The perineuronal nets in the lateral secondary visual cortex and their impact on remote visual fear memory

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Oslo, June 2015

Elise Holter Thompson
Abstract

The lifetime of a memory can be divided into four stages: acquisition, consolidation, storage and retrieval. While there is extensive knowledge about the molecules that underlie the early phases of memory formation, little is known about the mechanism of memory storage over long periods of time. In fact, most of the current memory research focuses on intracellular molecules, but there is a mismatch between the short half-life of these molecules and the need for a stable neuronal memory representation over time. A suggested candidate to provide the stability needed for memory storage is the extracellular matrix structure called the perineuronal net (PNN). The PNNs enwrap the soma, proximal dendrites and synapses. The PNNs are highly stable, yet modifiable structures, which in theory makes them candidates to maintain the integrity of synaptic connections and memories over time.

In order to test whether PNNs could be an integral part of memory stability, we used classical Pavlovian fear conditioning to create a remote memory in rats and experimentally degraded the PNN after memory acquisition. The PNNs were degraded by local injections of the bacterial enzyme chondroitinase ABC (chABC). This enzyme cleaves the glycosaminoglycan (GAG) side chains from the core protein, causing the PNN structure to disintegrate. Visual fear memory formation is initially dependent on plasticity in the amygdala, but previous findings have suggested that over time, the memory becomes dependent on the lateral part of the secondary visual cortex (V2L) as a result of a reorganization of the neuronal circuitry. We therefore injected chABC into the V2L at different time-points in the fear conditioning learning paradigm; either prior to training, or prior to retrieval of a remote memory (~30 days after training). While degrading the PNNs in V2L prior to training had no effect on the memory, the degradation of PNNs in V2L prior to the remote memory test disrupted the visual memory. This suggests that PNNs may play a vital role in memory storage and/or memory retrieval. The experiments conducted in this study is the first to have tested and shown the importance of PNNs in remote memory processing. Overall, my findings indicate that PNNs in V2L are critical for fear memory processing at remote time-points, but not for memory acquisition or early consolidation. Thus, we provide the first support for the hypothesis that the stability of PNNs contribute to long-term memory storage.
Table of contents

1 Introduction .................................................................................................................. 1
  1.1 Memory formation .................................................................................................. 1
    1.1.1 Pavlovian fear conditioning ............................................................................ 1
    1.1.2 Neuronal circuits in fear memory ................................................................. 3
    1.1.3 Cellular and molecular mechanisms of memory formation ...................... 4
  1.2 Remote memory storage ......................................................................................... 6
    1.2.1 Neuronal circuits of systems consolidation .................................................. 6
    1.2.2 Remote fear memory in secondary sensory cortex ....................................... 7
    1.2.3 Molecular mechanisms of remote memory .................................................. 8
    1.2.4 The perineuronal net .................................................................................... 9
    1.2.5 The perineuronal net and remote memories ................................................ 10
  1.3 Aim of study ............................................................................................................. 13

2 Materials and Methods ................................................................................................. 14
  2.1 Approvals and research animals ........................................................................... 14
  2.2 Intracortical injection of chABC ............................................................................ 14
  2.3 Fear conditioning .................................................................................................... 16
    2.3.1 Equipment ..................................................................................................... 16
    2.3.2 Visually cued fear conditioning .................................................................... 17
    2.3.3 Timing of the chABC-injection surgery ....................................................... 19
  2.4 Histology and immunohistochemistry ................................................................. 20
    2.4.1 Perfusion ...................................................................................................... 20
    2.4.2 Staining of the C-6-S antigen after chABC treatment for light microscopy ..... 20
    2.4.3 Dual fluorescent staining of PNNs and the C-6-S antigen ............................. 21
    2.4.4 Fluorescent M2-receptor staining ................................................................. 22
    2.4.5 Dual fluorescent staining with WFA and Nissl ............................................ 22
  2.5 Data analysis .......................................................................................................... 23

3 Results ......................................................................................................................... 24
  3.1 Methodological assessment ................................................................................... 24
  3.2 Fluorescent M2-receptor labeling indicates the V1/V2L border ......................... 24
  3.3 Perineuronal net distribution in V2L .................................................................... 26
  3.4 The role of PNN and V2L in fear memory ............................................................. 27
3.4.1 Histological verification of PNN removal ........................................... 27
3.4.2 Experiment 1: Removal of PNNs before testing .................................. 30
3.4.3 Experiment 2: Removal of PNNs before training .................................. 33
3.4.4 Experiment 3: Removal of PNNs before recent memory test .................. 38

4 Discussion ......................................................................................................... 41
4.1 Methodical considerations ........................................................................... 41
4.2 The role of PNNs in remote memory .............................................................. 42
  4.2.1 Lower levels of PNNs in V2L may reflect a higher level of plasticity .......... 42
  4.2.2 Experiment 1: Removal of PNNs before testing .................................... 43
  4.2.3 Experiment 2: Removal of PNNs before training .................................... 44
  4.2.4 Experiment 3: Removal of PNNs before recent memory test ............... 45
4.3 Neuronal circuit reorganization in fear memory storage over time ................. 45
4.4 Molecular reorganization in fear memory storage over time ......................... 47
4.5 Future perspectives ....................................................................................... 48
4.6 Conclusions .................................................................................................. 49

5 References ........................................................................................................ 51

6 Appendix .......................................................................................................... 61
6.1 List of abbreviations ..................................................................................... 61
6.2 Solutions used for immunohistochemistry and histochemistry .................... 63
  6.2.1 10X PBS ............................................................................................... 63
  6.2.2 T-PBS .................................................................................................. 63
  6.2.3 TNS ..................................................................................................... 63
  6.2.4 Tris-HCL 0.05M .................................................................................. 64
  6.2.5 4% Paraformaldehyde (PFA) ............................................................... 64
6.3 Immunohistochemistry and histochemistry protocols ................................ 65
  6.3.1 Staining of the C-6-S antigen after chABC treatment for light microscopy .. 65
  6.3.2 Dual fluorescent staining of PNNs and the C-6-S antigen ....................... 66
  6.3.3 Fluorescent M2-receptor staining ......................................................... 67
  6.3.4 Dual fluorescent staining with WFA and Nissl ................................. 68
1 Introduction

Our brain is constantly receiving and processing an incredible amount of information. Some of this information is stored as memories that may last for a few days, a week or beyond the end of an exam. Some information is stored even longer, lasting months and years; they become so called remote memories. One of the major mysteries in current neuroscience is the apparent discrepancy between the astonishing stability of our memories over time, and the short lifetime of molecules believed to underlie memory formation and their storage.

1.1 Memory formation

We can roughly divide the memory processing into four stages: acquisition, consolidation, storage and retrieval. A memory is in a labile state (short-term memory) in the immediate post-learning phase; both pharmacological agents and molecular disruption can disrupt the memory at this point (Dudai, 2004; E R Kandel, 2001; McGaugh, 2000). Within minutes or hours, the memory enters its more stable form (long-term memory) and becomes fairly resistant to manipulation (Nader, et al., 2000). The progression from labile short-term memory into stable long-term memory is often referred to as synaptic consolidation (Dudai, 2004; E R Kandel, 2001; G.E Müller & A. Pilzecker, 1900; Squire, E.R Kandel, 1999).

1.1.1 Pavlovian fear conditioning

Much of our knowledge about the mechanisms of memory processing is from studies of fear conditioning in animals. Fear conditioning has the advantage over other models for learning in that it creates a robust long lasting memory that can even last a lifetime (Gale, et al., 2004). Fear conditioning involves the pairing of an aversive stimulus (e.g. a foot shock or loud noise) with a neutral stimulus. The animal will learn to predict the onset of an aversive stimulus when it is exposed to the neutral stimulus in the future. The neutral stimulus can be a specific context (an environment where the foot shock is given), or it may be an explicit cue (e.g. a tone or a light). After fear conditioning, the previously neutral stimulus gains affective properties and initiates defensive responses in the animal (figure 1.1). In the nomenclature of classical Pavlovian fear conditioning, the neutral stimulus is referred to as the "conditional stimulus" (CS), the aversive stimulus is the "unconditional stimulus" (US), and the defensive
response to the CS is the "conditional response" (CR). The word “conditional”, meaning dependent, is used because the neutral stimulus needs to be in a dependent relationship with the US in order to obtain the correct response (Rescorla, 1967). The main CR in rodents fear conditioned with a light foot shock is freezing (D. C. Blanchard & Blanchard, 1972; R. J. Blanchard & Blanchard, 1969; Bolles, 1970). Freezing is a state where all movements stop except those related to respiration. Other physiological responses include increased blood pressure, hormone release and increased respiration rate. Freezing is often the measured parameter because it is easy to recognize, gives a reliable quantification and does not require advanced equipment.

When the CS is an explicit stimulus like a sound or a light, a “cued fear memory” is created. However, classical cued fear conditioning also always creates a contextual fear memory. This is because the context in which conditioning happens will be a location where the animal predicts the onset of an aversive stimulus, regardless of whether there is an additional explicit CS or not.

Figure 1.1: Illustration of a standard Pavlovian fear conditioning protocol with a light stimulus (yellow circle) as conditional stimulus (CS; middle panel) and a foot shock (red lightning bolts) as unconditional stimulus (US). In the memory retrieval test (right panel) the context is changed to avoid contextual freezing. One of the changes involves odor, one way to do this is to add a peppermint scented soap. If the rodent exhibits freezing (a defensive response) when exposed to the CS, it indicates that it has a memory of the CS being a prediction of the US.
1.1.2 Neuronal circuits in fear memory

The neuronal circuitry underlying fear memory formation was mapped out by a series of landmark lesion studies performed by Joseph LeDoux (Iwata, et al., 1986; LeDoux, et al., 1990, 1986, 1984). These early investigations clearly established the amygdala as the critical brain area for fear conditioning and pointed to amygdalar inputs to convey information about the CS and the US, and its outputs to control fear responses. Later studies using different approaches like lesions, pharmacology and optogenetics have all shown the need for a functional amygdala both for the formation and retrieval of fear memories (Janak & Tye, 2015). On the other hand, the hippocampus, a structure often associated with spatial learning and memory is necessary for contextual fear memories, but not for cued fear memory (Anagnostaras, et al., 1999; Clark & Squire, 1998). During fear conditioning, neurons signaling the US and the CS converge in the lateral amygdala, where the cellular plasticity necessary for fear memory formation is believed to occur (figure 1.2) (Johansen, et al., 2010). From the lateral amygdala, projections are sent either directly or indirectly to the central nucleus of amygdala, which in turn projects to the periaqueductal gray (PAG) responsible for the freezing response, while outputs to the lateral hypothalamus and the paraventricular nucleus of the hypothalamus mediate autonomic and hormonal fear responses (see figure 1.2) (for reviews Johansen et al., 2011; Sears, Schiff, & LeDoux, 2014). The neuronal circuits involved in the acquisition, consolidation and expression of fear memories have been extensively researched and the established evidence is generally acknowledged in the research community. However, much knowledge is still missing regarding the involvement of other neuronal circuits for the long-term storage of fear memories.
1.1.3 Cellular and molecular mechanisms of memory formation

The key structure in memory formation is the synapse. In order to form a memory it is believed that existing synapses must be activated and strengthened and additional synapses may be formed to support the memory (Nabavi, et al., 2014). An influential hypothesis called "Hebbian plasticity" proposes that memories are formed through strengthening of synaptic connections between neurons that fire in a correlated temporal manner (Hebb, 1949), often summarized as “cells that fire together, wire together” (Johansen, et al., 2014). In terms of fear conditioning, it is believed that synaptic inputs that relay information about the CS and the US can converge onto the same postsynaptic cells in the amygdala, and that the strong depolarization caused by the US can strengthen the weaker CS input via Hebbian plasticity. In other words, the foot shock causes a strong depolarization of cells that strengthen the co-
active and weaker CS inputs onto the same cells (Grace & Rosenkranz, 2002; Nabavi, et al., 2014; Gregory J. Quirk, et al., 1995). With the discovery of long term potentiation (LTP), an important step was made to understand the cellular and later molecular mechanisms of learning (Bliss & Lomo, 1973). Importantly, fear learning induces LTP in the amygdala, and interfering with LTP abolishes fear memory formation pointing to LTP as necessary for fear learning (Blair, et al.; Nabavi, et al., 2014; Rogan, et al., 1997; Schafe, et al.; Sears, et al., 2013). The NMDA-receptor dependent LTP starts with activation of a NMDA receptor which initiates a molecular signaling cascade inside the neuron leading to gene expression and protein synthesis (Bliss & Collingridge, 1993; J. Z. Tsien, et al., 1996; Wigström & Gustafsson, 1985). Blocking protein synthesis shortly after a learning has also been shown to inhibit the formation of long term fear memory (H. P. Davis & Squire, 1984; Hernandez & Abel, 2008; Schafe & LeDoux, 2000). Overall, the gene expression and protein synthesis that occurs within the first hours after fear conditioning ensures synaptic consolidation and is critical for fear memory formation (Bourtchouladze, et al.; Schafe & LeDoux, 2000). We have a broad understanding of the earliest steps in memory formation at both the neuronal circuit and molecular level. In contrast, we know very little about what happens next in the lifetime of a memory. In particular, we lack knowledge about what allows a memory to be stored for months and years (figure 1.3).

![Figure 1.3 The four phases of a memory as a function of time. There is a large amount of knowledge of mechanisms behind the initial phases, acquisition and consolidation, the mechanisms behind the storage phase is still largely a mystery.](image-url)
1.2 Remote memory storage

1.2.1 Neuronal circuits of systems consolidation

In comparison to synaptic consolidation, which takes place within minutes or hours after memory acquisition, the next phase in the memory process called systems consolidation is considerably slower and involves changes on a neuronal network level over months and years (Frankland & Bontempi, 2005; H. Wang, et al., 2006). Researchers early started focusing on systems consolidation after observing that amnesia patients had lost large part of their recent memories, while their remote memories were intact (Ribot, 1882). This observation led to the idea that recent and remote long-term memories depend on separate brain structures. In particular, it has been proposed that as time passes and the memory ages, a declarative memory becomes less dependent on the hippocampus where it was initially encoded and that cortical regions become increasingly involved in the memory processing instead (McClelland, et al., 1995; Squire & Alvarez, 1995). Exactly where the memory is “moved” to in the cortex as it ages is uncertain, but research indicates that the anterior cingulate cortex (ACC) is involved specifically for remote contextual fear memory (Einarsson & Nader, 2012; Einarsson, et al., 2014; Frankland, et al., 2004; Goshen, et al., 2011). By looking at expression of immediate early genes (IEG), genes transcribed shortly after neuronal activation, Frankland and co-workers (2004) found persuasive data regarding the cortical participation in remote memory storage (Frankland, et al., 2004). In line with the systems consolidation theory, the expression of IEG following contextual fear memory testing increased in the hippocampus after a recent memory test, but not in the cortex. Furthermore, after the remote memory test they found an increased IEG expression in specific parts of the cortex (ACC in particular) but no increase in the hippocampus. These results support the involvement of the cortex in remote memory processing and indicate that the hippocampus becomes less involved with time.

Remote declarative memories are generally less affected by hippocampal damage than recent memories as seen both in humans and other species (Anagnostaras, et al., 1999; Rempel-Clower, et al., 1996; Scoville & Milner, 1957), suggesting that remote declarative memories may instead depend on neocortical structures for remote memory retrieval. It should however be mentioned that conflicting studies using both spatial memory tasks and context fear conditioning (both models of declarative memory) have found that these memories depend on the hippocampus, regardless of the age of the memory (Broadbent, et al., 2006; Riedel, et al.,...
1999; Sutherland, et al., 2008). This is in conflict with the standard model for systems consolidation, and indicates that not all declarative memories become independent of the hippocampus with time. A different theory of memory storage is “Multiple Trace Theory” (Nadel & Moscovitch, 1997) and the related “Transformation Hypothesis” (Winocur & Moscovitch, 2011). These theories partly agree with the standard consolidation theory and concur that higher brain structures are increasingly involved in remote memory processing. These theories do however deviate from the systems consolidation theory in proposing that the involvement of new brain structures does not necessarily remove the original memory trace from where it was encoded. That is, more than one version of the memory can co-exist and be processed in different regions. Importantly, these are only theories and we currently know little about how different brain structures work together to process remote memories, specifically the mechanisms behind how memory traces can me “moved” between areas of the brain remains elusive.

1.2.2 Remote fear memory in secondary sensory cortex

As time passes, a contextual fear memory goes from being precise and hippocampus-dependent to become more generalized and cortex-dependent (Biedenkapp & Rudy, 2007; Wiltgen & Silva, 2007; Xu & Südhof, 2013). Recent studies indicate that cued fear memories also go through a similar reorganization process during systems consolidation, indicating that there is a time-dependent change in the circuits responsible for storage and retrieval of cued fear memories (Do-Monte, et al., 2015; Sacco & Sacchetti, 2010). One of these studies found that emotionally based remote memories may depend on secondary sensory cortex for storage and remote memory retrieval (Sacco & Sacchetti, 2010). Their results showed that animals with lesions to the secondary sensory cortices where unable to retrieve a cued fear memory encoded one month prior. Importantly, animals with the same lesions showed normal memory formation and retrieval after 24 hours, suggesting that these lesions specifically affected remote- rather than recent memory storage. The type of cued fear memory was linked to the placement of the lesion; auditory cued fear memories could not be retrieved if the secondary auditory cortex was damaged, while visually cued memories were abolished if the secondary visual cortex was damaged. The lesion was specifically aimed at the lateral area of the secondary visual cortex (V2L). The study proposes a unique model of the functional anatomy of remote memory storage. The importance of V2 has also been linked to long-term encoding of object recognition memory (López-Aranda, et al., 2009). These findings may suggest that
while the primary sensory cortex processes sensory input, the secondary sensory cortex might be capable of storing remote memories created by the input. Other studies have also found evidence of the involvement of the secondary sensory cortices in remote memory storage, especially emotionally significant memories, like those created by fear conditioning (Anderson, et al., 2003; Kwon, et al., 2012). It has been suggested that after an emotional experience, the secondary sensory might create a link between sensory input and its emotional meaning, and store this behaviorally significant association over a long time (Grosso, et al., 2015). However, the mechanism behind the storage of remote fear memories and the role of the secondary sensory cortices is still unknown.

1.2.3 Molecular mechanisms of remote memory

The scientific search for proteins and molecules that contribute to remote storage of memories has been challenging. In particular, there seems to be a conflict between the astonishing stability of many memories over time and the short half-life of most neuronal proteins. Some of the current theories on the topic of cellular mechanisms in remote memory storage include protein kinase M zeta (PKMζ), calmodium-dependent protein kinase II (CaMKII) coupled with NMDA receptor, protein phosphatase 2A, and cytoplasmic polyadenylation element binding protein (CPEB) (Eric R Kandel, 2012; Pi & Lisman, 2008; Sacktor, 2011; Sanhueza & Lisman, 2013). These are all intracellular molecules, most of which are found in the postsynaptic part of the neuron, that seem to play some part in the process of memory consolidation and storage. However, these intracellular molecules are regularly broken down, most of them only lasting a few days before new copies need to be made and transported to the correct location. In humans, a remote memory can last for decades, with the need of correct copying and intracellular transportation occurring thousands of times in order to preserve a memory. How can this highly dynamic internal environment support long-term storage of memories? It was recently proposed that the extracellular matrix aggregates termed the perineuronal net (PNN), which has low turnover and is therefore not reliant on constant copying to hold on to information, may be suited for contributing to memory storage (R. Y. Tsien, 2013). This hypothesis remains to be tested.
1.2.4 The perineuronal net

The perineuronal net (PNN) is a specialized form of extra cellular matrix (ECM) which is only found in the central nervous system (CNS), and only around subpopulations of neurons (Balmer, et al., 2009; Hendry, et al., 1988; Watanabe, et al., 1989). The PNN wraps around the cell soma and proximal dendrites and form a tight lattice-like structure around them. Composed of negatively charged chondroitin sulfate proteoglycans (CSPGs), hyaluronan, link proteins and tenascin-R (Carulli, et al., 2006; Deepa, et al., 2006; Kwok, et al., 2011), the PNN composition is very similar to that of cartilage (figure 1.4). The bacterial enzyme chondroitinase ABC (chABC) can be used to degrade the PNNs. This enzyme cleaves the glycosaminoglycan (GAG) side chains from the core protein, causing the PNN structure to disintegrate. Treatment with chABC in adult animals has been shown to lead to increased plasticity in several brain areas, e.g. the primary visual cortex (Pizzorusso, et al., 2002, 2006). The treatment is not specific to PNNs, and will also degrade ECM components in other parts of the tissue (Brückner, et al., 1998). However, mice with a deletion of the link protein CtrlI which is specifically located in the PNNs, show similar plasticity effects as found in chABC treated animals, supporting that the increased plasticity is due to the removal of the PNNs (Carulli, et al., 2010; Romberg, et al., 2013).

**Figure 1.4** The proposed structure of perineuronal nets (PNNs) on the cell membrane. The PNNs are composed of hyaluronan connected by link proteins with chondroitin sulfate proteoglycans (CSPGs). Tenasin-R further secures the tight PNN structure through its connection with the core proteins of CSPGs. HAS is hyaluronic acid synthetase, which are the molecules that secure the connection between the membrane and the PNN. Adapted from Kwok et al. (2011).
The PNNs are mainly found around a subtype of fast spiking GABAergic inhibitory neurons expressing parvalbumin (PV+) (Celio, 1986; Dityatev, et al., 2007; Härtig, et al., 1992). The assembly of PNNs is most significant at the end of juvenile brain development and is suggested to limit neuronal plasticity (Carulli, et al., 2006; Hockfield, et al., 1990; Pizzorusso, et al., 2002). Studies propose that parvalbumin (PV), a calcium binding protein, also contributes to regulating neuronal plasticity (Chevaleyre & Piskorowski, 2014; Donato, et al., 2013). Donato et al. found that the expression of PV decreases after environmental enrichment, a situation known to induce plasticity, and PV levels increase after fear conditioning in correlation with memory consolidation (Donato, et al., 2013). Moreover, PNNs seem to affect the level of PV expression in GABAergic neurons, thus suggesting that the up-regulation of PNNs and PV together causes restricted plasticity (Donato, et al., 2013; Yamada, et al., 2015). The PNNs may restrict plasticity by limiting the surface mobility and exchange of AMPA-receptors, a glutamatergic receptor thought to have a large role in facilitating LTP (Frischknecht, et al., 2009). Furthermore, the PNNs may also function as ionic buffers around neurons, for example the PV+-interneurons, to facilitate high spiking frequency of these neurons (Brückner, et al., 1993; Morris & Henderson, 2000).

1.2.5 The perineuronal net and remote memories

One of the reasons why the PNNs appear as a suitable structure for memory storage, is that the components have a very low turnover and are therefore not reliant on constant copying to hold on to information (Margolis, et al., 1975; R. Y. Tsien, 2013). The PNNs wrap around neurons and synapses leaving space for the synaptic connections in the holes in the structure (Hockfield & McKay, 1983; Zaremba, Guimaraes, Kalb, & Hockfield, 1989). The size of the holes in the net structure differs, most likely because of the size of the synapse. The potential influence that PNNs have on the size, placement, and structure of synapses may be a key to the role of PNN for memory storage. A fine regulation of PNNs is essential to allow for synaptic remodeling during plasticity processes. The PNNs can be remodeled in vivo by endogenously produced enzymes released in the extracellular environment. Upon LTP-induction, the extracellular matrix (ECM) is locally degraded by endogenous enzymes called matrix metalloproteinases (MMPs), and later regenerated by other enzymes (Dziembowska & Wlodarczyk, 2012; Ethell & Ethell, 2007; Meighan, et al., 2006). Because the neurons release the enzymes necessary to break down or build up the PNNs, enables a local regulation of the
cells synapse dynamics when needed. In other words, comparing the brain to a computer, storing memories in PNNs would be like storing your thesis on your hard drive. It does not require constant upgrading to stay there, but you can alter it when new paragraphs need to be added, or old ones have to be erased.

The proposed idea that PNNs can support remote memory storage has, to the best of my knowledge, not yet been tested. However, several lines of evidence point to the role of PNNs in memory processing (Gogolla, et al., 2009; Hylin, et al., 2013; Romberg, et al., 2013; Slaker, et al., 2015). The PNNs have been shown to influence memory created by cued and contextual fear conditioning (Hylin, et al., 2013), fear extinction (Gogolla, et al., 2009), object recognition memory (Romberg, et al., 2013) and cocaine-induced place preference memory (Slaker, et al., 2015). Gogolla and co-workers (2009) used chABC to remove the PNNs from the amygdala and looked at the effect on extinction of fear memories. In adult animals, extinction of a fear memory does not cause erasure of the previously acquired memory but rather creates a new memory that inhibits the fear response (Bouton & Bolles, 1979; Rescorla & Heth, 1975). In young animals where the PNNs are not yet developed, Gogolla and co-workers (2009) found that extinction caused complete erasure of the fear memory.
Furthermore, adult mice where PNNs were removed from the amygdala also showed erasure of the fear memory after extinction training indicating that the PNNs protect the fear memory against erasure (Gogolla, et al., 2009). In another study Hylin and co-workers (2013) found that removal of the PNNs in the hippocampus and medial prefrontal cortex before context-, and trace fear conditioning, disrupted recent long-term fear memories (Hylin, et al., 2013). While these studies show that PNN can influence relatively recent memories processing, the role of PNN in remote memory storage remains elusive.
1.3 Aim of study

The main objective of this study was to reveal whether perineuronal nets (PNNs) in V2L are involved in remote memory processing. In order to achieve this, we had the following secondary sub-goals:

a) develop a protocol for creating a remote visually cued fear memory,

b) determine the effect of PNN removal on recent fear memory,

c) determine the effect of PNN removal on remote memories, by removing the PNNs before fear conditioning or before memory retrieval

Degradation of PNNs was achieved by local injections of chABC into the V2L in rats. Both visually cued and contextual memories were examined in all fear conditioning experiments.
2 Materials and Methods

2.1 Approvals and research animals

The laboratory work was done at the Department of Biosciences, Faculty of Mathematics and Natural Sciences, the University of Oslo. Before initiation, the experiments were approved by the Norwegian Animal Research Committee (FDU). The animal facility and the experiments with the animals are in accordance with the Norwegian Animal Welfare Act and the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes. All participants have completed a course in Experimental Animal Research (similar to a FELASA C course) and are approved by the FDU to conduct research experiments with animals.

Experiments were conducted with male Sprague Dawley rats, ordered through the Norwegian Animal Research Center (NFS) at the Norwegian Institute of Public Health and Taconic Biosciences, Inc. (Denmark). In addition, 23 male Long Evens rats (locally bred) were used during the first phase pilot experiments to establish the fear condition protocol. Upon arrival, animals were eight to ten weeks old, weighing about 250g. The animals were acclimated to the vivarium for one to two weeks before training. Animals were maintained on a 12/12h light/dark cycle (lights on at 20.00) and the temperature in the housing facilities were kept at approximately 21 °C with humidity at 55 ± 10 %. The rooms have a ventilation rate of 5-20 times per hour. Rats were housed in groups of four, in polycarbonate cages (35x55x19 cm) with woodchip bedding (Scanbur) and plastic toys. Food and water were available ad libitum throughout the period of experiments. Experiments were conducted during the dark cycle.

2.2 Intracortical injection of chABC

Protease free Chondroitinase ABC (chABC) from Proteus vulgaris (AMS Biotechnology, Abingdon, UK) was diluted in 0.1M PBS to a final concentration of 61U/mL, and stored at -20°C in smaller aliquots. The aliquots were thawed immediately before pipette loading.

Surgery equipment was heat sterilized, work area was disinfected with 70% ethanol, and cotton swabs were autoclaved before surgery.
An intraperitoneal injection mixture of Ketamine and Medetomidin (Ketamine 75 mg/kg, Medetomidin 0.75 mg/kg) was used to anesthetize the animals.

Animals were head fixed in a stereotaxic frame with ear bars placed inside the external auditory canal, and a height-adjustable nose-clamp. After correct positioning, the midline of the skull was aligned with the frame enabling stereotaxic measurement of the coordinates.

The animal’s head was shaved from behind the eyes to right behind the ears, and the area was cleaned with 70% ethanol and chlorhexidine. A longitudinal incision in the skin was made and the skin, underlying membranes, and muscle tissue were moved aside to expose the skull. The skull was kept moist by applying a NaCl solution (0.9%).

Craniotomies were made bilaterally at two sites above V2L with a drill mounted to the stereotaxic frame. For infusions of chABC into V2L, a thin glass pipettes (opening approximately 20 µm) were filled with mineral oil and then assembled on a micro injector (NanoJect II, Drummond Scientific Company, PA, USA). The micro injector was mounted on the stereotaxic frame, filled with chABC and V2L injections were made at four locations (two in each hemisphere) using coordinates AP 5.8, ML 6.0, DV 3.0 and AP 7.4, ML 5.8 and DV 3.0, measured from bregma (AP=anterior-posterior, ML=mediolateral, DV=dorsoventral). Coordinates were based on the atlas of the rat brain by Paxionos and Watson (George Paxinos, 2007). All animals were bilaterally injected with chABC or artificial cerebrospinal fluid (aCSF, used as sham treatment) (Harvard Apparatus, Holliston, MA, USA).

In each location, we injected 0.5 µl chABC solution, divided into 10 smaller injections (50 nL x 10), with a 60s pause between each. After all 10 injections, 90 seconds passed before drawing back the needle.

The incision was sutured and an anti-bacterial ointment (Fucidin) was applied. The animals were given subcutaneous analgesic injections of Rimadyl (carprofen 5mg/kg) after surgery and on the following three days.

In order to achieve up to 16 operations in two days, we conducted the surgeries as a team. Dr. Mattis B. Wigestrønd conducted the anesthesia, placement of the animal in the stereotaxic frame and the correct positioning. Kristian K. Lensjø made the craniotomies and handled the injections. I sutured the incision, and looked after the animals in the post-operative days.

The animals recovered in their home cage six to seven days before fear conditioning/testing.
2.3 Fear conditioning

2.3.1 Equipment

Four standard fear conditioning chambers (interior: 30.5 cm x 24.1 cm x 21.0 cm; MED-Associates, St. Albans, VT, US) were used for conditioning and testing. The chamber is made of Plexiglas (door, back wall and roof) and aluminum (two side walls). It is placed inside a sound attenuating cubicle (1 x ENV-018MD Deep, extra tall MDF; MED-Associates). The removable floor in the chamber is made of 19 stainless steel rods (4mm in diameter) that are spaced 1.5 cm apart. The rods are connected to an electric pulse generator (MED-Associates) that delivers the foot shock. The strength of the shock is set manually, it was set to 0.30 mA. A house light (ENV-215M), a programmable audio generator (ANL-926), and a white stimulus light (ENV-221M) is mounted in the walls of the chamber (all from MED-Associates). A ventilation fan, a permanent fixture in the chamber, can be turned on and off.

K-limbic control and acquisition software (MED-Associates) control the fear conditioning protocols.

The training- and habituation context (figure 2.1) included a wall parting the chambers diagonally; the wall was made of one white and one black plastic board taped together with silver duct tape. The chamber’s original floor was used. It is made of stainless steel rods attaches to the electric pulse generator. The ventilation fan was on, and the house light was off. The light in the room where the chambers are located (the “fear conditioning room”) was on when the rats were placed in the chambers, but was otherwise turned off.

Figure 2.1: Training context
The altered context (figure 2.2) used during testing had no diagonal wall dividing the chamber, but a silver-striped back-wall. The floor was made of thinner metal rods with closer spacing than that used in the training context (36 rods, 3.2 mm diameter, spaced 7.9 mm apart). The floor was not connected to the electric pulse generator. Peppermint soap was added in the waste tray to change the smell in the chamber. The ventilation fan was off and so was the house light. The light in the “fear conditioning room” was off at all times.

![Figure 2.2: Altered context](image)

The rats movements in the chambers were filmed with infrared cameras placed in the ceiling of the isolation cubicles. The videos were recorded and saved on a DVD. The behavior (freezing) was scored offline with a digital stopwatch. Freezing is defined as cessation of all movement except that caused by respiration.

### 2.3.2 Visually cued fear conditioning

We established a fear conditioning protocol based on the protocol constructed by Sacco and co-workers (Sacco & Sacchetti, 2010). Training and testing was conducted at the same time of day (4:00 PM±2 hours). The protocol takes place on five days within one month (Figure 2.3).
**Habituation (day 1)**: On the day prior to training, the rats are habituated to the training context. They are in the chamber for five minutes with no stimulation.

**Training (day 2)** The rats are placed in the chamber and five minutes pass before the first light cue (CS). The CS is a six second continuous white light stimulus. The last two seconds of the CS is paired with an electric foot shock (0.3 mA). This is repeated seven times with a varied inter-trial interval (ITI). The ITI is between one, and two and a half minutes. After the final shock, there is a one-minute long exit stage before the animal is removed. The entire procedure lasts closer to 16 minutes.

**Altered context test (day 28)**: The animals explore the altered chamber for five minutes. For both day 28 and 29, contextual fear memory is quantified as the time spent freezing during the first five minutes after the door to the fear conditioning box is closed.

**Training context test (day 29)**: The rat explores the original training context for five minutes. This test, together with the altered context test, indicates the specificity of the contextual fear memory, i.e. generalized memory with freezing to both contexts, or specific freezing to the training context only.

**Light CS test (day 30)**: The rat is in the chamber for a little less than three minutes before the first CS. The CS is repeated six times, the ITI varies between one and four minutes. After the last CS, there is a one-minute long exit stage. The program lasts approximately 13 minutes.

The amount of time spent freezing during the six second CS is used as a measure of cued fear conditioning. Time spent freezing during the minute immediately preceding the first CS is used to define the baseline fear level during the CS test.

Following all fear conditioning sessions, the rats were placed in their home cage and returned to the vivarium. Waste trays, floors and walls were washed with water and dried between sessions.
2.3.3 Timing of the chABC-injection surgery

Experimental group 1 was injected with chABC in V2L seven days before day 28 of the protocol (altered context test) (figure 2.4). This group had intact PNNs in V2 during training, but the PNNs were absent during the memory retrieval tests.

![Diagram showing the timing of the chABC/aCSF injection surgery](image)

Figure 2.4: Experiment 1. Injection of chABC/aCSF before remote memory retrieval. For this experimental group the chABC/aCSF injection surgery took place six-seven days before day 28 of the protocol (altered context test).

Experimental group 2 was injected with chABC in V2L seven days before day 1 of the protocol (habituation) (Figure 2.5). Thus, this group lacked PNNs in V2L during fear conditioning training.

![Diagram showing the timing of the chABC/aCSF injection surgery](image)

Figure 2.5: Experiment 2. Injection of chABC/aCSF before conditioning. For this experimental group the chABC/aCSF injection surgery took place six to seven days before day one of the protocol (habituation).

Experimental group 3 was included to see if PNN removal from V2L had any effect on recent light-cued fear memories (figure 2.6). This modified protocol was identical to the remote fear conditioning protocol, but instead of performing the memory retrieval test one month after training, we now performed the memory tests one-to-four days after training. Like before, the chABC injection surgery was performed seven days before day one of the fear conditioning protocol. In all experiments, parallel control animals were injected with aCSF.
Figure 2.6: Experiment 3. Injection of chABC/aCSF before recent memory retrieval. In this experiment, the chABC/aCSF injection surgery took place six to seven days before day one of the protocol (habituation). The first of three consecutive tests was done 24h after training.

2.4 Histology and immunohistochemistry

2.4.1 Perfusion

Before sacrifice, animals were given an overdose of pentobarbital sodium (50mg/kg). When deeply anesthetized, and showing no response to pinching of the hind-legs, the animals were transcardially perfused with 0.9 % NaCl, followed by 4% paraformaldehyde in 0.01 M PBS. The brains were dissected out and placed in 4% paraformaldehyde. The tissue was left to post-fixate overnight in 4°C before being transferred to a cryoprotective 30% sucrose solution in 1X PBS for three days at 4°C. The tissue was then flash-frozen and cut into coronal sections (45 µm) using a cryostat (Ortomedic, Lysaker, Norway). Sections were transferred directly into a 0.01M PBS solution containing 0.02% sodium azide for later analysis.

2.4.2 Staining of the C-6-S antigen after chABC treatment for light microscopy

Staining was performed on free-floating sections. Sections were sampled from behind the posterior injection (AP 7.4), to in front of the anterior injection (AP 5.8). The sections were stained with a monoclonal anti-chondroitin-6-sulfate antibody (MAB 2035 Milipore). This antibody recognizes the six inner monosaccharides at the CS chain left on the core proteins after chABC cleavage, thereby confirming the activity of chABC (Brückner, et al., 1998).

Sections were blocked with 1.5% BSA (Sigma-Aldrich, USA), 0.3% Triton 100-X (Sigma-Aldrich, USA) in 1X PBS for one hour at room temperature, and incubated overnight in block solution with 1/1000 dilution of the primary antibody for chondroitinin-6-sulfate. The following day, the sections were incubated for two hours in block solution with 1/500 dilution of Anti-mouse-biotin-conjugated antibody (A-24522, Life). 1.5% H₂O₂ was then used to quench the endogenous peroxidase activity, before a one-hour incubation in ABC peroxidase
staining kit solution (Thermo Scientific, Rockford, IL, USA). Staining was visualized by adding 3,3’-Diaminobenzidine (DAB) solution (DAB pellets purchased from Sigma-Aldrich Chemie, Munich, Germany). By placing the sections in TNS (6g Trizma + 1L ddH₂O, to pH 7.4 with 1M NaOH) the DAB-reaction was stopped. Sections were mounted on Superfrost plus glass slides (Thermo Fisher Scientific, Oslo, Norway), and dried for several hours. The sections were dehydrated with 90 % and 100% ethanol, followed by xylene, cover slipped with Entellan (Merck Millipore, Darmstadt, Germany), and left to dry under fume hood overnight.

### 2.4.3 Dual fluorescent staining of PNNs and the C-6-S antigen

Several different staining methods can be used to visualize the PNN structure. The use of plant lecticans *Vicia villosa* agglutinin and *Wisteria floribunda* agglutinin (WFA) both label aggrecan-based PNNs selectively. These lectins have a high affinity for N-acetylgalactosamine (Brückner et al., 1993; Nakagawa, Schulte, & Spicer, 1986). Antibodies that bind chondroitin sulfate proteoglycans can also be used to visualize PNNs (Watanabe, et al., 1989). We used WFA to visualize PNNs as this is the most common method (Gogolla, et al., 2009; Hylin, et al., 2013; Pizzorusso, et al., 2002; Slaker, et al., 2015). Additionally, sections were labeled with monoclonal anti-chondroitin-6 sulfate antibody (MAB 2035 Milipore) to visualize the chABC treated area.

The sections were washed with agitation three times for five minutes in 1X PBS. Sections were incubated in a blocking solution (1.5% BSA, 0.3% Triton 100-X (Sigma), 1X PBS) for one hour at room temperature. Biotinylated WFA (L-1516, Sigma) (1:200 dilution) in blocking solution was then added and the sections incubated with agitation overnight at 4°C.

The sections were washed three times five minutes in 1X PBS, and then incubated overnight in a blocking solution (1.5% BSA, 0.3% Triton 100-X (Sigma) 1X PBS) with monoclonal anti-chondroitin-6-sulfate antibody (MAB 2035 Milipore) (1:1000 dilution).

The third day, the sections were washed three times five minutes in 1X PBS with agitation. After washing, sections incubated for two hours in Steptavidin Alexa488 and Anti mouse IgG Alexa594 (S11223 + A-21203, Life).

Following secondary antibody incubation, sections were rinsed three times for five minutes in 1X PBS with agitation and transferred to Superfrost plus glass slides and left to dry for
approximately one hour. Sections were washed in ddH$_2$O to remove excess salt (from PBS) and secured by a cover slip using ProLong Gold Antifade Reagent (Invitrogen, Madison, WI).

### 2.4.4 Fluorescent M2-receptor staining

In order to identify the approximate border between V1 and V2L we stained coronal sections with an antibody for the type 2 muscarinic acetylcholine receptor (M2R). It has been observed in mice that there is a sharp decline in M2R in layer 4 where V1 borders on V2L (Q. Wang, et al., 2011).

Sections were rinsed with agitation in 1X PBS three times five minutes and blocked in 1% BSA in 1X PBS before overnight primary antibody incubation.

Primary antibody solution is composed of 1:1000 Rabbit anti-M2R, 1% BSA and 0.02% azide in 1X PBS.

Sections were rinsed with agitation in 1X PBS three times five minutes before transferring to the secondary antibody solution (1:200 Alexa488 anti-rabbit, and 1% BSA in 1X PBS) and left to incubate for one hour.

Following secondary antibody incubation, sections were rinsed three times for five minutes in 1X PBS, transferred to Superfrost plus glass slides, and left to dry. Slides were washed briefly in ddH$_2$O to remove excess salt from PBS, and cover slipped (ProLong Gold Antifade Reagent, Invitrogen, Madison, WI). Slides were left to cure in a dark area overnight in room temperature before fluorescence imaging.

### 2.4.5 Dual fluorescent staining with WFA and Nissl

To map the extent of PNNs in V2L and find the distribution pattern between layers we labeled coronal sections with WFA as a marker for PNNs and fluorescent Nissl staining labeling cell bodies.

The sections were washed with agitation three times five minutes in 1X PBS. Sections were incubated in a blocking solution (1.5% BSA, 0.3% Triton 100-X (Sigma), 1X PBS) for one hour at room temperature. Biotinylated WFA (L-1516, Sigma) (1:200 dilution) in blocking solution was then added and the sections incubated with agitation overnight at 4°C.
Sections were rinsed with agitation in 1X PBS three times five minutes before transferring to the secondary antibody solution (Steptavidin Alexa488, S11223, Life) and left to incubate for one hour.

Following secondary antibody incubation, sections were rinsed three times five minutes in 1X PBS with agitation. Sections were then transferred to a 1:100 Neurotrace Fluorescent Nissl Stain dilution in 1X PBS and incubated for 30 minutes.

After incubation with Nissl staining, sections were transferred to a 0.1% Triton X-100 PBS solution and incubated for 10 minutes in RT.

After 2 times five minutes wash in 1X PBS sections were mounted on Superfrost plus glass slides, and left to dry for approximately one hour. Sections were washed in ddH$_2$O to remove excess salt (from PBS) and secured by a cover slip using FluorSave Reagent Calbiochem.

### 2.5 Data analysis

The brain sections were photographed using an Axiocam HRZ camera (Carl Zeiss, Oberkochen, Germany) through an Axioplan 2 microscope (Carl Zeiss, Oberkochen, Germany). For conversion of the M2-receptor staining into a color intensity map, we used the Jet LUT color processing function in ImageJ (red) (see figure 3.1d, blue= low, red = high). Prism 6 (Graphpad software) was used to conduct the statistical tests. All fear conditioning tests were analyzed using a two-way analysis of variance (ANOVA) with Bonferroni’s multiple comparisons test if a significant interaction effect was detected.
3 Results

In this project, a total of 23 male Long Evans (LE) and 99 male Sprague Dawley (SP) were used. Of these, 23 LE-rats and 16 SP rats were used to develop the visual fear conditioning protocol. Furthermore, two SP rats were used to analyze PNN distribution in the V2L.

3.1 Methodological assessment

Fear conditioning is a widely used learning paradigm for studying memory mechanisms. It has the benefit of minimal training to obtain long lasting memories, which makes it an ideal paradigm to experimentally study remote memories. In the current project, all rats showed increased freezing response to the CS as a result of fear conditioning training (see figures 3.6A, 3.9A and 3.11A), indicating that all animals learned to predict the foot shock when the light came on.

3.2 Fluorescent M2-receptor labeling indicates the V1/V2L border

The distribution of the type 2 muscarinic acetylcholine receptor (M2R) has been used in mice to aid in the anatomical identification of the V1/V2L border (Q. Wang, et al., 2011). When moving laterally from the V1 to the V2L there is a sharp decline in M2R immunolabeling that aligns well with the V1/V2L border. In our project, using rats, we found a similar decline in M2R expression at the lateral end of V1 (figure 3.1c). The M2R decline overlapped closely with the V1/V2L border as indicated in the rat brain atlas by Paxinos and Watson (George Paxinos, 2007). The intensity scale coloring of M2R (figure 3.1d, right bottom corner) illustrates how the amount of M2R is reduced in V2L compared to V1. Furthermore, staining the PNNs with WFA showed that there are less PNNs in V2L compared to V1 (figure 3.1b). Together, the immunohistochemistry indicates that V1 and V2 have a different composition of both PNNs and M2R.
Figure 3.1: Coronal section depicted in four ways; a) fluorescent labeling of nuclei with DAPI (blue), b) WFA staining used as a marker for perineuronal nets (green), c) labeling of the M2-receptor (red), d) the M2-receptor staining intensity (blue = low, red = high). The arrow points to where there is a sudden decline in M2-receptor staining intensity in the border between V1 and V2L.

Labeling of Chondroitin 6-sulfate (C-6-S) (red) was used to identify PNN degradation after chABC injection (figure 3.2). The C-6-S staining shows where the chABC enzyme cleaved the CSPGs. The antibody binds to the innermost sugar residues on the core proteins which can only be revealed by enzymatic cleavage. Together, the C-6-S and M2R staining were used to portray whether our chABC injection had correctly targeted V2L and not spread to V1. The M2R staining is not shown in the figure 3.2 but was used as the basis for the white lines superimposed on the image to specify the approximate location of V2L and V1. The staining illustrates that the chABC did not spread to the hippocampus, and was mainly contained within V2L, classifying it as a successful injection.
Figure 3.2: Coronal section with labeling of the Chondroitin 6-sulfate (C-6-S) antigen to visualize the chABC treated area (red) and counterstaining with DAPI (blue). The C-6.S antigen can only be revealed after enzymatic cleavage, and remains in the tissue for several months after chABC treatment. The lines indicating the placement of V1 and V2L were made based on M2-Receptor staining, which suddenly declines in the V1/V2L boarder (not shown in color).

3.3 Perineuronal net distribution in V2L

To examine the distribution of PNNs across the cortical cell layers of V2L we conducted a co-labeling with fluorescent Nissl staining (labeling cell bodies) to identify cortical layers together with WFA as a marker for PNNs. The staining showed that the PNNs in V2L are predominantly found in layer IV and V; there are a few in layers II and III, and even less in layer VI. Staining revealed a total absence of PNNs in layer one, as might be expected because of the very low cell number in this layer. The amount of PNNs in V2L is relatively low compared to other areas such as V1 (figure 3.1b). Other members of the Hafting-Fyhn lab, Charlotte Christensen and Kristian Lensjø, have conducted a quantitative analysis of PNN distribution and found that the number of PNNs in V2L is lower than in V1 (Christensen, Lensjø, Fyhn, unpublished observations).
Figure 3.3: Laminar PNN distribution in V2L. Nissl staining (labeling cell bodies) in red, WFA staining in green used as a marker for PNNs. A) Coronal section stained with WFA showing PNNs in the cortex. The red box indicates where V2L is on the section. B) A higher magnification for a closer view of the laminar PNN distribution V2L. The PNNs are predominantly located in layers IV and V; there are only a few nets in layers III and II, and even fewer in layer VI. No PNNs are detected in layer I.

3.4 The role of PNN and V2L in fear memory

In order to investigate the role of PNNs in fear conditioning we established a protocol for studying remote fear memories. The established a fear conditioning protocol is based on the protocol constructed by Sacco and Sacchetti (2010). To elucidate the role of PNNs in remote memory storage, we removed the PNNs in V2L by use of chABC at different time points in the fear conditioning protocol; specifically before training or before memory retrieval testing. The animals were give one week after surgery to fully recover before performing any behavioral training or testing. The chABC treatment causes the PNNs to degrade within three days before the PNN slowly regenerate over time (Brückner, et al., 1998). Lensjø (2013) described a more temporally accurate description of the reassembly of PNNs after chABC treatment in V1 and found that the nets are regenerated less than 10% after ten days and approximately 50% after one month.

3.4.1 Histological verification of PNN removal

All animals injected with chABC were sacrificed shortly after the light CS memory retrieval test and sections were stained with the C-6-S antibody to identify the chABC treated area. The
C-6-S antibody was visualized with 3,3’-Diaminobenzidine (DAB) solution. This method of staining ensures quick identification of the injected area without the need of a microscope. The C-6-S stained sections were examined by another researcher, blind to the behavioral performance of each animal, and the location of the degraded PNNs marked in a digital version of the Paxinos Rat Brain Atlas (-5, -5.6, -6.2, -7.0 and -7.8 compared to Bregma). The criteria used to classify a chABC injection as a successful bilateral hit, was that C-6-S staining had to be present in V2L in three out of five sections in both hemispheres (figure 3.4). Injections that failed to reach criteria were classified as missed injections. In some cases the C-6-S staining was also present outside V2L, these were also classified as successful hits if the above mentioned criteria were met. In the experiment where we removed PNN before training, eight rats were classified as having bilateral hits, while four rats were excluded from further analyses because they were classified as having missed injections. In the experiment where we removed PNN before testing, seven rats had bilateral hits, while five rats were excluded as having missed injections. Figure 3.6 shows the area with C-6-S labeling indicating the extension of chABC treatment superimposed on illustrations of brain sections (George Paxinos, 2007) from the animals operated before conditioning (3.4A), before testing (3.4B), and from those where we failed to bilaterally target the V2L (missed injections, 3.4C).
Figure 3.4: Labeling of the C-6-S antigen indicating the extension of chABC treatment superimposed on illustrations of brain sections (George Paxinos, 2007). For each individual rat, the gray color shows the area where chABC has been active. If there is an overlap between injections in different rats, this will darken the grey color. The red square indicates the location of V2L. A) Injection of chABC before training. B) Injection of chABC before testing. C) Missed injections.
3.4.2 Experiment 1: Removal of PNNs before testing

If PNNs in V2L are important for the remote storage of visual fear memories, one would expect that removing PNNs at remote time points would interrupt memory retrieval. To test this hypothesis, we used chABC to remove PNNs in V2L one week before remote memory testing (n= 12 aCSF, 12 chABC). The PNNs were largely degenerated at the time of testing (figure 3.7). We conducted three separate tests: altered context test, training context test and light CS test (figure 3.5). The altered context has a different smell, floor and sound than the training context, additionally the training context has a diagonal wall in the fear conditioning chamber that is removed in the altered context. The contextual fear memory normally goes from a specific to a generalized state with time. When it is generalized, both contexts will trigger the memory and initiate defense responses (e.g. freezing), because the animals cannot distinguish between them (Biedenkapp & Rudy, 2007; Wiltgen & Silva, 2007). We conducted the context tests in order evaluate if it the state of the contextual fear memory, in addition to the cued visual fear memory, was affected by PNN removal from V2L.

![Timeline for the procedures in experiment 1. The purpose of this experiment was to elucidate whether PNNs in V2 are important for remote storage of visual fear memories. The chABC injection took place one week before day 28 of the protocol (altered context test).](image)

The results from the context tests showed that the amounts of freezing behavior in both contexts were not different for chABC treated animals or the controls (see figure 3.6B). In detail, a matched two-way ANOVA revealed no significant main effect of chABC injection (F (2, 21) = 1.38, p = 0.27), no significant main effect context type (F (1, 21) = 1.84, p = 0.19), and no significant interaction (F (2, 21) = 0.77, p = 0.48)). Thus, injection of chABC in V2 before testing did not affect the strength- or specificity of contextual fear memory.

On the other hand, the light CS memory-retrieval test showed that the animals treated with chABC in V2L before testing had a significantly attenuated light cued fear memory (3.6C). In detail, a matched two-way ANOVA revealed a significant main effect of chABC injection (F
(2, 21) = 8.68, p = 0.002), a significant main effect of light CS (F (1, 21) = 163.2, p < 0.0001, and a significant interaction (F (2, 21) = 21.08, p < 0.0001). Tukey's multiple comparisons test further revealed that rats with bilateral chABC hits froze significantly less to the CS compared both to the saline group (p < 0.0001) and compared to the missed injection group (p < 0.0001).

To confirm that the chABC animals with reduced visual fear memory retrieval had normal memory acquisition prior to chABC injection, we constructed a training curve examining freezing levels during the original fear conditioning training session. Figure 3.6A shows that at this point, there was no significant difference in freezing between the controls and the animals later injected with chABC. In detail, a matched two-way ANOVA revealed a significant main effect of time (F (7, 147) = 129.5, p < 0.0001, no significant main effect of chABC injection (F (2, 21) = 0.81, p = 0.46) and no significant interaction (F (14, 147) = 0.53, p = 0.91). This indicates that the results from the “bilateral chABC” group in the light CS memory retrieval test was not caused by an inability to acquire the visually cued fear memory.

Histology results showed that five of the animals had missed injections, causing chABC not to be effective at all, or only in one hemisphere, these animals had behavioral results similar to the controls. This finding suggests that a unilateral/partial chABC injection has no effect, while a complete bilateral chABC treatment causes an attenuation of the remote visual memory.
Figure 3.6: The involvement of PNNs in V2L for the storage and retrieval of remote visual fear memory. A) Training curve for freezing levels to subsequent CS presentations during fear conditioning training. Freezing levels were similar in chABC- and control groups. B) Results from the altered context- and the training context test on day 28 and 29, respectively. The results indicate that there is no difference in the strength- or specificity of contextual fear memory between the groups. C) The results from the light CS memory retrieval test at day 30. Animals in the “bilateral chABC” group have a significantly lower freezing level to Light CS compared to both the controls (Saline) and the animals with a missed injection. Baseline freezing is similar in all three groups. n=7 bilateral chABC, n = 12 saline, n = 5 missed injection. Each dot in the figure represents one animal. Data analyzed using a two-way ANOVA with Tukey’s post-hoc test if a significant interaction effect was detected.
3.4.3 Experiment 2: Removal of PNNs before training

Given that our results in experiment 1 showed that intact PNN in V2L is critical for remote memory retrieval, we next asked whether PNN in V2L are also necessary for memory acquisition and the early consolidation phase. Importantly, PNNs gradually regenerate after chABC treatment and we therefore hypothesized that we could remove PNNs before fear conditioning training and that the regenerated PNNs might be sufficient to support memory retrieval 38 days later. We first confirmed that PNNs regenerate as expected by examining coronal sections from animals sacrificed either 38 or 10 days after chABC injection and labeled with WFA to visualize the PNNs. We found that PNNs were still largely disrupted 10 days after chABC injection, while 38 days after chABC injection, most of the PNNs had regenerated (figure 3.7) similar to Lensjø (2013). Notably, even if the PNNs gradually regenerate after chABC treatment, the C-6-S antigen remains in the brain for more than five months after chABC treatment as a marker for previous PNN removal (Brückner et al 1998).
Figure 3.7: PNN regeneration after chABC injection in V2L. Sections taken from animals sacrificed 10 and 38 days after chABC injection. WFA was used as a marker for PNNs are labeled in green, C-6-S antigen in red. The PNNs are still absent after 10 days after injection but are mostly regenerated after 38 days. The C-6-S antigen remains in the tissue as a marker of previous PNN removal long after chABC treatment.
In order to test whether PNNs in V2L are important for fear memory acquisition and early consolidation, we injected chABC one week before fear conditioning training (n= 12 aCSF, 12 chABC). One month after training, when the PNNs were expected to have regenerated, we conducted three separate tests: altered context test, training context test and light CS test (figure 3.8).

Similarly to what we found in experiment 1, chABC injection before training did not significantly affect remote contextual fear memory (3.9B). In detail, a matched two-way ANOVA revealed no significant main effect of chABC injection (F (2, 21) = 0.31, p = 0.74), a significant main effect context type (F (1, 21) = 9.55, p = 0.006), and no significant interaction (F (2, 21) = 1.73, p = 0.20). Thus, injection of chABC in V2 before training had no significant effect on the strength- or specificity o contextual fear memory.

Moreover, chABC injection before training did not significantly influence light CS memory-retrieval (3.9C). A matched two-way ANOVA revealed no significant main effect of chABC injection (F (2, 21) = 0.028, p = 0.97), a significant main effect of light CS (F (1, 21) = 38.2, p < 0.0001), and no significant interaction (F (2, 21) = 0.29, p = 0.75). Overall, these findings suggest that intact PNNs in V2L are not necessary for fear memory formation and that PNNs are sufficiently regenerated in V2L after chABC treatment to be able to support remote visual fear memory storage and/or retrieval. Alternatively, compensatory mechanisms might allow remote memory storage and/or retrieval in the case where PNNs are lacking during memory acquisition and the early parts of systems consolidation.

We also constructed a training curve examining freezing levels during the fear conditioning training session to confirm further that the chABC injection did not influence fear memory acquisition. Figure 3.9A shows that there was no significant difference in freezing between the controls and the animals when chABC was injected before training. In detail, a matched
two-way ANOVA revealed a significant main effect of time $F(7, 147) = 60.79$, $p < 0.0001$), no significant main effect of chABC injection ($F(1, 21) = 0.82$, $p = 0.37$), and no significant interaction $F(7, 147) = 1.09$, $p = 0.37$). In summary, this finding supports the idea that PNN in V2L is not necessary for visual fear memory acquisition. Our results in experiment 2 indicate that a visually cued fear memory is acquired normally in animals that lack PNNs in V2L during fear conditioning training.
Figure 3.9: The involvement of PNNs in V2L for the acquisition and early consolidation of remote visual fear memory. A) Training curve showing freezing levels to subsequent CS presentations during fear conditioning training. Freezing levels were similar in chABC- and control groups. B) Results from the altered context- and the training context-tests on day 28 and 29, respectively. The results indicate that there is no difference in the strength- or specificity of contextual fear memory between the groups. C) Results from the light CS memory retrieval test on day 30. The results indicate that there are no differences between the groups in the state of the light CS memory. Baseline freezing, and freezing to the light CS is similar in all three groups. n=8 bilateral chABC, 12 saline, 4 missed injection. Each dot in the figure represents one animal. Data analyzed using a two-way ANOVA. No significant results revealed.
3.4.4 Experiment 3: Removal of PNNs before recent memory test

In order to reveal if the PNNs in V2L are critical only for retrieval of visual remote fear memories, we asked whether PNNs in V2L are similarly important for the retrieval of a recent visual fear memory.

In order to test the role of PNNs in V2L for recent fear memory, we used chABC to degrade the PNNs before training and conducted the memory retrieval tests in the days immediately following the training session (see figure 3.10). The injection surgery took place seven days before training (n= 8 aCSF, n=7 chABC). Due to time constraints, I did not complete the histology analysis for this experimental group. I therefore cannot verify that the effectiveness of the chABC treatment and the results are only based on the assumption that all chABC injections were successful.

Figure 3.10: Timeline for the experimental procedure. The purpose of the experiment was to evaluate if recent visual fear memories can be created and retrieval without PNNs in V2L. The chABC injection took place one week before day 1 of the protocol (habituation). The memory retrieval tests were conducted in the days immediate following training.

All animals showed a normal response to the training procedure as shown in figure 3.7A, with no significant difference between the chABC and the control animals. This supports the finding in experiment 2 that PNNs in V2L are not required for visual fear memory formation.

As expected for a recent retrieval test (Biedenkapp & Rudy, 2007; Wiltgen & Silva, 2007), the rats in both treatment groups froze significantly more to the training context than to the altered context (figure 3.11B). In detail, a matched two-way ANOVA revealed no significant main effect of chABC injection (F (1, 13) = 0.0003, p = 0.99), a significant main effect context type (F (1, 13) = 33.44, p < 0.0001), and no significant interaction (F (1, 13) = 0.004, p = 0.95). In other words, at this early time (1-2 days after training), the rats have a more specific contextual memory and freeze only to the original training context. This contrasts with the contextual tests we performed at remote time points in experiment 1 (27 and 28 days after training, figure 3.6B), where there was no significant difference in freezing to the two
context types. In experiment 2, also at a remote time point (27 and 28 days after training, fig 3.9C), we did actually observe a significant main effect of context type (p = 0.006), but the difference in freezing levels was small with 19% freezing in the training context and 12% freezing in altered context. In the recent memory test, the freezing difference between the contexts was larger with 26% freezing in the training context and 4% freezing in the altered context. This time dependent loss of contextual memory specificity is well known (Biedenkapp & Rudy, 2007) and is probably related to the decreasing hippocampal involvement in memory processing over time (Anagnostaras, et al., 1999; Wiltgen & Silva, 2007; Winocur, et al., 2009).

Interestingly, unlike what we had seen at the remote time points, the rats were fully able to retrieve a more recent light cued fear memory in the absence of PNNs in V2L (assuming that chABC were in fact successful, figure 3.11C). A matched two-way ANOVA revealed no significant main effect of chABC injection (F (1, 13) = 55.70, p = 0.78), a significant main effect of light CS (F (1, 13) = 55.70, p < 0.0001), and no significant interaction (F (1, 13) = 0.01, p = 0.92). These findings suggest that while intact PNNs in V2L are necessary for the retrieval of visual fear memories at remote time points (29 days after training, figure 3.6C), rats do not require PNNs in V2L for the retrieval of recent memory (3 days after training, figure 3.11C).
Figure 3.11: The involvement of PNNs in V2L in recent visual fear memory formation and retrieval. A) Training curve showing freezing levels to subsequent CS presentations during fear conditioning training. Freezing levels were similar in chABC- and control groups. B) Results from the altered context- and the training context-tests on day 3 and 4, respectively. There is a significant difference between the levels of freezing on the two contexts, indicating that the animals have a specific contextual fear memory. C) The freezing levels during the light CS test. There is no difference between the chABC treated animals (n=7) and the controls (saline; n=8). Each dot in the figure represents one animal. Data analyzed using a two-way ANOVA.
4 Discussion

This study demonstrates that PNNs in V2L are critical for the retrieval of remote visual fear memories. We found that chABC injection in V2L caused an attenuation of a remote, one month old, visual fear memory when the injection took place seven days before remote memory retrieval. Removal of PNNs with injection of chABC conducted seven days before memory acquisition had no significant effect on either remote- or recent memory retrieval. Our results indicate that PNNs in V2L play an increasingly important role as the fear memory ages. During memory acquisition and in the first days after learning, the PNNs in V2L were not necessary. When we removed the PNNs in V2L at a later time point, when systems consolidation is believed to have occurred, we did however abolish the fear memory. How can chABC treatment influence remote memory? One theory suggests that remote memories are stored in the pattern of holes in PNNs (R. Y. Tsien, 2013). If this theory is correct, then perhaps breaking down the PNNs destabilizes this pattern and therefore disrupting the stored memory.

4.1 Methodical considerations

We used fear conditioning as a learning paradigm to create a remote memory. Fear conditioning is a standard method used to study memory processing. Our results are therefore comparable to findings from a large amount of other experiments. When using rodents in fear conditioning, it is common to present an auditory tone, rather than a light as the CS because it requires less CS-US pairings to create a robust memory. As a result, there is a much more extensive body of literature on mechanisms behind auditory-, compared to visually-cued fear memory processing. Nevertheless, studies in rodents using visual CS, have indicated that the general neuronal circuitry for visual- and auditory fear conditioning is similar, i.e. a subcortical CS pathway that converges with US information in the amygdala (Bergstrom & Johnson, 2014; M. Davis, 1986; Sananes & Davis, 1992; Tischler & Davis, 1983). Furthermore, secondary visual cortex has been shown to be essential for remote memory processing (Sacco & Sacchetti, 2010).

The borders of V2L can be challenging to identify and its lateral placement in the brain makes it a relatively difficult target for drug infusion. Ten of the chABC-injected animals were taken out of the treatment groups in the analysis because of a missed injection. The chABC
injection was done by using a thin glass pipette. This procedure ensures minimal tissue damage.

The chABC enzymatic treatment will cause all extracellular matrix structures with chondroitin sulfate proteoglycans (CSPGs) to be broken down, not only PNNs questioning if the effects we see are accounted for by the PNNs. However, using a link protein (CrtI1) knockout mice with genetically disrupted PNNs followed by parallel experiments using chABC in the same mouse line revealed similar results (Gogolla, et al., 2009; Romberg, et al., 2013). This indicates that the effect of chABC treatment in the current study is likely related to the removal of PNNs.

Initially we used fluorescent staining of chondroitin-6-sulfate (C-6-S) antibody to identify the brain regions where the chABC had been active. This was later replaced by 3,3’-Diaminobenzidine (DAB) solution as it can be seen without the use of a microscope. The DAB method proved to be an easier and faster way to detect the treated area. When the area was identified, we could compare the extent of the treatment to the behavioral results.

Due to the ten chABC injections that missed the V2L, we unfortunately ended up with unequal group sizes which can complicate statistical analysis. With unequal group sizes, it may be beneficial to use a one-way ANOVA with Welch's correction (McDonald, 2014). Importantly, when we re-analyzed our data with Welch's correction, we got the same results that we found using the matched two-way ANOVA.

4.2 The role of PNNs in remote memory

4.2.1 Lower levels of PNNs in V2L may reflect a higher level of plasticity

From the histological examinations we found a lower level of PNN in the V2L compared with the adjacent primary visual cortex (figure 3.3). Because PNNs are likely to act as a break on plasticity (Dick, et al., 2013; D. Wang & Fawcett, 2012), the relatively lower amount of PNNs in V2L might reflect a need for a more plastic neuronal circuitry to allow plasticity and information transfer from subcortical regions like the amygdala to V2L over time. At the same time, the PNN present in layer IV and V in V2L could stabilize the V2L circuit after information transfer, and allow long-term storage of memories. Overall, PNNs in V2L might
therefore be low enough to allow plasticity to occur, but sufficient to stabilize new synaptic connections and memory storage.

4.2.2 Experiment 1: Removal of PNNs before testing

In experiment 1, we detected that removal of PNNs with chABC in V2L seven days before remote memory testing attenuated the remote visually-cued fear memory. This experiment revealed that PNNs in V2L are necessary for remote memory retrieval, but given that PNNs were abolished in the V2L for an extended period, i.e. from day 21 to memory testing at days 28-30, it is not clear exactly what in the remote memory processing that was affected. Thus, the PNN removal might have influenced one or several stages of remote memory processing: 1) systems consolidation, 2) memory storage and/or 3) memory retrieval.

It is possible that the chABC treatment inhibited systems consolidation and the construction of a fear memory trace in V2L. This seems unlikely since systems consolidation would be expected to already have occurred at the time of chABC injection (21 days after memory acquisition) (H. Wang, et al., 2006). Alternatively, chABC treatment influenced memory storage by disrupting an established remote memory trace in V2L. In both cases, the chABC injections would have inhibited expression of the memory. As a third option, PNN removal might instead directly prevent the retrieval of the memory. In this case, chABC injection would cause a retrieval failure, where the memory trace in V2L is intact, but the information cannot be accessed due to the PNN removal. Examination these possibilities would require temporal controlled PNN manipulations, for example by more acute disruption of PNN structures, e.g. by pharmacological or genetic tools. A combination of chABC injection and physiological recordings in V2L during remote memory retrieval could also clarify how PNN influences memory processing.

The histology results from the chABC treatment showed that some of the injections were only partially successful. When correlating the behavioral results from the animals with a missed injection, we found that these animals had normal visually-cued fear memory. This result indicates that only complete removal of the PNNs in V2L causes attenuation of the memory. These findings may indicate that the fear memory trace is distributed across the large V2L area, rather than being confined to a restricted location. After PNN removal, induced plasticity are observed in adult animals (Pizzorusso, et al., 2002, 2006) indicating an opening for synaptic plasticity and thus a more unstable network. However, if enough neurons in the
network are surrounded by PNNs, perhaps they have the ability to compensate for the partial increased plasticity/destabilization of the network, and support memory storage.

We did not observe any impact on the remote contextual fear memory, indicating that the PNNs in V2L do not need to be intact for retrieving remote contextual fear memories. Recent findings on the mechanisms of contextual fear memories point to the importance of the anterior cingulate cortex in addition to the hippocampus in remote memory processing (Einarsson & Nader, 2012; Einarsson, et al., 2014; Frankland, et al., 2004; Goshen, et al., 2011).

Our results are consistent with the discovery of V2L’s involvement in remote visual emotional memories (Sacco & Sacchetti, 2010). In addition, our findings contribute to the increasing body of evidence indicating that PNNs are involved in memory processing (Gogolla, et al., 2009; Happel, et al., 2014; Hylin, et al., 2013; Romberg, et al., 2013; Slaker, et al., 2015). However, our study is the first to reveal a role for the PNNs in remote memories. How the PNNs influence memory remains elusive, but suggested hypotheses for the molecular mechanisms will be touched upon in section 4.4.

**4.2.3 Experiment 2: Removal of PNNs before training**

Injection of chABC one week prior to fear conditioning in experiment 2 appeared to have no influence on the remote visual fear memory or contextual fear memory. This indicates that PNNs in V2L are not necessary for the formation storage of fear memories during and shortly after learning. We observed that after chABC injection, the PNNs were almost completely regenerated after 38 days. Thus they were present at near normal levels during the remote memory test. We were surprised that the PNNs that are regenerated in V2L after chABC treatment may be sufficient to support remote visual fear memory storage and/or retrieval. However, we cannot exclude that compensatory mechanisms might support remote memory processing in the case where PNNs are lacking in the V2L during the early parts of systems consolidation and memory acquisition.
4.2.4 Experiment 3: Removal of PNNs before recent memory test

In experiment 3, we found that the PNNs in V2L are not important for the retrieval of a recent visual fear memory. These findings, combined with our results in experiment 1, allow us to propose that PNNs in V2L play an increasing role in the retrieval of memories as time passes and the memory ages. In contrast, other studies have observed an attenuation of recent memories when the PNNs are removed before training. Hylin and co-workers (2013) found that recent contextual and trace fear memories were attenuated when PNNs were removed from the hippocampus and the mPFC, respectively, before fear conditioning (Hylin, et al., 2013). Furthermore, Slaker and co-workers (2015) showed that when injecting chABC in the mPFC before cocaine induced conditioning place preference (CPP) training, the recent memory was attenuated. They concluded that the PNN surrounded neurons in mPFC are involved in the acquisition and/or consolidation of CPP memories (Slaker, et al., 2015). Rather than being in conflict with the previously reported role of PNNs in recent memory, our findings probably reflect a time dependent role for V2L in memory storage, which in turn translates into a time dependent role of PNNs in V2L (Sacco & Sacchetti, 2010).

4.3 Neuronal circuit reorganization in fear memory storage over time

Extensive research has been conducted on systems consolidation of declarative, hippocampus-dependent memories. It has been observed that declarative memories’ dependence on the hippocampus decreases with time, as they become increasingly supported by cortical structures (Anagnostaras, et al., 1999; Rempel-Clower, et al., 1996; Scoville & Milner, 1957) although see (Goshen, et al., 2011; Sutherland, et al., 2008). So far, little research has been done to investigate if the same is true for other types of memories but a recent study indicates that it may be the case for cued fear memories (Sacco & Sacchetti, 2010). The results from our experiments are in agreement with this recent discovery of how secondary sensory cortices are involved in remote- and not recent cued fear memory processing (Sacco & Sacchetti, 2010). How this time-dependent reorganization occurs is not clarified, but there are three hypothesis describing different scenarios for the temporal involvement of cortical structures (see figure 4.1) (Grosso, et al., 2015). The first hypothesis (4.1A) states that the sensory cortex is not involved in the initial processing of the memory at all, but engages with time during late consolidation and storage of the memory. However, several opposing physiological studies have observed early involvement of sensory cortices
after learning (Chavez, et al., 2013; G J Quirk, et al., 1997; Weinberger, 2004). The second hypothesis (4.1B) states that during learning, the sensory cortex is indeed active, but that it only encodes information designated for remote memory storage. In other words, the cortical structures are “tagged” during memory acquisition, and initiate remote memory storage (Lesburguères, et al., 2011), while other subcortical regions are responsible for storage of recent memories. The third scenario (4.1C) states that the sensory cortex is important for both recent and remote memory processing. However, studies have found that fear memory is not impaired when the sensory cortex is damaged shortly after learning (Campeau & Davis, 1995; LeDoux, et al., 1990; Rosen, et al., 1992; Sacco & Sacchetti, 2010). Researchers explain this by suggesting that other brain areas in addition to the sensory cortex are involved in memory encoding in the early stages after acquisition, and that several compensatory strategies can be used for recent memory retrieval. These alternative strategies diminish with time and the remote memory will therefore be lost if the sensory cortex is damaged (Grosso, et al., 2015).

Our experimental findings are relevant for the three alternative scenarios of cortical memory storage over time (Figure 4.1). Our observation that remote memory retrieval was attenuated by PNN manipulations in V2L, while neither memory formation nor recent retrieval were affected, suggests that there is indeed a time-dependent change in memory processing in V2L, supporting hypothesis A. However, we cannot fully exclude the other two scenarios, as both these could also be compatible with our data if the real change in memory processing over time is molecular rather than anatomical. For example, both scenario B and C are compatible with our data if one assumes that that recent memory storage/tagging in V2L occurs via intracellular mechanisms that are unaffected by chABC.
Figure 4.1: Three hypotheses of cortical engagement in memory processing. Hypothesis A states that the sensory cortex only is involved in the late consolidation and storage phase while other structures support the acquisition and early consolidation. Hypothesis B states that during acquisition and early consolidation, the sensory cortex is active, but that it only encodes information designated for remote memory storage and that other subcortical regions are responsible for storage of recent memories. Hypothesis C states that the sensory cortex is important for both recent and remote memory processing. Figure from Grosso et al. (2015).

4.4 Molecular reorganization in fear memory storage over time

Regardless of the extensive research on neuronal plasticity over the last decades, the molecular mechanism of long-term memory storage is still unknown. The early theorists advocated that memory storage was reliant on a stable molecule; much like gene expression relies on DNA (Crick, 1984). Later, the notion changed and researchers proposed that memory storage is reliant on learning-activated cellular proteins, such as PKMζ, CPEB, and CaMKII, with the ability convey their state on to the newer copies to preserve the
information. It was recently proposed that permanent alterations in the highly stable PNN structure could support remote memory storage (R. Y. Tsien, 2013). In his paper, Tsien proposes a theory stating that “very long term memories are stored in the pattern of holes in PNNs” (R. Y. Tsien, 2013). In light this, one may suggest that by removing the PNNs in our experiment, we possibly destroyed the “pattern of holes” and consequently the memory.

It is important to mention that the “pattern of holes” hypothesis has not been tested and that PNNs could in theory influence memory by other mechanisms. The PNNs may impact a memory trace more indirectly, for example by affecting the function of PV+-interneurons (Chevaleyre & Piskorowski, 2014; Del Río & DeFelipe, 1994; Donato, et al., 2013). Donato and co-workers (2013) reported that inducing plasticity with environmental enrichment causes a decrease in PV+-interneuron activity, while an increase in the activity of the PV+-interneuron was observed after fear conditioning, perhaps correlated with fear memory consolidation. The chABC injection removes the restriction on plasticity by removing the PNNs but it might additionally affect PV-expression. Yamada and co-workers (2015) found a decline in the level of PV fluorescence seven days after chABC injection in the hippocampus (Yamada, 2015). In our experiment, a possible reduction of PV, caused by chABC treatment, may have created a shift from high PV+-interneuron activity, created by fear memory consolidation, to low PV+-interneuron activity. This neuronal activity shift could have disrupted the established memory trace in V2L.

It is well known that intracellular molecules that make up the synaptic machinery are essential for creating a memory trace but due to their relatively short half-lives, these molecules need to be continuously transcribed, produced and transported to the correct location to sustain the memory. The effect of PNN removal may be due to their role in stabilizing the synaptic structure (and function), and ensure that its structural integrity stays the same. If the PNNs are broken down, the stabilizing factor is removed, distorting the consolidated state of the synapses, ultimately disrupting the memory.

4.5 Future perspectives

To specifically reveal if V2L is involved in the visual fear memory, we are about to perform a c-Fos expression analysis. The c-fos gene is one of several so-called “immediate early genes”, which are transcribed after a neuron has been depolarized as a part of its initial response (Hoffman, et al., 1993). Several studies have observed a marked increased expression of c-
Fos after fear conditioning, particularly in the amygdala (Radulovic, et al., 1998). If the animal is perfused 90 minutes after a test, a high amount of c-Fos protein will be found in the activated neurons. Staining the c-Fos protein with a fluorescent marker makes it possible to visualize which brain areas where activated. By doing this after a recent- and a remote light-CS memory test, we will reveal if neurons in V2L are specifically activated during memory retrieval. We also want to find the activity pattern of neurons wrapped in PNNs by using WFA to label PNNs together with c-Fos labeling. After finding the normal activity pattern, we wish to do the same c-Fos/PNN analysis, but this time when the PNNs have been removed before testing. The results will tell us if chABC treatment causes a change in the neuronal activity pattern. To investigate more directly the neuronal activity in V2L, we are also contemplating conducting in vivo electrophysiological recordings in awake behaving animals during visual fear memory retrieval. Furthermore, silencing of V2L at different time points of memory processing will identify the role of V2L in visual fear memory processing. In order to specifically target the PNNs and not alter extracellular matrix molecules, a genetic disruption of specific components of the PNNs would be essential to settle the role of PNNs for memory processing or brain plasticity in general.

4.6 Conclusions

In this study, we focused on the impact of PNNs on remote memory processing in V2L. We observed that chABC injection in V2L caused an attenuation of a remote visual fear memory when the injection took place seven days before remote memory testing. In contrast, we observed that recent visual fear memory retrieval was not affected by removal of PNNs from V2L. Importantly, because we observed no impact on the memory when the chABC injection took place before fear conditioning, our results indicate that PNNs in V2L are critical for remote fear memory retrieval, but not for memory acquisition or early consolidation. This study is the first to investigate the role of PNNs in remote memory processing.

The enzymatic treatment with chABC causes all extracellular matrix structures with CSPGs to be digested, not only the PNNs. It is therefore important to note that our results might not be specifically caused by removing PNNs, but by removing extracellular matrix in general, thereby suggesting that the ECM has a functional role in remote memory processing.

In addition to observing a possible role of PNNs in remote memory encoding, we found evidence of V2L’s involvement in remote visual fear memory processing. Our results indicate
that cued fear memories, like declarative memories, go through a time-dependent reorganization in the neuronal circuitry involved in memory encoding in accordance with what have been observed in other studies from secondary auditory cortex, prelimbic prefrontal cortex, various regions of the thalamus and amygdala (Do-Monte, et al., 2015; Kwon, et al., 2012; Sacco & Sacchetti, 2010).
5 References


## 6 Appendix

### 6.1 List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACC</td>
<td>anterior cingulate cortex</td>
</tr>
<tr>
<td>aCSF</td>
<td>artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>AMPA</td>
<td>$\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>C-6-S</td>
<td>chondroitin-6-sulfate</td>
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<tr>
<td>CaMKII</td>
<td>calmodium-dependent protein kinase II</td>
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<tr>
<td>chABC</td>
<td>Chondroitinase ABC</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>CPP</td>
<td>conditioning place preference</td>
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<tr>
<td>CR</td>
<td>conditional response</td>
</tr>
<tr>
<td>CREB</td>
<td>cytoplasmic polyadenylation element binding protein</td>
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<td>CS</td>
<td>conditional stimulus</td>
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<tr>
<td>CSPG</td>
<td>chondroitin sulfate proteoglycan</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
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<tr>
<td>GAG</td>
<td>glycosaminoglycan</td>
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<tr>
<td>HPC</td>
<td>hippocampus</td>
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<tr>
<td>ICM</td>
<td>intercalated cell mass</td>
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<tr>
<td>IEG</td>
<td>immediate early genes</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>ITI</td>
<td>inter-trail interval</td>
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<tr>
<td>LA</td>
<td>lateral amygdala</td>
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<tr>
<td>LTP</td>
<td>long-term potentiation</td>
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<tr>
<td>M2R</td>
<td>type 2 muscarinic acetylcholine receptor</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>mPFC</td>
<td>medial prefrontal cortex</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>PAG</td>
<td>periaqueductal gray</td>
</tr>
<tr>
<td>PKMζ</td>
<td>protein kinase M zeta</td>
</tr>
<tr>
<td>PNN</td>
<td>perineuronal net</td>
</tr>
<tr>
<td>PV</td>
<td>parvalbumin</td>
</tr>
<tr>
<td>SP</td>
<td>Sprague Dawley</td>
</tr>
<tr>
<td>US</td>
<td>unconditional stimulus</td>
</tr>
<tr>
<td>V1</td>
<td>primary visual cortex</td>
</tr>
<tr>
<td>V2L</td>
<td>lateral secondary visual cortex</td>
</tr>
<tr>
<td>WFA</td>
<td><em>Wisteria floribunda</em> agglutinin</td>
</tr>
</tbody>
</table>
6.2 Solutions used for immunohistochemistry and histochemistry

6.2.1 10X PBS

80g of NaCl

2.0g of KCl

14.4g of Na$_2$HPO$_4$

2.4g of KH$_2$PO$_4$

Dissolve in 800 ml dH$_2$O, adjust pH to 7.4, and adjust volume to 1L

Dilute 1:10 with dH$_2$O for 1X solution

6.2.2 T-PBS

0.3% Triton X-100 in 1 X PBS

6.2.3 TNS

6g Trizma-HCl

1L dH$_2$O, adjust pH to 7.4 with 1M NaOH
6.2.4  **Tris-HCL 0.05M**

6.06 g

1 L H₂O, adjust pH to 7.6

6.2.5  **4% Paraformaldehyde (PFA)**

40 g PFA

1 L 1X PBS

Heat to 50-60°C, leave with stirring until everything is dissolved (3-4 hours)

Filter before use
6.3 Immunohistochemistry and histochemistry protocols

6.3.1 Staining of the C-6-S antigen after chABC treatment for light microscopy

Primary antibody: Anti C-6-S clone MK302 (MAB 2035 Milipore)


1. Rinse sections 3x5 min in 1X PBS
2. Block 1 hour (1.5% BSA, 0.3% Triton, 1X PBS)
3. Anti C-6-S (1/1000) O/N at 4˚C in block solution
4. Rinse sections 3x5 min in 1X PBS
5. Prepare the DAB pellet: 15mL Tris-HCl to 1 pellet (10mg), in 50ml tube. Light shield and keep on roller/shaker for approx. 2 hours. Remains from this solution can be stored at 4°C and activated later by adding H2O2
6. Prepare ABC staining solution: 90µL Reagent A, 10 mL PBS, 90µL Reagent B. Incubate at RT for 30 min.
7. Rinse sections with T-PBS, 3x5 min at RT. (T-PBS: 0.3% triton X-100 in 1xPBS)
8. Quenching endogenous peroxidase: 2%H2O2 in ddH2O. Incubate for 5 minutes, rinse with T-PBS for 2x5 minutes
9. Add ABC solution to sections, incubate min 1 h at RT.
10. Rinse sections with TNS, 3x5 min (minimum) (TNS: 6g Trizma +1L ddH2O, to pH 7.4 with 1M NaOH).
11. Add 12µL H2O2 to DAB solution, filter it and add the DAB/H2O2 solution to the sections under fume hood. Incubate until desired color is observed (5-15 min)
12. Rinse minimum 3 times with TNS. Mount sections on slides using a fine brush and TNS/PBS. Leave to dry
13. Rinse in ddH2O
14. When sections are completely dry, dehydrate with 95% EtOH, then 100% etOH and finally xylene. Mount sections with DRX/entellan.
6.3.2 Dual fluorescent staining of PNNs and the C-6-S antigen

Primary antibodies: Biotinylated WFA (L-1516, Sigma) and Anti C-6-S clone MK302 (MAB 2035 Milipore).

Secondary antibodies: Streptavidin Alexa488 + Anti mouse IgG Alexa594 (S11223 + A-21203, Life).

1. Rinse sections 3x5 min 1X PBS
2. Block 1 hour (1.5% BSA, 0.3% Triton, 1X PBS)
3. Biotin-conjugated WFA (1/200) O/N at 4°C in block solution
4. Rinse 3x5 min 1X PBS
5. Anti C-6-S (1/1000) O/N at 4°C in block solution
6. Rinse 3x5 min 1X PBS
7. Streptavidin Alexa488 (1/1000) and anti-mouse IgG Alexa 594 (1/1000) in 1X PBS at RT 2h
8. Rinse 3x5 min 1X PBS
9. Mount, dry, rinse dH2O, dry, coverslip+FluorSave
6.3.3 Fluorescent M2-receptor staining

Primary antibody: Anti M2R (AMR-002 Alomone)

Secondary antibody: Anti rabbit Alexa 488 (A27034, Life)

1. Rinse sections 3x5 min 1X PBS
2. Block 1 hour (1,5% BSA, 0,3% Triton, 1X PBS)
3. Anti M2R (1:1000) over night at RT in block solution
4. Rinse 3x5 min 1X PBS
5. Anti rabbit Alexa 488 (1/1000) in 1X PBS at RT 2h
6. Rinse 3x5 min 1X PBS
7. Mount, dry, rinse dH2O, dry, coverslip+FluorSave
6.3.4 Dual fluorescent staining with WFA and Nissl

Primary antibody: Biotinylated WFA (L-1516, Sigma).

Secondary antibody: Streptavidin Alexa488 (S11223, Life)

1. Rinse sections 3x5 min 1X PBS
2. Block 1 hour (1,5% BSA, 0,3% Triton, 1X PBS)
3. Biotin-conjugated WFA (1/200) O/N at 4°C in block solution
4. Rinse 3x5 min 1X PBS
5. Streptavidin Alexa488 (1/1000)
6. Incubate in 1:100 Neurotrace Fluorescent Nissl Stain diluted in PBS for 30min at RT.
7. Incubate in 0.1% Triton X-100 PBS for 10min RT.
8. Wash 2x5 min in 1X PBS
9. Mount, dry, rinse dH2O, dry, coverslip+FluorSave