Acquisition of spatial search strategies and reversal learning in the Morris water maze depend on disparate brain functional connectivity in mice

Acquisition of spatial search strategies and reversal learning in the Morris water maze depend on disparate brain functional connectivity in mice

Running title: Acquisition and reversal spatial learning in mice depends on disparate brain functional connectivity

Authors:

Disha Shah[1,2,3], Marleen Verhoye[1], Annemie Van der Linden[1], Rudi D'Hooge[2]

Author affiliation:

[1] Bio-Imaging Lab, University of Antwerp, Univsersiteitsplein 1, 2610, WIlrijk, Belgium.

[2] Laboratory of Biological Psychology, KU Leuven, Tiensestraat 102, 3000, Leuven Belgium

[3] Laboratory for the Research of Neurodegenerative Diseases, VIB center for Brain and Disease Research, KU Leuven, O&N4 Herestraat 49 box 602, 3000, Leuven, Belgium

Keywords: Functional connectivity, Morris water maze, mouse brain, search strategy, spatial learning.

Abstract

Learning has been proposed to coincide with changes in connections between brain regions. In the present study, we used resting-state fMRI (rsfMRI) to map brain-wide functional connectivity (FC) in mice that were trained in the hidden-platform version of the Morris water maze. C57BL6 mice were investigated in a small animal MRI scanner following 2, 10 or 15 days of acquisition learning, or 5 days of reversal learning. Spatial learning coincided with progressive and changing FC between telencephalic regions that have been implemented in spatial learning (such as hippocampus, cingulate, visual and motor cortex). Search strategy assessment demonstrated that the use of cognitively advanced spatial strategies correlated positively with extensive telencephalic connectivity, whereas non-spatial strategies correlated negatively with connectivity. FC patterns were different and more extensive after reversal learning compared to after extended acquisition learning, which could explain why reversal learning has been shown to be more sensitive to subtle functional defects.

Introduction

The dominant hypothesis in contemporary neuroscience states that learning and memory are based on structural and functional changes in connectivity within and between brain regions (Kandel 2001; Eichenbaum 2008). Particular brain structures such as hippocampus have been implemented in different forms of learning that have been extensively studied in humans and other mammals (Kandel et al. 2014). Learning impairments of some sort occur in all major brain disorders, and the study of the mechanisms that underlie the brain's ability to store, update, retain and recall information has been a central topic in various research traditions (Tonegawa et al. 2015). Synaptic plasticity is the central cellular mechanism that changes connectivity between neurons, a process that could extend across several brain regions, and allows an organism to learn to solve a task or acquire knowledge (D'Hooge and De Deyn 2001; Latif-Hernandez et al. 2016; Pooters et al. 2017). Notably, it is supposed to alter functional connectivity between brain regions during learning (Nasrallah et al. 2016), which is the focus of the present study.

The Morris water maze remains the most widely used task to investigate spatial learning and memory, and model neurocognitive disorders in laboratory rodents (D'Hooge and De Deyn 2001). Spatial learning and memory in such animals have been suggested to represent a close equivalent to higher-

order cognitive functions in humans, in particular, episodic memory abilities (Morellini 2013). The task requires animals to use distal cues to navigate a circular pool and locate a hidden escape platform. Elaborate water maze protocols often include a phase of acquisition learning, during which the animals learn to use distal cues to locate the hidden platform, and a reversal phase, where the platform is relocated and the animals are required to learn the new platform position (Vorhees and Williams 2006).

Water maze learning has been shown to depend mainly on the integrity of telencephalic structures, most notably hippocampus (Eichenbaum et al. 1990; McNamara et al. 1993; Cho et al. 1998; Riedel et al. 1999; Martin and Clark 2007; Eichenbaum 2017). Involvement of hippocampus in the ability of rats and mice to solve the water maze by constructing an allocentric spatial map has been well established (Eichenbaum et al. 1990). More recent work indicated that the hippocampus acts as a central hub in a dynamic hippocampal-cortical network that is recruited during episodic memory acquisition and retrieval in humans as well as rodents (Benchenane et al. 2010; Watrous et al. 2013; Nasrallah et al. 2016). The hippocampus is extensively connected to cortical and subcortical structures and during the early phases of water maze learning in rats, connectivity increases in this hippocampocentric network and other relevant brain regions (Nasrallah et al. 2016). Connections between cingulate, retrosplenial, motor, and sensory cortices appear to be particularly relevant in the cognitive processes involved in spatial learning, such as attention, coupling of spatial processing to recognition memory, decision-making, navigation etc. (Warburton et al. 1998; Vann and Aggleton 2002, 2004; Knierim 2006; Keene and Bucci 2009; Godsil et al. 2013; Vogt and Paxinos 2014; Chersi and Burgess 2015; Pooters et al. 2017). Notably, neurons in visual cortex are known to play a role in spatial processing and exhibit location-specific firing activities that overlap with those of hippocampal neurons (Knierim 2006; Haggerty and Ji 2015). Moreover, subcortical structures such as caudate putamen and nucleus accumbens have also been found to be involved in specific stages of water maze acquisition and navigation (Mizumori et al. 2001; De Leonibus 2005; Woolley et al. 2013). Reversal learning and other aspects of cognitive flexibility (i.e., the ability to adapt learnt responses to changing environmental demands) has been shown to involve prefrontal cortex in humans and rodents. Rodent prefrontal cortex comprises a ventral i.e. prelimbic/infralimbic cortex, and a dorsal part i.e. anterior cingulate cortex (Uylings et al. 2003), which have been suggested to control cognitive

flexibility in a way homologous to prefrontal regions in primate brain (Dalley et al. 2004; Ragozzino and Rozman 2007; Goyal et al. 2008; Leber et al. 2008).

In the present study, we investigated the changes in functional connectivity (FC) between these relevant brain structures during an extended water maze protocol that has been used in many studies of spatial learning and neurocognitive impairment in rodents (Pooters et al. 2015). Resting-state fMRI (rsfMRI) was used to map FC based on the temporal correlations between BOLD signals of defined brain regions after 2, 10 and 15 days of acquisition training, and after reversal training. We correlated FC to the search strategy that mice use to locate the escape platform to identify brain connections that are crucial for task proficiency. The present work illustrates the ability of non-invasive MRI methodology to detect learning-induced changes in brain connectivity. This could inspire innovative approaches to investigate learning and memory processes longitudinally and *in vivo* in the healthy brain, and how these processes are affected by pathology. Obviously, we expected that the use of cognitively advanced spatial strategies would depend on extensive telencephalic connectivity. More specifically, we hypothesize that FC in the mouse brain changes during the course of extended training, and moreover that acquisition and reversal learning evoke different FC patterns."

Methods

Ethics statement and animals. Female C57BL/6 mice (C57BL/6, Jax mice strain, Charles River Laboratories), 12 weeks of age, were used, and all experimental procedures were performed in strict accordance with the European Directive 2010/63/EU on the protection of animals used for scientific purposes. The protocols were approved by the Committee on Animal Care and Use at KU Leuven, Belgium (permit number 2015-76), and all efforts were made to minimize animal suffering.

Spatial learning in the Morris water maze. The Morris water maze test was performed to assess spatial memory that relies on distal cues to locate a submerged platform (15 cm diameter) in an open circular swimming arena (150 cm diameter) filled with opaque water (non-toxic white paint, (26 ± 1 °C), as previously described (D'Hooge and De Deyn 2001) . The protocol included 2, 10 or 15 days of acquisition training, where each daily session consisted of 4 swimming trials (15 min interval

between trials) starting randomly from 4 starting positions. Swimming tracks were recorded using video hardware and Ethovision software (Noldus, The Netherlands). Mice that failed to find the platform within 120 s were guided to it and remain there for 15 s before being returned to their cages. Reference memory was determined by preference for the platform area when the platform is absent, and was tested by probe trials (100 s) after 5 and 10 acquisition sessions i.e. on days 6 and 11. For the probe tests the swimming trial started from the quadrant opposite to the target quadrant i.e. the platform area. After 10 days of acquisition training, when the mice have established a robust preference for the platform location, reversal training was performed during 5 days, during which the location of the platform was changed, thus requiring relearning and cognitive flexibility, and another probe trial was performed on day 16.

RsfMRI was performed in separate groups of mice after 2 days, 10 days or 15 days of acquisition training and after reversal training, i.e. 10 days acquisition followed by 5 days of reversal training. For each training protocol two control groups were added, yielding 3 groups for each training protocol (**Supplementary Figure S1 A and B**):

- Cage controls (N=10/training) were not in contact with water and remained in the same room as the swim controls and cognitive trained mice during the experiments.
- Swim controls (N=10/training) were placed in the Morris water maze after the platform was removed and swam on average as long as the cognitive trained group. This group should not have undergone extensive spatial memory training, since the platform was not present, and served to control for the motor aspect of training in the Morris water maze and stress due to aversion to water.
- The cognitive trained group (N=12/training) were subjected to standard protocol as described above, thus using distal cues to find the location of the platform and train spatial memory.

Analyses included calculating path length and % time spent in the target quadrant during the training sessions, and % time spent in each quadrant during the probe trials. Statistical analyses included One-way and Two-way ANOVA with Sidak correction for multiple comparisons ($p<0.05$). Spatial strategies were analysed using an in house program in MATLAB (MATLAB R2013a, The MathWorks Inc. Natick, MA, USA). We used an approach described previously to classify the search strategies

(Lo et al. 2014). Briefly, each track was classified to a particular strategy using binary support vector machine (SVM) classifiers and a previously described 9-category scoring system (Latif-Hernandez et al. 2016). The manner in which mice search for the hidden platform can be categorized into three main strategies, which can be further subdivided into several subcategories. Spatial strategies were calculated for the last day of each training session, as this time point is most comparable to when FC-MRI scans were acquired. Strategies were classified as previously described (Brody and Holtzman 2006; Latif-Hernandez et al. 2016). Spatial strategies include 1) spatial direct (swimming directly to the platform), 2) spatial indirect (Swimming towards the platform with one explorative loop), and 3) focal correct (searching for the platform in the correct quadrant). Non-spatial strategies include 4) scanning (searching in the center of the pool), 5) random (lack of preference for any quadrant of the pool), and 6) focal incorrect (searching in the wrong quadrant). Furthermore, mice could use repetitive strategies which include 7) chaining (circular swimming motions in the target annulus region), 8) thigmotaxis (peripheral looping), and 9) circling (swimming in tight circles, possibly with some direction). One-way ANOVA with Tukey correction for multiple comparisons ($p<0.05$) were performed for each training to assess which strategy was employed. Correlation between FC and spatial strategies was calculated using Pearson correlations (FDR corrected, $p<0.05$). Functional connections that were included were the ones that showed a significant group difference (swim controls vs. cognitive trained animals) for each of the training protocols.

Resting-state functional MRI procedures. RsfMRI allows the investigation of functional connectivity between brain regions at rest by measuring the temporal correlation of low frequency fluctuations (0.01-0.25 Hz) of the blood-oxygen-level-dependent (BOLD) signal in distinct brain regions. For the MRI handling procedures all mice were anesthetized with 2.5% isoflurane (IsoFlo, Abbott, Illinois, USA), which was administered in a mixture of 70% nitrogen (400 cc/min) and 30% oxygen (200 cc/min). During the rsfMRI imaging procedures, a combination of medetomidine (Domitor, Pfizer, Karlsruhe, Germany) and isoflurane was used to sedate the animals (Shah et al. 2016). After positioning the animal in the scanner, medetomidine was administered subcutaneously as a bolus injection (0.3 mg/kg), after which the isoflurane level was immediately decreased to 1%. Ten minutes before the rsfMRI acquisition, isoflurane was decreased to 0.4%. RsfMRI scans were consistently

acquired 40 min after the bolus injection, during which the isoflurane level was kept at 0.4%. After the imaging procedures, the effects of medetomidine were counteracted by subcutaneously injecting 0.1mg/kg atipamezole (Antisedan, Pfizer, Karlsruhe, Germany). The physiological status of all animals was monitored throughout the imaging procedure. A pressure sensitive pad (MR-compatible Small Animal Monitoring and Gating system, SA Instruments, Inc.) was used to monitor breathing rate and a rectal thermistor with feedback controlled warm air circuitry (MR-compatible Small Animal Heating System, SA Instruments, Inc.) was used to maintain body temperature at (37.0 ± 0.5) °C.

MRI procedures were performed on a 9.4T Biospec MRI system (Bruker BioSpin, Germany) with the Paravision 5.1 software (www.bruker.com). Images were acquired using a standard Bruker cross coil set-up with a quadrature volume transmit coil and a quadrature surface receive coil for mice. Three orthogonal multi-slice Turbo RARE T2-weighted images were acquired to render slice-positioning uniform (repetition time 2000 ms, echo time 33 ms, 16 slices of 0.4 mm with a gap of 0.1 mm). Field maps were acquired for each animal to assess field homogeneity, followed by local shimming, which corrects for the measured inhomogeneity in a rectangular VOI within the brain. Resting-state signals were measured using a T2*-weighted single shot EPI sequence (repetition time 2000 ms, echo time 15 ms, 16 slices of 0.4 mm with a gap of 0.1 mm, 300 repetitions). The field-of-view was (20 x 20) mm^2 and the matrix size (128 x 64), resulting in voxel dimensions of (0.156 x 0.312 x 0.5) mm^3.

MRI data pre-processing and analysis. Pre-processing of the rsfMRI data, including realignment, normalization and smoothing, was performed using SPM12 software (Statistical Parametric Mapping, http://www.fil.ion.ucl.ac.uk). First, all images within each session were realigned to the first image. This was done using a least-squares approach and a 6-parameter (rigid body) spatial transformation. For the rsfMRI data analyses, motion parameters resulting from the realignment were included as covariates to correct for variation in intensity related to possible movement that occurred during the scanning procedure. Second, all datasets were normalized to a study specific EPI template and co-registered to a study-specific anatomical T2-weighted template. The normalization steps consisted of a global 12-parameter affine transformation followed by the estimation of the nonlinear deformations. Finally, in plane smoothing was done using a Gaussian kernel with full width at half maximum of twice

the voxel size (0.31 X 0.62 mm²). All rsfMRI data were filtered between 0.01-0.25 Hz using the REST toolbox (REST1.7, http://resting-fmri.sourceforge.net).

Masks containing 4 voxels within the individual brain regions-of-interest (ROI) were defined using MRicron software (MRicron version 6.6, 2013, http://www.mccauslandcenter.sc.edu/mricro/; **Supplementary Figure S1 C**): medial prefrontal cortex (mPFC), cingulate cortex (Cg), retrosplenial cortex (Resp), hippocampus dentate gyrus (HC_DG), hippocampus CA1 region (HC_CA), sensory cortex (SC), caudate putamen (Cpu), thalamus (T), motor cortex (MC), and visual cortex (VC). These ROIs were then used for ROI-correlation analyses, where pairwise correlation coefficients between each pair of ROIs were calculated and z-transformed using an in-house program developed in MATLAB (MATLAB R2013a, The MathWorks Inc. Natick, MA, USA). Mean z-transformed FC matrices were calculated for each group. Statistical analyses of the rsfMRI data were performed for each protocol separately between cage controls and swim controls, and between swim controls and cognitive trained animals using One-way ANOVA with false discovery rate (FDR) correction for multiple comparisons ($p<0.05$).

Additionally, seed-based analyses were performed by computing individual z-transformed FC-maps of the left prefrontal cortex and left visual cortex using REST toolbox, resulting in FC-maps for each of these seed regions for each group. Statistical analyses of the FC-maps included a One-sample T-test for within group analyses, and included a Two-way ANOVA for between group analyses (Tukey correction for multiple comparisons).

Results

Spatial learning and memory in the extended Morris water maze protocol. After 2 days of acquisition training (**Figure 1A-D**), mice that were trained to find the location of the platform spent significantly more time in the target quadrant compared to swim controls (Two-way ANOVA, group effect $F_{1,20}=10.16$, $p=0.0046$). This effect became even more pronounced after 10 days of acquisition training (Two-way ANOVA, group effect , $F_{1,19}=107.1$, $p<0.0001$), 15 days of acquisition training (Two-way ANOVA, group effect , $F_{1,20}=257.3$, $p<0.0001$), and after reversal training (Two-way ANOVA, group effect $F_{1,20}=30.09$, $p<0.0001$,). Probe trials after 10 days acquisition show no significant group

effect between swim controls and trained mice (Two-Way ANOVA probe 1:$F_{1,76}$=0.02, p<=0.887, probe 2: $F_{1,74}$=0.6694, p=0.4159). However, post-hoc tests (Sidak correction for multiple comparisons) revealed that trained animals spent significantly more time in the target quadrant compared to swim controls during both probe tests (Two-way ANOVA, probe 1 p=0.0027; probe 2 p<0.0001). Swim controls tended to spend more time in the opposite (Two-way ANOVA, probe 1 p=0.009, probe 2 p=0.0005) and adjacent quadrants (Two-way ANOVA, probe 2 p=0.0007) as shown in **Figure 1E-F**. During the third probe trial of 15 days training (i.e., acquisition and reversal learning), trained animals spent significantly more time in the new target quadrant (Two-way ANOVA, acquisition: p<0.0001, reversal: p<0.0001), whereas swim controls spent more time in the opposite quadrant (Two-way ANOVA, acquisition: p<0.0001, reversal: p=0.007) as shown in **Figure 1G-H**.

The strategies that the mice applied to locate the platform were divided into spatial, non-spatial or repetitive strategies (Latif-Hernandez et al. 2016). Significant differences were observed in the applied strategy after 2 days of acquisition training (One-way ANOVA, p<0.0001), 10 days of acquisition training (One-way ANOVA, p=0.0034), 5 days of extended acquisition training (One-way ANOVA, p<0.0001), and 5 days of reversal training (One-way ANOVA, p<0.0001). For each training the predominant strategy, however, was different. Post-hoc comparisons (Tukey correction for multiple comparisons) revealed that after 2 days of training that non-spatial (p<0.0001) and repetitive (p=0.0004) strategies were used predominantly over spatial strategies, and that non-spatial strategies were applied more compared to repetitive strategies (p<0.0001) as shown in **Figure 1I**. After 10 days of training spatial (p=0.0024) and repetitive strategies (trend, p=0.08) were applied predominantly over non-spatial strategies (**Figure 1J**). After 5 continued days of acquisition training spatial strategies were applied predominantly over non-spatial (p<0.0001) and repetitive (p<0.0001) strategies (**Figure 1K**). Similarly, after 5 days of reversal spatial strategies were applied predominantly over non-spatial (p<0.0001) and repetitive (p<0.0001) strategies (**Figure 1L**).

Brain FC after 2 and 10 days of acquisition training

FC was assessed using ROI-based analyses between the frontal cortex, cingulate cortex, retrosplenial cortex, hippocampus (dentate gyrus DG and CA1 region), sensory cortex, caudate putamen, thalamus, motor cortex and visual cortex. After 2 days or 10 days of acquisition training, no significant differences were observed between cage controls and swim controls after correcting for

multiple comparisons (**Supplementary Figure S2)**. After 2 days of training, however, significant differences were observed between swim controls and trained mice in FC of cingulate and motor cortex (p=0.006), sensory and motor cortex (p=0.021), retrosplenial cortex and hippocampus (DG) (p=0.004), and hippocampus (DG) and visual cortex (p=0.031) as shown in **Figure 2**. After 10 days of acquisition training, a different pattern of FC changes was observed (**Figure 3**), and significant differences were observed between swim controls and trained mice in FC of prefrontal and cingulate cortex (p=0.017), cingulate and retrosplenial cortex (0.01), cingulate cortex and hippocampus (CA1) (p=0.008), hippocampus dentate gyrus and CA1 area (p=0.031), hippocampus (DG) and motor cortex (p=0.021), and caudate putamen and motor cortex (p=0.034) as shown in **Figure 2**.

Differences in brain FC between extended training and reversal learning

After 5 days of extended acquisition training or 5 days of reversal training, no significant differences were observed between cage controls and swim controls after correcting for multiple comparisons (**Supplementary Figure S2**). After 5 days of extended acquisition training, significant differences were observed between swim controls and trained group in FC of frontal cortex and hippocampus (DG, p=0.009 ; CA1, p<0.0001), frontal cortex and caudate putamen (p=0.015), frontal and motor cortex (p=0.002), frontal and visual cortex (p=0.026), retrosplenial cortex and hippocampus (DG) (p=0.021), CA1 and DG of hippocampus (p=0.033), and hippocampus (DG) and thalamus (p=0.001) as shown in **Figure 2**.

After 5 days of reversal training, significant differences were observed between swim controls and trained group in FC of frontal and visual cortex (p=0.022), cingulate and retrosplenial cortex (p=0.025), cingulate and visual cortex (p=0.026), retrosplenial cortex and hippocampus (DG) (p=0.024), retrosplenial cortex and visual cortex (p=0.012), hippocampus dentate gyrus and CA1 area (p=0.034), hippocampus (DG) and caudate putamen (p=0.028), hippocampus (DG) and motor cortex (p=0.025), hippocampus (DG) and visual cortex (p=0.03), hippocampus (CA1) and sensory cortex (p=0.013), hippocampus (CA1) and visual cortex (p=0.021), and sensory and visual cortex (p=0.012) as shown in **Figure 2**.

These results show that average FC of the prefrontal cortex with other brain regions substantially increases during extended acquisition training. During reversal training, however, it is the visual cortex that markedly increases its FC with other cortical and subcortical brain regions (**Figure 3**). This was

confirmed with a seed-based analysis of the left prefrontal cortex and left visual cortex, which demonstrated a significant difference between groups (Two-way ANOVA, $F_{2,53}$=9.0006, p<0.0004) as shown in **Figure 4**. Post-hoc comparison (Tukey's correction for multiple comparisons) of prefrontal cortex showed a significant difference with swim controls (prefrontal cortex: p=0.0001) and reversal learning (p=0.0037). Similarly, post-hoc comparison of the visual cortex showed a significant difference with swim controls (p=0.0006) and acquisition learning (p=0.0063).

Correlation between applied strategy and brain FC

Significant differences in FC were observed between swim controls and animals that were trained to find the platform in the Morris water maze. The way the brain reorganized after spatial training was different between early and later phases of acquisition training and between acquisition and reversal training (**Figure 3**). We wanted to investigate whether these different FC patterns after acquisition and reversal training were correlated to the use of different strategies during Morris water maze training (i.e., spatial, non-spatial and repetitive strategies). After 2 days of acquisition training there was a predominant use of non-spatial and repetitive strategies (**Figure 1I).** Overall, FC showed negative correlation with non-spatial strategy, which was statistically significant for FC of sensory and motor cortex (r= -0.814, p= 0.001). Overall positive correlations however were observed with the repetitive strategy, which was statistically significant for FC of cingulate and motor cortex (r= 0.725, p= 0.008) as shown in **Figure 5**.

After 10 days of acquisition training, mice mainly employed spatial and repetitive strategies (**Figure 1J**). Globally, FC showed positive correlation with the spatial strategy, which was statistically significant for FC of cingulate cortex and hippocampus CA1 region (r= 0.839, p= 0.005). In contrast to 2 days of training, the repetitive strategy showed overall negative correlations with FC, which was statistically significant for FC of cingulate cortex and hippocampus CA1 region (r= -0.637, p= 0.04), hippocampus dentate gyrus and motor cortex (r= -0.767, p=0.01), and caudate putamen and motor cortex (r= -0.768, p= 0.01) as shown in **Figure 5**.

After 5 days of continued acquisition training, mice primarily used spatial strategies (**Figure 1K**). Generally, FC showed a positive correlation with the use of the spatial strategy, which was statistically significant for FC of frontal cortex and hippocampus (CA1) (r= 0.696, p= 0.025), frontal cortex and

caudate putamen (r= 0.714, p= 0.02), frontal and visual cortex (r= 0.748, p= 0.013), and DG and CA1 regions of hippocampus (r=0.676 , p= 0.023) as shown in **Figure 5**).

After 5 days of reversal training, mice mainly engaged spatial strategies (**Figure 1L**). FC largely showed a positive correlation with the use of the spatial strategy, which was statistically significant for FC of visual cortex and cingulate cortex (r= 0.858, p= 3.6*10^-4), visual cortex and retrosplenial cortex (r= 0.613, p= 0.03), hippocampus CA1 and dentate gyrus (r= 0.710, p= 0.01), visual cortex and hippocampus CA1 (r=0.620 , p= 0.005), and visual cortex and hippocampus dentate gyrus (r=0.751, p= 0.03) as shown in **Figure 5**.

Discussion

We have mapped progressive changes in FC between relevant brain regions during extended spatial acquisition and reversal learning in the hidden-platform water maze task. Also, we investigated the relationship between task proficiency and brain FC, and finally, questioned whether reversal learning induces a different pattern of brain FC from extended training on the same platform location. Overall, connectivity between telencephalic regions increased during the course of the 15-day training period, even involving thalamus at the end of training. This illustrates increasing and/or persistent interaction between different cortical regions during spatial learning, also connecting to subcortical and diencephalic regions. Moreover, we did find a different pattern of brain connections in mice that continued on the same platform location compared to those that had to switch to another platform position (reversal group). Already after 2 days of acquisition, trained animals displayed prominent motor cortex FC with cingulate and sensory cortex, and hippocampus with retrosplenial and visual cortex, compared to swim controls. Increased cingulate cortex FC with motor cortex confirms the joint involvement of these regions in egocentric navigation and control of coordinated motor action (Chersi and Burgess 2015). These particular FCs already played a role during the initial phase of spatial learning, and were not significantly altered after 10 days of training or after reversal learning.

Significant and increasing connectivity between hippocampus and other telencephalic regions (and thalamus) further illustrates the prominent and central role of this brain region in spatial learning and memory. Already during the initial acquisition phase, hippocampal connections to other cortical regions appeared to be essential for spatial navigation, and further increased during the extended training period. Very early onwards, hippocampus was already significantly connected to retrosplenial

and visual cortex. This converges with reports that implicated retrosplenial cortex in aspects of spatial learning such as place recognition and allothetic/egocentric navigation (Vann and Aggleton 2002, 2004; Keene and Bucci 2009; Sherrill et al. 2013). Involvement of visual cortex is also obvious given its role in visual perception and visual action control, and the fact that it contains a large number of neurons that exhibit location-specific firing activity, which overlaps with that in hippocampal neurons (Goodale and Milner 1992; Wang et al. 2012; Haggerty and Ji 2015).

After a longer period of training (10 days of acquisition learning), there was a reorganization of functional connections. More specifically, cingulate cortex FC with prefrontal, retrosplenial and hippocampal areas became more important. Persistent recruitment of functional connections with the prefrontal cortex could indicate the importance of flexible and goal-directed behaviour (Dalley et al. 2004; Goyal et al. 2008). Increased cingulate cortex FC with retrosplenial and hippocampal areas suggests involvement of connections that support attention, working memory, memory consolidation, coupling of spatial processing and recognition memory, decision making, and goal-directed behaviour (Godsil et al. 2013; Leech and Sharp 2014; Vogt and Paxinos 2014). Other functional connections that were increased after 10 days of acquisition learning included FC between motor cortex and caudate putamen, which are involved in goal-directed guiding of locomotion (i.e., selection of future responses based on their past consequences) (Mizumori et al. 2001, 2005; Whitlock et al. 2008). These observations are consistent with a study in rats that described increased hippocampal FC with cingulate cortex, thalamus and striatum during initial stages of spatial learning, and with retrosplenial, sensory and visual cortex at later stages (Nasrallah et al. 2016). These data are also consistent with studies in humans, which showed increased hippocampal and striatal FC after learning in the virtual maze (Woolley et al. 2015). After 5 days of continued acquisition training, the role of the prefrontal cortex becomes more significant, suggesting increasing importance of goal-directed locomotion. After reversal training, we still observed FC between hippocampus and cingulate cortex, as well as between hippocampus and sensory cortex, suggesting that these connections continued to control attention, working memory, consolidation of memory processes, spatial processing, recognition memory, and decision making.

Notably, reversal learning involved a shift in FC patterns compared to acquisition training. There was still prominent prefrontal connectivity, since reversal learning represents an aspect of cognitive flexibility, which requires the involvement of prefrontal and cingulate regions (Dalley et al. 2004;

Ragozzino and Rozman 2007; Leber et al. 2008). In addition, visual cortex FC with frontal, cingulate, retrosplenial, and sensory cortex became prominent, as well as hippocampal FC with sensory cortex. Visual cortex is important for spatial memory and processing (Poucet et al. 2003; Tsanov and Manahan-Vaughan 2008), and its increased FC with other brain regions after reversal likely indicates intensified visual exploration and processing when previous navigational responses are no longer valid, and procedures and associations need to be updated.

Finally, we hypothesized that the deployment of specific search strategies during task performance evoke different FC patterns. Our analyses firstly confirm that each training phase has a specific pattern of strategy deployment, which changes as training proceeds and task proficiency increases, eventually leading to the preferred use of cognitively advanced, but highly efficient spatial search strategies. After 2 days of training, mice use mainly non-spatial and repetitive strategies, which coincided with specific profiles of negatively and positively correlated brain FC. More specifically, mice that consistently search for the platform in the wrong quadrant tend to express lower FC, specifically between motor and sensory cortex, which are involved in sensory information processing, coupling perception and action, and motor learning (Chersi and Burgess 2015). Accordingly, human studies have shown improved spatial acuity and discrimination on transcranial stimulation of sensory regions (Karim et al. 2006; Ragert et al. 2008). On the other hand, repetitive strategy use positively correlated with FC between cingulate and motor cortex. Repetitive strategies do not require the use of distal cues, but rather a previously learnt targeted motor routine to locate the platform (providing escape from the pool as positive reward). Increased FC between cingulate and motor cortex might relate to intensive motor control and reward-driven locomotion (Walton et al. 2002, 2003; Stevens et al. 2011; Holroyd and Yeung 2012).

After 10 days of training, mice mainly used spatial and repetitive strategies, which induced another typical pattern of positively and negatively correlated FC, specifically involving cingulate cortex and hippocampus. Use of spatial strategies is obviously hippocampus dependent (Garthe and Kempermann 2013), and consistent with FC between cingulate cortex and hippocampus, since both regions are crucially involved in spatial learning and memory (Martin and Clark 2007). Conversely, mice that use non-spatial strategies to locate the platform would be expected to display lower FC between hippocampus and cingulate cortex, as well as between other regions that are important for spatial navigation (e.g., motor cortex, hippocampal dentate gyrus and caudate putamen). After 5 days

of continued acquisition or reversal training, mice used predominantly spatial strategies, which positively correlated with specific FC changes. Overall, it may be noted that the use of efficient spatial strategies (and concomitantly high task proficiency) positively correlated with telencephalic FC, whereas non-spatial strategies were negatively correlated with FC. This illustrates the widespread interaction between brain regions that is required for those most advanced strategies, and for ultimate task proficiency. This might also relate to observations in mice during the advanced stages of progressive neuropathology, which display diminished telencephalic FC coinciding with impaired spatial learning and memory (Shah et al. 2013).

Funding

This research was supported by EU Seventh Framework Programme Imaging of Neuroinflammation in Neurodegenerative Diseases INMiND (grant agreement no. 278850), by the Molecular Imaging of Brain Pathophysiology BRAINPATH project (grant no. 612360) within the Marie Curie Actions-Industry-Academia Partnerships and Pathways (IAPP), by FLAG-ERA JTC 2015 - Ultrafast Functional Ultrasound (fUS) Imaging for Highly-Resolved Targeted Mapping of Functional Connectivity in the Awake Mouse Brain (grant agreement no. G.0D7615N), by the University Research Fund of Antwerp University BOF DOCPRO (grant agreement FFB150340), by the Institute for the Promotion of Innovation by Science and Technology (IWT) Flanders (grant agreement no. 13160 to D.S.), and by the Research Foundation Flanders (FWO, grant agreement no. G.0D76.14, G.0587.14 and 12R1119N).

References

Benchenane K, Peyrache A, Khamassi M, Tierney PL, Gioanni Y, Battaglia FP, Wiener SI. 2010. Coherent Theta Oscillations and Reorganization of Spike Timing in the Hippocampal- Prefrontal Network upon Learning. Neuron. 66:921–936.

Brody DL, Holtzman DM. 2006. Morris water maze search strategy analysis in PDAPP mice before and after experimental traumatic brain injury. Exp Neurol. 197:330–340.

Chersi F, Burgess N. 2015. The Cognitive Architecture of Spatial Navigation: Hippocampal and Striatal Contributions. Neuron.

Cho YH, Friedman E, Silva AJ. 1998. Ibotenate lesions of the hippocampus impair spatial learning but

not contextual fear conditioning in mice. Behav Brain Res. 98:77–87.

D'Hooge R, De Deyn PP. 2001. Applications of the Morris water maze in the study of learning and memory. Brain Res Rev.

Dalley JW, Cardinal RN, Robbins TW. 2004. Prefrontal executive and cognitive functions in rodents: Neural and neurochemical substrates. In: Neuroscience and Biobehavioral Reviews. p. 771–784.

De Leonibus E. 2005. A study on the role of the dorsal striatum and the nucleus accumbens in allocentric and egocentric spatial memory consolidation. Learn Mem. 12:491–503.

Eichenbaum H. 2008. Learning and memory. 1st Editio. ed. Boston University.

Eichenbaum H. 2017. On the Integration of Space, Time, and Memory. Neuron.

Eichenbaum H, Stewart C, Morris RG. 1990. Hippocampal representation in place learning. J Neurosci. 10:3531–3542.

Garthe A, Kempermann G. 2013. An old test for new neurons: Refining the morris water maze to study the functional relevance of adult hippocampal neurogenesis. Front Neurosci.

Godsil BP, Kiss JP, Spedding M, Jay TM. 2013. The hippocampal-prefrontal pathway: The weak link in psychiatric disorders? Eur Neuropsychopharmacol.

Goodale MA, Milner AD. 1992. Separate visual pathways for perception and action. Trends Neurosci.

Goyal N, Siddiqui S, Chatterjee U, Kumar D, Siddiqui A. 2008. Neuropsychology of prefrontal cortex. Indian J Psychiatry. 50:202.

Haggerty DC, Ji D. 2015. Activities of visual cortical and hippocampal neurons co-fluctuate in freely moving rats during spatial behavior. Elife. 4.

Holroyd CB, Yeung N. 2012. Motivation of extended behaviors by anterior cingulate cortex. Trends Cogn Sci.

Kandel ER. 2001. The molecular biology of memory storage: A dialogue between genes and synapses. Science (80-).

Kandel ER, Dudai Y, Mayford MR. 2014. The molecular and systems biology of memory. Cell.

Karim AA, Schüler A, Hegner YL, Friedel E, Godde B. 2006. Facilitating Effect of 15-Hz Repetitive Transcranial Magnetic Stimulation on Tactile Perceptual Learning. J Cogn Neurosci. 18:1577–1585.

Keene CS, Bucci DJ. 2009. Damage to the retrosplenial cortex produces specific impairments in

spatial working memory. Neurobiol Learn Mem. 91:408–414.

Knierim JJ. 2006. Neural representations of location outside the hippocampus. Learn Mem.

Latif-Hernandez A, Shah D, Ahmed T, Lo AC, Callaerts-Vegh Z, Van Der Linden A, Balschun D, D'Hooge R. 2016. Quinolinic acid injection in mouse medial prefrontal cortex affects reversal learning abilities, cortical connectivity and hippocampal synaptic plasticity. Sci Rep. 6.

Leber AB, Turk-Browne NB, Chun MM. 2008. Neural predictors of moment-to-moment fluctuations in cognitive flexibility. Proc Natl Acad Sci. 105:13592–13597.

Leech R, Sharp DJ. 2014. The role of the posterior cingulate cortex in cognition and disease. Brain.

Lo AC, De Maeyer JH, Vermaercke B, Callaerts-Vegh Z, Schuurkes JAJ, D'Hooge R. 2014. SSP-002392, a new 5-HT4 receptor agonist, dose-dependently reverses scopolamine-induced learning and memory impairments in C57Bl/6 mice. Neuropharmacology. 85:178–189.

Martin SJ, Clark RE. 2007. The rodent hippocampus and spatial memory: From synapses to systems. Cell Mol Life Sci.

McNamara RK, dePape GE, Skelton RW. 1993. Differential effects of benzodiazepine receptor agonists on hippocampal long-term potentiation and spatial learning in the Morris water maze. Brain Res. 626:63–70.

Mizumori SJ, Cooper BG, Leutgeb S, Pratt WE. 2001. A neural systems analysis of adaptive navigation. Mol Neurobiol. 21:57–82.

Mizumori SJY, Canfield JG, Yeshenko O. 2005. Parallel and interrelated neural systems underlying adaptive navigation. In: Integrative and Comparative Biology. p. 547–554.

Morellini F. 2013. Spatial memory tasks in rodents: What do they model? Cell Tissue Res.

Nasrallah FA, To XV, Chen DY, Routtenberg A, Chuang KH. 2016. Functional connectivity MRI tracks memory networks after maze learning in rodents. Neuroimage. 127:196–202.

Pooters T, Laeremans A, Gantois I, Vermaercke B, Arckens L, D'Hooge R. 2017. Comparison of the spatial-cognitive functions of dorsomedial striatum and anterior cingulate cortex in mice. PLoS One. 12.

Pooters T, Van Der Jeugd A, Callaerts-Vegh Z, D'Hooge R. 2015. Telencephalic neurocircuitry and synaptic plasticity in rodent spatial learning and memory. Brain Res. 1621:294–308.

Poucet B, Lenck-Santini PP, Paz-Villagrán V, Save E. 2003. Place cells, neocortex and spatial navigation: A short review. In: Journal of Physiology Paris. p. 537–546.

Ragert P, Vandermeeren Y, Camus M, Cohen LG. 2008. Improvement of spatial tactile acuity by transcranial direct current stimulation. Clin Neurophysiol. 119:805–811.

Ragozzino ME, Rozman S. 2007. The Effect of Rat Anterior Cingulate Inactivation on Cognitive Flexibility. Behav Neurosci. 121:698–706.

Riedel G, Micheau J, Lam AGM, Roloff EVL, Martin SJ, Bridge H, De Hoz L, Poeschel B, McCulloch J, Morris RGM. 1999. Reversible neural inactivation reveals hippocampal participation in several memory processes. Nat Neurosci. 2:898–905.

Shah D, Jonckers E, Praet J, Vanhoutte G, Delgado Y Palacios R, Bigot C, D'Souza D V., Verhoye M, Van Der Linden A. 2013. Resting state fMRI reveals diminished functional connectivity in a mouse model of amyloidosis. PLoS One. 8.

Shah D, Praet J, Latif Hernandez A, Höfling C, Anckaerts C, Bard F, Morawski M, Detrez JR, Prinsen E, Villa A, De Vos WH, Maggi A, D'Hooge R, Balschun D, Rossner S, Verhoye M, Van der Linden A. 2016. Early pathologic amyloid induces hypersynchrony of BOLD resting-state networks in transgenic mice and provides an early therapeutic window before amyloid plaque deposition. Alzheimer's Dement. 12:964–976.

Sherrill KR, Erdem UM, Ross RS, Brown TI, Hasselmo ME, Stern CE. 2013. Hippocampus and Retrosplenial Cortex Combine Path Integration Signals for Successful Navigation. J Neurosci. 33:19304–19313.

Stevens FL, Hurley RA, Taber KH. 2011. Anterior Cingulate Cortex: Unique Role in Cognition and Emotion. J Neuropsychiatry Clin Neurosci. 23:121–125.

Tonegawa S, Pignatelli M, Roy DS, Ryan TJ. 2015. Memory engram storage and retrieval. Curr Opin Neurobiol.

Tsanov M, Manahan-Vaughan D. 2008. Synaptic plasticity from visual cortex to hippocampus: systems integration in spatial information processing. Neuroscientist. 14:584–597.

Uylings HBM, Groenewegen HJ, Kolb B. 2003. Do rats have a prefrontal cortex? Behav Brain Res.

Vann SD, Aggleton JP. 2002. Extensive cytotoxic lesions of the rat retrosplenial cortex reveal consistent deficits on tasks that tax allocentric spatial memory. Behav Neurosci. 116:85–94.

Vann SD, Aggleton JP. 2004. Testing the importance of the retrosplenial guidance system: Effects of different sized retrosplenial cortex lesions on heading direction and spatial working memory. Behav Brain Res. 155:97–108.

528 Vogt BA, Paxinos G. 2014. Cytoarchitecture of mouse and rat cingulate cortex with human
529 homologies. Brain Struct Funct. 219:185–192.
530 Vorhees C V., Williams MT. 2006. Morris water maze: Procedures for assessing spatial and related
531 forms of learning and memory. Nat Protoc. 1:848–858.
532 Walton ME, Bannerman DM, Alterescu K, Rushworth MFS. 2003. Functional specialization within
533 medial frontal cortex of the anterior cingulate for evaluating effort-related decisions. J Neurosci.
534 23:6475–6479.
535 Walton ME, Bannerman DM, Rushworth MFS. 2002. The role of rat medial frontal cortex in effort-
536 based decision making. J Neurosci. 22:10996–11003.
537 Wang Q, Sporns O, Burkhalter A. 2012. Network Analysis of Corticocortical Connections Reveals
538 Ventral and Dorsal Processing Streams in Mouse Visual Cortex. J Neurosci. 32:4386–4399.
539 Warburton EC, Aggleton JP, Muir JL. 1998. Comparing the effects of selective cingulate cortex
540 lesions and cingulum bundle lesions on water maze performance by rats. Eur J Neurosci.
541 10:622–634.
542 Watrous AJ, Lee DJ, Izadi A, Gurkoff GG, Shahlaie K, Ekstrom AD. 2013. A Comparative Study of
543 Human and Rat Hippocampal Low-Frequency Oscillations During Spatial Navigation.
544 Hippocampus. 23:656–661.
545 Whitlock JR, Sutherland RJ, Witter MP, Moser M-B, Moser EI. 2008. Navigating from hippocampus to
546 parietal cortex. Proc Natl Acad Sci. 105:14755–14762.
547 Woolley DG, Laeremans A, Gantois I, Mantini D, Vermaercke B, Op de Beeck HP, Swinnen SP,
548 Wenderoth N, Arckens L, D'Hooge R. 2013. Homologous involvement of striatum and prefrontal
549 cortex in rodent and human water maze learning. Proc Natl Acad Sci. 110:3131–3136.
550 Woolley DG, Mantini D, Coxon JP, D'Hooge R, Swinnen SP, Wenderoth N. 2015. Virtual water maze
551 learning in human increases functional connectivity between posterior hippocampus and dorsal
552 caudate. Hum Brain Mapp. 36:1265–1277.
553
554

Figure legends

Figure 1: Training in the Morris water maze. A-D) Performance as % time spent in target quadrant ± standard error for swim controls and cognitive trained animals at 2 days of acquisition training (A), 10 days of acquisition training (B), 5 days of continued acquisition training (C) and 5 days of reversal training (D). **E-H)** Performance as % time spent in each quadrant ± standard error during the probe trials at day 6 (E) and day 11 (F) of the 10 days acquisition training and day 6 of the continued acquisition (G) reversal training (H). **I-L)** Use of spatial, non-spatial or repetitive strategies as % strategy used ± standard error for cognitive trained animals at 2 days of acquisition training (I), 10 days of acquisition training (J), 5 days of continued acquisition training (K) and 5 days of reversal training (L) *p<0.05, **p<0.01, ***p<0.001, corrected for multiple comparisons.

Figure 2: Functional connectivity increases after acquisition and reversal training. Upper panel: Mean FC-matrices of swim controls (lower half)) and trained animals (upper half) that were subjected to 2 days acquisition training, 10 days acquisition training, 5 days of continued acquisition training and 5 days of reversal training. X-and Y-axes represent brain regions. Color scale represents Pearson correlations i.e. strength of FC between each pair of brain regions. **Lower panel:** Binary matrix representing statistically significant differences between swim controls versus trained mice (lower half) and cage controls versus swim controls (upper half). X-and Y-axes represent brain regions. Abbreviations: mPFC= medial prefrontal cortex, Cg=cingulate cortex, Resp=retrosplenial cortex, DG=dentate gyrus of the hippocampus, CA1=CA1 region of the hippocampus, SC=sensory cortex, Cpu=caudate putamen, T=thalamus, MC=motor cortex, VC=visual cortex.

Figure 3: **Functional reorganization after acquisition and reversal learning.** Overview of functional connections that were significantly increased between swim controls and trained groups are shown in a binary representation overlaid on a sagittal representation of the mouse brain. Abbreviations: mPFC= medial prefrontal cortex, Cg=cingulate cortex, Resp=retrosplenial cortex, DG=dentate gyrus of the hippocampus, CA1=CA1 region of the hippocampus, SC=sensory cortex, Cpu=caudate putamen, T=thalamus, MC=motor cortex, VC=visual cortex.

Figure 4: FC-map of the prefrontal and visual cortex demonstrate difference between acquisition and reversal learning. A) Mean FC-maps of the prefrontal cortex of the swim controls, mice that underwent acquisition learning and mice that underwent reversal learning. FC-maps are shown as axial images overlaid on a T2-weighted anatomical MRI image. Color scale represents T-value i.e. strength of FC of the seed region with all voxels in the brain. **B)** Mean FC-maps of the visual cortex of the swim controls, mice that underwent acquisition learning and mice that underwent reversal learning. FC-maps are shown as axial images overlaid on a T2-weighted anatomical MRI image. Color scale represents T-value i.e. strength of FC of the seed region with all voxels in the brain. **C)** Mean FC derived from FC-maps of the prefrontal and visual cortex, representing mean FC of the seed region with other voxels in the brain for swim controls, mice that underwent acquisition learning and mice that underwent reversal learning. *p<0.05, **p<0.01, ***p<0.0001.

Figure 5: Correlation between FC patterns and strategies applied during Morris water maze training. Pearson correlations representing the correlation between applied strategies and FC are shown overlaid on a sagittal representation of the mouse brain**.** Thickness of the lines corresponds to the strength of the correlation. Positive correlations are shown in red, negative in blue. Abbreviations: mPFC= frontal cortex, Cg=cingulate cortex, Resp=retrosplenial cortex, HC-DG=dentate gyrus of the hippocampus, HC_CA=CA1 region of the hippocampus, SC=sensory cortex, Cpu=caudate putamen, T=thalamus, MC=motor cortex, VC=visual cortex.