THE EFFECT OF MANIPULATING THE EXPRESSION OF THE NR2B SUBUNIT OF THE NMDA RECEPTOR ON LEARNING AND MEMORY

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Abstract

Overexpression of the NR2B subunit of the NMDA receptor in the forebrain has been shown to improve learning and memory in mice (Tang et al 1999), which provides exciting implications for the enhancement of human cognition. However, it was first essential to establish replicability, and since the Tang et al (1999) study used only male mice we wished to investigate possible sex differences.

On the hidden platform watermaze, we found a trend for male NR2BOE mice to learn the task more quickly than male wildtype mice (as observed by Tang et al. 1999), but the opposite trend in female mice; female NR2BOE mice were slower to reach the hidden platform than female wildtype mice. This pattern of results was also observed on the spatial reference Y memory task and open field task (for anxiety), although not on the spatial working memory T maze task (despite a sex difference). However, wildtype and NR2BOE mice performed at similar levels on the novel object recognition task, the spatial novelty preference task, visible platform watermaze and visual discrimination task.

A battery of tests considering some species typical behaviours of mice demonstrated that wildtype and NR2BOE mice were comparable on tests of motor ability, strength, co-ordination, anxiety, burrowing and nesting. This suggests that our behavioural results are not due to a general impairment or enhancement of species typical behaviours.

We considered the possibility that the difference between the results of Tang et al (1999) and those we observed may be caused by age differences; hence we attempted to replicate our results on the hidden platform watermaze, spatial reference Y maze and open field test in age matched mice. However, the second cohort of NR2BOE mice performed at similar levels to wildtype mice, and at significantly improved levels compared to the mice of the first cohort.

We also considered the effects of knocking out the NR2B subunit on learning and memory, and NR1 subunit deletion within the hippocampus. On the spatial working memory T maze, these mouse strains performed similarly to their respective wildtype strains. Similarly, on a two beacon watermaze (with one indicating the platform position), mice lacking the NR2B subunit were able to locate the platform in a similar length of time.

To ensure that the null results we had observed in the second cohort were not due to loss of the NR2B protein overexpression in the forebrain, we performed polymerase chain reactions (PCR), quantitative real-time PCR, and Western blots. We ascertained that the transgene was indeed present and that NR2B mRNA and protein levels were elevated in the hippocampi of the NR2BOE mice.

In conclusion, it is unclear why the behaviours we observed in the NR2BOE mice are different to those published in the literature. It is possible that they may be due to differences in environmental enrichment, but the cause of the genotype by sex differences observed in the mice of cohort 1 is unclear. Nonetheless, we have advanced our knowledge of the effects of modifications in the levels of the NR2B subunit of the NMDA receptor on learning and behaviour.

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Abbreviations

AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
APV	2-amino-5-phosphonovaleric acid
CA	*Cornu ammonis*
CAMKII	Calmodulin kinase II
cAMP	Cyclic adenosine monophosphate
CREB	cAMP response element-binding protein
CS	Conditioned stimulus
DA	Dopamine
DG	Dentate gyrus
dNTPs	Deoxynucleoside triphosphates
dH_2O	Deionised water
EC	Entorhinal cortex
(f)EPSP	(Field) excitatory post synaptic potential
fMRI	Functional magnetic resonance imaging
HKG	House keeping gene
LTD	Long term depression
LTP	Long term potentiation

NMDA	*N*-Methyl-$_D$-aspartate
NMDAR	*N*-Methyl-$_D$-aspartate receptor
NR2BOE	NR2B overexpressor
PAG	Periaqueductal grey
PCR	Polymerase chain reaction
PET	Positron emission tomography
PKC	Protein kinase C
PKMζ	Protein kinase
PSD-95	Post synaptic density protein 95
TFRC	Transferrin receptor protein 1
ZIP	Zeta inhibitory protein

1. Introduction

A brain is a wonderful thing. We use it to think, to sense our environment, to act on what happens in our environment, and without our conscious input it regulates our bodies, ensuring that we are able to catch a ball that is thrown to us. However, it is something of "a riddle, wrapped in mystery, inside an enigma", to borrow the phrase from Winston Churchill. Although we have discovered many things regarding how our brains work, why we act in the way we do, and the mechanisms underlying these processes, there are still vast chasms in our knowledge. Although we know of a mechanism which is widely believed to underlie memory, we have been unable to prove that it actually does. Although we can predict the way in which people may think, act and react, our predictions are imperfect and are usually rather broad. But a little more progress is made each day, and as new techniques are produced and refined we become increasingly able to update our knowledge as to how our brains operate.

A particularly interesting area of neuroscience is that of learning and memory. Memories help mould our individuality. We are who we are because of our experiences, where we have been and what we have done. We can remember what school we went to, who our teachers were, where we now work and who we are friends with. But we do not remember everything; we still need to refer to an address book to remember an uncle's house number, and can sometimes be proved wrong in a debate when we were sure what we remembered was correct. And yet, although memories are so important to our individuality and our ability to live our lives normally, we only know so much about how memories are formed, where they are stored and how we remember them.

1.1.The anatomy of the brain

The human brain (Figure 1) can be divided into many different sections depending on the classification being used. During development, the spinal cord and brain start as a neural tube, areas of which become increasingly diverse in their functions as they develop. The brain consists of three regions that develop from the neural tube; the forebrain (prosencephalon), the midbrain (mesencephalon) and hind brain (rhombencephalon). The prosencephalon consists of two hemispheres (the right and left) and four lobes, the frontal, parietal, occipital and temporal lobes (Figure 1). Each lobe is responsible for diverse functions.

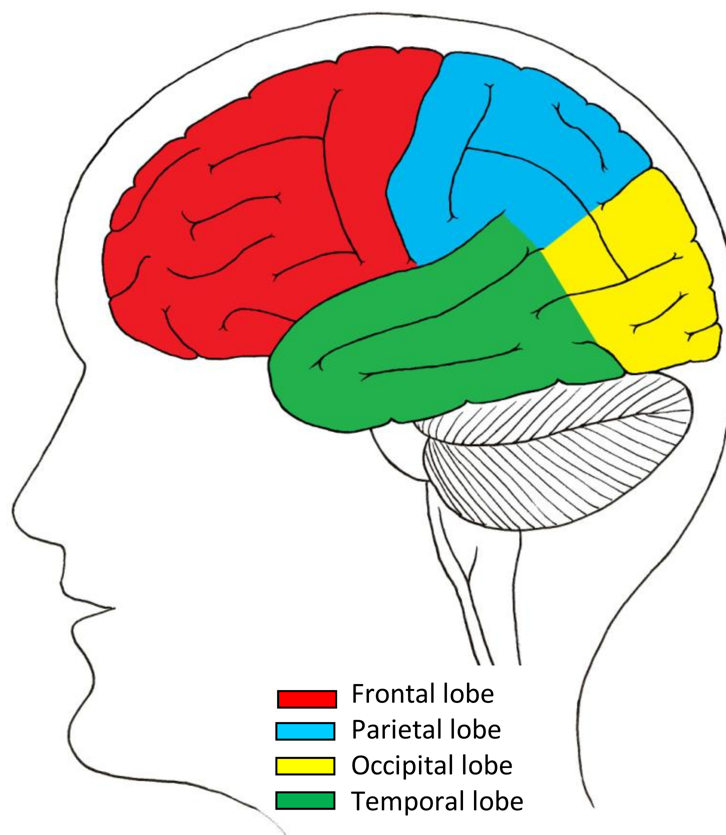

Figure 1 The lobes of the cortex

1.1.1. History of the elucidation of the hippocampal anatomy

The anatomy of the hippocampus was first observed in its crudest form thousands of years ago, by the scholars from the Alexandrian school of medicine. They are believed to have been among the first to have dissected the brain and identify the hippocampus, bestowing upon it its original Latin name of cornu ammonis (early Alexandrian neuroanatomists, circa 400BC (De Garengeot 1742)). Although this term is no longer used to describe the hippocampus itself, it has stood the test of time and is still used to refer to the subfields observed within the hippocampus (CA1, CA2 and CA3). The shape of the hippocampi have also been compared to that of a sea-horse (Arantius 1587) and a silk worm (Arantius; Duvernoy, 1729), although in rodents they are shaped more like bananas.

Although the early neuroanatomists were able to dissect the brain and view the hippocampal formation directly, they were not able to directly establish the functional links between the various regions and it was not for several centuries that the appropriate techniques were developed. By 1886, Camillo Golgi had developed a staining technique which, when applied to the hippocampus, illustrated a neat, organised formation of the constituent cells (Golgi 1886). Later, with the dawn of electron microscopy, closer studies of the hippocampus became possible (Hamlyn 1963). Tracing of neural circuits demonstrated the connections between the various areas of the hippocampal formation and cortex. Another technique that allowed the tracing of fibres within the hippocampus involved the injection of radioactive amino acids which were then transported in an anterograde fashion (ie. to the axon terminals) (Cowan et al 1972). These experiments helped to establish with which areas of the brain the hippocampus communicates, and how the hippocampus operates in conjunction with the other areas.

1.2.The hippocampus

1.2.1. Lobes of the cerebral cortex

The hippocampal formation resides within the temporal lobe (Sprengel et al 1998) (the shaded region in Figure 2). The hippocampal formation consists of several connecting regions, namely the hippocampus, the dentate gyrus (DG), the entorhinal cortex (EC), the presubiculum, the subiculum and the parasubiculum. The hippocampus itself consists of the three subregions: CA1, CA2 and CA3.

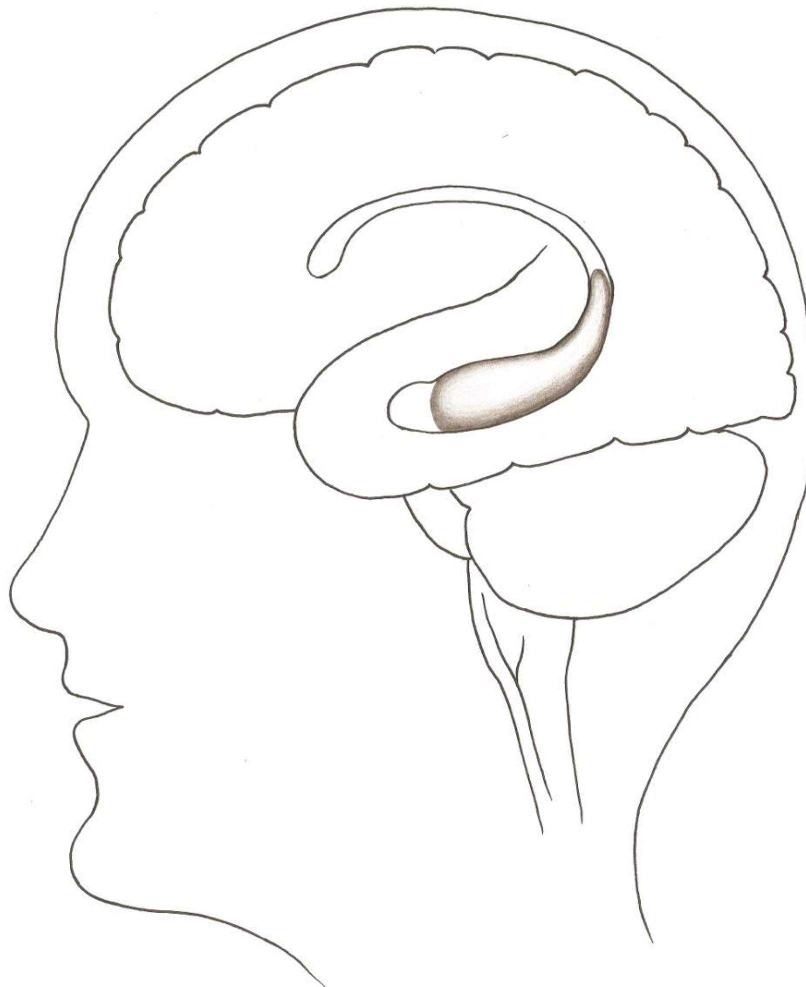

Figure 2 The human hippocampus. The hippocampus is indicated by the shaded area.

1.2.2. Connections of the hippocampal formation

Over time, it became clear that the different areas of the hippocampal formation were closely connected. Much study on the hippocampal formation has focussed on a pathway known as the tri-synaptic pathway. This takes signals from the EC directly to the dentate gyrus, which activate neurons in CA3, on to CA1, feeding directly back to the EC. It is known as the trisynaptic pathway as there are only three synapses involved; in the DG, CA3, and CA1. More recently, a disynaptic pathway has also been identified in which the entorhinal cortex forms synapses with pyramidal cells from CA2, which then make strong excitatory contacts with CA1 neurons (Chevaleyre & Siegelbaum 2010) (Figure 3, adapted from (Tsien et al 1996)). There are also direct connections from the EC to the CA3 cell layer, and from the EC to the CA1 cell layer. It has proved difficult to induce long term potentiation (LTP) in CA2 pyramidal neurons due to the effects of RGS14, a scaffolding protein that integrates secondary messenger cascades and suppresses LTP. However, when RGS14 is knocked out, robust LTP can be produced in CA2 neurons also (Lee et al 2010).

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Figure 3 Connections in the hippocampal formation. DG is the dentate gyrus, EC is the entorhinal cortex. The blue arrows are connections within the perforant pathway, the red arrow indicates the Schaffer collaterals.

1.2.3. Rodent hippocampus

Above, we have only considered the human brain and hippocampus. Naturally, rat and mouse brains are somewhat different (e.g. (Sprengel et al 1998)) not least in size. However, there are also considerable similarities, such that the equivalent hippocampal structure is easily identifiable in the rat brain (and similarly the mouse).

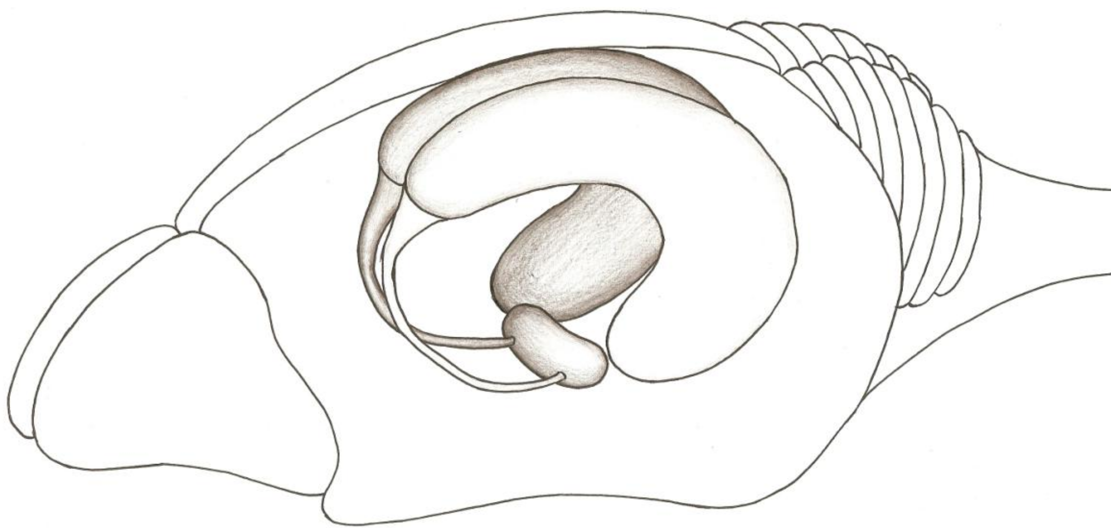


Figure 4 The rat brain. A rat brain with the hippocampi and amygdala (shaded areas) superimposed.

The positioning of the hippocampi in the brains of humans and rodents is different, such that they appear to hang down in rodents but appear to lie along the temporal lobe of humans. As a result, the different regions of the human hippocampus are split along the coronal axis (such that the front of the hippocampus is the anterior hippocampus and the back is the posterior hippocampus), and the rodent hippocampus is usually divided along the septotemporal axis (so the top of the hippocampus is referred to as the dorsal hippocampus, and the bottom is the ventral hippocampus). There is substantial evidence to suggest that the functions of the posterior hippocampus in humans are similar to the functions of the dorsal hippocampus in rodents (Bannerman et al 1999; Maguire et al 2000). There are strong similarities between the human and

rodent hippocampi (rat and mouse hippocampi are very similar (Squire 1992) although some differences exist (Sprengel et al 1998)), hence rodent hippocampi represent an excellent model for the human hippocampus.

1.2.4. Function of the hippocampus

Although the anatomy has been increasingly accurately dissected, the function of the hippocampus has remained a subject largely for speculation until the latter half of the 20th century. Although there was some evidence to suggest a role for the hippocampus in olfaction (Ferrier 1876) this has now been discredited as other roles for the hippocampus have been established (Scoville 1954). It has become increasingly apparent that the hippocampus is important for memory. However, further functional distinctions can be made even within the hippocampus; the dorsal and ventral portions of the hippocampus appear to have very different functions. There are also differences in the connections from the cerebral cortex, with the sensory cortices projecting to the dorsal two-thirds of the hippocampus (Witter & Groenewegen 1984) whereas the ventral hippocampus is more closely connected to subcortical areas (Kohler et al 1985) (such as the hypothalamus).

1.2.5. Spatial maps in the hippocampus

The major difference between the function of the dorsal and ventral hippocampus, at least in rodents, appears to be that the ventral hippocampus is crucial in anxiety and the dorsal hippocampus is essential for spatial memory. Although it is well known that complete ablation of the hippocampus in rats leads to a loss in their ability to solve the Morris water maze (Morris et al 1990) it would appear that selective ablation of the dorsal, but not ventral, hippocampus also prevents rats from solving this task (Bannerman et al 1999; Kjelstrup et al 2002; Moser et al 1995). This suggests that the dorsal hippocampus is required for spatial reference memory, but the ventral hippocampus

is not. By comparison, selective destruction of the ventral hippocampus makes rats less anxious, whereas selective dorsal lesions did not (Bannerman et al 1999; Kjelstrup et al 2002). Clearly the regions of the hippocampus were not all created equal (Bannerman et al 2004).

1.2.6. Location sensitive cells

It appears that the dorsal hippocampus forms a representation of the spatial environment. Certain cells in the dorsal hippocampus fire only when the animal is in a particular spatial location (known as the place field of the cell), and sometimes in a particular orientation (and sometimes only when combined with certain other stimuli) (O'Keefe & Dostrovsky 1971). These cells have been named place cells and it has been suggested that these cells provide the animal with a cognitive spatial representation (O'Keefe 1978). Similarly functioning cells have also been found in epileptic people who played a computer game in which a taxi collected passengers and navigated to a goal (Ekstrom et al 2003).

In addition, “grid cells” have been recorded in the entorhinal cortex; these cells fire over a triagonal or hexagonal array (Moser & Moser 2008). The more ventral the cell, the greater the area over which it fires, suggesting the dorsal entorhinal cortex may be important for more specific locations and the ventral entorhinal cortex may help with broader spatial locations (Brun et al 2008). Border cells that fire when the rat is close to a wall or edge in an environment have also been reported in the medial entorhinal cortex (Solstad et al 2008). The hippocampus receives much of its input from the entorhinal cortex and so may play a part in integrating these spatial signals.

1.2.7. Sequence completion

It has also been suggested that the hippocampus is important in remembering events in sequence (that is, not only recalling the memory, but remembering how it relates temporally to other events) (Agster et al 2002; Fortin et al 2002). There is evidence that there are differences between the hippocampi of the right and left hemispheres (Kohl et al 2011; Shinohara & Hirase 2009), and the NR2B subunit of the NMDA receptor is also asymmetrically distributed (Kiyama et al 1998) with up to 50% more NR2B in the left hippocampi of mice (Kawakami et al 2003; Kiyama et al 1998; Shinohara & Hirase 2009; Wu et al 2005).

Above we have considered evidence based in rodent models, but there are also differences in human hippocampi. One study considered the hippocampi of London taxi drivers. Taxi drivers must acquire a great deal of spatial knowledge and use it to navigate effectively, despite any obstacles such as road works or diversions. To become a licenced London taxi driver, it is necessary to pass a test which involves learning "The Knowledge". There are structural changes in the hippocampi of London taxi drivers such that the size of their posterior hippocampi (which is the equivalent of the dorsal hippocampus in rodents) is increased compared with normal, age matched non-taxi driving subjects. There also appeared to be a correlation with the length of time that the subject had spent as a taxi driver and the size of their posterior hippocampus, with more experienced taxi drivers having a greater posterior hippocampal volume (Maguire et al 2000). These results are believed to be due to the increased requirement of spatial memory by taxi drivers.

1.3.Learning and memory

But what do we mean when we refer to 'learning' and 'memory'? Learning is often defined as the acquisition of knowledge, behaviours, skills, values and so on. What is acquired is then retained, and what is retained is the memory of what was learned. Learning can be conscious or unconscious, and can be performed by all animals, including humans and even (in a simple forms) sea slugs.

Possibly the most famous experiment demonstrating learning and memory was conducted by Ivan Pavlov in the 1890's and 1900's. Whilst looking at the constitution of the saliva of dogs when presented with food, he noticed that the dogs tended to salivate before presented with the food. He then investigated this phenomenon more closely. He rang a bell when he presented the dogs with food, until they learned to associate the bell with food. After a while the dogs would salivate on hearing the bell in expectation of food, even in the absence of food. This is known as classical conditioning, and is an example of associative learning, in which the animal learns to associate two stimuli which are presented in a predictable, related manner; in the instance of Pavlov's dogs, the stimulus of the bell ringing predicts the presentation of food.

1.3.1. Forming and accessing memories

There are several steps to remembering anything. First of all, we have to acquire the memory. Then, once we have registered the item, we must store it. Finally, we must be able to recall the memory at a later point.

Acquisition involves transforming sensations into memory traces. We can have memories of what we heard at the opera, the smell of freshly cut grass, the taste of a strawberry or what we saw on the television. But we can have memories of anything we sense, so we can also have memories of dreams and hallucinations.

Precisely how memories are stored is somewhat controversial. However, it is widely believed that neurons in the brain form memories when they are active at the same time. They possess coincidence detection mechanisms that allow the cells to strengthen connections with communicating cells at their synapses, or points of contact, when the cells are simultaneously active. Thus synapses that have been previously activated at the same time will require far less stimulation subsequently to activate the connecting cells. Donald Hebb was the first to suggest this, and what is now referred to as Hebbian theory (Hebb 1949) or Hebb's Law is frequently summarized by the phrase "*Cells that fire together, wire together*". The molecular mechanisms underlying this process will be discussed in detail later.

Without effective recall, memories would be cached away, inaccessible, and unable to aid us. Usually, recall of a memory is easy and instantaneous. Occasionally, we have to think hard to retrieve a memory, for example of a word of French vocabulary that we learned many years ago. Often, if we are given a cue that we associate with that which we are trying to remember we find recall of the memory easier. Sometimes we are able to generate such cues ourselves. For example, we can generally conjure up the appropriate word or memory (as you might think of the first letter or number of syllables in a word you are trying to remember, or what you associate that word with). Most people are familiar with the feeling of a memory being just out of reach, or an evasive word being on the 'tip of my tongue'. Despite being unable to recall the word, people are often able to

supply words that sound similar or indicate the first letter or number of syllables of the word. Clearly certain elements of the memory are accessible, despite the inability to retrieve the desired word. There are two ways in which memories can be retrieved; by recognition (such as asking if a particular face has been seen before), or by free recall (such as when a memory is retrieved without any prompts to assist in its retrieval). Both methods have contributed considerably to studies of memory.

Above I have described forms of declarative memory (that is, memories that can be verbally declared, otherwise known as explicit memory), but there are also other types. Procedural (or implicit) memories can be accessed without conscious thought, and are particularly used to help us learn actions (such as learning how to trace a shape viewed only in a mirror). Declarative memory can be further subdivided into episodic memory (for episodes or events) and semantic memory (for concept-based knowledge), a division first noted by Tulving (Tulving 1984). Some experiments have shown that implicit and explicit memories can complement one another. Subjects who are shown a list of words and are then shown a second list of words, from which they are asked to recognize words that occurred in the first list, will occasionally miss a few words. If they are then presented with words for 35 milliseconds (which is usually enough time to allow recognition of a word) and asked what word was shown, they are able to identify words from the first list more readily than words that were not on the list, a phenomenon which is known as repetition priming. This repetition priming is seen for words from the first list that were not originally recognized on the second list, showing that the subjects have some form of implicit memory for the words on the first list that they are not always able to access explicitly (Jacoby 1982).

1.3.2. Short term memory

Short term memory can store things that have not been attended to for a short period (as any wife whose husband has not been listening can attest when, on demand, he can repeat what she has just said perfectly (Pratchett 2000) and items that are attended to, but are very quickly forgotten (in the case of rehearsing a phone number until the last number is dialled; this process is referred to as maintenance rehearsal. Despite repeated exposure to the string of numbers, and attention to them for a period, they do not often make it to long term memory).

Short term memory is generally considered to last for between a few seconds and around an hour. In this time, the memory is easily retrieved, but has not yet been committed to the long term memory store. It is possible to disrupt short term memory in rats using lidocaine (Daumas et al 2005), and electroconvulsive therapy (Bohbot et al 1996).

1.3.3. Long term memory

There are conflicting ideas concerning how long term memories are chosen to be stored in long term memory. It would not help us to remember every experience we have ever had indefinitely, although some people are able to do nearly this; they are said to be hyperthymestic. A similar phenomenon is eidetic memory, of which photographic memory is an example. People with photographic memory are able to recall pictures and pages of text in extraordinary detail indefinitely, after a short exposure. Many people consider these conditions to be desirable but it is unusual (found in around 5% of school children and probably a smaller proportion of adults (Gleitman 1991)). Hyperthymesticism may be undesirable as it may be more difficult for individuals to identify and recall memories appropriately. Hyperthymestic memories are usually almost entirely concerned with the person, their thoughts and actions, and what particularly interested them on that day. It has also been noted that one hyperthemestic, “A.J.”, was able to instantly recall her

personal past but had difficulty learning by rote at school (Parker et al 2006). AJ also commented that constantly remembering events from her life was like “a movie in her head that never stops”. She found the lack of control over remembering her memories burdensome (Parker et al 2006).

It is not clear by what mechanism a memory becomes integrated into long term memory. A single brief experience, particularly if it is associated with strong emotions, can be etched into someone’s memory very quickly. It seems that the element of surprise plays a part in the likelihood of an item being remembered. For example, someone witnessing the take-off of a space mission module might expect a loud noise at take-off, but may be surprised at the sheer volume of the sound, and would thus be more likely to remember the extreme volume. Also, seeing someone in an incongruous environment can cause considerable surprise and lead to particularly strong associations between the person and environment (for example, seeing a school friend who you have not seen for many years at your work place).

1.3.4. Habituation and associative memory

Quite different to the element of surprise is the process of habituation. Hearing a car alarm in a car park can be irritating at first, but quickly becomes ignored; this process is habituation, where the person gets used to the stimulus with repeated presentation and learns to ignore it. Once again, it is clear that surprise is important; when the car alarm first sounds, it is surprising and hence it is noticed, but as it continues it becomes increasingly predictable, and less surprising, until we do not consciously notice it. Our attention is only then drawn to it if it changes unexpectedly, such as when it suddenly stops. Once again, surprise draws attention to the stimulus. If, however, you were the owner of the car and had to turn off the alarm yourself, you would not be surprised when it was turned off because your actions were co-ordinated to stop the alarm sounding.

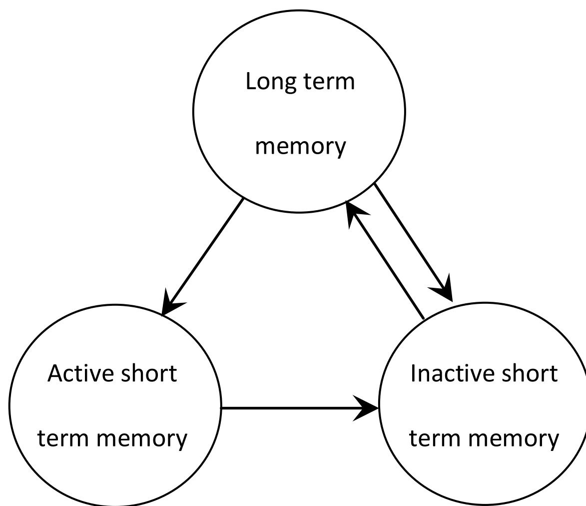


Figure 5 Representation of memory according to Wagner's theory.

Wagner has suggested a theory that may help explain these phenomena. He suggested a multicompartmental model of memory (Wagner 1981), in which new stimuli activate neuronal traces in the active short term memory, as shown in Figure 5. As time progresses, the active short term memory traces decay and become inactive, which in turn decay to a long term memory store. Long term memory traces can then be activated directly to the active short term memory store or activated indirectly to the inactive short term memory store. All stimuli experienced within a memory contribute to the memory, such that presentation of only a subset of the relevant stimuli can retrieve the associated memory traces from long term memory. Thus, if a mouse is placed in a novel context and subjected to an electric shock, the mouse will produce memory traces for the context and electric shock and if placed back in the same context will retrieve the associated memory traces of the electric shock to the inactive memory store.

The proportion of memory traces in the inactive short term memory represents the level of habituation towards the stimulus; if more traces are activated (such as immediately after the stimulus), more traces will decay to the inactive short term memory store, and so more habituation will be observed. Memory traces in the inactive memory store cannot be re-activated without first decaying to long term memory. Immediately after the initial presentation of a stimulus, traces for

that stimulus will be in the active short term memory store and then they decay to the inactive short-term memory store. Subsequent presentations of the stimulus while some of its memory traces have decayed to the inactive memory store leads to habituation (as traces that have decayed to the inactive state cannot be retrieved without first decaying to long term memory stores). By comparison, if all the memory traces are allowed to decay fully to the long term memory store between stimulus presentations, all of the memory traces for the stimulus will potentially be activated together and can be brought to the active short term memory store.

Wagner's model is not universally accepted (Holt & Maren 1999), but explains why mice lacking the GluRA AMPA receptor have improved long term memory alongside a short term memory impairment (Sanderson et al 2009).

1.3.5. Luria's patient, S.

The most famous patient with extraordinary memory was Solomon Veniaminovich Shereshevskii (1886–1958), who was studied extensively by Alexander Luria (who referred to Shereshevskii as S.) (Luria 1986). S. was sent to Luria by his boss (a newspaper editor), after he had noticed that S. did not take notes during meetings. Annoyed by his apparent inattention, his boss questioned him, only to find that he had a perfect memory of the meetings. Luria then studied S.'s memory over many years, during which time S. left his job as a newspaper reporter to become a professional mnemonist.

Although it is not entirely clear to what S. owed his fantastic memory, it is believed that his synesthesia played a large part. Synesthesia is a condition in which a single sensory stimulus is automatically and involuntarily sensed by more than one sensory system. Commonly, synesthetic

individuals see letters and numbers as being coloured. S., by comparison, experienced most stimuli in virtually all sensory pathways; different letters had a distinct colour, texture, taste, smell and sound. As a result, he associated many more sensations with a single stimuli, and was able to recall the stimuli very easily. He was able to "see" the item of interest, and so could simply read from what he saw, such that his infrequent mistakes were invariably attributable to the item being mis-read (for example, a 4 that looked like a 9) rather than being due to any fault in his memory. However, he did not recall everything perfectly, particularly before he trained as a professional mnemonist. The vast majority of the mistakes he made were those of omission. He reported that, when trying to remember items, he would imagine placing them along some path he knew well, such as between his home town and Moscow. When remembering, he would then walk back along the same path, noting the various items along the way. He reported that the omitted items were things he did not notice along the way; for example, he did not notice a small egg beside a large, white wall, or a dark item which had been 'placed' in a shadow.

However, this also interfered with his life. He found it difficult to extract the meaning of sentences, as there were many distracting influences. He is reported, in June 1953, as saying: "To this day I can't escape from seeing colours when I hear sounds. What first strikes me is the colour of someone's voice. Then it fades off… for it does interfere. If, say, a person says something, I see the word; but should another person's voice break in, blurs appear. These creep into the syllables of the words and I can't make out what is being said." He also reported having a poor memory for faces (Luria 1986). Clearly, although improved memory may seem desirable, it is not without its drawbacks.

1.3.6. Patient H.M.

Possibly the most studied and celebrated person in neuroscience is Henry Molaison (26th February 1926 - 2nd December 2008). As a child, Henry Molaison was an ordinary boy. But by the age of 10, he started to suffer from increasingly frequent and severe epileptic seizures (Scoville 1968). The epilepsy proved so debilitating despite anticonvulsant therapies that in 1953, the neurosurgeon William Scoville suggested experimental surgery to remove the areas of the brain in which the epilepsy was localized. On the 1st of September, 1953, Scoville removed parts of Henry Molaison's medial temporal lobes. The resection bilaterally removed the medial temporal lobe, "*extending posteriorly for 8cm from the mid points of the temporal lobes, with the temporal horns constituting the lateral edges of the resection*" (Scoville & Milner 1957). Hence the preperiform gyrus, uncus, amygdala, hippocampus, hippocampal gyrus and entorhinal cortex were removed, on both sides of the brain (Corkin et al 1997; Scoville 1968). However, an MRI study more than 40 years later found that only 5cm posteriorly had been removed (Corkin et al 1997). It was also discovered that his cerebellum was considerably atrophied, which is believed to be due to the anticonvulsant drugs he was taking (Corkin et al 1997).

From the perspective of controlling Molaison's epilepsy, the operation was a success; whilst it did not entirely stop seizures, the tonic-clonic seizures were reduced in frequency to around one a year, down from around one a day, and the minor seizures were also greatly reduced in frequency. Although Molaison seemed well and demonstrated above-average intelligence after his operation, his ability to form short term and long term memories had been destroyed; he suffered from severe anterograde amnesia (Milner 1968). He could not remember elementary details about his life, such as a discussion he had had just previously (Scoville & Milner 2000). As a result he was able to exist only in the present; from the time of his operation he was unable to form memories for more than a

few seconds. His impairment was investigated in considerable depth, and hence he became widely known in the neuroscience community as ‘H.M.’.

However, his ability to form memories and remember things was not completely destroyed. He could remember things from his childhood, although he suffered from temporally graded retrograde amnesia; that is, the closer memories were to the time of his operation the less clear and less numerous they became. And he was able to remember items such as a telephone number as long as he was able to rehearse it, although the minute he was distracted from his task, he forgot. After his operation, he moved house but for some years after could not reliably find it on his own (Scoville 1968).

Although his declarative memory was devastated, his procedural memory was intact. With practice, he became adept at tracing a shape he could see in a mirror despite not remembering having performed the task before. Although he could not remember many events from after the operation, when asked many years later who was assassinated in 1963 he was able to tell the questioner that it was the President (of the United States of America). He was unable to immediately recall the name of the president, but when told the President’s initials were ‘JFK’ he was able to supply the name ‘John Kennedy’ (Newhouse 2007). And although he had met Dr. Suzanne Corkin on multiple occasions and did not know who she was, when a nurse mentioned that she had been speaking to ‘Dr. Corkin’, H.M. asked ‘Suzanne?’; he had come to associate her first name and surname despite not recognising her when they regularly met (Newhouse 2007). Clearly, despite a massively decreased ability to form and retain memories, his ability to form some memories was not entirely destroyed. Also, his ability to learn implicit, procedural tasks whilst being unable to form declarative memories points to different underlying mechanisms for these forms of memory.

William Scoville went to considerable effort to ensure that this form of surgery was not replicated in other patients; in one paper, he stated "*We* [William Scoville and Brenda Milner] *have chosen to report these findings in full … partly as a warning to others of the risk to memory involved in bilateral surgical lesions of the hippocampal region.*" (Scoville & Milner 1957)

H.M. was not the only patient whose hippocampi were removed however, although his was the most extensive lesion created surgically. Others with lesions caused by strokes and smaller surgically induced lesions have also contributed to our understanding of the medial temporal lobe. The hippocampus is particularly vulnerable to a lack of oxygen, and so when the blood supply to the hippocampus is interrupted by a stroke the neurons quickly die. This is unfortunately a relatively common occurrence in stroke victims, but those who have participated in studies have helped our understanding of this region. Although H.M. has been of great help in helping us to understand how lesions of the medial temporal lobe prevent certain forms of memory, his lesions were rather broad and encompassed several brain areas. This made it difficult to ascertain precisely which areas were necessary for various components of learning and memory. Patients with smaller lesions (such as those caused by strokes or mini-strokes) have helped to establish that, for example, the amygdala is important in emotional memories, whereas the hippocampus itself is more important in declarative memory. Also, whereas H.M. suffered severe amnesia after bilateral hippocampal lesions, most individuals can suffer damage to either the left or right hippocampus without obvious ill effects (Milner 1966). In addition, studies of other patients with similar problems to those seen with H.M. have shown that there is some dissociation between episodic and semantic knowledge (Kumaran et al 2007).

1.3.7. Patient R.B.

Another patient, R.B., suffered a stroke which destroyed the CA1 region of his hippocampus. He suffered from marked anterograde amnesia whilst suffering very little retrograde amnesia (Zola-Morgan et al 1986). The lesions were very limited and, although there was a small amount of damage to R.B.'s brain elsewhere, the damage to the CA1 region was the only damage to areas involved with memory. This has helped us to establish that the hippocampus itself is important for memory, rather than another brain region that was destroyed in H.M.'s lesion.

1.3.8. Patient N.A.

Patient N.A. was a radar technician in the U.S. Airforce until he was accidentally stabbed with a miniature fencing foil. The foil went through his right nostril and primarily lesioned his left dorsomedial thalamus. Although his intelligence appeared unimpaired, he suffered severe anterograde and a degree of retrograde amnesia. His amnesia was not complete, and he retained sketchy memories of some faces and events from after the accident. It appears likely that this area may be involved in memory consolidation (Moriyoshi et al 1991).

However, the hippocampus is not the only area of the brain involved in memory formation and retention; procedural memories are believed to involve the striatum (Jog et al 1999), whereas emotional memories are disrupted by impaired function of the amygdaloid complex (Cahill et al 1995) and affected by adrenergic systems (McGaugh et al 1993). There is a clear distinction between the requirement of the hippocampus for declarative memories and the amygdala for emotional memories (Bechara et al 1995). Amnesic patients are capable of learning probabilistic associations despite their lack of declarative memory (in contrast to patients with Parkinsons disease, in which the neostriatum is damaged, who have impaired ability to learn probabilistic associations (Yin et al

2005)). Although there are several forms of memory which are stored elsewhere in the brain, in this thesis I will concentrate on declarative, hippocampal memory.

1.4.Neurobiology of memory

Whilst human subjects with lesions have provided us with much information regarding the neurobiology of memory, another method is to use animal models in which various areas of the brain have been surgically ablated, and investigate whether the animal can learn and/or remember. Perhaps a more appropriate method for considering human memory is to study humans in whom areas of the brain have been ablated, either surgically (for example, in an attempt to control epilepsy) or accidentally (such as in the case of stroke victims, or those suffering from brain damage such as Phineas Gage (Pocock G 2001) after a railway girder was driven through his head, destroying most of his forebrain). One technique, the Wada test, involves introducing an anaesthetic to a single hemisphere of the brain to produce a kind of passing, reversible lesion. It is also possible to induce reversible cold lesions, in which the area of interest is cooled until it becomes inactive, for example see George, Horel and Cirillo (George et al 1989). However, it is difficult to be certain that cold lesions are entirely reversible as they require the insertion of a cryogenic loop into or near the brain region of interest. Inserting the cryode would leave minor lesions in the tissue it passed through in order to reach its target; the same is true of most methods of generating a physical lesion. More recently developed techniques include functional magnetic resonance imaging (fMRI) (Blow 2008) and positron emission topography (PET) (Blow 2009), both non-invasive methods that allow the investigator to see how sections of the brain respond to the experiment a subject is given.

It is obviously unethical to permanently lesion human brains to help us understand the influence of different regions. Lesion studies certainly can aid understanding of the functions of different brain regions, but they cannot be used in isolation. It is entirely possible that the results observed in lesion studies are due to the effects of something other than the removal of the tissue. It is interesting to note that serially produced lesions do not always have the same effect as single lesions (Stein et al 1969).

1.4.1. Animals in studies of memory

Animals have been popular models in the field of learning and memory for hundreds of years. Primates are favoured as they are the most similar to humans, although rats and mice are probably the most common model used. For the past 40 or so years, rats especially have also been commonly used as a more practical model than primates. They are large enough to reproducibly create lesions in their brains, which has allowed many scientists to investigate the importance of regions of the brain, but small enough to have a short pregnancy and juvenile period. As a result, some popular learning and memory tasks have been developed specifically for rats, most notably the Morris water maze (Morris 1984). More recently, genetic manipulations of many different genes based in mice have led to mice being used increasingly for learning and memory tasks (Wong et al 2002).

1.4.2. Genetic models

Mice are also becoming particularly popular as genetic models, with many hundreds of mutated mouse lines being generated. Some of these have been of considerable interest to scientists within the field of learning and memory. One mutant mouse strain, which is one of the focuses of this thesis, overexpresses the NR2B subunit of the NMDA receptor under the control of a CaMKII promoter. These mice have been nicknamed Doogie mice, after Doogie Howser (a fictitious

child prodigy who obtained his M.D. aged 14, based on an individual who obtained his M.D. in the USA aged 21) as they are able to solve certain tasks more readily than their wildtype counterparts.

Doogie (NR2B overexpressing, or NR2BOE) mice are certainly not the only strain that have been reported to be smarter than wildtype mice; a strain with reduced GlyT1 levels (a transporter which re-uptakes glycine from the synaptic cleft) in the forebrain spent more time than wildtype mice exploring a novel object 2 hours after previously seeing two familiar objects (Singer et al 2007) (in a task of novel object recognition; mice prefer novelty and will spend more time exploring new stimuli). This result suggests that the mice could better remember which object they had seen previously, allowing them to investigate the novel object more closely, which would suggest improved memory in the mutant mice $GlyT1^{-/-}$ mice.

Unsurprisingly, not all strains with mutations have enhanced learning and memory; indeed, most mutant strains show impairments in memory rather than facilitations. One strain, in which post-synaptic density protein 95 (PSD-95) was knocked out, the process of long term potentiation (LTP) was enhanced at the expense of long term depression (LTD), both of which are processes believed to be important for learning and memory. The ability of these mice to solve the watermaze was impaired, suggesting their ability to learn about spatial relationships was disrupted (Migaud et al 1998). Another strain had a mutation in nonreceptor tyrosine kinase (*fyn*) and showed reduced LTP. They also were impaired on the watermaze, suggesting that this protein may also be important for LTP, although this may have been due to developmental disruption (Grant et al 1992).

From an evolutionary perspective, many of these results seem sensible. If a mutation has deleterious effects on the organism, individuals with the mutation are unlikely to pass it on to the

next generation unless they have some advantage that is of a greater magnitude than the harmful effect. So, mutations which prevent the organism from learning or remembering would be expected not to be perpetuated (such as with the *fyn* mutants or the PSD-95 knock-out mice). But the NR2BOE mutation appears to enhance memory, which would surely be advantageous and so would be predicted to have already been selected for evolutionarily. However, although it may aid memory, the mutation may have deleterious effects of greater magnitude than that of the enhancement of their memory. For example, mice overexpressing NR2B in the anterior cingulate cortex and insular cortex appear more sensitive to pain (Wei et al 2001), and there is evidence to suggest humans with schizophrenia may have overexpression of NR2B in their hippocampi (Gao et al 2000). These disadvantages could counter-act any positive effects of increased NR2B on memory. However, these evolutionary disadvantages do not detract from our ability to use these genetic manipulations to learn more about the mechanisms of learning in an environment in which natural selection is not an issue.

1.4.3. Sex differences

Some mutations appear to behave differently depending on the sex of the animal, such as in mice with low level p25 expression. In these mice, LTP was difficult to induce in males but not females, and males were impaired on the watermaze compared with females (Ris et al 2005). Similarly, in a mutant strain of mice in which the calcium/calmodulin kinase kinase β had been knocked out, females could solve the watermaze whilst males were impaired (Mizuno et al 2007). Whilst it is possible that these results were due to specific oestrogen levels, this seems unlikely as testing was carried out over six days, and the length of the rat oestrous cycle is four days; hence any potential beneficial effects of a particular point of the oestrous cycle would not apply a day or two later. Most mammals, including rats and humans, tend to cycle together when housed together (Handelmann et al 1980; McClintock 1978; 1984; Olton & Papas 1979; Stern & McClintock 1998).

Interestingly, however, when it is not the effects of the oestrous cycle that are being studied, sex differences are usually absent or small (Parra et al 1999).

1.4.4. Genetic background

One important consideration is that although there are many commercially available mouse strains, they vary greatly in their phenotypes (Wolfer & Lipp 2000). Some, such as the C57Bl/6 strain, is particularly favoured for behavioural experiments but others are comparatively unresponsive in behavioural tasks, such as the 129S2 strain which is less active and more anxious than the C57Bl/6 strain (Contet et al 2001). Unfortunately, when mutant mouse strains are generated, the strain into which the mutation is introduced is not necessarily a strain which is good to test behaviourally; indeed, the preferred strain (strain 129) for gene-targeting studies is particularly different and the animals are impaired on spatial learning tasks (Gerlai 1996). This frequently results in back-crossing of the mice onto a strain in which behaviour is easier to test, so that the strain has two backgrounds. It is then difficult, if not impossible, to rule out the possibility that any effect observed may be due to the background strain rather than the mutation (Gerlai 1996).

Another difficulty with using mutant models is that it is very difficult to be quite sure that any results seen are caused by the mutation itself and nothing else such as (in the example of gene deletions) redundancy (where a separate gene is able to counteract the absence of a deleted gene) or differential expression of a separate gene. Despite this, mutant mouse models are extremely helpful and informative, so even with the issues surrounding mutant models it is likely that they will prove popular for some time to come.

1.5. Neurobiology of Anxiety

The hippocampal formation is not only involved in memory formation and storage; it is also required for normal behavioural reactions to threats. Fear and anxiety are related but different reactions to threats, real or potential. Fear is the response to the presence of danger or a predator resulting in freezing, fight or flight behaviours. By comparison, anxiety arises from possible or potential threats and leads to increases in attention and arousal and inhibition of on-going behaviours. Both anxiety and fear can produce a reduction of movement. One important distinction is that anxiolytic drugs reduce anxiety but do not affect fear, allowing us to establish the role of different areas and receptors. The prefrontal cortex, periaqueductal grey, amygdala and hippocampus are involved in elements of fear and anxiety, but importantly the amygdala and septohippocamal system underlie anxiety (McNaughton & Corr 2004).

1.6. The NMDA receptor

It is important at this point to consider the NMDA receptor, which is believed to be very important for the formation of memories. It is a channel protein which is highly permeable to calcium (when open), and is closed at resting membrane potentials by a single Mg^{2+} ion (which is displaced when the membrane is depolarized). It also requires two co-agonists to open, glycine and glutamate. Overactivation of the NMDA receptor has been linked to excitotoxic pathology, particularly cell death (thought to be due to an excess of intracellular calcium (Lipton & Rosenberg 1994)).

The NMDA receptor (NMDAR) is a tetramer, two pairs of homodimers (Furukawa et al 2005; Stephenson et al 2008). Functional NMDARs can only be produced if the receptor contains one NR1 homodimer (Monyer et al 1992), which is expressed ubiquitously throughout the brain. An NR1 homodimer produces a functional receptor if it combines with an NR2 homodimer or NR2/NR3 heterodimer. There are believed to be four distinct NR2 subunits (NR2A, NR2B, NR2C and NR2D) and two NR3 subunits (NR3A and NR3B).

Differing NR2 subunits confer different properties upon the NMDAR. Although they all have similar calcium permeabilities, the properties of their voltage gated Mg^{2+} block vary, such that NR2A and NR2B containing NMDARs require a greater degree of membrane depolarization for the Mg^{2+} block to be removed compared with those containing NR2C and NR2D (Monyer et al 1994). In addition, their decay time constants are considerably different, with NMDARs containing NR2A having much shorter decay times than those containing NR2B (Monyer et al 1994). This fact aids identification of channels of interest for electrophysiological studies.

As this thesis is only concerned with the brain, all discussion will now regard NMDA receptors in the brain only. NR2 subunits have different expression profiles, such that (in adults) NR2A is found in the forebrain and cerebellum, NR2B is present in the forebrain (particularly hippocampus) but not the cerebellum, and NR2C is limited to the cerebellum (Monyer et al 1992). The different NR2 subunits are also differently expressed through development; for example, virtually no NR2A subunits can be found in neonates but the number of subunits increases with age until adulthood (Liu et al 2004). NR2B shows an opposite expression pattern with many NR2B receptors present at birth, increasing to maximal expression at 3 weeks (Liu et al 2004), after which expression decreases until it reaches normal adult levels (Monyer et al 1994). Although the changes

described above are from rodent studies, they also occur in humans, albeit on a longer (but relative) timescale (Santarelli et al 2003). This coincides with a decrease in the ability to produce LTP mediated by NMDA receptors (Crair & Malenka 1995).

As only NR2A and NR2B are present in the hippocampus, we will concentrate on these subunits.

1.6.1. Importance of NMDA receptors in the dentate gyrus

The dentate gyrus is a well-studied region of the hippocampus. It is the site in the brain at which most adult neurogenesis occurs (Gross 2000) and so has roused a good deal of interest in its function in learning and memory. It is believed that as new memories are formed throughout life, new cells may be needed in order to form the appropriate connections in order to record the new, distinct memory (Kee et al 2007; Kitamura et al 2009). New neurons produced in the dentate gyrus may allow temporal grading of memory storage, as when new neurons reach 6-8 weeks old they are preferentially incorporated into spatial memories (Kee et al 2007). In addition, when synapses are activated, the activation threshold for nearby synapses decreases. As a result, new memories are more likely to employ synapses close to those employed by a memory encoded in the last few minutes (Harvey & Svoboda 2007). This may allow temporal organisation of memories that were encoded soon after previous memories (Silva et al 2009).

Mice have been generated in which the NMDA receptor (or rather, the obligatory NR1 subunit of the NMDA receptor) has been knocked out altogether, and in which NR1 has been knocked out of certain regions (such as the dentate gyrus) only. It has been suggested that dentate gyrus (DG) NMDARs may be important in pattern separation, or the ability to tell apart similar contexts, as mice lacking NR1 in the DG did not differentiate between two similar contexts after one

had been used for fear conditioning (McHugh et al 2007). By comparison, knocking out NMDA NR1 subunits in the CA1 pyramidal cells of the hippocampus only led to impaired spatial learning but unimpaired non-spatial learning (Tsien et al 1996). Also, CA1 NMDA receptors may be of particular importance in consolidation (Shimizu et al 2000), and the hippocampus is also important in flexible learning (ie, the opposite of perseverance (Eichenbaum et al 1990)).

1.6.2. Splice variants

There are several splice variants that lead to the formation of functional NMDA receptors. There are nine known splice variants for the NR1 subunit, constructed from a 5' insertion and two 3' deletions (Anantharam et al 1992; Durand et al 1992; Hollmann et al 1993; Nakanishi et al 1992; Sugihara et al 1992) and an alternative splice-acceptor site (Sugihara et al 1992) from 22 exons. Terminology varies from paper to paper, so I will use the format used by Zhang et al (Zhang et al 1994). NMDARs expressing different splice variants appear to be differentially expressed in the brain; the most abundant form ($NR1_{011}$) lacks Insertion I (at the amino acid terminal) but contains Deletions I and II (carboxy-terminal cassettes), and the next most abundant form ($NR1_{111}$) is identical beside the inclusion of Insertion I (Standaert et al 1993). Just as different NR2 subunits confer different properties on the NMDA receptor, so different NR1 splice variants also affect the NMDA receptor channel properties (Durand et al 1992; Hollmann et al 1993; Standaert et al 1993). It has been suggested that, among other agonists, zinc may act as a positive modulator of NMDAR in certain brain regions due to its potentiating effects on some NR1 splice variants (Hollmann et al 1993), as the expression of some splice variants appears to vary between brain regions (Moriyoshi et al 1991; Nakanishi et al 1992). Spermine also potentiates NMDARs containing certain subunit/splice variant combinations; when $NR1_{011}$ forms a complex with NR2B, glycine affinity and current through the NMDAR are increased. However, $NR1_{011}$ combined with NR2A only produces an increased glycine

affinity, and $NR1_{011}$ permits neither to be potentiated (Zhang et al 1994). It is not yet clear how great an overall effect the different splice variants have *in vivo*.

Five different NR2B splice variants have also been found (Klein et al 1998; Sasner & Buonanno 1996; Tabish & Ticku 2004), with variations caused by non-coding exons-1 and -2. Although clear roles for the different splice variants have not been identified, it is possible that they may be associated with changes in spatial and temporal locations of the NR2B subunit (Sasner & Buonanno 1996).

1.6.3. Pharmacological studies

A common alternative to genetically knocking out a receptor (and indeed, the only selective alternative for many years) is pharmacological inhibition (or activation). AP5 is a selective competitive antagonist of the NMDA receptor, and chronic infusion has been shown to prevent spatial reference memory on the watermaze (Morris et al 1986a). What is more, the impairment in spatial learning has been found to be dependent on the dose of AP5 infused (Davis et al 1992). It was later shown that the impairment was caused by an inability to encode the memory, and that retrieval was unimpaired (Morris 1989). However, it should be noted that AP5 causes some motor deficits, which it has been argued could affect the ability of animals to perform the watermaze (Bannerman et al 2006).

There are many endogenous modulators of the NMDAR. It has been suggested that NR2B containing NMDA receptors can be selectively inhibited by platelet-derived growth factor (Beazely et al 2009), although ifenprodil is the most commonly used inhibitor of NMDARs containing NR2B. Ironically, although physiological concentrations of Mg block the NMDAR, at positive membrane

potentials Mg actually potentiates the NMDAR. Similarly, zinc ions can both potentiate (Zheng et al 1994) and inhibit the NMDAR (Traynelis et al 1998). Arachidonic acid also potentiates NMDARs (Miller et al 1992), and inhibiting phospholipase A_2 (the enzyme which catalyses the release of arachidonic acid and lysophospholipids from phospholipids) also inhibits LTP (Okada et al 1989). At pH 7.3 (physiologically relevant), the NR1 subunit of the NMDAR is inhibited by protons. It has been suggested that the inhibition of the NMDAR by protons may be neuroprotective in the event of ischemia and acidosis (Giffard et al 1990; Tang et al 1990; Traynelis & Cull-Candy 1990; Vyklicky et al 1990). However, in the presence of exon 5 (one of the NR1 splice variants), this tonic inhibition is somewhat reduced. Hence this splice variant may act as a modulator of NMDAR function (Traynelis et al 1995). As mentioned earlier, spermine (and other polyamines) (Zhang et al 1994) can also potentiate the NMDAR; it is possible that the method by which polyamines are able to potentiate the receptor are by relieving its tonic proton inhibition. Guanine nucleotides have the opposite effect of inhibiting the NMDAR (Hood et al 1990). Oestrogen is also a well-known promoter of NMDAR currents and LTP (Smith et al 2009a).

Frequently, modulators of receptor channel function will act through interactions with the extracellular N-terminus or intracellular C-terminus domains, and these regions are most frequently the areas that differ between different splice variants. All NMDAR subunits have a large extracellular N-terminus domain and a cytoplasmic C-terminus domain whose size varies depending on the subunit. The C-terminus domain allows the subunits to interact with intracellular proteins (Yamakura & Shimoji 1999), whilst on NR2A and NR2B, the N-terminus domain has binding sites for inhibitors such as ifenprodil. Extracellular agonist binding domains on NR1 and NR3 subunits allow glycine to bind with the subunit, whilst the binding site on NR2 binds primarily with glutamate (Mayer et al 1984).

1.6.4. The mechanisms of memory formation and LTP

Modulators of NMDA receptor function can affect NMDA receptor-dependent processes, such as the production of LTP. When a stimulus is experienced, the membranes of the cells that sense it depolarize. This wave of depolarization is then transmitted along the processes of the cell until it reaches a synapse, at which point the influx of calcium leads to the release of vesicles containing neurotransmitters. These neurotransmitters diffuse across the synaptic cleft and, where they encounter their binding sites in the membrane of the post-synaptic cell. This often causes a conformational change in the receptors to which they bind, and in the case of NMDA receptors, they open (when the membrane is sufficiently depolarized for the Mg^{2+} block to be removed) and allow the sodium and calcium ions to depolarize the post-synaptic cell, and for calcium ions to act as an intracellular signalling molecule activating secondary messenger cascades.

The neurotransmitter glutamate binds to N-methyl-$_D$-aspartate (NMDA) receptors. When the depolarization of the post-synaptic cell is of a sufficiently great magnitude, this leads to the recruitment of α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors. A greater number of these channels at the synapse means that fewer vesicles of neurotransmitter (and thus reduced stimulation of the pre-synaptic cell) are required to cause the same depolarization of the post-synaptic cell; that is, after recruitment of AMPA receptors, the threshold for post-synaptic cell depolarization is reduced. In the mean time, transporters in the pre- and post-synaptic membranes re-uptake the neurotransmitters remaining in the synaptic cleft, and/or enzymes in the synaptic cleft break them down.

AMPA receptor recruitment usually requires temporal or spatial summation of stimuli by pre-synaptic cells. For example, one pre-synaptic cell firing may be insufficient to depolarize the post-synaptic cell membrane, but if two pre-synaptic cells fire at the same time the post-synaptic cell

may depolarize and lead to AMPA recruitment. This is spatial summation (Figure 6 (b)). Temporal summation, by comparison, requires multiple stimuli from a single pre-synaptic cell for a long period (Figure 6 (a)). The accumulation of neurotransmitters in the synaptic cleft can then activate sufficient receptors to cause depolarization of the post-synaptic membrane and recruitment of AMPA receptors. After the AMPA receptors are recruited, firing of a single pre-synaptic cell is sufficient to depolarize the post-synaptic cell again.

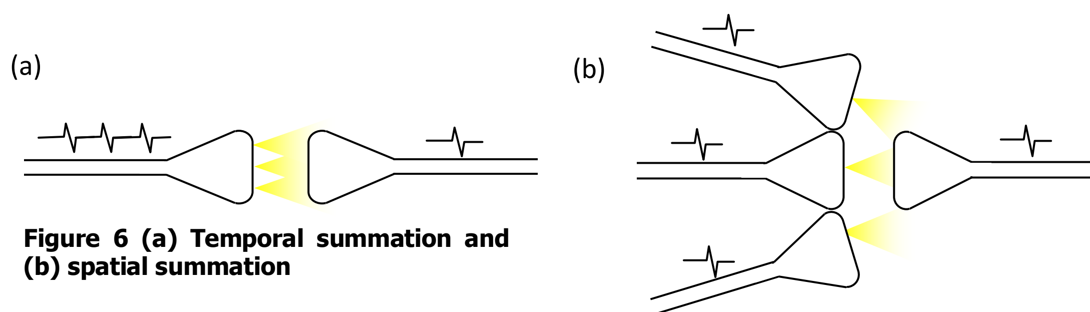


Figure 6 (a) Temporal summation and (b) spatial summation

1.7. NMDA receptor dependent LTP

Long term potentiation (LTP) has been recognized as the mechanism most likely to underlie learning and memory for the past four decades, since it was first discovered by Tim Bliss and Lømo (1973). They repetitively stimulated fibres in the perforant path (with a tetanus) to stimulate the DG in anaesthetized rabbits. The cells of the DG then produced an excitatory post synaptic potential (EPSP) of much greater magnitude than previously, which was maintained for over 6 hours. This process mirrors the formation and time course of memories. Although it is not possible with current techniques and technology to prove that LTP is the fundamental process underlying memory, it is strongly and very widely believed to be responsible.

LTP can be NMDA receptor dependent or NMDA receptor independent (Raymond 2007). Most work has been concerned with NMDA receptor dependent LTP, in which activated synapses

recruit AMPA receptors to the synapse to strengthen it and maintain the synapse strength. LTP has been classified further into short-term LTP, early LTP (lasting minutes to hours), late LTP (lasting weeks to months), although more recently LTP has been re-classified into forms LTP1, LTP2 and LTP3 (Raymond 2007). LTP1 rapidly decays and is protein synthesis independent, LTP2 requires protein synthesis but is gene transcription independent, whereas LTP3 requires protein synthesis and gene transcription.

Very little is known about which neurons and synapses are used to store a memory. However, using a viral vector, a protein has been overexpressed in the amygdala known as cyclic adenosine monophosphate (cAMP) responsive element-binding protein (CREB). This protein is important in gene transcription and is known to be important for the stability of synaptic potentiation and memories (as protein synthesis is necessary for LTP maintenance), and the amygdala is involved in the memory of emotional reactions. When the animals were subjected to tone fear conditioning, the likelihood that the neurons in the amygdala overexpressing CREB were recruited for the formation of the memory was greater than for neurons not over-expressing CREB (Han et al 2007; Han et al 2009). When the cells overexpressing CREB were then selectively killed, the animals appeared to lose the memory of the fear of the tone (Han et al 2009; Zhou et al 2009). This demonstrates that transcription promoted by CREB is required for long term memory maintenance.

CREB is not the only element required for the maintenance of long term memory. Maintenance of LTP is also mediated by an autonomously active isoform of protein kinase C (PKC) (Malinow & Malenka 2002), known as protein kinase M ζ (PKMζ) (Sacktor et al 1993). Applying zeta inhibitory peptide (ZIP), an inhibitor of PKMζ prevents maintenance of late (but not early) LTP in

hippocampal slices (Pastalkova et al 2006), leads to an inability for mice to remember paired place-shock associations caused by a storage deficit. Although long term memory is impaired by the application of ZIP, short term memory is not (Serrano et al 2008), suggesting that PKMζ is necessary for long term memory but not short term memory. Recent work has demonstrated disrupted ability to carry out learned sensorimotor skills after administration of ZIP, suggesting that there may be a common mechanism between the storage of declarative and procedural memories (von Kraus et al 2010).

As it has become possible to target mutations to specific cell types, there has been considerable interest in investigating the effects of varying protein levels in known cell populations to observe their effect on learning and memory. As the NR1 subunit is required for NMDA receptor formation, by knocking out the NR1 receptor it is possible to knock out NMDA receptors (mice in which the NR1 gene is knocked out globally have an embryonic lethal phenotype (Kutsuwada et al 1996)). Mice in which the NR1 subunit had been selectively knocked out in the dentate gyrus learned a fear conditioning paradigm normally (McHugh et al 2007) and showed normal spatial reference memory on a radial maze, but were impaired on the working memory component of the task (Niewoehner et al 2007). A similar phenotype was seen in mice in which NR1 had been knocked out in CA3 neurons, with spatial working memory being disrupted despite intact spatial reference memory (Nakazawa et al 2003). However, when NR1 was selectively knocked out from CA1 neurons, mice displayed impaired spatial reference memory (but did not affect non-spatial learning) (Tsien et al 1996); hence the NMDA receptor is important for working memory in the dentate gyrus and CA3 neurons, but for spatial working memory in CA1 neurons. It has also been suggested that NR1 receptors in the CA3 subfield may be important for acquiring rapid, one-trial memories (Nakazawa et al 2003).

Knocking out the NR2B subunit globally also causes death at birth (Kutsuwada et al 1996). However, NR2A only appears after birth (Monyer et al 1992), and global NR2A knockout mice have been successfully created. Mice lacking the NR2A subunit are able to perform tasks requiring spatial reference memory but are impaired on spatial working memory tasks (Bannerman et al 2008). Similarly, when NR2B was knocked out specifically in the hippocampus of mice, their spatial reference memory remained intact whereas their working memory was impaired (von Engelhardt et al 2008). However, when NR2B was knocked out throughout the forebrain, mice displayed impairments on spatial and non-spatial tasks (spatial reference memory in the watermaze and on the appetitively motivated Y maze, spatial working memory, non-spatial visible platform water maze, visual discrimination test, object recognition and a egocentric memory task), highlighting its importance in both hippocampus-dependent and –independent learning and memory (von Engelhardt et al 2008).

1.8. NR2BOE mice

NR2BOE mice overexpress the NR2B subunit of the NMDAR in the forebrain into adulthood, and so for each depolarisation of neurons with NR2B-containing NMDARs, more calcium should enter the cell, and for a longer period. From the discussion above, one could conclude that the increased time window during which calcium enters the cell could lead to increased coincidence detection, and so more cells strengthening their connections. Alternatively, the increased level of calcium could induce apoptosis and kill the cell. Depending on the precise level of excitation, perhaps both possibilities could occur more frequently. In the NR2BOE mice, it was hypothesised that "*enhanced signal detection by NMDA receptors should enhance learning and memory*" (Tang et al 1999), and so LTP was considered in these mice. Enhanced LTP was indeed found in brain slices from NR2BOE mice compared with wildtype mice, and protocols which normally produced only

minimal LTP produced robust LTP in NR2BOE mice (but not wildtypes). Importantly, NR2BOE and wildtype brain slices both showed similar levels of LTD (Tang et al 1999). There is a theory that NR2B-containing NMDARs may underlie LTD whereas NR2A underlie LTP (Liu et al 2004; Massey et al 2004), but numerous groups have demonstrated that NR2B-containing NMDARs produce LTP (Berberich et al 2005; Gardoni et al 2009) that LTD can occur in the absence of functional NR2B containing NMDARs (Morishita et al 2007) as well and so this theory appears unlikely (Rammes et al 2009; von Engelhardt et al 2008).

1.8.1. Genetic construction of Doogie mice

The linearised NR2B transgene in vector pJT-NR2B containing the CaMKII promoter was prepared and injected into C57B/6 zygotes. The F1 generation was then bred with CBF1 mice to produce the NR2BOE mice, and these mice were then back-crossed onto B6CBF1 mice to maintain the colony. A single copy of the transgene, i.e. heterozygous expression, defined the NR2BOE phenotype.

1.8.2. Behavioural characterisation of NR2BOE mice

Tang et al. then went on to consider the behaviour of the NR2BOE mice. Their hypothesis was that if they expressed greater levels of LTP, which is considered to underlie learning and memory, they should show enhanced learning and memory compared with normal (wildtype) mice. To consider this, they used the Morris water maze to test spatial reference memory, object novelty preference to test non-spatial memory, and fear conditioning to test non-spatial memory. In later work they also considered working memory. On all tests except working memory tasks, they found enhanced performance by the NR2BOE mice (Tang et al 1999). Other groups found that in memory tasks using olfaction (considered by some to be a more salient form for testing mice) that relied on NMDARs, mice overexpressing the NR2B subunit displayed improved memory (although not on tasks

that do not rely on NMDARs) (White & Youngentob 2004). However, in that task the mice were only tested at 24 hours; in the original study by Tang et al. the NR2BOE mice showed increased memory levels as judged by a preference for novel objects at 24 hours and 72 hours; it is possible that an improvement in memory may have been seen at 72 hours as well as 24 hours, had White et al. tested the mice at this interval. Recently rats overexpressing the NR2B subunit have also been produced, and they also appear to exhibit improved learning and memory (Wang et al 2009). These results accord with a study that has shown reversible improvements in cognitive function with associated upregulation of NR2B subunits caused by isoflurane anaesthesia (Rammes et al 2009).

Crucially however, Tang et al. used male mice only in their experiments. It is well known that there can be marked sex differences on many behavioural tests (Akinci & Johnston 1993; Cahill 2006; Cooke & Woolley 2005; Honack & Loscher 1993; Jonasson 2005; Smith et al 2009b). For this reason, we attempted to replicate the results of Tang et al with male and female mice. It is also useful to test mice on several different but complementary tasks to demonstrate that the results are due to improved memory per se and not simply due to the particular task used.

The age of the mice may also be significant, although NR2BOE were able to demonstrate improved learning and memory "during aging" (Cao et al 2007) (this paper also failed to mention the sex of the animals tested and presumably also used males only). Therefore the mice's age at the time of testing should not be an issue.

1.8.3. NR2B$^{\Delta HPC}$ mice

Mice overexpressing a receptor subunit can be informative, but cannot present a complete picture of the effects of the subunit. Whilst considering the influence of the NR2B subunit of the

NMDA receptor, we also looked at mice which lacked NR2B in the CA1 region and the dentate gyrus. Mice lacking the NR2B subunit globally lack the suckling response and so die within hours of birth (Kutsuwada et al 1996). These mice are characterized by decreased spine density, decreased long term depression and decreased learning and memory. The NR2B$^{\Delta HPC}$ mice were impaired on the hidden platform watermaze (testing spatial reference memory), spontaneous alternation (testing spatial working memory) and Pavlovian trace fear conditioning (testing corticohippocampal fear and contextual memory). There is a suggestion that their deficits may be at least partially caused by a tendency to persevere with incorrect behaviours, possibly due to an impairment in short term memory such that the mice are unable to remember places they have recently visited. In the hidden platform watermaze transfer tests (in which the platform is removed from the pool entirely and mice are free to swim for 60 seconds), the NR2B$^{\Delta HPC}$ mice tended to swim in the region of the platform for the entire time whereas wildtype mice swam in other areas of the pool after 45 seconds had elapsed (when it was clear that the platform was not in its usual position).

In order to test the hypothesis that mice could not remember recently visited places, we subjected them to a rewarded alternation task into which delays were introduced. If the knockout mice had more quickly degrading memory traces for recently visited places they might be expected to forget which arm they had just received a reward from when a delay was imposed.

We also gave them a reversal paradigm, in which they were trained to associate one of two identical beacons with an escape platform in the watermaze based on its spatial location. After a number of days, when the mice had learned the position of the platform relative to the beacons, the platform was placed under the platform that previously acted as a distracter. It was anticipated that

the knockout mice would persevere with their memory of the platform being associated with the first beacon, and so would take longer to reach the platform after reversal.

To replicate the findings of Tang et al. (1999), it was necessary to test the mice on the hidden platform watermaze in our own testing environment. This ensured that the results observed were replicable in our laboratory with our equipment and spatial cues. The hidden platform watermaze is dependent on the hippocampus, as lesions prevent mice from finding the platform effectively (Deacon et al 2002a; Reisel et al 2002). The hidden platform watermaze also requires NMDA receptors containing the NR2B subunit, as mice in which the NR2B subunit has been knocked out are unable to find the hidden platform as efficiently as wildtype mice (von Engelhardt et al 2008).

2. Chapter 2: NR2BOE mice, cohort 1

2.1.1. Replication of procedure

To replicate the findings of Tang et al. (1999), it was necessary to test the mice on the hidden platform watermaze in our own testing environment. This ensured that the results observed were replicable in our laboratory with our equipment and spatial cues. The hidden platform watermaze is dependent on the hippocampus, as lesions prevent mice from finding the platform effectively (Deacon et al 2002a; Reisel et al 2002). The hidden platform watermaze also requires NMDA receptors containing the NR2B subunit, as mice in which the NR2B subunit has been knocked out are unable to find the hidden platform as efficiently as wildtype mice (von Engelhardt et al 2008).

2.1.2. Sex specificity of behaviour

We wished to establish if the difference observed by Tang et al. (1999) was also present in females, or if it was sex specific and existed only in male mice. As has been noted previously, differing results for males and females have often been recorded, and it should not be assumed that behaviours observed in one sex will be present in the other. We used male and female mice, allowing us to directly compare their behaviours.

2.1.3. Causes of behaviours

In addition, we wanted to understand the underlying cause of the observed behaviours. In particular, we wished to test the hypothesis that the behaviours of the mice may be directed by their underlying anxiety levels or inherent thigmotaxic tendencies. It is possible that the results observed by Tang et al (1999) may be caused by some factor unrelated to memory such as anxiety, which could potentially confound behavioural observations and it was important to rule this out where

possible. The open field task was particularly apt for this purpose as it spatially resembles the watermaze (in that both are large, cylindrical arenas), and when brightly lit is particularly anxiogenic and hence ideal for considering anxiety.

2.1.4. Specificity of the effect

Also of considerable interest was the generality of the memory improvement. We wished to consider whether it applied to other spatial reference memory tasks, such as the appetitively motivated spatial reference Y maze. Additionally we wished to consider other tests of spatial working memory in order to test whether the memory of NR2BOE mice appeared enhanced across different forms of memory which use different neurobiological pathways. We also wanted to consider whether the memory improvement was visible on non-spatial tasks, on tasks such as novel object recognition which relies on the mouse's memory of an object, and non-spatial, associative tasks that do not require the hippocampus, such as the visible platform task and visual discrimination.

2.1.5. General methods

All experiments were carried out under the auspices of the Home Office (UK) during the light phase of a 12 hour cycle (lights on at 07:00), with free access to food and water unless otherwise stated. Behavioural testing was done using age-matched littermate wildtype and NR2BOE mice of both sexes, provided by Joe Tsien and bred in house in Oxford. The transgenic founders were produced from a C57B/6 zygote, intercrossed with B6/CBF1. Genotype was established using ear clips and/or tail clips. Mice were housed in littermate groups of 2 to 7 and tested during the light phase of the day, with male and female subjects being tested in an alternating sequence for all experiments (except where different sexes were run by different experimenters, in which case males and females were tested on seperate days at the same time and under identical testing conditions).

All tests on any individual mouse were conducted in different rooms. Where mice were tested by different experimenters, data from mice tested by all experimenters is presented together as analysis showed no significant effect of experimenter on the results.

2.1.6. Statistics

For experiments conducted over several days (such as the watermaze), repeated measures ANOVA was performed (with the statistical package SPSS or Sigmastat 3.5). Otherwise, a two-way ANOVA was conducted with gender and genotype as between subject factors (using the statistical package SPSS or Sigmastat 3.5) when data were normally distributed. Significant interactions were then investigated with a test of Simple Main Effects (SME).

2.2. Open field task

2.2.1. Introduction

One of the aspects to consider was the aversive nature of the watermaze, which relies on mice's innate preference to escape from water. A mouse's reaction to an aversive stimulus can be greatly affected by its anxiety. One possible explanation for the facilitated watermaze performance seen by Tang et al. (1999) reflects a reduction in anxiety by these animals and potentially reduced thigmotaxis. Hence it would be informative to observe the effect of an anxiogenic open field on the NR2BOE mice and controls before performing the hidden platform watermaze. The anxiogenic open field task was performed before the water maze to ensure that any thigmotaxic tendencies reflected anxiety rather than a behaviour learned on the water maze. One of the benefits of using the open field task over other tests of anxiety is that it allows consideration of thigmotaxic tendencies and resembles the watermaze as both involve round, white, bright open spaces.

2.2.2. Materials and Methods

Mice were 8 months of age when this task was conducted. This task was performed by other members of the laboratory for this cohort. The open field test comprised a large, brightly lit white metal drum (60cm high by 60cm diameter) with a camera mounted in the ceiling to allow tracking of the animal. The mouse was placed in the open field at the side, through a door which was immediately closed, and the animal was allowed to explore freely for 5 minutes. The latency to reach the centre of the open field (defined as a region with a radius of 10cm from the exact centre of the maze), the distance moved, and the time spent at the sides (thigmotaxis, defined as the mouse being within 5cm of the side walls), were measured. For this task, there were: male wildtypes = 12, female wildtypes = 20, male NR2BOE = 17, female NR2BOE = 17.

2.2.3. Results

2.2.3.1. Latency to centre

Male NR2BOE appeared less anxious than male wildtype mice, and female NR2BOE appeared more anxious than female wildtype mice. An ANOVA on the time to the centre was performed, with two between subjects factors (genotype and sex). There was no effect of genotype ($F(1,62)=1.190$, $p=0.280$) but a significant effect of sex ($F(1,62)=4.616$, $p=0.036$) and a genotype by sex interaction on latency to the centre ($F(1,62)=10.032$, $p=0.002$) as shown in **Error! Reference ource not found.**. SME revealed an effect of sex on wildtypes ($F(1,62)=13.298$, $p=0.001$) but not NR2BOE ($F(1,62)=0.554$, $p=0.46$), and there was an effect of genotype on males ($F(1,62)=8.003$, $p=0.006$) but not females ($F(1,62)=2.486$, $p=0.120$). Thus, male NR2BOE mice took less time than male wildtypes to reach the centre. By comparison, female NR2BOE mice took more time than female wildtypes to reach the centre. There were: male wildtype = 12, female wildtype = 20, male NR2BOE = 17, female NR2BOE = 17.

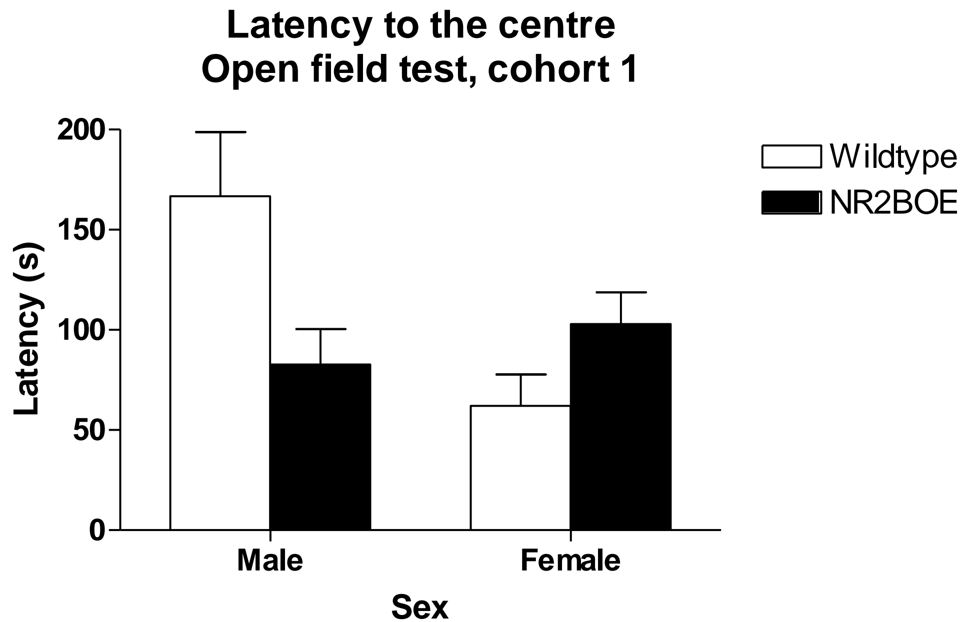


Figure 7 The latency of mice to reach the centre of the open field task was greater for wildtype male mice. Time of mice from cohort 1 to reach the middle of the open field (s), mean ± SEM, wildtype (☐) and NR2BOE (■) mice.

2.2.3.2. Time in centre

There was no difference between control and NR2BOE, or male and female, mice on the time spent in the centre of the open field test. An ANOVA with two between subjects factors (genotype and sex) was performed on the time spent in the centre. There was no effect of genotype (F(1,62)=1.014, p=0.318) and no genotype by sex interaction on time in the centre (F(1,62)=2.451, p=0.123) as shown in **Error! Reference source not found.**. There was a non-significant trend towards n effect of sex (F(1,62)=3.482, p=0.067), with females spending more time in the centre than males. There were: male wildtype = 12, female wildtype = 20, male NR2BOE = 17, female NR2BOE = 17.

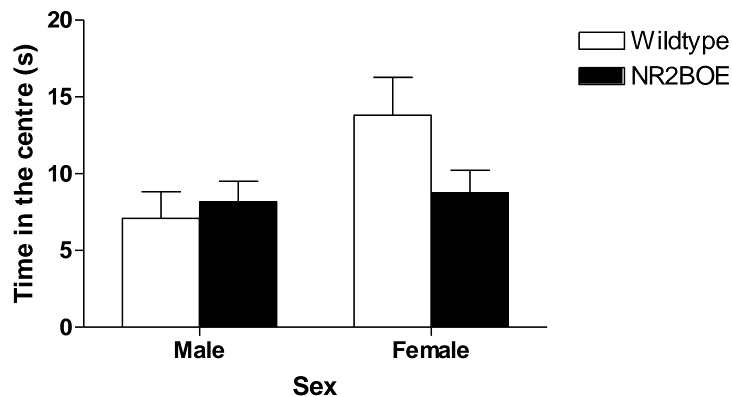


Figure 8 The time mice spent in the centre of the open field task was similar for wildtype and NR2BOE mice. Length of time mice from cohort 1 spent in the middle of the open field (s), mean ± SEM, wildtype (□) and NR2BOE (■) mice.

2.3. Hidden platform watermaze (spatial reference memory)

2.3.1. Introduction

The fixed location hidden platform watermaze task is a classical test of spatial reference memory for rodents, first developed by Morris (Morris 1981). It involves mice swimming in a tank of opaque water to a platform hidden a centimetre beneath the surface of the water. The platform remains in the same location, so that mice must learn to use extramaze cues in order to locate the platform. This task dependent on both the hippocampus and NMDA receptors, as ablating the hippocampus (Deacon et al 2002b; Morris et al 1982) or blocking NMDA receptors with amino-5-phosophovaleric acid (AP5) leads to impaired performance (Morris et al 1986a); however, see (Bannerman et al 1995; Saucier & Cain 1995). The spatial reference watermaze was thought to require the NR2A subunit, as mice in which the NR2A subunit is constitutively knocked out throughout the brain were impaired on the hidden platform watermaze (Sakimura et al 1995).

However, more recently it has been demonstrated in another knock out strain that NR2A is not needed for spatial reference memory in the watermaze, although mice lacking the NR2A subunit were impaired on spatial working memory in the watermaze (Bannerman et al 2008). It was postulated that the difference between the findings of Bannerman et al (2008) and Sakimura et al (1995) may be due to the different background strains into which the NR2A knock out was introduced, as it is well known that genes from the genetic background of strains can affect the behaviour of the mouse strain (Gerlai 1996).

Tang et al. (1999) found that the NR2BOE mice they tested on the hidden platform watermaze reached the platform in less time than wildtype littermates. This suggests that the NR2BOE mice had a better memory for the exact location of the platform, allowing them to escape the water more rapidly. The standard measure of memory on the watermaze is the length of time spent in the vicinity of the platform location on probe trials (when the platform is removed), with a greater time spent in the training quadrant considered a measure of improved learning and memory. Tang et al (1999) also showed that the NR2BOE showed a stronger preference for the training quadrant during the first transfer test after three sessions (although there was no difference between the wildtype and NR2BOE preference for the training quadrant on the second transfer test after 6 sessions). The first aim was to determine whether NR2BOE mice were also facilitated in acquiring the watermaze task in our laboratory with our spatial cues. Tang et al (1999) used only male mice for their tests (personal communication), so it was also of significant interest to establish whether this result would be present in female mice too.

2.3.2. Materials and Methods

Mice were between 8 and 11 months of age when they performed this task. The maze consisted of a 2m diameter circular tank, of depth 0.6m, elevated to a height of 0.6m, containing

water at 21°C ± 1°C at a depth of 0.3m. A platform (diameter 21cm) with a wire mesh across the top was submerged 1cm beneath the surface of the water. Both the platform and water maze were painted white. To escape from the maze, the mice had to find the submerged platform within 90 seconds of being placed in the water. After 90 seconds, the mouse was removed from the water and placed on the platform by the experimenter.

As the platform always remained at the same location, mice learn to navigate to the position of the platform using extramaze spatial cues. The water was made opaque by adding 2 pints of full-fat milk, which prevented the mice from seeing the platform and also facilitated tracking of the animals. A camera above the maze monitored swim paths, and the output was sent to a video recorder to allow on- and off-line analysis. Each animal's x and y coordinates were sampled by an Acorn (Ltd.) computer, allowing the experimenter to record the latency to find the platform, path length and percent time spent in each quadrant or at the sides, for each trial. The percentage of of time spent swimming at the sides of the water maze (defined as swimming within 12cm of the side walls) was also recorded, and analysed as representing thigmotaxis.

Each animal was trained to a fixed platform position in the centre of one of four quadrants (NW, NE, SE, SW). The quadrant ascribed to each animal was counterbalanced with respect to group, and remained constant throughout training. No animals received swimming pre-training. Each animal received 4 trials a day for 12 days.

The animal was placed at the edge of the maze facing the wall in one of 8 counterbalanced positions (N, NE, E, SE, S, SW, W, NW), and was allowed to swim until it reached the platform or 90s had elapsed. Animals that failed to find the platform within 90s were guided to or placed on the

platform. Animals were allowed 30s on the platform before they were removed, and the inter-trial interval was 15s as the mouse was removed and placed back in the water.

Probe trials were conducted 24 hours after each set of 12 trials. Probe trials were conducted as described above, but the platform was removed and the animals were allowed to swim freely for 60s before being removed. The percentage time spent in each quadrant (training quadrant, in which the platform had been present, adjacent quadrants and opposite quadrants) was analysed. Animals were considered to have learned the task if they spent the majority of their time swimming in the training quadrant.

For this task, there were: male wildtypes = 12, female wildtypes = 17, male NR2BOE = 17, female NR2BOE = 17. Note: three female wildtype mice which were tested on the open field were not tested on the hidden platform watermaze, as the animals were put down due to health concerns.

The males were tested by David Bannerman. Half of the females were tested by Amy Taylor, and the remaining females were tested by the author. Analysis was conducted by the author.

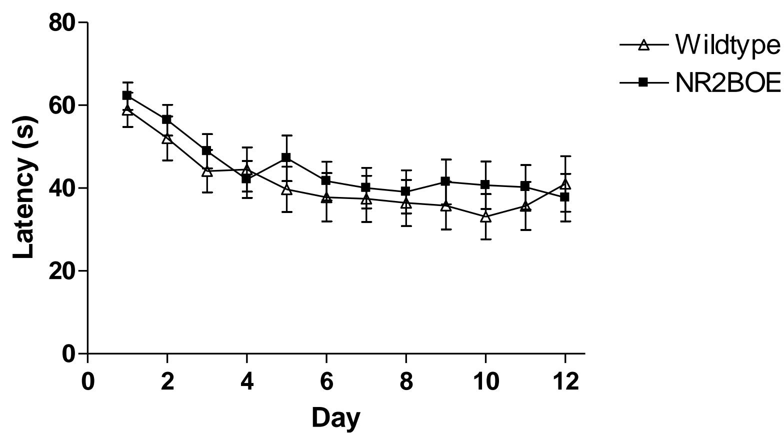
2.3.3. Results

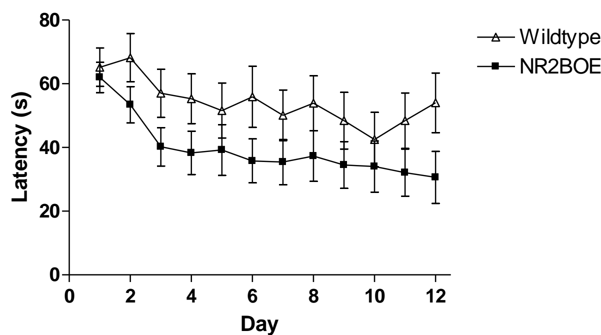
2.3.3.1. Latency

Both wildtype and NR2BOE mice learned to find the platform more quickly on later days. Wildtype female mice found the platform significantly faster than female NR2BOE mice and wildtype

male mice. A repeated measures ANOVA with two between subjects factors (genotype and sex) and one within subjects factor of block with 12 levels (corresponding to the 12 blocks, or days, of training) was performed. There was no effect of genotype on latency ($F(1,59)=0.064$, $p=0.801$) or sex ($F(1,59)=0.802$, $p=0.374$), but there was a significant genotype by sex interaction ($F(1,59)=7.008$, $p=0.01$) as shown in Figure 9 (a). The mean latencies were as follows: male wildtypes = 54.2±8.3, male NR2BOE = 39.4±7.0, female wildtypes = 32.3±6.6, female NR2BOE = 50.2±6.5. Simple main effects (SME) revealed that there was a significant difference between wildtype male and female mice ($F(1,59)=5.735$, $p=0.02$), but no significant difference between NR2BOE male and female mice ($F(1,59=1.695$, $p=0.198$). There was no difference between wildtype and NR2BOE male mice ($F(1,59)=2.62$, $p=0.111$) as shown in Figure 9 (b), but there was a non-significant trend for male NR2BOE mice to reach the platform more quickly than male wildtype mice, which is in the same direction as the result we wished to replicate. Tang et al (1999) found that the NR2BOE mice found the platform significantly more quickly than wildtypes across the six days of testing, reflected in a significant effect of genotype with post hoc analysis showing a significant difference at the third day of testing. In contrast, in our study there was a significant difference only between wildtype and NR2BOE female mice ($F(1,59)=4.643$, $p=0.035$) as shown in Figure 9 (c). This was driven by wildtype female mice finding the hidden platform significantly more quickly than the NR2BOE female mice. Post hoc analysis comparing the means of the NR2BOE and wildtype mice on day 3 only (two tailed independent samples t test) showed there was no difference between the latencies of the wildtype and NR2BOE mice to reach the platform (degrees of freedom = 61, two tailed $p = 0.465$). There were: male wildtype = 12, female wildtype = 17, male NR2BOE = 17, female NR2BOE = 17. Note: three female wildtype mice that had performed the anxiogenic open field were not tested in the Morris water maze, as they had been humanely killed due to illness.

There was a significant effect of block (F(11,649)=14.283, p<0.001) but no block by genotype (F(11,649)=0.785, p=0.655), block by sex (F(11,649)=0.953, p=0.489) or block by genotype by sex (F(11,649)=1.121, p=0.342) interaction. The effect of block was driven by significantly shorter latencies on later days (latencies on days 1 and 2 were significantly different to those on all other days, but there was no difference in latencies on days 6 to 12, suggesting that the mice had reached asymptotic levels of performance).





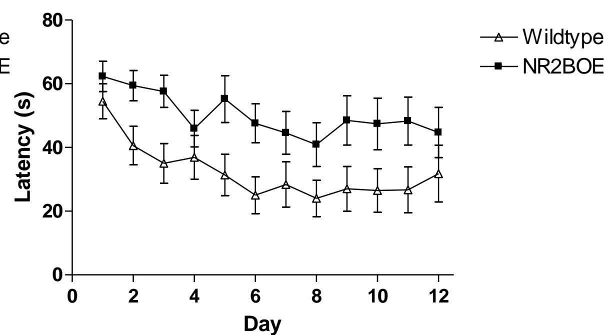


Figure 9 The latency of mice to reach the platform on the hidden platform watermaze decreased with practise. (a) Time of mice from cohort 1 to reach the hidden platform (s). (b) Latency of male mice from cohort 1 to reach the hidden platform. (c) Latency of female mice from cohort 1 to reach the hidden platform. Mean ± SEM during acquisition of the spatial reference memory task for wildtype (Δ) and NR2BOE (■) mice.

2.3.3.2. Distance

Similarly, the distance swum to reach the platform was similar for both genotypes, although wildtype female mice found the platform with significantly less distance travelled than female NR2BOE mice and wildtype male mice. A repeated measures ANOVA with two between subjects factors (genotype and sex) and one within subjects factor of block with 12 levels (corresponding to the 12 blocks, or days, of training) was performed. No effect of genotype ($F(1,59)=0.072$, $p=0.789$) nor sex ($F(1,59)=0.805$, $p=0.373$) was observed, although there was a significant genotype by sex interaction ($F(1,59)=7.436$, $p=0.008$), as shown in Figure 10 (a). The genotype by sex interaction was driven by wildtype males and NR2BOE females swimming greater distances to reach the platform than NR2BOE males and wildtype females respectively. The mean distances were as follows: male wildtypes =9.92 ± 1.43, male NR2BOE = 7.51 ± 1.13, female wildtypes = 6.36 ± 1.07, female NR2BOE = 9.3 ± 1.02. SME revealed that there was a significant difference between wildtype male and female mice ($F(1,59)=6.001$, $p=0.017$), but no significant difference between NR2BOE male and female mice ($F(1,59=1.848$, $p=0.179$). There was no difference between wildtype and NR2BOE male mice ($F(1,59)=2.761$, $p=0.102$) as shown in Figure 10 (b) although there was a trend for the NR2BOE mice to swim shorter distances to reach the platform; this effect is in the same direction as the effect observed by Tang et al. 1999. There was a significant difference between wildtype and NR2BOE female mice ($F(1,59)=4.954$, $p=0.03$) as shown in Figure 10 (c), with female NR2BOE mice swimming greater distances than female wildtype mice to reach the platform. There were: male wildtype = 12, female wildtype = 17, male NR2BOE = 17, female NR2BOE = 17.

There was a significant effect of block ($F(11,649)=26.074$, $p<0.001$), but no block by genotype ($F(11,649)=0.77$, $p=0.67$), block by sex ($F(11,649)=1.11$, $p=0.35$) or block by genotype by sex ($F(11,649)=1.409$, $p=0.164$) interaction. The effect of block was driven by reduced distances swum on later blocks (distances were significantly longer on days 1 and 2 compared with all other

days, but distances swum on days 7-12 were not significantly different, suggesting the mice had reached asymptotic levels of performance).

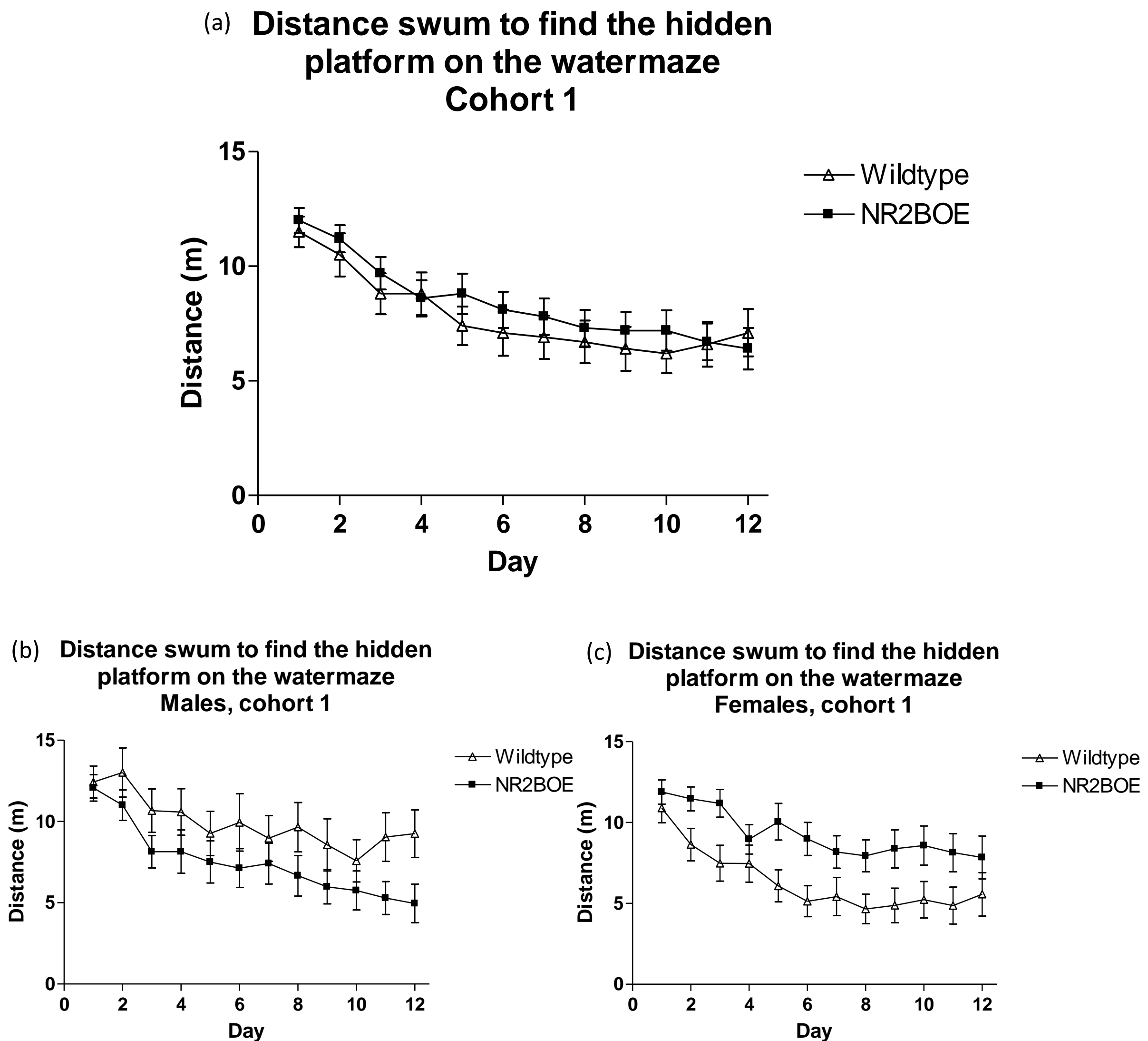


Figure 10 The distance swum by mice to reach the platform on the hidden platform watermaze decreased with practise. (a) Distance mice from cohort 1 swam to reach the hidden platform (m). (b) Distance male mice from cohort 1 swam to reach the hidden platform. (c) Distance female mice from cohort 1 swam to reach the hidden platform. Mean ± SEM during acquisition of the spatial reference memory task for wildtype (Δ) and NR2BOE (■) mice.

2.3.3.3. Thigmotaxis

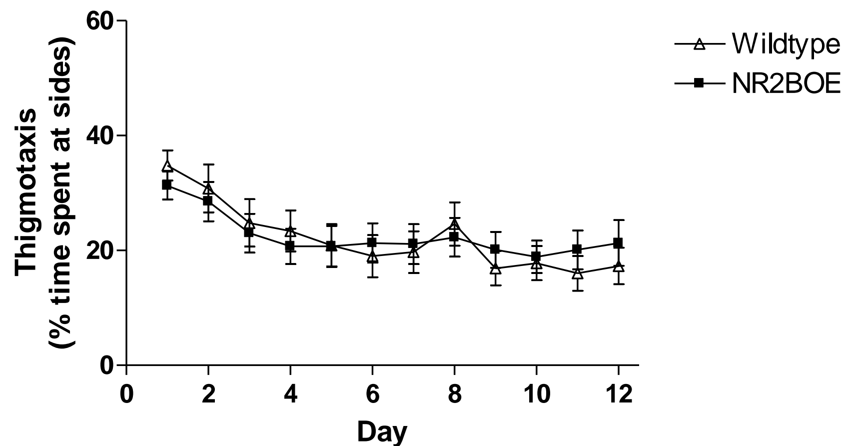
Wildtype and NR2BOE mice displayed similar levels of thigmotaxis, but wildtype female mice were significantly less thigmotaxic than female NR2BOE and wildtype male mice. A repeated measures ANOVA with two between subjects factors (genotype and sex) with one within subjects factor (block) with 12 levels (corresponding to the 12 days of trials) was performed. There was no effect of genotype on thigmotaxis ($F(1,59)=0.084$, $p=0.773$) nor sex ($F(1,59)=1.821$, $p=0.182$), although there was a significant genotype by sex interaction ($F(1,59)=7.688$, $p=0.007$) as shown in Figure 11. The mean percentage of time spent near the maze walls were as follows: male wildtypes = 32.1±5.68, male NR2BOE = 19.5±4.68, female wildtypes = 15.2±3.43, female NR2BOE = 25.4±4.63. SME revealed that there was a significant difference between male and female wildtype mice ($F(1,59)=7.763$, $p=0.007$), but no difference between male and female NR2BOE mice (F(1,59=1.119, $p=0.294$), hence the sex difference was driven by male wildtype mice being significantly more thigmotaxic than female wildtype mice, whereas male NR2BOE mice were less thigmotaxic than female NR2BOE mice. There was a trend towards a significant difference between wildtype and NR2BOE male mice ($F(1,59)=3.403$, $p=0.07$) and a significant difference between wildtype and NR2BOE female mice ($F(1,59)=4.286$, $p=0.043$). There were: male wildtype = 12, female wildtype = 17, male NR2BOE = 17, female NR2BOE = 17.

There was a significant effect of block ($F(11,649)=16.998$, $p<0.001$), driven by reduced thigmotaxis on later days. There was no block by genotype ($F(11,649)=1.611$, $p=0.091$), or block by genotype by sex ($F(11,649)=1.34$, $p=0.198$) interaction. There was, however, a significant block by sex ($F(11,649)=2.016$, $p=0.025$) interaction. There was a significant difference between wildtype and NR2BOE male ($F(1,59)=4.286$, $p=0.043$) and a trend towards a significant difference between wildtype and NR2BOE female ($F(1,59)=3.403$, $p=0.07$) mice. There was also a significant difference between wildtype male and female ($F(1,59)=7.763$, $p=0.007$) but not NR2BOE male and female

(F(1,59)=1.119, p=0.294) mice. Post hoc analysis showed a significant difference in time spent at the sides by male and female mice on day 8 (F(1,59)=6.346, p=0.014). Mice spent significantly longer swimming at the sides on days 1 and 2 than any other days, but there were no significant differences between times spent at the sides from days 9-12.

It was also of interest to consider if there were any initial differences in thigmotaxis between the wildtype and NR2BOE mice. Increased thigmotaxis in either genotype could be a result of two possible scenarios: 1) the mice swim at the sides because they cannot find the platform, or 2) the mice cannot find the platform because they swim at the sides. Hence we considered the percentage of time spent swimming at the sides for the first trial of the first day. An independent samples t test comparing the wildtype and NR2BOE mean percentage of time spent at the sides showed that there was no difference in thigmotaxis on the first trial of the first day (degrees of freedom = 61, two-tailed p = 0.577). An independent samples t test comparing the male and female mean percentage of time spent at the sides showed that there was a significant difference in thigmotaxis on the first trial of the first day, with female mice spending significantly longer at the sides (degrees of freedom = 61, two-tailed p = 0.015. Mean time spent at sides by: males =31.2% , females = 43.0%). Therefore the NR2BOE mice were not impaired in their tendency to swim away from the sides.

(a)



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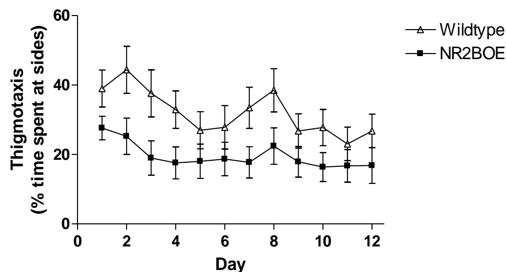

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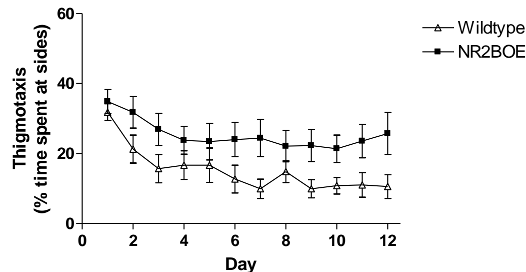


Figure 11 The % time mice spent swimming at the sides of the watermaze decreased with practise. (a) % of time mice from cohort 1 spent swimming at the sides of the watermaze on each trial. (b) % of time male mice from cohort 1 spent swimming at the sides of the watermaze on each trial. (c) % of time female mice from cohort 1 spent swimming at the sides of the watermaze on each trial. Mean ± SEM during acquisition of the spatial reference memory task for wildtype (Δ) and NR2BOE (■) mice.

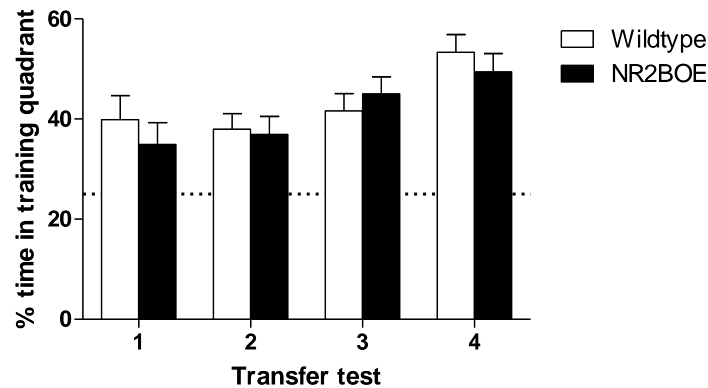
2.3.3.4. Transfer tests

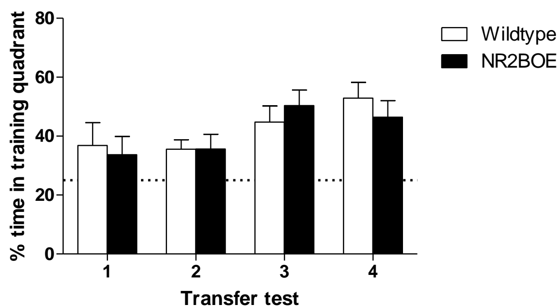
Wildtype male mice spent more of the transfer test swimming in the quadrant in which the platform was normally located compared with NR2BOE male mice. A repeated measures ANOVA of time spent in the training quadrant with two between subjects factors (genotype and sex) and one within subjects factor (transfer test number) with four levels (corresponding to the four transfer tests) was performed. There was no effect of genotype ($F(1,59)=1.77$, $p=0.188$), nor sex ($F(1,59)=0.112$, $p=0.739$), although there was a significant genotype by sex interaction ($F(1,59)=3.831$, $p=0.05$) as shown in Figure 12. There was a near significant difference between NR2BOE males and females ($F(1,59)=2.9$, $p=0.094$; there was no difference between wildtype males

and females ($F(1,59)=1.204$, $p=0.277$)). There was a significant difference between female wildtype and NR2BOE mice ($F(1,59)=5.968$, $p=0.018$) but not male wildtype and NR2BOE mice ($F(1,59)=0.18$, $p=0.673$). This included performance on the first transfer test conducted after 3 training sessions (the point at which Tang et al (1999) reported a significant group difference); a planned comparison of the mean time spent in the training quadrant by wildtype and NR2BOE male mice on the first transfer test using an independent samples t test showed that there was no difference between the two genotypes (degrees of freedom = 61, two tailed $p = 0.689$).

There was a significant effect of transfer test number ($F(3,177)=7.481$, $p<0.001$), but no genotype by transfer test number interaction ($F(3,177)=0.452$, $p=0.716$), no sex by transfer test number interaction ($F(3,177)=2.107$, $p=0.101$) and no genotype by sex by transfer test number interaction ($F(3,177)=0.215$, $p=0.886$). The effect of transfer test number was driven by mice spending longer in the training quadrant on later transfer tests. There were: male wildtype = 12, female wildtype = 17, male NR2BOE = 17, female NR2BOE = 17.

A one sample two tailed t test comparing the percentage of time spent in the training quadrant to chance levels of 25% revealed that wildtype mice were not significantly above chance on transfer test 1 (degrees of freedom = 28, $p=0.469$), but that they were performing significantly above chance on transfer test 2 (degrees of freedom = 28, $p=0.037$), 3 (degrees of freedom = 28, $p=0.002$) and 4 (degrees of freedom = 28, $p<0.001$). NR2BOE mice were not significantly above chance in the percentage of time spent in the training quadrant on transfer test 1 (degrees of freedom = 33, $p=0.735$) or 2 (degrees of freedom = 33, $p=0.265$), but were significantly above chance on transfer tests 3 (degrees of freedom = 33, $p=0.002$) and 4 (degrees of freedom = 33, $p=0.038$).

(a) 

(b) 

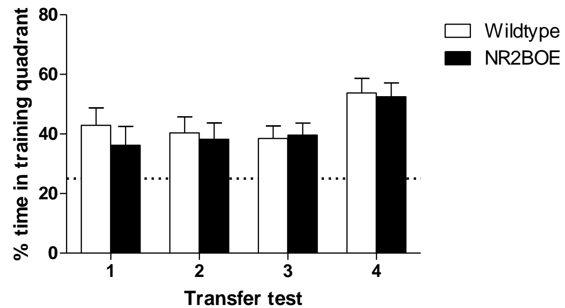
(c) 

Figure 12 The % of time spent in the training quadrants of the watermaze during transfer tests 1-4. (a) % of time (out of 60 seconds) spent swimming in the training quadrant by wildtype (open bars) and NR2BOE (hatched bars) mice, for (b) male and (c) female mice (mean ± SEM).

2.3.4. Discussion

On the open field task, male NR2BOE mice appeared less anxious than male wildtype mice as judged by their latency to reach the centre of the open field task, while female NR2BOE mice appeared more anxious than female wildtype mice. These results appear to mirror the latencies of mice to reach the hidden platform on the watermaze task in this study, suggesting that the thigmotaxis expressed by the mice may underlie their performance on the hidden platform watermaze. This in turn may be due to the anxiety levels in these mice. However, wildtype and NR2BOE mice do not appear to be significantly different in their inherent tendencies to swim at the sides of the watermaze.

We thus considered the possibility that the sex by genotype interaction on the water maze may have been a function of different anxiety levels. For example, male wildtype mice took longer to leave the side walls than male NR2BOE mice on the anxiogenic open field task. In contrast, if anything female wildtype mice tended to be quicker to leave the side walls than female NR2BOE mice. A very similar pattern of thigmotaxic behaviour with a significant genotype by sex interaction was also observed in the watermaze. As the hidden platform is located towards the centre of the maze, mice cannot find the platform if they swim at the sides of the maze. Completely thigmotaxic mice will therefore have less opportunity to find the platform on earlier trials, so learning may be retarded. Mice find large, open spaces aversive, and this is particularly true for anxious mice; hence, if female NR2BOE mice were more anxious than male NR2BOE mice they might be more inclined to swim at the sides of the maze and avoid the openness of the centre of the watermaze. This is particularly important to consider as the hidden platform watermaze is aversively motivated, as mice do not like swimming.

The results we obtained from the hidden platform watermaze were somewhat unexpected. In particular, whilst Tang et al (1999) found that male NR2BOE mice were able to solve the hidden platform watermaze more quickly than male wildtype mice, we replicated this finding only to some extent. Although generally speaking the effects were in the right direction, we failed to find significant effects (e.g. on transfer tests). We also found the opposite pattern of results in female mice; namely, NR2BOE female mice found the hidden platform less quickly than female wildtype mice. This sex difference was unexpected, and certainly worthy of further investigation. From these results, it is possible that the results observed by Tang et al. (1999) are replicable but gender specific, and our results from the open field task support the idea that this gender by genotype interaction may be caused by a difference in emotionality. As a result, it was of particular interest

that we consider a non-aversively motivated test of spatial reference memory, along with other tests of anxiety.

2.4. Y maze (spatial reference memory)

2.4.1. Introduction

To ensure that any improved performance on the watermaze was indeed a function of improved memory and not an artefact of the particular task, it was important to test the performance of the NR2BOE mice on other tests of spatial reference memory. The Y maze is excellent for this purpose as it also tests spatial reference memory but, unlike the aversively motivated water maze, is appetitively motivated. If the spatial reference memory of the NR2BOE mice is genuinely improved, one would expect this to be evident on other tests of spatial reference memory. Hence we wished to observe whether an improvement in spatial reference memory was also apparent on the spatial reference memory Y maze.

2.4.2. Methods and materials

The Y maze consisted of a wooden platform in the shape of a Y, painted black, with three identical arms (50cm × 9cm, surrounded by 0.5cm beading) and a central polygonal area 14cm in diameter. A metal food well was placed 5cm from the distal end of each arm. The maze was elevated to a height of 0.8m on a stand that could be rotated, in a well-lit laboratory that contained various prominent distal extramaze cues. The mice were maintained on chronic food deprivation at 85% of their free feeding body weight, and were habituated in the colony holding room to drinking semi-

sweetened condensed milk diluted with water at a ratio of 1:1 on the Y maze over several days prior to testing.

Testing took place in a room with several prominent extramaze cues. For each trial, the mouse was placed on one of the two possible start arms, decided by a pseudorandom sequence (such that each animal was not placed on the same arm on more than three consecutive trials, and started from each arm 5 times for each block of 10 trials), facing the experimenter. The target arm was counterbalanced such that approximately equal numbers of wildtype and NR2BOE mice were trained to each target arm. A reward of 0.1ml of sweetened condensed milk (diluted 1:1 with water) was placed in the food well on the goal arm only (defined by its position relative to the extramaze cues). The mouse was removed immediately if it entered the incorrect arm. A correct choice was recorded when the mouse directly entered the target arm without entering a non-rewarded arm. Each group of mice (by gender) were trained until their performance had plateaued, hence male mice received 10 trials each day for 12 days, and female mice received 10 trials each day for 16 days. On the 12th day of testing the male mice, post choice baiting was conducted (such that the reward was only placed on the correct arm after the mouse had chosen it). This ensured that the mice had indeed learned the position of the reward and were not using the smell of the reward itself in order to locate it. The maze was randomly rotated 120˚ between each trial to prevent identification of the correct arm by olfactory, visual or tactile cues unique to any particular arm. The number of correct choices made each day by each mouse was recorded. There were: male wildtypes = 12, male NR2BOE = 17, female wildtypes = 10, female NR2BOE = 9. The males were tested by Amy Taylor, the females were tested by the author.

2.4.3. Results

2.4.3.1. Males

Male NR2BOE mice displayed a non-significant trend towards improved performance on this task compared with controls. A repeated measures ANOVA, with one between subjects factor (genotype) and one within subjects factor (day) with 12 levels (corresponding to the 12 days of trials) was performed. There was a strong but non-significant trend towards an effect of genotype ($F(1,27)=3.902$, $p=0.059$), which was driven by improved performance by NR2BOE mice selecting the correct arm more often than wildtype mice. There was also a highly significant effect of day ($F(11,297)=44.266$, $p<0.001$) driven by improved performance on later days but no genotype × day interaction ($F(11,297)=1.313$, $p=0.216$). The effect of day was driven by an improvement in performance as the task continued, as shown in Figure 13.

Wildtype mice chose the correct arm at above chance levels from day 6 (where chance was defined as 50%; one way two-tailed t test on the % correct choices on each day, degrees of freedom = 11, $p=0.012$) whereas NR2BOE mice were above chance from day 3 (one way two-tailed t test on the % correct choices on each day, where chance was defined as 50%, degrees of freedom = 16, $p=0.037$). There were: wildtype = 12, NR2BOE = 17.

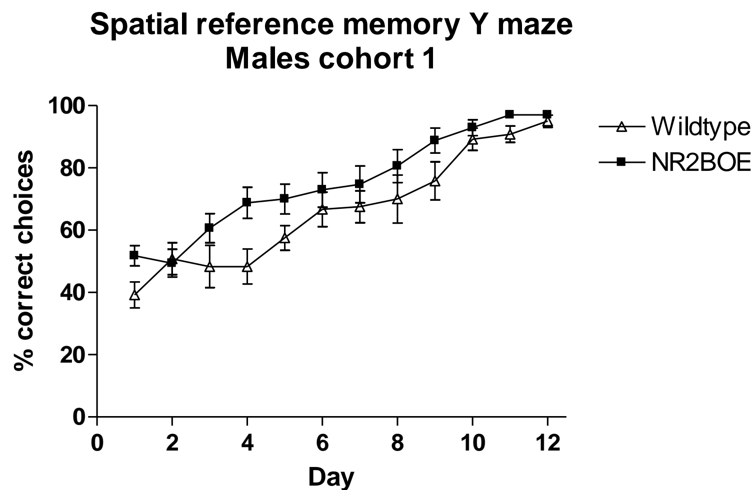


Figure 13 The percentage of correct choices made by male mice increased as they acquired the spatial reference memory Y maze task. NR2BOE male mice showed a non-significant tendency to make more correct choices than wildtype male mice from cohort 1. Mean ± SEM, wildtype (Δ) and NR2BOE (■).

2.4.3.2. Females

Female wildtype and NR2BOE mice displayed similar levels of performance on this task. A repeated measures ANOVA with one between subjects factor (genotype) and one within subjects factor (day) with 16 levels (corresponding to the 16 days of trials) was run. There was no significant effect of genotype ($F(1,17)=0.685$, $p=0.419$) but a significant effect of day ($F(15,255)=4.963$, $p<0.001$) which was driven by improved performance on later days. There was no genotype by day interaction ($F(15,255)=0.375$, $p=0.984$; Figure 14). Wildtype mice chose the correct arm at above chance levels from day 4 (one way two-tailed T test on the percentage of correct choices on each day, where chance was defined as 50%, degrees of freedom = 9, $p=0.034$) whereas NR2BOE mice were above chance from day 8 (one way two-tailed T test on the % correct choices on each day, where chance was defined as 50%, degrees of freedom = 8, $p=0.019$). There were: wildtype = 10, NR2BOE = 9.

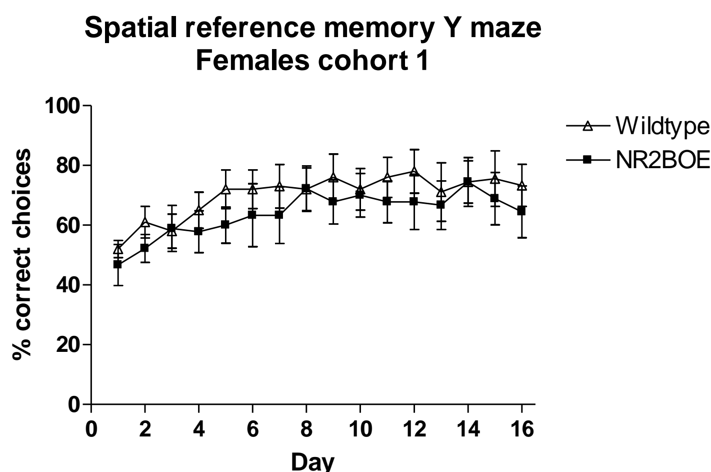


Figure 14 The percentage of correct choices made by female mice increased as they acquired the spatial reference memory Y maze task. Wildtype and NR2BOE female mice from cohort 1 showed similar levels of performance as they acquired the spatial reference memory Y maze task. Mean ± SEM, wildtype (Δ) and NR2BOE (■).

2.4.3.3. Combined male and female results (first 12 days)

In order to compare the males and females directly, the results from the first 12 days of testing (which was performed by both male and female mice) were analysed together. This allowed us to investigate whether there was an effect of sex or a genotype by sex interaction on performance on the Y maze.

Wildtype and NR2BOE mice displayed similar levels of performance on this task. A repeated measures ANOVA, with two between subjects factors (genotype and sex) and one within subjects factor (day) with 12 levels (corresponding to the 12 days of trials) was performed. There was no significant effect of genotype ($F(1,44)=0.071$, $p=0.792$) or sex ($F(1,44)=1.701$, $p=0.199$) and a non-significant trend towards a genotype × sex interaction ($F(1,44)=3.484$, $p=0.069$). This trend was driven by NR2BOE male mice making more correct choices than wildtype male mice and female mice, and female NR2BOE mice making fewer correct choices than female wildtype mice (mean correct choices ± SEM collapsed across 12 days for: wildtype males = 6.66±0.49. NR2BOE males = 7.54±0.4. Wildtype females = 6.89±0.63. NR2BOE females = 6.23±0.76). There was a significant effect

of day ($F(11,484)=33.769$, $p<0.001$), driven by improved performance as training continued. There was no genotype by day interaction ($F(11,484)=0.874$, $p=0.566$) but a significant sex by day interaction ($F(11,484)=6.722$, $p<0.001$), driven by a significant difference between males and females on days 10, 11 and 12 (with male mice performing significantly better; day 10: $F(1,44)=17.265$, $p<0.001$. Day 11: $F(1,44)=26.812$, $p<0.001$. Day 12: $F(1,44)=22.29$, $p<0.001$). There was no genotype by sex by day interaction ($F(11,484)=0.631$, $p=0.802$).

Wildtype male mice chose the correct arm at above chance levels from day 6 (one way two-tailed t test comparing the average number of correct choices made by wildtype male mice on each day compared to chance levels, where chance was defined as 50%; degrees of freedom = 11, $p=0.012$) wildtype female mice performed above chance from day 4 (degrees of freedom = 9, $p=0.034$), NR2BOE male mice were above chance from day 3 (two-tailed t test, degrees of freedom = 16, $p=0.037$) and NR2BOE female mice performed above chance from day 8 (two-tailed t test, degrees of freedom = 8, $p=0.019$). There were: male wildtypes = 12, female wildtypes = 10, male NR2BOE = 17, female NR2BOE = 9.

2.4.4. Discussion

We found that the male NR2BOE mice appeared to learn the task more readily than the male wildtype mice, although this did not quite attain statistical significance ($p=0.059$). These results correspond to our results on the hidden platform watermaze and those of Tang et al (1999). However, female wildtype and NR2BOE mice make similar correct choices. Thus the results from this task seem to support the hypothesis that the NR2BOE male mice have improved spatial reference memory, but it is surprising that no such effect was observable in female NR2BOE mice.

Thus it seems possible that spatial reference memory may be genuinely improved in male NR2BOE mice, and it is therefore worth considering whether this improvement is also present on tests of other forms of memory. Particularly, spatial reference memory and spatial working memory are believed to be supported by distinct underlying neurobiological processes (see (Bannerman et al 2008; Reisel et al 2002; von Engelhardt et al 2008)). Thus we next wished to investigate the effects of overexpressing NR2B on the rewarded alternation T maze task, which considers spatial working memory.

2.5. Spatial working memory on the T maze (rewarded alternation)

2.5.1. Introduction

There is a clear dissociation between spatial reference and spatial working memory (Bannerman et al 2008; Reisel et al 2002; Sanderson et al 2009; von Engelhardt et al 2008). The rewarded alternation task is NMDA receptor and hippocampus dependent, as lesions of the hippocampus impair spatial working memory (Lalonde 2002; Rawlins & Olton 1982) and disrupting the NMDA receptor lead to impairments in performance (Tonkiss & Rawlins 1991). Indeed, blockade of specifically hippocampal NMDA receptors has been shown to disrupt spatial working memory (McHugh et al 2008) and hippocampus specific NR2B deletion has also been shown to disrupt alternation behaviour (von Engelhardt et al 2008). Therefore it might be expected that NR2BOE mice would show improved learning and memory on this task. As mice prefer novelty, they usually spontaneously alternate at above chance levels (Jennings et al 2006), even when there is no reward for doing so, and so mice often perform significantly above chance from the very first trial.

2.5.2. Materials and methods

Mice were 10 months of age when they performed this task. Mice were maintained at 85% of their free feeding body weight and were rewarded with diluted sweetened condensed milk. Mice were habituated to the maze in a room other than that in which testing was to occur. The T maze consisted of a wooden platform with a start arm (47cm × 10cm) and two identical goal arms (37cm × 10cm) surrounded by walls 10cm high. The maze was painted grey and was elevated to a height of 1m. A metal food well was placed 2cm from the end of each goal arm. The laboratory was well lit with prominent distal extramaze cues.

Each trial consisted of a sample run and a choice run. 0.1ml diluted, sweetened condensed milk was placed in both food wells. For the sample run, each mouse was placed in the start arm, facing the end wall, and was allowed to explore the maze. The mouse was prevented from accessing one arm; which arm was determined by a pseudorandom sequence such that equal numbers of left and right turns were made, with no more than three consecutive left (or right) turns. Once the mouse had obtained the reward, it was removed from the maze, along with the blockade, and the mouse was replaced in the start arm. The mouse was rewarded for selecting the previously unsampled arm; that is, for alternating. The time interval between the sample and choice run was approximately 10s, and the intertrial interval was approximately 10 minutes. The number of correct choices made each day by each mouse was recorded. Mice received 10 trials a day, with 30 trials in total. There were: male wildtypes = 12, male NR2BOEs = 17, female wildtypes = 9, female NR2BOEs = 9.

2.5.3. Results

Wildtype and NR2BOE mice alternated at similar levels, although male mice alternated significantly more than female mice. A repeated measures ANOVA with two between subjects

factors (genotype and sex) and one within subjects factor (day) with three levels (corresponding to the three days of testing) was performed. There was no significant effect of genotype on the number of times mice alternated ($F(1,43)=0.097$, $p=0.757$), although there was a significant effect of sex ($F(1,43)=15.921$, $p<0.001$; Figure 15). Male mice alternated more than female mice (mean male alternations = 8.32±0.28 (out of 10), mean female alternation = 6.91±0.34 (out of 10); Figure 15 (b) and (c)). There was also a significant effect of day ($F(2,86)=5.601$, $p=0.005$) and a sex by day interaction ($F(2,86)= 6.106$, $p=0.003$), but no genotype by sex interaction ($F(1,43)=1.144$, $p=0.291$), no genotype by day interaction ($F(2,86)=0.327$, $p=0.722$) or a genotype by sex by day interaction ($F(2,86)=1.26$, $p=0.289$). Simple main effects reveal that the sex by day interaction was driven by a significant difference between males and females on days 2 and 3 ($F(1,43)=12.531$, $p=0.001$ and $F(1,43)=67.152$, $p<0.001$ respectively), although there was no difference between male and female alternation rates on day 1 ($F(1,43)=0.312$, $p=0.579$).

Wildtype male mice alternated at significantly above chance levels on all 3 days (where chance was defined as 50%, one way two-tailed t test performed on the number of alternations on each day, degrees of freedom = 11, day 1 $p=0.016$, day 2 $p<0.001$, day 3 $p<0.001$) as did wildtype female mice (degrees of freedom = 8, day 1 $p=0.007$, day 2 $p<0.001$, day 3 $p=0.001$). Similarly NR2BOE male mice alternated at above chance levels on all 3 days (one way two-tailed t test, degrees of freedom = 16, day 1 $p<0.001$, day 2 $p<0.001$, day 3 $p<0.001$) although NR2BOE female mice alternated at above chance levels only on days 1 and 3 (one way two-tailed t test, degrees of freedom = 8, day 1 $p=0.009$, day 2 $p=0.069$, day 3 $p=0.001$). Male wildtypes = 12, female wildtypes = 9, male NR2BOEs = 17, female NR2BOEs = 9.

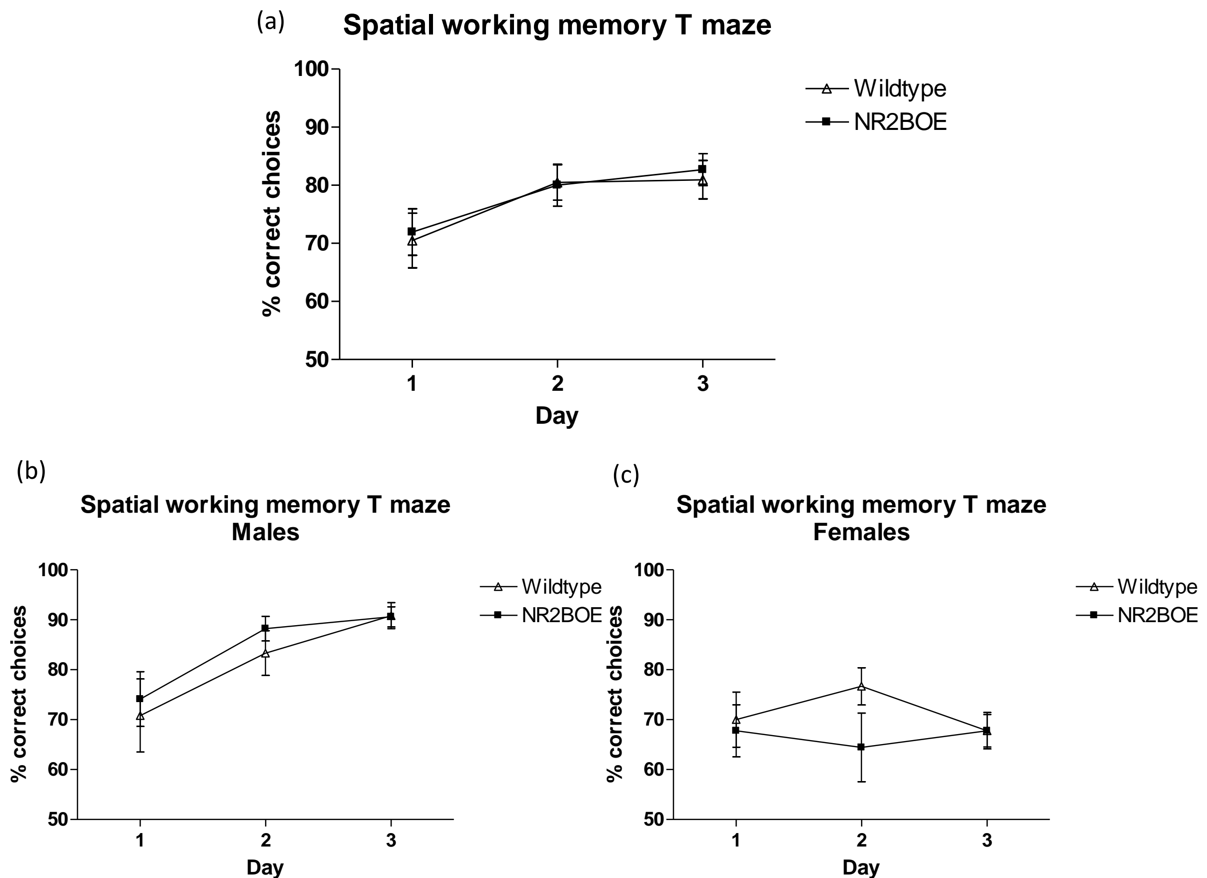


Figure 15 Male mice performed significantly better than female mice on the spatial working memory T maze test. (a) Wildtype and NR2BOE mice from cohort 1 displayed similar levels of performance. There was also not a significant difference between wildtype and NR2BOE male (b) or female (c) mice in the number of correct choices made. Mean ± SEM, Wt = wildtype (Δ) and NR2BOE (■).

2.5.4. Discussion

Previously an improvement in spatial working memory has been found on this task in these mice (Cao et al 2007). However, we found no effect of genotype on this task (as wildtype and NR2BOE mice alternated to a similar extent), although there was an effect of sex such that male mice alternated more than female mice. This suggests that working memory is not impaired or improved in the NR2BOE mice, but may point towards a difference on spatial working memory in female and male mice. As short and long term memory are believed to rely on dissociable mechanisms, improved long term memory without a similar improvement in short term memory as observed in the male mice is perfectly plausible (although it should be noted that the lack of effect of genotype is still inconsistent with the observations of Cao et al.).

2.6. Novel object recognition

2.6.1. Introduction

As mice tend to prefer novelty to familiar stimuli, we also wished to consider novelty preference. When mice are exposed to two stimuli, one of which they have previously experienced and one of which is new, they will tend to spend more time sampling the previously unsampled object. However, the time spent investigating the novel object falls to chance levels when mice can no longer differentiate between the familiar and novel objects. Tang et al. demonstrated that spatial reference memory on the hidden platform water maze was improved in the NR2BOE mice (Tang et al 1999), but they also showed that the NR2BOE mice had a stronger novelty preference than controls 24 hours after exposure to the familiar objects. This hints at the NR2BOE mice expressing greater memory which is not limited to spatial reference memory. As a result it was of interest to replicate this finding. As mentioned previously, Tang et al (1999) only used male mice and so it was also of interest to observe the effect of NR2BOE on female mice on this task.

2.6.2. Materials and methods

Mice were habituated to a grey box (measuring 40cm × 40cm × 40cm) by placing them inside once a day for 10 minutes for four consecutive days. On the fourth day, two identical objects (A_1 and A_2) were placed in the box, 10cm from the top and side. Mice were replaced in the box and were allowed to investigate freely the objects. On the test trial, another identical object (A_3) and a novel object (B_1) replaced the objects from the sample exposure. There was a delay between the sample and choice trials of either 1 hour or 24 hours. Each mouse performed the task twice; once with a 1 hour delay between the first exposure and test trial, and once with 24 hour between the first

exposure and the test trial, in a fully counterbalanced manner (such that equal numbers of mice from each genotype and sex were exposed to each of the novel objects at 1 hour or 24 hours first). The position of the novel object was also counterbalanced such that equal numbers of mice from each genotype and sex were exposed to the novel object on the left or right. The four objects were paired A-B and C-D; which object within a pair was the familiar and novel object was also counterbalanced such that equal numbers of mice from each genotype saw, for example, object A_3 or object B_3 as the novel object). The experiment was also counterbalanced with respect to which object pair (A-B or C-D) was allocated to the 1h or 24h condition. Mice were tracked by a camera and with Ethovision software, which measured the amount of time mice spent exploring the object if their noses (but not their centre of mass) were in a zone extending 1cm from the object. The primary measure of performance was the amount of time mice spent investigating each object. The latency to contact each object, the distance moved, and time spent in the middle, outside and edges of the box were measured, along with latency to enter and the distance moved in each area. The number of times each object was approached was also measured. The objects used were cans of Carlsberg beer, bottles of Orangina, 500ml sized tins with the labels removed and a wooden cuboid-shaped block. There were: male wildtypes = 6, male NR2BOEs = 6, female wildtypes = 6, female NR2BOEs = 6.

2.6.3. Results

2.6.3.1. Distance travelled during habituation to the box

The distances mice moved in the box during habituation were subject to a genotype by sex interaction, with wildtype female mice moving greater distances and wildtype male mice moving shorter distances. A repeated measures ANOVA was conducted with two between subjects factors (genotype and sex) and one within subjects factor (day) with four levels (corresponding to the four

days of exposure) was performed. There was no effect of genotype (F(1,20)=0.468, p=0.502) but there was a significant effect of sex (F(1,20)=11.865, p=0.003) and a significant genotype by sex interaction (F(1,20)=5.507, p=0.029) as shown in Figure 16. This was driven by greater distance travelled by female wildtype mice and less distance travelled by male wildtype mice (male wildtype mice mean distance = 2269.4±117.7, male NR2BOE mean distance = 2622.0±120.2), female wildtype mice mean distance = 3497.4±210.1, female NR2BOE mean distance = 2854.8±119.2). Simple main effects revealed a significant difference between wildtype male and female mice (F(1,20)=16.769, p=0.001) but not NR2BOE mice (F(1,20)=0.603, p=0.447). The difference between female wildtype and NR2BOE mice was also significant (F(1,20)=4.592, p=0.045), but not for male wildtype and NR2BOE mice (F(1,20)=1.383, p=0.253). There was a significant effect of day (F(3,60)=34.011, p<0.001), due to mice moving shorter distances on later days as the mice habituated to the environment. There was no genotype by day interaction (F(3,60)=1.147, p=0.338), sex by day interaction (F(3,60)=1.8, p=0.157) or a genotype by sex by day interaction (F(3,60)=1.226, p=0.308). Mean distances moved on each day are given in Table 1. There were: male wildtypes = 6, female wildtypes = 6, male NR2BOE = 6, female NR2BOE = 6.

Distance travelled (cm)	Day			
	1	2	3	4
Male	2996.6±107.3	2236.2±188.6	2127.66±166.9	2422.3±118
Female	3991.6±253.3	2980.3±190.8	2834±243.6	2898.5±200.2

Table 1 Distance moved by male and female mice during habituation to the box used for novel object recognition.

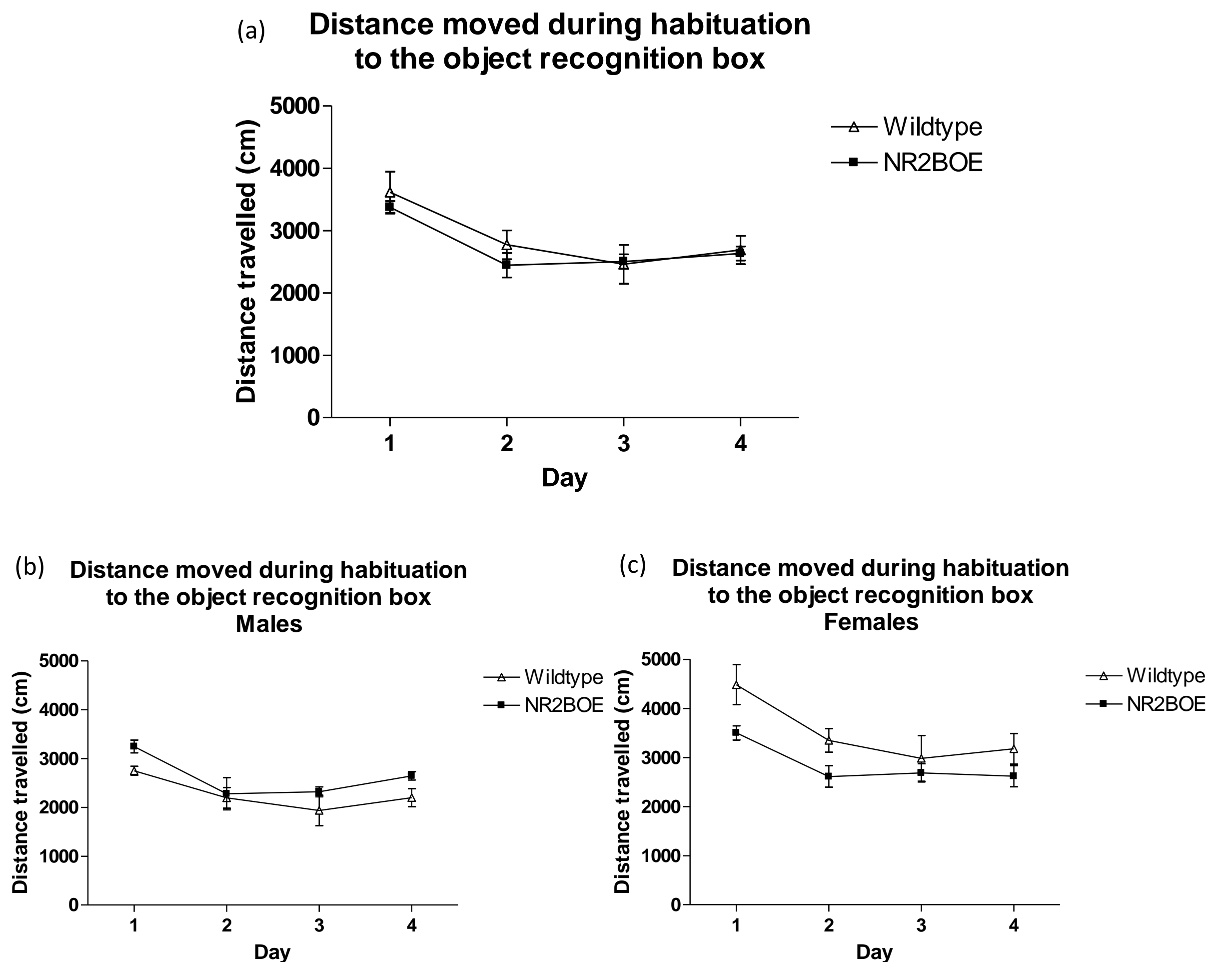


Figure 16 Female mice moved significantly longer distances than male mice during habituation to the box in which novel object recognition was to occur. (a) Wildtype and NR2BOE mice displayed similar levels of performance when sex was not included as a factor. There was not a significant difference in the distances moved by wildtype and NR2BOE male (b) mice, although there was a significant difference between the wildtype and NR2BOE female (c) mice. Mean ± SEM, Wt = wildtype (Δ) and NR2BOE (■).

2.6.3.2. Time spent investigating the objects during familiarisation

There was no preference for either of the objects during familiarisation for wildtype and NR2BOE mice, although female mice spent longer investigating the objects than males. A repeated measures ANOVA with two between subjects factors (genotype and sex) and one within subject factors with two levels (1h and 24h) on the amount of time spent investigating the objects showed that there was no effect of genotype ($F(1,20)=0.133$, $p=0.720$) although there was a significant effect of sex ($F(1,20)=5.98$, $p=0.024$) driven by males spending less time investigating the objects than females (mean amount of time (s) spent investigating the object ± SEM for: males = 105.4±7.2.

Females = 137.2±10.4). There was no genotype by sex interaction (F(1,20)=0.959, p=0.339). There was also no effect of training (F(1,20)=0.173, p=0.682), no genotype by training interaction (F(1,20)=0.001, p=0.991), no sex by training interaction (F(1,20)=1.213, p=0.284) and no genotype by sex by training interaction (F1(1,20)=0.035, p=0.853).

A discrimination ratio was calculated for each mouse to establish that mice were not displaying a preference for either the right or left object by dividing the time spent investigating the left object by the total time spent investigating both the left and right objects (object 1 / (object 1 + object 2). A repeated measures ANOVA with two between subjects factors (genotype and sex) and one within subjects factor with two levels (1h and 24h) analysing the discrimination ratios showed that there was no effect of genotype (F(1,20)=0.788, p=0.385), sex (F(1,20)=1.125, p=0.302) or a genotype by sex interaction (F(1,20)=0.005, p=0.944). There was no effect of training (F(1,20)=1.723, p=0.204), nor a genotype by training effect (F1,20)=0.006, p=0.94), nor a sex by training effect (F(1,20)=0.945, p=0.343), nor a genotype by sex by training interaction (F(1,20)=1.51, p=0.233). There were: male wildtype = 6, female wildtype = 6, male NR2BOE = 6, female NR2BOE = 6.

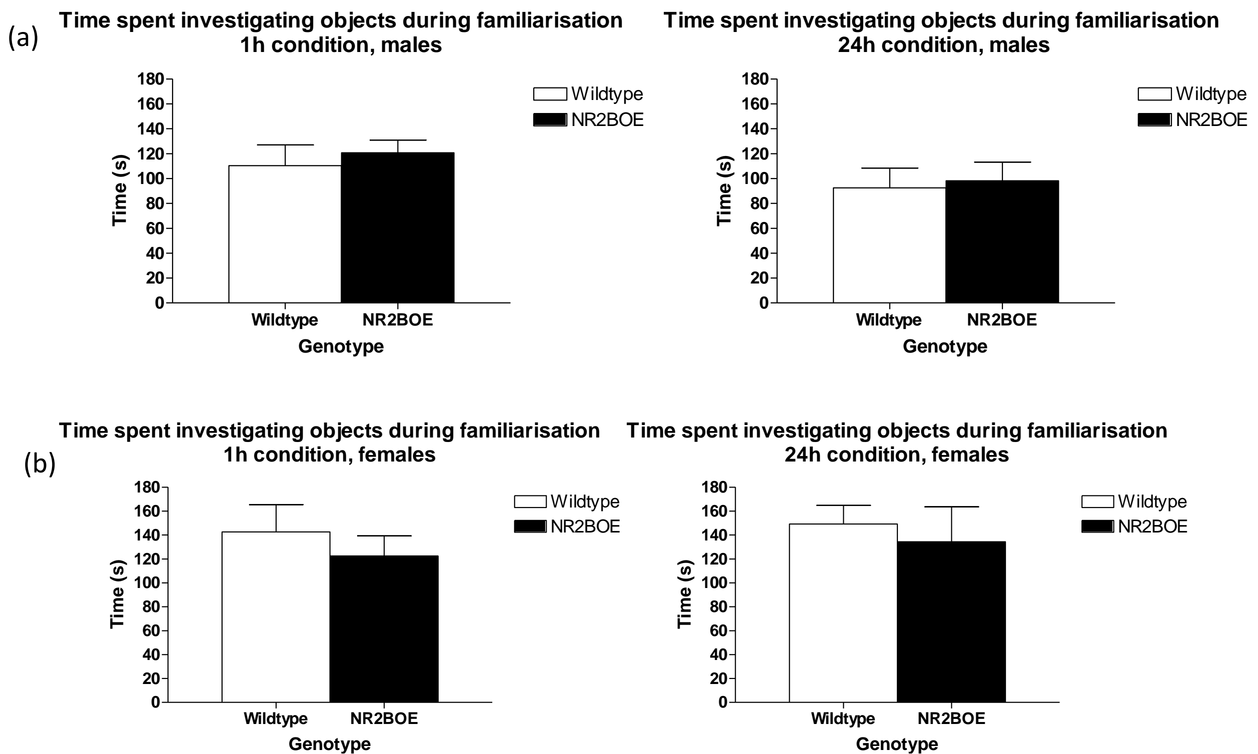


Figure 17 Wildtype and NR2BOE mice spent equivalent lengths of time investigating the objects during familiarisation. There was no difference between the time wildtype and NR2BOE male (a) or female (b) mice spent investigating the objects. Mean ± SEM, Wt = wildtype (□) and NR2BOE (■).

2.6.3.3. Time spent investigating the objects on the test trial

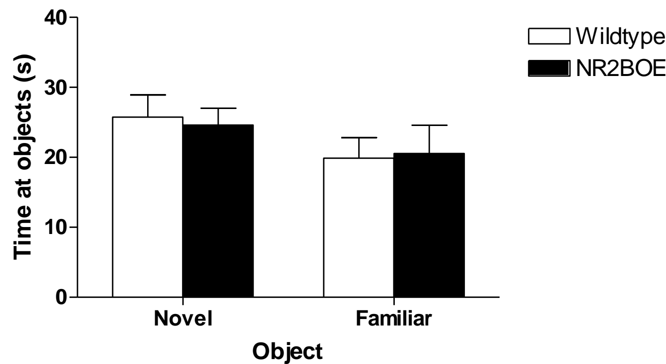
Wildtype and NR2BOE mice spent similar lengths of time investigating the objects. A repeated measures ANOVA with two between subjects factors (genotype and sex) and one within subjects factor with two levels (delay condition, with the conditions of 1h and 24h delays) analysing the amount of time investigating both objects showed that there was no effect of genotype ($F(1,20)=0.007$, $p=0.932$), sex ($F(1,20)=2.635$, $p=0.12$) nor a genotype by sex interaction ($F(1,20)=2.601$, $p=0.122$). There was no effect of delay condition ($F(1,20)=2.707$, $p=0.116$), nor a genotype by delay condition interaction ($F(1,20)=0.061$, $p=0.807$), nor a sex by delay condition interaction ($F(1,20)=1.45$, $p=0.243$), not a genotype by sex by delay condition interaction ($F(1,20)=0.149$, $p=0.703$).

2.6.3.4. Discrimination ratio

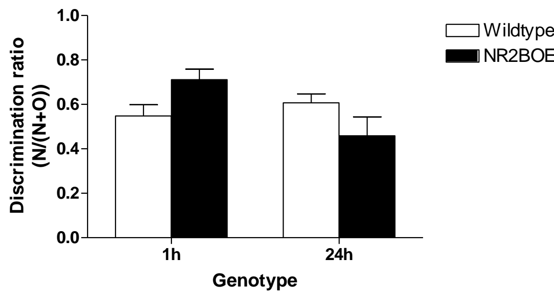
Both wildtype and NR2BOE micespent more time investigating the novel object at the 1h time interval. A repeated measures ANOVA with two between subjects factors (genotype and sex) and one within subjects factor with two levels (delay condition, with the conditions of 1h and 24h delays) analysing the discrimination ratio (time investigating novel / (time investigating novel + time investigating familiar)) showed that there was no effect of genotype ($F(1,20)=0.138$, $p=0.715$), sex ($F(1,20)=0.1$, $p=0.755$) or a genotype by sex interaction ($F(1,20)=0.05$, $p=0.826$). There was no effect of delay condition ($F(1,20)=0.299$, $p=0.591$) on the preference of the mice for the novel over the familiar object, no genotype by delay condition effect ($F(1,20)=1.611$, $p=0.219$), no sex by delay condition effect ($F(1,20)=2.769$, $p=0.112$) but a significant genotype by sex by delay condition effect ($F(1,20)=5.31$, $p=0.032$). The mean discrimination ratios were: wildtype males 1h = 0.547±0.051, wildtype males 24h = 0.607±0.04, wildtype females 1h = 0.581±0.07, wildtype females 24h = 0.584±0.076, NR2BOE males 1h = 0.711±0.047, NR2BOE males 24h = 0.459±0.085, NR2BOE females 1h = 0.568±0.038, NR2BOE females 24h = 0.662±0.113. There was a significant difference between wildtype and NR2BOE male mice at the 1h interval ($F(1,20)=4.565$, $p=0.045$), and between NR2BOE male mice at the 1h and 24h intervals ($F(1,20)=8.364$, $p=0.009$).

A two tailed t test comparing the discrimination ratio to chance levels (counted as 0.5) for the 1h condition showed that NR2BOE mice (degrees of freedom = 11, $p=0.003$) but not wildtype mice (degrees of freedom = 11, $p=0.166$) were significantly above chance. For the 24h condition, wildtype (degrees of freedom = 11, $p=0.039$) but not NR2BOE mice (degrees of freedom = 11, $p=0.431$) were significantly above chance. There were: male wildtype = 6, female wildtype = 6, male NR2BOE = 6, female NR2BOE = 6.

(a)



(b)



(c)

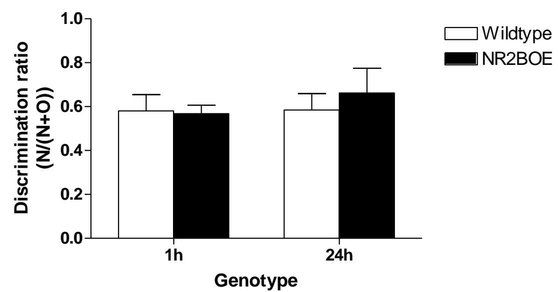


Figure 18 Wildtype and NR2BOE mice spent similar lengths of time investigating the novel object. (a) Wildtype and NR2BOE preferences for the novel object were similar for male (b) and female (c) mice. Discrimination ratio was calculated as (time investigating novel / (time investigating novel + time investigating familiar)). Chance levels are a ratio of 0.5. Mean ± SEM, Wt = wildtype (□) and NR2BOE (■).

2.6.4. Discussion

We expected wildtype mice to spend more time investigating the novel object at levels that reflected the strength of the memory of the familiar object, such that they would spend more time investigating the novel object if their memory of the familiar object was strong. Indeed, this was observed by Tang et al. (1999). Wildtype and NR2BOE mice spent similar amounts of time investigating the novel and familiar objects, suggesting that they had similar novelty preferences and memories of the familiar object that were equivalent in strength, although both genotypes spent more time in total investigating the novel object when the delay was 1h rather than 24h. There was also no effect of sex, suggesting that male and female mice had memories of similar strength on this

task. This is in opposition to what was found by Tang et al (1999), who found a preference for the novel object at 24 hours by the NR2BOE male mice, suggesting improved memory on this task by the male NR2BOE mice. We also found no effect of delay on the novelty preferences of the wildtype and NR2BOE mice, such that their memory appeared similar when they were exposed to the novel object 1h or 24h after their exposure to the familiar objects.

2.7. Spatial novelty preference test

2.7.1. Introduction

The results from the spatial working memory task suggest that working memory is unimpaired and not enhanced in the NR2BOE, in comparison to those of Cao et al (2007), who found that the NR2BOE mice performed significantly better than wildtypes on the spatial working memory T maze. However, although not statistically significant, the results from the hidden platform watermaze and spatial reference memory Y maze suggest that the male NR2BOE may have facilitated learning and memory. Despite this, we found no significant difference between wildtype and NR2BOE on their preference for novel objects either 1 hour or 24 hours after exposure to a familiar object. Hence we wished to consider the performance of the NR2BOE on the spatial novelty preference Y maze task.

The paradigm we used involves blocking access to one arm on 5 familiarisation trials, after which the blockade is removed to allow access to all three arms for a test trial. Mice should spend more time in the previously unsampled arm in accord with their preference for novel over familiar stimuli (Sanderson et al 2009). The Sanderson et al. (2009) study found that GluA1$^{-/-}$ mice had

impaired short term but had enhanced long term memory, and they postulated that the GluA1$^{-/-}$ mice may experience less habituation to stimuli in the short term than their wildtype controls. Hence they would be unable to differentiate between novel and familiar stimuli in the short term as they would fail to habituate to familiar stimuli, but in the long term would have stronger memories of the familiar stimuli as the memory traces of the familiar stimuli become fully inactive and would hence spend more time considering the novel stimuli. This argues for dissociable mechanisms for short and long term memory, in which they compete with each other.

It is possible to consider short term and long term memory on the novelty preference Y maze task by varying the time between the final exposure and the test. We chose intervals of 1 minute and 24 hours; for the one minute interval before the test, the short term memory should exert maximal influence over the mouse's performance on the task. However, at 24 hours, short term memory may have decayed so mice will be dependent on their long term memory.

The intertrial interval between familiarisation exposures was also varied; mice were replaced in their home cages for either 1 minute or 24 hours between exposures. The purpose of this was to establish how overexpressing NR2B affected acquisition of the task.

2.7.2. Materials and methods

A Y maze with clear plastic sides (size 30cm × 8cm × 13cm) on a white plastic floor was used. Woodchips (identical to those used in the home cage) were scattered on the floor of the maze. Between trials the woodchips were mixed and redistributed to avoid the interaction of fixed olfactory cues specific to any arm.

Mice were placed in the start arm (defined as the arm closest to the experimenter), and were allowed to explore the start arm and the "other" arm for 2 minutes. The third arm (designated "novel" arm, which was counterbalanced between animals) was blocked off. The mouse was then removed and replaced in the home cage for a delay of either 1 minute or 24 hours (see

Table 2), after which it was returned to the maze for another 2 minutes. This was repeated 4 times (such that mice were exposed to the maze for a total of 5 × 2 minutes) with set rest periods in the home cage between each exposure. The number of arm crossings made were measured during habituation and the test (a crossing was considered to have occurred when all four paws passed the start of the arm), and additionally we measured the amount of time mice spent in each arm for the test.

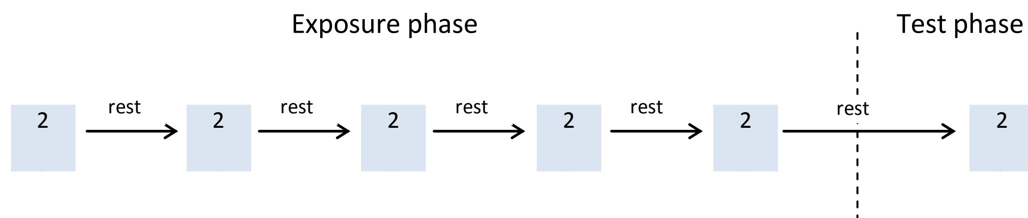


Figure 19 Mice were exposed to the maze 5 times for 2 minutes and for a further 2 minutes for the test. In between the exposures and the test, mice were returned to rest in their home cages.

The test phase consisted of removing the blockade and placing the animal in the maze for 5 minutes, timed from when the mouse left the start arm, after resting in the home cage for either 1 minute or 24 hours from the end of the previous exposure trial. The length of time spent in each arm and the number of crossings between arms were recorded. All mice performed the task with all four possible conditions (SS, SL, LS and LL) as described in

Table 2 in four novel testing rooms according to a fully counterbalanced, within subject design (such that equal numbers of mice from each group (wildtype male, wildtype female, NR2BOE male, NR2BOE female) were tested on each condition in each testing environment). Males were tested by

Dr Chris Barkus. There were: male wildtypes = 17, male NR2BOEs = 12, female wildtypes = 20, female NR2BOEs = 17.

Time before test	Condition between exposures	
	Short	Long
Short	Exposure: 1m – 1m – 1m – 1m – 1m	Exposure: 24h – 24h – 24h – 24h – 24h
Long	Exposure: 1m – 1m – 1m – 1m – 1m	Exposure: 24h – 24h – 24h – 24h – 24h

Table 2 The four conditions for exposure to the spatial novelty prefence Y maze task. Mice either spent 1 minute between exposures (S exposure) or 24 hours (L exposure) in the home cage. After 5 exposures, mice spent either 1 minute (S test) or 24 hours (L test) in their home cage before being re-exposed to the Y maze for the test. This resulted in 4 conditions: SS, SL, LS and LL.

2.7.3. Results

Female mice appeared more active than male mice on some measures of activity on this task, but wildtype and NR2BOE largely expressed a similar preference for the novel arm.

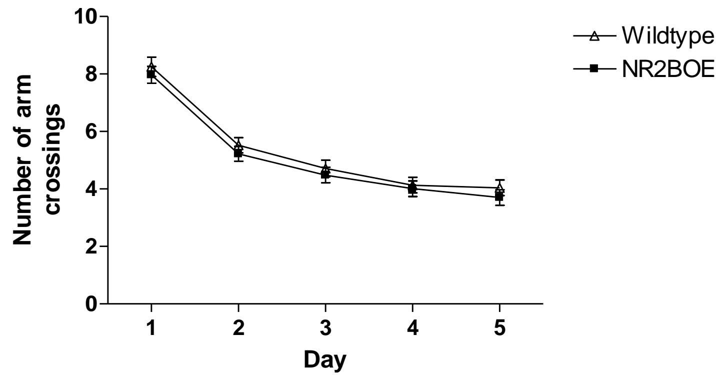
Mice were 12 months of age when they performed this task. The time spent in the novel arm was expressed as the fraction of time spent in the novel arm by the amount of time spent in both the novel and “other” arms. This discrimination ratio demonstrated a preference for the novel arm when the value was greater than 0.5.

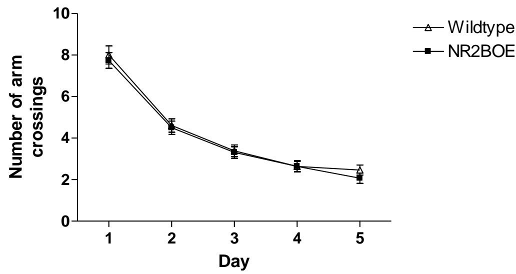
2.7.3.1. Habituation

Control and NR2BOE mice habituated similarly during the exposures as measured by the number of arm entries made. A repeated measures ANOVA was performed with two between subject factors (genotype and sex) and two within subjects factor (trial and ITI) with five and two levels respectively (corresponding to the five exposures and to the long and short ITI). There was no effect of genotype ($F(1,120)=1.7$, $p=0.195$; Figure 20 (a)), although there was a significant effect of

sex ($F(1,120)=27.685$, $p<0.001$) on the number of arm entries made by mice during the habituation trials. The effect of sex was due to females making more arm crossings than males (average arm crossing for: males = 4.38±0.14, females = 5.94±0.14). There was no genotype by sex interaction ($F(1,120)=1.109$, $p=0.294$), genotype by ITI interaction ($F(1,120)=0.395$, $p=0.531$) nor a genotype by sex by ITI interaction ($F(1,120)=0.124$, $p=0.725$). There was, however, a significant effect of ITI ($F(1,120)=65.591$, $p<0.001$) driven by fewer arm crossings at the 1min ITI compared to the 24h ITI (mean number of arm crossings ± SEM for: 1min ITI = 4.15±0.13, 24h ITI =6.28±0.14). There was also a significant sex by ITI interaction ($F(1,120)=12.373$, $p=0.001$), driven by a significant difference between males and females at the 1min ITI ($F(1,120)=4.848$, $p=0.03$) and the 24h ITI ($F(1,120)=28.66$, $p<0.001$) and by a significant difference between males at the 1min and 24h intervals ($F(1,120)=9.725$, $p=0.002$) and females at the 1min and 24h intervals ($F(1,120)=73.263$, $p<0.001$). Mean number of arm crossings ± SEM for 1min intervals: males = 3.79±0.3. Females = 4.46±0.3. Mean number of arm crossings ± SEM for 24h intervals: males = 4.97±0.42. Females = 7.42±0.36.

There was a significant effect of day ($F(4,480)=212.41$, $p<0.001$) driven by fewer arm crossings on later days. There was no genotype by day interaction ($F(4,480)=0.095$, $p=0.984$) or a genotype by sex by day interaction ($F(4,480)=0.554$, $p=0.696$) although there was a significant sex by day interaction ($F(4,480)=3.323$, $p=0.011$). This was a result of non-significant differences only between days 4 and 5 for males, and days 3 and 4 for females. There were: male wildtype = 17, female wildtype = 20, male NR2BOE = 12, female NR2BOE = 17.





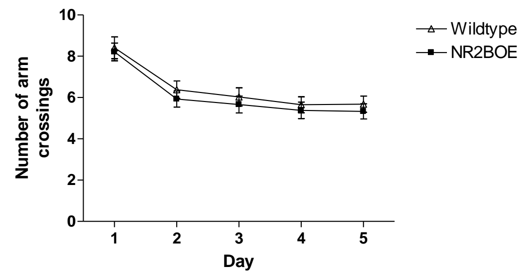


Figure 20 Female mice made significantly more arm crossings than male mice during habituation to the spatial novelty preference Y maze. (a) Wildtype and NR2BOE mice did not differ significantly in the number of arm crossings made. There was no difference in the number of arm crossings between the two genotypes at 1h (b) or 24h (c) ITIs. Mean ± SEM, Wt = wildtype (Δ) and NR2BOE (■).

2.7.3.2. Time in novel arm

There was no difference between the amount of time spent by wildtype and NR2BOE mice in the novel arm. A repeated measures ANOVA with two between subject factors (genotype and sex) and two within subject factors (ITI and delay condition), each of which had two levels (1min and 24h) was performed on the amount of time spent in the novel arm. There was no effect of genotype ($F(1,58)=2.166$, $p=0.146$) or sex ($F(1,58)=0.047$, $p=0.83$), nor a genotype by sex interaction ($F(1,58)=0.114$, $p=0.736$). There was also no effect of ITI ($F(1,58)=0.055$, $p=0.816$), no genotype by ITI interaction ($F(1,58)=0.852$, $p=0.36$), no sex by ITI interaction ($F(1,58)=0.404$, $p=0.527$) and no genotype by sex by ITI interaction ($F(1,58)=0.075$, $p=0.785$). There was a near-significant effect of delay condition ($F(1,58)=3.81$, $p=0.056$), caused by mice spending a greater amount of time in the

novel arm when there was a 1min delay compared to the 24h delay (mean time (s) in the novel arm ±SEM for: 1min delay = 51.6±2.2, 24h delay = 46.1±1.8). There was no genotype by delay condition interaction ($F(1,58)=0.151$, $p=0.699$), no sex by delay condition interaction ($F1,58)=2.402$, $p=0.127$) and no genotype by sex by delay condition interaction ($F(1,58)=1.449$, $p=0.234$). Finally, there was no ITI by delay condition interaction ($F(1,58)=0.007$, $p=0.932$), no ITI by delay condition by genotype interaction ($F(1,58)=0.334$, $p=0.566$), no ITI by delay condition by sex interaction ($F(1,58)=1.228$, $p=0.272$) and no genotype by sex by ITI by delay condition interaction ($F(1,58)=1.509$, $p=0.224$).

An independent samples t test was conducted on the mean time in the novel arm for the wildtype and NR2BOE mice across all ITIs and delay conditions. The discrimination ratios for wildtype mice (degrees of freedom = 131, two tailed significance < 0.001) and NR2BOE mice (degrees of freedom = 115, two tailed significance < 0.001) were significantly above chance. There were: male wildtype = 17, female wildtype = 16, male NR2BOE = 12, female NR2BOE = 17.

There was no difference between wildtype and NR2BOE mice in the discrimination ratio for the time spent in the novel and familiar arms, and both groups displayed a novelty preference on this task. A repeated measures ANOVA was conducted with two between subject factors (genotype and sex) and two within subject factors (ITI and delay condition) each with 2 levels (corresponding to the 1min and 24h conditions respectively) was performed. There was no effect of genotype ($F(1,58)=0.579$, $p=0.45$) or sex ($F(1,58)=0.003$, $p=0.953$) and no genotype by sex interaction ($F(1,58)=0.675$, $p=0.415$). There was no effect of ITI ($F(1,58)=0.462$, $p=0.499$, no genotype by ITI interaction ($F(1,58)=0.201$, $p=0.655$), no sex by ITI interaction ($F(1,58)=0.046$, $p=0.831$), nor a genotype by sex by ITI interaction ($F(1,58)=0.272$, $p=0.604$. There was also no effect of delay condition ($F(1,58)=0.256$, $p=0.615$), nor was there a genotype by delay condition ($F(1,58)=0.442$,

p=0.723), although there was a significant sex by delay condition interaction (F(1,58)=4.405, p=0.04) but no genotype by sex by delay condition (F(1,58)=1.121, p=0.294) interaction. The sex by delay condition interaction was caused by a near significant difference between females on the two delay conditions (F(1,58)=3.685, p=0.06; mean discrimination ratio for females at: 1min delay = 0.62±0.03, 24h delay = 0.54±0.02). There was also no genotype by sex by ITI by delay condition interaction (F(1,58)=1.471, p=0.23). Finally, there was no ITI by delay condition interaction (F(1,58)=1.138, p=0.291), no ITI by delay condition by genotype interaction (F(1,58)=0.746, p=0.391, and no ITI by delay condition by sex interaction (F(1,58)=0.472, p=0.495).

A two way independent samples t test was conducted on the mean discrimination ratio for the wildtype and NR2BOE mice across all ITIs and delay conditions. The discrimination ratio for wildtype mice (degrees of freedom = 131, two tailed significance < 0.001) and NR2BOE mice (degrees of freedom = 115, two tailed significance = 0.004) was significantly above chance. There were: male wildtype = 17, female wildtype = 16, male NR2BOE = 12, female NR2BOE = 17.

2.7.3.3. Number of arm entries

Control mice and NR2BOE mice made similar numbers of arm entries. A repeated measures ANOVA with two between subjects factors (genotype and sex) and two within subjects factors (ITI and delay condition) with two levels each (1min and 24h for each) was performed on the discrimination ratio for the arm entries to the novel and familiar arms (N/(N+F)). There was no effect of genotype (F(1,58)=0.043, p=0.837), a trend towards an effect of sex (F(1,58)=3.778, p=0.074), and a trend towards genotype by sex interaction (F(1,58)=3.778, p=0.057). The trend towards an effect of sex was due to males making proportionally more arm entries to the novel arm than females (F(1,58)=3.299, p=0.074. Mean discrimination ratio ± SEM for: males = 0.58±0.01, females = 0.54±0.01). The trend towards a genotype by sex interaction was due to a significant difference

between NR2BOE males and females ($F(1,58)=6.551$, $p=0.013$), with male NR2BOE mice making proportionally more arm crossings to the novel arm than female NR2BOE mice (mean discrimination ratio ± SEM for NR2BOE: males = 0.6±0.02, females = 0.52±0.02). There was no effect of ITI ($F(1,58)=0.149$, $p=0.701$), nor genotype by ITI effect ($F(1,58)=0.162$, $p=0.688$), no sex by ITI effect ($F(1,58)=0.814$, $p=0.371$) and no genotype by sex by ITI effect ($F(1,58)=1.001$, $p=0.321$). There was no effect of delay condition ($F(1,58)<0.001$, $p=0.996$), no genotype by delay condition effect ($F(1,58)=0.439$, $p=0.51$), a trend towards a sex by delay conditioning effect ($F(1,58)=2.841$, $p=0.097$), no genotype by sex by delay condition effect ($F(1,58)=0.901$, $p=0.347$) and no genotype by sex by ITI by delay condition effect ($F(1,58)=1.323$, $p=0.255$). Finally, there was no ITI by delay condition interaction ($F(1,58)=0.212$, $p=0.647$), no ITI by delay condition by genotype interaction $F(1,58)=1.018$, $p=0.317$) and no ITI by delay condition by sex interaction ($F(1,58)=1.009$, $p=0.319$). There were: male wildtype = 17, female wildtype = 16, male NR2BOE = 12, female NR2BOE = 17.

There was no difference between the number of arm entries made to the novel arm by wildtype and NR2BOE mice. A repeated measures ANOVA with two between subject factors (genotype and sex) and two within subject factors (ITI and delay condition), each of which had two levels (1min and 24h) was performed on the number of arm crossings to the novel arm. There was no effect of genotype ($F(1,58)=1.174$, $p=0.283$) nor sex ($F(1,58)=0.32$, $p=0.574$) and no genotype by sex interaction ($F(1,58)=2.282$, $p=0.136$). There was a significant effect of ITI ($F(1,58)=4.944$, $p=0.03$), driven by more arm crossings made to the novel arm at the 24h ITI compared to the 1min ITI (mean number of crossings to the novel arm ± SEM at ITI: 1min = 3.4±0.1, 24h = 3.8±0.2). However, there was no genotype by ITI interaction ($F(1,58)=0.992$, $p=0.323$), no sex by ITI interaction ($F(1,58)=0.424$, $p=0.517$) and no genotype by sex by ITI interaction ($F(1,58)=0.024$, $p=0.876$). There was also no effect of delay condition ($F(1,58)=1.234$, $p=0.271$), no genotype by delay condition interaction ($F(1,58)=0.219$, $p=0.642$), no sex by delay condition interaction ($F(1,58)=0.219$, $p=0.642$) and no

genotype by sex by delay condition interaction (F(1,58)=0.201, p=0.656). Finally, there was no ITI by delay condition interaction (F(1,58)=0.613, p=0.437), no genotype by ITI by delay condition interaction (F(1,58)=0.613, p=0.437), no sex by ITI by delay condition interaction (F(1,58)=2.485, p=0.12) and no genotype by sex by ITI by delay condition interaction (F(1,58)=2.485, p=0.12). There were: male wildtype = 17, female wildtype = 16, male NR2BOE = 12, female NR2BOE = 17.

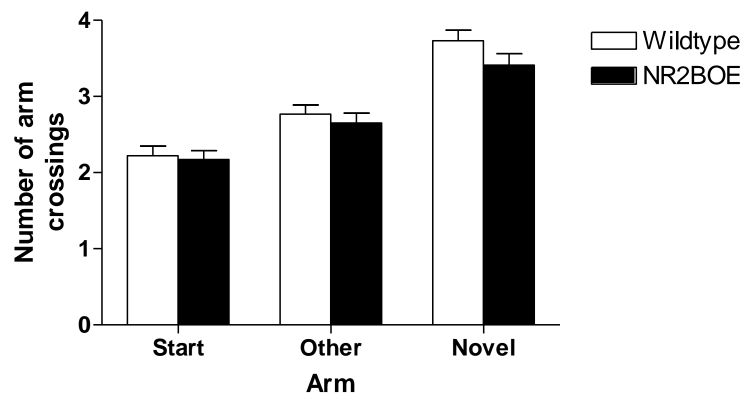


Figure 21 Wildtype and NR2BOE mice made similar numbers of arm crossings during the spatial novelty preference test. There was also no effect of sex. Mean ± SEM, Wt = wildtype (□) and NR2BOE (■).

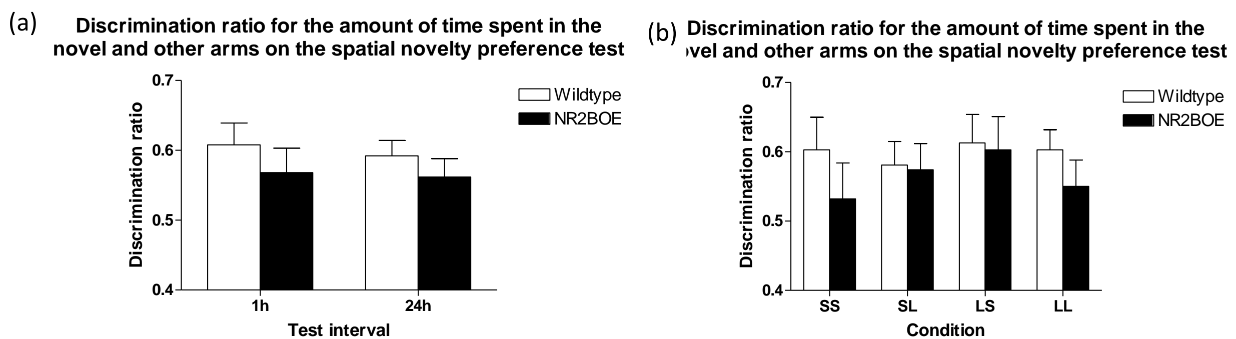


Figure 22 There was no effect of genotype or sex on the discrimination ratio for the time spent in the novel arm. (a) The test interval also had no effect on the discrimination ratio for the amount of time spent in the novel arm. (b) There was also no effect of genotype on the discrimination ratio for any of the four ITI and test conditions. S = 1min, L = 24h, mean ± SEM, Wt = wildtype (□) and NR2BOE (■).

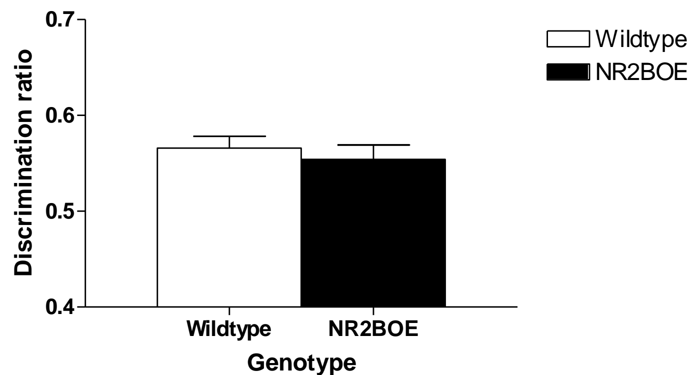


Figure 23 There was no effect of genotype on the discrimination ratio for the number of arm crossings made. There was, however, a significant effect of sex. Mean ± SEM, Wt = wildtype (□) and NR2BOE (■).

2.7.4. Discussion

These results suggest that the wildtype and NR2BOE mice show similar spatial novelty preferences, presumably as a result of motivation levels to explore novelty and memories of the familiar arm being similar for both genotypes. If the memory of the familiar arm was greater for the NR2BOE mice, one would expect them to spend more time in the novel arm. As wildtype and NR2BOE mice spent similar lengths of time in the novel arm, it appears that they remember the familiar arm and so spend more time investigating the novel arm. Also, as they spend equivalent lengths of time in the novel arm, it suggests that the novelty preference of the NR2BOE mice is not significantly different to that of wildtype mice. This accords with the results from the object recognition task observed here, such that the wildtype and NR2BOE mice expressed comparable preferences for the novel object.

Two further control experiments were conducted, the visible platform watermaze and the visual discrimination task. The visible platform watermaze is an ideal control task for the hidden platform watermaze, and the visual discrimination task is an excellent control for appetitively

motivated spatial tasks. Both test the visual acuity of the mice; it is believed that the hidden platform watermaze and spatial reference Y maze are solved primarily on the basis of visual cues, so if the vision of the mice was altered this could affect their performance on these tasks. The visible platform watermaze also acts as a control for sensorimotor and motivation on the hidden platform watermaze.

2.8. Visible platform watermaze

2.8.1. Introduction

Improved performance on the hidden platform watermaze could be due to improved learning and memory by the NR2BOE, but could also be due to other possibilities. In order to rule out the possibility that the difference in performance between the NR2BOE and control mice would be due to, for example, innate thigmotaxic behaviour on the watermaze, we conducted a visible platform watermaze task.

It was desirable to observe the behaviour of the NR2BOE mice on the visible platform for two principal reasons: 1) to ensure that there was no difference between the two genotypes in terms of motivation or motor performance and 2) to determine whether the mice would demonstrate any innate thigmotaxic tendencies. If female NR2BOE mice were thigmotaxic as suggested by their tendency to stay close to the sides on the watermaze and open field task, they would be expected to display this behaviour on a non-spatial watermaze task. Thigmotaxis on the hidden platform watermaze would tend to prevent mice from reaching the hidden platform, which was always located towards the centre of the maze. Hence the visible platform watermaze served

primarily as a control for the hidden platform watermaze task. The major difference between the two tasks is that the hidden platform watermaze is hippocampus dependent whereas the visible platform watermaze is not (Morris et al 1986b); thus, it is comparatively simple to establish that behaviours observed on the hidden but not visible platform watermaze are due to hippocampal effects.

2.8.2. Materials and methods

Mice were 11 months of age (ie, a similar age to that when they performed the hidden platform watermaze task) when they conducted the visible platform watermaze task. This task was conducted with a separate cohort of experimentally naive mice in the same watermaze as before, with a platform whose location was indicated by a large plastic container placed on the centre of the platform. The white plastic container was an old Noyes (Inc.) food pellet container and was cylindrical (152mm diameter, 250mm in height) with 5 stripes, 18mm thick, around the circumference made with black electrical tape. It was balanced upside down on the platform such that the neck of the container (100mm diameter, 25mm in height) was partially submerged. A circle of white paper was attached to the bottom of the container to conceal the electrical tape from the camera and hence prevent the tracking system from following the platform rather than the mice. The spatial position of the platform was changed for each trial, and mice were placed in the maze from a different start location for each trial. This prevented mice from using a spatial solution to find the platform. Mice were allowed 90s in which to reach the platform; if they did not reach the platform in this time they were led to, or placed on, the platform. Once on the platform, the mouse was allowed to rest for 30s before being removed. As before, mice were tracked and latency to reach the platform, path length, swim speed and thigmotaxis were recorded. Mice were given 4 trials a day for 6 days, and were run in a pseudorandomised interleaved sequence so that male and

female (and wildtype and NR2BOE) mice ran the task in an interleaved sequence. There were: male wildtypes = 12, male NR2BOEs = 12, female wildtypes = 12, female NR2BOEs = 12.

2.8.3. Results

2.8.3.1. Latency

NR2BOE and wildtype mice performed similarly on this task. A repeated measures ANOVA with two between subject factors (genotype and sex) and one within subjects factor (day) with 6 levels (corresponding to the 6 days of training) was performed. There was no effect of either genotype ($F(1,44)=2.369$, $p=0.131$) or sex ($F(1,44)=0.732$, $p=0.397$) on latency to reach the platform, nor was there a genotype by sex interaction ($F(1,44)=0.275$, $p=0.603$). There was an effect of day ($F(5,220)=132.718$, $p<0.001$), driven by improved performance on later testing days, but no genotype by day ($F(5,220)=0.769$, $p=0.554$), sex by day ($F(5,220)=0.298$, $p=0.913$) or genotype by sex by day ($F(5,220)=0.622$, $p=0.683$) interaction. There were: male wildtype = 12, female wildtype = 12, male NR2BOE = 12, female NR2BOE = 12.

2.8.3.2. Distance

There was no difference between the distance swum by wildtype and NR2BOE mice on the visible platform task. A repeated measures ANOVA with two between subject factors (genotype and sex) and one within subjects factor (day) with 6 levels (corresponding to the 6 days of training) was performed. There was no effect of genotype ($F(1,44)=0.164$, $p=0.687$) or sex ($F(1,44)=0.761$, $p=0.388$) on the distance swum to reach the platform as shown in Figure 24 (a) and (b), nor was there a genotype by sex interaction ($F(1,44)=1.18$, $p=0.283$). There was a significant effect of day ($F(5,220)=148.323$, $p<0.001$) driven by improved performance on later days (evidenced by shorter

distances swum to reach the platform) but no genotype by day effect (F(5,220)=0.796, p=0.554), sex by day (F(5,220)=0.298, p=0.913) or genotype by sex by day interaction (F(5,220)=0.622, p=0.683). There were: male wildtype = 12, female wildtype = 12, male NR2BOE = 12, female NR2BOE = 12.

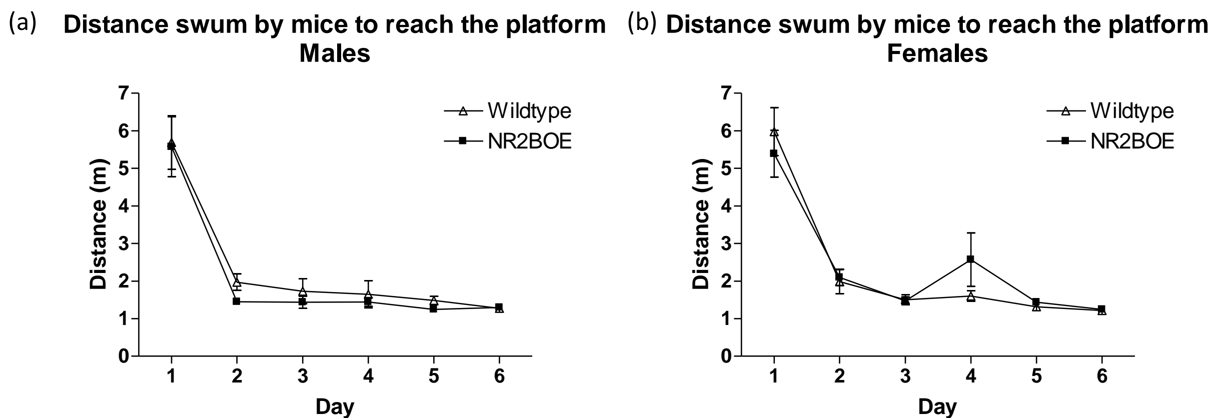


Figure 24 Mice reached the platform significantly faster as they acquired the visible platform task. There was no effect of genotype on the distances to reach the platform, for males (a) or females (b). Mean ± SEM, Wt = wildtype (Δ) and NR2BOE (■).

2.8.3.3. Thigmotaxis

Wildtype and NR2BOE spent less time swimming at the sides on later blocks. A repeated measures ANOVA with two between subject factors (genotype and sex) and one within subjects factor (day) with 6 levels (corresponding to the 6 days of training) was performed. There was no effect of genotype (F(1,44)=0.718, p=0.401) or sex (F(1,44)=0.454, p=0.504) on the distance swum to reach the platform, nor was there a genotype by sex interaction (F(1,44)=0.079, p=0.78). There was a significant effect of day (F(5,220)=86.07, p<0.001) driven by improved performance on later days (evidenced by less % time spent swimming at the sides on later days) but no genotype by day effect (F(5,220)=0.953, p=0.447) or sex by day (F(5,220)=1.188, p=0.316). However, there was a significant genotype by sex by day interaction (F(5,220)=3.229, p=0.008) driven by a significant difference between male wildtypes spending significantly longer at the sides than female wildtypes on day 2 (F(1,44)=7.92, p=0.007; mean % time at sides ± SEM for wildtype: males = 8.8±1.45. Females =

4.19±0.83) and male NR2BOE mice spending significantly longer at the sides than female NR2BOE mice on day 6 ($F(1,44)=4.24$, $p=0.045$; mean % time at sides ± SEM for NR2BOE: males = 7.13±1.27. Females = 4.22±0.89). Female NR2BOE mice also spent significantly longer at the sides than female wildtype mice on day 2 ($F(1,44)= 6.006$, $p=0.018$; mean % time at sides ± SEM for female: wildtypes = 4.19±0.83. NR2BOE = 8.2±1.25) and male NR2BOE mice spending significantly less time at the sides than male wildtype mice on day 2 ($F(1,44)=4.67$, $p=0.036$; mean % time at sides ± SEM for male: wildtypes = 8.8±1.45. NR2BOE = 5.26±1.01). These effects are in the same direction as those observed on the open field task, and the hidden platform watermaze (with male NR2BOE mice spending proportionally less time at the sides compared to male wildtype mice, and female NR2BOE mice spending more time at the sides than female wildtype mice). There were: male wildtype = 12, female wildtype = 12, male NR2BOE = 12, female NR2BOE = 12.

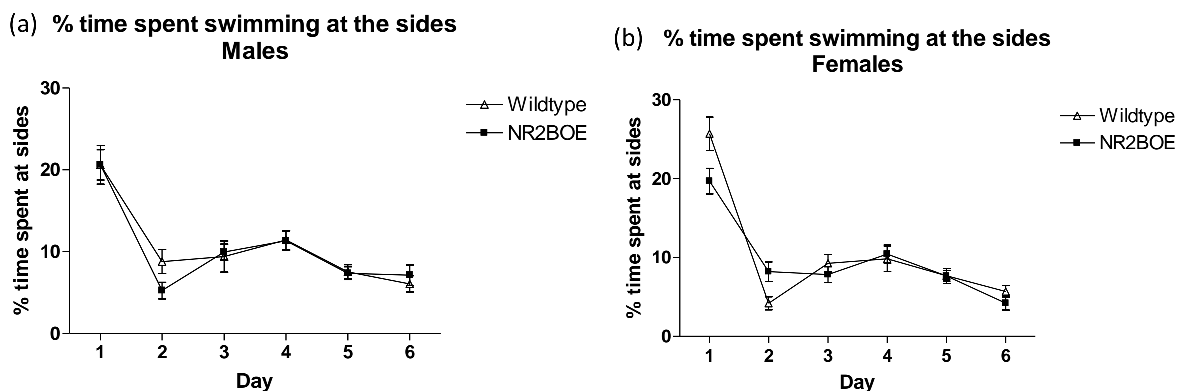


Figure 25 Mice spent less time swimming at the sides of the watermaze on the visible platform task on later days of training. There was no effect of genotype on the distances to reach the platform, for males (a) or females (b). Mean ± SEM, Wt = wildtype (Δ) and NR2BOE (■).

2.8.4. Discussion

As NR2BOE mice and wildtype mice had similar latencies to reach the platform, and swam similar distances, it is fair to state that the NR2BOE mice are unimpaired on the visible platform watermaze task. It is interesting to note that although there was no overall effect of genotype on thigmotaxis, there is a genotype by sex interaction on day 2. Interestingly, this interaction mirrored

the interaction observed for thigmotaxis on the hidden platform watermaze and open field task, as female wildtype mice spent significantly more time at the sides than male wildtype mice. These results suggest that the NR2BOE mice are not generally more thigmotaxic on the watermaze than wildtype mice. However, it is unclear why the genotype by sex interaction was replicated on only one day of this task; it may simply be a reflection of the ease with which the mice are able to complete this task relative to the hidden platform watermaze.

2.9. Visual discrimination

2.9.1. Introduction

Tasks testing spatial working and spatial reference memory test the ability of mice to coordinate landmarks from their surroundings with their target location. As a result it was of considerable importance to establish that the sight of the mice was not impaired, as this could certainly lead to altered performance on the spatial memory tasks. Thus mice were also tested on the appetitively motivated visual discrimination task, in which they were rewarded for always selecting a specific visually guided arm (one of which was grey, whilst the other was painted with black and white stripes). This task is not hippocampal dependent, thus the visual discrimination task serves as a control for the spatial memory tasks that *are* hippocampal dependent. It also acts as a control for differences in motivation on appetitive tasks, ensuring that wildtype and NR2BOE mice do not act differently due to different preferences for the food reward.

2.9.2. Materials and methods

Mice were between 10 and 12 months of age when they completed this task. They were maintained at 85% of their free feeding body weight and were rewarded with diluted sweetened condensed milk. During habituation the maze contained neutral inserts rather than those used for visual discrimination; for testing, the stripy and grey inserts were used to test visual discrimination. In a well lit laboratory, mice were trained to always enter either the grey or striped arm of a T maze to obtain a reward. The maze consisted of a wooden platform with a start arm (47cm × 10cm) and two visually distinct goal arms (28cm × 10cm), surrounded by walls 30cm high. One arm was painted dark grey, the other consisted of black and white stripes 2cm wide. The stem of the maze was painted light grey, and the maze itself was elevated to a height of 1m. A metal food well was placed 2cm from the end of each goal arm.

Each trial consisted of a single run. 0.1ml diluted, sweetened condensed milk was placed in the food well of the target arm only. Each mouse was placed in the start arm, facing the end wall, and was rewarded for selecting the correct arm; half were trained to always select the grey arm, the other half always had to select the black and white arm. The target arm was swapped with the unbaited arm such that for half of the trials mice had to make a right turn for the correct arm, and for half a left turn was necessary. The position of the correct arm was determined by a pseudorandom sequence in which there were no more than three consecutive trials requiring turns in the same direction. The number of correct choices made by each mouse each day was recorded. The intertrial interval was approximately 10 minutes. Mice received 10 trials a day, with 70 trials in total. There were: male wildtypes = 12, male NR2BOEs = 17, female wildtypes = 12, female NR2BOEs = 10.

2.9.3. Results

NR2BOE and wildtype mice performed similarly on this task. A repeated measures ANOVA with three between subjects factors (genotype, sex and arm, where arm represented the grey or stripey arm to which mice were trained for a reward) and one within subjects factor (day) with 7 levels (corresponding to the 7 days of training) was performed. There was no effect of genotype (F(1,43)=1.601, p=0.213), sex (F(1,43)=0.109, p=0.743) or a genotype × sex by interaction (F(1,43)=0.267, p=0.608; Figure 26 (a) and (b)) on the number of correct choices made. There was a significant effect of arm (F(1,43)=7.607, p=0.009) and of block (F(1,43)=203.373, p<0.001). The effect of arm was driven by mice making more correct choices when trained to the grey, rather than black and white, arm (mean correct choices (out of 10) ± SEM for: grey = 7.85±0.24, black and white = 7.49±0.28). The effect of block was driven by improved performance on later days of training. There was no genotype by block interaction (F(1,43)=1.214, p=0.277), a significant sex by block interaction (F(1,43)=29.391, p<0.001), but no arm by block (F1,43)=0.159, p=0.692) and no genotype by sex by block interaction (F(1,43)=1.533, p=0.222). The sex by block interaction was driven by a significant difference between male and female performances on days 1, 4, 6 and 7. On these days, male mice performed significantly better than female mice (see Table 3).

The effect of arm may have been due to the aversive nature of a bright white part of the environment to mice. Importantly, there was no arm by genotype interaction (F(1,43)=0.249, p=0.62), although there was a significant sex by arm interaction (F(1,43)=16.949, p<0.001), driven by a significant difference between females making significantly more correct choices to the grey arm than the black and white arm (F(1,43)=7.334, p<0.001; female mean correct choices to the grey arm = 8.26±0.33, female mean correct choices to the black and white arm = 7.12±0.41). There was not a significant difference between the number of correct choices made by male mice to the grey and black and white arms (F(1,43)=1.056, p=0.31). There was no arm by block (F(1,43)=0.159, p=0.692)

or genotype by sex by arm interaction (F(1,43)=0.345, p=0.56), and no genotype by sex by arm by block interaction (F(1,43)=3.445, p=0.07).

These results suggest there is no difference between wildtypes and NR2BOE, or males and females, on their ability to acquire a visual discrimination task. There were: male wildtype = 12, female wildtype = 12, male NR2BOE = 17, female NR2BOE = 10.

Correct choices (out of 10)	Day						
	1	2	3	4	5	6	7
	*			*		*	*
Male	6.90±0.31	6.52±0.24	7.34±0.19	7.52±0.23	8.38±0.21	8.52±0.18	8.45±0.16
Female	5.27±0.27	5.95±0.46	6.86±0.36	8.32±0.27	8.91±0.23	9.36±0.21	9.14±0.22

Table 3 Number of correct choices (out of 10) male and female mice made on each day of the visual discrimination task. * indicates that there was a significant difference between the performance of male and female mice on that day.

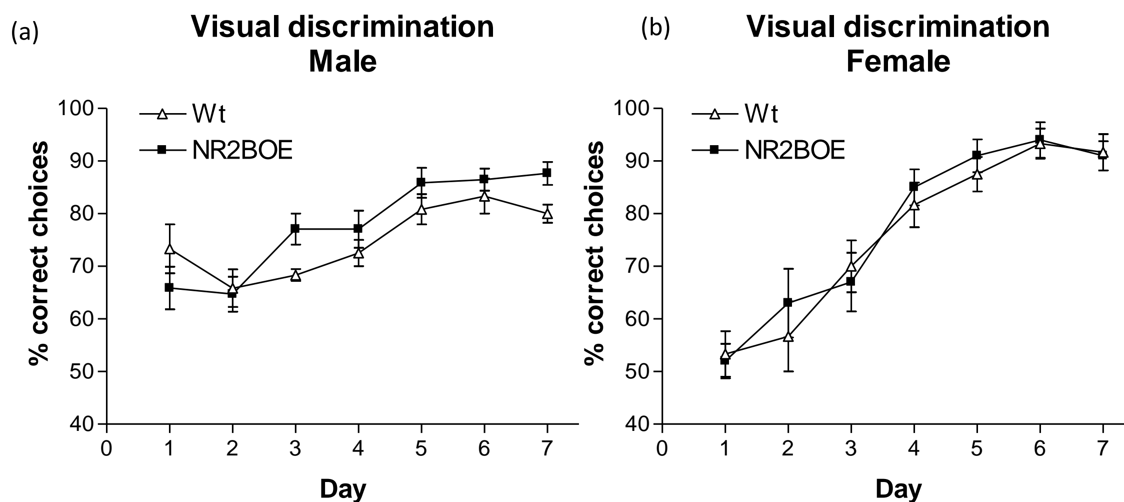


Figure 26 Mice made more correct choices as they acquired the visual discrimination task. There was no effect of genotype on the number of correct choices made for males (a) or females (b). Mean ± SEM, Wt = wildtype (Δ) and NR2BOE (■).

2.9.4. Discussion

The lack of an effect of genotype or sex suggests that there is no difference between NR2BOE and wildtype mice, nor male and female mice, in their ability to make choices based on simple visual stimuli. This demonstrates that the visual acuity of the mice is not noticeably different and therefore any differences in performance on other spatial tasks cannot be attributed to differential ability to perceive visual cues.

2.10. Conclusions

It is notable that the NR2BOE male mice did indeed appear to replicate the findings of Tang et al (1999) on the hidden platform and spatial reference Y maze, albeit to a lesser extent to that observed by Tang et al (1999). It is possible that the finding of improved learning and memory in the NR2BOE mice is indeed replicable, but that the effect is gender specific; that is, an improvement in learning and memory is present in male, but not female, NR2BOE mice. The neurobiological reasons underlying such a behavioural difference are not clear; it is not obvious why this effect of sex should exist, nor why the effect was not more obvious or found on tasks such as the novel object recognition and spatial novelty preference tasks. It is, of course, possible that these sex differences arose due to effects specifically related to the tasks in question; however, it is not clear what factor could cause a sex difference on the hidden platform watermaze, open field task and rewarded alternation task but not the other tasks performed here. However, the sex difference is certainly of interest and worthy of further investigation.

Another task in which the NR2BOE mice demonstrated greater memory than wildtype mice in the Tsien laboratory was on the novel object recognition task (Tang et al. 1999). They found that although wildtype and NR2BOE mice had similar preferences for the novel object 1h after their exposure to the familiar objects, NR2BOE mice displayed a significantly greater preference for a novel object at 24 hours. However, we did not observe any significant differences between the preference that wildtype and NR2BOE mice had for the novel object after 24 hours. This is unlikely to be attributable to a floor effect as at 24 hours wildtype mice still expressed an above chance discrimination ratio (preferring the novel object to the familiar one), whilst the corresponding discrimination ratio for NR2BOE mice was not significantly different to chance. Nor can these results be attributed to a ceiling effect as mice spent less than 60 out of 300 seconds investigating the objects during the test. This is in comparison to the difference in exploratory preference observed between the genotypes by Tang et al. (1999) at 24 hours. Hence these results did not replicate the findings of Tang et al. (1999), which may be a reflection of the differences between the conditions in the two laboratories.

Our mice also performed a spatial novelty preference task. This allowed us to consider the spatial reference memory of mice at 1h and 24 hour intervals (as in the novel object recognition task), while simultaneously investigating their short term and long term memory. This task was of particular interest as a previous study has demonstrated that although $GluRA^{-/-}$ mice are impaired at the 1h (short term memory) condition, their preference for the novel arm (presumably driven by their memory of the familiar arm) was facilitated at 24 hours. This points to competitive mechanisms underlying short and long term memory, and it was of interest to investigate if a similar pattern of results was observable in the NR2BOE in view of the greater memory found by Tang et al (1999).

However, there was not a significant difference between wildtype and NR2BOE mice in the discrimination ratio (time spent in the novel arm divided by the total time spent in the novel and other arms), nor was there any effect of sex. There was also no effect of ITI. This is in agreement with the results observed in the novel object recognition task (which provided no evidence of improved learning and memory in the NR2BOE mice).

The differences between these results and those observed by Tang et al. (1999) could potentially be explained by the differences in ages between the mice (which were tested at 8+ months of age) and mice tested by Tang et al (which were tested at 3-4months of age). As a result, it was desirable to replicate some of these tests in younger mice, which were comparable in age to those used by Tang et al. In addition, it was of considerable interest to investigate the suggestion of different anxiety levels in the NR2BOE mice as a potential explanation of the differences in their performance on anxiogenically motivated tasks. As a result, mice first underwent a battery of tests designed to consider the innate behaviours of the NR2BOE mice under different conditions.

3. Chapter 3: NR2BOE mice, test battery

3.1. Background

A battery of tests was conducted as part of a comprehensive analysis of the behavior of these animals particularly to consider the innate behaviours and tendencies of the NR2BOE mice. Some of these tests are concerned with species typical behaviours normally exhibited by mice, some consider how anxious the animals are and some are used to investigate the motor abilities and strength of the mice. Generalised increased levels of anxiety caused by approach/avoidance conflicts can lead to different behaviours being exhibited on other tests, particularly those concerned with the cognition of the animals. It is important to establish that there is no difference in innate behaviours or anxiety levels that may confound any differences observed on cognitive behavioural tests.

In particular, thigmotaxis on the aversively motivated hidden platform watermaze and aversive open field task, are associated with anxiety such that more anxious animals tend to spend more time at the sides (Simon et al 1994; Treit & Fundytus 1988). There was a genotype by sex interaction on the thigmotaxis expressed by the mice in the hidden platform watermaze, open field task and visible platform watermaze, such that the NR2BOE males tended to spend less time at the sides than wildtype males and NR2BOE females tended to spend more time at the sides than wildtype females. It is therefore of great interest to establish if there is a genotype by sex interaction between the anxiety levels of these mice which might explain why we observed these results on the watermaze and open field task.

3.2. General methods for behavioural testing

The first cohort of mice were tested by other members of the group (males were tested by Katie Vernon, females were tested by Dr James Groves). The second cohort was tested by the author. The first cohort were 5-6 months of age, the second cohort were 5-10 months of age unless otherwise stated in the text. As the previous literature suggests that the improvement in learning and memory is consistently present as the mice age (Cao et al 2007), it is not believed that the difference in ages of the mice would affect our results. Where the number of crossings was measured, a crossing was defined as all four paws crossing into the other section. For the anxiety tasks, we measured defecation and urination (which are usually considered indicators of anxiety); however, when analysed with Mann-Whitney U tests (as the data were not normally distributed) there was no effect of genotype or sex, so these data have not been presented. All data were analysed and are presented by the author.

3.3. Statistics

Results were analysed with ANOVA with genotype and sex as between subjects factors when the data were normally distributed, using the statistical packages SPSS or Sigmastat 3.5. When data were non-normally distributed and could not be rendered normally distributed with a transformation they were analysed using a non-parametric Mann-Whitney test.

3.4. Anxiety tests

3.4.1. Black/white alleys

3.4.1.1. Introduction

Mice have a natural aversion for bright, open areas, and will tend to prefer dark environments. When mice are particularly anxious and are introduced to an alley, half of which is painted black and half of which is painted white, they will tend to avoid the white half of an alley due to the approach/retreat conflict; mice tend to explore novel environments but will experience more conflict about doing so in a potentially dangerous environment. Benzodiazepines reduce anxiety of rats on this task (McHugh et al 2004). Therefore this task can help establish whether the NR2BOE mice are more or less anxious than wildtype mice. This task is similar to the light/dark box developed by Crawley (Crawley 1981).

3.4.1.2. Materials and methods

Mice were aged between 5 and 10 months for this task, the light-dark box, successive alleys and hyponeophagia (ie anxiety tests). The maze consisted of a long alley (120cm × 9cm × 29 cm), half white and half black. Mice were placed facing the end of the black arm, and were allowed to explore the maze for 2 minutes. Latency to enter the white portion and the total time spent in the white portion were measured, along with the number of arm crossings made. There were: male wildtype = 30, female wildtype = 34, male NR2BOE = 35, female NR2BOE = 35.

3.4.1.3. Results

There were no indications of differing anxiety levels between wildtype and NR2BOE mice from the black white alleys, although there was a suggestion of differing activity levels.

3.4.1.3.1. Latency to enter white alley

There was no difference between control and NR2BOE mice on the latency to enter the white alley. An ANOVA with two between subjects factors (genotype and sex) was performed on the latency to enter the white alley. There was no effect of genotype ($F(1,130)=1.27$, $p>0.2$), sex ($F(1,130)=2.02$, $p=0.158$), nor a genotype by sex interaction ($F(1,130)=2.687$, $p=0.104$). There were: male wildtype = 30, female wildtype = 34, male NR2BOE = 35, female NR2BOE = 35.

3.4.1.3.2. Number of crossings between alleys

There was no difference between control and NR2BOE mice on the number of crossings between the black and white alleys. Although the data violated assumptions of normal distribution, the data for the number of crossings were rendered normally distributed by a square root transformation. An ANOVA with two between subjects factors (genotype and sex) was performed on the square root of the number of crossings between alleys. There was no effect of genotype ($F(1,130)=2.238$, $p=0.137$) but there was a significant effect of sex ($F(1,130)=12.405$, $p=0.001$) and a significant genotype by sex interaction ($F(1,130)=13.445$, $p<0.001$). Simple main effects showed that there was a significant effect of sex on the NR2BOE mice ($F(1,130)=27.107$, $p<0.001$) but not the wildtype mice ($F(1,130)=0.01$, $p>0.2$; square root of the mean arm crossings for: wildtype males = 2.15 ± 0.15. Wildtype females $=2.18\pm0.16$. NR2BOE males = 3.38 ± 0.35. NR2BOE females = 1.66 ± 0.21), driven by more arm crossings by male NR2BOE mice than female NR2BOE mice. There was a significant effect of genotype on male mice ($F(1,130)=12.905$, $p<0.001$) but not female mice $F(1,130)=2.436$, $p=0.121$). This suggests that male NR2BOEs may be more active than male wildtypes but that female wildtypes and NR2BOEs do not differ significantly. There were: male wildtype = 30, female wildtype = 34, male NR2BOE = 35, female NR2BOE = 35.

3.4.1.3.3. Time spent in the white section

There was no difference between the amount of time spent by wildtype and NR2BOE mice in the white section of the alley. An ANOVA with two between subjects factors (genotype and sex) was performed on the time spent in the white alley. There was no effect of genotype (F(1,130)=0.518, p=0.447), sex (F(1,130)=0.265, p=0.608) or a genotype by sex interaction (F(1,130)=0.919, p=0.339). There were: male wildtype = 30, female wildtype = 34, male NR2BOE = 35, female NR2BOE = 35.

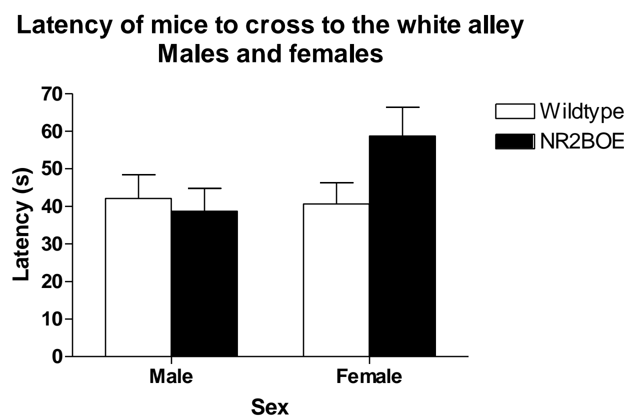


Figure 27 Latency of NR2BOE mice to enter the white alley of the black white alley. Wildtype (□) and NR2BOE (■) mice.

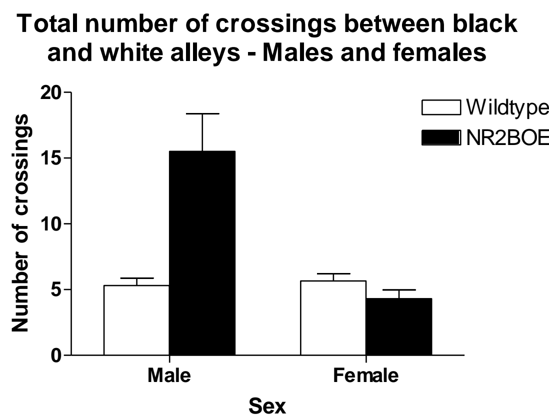


Figure 28 Number of crossings between the black and white alleys of the black white alley. Wildtype (□) and NR2BOE (■) mice.

3.4.2. Successive alleys

3.4.2.1. Introduction

The successive alleys are essentially a modified version of the elevated plus maze (McHugh et al 2004) but allows for easier interpretation as its linear form removes the central square between the open and closed arms. Instead of having alleys in a + configuration (one half of which is enclosed and one half of which is exposed), the successive alleys involve a linear set of alleys which are suspended above the floor as depicted in Figure 30. The first quarter of the alley is enclosed by tall walls and is painted dark grey, whilst the rest of the alley has no walls and the sections are painted in progressively brighter colours (dark grey, light grey, white and brilliant white). Coincident with the increased brightness of the maze, the alleys become progressively thinner with smaller beading at the edges until the alley becomes a very slim, white projection. The latency of mice to leave the enclosed first alley and enter the progressively more exposed alleys, and the length of time spent by mice in each exposed section, reflects how anxious they are. The less anxious the animal is, the shorter the latency to enter the exposed alleys and the more time spent in the exposed alleys (Contet et al 2001).

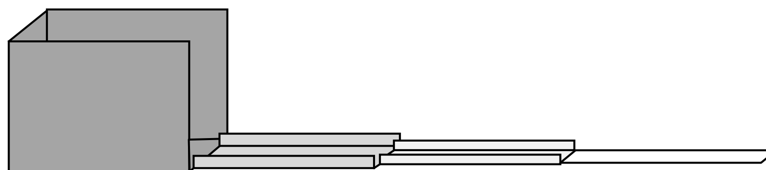


Figure 30 The successive alleys Mice are placed in the enclosed, dark grey end. The amount of time spent in the more exposed, anxiogenic alleys reflects how anxious the mouse is.

3.4.2.2. Materials and methods

The successive alleys consisted of a succession of 4 linearly arranged alleys that become increasingly narrow and exposed, elevated 70cm above the floor. The first, enclosed (dark grey) alley was 25cm long × 26cm high × 8cm wide, proceeding to the second (grey) alley (24cm long × 8cm wide × 2cm high beading). This leads to the third (white) alley (25cm long × 3.5cm wide × 0.5cm

beading), and finally to the fourth (bright white) alley (25cm long × 2cm wide × 0.2cm beading). The mouse was placed facing the closed end in the enclosed (first) alley, and was allowed to explore freely for 5 minutes. The time in each arm and the latency to cross into each arm were measured. Increased time spent in the more exposed alleys suggests reduced anxiety. As very few mice ventured beyond the second alley, it was not possible to analyse the latency to enter or time spent in the third and fourth alleys in a meaningful manner. Hence only time in the first alley and latency to leave the first alley were analysed. There were: male wildtype = 29, female wildtype = 34, male NR2BOE = 36, female NR2BOE = 35.

3.4.2.3. Results

Wildtype and NR2BOE mice had comparable latencies to enter exposed alleys on the successive alleys, and spent similar lengths of time in the first alley. As the time spent in the remaining three alleys is equal to the total amount of time on the task minus the amount of time spent in alley one, and as the amount of time spent in the remaining alleys was highly skewed, these data have not been presented as their analysis is not very meaningful.

3.4.2.3.1. Time in the first alley

Wildtype mice appeared to spend less time in the first alley than NR2BOE, although this did not reach statistical significance. An ANOVA with two between subject factors (genotype and sex) was performed on the time spent in the first alley. There was a trend towards an effect of genotype on the length of time spent in the first alley ($F(1,130)=3.106$, $p=0.08$), but no effect of sex ($F(1,130)=0.747$, $p=0.389$) and no genotype by sex interaction ($F(1,130)=0.399$, $p=0.529$). Mean time (s) in first alley by: wildtype mice = 267.7±4.98, NR2BOE mice = 277.91±3.48 seconds. There were: male wildtype = 29, female wildtype = 34, male NR2BOE = 36, female NR2BOE = 35.

Figure 31 Time mice spent in the first alley of the successive alleys. Mean ± SEM for wildtype (□) and NR2BOE (■) mice.

3.4.2.3.2. Latency to enter the second alley

Wildtype and NR2BOE mice had similar latencies for entering the second alley. An ANOVA with two between subject factors (genotype and sex) was performed on the latency to enter the second alley. There was no effect of genotype ($F(1,130)=2.153$, $p=0.145$), sex ($F(1,130)=1.008$, $p=0.317$) or a genotype by sex interaction ($F(1,130)=0.316$, $p=0.575$).

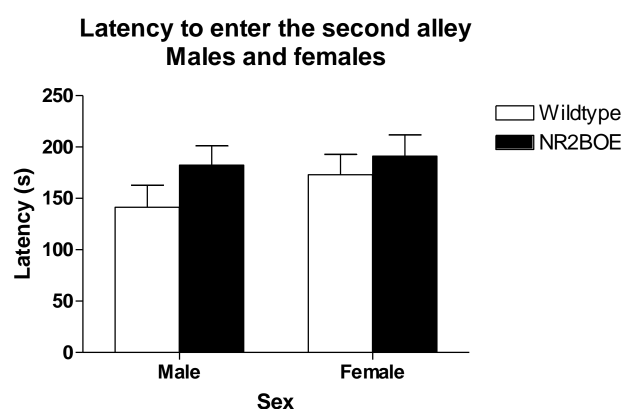


Figure 32 Latency of mice to enter the second alley of the successive alleys. Wildtype (□) and NR2BOE (■) mice.

3.4.3. Light/dark box

3.4.3.1. Introduction

This task was designed by Crawley (1981) to assess the anxiolytic effects of drugs. Since then it has been developed and refined considerably such that it is now a standard behavioural test of anxiety (Bourin & Hascoet 2003). It consists of two paradigms; one in which the mouse is first placed in the dark side of the box, the second in which it is placed in the anxiogenic, brightly lit light side of the box. As mentioned previously, anxiety is a result of conflicts; in this case, the desire to explore the exposed, open section and the concern that the exposed section may be dangerous. The paradigm in which the mouse starts in the smaller, dark section of the box is particularly good for considering the conflicts between leaving the safe, dark area and investigating the novel area, usually measured as the latency to leave the dark box and time spent in the light box. The paradigm in which the mouse starts in the light box underlies similar principles, but can be confounded as frequently mice sometimes freeze when placed in the light box. Freezing is a behaviour induced by fear, which relies on the amygdala, as opposed to anxiety which is regulated by the hippocampus.

3.4.3.2. Materials and methods

The light-dark box consisted of an enclosed box painted dark grey, attached to a larger, brightly lit box with three closed walls painted white, an open top and a clear plastic window facing the experimenter (dimensions of dark box; 16.6cm × 21cm × 21.3cm. Dimensions of light box; 30.5cm × 21cm × 21.3cm). The two boxes were connected by a small square hole (3.5cm × 3.5cm). The aversiveness of the light box was increased by placing a 60W angle poise lamp 45cm from the floor of the box. This test consists of two trials; one involves placing the mouse in the light portion of the box, facing the end of the box (away from the connecting hole). The light portion was twice the size of the dark portion, so a preference for the dark environment cannot be explained by there being a greater area to explore.

The second paradigm involves placing the mouse in the dark box, facing the end of the box (away from the entrance to the light box). A shorter latency to cross to the light box indicates increased exploratory drive and decreased anxiety. In both paradigms, the mouse was allowed to explore freely for 5 minutes, and the latency to enter the opposite box, number of crossings between the boxes and amount of time spent in each box were recorded. There were: male wildtype = 30, female wildtype = 34, male NR2BOE = 36, female NR2BOE = 35.

3.4.3.3. Results

3.4.3.4. Starting in light

NR2BOE crossed to the dark box less readily than wildtypes, which could be considered as indicating decreased anxiety (but see Discussion). A log10 transformation rendered the data for the latency to cross normally distributed. An ANOVA with two between subjects factors (genotype and sex) on the latency to enter the dark section was performed. There was a significant effect of genotype ($F(1,131)=5.166$, $p=0.025$) , but no effect of sex ($F(1,131)=0.013$, $p=0.873$) and no genotype × sex interaction ($F(1,131)=0.026$, $p=0.873$). The effect of genotype was caused by NR2BOE mice having a greater latency to enter the dark (mean latency (log10) ± SEM for: wildtypes = 1.15±0.05, NR2BOE = 1.33±0.06).

Using an ANOVA with two between subjects factors (genotype and sex) on the time spent in the dark, we found a near significant effect of genotype ($F(1,131)=3.438$, $p=0.066$), but no effect of sex ($F(1,131)=2.539$, $p=0.113$), nor a genotype by sex interaction ($F(1,131)=0.179$, $p=0.673$). The trend towards an effect of genotype was caused by the NR2BOE mice spending more time in the

light section than wildtype mice (time spent in dark section (s) ± SEM for: wildtypes = 211.6±7.69, NR2BOE = 190.4±11.4).

An ANOVA with two between subjects factors (genotype and sex) on the number of crossings made between the light and dark boxes showed that there was no effect of genotype (F(1,131)=0.011, p=0.917), sex (F(1,131)=0.668, p=0.415) or a genotype by sex interaction (F(1,131)=0.103, p=0.749). There were: male wildtype = 30, female wildtype = 34, male NR2BOE = 36, female NR2BOE = 35.

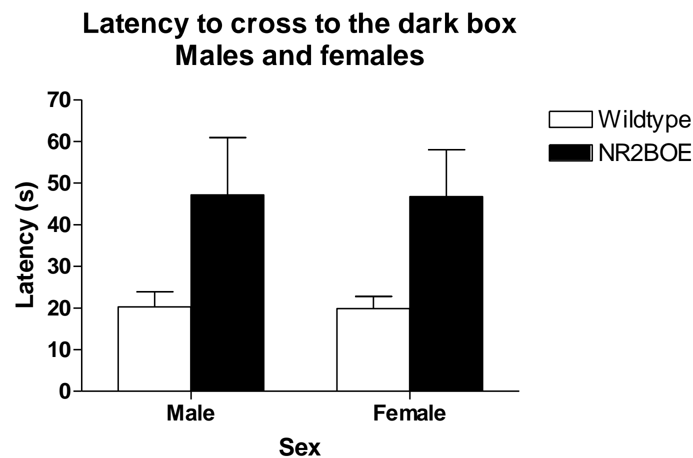


Figure 33 Latency of mice to cross from the light box to the dark box. Wildtype (□) and NR2BOE (■) mice.

3.4.3.5. Starting in dark

Wildtype and NR2BOE mice had similar latencies to cross from the dark box into the light box. An ANOVA with two between subjects factors (genotype and sex) on the latency to enter the light was performed. There was no effect of genotype (F(1,131)=0.031, p=0.861) or sex (F(1,131)=0.097, p=0.756), and no genotype by sex interaction (F(1,131)=0.002, p=0.961).

In addition, an ANOVA with two between subjects factors (genotype and sex) was performed on the amount of time spent in the dark section. There was no effect of genotype ($F(1,131)=0.926$, $p=0.338$) or sex ($F(1,131)<0.001$, $p=0.998$), and no genotype by sex interaction ($F(1,131)=0.119$, $p=0.731$).

Finally, an ANOVA with two between subjects factors (genotype and sex) was performed on the number of arm crossings made. There was no effect of genotype ($F(1,131)=0.067$, $p=0.797$) or sex ($F(1,131)=0.05$, $p=0.946$), nor a genotype by sex interaction ($F(1,131)<0.001$, $p=0.985$). There were: male wildtype = 30, female wildtype = 34, male NR2BOE = 36, female NR2BOE = 35.

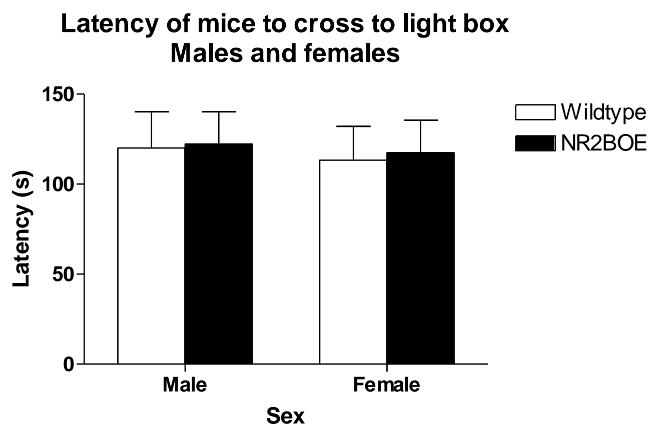


Figure 34 Latency of mice to cross from the dark box to the light box. Wildtype (□) and NR2BOE (■) mice.

3.4.4. Hyponeophagia

3.4.4.1. Introduction

This task considers the aversion of mice to novel foodstuffs in a potentially aversive environment. Anxious mice will tend to display reduced willingness to eat the novel foodstuff in an anxiogenic environment and so demonstrate a greater latency to eat the novel foodstuff when hungry, as a conflict will arise between the desire to try the novel food stuff and the possibility that the environment may be too dangerous to eat in. This is due to the possibility that a previously unsampled foodstuff may be poisonous and that the environment may contain danger, thus wariness of novel food and especially novel and potentially dangerous environments is evolutionarily sensible. Thus, this task is well suited to observe the natural anxiety of mice, as anxious animals will be much more wary of the foodstuff and therefore have a greater latency to eat it. This task has been shown to be sensitive to the effects of benzodiazepines (Britton & Britton 1981) and lesions of the amygdala, hippocampus, subiculum and nuculus accumbens (Burns et al 1996), although the effects of the amygdala lesions noted in the Burns study may be an artefact of the measures used rather than a genuine effect of anxiety differences (McHugh et al 2004).

3.4.4.2. Materials and methods

This test exploits the natural hesitancy of a mouse to eat a novel foodstuff in a novel environment. Mice that had had no contact with milk previously were food deprived overnight. The following day, each mouse was placed on a white perspex board (measuring 30cm × 30cm × 0.5cm) with a food well (1.2cm diameter, 0.9cm high) containing 0.2ml diluted (50:50 milk:water) condensed milk (Nestle). A clear jug (diameter 15cm, height 13cm) was placed over the mouse and food well for 2 minutes, forcing the mouse into close proximity to the new foodstuff. If the mouse did not drink the milk, it was returned to a cage for 5 minutes before being replaced for a further 2 minutes. No mouse was exposed to the milk for more than 6 minutes. Latency to contact the milk

and latency to drink the milk were measured. A longer latency to drink the milk indicates increased anxiety. There were: male wildtype = 30, female wildtype = 34, male NR2BOE = 37, female NR2BOE = 34.

3.4.4.3. Results

NR2BOE contacted and drank the milk less readily than wildtypes, which could be interpreted as indicating that they may be more anxious. An ANOVA with two between subjects factors (genotype and sex) was performed on the latency to contact the milk. There was an effect of genotype ($F(1,131)=9.487$, $p=0.003$) but no effect of sex ($F(1,131)=0.036$, $p=0.85$), and no genotype × sex interaction ($F(1,131)=1.098$, $p=0.297$) on the latency of mice to contact the milk; however, the data violated assumptions of normal variance and normal distribution. When a Mann-Witney U test was performed, the effect of genotype remained significant ($U_A=1167$, $z=-2.13$, $p=0.02$). The effect of genotype was driven by the NR2BOE mice taking longer to contact the milk than wildtype mice (average latency (s) to contact milk for: wildtype mice = 4.67±0.5, NR2BOE mice = 7.8±0.85). There were: male wildtype = 30, female wildtype = 34, male NR2BOE = 37, female NR2BOE = 34.

There was also an effect of genotype on the latency to drink the milk ($F(1,131)=5.443$, $p=0.021$), driven by a greater latency by NR2BOE mice to drink the milk (average latency (s) to drink for: wildtype mice = 33.67±7.44, NR2BOE mice = 69.62±12.61). However there was no effect of sex ($F(1,131)=0.491$, $p=0.485$) and no genotype by sex interaction ($F(1,131)=0.174$, $p=0.677$) although again the data were not normally distributed. Non-parametric analysis revealed that the effect of genotype was significant ($U_A=1023.5$, $z=-0.89$, $p=0.045$). There were: male wildtype = 30, female wildtype = 34, male NR2BOE = 37, female NR2BOE = 34.

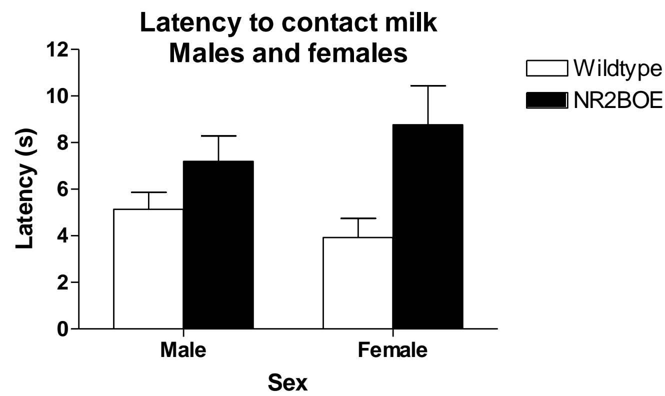


Figure 35 Latency of mice to contact condensed milk. Wildtype (□) and NR2BOE (■) mice.

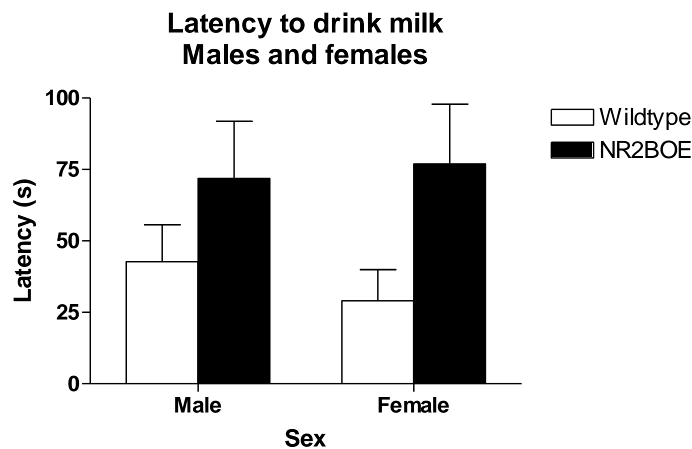


Figure 36 Latency of mice to drink condensed milk. Wildtype (□) and NR2BOE (■) mice.

Lastly we considered the latency to drink the milk after first contacting it. An ANOVA with two between subjects factors showed that there was a significant effect of genotype ($F(1,131)=4.74$, $p=0.031$) but no effect of sex ($F(1,131)=0.533$, $p=0.467$) and no genotype by sex interaction ($F(1,131)=0.126$, $p=0.723$). The effect of genotype was driven by a greater time between contacting and drinking the milk for NR2BOE mice (average latency (s) for: wildtype mice = 29±7.3, NR2BOE mice = 61.8±12.3). There were: male wildtype = 30, female wildtype = 34, male NR2BOE = 37, female NR2BOE = 34.

3.5. Motor function and coordination tests

3.5.1. Rotorod

3.5.1.1. Introduction

The rotorod tests balance and motor ability. The mouse is placed on the hub of an accelerating rotating rod and the latency of the mouse to fall from the rod is measured. Mice with impaired motor ability will tend to fall from the rod at slower rotation speeds than those whose motor ability is intact. Thus, this test is a good method for observing the motor ability of mice and is frequently used to assess drug and brain lesion effects in rodents (Crawley 1999; Cunningham et al 2005; Deacon et al 2006; Guenther et al 2001; Jennings et al 2006; Jones & Roberts 1968).

3.5.1.2. Materials and methods

This is a test of motor co-ordination. The mouse was placed on a slowly rotating hub between flanges that were 30cm in diameter and 4.5cm apart, at an elevation of 17cm, whose speed of rotation gradually increased from 4 rpm at a rate of 20rpm. The length of time for the mouse to fall from the rotating rod, and the speed of rotation of the rod when the mouse fell, were measured; a longer latency to fall suggests greater motor co-ordination. If mice fell within 10 seconds of being placed on the rotorod, the trial was restarted. If the mouse fell from the hub within 10 seconds on three trials, the mouse was not replaced and a fall from the minimum speed (i.e. 4rpm) was recorded. There were: male wildtype = 17, female wildtype = 23, male NR2BOE = 24, female NR2BOE = 23.

3.5.1.3. Results

Wildtype and NR2BOE mice performed similarly on the rotorod. An ANOVA with two between subjects factors (genotype and sex) was performed on the rotation speed at which mice fell from the rotorod. There was no effect of genotype ($F(1,83)=1.809$, $p=0.182$) or sex ($F(1,83)=1.568$, $p=0.241$), nor a genotype by sex interaction ($F(1,83)=0.085$, $p=0.771$). This suggests that NR2BOE and wildtype mice are equivalent in their motor co-ordination on the rotorod. There were: male wildtype = 17, female wildtype = 23, male NR2BOE = 24, female NR2BOE = 23.

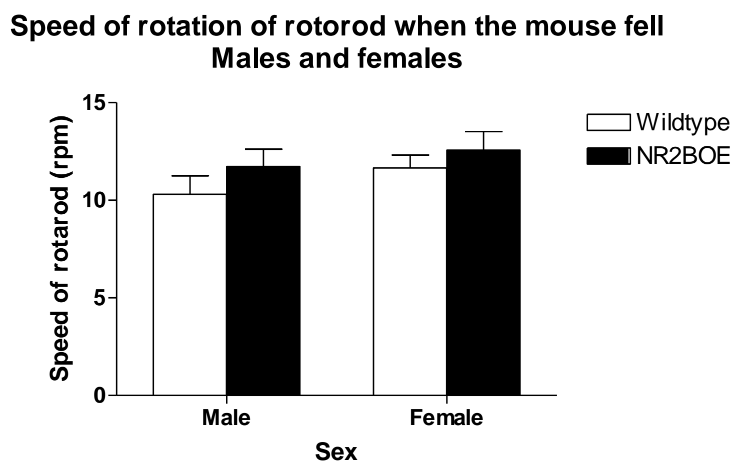


Figure 37 Speed of rotation of the rotorod when the mice fell. Wildtype (□) and NR2BOE (■) mice.

An ANOVA with two between subjects factors (genotype and sex) was performed on the weights of the mice. Interestingly, there was a significant effect of genotype on weight ($F(1,83)=4.707$, $p=0.033$) as well as a significant effect of sex on weight ($F(1,83)=172.068$, $p<0.001$), with females significantly lighter than males. There was also a genotype by sex interaction ($F(1,83)=5.364$, $p=0.023$), driven by male NR2BOE being significantly lighter than male wildtypes ($F(1,83)=9.382$, $p=0.003$). Increased weight of a mouse is sometimes associated with decreased ability to perform the rotorod task, although there was no evidence of this here. There were: male wildtype = 17, female wildtype =23 , male NR2BOE = 24, female NR2BOE = 23.

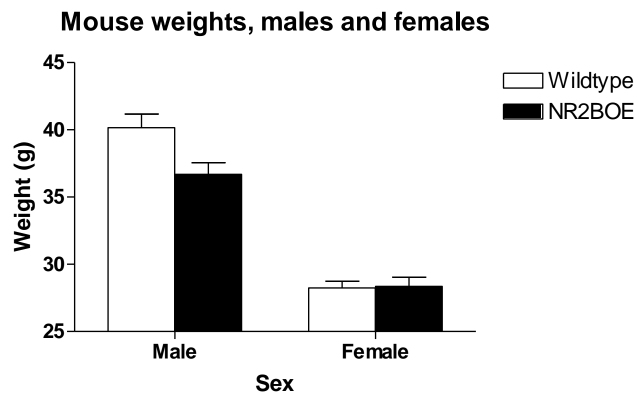


Figure 38 Mouse weights when the rotorod was conducted. Wildtype (□) and NR2BOE (■) mice.

3.5.2. Horizontal bar

3.5.2.1. Introduction

The horizontal bar consists of a 30 centimetre long thick metal wire which is suspended at the ends, 30 centimetres above the tabletop, by two wooden supports. To avoid falling, mice will hold on to the wire strongly and sometimes move along it to reach the wooden supports at the ends. Mice who are impaired in their strength or motor ability will tend to fall from the wire in less time than mice whose strength and motor ability are unimpaired (Betmouni et al 1999; Cunningham et al 2005). Hence this task was also of interest for investigating the strength and motor ability of the NR2BOE mice.

3.5.2.2. Materials and methods

This is a test of motor co-ordination and strength. The forepaws of the mouse were placed on a thick horizontal wire, 2cm in diameter and 38cm in length, suspended at each end by wooden supports, 49cm above the work surface. The test was ended when the mouse placed a forepaw on one of the supports, fell from the bar, or if it did not fall from the bar within 30 seconds. Reaching the support or holding on to the bar for the full 30 seconds was scored as a pass, whereas falling

within the 30 seconds was recorded as a fail. The length of time spent on the horizontal bar was measured, along with a 5 point scale indicating the length of time before the mouse fell. 1 point: fell within 0-5 seconds. 2 points: fell within 6-10 seconds. 3 points: fell within 11-20 seconds. 4 points: fell within 21-30 seconds. 5 points: did not fall. There were: male wildtype = 17, female wildtype =23 , male NR2BOE = 24, female NR2BOE = 22.

3.5.2.3. Results

There was no difference between the performance of NR2BOE and wildtype mice on the horizontal bar. An ANOVA with two between subjects factors (genotype and sex) was performed on the weights of the mice. Out of 86 mice, only one female NR2BOE mouse fell within 30 seconds. Females were significantly smaller than males ($F(1,82)=176.089$, $p<0.001$) and NR2BOE mice were also significantly smaller ($F(1,82)=4.289$, $p=0.042$) than wildtype mice. There was a trend towards a genotype by sex interaction ($F(1,82)=2.985$, $p=0.088$).

A short time spent on the bar can either represent improved agility in that the mice are able to reach the posts on either side of the bar within 30 seconds, or it can reflect reduced strength as mice can fall from the bar within 30 seconds. If the mouse which fell is excluded from the analysis, the latency to fall from the horizontal bar reflects only relative abilities of the mice to reach the posts.

As the latencies to fall or reach the end of the bar were not normally distributed, separate non-parametric Mann-Witney U tests were performed for genotype and sex. There was no effect of genotype ($U_A=974$, $z=-0.46$, $p=0.323$) although there was a significant effect of sex ($U_A=684$, $z=2.04$, $p=0.021$) on the latency to fall from (or reach the end of) the horizontal bar, suggesting that performance of wildtype and NR2BOEmice were similarly able on the horizontal bar. When

genotypes were compared only for male or female mice, there was no effect of genotype for male (UA=232.5, z=-0.74, p=0.23) or female (UA=282, z=-0.37, p=0.356) mice.

An issue with this task is that the scores were subject to a ceiling effect. Only one mouse (a NR2BOE female who fell and scored 4) did not score 5 out of 5, hence the data were not normally distributed and cannot sensibly be interpreted except to say that none of the mice appear to have a strength impairment. The ceiling effect was not so severe on the times spent on the bar, although a substantial number of mice spent the maximum time on the bar (64 out of 86) so the data is subject to a substantial ceiling effect. There were: male wildtype = 17, female wildtype =23 , male NR2BOE = 24, female NR2BOE = 22.

It is inadvisable to perform multiple Mann-Whitney U tests on a single set of data due to the increasing likelyhood of type 1 errors (or false positive results). However, the analyses have produced negative results and so the possibility of false positive results has not presented a problem.

3.5.3. Multiple static rods

3.5.3.1. Introduction

The multiple static rods consist of three wooden rods suspended about a metre above the ground, each of which has a smaller diameter than the last. The mouse is placed on the end of each rod facing outwards away from the bench (starting with the thickest), and must then orientate by 180˚ until it is facing the bench. It must then pass along the rod until it reaches the bench. If the mouse is able to do so, it is then placed on the next rod with a smaller diameter to repeat the

actions. This task requires motor co-ordination to perform and mice with neurodegenerative or inflammatory brain diseases are impaired in their performance of this task (Cunningham et al 2005; Deacon et al 2006; Guenther et al 2001; Jennings et al 2006), and so is a good method for establishing the motor co-ordination of the NR2BOE mice.

3.5.3.2. Materials and methods

This is an anxiety driven test of motor co-ordination. The mouse was placed on the end of the first of three rods, facing outwards. Each rod had a smaller diameter than the last (diameters: rod 1 = 3.3cm, rod 2 = 2.1cm, rod 3 = 0.8cm), elevated to a height of 60cm (below which padding was placed in case the mice fell), and each rod was 60cm long. The latency to orientate 180° and the latency to reach the platform were recorded. If the mouse reached the platform within 180s, it was placed on the next rod in the same manner after 5 minutes rest in the home cage. If it failed to reach the platform and fell from the rod, it was not placed on any of the rods with a smaller diameter. The time to orientate and latency to reach the end of the rod (or fall) were measured.

3.5.3.3. Results

No significant differences were found between the performance of NR2BOE and wildtype mice, or between males and females, on the multiple static rods. For most of the times to orientate, and times to reach the end of the rod, the data were not normally distributed. However, the time to orientate on rod one was rendered normally distributed when a log10 transformation was applied. An ANOVA with two between subjects factors (genotype and sex) was performed on the time to orientate. Where data were not normally distributed, the non parametric Mann-Witney U test was performed. There was no significant difference between wildtypes and NR2BOEs time to orientate on ($F(1,78)=0.531$, $p=0.469$) or reach the end of ($UA=804$, $z=0.29$, $p=0.386$) rod one. There was also

no effect of sex (rod 1: orientation = UA=953.5, z=-1.17, p=0.121, time to reach end = UA=849, z=-0.19, p=0.425).

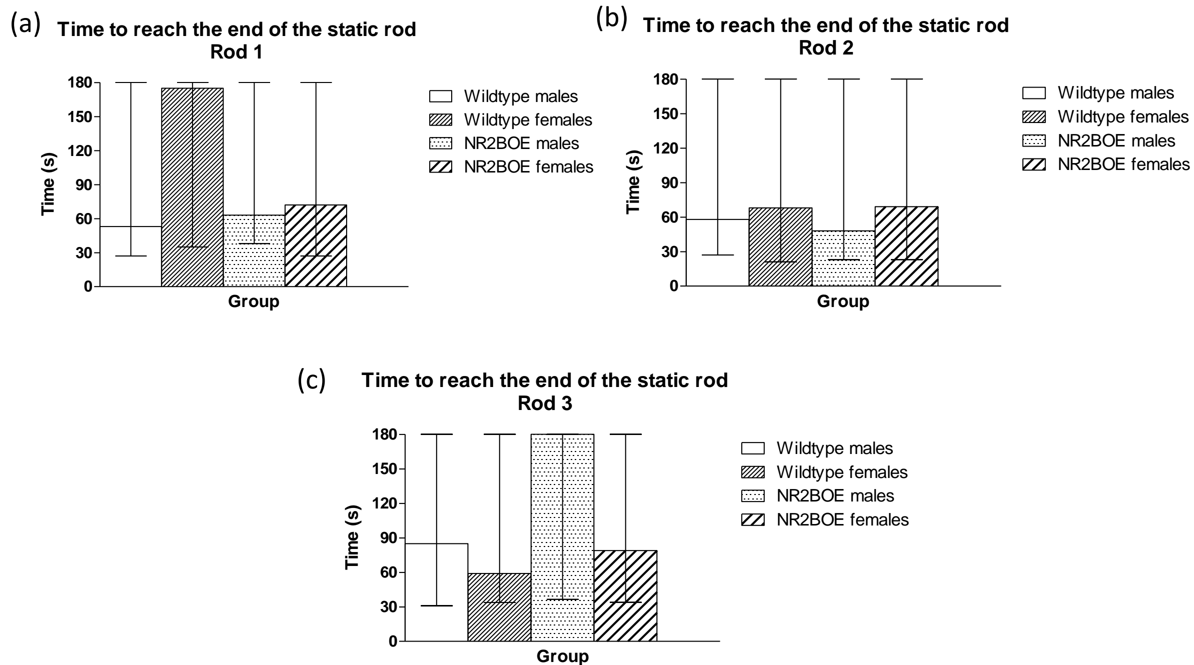


Figure 39 Time to reach the end of the static rods. Time to reach the end of (a) rod 1, (b) rod 2 and (c) rod 3. Median ± interquartile range.

3.6.Species typical behaviours

3.6.1. Burrowing

3.6.1.1. Introduction

This test capitalises on the natural tendency of rodents to burrow. It is likely that rodents developed this tendency to burrow as a means of obtaining shelter from predators and weather. When given a plastic tube filled with material such as sand, food pellets or baking beads overnight, mice will tend to displace the material. The precise reason for doing so is not entirely clear as they will still burrow even when an identical but empty tunnel (which acts as a ready-made shelter) is

placed alongside the burrow. We classically use food pellets as our burrowing material, so it could be argued that mice displace the food pellets in order to hoarde food. However, mice displace the food pellets such that they gather around the entrance to the burrow and do not move them to a selected spot as usually occurs during food hoarding. Similarly, the pellets are kicked out with the hind paws rather than being carried out in the mouth, further suggesting that the pellets are being burrowed rather than hoarded (Jennings et al 2006).

Burrowing has proved to be a sensitive test for the development of prion disease in mice (Deacon et al 2001), and to hippocampal and prefrontal cortex lesions (Deacon et al 2002c; Deacon et al 2003; Guenther et al 2001). It is of interest to observe whether subtle differences exist between the NR2BOE and wildtype mice and their tendency to burrow which can be observed on this task, as in the Deacon et al (2001) paper.

3.6.1.2. Materials and methods

The burrows were filled with 200g of food pellets in a cage, in which mice were housed singly overnight. The following morning the burrows were removed and weighed with the pellets that remained in them. The weight of mice before testing, the weight of food displaced in 2h and the weight of food displaced overnight were recorded. There were: wildtype male = 5, wildtype female = 23, NR2BOE male = 7, NR2BOE female = 23.

3.6.1.3. Results

The weight of the chow pellets remaining in the tunnel was measured twice; after 2 hours and after 18 hours (overnight). As with the horizontal bar task, this task suffers from a ceiling effect when mice are allowed to burrow overnight, but tends to be much more sensitive to potential

differences after 2 hours of burrowing. An ANOVA with two between subjects factors (genotype and sex) was performed on the weight of pellets displaced after 2 hours. The data for the weight of pellets displaced overnight are not presented as the results were subject to a ceiling effect, with nearly all mice displacing all the pellets. There was no significant effect of genotype on the weight of pellets displaced after 2 hours ($F(1,54)=0.652$, $p=0.423$) although there was a significant effect of sex ($F(1,54)=8.856$, $p=0.004$) with males displacing a significantly larger weight of pellets than females. Mean weight displaced by males = 182.5±7.86g, mean weight displaced by females = 122.4±10.23g. There was not a significant genotype by sex interaction on the weight of pellets displaced ($F(1,54)=0.196$, $p=0.66$). There were: wildtype male = 5, wildtype female = 23, NR2BOE male = 7, NR2BOE female = 23.

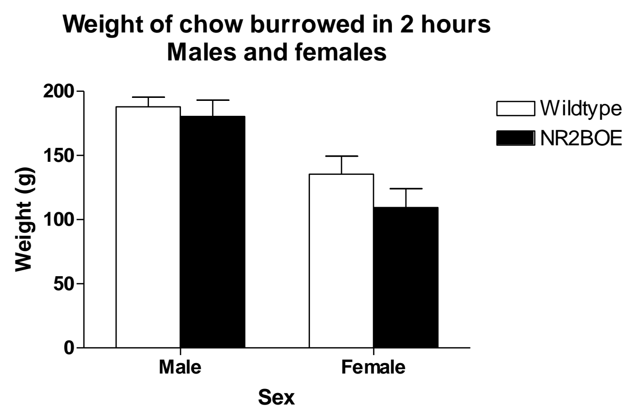


Figure 40 Burrowing for 2 hours. Wildtype (□) and NR2BOE (■) mice.

3.6.2. Nesting

3.6.2.1. Introduction

Mice habitually attempt to make nests in order to conserve heat. Disruptions to the pathways in the brain can disturb a mouse's instinct to make a nest, hence observing the willingness

of a mouse to create a nest, and the quality of the nest can reflect some neural disruptions (Jennings et al 2006) and lesions of the hippocampus (Deacon et al 2002c). We tested mice by providing them with an empty cage overnight, containing only a little sawdust and a pressed cotton nestlet. The ability of the mice to then use this nestlet to produce a nest was recorded.

3.6.2.2. Materials and methods

Mice were singly housed overnight in an empty cage, with a single nestlet. The amount of nestlet shredded, and the size of the walls of the nest, were scored on a scale of 1-5 (1 point: untouched nestlet (90% untouched). 2 points: nestlet is partially torn (50-90% is intact). 3 points: nestlet shredded, but without an identifiable nest site. 4 points: an identifiable, but flat, nest. 5 points: the nestlet has been >90% shredded and there is an identifiable nest with walls at least the height of the mouse for at least 50% of the circumference of the nest). There were: male wildtype = 5, female wildtype = 5, male NR2BOE = 7, female NR2BOE = 6.

3.6.2.3. Results

Wildtype and NR2BOE mice made recognisable nests of similar quality. A Mann-Whitney U test was performed on the nesting scores, as the data were non-parametrically distributed. Conducting separate Mann-Whitney tests will tend to produce false positives; as the results were negative, this was not an issue. There was no significant effect of genotype (UA=82, z=-1.02, p=0.154) or sex (UA=52.5, z=0.8, p=0.212) on the ability of NR2BOE and wildtype mice to make a nest. When analysed by sex, there was still no effect of genotype for males (UA=18, z=0, p=0.5) or females (UA=23, z=-1.37, p=0.085). There were: male wildtype = 5, female wildtype = 5, male NR2BOE = 7, female NR2BOE = 6.

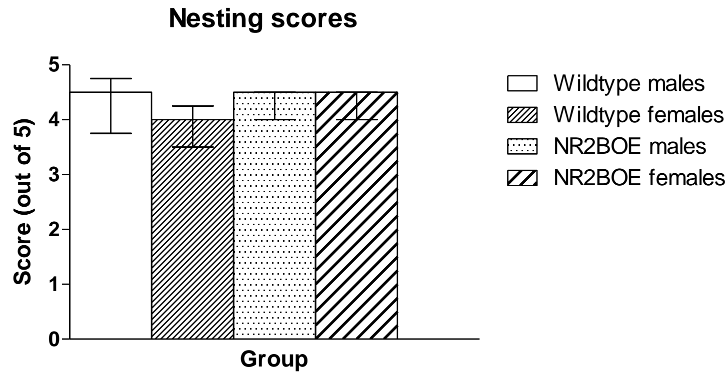


Figure 41 Nesting scores. Out of a maximum of 5. Wildtype (□) and NR2BOE (■) mice.

3.7. Conclusions

Overall, there was very little difference between wildtype and NR2BOE mice. In particular, there was no difference between the two genotypes on the black/white alleys task, successive alleys task, light/dark box starting in the dark, the rotorod, the horizontal bar, multiple static rods, burrowing, or nest making. There was a suggestion that male NR2BOE mice may be hyperactive compared to male wildtype and female mice on the black/white alleys task. Also, on the light/dark box starting in the light, there was a suggestion that NR2BOE mice may be less anxious when the mice started in the light section; however, this could be confounded by fear reactions which cause the mice to freeze. Although anxious mice would be expected to spend less time in the light section for both tasks in the light/dark box, these fear behaviours confound interpretation of the results regarding anxiety. By comparison, when the mice started in the dark, there was no difference in latencies between wildtype and NR2BOE mice which would be most readily interpreted as reflecting similar anxiety levels between genotypes. Finally, NR2BOE mice appeared more anxious than wildtypes on the hyponeophagia task, as they took longer to contact and drink the novel foodstuff, which is considered to reflect the anxiety of the animal as it resolves the approach/avoid conflict between the food and the aversive environment.

These results paint a somewhat confusing picture. On some tasks the NR2BOE mice did demonstrate a significant difference in their performance compared to the wildtype mice, but significant differences were mostly contradictory (NR2BOE mice appeared more anxious than wildtype mice on some tasks, but less anxious on other tasks). It is possible that the results were due to peculiarities of the tasks performed, but it appears unlikely that they truly reflected anxiety as it would be expected that the effects would be in the same, rather than opposite, directions. NR2BOE mice appeared more anxious on the hyponeophagia task, but there was no difference in anxiety levels between the genotypes on the black/white alleys, successive alleys or light/dark box starting in the dark. In the absence of a clear expression of greater or reduced anxiety, it seems unlikely that there is any significant overall difference in the anxiety levels of wildtype and NR2BOE mice.

As mentioned previously, Tang et al (1999) only used male mice for their study. As we used male and female mice, we were able to consider the effect of sex. There was a significant effect of sex on the burrowing task (males burrowed more than females). However, there was not a significant effect of sex on the open field test, black/white alleys, successive alleys, light/dark box, hyponeophagia, rotorod, horizontal bar, multiple static rods or nesting. Therefore this leads us to believe that there is no substantial, replicable difference between the performance of male and female mice on these tests of innate, species typical behaviours.

These results allow us to express greater confidence that the differences observed on cognitive tests are not merely a result of differences between the innate behaviours of the wildtype and NR2BOE mice. However, they do not help interpretation of the results of the mice of cohort 1, where significant differences were observed between both genotypes and genders on certain tasks.

Hence it was desirable to return to these tasks and consider the possible causes of the observed results and their replicability.

4. Chapter 4: NR2BOE mice, cohort 2

The results from the first cohort of NR2BOE mice presented a somewhat confusing picture. When the first cohort performed the hidden platform watermaze they were 11 months of age, and it is possible that differences between these observed findings with the NR2BOE mice compared to previous studies (Tang et al 1999) could be attributed to age. However, previous studies suggest that the improvement in learning and memory observed by Tang et al (1999) is also present in older animals on novel object recognition, contextual and conditioned fear, spatial working and spatial reference memory (Cao et al 2007). It should be noted that the effects we observed in the first cohort of NR2BOE mice were not statistically very strong, and indeed the improvement on the watermaze in the male mice was a trend that did not attain statistical significance; hence confirming the replicability of these results was important. Indeed, there was a statistical difference between the performance of the female mice on the hidden platform watermaze – but in the opposite direction to that observed in the male mice. This suggests that sex differences may be present. Another aspect we wished to consider was the general performance of the mice; the mean latency to reach the hidden platform for both genotypes on the last day of training was 39.2 seconds (and a mean path length of 6.7m). This is remarkably slow compared to the latencies after training of many other mouse strains (Bannerman et al 2008; Bannerman et al 1999; Kellendonk et al 2006; Malleret et al 2001; Migaud et al 1998; Mizuno et al 2007; Serrano et al 2008; Shimizu et al 2000; Silva et al 1992a; Silva et al 1992b). To ensure replicability of these results, and to address the concerns mentioned above, a second cohort of mice performed some of the same tasks as the first cohort of NR2BOE. The second cohort of mice were a similar age to the mice tested in the study by Tang et al (1999). Behavioural tasks were performed on NR2BOE and wildtype littermate controls. All tests were conducted on cohort 2 by the author and were carried out exactly as described in the Materials and Methods section of the NR2BOE 1st cohort chapter. As noted previously, males and females

were run alongside each other for each experiment to prevent any confounds between sexes (except where it has been explicitly stated that different sexes were tested by different experimenters). The ages of the mice for the different tasks are provided in Table 4.

Mouse ages (months)	Behavioural test					
	HPW	OFT	SRMYM	SWMTM	SNPYM	VD
1st cohort	8-11	8	10-11	10	12	10-12
2nd cohort	4	4.5	10-13	-	-	12

Table 4 The ages of mice from the two cohorts when they performed each behavioural task. HPW = hidden platform watermaze; OFT = open field test; SRYM = spatial reference memory Y maze; SWMTM = spatial working memory T maze; SNP = spatial novelty preference Y maze; VD = visual discrimination.

4.1. Background

4.1.1. Hidden platform watermaze (spatial reference memory)

With the first cohort, we found that male NR2BOE mice had non-significantly shorter latencies than wildtype male mice for finding the hidden platform on the watermaze, whilst female NR2BOE had significantly longer latencies than female wildtype mice. It appeared that these results may be due to thigmotaxis, as female NR2BOE mice spent longer than wildtype female mice at the side walls on the open field task and in the visible platform watermaze as well as the hidden platform watermaze. Mice from the first cohort were 8-11 months old when they performed this task, and so we repeated the hidden platform watermaze with a second cohort of NR2BOE and wildtype littermate mice which were 4 months old. It was of great interest to consider the general levels of performance of the mice and to ensure the sex differences observed previously were replicable. There were: male wildtype = 12, female wildtype = 12, male NR2BOE = 12, female NR2BOE = 12.

4.1.2. Open field task

We had found with cohort 1 that the thigmotaxis observed on the open field predicted latencies on the hidden platform watermaze (such that female NR2BOE mice, which had a greater latency to reach the centre of the open field task were significantly more thigmotaxic than female wildtype mice and were subsequently impaired on watermaze acquisition). It would be valuable to see if this effect was seen in younger mice also, equivalent to the ages of the mice used by Tang et al. (1999). We performed the open field task with the second cohort of mice after testing them on the hidden platform watermaze to ensure that the behaviours they exhibited on the hidden platform watermaze were not a result of behaviours learned on the open field task. There were: male wildtype = 12, female wildtype = 12, male NR2BOE = 12, female NR2BOE = 13.

4.1.3. Y maze (spatial reference memory)

The hidden platform watermaze is an aversively motivated test of spatial reference memory. Hence it was of interest in the first cohort to establish whether the enhancement in memory observed in the NR2BOE mice was present on other tests of spatial reference memory, such as the appetitively motivated spatial reference memory Y maze. In the first cohort, there was a non-significant effect of genotype for the male mice, such that the NR2BOE male mice made non-significantly more correct choices than wildtype males.

Mice were between 10 and 13 months of age when they performed this task, which was comparable to the ages of the mice of the first cohort when they performed this task. Unlike cohort 1 (where mice were grouped by sex and ran the task until performance plateaued), all mice in cohort 2 (males and females) performed this experiment for 16 days to allow comparisons between male

and female mice across all 16 days. Male and female mice ran the task together in an interleaved sequence. There were: male wildtype = 4, female wildtype = 5, male NR2BOE = 7, female NR2BOE = 6.

Repeated measures ANOVA tests were performed (with the statistical package SPSS or Sigmastat 3.5) on the data from the hidden platform watermaze and spatial reference memory Y maze. For the open field task data, a two-way ANOVA was conducted with gender and genotype as between subject factors (using the statistical package SPSS or Sigmastat 3.5) when data were normally distributed. Significant interactions were then investigated with a test of Simple Main Effects (SME).

4.2. Results

4.2.1. Hidden platform watermaze (spatial reference memory)

Wildtype and NR2BOE mice did not have significantly different latencies, distances swum or % time spent swimming at the sides. In addition, the performance of the mice of the second cohort was significantly better than the performance of the mice of the first cohort.

4.2.1.1. Latency

There were no differences between the latencies of NR2BOE and wildtype mice to reach the hidden platform. An ANOVA with two between subjects factor (genotype and sex) and one within subject factor (block) with 12 levels (corresponding to the 12 blocks, or days, of training) was performed. There was no effect of genotype on latency ($F(1,44)=0.374$, $p=0.544$; Figure 42) or sex ($F(1,44)=1.777$, $p=0.189$), and no genotype by sex interaction ($F(1,44)=0.239$, $p=0.627$).

There was a highly significant effect of block (F(11,484)=91.268, p<0.001), but no genotype by block (F(11,484)=1.465, p=0.141) or genotype by sex by block interaction (F(11,484)=0.708, p=0.731) although there was a trend towards a sex by block interaction (F(11,484)=1.748, p=0.061). This was caused by a significant difference between the latencies of male and female mice on blocks 4 (F(11,484)=6.719, p=0.013), 7 (F(11,484)=5.453, p=0.024) and 12 (F(11,484)=5695, p=0.021). There were: male wildtype = 12, female wildtype = 12, male NR2BOE = 12, female NR2BOE = 12.

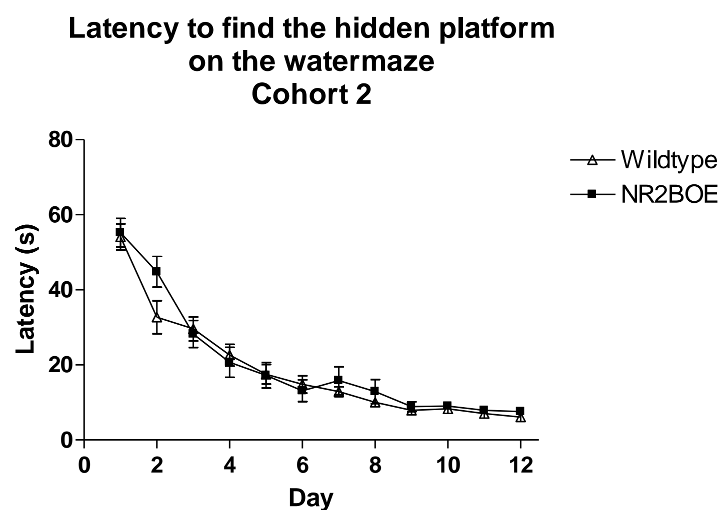


Figure 42 The latency of mice to reach the platform on the hidden platform watermaze decreased with training. Time of mice from cohort 2 to reach the hidden platform (s), mean ± SEM during acquisition of the spatial reference memory task for wildtype (Δ) and NR2BOE (■) mice.

4.2.1.2. Distance

There were no differences between the distances swum by wildtype and NR2BOE mice to reach the hidden platform. An ANOVA with two between subjects factor (genotype and sex) and one within subject factor of block with 12 levels (corresponding to the 12 blocks, or days, of training) was performed. There was no effect of genotype (F(1,44)=0.001, p=0.972) nor sex (F(1,44)=1.612, p=0.211), nor a genotype by sex interaction (F(1,44)=0.021, p=0.884) on distance swum as shown in Figure 43.

There was a significant effect of block (F(11,484)=100.668, p<0.001), and a trend towards a genotype by block interaction (F(11,484)=1.753, p=0.06) driven by a trend towards a difference between distances swum by wildtype and NR2BKO mice on day 12 (F(11,484)=3.539, p=0.067. There was also a significant sex by block interaction (F(11,484)=2.011, p=0.026) driven by a significant difference between distances swum by male and female mice on days 4 (F(11,484)=5.288, p=0.027), 7 (F(11,484)=8.68, p=0.005) and 12 (F(11,484)=6.453, p=0.015). However, there was no genotype by sex by block interaction (F(11,484)=0.497, p=0.906). There were: male wildtype = 12, female wildtype = 12, male NR2BOE = 12, female NR2BOE = 12.

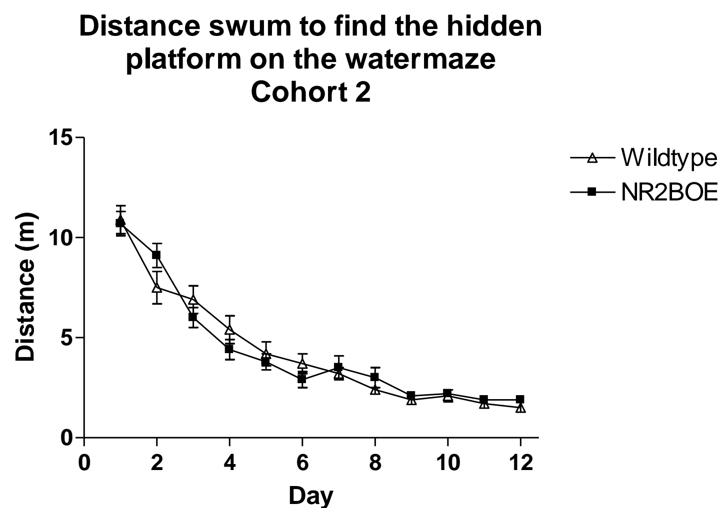


Figure 43 The distance swum by mice to reach the platform on the hidden platform watermaze decreased with training. Distances swum by mice from cohort 2 to reach the hidden platform (m), mean ± SEM during acquisition of the spatial reference memory task for wildtype (Δ) and NR2BOE (■) mice.

4.2.1.3. Thigmotaxis

There was no difference between the time spent at the sides by wildtype and NR2BOE mice. An ANOVA with two between subjects factor (genotype and sex) and one within subject factor of block with 12 levels (corresponding to the 12 blocks, or days, of training) was performed. There was no effect of genotype on thigmotaxis (F(1,44)=0.012, p=0.913) nor sex (F(1,44)=2.42, p=0.127), nor a genotype by sex interaction (F(1,44)=1.274, p=0.265) as shown in Figure 44.

There was a highly significant effect of block (F(11,484)=80.284, p<0.001) but no genotype by block (F(11,484)=0.245, p=0.994) or genotype by sex by block interaction (F(11,484)=0.44, p=0.938). There was a significant sex by block interaction however (F(11,484)=2.429, p=0.006) caused by a significant difference between male and female mice on days 2 (F(11,484)=5.819, p=0.02), 5 (F(11,484)=4.446, p=0.041) and 9 (F(11,484)=5.61, p=0.022). There were: male wildtype = 12, female wildtype = 12, male NR2BOE = 12, female NR2BOE = 12.

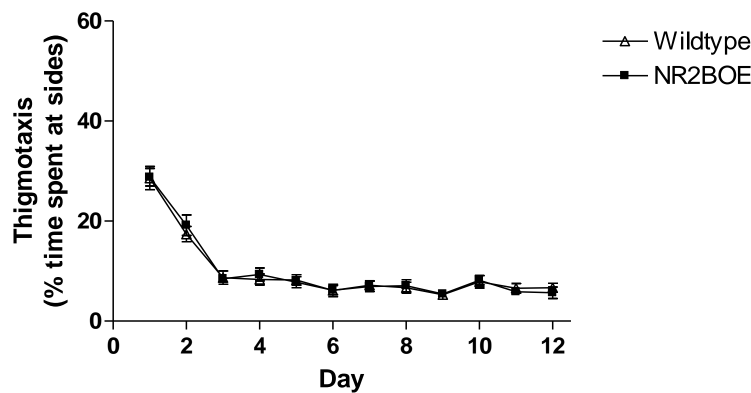


Figure 44 The % of time mice spent swimming at the sides of the watermaze decreased with training. The % time mice from cohort 2 spent swimming at the walls of the watermaze, mean ± SEM during acquisition of the spatial reference memory task for wildtype (Δ) and NR2BOE (■) mice.

4.2.1.4. Transfer tests

There was no difference between the ability of control and NR2BOE mice (or male and female mice) to remember the position of the platform on transfer tests. An ANOVA of the % time spent in the training quadrant with two between subjects factors (genotype and sex) and one within subjects factor (transfer test number) with four levels (corresponding to the four transfer tests) was performed. There was no effect of genotype (F(1,44)=0.237, p=0.629) or sex (F(1,44)=0.048, p=0.827), nor a genotype by sex interaction (F(1,44)=0.036, p=0.851; Figure 45). There was a significant effect of transfer test number (F(3,132)=7.273, p<0.001) reflecting an increase in the amount of time spent in the training quadrant on the later tests, presumably reflecting learning of

the platform position, but no genotype by transfer test number interaction (F(3,132)=0.575, p=0.632), sex by transfer test number interaction (F(3,132)=1.594, p=0.194) or a genotype by sex by transfer test number interaction (F(3,132)=0.202, p=0.895). There were: male wildtype = 12, female wildtype = 12, male NR2BOE = 12, female NR2BOE = 12.

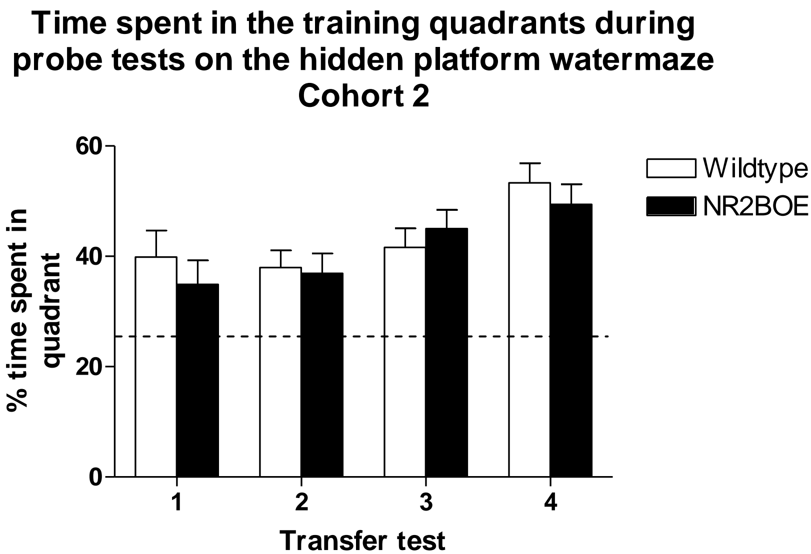


Figure 45 The % of time spent in the training quadrant, in which the platform was normally present, by mice during transfer tests 1-4. The % of time spent swimming in the training quadrant (out of 60 seconds) my mice from cohort 2, by wildtype (□) and NR2BOE (■) mice (mean ± SEM). The dashed line indicates chance levels (25%).

4.2.1.5. Both cohorts

When the results from both cohort 1 (chapter 2) and cohort 2 were combined, mice had shorter latencies and distances to reach the platform on later blocks. An ANOVA with three between subjects factor (genotype, sex and cohort) and one within subject factor of block with 12 levels (corresponding to the 12 blocks, or days, of training) was performed on latencies to reach the platform. Analysis of escape latencies revealed that there no effect of genotype F(1,103)=0.172, p=0.679; Figure 46 (a)) or sex (F(1,103)=0.096, p=0.758), although there was a significant genotype × sex interaction (F(1,103)=5.656, p=0.019). The genotype by sex interaction was driven by wildtype

males showing a trend towards being slower to find the platform than wildtype females ($F(1,104)=3.468$, $p=0.065$) and a significant difference between wildtype and NR2BOE females ($F(1,104)=4.067$, $p=0.046$; mean latency (s) ± SEM for: wildtype males = 35.9±5.9. Wildtype females = 28.8±4.5. NR2BOE males = 30.5±4.7. NR2BOE females = 38.7±5.1). There was a significant effect of day ($F(11,1133)=71.893$, $p<0.001$), driven by reduced latencies on later days, but no genotype × day ($F(11,1133)=0.99$, $p=0.453$), sex × day ($F(11,1133)=1.034$, $p=0.413$) or genotype × sex × day interaction ($F(11,1133)=1.462$, $p=0.14$). There was also a significant effect of cohort ($F(1,103)=44.789$, $p<0.001$) driven by significantly shorter latencies overall for mice from cohort 2 (mean latency for cohort 1 = 43.2±3.6 seconds, mean latency for cohort 2 = 20.6±2.3 seconds). There was no genotype by cohort ($F(1,103)<0.001$, $p=0.993$) or sex by cohort interaction ($F(1,103)=1.424$, $p=0.236$), although there was a significant genotype by sex by cohort interaction ($F(1,103)=4.22$, $p=0.042$) driven by a significant difference between wildtype males and females of cohort 1 ($F(1,103)=9.186$, $p=0.003$; mean latency (s) ± SEM for cohort 1 wildtype: males = 54.2±8.3, females = 32.3±6.6) between the cohort 1 female wildtype and NR2BOE mice ($F(1,103)=7.43$, $p=0.008$) and between the cohort 1 male wildtype and NR2BOE mice ($F(1,103)=0.001$, $p=0.969$), and between the mice of cohorts 1 and 2 for wildtype males ($F(1,103)=21.937$, $p<0.001$), wildtype females $(1,103)=3.079$, $p=0.082$), NR2BOE males ($F(1,103)=8.895$, $p=0.004$) and NR2BOE females ($F(1,103)=14.913$, $p<0.001$). There were: male wildtype = 24, female wildtype = 29, male NR2BOE = 29, female NR2BOE = 29.

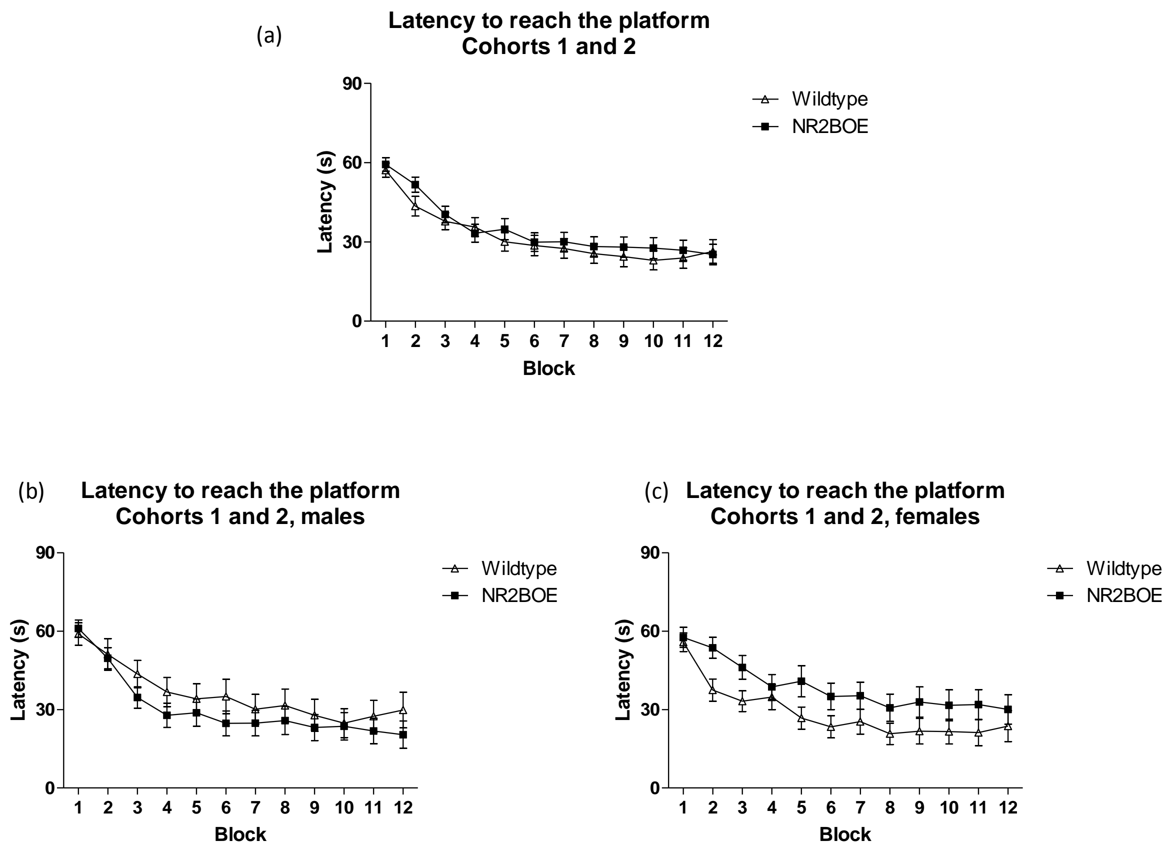


Figure 46 Wildtype and NR2BOE mice learned to find the hidden platform more quickly with practise, and did not perform significantly differently. (a) Time of mice from both cohorts to reach the hidden platform (s). (b) Latencies for male mice from both cohorts to reach the hidden platform. (c) Latencies for female mice from both cohorts to reach the hidden platform. Mean ± SEM during acquisition of the spatial reference memory task for wildtype (Δ) and NR2BOE (■) mice.

4.2.1.6. Both cohorts, distances

Latency measurements can be confounded by the speed at which animals swim, so path length was also analysed. An ANOVA with three between subjects factors (genotype, sex and cohort) and one within subject factor of block with 12 levels (corresponding to the 12 blocks, or days, of training) was performed. There was no effect of genotype ($F(1,103)=0.037$, $p=0.848$) or sex ($F(1,103=0.092$, $p=0.762$) on distances swum, although there was a significant genotype × sex interaction ($F(1,103)=5.059$, $p=0.027$). This was driven by a trend towards a difference between female wildtype and NR2BOE mice ($F(1,103)=3.109$, $p=0.081$) and between wildtype male and

female mice ($F(1,103)=3.13$, $p=0.08$; mean distance (m) ± SEM for: wildtype males = 6.96±0.99. Wildtype females = 5.82±0.73. NR2BOE males = 6.05±0.77. NR2BOE females = 7.31±0.81). There was a highly significant effect of cohort ($F(1,103)=45.963$, $p<0.001$) caused by the first cohort swimming significantly further to reach the platform than the second cohort (mean distance swum (m) ± SEM for: cohort 1 = 8.12±0.59, cohort 2 = 4.43±0.36), although there was no genotype by cohort ($F(1,103)=0.047$, $p=0.829$) or sex by cohort interaction ($F(1,103)=1.32$, $p=0.253$). There was, however, a significant genotype by sex by cohort interaction ($F(1,103)=5.512$, $p=0.021$). This was driven by significant differences between cohort 1 female wildtype and NR2BOE mice ($F(1,103)=7.895$, $p=0.006$), between cohort 1 male wildtype and NR2BOE mice ($F(1,103)=4.574$, $p=0.035$), between cohort 1 wildtype male and female mice ($F(1,103)=9.543$, $p=0.003$), between wildtype male mice of cohorts 1 and 2 ($F(1,103)=22.51$, $p<0.001$), between NR2BOE male mice of cohorts 1 and 2 ($F(1,103)=8.762$, $p=0.004$) and NR2BOE female mice from cohorts 1 and 2 ($F(1,103)=17.512$, $p<0.001$).

There was a significant effect of block ($F(11,1133)=102.521$, $p<0.001$) driven by shorter distances travelled on later blocks. However, there was no genotype by block ($F(11,1133)=1.005$, $p=0.44$), sex by block ($F(11,1133)=1.15$, $p=0.319$) or genotype by sex by block interaction ($F(11,1133)=1.521$, $p=0.118$). There was, however, a significant cohort by block interaction ($F(11,1133)=7.138$, $p<0.001$). This was due to a significant difference between the mice of cohorts 1 and 2 on all but day 1 ($F(11,1133)=2.803$, $p=0.097$). There was no sex by cohort by block interaction ($F(11,1133)=0.01$, $p=0.919$), and no genotype by sex by cohort by block interaction ($F(11,1133)=0.588$, $p=0.445$). There were: male wildtype = 24, female wildtype = 29, male NR2BOE = 29, female NR2BOE = 29.

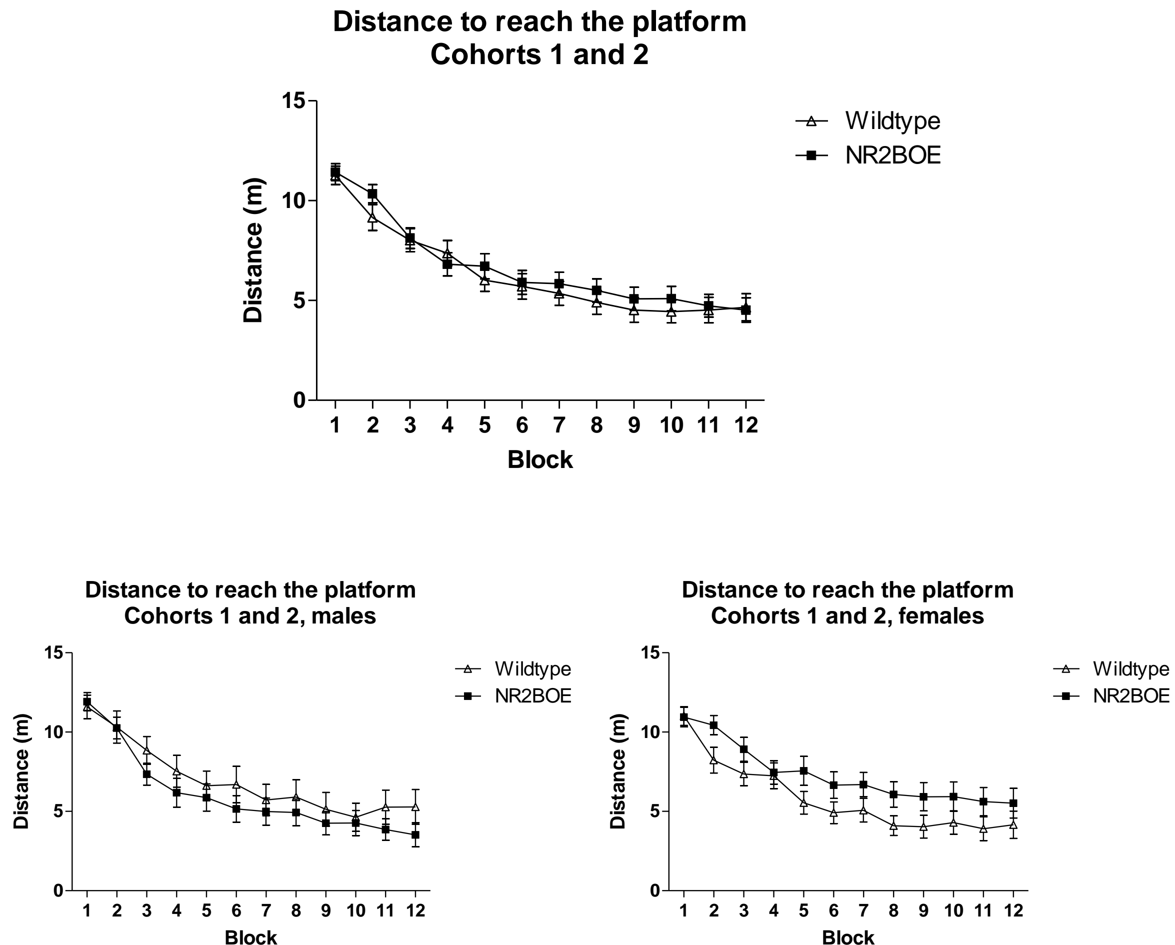


Figure 47 Wildtype and NR2BOE mice swam shorter distances to reach the hidden platform on later blocks. (a) Distances swum by mice from both cohorts to reach the hidden platform (s). (b) Distance swum by male mice from both cohorts to reach the hidden platform. (c) Distance swum by female mice from both cohorts to reach the hidden platform. Mean ± SEM during acquisition of the spatial reference memory task for wildtype (Δ) and NR2BOE (■) mice.

4.2.1.7. Both cohorts, swim speeds

Swim speeds were also considered. Wildtype and NR2BOE mice swam faster on later days. A repeated measures ANOVA with three between subjects factors (genotype, sex and cohort) and one within subjects factor of block with 12 levels (corresponding to the 12 blocks, or days, of training) was performed. There was no effect of genotype ($F(1,103)=0.006$, $p=0.938$) or sex ($F(1,103)=0.408$, $p=0.524$), although there was a significant genotype by sex interaction ($F(1,103)=4.338$, $p=0.04$). This interaction was caused by a trend towards a difference between wildtype male and female mice ($F(1,103)=3.556$, $p=0.062$). There was a highly significant effect of cohort ($F(1,103)=15.22$, $p<0.001$),

although there was no genotype by cohort (F(1,103)=0.197, p=0.658), sex by cohort (F(1,103)=1.472, p=0.228) or genotype by sex by cohort interaction (F(1,103)=0.278, p=0.599).

There was a significant effect of block (F(11,1133)=6.032, p<0.001), block by genotype interaction (F(11,1133)=1.8, p=0.05) and block by cohort interaction (F(11,1133)=2.376, p=0.007). The effect of block was caused by mice swimming faster on later blocks, whilst the genotype by block interaction was caused by a significant difference between wildtype and NR2BOE mice on day 2 (F(11,1133)=5.1, p=0.026). The cohort by block interaction was a result of a significant difference between the wildtype mice on cohorts 1 and 2 (F(11,1133)=9.066, p=0.003) and the NR2BOE mice on cohorts 1 and 2 (F(11,1133)=6.234, p=0.014). However, there was no genotype by block interaction (F(11,1133)=1.356, p=0.188), no genotype by sex by block interaction (F(11,1133)=0.991, p=0.452) and no genotype by sex by cohort by block interaction (F(11,1133)=0.697, p=0.743). There were: male wildtype = 24, female wildtype = 29, male NR2BOE = 29, female NR2BOE = 29.

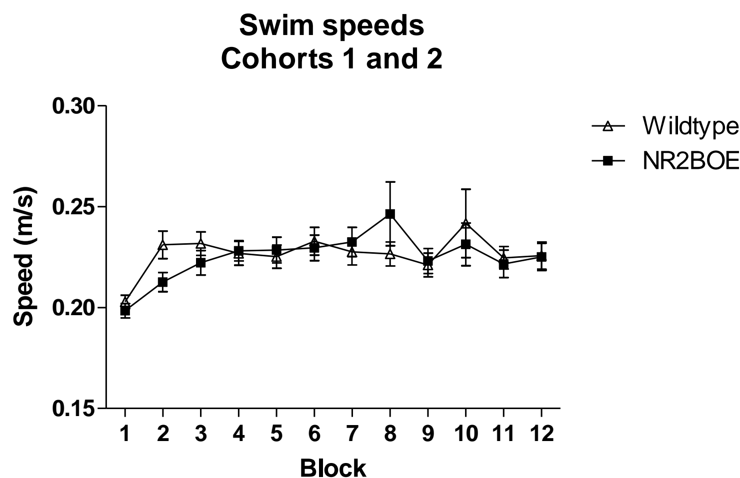


Figure 48 Swim speeds for mice from cohorts 1 and 2 on the watermaze. Mean ± SEM for wildtype (Δ) and NR2BOE (■) mice.

4.2.1.8. Both cohorts, thigmotaxis

We also considered the % of time the mice spent swimming at the sides of the watermaze (thigmotaxis;). An ANOVA with three between subjects factor (genotype, sex and cohort) and one within subject factor of block with 12 levels (corresponding to the 12 blocks, or days, of training) was performed. There was no effect of genotype ($F(1,103)=0.052$, $p=0.819$) or sex ($F(1,103)=0.748$, $p=0.389$) on the % of time spent swimming at the sides, but there was a significant genotype × sex interaction ($F(1,103)=4.716$, $p=0.032$). Significant main effects revealed that the genotype by sex interaction was caused by a near significant difference between wildtype male and female mice ($F(1,103)=3.636$, $p=0.059$). There was a highly significant effect of cohort ($F(1,103)=30.6$, $p<0.001$), driven by mice in cohort 1 spending more time at the sides than mice from cohort 2. There was no genotype by cohort interaction ($F(1,103)=0.074$, $p=0.787$) nor was there a sex by cohort interaction ($F(1,103)=2.15$, $p=0.146$). There was a significant genotype by sex by cohort interaction ($F(1,103)=6.801$, $p=0.01$) driven by a significant difference on cohort 1 for the male wildtype and NR2BOE mice ($F(1,103)=7.27$, $p=0.008$), on cohort 1 for the female wildtype and NR2BOE mice ($F(1,103)=5.77$, $p=0.018$), on cohort 1 between wildtype males and females ($F(1,103)=131.186$, $p<0.001$), for wildtype male mice between the 1st and 2nd cohorts ($F(1,103)=21.701$, $p<0.001$), NR2BOE male mice between the 1st and 2nd cohorts ($F(1,103)=4.424$, $p=0.038$) and NR2BOE female mice between the 1st and 2nd cohorts ($F(1,103)=10.687$, $p=0.001$).

There was a significant effect of block ($F(11,1133)=59.695$, $p<0.001$) driven by mice exhibiting less thigmotaxis on later blocks, but no genotype by block ($F(11,1133)=0.716$, $p=0.724$) interaction. There was a trend towards a sex by block ($F(11,1133)=1.791$, $p=0.051$) interaction, and there was a cohort by block interaction ($F(11,1133)=4.809$, $p<0.001$). The sex by block interaction was due to a significant difference on day 8 between males and females ($F(11,1133)=3.985$, $p=0.049$), and the cohort by block interaction was caused by a significant difference between the

cohorts on all blocks except block 1 (F(11,1133)=3.743, p=0.056). There was no genotype by sex by block interaction (F(11,1133)=0.779, p=0.662) and no genotype by block by cohort interaction (F(11,1133)=1.37, p=0.181). There was, however, a significant sex by block by cohort interaction (F(11,1133)=1.977, p=0.027), although there was no genotype by sex by cohort by block interaction (F(11,1133)=1.088, p=0.367). The sex by cohort by block interaction was driven by a significant difference between mice from cohorts 1 and 2 for male mice on all blocks except block 1 (F(11,1133)=3.472, p=0.065) and for female mice on all blocks except blocks 1 (F(11,1133)=0.726, p=0.393) and 2 (F(11,1133)=1.527, p=0.219), as well as a significant difference between male and female mice of cohort 1 on block 2 (F(11,1133)=4.128, p=0.045), 7 (F(11,1133)=5.419, p=0.022) and 8 (F(11,1133)=10.413, p=0.002). There were: male wildtype = 24, female wildtype = 29, male NR2BOE = 29, female NR2BOE = 29.

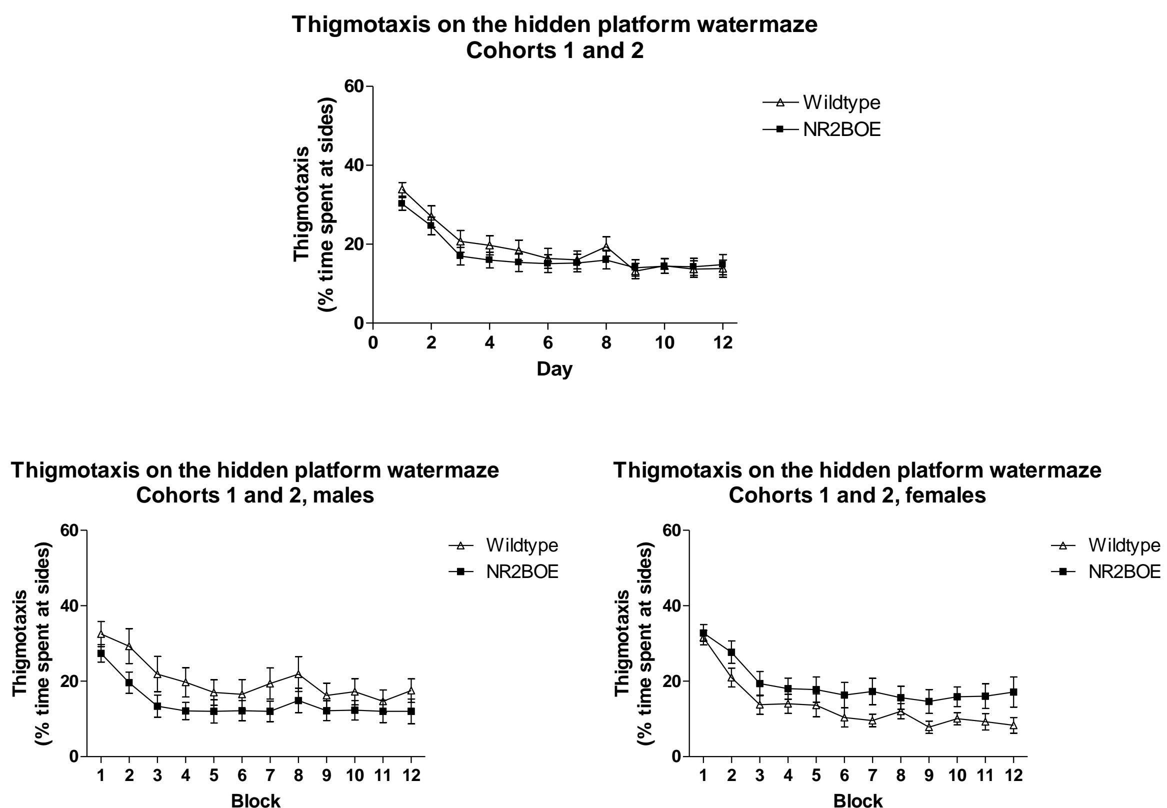


Figure 49 Wildtype and NR2BOE mice spent a smaller % of their time swimming at the sides on later blocks. (a) % time at the sides for mice from both cohorts (s). (b) % time at the sides for male mice from both cohorts. (c) % time at the sides for female mice from both cohorts. Mean ± SEM during acquisition of the spatial reference memory task for wildtype (Δ) and NR2BOE (■) mice.

4.2.1.9. Both cohorts, transfer tests

There was no difference between the amount of time wildtype and NR2BOE mice spent in the training quadrant on the transfer tests. A repeated measures ANOVA was performed on the % time spent in the training quadrant on transfer tests with three between subjects factors (genotype, sex and cohort) and one within subjects factor of transfer test number, with 4 levels corresponding to each transfer test. There was no effect of genotype ($F(1,103)=1.062$, $p=0.305$) or sex ($F(1,103)=0.058$, $p=0.809$) nor a genotype by sex interaction ($F(1,103)=1.514$, $p=0.221$). There was a highly significant effect of cohort ($F(1,103)=21.14$, $p<0.001$) caused by mice from cohort 2 spending more time in the training quadrant than mice from cohort 1 (mean time in the training quadrant (s) ± SEM for cohort: 1 = 31.5±2.2, 2 = 42.4±2.6). There was no genotype by cohort interaction ($F(1,103)=0.122$, $p=0.728$), nor a sex by cohort interaction ($F(1,103)=0.302$, $p=0.584$) or a genotype by sex by cohort interaction ($F(1,103)=0.964$, $p=0.336$).

There was a significant effect of transfer test number ($F(3,309)=13.033$, $p<0.001$) but no genotype by block interaction ($F(3,309)=0.492$, $p=0.688$) or sex by block interaction ($F(3,309)=1.734$, $p=0.16$). However, there was a trend towards a cohort by block interaction ($F(3,309)=2.558$, $p=0.055$), caused by significant differences between the mice of cohort 1 and 2 on transfer tests 1 ($F(3,309)=9.76$, $p=0.002$), 2 ($F(3,309)=6.947$, $p=0.01$) and 4 ($F(3,309)=25.078$, $p<0.001$). There was no genotype by sex by block interaction ($F(3,309)=0.034$, $p=0.991$), no genotype by cohort by block interaction ($F(3,309)=0.562$, $p=0.641$), no sex by cohort by block interaction ($F(3,309)=1.929$, $p=0.125$) and no genotype by sex by cohort by block interaction ($F(3,309)=0.341$, $p=0.796$).

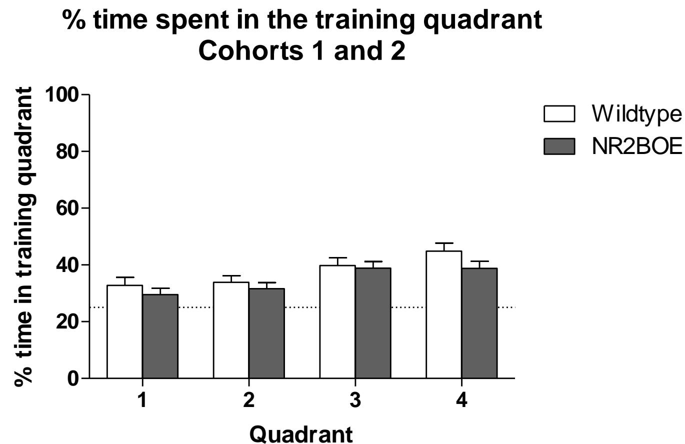


Figure 50 The % of time spent in the training quadrant, in which the platform was normally present, by mice during transfer tests 1-4. The % of time spent swimming in the training quadrant (out of 60 seconds) my mice from cohorts 1 and 2, by wildtype (□) and NR2BOE (■) mice (mean ± SEM). The dashed line indicates chance levels (25%).

4.3. Open field task

As the results of the open field task seemed to predict the results seen in the hidden platform watermaze in the first cohort of NR2BOE, to confirm reliability we repeated the open field task. Mice were 4.5 months of age when they ran this task.

4.3.1.1. Cohort 2, latency to centre

Male control and NR2BOE mice had similar latencies to reach the centre, but female NR2BOE took less time to reach the centre than female wildtype mice. An ANOVA with two between subjects factors (genotype and sex) was performed on the latency of mice to reach the central area. There was no effect of genotype ($F(1,45)=1.266$, $p=0.266$) but there was a significant effect of sex ($F(1,45)=11.785$, $p=0.001$; driven by a shorter latency to the centre for males compared to females) and a trend towards a genotype by sex interaction on latency to the centre ($F(1,45)=3.898$, $p=0.055$; Figure 51). Simple main effects revealed an effect of sex on wildtypes ($F(1,45)=14.338$, $p<0.001$) but

not NR2BOE (F(1,45)=1.085, p=0.303), and there was an effect of genotype on females (F(1,45)=4.9, p=0.032) but not males (F(1,45)=0.354, p=0.555). Thus, female wildtype mice were slower to reach the centre than female NR2BOE mice. Male wildtype mean = 32.9±19.46, female wildtype mean = 119.6±15.51, male NR2BOE mean = 46.5±13.88, female NR2BOE mean = 69.9±14.9. There were: male wildtypes = 12, female wildtypes = 12, male NR2BOE = 12, female NR2BOE = 13.

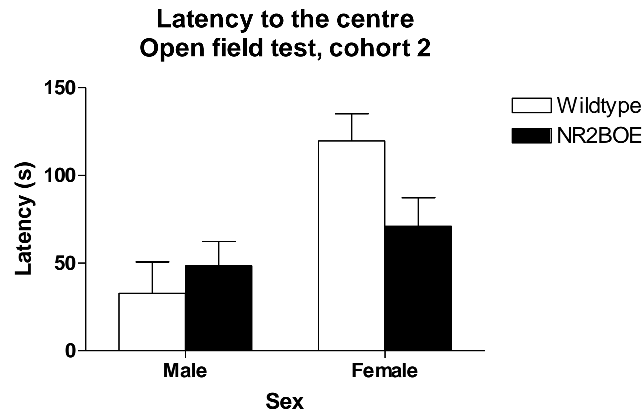


Figure 51 The latency of mice to reach the centre of the open field was greatest for wildtype female mice. Time of mice from cohort 2 to reach the middle of the open field (s), mean ± SEM, wildtype (□) and NR2BOE (■) mice.

4.3.1.2. Cohort 2, time in centre

Wildtype and NR2BOE mice spent comparable lengths of time in the central 10cm diameter area of the open field. An ANOVA with two between subjects factors (genotype and sex) was performed. There was no effect of genotype (F(1,45)=0.613, p=0.438) or sex (F(1,45)=2.36, p=0.131) or genotype by sex interaction on time time spent in the centre (F(1,45)=0.174, p=0.678; Figure 52). There were: male wildtypes = 12, female wildtypes = 12, male NR2BOE = 12, female NR2BOE = 13.

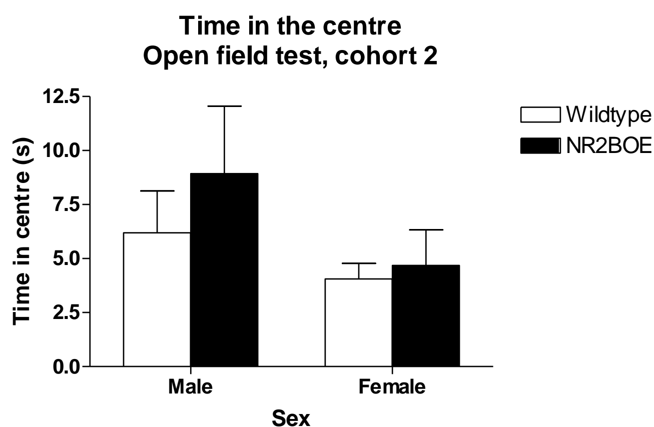


Figure 52 The time mice spent in the centre of the open field was similar for wildtype and NR2BOE mice. Length of time mice from cohort 2 spent in the middle of the open field (s), mean ± SEM, wildtype (☐) and NR2BOE (■) mice.

4.3.1.3. Cohort 2, time at the edges (thigmotaxis)

There was no difference between control and NR2BOE mice on the time spent within 5cm of the sides of the open field. However, male mice spent significantly less time than females at the sides. An ANOVA with two between subjects factors (genotype and sex) was performed. There was no effect of genotype ($F(1,45)=0.328$, $p=0.569$) but a significant effect of sex ($F(1,45)=8.611$, $p=0.005$) driven by female mice spending more time at the sides than male mice, but no genotype by sex interaction on the time spent at the sides ($F(1,45)=0.754$, $p=0.39$;

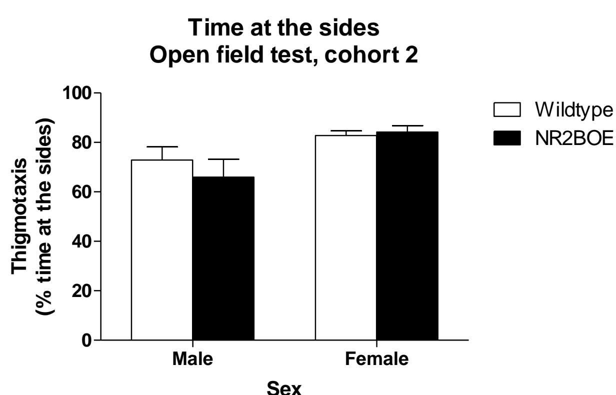


Figure 53). The mean time spent at the sides by males = 208.6±13.22 seconds, mean time spent at the sides by females = 250.5±4.72 seconds. There were: male wildtypes = 12, female wildtypes = 12, male NR2BOE = 12, female NR2BOE = 13.

Figure 53 Female mice spent more time at the sides of the open field than males. The % of time (out of 300 seconds) mice from cohort 2 spent in the middle of the open field (s), mean ± SEM, wildtype (□) and NR2BOE (■) mice.

4.3.1.4. Both cohorts, latency to centre

Mice from the first cohort took longer to reach the centre of the open field than mice from the second cohort. An ANOVA with three between subjects factors (genotype, sex and cohort) was performed. There was no effect of genotype ($F(1,107)=2.029$, $p=0.157$) or sex ($F(1,107)=0.22$, $p=0.64$) and no genotype by sex interaction ($F(1,107)=1.294$, $p=0.258$;

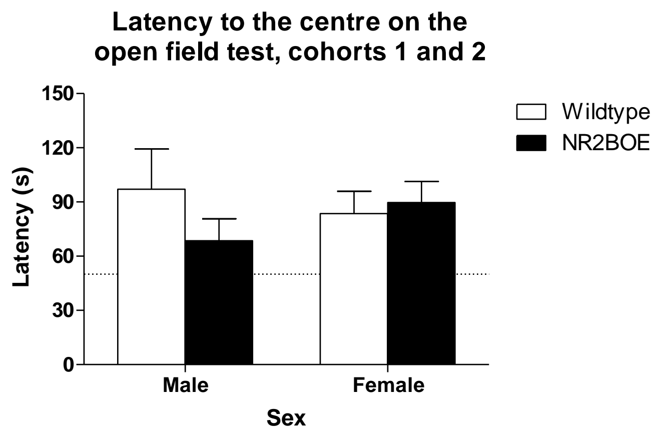


Figure 54). Male wildtype mean = 97±22.3, female wildtype mean = 83.6±12.3, male NR2BOE mean = 68.6±12.1, female NR2BOE mean = 89.7±11.6.

There was a significant effect of cohort (F(1,107)=7.161, p=0.009), driven by mice from the first cohort taking longer to reach the centre than mice from cohort 2 (mean latency (s) ± SEM for mice from cohort: 1 = 98.74±19.73. 2 = 67.99±15.85). There was no genotype by cohort (F(1,107)=0.036, p=0.85) interaction, although there was a sex by cohort interaction (F(1,107)=13.307, p<0.001), driven by a significant difference between the latency of males from cohorts 1 and 2 to reach the centre of the open field (mean latency (seconds) for cohort 1 males = 117.52±18.25, mean latency for cohort 2 males = 40.29±11.31). There was also a significant genotype by sex by cohort interaction (F(1,107)=12.598, p=0.001), due to a significant difference between the wildtype and NR2BOE male mice of cohort 1 (F(1,107)=10.089, p=0.002), between the wildtype male and female mice of cohort 1 (F(1,107)=16.763, p<0.001), between the wildtype male and female mice of cohort 2 (F(1,107)=9.606, p=0.002) and between wildtype male mice of cohorts 1 and 2 (F(1,107)=22.824, p<0.001). There were: male wildtypes = 25, female wildtypes = 32, male NR2BOE =29, female NR2BOE = 29.

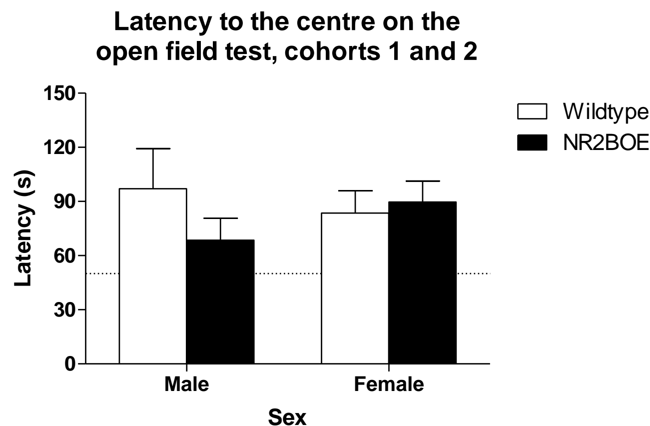


Figure 54 The latency of mice to reach the centre of the open field task was not significantly different for wildtype and NR2BOE mice. Time of mice from both cohorts to reach the middle of the open field (s), mean ± SEM, wildtype (□) and NR2BOE (■) mice.

4.3.1.5. Both cohorts, time in centre

There was no effect of genotype on the length of time mice spent in the centre of the open field. An ANOVA with three between subjects factors (genotype, sex and cohort) was performed.

There was no effect of genotype (F(1,107)=0.01, p=0.921) or sex (F(1,107)=0.025, p=0.873) and no genotype by sex interaction on time in the the centre (F(1,107)=2.065, p=0.154) as shown in

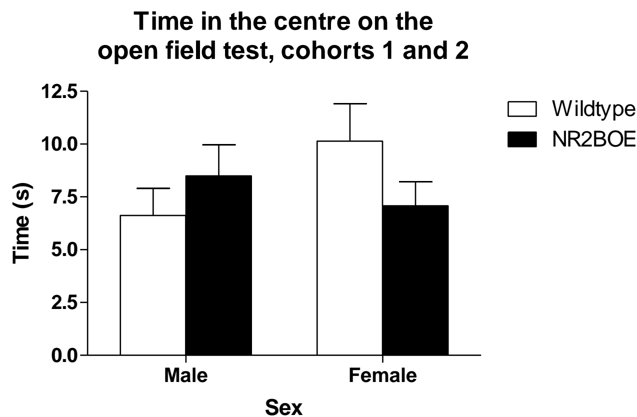


Figure **55**. There was a significant effect of cohort (F(1,107)=5.935, p=0.016), driven by mice from the first cohort spending more time in the centre than mice from the second cohort (mean time (s) ± SEM spent in the centre by mice from cohort: 1 = 10.59±2.03, 2 = 5.97±1.85). There was no genotype by cohort interaction (F(1,107)=1.63, p=0.205) although there was a significant sex by cohort interaction (F(1,107)=5.716, p=0.019), driven by a significant difference between the length of time spent in the centre by the females of cohorts 1 and 2 (F(1,107)=12.22, p=0.001). There was no genotype by sex by cohort interaction (F(1,107)=0.494, p=0.484). There were: male wildtypes = 25, female wildtypes = 32, male NR2BOE =29, female NR2BOE = 29.

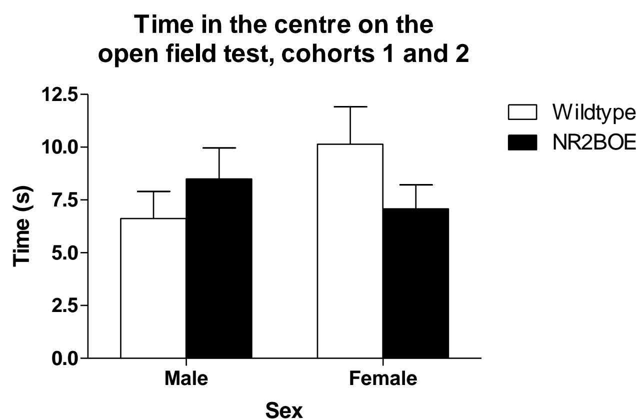
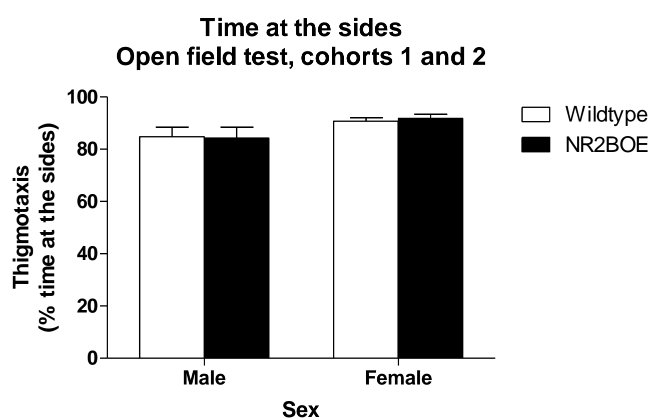


Figure 55 The length of time mice spent in the centre of the open field task was similar for wildtype and NR2BOE mice. Time mice from both cohorts spent in the middle of the open field (s), mean ± SEM, wildtype (□) and NR2BOE (■) mice.

4.3.1.6. Both cohorts, time at the edges (thigmotaxis)

There was no difference between control and NR2BOE mice on the time spent within 5cm of the sides of the open field. However, male mice spent less time at the sides than female mice. An ANOVA with three between subjects factors (genotype, sex and cohort) was performed. There was no effect of genotype (F(1,107)=0.248, p=0.62), a significant effect of sex (F(1,107)=9.359, p=0.003) caused by females spending more time at the sides than males (mean time at the sides (s) ± SEM for: males = 250.5±8.4, females = 269.5±3.2) but no genotype by sex interaction on time at the edges



(F(1,107)=1.521, p=0.22;

Figure **56**). There was a highly significant effect of cohort (F(1,107)=93.804, p<0.001), driven by mice from cohort 1 spending significantly longer at the sides than mice from cohort 2 (mean time (s) at the sides ± SEM for mice from cohort: 1 = 275.1±15.6, 2 = 229.4±12.8). There was no genotype by cohort interaction (F(1,107)=0.656, p=0.42) although there was a significant sex by cohort interaction (F(1,107)=13.232, p<0.001). Simple main effects showed that there was a significant difference between the male and female mice of cohort 2 (F(1,107)=19.82, p<0.001; mean time at the sides (s) ± SEM for cohort 2: males = 208.6±13.2, females = 250.5±4.7) and between the different sexes of both cohorts (males of cohort 1 and 2: F(1,107)=84.798, p<0.001. Females of cohort 1 and 2: F(1,107)=19.18, p<0.001. Mean time at the sides (s) ± SEM for males of cohort: 1 = 292.3±1.1, 2 = 208.6±13.2. Mean time at the sides (s) ± SEM for females of cohort: 1 = 288.5±1.5, 2 = 250.5±4.7).

There was no genotype by sex by cohort interaction (F(1,107)=0.56, p=0.456). There were: male wildtypes = 25, female wildtypes = 32, male NR2BOE =29, female NR2BOE = 29.

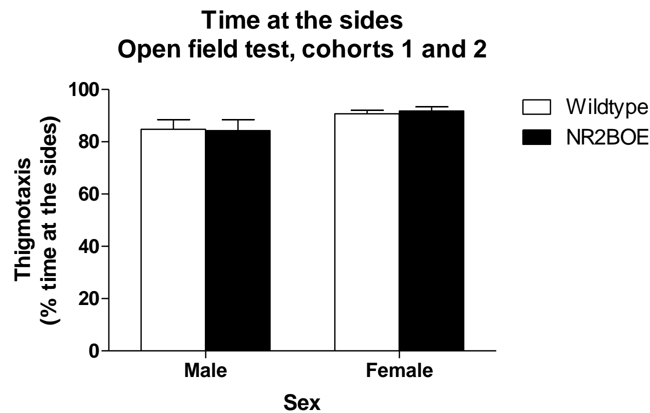


Figure 56 The time mice spent at the sides of the open field task was greater for female mice than males. Time mice from both cohorts spent in the middle of the open field (s), mean ± SEM, wildtype (☐) and NR2BOE (■) mice.

4.4. Y maze (spatial reference memory)

4.4.1. Second cohort

Mice made more correct choices on later days as they learned this task. An ANOVA with two between subjects factors (genotype and sex) and one within subjects factor (day) with 16 levels (corresponding to the 16 days of trials) was performed. There was no effect of genotype (F(1,18)=2.002, p=0.174;

Figure **57**) or sex (F(1,18)=1.091, p=0.31), nor a genotype by sex interaction (F(1,18)=0.292, p=0.595). There was a significant effect of day (F(15,270)=33.433, p<0.001) caused by more correct choices being made on later days, and a genotype by day interaction (F(15,270)=1.731, p=0.045) caused by significant differences between wildtype and NR2BOE mice on days 4 (F(1,18)=10.888, p=0.004) and 5 (F(1,18)=6.331, p=0.022; mean correct choices on day 4 for: wildtypes = 8.67±0.29, NR2BOE = 6.46±0.49. Mean correct choices on day 5 for: wildtypes = 8.89±0.35, NR2BOE = 7.08±0.52). There

was no sex by day interaction (F(15,270)=0.4, p=0.978), or genotype by sex by day interaction (F(15,270)=1.528, p=0.095). There were: male wildtype = 4, female wildtype = 5, male NR2BOE = 7, female NR2BOE = 6.

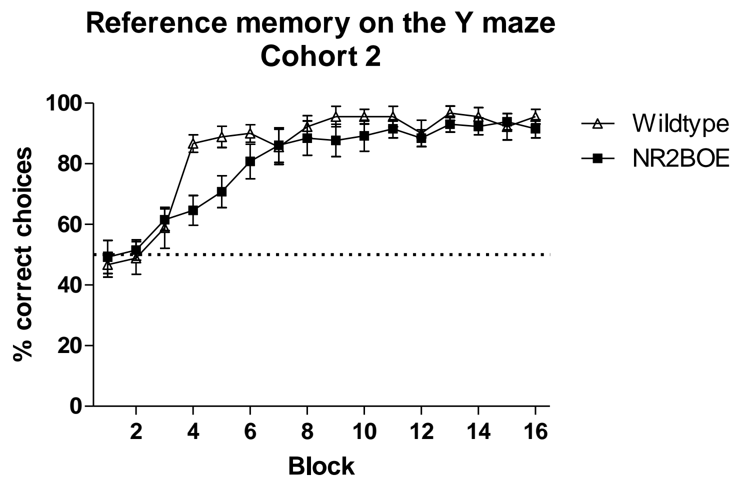
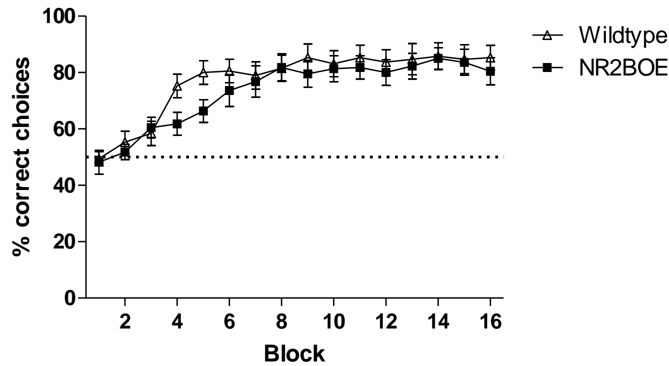


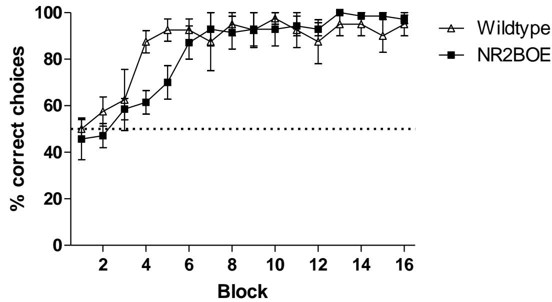
Figure 57 The number of correct choices made by mice increased as they acquired the spatial reference Y maze. Wildtype and NR2BOE mice from cohort 2 displayed improved performance as they learned the task. Mean ± SEM, Wt = wildtype (Δ) and NR2BOE (■), the dotted line indicates chance levels (50%).

4.4.2. First and second cohorts combined (16 days)

Mice made more correct choices on later days. An ANOVA, with three between subjects factors (genotype, sex and cohort) and one within subjects factor (day) with 16 levels (corresponding to the 16 days of trials). When all data across all days was analysed, there was no effect of genotype (F(1,35)=1.332, p=0.256), sex (F(1,35)=0.363, p=0.55) and no genotype by sex interaction (F(1,35)=0.097, p=0.757). However, there was a significant effect of cohort (F(1,35)=6.286, p=0.017) driven by the second cohort making significantly more correct choices (mean number of correct choices made ± SEM for cohort: 1 = 7.02±0.36, 2 = 8.19±0.3). There was no genotype by cohort interaction (F(1,35)<0.001, p=0.995) and no sex by cohort interaction (F(1,35)=0.097, p=0.757).

There was a significant effect of day ($F(15,525)=30.922$, $p<0.001$), but no genotype by day interaction ($F(15,525)=1.264$, $p=0.22$), sex by day interaction ($F(15,525)=0.346$, $p=0.99$) or genotype by sex by day interaction ($F(15,525)=1.319$, $p=0.185$). The trend towards sex by day interaction was driven by a significant difference between males and females on days 4 ($F(1,35)=6.624$, $p=0.014$), 5 ($F(1,35)=6.018$, $p=0.019$), 6 ($F(1,35)=6.424$, $p=0.016$), 7 ($F(1,35)=5.934$, $p=0.02$), 8 ($F(1,35)=8.043$, $p=0.008$), 9 ($F(1,35)=9.237$, $p=0.004$), 10 ($F(1,35)=12.772$, $p=0.001$), 11 ($F(1,35)=16.632$, $p<0.001$), 12 ($F(1,35)=6.827$, $p=0.013$), 13 ($F(1,35)=15.482$, $p<0.001$), 14 ($F(1,35)=10.899$, $p=0.002$), 15 ($F(35)=9.053$, $p=0.005$) and 16 ($F(1,35)=16.921$, $p<0.001$). Males performed significantly better than females on these days. There were: male wildtype = 4, female wildtype = 15, male NR2BOE = 7, female NR2BOE = 15. Note that the male mice from cohort 1 performed this task for 12 days rather than 16 days, and so have not been included in this analysis.





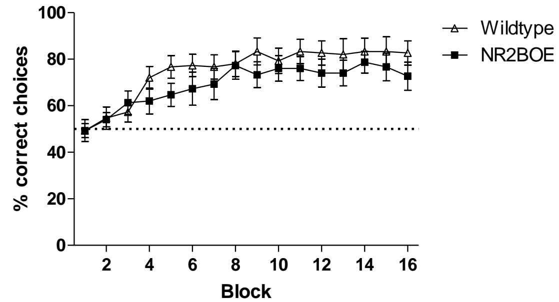


Figure 58 The number of correct choices made by mice increased as they acquired the spatial reference Y maze on 16 days. Wildtype and NR2BOE mice from both cohorts displayed similar levels of performance. Mean ± SEM, Wt = wildtype (Δ) and NR2BOE (■), the dotted line indicates chance levels (50%).

4.4.3. First and second cohorts combined (first 12 days only)

As the male mice from the first cohort were only tested for 12 days, we also analysed the results for all mice for the first 12 days only. There was no difference between the control and NR2BOE mice in solving this task when the results from the first 12 days of testing only were analysed. An ANOVA with three between subjects factors (genotype, sex and cohort) and one within subjects factor (day) with 12 levels (corresponding to the 12 days of trials) was performed on the number of correct choices made. There was no effect of genotype ($F(1,62)=0.454$, $p=0.216$) or sex ($F(1,62)=1.563$, $p=0.216$), nor a genotype by sex interaction ($F(1,62)=1.293$, $p=0.26$).

There was a significant effect of day ($F(11,682)=59.583$, $p<0.001$), driven by an improvement in performance on later days. There was no genotype by day interaction ($F(11,682)=1.202$, $p=0.282$) or genotype by sex by day interaction ($F(11,682)=1.095$, $p=0.362$), although there was a significant sex by day interaction ($F(11,682)=2.703$, $p=0.002$) driven by a significant difference between male and female mice on days 10 ($F(1,62)=9.286$, $p=0.003$), 11 ($F(1,62)=10.015$, $p=0.002$) and 12 ($F(1,62)=10.328$, $p=0.002$; mean number of correct choices (out of 10) on day 10: males = 9.23±0.19, females = 7.77±0.38. Day 11: males = 9.43±0.14, females = 7.97±0.37. Day 12: males = 9.48±0.13, females = 7.83±0.4). Male mice chose the correct arm significantly more times than females on these days.

There was a significant effect of cohort ($F(1,62)=9.138$, $p=0.004$), driven by cohort 2 making more correct choices than cohort 1 (mean correct choices by cohort 1 = 6.94±0.29, mean correct choices by cohort 2 = 7.8±0.34). There was no genotype by cohort ($F(1,62)=0.995$, $p=0.322$) or sex by cohort ($F(1,62)=0.114$, $p=0.737$), nor a genotype by sex by cohort ($F(1,62)=1.289$, $p=0.261$) interaction. There was also no genotype by sex by day by cohort interaction ($F(11,682)=1.015$, $p=0.413$). Male wt = 16, female wt = 15, male NR2BOE = 24, female NR2BOE = 15.

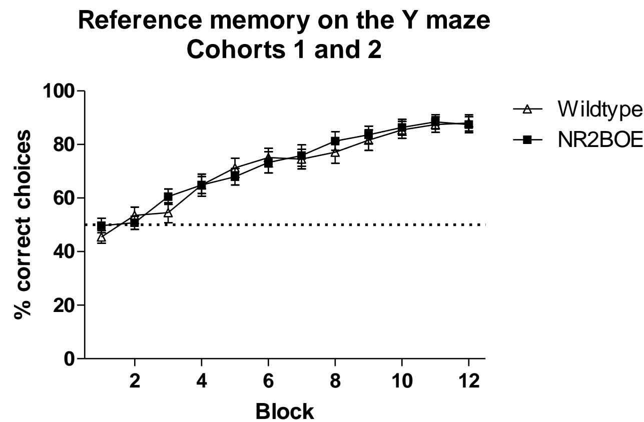


Figure 59 The number of correct choices made by mice increased as they acquired the spatial reference Y maze on 12 days. Wildtype and NR2BOE mice from both cohorts displayed similar levels of performance. Mean ± SEM, Wt = wildtype (Δ) and NR2BOE (■), the dotted line indicates chance levels (50%).

4.5. Discussion

Previously we had found that NR2BOE male mice were faster than male wildtype mice at finding the hidden platform of the watermaze. By contrast, NR2BOE female mice were significantly slower than wildtype female mice at reaching the platform. However, when we replicated the hidden platform watermaze with cohort 2, in mice that were of a comparable age to those used by Tang et al. (1999), we found that all mice had significantly shorter latencies for reaching the hidden platform than for the mice in cohort 1. Similarly, performance on the spatial reference memory Y maze task was also considerably improved in the second cohort of mice. When the two cohorts were combined there was not a significant effect of genotype or sex on the latency of mice to reach the centre of the open field, nor on their latency to reach the hidden platform, nor on the number of correct choices made on the spatial reference memory Y maze.

It is unclear what caused this improvement in performance on the watermaze and spatial reference memory Y maze by both wildtype and NR2BOE mice in cohort 2. This finding is particularly surprising in view of the fact that the results from older mice fairly closely replicated those of Tang et al, but that the results observed in mice of an equivalent age were considerably different.

As a result, it was fundamentally important to establish that the NR2B overexpression had not been lost, for example between generations. We sought to do this using the brains from the mice of cohort 2, as described in chapter 6.

5. Chapter 5: NR2B$^{\Delta HPC}$ and GluN1$^{\Delta HPC}$ mice

5.1. Background

In order to generate mice in which the NR2B subunit was knocked out specifically in the CA1 and dentate gyrus regions, exon 9 of the NR2B gene was flanked by loxP sites in progenitor mice. In the presence of Cre recombinase (expression of which is controlled by a tissue specific promoter), the area flanked by the loxP sites is removed, which prevents the functional formation of the NR2B subunit, thereby knocking it out in the regions expressing Cre. This allows region- and time- specific knockout of particular genes (Lewandoski 2001). Recombination of unrelated, endogenous mouse genes with regions similar to the lox sites can sometimes occur, but it is rare and so seldom interferes with the desired knockout (Lewandoski 2001).

In order to ensure the knockout was successful, NMDAR-mediated EPSPs were induced by stimulating the Schaffer collaterals. NMDARs containing NR2B have a slower decay time than NMDARs containing NR2A, and are sensitive to block by the NR2B antagonist ifenprodil. As expected, hippocampal slices from the NR2B$^{\Delta Fb}$ mice (in which NR2B was knocked out throughout the forebrain) were insensitive to ifenprodil compared to control slices, and also had much faster decay kinetics, confirming the loss of NR2B. *In situ* hybridisations confirmed the absence of NR2B mRNA in the CA1 pyramidal cells (Figure 60, from Von Engelhardt et al (von Engelhardt et al 2008) supplementary information).

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Figure 60 *In situ* hybridization with NR2B$^{\Delta HPC}$ mice. Radioactive in situ hybridization on coronal sections derived from an adult NR2B$^{2lox/2lox}$ mouse showed expression of NR2B mRNA in the principal cell layers of the hippocampus, in different cortical layers, thalamus, caudate putamen and amygdala in wildtype mice. In NR2B$^{\Delta HPC}$ mice, expression of NR2B mRNA was substantially reduced in the dorsal CA1 region and the entire dentate gyrus of the hippocampus. A: amygdala; CPu: caudate putamen; Hipp: hippocampus; SCx: somatosensory cortex; T: thalamus; VCx: visual cortex .Scale bar: 2.5 mm. Figure from Von Engelhardt et al (2008) supplementary information.

Several behavioural tasks had been performed previously with the NR2B$^{\Delta Fb}$ mice and another strain (in which NR2B had been ablated from the hippocampus only; NR2B$^{\Delta HPC}$; see von Engelhard et al, 2008 (von Engelhardt et al 2008)). The NR2B$^{\Delta Fb}$ mice were impaired across a range of tasks, whereas the impairment displayed by NR2B$^{\Delta HPC}$ mice was more subtle, suggesting that much of the impairment in NR2B$^{\Delta Fb}$ mice may be due to the lack of extrahippocampal NR2B. The NR2B$^{\Delta Fb}$ mice were impaired on appetitive and non-appetitive spatial working memory tasks (rewarded and spontaneous alternation respectively) and appetitively and aversively motivated spatial reference memory tasks (the Y maze and watermaze respectively), egocentric spatial memory (in which mice

learn to make a turn in a particular direction relative to themselves, e.g. always turn right), object recognition and visual discrimination tasks. If the deficit observed on visual discrimination tasks reflected an impairment in the mice's vision, this may explain the deficits on spatial tasks which are believed to rely on visual cues. However, as the NR2B$^{\Delta Fb}$ mice were also impaired on egocentric tasks (which are solved independently of visual cues) it is likely that the deficit is due to a broader impairment than simply vision. As the NR2B$^{\Delta Fb}$ mice were impaired on all learning and memory tasks on which they were tested, it is impossible to accurately attribute the cause as it is impossible to know whether the impairment was due to learning and memory or another attribute, such as a sensorimotor or motivational impairments. Thus the NR2B$^{\Delta HPC}$ mice were of considerable interest, as they had undergone much less comprehensive behavioural analysis than the NR2B$^{\Delta Fb}$ mice, and a more specifically delineated impairment observed in the NR2B$^{\Delta HPC}$ mice may help to establish the areas responsible for the different elements of memory and behaviour.

The NR2B$^{\Delta HPC}$ mice were slightly hyperactive and less anxious compared to wildtype littermates (NR2B$^{2lox/2lox}$), although the differences were much reduced compared to those seen in the NR2B$^{\Delta Fb}$ mice. The NR2B$^{\Delta HPC}$ mice were comparable to wildtypes on tasks of motor co-ordination and on the hidden platform watermaze, although on the second transfer test (in which the platform is removed from the pool entirely) the NR2B$^{\Delta HPC}$ mice spent most of their time swimming in the location in which the hidden platform had been located (training quadrant), and significantly more time than wildtype mice. When this transfer test was analysed in detail, a significant interaction was apparent between the genotypes and time bins, such that NR2B$^{\Delta HPC}$ mice appeared to be spending more time swimming in the training quadrant in the final 15 seconds of the transfer test compared to controls. This suggests that the NR2B$^{\Delta HPC}$ mice were making perseverance errors, possibly due to an inability to remember where they had just been swimming. It is also possible that the perseverance errors were a result of mismatch anxiety caused by a difference between the expected

outcome and the stimuli received from the environment; the mice have memories of the platform position from previous tasks and so swim to the expected location, but are unable to coordinate the lack of a platform at the expected location with the perception that the platform is not present. The NR2B$^{\Delta HPC}$ mice were also impaired on a reversal task in the watermaze, in which the hidden platform was moved to the opposite quadrant of the pool.

Other tasks performed with the NR2B$^{\Delta HPC}$ mice included a spatial working memory task (spontaneous alternation on the T maze), on which NR2B$^{\Delta HPC}$ mice were slightly but significantly impaired compared to wildtype mice. The impairment was not as great as that observed in NR2B$^{\Delta Fb}$ mice. However, as these behavioural assessments were preliminary it was of considerable interest to consider the behaviour of the NR2B$^{\Delta HPC}$ mice more comprehensively.

We also considered the behaviour of a strain of mice in which the NR1 subunit of the NMDA receptor had been knocked out in the dentate gyrus and CA1 region of the hippocampus (GluN1$^{\Delta HPC}$ mice). The NR1 subunit is the obligatory subunit for the formation of functional NMDA receptors, so knocking out NR1 knocks out NMDA receptors in that region. When compared to the NR2B$^{\Delta HPC}$ mice, these mice allowed us to establish the effect of removing NMDA receptors or only a subset of NMDA receptors in the hippocampus.The GluN1$^{\Delta HPC}$ mice were generated by combining two mouse strains, one of which had *loxP* flanking exons 11-18 of the GluN1 gene (*Grin1*), the other strain expressing tTA dependent Cre recombinase driven by the α-Ca^{2+}-calmodulin dependent protein kinase II/NR2C hybrid promoter. Pregnant mothers were maintained on doxycyclin (50mg/l) in their drinking water to restrict expression of Cre to the hippocampus, and pups were not exposed to doxycyclin, which led to Cre expression and so ablation of the *Grin1* gene. Knockout of the NR1 subunit was confirmed by examining LTP at CA3-CA3 and CA3-CA1 synapses using electrophysiological analysis. The NR1

receptor was knocked out in these mice specifically in the hippocampus and dentate gyrus only (see Figure 61 from manuscript in preparation, Bannerman et al 2011).

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Figure 61 Hippocampal slices from control (top) and GluN1$^{\Delta HPC}$ (bottom) mice. The NR1 subunit was present at greatly reduced levels in the CA1 region of the hippocampus and almost completely absent from the dentate gyrus. DG = dentate gyrus.

The GluN1$^{\Delta HPC}$ mice have been shown previously to be able to acquire the spatial reference memory task, the Morris water maze (manuscript in preparation). On the transfer tests, in which the platform is removed, GluN1$^{\Delta HPC}$ mice again actually spent longer swimming in the area the platform had been (comparable to the results observed in the NR2B$^{\Delta HPC}$ mice). Despite this, which could be interpreted as demonstrating superior spatial reference memory on the watermaze, they were unable to acquire the spatial reference component of the radial arm maze. On a form of the watermaze with two identical beacons (one of which indicated the platform position and one of which did not), GluN1$^{\Delta HPC}$ mice were unable to accurately select the beacon indicating the platform

position, despite knowing the exact spatial location of the platform. It has been suggested that HPC NMDA receptors are important in disambiguating similar, competing spatial memories and inhibiting incorrect behavioural responses (manuscript in preparation). It is also interesting to note that, in addition to the comparable results for the NR2B$^{\Delta HPC}$ mice and GluN1$^{\Delta HPC}$ mice on the watermaze transfer tests, both strains appeared to be impaired on spatial working memory (NR2B$^{\Delta HPC}$ mice demonstrated a significant impairment on a task of spontaneous alternation (von Engelhardt et al 2008), whereas the GluN1$^{\Delta HPC}$ mice made more working memory errors on the radial arm maze (Niewoehner et al 2007). Thus it could be that the GluN1$^{\Delta HPC}$ and NR2B$^{\Delta HPC}$ mice are unable to sufficiently inhibit incorrect memories in the light of unexpected environmental stimuli.

All experiments were carried out under the auspices of the Home Office (UK). For the behavioural testing of the CA1 and DG NR2B KO mice, tests were performed using age-matched littermate wildtype and NR2B$^{\Delta HPC}$ mice, and littermate wildtype and GluN1$^{\Delta HPC}$ mice, of both sexes. Mice were housed in littermate groups of 1 to 4 and tested during the light phase of the day.

5.2. Materials and Methods

5.2.1. Delays on the T maze, rewarded alternation (spatial working memory)

This task was performed as described for the NR2BOE mice (see chapter 2 (p67-69) for details).

The intertrial interval was approximately 10 minutes, and mice received 8 trials a day. The mice were trained in this manner for 10 days (80 trials), after which trials with a 20 second delay were introduced (between the sample trial and the choice trial), interleaved with normal, “no delay”

trials (for 5 days for the NR2B$^{\Delta HPC}$ mice and for 7 days for the GluR1$^{\Delta HPC}$ mice, four trials for each condition per day, such that NR2B$^{\Delta HPC}$ mice ran a total of 20 trials for each condition and GluR1$^{\Delta HPC}$ mice ran a total of 28 trials per condition). For each delay condition (four trials with no delay, four with a 20 second delay), mice turned left twice and right twice according to a pseudorandom sequence, which also interleaved delay conditions. This ensured that the mouse made equal numbers of turns in each direction on each day, and at each delay condition, with no more than two consecutive trials of either no delay or delay condition, and no more than three consecutive trials with a turn in either direction. The time interval between the sample and choice run was approximately 3 seconds for the "no delay" trials and 20 seconds when a delay was introduced.

5.2.2. Spontaneous alternation

This task was as described for the rewarded alternation task, except that mice were not food deprived and there was no food reward for alternating. There was also a dividing central partition equidistant between the two arms that prevented mice from visually sampling the other arm. Both arms were open so that mice could freely choose the first arm, and once a mouse had entered either arm (when all four paws had passed the start of the arm) a door closed the arm and the mouse was allowed to explore the arm for 30 seconds. The mouse was removed, the door was raised to allow access to both arms once more, and the mouse was returned to the start arm and was once again free to explore. The mouse was counted as having alternated if the arm it entered on the choice run was the opposite one to the one it had selected on the sample run. The intertrial interval was approximately 10 minutes, and mice received 4 trials a day for three days, giving a total of 12 trials. Both the NR2B$^{\Delta HPC}$ and the GluN1$^{\Delta HPC}$ strains of mice performed this task.

5.2.3. Spatial discrimination using visible beacons in the hidden platform watermaze (spatial reference memory)

The maze consisted of a 2m diameter circular tank, of depth 0.6m, elevated to a height of 0.6m, containing water at 21°C ± 1 at a depth of 0.3m. The platform (diameter 21cm) had a wire mesh across the top and was submerged 1cm beneath the surface of the water (Figure 62). Both the platform and watermaze were painted white. To escape from the maze, the mice had to find the submerged platform within 90 seconds of being placed in the water. The position of the platform was indicated by a spherical, black float supported over the platform (S+). A circle of white filter paper was laminated and adhered to the top of the beacon to prevent the computer tracking the beacon as opposed to the mice. If the mouse had not reached the platform within 90 seconds, the mouse was removed from the water and placed on the platform by the experimenter.

5.2.3.1. Pretraining

Animals were trained to swim to a single beacon for three days as pre-training. The platform location varied from trial to trial but was always indicated by the beacon. Mice were placed in the pool from one of eight positions, counterbalanced across the 3 days (8 trials per day), such that for equal number of trials the starting location of the mouse was in the same quadrant as the platform or the opposite quadrant to the platform, or the quadrants adjacent to the platform.

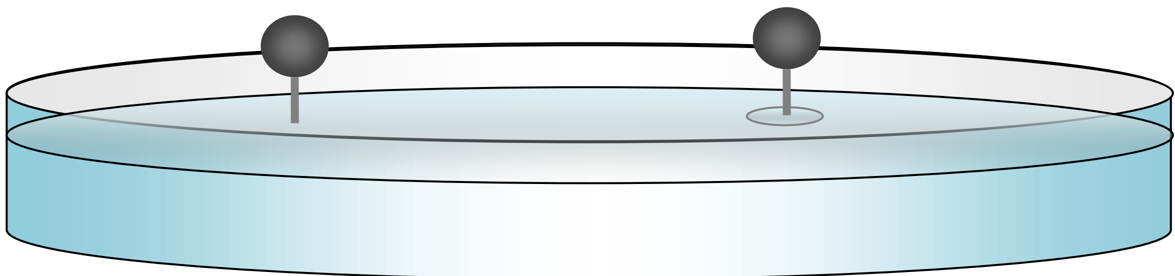


Figure 62 The hidden platform false beacon task. Two beacons in a constant position either always act as a marker for the position of the hidden platform, or as a distractor in the opposite quadrant to the platform. The mouse must learn the spatial relationships between the two beacons in conjunction with the extramaze cues in order to remember which is the distractor and which is the platform and thereby reach the platform quickly on later trials.

5.2.3.2. Spatial discrimination training

During spatial discrimination training, the platform position was always fixed. Another identical beacon which was held in place on a thin metal pole which was not attached to a platform was introduced to the quadrant opposite the platform (S-). Mice were able to use the beacons and the extramaze cues to navigate to the platform. The first platform approached was recorded (S- or S+), as well as the total number of approaches to the S- made before the platform was reached. An approach was noted as having been made if the mouse swam underneath the filter paper obscuring the beacon from the camera.

Trials were grouped and analysed per 3 days of trials. Across the three days, 24 trials were counterbalanced such that each mouse started swimming from 8 positions close to the target beacon (S+), 8 positions near the opposite beacon (S-), and 8 positions equidistant between the two beacons (Eq). No more than two consecutive trials started from close to the same beacon. Mice were allowed to swim until they reached the platform or 90s had elapsed. Animals that failed to find the platform within 90s were guided to, or placed on, the platform. Animals were allowed 30s on the platform before they were removed, and the inter-trial interval was approximately 10 minutes. For each trial, the length of time to reach the platform, thigmotaxis, the distance travelled, swim speed, the first beacon approached (correct or incorrect) and number of times the incorrect beacon was approached (errors) were recorded. This was then analysed to give the choice accuracy and number of errors made when the mouse started swimming from different start locations, and the number of trials (out of 24) in which the S+ was approached directly from each start position. Mice performed 8 trials a day for 21 days during the acquisition of the spatial discrimination training.

5.2.3.3. Probe trial

Probe trials were conducted on day 10 (after 72 trials) and day 16 (after 120 trials). For probe trials, both beacons and the platform were removed and the animals were allowed to swim freely for 60s before being removed. The percentage time spent in each quadrant (training quadrant, in which the platform had been present, adjacent quadrants and opposite quadrant) was analysed. Animals were considered to have learned the task if they spent the majority of their time swimming in the training quadrant.

5.2.3.4. Reversal

After 21 days, the platform position was reversed (to the position of the S-) for a further 12 days. Trials were conducted otherwise as indicated above, except with platform position swapped. The primary aim was to see if the NR2B$^{\Delta HPC}$ mice were able to learn the new platform position at the same rate as control mice, as in previous tests of spatial reference memory on the watermaze (without the beacons), the NR2B$^{\Delta HPC}$ mice showed a persistent preference for the old platform location. For example, in the transfer test at the end of acquisition training (in which the platform is removed from the pool), NR2B$^{\Delta HPC}$ mice spent significantly more time swimming in the platform location in the last 15 seconds of the task than controls. Furthermore, when the platform was moved to the opposite quadrant of the pool (reversal; see supplementary information of (von Engelhardt et al 2008)) the NR2B$^{\Delta HPC}$ took significantly longer to find the new position of the platform compared with controls. This was thought to correspond to perseverance errors, suggesting that NR2B$^{\Delta HPC}$ mice may have less flexible memories than control mice (von Engelhardt et al 2008).

5.2.3.5. Probe tests

A further probe task was performed after 264 trials, after the last day of reversal training had been conducted. As before, both beacons and the platform were removed and the animals were

allowed to swim freely for 60s. The percentage time spent in each quadrant was analysed and mice were considered to have learned the task if they spent the majority of their time in the training quadrant.

5.3. Results

5.3.1. GluN1 $^{\Delta HPC}$ T maze (spatial working memory)

5.3.1.1. Spontaneous alternation

There were no significant differences between the spontaneous alternation rates of control and GluN1$^{\Delta HPC}$ mice when results were collapsed across the four days (

Figure **63**). A repeated measures ANOVA with genotype and sex as between subject factors was performed on the alternation rate. There was no effect of genotype ($F(1,21)=0.003$, $p=0.954$) or sex ($F(1,21)=1.421$, $p=0.247$) or a genotype by sex interaction ($F(1,21)=0.498$, $p=0.488$). Control mean = 8.38±0.71, GluN1$^{\Delta HPC}$ mean = 8.25±0.74, male mean = 8.92±0.62, female mean = 7.67±0.78. When sex was removed as a factor, there was still no effect of genotype, confirmed by an independent samples t test of the difference between the means of the two genotypes. Wildtype males = 7, wildtype females = 6, GluN1$^{\Delta HPC}$ males = 6, GluN1$^{\Delta HPC}$ females = 6.

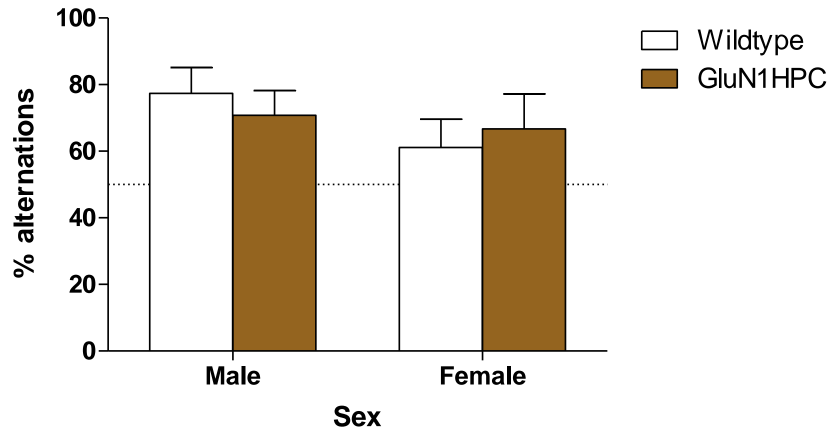
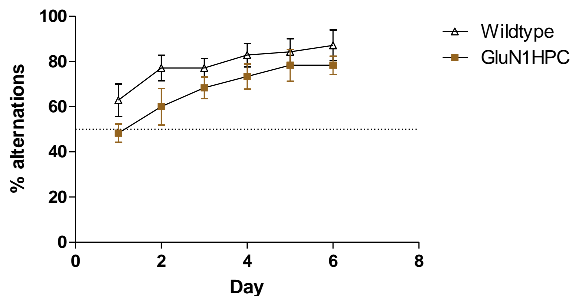


Figure 63 Mice alternated spontaneously on the T maze. Mean ± SEM during acquisition of the spontaneous alternation task for wildtype (Δ) and GluN1$^{\Delta HPC}$ (■) mice, the dotted line indicates chance levels (50%).

5.3.1.2. Rewarded alternation - GluN1$^{\Delta HPC}$

The GluN1$^{\Delta HPC}$ mice were significantly impaired compared to control mice on rewarded alternation (



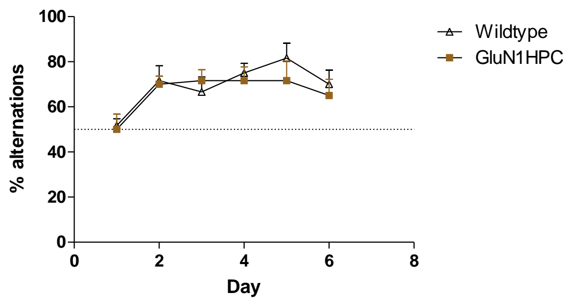
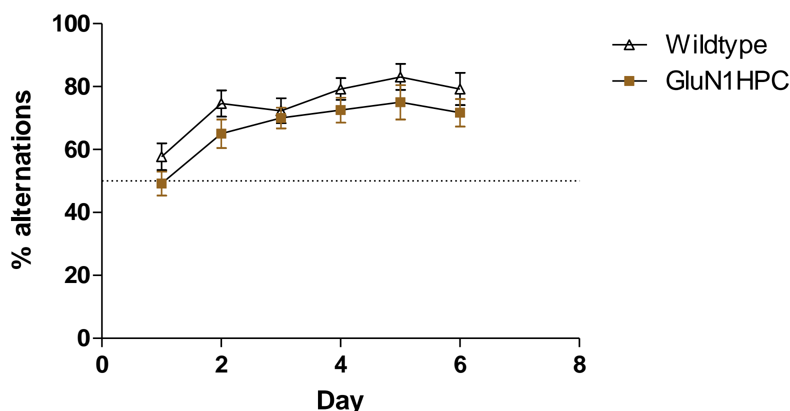
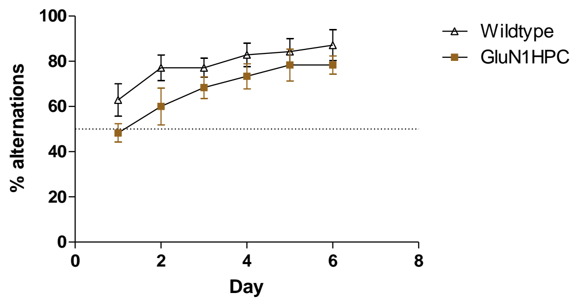


Figure 64). For the rewarded alternation task, a repeated measures ANOVA was performed, with genotype and sex as between subject factors, and day (6 levels corresponding to the 6 days of training) as the within subjects factor. There was a significant effect of genotype on the training days ($F(1,21)=5.926$, $p=0.024$). This was driven by wildtype mice making significantly more correct choices than GluN1$^{\Delta HPC}$ mice (wildtype mean correct choices (out of 60) = 44.6±1.24, GluN1ΔHPC mean correct choices (out of 60) = 40.3±1.3). There was also a significant effect of day ($F(5,105)=9.984$, $p<0.001$) but no genotype by day interaction ($F(5,105)=0.202$, $p=0.961$). There was a trend towards an effect of sex ($F(1,21)=3.372$, $p=0.08$), but no genotype by sex interaction ($F(1,21)=2.067$, $p=0.165$) or a sex by day interaction ($F(5,105)=0.949$, $p=0.453$). There was also no genotype by sex by day interaction ($F(5,105)=0.415$, $p=0.838$). Wildtype males = 7, wildtype females = 6, GluN1$^{\Delta HPC}$ males = 6, GluN1$^{\Delta HPC}$ females = 6.





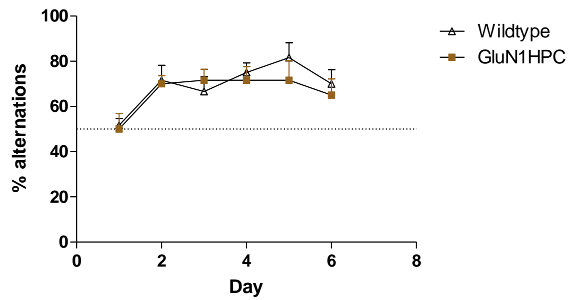
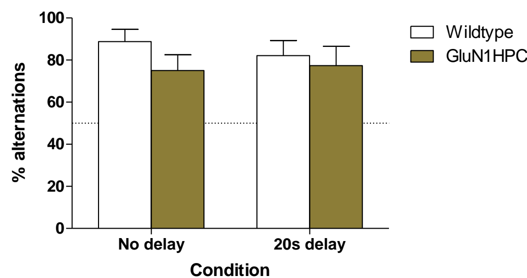


Figure 64 GluN1$^{\Delta HPC}$ mice were significantly impaired compared to wildtype mice on acquisition of the rewarded alternation task. Mean ± SEM during acquisition of rewarded alternation for all wildtype (Δ) and GluN1$^{\Delta HPC}$ (■) mice (a), and for male (b) and female (c) mice, the dotted line indicates chance levels (50%).

5.3.1.3. With and without a 20s delay between the sample and choice run

The GluN1$^{\Delta HPC}$ mice were significantly impaired compared to the wildtype mice on the rewarded alternation task when there was no delay between the sample and choice run, but there was no difference between the two genotypes when there was a 20 second delay between the sample and choice runs (



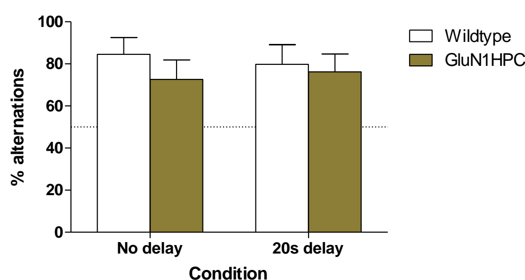
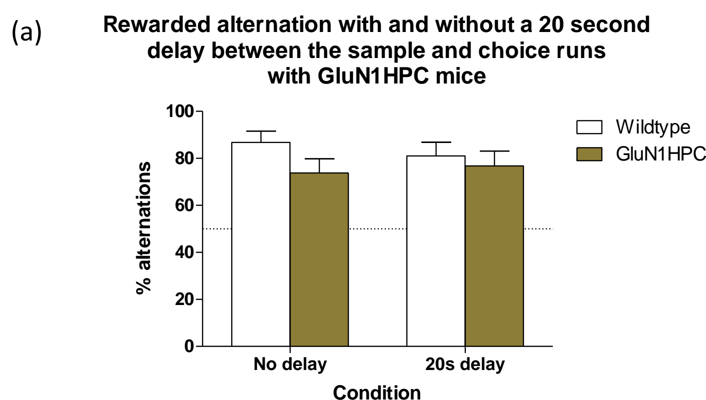


Figure 65). Performance of wildtype mice was worse at the delay condition compared to no delay condition. A repeated measures ANOVA with two between subjects factors of genotype and sex and one within subjects factor of condition (no delay and 20 second delay), was performed. There was a trend towards a significant effect of genotype ($F(1,21)=3.031$, $p=0.096$) driven by wildtype mice making more correct choices than GluN1$^{\Delta HPC}$ mice (control mean correct choices (out of 28) = 23.5±0.89, GluN1$^{\Delta HPC}$ mean correct choices (out of 28) = 21.1±1.08). However, there was no effect of sex ($F(1,21)=0.273$, $p=0.607$), no genotype by sex effect ($F(1,21)=0.025$, $p=0.877$), and no effect of condition ($F(1,21)=0.552$, $p=0.466$). Importantly, there was also a significant genotype by condition interaction ($F(1,21)=5.604$, $p=0.028$) driven by wildtype mice making more correct choices than GluN1$^{\Delta HPC}$ mice when there was no delay ($F(1,21)=6.313$, $p=0.02$) and a significant difference between control mice on the different delay conditions ($F(1,21)=5.022$, $p=0.036$; mean correct choices (out of 28) at no delay: wildtype = 24.31±0.76; GluN1$^{\Delta HPC}$ = 20.67±1.18. Mean correct choices (out of 28) at the 20 second delay: wildtype = 22.69±1.02; GluN1$^{\Delta HPC}$ = 21.5±0.99). There was no sex by condition effect ($F(1,21)=0.175$, $p=0.68$) and no genotype by sex by delay effect ($F(1,21)=0.009$, $p=0.927$). Wildtype males = 7, wildtype females = 6, GluN1$^{\Delta HPC}$ males = 6, GluN1$^{\Delta HPC}$ females = 6.



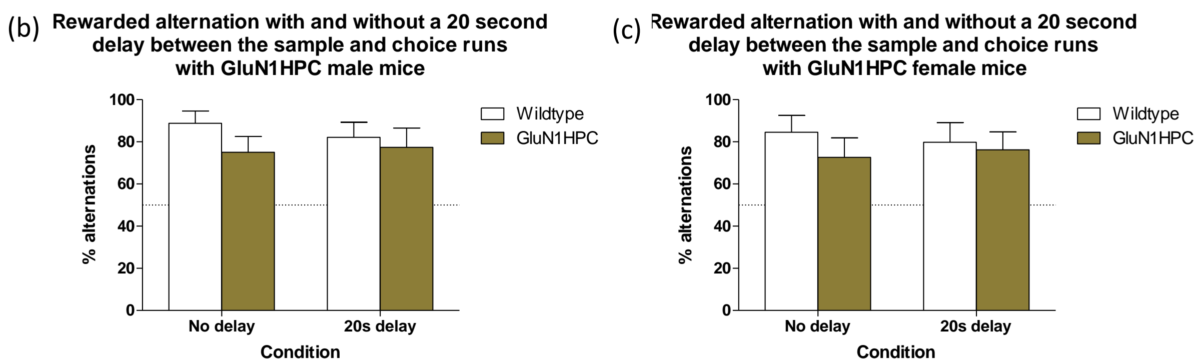


Figure 65 Wildtype and GluN1$^{\Delta HPC}$ mice alternated at similar levels when a 20 second delay was introduced between the sample and trial run, but GluN1$^{\Delta HPC}$ mice were still impaired compared to wildtype mice when there was no delay. Mean ± SEM for all wildtype (□) and GluN1$^{\Delta HPC}$ (■) mice on the rewarded alternation T maze with and without delays (a), and for male (b) and female (c) mice, the dotted line indicates chance levels (50%).

5.3.2. NR2B$^{\Delta HPC}$ T maze (spatial working memory)

5.3.2.1. Rewarded alternation

Wildtype and NR2B$^{\Delta HPC}$ mice alternated at above chance levels on the spatial working memory rewarded alternation T maze task.

5.3.2.2. Training days

For the training days, a repeated measures ANOVA with two between subjects factors (genotype and sex) and one within subjects factor (day) with 10 levels (corresponding to each day of testing) was performed. There was no effect of genotype (F(1,17)=1.841, p=0.193), no effect of sex (F(1,17)=0.212, p=0.651) although there was a significant effect of day (F(9,153)=2.061, p=0.036) and a genotype by day interaction (F(9,153)=2.596, p=0.008;

Figure **66**). This effect of day was driven by mice alternating more on later days, and the genotype by day interaction is driven by a significant difference between control and NR2B$^{\Delta HPC}$ mice on day 7 only (analysis of simple main effects; F(1,19),=6.505, p=0.02), control mean = 8.1±0.52,

NR2B$^{\Delta HPC}$ mean = 6.27±0.49). There was no genotype by sex interaction (F(1,17)=1.954, p=0.18) nor a sex by block (F(9,153)=1.408, p=0.189) or a genotype by sex by block interaction (F(9,153)=1.477, p=0.161). Wildtype = 10, NR2B$^{\Delta HPC}$ = 11.

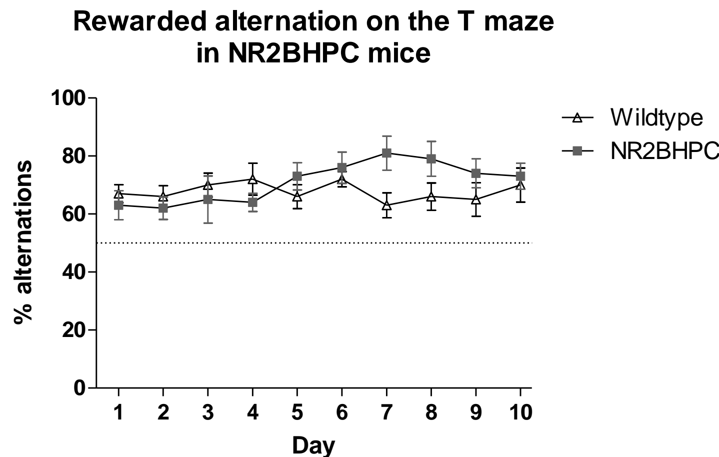


Figure 66 Wildtype and NR2B$^{\Delta HPC}$ mice alternated on this task. Mean ± SEM for wildtype (Δ) and NR2B$^{\Delta HPC}$ (■) mice, the dotted line indicates chance levels (50%).

5.3.2.3. With and without a 20 second delay

Wildtype and NR2B$^{\Delta HPC}$ mice perfomed similarly on this task. For the delay/no delay paradigm, a repeated measures ANOVA with two between subjects factors (genotype and sex) and one within subject factor (delay condition) with two levels (no delay, 20 second delay) was performed. There was no effect of genotype (F(1,17)=1.786, p=0.199) or sex (F(1,17)=2.628, p=0.123), no genotype by sex interaction (F(1,17)=2.961, p=0.103), a highly significant effect delay of condition (F(1,17)=31.714, p<0.001) but no condition by genotype interaction (F(1,17)=0.01, p=0.923;

Figure **67**). The effect of condition was driven by mice making significantly more correct choices on the no delay condition (mean correct choices (out of 20) with no delay: wildtype = 16.3±1.05, NR2B$^{\Delta HPC}$ =15.6±0.86. Mean correct choices (out of 20) with a 20 second delay: wildtype = 13.5±1.02,

NR2B$^{\Delta HPC}$ = 12.55±0.78). There was also no genotype by sex by condition interaction (F(1,17)=0.177, p=0.679). Wildtype = 10, NR2B$^{\Delta HPC}$ = 11.

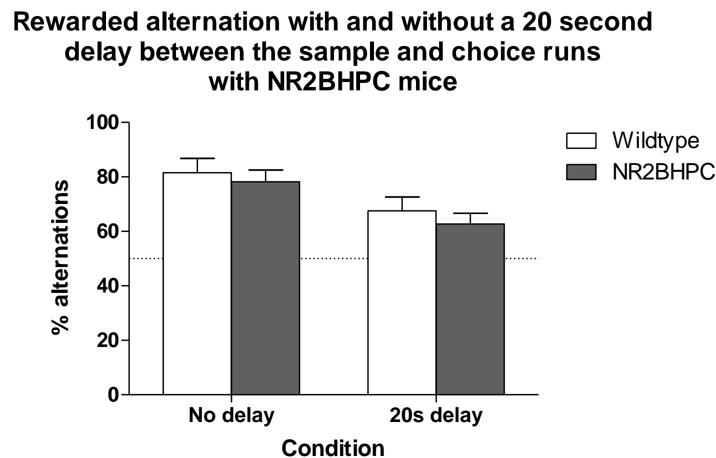


Figure 67 Wildtype and NR2B$^{\Delta HPC}$ mice alternated at similar levels when there was no delay, and when there was a 20 second delay, between the sample and choice runs. Mean ± SEM for wildtype (□) and NR2B$^{\Delta HPC}$ (■) mice, the dotted line indicates chance levels (50%).

5.4. NR2B$^{\Delta HPC}$ - Spatial discrimination using visible beacons in the hidden platform watermaze (spatial reference memory)

5.4.1. Pretraining – latency

There was no difference between control and NR2B$^{\Delta HPC}$ mice on latency to reach the platform during pretraining (

Figure **68**). A repeated measures ANOVA with two between subjects factors (genotype and sex) and one within subjects factor (day) with three levels (corresponding to the 3 days of pretraining) was performed on the latency to reach the platform. There was no effect of genotype on latencies (F(1,16)=0.011, p=0.916) no effect of sex (F(1,16)=0.065, p=0.803) and no genotype by sex interaction (F(1,16)=0.386, p=0.543). There was a significant effect of day on latencies

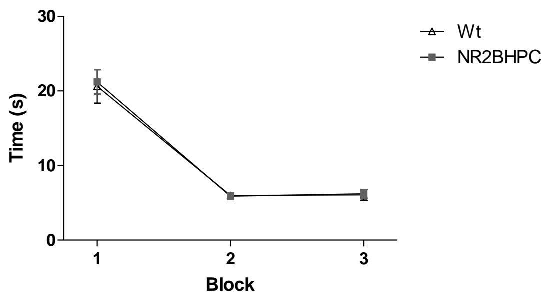
($F(2,32)=83.683$, $p<0.001$) but there was no genotype by day interaction ($F(2,32)=0.02$, $p=0.98$) or sex by day interaction ($F(2,32)=0.175$, $p=0.84$) nor a genotype by sex by day interaction ($F(2,32)=1.044$, $p=0.364$). Wildtype = 9, NR2B$^{\Delta HPC}$ = 11. One wildtype mouse that performed the rewarded alternation spatial working memory task was culled due to deteriorating health and so did not perform the spatial discrimination beacon watermaze.

5.4.2. Pretraining – distance

There was no difference between control and NR2B$^{\Delta HPC}$ mice on distances swum during pretraining (

Figure 68). A repeated measures ANOVA with two between subjects factors (genotype and sex) and one within subjects factor (day) with three levels (corresponding to the 3 days of pretraining) was performed on the distances swum. There was no effect of genotype on distance ($F(1,16)=0.101$, $p=0.754$) or sex ($F(1,16)=0.071$, $p=0.794$) although there was a trend towards a genotype by sex interaction ($F(1,16)=3.846$, $p=0.068$). There was an effect of day ($F(2,32)=84.055$, $p<0.001$) but there was no genotype by day interaction ($F(2,32)=0.45$, $p=0.642$) or sex by day interaction ($F(2,32)=321$, $p=0.728$) although there was a trend towards a genotype by sex by day interaction ($F(2,32)=3.144$, $p=0.057$). The genotype by sex by day interaction was caused by significant differences between male wildtype and NR2BOE on the different days, and female wildtype and NR2BOE mice on different days (the only non-significant comparisons were between male wildtype mice on days 2 and 3 and male NR2B$^{\Delta HPC}$ mice on days 2 and 3, and between female wildtype and NR2B$^{\Delta HPC}$ mice on days 2 and 3 and female wildtype and NR2B$^{\Delta HPC}$ mice on days 2 and 3). Wildtype male = 6, wildtype female = 3, NR2B$^{\Delta HPC}$ male = 8, NR2B$^{\Delta HPC}$ female = 3.

(a) (b)



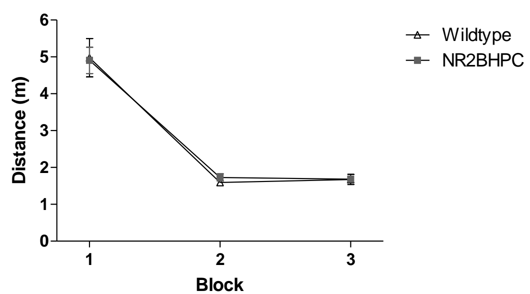
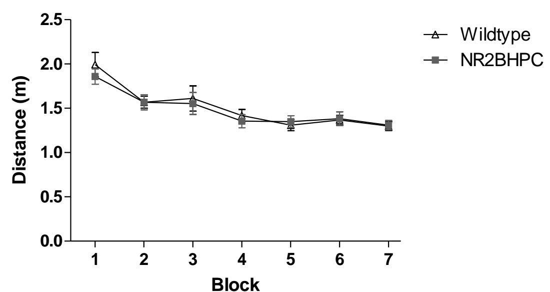


Figure 68 The latency and distance swum by wildtype and NR2B$^{\Delta HPC}$ mice to reach the platform on the pretraining trials decreased at similar rates. (a) Latency to reach the platform during pretraining. (b) Distance swum to reach the platform during pretraining. Mean ± SEM for wildtype (Δ) and NR2B$^{\Delta HPC}$ (■) mice.

5.4.3. Acquisition of the spatial discrimination – latencies

There was not a significant difference between control and NR2B$^{\Delta HPC}$ mice on latency,



distance or swim speed during acquisition (

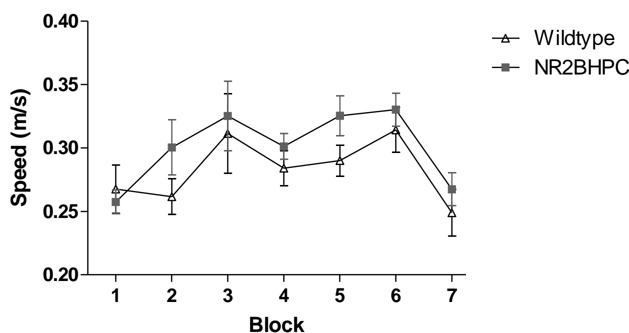


Figure 69). A repeated measures ANOVA with two between subjects factors (genotype and sex) and one within subjects factor (block) with seven levels (corresponding to the 7 blocks of

acquisition) was performed on the latency of mice to reach the platform. There was no effect of genotype on the latencies (F(1,16)=0.833, p=0.375) although there was a significant effect of sex (F(1,16)=18.215, p=0.001) caused by male mice taking more time to reach the platform than female mice (mean latency (s) ± SEM for: males = 5.72±0.3, females = 4.32±0.32). There was a non-significant trend towards a genotype by sex interaction (F(1,16)=3.282, p=0.089) caused by wildtype males being significantly slower than wildtype females (analysis of simple main effects, F(1,16)=17.711, p=0.001; mean latency (s) ± SEM for wildtype: males = 6.27±0.42, females = 4.16±0.36) and male wildtype mice being significantly slower than male NR2BΔHPC mice (F(1,16)=6.095, p=0.025; mean latency (s) ± SEM for male: wildtype = 6.27±0.42, NR2BΔHPC = 5.32±0.37). There was a significant effect of block on latency (F(6,96)=27.686, p<0.001) but no genotype by block interaction (F(6,96)=0.27, p=0.95), sex by block interaction (F(6,96)=0.902, p=0.497) or genotype by sex by block interaction (F(6,96)=1.628, p=0.148). Wildtype male = 6, wildtype female = 3, NR2B$^{\Delta HPC}$ male = 8, NR2B$^{\Delta HPC}$ female = 3.

5.4.4. Acquisition of the spatial discrimination – distances

There was no difference between the distances control and NR2B$^{\Delta HPC}$ mice swam to reach the platform (

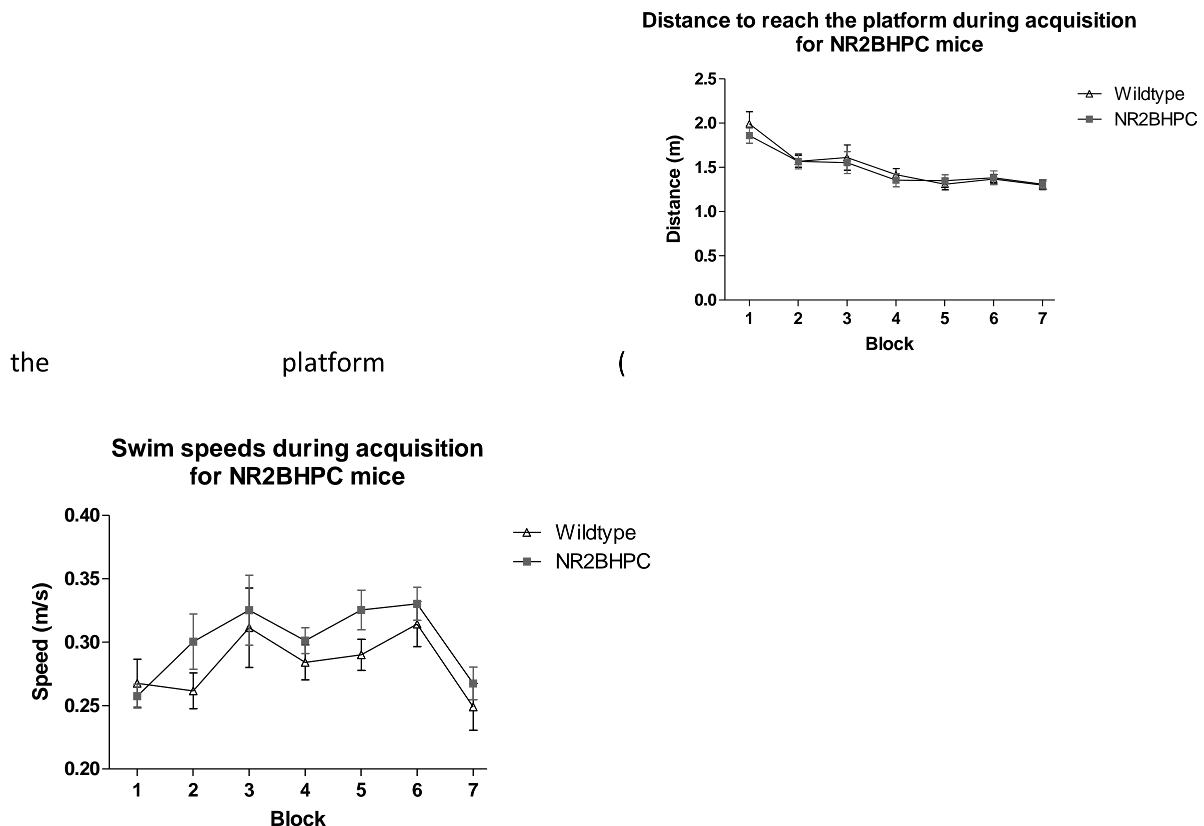
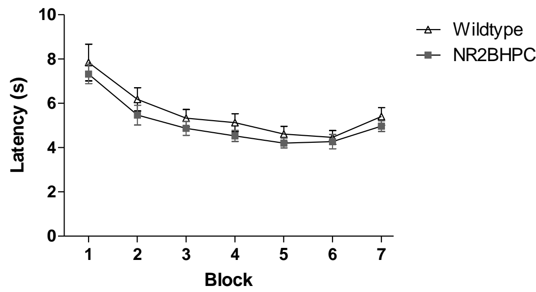
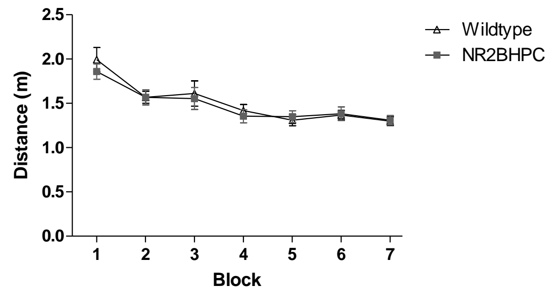


Figure 69). A repeated measures ANOVA with two between subjects factors (genotype and sex) and one within subjects factor (block) with seven levels (corresponding to the 7 blocks of acquisition) was performed on the distance swum. There was no effect of genotype (F(1,16)=0.246, p=0.627) although there was a significant effect of sex (F(1,16)=10.034, p=0.006) caused by males swimming further than females (mean distance (m) ± SEM for: males = 1.57±0.07, females = 1.33±0.06). There was no genotype by sex interaction (F(1,16)=0.017, p=0.898) on the acquisition trials. There was a significant effect of block (F(6,96)=15.806, p<0.001) caused by mice swimming shorter distances on later blocks but no genotype by block interaction (F(6,96)=0.257, p=0.955) or sex by block interaction (F(6,96)=0.928, p=0.478) or genotype by sex by block interaction (F(6,96)=0.688, p=0.66). Wildtype male = 6, wildtype female = 3, NR2BΔHPC male = 8, NR2BΔHPC female = 3.

5.4.5. Acquisition of the spatial discrimination – swim speeds

There was no difference between control and NR2B$^{\Delta HPC}$ mice on swim speed during acquisition. A repeated measures ANOVA with two between subjects factors (genotype and sex) and one within subjects factor (block) with seven levels (corresponding to the 7 blocks of acquisition) was performed on swim speeds. There was no effect of genotype ($F(1,16)=0.249$, $p=0.625$) although there was a non-significant trend towards an effect of sex ($F(1,16)=4.131$, $p=0.059$) caused by female mice swimming faster than males (mean speed (cm/s) ± SEM for: males = 0.28±0.01, females = 0.32±0.02) but no genotype by sex interaction ($F(1,16)=2.675$, $p=0.121$) on the acquisition trials. There was a significant effect of block ($F(6,96)=5.621$, $p<0.001$) driven by mice swimming more rapidly on intermediate trials (mean swim speed (cm/s) ± SEM on: block 1 = 26.2±1; block 2 = 28.3±1.4; block 3 = 31.9±2; block 4 = 29.4±0.8; block 5 = 30.9±1.1; block 6 = 32.3±1.1; block 7 = 25.9±1.1). There was no genotype by block interaction ($F(6,96)=0.806$, $p=0.568$), sex by block interaction ($F(6,96)=1.286$, $p=0.271$) or genotype by sex by block interaction ($F(6,96)=0.22$, $p=0.97$). Wildtype = 9, NR2B$^{\Delta HPC}$ = 11.





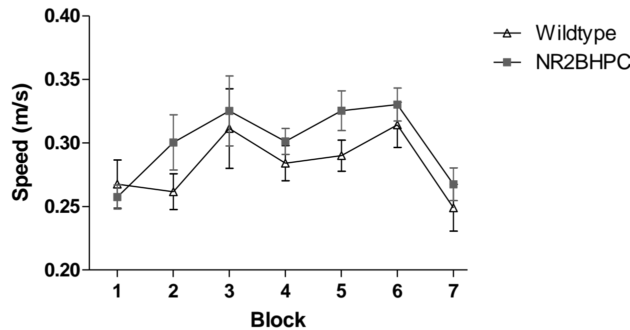


Figure 69 The latencies, distances and speeds swum by wildtype and NR2B$^{\Delta HPC}$ mice to reach the platform on the acquisition trials were similar. (a) Latency to reach the platform during acquisition. (b) Distance swum to reach the platform during acquisition. (c) Swim speeds to reach the platform during acquisition. Mean ± SEM for wildtype (Δ) and NR2B$^{\Delta HPC}$ (■) mice.

5.4.6. Acquisition of the spatial discrimination – first choice accuracy

There was no difference between wildtype and NR2B$^{\Delta HPC}$ mice in the accuracy of their first choices. A repeated measures ANOVA with two between subjects factors (genotype and sex) and two within subjects factors (block, with 7 levels corresponding to each of the 7 blocks of acquisition, and start location with 3 levels corresponding to starting locations close to the S-, to the S+ and equidistant between the two beacons) was performed. There was no effect of genotype ($F(1,16)=0.248$, $p=0.625$) and no genotype by sex interaction ($F(1,16)=0.52$, $p=0.481$) on the accuracy of mice in selecting the beacon with the platform, although there was a significant effect of sex ($F(1,16)=13.771$, $p=0.002$) caused by males being less accurate than females (mean number of accurate choices per block ± SEM for: males = 4.94±0.13, females = 6.23±0.18).

There was a significant effect of block ($F(6,96)=13.133$, $p<0.001$), a non-significant trend towards a block by sex interaction ($F(6,96)=2.172$, $p=0.052$) caused by a significant difference between males and females on blocks 2 ($F(1,16)=9.761$, $p=0.007$), 3 ($F(1,16)=4.672$, $p=0.046$), 4 ($F(1,16)=18.054$, $p=0.001$), 5 ($F(1,16)=9.44$, $p=0.007$) and 6 ($F(1,16)=8.452$, $p=0.01$). However, there was no genotype by block interaction ($F(1,16)=0.67$, $p=0.674$) or genotype by sex by block interaction ($F(1,16)=0.732$, $p=0.625$).

There was a highly significant effect of start position ($F(2,32)=44.479$, $p<0.001$), caused by greater accuracy when mice were released from positions closer to the platform (S+ starts). There was no genotype by start position interaction ($F(2,32)=0.57$, $p=0.571$) and no sex by start position interaction ($F(2,32)=0.968$, $p=0.391$), and no genotype by sex by start position interaction ($F(2,32)=0.166$, $p=0.848$).

There was a trend towards a block by start position interaction ($F(12,192)=1.695$, $p=0.07$), caused by mice making significantly more incorrect choices when starting from positions near the S- on day 1 compared with all other days. The number of correct choices made from starting positions near the S+ and equidistant between the two beacons were not significantly different on the first three days (nor on the last 3 days). There was no genotype by block by start position interaction ($F(12,192)=0.356$, $p=0.977$), no sex by block by position interaction ($F(12,192)=0.186$, $p=0.999$) and no genotype by sex by block by start position interaction ($F(12,192)=0.415$, $p=0.956$). Wildtype male = 6, wildtype female = 3, $NR2B^{\Delta HPC}$ male = 8, $NR2B^{\Delta HPC}$ female = 3.

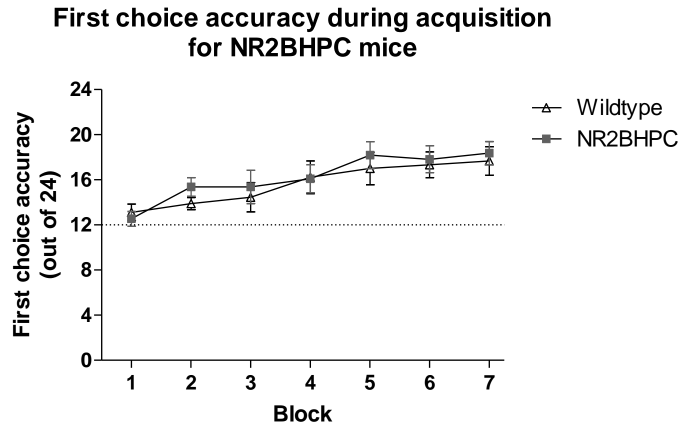


Figure 70 First choice accuracy during acquisition was similar for wildtype and NR2B$^{\Delta HPC}$ mice. Mean ± SEM for wildtype (Δ) and NR2B$^{\Delta HPC}$ (■) mice, the dotted line indicates chance levels (12 out of 24).

5.4.7. Acquisition of the spatial discrimination – total errors

There was no significant difference between wildtype and NR2B$^{\Delta HPC}$ mice in the total number of errors made. A repeated measures ANOVA with two between subjects factors (genotype and sex) and two within subjects factors (block, with 7 levels corresponding to each of the 7 blocks of acquisition, and start location with 3 levels corresponding to starting locations close to the S-, to the S+ and equidistant between the two beacons) was performed. There was no effect of genotype ($F(1,16)=0.357$, $p=0.558$) and no genotype by sex interaction ($F(1,16)=1.129$, $p=0.304$) on the total number of errors made by mice before reaching the platform, but there was a significant effect of sex ($F(1,16)=20.197$, $p<0.001$). This was caused by males making more errors than females (mean incorrect approaches made ± SEM for: males = 3.95±0.24, females = 2.16±0.24).

There was a significant effect of block ($F(6,96)=20.179$, $p<0.001$) resulting from significantly more errors being made on the first blocks, but there was no genotype by block ($F(6,96)=0.297$, $p=0.937$) or sex by block ($F(6,96)=0.487$, $p=0.817$) or genotype by sex by block ($F(6,96)=1.069$, $p=0.387$) interaction.

There was also a significant effect of start position ($F(2,32)=45.789$, $p<0.001$), caused by more errors made when mice were released from positions close to the S-. There was no genotype by start position interaction ($F(2,32)=0.019$, $p=0.981$) although there was a non-significant sex by start position interaction ($F(2,32)=3.124$, $p=0.058$) but no genotype by sex by start position interaction ($F(2,32)=0.533$, $p=0.592$).

There was a significant block by start position interaction ($F(12,192)=6.568$, $p<0.001$), caused by a significant difference between the blocks when mice were released from positions close to the S- ($F(12,192)=4.417$, $p=0.016$) and positions equidistant between the beacons ($F(12,192)=4.177$, $p=0.02$) and significant differences between the start positions on all 7 blocks. There was no genotype by block by start position interaction ($F(12,192)=1.085$, $p=0.375$) and no genotype by sex by block by start position interaction ($F(12,192)=0.975$, $p=0.474$). There was, however, a significant sex by block by start position interaction ($F(12,192)=2.627$, $p=0.003$) driven by a significant difference between blocks for females released from the equidistant positions ($F(12,192)=2.933$, $p=0.058$) and between blocks for males released from positions close to the S- ($F(12,192)=6.261$, $p=0.005$) and between males and females released from positions close to the S- on days 1 ($F(1,16)=6.431$, $p=0.022$), 4 ($F(1,16)=7.551$, $p=0.014$) and between males and females released from points equidistant between the beacons on days 4 ($F(1,16)=19.05$, $p<0.001$), 5 ($F(1,16)=7.363$, $p=0.015$) and 6 ($F(1,16)=5.483$, $p=0.032$). Wildtype male = 6, wildtype female = 3, $NR2B^{\Delta HPC}$ male = 8, $NR2B^{\Delta HPC}$ female = 3.

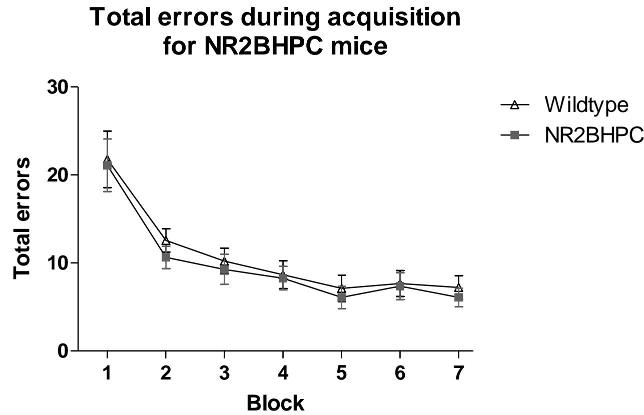


Figure 71 Total errors made during acquisition were similar for wildtype and NR2B$^{\Delta HPC}$ mice. Mean ± SEM for wildtype (Δ) and NR2B$^{\Delta HPC}$ (■) mice.

5.4.8. Transfer tests – % time spent in each quadrant

Wildtype and NR2B$^{\Delta HPC}$ mice spent more time in the training quadrant than would be expected by chance (Figure 72). A repeated measures ANOVA with two between subjects factors (genotype and sex) and two within subjects factors (transfer test, with 2 levels corresponding to the two transfer tests, and quadrant with 4 levels corresponding to the 4 quadrants) was performed on the % time spent in the quadrants.

There was a significant effect of quadrant ($F(3,48)=20.879$, $p<0.001$) caused by mice spending longer in the training quadrant than the other quadrants (mean % time ± SEM spent in quadrant: adjacent left = 18.7±2, training = 46.4±2.8, adjacent right = 23.3±1.5, opposite = 11.4±1.3) but no genotype by quadrant interaction ($F(3,48)=0.319$, $p=0.811$) or sex by quadrant interaction ($F(3,48)=1.036$, $p=0.385$), and no genotype by sex by quadrant interaction ($F(3,48)=0.24$, $p=0.868$). There was no genotype by transfer test effect ($F(1,16)=0.487$, $p=0.495$) or sex by transfer test effect ($F(1,16)=0.002$, $p=0.968$). There was no quadrant by transfer test effect ($F(3,48)=0.256$, $p=0.857$) or genotype by transfer test by quadrant effect ($F(3,48)=0.664$, $p=0.578$), sex by transfer test by quadrant effect ($F(3,48)=0.79$, $p=0.505$) or genotype by sex by transfer test by quadrant effect ($F(3,48)=0.488$, $p=0.692$).

When a two tailed t test comparing the % of time wildtype and NR2B$^{\Delta HPC}$ mice spent in the training quadrant, there was no difference between the genotypes (degrees of freedom = 19, two tailed p=0.741). Two separate t tests comparing the % of time each genotype spent in the training quadrant to chance levels (25%) revealed that the wildtype and NR2B$^{\Delta HPC}$ mice were significantly above chance (two tailed significance for wildtype mice (degrees of freedom = 8) on transfer test: 1 = 0.005, 2 = 0.023. Two tailed significance for NR2B$^{\Delta HPC}$ mice (degrees of freedom = 10) on transfer test: 1 = 0.006, 2 = 0.001). Wildtype male = 6, wildtype female = 3, NR2B$^{\Delta HPC}$ male = 8, NR2B$^{\Delta HPC}$ female = 3.

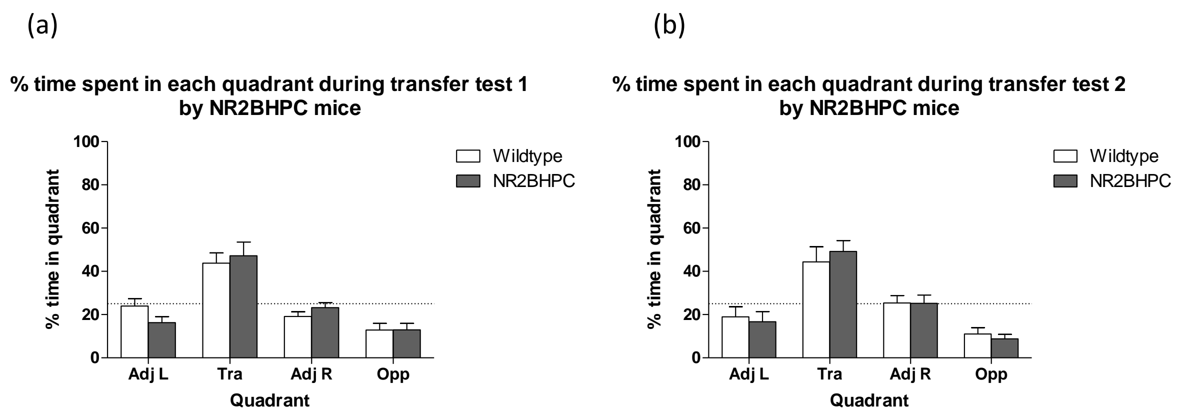


Figure 72 Wildtype and NR2B$^{\Delta HPC}$ mice spent significantly above chance % of time in the training quadrant. Mean ± SEM for wildtype (Δ) and NR2B$^{\Delta HPC}$ (■) mice, the dotted line indicates chance levels (25%).

5.4.9. Transfer tests – number of annulus crossings

There was no difference between the number of annulus crossings by wildtype and NR2B$^{\Delta HPC}$ mice in the training quadrant on transfer tests (**Error! Reference source not found.**). A repeated easures ANOVA with two between subjects factors (genotype and sex) and two within subjects factors (transfer test with two levels corresponding to the two transfer tests, and quadrant annulus

crossings with 4 levels corresponding to the 4 quadrants) was performed on the number of annulus crossings in each quadrant. There was no effect of genotype ($F(1,16)=0.379$, $p=0.547$) or sex ($F(1,16)=0.178$, $p=0.679$) although there was a trend towards a genotype by sex interaction ($F(1,16)=3.179$, $p=0.094$). There was a highly significant effect of quadrant ($F(3,48)=10.554$, $p<0.001$) driven by mice making more annulus crossings in the training quadrant, but no genotype by quadrant effect ($F(3,48)=1.385$, $p=0.259$) and no sex by quadrant effect ($F(3,48)=1.397$, $p=0.255$), and no genotype by sex by quadrant effect ($F(3,48)=0.255$, $p=0.857$).

There was no effect of transfer test ($F(2,32)=1.532$, $p=0.232$) caused by mice making more annulus crossings on later transfer tests, and there was no genotype by transfer test effect ($F(2,32)=0.516$, $p=0.602$), sex by transfer test effect ($F(2,32)=0.149$, $p=0.862$) or genotype by sex by transfer test effect ($F(2,32)=1.028$, $p=0.369$). Finally, there was a significant quadrant by transfer test effect ($F(6,96)=2.53$, $p=0.026$), but no genotype by quadrant by transfer test effect ($F(6,96)=0.624$, $p=0.71$) or sex by quadrant by transfer test effect ($F(6,96)=0.922$, $p=0.482$) or genotype by sex by quadrant by transfer test effect ($F(6,96)=1.105$, $p=0.365$).

A two tailed t test comparing the total number of annulus crossings made by wildtype and NR2B$^{\Delta HPC}$ mice revealed that the genotypes were not significantly different on the first (degrees of freedom = 18, $p=0.303$) or second (degrees of freedom = 18, $p=0.262$) transfer tests. Wildtype male = 6, wildtype female = 3, NR2B$^{\Delta HPC}$ male = 8, NR2B$^{\Delta HPC}$ female = 3.

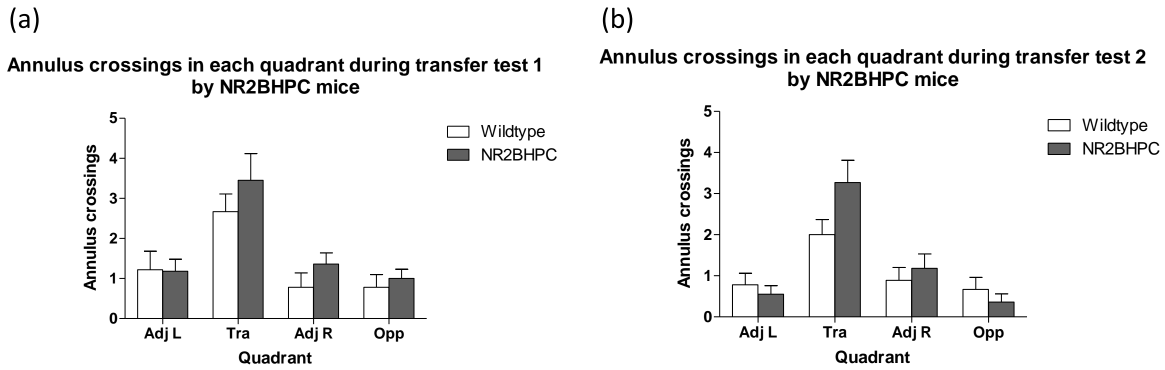


Figure 73 Wildtype and NR2B$^{\Delta HPC}$ mice made similar numbers of annulus crossings at the position in which the platform was normally located. Mean ± SEM for wildtype (Δ) and NR2B$^{\Delta HPC}$ (■) mice.

5.4.10. Reversal – latency

There was also no difference between the latencies of controls and NR2B$^{\Delta HPC}$ after reversal of the platform position (

Figure **74**). A repeated measures ANOVA with two between subjects factors (genotype and sex) and one within subjects factor (block) with four levels (corresponding to the four blocks of reversal training) were performed on the latencies of the mice. There was no effect of genotype ($F(1,16)=0.013$, $p=0.91$) or sex ($F(1,16)=0.737$, $p=0.403$) nor a genotype by sex interaction ($F(1,16)=0.661$, $p=0.428$). However, there was a significant effect of block ($F(3,48)=17.845$, $p<0.001$) driven by shorter latencies on later blocks, but no block by genotype ($F(3,48)=0.146$, $p=0.932$) or sex by day ($F(3,48)=0.045$, $p=0.987$) or genotype by sex by day interaction ($F(3,48)=0.535$, $p=0.66$). Wildtype male = 6, wildtype female = 3, NR2B$^{\Delta HPC}$ male = 8, NR2B$^{\Delta HPC}$ female = 3.

5.4.11. Reversal – distance

There was no difference between the distances swum by control and NR2B$^{\Delta HPC}$ mice after reversal of the platform position (

Figure **74**). A repeated measures ANOVA with two between subjects factor (genotype and sex) and one within subjects factor (block) with four levels (corresponding to the four blocks of reversal training) was performed on the distances swum. There was no effect of genotype (F(1,16)=0.23, p=0.638) or sex (F(1,16)=0.141, p=0.712) and no genotype by sex effect (F(1,16)=0.002, p=0.964) after reversal. There was a significant effect of block (F(3,48)=17.75, p<0.001) but no genotype by block (F(3,48)=0.521, p=0.67) or sex by block interaction (F(3,48)=0.232, p=0.874). There was also no genotype by sex by block interaction (F(1,48)=1.862, p=0.149). Wildtype male = 6, wildtype female = 3, NR2B$^{\Delta HPC}$ male = 8, NR2B$^{\Delta HPC}$ female = 3.

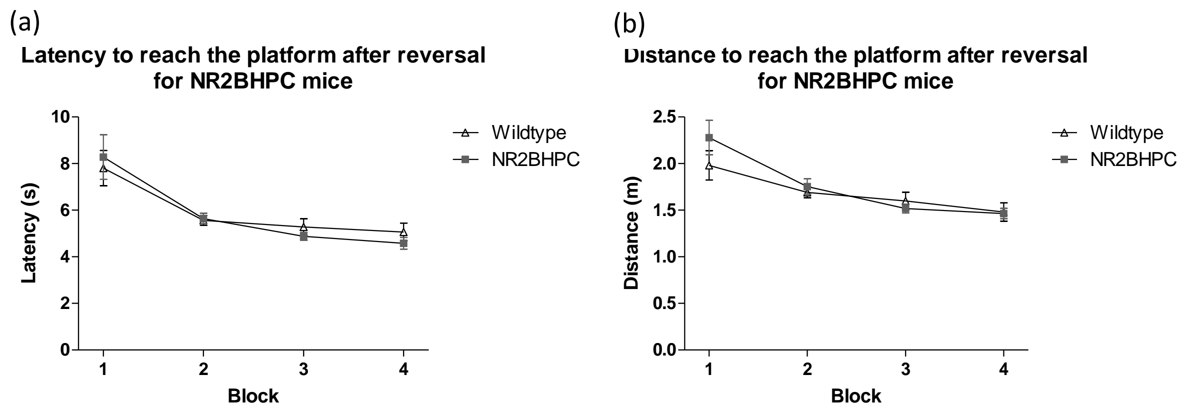


Figure 74 The latencies and distance swum by wildtype and NR2B knock out mice to reach the platform after reversal of the platform position decreased at similar rates. (a) Latency to reach the platform after reversal. (b) Distance swum to reach the platform after reversal. Mean ± SEM for wildtype (Δ) and NR2B knock out (■) mice.

5.4.12. Reversal – first choice accuracy

Wildtype and NR2B$^{\Delta HPC}$ had similar levels of first choice accuracy after reversal. There was no effect of genotype on first choice accuracy after reversal (F(1,16)=2.772, p=0.115) although there was a significant effect of sex (F(1,16)=20.4, p<0.001) caused by males being less accurate than females (mean first choice accuracy ± SEM for: males = 3.98±0.18, females = 5.67±0.28). There was no genotype by sex interaction (F(1,16)=1.28, p=0.275).

There was a significant effect of block ($F(3,48)=41.516$, $p<0.001$) caused by mice making fewer errors on later days. There was no genotype by block interaction ($F(3,48)=1.119$, $p=0.351$) but a significant sex by block interaction ($F(3,48)=8.018$, $p<0.001$) caused by a significant difference between males and females on block 2 ($F(3,48)=24.217$, $p<0.001$), 3 ($F(3,48)=13.892$, $p=0.002$) and 4 ($F(3,48)=14.01$, $p=0.002$). There was no genotype by sex by block interaction ($F(3,48)=1.911$, $p=0.14$).

There was a significant effect of start position ($F(2,32)=30.659$, $p<0.001$) caused by mice making more correct choices from positions close to the S+, although there was no genotype by start position effect ($F(2,32)=0.07$, $p=0.932$). There was a trend towards a sex by start position interaction ($F(2,32)=3.08$, $p=0.06$) caused by a significant difference between the start positions for males ($F(2,32)=30.506$, $p<0.001$) and females ($F(2,32)=3.768$, $p=0.047$) and significant differences between males and females when starting from near the S- ($F(2,32)=17.918$, $p=0.001$) and when equidistant between the beacons ($F(2,32)=10.837$, $p=0.005$). There was no genotype by sex by starting position interaction ($F(2,32)=0.187$, $p=0.83$).

There was a significant block by start position interaction ($F(6,96)=2.254$, $p=0.044$) caused by significant effects between the blocks for mice starting from each start position (starting from: near S- ($F(6,96)=9.512$, $p=0.001$), near S+ ($F(6,96)=8.65$, $p=0.002$) and equidistant ($F(6,96)=17.483$, $p<0.0001$)), and for all start positions on block 1 ($F(6,96)=14.385$, $p<0.001$), 2 ($F(6,96)=10.449$, $p=0.001$), 3 ($F(6,96)=11.366$, $p=0.001$) and 4 ($F(6,96)=17.278$, $p<0.001$). There was no genotype by block by start position ($F(6,96)=0.389$, $p=0.884$), sex by block by start position ($F(6,96)=0.84$, $p=0.542$) or genotype by sex by block by start position ($F(6,96)=1.264$, $p=0.281$) interaction.

Importantly, an independent sample two tailed t test showed that the wildtype and NR2B$^{\Delta HPC}$ mice were not significantly different on their first choice accuracy for the first block after the reversal (degrees of freedom = 18, p=0.75) but were significantly below chance (where chance was considered to be 12 out of 24 trials: wildtype degrees of freedom = 8, p=0.004. NR2B$^{\Delta HPC}$ degrees of freedom = 10, p=0.002). Wildtype male = 6, wildtype female = 3, NR2B$^{\Delta HPC}$ male = 8, NR2B$^{\Delta HPC}$ female = 3.

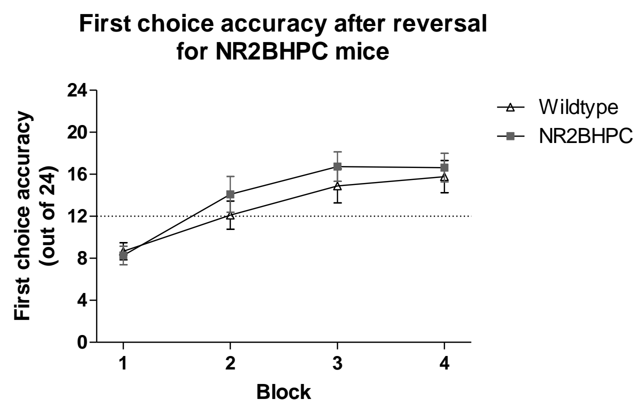


Figure 75 First choice accuracy after reversal was similar for wildtype and NR2B$^{\Delta HPC}$ mice. Mean ± SEM for wildtype (Δ) and NR2B$^{\Delta HPC}$ (■) mice, the dotted line indicates chance levels (12 out of 24).

5.4.13. Reversal – total errors

As discussed previously, we were interested to see if the NR2B$^{\Delta HPC}$ would be as capable as control mice at finding the platform when the platform position was reversed. As we have found that they tend to make perseverance errors, we predicted that they would be more likely to approach the S- beacon (formerly the S+ beacon) after reversal. However, this was not what we observed. There was a trend towards an effect of genotype on the total number of errors made (F(1,16)=3.083, p=0.098) caused by NR2B$^{\Delta HPC}$ mice making fewer errors than wildtype mice (mean total errors ± SEM for: wildtypes = 4.82±0.44, NR2B$^{\Delta HPC}$ =4.14±0.38) and a significant effect of sex

($F(1,16)=12.657$, $p=0.003$) caused by males making more errors than females (mean total errors ± SEM for: males = 5.04 ± 0.34, females = 3.07 ± 0.5). However, there was no genotype by sex interaction ($F(1,16)=0.725$, $p=0.407$).

There was a significant effect of block ($F(3,48)=30.019$, $p<0.001$) caused by mice making more errors on earlier blocks, although there was no genotype by block interaction ($F(3,48)=0.176$, $p=0.912$) or sex by block interaction ($F(3,48)=1.038$, $p=0.384$) nor a genotype by sex by block interaction ($F(3,48)=0.686$, $p=0.565$).

There was a significant effect of start position ($F(2,32)=40.022$, $p<0.001$) but no genotype by start position interaction ($F(2,32)=0.254$, $p=0.778$), no sex by start position interaction ($F(2,32)=1.56$, $p=0.226$) and no genotype by sex by start position interaction ($F(2,32)=0.098$, $p=0.907$).

There was a significant block by start position interaction ($F(6,96)=6.144$, $p<0.001$) caused by mice making significantly different numbers of errors across the different blocks when released from near the S- ($F(6,96)=6.067$, $p=0.007$), near the S+ ($F(6,96)=5.685$, $p=0.009$) and equidistant between the beacons ($F(6,96)=8.781$, $p=0.002$). There was also a significant difference between the total number of errors mice made between different starting locations on block 1 ($F(6,96)=9.626$, $p=0.002$), block 2 ($F(6,96)=9.814$, $p=0.002$), block 3 ($F(6,96)=10.733$, $p=0.001$) and block 4 ($F(6,96)=16.673$, $p<0.001$). However, there was no genotype by block by start position interaction ($F(6,96)=0.374$, $p=0.894$), sex by block by start position interaction ($F(6,96)=0.564$, $p=0.758$) or genotype by sex by block by start position interaction ($F(6,96)=0.221$, $p=0.969$). Wildtype male = 6, wildtype female = 3, $NR2B^{\Delta HPC}$ male = 8, $NR2B^{\Delta HPC}$ female = 3.

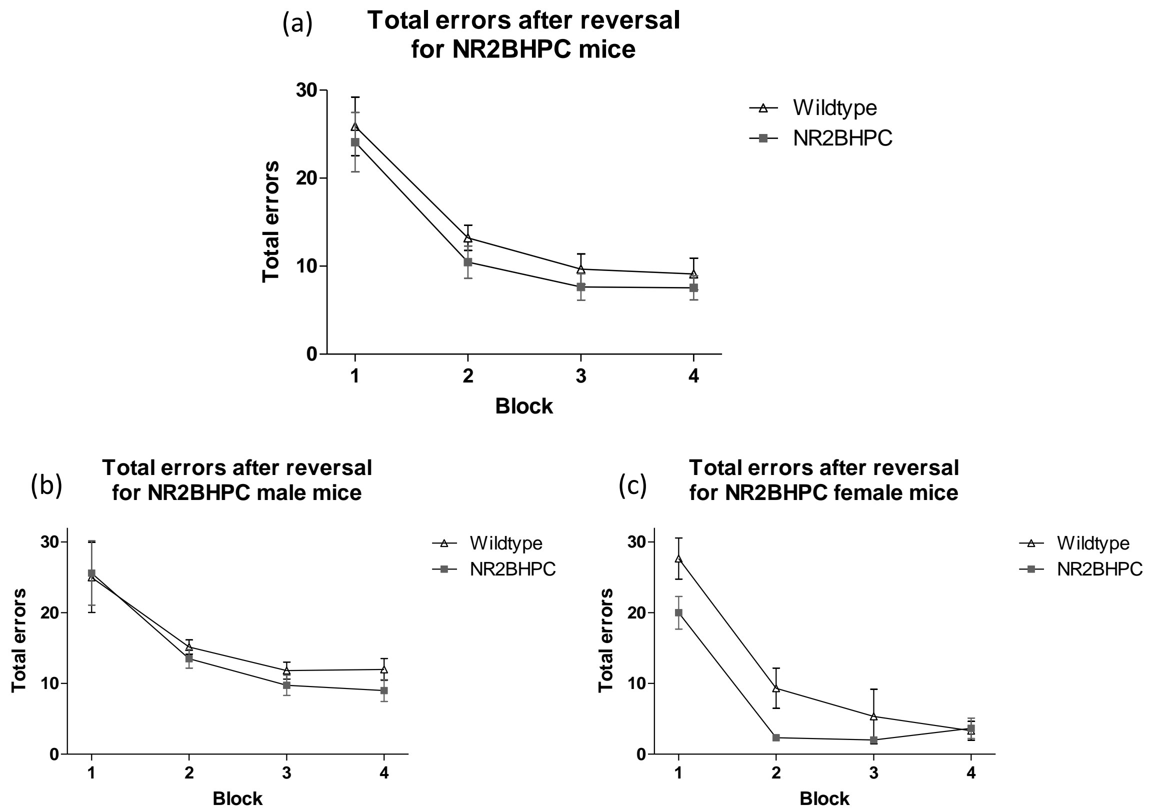


Figure 76 Total errors after reversal for wildtype and NR2B$^{\Delta HPC}$ mice. Mean ± SEM for all wildtype (Δ) and NR2B$^{\Delta HPC}$ (■) mice (a), for male mice (b) and for female mice (c).

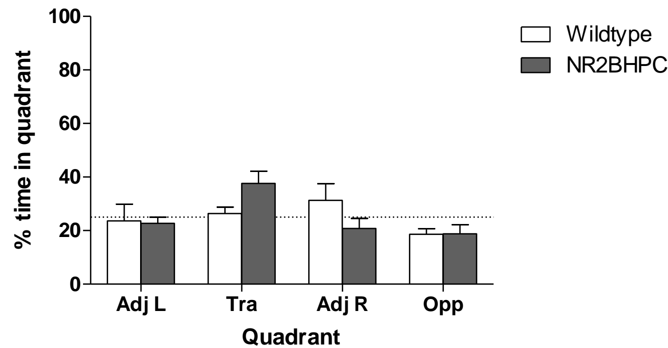
5.4.14. Errors from S- position

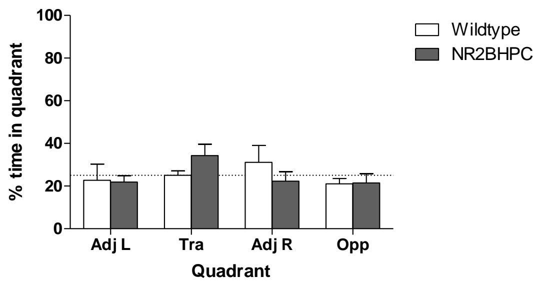
We had considered that the NR2B$^{\Delta HPC}$ mice may make more perseverance errors, and so we compared the number of errors made by mice when released from positions close to the S- after reversal. If the NR2B$^{\Delta HPC}$ mice perseverated, we would expect to see them making significantly more errors than wildtype mice after the reversal. A repeated measures ANOVA with two between subjects factors (genotype and sex) and one within subjects factor (errors, with four levels corresponding to the four blocks after reversal) were performed on the number of errors made when mice were released from positions close to the S-. There was no effect of genotype ($F(1,16)=1.473$, $p=0.243$) and no genotype by sex interaction ($F(1,16)=0.184$, $p=0.673$), although there was a significant effect of sex ($F(1,16)=6.734$, $p=0.02$) caused by males making significantly

more errors than females (mean number of errors ± SEM for: males = 8±0.73, females = 5.17±1.23). Wildtype male = 6, wildtype female = 3, NR2B$^{\Delta HPC}$ male = 8, NR2B$^{\Delta HPC}$ female = 3.

5.4.15. Transfer test – % time in quadrants

There was a no difference between the % of time spent in each quadrant by wildtype and NR2B$^{\Delta HPC}$ mice on the third transfer test. A repeated measures ANOVA with two between subjects factors (genotype and sex) and one within subjects factor (quadrant, with 4 levels corresponding to each of the 4 quadrants) was performed on the % time spent in each quadrant. There was no genotype by sex interaction ($F(1,16)=0.01$, $p=0.92$). There was a significant effect of quadrant ($F(3,48)=3.359$, $p=0.026$) driven by mice spending longer in the training quadrant than the other quadrants, but there was no genotype by quadrant effect ($F(3,48)=1.896$, $p=0.143$) and no sex by quadrant effect ($F(3,48)=0.911$, $p=0.442$), and no genotype by sex by quadrant effect ($F(3,48)=0.167$, $p=0.918$). An independent samples t test revealed a significant difference between the % time spent in the training quadrant by wildtype and NR2B$^{\Delta HPC}$ mice (degrees of freedom = 18, two-tailed p = 0.044), with NR2B$^{\Delta HPC}$ mice spending longer in the training quadrant than wildtype mice. Wildtype male = 6, wildtype female = 3, NR2B$^{\Delta HPC}$ male = 8, NR2B$^{\Delta HPC}$ female = 3.





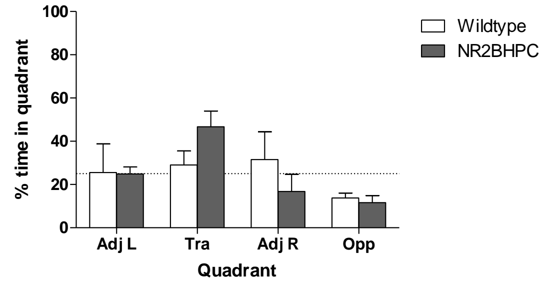


Figure 77 The % time spent in each quadrant by wildtype and NR2B$^{\Delta HPC}$ mice. Mean ± SEM for wildtype (Δ) and NR2B$^{\Delta HPC}$ (■) mice, the dotted line indicates chance levels (25%).

5.4.16. Transfer test – number of annulus crossings

There was a no difference between the number of annulus crossings by wildtype and NR2B$^{\Delta HPC}$ mice in the training quadrant on the third transfer test. A repeated measures ANOVA with two between subjects factors (genotype and sex) and one within subjects factor (quadrant annulus crossings with 4 levels corresponding to the 4 quadrants) was performed on the number of annulus crossings in each quadrant. There was no effect of genotype ($F(1,16)=0.19$, $p=0.891$) or sex ($F(1,16)=0.062$, $p=0.806$) and no genotype by sex interaction ($F(1,16)=2.685$, $p=0.121$). There was no effect of quadrant ($F(3,48)=0.784$, $p=0.509$) or genotype by quadrant effect ($F(3,48)=1.214$, $p=0.315$) and no sex by quadrant effect ($F(3,48)=0.527$, $p=0.666$), and no genotype by sex by quadrant effect

(F(3,48)=0.768, p=0.508). Wildtype male = 6, wildtype female = 3, NR2B$^{\Delta HPC}$ male = 8, NR2B$^{\Delta HPC}$ female = 3.

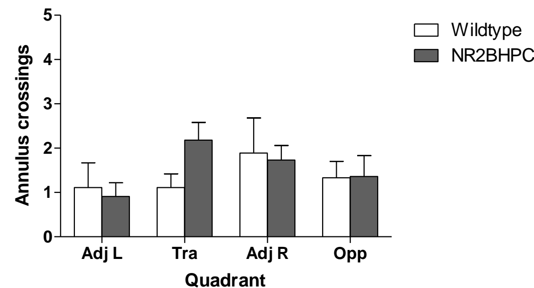


Figure 78 The number of annulus crossings were similar for wildtype and NR2B$^{\Delta HPC}$ mice. Mean ± SEM for wildtype (Δ) and NR2B$^{\Delta HPC}$ (■) mice.

5.5. Conclusions

The impairment conferred upon mice lacking the NR2B subunit in the hippocampus appears, from these results, to be subtle. A task requiring working memory (the T maze) and a task using spatial reference memory (the watermaze) both found that NR2B$^{\Delta HPC}$ mice solve the tasks as readily as wildtype mice. They also alternated at a rate similar to wildtype mice when the choice run was delayed by 20 seconds, and learned not to persevere by swimming to the previously rewarded S-beacon when the platform position was reversed. Indeed, they may have an enhancement rather than an impairment to their memory, as the NR2B$^{\Delta HPC}$ mice made more annulus crossings during transfer tests than wildtype mice. This could suggest that the NR2B$^{\Delta HPC}$ mice have a more accurate spatial representation of the watermaze and therefore are able to search more precisely than wildtype mice. However, as mentioned previously, this may be partially explained by inflexibility of memories; as the mice obtain the spatial representation, they may be less capable of altering it in light of new conditions and information.

By contrast, in mice in which the NR1 subunit has been knocked out in the hippocampus, there was a significant impairment compared to wildtype mice in their ability to alternate on the rewarded alternation task but not the spontaneous alternation task. However, the difference on the rewarded alternation task between NR1 knockout mice and wildtype mice disappeared when they were subjected to a 20 second delay between the sample and choice runs. This would seem to suggest that NMDA receptors in the hippocampus are necessary for the rewarded alternation task but not spontaneous alternation task. This difference could be due to the different underlying motivations for the spontaneous and rewarded alternation tasks; spontaneous alternation makes use of a mouse's innate preference for novelty to test working memory, whereas rewarded alternation is appetitively motivated. If the GluN1$^{\Delta HPC}$ mice were less motivated by a food reward than wildtype mice, they might be expected to perform less well on the task, potentially explaining the difference; however, this would seem unlikely. The performance of the control mice is better than that of the GluN1$^{\Delta HPC}$ mice when there was no delay but was equivalent at the 20 second delay condition, suggesting that the wildtype mice fell to the same performance levels as the GluN1$^{\Delta HPC}$ mice when the delay was imposed as shown in Figure 67.

In previous work, the GluN1$^{\Delta HPC}$ mice showed an impairment in spatial working memory on the radial arm maze task (Niewoehner et al 2007), similar to the spatial working impairment seen in mice lacking NR1 in the CA3 pyramidal cells (Nakazawa et al 2003), and another group has found that NR1 elimination from 40-50% of cortical and hippocampal interneurons leads to a deficit in spatial working memory (Belforte et al 2010). By comparison, mice lacking NR1 in the CA1 pyramidal cells display a spatial reference memory impairment, but not a spatial working memory impairment) (Tsien et al 1996). Thus these results suggest that GluN1$^{\Delta HPC}$ mice are impaired on spatial working memory (with rewarded alternation), which is in agreement with the published literature. However,

it is slightly more difficult to understand how the finding that they are unimpaired on the spontaneous alternation version of the spatial working memory T maze fits.

It is interesting that the NR2B$^{\Delta HPC}$ mice are slightly impaired on the rewarded alternation task whereas GluN1$^{\Delta HPC}$ mice are significantly impaired; it suggests that the NR1 subunit is necessary for spatial working memory, but that NR2B is not. As NR2A is the only other subunit found in the adult hippocampus it would seem to suggest that NR2A is important for spatial working memory, and indeed this is supported by the finding that mice lacking the NR2A subunit are impaired on spatial working memory tasks (Bannerman et al 2008). However, it seems most likely that, since the GluN1$^{\Delta HPC}$ mice lack NMDAR containing either NR2A or NR2B whilst NR2B$^{\Delta HPC}$ mice lack NMDAR containing NR2B only, these observations are probably a reflection of the magnitude of loss of functional NMDARs in the hippocampi of these mice.

It has been suggested in the past that the NR2B subunit is required for long term depression, whereas the NR2A subunit is necessary for long term potentiation (Liu et al 2004; Massey et al 2004), although more recently published work suggests this is not the case (Berberich et al 2005; Gardoni et al 2009; Morishita et al 2007; Rammes et al 2009). Nonetheless, LTP and LTD may work together to allow more precise manipulation of connections by allowing greater refinement of the neuronal connections. If NR2B was necessary for this, it might be expected that mice lacking NR2B would be less able to alter memories that have become out-dated and might thus be more likely to perseverate. This might be displayed in increased perseverance errors. Indeed, this appeared to be the case in the study in which the NR2B$^{\Delta HPC}$ mice perseverated (von Engelhardt et al 2008).

It is interesting to note that NR2B$^{\Delta HPC}$ mice made more annulus crossings than wildtype mice. There are two possible interpretations of this finding: 1) the NR2B$^{\Delta HPC}$ mice may have a more precise spatial representation of the watermaze and are therefore better able to recall the precise location of the platform, or 2) the NR2B$^{\Delta HPC}$ mice are more likely to continue searching in the area the platform is normally located in even when it is clear that it is no longer present. If 1) was true, one would expect that, in order to escape the aversive environment of the watermaze more quickly, NR2B$^{\Delta HPC}$ mice would swim directly to the platform as rapidly as possible. This would result in shorter escape latencies for the NR2B$^{\Delta HPC}$ mice; as there was no significant difference between the escape latencies for control and NR2B$^{\Delta HPC}$ mice, it seems more likely that 2) is more accurate. This is in agreement with previous findings that mice lacking NR2B in the HPC are impaired at spatial reversal tasks in the watermaze (von Engelhardt et al 2008).

Another issue with these mice is that the genetic manipulations used were knock-outs, such that the protein was entirely absent in the hippocampus from birth. One difficulty with such models is that it is difficult to establish with negative results whether there was no difference as the protein genuinely does not affect the factor in question or because of a degree of redundancy in the system, where other mechanisms substitute their function to restore normal behaviour. One elegant way to circumvent this problem is to generate mice in which the gene can be selectively turned on or off (Lewandoski 2001) by use of an agent such as tamoxifen (Arin et al 2001). Thus the gene knockout is known to be present at the specific time period whilst allowing minimal time for substitution of function to affect results, whilst also allowing the animal to grow to adulthood without the gene loss affecting maturation. It may be valuable in the future to consider replicating the NR2B hippocampal knockout mouse model used here in such a manner to further clarify the effects of the loss of NR2B in the HPC.

6. Chapter 6: Molecular biology

The results from the behavioural tasks are not consistent with what was already known about the behavioural phenotype of the NR2BOE mice. A particular concern was that the molecular phenotype of overexpression of NR2B may have been lost between generations. Each generation was produced by only a few (around 5) male mice of the previous generation. If any of these sires were incorrectly genotyped and thus considered an overexpressor rather than a wild type mouse, all offspring would be wildtype (as NR2BOE had heterozygous overexpression of the gene). Another possibility is that the sires may have all carried the transgene, but that the overexpression of NR2B has been lost, either as the result of an acquired null mutation in the transgene, or as the result of compensatory changes at the mRNA or protein level. Upregulation of the NR2A subunit, which is also present in the hippocampus and can form functional NMDA receptors with NR1, could lead to increased signalling through NR2A containing NMDA receptors rather than NR2B containing NMDA receptors. Neurons which overexpressed the NR2B subunit could allow more Ca^{2+} enter the cell than it is capable of processing, potentially leading to apoptosis in the neurons in which NR2B was overexpressed. Clearly it would be invaluable to establish the protein levels of NR2B in the hippocampi of these mice to ascertain whether the NR2BOE do express more NR2B mRNA and protein.

6.1. Choice of methodology

There are several methods for establishing mRNA and protein levels. Below is a brief outline of the reasons for using qPCR and western blots in this thesis.

6.1.1. mRNA

6.1.1.1. In situ hybridisation

This technique involves fixing a sample of the tissue of interest, mounting it and applying a complementary RNA strand. This probe strand hybridises with the RNA of interest and is then labelled. The mRNA can then be measured using immunohistochemistry (in which the probe is labelled using an antibody conjugated to a marker), autoradiography (in which a radioactive marker is used to label the probe) or fluorescence microscopy (in which fluorophores are used to label the probe, allowing the sample to be considered under a microscope).

6.1.1.2. qPCR

Quantitative, or real time, PCR is a method in which the number of copies of mRNA is amplified and measured in real time using fluorescent markers attached to probes for the mRNA sequence of interest. It is possible to establish the starting quantity of mRNA by noting how many PCR cycles are required for the number of copies of mRNA to pass a given threshold, and comparing this to the number of cycles required to reach threshold for known quantities of mRNA.

1.1.1.1. Semiquantitative and end point PCR

For this method, after PCR has been performed, quantitative measurements are made of the templates on agarose gels. This method is less precise than real time PCR, and for this reason we did not use end point PCR to measure mRNA levels.

6.1.2. Protein

6.1.2.1. ELISA

Enzyme-linked immunosorbent assay (Elisa) is a method for detecting the presence of a protein using spectrophotometry. An unknown quantity of an antigen is attached to the surface of the ELISA well, to which an antibody is added which detects the antigen of interest. Unbound antibodies are then washed off. An enzyme is linked to the antibody, which then converts a substrate of interest to a detectable signal. The amount of signal produced is proportional to the amount of bound antibody, which in turn is proportional to the amount of antigen present in the sample.

Alternatively, an antibody specific to the antigen of interest can be attached to the surface of the well, and the antigen can then be added. The antigen adheres to the antibody, which leaves it attached to the well surface. An antibody conjugated to an enzyme to a different epitope on the protein can then be added. The substrate for the enzyme is added, and the intensity of the signal is proportional to the amount of secondary enzyme, which is in turn proportional to the amount of antigen bound.

This method is sensitive, but is subject to ceiling effects when the samples loaded are too concentrated; however, it is possible to dilute them. If the antigen of interest does not easily bind to the wells of the ELISA plate, this technique may not be optimal.

6.1.2.2. Western blotting

This technique is very popular for establishing the presence or absence of a protein of interest. It is also possible to establish protein amount quantitatively. Briefly, the protein of interest is loaded onto a gel and is separated based on its size to charge ratio. It is then bound to a

membrane, to which is applied a primary antibody directed against the protein of interest. A secondary antibody which is conjugated to a marker, directed against the primary antibody. This results in the band of interest being labelled, the size of which can be measured to establish protein concentration.

Both western blots and ELISA are valid methods for establishing protein concentration. We opted to use western blots due to greater experience with the technique.

6.2. Genotyping

In order to overexpress the NR2B subunit, mice containing an additional copy of the NR2B coding sequence under the control of the CamKII promoter were produced. To produce the mice, the linearized NR2B transgene containing the CaMKII promoter was injected into the nuclei of C57B/6 zygotes. The first step was to ensure that the transgene was indeed present in the NR2BOE mice tested in this thesis.

The mice were genotyped using the polymerase chain reaction (PCR). PCR amplifies the quantity of DNA (or RNA) in a sample by repeatedly heating the sample to certain levels (thermal cycling). Added to the sample of interest are primers, short strands of DNA sequence complementary to the ends of the DNA to be amplified, a DNA polymerase which extends the DNA sequence from these primers, and dinucleotides (dNTPs), the individual nucleotides from which the new DNA is synthesised. The sample is heated until the DNA strands separate (typically >90°C), after which it is cooled to 50-70˚C to allow the primers to anneal, or attach, to the single stranded DNA. The sample is then heated slightly (typically to 72°C) to allow primer extension by the DNA

polymerase. The cycle is then repeated, allowing further rounds of amplification, using the freshly-synthesised DNA as template (this time with double the number of strands available to use as templates, assuming 100% replication efficiency; Figure 79).

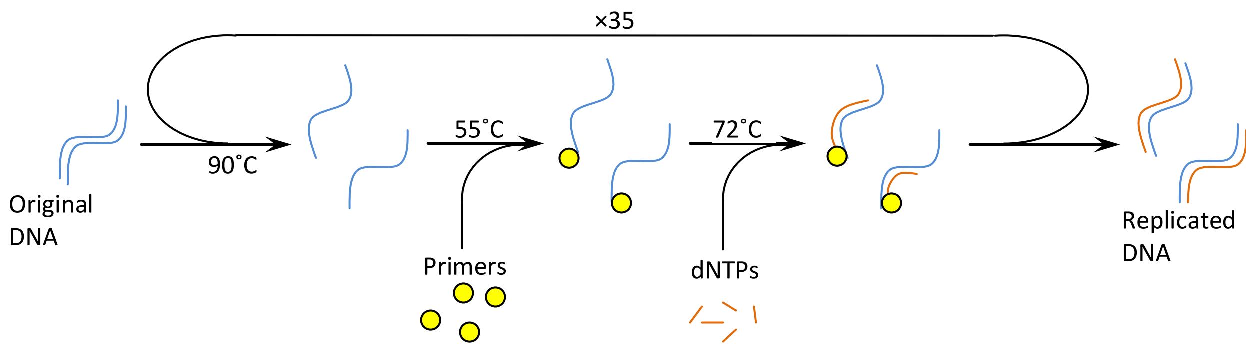


Figure 79 The process of the polymerase chain reaction.

PCR allows the amplification of DNA such that it is possible to greatly increase the amount of DNA in a sample. This allows easy detection of genes of interest, in this case the 3' region at the end of the NR2B transgene, containing an artificial polyA sequence.

6.3.Method

6.3.1. DNA extraction

Genomic DNA (gDNA) was obtained from mice from ear clips (used to identify individual animals) or tail clips. gDNA was extracted from this tissue using the Nucleon Genomic DNA Extraction Kit (Tepnel Life Sciences). Briefly, proteinase K was added to the samples, which were

mixed and incubated at 55˚C for 1 hour (3 hours for tail clips) in order to disrupt and lyse the tissue. The DNA was spun at 2,000g for 10 minutes and precipitated in propan-2-ol. It was then washed with 70% ethanol, centrifuged at 2,000g for 2 minutes to pellet the DNA, and the supernatant was discarded. The DNA pellet was left to dry, and was then resuspended in deionised H_2O (dH_2O) overnight.

6.3.2. Genotyping the DNA

The conditions described here were the optimal ones obtained for this protocol. The variables changed in order to optimise the procedure are listed in Table 5.

Polymerase chain reactions (PCR) were carried out in 10µl reaction volumes. Each reaction included 3µl gDNA template, 1µl primers mixture (5pmol of forward and reverse primers), 0.05µl (1 Unit) HotStar Taq (Qiagen, West Sussex UK) 1µl HotStar Taq PCR buffer (containing 1.5 mmol/L magnesium chloride [$MgCl_2$], Tris-Cl, potassium chloride [KCl], and ammonium sulphate [$(NH_4)_2SO_4$], pH8.7), 1µl deoxyribosenucleotide triphosphates (dNTPs) (2 mmol/L deoxyadenosine triphosphate [dATP], 2mmol/L deoxyguanosine triphosphate [dGTP], 2mmol/L deoxycytosine triphosphate [dCTP] and 2mmol/L deoxythymidine triphosphate [dTTP]), and 3.4µl Milli-Q-purified water. The primers used were those that the Tang et al group used (Tang et al 1999); 5'-AGA GGA TCT TTG TGA AGG AAC-3' and 5'-AAG TAA AAC CTC TAC AAA TG-3'. PCR was performed using a Peltier Thermal Cycler (PTC-255, MJ Research), using a variation of a TouchDown (TD) program for 40 cycles (95˚C for 30s, 62˚C for 30s (-0.5˚C each cycle until 55˚C), 72˚C for 2mins).

PCR products were run on agarose gels, alongside a 2kb Hyperladder (bands every 100bp until 1000, then every 200bp until 2000; Bioline). 3% weight/volume (w/v) gels were used (18g agarose in 600ml TAE buffer (242g Tris, 37.2 EDTA, 57.1ml glacial acetic acid made up to 5L)) containing 10µl ethidium bromide at a concentration of 1:60,000, at 200V (Consort E865 Powerpack) submerged in TAE buffer in a buffer tank for half an hour. The presence of a band at 400bp (Mayford et al 1995) indicated the presence of the transgene (i.e., an NR2B overexpressing mouse), whereas absence of a band indicated the absence of the transgene (i.e. a wild-type mouse). A positive control gene was also run on the samples to ensure that DNA had been successfully extracted. However, this prevented the formation of the band indicating the presence of the NR2B transgene due to the formation an excessively large dimer-dimer band when both primers were run together. Hence the control primer was run separately to ensure successful DNA extraction.

A Nanodrop (ND-1000 Spectrophotometer, Nanodrop Technologies) was used to establish the concentration and purity of each sample (ng/µl). The spectrophotometer creates a liquid column from the homogenous sample, and uses the absorbance of light at 260nm to calculate DNA concentration. It does so by using Beer's Law:

$$A = \varepsilon l c$$

Where A = transmission of light through the solution, ε is the molar absorptivity of the absorber in the liquid, l is the distance the light travels through the solution and c is the concentration of the absorbing species. This allows the program to establish the concentrations of the DNA and other solutes; different solutes absorb different wavelengths, with impurities (such as salts) giving ratios at 260:230 nm wavelengths that are outside of the usual range of 1.8-2.2 (which is the common range for accepted pure DNA).

The samples were mostly <10ng/µl and so were loaded undiluted. Samples that were too concentrated were diluted. In order to ensure that the reaction was working correctly, primers were added for some reactions which identified the doublecortin gene (present in both wildtype and NR2BOE mice) as a positive control, and a well in which distilled H_2O was added instead of DNA acted as a negative control. The PCR was performed three times to avoid potential errors.

Table 5 gives the steps taken to establish optimal conditions for the genotyping.

Variable	**Reason**
DNA concentration	When DNA was too concentrated when run on the gel, it appeared smeared, hence bands were not discernable. DNA was diluted to 10ng/µl if they were more concentrated to prevent this occurring
Control primer	To ensure that the DNA amplification and primer bonding was occurring, a control primer was also used.
Magnesium concentration	The concentration of magnesium in the PCR reaction mixture can affect the efficacy of the reaction. By varying the concentration of magnesium it was established that a concentration of 1.5mM was optimal.
Reaction volume	Increasing the reaction volume can help reactions work. However, increasing the reaction volume produced no effect, and so 10µl reaction volumes were used.
Taq polymerase	Various taq polymerases catalyse polymerisation of different reactions with different affinities. By varying the Taq polymerase used it was established that HotStar taq was optimal.
PCR program	Different PCR programs use differing heats and lengths of time for the reaction chambers. After varying the PCR program used for DNA amplification, it was established that the optimal program was a Touch Down program (details in text above).

Table 5 The variables considered for the optimisation of PCR for the NR2BOE mice.

6.4.Results

Error! Reference source not found. shows a representative gel. Once the protocol was ptimised, the NR2BOE DNA gave a clear band at around 400bp, whereas the wildtype DNA produced no band.

Figure 80 A representative gel.

Thus it is clear that the transgene is present in the NR2BOE mice, therefore it is evident that the transgene is still present in the cohort.

6.4.1. mRNA analysis

It is possible to accurately quantitatively measure the amount of mRNA in a sample using quantitative real-time polymerase chain reaction (qPCR) analysis. To measure the amount of mRNA transcript in real time, the mRNA is converted to complementary DNA (cDNA) by reverse transcription. The DNA fragment of interest is then amplified from cDNA template using PCR (as explained in the genotyping section above) and probes that fluoresce depending on the number of copies made are included in the reaction. The fluorescence of these probes can be determined using a real-time PCR machine to give a measure of the number of copies of the transcript of interest in

the reaction in each sample, and by extrapolation the amount that was present in the original sample. We used Taqman probes, which bind specifically to the NR2B sequence, to measure NR2B mRNA in the hippocampi of the NR2BOE mice (as well as mRNA abundance of an endogenous control gene, TFRC). Taqman probes have attached a fluorescent reporter and quencher (in close proximity to each other) and anneal specifically to a given target sequence (in this case NR2B or TFRC). The probe is cleaved as the primer is extended, allowing the reporter to escape the quencher and thus fluoresce. The greater the number of copies of the gene, the more probe is cleaved and thus the greater the fluorescence.

There are two commonly used methods for quantitatively measuring RNA; the number of cycles for the level of fluorescence to cross the cycle threshold (C_T), and the standard curve method (which can be used to calculate the relative or absolute differences between mRNA levels). The standard curve method was used here as it allows for absolute quantification and is less sensitive to differences in PCR efficiency between the target and control assays. The standard curve technique uses known samples (of known concentrations for establishing absolute mRNA levels) to compare to the mRNA concentrations of the unknown samples. The standard curve method allows the absolute quantification of the mRNA in the unknown samples through direct comparison of fluorescence levels (Figure 81).

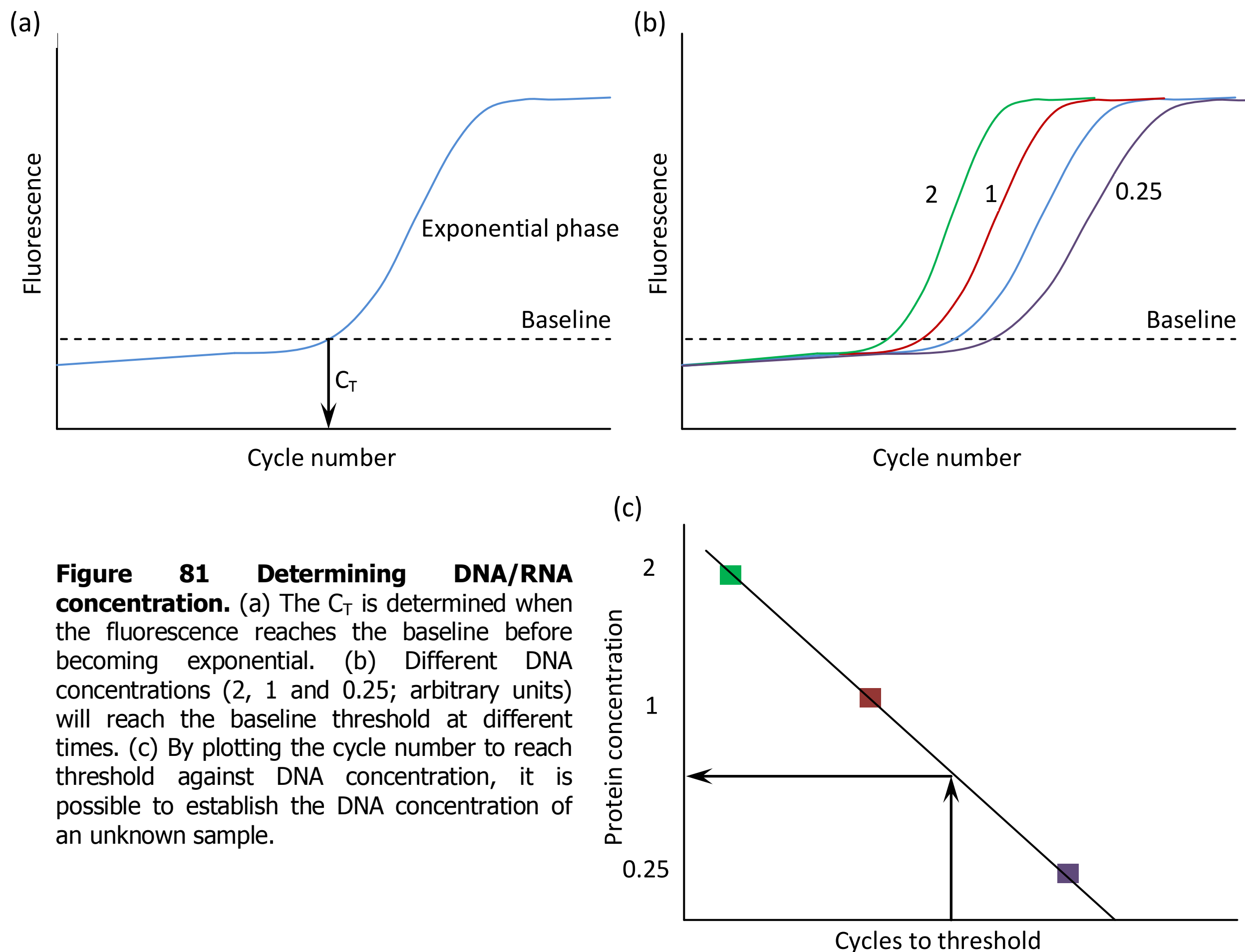


Figure 81 Determining DNA/RNA concentration. (a) The C_T is determined when the fluorescence reaches the baseline before becoming exponential. (b) Different DNA concentrations (2, 1 and 0.25; arbitrary units) will reach the baseline threshold at different times. (c) By plotting the cycle number to reach threshold against DNA concentration, it is possible to establish the DNA concentration of an unknown sample.

Often, at least one endogenous control gene is used to control for experimental errors, for variations in mRNA quantity and quality and/or for overall group differences in gene expression (Tunbridge et al 2010). For the mRNA analysis of the NR2B expression, the TFRC (transferrin receptor 1) gene was used as an endogenous control gene, which did not differ between the two genotypes. The quantity of NR2B was normalised to TFRC by dividing the NR2B mRNA, derived from the standard curve, to that of the TFRC mRNA.

NR2B reverse transcription and real-time PCR analysis was kindly conducted by Dr Liz Tunbridge (Dept. Psychiatry, Oxford University, UK).

RNA extractions were performed (by the author) using RNeasy mini kits (Qiagen). The right hippocampi were placed in 1.5ml reaction tubes with 600µl buffer RTL. The sample was then homogenised for 10 minutes and centrifuged for 3 minutes. The supernatant was placed in a separate reaction tube, to which 600µl of 70% ethanol was added and mixed. This was placed on a fractionation column and centrifuged for 15 seconds at 8,000g. 700µl of buffer was added to wash the column (which was spun for 15 seconds at 8,000g). This step was repeated (at 8,000g for 2 minutes), and the column was placed in a separate reaction tube. 50µl of water was added and the column was spun at 8,000g for 1 minute to obtain the eluted RNA.

250ng mRNA was treated with RQ1 DNase (Promega) in a volume of 30µl containing RNA, 1µl DNase (1u/µl) and 1.5µl RNAsin (Promega, 40u/µl). Samples were incubated for 30 min at 37°C, then 70°C for 6 min to stop the reaction.

For the reverse transcription step, 30µl of MMLV reverse transcription mastermix (50mM Tris-HCl, 75mM KCl, 3mM $MgCl_2$, 10mM dNTPs, 180ng Oligo dT, 108 units RNAsin, 1,200 units MMLV; Promega) was added to each sample. The mixture was incubated at 42˚C for 1 hour, followed by 70˚C for 10 minutes.

The real-time qPCR used gene expression assays from Applied Biosystems (NR2B gene; Mm00433820 m1. TFRC; Mm00441941 m1) according to the manufacturer's instructions. Samples were tested in triplicate and averaged to give a mean value. Two negative controls were conducted,

one without the reverse transcription enzyme and one with water in the place of cDNA, which were both negative for NR2B and TFRC.

Standard curves were constructed from pooled cDNA from several samples at concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.56 and 0.78ng. The number of PCR cycles needed for the mRNA levels to reach threshold along with the ratio of NR2B to TFRC were measured.

6.5. Results

Both NR2B and the control gene, TFRC, were successfully amplified (Figure 84 and Figure 87) and were in the range of the standard curve (Figure 84). The standard curves also satisfied quality control for PCR amplification efficiency (amplification efficiency was between 90-110%). The negative controls were not successfully amplified (Figure 83 and Figure 86), suggesting that the target RNA only was being amplified and that the samples were not contaminated.

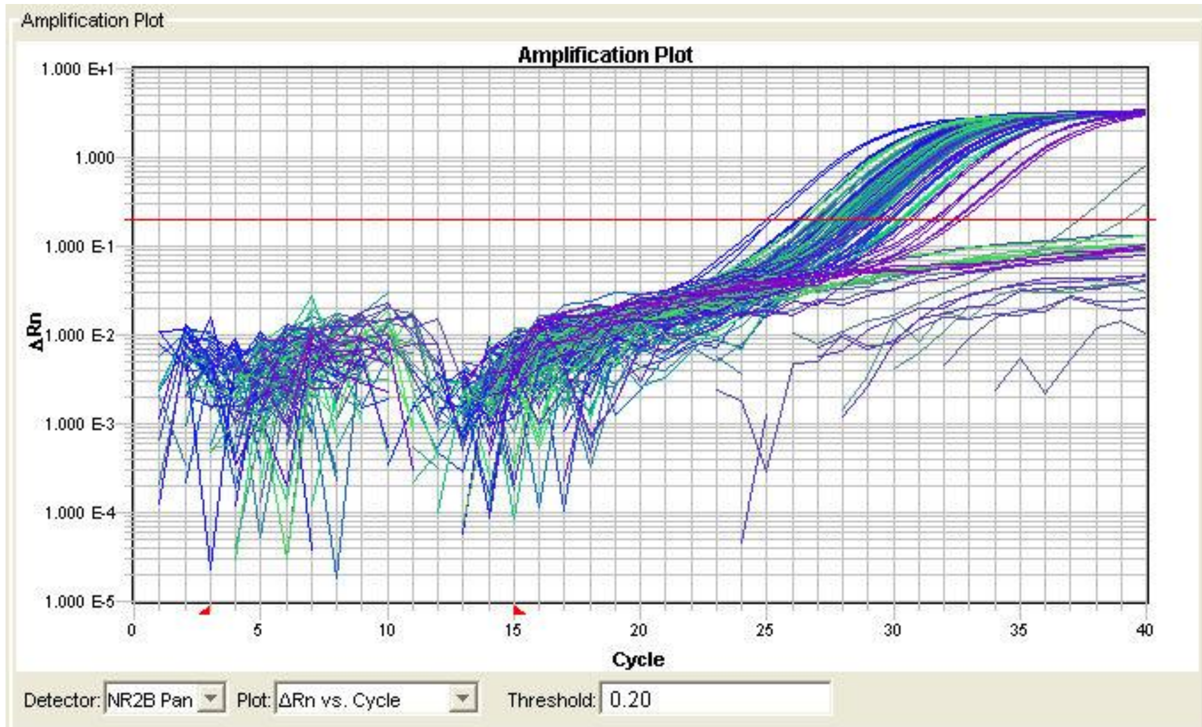


Figure 82 NR2B amplification plot. Mouse mRNA samples and negative controls were amplified.

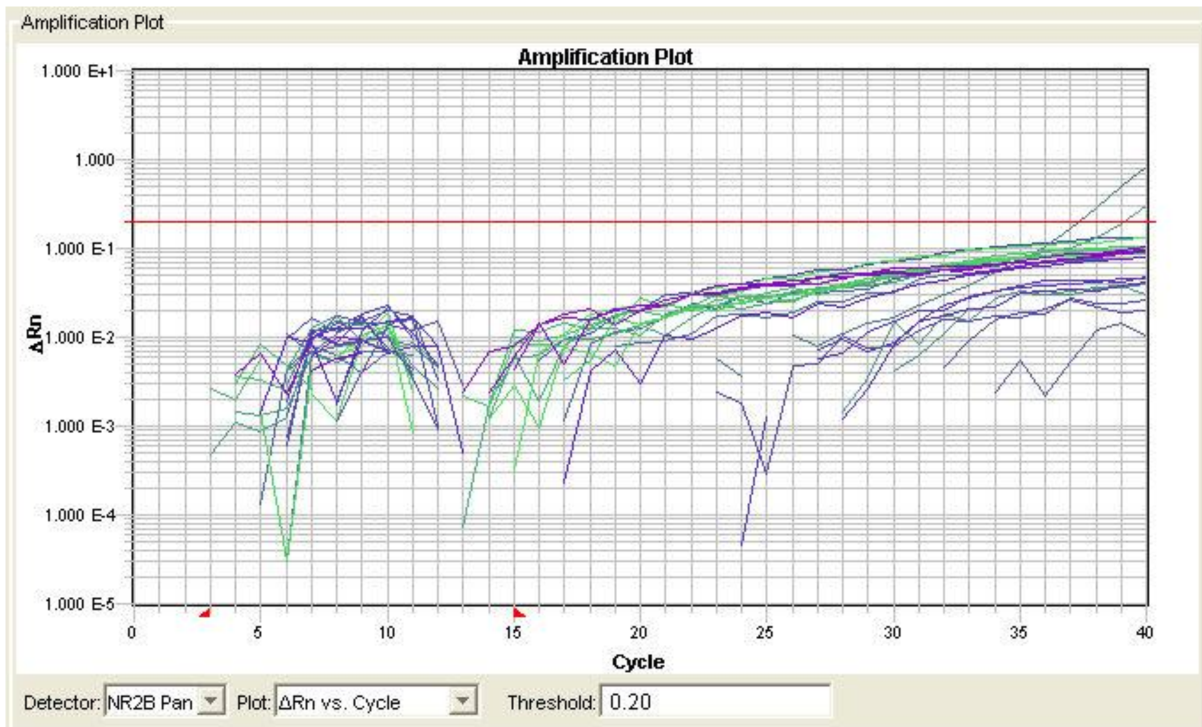


Figure 83 NR2B negative control amplifications

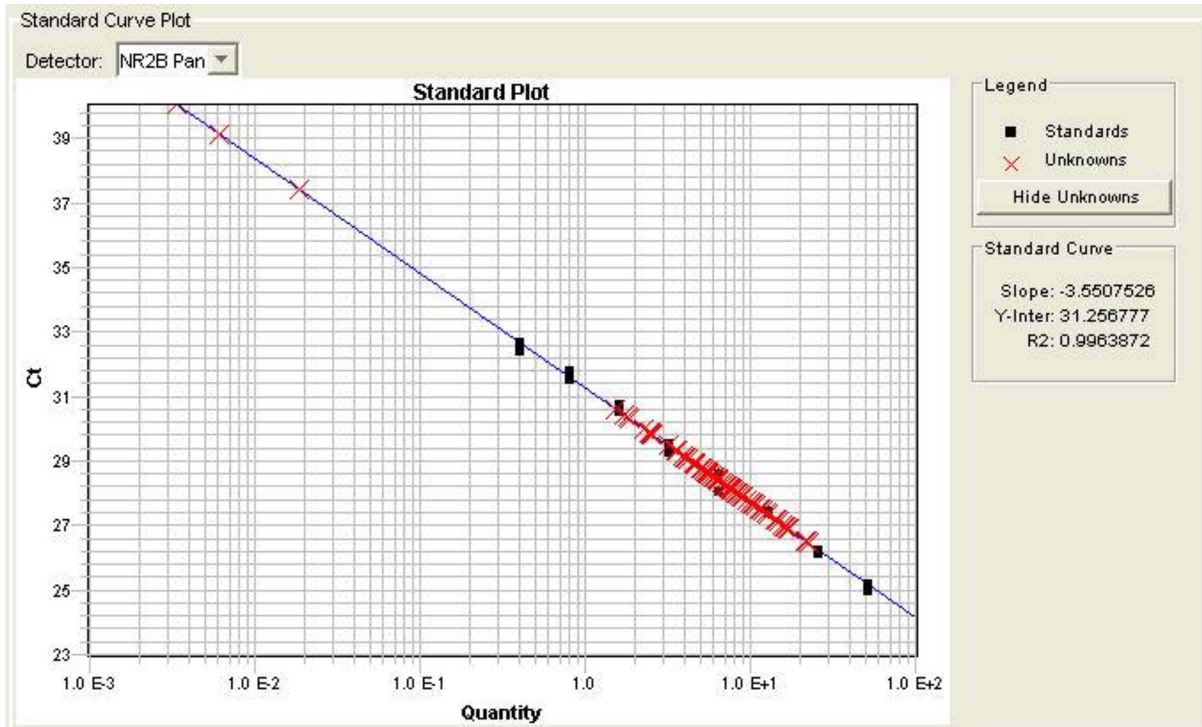


Figure 84 Standard curve for NR2B amplification

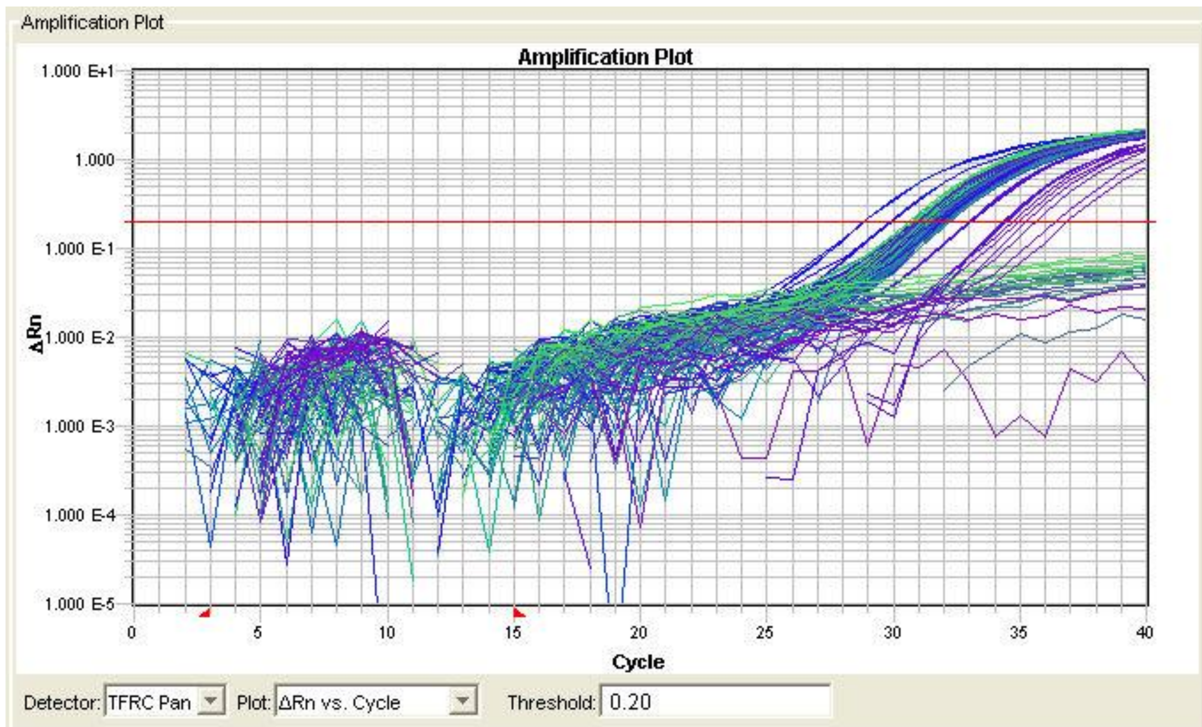


Figure 85 TFRC amplification plot. Mouse mRNA samples and negative controls were amplified.

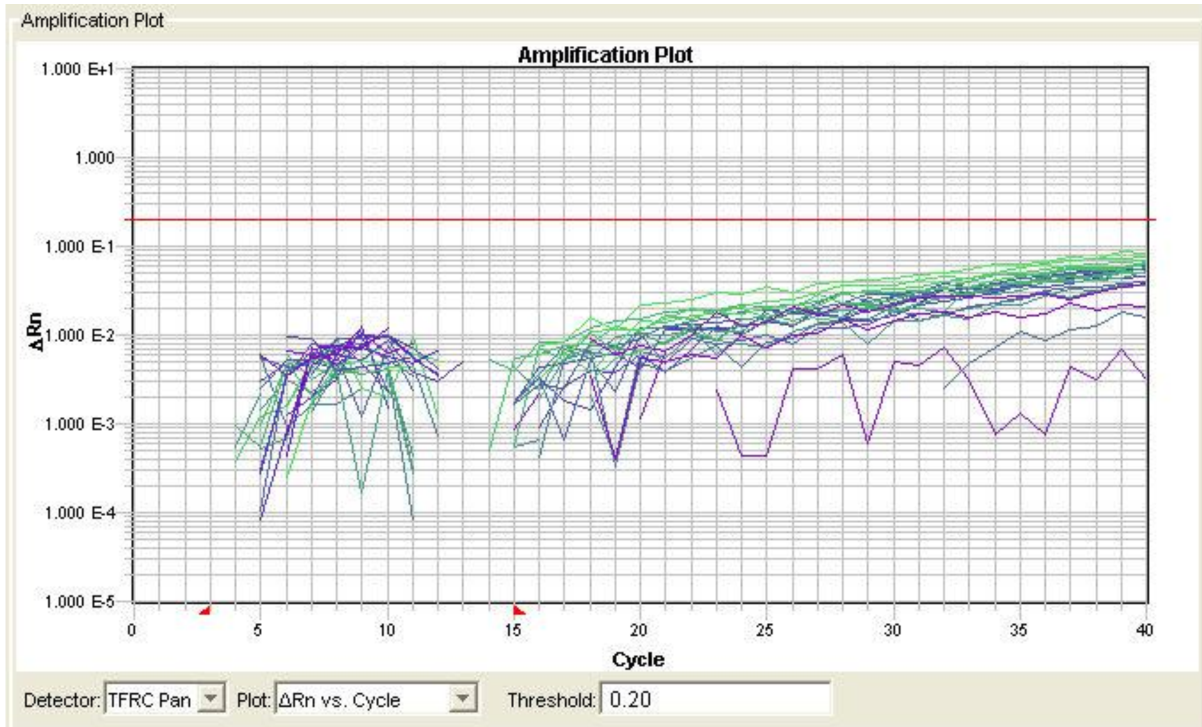


Figure 86 TFRC negative control amplifications

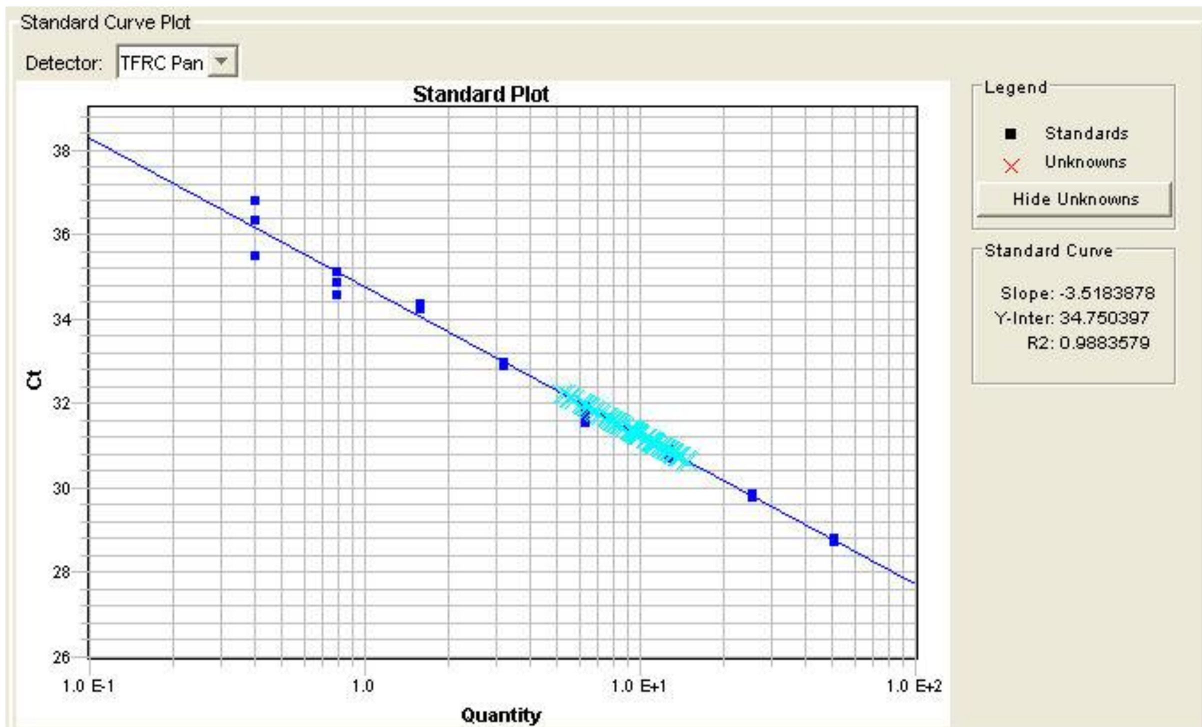


Figure 87 Standard curve for TFRC amplification

An ANOVA was performed with genotype and sex as between subject factors on the normalised ratio of NR2B mRNA to TFRC mRNA. There was more NR2B mRNA in the NR2BOE HPC than the wildtype HPC, and more NR2B mRNA in male HPC than female HPC. mRNA from NR2BOE samples took fewer cycles to reach threshold and showed a larger NR2B mRNA concentration (Figure 82). There was a significant effect of genotype ($F(1,32)=18.898$, $p<0.001$) and a trend towards an effect of sex ($F(1,32)=3.34$ $p=0.078$). There was, however, no sex by genotype interaction ($F(1,32)=0.676$, $p=0.418$). The mean normalised ratios were as follows: wildtypes = 0.65±0.07, NR2BOE = 1.32±0.13, males = 1.09±0.13, females = 0.76±0.11. Wildtype males = 0.74±0.09, wildtype females = 0.56±0.1, NR2BOE males = 1.47±0.18, NR2BOE females = 1.09±0.16.

This was reflected in the raw data for the NR2B mRNA values (genotype: $F(1,32)=9.494$, $p=0.004$. Sex: $F(1,32)=2.142$, $p=0.154$. Genotype by sex interaction ($F(1,32)=0.954$, $p=0.337$. Mean wildtype = 6.9±0.9, mean NR2BOE = 12.5±1.4) but there was no effect of genotype or sex on the raw data for the TFRC mRNA values (genotype: ($F(1,32)=0.742$, $p=0.396$. Sex: $F(1,32)=0.007$, $p=0.935$. Genotype by sex interaction: $F(1,32)=0.009$, $p=0.927$. Mean wildtype = 10.6±0.6, mean NR2BOE = 9.7±0.7). The lack of a genotype or sex effect on the TFRC values suggests that the total amount of mRNA was comparable for all groups, therefore any differences in NR2B values are likely to be a genuine result of different NR2B mRNA levels.

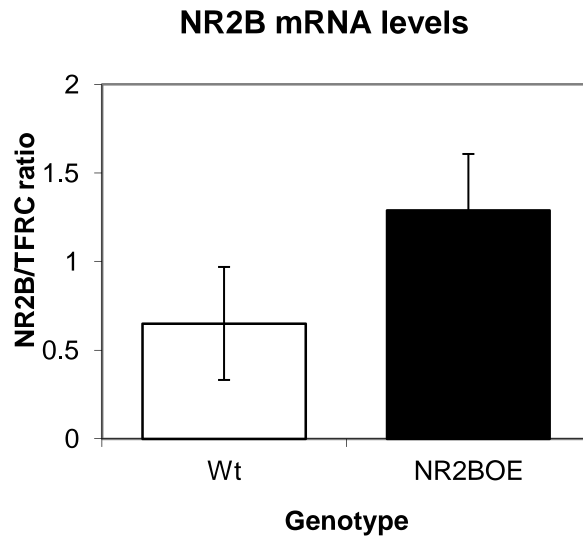


Figure 88 NR2B mRNA levels in wildtype and NR2BOE HPC. There was significantly more NR2B mRNA in NR2BOE HPC compared to wildtype HPC.

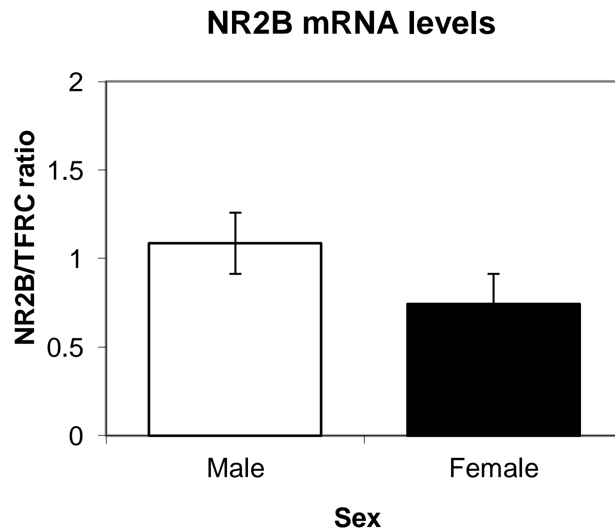


Figure 89 NR2B mRNA levels in male and female HPC. There was significantly more NR2B mRNA in male HPC compared to female HPC.

6.6. Western blots

Western blots represent a way of separating out proteins of different sizes by gel electrophoresis, transferring to a protein-binding membrane, and then detecting the protein of interest using a primary antibody specifically targeted to that protein. It involves placing the samples of interest in separate wells at the top of a gel, across which a charge is passed. Proteins then separate down the gel dependent on their size. Once the proteins have been separated, they are transferred to a membrane (polyvinylidene difluoride, or PVDF) by passing a charge across the gel and membrane. Primary antibodies can then be applied to the membrane to bind the protein of interest, which is then incubated with a secondary antibody directed against the primary antibody. The secondary antibody is linked to an enzyme, in this case horseradish peroxidise (HRP) which cleaves chemiluminescent agents to produce luminescence at the relevant location on the membrane that reflects the amount of protein present.

In the original study in which the NR2BOE mice were described (Tang et al 1999), Western blot analysis showed that the NR2BOE mice had about twice as much NR2B in the cortex than wild type mice. Unfortunately, little detail regarding the exact protocol used was included, although other Western blots performed by the same group (Cao et al 2007) have involved pooling samples together.

6.6.1. Method

The below method describes the optimised method used. All reagents were purchased from Invitrogen unless otherwise stated. There was some difficulty in optimising the procedure, primarily due to the difficulty in obtaining primary antibodies to bind specifically and exclusively to NR2B. Numerous antibodies that reportedly bind NR2B in practise were either prohibitively non-specific or even did not bind NR2B at all (such as ab109 from AbCam). Thus optimisation took a significant length of time. The variables altered in order to establish the optimal protocol are listed in Table 6.

Mice were killed by a schedule 1 method, and their brains were quickly removed. These were then divided into the left and right hemispheres (studies suggest NR2B is present at higher levels in the left hemisphere (Kiyama et al 1998)). The left and right cerebellum, forebrain and hippocampi were quickly dissected out and snap-frozen in isopentane. Samples remained on dry ice after freezing and were stored subsequently at -80°C. We investigated the protein levels of NR2B in the left HPC of each mouse, and mRNA levels of NR2B in the right HPC of each mouse.

The hippocampi of each mouse were separated into the left and right hippocampi, and the left hippocampi were then used for protein analysis. In order to ensure that the results were comparable to those of Tang et al, the protein from the mice was pooled into one of four groups (wildtype male, wildtype female, NR2BOE male or NR2BOE female). Hippocampi from 48 mice were pooled, such that within each group there was tissue from 12 mice. Within each group, tissue was subdivided into one of two pools (such that for each group n = 2) with tissue from 6 mice contributing to each pool. Equal amounts of protein from each sample were added to the pool to ensure that all mice were contributing equally to the signal. See Figure 90.

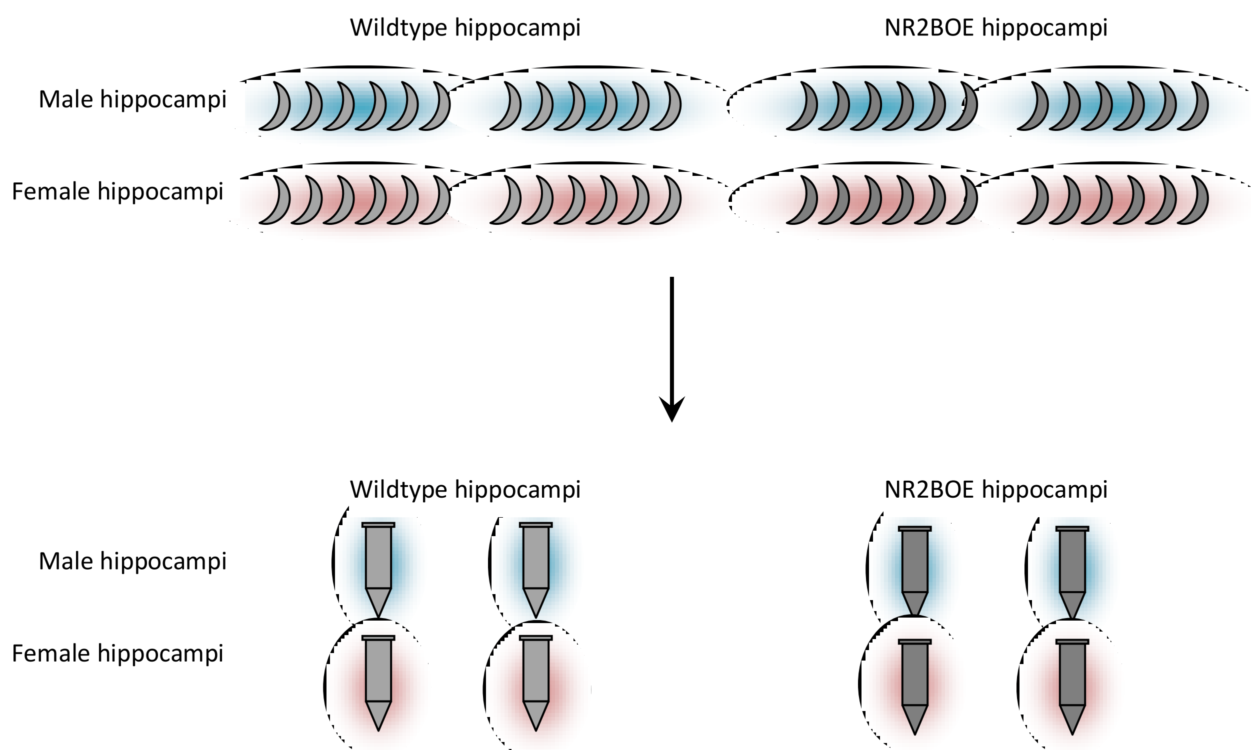


Figure 90 Left hippocampi from all mice were pooled. The left hippocampi of all 48 mice were put into one of eight groups, such that there were four wildtype and four NR2BOE samples, of which two from each group were male and two were female.

Samples were homogenized in approximately 300µl of lysis buffer (1ml RIPA buffer:1µl protease inhibitor (P8340-1ML from Sigma), with 25µl of lysis buffer per mg of tissue) using 5mm diameter stainless steel beads in a TissueLyser LT homogenizer (Qiagen) at 50Hz for 5 minutes, and were then spun at 18,000rpm to eliminate foam and to spin out any tissue debris. The protein concentration of each sample was then established using the BCA method (Pierce), alongside BSA control samples at concentrations of 4, 2, 1, 0.5, 0.25, 0.125 and 0.0675mg/ml and a blank containing lysis buffer but no protein. 20µl of each sample was diluted in 180µl of 1M NaOH, and 25µl of this was then added to the compatability reagent and was incubated at 37°C for 15 minutes. 1ml of the BCA working reagent was added and the mixture incubated at 37°C for 30 minutes. 200µl of each sample was pipetted in triplicate into a 96 well plate, and absorbance at 562nm was measured with a spectrophotometer (SpectraMAX 190, Molecular Devices). The values obtained

were compared against the BSA control values to establish samples protein concentrations. Samples were diluted to 1mg/ml in lysis buffer before being frozen to prevent the precipitating when thawed.

Standard curves were used to establish the optimal protein concentration and antibody concentrations to be used for western blotting. 4µg of protein in each sample was considered optimal using a standard curve ().

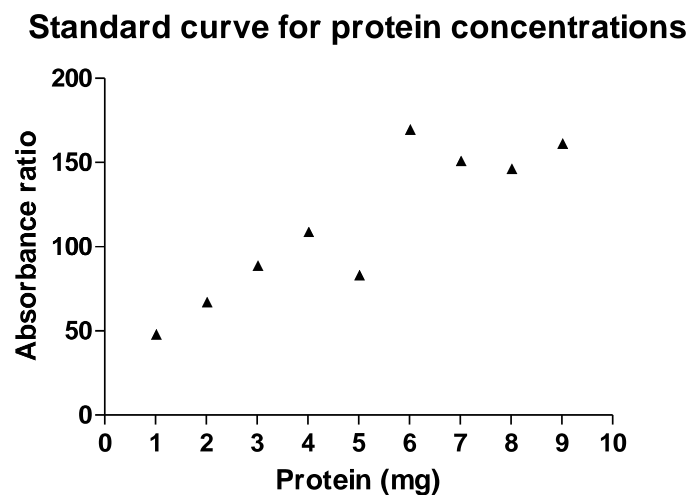


Figure 91 The standard curve for protein concentrations. 4mg/µl was considered optimal.

1 part in 10 of sample reducing agent was added to the sample protein along with 1 part in 4 of LDS sample buffer. The sample was then made up to 10µl with RIPA buffer (Thermo Fisher Scientific), and was incubated at 95°C for 10 minutes to denature the protein. This was then loaded onto a pre-set 12 well 3-8% Tris-Acetate gel. A ladder (Seeblue Plus2 prestained ladder) was loaded into the first well and the samples were loaded into the following 8 lanes. It was then run at 200V for 35 minutes in SDS running buffer. Afterwards, the gel was removed from its case, placed on filter paper soaked in transfer buffer (containing 1 part in 5 of methanol, Fisher Scientific), and a PVDF membrane (Immobilon P, Millipore) that had been soaked in methanol for 5 minutes was placed

over the gel. Another filter paper was placed over the membrane, and the gel sandwich was left to transfer in the transfer buffer overnight at 30V.

The samples were loaded onto a gel (see Figure 92).

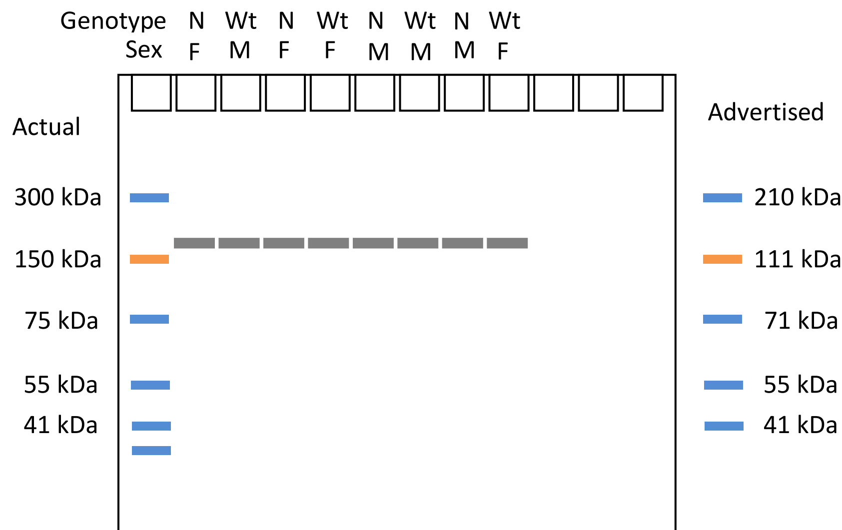


Figure 92 Set up of the gel for the NR2B blots. Wt = wildtype, N = NR2BOE, M = Male, F = Female.

Once the protein was transferred, the PVDF blot was placed in PBS containing 0.1% Tween (VWR) and 5% BSA (or 10% when blots had a lot of background noise; Sigma) for 1 hour to block the membrane. The blot was rinsed and washed in PBS-Tween three times before being transferred to PBS-Tween containing the NR2B primary antibody (M265 from Sigma, labelling the NR2B protein at 180kDa) at a concentration of 1:1000. The blot was washed again as before, before being placed in PBS-Tween containing the HRP-conjugated secondary antibody (anti-rabbit IgG raised in goat, 172-1019 from Biorad) for an hour. This was then washed as previously, and then washed in PBS without Tween twice for five minutes and once for 15. Then ECL detection reagent (GE Healthcare) was prepared according to manufacturer's guidelines and applied for 5 minutes. The blot was carefully enclosed in saran wrap, placed in a light-safe photographic cassette and apposed to photographic film (CL-Xposure film, Thermo Fisher Scientific). Exposure time was optimised to ensure that bands

of interest were visible but not saturated. The film was exposed in developer solution (Phenisol, Ilford) and fixed in a fixative solution (Hypam, Ilford). The band of interest (the antibody staining NR2B) is at approx. 180kDa, the size of the NR2B subunit.

To remove NR2B antibody, the blot was placed in Restore stripping buffer (Thermo Fisher Scientific) for half an hour, before repeating the method described above with the application of antibodies, this time using a tubulin specific primary antibody (1:8000, ab59680 from Abcam). The secondary antibody (anti-rabbit IgG raised in goat, 172-1019 from Biorad) was applied at a concentration of 1:2000.

Tubulin was probed for as well as NR2B as a control. Tubulin is a cytoskeletal protein expressed ubiquitously in the body, and its expression is unrelated to the expression of the NR2B protein. Tubulin levels correspond fairly closely to the amount of protein in the sample, thus tubulin levels can be compared across samples to ensure that the amount of protein loaded is approximately equivalent. This allows a greater degree of certainty that any variations observed are due to genuine differences in NR2B protein expression levels and not simply due to larger amounts of protein being loaded onto the gel.

The films were then scanned on a grey scale as the films themselves were blue, and the images were colour inverted and analysed using ImageJ (NIH). An area was chosen that covered the band of interest for one sample, and all measurements taken were from an area of the same size. The absorbance for each band was recorded for each sample. The NR2B absorbances for each sample were divided by the corresponding tubulin absorbances to give a relative NR2B protein level.

The ratio of NR2B to tubulin for NR2BOE and wildtype mice, and male and female mice, was then compared.

The ladder used was the Seeblue Plus2 prestained ladder; comparison against another ladder (Precision Plus Protein Kaleidoscope standard from Biorad) indicated that the sizes indicated by the ladder were not quite as advertised. The advertised and actual (approximate) sizes are shown on the diagram below (Figure 92).

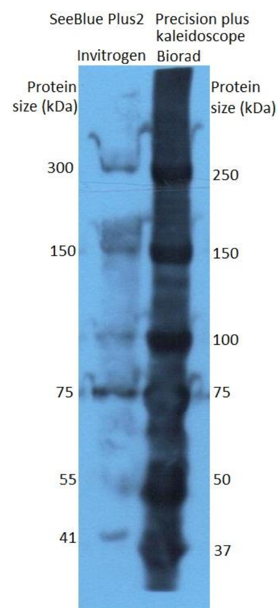


Figure 93 Comparison of the Invitrogen SeeBlue Plus2 and Biorad Precision plus kaleidoscope ladders

The below table gives the steps taken to establish optimal conditions for the western blots.

Variable	Reason
Protein concentration	To establish the optimal protein concentration to load onto the gels, a protein concentration curve was produced. It was established that 4mg protein per well was optimal and hence this concentration was used for the western blots.
Ladder	Ladders can behave differently depending on the gel through which they are run. The first ladder used did not appear to be labelling the gel at the expected weights. Hence a ladder was used which appeared to label more accurately.
Correct antibody	Several primary antibodies directed towards the NR2B protein were tried as several (ab109 and ab52033 from AbCam, and AB1557P from Millipore) did not bind at the correct molecular weight or bound to protein non-specifically. The ab65875 antibody from AbCam appeared to target the correct protein most specifically, and so this was used for the blots.
Antibody concentration	The concentration of the antibodies used affected the amount of antibody bound to the protein in the ban, and therefore the signal strength. The optimal strength for the NR2B antibody was 1:1000, and 1:2000 for the secondary antibody. The optimal strength for the tubulin primary antibody was 1:8000
Number of washes	Washing the blot helps reduce non-specific antibody binding and hence signal to noise ratio. Increasing the number of washes reduced the background noise.

Variable	Reason
Blocking agent	Differing blocking agents can be used to prevent non-specific antibody binding and improving the signal to noise ratio of the blot. Different blocking agents were tried, and BSA appeared to produce the best results.
Blocking agent concentration	Differing concentrations of the blocking agents are used to provide a differing signal to noise ratio. Varying the concentration of BSA showed that 5% was optimal.
Membranes	Differing membranes produced subtly different results. After observing results with different membranes, it was established that PVDF gave the best results.
Staining	The diaminobenzidine (DAB) staining method was used originally, but later the ECL method was employed as it is less toxic.
Film exposure	In order to differentiate between the different bands, the film had to be exposed to the signal for a length of time that varied depending on the strength of the signal. The time was varied to ensure that the signal was visible but non-saturated.

Table 6 The variables that were altered to ensure the optimal conditions for the western blots.

6.6.2. Results

The ideal antibody identifying the protein of interest for Western blots should a) bind strongly to the protein of interest, and only the protein of interest, b) be easily removable when not bound to the protein of interest, and c) be completely removed by stripping buffers, allowing the blots to be re-probed using other antibodies. This would give a single band against minimal

background noise, allowing the size of the band (representing the quantity of protein) to be accurately established.

Unfortunately, we encountered considerable difficulty obtaining an antibody that would fulfil all (or even any) of these requirements. The first antibody used (ab109, AbCam) produced a band at the wrong molecular weight (as judged against a ladder; see Figure 92). Other antibodies tried did not produce a clear band without excessive background binding (such as AB1557P and ab1557 from Millipore, ab65875 from AbCam; see Figure 94), and so we decided to use M265 (Millipore) as this gave the clearest specific band with the least background.

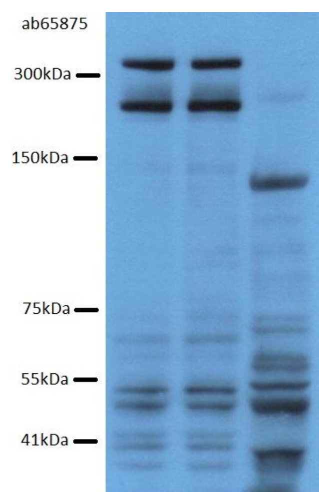


Figure 94 NR2B antibody ab65875 from AbCam. The first two lanes are hippocampal tissue, the third lane contains liver tissue (which does not express the NR2B protein. There are two clear bands at around 200 and 400 kDa in the HPC samples, presumably corresponding to the NR2B protein and possibly an NR2B dimer. However, the antibody appears to bind rather indiscriminately as shown by the large number of other bands in all three lanes, particularly the strong band at 140kDa in the liver sample.

However, this antibody did not bind as specifically as we would hope; it bound to protein at 180kDa, in agreement with the size of the NR2B protein, but it also produced a band at

approximately 300kDa. It is possible that this band represented a NR2B homodimer, or possibly an NR1-NR2B heterodimer.

It was not clear how the western blots were prepared for the original Tang et al study. Other studies performed in the same group have pooled brain samples together (such that hippocampi from all the overexpressors were homogenised together, and all wildtype hippocampi were homogenised together) (Cao et al 2007). The issue with carrying out Western blots on grouped samples is that it is impossible to identify individuals. Thus, one outlier could potentially skew the results and make samples appear significantly different (especially considering the numbers used in this study were small). Additionally, the tissue samples from individual mice will each contribute varying amounts of signal, depending on the total tissue mass each sample contributed to the overall protein volume. However, on the basis of the published results, there is no reason to suspect this occurred as the electrophysiological data also suggest an overexpression of NR2B (Tang et al 1999). To allow comparison of the results with those of Tang et al, the protein extracts were grouped.

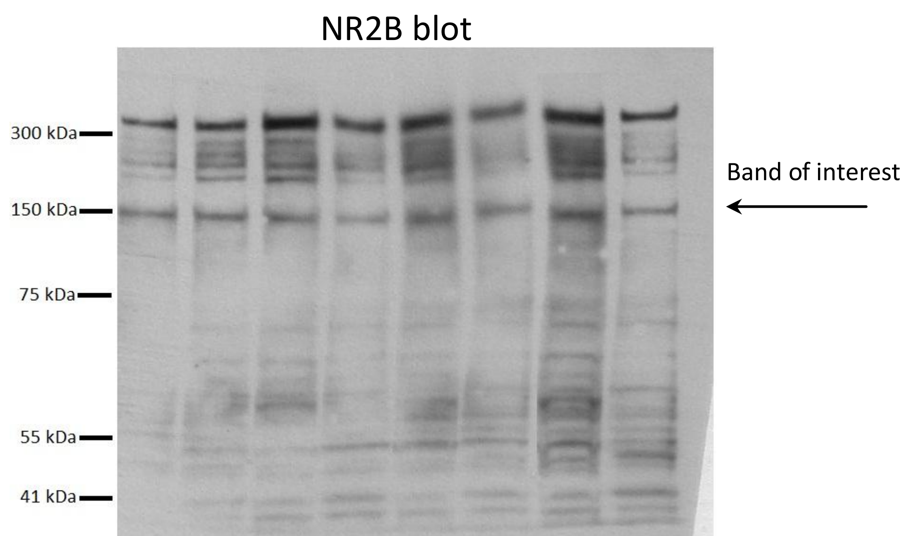


Figure 95 NR2B blot. The NR2B subunit is approx. 180kDa in size, indicated by the arrow.

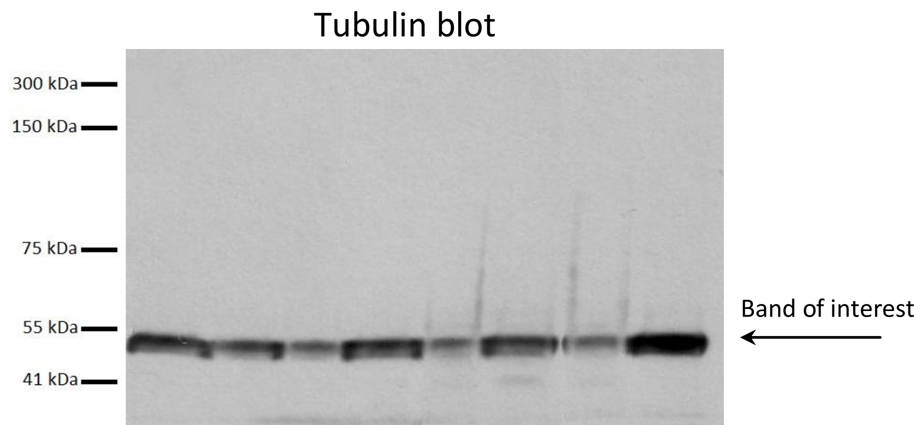


Figure 96 Tubulin blot. Tubulin is approx. 55kDa in size, indicated by the arrow

An ANOVA was performed with genotype and sex as between subjects factors. NR2BOE mice showed a near-significant overexpression of NR2B protein compared with wildtype mice (Figure 9). There was a trend level significant difference in NR2B levels between genotypes, such that the NR2BOE mice showed higher levels of hippocampal NR2B protein, compared with wildtype mice ($F(1,7)=7.248$, $p=0.055$). Although there appeared to be more NR2B protein expressed in male mice than female mice (see figure 10), the difference did not reach significance ($F(1,7)=4.513$, $p=0.101$). Although the n's were small, there was no indication of a significant genotype by sex interaction ($F(1,7)=1.096$, $p=0.354$). Mean NR2B protein expression in; wildtypes = 113.7±4.12, NR2BOE = 142.7±16.06, males = 140.2±14.43, females = 116.2±10.55; wildtype males = 116.1±6.67, wildtype females = 111.2±6.75, NR2BOE males = 164.4±6.45, NR2BOE females = 121.1±23.94. Mean tubulin protein expression in; wildtypes = 196.6±17.5, NR2BOE = 154.6±13.5, males = 156.6±10.4, females = 194.7±20.8; wildtype males = 174.2±4.7, wildtype females = 219.1±28.4, NR2BOE males = 139.0±1.2, NR2BOE females = 170.3±24.4. Mean NR2B:tubulin ratio in; wildtypes = 0.59±0.06, NR2BOE = 0.96±0.16, males = 0.93±0.15, females = 0.63±0.13; wildtype males = 0.67±0.06, wildtype females = 0.52±0.1, NR2BOE males = 1.18±0.04, NR2BOE females = 0.75±0.25.

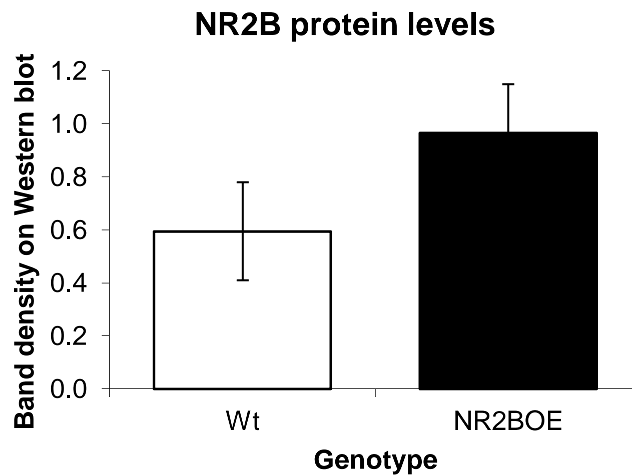


Figure 97 NR2B protein levels. The band density for the NR2B blots normalized to the band density for the tubulin blots, comparing wildtype and NR2BOE groups.

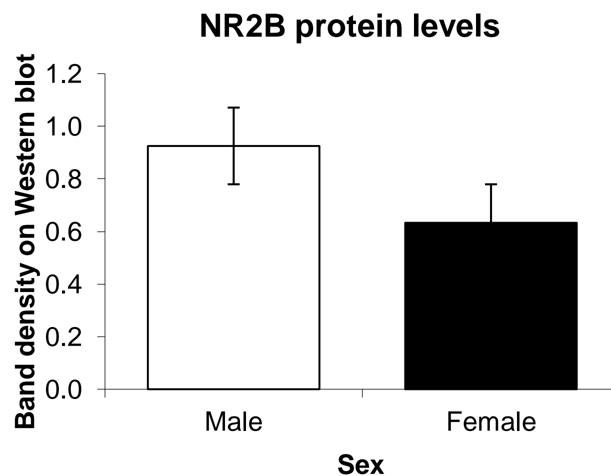


Figure 98 NR2B protein levels. The band density for the NR2B blots normalized to the band density for the tubulin blots, comparing male and female groups.

6.7. Conclusions

The genotyping data shows that the transgene is present in the NR2BOE mice (and not the wildtype mice) as predicted. The qPCR analysis demonstrated that the NR2B mRNA was indeed being

overexpressed in the NR2BOE mice, and the western blot data show that this led to a near-significant overexpression of the NR2B protein. The protein expression is only 1.6 times greater in the NR2BOE, slightly less than twice as much as reported by Tang et al. However, the mRNA expression was double in the NR2BOE HPC (on average 1.98 times greater). It is possible that the discrepancy between the greater relative quantities of NR2B mRNA in the NR2BOE could be due to the fact that the protein analysis was performed only using left hippocampi whereas the mRNA analysis used only right hippocampi. NR2B levels are known to be greater in the left hippocampi (Kiyama et al 1998), so there is no reason to believe that this had a significant impact on the protein expression levels. However, mRNA levels do not often correlate well with protein (Greenbaum et al 2003; Pascal et al 2008), so it is possible that it is merely a result of noise. Also, NR2B could be downregulated by increased protein degredation or turnover, which could explain a decrease in NR2B protein levels. Further investigations would be needed to establish a cause.

Our findings allow us to express confidence that the NR2BOE mice do indeed overexpress NR2B as intended by the insertion of the transgene. Although there is not a significant difference between the NR2B protein expression levels between the male and female mice, there does appear to be a trend towards more NR2B in the hippocampi of male mice. This may provide some explanation as to why there are clearer behavioural differences between the male mice than female mice.

7. Chapter 7: Discussion

It is clear that in the hippocampus, the NR2B subunit of the NMDAR has a significant but subtle effect on the overall behaviour of the organism. Contrary to what has been published previously (Tang et al 1999), we did not find a consistent significant improvement in the learning and memory of NR2BOE mice, despite establishing that the NR2B overexpression was still present at the genetic, mRNA and protein levels (albeit only a slight overexpression). It is important to note that this is despite the fact that the mice we used were derived from the same mice used in the Tang et al. (1999) study. Similarly, in mice lacking the NR2B subunit in the CA1 and DG, we did not see an overt behavioural impairment.

7.1.Genetics

One concern was that the NR2BOE transgene may have become mutated or lost over the course of a generation. The transgene was originally introduced into C57B/6 mice, which were then crossed with CBA mice to produce a first generation mouse line in which behavioural testing would be possible (as some mouse strains do not show good or consistent learning behaviours); specifically, it was stated that "*We found that F2 wild-type mice on this hybrid background consistently showed excellent learning behaviors, which are critical to any comparative behavioral studies*." (Tang et al 1999). This mating strategy is unusual as most groups consider C57B/6 mice to be optimal for behavioural studies. Subsequent generations were produced by crossing transgenic (NR2BOE) male mice with CBA/C57B/6 female mice. As a result, the different generations of mice should have roughly equivalent numbers of CBA and C57B/6 genes (approximately 50% of the animals genes should be from each line). However, in first generation crosses, genes from each

strain tend to be clustered together; in later generation crosses, these genes will become increasingly intermixed. This should not, theoretically, cause significant differences in behaviour, although it should be noted that it may potentially have some effect (Gerlai 1996). It is also possible that a mutation may have occurred between generations in an unrelated (or tangentially related) gene which could affect the behaviour of the mice without actually affecting the levels of NR2B. However, it is extremely unlikely that a single mutation would systematically occur in, and therefore affect, all prodigy from multiple breeding pairs and multiple litters in a single generation.

In the NR2BOE, we found an upregulation of NR2B at the DNA level using PCR, at the mRNA level using realtime PCR, and at the protein level by using Western blot analysis. However, it has already been mentioned that there are differences in the amount of NR2B in the right and left hemispheres (Kawakami et al 2003; Kiyama et al 1998; Shinohara & Hirase 2009; Wu et al 2005), and it should be noted that the Western blots were performed on protein extracted from the left hippocampi of the mice whereas mRNA realtime PCR was performed on extracts from the right hippocampi. Although this means that the data regarding the NR2B overexpression cannot be directly compared between the protein and mRNA data, as the data from both hippocampi suggest an overexpression of NR2B it is fair to assume that the genetic upregulation affects both hemispheres. It would also be impossible to accept that any effects seen in the behaviour of the mice were a result of NR2B overexpresion if there was no upregulation of the NR2B protein, so the results of the molecular biology allow us to place confidence in the belief that the behavioural results are genuinely caused by upregulation of the NR2B subunit. More importantly, we are able to confidently assert that the lack of significant results was not because NR2B was not being overexpressed.

7.2.Environmental enrichment

It has long been known that environmental enrichment promotes the ability of rats to solve problems (Hebb 1947; Rampon et al 2000a; Rampon et al 2000b), and a study by the group who originally found memory enhancements in the NR2BOE mice demonstrated that wildtype mice showed comparable enhancements in learning and memory to the NR2BOE when exposed to environmental enrichment (Tang et al 2001). Unfortunately the level of enrichment in the standard home cages in the original study by Tang et al (1999) is not discussed in detail. The NR2BOE in our studies were all housed in groups of 2 to 7 mice, with wood chips, a cardboard house, compressed cotton pads and a pine stick to chew (the wood chips, cardboard house, cotton pads and chew stick were replaced each week where necessary). Some groups house their animals in much more sparse environments (wire cages with no environmental enrichment are no longer legal to house animals in the UK), and hence in considerably less environmentally enriched cages. This could be exacerbated further by individually housing males to prevent the mice fighting, as is frequently done, despite mice being sociable animals that normally live in colonies. If the Tsien group house their mice singly or in considerably less enriched environments, the difference between their wildtype mice and NR2BOE mice is likely to be greater than that observed in this thesis. Similarly, if the conditions in which the mice were housed were considerably more enriching, we would expect to find a negligible behavioural difference between the memories of wildtype and NR2BOE mice. The two cohorts of mice were housed under exactly the same conditions, so differences in housing conditions between cohorts 1 and 2 cannot account for the differences we have observed. Hence it is possible that these results differ from those of Tang et al (1999) simply because the mice in our laboratories were exposed to greater environmental enrichment.

Other studies have demonstrated that different environments can change the neural wirings of the brain. In one study, mice were kept in ventilated cages that were subject to either high or low

ventilation. The mice kept in high ventilation cages were more aggressive to unfamiliar mice, and had alterered neuroanatomy of the olfactory bulb (specifically, there were reduced numbers, but increased volumes, of lateral P2 glomeruli in the olfactory bulb) compared with mice kept in low ventilation cages (Oliva et al 2010). If ventilation rate can cause neuroanatomical changes in mice, other environmental differences may have a much greater impact on experimental results than is currently recognised. It would make comparisons of experimental techniques (and especially differences in experimental results) more accurate if housing between laboratories was more standardised. However, this could be difficult to implement widely as some practises (such as maintain colonies in individually ventilated cages) may be impractical or too costly for some groups.

7.3.Effects of NR2B overexpression on anxiety

It has been shown previously that there is no effect of NR2B overexpression on anxiety (Li et al 2009). On the hyponeophagia task we found the NR2BOE mice appeared more anxious, but were less anxious on the light/dark box starting in the light. As pointed out previously, the results from the light/dark box starting in light could have been caused by several of the NR2BOE mice freezing, which is a reaction to fear (controlled by the amygdala) rather than anxiety (controlled by the hippocampus (McHugh et al 2004)). As such, a greater latency to enter the dark box would reflect an increased reaction to fear when mice froze. It seems premature to conclude that the NR2BOE mice are more anxious than wildtype mice as there was no significant difference between wildtype and NR2BOE anxiety levels on a further three tasks. In the face of such conflicting evidence, it is tempting to conclude that there is no significant overall difference in the anxiety levels of wildtype and NR2BOE mice. Hence if there is no difference in anxiety levels between the two genotypes, it is fair to assume that behavioural differences between the mice are not a direct result of anxiety differences.

7.4. GluN1$^{\Delta HPC}$ mice

Recent investigations with the GluN1$^{\Delta HPC}$ mice have suggested that the granule cell layer reduces in these mice after a few months of age (paper submitted to Nature Neuroscience). If this is indeed the case, while it is clear that NR1 certainly does not exist in these cells, the fact that nothing else does either due to their absence creates some difficulties. It is not possible to say unequivocally that behavioural differences observed in these mice are due to a lack of NR1 as opposed to the absence of any other function that these cells perform in wildtype animals.

Previous studies have shown that mice lacking NR1 in the CA3 region or the dentate gyrus show a spatial working memory deficit, whereas mice lacking NR1 in the CA1 region show a spatial reference memory deficit; this suggests a role for NR1 in spatial working memory in the CA3 region, but a greater role in spatial reference memory in the CA1 region. The mice we tested were lacking NR1 in the CA1 region and the dentate gyrus. There was no effect of NR1 deletion in CA1 and DG on the ability of mice to spontaneously alternate, although the mice were impaired on rewarded alternation tasks compared to wildtype mice (unless a delay between sample and choice runs was introduced). This is in accord with the previous literature which describes a spatial working memory impairment in mice lacking NR1 in the dentate gyrus. However, it is unclear why the impairment was present on the rewarded alternation task but not the spontaneous alternation task. This difference may be task sensitive as an impairment on the rewarded, but not spontaneous, alternation task has been noted in other mice also (paper submitted to Nature Neuroscience). It is possible that the molecular pathways that discern between spontaneous and rewarded alternation differ, despite both being forms of spatial working memory. Spontaneous alternation relies on the novelty

preference of the animal whereas rewarded alternation is an appetitively driven task, hence it is possible that the novelty preference of the GluN1$^{\Delta HPC}$ mice is unimpaired whilst appetitive drive is reduced. However, given the ability of the mice to acquire the spatial working memory but not spatial reference memory on the radial arm maze (manuscript in preparation) this seems unlikely.

7.5.NR2B$^{\Delta HPC}$ mice

Previous studies have found that loss of NR2B from different areas of the brain can lead to different behavioural effects. When NR2B was knocked out in the entire forebrain, mice were unable to perform spatial reference memory tasks and spatial working memory tasks. They were also impaired on several other tasks, and appeared less anxious than wildtype mice (von Engelhardt et al 2008). However, the NR2B$^{\Delta HPC}$ mice were able to perform spatial reference memory tasks at levels comparable to those in wildtype mice, although they were impaired on reversal and spontaneous alternation (von Engelhardt et al 2008). In light of this, we considered the possibility that the NR2B$^{\Delta HPC}$ mice may suffer from an inability to inhibit inappropriate associations being formed. The NR2B$^{\Delta HPC}$ mice may be capable of forming an association between the beacon and the platform but may have difficulty in preventing the beacon/platform association being formed with the S- beacon as well as the S+.

However, we did not find a significant difference between wildtype and NR2B$^{\Delta HPC}$ mice in their ability to solve this task. When the platform position was reversed, both wildtype and NR2B$^{\Delta HPC}$ mice were able find the new platform location with comparable levels of accuracy. Interestingly, on the final transfer test, the NR2B$^{\Delta HPC}$ mice made significantly more annulus crossings, possibly pointing to a more precise memory of the platform location, although this could also be an

indication of greater perseverance of NR2B$^{\Delta HPC}$ mice to swim in the area in which the platform once was located. In the von Engelhardt study the position of the platform was not visible, which may be why the NR2B$^{\Delta HPC}$ mice appeared impaired in their study whilst we found them to be unimpaired; in the visible beacon hidden platform experiment, there were two possible locations of the platform whereas in the von Engelhardt study there were countless possible platform locations.

7.6. Sex differences

Another issue considered was the possibility that there may be effects of sex on the behaviour of the NR2BOE mice. Although there was a sex difference on the watermaze, the open field task and the reference memory Y maze in the first cohort, there were very few sex differences of note when the results from both the first and second cohorts were combined.

The hidden platform watermaze was conducted in two cohorts of mice, and in the first iteration there was a significant genotype by sex interaction, whereby the female NR2BOE were significantly slower at reaching the platform than female wildtype mice, whereas male NR2BOE were significantly faster than male wildtype mice. However, this sex difference disappeared when the experiment was repeated and when results from both cohorts were grouped. It is not clear why this difference was not significant in the second cohort (or why the difference between the two genotypes was significant in the first cohort).

In addition, significant sex by cohort interactions were observed in several experiments. One potential explanation for this is that it is the result of different experimenters handling and directing

the experiments in the first cohort; hence the first cohort received subtly different experimental environments compared to the second cohort. However, this appears unlikely as analysis showed no significant differences between the performances of female mice of the first cohort when analysed by experimental handler. In addition, in the first cohort, the performance of the female mice was signficantly worse compared to the male mice, but in the second cohort the performance of the males and females was significantly improved. Half of the female mice from the first cohort (in addition to all of the mice in the second cohort) were tested by the author, so if the results were purely due to experimenter diffferences one would expect to observe the same results in the mice tested by the same experimenter. Thus half of the female mice of the first cohort would be expected to perform at the same level as the mice of the second cohort. In practice, we found that in the first cohort the female mice were significantly impaired compared to male mice on several tests, whereas experimenter differences would predict that the females tested by the author would perform at levels equivalent to the second cohort, and thus prove significantly enhanced compared to the males of the first cohort. Therefore it appears unlikely that differences between experimenters can explain the results observed in this thesis.

It was not clear why the performance in the second cohort of mice should be so different to the performance of the first cohort; the second cohort reached the platform of the hidden platform watermaze significantly faster than the first cohort, were significantly faster to enter the centre of the open field task and made more correct choices on the spatial reference memory Y maze task. We considered the possibility that the gene was no longer overexpressed and had been lost between generations, but multiple molecular biology techniques indicated that it was still present, and indeed overexpressed as intended. As mentioned earlier, environmental enrichment can significantly affect the performance of these mice, but the two cohorts were housed in identical conditions.

It is also possible that the difference is related to age. This is particularly pertinent as the mice tested by Tang et al (1999) were around 3-4 months of age, whereas the mice in the first cohort for this thesis were around a year of age when tested on the watermaze. However, the second cohort were of a similar age to the mice of Tang et al (1999) but still performed at vastly improved rates compared to the mice in Tang et al (1999). In addition, the mice of the first and second cohorts were of a similar age when they performed the spatial reference Y maze but were still significantly different in their levels of performance. It is also unclear why there should be any interactions between age and gender or genotype.

7.7.Further investigations

It might also be valuable to consider, in further investigations, whether NR1 is also upregulated in the NR2BOE; Tang et al. found a slight upregulation of NR1 in parallel with the increased NR2B receptors (Tang et al 1999). If NR1 was upregulated in NR2BOE mice, it is possible that the mice would have more functional NMDARs than controls. However, as mentioned previously, NMDARs require NR2 subunits to form functional receptors, so it would be interesting to observe whether NR2A (the only other NR2 subunit present in the hippocampus) levels also differed compared to controls. However, although this would indicate the presence of the protein and suggest the quantities of the relevant proteins, it would not provide evidence as to whether there were more functional receptors in the mutant animals; it would be necessary to use electophysiological techniques in order to establish this. Electrophysiology would also provide additional data indicating whether functional NR2B containing NMDA receptors were overexpressed as suggested by the Western blot analysis.

7.8. Wider implications

Mice are used frequently as a model organism due to their low maintenance costs and ability to breed rapidly. They also fairly closely parallel humans in most physiological systems, and as they are mammals they are more closely related to humans than other models such as yeast and worms. If it were possible to improve learning and memory in mice by upregulating a receptor subunit which is also found in humans, it is possible to imagine the exciting possibility of improving human memory by similarly increasing the abundance of this subunit. It is also highly unlikely that evolution would not have already selected for increased NR2B expression unless there was an evolutionary cost to doing so. It is not clear what these costs may be, although it is possible that increasing NR2B may, for example, increase neurotoxicity or upregulate pain sensations (Wei et al 2001).

It would be highly unethical to start manipulating the levels of NR2B in humans based purely on the not wholly substantial previous literature, and before the results from previous experiments could be extrapolated to humans a great deal more information would be needed about the reliability of these results, the potential side effects and the variation across species. We contributed to establishing the reliability of the results but were unable to replicate the previous findings convincingly. This may have been due to unspecified differences between our laboratory and the Tsien laboratory, or possibly due to previously unknown effects of NR2B variation. As we found very little effect of up regulating and knocking out the NR2B subunit of the NMDA receptor, it seems premature to believe that modulating the abundance of NR2B in people's brains may be a viable or desirable method for improving our learning and memory capabilities.

Whilst increasing a person’s ability to learn and remember seems a highly desirable aim, this work suggests that it may not be achieved through upgregulation of the NR2B subunit. These results cannot be immediately translated across to man, as there are large differences between human and mouse brains. However, the ability to modulate the level of a protein receptor in specific, restricted areas of the brain is likely to help us piece together more information, which may help us gradually solve the puzzle that is the brain.

8. References

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