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Spatial representation from birth to old age: Insights from comparative neurobiology and behavioral genomics.

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“It is imperfection - not perfection - that is the end result of the program written into that formidably complex engine that is the human brain, and of the influences exerted upon us by the environment and whoever takes care of us during the long years of our physical, psychological and intellectual development.”

(Rita Levi-Montalcini)

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Abstract

Finding one's way back to a safe refuge or recalling the best place to find food is essential to all animals including human beings. We engage in future actions based on past events. So how does our brain compute such important cognitive tasks? Is it an innate ability we have from birth that is hardwired into the blueprint of our brains? And what happens if for some reason we realize that we are unable to perform these cognitive abilities in old age or due to a neurological disorder?

The hippocampus is the main area of the brain involved in memory and learning. Animal studies show evidence of its role in spatial navigation and memory. The complex network of spatial cells in the hippocampus, all participate in constructing a cognitive map in the brain, where an animal stores information about the external environment and uses it to engage in future actions. However, despite the importance in its function, the hippocampus is also one of the first areas of the brain to be affected by aging and other neurological disorders.

The present thesis used the help of various animal models to answer three questions: first whether hippocampal function is present immediately at birth, second whether genes can regulate hippocampal activity and third whether a sensitive task such as reorientation can highlight hippocampal alteration caused by age. To answer the first question, we used the domestic chick that has the advantage of being tested after hatching. We show evidence that a change in environmental shape can alter hippocampal activity in naïve chicks, suggesting that hippocampal function is present already in early stages of life. Furthermore, we investigated if genes regulate hippocampal activity. We used a mouse model that carried one half of the Williams syndrome deletion, a disorder known for its hippocampal deficit. We show evidence that genes on the proximal deletion of Williams syndrome deletion, can alter reorientation and episodic memory, two hippocampal related functions. Finally, we aimed to find an appropriate task to highlight the allocentric difficulty that arises in age. We used aged animals of two species (mice and rats) and tested them in the reorientation paradigm. We show that this simple task has potential to be a better suited assay to evaluate hippocampal behavior.

Introduction

Finding our way toward a food source or shelter and storing this information in our memory is of major importance to all animals. Given the importance of these abilities, it is essential to understand the neural mechanisms that are behind such cognitive capacities and how they change throughout our lives. There are various strategies an animal can implement to find a location: one is to keep track of their movement with respect to the external environment, updating the route while moving. This doesn't require memorizing landmarks or other cues, but relies only on precision. However, the constant updating leads inevitably to errors and isn't an efficient strategy to put in place when one loses its way. When an animal is disoriented and loses its bearings, it relies heavily on the geometric properties of the environment to recall a target location rather than non-geometric cues. This strategy called reorientation; has proved to be a widely conserved ability. Reorientation 's validity across animal species allows us to test spatial representations not only in different animals, but also at different time points in development. Navigation by boundary geometry depends on the correct firing of hippocampal place and boundary cells in rats, and is a sensitive task to measure physiological changes in hippocampal function. This is of main concern especially when spatial navigation and memory, both known to be dependent on hippocampal function, are compromised in age-related disorders, such as Alzheimer's disease, or in neurodevelopmental disorders. The aim of my dissertation research was to use various animal models to investigate spatial cognition and memory in relation to hippocampal function at different ages starting from birth to old age. At birth, mammals such as rats or mice, classically used in behavioral testing, cannot be tested on any kind of behavioral paradigm including the reorientation task. To overcome this hurdle, we chose to use a precocial species such as the domestic chick, and measured hippocampal activity using a neural marker: c-Fos. Given the difficulty in testing adult chickens, we used a different animal model, well documented in literature in regard to hippocampal related aging: Rattus Norvegicus. We tested rats in adulthood and in old age on the same reorientation task to have a direct comparison of hippocampal ability in using geometry. We later extended the applicability of the reorientation task to a genetically modified mice model, with gene deletions that characterize hippocampal deficit. These mice carry one half of the WSCR (Williams syndrome critical region), and were tested not only in reorientation but also on episodic memory, two strictly related functions regulated by the hippocampus. We tested mice on two tasks to understand whether their specific deletion would alter one or more hippocampal functions. We show interesting findings on how various animal models, ranging from birds to mutant mice can provide suggesting evidence on hippocampal development and function.

The hippocampus and spatial cognition/navigation

The curiosity to comprehend how we perceive our surrounding space has intrigued researchers and scientists throughout history. Important questions such as: how do we go from one place to another? How do we remember locations? Is this cognitive ability confined to only human beings? Is the perception of space an innate ability or is it acquired through experience and furthermore continue to puzzle scientists. The first scientific notion of how our brain navigates in the external world comes from Tolman's experiments. He was the first to suggest that animals navigated forming internal representations of the external environment. Tolman conducted a series of experiments on rats in complex mazes, and observed that when given a choice they did not randomly chose to go left or right, but took a decision according to a structured map, a cognitive map based on previous trials in the maze (Tolman, 1948). The essential characteristic of a cognitive map according to Tolman was the ability to make short cuts along the way in order to take the shortest route toward the goal. In the late 70's, John O'Keefe and Nadel further developed his idea by proposing the "cognitive map theory" (O'Keefe & Nadel, 1978). They suggested the mental representations Tolman was referring to were formed by a specific brain structure: the hippocampus. They thought the hippocampus of rodents represents the environment along with its locations, landmarks and contexts forming a map that animals used to navigate from one location to another and stored this information for the future (Bennett, 1996).

The role of the hippocampus has been analysed in human and non-human animals in its role in memory and spatial navigation. To further understand whether a cognitive map was present in other animal species, at first comparative neuroanatomists suggested behaviours such as homing, migration, and territoriality as evidence of cognitive based mapping. Therefore, it is safe to assume that species demonstrating these behaviours have a homologue to the mammalian hippocampus(O'Keefe & Nadel, 1978). The hippocampus is a highly conserved structure, and although may differ in anatomical organization, preserves the same function in mammals and non-mammals (Broglio et al., 2015). Lesion studies in birds, mammals and humans, all produce deficits in spatial tasks and spatial memory (Squire, 1992). Evidence from neuroanatomical studies show that a common brain structure called "pallium" evolved from fish, and specialized in forming map-like representations of space in mammals and birds (Broglio et al., 2015; Jarvis et al., 2005; Rodríguez et al., 2002). In particular, the avian brain, although different in anatomy, has the most similarities in function to the mammalian brain. Similarly to rodents, chicks also show two types of spatial learning: hippocampus and non-hippocampus based (Mayer et al., 2016). Studies on chicks have shown they rely on boundary geometry to orient (allocentric) but are also capable of using non-geometric cues (egocentric) to find a goal location. In this scenario, the use of a precocial species such as the domestic chick (Gallus gallus domesticus) to investigate early hippocampal function is very useful. The evidence of homologies across species, gives the knowledge and tools to find useful neurobiological markers that can

allow us to mark hippocampal activity and enable the use of multiple animals as models to investigate hippocampal function. We can therefore overcome the limitations in testing only mammals to study human disorders, such as the inability to test immediately after birth for example.

The hippocampus, as previously mentioned, engages in spatial representations in humans and non-humans, but what remains to be clarified is how it actually forms these complex mental constructs. Generally speaking, an animal can implement various strategies to move from one location to another. One very simple way is to keep track of its self-motion along the way (Burgess, Maguire, & O'Keefe, 2002; Lee & Spelke, 2010). This mechanism requires no memory of the environment or other cues, but only precision in keeping track of its movement. The neural correlates of path integration in rodents lie in the entorhinal cortex, and keep track of the animal's heading along a route. In more complex environments where one has to recall and go back to a target location this system is integrated by more sophisticated strategies. To remember a target corner for example, an animal can use the geometric shape of the environment (geometric information), or visual cues such as pattern walls or salient objects (non-geometric information). Lee and Spelke point out two distinct mechanisms that process spatial properties of the external environment: the first uses objects and patters, the other creates 3D layouts based on its geometric properties (Lee & Spelke, 2010). Experiments in disoriented animals all show that when an animal loses its reference points, it relies mainly on geometric rather than other types of non-geometric cues (Lee, 2017).

The geometric layout of the environment is often a stable and constant type of information that animals rely upon especially when disoriented (Cheng & Newcombe, 2005; Gallistel, 1990). Cheng in the late 80's tested rats in an enclosed rectangular arena. The task was simple: after habituation and a few learning trials animals had to find food in a corner of the rectangular arena (Cheng, 1986). To aid their searches they could rely on features such as striped panels or odours. He observed that rats made rotational errors in their searches, meaning 180° rotation error from the correct corner. In a rectangular arena, with featural cues on the wall, rats searched in the correct corner and in the geometrically opposite corner, narrowing down their searches from 4 to 2 corners. However, they failed to narrow their searches from two to one corner in spite of distinctive panels and odours to help them. This lead Cheng to believe and develop the "geometric module"(Cheng & Newcombe, 2005). The validity of Cheng's observations was later confirmed by similar experiments in both human children (Lee & Spelke, 2011) and non-human animals (Lee, Spelke, & Vallortigara, 2012; Lee et al., 2015; Sovrano, Bisazza, & Vallortigara, 2002) all show standing evidence that disoriented animals rely on the geometric shape of the environment, rather than other more salient cues to find the target location(Cheng & Newcombe, 2005). Not only can rats use geometry in a rectangular shaped arena, but can infer geometric properties from separate objects as in the case of Benhamou and Pouchet (Benhamou & Poucet, 1998). They tested rats in a circular swimming pool where cues were distinct landmarks sticking from the water. They observed that rats can use geometry from separate objects and as in

experiments in the rectangular arena, featural information is either ignored or used less. Like rats, other species such as the avian species, mostly chicks and pigeons, have also shown to prefer geometric based navigation. Chicks in a reference memory paradigm in a rectangular arena, in absence of featural cues use geometry. However, when features such as coloured panels were presented near the target, chicks used features. This shows that they can use both geometry and features to navigate (Vallortigara et al., 1990). Similar experiments have been conducted in human children and adults and what is interesting to observe is that spatial behaviour is modulated by development. As observed in animals, very young children (18-24 months) use geometry of a rectangular room to concentrate searches of a hidden object in the correct corner and the diagonally opposite corner. However, experiments in human adults show that the geometric module is often substituted by egocentric based strategies. Wang and Spelke argue that this shift in behavioural preference in development might be due to the acquisition of spatial language that takes over the more innate ability of geometric based navigation (Wang & Spelke, 2002).

Behavioural data from animals and humans have highlighted multiple neural structures that regulate spatial behaviour (Hartley et al., 2014; Lee & Spelke, 2010). The distinction between geometric cues and featural cues are mediated by separate brain structures. The hippocampus seems to be involved in processing layout geometry but not features such as colours or odours. Its role in spatial function is widely known thanks to the discovery of place cells in the hippocampus and grid, boundary and head-direction cells in the entorhinal cortex of rats (Hafting et al., 2005). Place cells in the rodent hippocampus create a cognitive map in the brain, a spatial representation that includes position of landmarks as well as distances and orientations estimated by the animal (Mizumori et al., 2008; Moser, Kropff, & Moser, 2008). When the external environment changes, place cells put together new pieces of information and update the old map, a mechanism called “remapping” (Lever et al. 2002; O’Keefe and Burgess, 1996). Research in rodents show evidence that place cells are sensitive to change of environmental shape. Lever and colleagues, 2002 placed rats in two unfamiliar environments in a circular and square shaped arena. Over days of exposure to the two environments, place cells fired in a different pattern showing to be sensitive to the change in shape of the arena (Lever et al., 2002). Place cells in the hippocampus receive inputs from boundary cells in the entorhinal cortex, which are sensitive to sensory information about the distance of the animal from the walls of the environment (Lever, Burton, Jeewajee, O’Keefe, & Burgess, 2009). Moreover, objects if placed near to the walls of the arena and not inside activate place cells and head direction cells (Hartley et al., 2014; Lever et al., 2002). In fact, hippocampal lesions impair the ability to use geometric cues, but do not affect the use of objects in navigation (McGregor., 2004). Supporting the hippocampus is also the neighbouring entorhinal cortex, that participates in encoding geometric properties of the environment. Border cells, neurons inside the entorhinal cortex, along with boundary cells define the borders of the environment in relation to the inside locations (Stensola et al., 2012). Similar properties in hippocampal function were seen by Vallortigara, Sovrano and Pagani. They tested chicks restricted to one eye in a reorientation task. They concluded that when the right eye was closed,

(use of the left hemisphere) they relied mainly on featural cues. However, when the left eye was closed (use of the right hemisphere) they used geometry to navigate (Vallortigara, Pagni, & Sovrano, 2004). A Similar disassociation is seen also in humans: functional neuroimaging studies have shown right posterior hippocampus for geometry and right dorsal striatum for landmark related location (Doeller, King, & Burgess, 2008). The hippocampus has a goal independent representation of space that depends on the firing of place cells. This type of learning provides an immediate association between a goal (an object) and its context (environment). In humans, spatial cognition is extended to a larger area of the brain called PPA, (parahippocamapal place area), which surrounds the hippocampus and is connected tightly to the limbic system. Behavioural data in humans is controversial on whether this area is restricted to only geometric properties or landmarks or both. However, lesions in this region lead to spatial deficits suggesting an overall important role in spatial representations and memory (Cheng & Newcombe, 2005).

The hippocampus and long term memory: Episodic memory

The hippocampal function is not limited to only spatial cognition but also to more complex functions such as recalling and retrieving long term memories (Eichenbaum, 2017; Nadel, Hoscheidt, & Ryan, 2012; Squire, 1992). The role of the hippocampus in memory started triggering researchers interest after the case of patient HM. Henry Molaison, better known as patient HM, suffered from major epileptic seizures. In 1953, HM got surgery where parts of the temporal lobes were removed in order to treat his epilepsy. The resection included the removal of major parts of the hippocampi. Although his seizure attacks reduced, surprisingly he was not able to form new memories or retain the most recent ones (Dossani, Missios, & Nanda, 2015). Similarly patients that lost parts of the temporal lobes also showed the same pattern, suggesting the hippocampus beeing essential in forming new memories (Dossani, Missios, and Nanda, 2015) and in retrieving old ones (Burgess et al., 2002).

Recalling personal memories is an essential part of life, as they characterize each individual's personal history. First defined by Tulvig, (Tulving & Thomson, 1973), episodic memory is the conscious recollection of what, where and when of a past event. This memory, along with semantic memory belongs to the class of long term memories. Thanks to behavioural findings in animals, this ability is now known to be present also in non-human animals. There is behavioural evidence that animal possess episodic-like memory (Dere et al, 2005; Eacott, 2004; Roberts & Feeney, 2009; Templer & Hampton, 2013). Although the same definition by Tulvig cannot be applied to animals, several attempts in behavioural research have underlined a few prerequisites to define episodic memory in non-humans: the ability to spontaneously communicate their past, keep track of their own recent behaviour and finally conscious recollection like memory (Dere, 2005). Scrub jays can remember what they catch, where and when: they search first in sites that contain perishable food, such as worms, but only if the food has been stored there recently, but if days have gone by, jays search

directly in sites that contain non-perishable food, such as peanuts (Clayton & Dickinson, 1998; Nyblade et al., 1998). Likewise, rodents, non-human primates, dolphins, pigeons, magpies, honeybees and many other animals possess this cognitive ability called episodic-like memory. Episodic like memory in animals like in humans has an evolutionary value because they recall facts of past events in order to decide and plan future events (Dere, 2005). From an experimental point of view, accessing episodic memory in animals has major applications. Brain injured individuals, Alzheimer's disease patients and other disorders that selectively impair the hippocampus, all disrupt memory formation and retrieval (Small, Fratiglioni, Viitanen, Winblad, & Bäckman, 2000; Walker et al., 2011). Having an animal model which can be tested on this cognitive ability would highlight anatomical, physiological and molecular mechanisms that could not be possible through human studies.

The neural correlates of memory, especially episodic memory lie in the hippocampus (Burgess et al., 2002; Knierim, 2015). The anatomical and functional distinction of the hippocampus allows it to link together space and time. The hippocampus, being so largely connected to other brain areas, has an important role of binding together spatial and temporal information and creating complex contexts to which a specific memory is associated. To aid its complex function is also its anatomical differentiation: the posterior hippocampus is involved in retrieving detailed spatial relational information, whereas the anterior mainly involved in retrieving contexts and memory (Nadel et al., 2012; Strange et al., 2014).

The hippocampus provides a spatio-temporal context where a certain memory's what, where and when are stored and bind together. The formation of a cognitive map (spatial component) is essential to support episodic memory. In order to recall where you left your glasses last night, the hippocampus must distinguish countless other memories that include locations of your glasses. In order to do so, it assigns a context to a certain memory by manipulating the where and when. When the change of where and when crosses a threshold, then the hippocampus forms a new context. For example, if the configuration of furniture remains the same every time you walk into your office, then those factors will become indicators of the context (or "landmarks"), and hippocampal cells would likely map relative to the boundaries of your office. If, one day, the furniture configuration changes, the change would trigger the hippocampus to assign distinct context representations to the "old" vs. "new" office configurations. Additionally, if the office chair, changes locations continuously, it would be treated as an item to be mapped in the office context (Ekstrom, Ranganath, & Plant, 2017).

The relationship between place cells (space) and time cells (time) provide the representation of a unique context for a memory and bind spatial and temporal components together. Time cells in the rat CA3 region fire at specific time points as an animal experiences a predictable sequence of events, even when the animal remains in the same location (Macdonald, Lepage, Eden, & Eichenbaum, 2011). Moreover, just as

place cells remap when an animal is moved to a new spatial context, hippocampal time cells "retime" when the temporal structure of a task, or the current behavioural context is changed.

The hippocampus as shown by neural data has an important role in binding information but also connecting different brain areas. The heavy workload of integration; the hippocampus requires constant production of new neurons to answer its highly demanding cognitive needs. In fact, evolution has provided this only structure in the entire brain the faculty of producing new neurons throughout adulthood. Surprisingly, extensive use of the hippocampus can increase the number of neurons and therefore produce an increase in volume. London taxi drivers, who use the hippocampus extensively on a daily basis, have an overall larger hippocampal volume compared to controls (Maguire et al., 2000). On the other hand, decrease of use causes neuronal death and shrinkage. This occurs in age – related disorders such as Alzheimer's disease, dementia and mild cognitive impairment (Lithfous, Dufour, & Després, 2013). The neuronal plasticity that characterizes hippocampal function is also the cause of its vulnerability toward aversive stimuli such as stress, anxiety and aging.

Hippocampal vulnerability and sensitivity to age related and genetic disorders

"The plasticity of the hippocampus is the reason for its vulnerability" (McEwen, 1994). For decades it has been known that conditions such as ischemia, hypoglycemia, epileptic seizures, neurodegenerative disorders affect the hippocampus (Bartsch, 2012). Given the hippocamapal role in important cognitive functions such as memory and spatial navigation, damage to this small area, due to disease or simply physiological aging, can lead to severe deficits in cognitive function. Therefore, investigating the neural mechanisms that cause hippocampal degeneration and impairment are of keen importance. The longevity of our population goes hand in hand with an increase of neurodegenerative disorders such as: Alzheimer's, Parkinson's disease and senile dementia to name a few. All these disorders are characterized by a general death of neurons (neurodegeneration) in the central nervous system. In this scenario, the hippocampus is the most affected, leading to loss of memory as one of the most salient cognitive declines. In Alzheimer's disease (AD) elderly individuals show a general difficulty in spatial navigation and memory, and this is often correlated with a structural alteration inside the hippocampal formation. Braak and colleagues showed pathological staging of AD starts in the entorhinal cortex and hippocampus and then spreads to other areas of the brain (Braak & Braak, 1995). This suggesting that a measure of early damage to the hippocampus could help diagnose the disorder before its clinical manifestations.

The most obvious clinical signs of AD include loss of memory and inability to recall locations (Head & Isom, 2010). Elderly individuals acquire spatial information less and preferably take familiar routes and environments as a coping strategy to avoid errors (Lithfous et al., 2013). In a spatial task using a virtual environment, old individuals show preference in using an egocentric to allocentric strategy. This difficulty in

forming cognitive maps, and using hippocampus based strategies in aged individuals, is a reflection of structural changes inside the brain. Normal aging causes a general shrinkage of cortical structures, with the hippocampal formation being the most sensitive (Raz et al., 2005). Similar findings are present in aged animals. Lesion studies in rodents have shown that hippocampal lesions affect allocentric but not egocentric strategies, suggesting the hippocampus being involved in place learning rather that response learning. Mostly, animal models of normal and pathological aging have highlighted the neuropathological mechanisms of normal aging in AD disorder. Place cell specificity was reduced in old compared to young rats, suggesting an age-related difference in hippocampal neurons caused by aging. Moreover, as in old humans they show a tendency of using egocentric strategies rather than allocentric in a Morris water maze task (Barnes, Nadel, & Honig, 1980). Likewise, mutant mice models of AD disorder show a decline in spatial performance and a reduced place cell firing pattern (Chishti et al., 2001; Walker et al., 2011).

A similar deficit in allocentric navigation is observed in a genetic and neurodevelopmental disorder called Williams syndrome (WS). What is interesting to observe, that although having a different etiology from AD, similar difficulties in spatial navigation and memory are observed in both disorders. WS is a rare genetic disorder caused by the deletion of around 20 genes on human chromosome 7 (Mervis et al., 2000). Affected patients show a very unique cognitive and behavioral phenotype, with relatively strong social and verbal skills but severe difficulties in visuo-spatial tasks.They fail in both egocentric (Bernardino et al., 2013) and allocentric spatial tasks (Ferrara & Landau, 2015; Lakusta, Dessalegn, & Landau, 2010). Bernardino and colleagues performed a screen based and 3D task to test whether WS patients could use their own body (egocentric) or external objects (allocentric) to correctly distinguish the position of an object in the environment. Results show that in both tasks there is a failure when compared to controls (Bernardino et al., 2013). Allocentric navigation uses not only external references such as objects or landmarks, but also environmental geometry to orient. Lakusta and colleagues, 2010 showed that WS patients were not able to use the geometric properties of a rectangular arena to reorient, but can improve when a blue wall is added to the arena. This provides evidence that while incapable of using boundary geometry, they can improve when a visual landmark is added to the arena (Lakusta et al., 2010). While spatial memory is completely altered, object memory (physical properties) is relatively conserved in WS individuals. Vicari et al. 2005 designed a task where one had to recall a succession of objects that were presented, and WS scored equally as their controls. However, in a visuo-spatial task where they were asked to recall the spatial collocation of an object, WS failed (Vicari, Bellucci, & Carlesimo, 2005). These results suggest a difficulty in linking together elements of complex memories such as episodic memories. Neural substrates of spatial representation seem to be affected by genetic deficit therefore causing impairment in behavioural tasks (Bostelmann et al., 2017;Lakusta et al., 2010). MRI scans of WS patients show an overall smaller hippocampal volume compared to controls, suggesting the hippocampal formation strongly involved in their visuo-spatial deficit (Meyer-Lindenberg, 2005).

Williams syndrome therefore, represents a good model to study hippocampal function as it contains an intrinsic deficit related to its gene deletion. In our work we try to provide a multidisciplinary approach using different animal models from the domestic chick to mutant mice models with selective hippocampal deficits, to study the development and physiological aging of the hippocampus. Our findings show alternatives to the extensive use of rodents in behavioural research, and extend the knowledge of how the conserved hippocampal function across animals, allows us to study human disorders such as aging and neurodevelopmental disorders not only in mammals but also other animal species.

Aims of the study

The main aim of our study was to investigate spatial representations and memory and its neural correlates through development starting from birth to old age.

Our first question was whether hippocampal mapping of spatial geometry is present immediately at birth or whether it requires prior experience to trigger its function. To test early hippocampal function, we used a precocial animal species: the domestic chick (Gallus gallus domesticus). We designed a simple behavioural paradigm **(Chapter 1)** to test whether a change in environmental shape (square vs. rectangle) can be detected by the hippocampus of new-born chicks. We later investigated hippocampal activation by measuring the quantity of c-Fos, an immediate early gene, activated and later transcribed into a protein when a neuron detects novelty. We chose the chick as the appropriate animal model, because unlike rodents it allows behavioural testing immediately after hatching. Given the difficulty in maintaining and testing adult chickens, they fail to be a good model to study hippocampal development in adulthood and senility.

We later wanted to investigate whether two hippocampal functions, reorientation and episodic memory are regulated by genes. Reorientation is a well-established paradigm but what still remains to be understood is whether a particular set of missing genes can alter its behavioural outcome. Episodic memory on the other hand, has never been investigated in this disorder, therefore given its importance we aimed to investigate if this strictly related hippocampal function, is also altered in relation to gene manipulations. In order to do so, we used a disorder characterized by hippocampal deficit: Williams syndrome (WS). WS is a rare neurodevelopmental disorder, caused by a deletion of around 20 genes on chromosome 7. This disorder is a good model to study hippocampal deficit, because it allows us to understand the relationship between a selective gene deletion and hippocampal function **(Chapter 2)**. We used a mutant mouse model that carries one half of the Williams syndrome critical region (Gtf2i-Limk1). This deletion reproduces many behavioural features of WS. However, there is no evidence whether it includes the typical deficit in spatial and memory tasks observed in clinical patients. We tested mutant mice in reorientation (in two conditions to test correct use of environmental geometry and use of landmark alone) and episodic-like memory in its three components “what”, “where” and “when”. We compared performance of genetically modified mice to controls in all conditions.

Finally, we aimed to investigate the last stage of hippocampal development: aging **(Chapter 3).** There is evidence that aging causes difficulty in using allocentric strategies in both animals and humans (Barnes et al., 1980; Lithfous et al., 2013). Given the sensitivity of the hippocampus to aging, we aimed to investigate if a simple task such as reorientation could highlight deficit in function. Spatial tasks in animals such as Morris water maze, and Barnes maze aren’t able to isolate hippocampal function in relation to boundary based navigation. These tasks often risk overtraining animals that show good performance mainly because of

repetitive trials. In order to overcome this, we used the reorientation paradigm on two different species: mice (Mus Musculus) and rats (Rattus Norvegicus) on their ability to use geometry and landmark separately. We compared performance between old and young animals of each species.

Chapter 1: Representation of environmental shape in the hippocampus of domestic chicks (Gallus gallus).

The present knowledge on hippocampal function comes mostly from adult animals. There are few studies that investigate whether spatial representations are present already at birth or whether the hippocampus requires external input to function. There is evidence in rodents that border cells in the entorhinal cortex have adult like firing rates at 16-18 days after birth (Bjerknes, Moser, & Moser, 2014). However, there are no findings whether neurons inside the hippocampal formation have adult like firing at birth or in earlier stages of life.

We decided to use the domestic chick (Gallus gallus domesticus) to investigate if a simple change in environmental shape can affect hippocampal activity. The avian brain represents a good alternative to study immediate function. Chicks, can be tested immediately after hatching and represent an alternative animal model to overcome the limitations of rodent testing, that become autonomous only a month after birth.

Phylogenetically, the avian species share more similarities with mammals than other species. Mammals and birds share the same hippocampal functions such as spatial navigation and memory. Hippocampal lesions in birds impair large-scale navigation and cause impairments in the dry Morris water maze task. Moreover, chicks like rats are sensitive to environmental geometry (Mayer et al., 2016; Lee, Spelke, and Vallortigara, 2012). What remains controversial is the correspondence between structures: in rodents, the hippocampus is divided into distinct regions, but in birds, the same structure has a lamellar structure with neurons packed together but without any clear anatomical boundary. Some studies using molecular markers and immunohistochemistry have underlined similarities in hippocampal regions between birds and mammals but they remain still incomplete(Gupta, Maurya, Saxena, & Sen, 2012).

Given the similarities in function to the mammalian hippocampus, across species experiments allow neuroanatomical comparisons of the hippocampal formation to narrow the gap between the structural differences between the two species. In this scenario, based on the sensitivity to environmental shape present also in chicks, we wondered whether the chick hippocampus could detect a change in arena shape in its very early stages of life. We trained naïve chicks in a square-square apparatus and on test day replaced a square with a rectangle. We measured hippocampal activity by counting neurons that produced c-Fos, an immediate early gene that is produced when the hippocampus is stimulated by novelty and learning. Our results show that neurons in the chick hippocampus are activated by a change of shape. We provide further evidence of similarities between chick and mammalian hippocampus and show that hippocampal activity starts immediately at birth in the avian brain.

Chapter 2: Proximal deletion (Gtf2i-Limk1) in Williams syndrome affect reorientation and episodic like memory

Williams syndrome (WS) is a rare neurodevelopmental disorder that affects around 1/7500 new-borns. An error during meiosis gives rise to a deletion on chromosome 7. This deleted region, also called WSCR (Williams syndrome critical region) contains 26-28 missing genes characterizes these patients with a unique profile that includes specific physical, behavioural, and cognitive abnormalities along with structural deficits in the brain. Functional imaging studies point out an have an abnormal hippocampal structure in WS patients (Meyer-Lindenberg, 2005) Moreover, they also show a different activation pattern in hippocampus-dependent learning and hippocampal physiology compared to controls.

Some genes of the WSCR have been linked to visuo-spatial deficits: knockout mouse models for LIMK1 and CLN2 showed functional alterations of hippocampal formation (Gray et al., 2006).Their hippocampal deficit is observed not only in anatomical structure but it also corresponds to a behavioural deficit mainly in difficulty in viso-spatial tasks (Donnai & Karmiloff-Smith, 2000).). Moreover, hippocampal related behaviours, in particular verbal long-term memory and spatial navigation are impaired due to their inbred genetic deficit (Lakusta et al., 2010). These preclinical results strongly suggest involvement of the hippocampus in the pathophysiology of WS and make it a good model to study hippocampal related deficits.

In our experiments we used a mouse model contained one half of the WSCR deletion (Gt2fi-Limk1). This model reproduces most of the clinical and behavioural aspects of Williams syndrome (Li et al., 2009). We investigated whether there is an effect of gene deletion on spatial reorientation and memory. We show evidence that the proximal deletion of the WSCR (Gt2fi-Limk1) is correlated to a deficit in hippocampal function that is seen in both behavioural tasks. In reorientation, mutant mice show failure in using geometry and landmark. In episodic memory they show a deficit in the temporal component (what+where), while conserve the what component.

Chapter 3: Aging impairs boundary based navigation and landmark use in mice (Mus musculus) and rats (Rattus Norvegicus).

Allocentric along with egocentric navigation are the two main strategies animals can use to find a target location. Adult animals preferably use an allocentric hippocampus based strategy following disorientation. In particular, the geometry of the external environment is a salient cue for animals' navigation (Lee & Spelke, 2010).

Aging however, causes difficulty in using allocentric strategies and behavioural tests in elderly individuals show a switch in preference toward other extra-hippocampal strategies. Likewise, animal studies with aged animals also show significant impairments in using allocentric strategies in Morris water maze tasks (Barnes et al., 1980; Fellini, Schachner, & Morellini, 2006). This behaviour is strongly related to a hippocampal

deficit that arises in age. Normal aging causes some significant changes in brain structure: especially the hippocampus.(Raz et al., 2005).

We aimed to see if a simple behavioral task such as the reorientation paradigm could highlight hippocampal deficits. Therefore, we tested old animals of two different animal species: mice (Mus musculus) and rats (rattus norvegicus) on their ability to use environmental geometry and landmark to reorient. Given the sensitivity of boundary geometry to hippocampal function, we show how this sensitive task can be used to test hippocampus related behaviors in aged animals.

Chapter 1: Representation of environmental shape in the hippocampus of domestic chicks (*Gallus gallus*)[1.]

1. Mayer, U., Bhushan, R., Vallortigara, G., & Lee, S. A. (2017). Representation of environmental shape in the hippocampus of domestic chicks (Gallus gallus). *Brain Structure and Function*, 1–13. https://doi.org/10.1007/s00429-017-1

Abstract

The hippocampus plays an important role in spatial encoding and memory across various vertebrate species. In rodents, hippocampal neurons are particularly sensitive to a change in environmental geometry. Given the similarities in function between the mammalian and avian hippocampi, we aimed to measure whether arenas varying in geometric shape (square and rectangle) can differentially activate hippocampal cells in the domestic chick (*Gallus gallus domesticus*). Chicks exposed to both a square and a rectangular arena exhibited a significantly higher neural activation (as measured by c-Fos expression) than those exposed twice to just the square or just the rectangle (both of which were significantly higher in activation than a one-environment control group). For the first time in an avian species, we show that exposure to two arenas of different geometric shape activates the hippocampus to a greater degree, suggesting a possible effect of spatial remapping.

Introduction

Animal spatial navigation is mediated by internal 'maps' of the environment consisting of allocentric representations of locations and their spatial relationships (Tolman, 1948). The hippocampus (Hp) is a phylogenetically ancient part of the vertebrate brain (Butler & Hodos 2005). In all vertebrate groups, the hippocampal pallium homologue is involved in the use of map-like, relational representations of the environment that provide stable allocentric reference for flexible navigation (Rodríguez et al., 2002; Broglio et al., 2015). These similarities suggest a common evolutionary ancestry of the functional properties of the hippocampus, which are retained through the independent evolution of vertebrate lineages. Although the spatial mapping function of the hippocampus is shared across both mammals and non-mammals, the understanding of avian hippocampus at the neuronal level is very limited. In particular, because the avian hippocampus lacks a layered structure, the anatomical subdivisions that correspond to the mammalian hippocampal regions are still highly debated (Atoji et al., 2006; Gupta et al., 2012; Herold et al., 2014; Striedter 2016). Nevertheless, the question of how such seemingly different structures can contribute to similar functions makes it worthwhile to further investigate the avian hippocampus in ways that can be directly compared to its mammalian counterpart.

In mammals, internal 'maps' of the environment are generated through a critical contribution of hippocampal place cells (O'Keefe & Dostrovsky 1971). These neurons fire when the animal passes through a specific part of its environment, a phenomenon that has been investigated in detail in hundreds of experiments over four decades (Barry & Burgess 2014; Moser et al., 2014). Spatial coding in mammals is heavily dependent on the inputs from spatial boundaries (Lee, 2017). Change in the environmental shape cause remapping of the hippocampal place cells to new preferred firing fields or activation of new cell populations (Muller & Kubie 1987; O'Keefe & Burgess 1996; Lever et al., 2002). Although the above studies on rodent measured neural activity using electrophysiology, environmental novelty can also be observed using immediate early genes (IEG's) as neuronal activity markers (Kubik et al., 2007). IEG's are rapidly expressed when neuronal activity increases and play an important role in memory consolidation (Lanahan and Worley 1998; Jones et al. 2001; Guzowski 2002; Barry & Commins 2011). The availability of this alternative measure of neural activity is especially important in the study of the avian hippocampal formation and how it represents multiple environments.

Despite the structural differences, the hippocampus is crucial to spatial navigation in birds, just as it is in mammals (Bingman & Able 2002; Smulders 2006; Mayer et al., 2013). Hippocampal lesions in birds impair large-scale navigation (Bingman et al., 1985, 2005), disrupt orientation in the 'dry Morris water maze' (Watanabe & Bischof 2004; Watanabe et al., 2008) and interfere with the use of boundary geometry of the environment (Tommasi et al., 2003; Vargas et al., 2004; Bingman et al., 2006). In pigeons, hippocampal neurons comparable to place cells were found using electrophysiology, but their responses were not as

spatially selective as in rats (Hough & Bingman 2004; Siegel et al., 2005; Bingman & Sharp 2006). The importance of the avian hippocampus for spatial navigation has been further confirmed with IEG's experiments (Smulders & DeVoogd 2000; Bischof et al., 2006; Mayer et al., 2010; Mayer & Bischof 2012). Our most recent study using IEG's in domestic chicks trained in a standard reference memory task (Vallortigara et al., 1990), further confirmed hippocampal involvement in goal-based navigation by the shape of a rectangular arena (Mayer et al., 2016), suggesting similar sensitivity of avian hippocampus to the boundary space. Given the fundamental importance of boundary geometry for spatial orientation abilities across vertebrates, including domestic chicks (Vallortigara et al., 1990; Lee et al., 2012), in the present study we aimed to address whether the hippocampus of newly-hatched chicks would respond to a change in environmental shape in a way that is similar to remapping effects observed in the mammalian hippocampus.

General Methods

Rationale of the experimental design

A standard procedure to map neuronal activity in brain sections is to quantify cells containing IEG products. The underlying assumption is usually that the experimental task should activate a greater number of cells in the region of interest of the experimental animals compared to the controls. However, the differences in neural coding associated with the differing environmental conditions can also involve variations in which neuronal populations, within the same region, are activated by two different experiences (Chaudhuri et al., 1997; Vazdarjanova & Guzowski 2004; Guzowski et al., 2005). Thus, the involvement of brain structures in which two different neural populations are activated in the control and experimental conditions might be overlooked, if the two populations are approximately of the same numerosity.

Prior to the current study, a series of pilot experiments revealed equally high activity levels in the hippocampus of chicks that were habituated to visit a square-shaped arena over multiple days and then exposed either to a new rectangular arena or to an identical square environment to that seen in habituation. Both conditions elicited similar hippocampal activation, with no differences in activated cell counts between the two groups (unpublished results). These surprising results led us to hypothesize that distinct neural populations might be activated in the two conditions, but since they were of similar numerosity we could not detect the difference.

To overcome this limitation, the design of the present experiment involved exposing different groups of chicks, either two times to the same shape environment or to two different environmental shapes. By considering the time course of c-Fos expression (an immediate early gene product with protein peak level between 1-2h after behaviourally induced activation), we expected to observe highest number of c-Fos activated cells in the group of chicks which were exposed to two different environmental shapes, because the two different experiences within a short period would activate different (potentially overlapping)

populations of neurons, both containing high level of c-Fos at 1-2h after exposure. On the contrary two repeated exposures to the same environmental shape within a short period would induce an overlap of neuronal populations (Guzowski et al., 2005) and therefore activate lower densities of cells.

Subjects

Thirty-four laboratory-hatched, male domestic chicks (Gallus gallus domesticus), of the Aviagen ROSS 308 strain, were used. We excluded all female chicks from our study to avoid interference of female hormones on behaviour and brain activity. Fertilized eggs were obtained from a local commercial hatchery (Agricola Berica, Montegalda (VI), Italy) and incubated under standard conditions in darkness. After hatching, chicks were maintained individually in metal cages (22.5 x 40 x 30cm^3) at room temperature of 30–32°C with LED illumination from above at a day/night cycle of 14h light and 10h dark. Chicks were deprived of food in the evening before each day of training. After the training sessions chicks received food ad libitum for at least 4 h before it was removed again in preparation for the next day of training. Water was available ad libitum during the entire training period. All experiments were carried out in accordance with ethical guidelines current to European and Italian laws. The experimental procedures were licensed by the Ministero della Salute, Dipartimento Alimenti, Nutrizione e Sanità Pubblica Veterinaria (permit number 25587).

Apparatus

Chicks were trained to forage for mealworms in two square-shaped chambers (60cm x 60cm x 60cm) connected by a door (15cm x 15cm) that they had to walk through to get a mealworm (Tenebrio molitor larvae) (Fig.1). Only the first chamber (base arena) contained a water dish, which was positioned in the centre whereas the second chamber (experimental arena) was empty. The wall between the two arenas could slide vertically (max. 15cm). This way the door appeared in the centre of the wall when it was elevated and disappeared below the floor when the wall was slid down again. All inner surfaces were white and the arenas were homogeneously illuminated through a 10cm hole in the centre of the ceilings with a 40W warm light bulbs. The room outside of the arenas was dark. For the test phase, the experimental arena was replaced either with a new square shaped arena or a rectangular arena (L x W x H: 80cm x 45cm x 57.6cm). All arenas had identical overall area of the inner surfaces (21600cm^2), thus the reflections of the light from the walls were balanced. The surface areas of the floors on which chicks could move around were also identical: 3600cm^2 (square: 60cm x 60cm; rectangle: 45cm x 80cm).

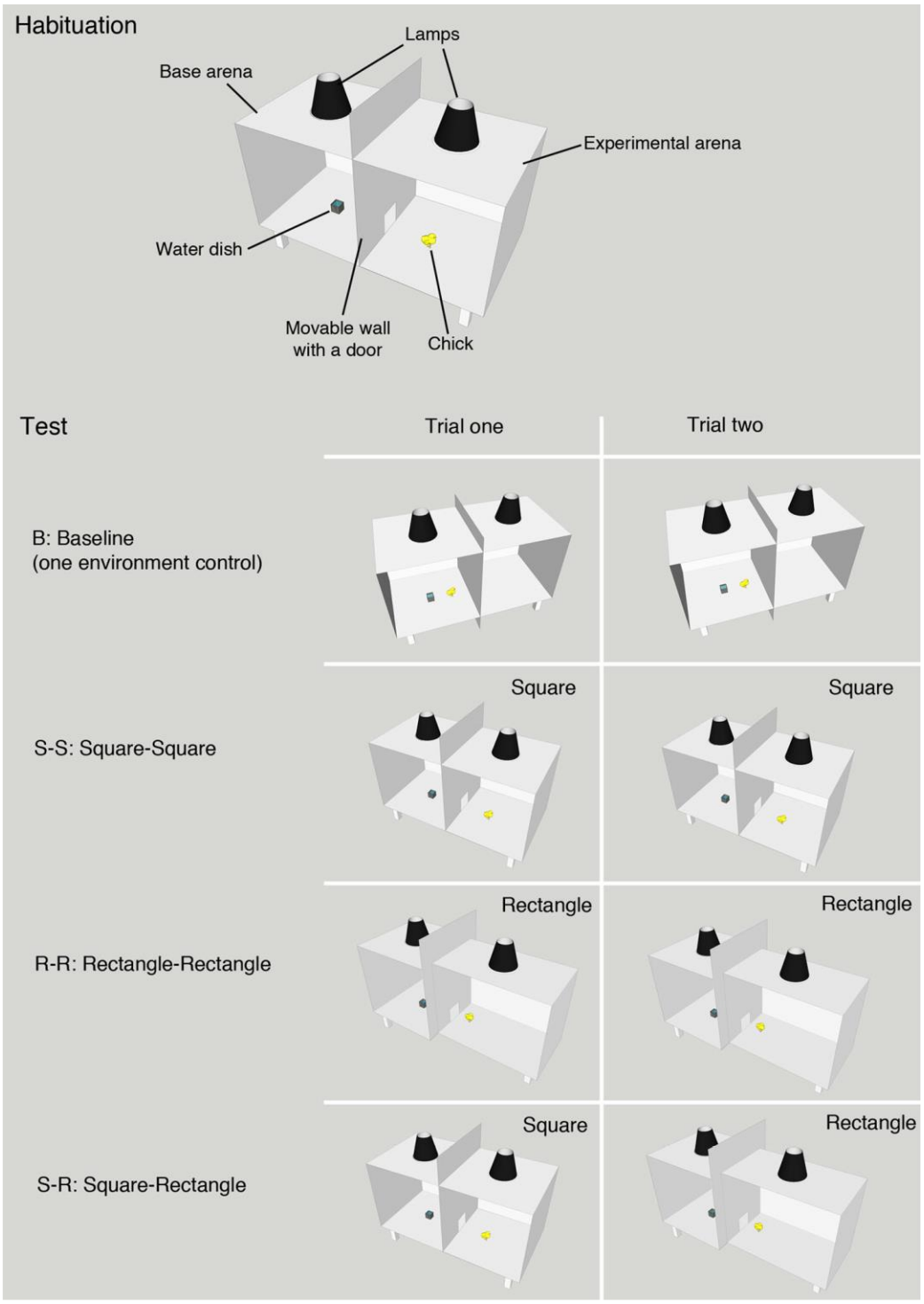

Figure 1: Schematic representation of the experimental setup and procedures. The base arena (square shaped) contains a water dish in the centre and is connected to the experimental arena. During habituation, chicks were trained to forage for mealworms by traversing between the base arena and an identical square-shaped arena, which were connected by a door opening which appeared in the centre of the wall when it was elevated. Chicks were repeatedly habituated to this procedure multiple times over multiple days (see ''Methods'' for details). On the morning of testing, chicks were placed in the base arena for 5 h, prior to the test session. For the test session, chicks were divided into four groups. The baseline group stayed in the base arena, where they received four worms with an interval of 1 min. One hour after receiving the last worm, the chicks were perfused. The other three groups performed two experimental trials with the following sequence: Trial 1: chicks entered the experimental arena, found a worm and stayed there for 1 min; and then, they came back to the base arena, found another worm and stay there for 1 min. Trial 2: chicks entered the experimental arena, found a worm and stayed there for 1 min; and then, they came back to the base arena, found another worm and stayed there for 1 h until perfusion. The square–square (S– S) group visited a new square-shaped arena (identical to the one used during habituation) twice, both in trials 1 and 2. The rectangle– rectangle (R–R) group visited a rectangular arena twice, in both trials

1 and 2. The square–rectangle (S–R) group visited a square arena in the first trial and a rectangular one in the second trial. All arenas had identical inner wall and floor surface areas

Habituation Training

On the first day after hatching male chicks were placed in individual home cages with food and water provided *ad libitum* (see *Subjects* section for details). On day four, chicks were familiarized with the experimental environment. Each chick was individually placed in the base arena (which was not covered from above) with a water dish in the centre. They were allowed 5–10 min to explore the environment and find mealworms which were placed nearby. To encourage exploration, chicks were trained to walk through the door when it appeared, to the experimental arena, by placing a mealworm directly in front of the door in the new environment. The door was then closed and after one minute it was opened again to allow the chick to go back in the first arena and to find the next reward. This procedure was repeated at least three times with each chick. After the habituation training chicks were moved back to their home cages where they remained until the next day. Transport from the home cage to the experimental arena and back occurred in a closed, plastic box (32 cm long, 18 cm wide, 30 cm high).

On the following three days, chicks underwent 6 daily sessions of habituation training (3 in the morning and 3 in the afternoon). One session contained 5 trials, in which the chicks needed to walk through the open door to find a reward in the experimental arena and when the door was opened again after one minute to walk back to the base arena to receive another worm. The arenas were now covered from above and the light in the experimental room was off. The worms were delivered at random positions through the central light bulb holes in the ceilings of the arenas when the chick was in the other arena. Between the sessions, the chick remained in the base arena, which contained a water dish in the centre. The intervals between each trial were 1min, between sessions 30min, and between morning and afternoon sessions 2h. On the fifth day of training, the morning session was carried out as usual. After the end of the morning session the chick remained in the base arena until the beginning of the test session, which was performed in the afternoon (no training session was performed in the afternoon of the test day). Chicks remained in the base arena continuously for 5h after the morning training session, before the test session began. This ensured that c-Fos expression due to brain activation in response to the base arena was at baseline by the time of perfusion after the test (Zangenehpour and Chaudhuri 2002). Thus, we did not expect our measures to be influenced by any activity associated with experiencing the base arena.

Test Session for c-Fos Labelling

Chicks were divided into four experimental groups. The test session was performed 5h after the morning session and consisted of two trials for each experimental group (except the baseline control group). The baseline group (n=10) was a control condition to measure hippocampal baseline activity. The chicks of this group remained in the base arena where they received four worms randomly placed in the environment

with a 1min interval between each worm. For the three experimental groups, which differed only in the shape of the experimental arena used during test, the chick went from the familiar base arena to the experimental arena and back twice, receiving four worms total. The second group (square-square: n=8) was exposed twice to a novel square shaped arena (the experimental arena). The inter-trial interval was 1 min. The third group of chicks (rectangle-rectangle: n=8) was exposed two times to a novel rectangular experimental arena, with the same procedure as the second group. The fourth experimental group (square-rectangle: n=8) was exposed to both the square and rectangular arenas. Thus, in the first trial, the experimental arena was a square. When the chick went back to the base arena, the square arena was replaced with the rectangular one for the second trial. After the second trial, the chicks remained in the base arena until perfusion.

Immunohistochemistry

Seventy minutes after the test session, subjects were overdosed with an intramuscular injection of 0.8ml Ketamine/Xylazine Solution (1:1 Ketamine 10 mg/ml + Xylazine 2 mg/ml). The chicks were perfused transcardially with phosphate buffered saline (PBS; 0.1mol, pH=7.4, 0.9% sodium chloride, 4°C) and paraformaldehyde (4%PFA in PBS, 4°C). The skull with the brain was post-fixed in 4% PFA/PBS solution for 7 days. For the removal of the brain from the skull procedures described in chick's brain atlas Kuenzel & Masson (Kuenzel and Masson 1988) were applied to ensure that the coronal brain sections of all brains had the same orientation (45°). After removing the brains from the skull, the left and the right hemispheres were separated and processed separately. Each hemisphere was embedded in gelatine (7%) containing egg yellow, post-fixed for 48h in 4% PFA/PBS containing 20% sucrose at 4 °C, and further 48h in 30% sucrose in 0.4% PFA/ PBS. The brain hemispheres were frozen at -80°C covered with O.C.T (Tissue-Tek freezing medium). Four series of 40μm coronal sections were cut on a Cryostat (Leica CM1850 UV) at -20°C. The sections were collected only from the regions of interest A(nterior) 7.8 to A 5.4 (Kuenzel and Masson 1988). The sections of the first series were used for processing and labelling. The sections of the other series were kept as backup or for testing antibody specificity (processing without the primary antibody). Washing in PBS (3x5min) was performed between each of the following reaction steps. Endogenous peroxidase activity was depleted in 0.3% H_2O_2 in PBS for 20 min. After incubation with 3% normal goat serum (S-1000; Vector Laboratories, Burlingame, CA, USA) in PBS for 30 min, the sections were treated for 48h at 4°C with an anti-c-Fos antibody solution (1:2000; mouse monoclonal, E-8, sc-1669, Santa Cruz, CA, USA), followed by biotinylated anti-mouse in PBS (1:2000; BA-9200, made in goat; Vector Laboratories) for 60min at room temperature. The ABC kit was used for signal amplification (Vectastain Elite ABC Kit, PK 6100; Vector Laboratories) and VIP substrate kit for peroxidase (SK-4600; Vector Laboratories) for visualisation of c-Fos-immunoreactive (-ir) neurons. Sections were serially mounted on gelatine-coated slides, dried at 50 °C, counterstained with methyl green (H-3402; Vector Laboratories) and cover slipped with Eukitt (FLUKA).

Brain analysis

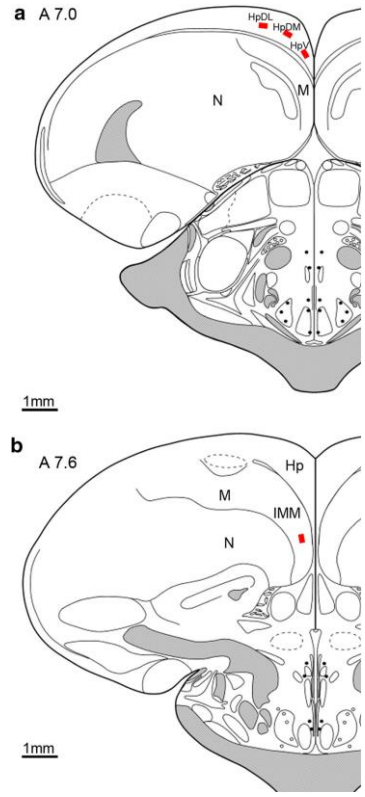

Figure 2: Typical placements of counting enclosures (red rectangles) inside the three subdivision of hippocampus (a) and the intermediate medial mesopallium (b). Those drawings of the coronal sections were adapted from Kuenzel &Masson (1988). Hp hippocampus, HpVM ventro-medial hippocampus, HpDM dorso-medial hippocampus, HpDL dorso-lateral hippocampus, M Mesopallium, IMM intermediate medial mesopallium, N nidopallium

Brain sections were examined with a Zeiss microscope (objective: 20x with a numerical aperture of 0.5) and a digital camera (Zeiss AxioCam MRc5). Contrast and exposure time of the camera were adjusted so that the image on the screen matched the view under the microscope (eyepiece 10x, overall magnification 200x). Successful immunostaining produces dark, purple-black stained nuclei, which can easily be discerned from background and non-activated cells, which were stained light green (see Fig.3). The imaging software ZEN (Zeiss) was used for counting of c-Fos-ir neurons on a computer screen. For this purpose, a counting grid (150 x 250 mm^2), was positioned over the different sample areas (see below). Counting was performed blind to the experimental condition. Every activated c-Fos-ir cell within each sample area was marked on the screen using the ZEN software, which computed the total counts.

To estimate labelled cell density within the hippocampus, five to eight sections of each hemisphere were selected from that part of hippocampus extending from A(nterior) 7.0 to A 6.0 (determined by the shape and anatomical organization matched to the atlas of Kuenzel and Masson, 1988). The hippocampus of each section was parsed into three subdivisions: the ventral hippocampus (HpVM), the neighbouring dorsomedial hippocampus (HpDM) and the dorsolateral hippocampus (HpDL) (Fig. 2a). For cell counting of each subdivision across the sampled sections, the counting grid was placed in a way such that it covered as many activated neurons as possible while keeping a minimum distance of 20μm to the edge of the brains section and the border of a neighbouring subdivision. Typical placements are schematically shown in Fig.2a. Labelled cell density was estimated also in the intermediate medial mesopallium (IMM) as control region. Here we relied on previous anatomical descriptions of this region (Ambalavanar et al. 1993). Five brain slices were selected from a region where the shape of IMM corresponded to what is depicted on plate A7.6 of the Kuenzel and Masson atlas. The counting grid was positioned inside the IMM according to the drawings (Ambalavanar et al. 1993), see also Fig.2b.

After completing the cell counts, the mean values (derived from the five sections) for the three subdivisions were calculated for each hemisphere and cell densities were standardized to 1mm^2. Because no significant lateralization effect was found for any subdivision of the experimental groups, the measured values from the two hemispheres were pooled for further analysis. Cell counts pooled from the 3 Hp subdivisions were further averaged to estimate overall Hp activity. Thus, the overall estimate of hippocampal activity of an individual bird was based on an average from all 30-48 counted areas (15-24 from each hemisphere). The calculated neuronal activity in IMM for each individual chick was based on 10 counted areas (5 sections, 2 hemispheres). The resulting individual bird means were considered overall indicators for the number of c-Fos-ir neurons and were employed for further statistical analysis.

Statistical Analysis

Differences between groups in their Hp and IMM activation were tested with two independent univariate ANOVA's. Because the Levene's test for the Hp analysis revealed a significant violation of equality of variances and Kolmogorov–Smirnov test (K–S) showed that the distribution of residuals was significantly different from normality a logarithmic transformation (log(x+10)) was applied before running the statistical analysis. This procedure increased the equality of variances and normality of the residuals as follows: Levene's test before log transformation: $F(3.29)=7.022$, $p<0.01$; Levene's test after log transformation: $F(3.29)=1.139$, $p=0.35$; K-S before log-transformation: $D(33)=0.164$, $p<0.03$; K–S after log-transformation: $D(33)=0.137$, $p=0.122$. For the IMM violation of variances was not present (Levene's test: $F(3.29=0.574$, $p=0.64$) and the residuals were normally distributed (K-S: $D(33)=0.089$, $p=0.2$) therefore no transformation was required. The post-hoc comparisons were carefully planned based on the a priori expectations derived from the pilot studies (see the section 'Rationale of the experimental design'*).* For analyses of Hp activation, three independent t-tests (two-tailed) were carried out. First, based on our pilot study, we expected no significant differences to emerge from the second comparison between the two same-shape conditions, the square-square and rectangle-rectangle groups. Second, we expected lowest levels of activity in the baseline condition and planned to compare the baseline condition with one of the same-shape groups, the one which would show the second lowest activity level. The third planned comparison was between the square-rectangle group (in which we expected highest levels of c-Fos-ir neurons) with one of the other groups that would show the second highest levels of activity.

Results

We processed all 34 brains, however during the staining procedure the hippocampus of one brain was damaged and could not be used for counting. This brain was excluded from further analysis resulting in n=7 for the square-rectangle group. In all animals, the hippocampal slices contained high numbers of stained

nuclei with exception of those from the baseline group in which hippocampus was often devoid of activation (Fig.3). The brains also showed some individual variability of c-Fos-ir cell distribution, although in most of the cases high number of activated cells were visible in the dorso-lateral Hp. Counts in the subdivisions confirmed this observation, showing highest numbers of c-Fos-ir neurons in the dorsal region; the trends between the groups, however, were similar in all subdivisions (Table 1). In all subdivisions, the highest densities of c-Fos-ir 6 cells were present in the square-rectangle group, intermediate densities in the square-square and rectangle-rectangle group and the lowest in the baseline condition. For statistical analysis, the Hp subdivisions were lumped together (Fig.4a).

The density of c-Fos-ir cells within Hp of the baseline group (mean ± s.e.m.: 161.6 ± 42.4 cells/mm^2; n=10) was approximately 38% lower than in the square-square group (mean ± s.e.m.: 427.2 ± 85.9 cells/mm^2; n=8). The density of c-Fos-ir cells was almost identical in the rectangle-rectangle group (mean ± s.e.m.: 433.9 ± 77 cells/mm^2; n=8) compared to the square-square. The density of c-Fos-ir cells was highest in the square-rectangle group (mean ± s.e.m.: 865.9 ± 188.9 cells/mm^2; n=7) which was approximately 50 % higher than in the rectangle-rectangle group (Fig.4a). Statistical analysis revealed significant between group differences in the number c-Fos-ir cells in the Hp (ANOVA: $F(3,29)=10,892$, $p<0.01$). T-test planned comparisons revealed a significant difference between the baseline and the square-square condition ($T(16)=-3,151$; $p<0.01$), no significant difference between the square-square and the rectangle-rectangle group ($T(14)=-0.179$; $p=0.86$) and a significant difference between the rectangle-rectangle group and the square-rectangle group $T(13)=-2,189$; $p<0,05$). Differences between the groups were not present in the IMM: ANOVA: $F(3,29)=0.051$, $p=0.98$.

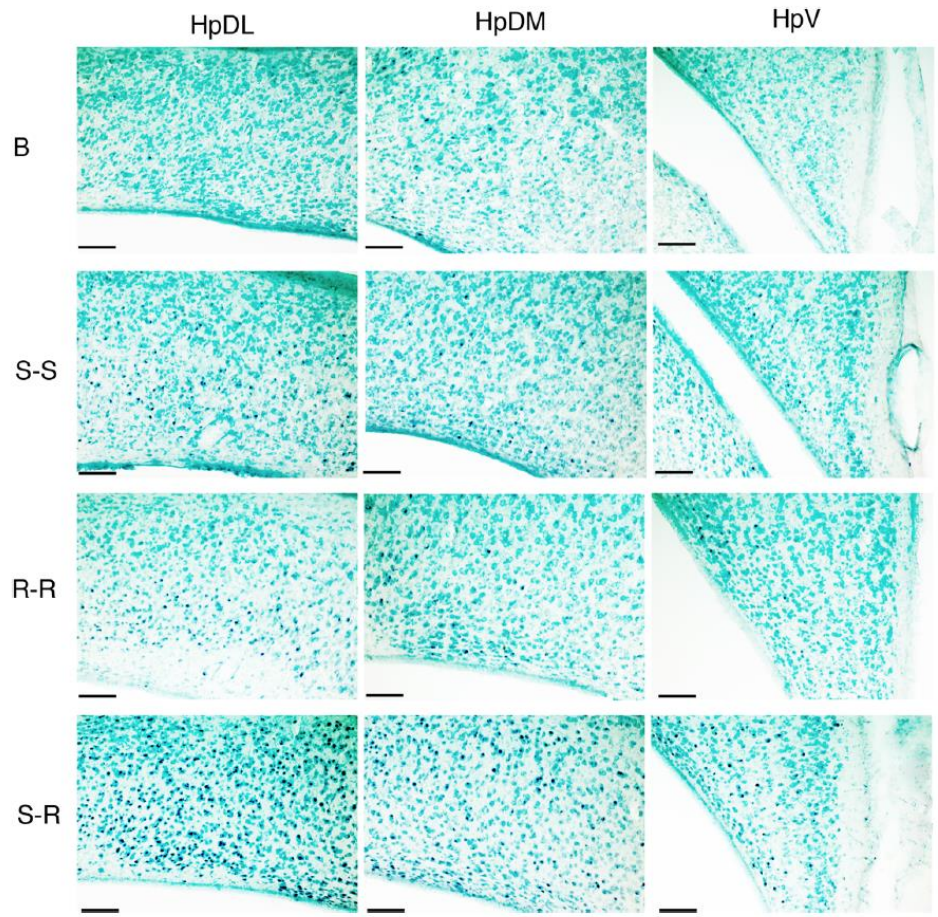

Figure 3: Photomicrographs of hippocampal activations, showing the dorso-lateral (HpDL), dorso-medial (HpDM) and ventral (HpV) parts of one exemplary coronal section from each group of chicks. c-Fos-ir cells are stained black after the immunohistochemical procedure. The non-activated cells are counterstained in green. B (base-arena control group), S–S (square–square group), R–R (rectangle– rectangle group), S–R (square– rectangle group)

Table 1: Measured c-Fos-ir cell densities observed in all three hippocampal subdivisions (HpVM—ventro-medial hippocampus; HpDM—dorso-medial hippocampus; HpDL—dorso-lateral hip- pocampus) and in the intermediate medial mesopallium (IMM) for the different groups of chicks: B (base-arena control group), S–S (square–square group), R–R (rectangle–rectangle group), S–R (square–rectangle group)

R–R

	B	S–S	R–R	S–R
HpVM	58.2 ± 18.6	216.2 ± 63.7	167.2 ± 30.3	308.6 ± 104.9
HpDM	123.7 ± 39.1	321.0 ± 73.4	322.3 ± 84.3	735.0 ± 184.7
HpDL	330.5 ± 88.8	713.9 ± 174	812.2 ± 141.6	1554.2 ± 322.9
IMM	208.1 ± 62.6	202.9 ± 52.3	226.7 ± 49.1	228.9 ± 55.3

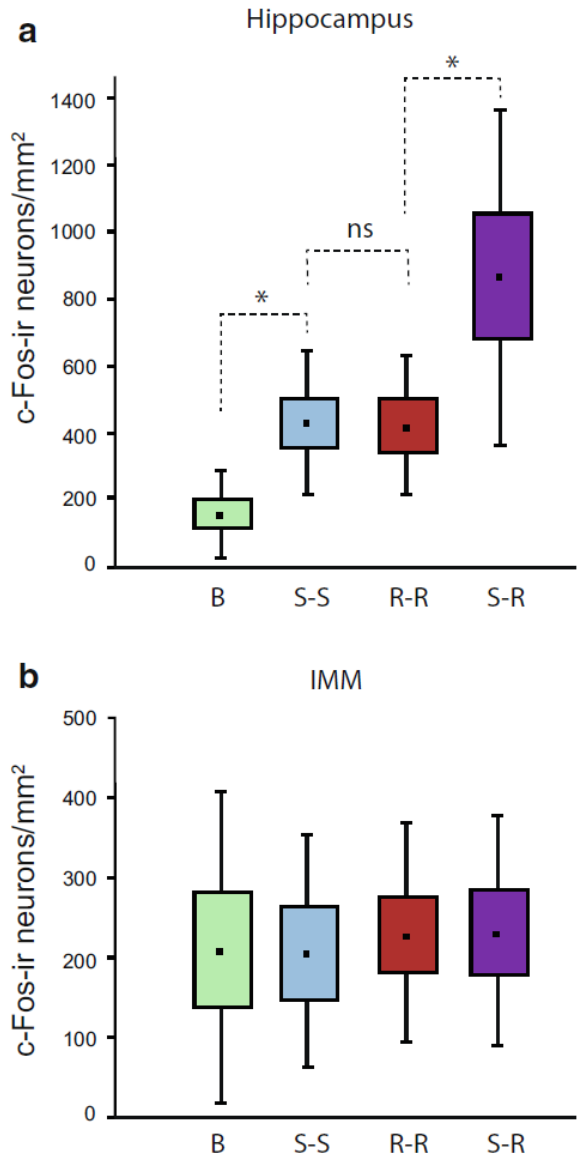

*Figure 4: Measured c-Fos-ir cell densities in hippocampus (a) and intermediate medial mesopallium (b) in four groups of chicks: B (base-arena control group), S–S (square–square group), R–R (rectangle–rectangle group), S–R (square–rectangle group). Graph-plot: mean (black square), SEM (box) and SD (whisker) (*p\0.05; **p\0.01). Densities of c-Fos-ir neurons per mm2 are represented on the Y-axi*

Discussion

The key finding of our study is that even in the earliest stages of life, hippocampal response of domestic chicks is strongly influenced by the geometric layout of environmental boundaries. The number of activated cells in the hippocampus did not differ between chicks exposed twice to an arena having a familiar square shape or a novel rectangular shape (both had higher activation than a control group exposed only to the base arena). However, if chicks were exposed to both environmental shapes in two consecutives trials, the number of c-Fos-ir cells was doubled. The effect was region-specific: no differences were present in IMM.

An explanation of the increased number of cells in the square-rectangle group is that different populations of cells represented the environments of two shapes. Although our method does not allow us to

distinguish whether a given c-Fos-ir neuron was activated two times by two events occurring close together in time or only once. Presumably, two repeated exposures to same-shape arenas would induce highly overlapping activity of the neuronal network representing the shape. On the contrary, exposures to two test arenas differing in shape would cause mostly non-overlapping activation. Thus, the results confirmed this hypothesis by showing that the density of c-Fos-ir neurons in the square-rectangle group is almost twice as high compared to rectangle-rectangle group. This finding is consistent with studies of the mammalian hippocampus. IEG expression in CA1 of rats exposed either to two different environments or twice to the same environment, revealed that the repeated exposure group had a higher number of double-labelled cells expressing two activations at two different time points (Nakamura et al., 2010). Moreover, when mRNA of different IEGs visualised hippocampal activation at two time points, different environments induced responses in different populations of CA3 neurons (Vazdarjanova & Guzowski 2004). After two exploration sessions across three conditions for which the second environment was unchanged, slightly modified, or novel, the highest degree of overlap in activated neurons in the two sessions was found in animals exposed to the same environment twice; animals exposed to two different environments exhibited a low degree of overlap, and an intermediate degree of overlap was observed for the slightly modified environment. The recruitment of an entirely new ensemble in area CA3 of the hippocampus, suggests that at least this subfield clearly delineates between different. Future studies could capitalize on the evidence obtained here and further investigate this hypothesis, adapting for chicks the protocols developed for discriminating two time points in immediate early gene induction (Guzowski et al., 1999, 2005).

The lack of differences between the square-square and rectangle-rectangle group might seem surprising, in which the chicks were familiar with the square but unfamiliar with the rectangular shape. IEG expression is known to be highest during early learning (Anokhin & Rose 1991) and to diminish following extended training (Kelly & Deadwyler 2002), and this is also the case for birds engaged in spatial learning tasks (Mayer et al., 2010). Thus, one might expect hippocampal c-Fos production in response to square (but not rectangular) arenas, to be reduced to a minimum after repeated habituation in the square. This was not the case, however: both square-square and rectangle-rectangle groups showed higher c-Fos-ir neurons density than the one-environment control group, but there was no difference in activation between them. Thus, the activity enhancement of the hippocampus was triggered by the change from the base arena (for which c-Fos expression was at baseline, see Methods) to the experimental arena, regardless of whether the shape of the experimental arena was familiar or novel. This would suggest two things: that the habituation to the square experimental arena during training did not reduce IEG expression and that the novelty of being in a rectangular environment for the first time did not induce hippocampal activation to a greater degree than to the square environment. At least one study with rats found no difference in the proportion of hippocampal cells displaying Arc mRNA between animals exposed to an environment for the first time or after nine daily sessions (Guzowski et al., 2006). Future studies are needed to understand the effect of

habituation over multiple days of exposure to the same environment. However, the lack of difference between the square-square and rectangle-rectangle group invites the interpretation of the higher activity in the square-rectangle group as a consequence of two different neural representations based on environmental shape, rather than simply on novelty.

Why is hippocampal c-Fos expression increased when chicks visit experimental arenas of different shapes? Here we would like to consider if this activity can be related to remapping mechanisms comparable to those known in mammals (Fyhn et al., 2007; Moser et al., 2008; Barry et al., 2012). Remapping in mammals was first studied in electrophysiological recordings of hippocampal neurons. Hippocampal place cells 'remap' and alter, activate, or inactivate their preferred firing fields after changes of environmental shape (Muller & Kubie 1987; O'Keefe & Burgess 1996; Lever et al., 2002). Such changes in the firing of a single cell are of course dependent on the inputs that it receives within a given neuronal network. Therefore, given that at least a part of this network is within hippocampus, this area should contain different, potentially partially overlapping, populations of cells that respond to the different shapes. Our findings are consistent with studies in mammals which show that different hippocampal cells are activated by different environments (Vazdarjanova & Guzowski 2004; Guzowski et al., 2005; Barry & Commins 2011). Although we did not measure two time points independently, the two-fold increase in hippocampal activity in the square-rectangle group is consistent with a largely non-overlapping representation of two neuronal populations of cells within the hippocampus of chicks. Thus, it is possible that remapping-like mechanisms exist also in birds to the extent that environmental differences induce a new pattern of neural activity in the hippocampus. Unfortunately, few studies have reported hippocampal place coding at the level of single cells in freely moving birds. Bingman and collaborators studied place-related responses in pigeons finding different location related activities, such as location cells, grid like cells, arena-off cells and path cells, which were not as specific as in mammals (Bingman & Sharp 2006). These effects are not necessarily a reflection of variations in biological organization, but may emerge as a consequence of testing procedures and behavioural differences (Bingman & Sharp 2006). The existence of some forms of location specific response in pigeons, together with the results from the present study, suggest that also remapping related activities might be present in bids.

In conclusion, here we present the first evidence of hippocampal representation of environmental shape in birds. Our study adds to a series of experiments showing astonishing functional similarities between the mammalian and avian hippocampi (Colombo & Broadbent 2000; Vargas et al., 2004; Bingman & Sharp 2006; Mayer et al., 2013, 2016). The results suggest functional similarities not only at the anatomical, but also at the neural level. Many questions remain, but the study opens doors for expanding knowledge of hippocampus across evolution.

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Chapter 2: Proximal deletion of Williams syndrome critical region causes deficits in reorientation and episodic-like memory in Williams syndrome mouse model.

Abstract

Williams syndrome is a rare genetic disorder caused by a deletion of around 20 genes on chromosome 7. This deletion, also called Williams syndrome critical region (WSCR), generates a unique cognitive and behavioural profile characterized by severe visuo-spatial deficits and long term memories. Mouse models of this disorder provide the possibility of gaining deeper insight as to the connection between genetic modifications and cognitive-behavioral deficits. In this study, we aimed to better characterize a mouse model that had one half of the WSCR deleted. These mice also called PD mice (partially deleted mice) reproduce most of the phenotypes observed in WS patients including spatial and social deficit. To further understand the interaction between gene deletion and behaviour, we tested PD and wild type controls in the following behavioural tasks: reorientation (using boundary geometry, and landmark) and episodic-like memory. Our results provide evidence that PD mice reproduce also the spatial and memory deficit of clinical patients. In particular, we observed a failure in spatial tasks and an impairment in the temporal component of episodic memory. We conclude that the PD genetic deletion may be sufficient in causing a hippocampal deficit assessed through behavioural testing in mice models. These results provide suggestive evidence of the PD deletion (Gtfi2-Limk1) being related to hippocampal function and set the stage for a more detailed study on the effects of the PD deletion on the hippocampus.

Introduction

Williams syndrome (WS) is a rare neurodevelopmental disorder that affects around 1/7500 new-borns (Donnai & Karmiloff-Smith, 2000). An error during meiosis gives rise to a deletion on chromosome 7q11.23. This deleted region, also called WSCR (Williams syndrome critical region), contains 26-28 missing genes (Donnai & Karmiloff-Smith, 2000). WS patients have a unique clinical and cognitive profile with some strong abilities (preservation of the language, facial recognition, social and interpersonal skills) and weaknesses (anxious behaviour in novel contexts and a severe deficit in spatial and memory tasks). Their altered behaviour, in particular their spatial deficit is associated to hippocampal abnormalities. WS patients show abnormal function and metabolism of the anterior hippocampal formation despite preserved volume and subtle altered morphology in compared to healthy controls. Furthermore, resting state cerebral blood flow was significantly reduced bilaterally in the hippocampal formation of WS patients in response to visual stimuli (Meyer-Lindenberg, 2005).

Hippocampus related behaviours such as spatial navigation and memory are severely impaired in WS patients (Ferrara & Landau, 2015; Lakusta et al., 2010). WS individuals show an extensive visuo-spatial deficit (Gray et al., 2006; Tassabehji et al., 2005). Difficulty in recalling routes and the tendency to get lost in new unfamiliar environments has been often reported by WS family members (Farran, Blades, Boucher, & Tranter, 2010). Navigation ability is altered in WS patients: Bernardino and colleagues performed a screen based and 3D task to test whether patients could use their own body (egocentric) or external objects (allocentric) to correctly distinguish the position of an object in an environment. Results show that in both tasks there is an impairment compared to controls (Bernardino et al., 2013). Allocentric navigation uses not only external references such as objects or landmarks but is also particularly reliant on environmental geometry to orient (Lee, 2017). Lakusta and colleagues showed WS patients were not able to use the geometric properties of a rectangular arena to reorient, but can improve when a blue wall is added to the arena (Lakusta et al., 2010) suggesting that while geometry based navigation is impaired, feature use is spared.

Not only spatial memory but other branches of memory including short and especially long term memory are also affected in WS patients (Vicari, Brizzolara, Carlesimo, Pezzini, & Volterra, 1996). However, most tasks assessed verbal and spatial memory (Rhodes, Riby, Fraser, & Campbell, 2011) and observed a general deficit mainly in long term-memory. More recently, Vicari et al designed a task where the subject had to recall a succession of presented objects, and WS scored equally as controls suggesting normal memory for recalling objects (physical properties). However, in a visuo-spatial task where they were asked to recall the spatial collocation of an object, WS patients failed (Vicari, Bellucci, & Carlesimo, 2005). These results suggest an overall difficulty in linking together what object they saw and where/which location it was

collocated. Given, the failure in Vicari's task, we wondered whether this might lead to an overall difficulty in linking elements of complex memories such as long-term episodic memories.

Episodic memory, first defined by Tulvig, is the conscious autobiographical recollection of what, where and when of a personal memory. This is an important branch of long-term memories, essential to all human beings (Tulving & Thomson, 1973) and animals (Dere et al., 2006; Templer & Hampton, 2013) as they encapsulate personal memories of the past that are imperative to plan future actions. Brain-injured amnesic individuals, i.e., with selective hippocampal lesions, or damage to the frontal lobes and diencephalic structures, such as the dorsomedial thalamus, and the mammillary bodies, cause severe deficits to mainly episodic memory while sparing semantic memory (Aggleton & Brown, 1999). Healthy aged individuals and variety of neuropsychiatric diseases including Alzheimer's disease, all show an overall damage to recall and retrieve personal memories. (Small et al., 2000). Thus, there is a need for animal models of episodic memory, since animal studies have the advantage of underlying the anatomical, pharmacological, physiological, genetic and molecular mechanisms that aren't possible in human studies.

In this scenario, WS, given its clear genetic deletion and hippocampal abnormalities, allows us to investigate hippocampal function and the relationship between genotype and phenotype. Many mice models of WS have been designed from single gene to multiple gene knockouts. The first allows the study of a gene in both homozygous and heterozygous state, and leads to potential correlations between genotype and phenotype. Single knockout mouse models for LIMK1 (Meng et al., 2002) and CYLN2 (Hoogenraad et al., 2002), two genes present on the WSCR, show abnormalities in hippocampal physiology and structure. Both mouse strains show reduced long term potentiation and learning impairments. In addition, hippocampal neurons of LIMK1 mice have a different pattern of dendritic spines with thinner necks and smaller heads compared to controls. However, Williams syndrome is a multigene deletion disorder, and therefore in order to understand the complexity, multiple gene knockouts are more reliable in reproducing more aspects of the disorder. Given the limitations of single knockouts, more recently multiple knockouts that include distal and proximal regions of the WSCR have been generated (Li et al., 2009).

In the present study, we aimed to fill a gap in the literature using a mouse model that contained the proximal deletion of the WSCR (Gtf2i-Limk1). These mice reproduce abnormal social behaviour and anxiety seen in WS patients. However, there is no evidence whether they are impaired in spatial tasks and in complex memories such as episodic-like memory (Li et al., 2009; Osborne, 2010). Given the severe difficulty in visuo-spatial tasks in WS patients, we wondered if the PD deletion could be linked to these specific deficits. Although single genes have been studied separately, there is no knowledge whether the interaction of more genes together, as that on the PD region could together trigger spatial impairment. Therefore, we aimed to investigate two hippocampal related behaviours: the capacity to successfully reorient using environmental

geometry and landmark (Cheng, 1986; Hartley et al., 2014; Lee et al., 2015) and the ability to recall episodic like memory (Dere et al., 2005; Eacott, 2004).

General Methods

Animals: Williams syndrome F0 mice were bought from Jackson Laboratories. This mutant mouse strain carries a deletion corresponding to the proximal half (G2fi-Limk1) of the WSCR on mouse Chromosome 5G2 (figure 1). Two to five mice were housed per cage in a room with a 12 h light–dark cycle (lights on at 7 AM, off at 7PM) with access to food and water ad libitum. Two weeks before testing animals were transferred into a ventilated cabinet and kept in the testing room until the end of the experiments. Each subject was tested in Experiment 1 followed by Experiment 2. After completing both experiments all mice were sacrificed.

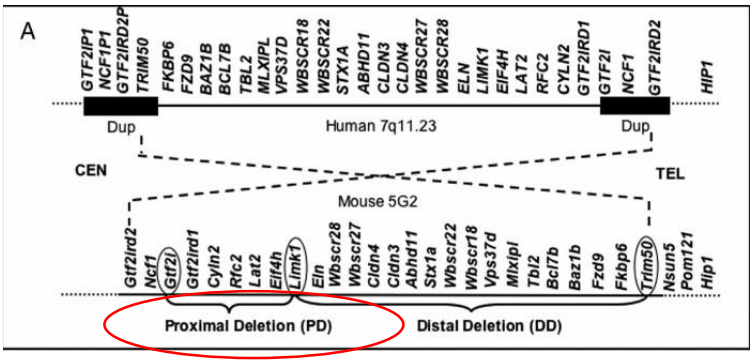

Figure 1: Williams's syndrome genomic region in human and mouse. In red is the proximal deletion (Gtf2i-Limk1), that is deleted on chromosome 5 in the correspondent mouse model. Adapted from (Li et al., 2009).

Experiment 1: Episodic-like memory

We designed a three-step task to investigate the three elements of episodic-like memory in animals: "what", "where" and "when"(Dere et al., 2005). This test is a modified version of the object recognition task, where given the natural curiosity of rodents to explore new objects we can test whether they can distinguish between old and new object. We manipulated the objects we used and their positions and after two trials we tested mice after 60'. This interval, used in other studies is sufficient for mice to "forget" the first seen position or object, but recall the most recent seen object or position. In this view the first object or position becomes novel and attracts the animal, that explores it more than the most recent object/position that becomes familiar and less attractive.

Apparatus: Preliminary experiments using the object recognition task were used to find the most appropriate objects that were salient and distinguishable to animals. We used a squared arena (40 x 40 x 20 cm) made of hard plastic, elevated 80 cm above the floor. One bright central light (round; diameter, 10 cm) illuminated the circular testing space from above. A camera was mounted on the ceiling and recorded animal behaviour.

Procedure: all animals were tested in the following order: Place test (where+when), object test (what), episodic memory test (where+what+when). Each test had two sample trials and one test trial. Each trial including the test trial lasted for 5 minutes. An interval of 60 minutes was maintained between sample and test trials.

Place Test: (n=21 WT & 12 PD). This test aims to investigate the "where+when" component. In the first sample trial the animal explored an object placed in a corner of the arena, (old position); in the second sample trial an identical object was placed in opposite corner (new position) of the arena. Following a delay of 60 minutes after the two sample trials, the animal performed the test trial. During the test trial, both objects were placed in the arena in the same position of previous trials and the mouse explored them for 5 minutes.

Object test: (n=12 WT & 8 PD) This test aims to investigate the "what" component. In the first trial the animal was left free to explore two identical objects. In the second trial, objects were replaced with a pair of different objects in the same position. During the test trial, animals explored one object of the first trial (old object) and one object of the second trial (new object).

Episodic Memory Test: (n=29 WT & 11 PD) In the last test we test all three components "what", "where" and "when" of episodic-like memory. In the first sample trial the mouse explored two identical objects. In the second sample trial, the objects were replaced with a pair of different objects placed in the opposite side of the arena. After 60 minutes the mouse performed the test trial: the animal encounters one object of the first trial and one object of the second trial situated in the same position of the previous trials (called respectively "Old stationary" and "New stationary"), and one object of the first trial and one object of the second trial displaced in a different position (called respectively "Old Displaced" and "New Displaced").

Predictions: We predicted that wild type controls would spend more time with the "old object" and "old position" compared to more recent ones. On the other hand, we expected PD mice to show difficulty in recalling the older objects and positions due to their genetic deficit.

Statistical analysis: All tests were recorded and coded offline using Ethovision 9.0. We considered exploration when the animal was in a 3cm range from the object. For each test, we recorded the time spent with the object in seconds and then calculated the proportion of time. For place and object test we used an independent t-test to compare behavior of PD and WT mice. We later investigated behavior of each group separately doing a paired t-test between the two dependent variables: old position vs new position in place test, old object vs new object in the object test. For episodic memory we did a multifactorial ANOVA with "old stationary", "new stationary", "new displaced" and "old displaced" as dependent variables and "genotype" as the independent variable.

In order to investigate the effect of genotype on behavior, a small number of mice were chosen and matched to control littermates for each test according to age and sex. We analyzed place and object test using independent t-tests, and episodic memory with multifactorial ANOVA.

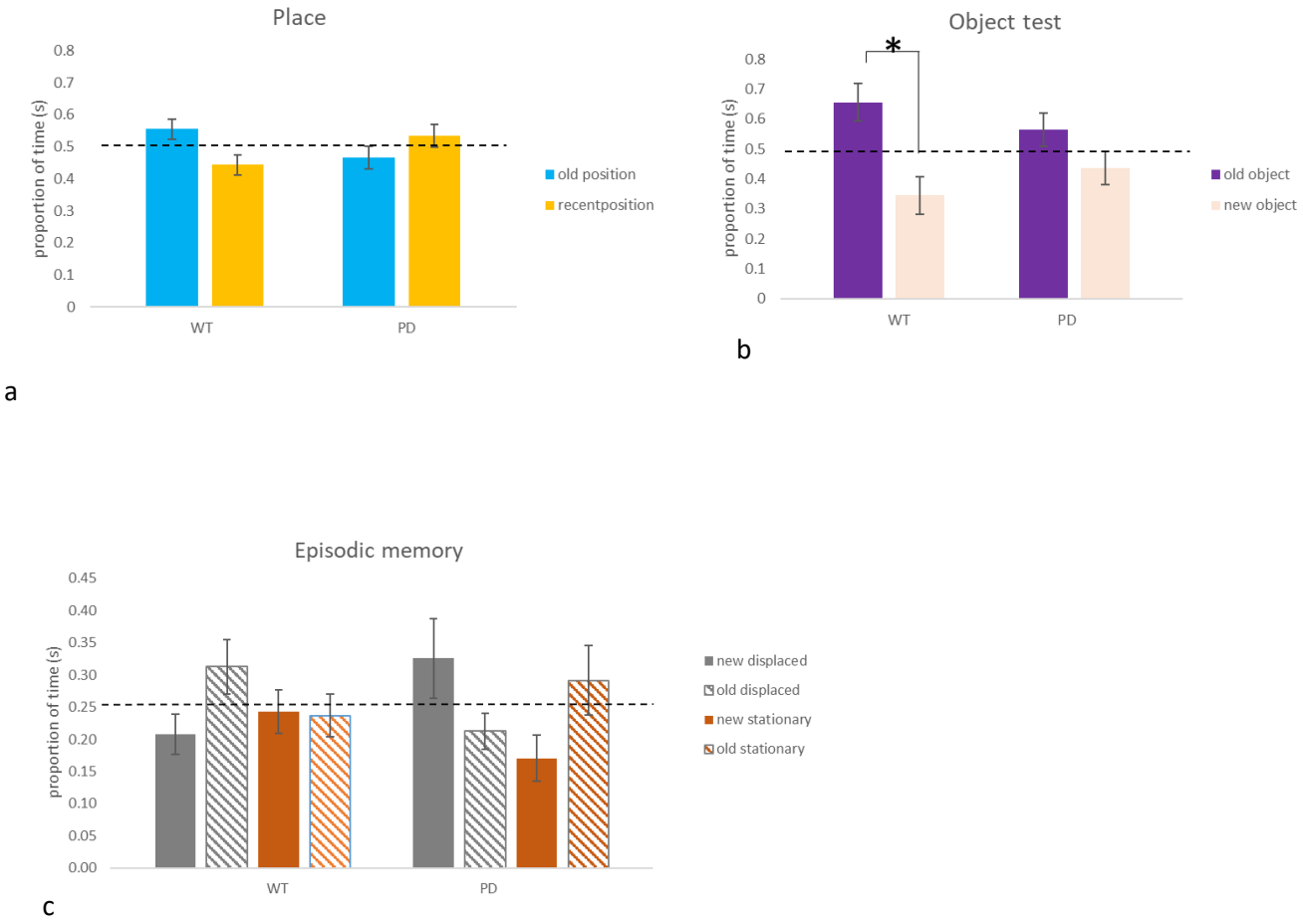

*Figure 2: Results of episodic memory testing in PD mice and control mice: a) place test b) object test c) Episodic memory. Error bars represent standard error of the mean SEM. * indicate p< 0.05 between means.*

Results wild type mice: Wild type animals show a tendency although not significant toward the old position compared to the new position (paired t-test: t (20) = 1.7, p=0.09), suggesting overall recall of where+when elements of episodic memory. In the object test, they show a significant preference for the old object compared to the new object (paired t-test: t (11) =2,5, p=0,029), and therefore correct memory for recalling objects. Their preference for the old object is significantly above chance (t-test against chance of 0, 50: t (11) =2,5, p=0,029). In the episodic memory test, we do not observe any statistical significance in exploration between objects. However, although not statistically significant, observing the means we do observe a preference toward the old displaced over the other objects.

Results PD mice: In PD mice (figure 2a) we observe no statistical significance in exploration time between old and new position concerning place test (t (11) = -1,4, p=0,2). Likewise, PD mice show no significant preference

toward the “old object” in object test (t (7) = 1,1, p= 0.2) (figure 2b). In episodic memory we observe no significant difference in exploration between the four objects (figure2c).

Results PD mice vs WT mice: In order to see whether genotype had an effect on behavior, we matched PD mice to control littermates and compared their behavior for place (n=10) object (n=7) and EM (n=11). Comparison between PD and control littermates showed no significant interaction for place test, suggesting a marginal difference between groups in recalling the old (t-test (t (18) = 1,9, p=0.064) and new position (t (18) =1,9, p=0.064). Comparison for object memory does not show difference between groups (t (12) =0,4, p=0,6), suggesting no difference between WT and PD mice. However, differences between PD and controls are seen in episodic-like memory test: we observed a difference in exploration between groups in exploring old displaced object (F (1,22) =4,6, p=0.04, η^2=0.19) and new displaced object (F (1,20) = 4,5, p=0.046, η^2=0.18). WT mice show a higher exploration of the old displaced object compared to PD mice (t (20) =2,1, p=0,04). PD mice on the other hand, show a preference for the new displaced object (t (20) =2,1, p=0,40).

Interpretation and discussion experiment 1: Our task has the advantage of evaluating each component of episodic memory collapsed together and in isolation. We observe hypothesized results in control mice in all three tasks, indicating correct memory for what, where and when. In PD mice on the other hand, we observe a tendency toward the “New position” in place test (figure 2a) and “New displaced object” in the episodic memory test (figure 2c), suggesting difficulty in recalling where and when as they spend more time with the most recent object seen in the most recent position. However, the what component of memory is relatively spared. Although not significant, we see them spending more time with the “Old object” compared to the “New Object” in the object test (figure 2b). This result is consistent with clinical evidence from WS patients that show correct object memory (Landau, Hoffman, & Kurz, 2006; Vicari et al., 2005). Although inconclusive at the moment, we believe that future analysis perhaps with a larger sample size would improve and confirm our findings.

Experiment 2: Reorientation

Experiment 2 aimed to see whether mutant PD mice were able to use external environmental cues such as boundary layout (rectangular arena) or a landmark (striped wall) to map space, when they were not able to track their own egocentric movements due to disorientation.

Apparatus: The experiments took place in a circular testing space surrounded by black curtains. One bright central light (round; diameter, 10 cm) illuminated the circular testing space from above. A camera was mounted on the ceiling and recorded animal behaviour. At the centre of the testing space was either a uniformly coloured white rectangular arena (40 x80x20 cm) or a square arena (40x40x20 cm) with three white walls and one striped black/white, featurally distinctive wall (stripe thickness, 4.5 cm). The arena was

filled with 5 mm of water. In each corner was a black box (8x8x 12 cm), with an opening (7.5 x 7.5 x4 cm) on one side.

Procedure: We adapted this test according to (Lee et al., 2015). The mouse was removed individually from its home cage in a covered cylinder and transported to the testing arena within the same room. In boundary geometry goal corners (open refuges) were the correct corner and rotationally correct corner. In the landmark condition goal corners were either close to the striped wall or far from the striped wall. The mouse was released from the centre of the arena and allowed to explore the environment until it found either of the target holes and took refuge inside. After 60 s it was removed and disoriented for 30 s in the covered cylinder (0.3 rotations/s in one direction and then the other). Meanwhile, the arena was rotated 90°with respect to the rest of the environment and the position of the experimenter, the target boxes were rinsed with water, and were closed shut. The mouse was released from the centre again and given 1 min to explore the arena. After 1 min, the mouse was removed from the testing arena, again using the covered cylinder, and placed back inside its home cage. Testing was conducted across 2 consecutive days (3trials/day) per condition (boundary geometry and landmark). Behavioural measures were coded offline using Ethovision 9.0.

Predictions: We expected wild type animals to use boundary geometry and landmark correctly; spending more time in the correct corners compared to the incorrect ones (Lee et al., 2015). We expected PD mice to fail in geometry (Lakusta et al., 2010)and landmark use (Ferrara & Landau, 2015), based on evidence from WS patients.

Statistical analysis: All tests were recorded and coded offline using Ethovision 9.0. We considered exploration when the animal was in a 3cm range from the refuge. For each test, we recorded the time spent close to the refuge in seconds and then calculated the proportion of time. All data was analyzed using Excel and SPSS. We defined our dependent variable “accuracy” as proportion of time spent in the correct + geometrically correct corner for boundary condition, and correct + featurally equivalent corner for the landmark condition. First a univariate ANOVA with “accuracy” as a dependent variable and condition (boundary or landmark) and genotype (WT or PD) as independent variables, gave no statistically significant effects. We later analyzed each group WT and PD separately for each condition.

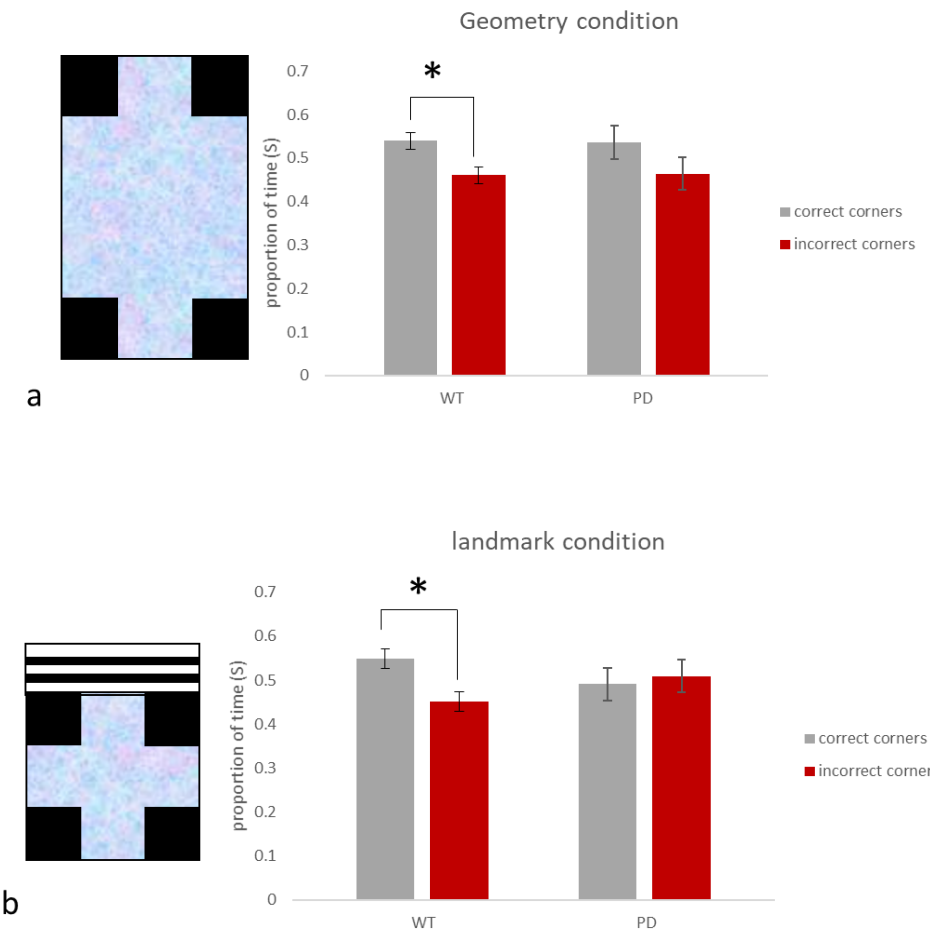

Figure 3: Reorientation using a) boundary geometry and b). * indicates comparison between open and close corners in a single group p<0,05. WT-wildtype, PD-proximal deletion mice

Results Wild type mice: Adult mice (n=29) show correct use of geometry spending significantly more time in the correct corners compared to the incorrect corners (t (28) =2,03, p=0.05) (figure 3a). Likewise, they (n=23) also show correct use of landmark by spending more time in the correct corners compared to the incorrect ones (t (22) =2,09, p=0,047) (figure 3b).

Results PD mice: PD mice show failure in both geometry (n=12) (t (11) = 0.8, p=0,4) and landmark use (n=13) (t (12) =0.64, p=0,9) (figure 3a, b).

Results Wild type vs PD mice: Comparison with control littermates (n=12/group) showed no statistical difference in landmark condition (t (22) = 1,4, p=0,9). Likewise, no difference between groups was observed in geometry condition (t (22) =1,4, p=0,1).

Discussion and Interpretation of Experiment 2: Unlike wild type mice, PD mice fail to successfully use boundary geometry and landmark to reorient. This is consistent with previous findings in WS patients, that show failure in using environmental shape to find a target corner(Lakusta et al., 2010). The comparison

between PD and WT littermates fails to show statistical difference in use of geometry. Therefore, we suggest that our results need to be further confirmed by increasing the sample size in order to provide statistical power to our findings.

Conclusions and Discussion

Williams syndrome mutant mice show a different behavioral pattern in both tasks compared to controls. Here we have shown evidence the PD mouse model represents also spatial deficit and memory impairment observed in clinical patients.

In episodic memory, we observe an overall tendency of PD mice to go toward the more familiar position ("New position" figure 2a) and object ("New Displaced" figure 2c). Therefore, we cannot rule out the possibility that PD mice may have a familiarity preference, as they explore the position they recall and are more comfortable with due to their inbred anxiety and fearful nature. Anxious like behavior was observed in PD mice (Li et al., 2009), suggesting it as a possible cause of their familiarity preference. Likewise, WS patients manifest anxious behavior in unfamiliar situations (Gray et al., 2006) and tend to prefer well-known environments. We also show that while place memory is altered, object memory is relatively spared in PD mice. They show a small tendency, although not statistically significant, toward the "old object" which could suggest intact memory in recalling items (figure 2b). Correct memory for objects (physical properties of objects) is observed in Williams syndrome patients (Landau et al., 2006; Vicari et al., 2005), suggesting the memory for physical properties of objects isn't impaired. Most difficulty in PD mice is seen when all three elements (what, where and when) of episodic memory are bound together, as seen in the incorrect preference toward the "New Displaced" object in the episodic memory test. This suggests that while separate elements of EM such as the "what" component is relatively conserved, the hippocampal function of binding and providing a spatio-temporal context seems to be affected.

Hippocampal deficit of PD mice is seen also in experiment 2. The failure observed in both conditions (geometry and landmark) is inconsistent from findings in WS patients. Ferrara & Landau, 2015, show that increasing the size of the arena and highlighting the geometric properties (i.e. adding small lights) helps WS patients to use boundary geometry and in fact in this version show success in geometric navigation. We speculate whether this mechanism could apply also to mutant mice, that could succeed if the geometric properties were highlighted. A modified version of the reorientation paradigm of Lee, 2015 could be of help to aid PD mice's searches in the correct corners. Based on (Lakusta et al., 2010) we expected PD mice to improve performance when a landmark such as a striped wall was introduced. Unlike our hypothesis, PD mice did not succeed in landmark use. Failure observed in PD mice could be due to an insufficient sample size. We expect to observe landmark success in PD mice by increasing the number of animals.

Allocentric spatial and episodic memory deficits are coherent with structural impairments observed in the hippocampal formation of patients (Meyer-Lindenberg, 2005). However, although brain skull morphometry tests show a smaller skull and brain volume in PD mice, no studies were performed on the hippocampal structure alone. The analysis of this structure and its connectivity can provide more evidence of a PD hippocampal deficit that is seen not only in behavior, but also in the anatomical structure. Moreover, given the difficulty in breeding these animals, often behavioral measures lacked power for statistical analysis. We aim to add more subjects in the future to confirm our observations.

We suggest our interpretations should be taken with caution, given matches between PD and control mice do not show a significant difference in experiment 1 and 2 and further analysis should be carried out to confirm our findings. Our results however, suggest a genetic related deficit (GTf2i- Limk1) behind spatial navigation and memory. Single knockout gene models for Limk1 and CLIP2, two genes on the PD region have been associated with hippocampal dysfunction (Hoogenraad et al., 2002; Meng et al., 2002), in terms of reproducing anxious-like behavior and difficulty in spatial tasks. However, we show evidence that the interaction of more genes can cause deficit in hippocampal behavior. To further test our hypothesis, future studies on DD (distal deleted) mice should be carried out to isolate the spatial impairment to PD deletion alone.

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Chapter 3: Boundary based navigation and landmark use in mice (Mus musculus) and rats (Rattus Norvegicus) are impaired by age.

Abstract

Normal aging produces an overall difficultly in performing spatial tasks. The large deficit in spatial navigation is supported by a significant decrease in hippocampal volume and function. However, in case of disorientation we wonder what strategy aged animals would put in place. Adult animals use allocentric strategies to find a target location. In particular, the geometric properties of the environment over shadow non-geometric cues following disorientation. In this study, we aimed to test whether this highly conserved cognitive function is altered by age. We used old animals of two species (mice and rats) and tested their ability to use geometry (hippocampus based) and landmark (extra hippocampus based) separately. Given the sensitivity of hippocampal place cells to boundary geometry, we hypothesized that a decline in hippocampal function can be assessed using a classical reorientation task. We show evidence of failure in using landmark and geometry in aged animals, consistent with the existing literature on an age related decline in the hippocampus. We suggest the reorientation task to be useful in underlying early deficits in the hippocampus and can be used in animal models of aging and age-related neurodegenerative diseases such as Alzheimer's disease.

Introduction

When an animal loses its sense of direction, it relies on external cues to find its way back toward a target location. To do so it can put in place two navigation strategies: egocentric and allocentric (Burgess, 2006). An egocentric navigation, is for example keeping track of one's movement constantly updating along the way. This however, has one main limitation: the accumulation of errors along the route and is inefficient when an animal loses its bearings. To recall a target corner of an arena animals mostly uses external cues such as landmarks and geometric properties of the environment to get back on track.

Animals, following disorientation, use prevalently an allocentric hippocampus based strategy to find a key location. External geometric cues such as the shape of the environment, are preferred over non-geometric cues to aid navigation. Cheng was the first to observe that rats navigated using the shape of a rectangular arena. His findings were replicated in other animals (Lee & Spelke, 2011; Lee et al., 2012; Sovrano et al., 2002) providing strong behavioural evidence for geometric based navigation. Navigating using lengths and distances seems to be an innate ability present in all animals. Rearing studies in fish and chicks, where animals were exposed to a circular arena at birth showed the same preference to geometry as those reared in a rectangular arena (Brown, Spetch, & Hurd, 2007; Chiandetti & Vallortigara, 2007).

The neural correlates of geometric navigation lie in hippocampal place cells and entorhinal boundary cells. Place cells are sensitive to geometric boundaries and their firing depends solely on geometric shape rather than non- geometric cues (Keinath, Julian, Epstein, & Muzzio, 2017). Place cell function is strictly related to boundary cell input, that provide key information such as the distance from boundaries represented by the walls of the arena in an environmental setup (Lever et al., 2009). Boundary cells have adult like firing rates already in the early days of rat pups, supporting the innate ability of geometric based navigation (Bjerknes et al., 2014).

Given the inbred ability to use lengths and distances of a rectangular arena, we wonder whether this cognitive ability remains unaltered throughout development until senility. Hippocampal function declines in age and so do place learning strategies. Allocentric navigation is often replaced by response learning egocentric strategies instead. The switch in preference is related to the structural deficits in the hippocampus, that are more vulnerable to the effect of aging than other brain structures. In a virtual analog of the Morris water maze, aged individuals took more time and retained less accurate knowledge of the hidden platform than younger individuals (Moffat, Kennedy, Rodrigue, & Raz, 2007; Moffat & Resnick, 2002). Moreover, when asked to navigate toward a target landmark, elderly individuals travelled longer distances to reach specific landmarks and acquired less knowledge about the spatial layout (Head & Isom, 2010). This suggests that elderly individuals have difficulty in the construction and use of hippocampal cognitive maps, and therefore use alternative strategies such as egocentric or path integration to navigate(Lithfous et al.,

2013). Likewise, aged rats also show a reduced specificity for place learning and preference for egocentric strategies in a Barnes maze task (Barnes et al., 1980).

In previous studies classical spatial assays in animals, such as Morris water maze, or Barnes Maze have been used to measure spatial behavior. These tasks although extensively used have some limitations especially in the interpretation of results. They require the use of a combination of multiple cues (such a various sources of distal cues around the room) and the extensive learning and lack of precision make these tests unideal for evaluating hippocampal function alone. Moreover, they fail to isolate the use of use of geometric cues from other sources of information.

Therefore, we aimed to detect deficits in hippocampal place learning in aging using a shorter, simpler measure. The reorientation paradigm is a sensitive task to test the use of boundary geometry (hippocampal place and entorhinal boundary activity) and landmark (extra hippocampal activity) separately in a controlled space and isolated from all other cues. Deficits in hippocampal place learning might be elucidated by the reorientation task. In order to test this hypothesis, we tested two different animal species: mice and rats to evaluate how aging alters navigation strategies. Rodents are classical animal models used in testing, but show different behavioral patterns. In this scenario the use of both species can underline specie-differences, and given the possibility of genetic manipulations in mice, can provide insights on future lines of research that can investigate the genetic relationships between genes and boundary based geometry.

General Methods

We tested two animal species (mice and rats), in two age groups (young and old) on their ability to use boundary geometry (experiment 1) and landmark (experiment 2).

Subjects

Male Lister Hooded rats (Rattus norvegicus, Harlan Olac, Bicester,87 England) were housed in pairs with access to water and food ad libitum. They were held on a 8- 12-hr light/dark cycle; testing occurred during the light phase. Rats were food deprived 3-4 hours before testing to induce motivation towards reward. No animals dropped below 95% of 90 free-feeding weight. Animals were kept in the animal facility until the age of 24 months and the animals that survived were tested again.

Mice were obtained from Jackson Laboratories (USA). Mice were housed in groups of 2–4 in standard Technoplast Type II cages, which were cleaned weekly and filled with fresh bedding, cardboard domes, and strips of paper (for enrichment). Mice were checked daily for their condition, weight, and visible injuries to ensure the absence of aggressive behaviours and general well-being. Animals were bred and kept in the animal facility in which the temperature was maintained at 21–23 °C. The mice were provided with a grid (13

x 25 mm) full of standard food pellets and a bottle of water (food and water checked daily), and put on a 12–hr light/dark cycle (lights on from 7:00 a.m. to 8:00 p.m.). Two weeks before testing animals were transferred by means of a ventilated cabinet and kept in the testing room until the end of the experiments.

Apparatus

Rats: The arena was raised 86cm above the ground and surrounded by black curtains to avoid extra-maze cues. A bright light 45Wwas suspended above the arena. Close to the light we placed a video camera to record all the experiments. Arenas (rectangular: 120 x 60cm; square: 85 x 85cm; height 50cm) were constructed from black medium-density fibreboard. The features were 0.5cm thick polyurethane panels, in alternating black and white stripes (thickness 10cm). In each corner of the arena was a white ceramic cylindrical feeder (diameter 8cm, height 4.2cm). Chocolate chips (Kellogg's coco pops) were used as a reward. During disorientation, animals were placed inside a light-tight box (22 x 15 x 21cm).

Mice: The experiments took place in a circular testing space surrounded by black curtains. One bright central light (round; diameter, 10 cm) illuminated the circular testing space from above. A camera was mounted on the ceiling and recorded animal behaviour. At the centre of the testing space was either a uniformly coloured white rectangular arena (40 x80x20 cm) or a square arena (40x40x20 cm) with three white walls and one striped black/white, featurally distinctive wall (stripe thickness, 4.5 cm). The arena was filled with 5 mm of water. In each corner was a black box, one of which had an opening on one side.

Design

Rats: Testing lasted two days, three trials per day. Goal corners and release points were chosen randomly. One day before testing, rats were provided with some chocolate chips in their cages. Before the first trial of each day, animals received a familiarization trial during which they were given three minutes to explore the arena and eat one chocolate chip placed at its centre. For each test trial, chocolate chips were added to one feeder. The animal was allowed to explore until it had eaten a piece of chocolate, at which point it was removed for 15s, before being placed back in to the arena from the same starting point. The animal was allowed to explore the arena until it had eaten a second piece of chocolate. This was to discourage the use of an alternation strategy, documented in rats as a method of foraging (Olton & Schlosberg, 1978). The animal was then disorientated for 30s by placing it in a dark, covered box and rotating the box. Disorientation involved clockwise and then anticlockwise rotations (at least 720° in each direction). During this time, the feeder containing chocolate was removed from the arena and replaced with an identical, but empty, feeder. The arena was cleaned with 15% ethanol and rotated 90° clockwise to counteract the use of possible uncontrolled extra-maze cues. The animal was placed back into the arena from a randomly selected wall and allowed to search for 60s. Behaviour was coded manually offline. When the animal was within 5cm range from the feeder it was considered inside the zone.

Mice: The mouse was removed individually from its home cage in a covered cylinder and transported to the testing arena within the same room. In boundary geometry condition goal corner and rotationally correct corner were rewarded. In the landmark condition correct and featurally correct corner were rewarded. The mouse was released from the centre of the arena and allowed to explore the environment until it found either of the target holes and took refuge inside. After 60 s it was removed and disoriented for 30 s in the covered cylinder (0.3 rotations/s in one direction and then the other). Meanwhile, the arena was rotated 90°with respect to the rest of the environment and the position of the experimenter, the target boxes were rinsed with water, and were closed shut. The mouse was released from the centre again and given 1 min to explore the arena. After 1 min, the mouse was removed from the testing arena, again using the covered cylinder, and placed back inside its home cage. Testing was conducted across 2 consecutive days (3trials/day) per condition (boundary geometry and landmark).

Statistical analysis

All tests were recorded and coded offline using Ethovision 9.0 for mice and manually for rats. We considered active exploration when the animal was in a 3cm range from the refuge or feeder. For each test, we recorded the time spent close to the refuge in seconds and then calculated the proportion of time. We defined our dependent variable “accuracy” as proportion of time spent in the correct + geometrically correct corner for boundary condition, and correct +featurally equivalent corner for the landmark condition.

We analysed each animal species separately, because based on literature on rodent behavior we expected some difference in between mice and rats. We later analysed mice and rats separately for each condition. In each group we used a paired t-test to see statistical difference between correct and incorrect corners. Later we analysed the age effect comparing performance between old and young animals of each species separately by a univariate ANOVA with “accuracy” as a dependent variable and condition (boundary or landmark) and age (young or old) as independent variables.

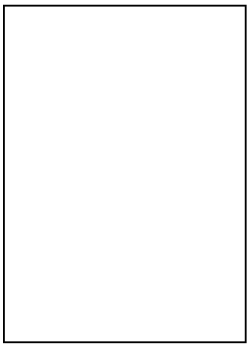
Results experiment 1: use of boundary geometry

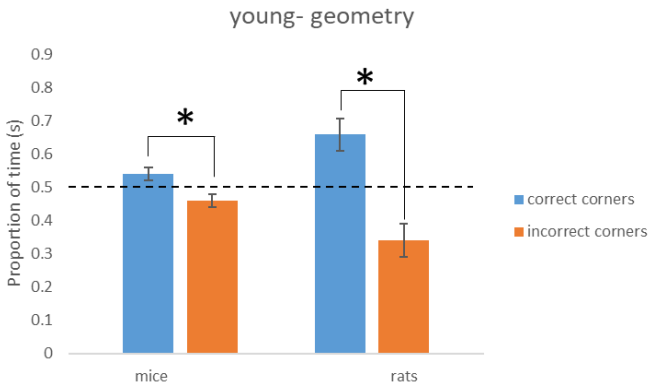
Experiment 1 tested the use of boundary geometry using a rectangular arena. We expected young animals to spend more time in the correct compared to the incorrect corners. We expected old animals to show failure in geometry due to a decline in hippocampal function caused by aging.

Young animals: Young mice (29 mice aged 3-5months), spend significantly more time in the open compared to the closed corners (t (28) =2,03, p=0,05). Searches were significantly above chance of 0,50 (t (28) =2,06, p=0,05). Likewise, young rats (n= 5 aged 6 months) spend significantly more time in the correct (correct + geometrically correct corners) compared to the incorrect corners (paired t-test: t (26) =3,2, p=0,03) (figure 1a). Furthermore, to make sure searches of young rats were not random, we performed a t-test

against chance of 0,50 and found that searches in correct corners were significantly above chance (t (26) =3,25, p= 0,03).

a.





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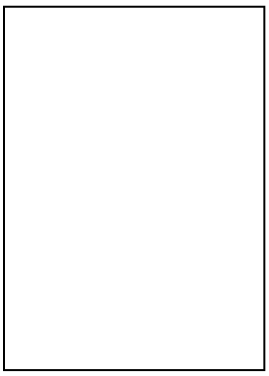


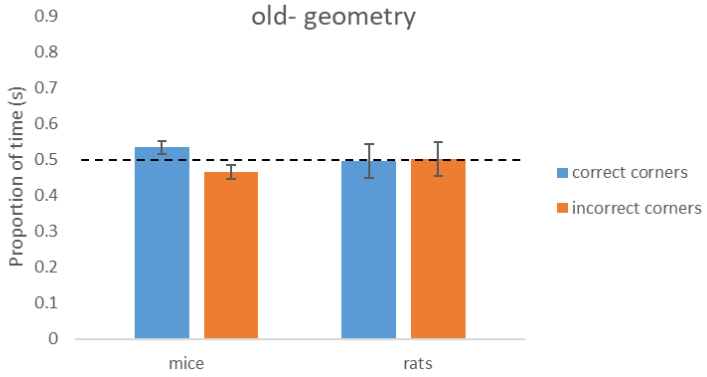


*Figure 1: Experiment 1: geometry condition. a) geometry in young animals. b) geometry in old animals. Correct corners (correct + rotationally correct) incorrect corners (near+far). *p<0,05*

Old animals: Old mice (n=12) do not show a significance preference (t (11)=1,8, p=0,09) for the correct versus incorrect corners. Likewise, old rats (n=7) fail in using boundary geometry (t (30)=-0,52, p=0,09) to reorient (figure 1b).

Young vs old: There was no statistical significance in searches between young and old mice. We compared performance of young and old rats and found a significant age affect. A univariate ANOVA showed an effect of age in boundary geometry F (1,56) =5,6, p= 0,021, η2 = 0,91. Young rats spent more time in in correct corners compared to old t (56) = 2,3, p=0,021.

Discussion experiment 1: Our results replicate other findings that show correct use of geometry in adult rats and mice (Cheng & Newcombe, 2005; Lee et al., 2015; Sovrano et al., 2002). Unlike young animals, aged animals of both species (mice and rats) fail in geometric based navigation. We confirm previous findings in boundary geometry seen in old mice (Fellini et al., 2006). We show evidence for the first time, that also aged rats are impaired in using geometry. Previous tasks in rats showed an overall difficulty in using

allocentric strategies (Barnes et al., 1980) but none tested the reorientation paradigm in old rats. The overall deficit, namely in using hippocampal based navigation, is seen also in elderly individuals (Moffat & Resnick, 2002).

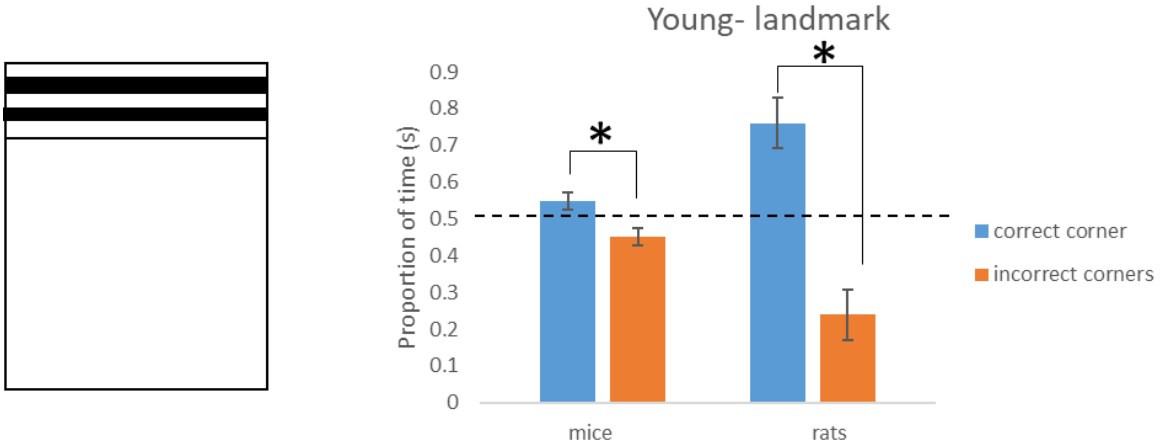
Results experiment 2: Use of landmark

Experiment 2 tested the use of a visual landmark (striped wall) in reorientation. We observed no difference in behaviour when goals were close to the feature compared to far from the feature in both animal species. Therefore, we collapsed all trials together and averaged the proportion of time across trials.

Young animals: Young mice (n=23) spend significantly more time in the open corners (correct and featurally equivalent compared to the incorrect corners (t (22) =2,09, p=0.04). Searches were significantly above a chance level of 0,50 (t (22) =2,09, p=0,04). Likewise, young rats (n=5) spend significantly more time in the correct corners (correct + featurally equivalent corner) compared to incorrect ones (t (20) =3,7, p=0,01). Searches in the correct corners were significantly above chance of 0,50 (t (20) =3,7, p=0,001) (figure 2a).

Old animals: Old mice (n=23) (t (22) = -0.5, p=0,59) and rats (n=8) (t(22)= -1,6, p=0,113)showed no statistical significance in searches between correct and incorrect corners (figure 2b).

Young vs old: No age effect was seen between performance of young and old mice in landmark use. However, an age effect was seen between young and old rats: F (1, 43) = 10,2, p=0.03, η2 =0,193. Accuracy (correct corner + featurally equivalent) was significantly higher in young rats compared to old ones. An independent t-test showed young animals searched more in the correct and featurally equivalent corners than old rats (t (43) =0,5, p= 0,03).



a.

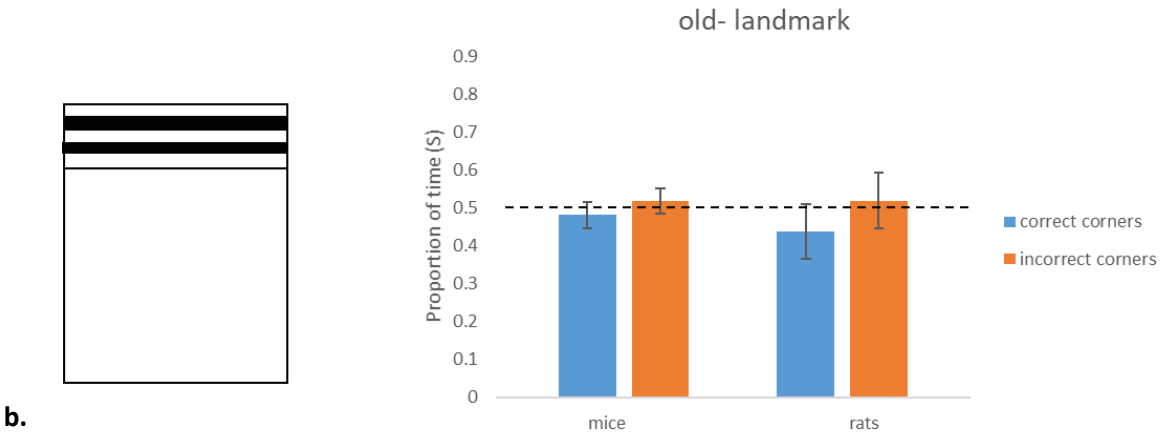


*Figure 2: Use of landmark to reorient. In experiment 2: landmark condition. a) landmark in young animals. b) landmark in old animals. Correct corners (correct + featurally correct) incorrect corners (near+ incorrect). *p<0,05*

Discussion experiment 2: Young rats and mice show success in using a landmark (striped wall) to navigate (figure 2a). Old animals on the other hand, show difficulty in using a landmark such as a striped wall to reorient. Unlike mice, where the effect isn't significant, it is clearly seen in rats. In mice however, we can observe a trend toward the same direction and believe that an increase in sample size can give significant results. As observed in animals, a diminution in using landmark knowledge is also seen in elderly individuals (Head & Isom, 2010; Moffat et al., 2007) suggesting that the hippocampus along with other brain areas are altered by age.

Conclusions and Discussion

The overall failure in both geometry and landmark use made us wonder if age affected other abilities such as eyesight, that could interfere with performance. To rule out this possibility, we tested mice in the light and dark box. In this test, they show preference for the dark compartment because of their natural aversion toward bright lights, confirming no alteration in vision. Likewise, in rats we measured their attraction toward the striped walls by seeing the amount of time they spent close to the feature in each trial. We observe an overall attraction toward the striped wall but failure to use it as a spatial cue.

In our experiments, we can observe a difference in behaviour in young vs old animals, mainly due to the effect of age. Moreover, our results suggest that spatial representations, particularly hippocampal boundary-based spatial mapping but also disoriented landmark use, are altered in two rodent species. Anatomical analysis could point out if there are differences in brain regions between mice and rats that could point out how aging differs in species.

Because boundaries are crucial to hippocampal spatial representation (Lee, 2017), testing boundary geometry in aged animals could be an early indication of an age related impairment in the hippocampal formation. We show evidence that the reorientation paradigm has potential to be a useful behavioural

measure of hippocampal function, in terms of its capacity to use geometric cues. Unlike other spatial tasks in rodents, such as the Morris water maze or Barnes maze, this task evaluates geometric from non-geometric based navigation and isolates boundary based navigation from other strategies. Moreover, extensive learning and stress in the Morris water maze represent external factors that influence negatively animal's behaviour. These limitations are overcome in the reorientation paradigm, that takes only two days of testing inducing the minimum stress to the animal.

Given the wide literature of decline in spatial navigation in aging in animals (Lithfous et al., 2013), we decided to test aged animals of two species (mice and rats) to underline whether a different aging pattern might be present. Rats, in particular have long been used in behavioural studies, and given their long life span we decided to use them to investigate aging effects. Mice on the other hand, are less common in behavioural tests, but represent an equally important animal model. The sequencing of their genome makes them an essential tool to study genotype-phenotype relationships. There are many mutant mice models to study aging pathologies such as Alzheimer's and we suggest that the reorientation task might be more appropriate to evaluate spatial decline than other more traditional tasks. Our task could be successfully implemented in mutant mice models as a behavioural measure of various stages of hippocampal decline.

We suggest that an allocentric task, specific to hippocampal activity should be implemented in clinical use to evaluate early signs of spatial deficit. The geometric module is more difficult to test in humans as the acquisition of spatial language overrules geometric based navigation (Wang & Spelke, 2002). Nevertheless, future research, perhaps using a 3D virtual reality task, could highlight subtle alterations of hippocampal function. Testing navigation in humans is a challenge, mainly due to the acquisition of other abilities that interfere and fail to isolate the ability to use boundary geometry alone. To overcome these issues, virtual reality environments that are strictly controlled for external cues and interference can substitute experimental setups. This type of task in a clinical setup could be helpful in early diagnosis of disorders such as Alzheimer's disease, where the onset of clinical manifestations arise long after the pathogenesis of the disorder. The window between beginning of the disorder to clinical diagnosis is critical and a specific behavioural task could be the key to an early diagnosis.

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General Conclusions and Discussion

The most innovative outcome of this dissertation is the successful use of different animal models to test different functions at specific age points of the hippocampus. The second interesting finding is the use of the reorientation task as a measure of hippocampal function, in particular the ability to use environmental geometry as an indicator of proper function of hippocampal neurons.

Our results together show evidence that the hippocampus has adult-like activity starting already in early stages of life **(chapter 1)**. Hippocampal response of domestic chicks is strongly influenced by the geometric layout of environmental boundaries. This suggests that a change of environmental shape can be detected by the hippocampus of naïve chicks. This experiment shows that the domestic chick can be a useful model to study early hippocampal function. It also increases our knowledge on the similarities in hippocampal function between birds and mammals (Gupta et al., 2012; Jarvis et al., 2005;Rodríguez et al., 2002). The second most intriguing finding of this study is that a mechanism similar to "remapping" seen in rodents, (O'Keefe & Burgess, 1996) is also present in the avian species. This increases the similarities between the two species, especially in relation to place cells and their sensitivity to geometry (Keinath et al., 2017) that are possibly present also in birds. Future research in the direction of single cell recordings might evidence place like cells confirming our initial suppositions of their existence. We believe that our findings might lay the ground to future studies that could narrow the gap between avian and mammalian hippocampus and provide more ground on the use of the avian brain as a good model to study also mammalian disorders.

The sensitivity to environmental shape is seen not only in chicks, but also in other animal species. We used the reorientation task as a measure of hippocampal sensitivity to geometry in genetically modified mice and aged rats. The reliability of geometric properties is a sensitive test to measure hippocampal deficit. Therefore, we used this task **(chapter 2)** in our mutant mouse model to investigate whether the selected gene deletion could interfere with hippocampal functions. We chose to use the PD mouse as a model to study hippocampal deficit and submitted mice to two behavioral tasks to evaluate different functions, regulated by the hippocampus. The PD deletion regulates two strictly related hippocampal functions: reorientation and episodic memory. Moreover, genes on this deletion (Gtf2i-Limk1) cause difficulty in using geometric cues and also difficulty in binding the temporal and spatial information of episodic memory. The results in our mice models, mimic the same behavioral deficit observed in WS patients in reorientation and memory (Lakusta et al., 2010; Landau et al., 2006; Vicari et al., 2005). We observe a specific deficit in the temporal component of episodic memory, suggesting that while memory for objects is preserved (what), what lacks is the capacity to collocate them in a spatio-temporal context (where). Their hippocampal deficit is reflected also in their difficulty in using environmental geometry of the rectangular arena (long vs. short arm) to recall correct corners. This is consistent with previous findings in WS patients, that show failure in using environmental

shape to find a target corner (Lakusta et al., 2010). We hypothesized, based on studies in clinical patients, that a landmark would improve performance. But on the contrary, no improvement was observed in mutant mice, showing repeated failure in all trials. Spatial and memory deficits are coherent with structural impairments observed in the hippocampal formation in patients (Meyer-Lindenberg, 2005) and single gene knockouts of the PD region (Hoogenraad et al., 2002; Meng et al., 2002). Anatomical and structural parameters of PD mice hippocampus, could help correlate the behavioral deficit to brain abnormalities in the future.

Not only genetic alterations, but also age alters hippocampal function. The reorientation task is an instilled cognitive function in all animal species. We used this behavioral measure to evaluate hippocampal deterioration. We tested this hypothesis using young and aged animals **(chapter 3)**. Young animals, both mice and rats, show correct use of boundary geometry and landmark use. However, old rats and mice show failure in both conditions over repeated trials. These results suggest that hippocampal allocentric ability is altered by age. We also show evidence of the validity of the reorientation paradigm, and suggest it as potential behavioral measure to evaluate hippocampal function. Moreover, we conclude that a similar deficit in function is observed in two species, suggesting that a similar deterioration might include also other non-human animals and humans. The potential of this behavioral measure could develop into an important assay of early degeneration of the hippocampus. Neurodegenerative disorders such as Alzheimer's disease start in this specific brain region and spread to other areas of the central nervous system. Therefore, we hope our findings could help the development of behavioral tasks to measure reorientation in aged individuals, as it could be an inexpensive and non-invasive measure for early diagnosis.

In conclusion, although our findings in PD mouse models and aged animals are still inconclusive due to lack of animals, we show evidence of how important cognitive functions such as spatial navigation and memory are tightly related to intrinsic and physiological factors such as genes and senescence. We believe that an increase in sample size would provide more significant results and confirm the trends we observed in our behavioral assays.

Concluding Remarks

This dissertation shows suggesting evidence of how various aspects of hippocampal function can be studied using different animal species.

We highlight two key findings: first a “remapping” mechanism is present also in the avian brain, narrowing the gap between similarities of mammalian and avian hippocampus, second the reorientation task as a reliable behavioral measure to evaluate spatial memory in genetically modified mice and aged rats. This task could be applied to test hippocampal function in mice models of AD and set the ground for applications in clinical practice as a sensitive measure to evaluate hippocampal deterioration and early diagnosis in aged individuals, prone to develop neurodegenerative disorders. Finally, we also point out the PD mouse as a model to study hippocampal function in relationship to gene regulation. Further studies with this model would confirm the predictions we observed in our studies.

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