Noradrenergic effects on memory accuracy over time

Abstract

Over time memories are believed to undergo a time-dependent reorganization in the brain through a process known as systems consolidation during which they become less dependent on the hippocampus and more dependent on the neocortical structures. This reorganization is accompanied by a generalization of the memory, meaning that it becomes less detailed and less accurate. However, emotionally arousing or highly stressful experiences seem to escape this pattern and be remembered stronger and longer even at remote time points. It has been shown that noradrenaline can alter the process of systems consolidation and maintain memories accurate and episodic-like. Nevertheless, it remains unclear how it can alter this long-term trajectory of memory. Thus, in this research project, we aimed to investigate the effects of yohimbine, a noradrenergic stimulant, on the accuracy of memory at the level of the engram neurons within the hippocampus. For this, we used FosxTRAP2xROSA transgenic mice to permanently label neurons activated over a time window. We trained them on an object-in-context task to form an episodic-like memory. The accuracy of the object-context association was tested at 3 days (for recent memory) and at 14 days (for remote memory). After testing and perfusing the mice, hippocampal slices were immunostained and imaged. The tdTomato- and c-Fos-expressing cells (i.e., active during training and testing, respectively) and their overlap (i.e., reactivated cells) were counted. The latter were then correlated with the discrimination index of each mouse to assess memory performance. We found that yohimbine-treated mice showed enhanced object-in-context memory accuracy 14 days after training while at recent memory recall, no differences were observed between the groups. We found no group differences in the percentage of c-Fos+ and tdTomato+ cells at both time points. The discrimination index and the reactivation rate during the remote memory testing of the 0.3 mg/kg yohimbine group were negatively correlated whilst in the recent memory recall we did not find a correlation in any of the groups. A crucial finding was that the reactivation rate of the hippocampal neurons in the vehicle group at recent memory recall was significantly higher compared to the reactivation at remote memory recall of the same group. This was not the case for the 0.3 mg/kg yohimbine-treated group, indicating that yohimbine administration post-training affects only remote memory recall since at a recent time point, memory is still dependent on the hippocampus and thus maintained accurate.

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4-OHT – 4-Hydroxytamoxifen

ACC – Anterior Cingulate cortex

BLA – Basolateral amygdala

CCD – Central Authority for Scientific Procedures on Animals

CORT – Corticosterone

DG – Dentate gyrus

DI – Discrimination index

GR – Glucocorticoid receptor

HPA - Hypothalamic–pituitary–adrenal axis

HPC – Hippocampus

I.P. – Intraperitoneal

IEG – Immediate early gene

IHC - Immunohistochemistry

LBDG – Lower blade dentate gyrus

LC – Locus Coeruleus

mPFC – Medial Prefrontal cortex

NA – Noradrenaline

NDS – Normal donkey serum

NTS – Nuclei of the solitary tract

OiC – Object in context

PBS – Phosphate-buffered saline

PFA – Paraformaldehyde

PFA – Paraformaldehyde

RR – Reactivation rate

RT – Room temperature

SEM – Standard error mean

TRAP – Targeted recombination in active neurons

UBDG – Upper blade dentate gyrus

1. Introduction

Emotionally arousing or highly stressful experiences are remembered stronger and longer in contrast to everyday situations. Accordingly, the strength of a memory is related to its emotional significance which allows for behavioural adaptation upon stressful or threatening situations, providing survival benefits (McGaugh, 2013). For example, the memory of past situations helps a mouse to avoid dangerous locations and return to places where it found food (Lamothe-Molina, et al., 2022). Strengthening of these significantly important memories involves the cooperated actions of two essential hormone systems. These are noradrenaline (NA) and cortisol (in humans; corticosterone in rodents, CORT). They are not only important for the accurate and acute response at the moment of stress or danger but also for the future as they cause long-term behavioural changes (Bahtiyar, Gulmez-Karaca, Henckens, & Roozendaal, 2020).

1.1 Effects of stress hormones on memory

In stressful or emotionally arousing situations, the release of NA reaches its peak which results in increased attention or feeling of anxiety. NA is released from the adrenal medulla and the sympathetic nerve endings; however, it is unable to cross the blood-brain barrier and hence, it first needs to bind to the G-protein coupled receptors (adrenergic receptors). In addition to this, the locus coeruleus (LC) and nuclei of the solitary tract (NTS) release NA directly into the brain. Corticosterone, on the other hand, is released from the adrenal cortex after the activation of the hypothalamic-pituitary-adrenal (HPA) axis. It can pass through the blood-brain barrier thereby activating two types of receptors –mineralocorticoid and glucocorticoid receptors in the brain (Bahtiyar et al., 2020).

It is well-known that NA and CORT improve memory consolidation, a process in which a short-term memory turns into a long-term one. Although both hormones act synergistically on the strength of memory, it has been recently shown that they may exert opposite effects on memory accuracy. This was investigated in rats by administering either yohimbine, a NA stimulant, or corticosterone immediately after training on the inhibitory avoidance discrimination task (Roozendaal & Mirone, 2020). Yohimbine-injected rats showed improved memory strengthening and accuracy compared to vehicle-treated rats, whereas corticosterone-injected rats displayed stronger and more generalized inhibitory avoidance memory.

A critical brain region that orchestrates the effects of NA and CORT on memory is the amygdala. A large body of literature provides experimental evidence to support the involvement of the amygdala in establishing stress effects on memory in rodents, as well as in humans (Roozendaal, McEwen, & Chattarji, 2009) (Cahill, Prins, Weber, & McGaugh, 1994). Hamann and colleagues (1999) found that amygdala activity is linked to an improved episodic memory but only on the occasion of emotionally pleasant stimuli. In animals, the role of the amygdala and particularly the basolateral amygdala (BLA) has been investigated mostly using the paradigms that include aversive stimuli such as fear conditioning. Furthermore, Roozendaal and colleagues (2008) showed that rats receiving NA infusion into the BLA shortly after object recognition training displayed an improved memory of the object recognition 24 hours later. Alongside, the administration of NA into the BLA immediately after training on contextual fear conditioning or inhibitory avoidance task showed enhanced memory (Barsegyan, McGaugh, & Roozendaal, 2014) (Ferry, Roozendaal, & McGaugh, 1999). Post-training systemic yohimbine administration enhanced the memory accuracy and this was indicated by

the high latency specific for the shock context and low latency specific for the non-shock context meaning that the accuracy of the memory was maintained (Roozendaal & Mirone, 2020). Altogether these results support the notion that the BLA has an essential role in orchestrating the memory-enhancing effect of NA and CORT (Bahtiyar et al., 2020).

1.2 Systems consolidation

Over time episodic memories (or episodic-like memories in animals) are believed to undergo a time-dependent reorganization in the brain through a process known as systems consolidation (Krenz, Sommer, Alink, Roozendaal, & Schwabe, 2021). Episodic memory refers to the conscious recollection of past experiences with temporal and spatial context (Yonelinas, Yonelinas, Ranganath, Ekstrom, & Wiltgen, 2019). Initially, when episodic memory is acquired, the information is majorly dependent on the hippocampus (Winocur & Moscovitch, 2011). The hippocampus is important for the processing and organization of this temporal and spatial input information (Lamothe-Molina, et al., 2022). The detailedness and accuracy of episodic memories are maintained by the continuous involvement of the hippocampus. However, episodic-like memories are dependent on the hippocampus only for a limited time. After that, they become more dependent on the neocortical regions, for instance, the medial prefrontal cortex (mPFC) and the anterior cingulate cortex and less dependent on the hippocampus (Winocur & Moscovitch, 2011). The increased dependency on the cortex causes accurate, episodic memories to become more generalized and gist-like (Dandolo & Schwabe, 2018). Depending on the type of memory, it can be retrieved independently of the hippocampus at remote times, however, there is still controversy on how much the hippocampus is still involved in the recall of memories over time (Winocur & Moscovitch, 2011) (Dandolo & Schwabe, 2018).

1.3 Noradrenaline and system consolidation

Although memory reorganization has been described in different studies, stressful or highly arousing experiences seem to escape the time-dependent relocation from the hippocampus to the neocortical structures. It has been shown that post-training coadministration of noradrenaline with beta-adrenoreceptor antagonists into the amygdala blocks the effects of NA on memory (Roozendaal & McGaugh, 2011). In a remote memory test, Atucha and colleagues (2017) found that after 28 days, rats that had NA administered into the BLA post-training still show accurate memory and shock-context association. Conversely, the saline-treated rats did not have an accurate representation of the memory, and this has been shown by the lack of discrimination of the shock context. Similarly, a study in humans, where participants received yohimbine orally, showed high hippocampal dependency after 28 days and a decrease in involvement of neocortical structures compared to the placebo group (Krenz, Sommer, Alink, Roozendaal, & Schwabe, 2021). However, it is still unclear through what neuronal mechanisms NA affects the process of systems consolidation.

1.4 Memory engrams

Memories are believed to be encoded as engrams in the brain (Josselyn, Kohler, & Frankland, 2015). The engram represents physical changes in the brain that involve strengthening the synapse connections between engram neurons. Engram neurons are the neurons activated during learning. The process of memory consolidation involves not only functional and structural changes of these engram neurons but also epigenomic and transcriptional ones which result in stabilization of the engram (Gulmez-Karaca, Kupke, & Oliveira, 2021). During recall, the engram neurons, or a part of them are reactivated, which was shown to be both necessary

and sufficient to induce memory recall (Tonegawa, Morrissey, & Kitamura, 2018). This is implemented in rodent studies and benefits the identification of the engram neurons that were activated at the time of learning. Nevertheless, how the dynamics of engram neurons change over time, and how stress hormones affect the memory-engram-mediated system consolidation determining memory quality remains unclear.

1.5 Aim and methodology

As it has been mentioned above, over time memories become more dependent on neocortical structures and less on the hippocampus which results in less accurate and more generalized memories. NA has been shown to act on memory strength as well as its accuracy at remote time points, suggesting its involvement in slowing down or reversing the process of system consolidation (Atucha et al., 2017). However, the exact cellular mechanisms of how NA affects the systems consolidation of memories and their dependency on the hippocampus at remote time points remains unclear. In this research project, we aim to study the effect of yohimbine, a noradrenergic stimulant, on the accuracy of recent and remote memory at a cellular level (engram neurons). To reach our goal the following research questions are formulated:

1) What are the effects of post-training systemic yohimbine administration on the cellular activation of the hippocampus after training?
2) What are the effects of post-training systemic yohimbine administration on the cellular activation of the hippocampus after retention testing?
3) What are the effects of post-training yohimbine systemic administration on the reactivation of hippocampal engram neurons at recent versus remote memory recall testing?
4) How does cellular activation or reactivation of hippocampal neurons correlate with the memory accuracy of individual mice?

Our main hypothesis is that NA will enhance the accuracy of episodic-like memories over time. Thus, we expect that mice that receive yohimbine (a noradrenergic stimulant) injection immediately after training will show a more accurate memory compared to the control group. To test this hypothesis, a set of behavioural experiments and cellular imaging are conducted. We use a transgenic mouse line FosxTRAP2xRosa where two transgenes are expressed. Specifically, upon neuronal activity, immediate early genes (IEGs), including the c-Fos gene, are transcribed. In the TRAP construct, Cre recombinase expression is dependent on the promoter of the immediate early gene and thus upon neuronal activity, the Cre recombinase can be expressed. Additionally, only in the presence of 4-hydroxytamoxifen (4-OHT), Cre recombinase can enter the nucleus and induce recombination. Therefore, neuronal activity and the presence of 4-OHT result in the permanent labelling of these neurons at a certain time frame by tdTomato (a red fluorescent protein). In figure 1 below, the principle of the TRAP system is depicted.

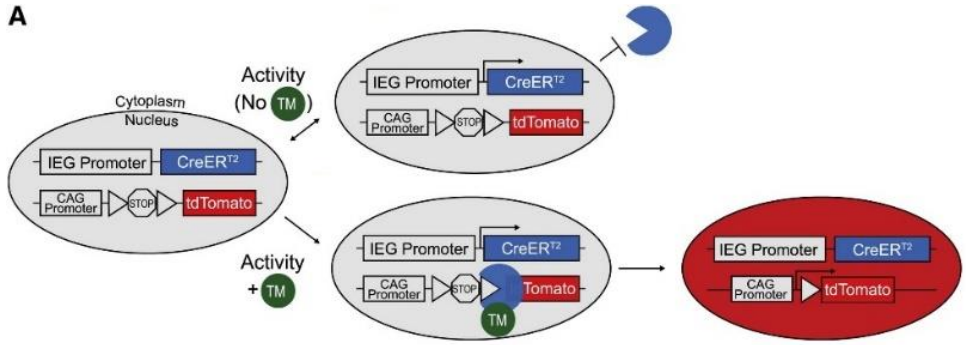

Figure 1. Targeted recombination in active neurons (TRAP) model. The TRAP system expresses two transgenes, one of which is the CreER^{T2} that is under the control of the IEG promoter (i.e., c-Fos). The second one can initiate the expression of an effector gene, in this case- tdTomato. Thus, in active neurons, and in the presence of tamoxifen, recombination can occur leading to the expression of tdTomato and the presence of a red fluorescent signal (Guenthner, Miyamichi, Yang, Heller, & Luo, 2013).

The experimental timeline in this research project starts with training and testing the mice on the object-in-context (OiC) task in which mice form an episodic-like, object-context associative memory. Mice were trained on the OiC task and then the retention of this memory was tested either 3 days (recent memory) or 14 days (remote memory) later. In the training phase, mice acquire new information (i.e., an association of an object with a certain context). Immediately after training, mice receive a systemic intraperitoneal (i.p.) yohimbine injection or saline injection. The testing phase includes switching the objects that were previously associated with the context. The memory accuracy for the object-context association is determined based on the discrimination index. The discrimination index indicates how accurately mice remembered the object-context association based on the exploration time of the non-associated and associated object in a context (further information can be found in the materials and methods section).
After sacrificing the mice, promptly after memory testing, immunohistochemistry is performed to stain the c-Fos expressing cells (i.e., cellular activation during memory recall) in the hippocampus. Following the imaging of the brain slices on a fluorescent microscope- c-Fos expressing cells, tdTomato expressing cells (i.e., cellular activation during training) and their overlap (i.e., reactivation rates of engram cells) were counted. Based on the reactivation rates, we evaluated whether the same neuronal ensembles were active at the time of the training and testing. Lastly, neuronal readouts were correlated with the memory performance of the animals.

2. Materials and methods

This research aims to investigate the effects of yohimbine administration, a noradrenergic stimulant, on the accuracy of memory and neuronal representations over time. This effect was tested by systemic administration of yohimbine immediately after the mice were trained on the object-in-context task. Depending on whether the recent or remote memory is tested, the period between the training and testing session was 3 days and 14 days, respectively. The mice were sacrificed via transcardial perfusions one hour after the testing and then the brains were examined by performing histological analysis, immunohistochemistry, and microscopy.

2.1 Animals

Eight-week-old, male FosxTRAP2xROSA mice were used in this research project. This is a genetically modified mouse line in which 4-hydroxytamoxifen administration after the training led to fluorescent labelling of all c-Fos-expressing neurons activated at the time of training.
During the experiments, mice were single housed in a room without music or females to avoid any interference with their behaviour. The animals were maintained under a 12-hour light/ 12-hour dark cycle with all training and testing sessions being conducted during the light cycle (7:00 am to 7:00 pm). The experimental process was in accordance with the European Union Directive 2010/63/EU and certified by the CCD (Central Authority for Scientific Procedures on Animals).

2.2 Object-in-context

Mice were first handled by the experimenter to habituate them to the experimenter and experimental room. This has been done for 5 consecutive days and each mouse was handled for 5 minutes. Over the course of the next 3 days, mice were habituated to two square contexts (X and Y; 40 cm diameter). One of the boxes (contexts) had grey walls with white dots and sand for bedding while the second box had plain grey walls with sawdust. Both were different from the training contexts. Habituation to each context lasted 10 minutes. One day after habituation, the mice underwent object-in-context training which forms an episodic-like memory where mice learn to associate a certain context with a particular set of identical objects. During the training day, each mouse was placed in two contexts (A+B) for 10 minutes each with a pair of identical objects inside. Context A and B were round boxes, 40 cm in diameter with the same walls and bedding as in the habituation context. After spending 10 minutes inside context A, mice were immediately placed into context B for 10 minutes where a different pair of identical objects was placed. The objects used were two light bulbs and two empty jars. Immediately after completing the training session in contexts A and B, each mouse was injected with 4-hydroxytamoxifen (50 mg/kg; preparation is described in Appendix C) and with a second injection which was different for the three treatment groups: high-dose yohimbine (1 mg/kg), low-dose yohimbine (0.3 mg/kg) or vehicle (saline). All injections were performed intraperitoneally and in the fume hood. The delay between training and testing was 3 days for the recent and 14 days for the remote memory. During the testing, each mouse was placed into one of the training contexts, for 5 minutes, however, the box (context) contained one object from each of the two pairs used in the training session. Combinations of objects and contexts, as well as the orders of context exposure, were fully randomized across animals.
To determine how accurately the mice remembered the object-context association, a discrimination index (DI) was calculated based on the time each mouse spent exploring the object not associated with the testing context in comparison with the one they saw before in the context. The DI is calculated as follows:

$$DI = \frac{Exploration\ of\ non-associated-associated\ object}{Total\ time\ spent} * 100\%$$

A large DI indicates that mice spent more time exploring the non-associated object and thus the object-context association accuracy was high. When mice do not remember which object was associated with the context, they would sniff both objects and spend equal time around them and the DI will be close to or 0. Thus, when the DI% is greater than 0 the memory is considered accurate.

2.3 Brain collection and histology

Sixty minutes after the retention testing, the mice received an overdose of sodium pentobarbital (40-50 mg, i.p.) and were then sacrificed by transcardial perfusion where the blood was first exchanged with 1x phosphate-buffered saline (PBS) and then with 4% paraformaldehyde (PFA). The brains were collected in 4% PFA and post-fixed overnight. The 4% PFA was then replaced with a 30% sucrose solution diluted in 1x PBS. Once the brain sunk into the sucrose, it was ready for sectioning as this ensured it is impregnated with sucrose and thus cryoprotected.

Brain histology was performed using a sliding microtome with coronal slices at a thickness of 20 µm. All slices were collected in a well plate and stored in 0.01% sodium azide diluted in 1x PBS to prevent contamination and moulding. Due to the light sensitivity of the tdTomato fluorescent signal, the brains and the plates were kept wrapped and stored in the dark.

2.4 Immunohistochemistry

In order to determine whether the same engram neurons that were active during training were also active during the testing, immunohistochemistry (IHC) was performed which aims to immunostain all c-Fos expressing cells active at the time of testing. All the steps described below are performed with a minimum amount of light to ensure the signal of the tdTomato will be maintained. During the washing steps and incubation steps, the plates were always kept in the dark. Out of all the collected slices from each brain, in total 4-5 slices from the hippocampus were collected for immunohistochemistry in a 24-well plate. The slices were washed 3 times for 5 minutes in 1x PBS to remove the sodium azide. Consequently, the slices were permeabilized for 5 minutes on the rotator using 400 µL 0.1% Triton-X (Sigma-Aldrich) in 1x PBS. By adding Triton-X, the lipids on the membranes are broken down and thus antibodies can enter the cell for antigen binding. To reduce the non-specific binding of the antibodies, 400 µL of blocking solution was pipetted into the wells (8% normal donkey serum (Sigma Aldrich) and 0.3% Triton-X in 1x PBS). The blocking step continued for 50 minutes at room temperature (RT), on a rotator and then the slices were washed three times in 1x PBS.

The IHC aimed to stain all c-Fos-expressing cells indicating active cells at the time of the testing and thus the primary antibody used was against c-Fos (anti-c-Fos antibody produced in guinea pig; 226-308, Synaptic systems). It was diluted 1:1000 in 1x PBS, 2% normal donkey serum (NDS) and 0.3% Triton-X and then 0.3 mL of the solution was added to each well. The slices were incubated overnight at 4 °C in the dark. On the second day of the immunohistochemistry, slices were washed three times for 5 minutes with 1x PBS to remove the excess primary antibody. The following step was incubation for 2 hours at RT with the secondary antibody (Donkey, anti-pig Alexa 647, 706-605-148, Jackson ImmunoResearch) which was diluted 1:750 in 1x PBS, 2% NDS and 0.3% Triton-X.

For the staining of the cell nuclei, DAPI (62248, Thermo Scientific) was used in a 1:5000 dilution and the incubation lasted for 1 minute. Before mounting, the slices were washed once with 1x PBS. After mounting on glass slides and air-drying them, the slices were covered with

FluorSafe and cover slipped. They were stored in the dark at RT for 1 day to dry and then moved to 4 °C for long-term storage.

2.5 Microscopy and Image analysis

The stained slices containing the hippocampus were imaged on a Leica DMI 6000B microscope. Three slices were imaged per brain region for each animal and the magnification used was 20x for the hippocampus. Each slice was measured using three different channels – DAPI (nuclei of the cells), tdTomato (all c-Fos-expressing active cells at the time of training) and c-Fos (all active cells expressing c-Fos at the time of testing). The excitation peaks were 359 nm, 555 nm and 647 nm while the emission peaks were 457 nm, 582 nm and 671 nm, respectively. The exposure was set to 11 ms and the autofocus channel was set to DAPI. Auto stitching function was also applied at a rate of 20%.

The image analysis was performed using Fiji (version 2.9.0/1.53t) which is a distribution of the Image G software (Schindelin, et al., 2012). We first merged all tile images and then combined them into a stack. However, sometimes images can be impacted by shading which affects the process of analysis. Thus, the BaSiC plugin was used to 1) calculate the average distribution of the signal over the entire stack and 2) correct for it (Peng, et al., 2017). Furthermore, the tiles from each stack were stitched together column-by-column and corrected for shading. The detailed step-by-step protocol is described in Appendix A. Once the pre-processing has been completed, the background-subtracted images were analyzed. A threshold was set based on the image in the c-Fos channel to remove as much as possible of the background signal and leave only the cells that express a specific c-Fos signal. This threshold value varied between the different batches of mice from 80 to 130, however, it was the same for all animals part of the same batch.

The analysis of the hippocampal slices included the lining of 11 regions of interest, as illustrated in Figure 2. These are the cellular layers: Cornu Ammonis 3 and 1 (CA3 and CA1), the upper blade of the dentate gyrus (UBDG) and lower blade of the dentate gyrus (LBDG), as well as the dendritic layers of CA3 (5-6), UBDG (7), the hilus (8), LBDG (9) and CA1 (10-11).

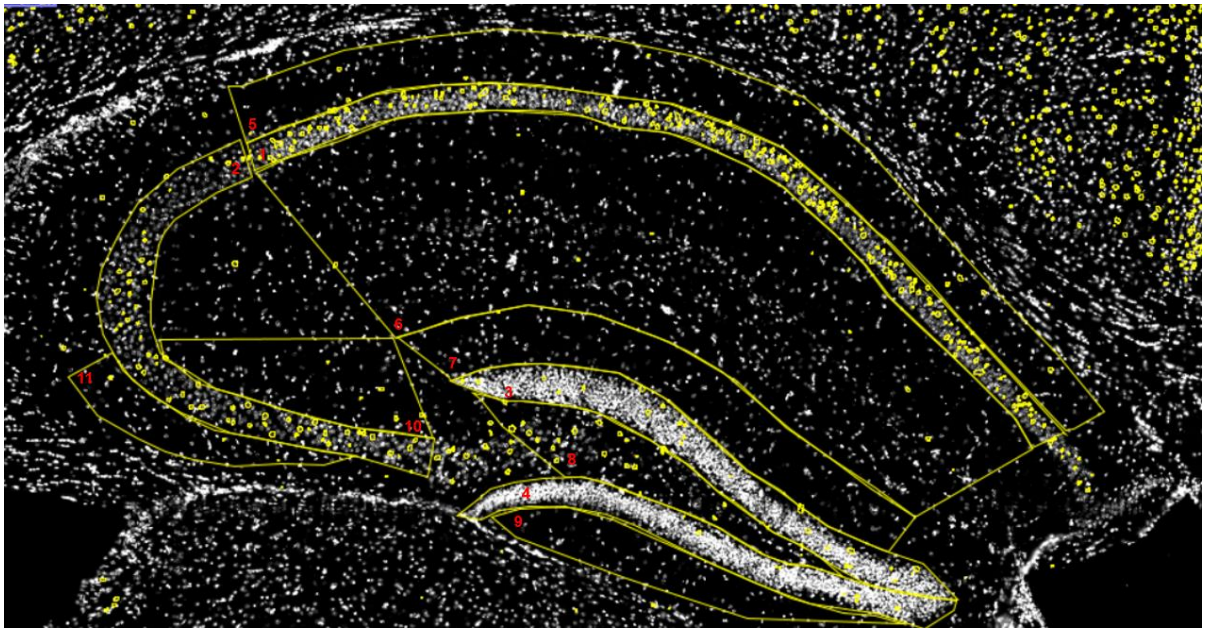

Figure 2. Hippocampal regions numbered. Labelled from 1-4: Cornu Ammonis 3 and Cornu Ammonis 1 (CA3 and CA1), upper blade of the dentate gyrus and lower blade of the dentate gyrus, respectively. Labelled 5-11 are the dendritic layers of the 4 regions.

The cell counting was performed manually. The positive c-Fos cells, the positive tdTomato cells and the overlapping cells (both c-Fos and tdTomato positive cells) were counted. Additionally, the size of the area of all 11 regions was taken. The counted cells in each region were then corrected to obtain the number of cells per 100.000 μm^2. The percentage of positive cells was calculated as well as the reactivation rate of the cells in the 11 areas (as shown in Appendix B). The average of the 3 slices was taken to obtain data per animal.

2.6 Statistics

To compare the control versus the yohimbine group (for neuronal readouts), an unpaired T-test or (non-parametric equivalent – Mann-Whitney test) was performed based on the normal distribution of the data. To compare the three treatment groups for memory testing, one-way ANOVA was applied. To determine the correlation between the DI% and the reactivation rate of the hippocampal neurons, simple linear regression graphs were used. Two-way ANOVA was performed to compare the effects of both the time point and the treatment. A p-value lower than 0.05 was considered statistically significant.

2.7 Yohimbine

Yohimbine (17-hydroxyyohimban-16-carboxylic acid methyl ester hydrochloride), a noradrenergic stimulant, was used in two different concentrations for the treatment groups – a high dose of 1 mg/kg and a low dose of 0.3 mg/kg. Yohimbine induces the release of noradrenaline in amongst others, the hippocampus, and the prefrontal cortex. It exerts its function by blocking the alpha-2-adrenergic receptors (present on presynaptic noradrenaline neurons) which leads to disruption of the negative feedback inhibition loop and eventually higher NA release (Szemeredi, et al., 1991).

The preparation of the rest of the solutions and buffers used in this research project can be found in Appendix C.

3. Results

In this research project, we tested the effects of yohimbine, a noradrenergic stimulant, on the accuracy of OiC memory and its neuronal representatives (i.e., engram neurons) over time. For this purpose, we used the FosxTRAP2xROSA transgenic mouse line which utilizes the system of **T**argeted **R**ecombination in **A**ctive **N**eurons. TRAP is both an activity-dependent and tamoxifen-dependent system. This means that only when a neuron is active and tamoxifen (or its derivatives such as 4-hydroxytamoxifen) is present, the red fluorescent protein tdTomato can be expressed. This allows for the permanent labelling of neurons activated within a time frame. We trained FosxTRAP2xROSA mice on the object-in-context task, a behavioural paradigm that forms an episodic-like memory. The task consists of two phases – training and testing which are performed in a certain time frame (Figure 3).

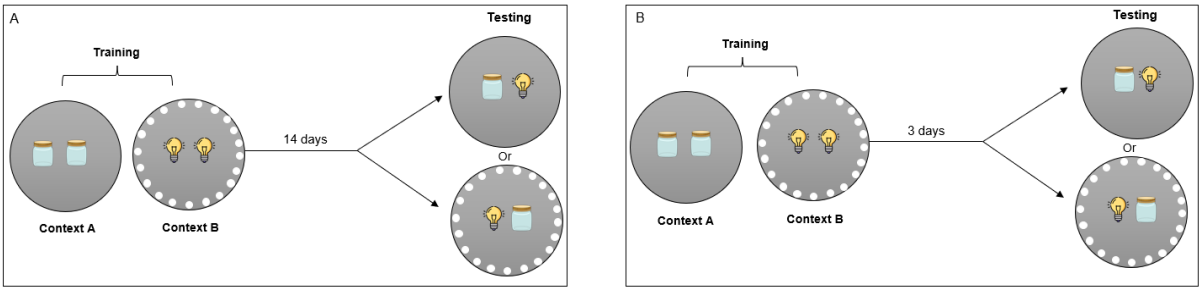

Figure 3. Experimental design of the object-in-context task for memory testing. Each mouse spends 10 minutes in context A and another 10 minutes in context B. The testing takes place 14 days or 3 days later in one of the contexts, chosen randomly, that contains one object from each of the two pairs introduced during the training.

Previously, we found that mice which underwent OiC training had significantly more tdTomato-expressing cells in the hippocampus as well as in the prefrontal cortex compared to the home cage- confirming that the TRAP labelling is neuronal activity-dependent (data not shown). Here, we tested the tamoxifen dependency of TRAP labelling. For this, we trained mice on the OiC task, however, three mice from the batch did not receive a 4-OHT injection and instead, we administered 0.9% saline. We found almost no tdTomato-positive cells in the hippocampus and mPFC of mice that received only saline injection (fig. 4*A*). In comparison, mice that were administered with 4-OHT displayed a higher number of tdTomato+ cells in both the mPFC and hippocampal regions (fig. 4*B*). This confirms that the TRAP system is not only activity-dependent but also tamoxifen dependent.

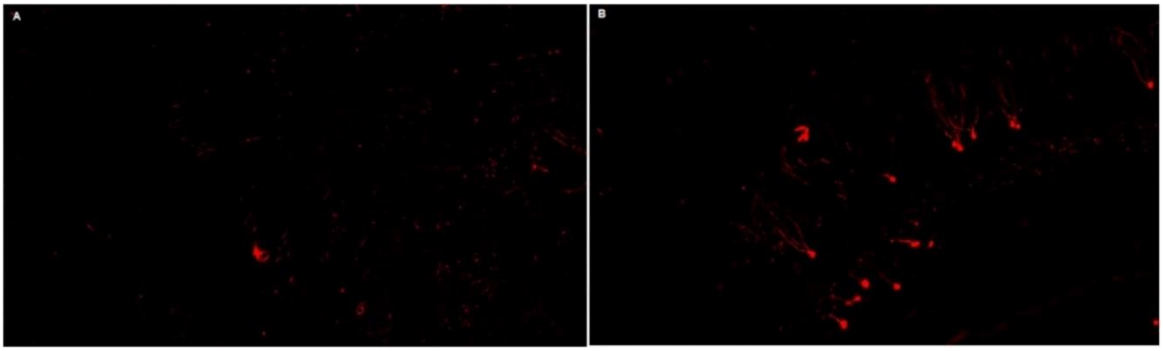

Figure 4. TRAP is dependent on the presence of tamoxifen. (**A)** The absence of 4-OHT led to almost no tdTomato signal in the c-Fos expressing neurons, active at the time of training. **(B)**

Active neurons (expressing c-Fos) in the tamoxifen-injected mouse were permanently labelled with tdTomato.

3.1 Post-training noradrenergic effects on remote OiC memory

After confirming the specificity of the TRAP system, we tested the effects of post-training yohimbine administration on memory accuracy at the remote retention test. To this end, we tested mice on the OiC task 14 days after the training. The DI indicating the difference in exploration time of the non-associated versus associated object was calculated. The high DI implies that the mouse spent more time exploring the non-associated object and thus displayed accurate memory in contrast to a low DI which meant that the mouse spent equal time investigating both objects hence poor memory accuracy. The DI of each mouse in the control or the 0.3 mg/kg yohimbine group was compared to identify the effect of the pharmacological manipulation on memory accuracy. We found that the systemic yohimbine administration in a dose of 0.3 mg/kg resulted in a significant increase in the DI (M= 18.68, SEM= 5.24), thus indicating memory accuracy on the OiC task testing at a remote time point compared to the control group (M= -6.34, SEM= 5.55) (Unpaired T-test: n= 11-13 animals per group, p= 0.0038 (**)) (Fig. 5).

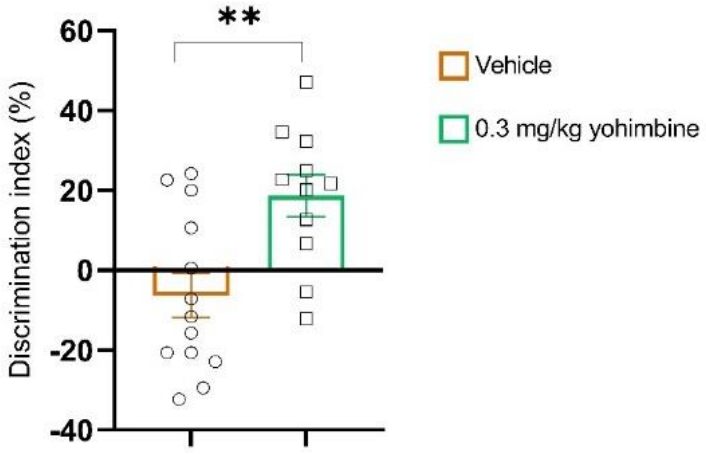

Figure 5. Discrimination index (%) of individual mice during the testing phase. The 0.3 mg/kg yohimbine injected mice showed a significantly greater DI compared to the control group during the recall session 14 days later. n= 11-13 per group, p= 0.0038 (**). The error bars represent the standard error mean (SEM).

Apart from the behaviour, we also analysed the noradrenergic effects on memory accuracy at the level of engram neurons. To test the remote memory accuracy, we tested mice on the OiC task 14 days after the training and perfused them 1 hour post-testing for immunohistological analysis of the brain sections. The activated neurons during the training were labelled by tdTomato signal while the neurons active during the recall session were stained using anti-c-Fos primary antibody followed by Alexa 647 secondary antibody. This way, the neurons active at the two phases of the OiC task are labelled separately, however, neurons that were reactivated during the testing showed an overlapping tdTomato and c-Fos signal. Figure 6 shows representative images of the immunohistochemistry staining in the saline-treated and yohimbine-treated animals.

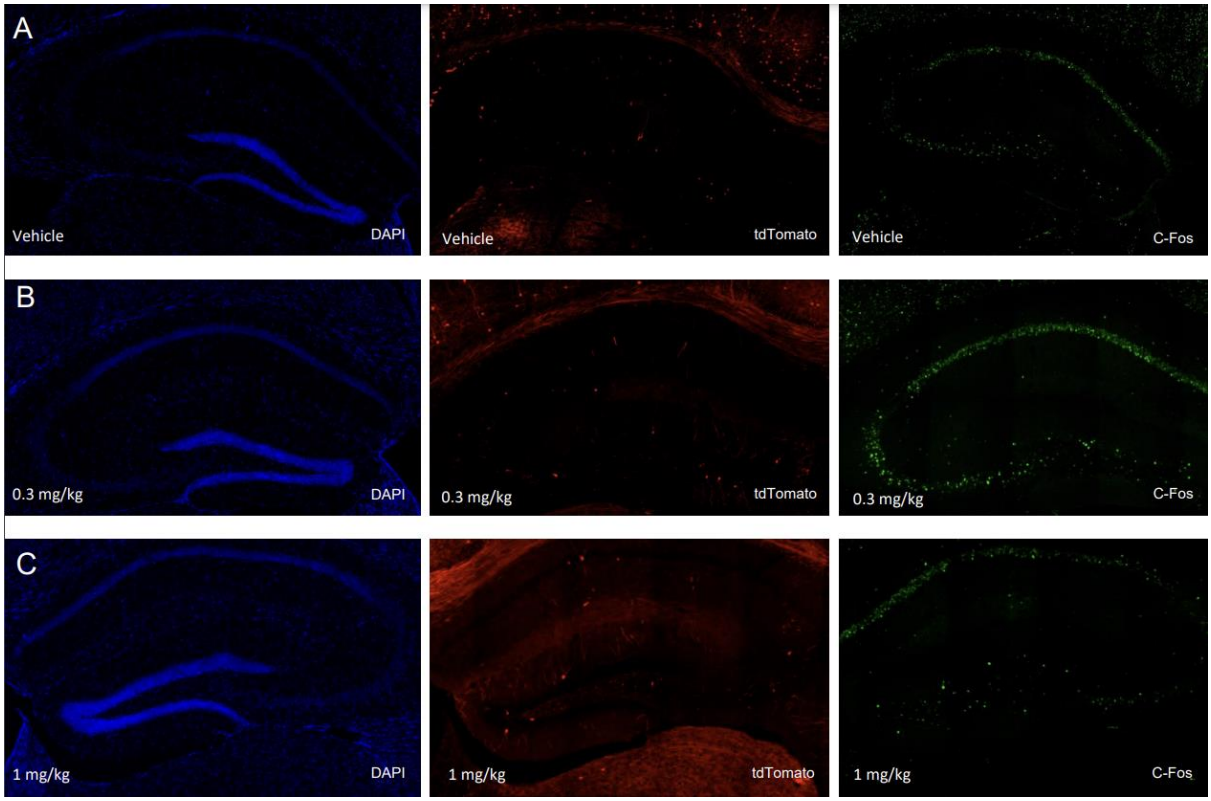

Figure 6. Representation of the IHC results from remote memory testing. DAPI, tdTomato and c-Fos channels of vehicle-treated FosxTRAP2xROSA mice (A), 0.3 mg/kg yohimbine injected mice (B) and 1 mg/kg yohimbine injected mice (C).

We assessed the number of c-Fos and tdTomato-positive cells in each region of the hippocampus. Since we only detected a significant remote memory accuracy enhancement in the 0.3 mg/kg yohimbine-treated group compared to the controls, we only analysed the two experimental groups for the neuronal readouts. As shown in Figure 7, the 0.3 mg/kg yohimbine-treated mice displayed a significantly higher percentage of c-Fos expressing neurons in the CA3 region (M= 5.976, SEM= 1.172) in comparison to the control group (M= 3.759, SEM= 0.4604), (Mann-Whitney test: n=9-11 per group, p=0.0381 (*)). There was not a significant difference detected in the dentate gyrus (nor with the individual analyses of the upper or lower DG blade) and the whole hippocampus between the two treatment groups. The percentage of the tdTomato-expressing neurons was not significant in any of the hippocampal regions (Unpaired T-test, n= 9-11 per group, p>0.05). The percentage of c-Fos-activated cells was higher compared to the percentage of tdTomato-positive cells which could be a result of the low sensitivity of the TRAP system (Fig. 7*B*).

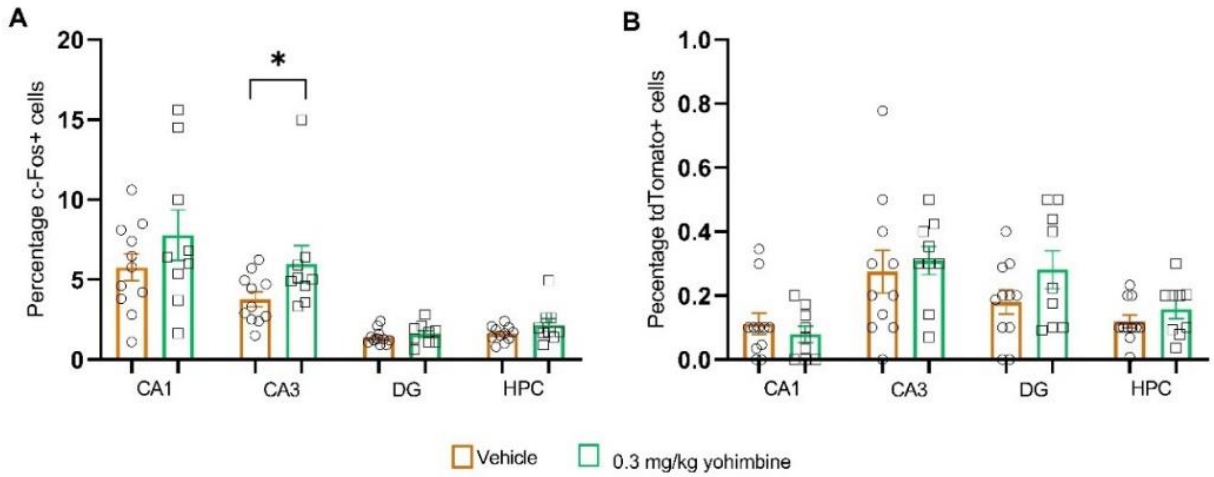

Figure 7. Noradrenergic effects on the percentage of c-Fos+ and tdTomato+ cells at remote memory testing. **(A)** The percentage of c-Fos positive neurons, active at the time of testing in the three hippocampal regions – CA1, CA3 and dentate gyrus, as well as in the hippocampus in general. The percentage of c-Fos+ cells in the CA3 region of the low-dose yohimbine-treated mice is significantly higher compared to the control group (n= 9-11 per group, p=0.0381(*)). **(B)** Percentage of tdTomato-positive cells in CA1, CA3, DG and hippocampus (total). No significant differences were observed. The error bars represent the standard error mean (SEM).

The percentage of tdTomato-positive cells in the upper and lower blade of the DG was also compared within the two groups. The 0.3 mg/kg yohimbine-injected mice displayed a statistically higher percentage of tdTomato labelled neurons in the lower blade of the DG (M= 0.158, SEM= 0.027) compared to the saline-treated mice (M= 0.082, SEM= 0.022), (Unpaired T-test, p= 0.0398) (Fig. 8*B*).

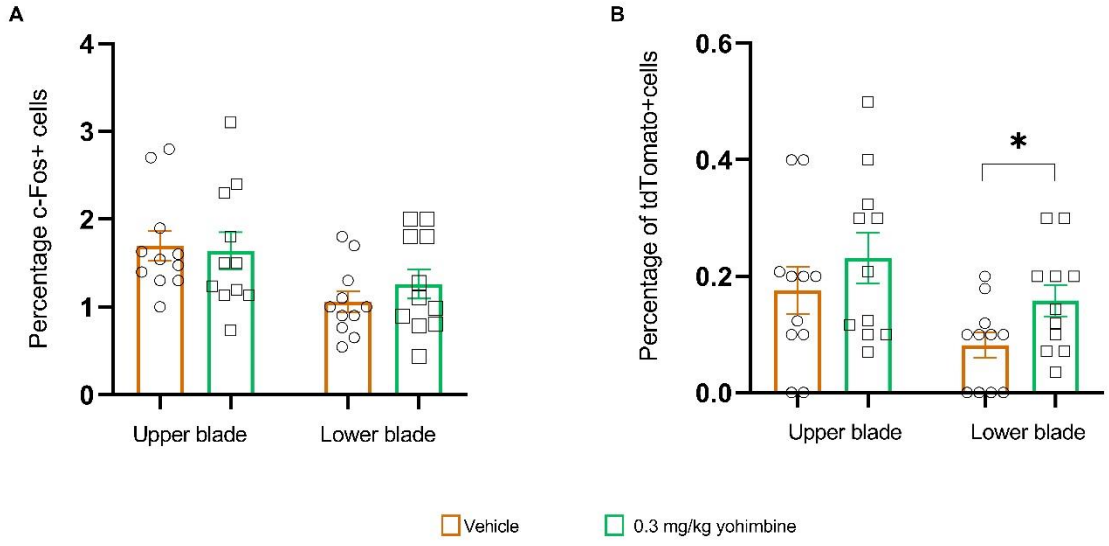

Figure 8. Percentage of c-Fos+ and tdTomato+ cells in the upper blade and the lower blade of the dentate gyrus at remote memory recall. (**A)** No significant differences were observed in the c-Fos positive cells percentage. **(B)** The 0.3 mg/kg yohimbine-treated animals showed a higher percentage of tdTomato-positive cells in the lower blade (Unpaired t-test, n=11, p=0.0398(*)). The error bars represent the standard error mean (SEM).

To determine what fraction of the neurons active during the training was also active during (or after) the retention test, the reactivation rate (RR) was calculated and then compared between the vehicle and the low-dose yohimbine group. The RR represents the number of overlapping neurons divided by the number of tdTomato-positive cells * 100%. Although we did not find any significant difference between the two groups for the different hippocampal regions (Unpaired T-test for CA3 region data and Mann-Whitney test for CA1 and DG: n= 6-10, p>0.05), we found that the RR in the whole hippocampus (including all regions combined) of mice that received a post-training yohimbine injection of 0.3 mg/kg (M= 21.22, SEM= 2.58) was significantly increased compared to the saline-injected mice (M=11.85 SEM=3.027), (Mann-Whitney test: n=10; *p*= 0.0042(**)), (Figure 9).

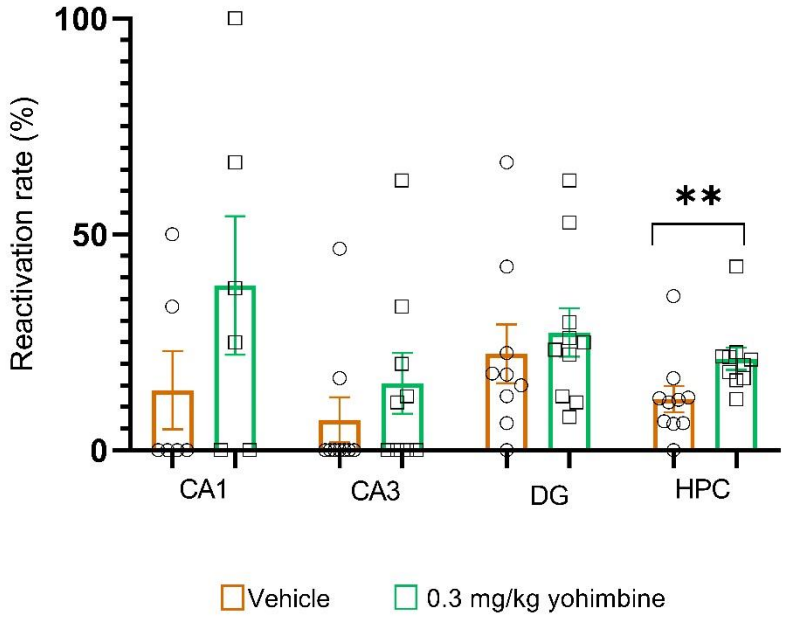

Figure 9. Reactivation rate of the neurons in the regions CA1, CA3, DG and the hippocampus (HPC) at remote memory recall. The reactivation rate in the hippocampus is significantly higher in the yohimbine-injected animals compared to the control group. n=10, p= 0.0042 (**). The error bars represent the standard error mean (SEM).

The RR of the neurons in the hippocampus and the DI of each mouse were then compared. We found no correlation between the RR and the DI in the vehicle group (p= 0.38) (Figure 10*A*). The RR was also compared with the DI for the yohimbine-treated mice (0.3 mg/kg). There was a negative correlation that was close to significance (p=0.0572), however, this was majorly driven by a single data point with high RR and low DI (42.5% and -12.11%, respectively).

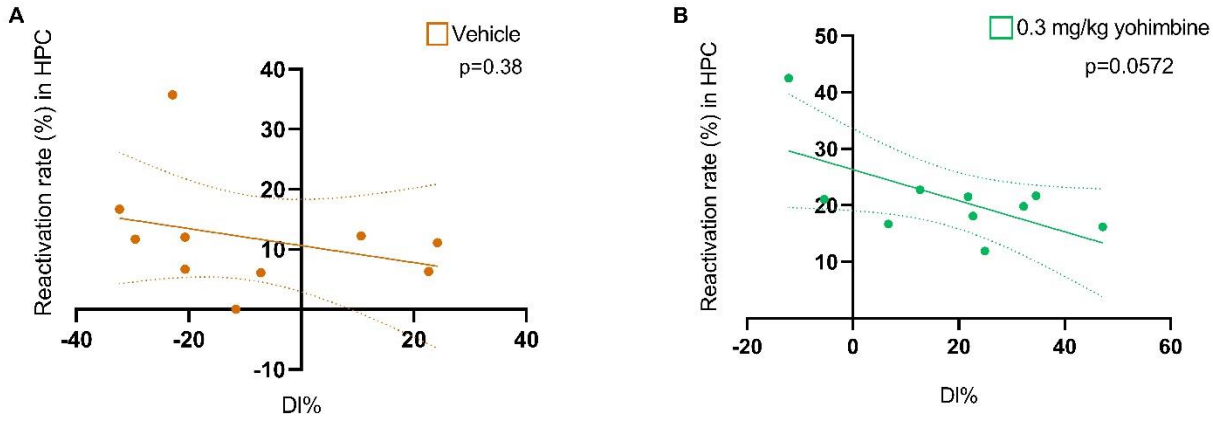

Figure 10. Correlation between RR and DI during remote memory recall. (A) No correlation was found between the reactivation rate and the discrimination index of saline-treated mice (n= 10, p= 0.38). (B) In the 0.3 mg/kg yohimbine injected mice, a trend for negative correlation was observed (n=10, p= 0.0572).

Overall, this set of experiments showed that the DI of 0.3 mg/kg yohimbine-injected animals was significantly higher than that of the vehicle group. When compared with the reactivation rates, we found no correlation between the DI and RR in the saline-injected mice and a negative correlation in the low-dose yohimbine-administered animals.

3.2 Post-training noradrenergic effects on recent OiC memory

To test the recent memory accuracy, we tested mice on the object-in-context task 3 days after training as described above (Fig. 3*B*). The behaviour of each mouse was assessed and based on the exploration time that the animal spent on the non-associated versus associated object, a discrimination index was calculated. We compared the systemic effects of 0.3 mg/kg and 1 mg/kg yohimbine on the performance of every mouse. These results show no statistical difference between the low-dose yohimbine (M= 3.076, SEM= 5.57), high-dose yohimbine (M= 12.81, SEM= 5.96) and the control group (M= 7.93, SEM= 4.57) (Ordinary one-way ANOVA: n=15-16 per group, $F_{2,43} = 0.473$, p= 0.45) (Fig. 11).

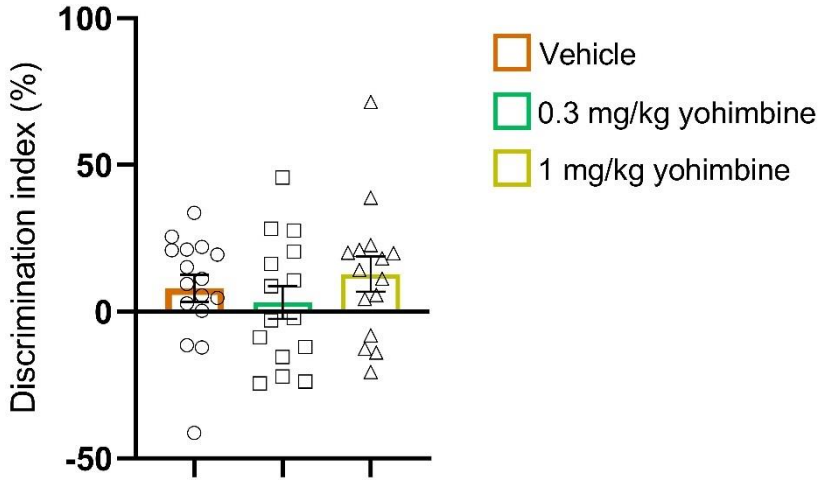

Figure 11. Discrimination index (%) of individual mice during the testing phase. No significant difference was observed between the three groups n= 15-16, p= 0.454. The error bars represent the standard error mean (SEM).

We also analysed the noradrenergic effects on recent memory accuracy at the level of engram neurons. To investigate this, mice were tested on the OiC task 3 days after the training and perfused one-hour post-testing for immunohistochemical analysis of the brain sections. The number of c-Fos-expressing cells (active during the testing) and the tdTomato-positive cells (c-Fos-expressing cells activated during the training), were counted. Figure 12 shows representative images of the immunohistochemistry staining in the saline-treated and yohimbine-treated animals.

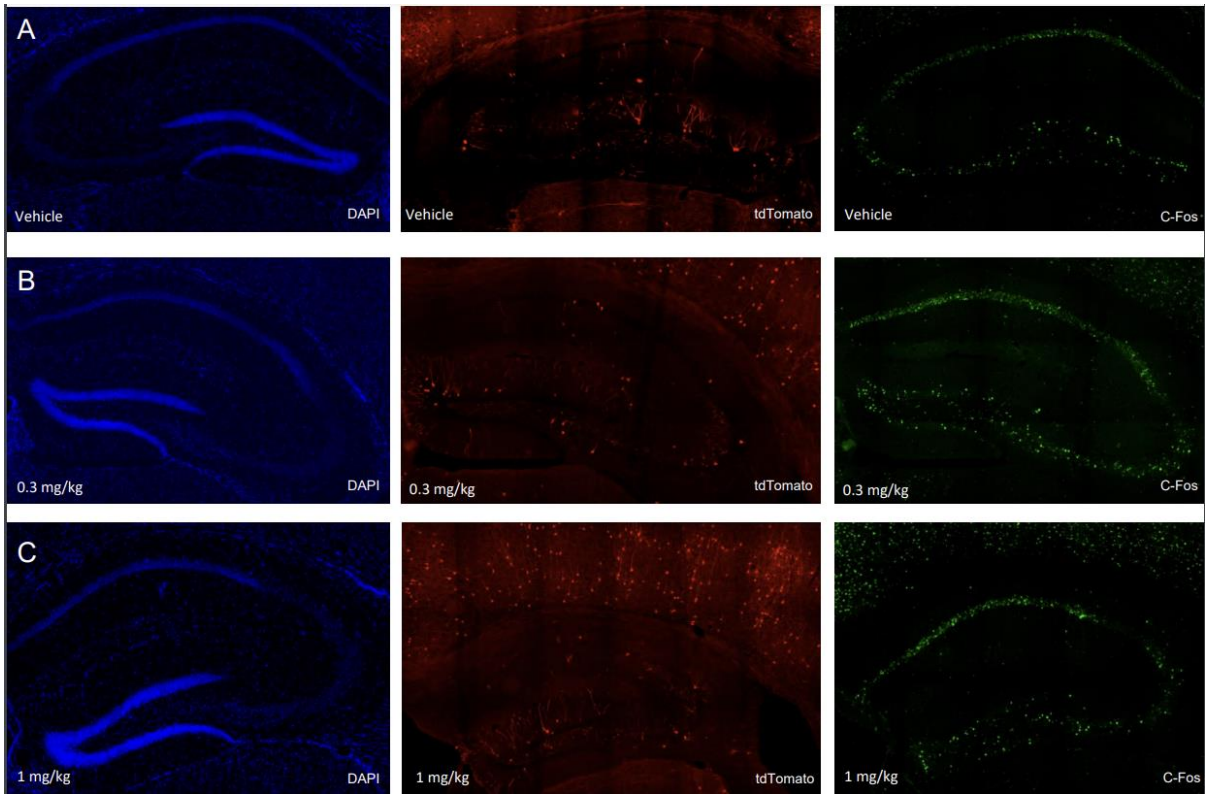

Figure 12. Representation of the IHC results from recent memory testing. DAPI, tdTomato and c-Fos channel of vehicle-treated FosxTRAP2xROSA mice (A), 0.3 mg/kg yohimbine injected mice (B) and 1 mg/kg yohimbine injected mice (C).

First, we assessed the percentage of c-Fos+ cells in the three treatment groups (saline, 0.3 mg/kg yohimbine and 1 mg/kg yohimbine). We then analysed the CA1, CA3 and DG regions of the hippocampus as well as the hippocampus as a whole (fig. 13*A*). We did not find any significant differences between the percentage of cFos-positive cells in the three groups in the different regions (ordinary one-way ANOVA: n= 3-7 per group, $F_{2,13} = 0.7811$, p= 0.94). In figure 13*B*, the percentage of tdTomato+ cells is shown. We found no significant difference between the three experimental groups (ordinary one-way ANOVA: n= 3-7 per group, p= 0.28). Similarly, to the remote memory analysis, the percentage of tdTomato+ cells was much lower in comparison with the percentage of c-Fos cells which again indicates the low labelling sensitivity of the TRAP system.

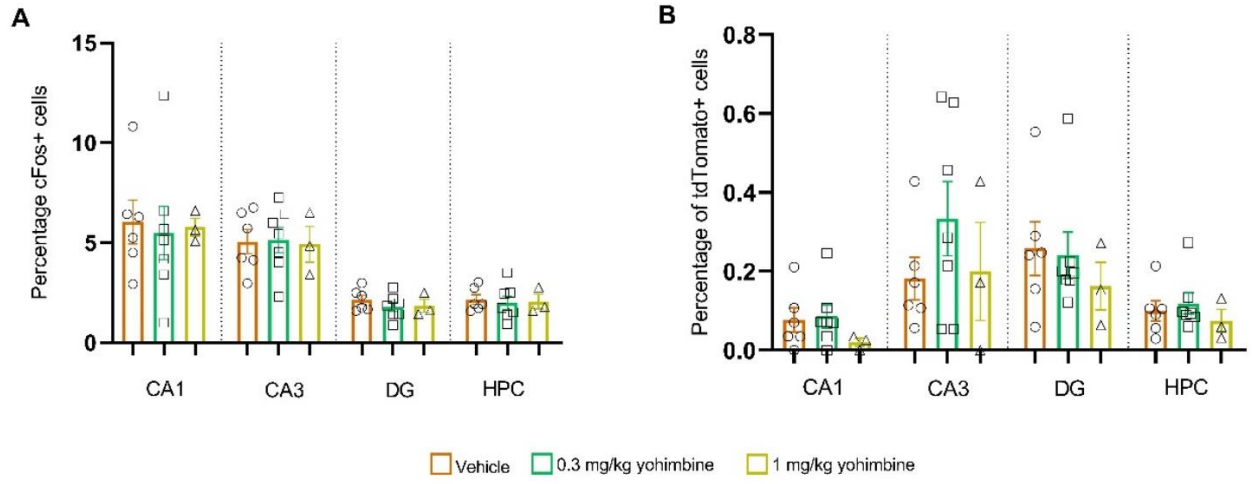

Figure 13. Noradrenergic effects on the percentage of c-Fos+ and tdTomato+ cells at recent memory testing. **(A)** The percentage of c-Fos positive neurons, active at the time of testing in the three hippocampal regions – CA1, CA3 and dentate gyrus, as well as in the hippocampus in general. **(B)** Percentage of tdTomato cells in CA1, CA3, DG and hippocampus (total). No significant differences were observed (n= 3-7 per group, p> 0.05). The error bars represent the standard error mean (SEM).

To identify what percentage of neurons active during the training was also active during the testing phase, the RR was calculated again. Although the vehicle group showed a higher reactivation rate in CA3, DG and the hippocampus compared to the yohimbine-treated groups, we found no statistical difference in the RR between the three treatment groups in none of the analysed regions (Ordinary one-way ANOVA or Kruskal-Wallis test were performed depending on the distribution of the data) (Fig. 14*A*). Furthermore, given the very small sample size, this might change with the analysis of more animals. Consequently, the percentage of overlapping cells was also compared in the DG and the whole hippocampus. The control group displayed a higher percentage of overlapping cells in the DG whereas the 0.3 mg/kg yohimbine group showed a slightly higher overlapping proportion in the whole hippocampus (Figure 14*B*). Both findings were not statistically significant, however, the results should be taken with precaution given the low tdTomato expression and the small sample size.

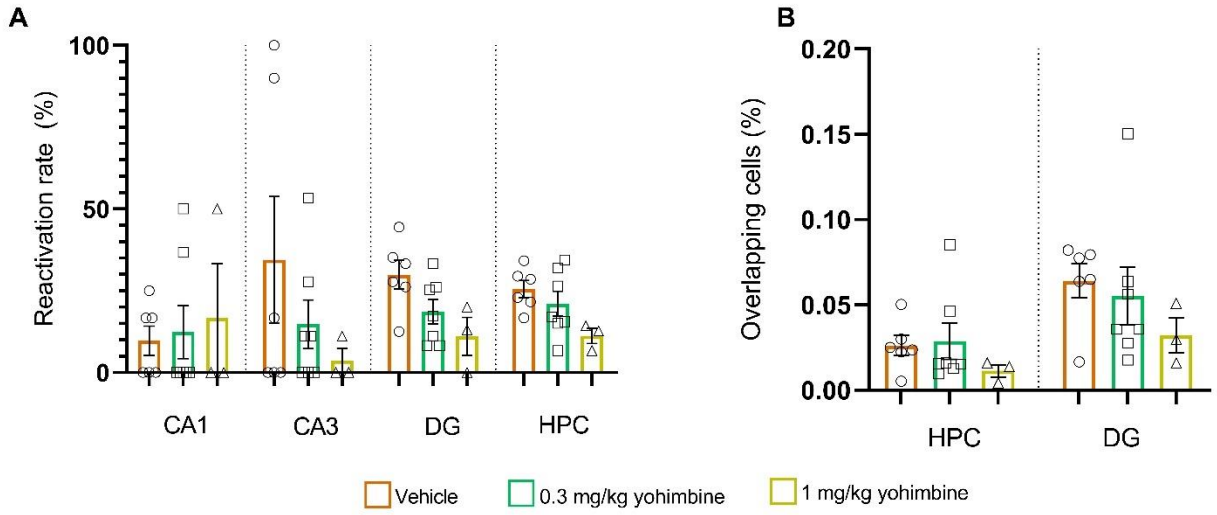

Figure 14. The RR and the overlapping cells (%) at recent memory testing. **(A)** No significant difference was found between the reactivation rates within the three treatment groups. **(B)** The

percentage of overlapping cells does not differ between the saline-treated versus yohimbine-treated mice. Error bars present the standard error mean (SEM). n= 3-7, p> 0.05.

The final step of the analysis was a comparison between the RR of the neurons in the hippocampus and the DI of each animal at a recent time point. The two graphs in figure 15 illustrate the correlation between RR and DI for the vehicle group (fig. 15*A*) and 0.3 mg/kg yohimbine-injected animals (fig. 15*B*). Due to the low number of animals from the 1 mg/kg yohimbine group, the results were not presented. We found no significant correlation between the RR and the DI in both groups (Simple linear regression: n= 6-7 mice, p= 0.213 for control and 0.44 for 0.3 mg/kg yohimbine-treated).

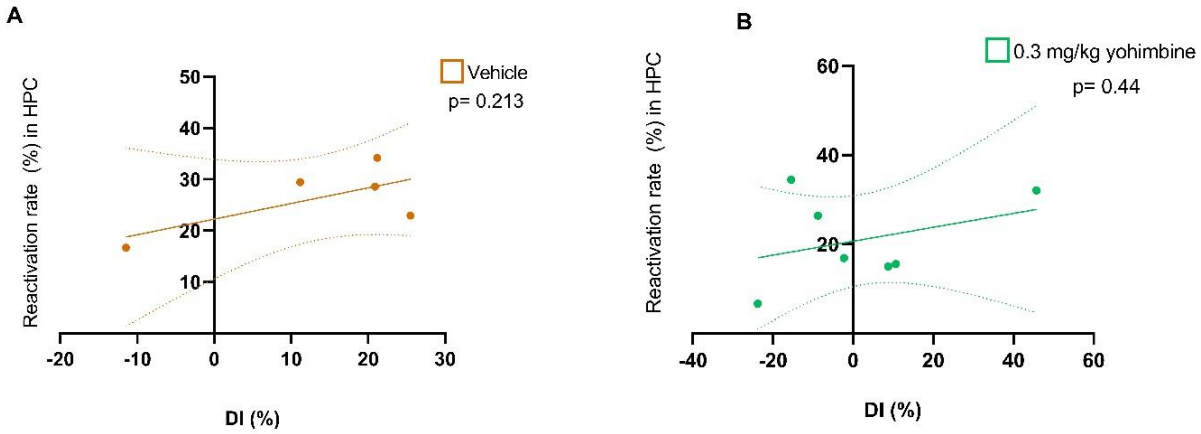

Figure 15. Correlation between RR and DI during recent memory recall. No correlation was observed in the control group **(A)** and the 0.3 mg/kg yohimbine group **(B).**

This set of experiments demonstrated that at recent memory recall the two treatment groups do not significantly differ from the control group regarding their DI. When the DI was compared with the RR of the individual mice, we found no significant correlation between them in either the vehicle nor the 0.3 mg/kg yohimbine group.

The recent memory recall analysis showed no significant difference in the reactivation rates of hippocampal neurons between the control group and the 0.3 mg/kg or 1.0 mg/kg yohimbine-treated mice. However, during the remote memory recall this hippocampal neuronal reactivation was significantly higher for the 0.3 mg/kg yohimbine group in contrast to the vehicle group. Thus, we decided to investigate whether the time point (recent versus remote) can affect the reactivation rates of the neurons in the HPC. We performed a two-way ANOVA test to compare the results of both time point analyses as well as see if the yohimbine administration had any effect. We found that the vehicle group displayed a significantly higher reactivation rate of hippocampal neurons at a recent memory recall compared to the remote memory recall (two-way ANOVA, n= 5-10 per group, p= 0.029(*)). In contrast, the reactivation rate in the 0.3 mg/kg yohimbine-injected mice was similar at both time points (two-way ANOVA, n= 7-10 per group, p> 0.99) (Fig. 16).

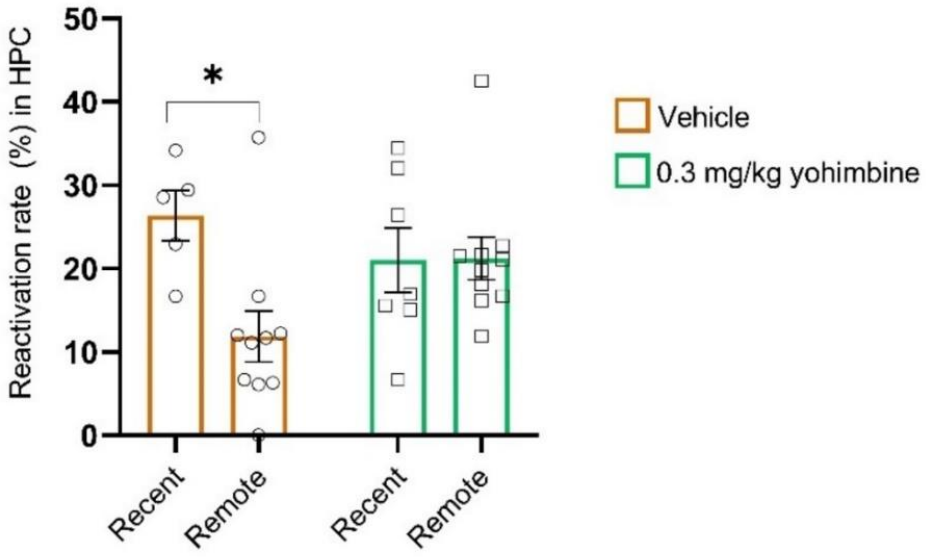

Figure 16. Reactivation rate of HPC neurons in recent versus remote memory. The two-way ANOVA test shows a statistical difference between the recent and remote memory recall of the saline-injected animals (n= 5-10 per group, p= 0.029(*)). The low-dose yohimbine-injected mice showed similar RR at both recent and remote recall sessions.

We observed that while the 0.3 mg/kg yohimbine group showed similar RR at the 3- and 14-day recall, the control group displayed high reactivation at recent memory recall, but this was decreased at the remote memory recall.

4. Discussion

Although an extensive body of literature shows that memories become less dependent on the hippocampus and more dependent on the neocortical structures over time, it has been suggested that emotionally arousing experiences escape this pattern. Instead of becoming more generalized and gist-like which happens to memories during the relocation from the hippocampus to the cortex, highly arousing or stressful memories remain accurate and episodic-like by maintaining their dependency on the hippocampus (Dandolo & Schwabe, 2018) (Krenz et al., 2021). NA was shown to play an important role in this process of maintaining memories accurate by increasing the connectivity with the hippocampus (de Voogd, Fernandez, & Hermans, 2016), however, the cellular mechanisms underlying these effects, especially at the level of memory-representing engram neurons remains unknown.
In this research project, we aimed to investigate the effects of post-training administration of yohimbine, a noradrenergic stimulant, on the accuracy of remote and recent memory at the level of the engram neurons. To label the engram neurons active during the training phase but also during the testing, FosxTRAP2xROSA mice were used. This allowed the permanent labelling of neurons activated over a time window. We trained mice on the OiC to form an episodic-like memory and the accuracy of this memory was evaluated based on the discrimination index. The DI reflects the time mice spent exploring the non-associated object compared to the associated one. In animals where the DI was greater than 0, memory was considered accurate since it implied that the mouse spent more time exploring the object that was not associated with the specific context during the testing. Our results suggest that systemic administration of yohimbine, a noradrenergic stimulant, can enhance memory performance at a remote time point. Interestingly, this yohimbine effect was not seen at a recent time point, which suggests that the better memory performance at the remote time point is not simply caused by a more robust consolidation process shortly after the training session and may indicate that NA acts through different mechanisms on remote and recent memories.

4.1 Noradrenergic effects on memory accuracy – remote versus recent

We found that the 0.3 mg/kg yohimbine-injected mice displayed a statistically greater DI compared to the vehicle group at a remote testing point. In contrast, there was not a statistically significant difference between the yohimbine groups (either 0.3 mg/kg or 1 mg/kg) and the control group at recent memory testing. These results suggest that the administration of yohimbine results in enhanced memory accuracy only at a remote time point. The remote memory findings are in agreement with previous research from Atucha and colleagues (2017) where rats were trained and 28 days later tested on the inhibitory avoidance discrimination task. The authors found that saline-treated rats could not accurately distinguish between the shock and non-shock context at this remote time point while rats that had received an NA infusion bilaterally into the amygdala were able to accurately discriminate between the two contexts. Moreover, when the retention test was performed at a recent time point (2 days after), both the control and the NA-treated group displayed accurate memory. This can imply that regardless of the pharmacological manipulation (including systemic administration of yohimbine), a recent memory recall is maintained accurate because of the dependency on the hippocampus.
The following aspect of the analysis was the reactivation rate of the neurons. The reactivation rate represents the overlapping cells divided by the tdTomato-positive cells. We found that 0.3 mg/kg yohimbine-injected mice displayed a significantly higher reactivation rate in the hippocampal neurons compared to the saline group at 14 days retention test. In contrast, at 3-day retention testing, we did not observe any significant differences between the yohimbine groups and the control group regarding the percentage of overlapping cells or the reactivation

rate. Based on these findings, it is plausible to conclude that post-training administration of yohimbine (0.3 mg/kg) induces accurate memory on remote experiences but not on recent ones. The study from Atucha (2017) suggested that at the 2-day retention test, the saline-treated and NA-treated rats showed equally accurate memories, however, in our case the memory accuracy is not strong at the 3-day retention testing.

The reactivation rate of the hippocampal neurons in each mouse was compared with the discrimination index calculated during the testing. This was done to find whether the findings at the cellular level correspond with the behaviour of each animal during the testing phase of the OiC task. At the 14-day recall session, the vehicle group showed no correlation between the reactivation rate and the discrimination index meaning that some saline-treated animals showed low reactivation rates in the hippocampus even though their DI was high. On the other hand, mice that were administered 0.3 mg/kg yohimbine after training showed a strong trend for a negative correlation between the DI and the RR. This was unexpected because the high discrimination index indicates high memory accuracy, but it appears that some mice treated with yohimbine displayed inaccurate memory after 14 days despite showing a high reactivation rate (indicating that part of the engram was again active during the retention testing). However, after careful inspection of the data, we noticed that this strong trend was driven by a single data point showing a negative DI with high RR (-12,11% and 42.5%, respectively) and without that data point there was no correlation. Moreover, this trend was only found when looking at the RR of the whole hippocampus and not the individual subregions. Nonetheless, this was not according to our expectations as we hypothesized that the systemic administration of yohimbine will enhance memory accuracy and thus yohimbine-injected mice will display a higher reactivation rate with a trend for strong correlation with high DI.

The reactivation rate was compared with the DI also for the animals that underwent a 3-day retention test. We found no correlation between the RR and the DI of individual mice from the vehicle group and the 0.3 mg/kg yohimbine group. This is accompanied by the lack of difference in the discrimination index between the 3 groups of animals as well as by the similar results obtained from the reactivation rate and DI between the saline-treated and 0.3 mg/kg-treated mice. This lack of yohimbine on enhancing memory accuracy at the recent time point can possibly be explained by the fact that we implemented a strong version of the OiC task where mice were trained for 10 minutes in two different contexts. Thus, it is possible that due to the intense training, the memory performance was affected already in the vehicle group regardless of the systemic yohimbine administration. Other studies have shown that yohimbine can enhance memory accuracy of recent memory if animals are weakly trained. For example, preliminary findings on a study in which rats were injected with yohimbine (1 mg/kg) immediately after training on weak fear conditioning protocol indicated that the yohimbine enhanced memory accuracy. Other preliminary findings in which the OiC task was implemented with a training duration of 5, instead of 10 minutes showed that systemic administration of yohimbine immediately after training resulted in enhanced memory performance compared to the saline-treated group.

Additionally, we found significantly higher RR of hippocampal neurons at recent memory recall compared to remote memory recall in vehicle-treated mice. In contrast, the RR of the 0.3 mg/kg yohimbine-injected mice was similar at both time points. These results suggest that at a recent time point, the memory is still accurate due to the hippocampal dependency as we do not observe a difference between the vehicle group and the 0.3 mg/kg yohimbine. This is also in agreement with the abovementioned study from Atucha (2017) where it was found that at a 2-day retention test, the vehicle and the NA-treated animals showed equally accurate memories. In contrast to the recent memory recall finding, our results from the remote memory

recall show that the reactivation of neurons was significantly lower in the control group which could be due to the process of system consolidation and the decrease in hippocampal dependency. Since this difference in reactivation percentage was not found in the 0.3 mg/kg yohimbine group, it can be implied that administration of yohimbine induced the dependency on the hippocampus thereby preserving the memory accurate in comparison with the control group.

4.2 Neuronal activity pattern – remote versus recent

Besides the behavioural analysis, we also assessed the activity pattern of neurons 1) activated during learning, 2) activated during recall and 3) activated in both episodes (i.e., reactivated neurons**).**

In the remote memory testing, we found that the percentage of c-Fos positive cells in the CA3 region of yohimbine-injected animals was significantly higher compared to the control group, however, no significant differences were found between the groups in the other hippocampal regions as well as in the whole hippocampus. During the analysis of the IHC and microscopical results, the n-number of animals increased and the significance of the proportion of the c-Fos+ cells in the 0.3 mg/kg yohimbine-injected animals diminished. This means that none of the two groups showed a significantly higher percentage of c-Fos positive cells in any hippocampal regions (or the hippocampus as a whole). Similarly, during the recent memory testing, we found no difference in the percentage of c-Fos positive cells of the control group and the two groups that received yohimbine in a low or a high dose. This trend was observed in the individual hippocampal subregions (CA1, CA3, DG) but also in the entire hippocampus (which includes the sum of all eleven analyzed subregions). These findings may suggest that the administration of a low dose of yohimbine (0.3 mg/kg) increases the activation of neurons at a remote time point but not at a recent one.

Furthermore, we investigated the percentage of tdTomato-positive cells at both remote and recent time point. However, no significant differences were observed with only one exception. The lower blade of the DG in the low-dose yohimbine-treated mice showed significantly higher tdTomato count compared to the control but this difference diminished in the upper blade and was not seen when the neurons of both blades were summed together. A very important observation was that the percentage of tdTomato-positive cells was very low in all treatment groups as well as in both recent and remote memory recall. All results displayed that the positive tdTomato cells account for less than 1%. Guenthner and colleagues (2013) found that ArcTRAP showed higher background recombination and higher tamoxifen-induced recombination compared to FosTRAP. This may suggest that FosTRAP has lower sensitivity compared to ArcTRAP and thus does not induce the labelling of a higher proportion of neurons. Arc is another immediate early gene (IEG) and hence it is expressed at the time of learning together with c-Fos. A mouse line in which Cre recombinase is expressed under the Arc promoter can be utilized to check whether a higher tdTomato expression will be obtained. However, it is possible that the TRAP system itself has a low sensitivity for labelling and may not be as effective as immunohistochemistry for c-Fos expression.

It is important to also note the limiting factors in this analysis of the recent memory testing: (1) the low number of high-dose yohimbine-injected animals, (2) the large standard error mean found within the groups and (3) the overall low number of animals analyzed so far. Only a proportion of the animals that were tested on the OiC task as reported here. The analysis of the rest of the animals is still ongoing. Therefore, by increasing the number of animals, the variability may decrease and a higher statistical power in the comparisons will be obtained. The higher experimental group sizes would also allow for the assessment of correlation plots

between the DI and the reactivation rates of the neurons. Furthermore, our initial aim was to analyze the 3 brain regions of interest – the hippocampus, prefrontal cortex and amygdala. However, during the course of the experiments, the hippocampus was prioritized. Further understanding of the mechanisms through which NA affects remote and recent memory is needed and thus in the future, the prefrontal cortex and the amygdala need to be included in the analysis to investigate the connectivity between the three regions. A possible direction is reinvestigating the accuracy at 3- and 14-days retention test in the ArcTRAP model and not FosTRAP one to determine whether the tdTomato counts are more similar to the counts of c-Fos expressing neurons. The latter is essential for the accurate reactivation rate calculation and therefore it may lead to different findings. There are also several more scientific limitations of the study which might direct future directions: (1) using immediate early gene expression for the assessment of neuronal activity has certain specific limitations because it takes time (about 1 hour) before peak levels of activity are seen. Moreover, the TRAP system has an activation window that is even less specific in time. Therefore, it is difficult to determine with precision whether these neurons were activated by the encoding or recall of the experience or whether changes in activation patterns might be caused by post-test effects (for example new consolidation processes). (2) We focused here on c-Fos expression as a marker of neuronal activity because of the availability of the FosTRAP mice. However, it could be argued that other neuronal activity markers (e.g., CREB) would give a better impression of the allocation of neurons to a memory engram (Lisman, Cooper, Sehgal, & Silva, 2018). (3) Even if we would have found significant correlations between memory performance and neuronal activation patterns, it is important to realize that these do provide causal evidence. More mechanistic studies, e.g., direct manipulations of neuronal activity, are necessary to determine a direct causal relationship.

5. Conclusion

In this research project, we aimed to study the effects of systemic post-training yohimbine administration on the accuracy of both recent and remote memory at the level of engram neurons. Several research questions were implemented to reach our aim. The most important findings were that yohimbine-treated mice showed an enhanced memory accuracy 14 days after training on the OiC task which was supported by the significant difference in the discrimination indexes between the control and the yohimbine (0.3 mg/kg) group. In contrast, we found no differences between the yohimbine group and the control in the accuracy of OiC memory at a recent time point, suggesting that NA effects on recent and remote memories are established through different mechanisms. Furthermore, we found no correlation between the RR and DI of individual mice in both groups at recent time point. In comparison, at a remote memory recall, the yohimbine-injected group showed a negative correlation while the control group – did not correlate. At a cellular level, regarding the percentage of tdTomato+ and c-Fos+ cells, the vehicle group and the yohimbine-treated group showed no difference in both recent and remote memory recall. However, due to the limitations of the FosTRAP system and the low n-number of animals analyzed so far on the recent memory recall, these results cannot be considered reliable. An important finding was that while the 0.3 mg/kg yohimbine-injected group maintained similar RR at the remote and recent time point, the saline-injected mice displayed a significantly higher reactivation of hippocampal neurons at a recent time point, suggesting that yohimbine effects are only observed at a remote time point when the process of systems consolidation has begun and the memory is not fully dependent on the hippocampus. Although more data is still needed to reach our aim, the hypothesis that was set at the beginning: "*The administration of yohimbine immediately after training results in an accurate, episodic-like memory compared to the control group*" was only confirmed by the remote memory testing data. Our current findings may indicate that NA acts through different mechanisms on remote and recent memories. Therefore, further understanding of the process of systems consolidation is required.

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Appendices

Appendix A: Image pre-processing protocol

If a setting is not specifically mentioned in the protocol, we used the default settings.

1. In all folders that contain tile images for each slice of every animal create a new folder named ‘Corrected’.
2. Start with the first slice and open all the tile images from channel ch00 in ImageJ
3. In ImageJ, go to Image - Stacks - Images to Stack, and press ‘Ok’. ImageJ then makes one stack of all the pictures that are open and names it ‘Stack’.
4. Then go to Plugins – BaSiC. At ‘processing stack’ you should fill in the newly created ‘stack’ file. If you then press OK, ImageJ will calculate the average distribution of the signal over your entire stack and correct for it. It then creates a ‘Corrected’ stack.
5. Save this corrected stack via File - Save as - Image sequence and click "Use slice labels as file names". Save these corrected photos in the ‘Corrected’ folder you created
6. Carry out steps 2-5 for the other two channels (ch01 and ch02).

Next, we need to stitch the tiles. It is therefore important to know the dimensions of your photo.

7. Go to Plugins - Stitching - Grid/Collection stitching.
8. First fill in how you want to stitch your tiles. Select ‘Grid: column-by-column.
9. Next you must fill in your grid size; how many tiles you have in the x and y directions. The other settings are:
    - First file index i: 0
    - Directory: here you enter the path to the ‘Corrected’ folder you have created in step 1. You select it via Browse.

File names for tiles: here you enter the file name of 1 of the tiles that you want to stitch, and replace the exact tile number with {ii} or {i} (depending on whether it has 2 or 1 digit). This will search for all files with that name.

Uncheck all the boxes below and set Computation parameters to ‘Save computation time’.

This way you will see a file "Fuse" in which all your tiles are stitched, and you can immediately see whether this went well.

10. Repeat step 9 for ch01 and ch02.
11. While all the fused files for one slice are open, go to Image - Stacks - Images to Stack, and press Ok. Now the images are stitched fully and corrected for shading. Save this file for further analysis.

Appendix B: Calculation formulas

To calculate the percentage of c-Fos+ neurons:

$$\frac{cFos + cells}{total\ number\ DAPI\ cells} * 100\%$$

To calculate the percentage of tdTomato+ neurons:

$$\frac{tdTomato + cells}{total\ number\ DAPI\ cells} * 100\%$$

To calculate the RR of the neurons:

$$\frac{Overlap\ cells}{tdTomato + cells} * 100\%$$

Appendix C: Buffers and Solutions preparation

1) 4-Hydroxytamoxifen preparation

The 4-OHT was used to label the active neurons at the time of training, and it was administered via intraperitoneal injection. The volume injected in each mouse was different and dependent on the weight of the animal. The final concentration of the 4-OHT was 10 mg/ml and the dosage used was 50 mg/kg per mouse. For 50 mg of 4-hydroxytamoxifen powder, 0.5 mL of 100% ethanol was added. The tube was sonicated for 1 hour at 55°C and in the meantime, it was vortexed thoroughly every 15 minutes. Then 4.5 ml of 100% corn oil was added and the tube was sonicated for one hour at 55°C. The 4-OHT was stored in the freezer and moved to 4 °C a day before the training sessions. On the day of training, the 4-OHT was sonicated at 50 °C for 30 minutes. The 4-hydroxytamoxifen is light-sensitive and thus, the tube needs to be wrapped in aluminium foil and always kept in the dark.

2) Yohimbine

To prepare the 1 mg/kg yohimbine, 1 mg of yohimbine was dissolved in 10 ml saline (0.9%) obtaining a stock of 0.1 mg/ml. From the 0.3 mg/kg solution, the 1 mg/kg stock was diluted with saline. They were prepared right before the onset of the experiment.

3) 4% PFA

Forty grams of PFA powder was added to 300 ml of MilliQ water. The mixture was stirred and heated to 60°C and once the temperature was reached, 1 pellet of NaOH was added. The temperature was then lowered to 20°C and 12.8 g of Na2HPO4.2H2O (Sodium hydrogen phosphate dihydrate) was added followed by 3.8 g of NaH2PO4. H2O (Sodium phosphate monobasic monohydrate). The solution was filled up to 1L with MilliQ and the pH was adjusted to 7.2. The PFA was frozen after preparation and then stored at 4°C for one day before perfusions. Considering the safety data sheet of the chemicals used, the procedure was executed in a fume hood, and gloves and a face mask were also worn.

4) 30% Sucrose

Thirty grams of sucrose powder are dissolved in 100 ml of 1x PBS.