, and this activity is similar to that observed when the HPC is on. *A priori* predictions suggest that there should be no difference in co-labeled cells between groups AA and AxA. A one-tailed independent-samples t-test confirmed this prediction (p = 0.103). The results for group AxA are outlined in Figure 6.4. A confocal image of a representative section for anterior RSD from an animal in group AxA are depicted in Figure 6.8 C.

There was no significant correlation between the number of HPC Arc labeled cells and the number in RSD (one-tailed paired-samples t-test; r = -0.165; p = 0.377). When comparing the number of cells labeled for Arc alone, a one-tailed paired-samples t-test failed to find a significant correlation between HPC and RSD Arc mRNA labeling (r = 0.424; p = 0.201). This indicates that the Arc observed in RSD was not due to residual HPC activity.

Group AxB

The average absolute number of cell counted for group AxB was 6,385,405.79 ($\pm$ 984,635.62) Of these cells, 81,053.38 ($\pm$ 14,992.71) were expressing any IEG. 69,911.67 ($\pm$ 14,848.61) were expressing H1a, 26,778.95 ($\pm$ 5,864.51) Arc, and 15,636.95 ($\pm$ 3,330.28) were co-labeled.

A one-tailed independent samples t-test with equal variance assumed as used to compare cellular IEG labeling in groups AB (Chapter 5) and AxB. Group AB had significantly more cells labeled with IEGs compared to group AxB (p = 0.031) and significantly more cells labeled for Arc mRNA (p = 0.009). A paired-samples t-test also showed that rats in group AxB had significantly more H1a mRNA labeled cells than Arc labeled cells (p = 0.017). In

addition, group AxB had significantly fewer co-labeled cells compared to rats in group AB (p = 0.035). These results are consistent with those observed in group AxA, where HPC inactivation significantly reduced RSD IEG labeling. However, they do not comply with the hypothesis that there are no differences in IEG labeling between groups AB and AxB, as there was a significant reduction in co-labeled cells. The result from group AxB are outlined in Figure 6.5.

There was no significant correlation between HPC Arc labeling and total RSD Arc mRNA labeling (r = 0.422; p = 0.239) nor between all HPC Arc and RSD cells expressing Arc alone (not co-labeled; r = 0.564; p = 0.161).

Groups BcB and BxB – novel context exposure

The average absolute number of cells counted for group BcB was 926,804.17 ($\pm$ 93,057.7) where of 202,708.62 ($\pm$ 22,217.91) were expressing any IEG. 152,123.95 ($\pm$ 17,730.39) were expressing H1a, 137,457.35 ($\pm$ 16,849.85) Arc, and 86,872.99 ($\pm$ 11,331.07) were co-labeled. For group BxB, average absolute number of cells counted were 6,409,029.75 ($\pm$ 31,9017.89) were of 61,607.36 ($\pm$ 16,055.73) were expressing any IEG. 57,806.23 ($\pm$ 14,923.78) were expressing H1a, 8,652.88 ($\pm$ 3,610.84) Arc, and 4,851.75 ($\pm$ 2,711.93) were co-labeled.

When comparing group BcB to groups AA and AB (Chapter 5), a one-way ANOVA found between-groups difference of total cells labeled ($F_{(2,15)}$ = 9.77; p = 0.003), H1a mRNA ($F_{(2,15)}$ = 34.64; $p < 0.001$) and co-labeled cells ($F_{(2,15)}$ = 13.5; p = 0.001) but not Arc mRNA ($F_{(2,15)}$ = 3.14; p = 0.07). *Post-hoc* Fisher LSD revealed that group BcB had significantly more cells labeled for IEGs compared to group AA (p = 0.001) and AB (p = 0.015), more H1a mRNA labeled cells compared to group AA ($p < 0.001$) and AB ($p < 0.001$), and more co-labeled cells compared to group AA ($p < 0.001$) and AB (p = 0.001). Group BcB also had significantly more Arc mRNA labeled cells compared to group AA (p = 0.046) and

approached significance compared to group AB ($p = 0.067$). This indicates that if rats are exploring a novel context, the proportion of cells activated is significantly higher than when they are exploring a familiar context. In addition, the number of co-labeled cells was significantly elevated compared to groups AA and AB, indicating that parts of the cellular firing pattern when exposed to the novel context the first time, are re-activated when the animal re-enters the same context. A comparison of groups AA, AB, and BcB are depicted in Figure 6.6. Confocal images of representative areas from dorsal CA1 and anterior RSD are depicted in Figure 6.8 A and B.

A one-way ANOVA revealed between-groups differences between groups BcB and BxB in total number of cells labeled with IEGs ($F_{(1,9)} = 30.98$; $p = 0.001$), in H1a mRNA ($F_{(1,9)} = 16.38$; $p = 0.004$), Arc mRNA ($F_{(1,9)} = 69.89$; $p < 0.001$) and in co-labeled cells ($F_{(1,9)} = 70,22$; $p < 0.001$) where group BxB showed significantly fewer cells labeled for both IEG compared to group BcB. An independent-samples two-tailed t-tests showed that group BxB had significantly fewer co-labeled cells compared to group AxA ($F_{(1,9)} = 2.28$; $p < 0.001$). These results are depicted in Figure 6.7.

Paired-samples t-tests showed that group BxB had significantly more H1a mRNA labeled cells compared to Arc mRNA ($p = 0.011$), and significantly more H1a mRNA labeled compared to co-labeled cells ($p = 0.012$), as well as more Arc mRNA labeled compared to co-labeled cells at the significant levels of $p = 0.059$.

There was no significant correlation between HPC Arc mRNA labeling and all Arc mRNA labeling in RSD ($r = -0.163$; $p = 0.418$), nor with RSD Arc mRNA alone ($r = -0.173$; $p = 0.414$).

A one-way ANOVA showed a significant between-groups effect of proportion of IEG expressing cells being co-labeled ($F_{(3,20)} = 34.53$; $p < 0.001$). *Post-hoc* Fisher LSD revealed that group BxB had significantly fewer co-labeled cells compared to groups AxA ($p = 0.001$)

and groups AxB ($p = 0.004$), as well as group BcB ($p < 0.001$). There was no significant difference between groups AxA and AxB ($p = 0.688$). Group BcB also had significantly more co-labeled cells compared to group AxA ($p < 0.001$) and AxB ($p < 0.001$). These results resemble those that were presented for groups AxA and AxB, where the IEG response was significantly lowered after HPC inactivation. These results are depicted in Figure 6.9.

6.4 Discussion

The aim of the current study was to determine whether, in very familiar contexts, the RSD context-specific activation patterns depend on activity in HPC. Rats explored either one or two different contexts over 12 days and on the critical test-day HPC was bilaterally inactivated following exposure to the first context, but before exposure to the second context. In terms of rearing during context exposure, groups AxA and AxB remained similar to groups AA and AB. There was no change in their rearing behaviour following HPC inactivation. Groups BcB and BxB reared significantly more than any of the other groups in both contexts. If HPC inactivation had caused loss of memory for contexts A and B, then rats would likely show an increase in rearing due to context novelty. Group BcB reared significantly less when they explore the same context a second time on test day, a result that is consistent with the behaviour reported in Chapter 2. In Chapter 2 it was shown that during the first 3 days of context exploration, rats reared significantly less in the second context compared to the first context, independent of group. Therefore, if a rat remembers exploring the context the first time, one would expect a decline in rearing behaviour during the second exploration. Group BxB failed to show such a decline in rearing. This finding is consistent with the idea that inactivation of HPC in group BxB eliminated the memory of having just explored the new context a few minutes before exploring the same new context. Therefore this recent context memory may have been lost, consistent with it still being HPC

dependent. As no such difference was seen in groups AxA and AxB, one may infer that their memory for the contexts was no longer HPC-dependent.

Rats in group AxA and AxB had significantly fewer Arc mRNA labeled cells compared to their control groups AA and AB. Groups AA and AB did not differ in the number of co-labeled cells after 12 days of exploration, and neither did group AxA and AxB. However, group AxB had significantly fewer co-labeled cells compared to group AB. This was unexpected and may not be congruent with the predictions that animals that did not show behavioural effects of HPC inactivation should also have the same cellular firing pattern as compared to their respective control group. As the drop in co-labeled cells was only observed in animals that were exposed to two different contexts, this effect may be due to context discrimination based on input from non-HPC systems. Another idea is that a proportion of the co-labeled cells is caused by pre-activation of cells that were initially Context B specific. This would lead to more cells labeled with both H1A and Arc. With the repeated exploration of two different contexts in succession over 12 days, it is possible that before entering the second context, some of the context-specific (Arc) cells activate during the rest or even during the time in the first context. This out-of-context pre-activation may continue to depend on HPC activity. As expected, group BcB had significantly higher levels of IEG labeling compared to groups AxA and AxB, indicating an increased activation following exploration of a novel context compared to a very familiar one. In addition, the number of co-labeled cells were significantly increased, indicating that the neuronal firing pattern that was associated with exploring the context the first time, was largely re-activated when exploring the second context. These were expected results, as co-labeled cells in group AA following 3 days of exploration were also significantly higher than following 12 days of exploration (Chapter 5). A high proportion of co-labeled cells was not observed in group BxB, where the IEG response was almost completely gone in RSD following HPC inactivation.

Considering these results together with the behavioural ones, one can suggest that the memory for the contexts is reflected by the co-labeled cells, as these remained stable in groups that did not show any behavioural effect of HPC inactivation (group AxA and AxB) but were almost completely gone in the group whose behaviour indicated loss of memory (group BxB). Also, the much greater reduction in Arc label in group BxB compared to AxA and AxB suggests that RSD Arc activation by new information depends upon HPC output to RSD, but a significant amount of RSD Arc activation with very familiar information is triggered by non HPC inputs to RSD, possibly other cortical inputs. This may be a reflection of non HPC memory consolidation in this cortical region.

HPC overshadowing occurs when the HPC and at least one non-HPC memory system is able to support a memory, but the memory is lost following HPC removal. In this chapter, it was demonstrated that the memory for a context is indeed dependent on the HPC, at least while it is still novel. With multiple, distributed exposures to the same context, it becomes HPC independent and is therefore immune to HPC inactivation. The main difference in RSD IEG labeling, following HPC inactivation and testing of a HPC dependent memory vs. a HPC independent memory, is found in the proportion of co-labeled cells. While rats with HPC independent memories had no change in co-labeled cells compared to control animals, rats with HPC dependent memories had significantly fewer co-labeled cells. In fact, they had hardly any co-labeled cells at all. This pattern of activation had never before been demonstrated, and provides an interesting basis to form a theory which can explain both HPC overshadowing and the cellular activation pattern reported here. Possible theories will be discussed in more detail in Chapter 7.

CHAPTER 7: GENERAL DISCUSSION

7.1 Summary of the thesis

The aim of this thesis is to investigate the processes that may underlie the phenomenon of HPC overshadowing of context memories, or between-systems interaction more generally, using a new approach. When overshadowing is overcome by distributed context learning episodes, processes must exist outside of the HPC that function effectively in the absence of HPC input. Furthermore, these processes must be able to explain why some memories that are lost following HPC damage, can be learned in the absence of the HPC just as quickly as in its presence. Maren at al. (2007) showed that context fear conditioning was lost following post-learning HPC damage, but could be learned as quickly without a HPC as with an intact HPC. Lehmann et al. (2009) replicated Maren et al.'s findings, but importantly showed that following multiple, distributed context-shock pairings the context fear memory survived complete HPC damage, that is, there was no retrograde amnesia. The context fear memory had become HPC independent. In earlier work I showed that context fear memory was lost when the HPC was inactivated following three context-shock pairings which were distributed over three consecutive days, but context fear memory was not lost after 6 pairings distributed over the same time interval (Gulbrandsen et al., 2013). In the current work, I chose to use a simple task, basing my behavioural component on the rat's natural rearing behavior during context exploration. The results of Chapter 2 showed that if exposed to a novel context, rats spend a great amount of time exploring it, reflected in the high levels of rearing. Following multiple re-exposures to the same contexts, the rearing levels declined and after 10-12 days of exploration rearing had reached an asymptote. The results of Chapter 4 showed that in CA1 when the same context was explored twice there was more overlap in the subpopulation of cells activated than

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when two different contexts were explored. When the amount of context exploration was extended from 3 days to 12 days this difference in overlap in activated cell subpopulations disappeared. The results in Chapter 5 showed that the activation of cells in RSD followed the same patterns as CA1. There was significantly more overlap between subpopulations of cells activated when the animal was exposed twice to one context compared to two different contexts. As in CA1, this effect was present after 3 days of exploration and gone after 12 days of exploration. The lack of a difference in co-labeled cells following 12 days of exploration, was a novel and unpredicted finding, and will be discussed in more detail later on in this chapter.

Chapter 4 and 5 established that CA1 and RSD create neuronal firing patterns that differentiate different contexts when exposed to two different contexts, providing support for the idea that RSD responds to contextual information in a very similar way to CA1. These observations encouraged the idea that RSD could be further investigated in relation to the hypothetical mechanisms underlying HPC overshadowing. In Chapter 6 some of the main aspects of HPC overshadowing were examined. A memory that was initially HPC dependent appeared to be lost after HPC inactivation. This is demonstrated in group BxB vs BcB, as group BcB showed the typical drop in rearing when re-entering the context, while group BxB did not. Thus the rats with HPC inactivated did not show a decrease in rearing on second exploration of a new context, consistent with the idea that they did not remember the context. However, following many distributed exploration sessions the memory did not depend on HPC, demonstrated by no effect of HPC inactivation on behaviour (groups AxA and AxB). If the context memories were lost, then the post-activation context should have been treated as novel and rearing should have been triggered. This provided the foundation for evaluation of neuronal activation patterns in RSD. Consistent across all groups that had intra-HPC infusions of muscimol, the number of Arc mRNA expressing cells was significantly

reduced compared to both comparable control groups and to H1a mRNA labeling. The main difference between animals that likely had a HPC independent memory (groups AxA and AxB) compared to the animals that did not (group BxB), was the presence of co-labeled cells. Groups AxA and AxB did not differ in proportion of co-labeled cells from control groups AA and AB, however, group BxB had significantly fewer co-labeled cells compared to all other groups. In fact, there were hardly any co-labeled cells present at all. This provides an interesting foundation when trying to compose a theory for HPC overshadowing which explains both behavioural and physiological findings.

7.2 Further considerations

There are earlier demonstrations of context discrimination in CA1 using IEGs. Guzowski et al. (1999) reported that following exploration of a novel context, approximately 38% of CA1 neurons showed IEG labeling. In the current experiment, approximately 26% of cells in CA1 were active when exploring a novel context (group BxB; Chapter 6). Guzowksi et al. (1999) demonstrated that when exploring the same context twice, 90% of the neurons activated in the first exploration were re-activated in the second exploration. In my experiment, if a cell was active during exploration of the first context, the chance of it being re-activated was 49% (± 2%). This is a much smaller effect than what was presented by Guzowski et al. (1999). There are a few key differences which may help explain these different outcomes. The major difference is in how cells were counted. Guzowski et al. (1999) collected confocal image stacks from a very small region in dorsal CA1 only. From these stacks, only cells morphologically evaluated as neurons – and not glia – were exhaustively counted. This allowed them to investigate the IEG labeling in an average of 120 neurons per animal. In the current study, random confocal image stacks were collected from the entire CA1 – from the full septal to temporal extent – in a method using unbiased

stereology methods using a well-established software system. This allowed for the analysis of over 1000 cells per animal. However, the label that was used for cellular nuclei – DAPI – does not distinguish between neurons and glia. To avoid any bias in in cell counting, all cells including glial cells were included in the total cell count in current work. Although this should not create any problems when comparing activation between the different groups in the current study, it should be taken into consideration when comparing the numbers obtained here, to those obtained by Guzowski et al. (1999). In their paper from 1999, based upon morphological criteria, Guzowski et al. (1999) estimated that approximately 20% of the DAPI expressing neurons in the CA1 were glia cells. Similarly, Demchuck (2014) applied a counter-label with NeuN to distinguish neurons from glia and showed that approximately 16% of CA1 neurons were glia. Taking this into consideration, the number of IEG expressing cells in the current thesis was still less than previously reported by Guzowski. However, the current thesis evaluated all of CA1, and not just a small portion of dorsal CA1.

Another possible explanation in the difference in proportion of co-labeled cells between the current study and the one conducted by Guzowski et al (1999) lies in the size of the contexts explored. In Guzowski et al. (1999) the rats were exploring two 3900 cm^2 arenas, while in the current experiments the Plexiglas cage that the rats were held in where only 482.6 cm^2. As CA1 place-fields in the dHPC 0.98 ± 0.03 m. It is therefore reasonable to predict that the rats in the study by Guzowski et al. (1999) had more place-fields established and therefore had a higher number of cells activated during the exploration sessions.

The change in proportion of co-labeled cells in rats who explored the same context twice each day from 3 days of exploration to 12 days is an interesting one, as it has not previously been reported. The HPC has been shown to be essential to remembering unique sequences of events as well as the ability to disambiguate sequences that share common events in rats (Fortin et al., 2002; Kesner et al., 2002; Agster et al., 2002), and it has been

suggested that the HPC is necessary for temporal context discrimination (Kesner et al., 2002). As the drop in co-labeled cells after extensive context exploration was only observed in rats who explored the same context twice each day, it is possible that the learned sequence of entering the same context twice within a relatively short time-frame generates separate cellular activation patterns in order to separate the two experiences temporally. It should also be noted that although there was a change in the cellular activation pattern, this does not mean that the information about the different contexts were lost. Previous electro-physiological studies have shown that information about context can be stored in the properties of the cellular firing patterns (Leutgeb et al., 2005). These kind of changes cannot be detected using IEGs.

Chapter 5 describes the first experiment to investigate context discrimination in RSD using IEGs and dISH. The findings support the hypothesis that cells in RSD create two different neuronal activation patterns in response to exploration of two different contexts in a manner that resembles CA1. As in CA1, this difference was not apparent after 12 days of exploration. The reason for this was the significant drop in proportion of co-labeled cells in rats who explored the same context twice each day from 3 days to 12 days. Agster et al. (2002) demonstrated that rats can learn sequences in the absence of the HPC, even when there was a 15 min delay in the presentation of items in the sequence. As previous research has established that the RSC is important in the acquisition of temporal order (Bowers et al.,1988). It is therefore possible that the loss of the "A-B effect" is a result in the sequence of context exploration being learned, and the cellular firing pattern no longer representing the context *per se*, but is concerned with differentiating between the two context exposures.

Another novel finding from Chapter 5 is the coherence between CA1 and RSD activity. After 3 days of exploration there was no correlation between IEG labeling in CA1 and RSD, but after 12 days of exploration the activation in these two regions was

significantly correlated. As output from RSC has also been implicated in the generation of HPC theta rhythm (Destrade & Ott, 1982), which is thought to be important in spatial memory processing (Pan & McNaughton, 1997; Skaggs & McNaughton, 1996), as well as in the stabilization of HPC place fields (Mankin et al., 2012). This indicates that the RSD is not necessarily driven by CA1, but the two structures are a part of a reciprocal network where each region provides important information necessary for successful completion of complex spatial tasks. In a similar sense, damage to the HPC impairs performance on certain spatial tasks, such as the Morris water task, so does damage to the RSC. If RSC is indeed important in stabilizing the place-signal in CA1, then logic predicts that damage to RSC makes solving a spatial navigation task close to impossible.

Chapter 6 demonstrated that context exploration, indexed by rearing, is initially HPC dependent, but following multiple, distributed exploration sessions, the context memory becomes HPC independent, surviving HPC inactivation. These are the common behavioural observations which are linked to HPC overshadowing. Setting up the experiment, I had strong predictions that the IEG labeling in rats who were exploring very familiar contexts would be indistinguishable from that of the relevant HPC active control groups. This turned out to be only partially true. In rats exploring very familiar contexts, the number of Arc mRNA labeled cells was significantly reduced following HPC inactivation. However, this did not necessarily mean a subsequent decrease in co-labeled cells. Only rats who were exploring two different contexts each day showed a decrease in co-labeled cells compared to the rats in the respective control group. One possible explanation for this is that following HPC inactivation, a part of the information entering the RSD is lost. As rats in group that were exploring two different familiar contexts had less overlap in cellular firing patterns than if they would have entered the same familiar context twice. If the control group is coding for the temporal sequence of events, then the rats with HPC inactivation would have

lost some of this information. If that is the case, this would be consistent whether or not the rats explored the same context twice or two different contexts. However, when the same context was entered twice, this led to a greater overlap in cellular firing patterns, and therefore there was no decrease in co-labeled cells in the HPC inactivated group compared to the control group.

Also noteworthy was the almost complete lack of co-labeled cells following HPC inactivation in animals that were exploring a relatively novel context. Behaviourally, this group demonstrated a loss of memory for a recently visited novel context indexed by rearing behaviour following HPC inactivation. As this group was exploring a relatively novel context, it is reasonable to assume that the networks which had become stable in groups that had 12 days of exploration, was not present. Therefore, effects related to phenomenon such as temporal sequences were not yet present. In addition, as the memory for entering the context the first time was lost following HPC inactivation, co-labeling of cells was not expected. Also, the much greater reduction in Arc label cells when HPC was inactivated after exploring a novel context compared when the context was a highly familiar one suggests that RSD Arc mRNA activation by new information depends upon HPC output to RSD, but a significant amount of RSD Arc activation with very familiar information is triggered by non HPC inputs to RSD, possibly other cortical inputs. This may be a reflection of non HPC memory consolidation in this cortical region.

Previous experiments have shown that rats that were trained in context fear conditioning once per day for 12 days were able to discriminate between the fearful and a non-fearful context even without an intact HPC (Lehmann et al., 2009). According to the results obtained in Chapter 6, RSD may not be able to contribute to the discrimination of the two contexts, as there was very sparse Arc mRNA labeling unique to the second context (not co-labeled). It is therefore possible that the RSD only contributes to part of the memory, and

that similar – or even stronger – configurations of pattern completion may be observed elsewhere in the neocortex. As the RSC serves as a relay structure between the HPC and prefrontal cortex (Vertes, 2006; Hoover & Vertes, 2007), and this region has been implicated in many forms of learning and memory (see Miller & Cohen, 2001 for a comprehensive review), and could therefore be an interesting site to continue the investigation of mechanisms such as the ones described in this thesis.

Another interesting result was presented in Chapter 6, that following HPC inactivation, RSD Arc mRNA labeling in response to exploration of a novel context was mainly absent. This lack of Arc mRNA labeling following HPC inactivation has been previously demonstrated, but the persistence of Arc mRNA activation in RSD in very familiar contexts has not been previously described. Kubik et al. (2012) showed that when inactivating HPC with muscimol following 3 days of training in Morris water task, the memory for the task was lost and so was Arc labeling in RSC. Although no paper to date has shown that solving the Morris water task could become a HPC independent task following enough training, it is likely that any spatial memories formed outside the HPC after 3 days of training are not strong enough to allow for recall in the absence of the HPC. This has also been demonstrated in context fear conditioning, where rats showed significantly less freezing when the HPC was inactivated after 3 training sessions on 3 consecutive days compared to controls and if they were trained a total of 6 sessions over the same time interval (Gulbrandsen et al., 2013).

7.3 Final conclusion

Multiple, distributed training sessions allowed for context memory consolidation to take place in circuitry outside the HPC (Sutherland et al., 2010). If part of the network that was originally involved in an event – such as the HPC – is inactivated prior to proper extra-

HPC consolidation, the context memory cannot be recalled. In the current thesis this was demonstrated in group BxB in Chapter 6. The second time rats in this group entered the novel context, they reared just as much as the first time they entered the environment. This differed from group AA and AB, where animals reared significantly less the second time they entered a context on their first day of exploration (Chapter 2). However, when rats had been habituated for 12 days prior to HPC inactivation, there was no difference in rearing behaviour when the HPC was inactivated, consistent with the idea that the memory for the context was now HPC independent.

Cellular activation patterns also shifted in conditions that would favour a context memory being HPC dependent to being independent. Cellular activation in CA1 and RSD went from pattern separating between two different contexts to showing no differential context activation. This shift may be due to the HPC moving from encoding details about the contextual information, to encoding information related the sequence of events. After 12 days of exploration, the time-point associated with the establishment of a HPC independent context memory, the correlation between IEG labeling in CA1 and RSD was highly significant. After 12 days of exploration, when the HPC was inactivated, the level of co-labeled cells stayed the same as in the control condition, however, Arc alone activation was almost non-existent. This was not the case when a novel context was explored, as both co-labeled and Arc only labeled cells were significantly lower than in any other condition. As the only notable difference in IEG labeling between rats that were tested for a HPC independent memory vs. rats tested for a HPC dependent one was the presence of co-labeled cells, it is therefore reasonable to believe that the activation of the co-labeled cells is what drives the HPC-independent memory. The basis for this should be investigated.

To conclude, the aim of the current thesis was to develop an experimental approach to investigate the mechanisms underlying HPC overshadowing at a systems level and to

evaluate whether the cellular processes of pattern separation and pattern completion might plausibly be an explanation for these and related phenomena. This thesis finds support for context discrimination in RSD and a possible mechanism partly supporting the retrieval of a HPC-independent memory.

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Tables

Table 2.1 An outline of the training procedures for each of the behavioural groups

Group	Habituation	Test Day
AA (*n* = 5)	A (10 min) – home (20 min) – A (10 min)	A (5 min) – home (20 min) – B (5 min)
AB (*n* = 5)	A (10 min) – home (20 min) – B (10 min)	A (5 min) – home (20 min) – B (5 min)

Table 3.1 Surgical coordinates

Site	Anterioporterior	Mediolateral	Dorsoventral
Dorsal HPC	-3.5	± 2	-3.7
Ventral HPC	-5.6	± 5.2	-6

Table 4.1. Training and testing paradigm. Groups 1 and 2 were trained for 3 days and tested on day 4, while groups 3 and 4 were trained for 12 days and tested on day 13. HC = home cage. Group 5 served as home cage controls, and were not subjected to behavioural training.

Group	Exploration	Training paradigm	Test day paradigm
AA (*n* = 6)	3 days	A (10 min) – HC (20 min) –B (10 min)	A (5 min) – HC (20 min) – B (5 min)
AB (*n* = 4)		A (10 min)- HC (20 min) – A (20 min)	A (5 min)- HC (20 min) – B (5 min)
AA (*n* = 5)	12 days	A (10 min) – HC (20 min) –B (10 min)	A (5 min) – HC (20 min) – B (5 min)
AB (*n* = 5)		A (10 min)- HC (20 min) – A (20 min)	A (5 min)- HC (20 min) – B (5 min)
HC (*n* = 7)	N/A	N/A	Home cage control

Table 6.1 Behavioural protocol for training and testing day. HC = home cage. Sham infusion was a simulation of intra-cranial infusions. All steps were the same as an actual infusion with the exception of the infusion cannulae not being implanted. N's are final numbers after quantification of HPC inactivation

Group	Habituation	Test day
AxA ($n = 5$)	A (10 min) – sham infusion (10 min) – HC (10 min) – A (10 min)	A (5 min) – Muscimol infusion (10 min) – HC (10 min) – A (5 min)
AxB ($n = 6$)	A (10 min) – sham infusion (10 min) – HC (10 min) – B (10 min)	A (5 min) – Muscimol infusion (10 min) – HC (10 min) – B (5 min)
BcB ($n = 6$)	A (10 min) – HC (20 min) – A (10 min)	B (5 min) - HC (20 min) – B (5 min)
BxB ($n = 4$)	A (10 min) – sham infusion (10 min) – HC (10 min) – A (10 min)	B (5 min) – Muscimol infusion (10 min) – HC (10 min) – B (5 min)
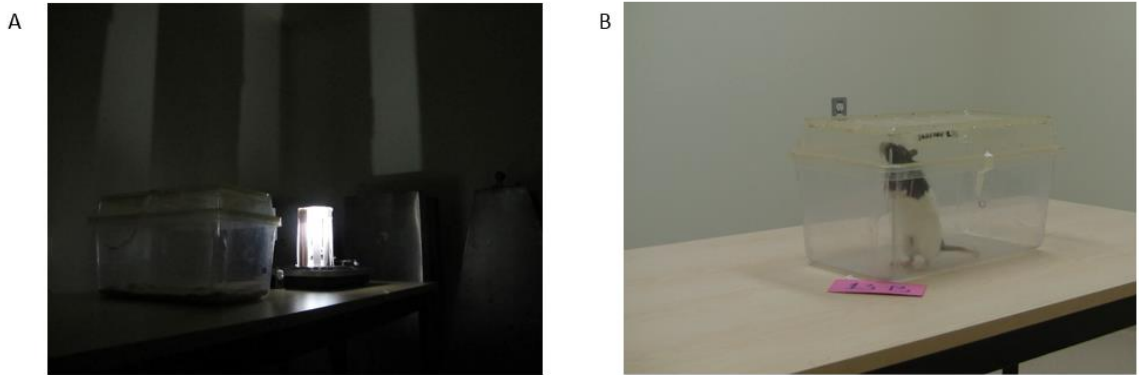
Figure 2.1 A. Room 1. Rats were placed in a Plexiglas container with a high lid and bedding in the middle of a dark room. On the table in front of the container there was a turntable equipped with an LED light covered by a cup wit vertical slits in it. The turntable would be turning giving the appearance of vertical lines rotating on the walls. Between the container and turntable there was a small jar with a cotton ball in it. 3 drops of Tea tree oil would be dripped on the cotton ball prior to testing. In addition, a small speaker was placed on the side of the room playing music at ~75 dB. B. Room 2. Rats were placed in a Plexiglas container with no bedding and a high lid in the middle of a lit room. With the exception of the ID card and camera, there were no external cues within the room. There were no smells or sounds introduced to the room.
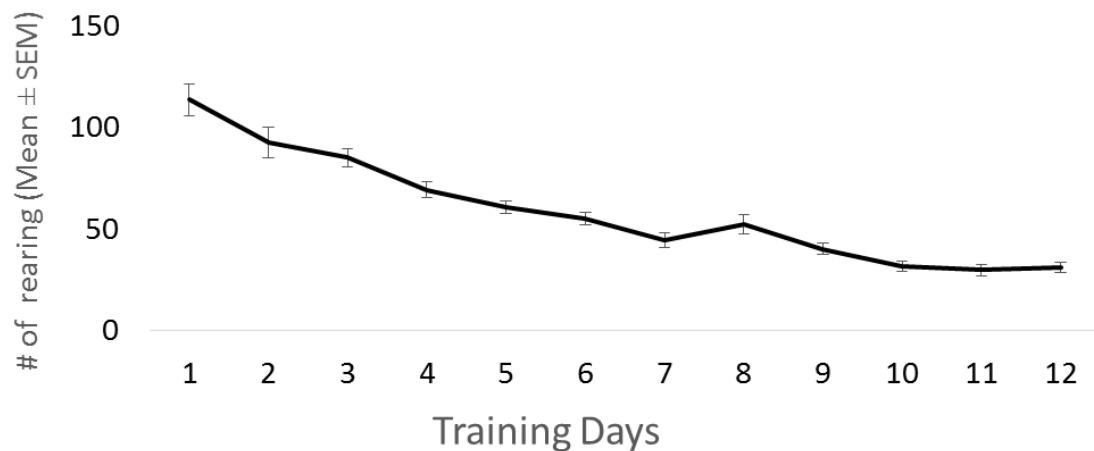
Figure 2.2 Mean number of rearing on each training day, independent of group or context. There was steady decline of rearing across training days, until day 10 when the behaviour reached an asymptote and there were no further decline.
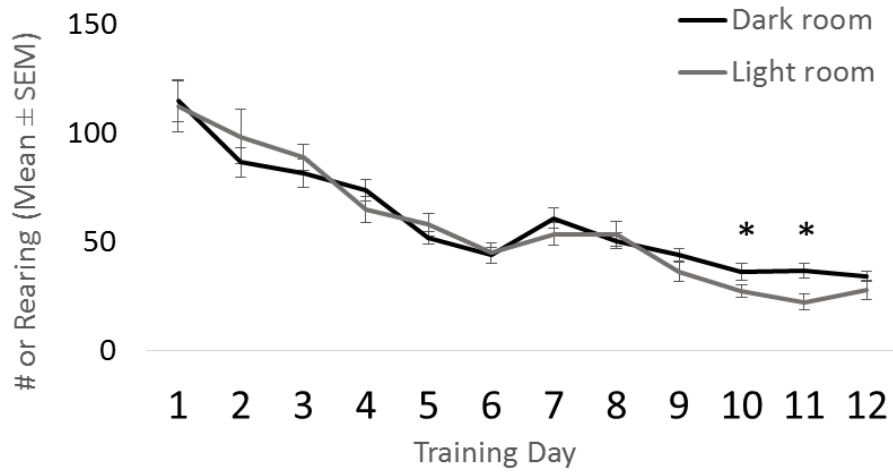
Figure 2.3 Total number of rearing on each training day in the dark vs. the light room. Which testing room was used did not affect the habituation response to context exploration. The only exception was on days 10 and 11 where rats in the dark room reared more than in the light room. (* = $p < 0.05$)
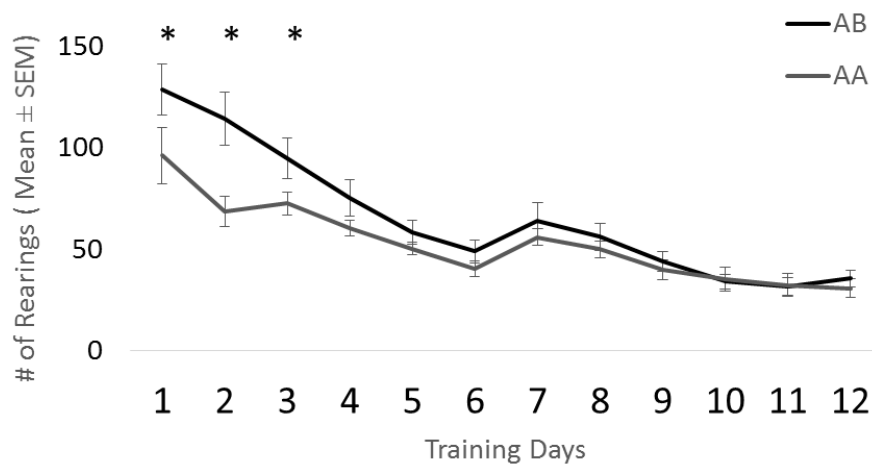
Figure 2.4 Rearing behaviour by group. On exploration days 1-3, rats in group AA reared significantly less than rats in group AB. (* = $p < 0.05$)
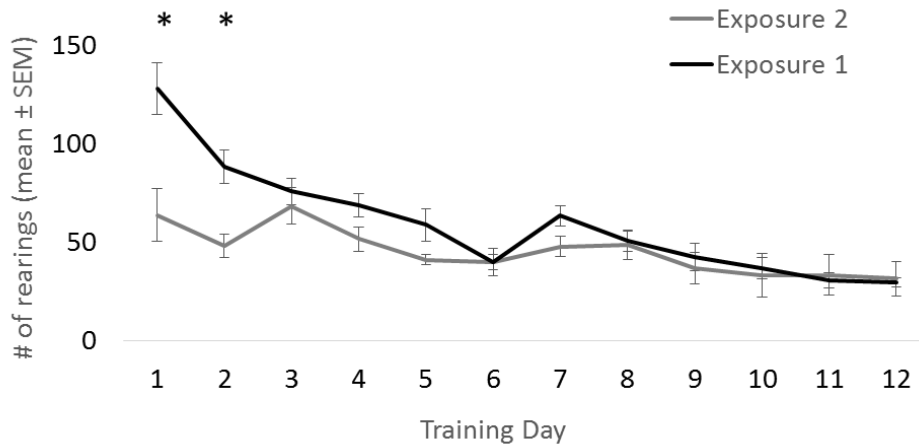
Figure 2.5 Rearing behaviour in group AA within each session across training days. On exploration days 1 and 2 rats reared significantly less in the second context compared to the first context, indicating a short-term habitual response. (* = $p < 0.05$)
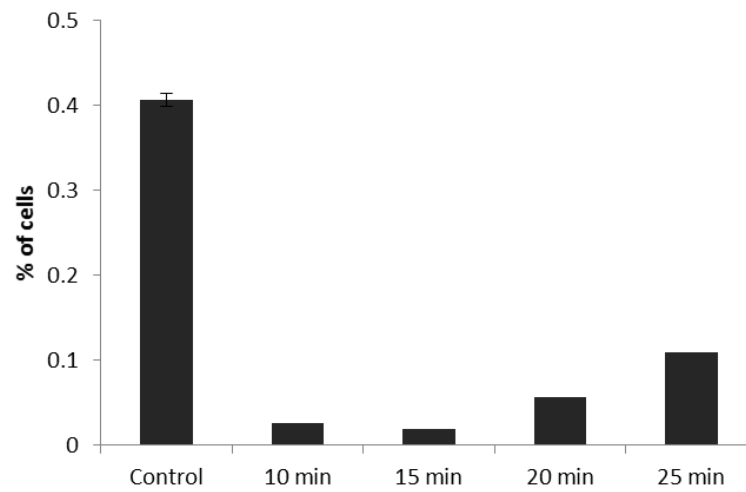
Figure 3.1 Arc mRNA labeling (± SEM) at different time-points after completed infusion of muscimol. The values are presented as proportion of total cells counted. The control data was collected from the control hemisphere of each animal used in the study ($n = 4$).
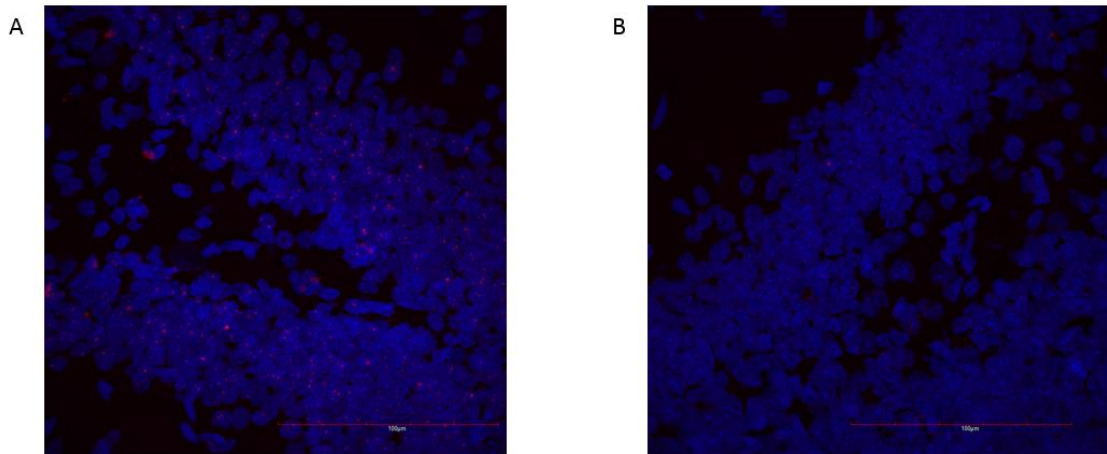
Figure 3.2 A. Dorsal DG following MECS in control hemisphere. DAPI stain for cellular nuclei are in blue and Arc mRNA are in red. The image was captured at 60x using a confocal microscope. The image is a collapse of 1 μm slices taken through the depth of the section, ~ 35 μm. B. Same as A, but from the contralateral hemisphere in the same section. This hemisphere was infused with muscimol. The representation is 10 min following end of infusion.
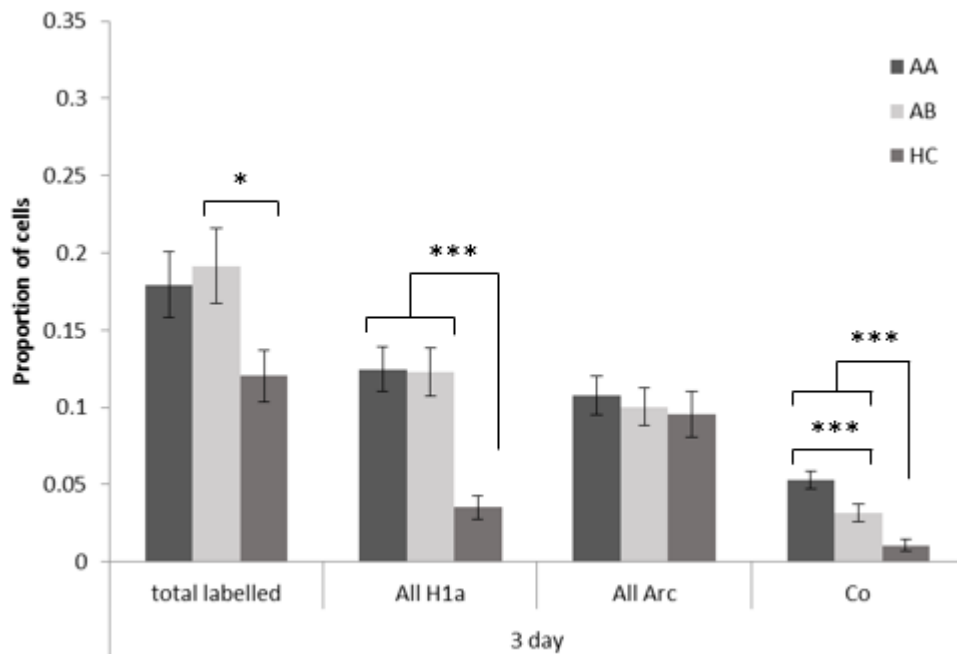
Figure 4.1 Average proportion of cells counted (total labeled with IEG/nucleus counted) expressing any IEG, any H1a, any Arc, and both H1a and Arc (co) after 3 days of habituation (± SEM). Home-cage control animals (HC) showed significantly less H1a and co-labeled cells compared to groups AA and BB, and significantly less total cells expressing IEGs compared to group AB. In addition, group AA had significantly more co-labeled cells compared to group AB. There were no differences in Arc labeling between the groups. (* = $p < 0.05$; *** = $p < 0.001$)
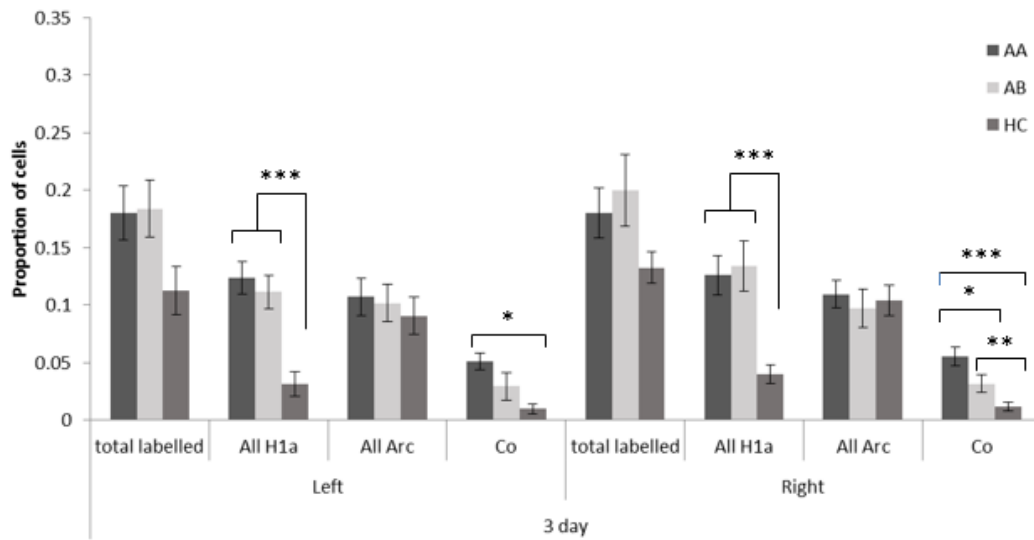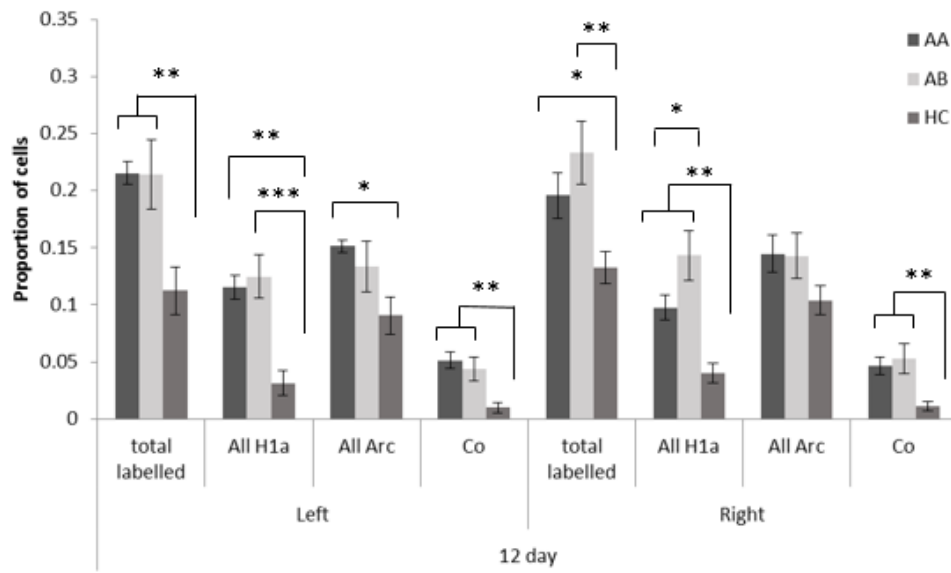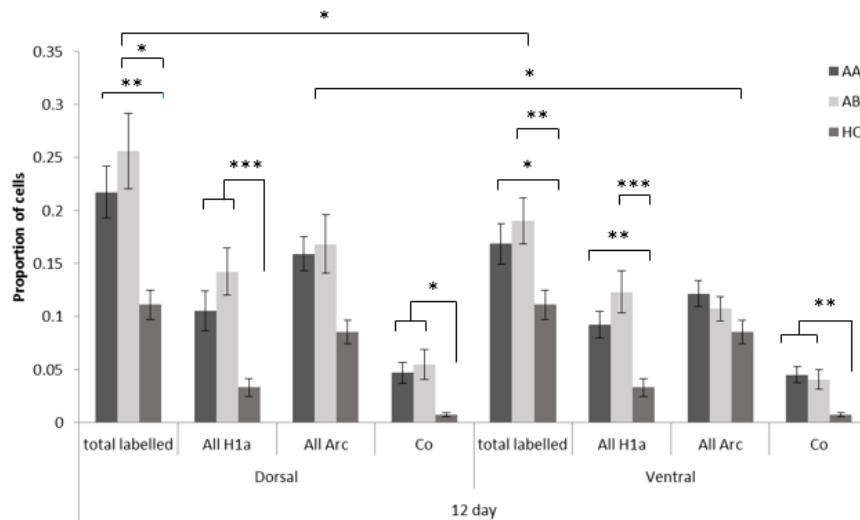
Figure 4.2 Average proportion of cells counted (total labeled with IEG/nucleus counted) expressing IGEs in the left and right CA1 after 3 days of habituation ($\pm$ SEM). Co = co-labeled cells; HC = home-cage control animals. There were no significant effects between the left and right CA1. However, in the left CA1 home-cage control animals had significantly fewer cells expressing H1a mRNA compared to groups AA and AB, and fewer co-labeled cells compared to rats in group AA. In the right hemisphere, home-cage control animals had significantly fewer cells expressing H1a mRNA and fewer co-labeled cells compared to both group AA and AB. In addition, animals in group AA had significantly more co-labeled cells than animals in group AB. (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$)
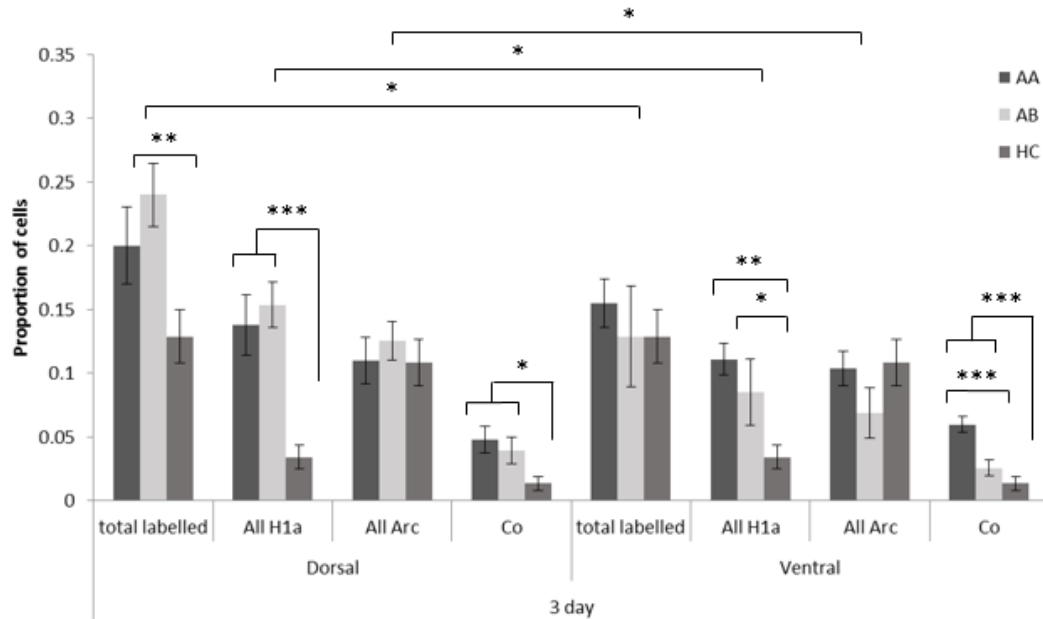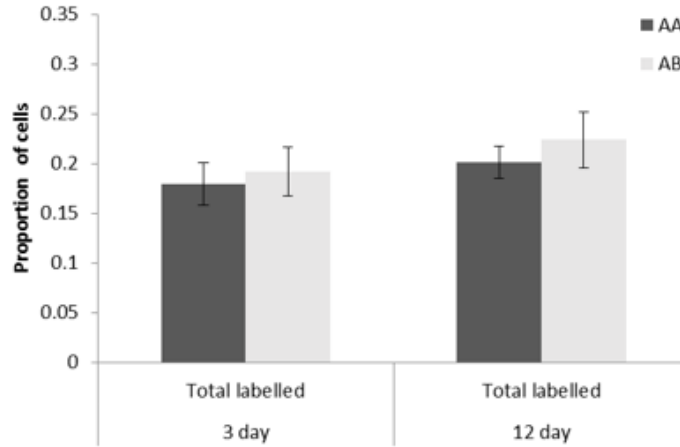
Figure 4.3 Average proportion of cells counted (total labeled with IEG/nucleus counted) expressing IGEs in the left and right CA1 after 3 days of habituation (± SEM) in dorsal and ventral CA1. Co = co-labeled cells; HC = home-cage control animals. Group AB had significantly more total cells labeled, H1a, and Arc labeled cells in the dorsal CA1 compared to the ventral CA1. Home-cage control animals had significantly less total cells labeled compared to animals in group AB in dorsal CA1. In addition, home-cage animals had significantly less H1a and co-labeled cells compared to both group AA and AB in dorsal and ventral CA1. Animals in group AA had significantly more co-labeled cells than animals in group AB in ventral CA1 and not in dorsal CA1. (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).
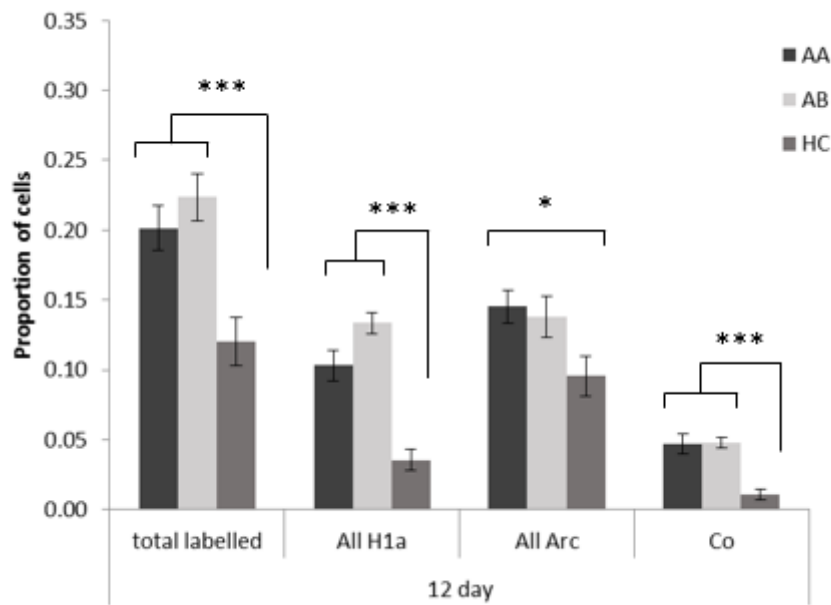
Figure 4.4 Average proportion of cells counted (total labeled with IEG/nucleus counted) expressing IGEs in the left and right CA1 after 12 days of habituation (± SEM) Home-cage (HC) control animals had significantly less total cells labeled, H1a, and co-labeled (Co) cells compared to both group AA and AB, as well as less Arc than animals in group AA. (* = $p <$ 0.05; *** = $p < 0.001$).
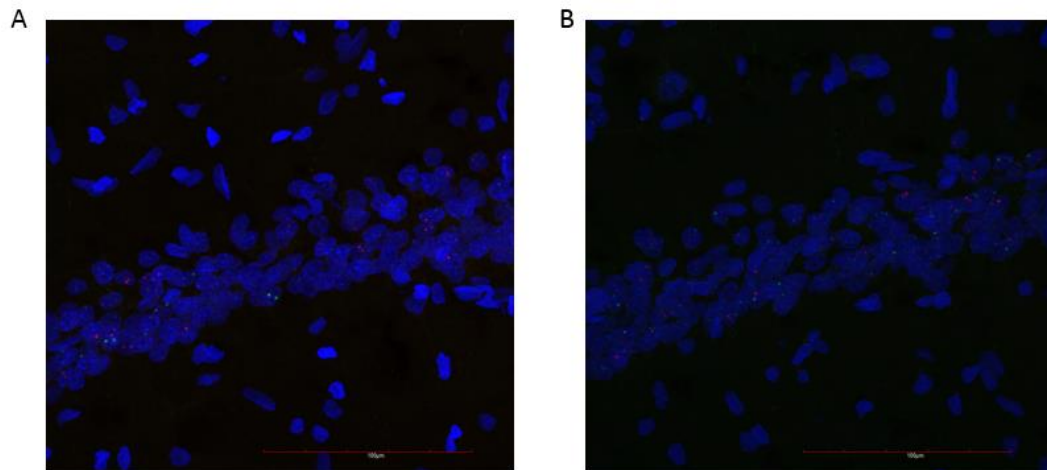
Figure 4.5 IEG labeling in dorsal CA1 after 12 days of habituation. DAPI stain for cellular nuclei are in blue, H1a mRNA are in green, and Arc mRNA are in red. The images was taken using a confocal microscope at 60x magnification. The images are a compressed stack of images separated by 1 μm throughout the depth of the section (~35 μm). A is from an animal in group AA and B is from an animal in group AB.

Figure 4.6 Average proportion of cells counted (total labeled with IEG/nucleus counted) expressing IGEs in the left and right CA1 after 12 days of habituation (± SEM). In the left hemisphere, home-cage (HC) control animal had significantly fewer cells expressing IEGs in all measures. In right CA1, home-cage control animals had significantly fewer cells expressing any IEG, H1a mRNA and co-labeled (Co) cells. (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).
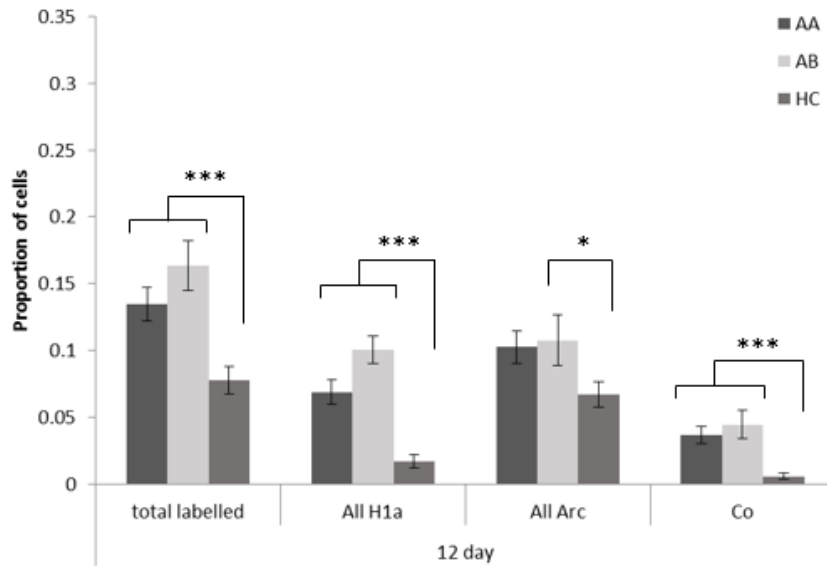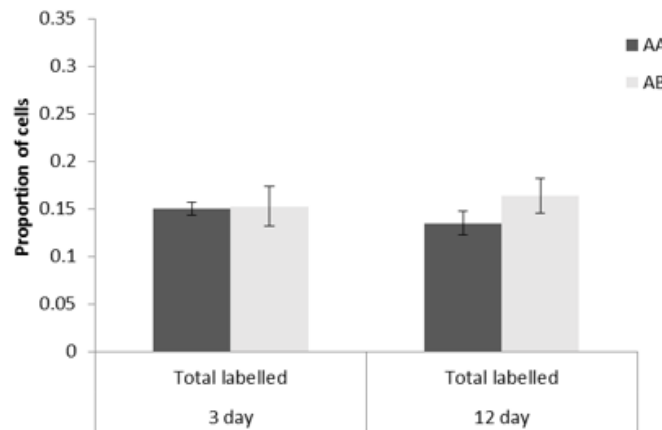

Figure 4.7 Average proportion of cells counted (total labeled with IEG/nucleus counted) expressing IGEs in CA1 after 12 days of habituation (± SEM) in dorsal and ventral CA1.. Group AB had significantly more total labeled cells and Arc in dorsal CA1. Home-cage (HC) control animals had significantly less total cells labeled, H1a, and co-labeled (Co) cells in both dorsal and ventral CA1. (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

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Figure 4.8 Average proportion of cells counted (total labeled with IEG/nucleus counted) expressing IGEs in CA1 after 3 and 12 days of exploration . There were no difference between groups at either time-point, nor between the different time-points.
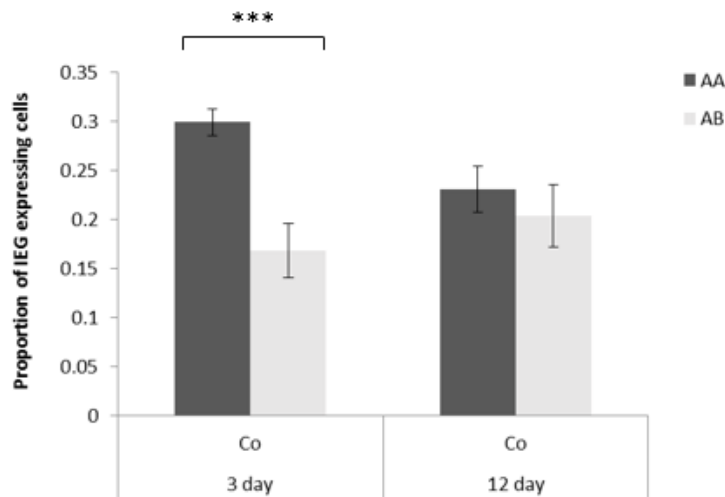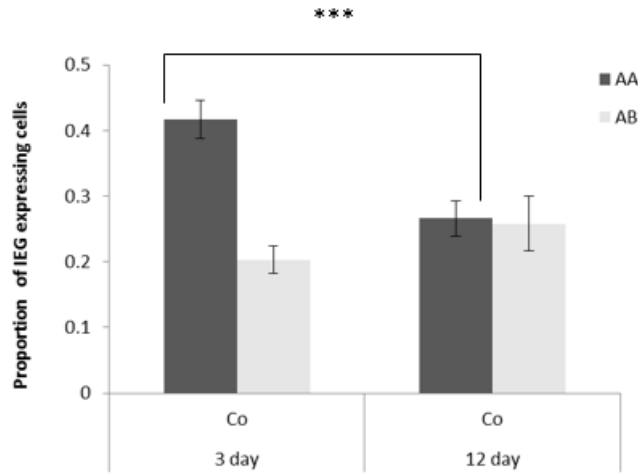

Figure 4.9 Average proportion of cells expressing IGEs being co-labeled after 3 and 12 days of exploration (co-labeled/total labeled). After 3 days group AA had significantly more co-labeled cells than group AB. There were no difference between the groups after 12 days. Group AA had less co-labeled cells after 12 days when compared to 3 days at the significance level of 0.053 (no *). There were no difference with time and habituation in group AB. (*** = $p < 0.001$).
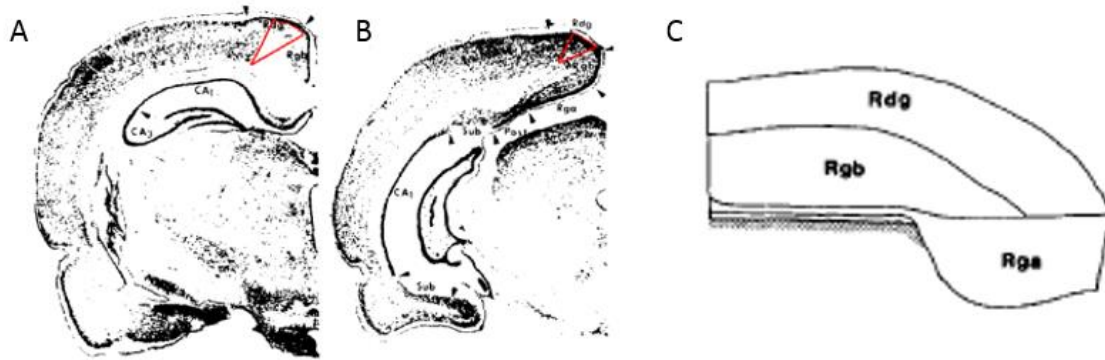
Figure 5.1 Maps of RSD as described by Wyss and van Groen (1992). Coronal sections of anterior (A) and posterior (B) RSD, as well as an unfolded, schematic map of RSD (C) is shown.
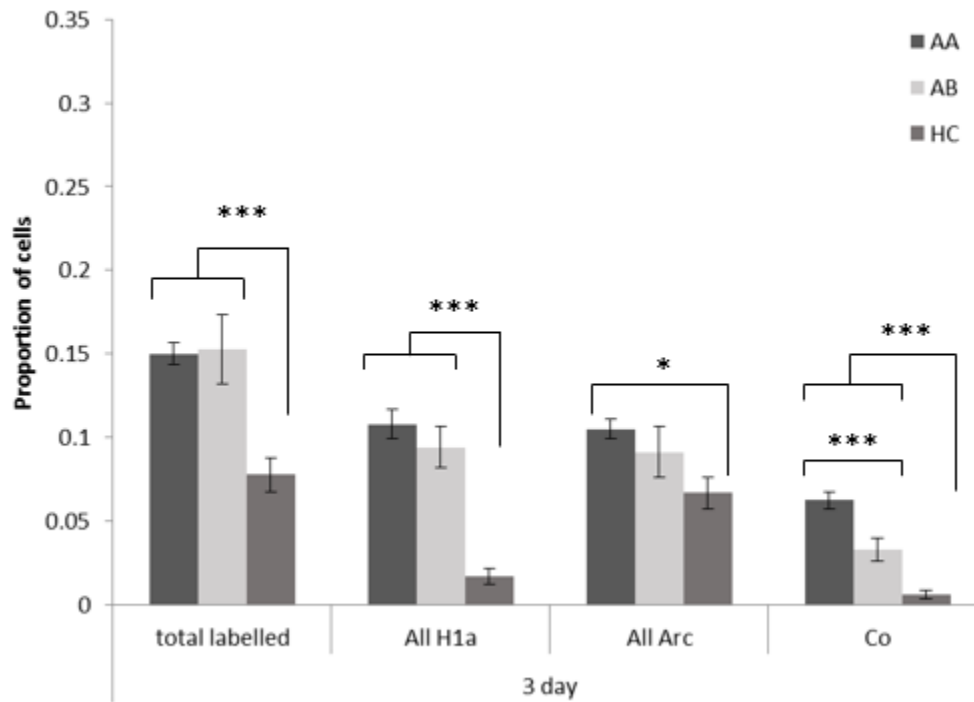
Figure 5. Average proportion of cells counted (total labeled with IEG/nucleus counted) expressing IGEs in RSD after 3 days of habituation (± SEM). Home-cage (HC) control animals had significantly fewer IEG positive cells across all categories. In addition, group AB had significantly fewer co-labeled (Co) cells compared to group AA. (* = $p < 0.05$; *** = $p < 0.001$).

Figure 5.3 Average proportion of cells counted (total labeled with IEG/nucleus counted) expressing IGEs in RSD after 12 days of habituation (± SEM)Home-cage (HC) control animals had significantly fewer IEG positive cells in all categories. Co = co-labeled cells. (* = $p < 0.05$; *** = $p < 0.001$).

Figure 5.4 Average proportion of cells counted (total labeled with IEG/nucleus counted) expressing IGEs in RSD after 12 days of habituation (± SEM) after 3 days and12 days of exploration. There were no significant differences between groups, nor within groups at the different time-points.

Figure 5.5 Average proportion of cells expressing IGEs being co-labeled after 3 and 12 days of exploration (co-labeled/total labeled). After 3 days of exploration, group AA had a significantly greater proportion of co-labeled (Co) cells compared to group AB, although this was not the case after 12 days. The lack of between-groups difference after 12 days of exploration was due to the significant decrease in co-labeled cells in group AA from 3 days to 12 days. (*** = $p < 0.001$).
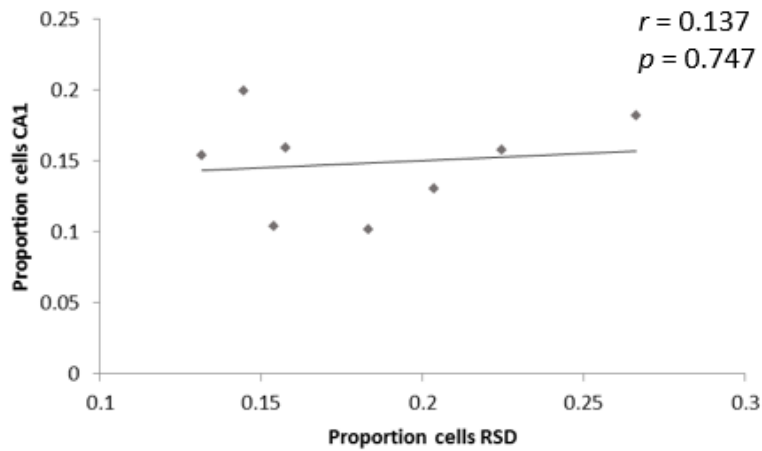
Figure 5.6 The correlation between IEG labeling on CA1 and RSD following 3 days of habituation.
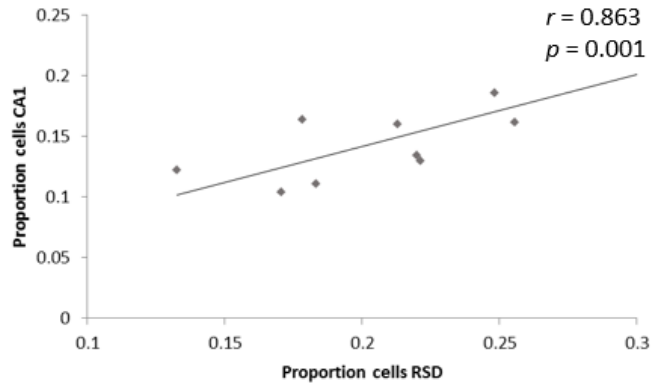
Figure 5.7 The correlation between IEG labeling in CA1 and RSD after 12 days of habituation.
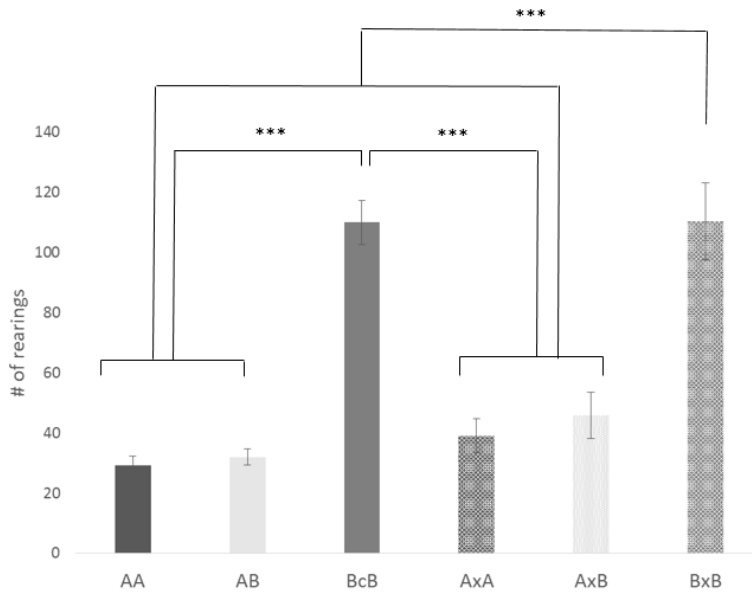
Figure 6.1 Average number of rearing in the two different contexts on test day (day 13; ± SEM). The data points include the groups from chapter 2, 4 and 5 (groups AA and AB) and Chapter 6. Groups BcB and BxB reared significantly more than any of the other groups, and were not statistically significant from each other. (*** = $p < 0.001$).
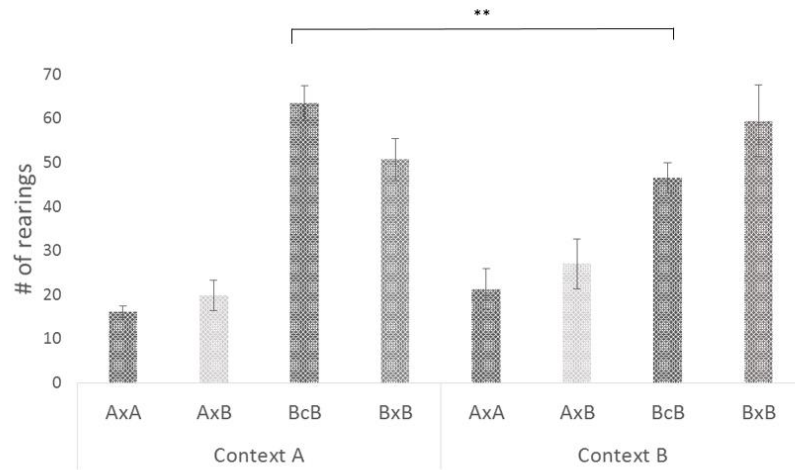
Figure 6.2 Average number of rearing during exploration on test-day in groups that were injected with muscimol before entering Context B. Animals in group BcB reared significantly more in Context A compared to Context B. (** = $p < 0.01$).
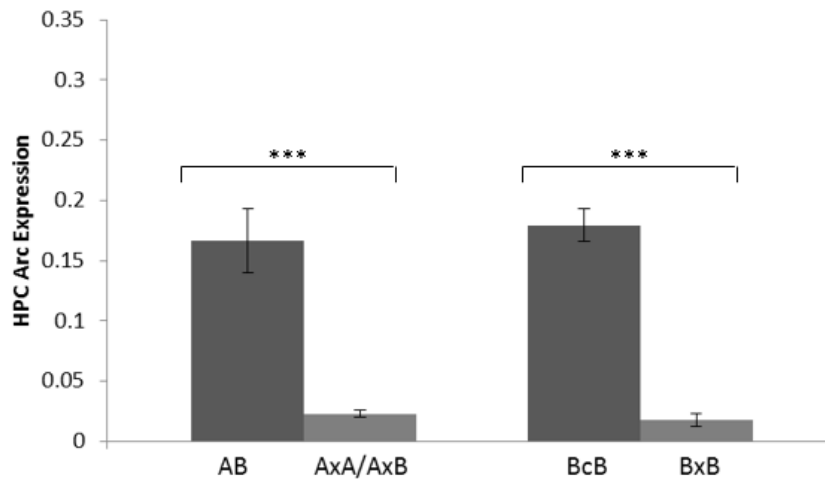
Figure 6.3 Proportion of cells in HPC expressing Arc (total Arc labeld/total labeled) on test day (±SEM) in control groups (AB and BcB) and groups that were inactivated using muscimol (AxA/AxB and BxB). The control groups had significantly more Arc expression compared to groups that had intra-HPC injections of muscimol.. (*** = $p < 0.001$).
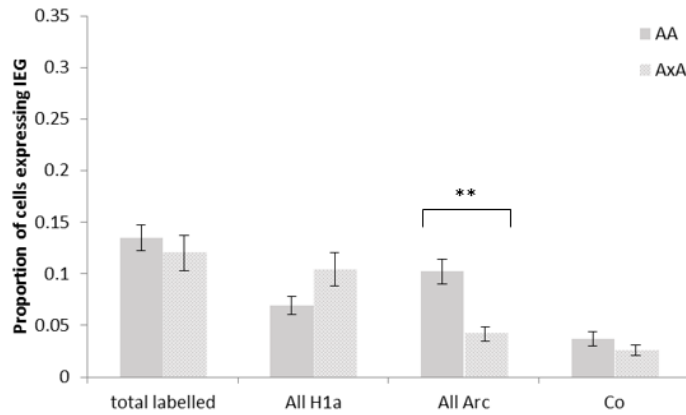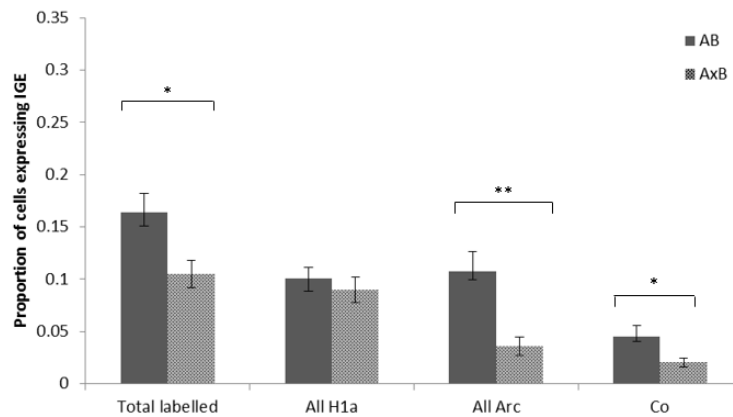
Figure 6.4 Average proportion of cells in RSD expressing any IEGs (total cells labeled/total cells counted) as well as average H1a, Arc and co-labeled (Co) cells in group AA (chapter 5) and group AxA (± SEM) Group AA had significantly more Arc labeling than group AxA. (** = $p < 0.01$).

Figure 6.5 Average proportion of cells in RSD expressing any IEGs (total cells labeled/total cells counted) as well as average H1a, Arc and co-labeled (Co) cells in group AB (chapter 5) and group AxB (± SEM) Group AxB had significantly fewer cells expressing any IEG as well as significantly fewer Arc labeled cells. In addition, an independent-samples t-test showed that groups AxB had significantly fewer co-labeled (Co) cells compared to group AB. (* = $p < 0.05$; ** = $p < 0.01$).
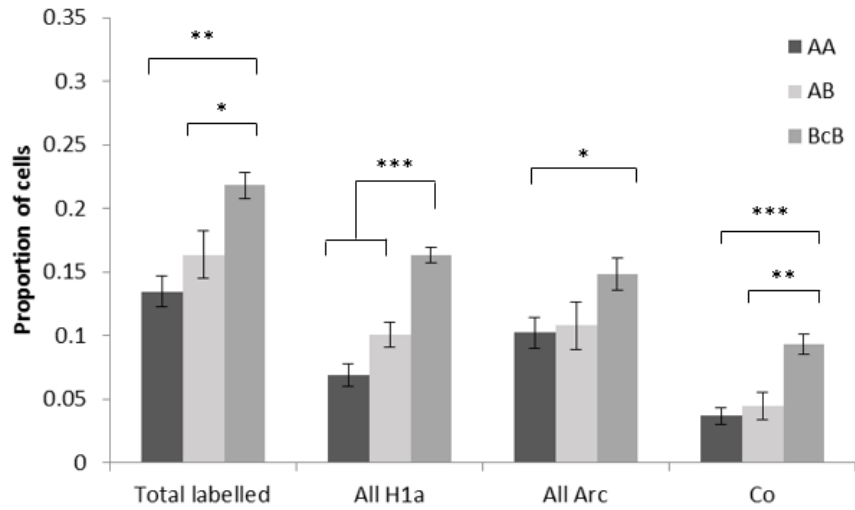
Figure 6.6 Average proportion of cells in RSD expressing any IEGs (total cells labeled/total cells counted) as well as average H1a, Arc and co-labeled (Co) cells in group BcB compared to groups AA and AB from chapter 5 (± SEM). Group BcB had significantly more cells expressing any IGE, more H1a, Arc and co-labeled cells compared to groups AA and AB. (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).
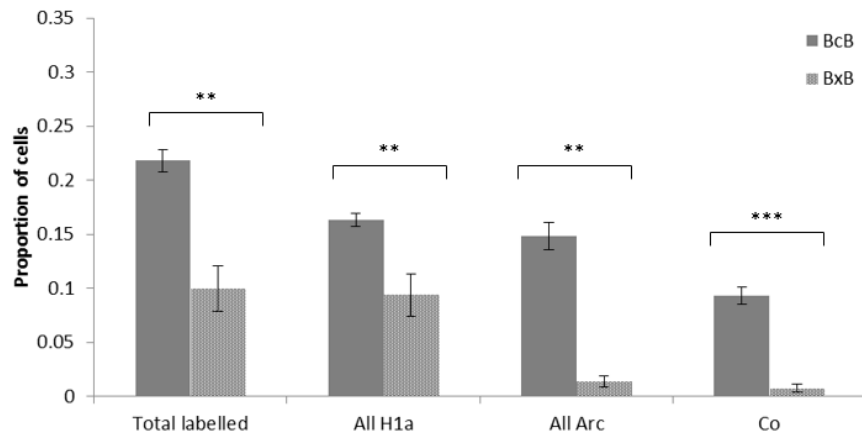

Figure 6.7 Average proportion of cells in RSD expressing IEGs in groups BcB and BxB (± SEM). BcB had significantly more IEG labeling in all categories compared to group BxB.(** = $p < 0.01$; *** = $p < 0.001$).
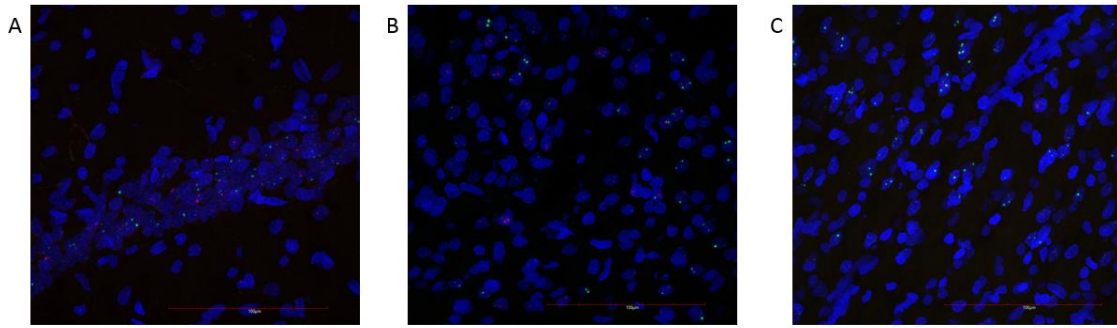
Figure 6.8 A. IEG labeling in dorsal CA1 in an animal from groups BcB. Cell nuclei are colored in blue, H1a mRNA in greed, and Arc mRNA in red. B. IEG labeling in anterior RSD in an animal from group BcB. C. IEG labeling in anterior RSD in an animal from group AxA. All images were taken on a confocal microscope and is the collapsed z-stack of the full thickness of the section (~35µm). All images were taken at 60x magnification.
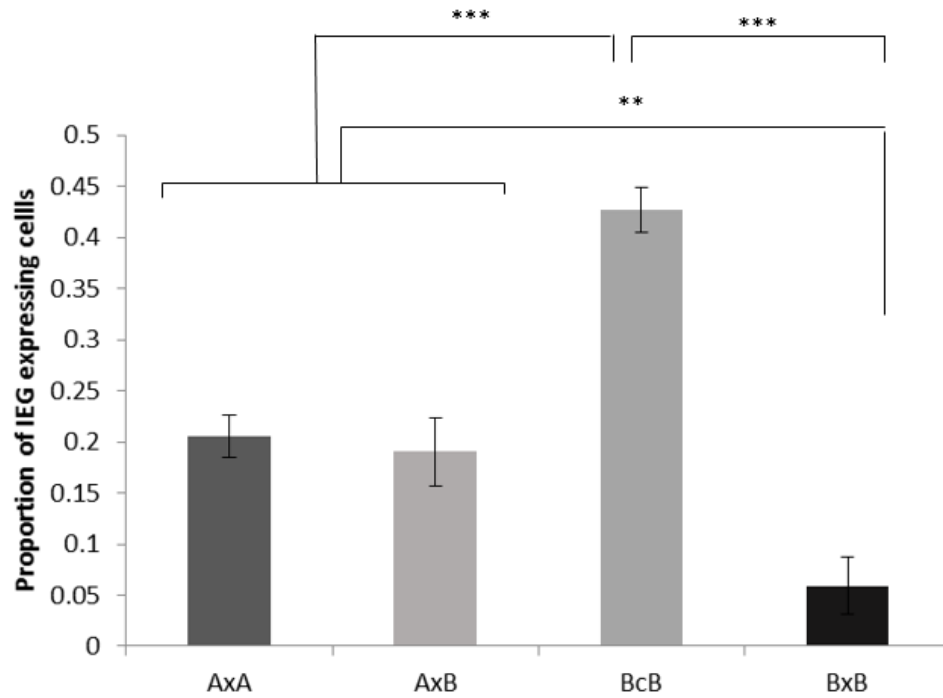

Figure 6.9 The proportion of IGE expressing cells in groups AxA, AxB, BcB and BxB expressing both H1a and Arc. Group BxB had significantly less co-labeled cells compared to the other three groups. In addition, group BcB had significantly more co-labeled cells compared to the other groups.