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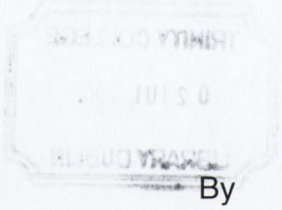
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# MOLECULAR AND CELLULAR MECHANISMS UNDERLYING SIGNAL TRANSDUCTION IN THE HIPPOCAMPAL FORMATION: THE ROLE OF BRAIN-DERIVED NEUROTROPHIC FACTOR

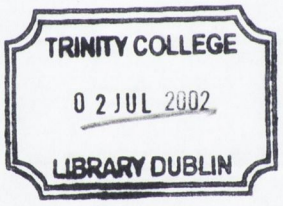


By

Kendra Shaw

A dissertation submitted for the degree of Doctor of Philosophy of the University of Dublin, Trinity College, Dublin 2, Ireland.

This research was conducted in the Department of Psychology.
September 2001.

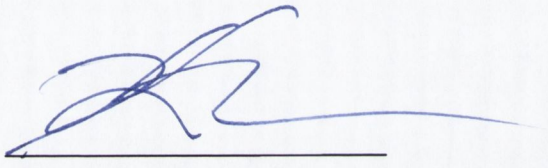


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## Summary

We investigated the possibility that BDNF might play a role in the modulation of synaptic function in the hippocampal formation. Our experiments have focused on the regulation of endogenous BDNF systems in spatial learning and on parallels between the intracellular signalling pathways in learning and long-term potentiation (LTP). We used the watermaze as our spatial learning task; the watermaze is a hippocampal-dependent task where the rats had to navigate to a platform submerged in water by learning the spatial configuration of the testing room. Typically rats were trained on the watermaze for up to 8 days: 5 days learning with 3 days retention testing after a 3-day rest period. Behavioural and endotoxic stressors, exercise and cyclooxygenase (COX) inhibition were used as means of manipulating endogenous hippocampal BDNF levels. We measured BDNF concentration in the dentate gyrus of rats and found correlations between decreased BDNF concentration, spatial learning deficits, a block of LTP induction, behavioural and endotoxic stress and cyclooxygenase inhibition. We also examined the relationship between BDNF mRNA and learning and have shown that BDNF plays an important role in the encoding of memory. We have also shown a possible interaction between BDNF and COX by suggesting that BDNF regulates COX via extra-cellular regulating kinases. This possible interaction, that we have modelled, implicates a regulatory role for prostaglandins, in particular PGE2, in spatial learning and LTP.

Acknowledgements

I would like to thank my family, friends and colleagues for their endless support and encouragement that gave me the strength to finish my thesis. I would particularly like to mention my parents, Lynda and Derek, and my sister Vanessa without whom the experience would have been a struggle. I would also like to thank my supervisor, Shane O'Mara for his excellent guidance, visionary ideas, ambition and great sense of humour; I have learned a lot from him and have been inspired by him to continue in science. I would also like to thank Sean Commins for his unyielding patience and friendship. I would like to thank those who have provided their technical expertise and assistance with assays and *in situ* hybridisation, in particular Martina Gooney, Marina Lynch Mark Webber, and Steve Hunt. Finally, I would like to thank my lab group: Sean Commins, Richard Roche, Olivier Gobbo, Michael Anderson, David Delany, and Meave Moangagang for all their help throughout the years.

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**Abbreviations**

| | |
|---|---|
| BDNF | Brain-derived neurotrophic factor |
| $Ca^{2+}$ | Calcium |
| COX | Cyclooxygenase |
| CS | Conditioned stimulus |
| CR | Conditioned response |
| DG | Dentate gyrus |
| EC | Entorhinal cortex |
| ERK | Extra cellular-signal-regulated kinase |
| FEPSP | Field potential |
| GR | Glucocorticoid Receptor |
| HFS | High frequency stimulation |
| HPA | Hypothalamic-Pituitary-Adrenal axis |
| IBU | Ibuprofen |
| IL 1/6 | Interleukin 1/6 |
| IP | Intraperitoneal |
| LPS | Lipopolysaccharide |
| LTD | Long term depression |
| LTM | Long term memory |
| LTP | Long term potentiation |
| MAP | Mitogen-activated protein |
| MC | Motor controls |
| MRI | Magnetic Resonance Imaging |
| MR | Mineralcorticoid receptor |
| mRNA | Messenger ribonucleic acid |
| NGF | Nerve growth factor |
| NMDA | N-methyl-D-aspartate |
| NSAID | Non-steroidal anit-inflammatory |
| NT 3/4/5 | Neurotrophic factor 3/4/5 |
| STM | Short term memory |
| TNF | Tumour necrosis factor |
| TrkA/B/C | Tyrosine Kinase A/B/C |
| UCS | Unconditioned stimulus |
| UCR | Unconditional response |

# CHAPTER 1

# Literature Review

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## The Hippocampus and Memory

The neurosurgical treatment of intractable epilepsy was in its infancy during the 1950's, and many novel and experimental techniques were attempted by differing neurosurgeons. Before an epileptic seizure patients experience an "aura", a warning that the seizure is about to occur. The famous Canadian neurosurgeon Wilder Penfield thought if he could provoke this aura with a mild electric current on the brain, then he could locate the source of the seizure activity and could remove the damaged tissue. The surgery to locate the source of a patient's epilepsy was conducted while patients were fully conscious, though under local anaesthetic. His technique was often successful, but his experimental surgery led him to an even more dramatic discovery. Stimulation anywhere on the cerebral cortex could bring responses of one kind or another, but he found that only by stimulating the temporal lobes patients would report recollections of past experiences. These memories were much more distinct than usual memory, and were often about things unremembered under ordinary circumstances (Penfield, 1958). Stimulation of the same area again, resulted in the exact same memory being recalled. This provided the first evidence that the temporal lobes are, in some way, implicated in memory traces or engrams.

Further neuropsychological evidence that the temporal lobes, and more specifically the hippocampal formation was important in learning and memory came from the neurosurgical patient H.M. who underwent surgery for intractable and severe epilepsy in 1953, a practice still used today to control epilepsy (see Figure 1 for the extent of the ablation). After the surgery, H.M. who used to experience at least one convulsant seizure each week and several smaller seizures daily, no longer needed anti-convulsant medication to control his seizures as they all but disappeared. Subsequent neuropsychological testing revealed that the removal of HM's medial temporal lobes had little effect on his perceptual abilities, IQ, or personality but he was unable to form new memories (Milner, 1968). This type of memory loss is called anterograde amnesia. Brenda Milner and her colleagues have spent the past 50 years testing H.M. in an effort to gather information concerning the organisation of memory.

Neuropsychological testing found that H.M.'s short-term memory (STM) and long-term memory (LTM) were both intact. He could easily recall information about his life 2 years prior to the surgery (evidence of LTM) and had a digit-span of 6 (evidence of STM). Typically, the normal population can hold up to $7 \pm 2$ items of information in STM before forgetting. An example of using STM is when you look up a phone number in the phone directory, repeat the number to yourself,

and dial the number. If asked to recall the number after a few minutes it is unlikely that you will be able to do so. The explanation for this forgetting is that there was not enough time to repeat and rehearse the numbers for memory consolidation and the information to be transferred to LTM, and therefore STM rather than LTM is used to complete the task. H.M. falls within this normal range as his digit span is 6, suggesting that his STM is intact (Milner, 1965). A variation of the digit- span test is the digit-span +1 and it is used to assess the transfer of information from STM to LTM. In the digit-span +1 test the psychologist asks the patient to repeat 5 digits that the psychologist has just spoken. The psychologist then adds one digit e.g. (1,2,3,4,5, + 6) and the patient must then recall the first 5 digits and the newly added number. This task allows for enough time to repeat and rehearse the first digits, which results in the consolidation of memory, and the information is transferred from STM to LTM, a task that requires the hippocampus. Normal subjects can repeat up to 15 digits whereas H.M. can only repeat 8 suggesting that he has a deficit in the transfer of information from STM to LTM, which ultimately results in the inability to form new LTM's (Drachman & Arbit, 1966).

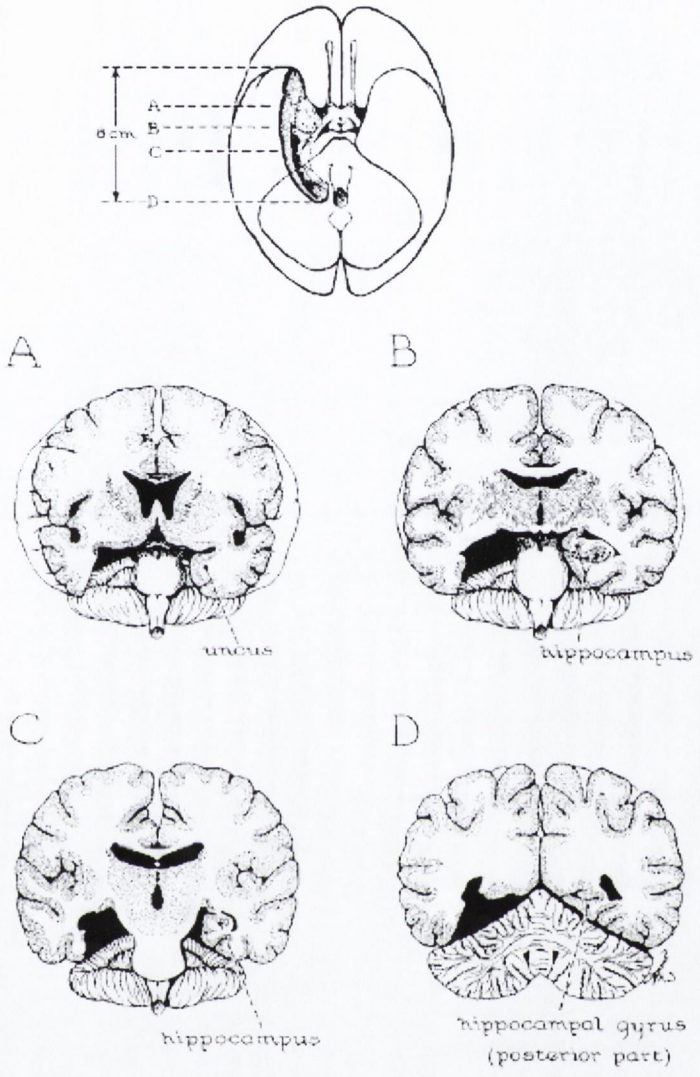



Figure 1. Diagram showing the surgeon's estimate of H. M.'s medial temporal lobe resection (Scoville and Milner, 1957, their Fig. 2, p 13). The inset at the top is a ventral view of the human brain showing the predicted rostrocaudal extent of the ablation. A through D are drawings of coronal sections, arranged from rostral (A) to caudal (D), showing the predicted extent of the lesion. Note that although the lesion was made bilaterally, the right side is shown intact to illustrate structures that were removed.

Further psychological testing found that H.M. could indeed learn certain tasks and could recall the tasks within the normal range. H.M. could be classically conditioned and learn new motor skills, e.g. mirror-drawing (Milner, 1965). Classical conditioning is a type of associative learning in that the subject, over time, elicits a response to an innocuous stimulus. We can be classically conditioned to blink our eyes to the sound of a tone. Initially, a puff of air is blown into the eye (termed the unconditioned stimulus: US) which naturally elicits an eye blink (termed the

unconditioned response: UCR). A sound of a tone (termed the conditioned stimulus: CS) is then introduced, with the tone preceding the puff of air. After repeated pairings, the tone (CS) is presented alone which causes the subject to blink, as an association has been learned between the tone and the puff of air. The eye blink in the absence of the puff of air is now termed a conditioned response (CR) as the response has been conditioned or influenced by the prior paring of the CS with the UCR (see Figure 2).

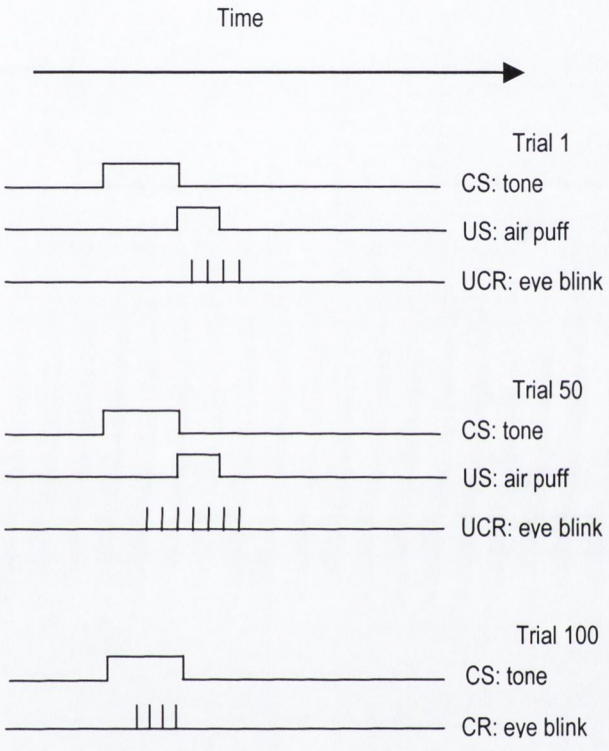



Figure 2: Development of a conditioned eye blink. In trial 1, the subjects blinks (vertical lines) only after the puff of air. In Trial 50, the subject blinks before the air puff. By trial 100, the subject blinks at the onset of the tone.

H.M. learned this task and when tested two years after initial learning, he still showed the conditioned response to the puff of air (Woodruff-Pak, 1993). These simple reflexive learning tasks do not require conscious recall or complex cognitive processes and there is an automatic or

reflexive element to the learning. It seems that there are two types of learning and memory, implicit and explicit. Implicit memory is knowledge about how we do we do things; it is the memory for motor and perceptual skills that are unavailable to consciousness, for example, how to ride a bike (Graf & Schacter, 1987; Schacter et al., 1993). Explicit memory is knowledge about autobiographical events and facts and it is available to consciousness, for example, remembering your mother's favourite perfume. Implicit memory does not require deliberate recall and involves various perceptual and reflexive pathways specific to the particular sensory and motor systems involved in the task (e.g. auditory-cerebellar pathways), it is not dependent on awareness and accumulates slowly through repetition (for a review, see Gormezano *et al.*, 1987). Implicit memories cannot usually be expressed whereas explicit learning is faster than implicit learning. Explicit learning is flexible and applicable to new situations and is most successful when two associated events occur at the same time. We recognise, for example, the face of an acquaintance more easily when we see that person in a particular context, thus the facial stimuli and the contextual stimuli act together to help us recognise the person. Neuropsychological testing of H.M. revealed that he is unable to form new memories and thus, has not acquired any new autobiographical facts since his surgery (Milner, 1965). Based on this work with H.M. and other people with temporal lobe lesions (i.e. R.B.), it has become apparent that the structures within the temporal lobe are important for the formation of explicit memories. In the medial temporal lobe there is a group of interconnected structures (e.g. thalamus, amygdala, hippocampus, mammillary bodies) that appear to be of great importance for the consolidation of explicit memories.

Subsequent research has confirmed that damage to the hippocampal formation causes deficits in spatial and mnemonic processing in humans (Eichenbaum, 2001), monkeys (Zola-Morgan & Squire, 1990; Zola *et al.*, 2000), and rats (Morris *et al.*, 1982) suggesting that the hippocampal formation plays a vital role in certain types of learning and memory (Squire, 1992; Morris *et al.*, 1982; Mishkin, 1978). Based on these animal studies and magnetic resonance imaging (MRI) studies, the hippocampal formation has emerged as the critical structure for explicit memory storage (Corkin *et al.*, 1997; Squire, 1992). Mishkin (1978) was one of the first people to mimic the lesions sustained by H.M. in monkeys by lesioning the amygdala, hippocampal formation and the surrounding cortical regions. Mishkin (1978) used a delayed non-matching to sample task to test the memory of the monkeys. This task required the monkeys to remember an object across a delay of up to 10 minutes. Following this delay, recognition was tested by presenting the monkeys with a two-choice test. As the test is non-matching, the monkeys had to choose the new

object instead of the sample object presented at the first trial. When the monkeys made the correct choice, they were rewarded. The monkeys with bilateral medial temporal lobe lesions were impaired on the delayed non-matching to sample task in a time dependent manner; this was very similar to H.M.'s performance on the delayed non-matching to sample task (Sidman *et al.*, 1968). In a subsequent study Zola-Morgan & Squire (1985) found that monkey's with bilateral medial temporal lobe lesions had intact immediate memory (STM) but memory was impaired when the number of stimuli to recall exceeded 7 ±2, they also reported that distraction exacerbated this memory deficit. After hippocampal lesions, rats have grave difficulties learning spatial tasks (Kesner *et* al., 1991; Morris et al., 1982; Oliveira *et al.*, 1997). In one such experiment, rats with hippocampal lesions were tested for acquisition and retention of a cheese board spatial task (dry land version of a watermaze task) and were impaired in both acquisition and retention of the task as measured by increased distances travelled to find the correct food location. These results suggest that the hippocampus is required for optimal learning and performance of the cheese board spatial task (Kesner *et al.*, 1991). Likewise, hippocampal lesioned rats were impaired on both the acquisition and retention of a spatial navigation task (i.e. Morris watermaze; Marston *et al.*, 1993); however, rats with hippocampal lesions can learn a left/right discrimination task in an elevated T-maze but are unable to transfer strategies learned during the transfer test (Oliveira *et al.*, 1997). These examples show that the hippocampus is critical for the transfer of spatial information from STM to LTM stores for permanent storage. Conversely, hippocampal lesions do not disrupt implicit learning; Solomon and Moore (1975) found that rabbits with hippocampal lesions learned the conditioned eye blink task as quickly as non-lesioned control animals.

## Memory Stores

Studies on retention and disruption of memory suggest that memory storage happens instages. The store theory of memory proposes that we have 3 stores, namely the sensory store, the short- term memory store and the long-term memory store. Briefly, the memory store theory purports that a stimulus activates the corresponding sensory store; if we attend to this input it is then transferred to the STM store. The STM store is small and has a limited capacity, usually 7± 2 bits of information. The information in this store fades within a few minutes unless rehearsed. Once in the STM store, if the information is rehearsed and repeated often enough it is then transferred the LTM store. LTM is much larger and enduring compared to the STM store (see Figure 3). Some theorists believe that LTM consists of an intermediate LTM and a true LTM.

Intermediate LTM is relatively sensitive to disruption whereas true LTM is not. The stability of this LTM depends on the quality and quantity of processing in the STM store. Retention of memory can also suffer due to search and retrieval mechanism failure but with time both the storage of memory and the ability to retrieve it diminish. (Atkinson & Shiffrin, 1968; Squire *et al.*, 1993).

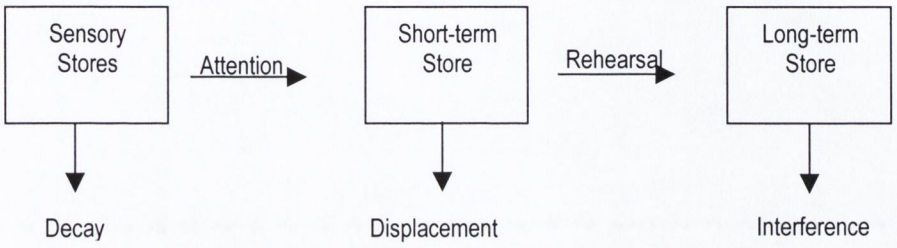


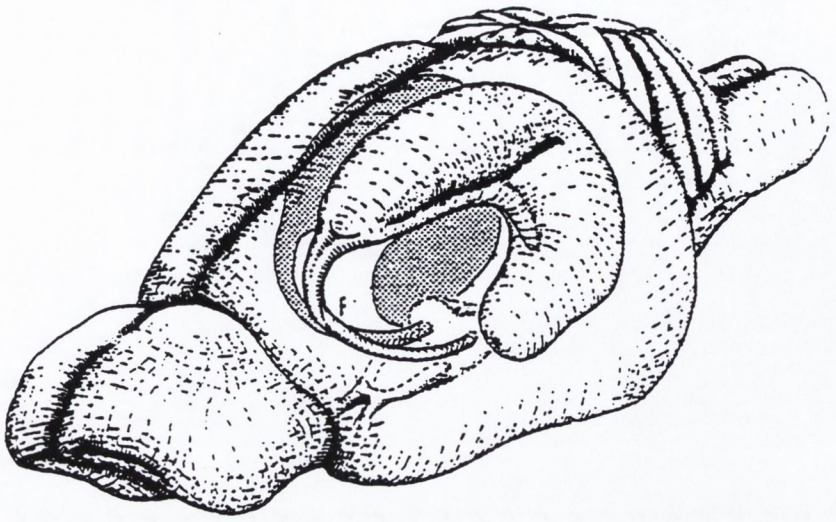

Figure 3. The multi-store model of memory

## The Anatomy of the Hippocampal Formation

Based on the literature reviewed above, the hippocampal formation is clearly important for the transfer of STM to LTM. The hippocampus (areas CA1 and CA3, sometimes called Ammons Horn), the dentate gyrus and subiculum comprise the hippocampal formation (see Figure 4a). It should be mentioned at this point that in discussions related to memory, "it is customary to use the term 'hippocampus' as synonymous with 'hippocampal formation'" (FitzGerald & Folan-Curran 2002). The pyramidal cells of the hippocampus possess extensive apical and basal dendrites and axons that bifurcate, sending projections running both anterior and posterior in the alveus, a white fibre layer that covers the surface of the hippocampus. Many pyramidal cells, therefore, project to the septal nuclei and the subiculum (Amaral *et al.*, 1991), as well as other structures (see Figure 4b for projections within the hippocampal formation). The principle output of the hippocampus proper is to the subiculum (O.Mara *et al.*, 2001), a strip of cortex lying between the entorhinal cortex and Ammon's horn. Subicular cells send axons through the alveus and the pre-commissural fornix (rostral to the anterior commissure) and the post-commissural fornix (caudal to the anterior commissure) to reach the septal nuclei and the hypothalamus, especially the mammillary body. Septo-hypothalamic fibres, running in the medial forebrain bundle, provide an additional means by which hippocampal output can influences can influence the hypothalamus. Descending projections from the mammillary body to the pons may provide an important pathway linking hippocampal output to the cerebellum, permitting hippocampal control of behaviour. The major afferent input to

the hippocampal formation arises in the entorhinal cortex which sends fibres to the dentate gyrus and to Ammon's horn via the perforant path, which runs through the subiculum (see Figures 4b & c). The entorhinal cortex, a structure closely associated with hippocampal formation, is the posterior part of the pyriform cortex (Amaral & Witter, 1995). The entorhinal cortex, in turn, receives input from virtually the entire neocortex via a complex multisynaptic pathway (Witter *et al.*, 2000). Thus, the hippocampal formation may be one of several output pathways by which the neocortex can influence behaviour. In addition to the perforant pathway, the hippocampal formation receives numerous inputs which enter rostrally via the fornix or caudally via the entorhinal cortex and subiculum. Prominent among these are cholinergic afferents from the medial septal nuclei and the nuclei of the diagonal band, serotonergic afferents from the midbrain, and noradrenergic afferent from the locus coeruleus in the pons. Finally, the right and left sides of the hippocampal area CA3 are strongly interconnected via the hippocampal commissures (Vanderwolf & Cooley, 1990).

4a




4b

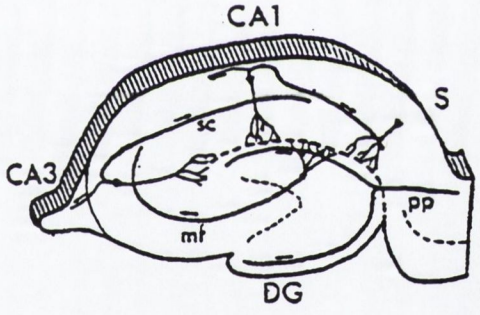



Figure 4a: shows the hippocampal formation in the rodent brain; 4b is showing a coronal section of the hippocampal formation, showing the various sub-regions. DG ; dentate gyrus, CA3 & CA1, Ammons horn regions 1 & 2, S; subiculum, pp; perforant pathway, mf; mossy fibres, sc; Schaffer collaterals.

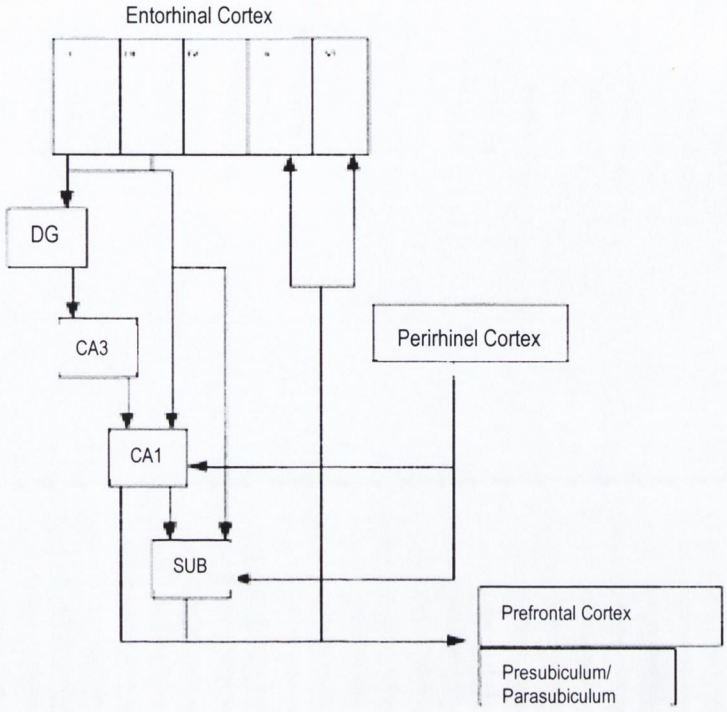



Figure 4c: Schematic representation of the major projections through the hippocampal formation (from O'Mara *et al*, 2001. their Fig. 3 page 6).

## Hippocampal-Dependent Spatial Learning

Spatial memory is the ability to remember the spatial layouts of the environment (Gallistel,1990). There are several types of knowledge required for spatial learning and these include: knowledge of object location; knowledge of your own location in relation to the objects; knowing how to get from one location to another; general geographical knowledge, and knowledge of spatial configurations of familiar objects. While there are many spatial maps or layouts that we learn there are only two types of spatial representation: egocentric and allocentric representation; a spatial representation is a cognitive construct of the animals environment. Egocentric representation is essentially a body-centred representation where distance, orientation or directional information is represented in relation to the current position of the body (Taube *et al.*, 1996). This form of representation occurs in the parietal cortex and is beyond the scope of this thesis. Allocentric representation is an environment-centred representation where information about the environment is encoded in terms of cognitive maps or vectors; this form of representation occurs in the hippocampus (Tolman, 1948; O'Keefe & Nadel, 1987; Poucet, 1993).

## The Role of the Hippocampus in the Representation of Space

O'Keefe and Dostrovsky (1971) implanted electrodes in the hippocampus of freely-moving rats while they explored a maze and they found a correlation between the firing of single neurons and the position of the rat in the maze. These location-specific neurons were called "place cells". O'Keefe (1979) defined a place cell as a cell that constructs the concept of a location in an environment by connecting many multi-sensory inputs each of which can be perceived when the animal is in a particular place in the environment. To make correct choices on a spatial learning task, animals must recognise the environment in order to call up the correct cognitive map, confer an orientation onto the map appropriate to the direction form which the animal entered the environment, and track its own movement through the environment in order to update its internal representation of direction and position (Huxter *et al.*, 2001). The memory of a place in rats then, relies in part on contextual stimuli or input which is an important component of explicit memory. Both spatial representation in rats and explicit memory are hippocampal-dependent and it for this reason that rodent spatial learning paradigms are used to help us in our quest to understand human explicit learning and memory function.

## The Watermaze.

The watermaze task addresses the issue of whether or not animals can learn about a position in space which is not marked by a local cue. The watermaze is an apparatus where rats are trained to find a platform submerged in water (see Figure 5). This hidden platform can only be found by using distal extramaze cues. Animals are required to learn to swim from the start position to the escape platform. Rats are placed in the watermaze facing the side walls and the trial is started as soon as the rats is released. If the rat doesn't find the platform within 60 seconds the rats is guided to the platform by the researcher. The rat remains on the platform for 20 seconds. The basic task involves a 5 day acquisition phase where the rat learns the location of the hidden platform, followed by a 3 day rest period where the rat remains in the housing unit and then a 3 day retention phase where long-term memory of the platform's location is assessed. On the final day of the retention phase a probe test is conducted, this is where the platform is removed from the watermaze and the rat is allowed to swim freely for 60 seconds. The probe test allows to assess if the rat has learned the location of the platform, this is indicated by the amount of time the rat spends in the quadrant where the platform was. There is no universally accepted standard and the

task described above is only one example of the many variations on the basic task ( see Stewart & Morris, 1993 for a review of the various watermaze protocols).

Successful acquisition of the watermaze task requires that a rat first learn a set of general behavioural strategies that allow it to cope with the conditions of the task and acquire information about the specific spatial location of the platform that is hidden beneath the surface of the water (Morris *et al,* 1982; Stewart & Morris, 1993). Extramaze cues are essential for learning this task; the rat learns the location of the platform in relation to these distal cues. If curtains are drawn around the watermaze after several trials of learning the animal cannot find the platform but if curtains are only partially drawn around the watermaze the animals will be able to find the platform (Stewart & Morris, 1993). The watermaze is widely and preferentially used to the radial arm maze as there are no odour cues, no motivational problems, and the animals learn quickly (Hölscher & O'Mara, 1997).

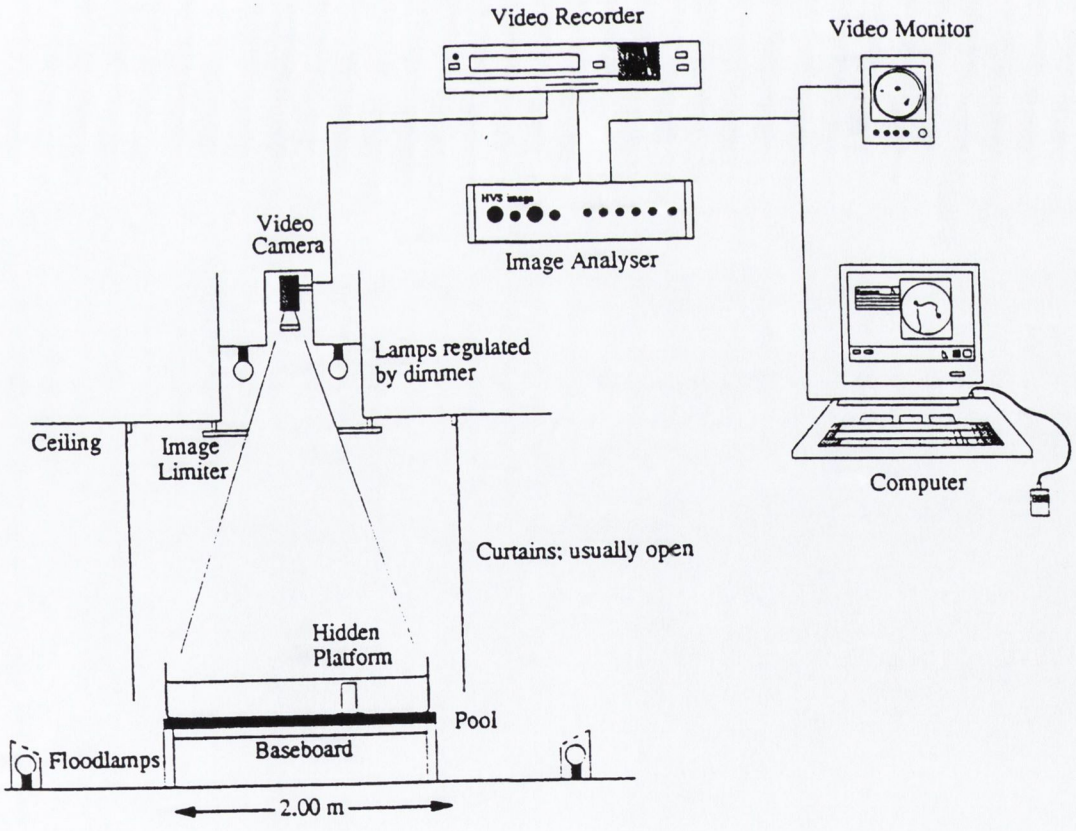



Figure 5: Typical arrangement of equipment for automatic tracking of rats in the watermaze. Reprinted from Stewart & Morris (1993).

## Spatial Navigation

There are several strategies that an animal can use to find its way in this environment. When the rat has no information about the location of the platform it randomly swims until it accidentally finds the platform or is placed on it by the researcher. Once on the platform the rat can use the spatial cues outside of the watermaze and apply strategies to find the hidden platform upon re-entry into the watermaze. An animal may rely on the motor feedback from finding the platform, a strategy called Praxic navigation. Rather than relying on random navigation or praxic navigation, a more useful strategy for the rat to use is taxon navigation (Redish, 1999), where the animal uses a single cue to orientate itself toward the platform. After several trials in the watermaze, the rat may use route navigation or path integration to find the platform. Path integration it is a process by which an animal is able to keep track of its position relative to its start position. Distance, temporal and kinaesthetic feedback are important in this strategy. Path integration requires monitoring the distance travelled along a straight line and the angles at which turns are made. These vectors are then added and the animal uses this calculation to find the location (for a review see Redish, 1999). A final strategy used in spatial navigation is the cognitive map, a phrase first used by Tolman (1948), who defined a cognitive map as a representation of the environment which indicates the routes, paths and environmental relationships that an animal uses in making a decision about where next to move. Having a mental representation of an environment allows for novel short cuts to be made by the animal (Tolman, 1948; O'Keefe & Nadel, 1978).

Poucet (1993) believes that cognitive maps are first formed with topographical information and later refined by metric information. Topographical information is the knowledge of specific relationships between objects, for example knowing if objects are joined. Metric information is qualitative information about specific angular directions and distances of objects from one another. A cognitive map then is formed when an animal integrates the views of the area into a complete representation by specifying the relationship between places with in a certain area. A single local view of an object in the area may reactivate the memory for the total area. Essential to the formation of a cognitive map is exploration, by travelling through a novel environment an animal forms a location-dependent representation of connected places. Initially due to lack of exploration only certain routes are represented but, with increased exploration a more complex map is formed and finally a location-dependent cognitive map is formed so that the animal can utilise short cuts by linking topographical information with metric information (Redish, 1999).

## Mechanisms of Storing Information in the Brain

It is of great interest and importance to understand how such experiences lay down lasting traces in the brain and to understand the mechanisms by whihc traces endure. Information storage in the brain during learning and memory has long been considered to derive from long-term changes in the strength of synaptic connections between neurons. Donald Hebb (1949) proposed a simple mechanism for storing information in the brain, suggesting that the changes required for learning take place at the synapse and it is the change in the synaptic strength that constitute memory traces. Hebb essentially proposed that when the axon of neuron A repeatedly takes part in firing neuron B, some growth process or metabolic change occurs in one or both cells that increases the subsequent ability of axon A to excite neuron B. According to this Hebbian learning rule, coincident activity in the presynaptic and postsynaptic neuron is critical for strengthening the connection between them. This type of synapse, one that is strengthened through coincident activation of pre and post synaptic terminals, is termed the Hebbian synapse.

In an extremely important experiment, Bliss and Lomo (1973) implanted stimulating electrodes in the entorhinal cortex (EC) and recording electrodes in the dentate gyrus (DG) of the hippocampus (see Figure 6). They then stimulated the EC-DG projection at the rate of one pulse every 20 seconds to establish a baseline response called a field potential (fEPSP). They then stimulated the EC-DG projection at a very high rate (typically a train of 50 - 100 stimuli at 100 Hz or more; Bliss & Collingridge, 1993). Following this high-frequency stimulation (HFS) they stimulated this pathway again at the baseline rate. Baseline responses more than doubled after HFS and remained well above the baseline response for a long time (typical experiments record for between 30 minutes and 4 hours; Bliss & Collingridge, 1993).

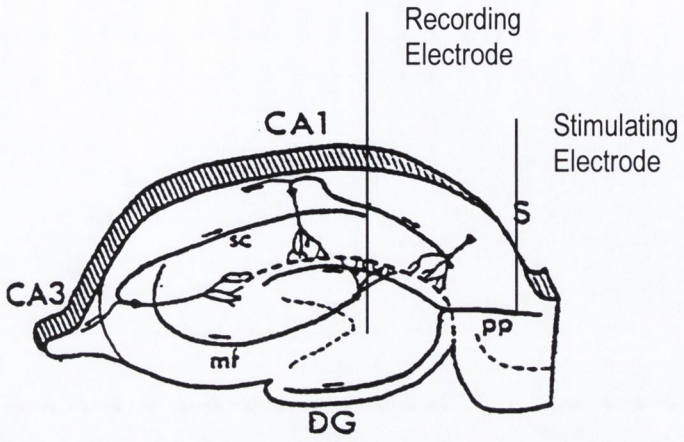



Figure 6: Representation of stimulating and recording from the EC-DG projection in the hippocampal formation DG ; dentate gyrus, CA3 & CA1, Ammons horn regions 1 & 2, S; subliculum, pp; perforant pathway, mf; mossy fibres, sc; Schaffer collaterals.

The experiment by Bliss and Lomo (1973) is important as it demonstrates that neurons in the hippocampus are plastic- that is, by applying brief, high-frequency electrical stimulation to an excitatory pathway in the hippocampus there is an increase in the strength of synaptic transmission in that pathway. This activity-dependent enhancement of synaptic strength has come to be known as long term potentiation (LTP). LTP is readily induced in the hippocampus, a part of the brain that is involved in memory and it is believed that LTP is a possible mechanism of synaptic plasticity that contributes to the formation of memories (Bliss & Lomo, 1973; Bliss & Collingridge, 1993).

Learning the location of the hidden platform is hippocampal dependent (Morris et al, 1982; Morris et al., 1996; Marston *et al.*, 1993; Good & Honey, 1997). Good and Honey (1997) induced excitotoxic lesions in both the hippocampus proper (areas CA3 & CA1 and dentate gyrus) and the entorhinal cortex-subiculum region of rats. Both groups of rats were then trained on the watermaze and were found to perform significantly worse than the controls, spending significantly less time in the platform quadrant. Also, studies have shown that drugs that block LTP also block learning in a variety of hippocampal-dependent situations. Morris *et al.* (1986) introduced a method to test the proposed link of LTP with spatial learning; they reasoned that if NMDA-dependent LTP in the hippocampus was a neural mechanism required for learning, then blocking the induction of LTP by an NMDA antagonist would prevent animals from learning spatial tasks. They used chronic intraventricular infusion of AP5, an NMDA-receptor blocker, in rats that were learning the

watermaze task. These rats were unable to learn the task normally, suggesting that NMDA receptor activation is important in both LTP and learning. A subsequent AP5 experiment showed that an AP5- induced learning deficit can be avoided if rats are pretrained in watermaze different to the test watermaze before AP5 administration (Bannerman et al., 1995). A possible explanation for these results is that while NMDA receptors are required for some components of spatial learning they may not be important for encoding the spatial representation of a certain setting (Bannerman et al., 1995). These studies show that by blocking NMDA receptors rats are unable to learn the task normally, unless they have been trained prior to drug administration, suggesting that NMDA receptor activation is important in both LTP and learning.

Even though no one has shown that LTP is the mechanism for learning in the hippocampus it is generally accepted that LTP may engage similar biological mechanisms to those that underlie memory, as memory and LTP are both hypothesised to engage activity-dependent mechanisms in the brain (Martin et al., 2000; O'Mara *et al.*, 2000). Furthermore, LTP is the predominant contemporary expression of the Hebbian hypothesis- that the brain encodes memories as activity-dependent changes in the behaviour of distributed synapses- is stated by Bliss and Collingridge (1993): "*External events are represented in the brain as spatio-temporal patterns of neural activity, and it is these patterns of activity which must be the locus of synaptic change. The location of the storage, engram of learning and memory, must therefore be found among those synapses which support activity-dependent changes in synaptic efficacy.*" Many molecular signals are involved in aiding the synaptic plasticity indicative of LTP and while NMDA-receptor activation and calcium influx are crucial for LTP and spatial learning (Cain, 1998; Morris et al., 1989; Lynch, 1998; Neveu & Zucker, 1996) new research suggests that neurotrophins may also play a role in hippocampal-dependent learning and LTP (Messaoudi et al., 1998; Kesslak et al., 1998; Narisawa-Saito & Nawa, 1996; Lu B. (2001).

## Neurotrophins

The neurotrophin family comprise small, homodimeric polypeptides (120 amino acid residues) with a varied range of biological activities that influence the generation, differentiation, survival and regeneration of neurons (Thoenen, 1991). The neurotrophins include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), NT-3 and NT-4/5 and they exert their effects through tyrosine kinase receptors (Trk receptors) (Glass & Yancopoulos, 1993; Segal & Greenberg, 1996). In 1953, Rita Levi-Montalcini in her experiments showed that a soluble factor was responsible for the stimulation of neuronal growth (Levi-Montalcini & Hamburger, 1953) and

the following year Seymore Cohen joined Levii-Montalcini's team and isolated the soluble factor, identified it as a protein and called it nerve growth factor (Cohen et al., 1953). In 1956, snake venom containing NGF was injected into the yolk of 6-8 day old chick embryos for 3-5 days; this resulted in the overgrowth of sensory and sympathetic ganglia and excessive production of their fibres (Levi-Montalcini & Cohen, 1956). Almost thirty years after the discovery of NGF, BDNF was isolated and characterised (Barde, 1982).

BDNF, NT3 and NT4/5 mRNA levels increase with postnatal age and are widely expressed in the brain throughout life suggesting, in addition to nervous system development, these neurotrophins regulate the structure and function of a mature nervous system (Thoenen, 1995; Oppenheim *et al.*, 1992; Yan *et al.*, 1992; Freidman *et al.*, 1993). Neurotrophins and their receptors are highly expressed in the cortex; NGF and its receptor, TrkA, is largely expressed in striatal and basal forebrain cholinergic neurons whereas BDNF and NT-4/5 and their common receptor, TrkB, are highly expressed in the areas CA1, CA3, and the dentate gyrus of the hippocampal formation (see Figure 7), cerebellum and cerebral cortex (Chao, 1992; Lindsay *et al*, 1994; & Thoenen, 1995; Schmidt-Kastner *et al.*, 1996). Neurotrophins are found in both the peripheral and central nervous systems.

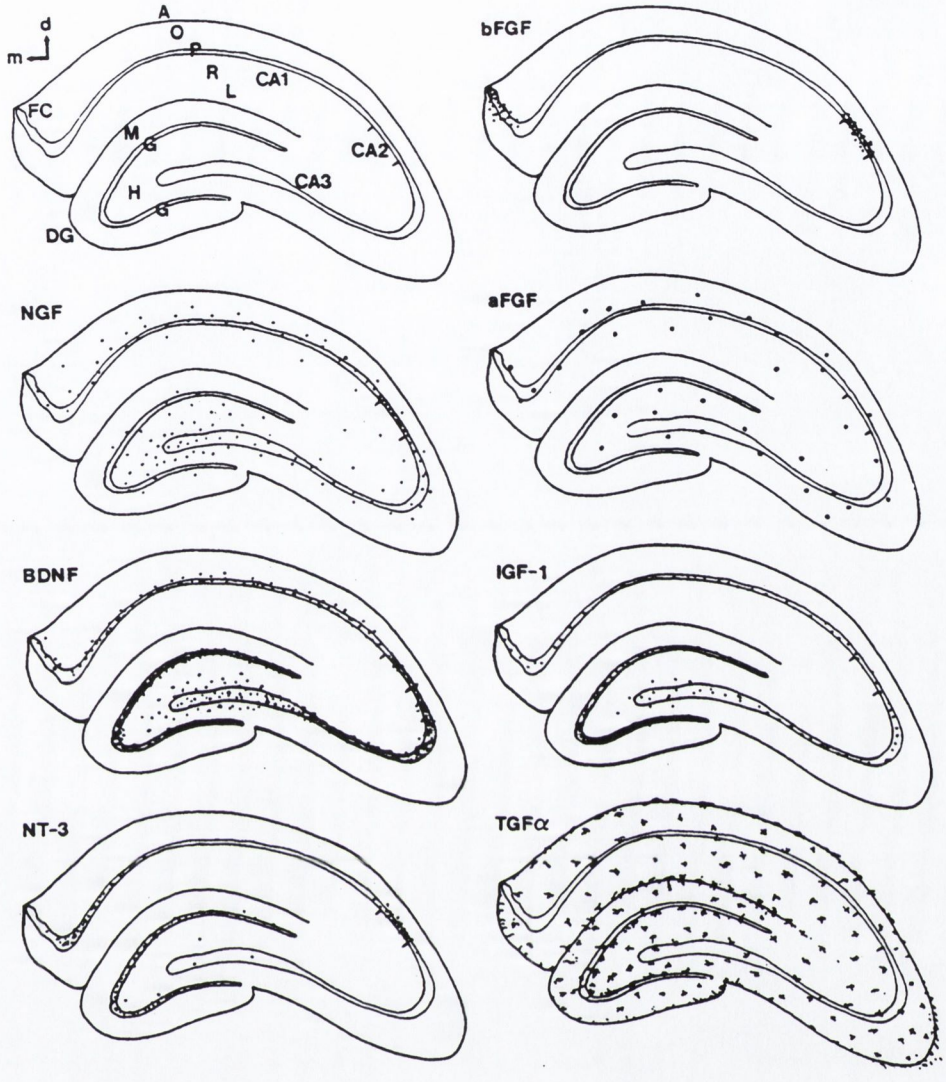



Figure 7: Coronal sections of the right side of the rat brain illustrating the distribution of neurotrophic factors in this structure. All distributions are in neurons, with the exception of TGFα precursor which is localised in glia. DG; dentate gyrus, M; molecular layer; G; granule cell layer; H; hilar layer; Ammon's horn includes the CA1, CA2, & CA3 regions each with an alveus (A), stratum oriens (O), pyramidal layer (P), stratum radiatum ( R), and stratum lacunosum (L). FC; fasciola cinerium. BDNF is the most prominent trophic factor in the hippocampus and is particularly concentrated in the DG.

One of the best described functions of the neurotrophins is in the regulation of specific neurons. During development it is proposed that neurotrophins are released from target tissue in limited quantities, which results in competition between innervating neurons. Neurons that successfully form synaptic pathways survive and the neurons that fail die (Oppenheim, 1991), a process that is essential to the developing nervous system. Neurotrophins exert their effects by either anterograde and/or retrograde signalling. Anterograde expression is when the protein is

produced by the pre-synaptic neuron, is released and subsequently influences the post-synaptic neuron by attaching to its receptor. Retrograde neurotrophic expression is when the post-synaptic neuron sends the protein back to the pre-synaptic neuron to stimulate further changes in the pre-synaptic neuron. Retrograde axonal transport of proteins from the nerve terminal to the cell body is important as it allows information about the nerve terminal to be transmitted to the nucleus and for the cell to respond appropriately (see Figure 8). The neurotrophins, especially BDNF, can rescue motor neurons from cellular death, rescue axotomized neonatal rat neurons and can prevent axotomy in injured adult motor neurons (Oppenheim *et al*, 1992; Yan *et al*, 1992; Freidman *et al*., 1993). BDNF synthesis, which is low in a normal nerve is increased in the nerve distal to the injury (Meyer *et al*., 1992) suggesting the possibility that retrograde transportation may occur. Retrograde transportation of BDNF is increased upon nerve lesion making BDNF more available to the damaged nerve, enabling survival (Meyer *et al*., 1992). Based on these findings many researchers have investigated the clinical effects of BDNF on degenerative neuromuscular conditions but without much success. For example, a clinical study investigating the intrathecal and subcutaneous administration of BDNF found no beneficial effect on either respiratory function or survival of patients with Amyotrophic Lateral Sclerosis (ALS) (Rothstein, 1996).

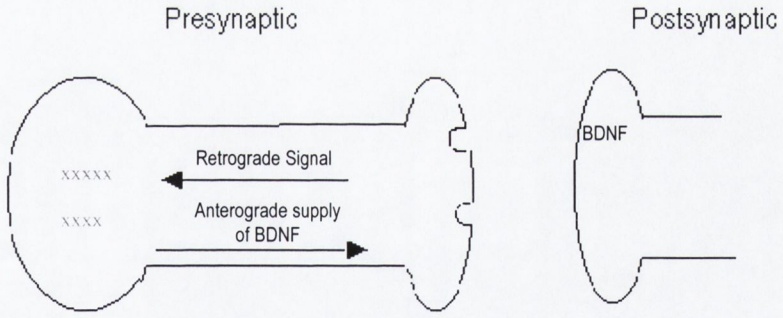



Figure 8: A model of BDNF signalling and retrograde axonal transport. BDNF is secreted by target postsynaptic cells (anterograde signalling). BDNF binds to the presynaptic neuron and is transported to the nucleus (retrograde signalling).

The physiological significance of retrograde axonal transport of BDNF has not been resolved (Reynolds et al., 2000). The possible purposes of the retrograde transport of BDNF include: a means of getting BDNF to the cell body where it or a by product can exert its trophic

effect (DiStefano et al., 1992; Curtis *et al.*, 1995; Hendry *et al.,* 1974); a means of getting the receptor to the cell body so it can act as the trophic message; an appropriate second messenger, which is subsequently transported to the cell body, may be generated at the nerve terminal (found by Heumann et al., 1981 for NGF); enabling a short-lived or unstable second messenger to reach the cell body (Hendry 1980); providing a means to regulate the levels of BDNF in the target and serving as a mechanism to remove BDNF from the terminal (Canossa et al., 1997; Kruttgen et al., 1998); inhibiting active cell death induced by another molecule or inhibiting the retrograde axonal transport of another molecule.

## Neurotrophins and synaptic plasticity

In addition to the survival and regeneration of neurons, more recent studies have indicated that neurotrophins might play a role as molecular signals during activity-dependent synaptic plasticity. Application of neurotrophins on rat hippocampal tissue enhances the release of the neurotransmitters, glutamate and acetylcholine (Knipper *et al.*, 1994a; Knipper *et al.*, 1994b). BDNF and NT-3 enhance both spontaneous synaptic activity and synaptic transmission in hippocampal neurons (Lessmann *et al.*, 1994; Kang & Schuman, 1995).

The BDNF gene has five short different exons (coding regions) that encode the mature BDNF protein. Each of the first four 5′ exons have separate promoters and alternative use of these promoters and differential splicing dives rise to eight different BDNF mRNA's. BDNF messages can be transcribed from any of the first four promoters while a precursor form of the BDNF protein is encoded within exon V and undergoes cleavage to yield the mature BDNF protein. Calcium influx and CREB lead to the formation of mature BDNF transcripts that contain exon III (Tao *et al.*, 1998). Although the synthesis of BDNF is rather complex there is only one type of mature BDNF protein, suggesting that BNDF protein can be produced under a variety of conditions.

BDNF mRNA levels increase substantially after an epileptic seizure: a 6-fold increase of BDNF mRNA in the dentate granule cells has been reported within 30 minutes after a seizure *in vivo* (Isackson *et al.*, 1991; Ernfors *et al.*, 1991). The induction of LTP in the CA1 pyramidal cell layer of the hippocampus evoked significant increases BDNF mRNA in CA1 neurons as measured by *in situ* hybridisation (Patterson *et al.*, 1992). Several experiments support a role for BDNF in LTP using both *in vitro* and *in vivo* assays in immature and mature hippocampal tissue. Following LTP induction, Castren *et al.* (1993) found BDNF mRNA expression in the hippocampus is unregulated, suggesting an interaction between BDNF expression and synaptic plasticity.

Electrically-stimulated immature hippocampal CA1 slices (post-natal day 12-13) treated with exogenous BDNF resulted in LTP while short-term potentiation (STP) was only inducible in untreated control slices (Figurov *et al.*, 1996). When AP5 (a NMDA blocker) was applied to the immature CA1 BDNF-treated slices, LTP was blocked, indicating that BDNF-facilitated LTP is in immature neurons is NMDA-dependent. TrkB mRNA, the gene for the BDNF receptor, is also highly expressed in CA1 and CA3 at postnatal days 6-13 (Dugich-Djordjevic, 1993). In adult slices, LTP was induced in both BDNF-treated and control tissue, this may have be due to high endogenous BDNF levels in the adult tissue. The BDNF-LTP effect was prevented when the tissue was pre-treated with TrkB-IgG (a specific scavenger for BDNF) (Figurov *et al.*, 1996).

The exogenous application of BDNF to immature hippocampal neurons and endogenous BDNF in mature hippocampal cells seems to facilitate LTP. Korte *et al.* (1995) investigated the influence of the BDNF gene on LTP; they used mice that had the BDNF gene deleted to see if BDNF-mutant mice could produce LTP in area CA3. LTP was induced 87% of the time in wild-type (+/+) mice, 27% of the time in heterozygous BDNF-mutant mice (+/-) and not al all in homozygous BDNF-mutant (-/-). They concluded that more than 50% of the endogenous or wild-type BDNF level is required for LTP. This suggests that an inability to generate BDNF is what causes the failure to induce LTP rather than developmental or neuronal abnormalities.

These effects may arise from post-translational modifications of neuronal signalling proteins, the consolidation of these short-term effects to long-term changes may require changes at the level of gene expression and neuronal morphology and/or connectivity. Longer-term regulation of neuronal activity by neurotrophins may also involve changes in neuronal form and connectivity. Engert & Bonhoeffer (1999) investigated such morphological effects of LTP on the postsynaptic dendrite. Pyramidal neurons in area CA1 were impaled with an intracellular recording electrode and filled with a fluorescent dye. EPSPs were evoked by electrical stimulation of the Schaffer collaterals. The postsynaptic neuron was monitored for morphological changes before, during and after the induction of LTP. They found that spines grow 20-40 minutes after LTP, but it is not known if the new spines contained synapses or if neurotrophins played a role in this new growth.

## Synaptic Plasticity and BDNF: Mechanism for Learning and Memory

As mentioned earlier LTP is regarded as a good biological model for learning and memory but for this to be so, according to Martin *et al.* (2000), certain criteria must be met. The first of the

four criteria is detectability; this simply states that if an animal displays a memory for a previous event/ experience there should be a detectable change in synaptic efficacy. The second criterion is mimicry; simply, if it was possible to induce a specific spatial pattern of synaptic activation the animal should show an 'apparent' memory for a non-occurring experience. This would show that changes in synaptic weights are enough to induce memory. The third criterion is anterograde alteration, by blocking the mechanisms that induce or express changes in synaptic weights during learning will impair the learning and memory of the event/experience. The fourth criterion is retrograde alteration and it is essentially the reverse of anterograde alteration, essentially if you alter the mechanisms required for learning a particular event/ experience, after learning has occurred, the memory of that event/ experience will be altered. Although there is no formal equivalence between learning and LTP research shows that both learning and LTP induce synaptic changes and to date there are yet to be any credible alternatives to LTP that can account for these synaptic changes that occur during learning.

One way of providing evidence to support LTP as a convincing model for learning and memory is to compare cellular changes after LTP or an appropriate learning task, for example blocking BDNF blocks LTP and spatial learning in rats. Mizuno *et al.* (2000) found animals trained on the radial arm maze had a significant increase in BDNF mRNA in the hippocampus but when the rats were continuously infused with antisense BDNF oligonucleotide (a BDNF scavenger) intracerebraventrically (icv) there was an impairment of learning. They suggest that learning is associated with an alteration on BDNF mRNA in the hippocampus. Memory formation has both activity-dependent changes (LTP & LTD) and morphological changes (synaptogenesis & neural growth). BDNF modulates both forms of changes and thus has been implicated in memory formation (Thoenen, 1995; Lo, 1995). Mizuno *et al.* (2000) showed that previous training on the radial arm maze has no residual effect on BDNF mRNA levels suggesting that levels may be turned over between training trials. BDNF mutant mice are also significantly impaired compared with wild-type mice in learning the watermaze task further linking BDNF to learning (Linnarsson *et al.*, 1997). Similarly, Kesslak *et al.* (1998) assessed the BDNF mRNA levels in watermaze trained animals, yoked motor controls (MC) and sedentary controls (SC). Watermaze training consisted of 6 days; on days 3 and 6 learners had higher BDNF mRNA levels in the hippocampal formation compared to MC and SC. The correlation between learning and BDNF mRNA levels on day 3 was very high and by day 6, when the task was well learned, BDNF mRNA levels were reduced. The increased levels of BDNF mRNA may promote the structural modifications associated with the

formation of new synaptic connections for the storage of the memory for the watermaze task (Barde, 1994). The MC's also showed a BDNF mRNA increase but it was suggested that this increase was due to exercise as the non-learning sedentary controls did not show an increase.

## Possible BDNF pathway for synaptic plasticity

Eric Kandel pioneered work on understanding the underlying cellular mechanisms for associative learning in *Aplysia*, a small sea slug (see Kandel & Hawkins, 1992 for a review). Briefly, Kandel discovered that ERK, extra cellular-signal-regulated kinase, activation is important for serotonin induced long-term facilitation of the Aplysia sensory-motor synapse. ERKs are enzymes that are activated by cyclic AMP (cAMP) which removes a phosphate group from a high-energy phosphate compound (such as ATP) and transfers it to another substance, frequently resulting in further intracellular activation. cAMP and $Ca^{2+}$, for example, stimulate ERK activation in hippocampal neurons, a process that is required for the trascription of genes implicated in LTP (English & Sweatt, 1997; Impey *et al.*, 1998). Furthermore, Gooney and Lynch (2001) found that tetanised DG tissue increased ERK activation and BDNF, suggesting that both are required for synaptic plasticity; they investigated if there is a relationship between ERK and BDNF expression and found that the ERK inhibitor, UO126, inhibited the BDNF-induced enhancement of release typically found during the induction of LTP. Further evidence of a relationship between ERK and BDNF was provided by Han & Holtzmann (2000) who found that pharmacological inhibition of ERK inhibits the ability of BDNF to prevent tissue loss in neonatal brain from hypoxic-ischemic injury *in vivo*.

Behavioural data also provide compelling evidence for a role of ERK in learning. Hippocampal ERK is activated in rats who have learned the watermaze task and when given a MEK blocker they were unable to form a long-term memory of the task (Blum *et al.*, 1999). Atkins *et al.* (1998) found that fear-conditioning elicited a NMDA-receptor dependent ERK activation in the hippocampus of rats. They used a selective MEK inhibitor, an upstream mechanism of ERK, and found contexual and cue learning impairment; this inhibition of associative learning was correlated with inhibition of ERK activation and the induction of LTP. Gooney *et al* (in press) found that LTP and spatial learning is associated with increased phosphorylation of TrkB and ERK in dentate gyrus suggesting a role for BDNF in learning. Activation of TrkB by BDNF stimulates a cascade of signalling events including the Raf pathway. The end product of BDNF-induced stimulation of cells

is probably an increase in protein synthesis needed for the maintenance of LTP (Finkbeiner *et al.*, 1997; Krug *et al.*, 1984).

## Activity-dependent BDNF Pathway

Neurotrophin binding to the Trk receptor at the cell membrane results in the formation of Trk dimers and activation of their tyrosine activity (see Figure 9). This results in the formation of many intracellular and membrane-associated proteins. Upon phosphoralation, phospholipase C (PLCγ) catalyzes the hydrolysis of membrane phosphatidylinositol. Phosphatidylinositol, is a constituent of cell membranes and is a precursor of the intracellular messenger molecules, inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 mobilizes intracellular $Ca^{2+}$. The most prominent ras-dependent pathway is the MAP kinsae cascade. The MAP pathway is composed of kinases that are serially phosphorylated and activated. The initial member of this cascade is raf, which activates MEK, which in turn phosphorylates and activates the MAP kineses (ERK1 and ERK2). The activation of the MAP kinase causes their translocation to the nucleus where they phosphorylate a number of immediate early gene transcription factors i.e. *c-fos* and CREB (Johnson, 1999). The research described above indicates the very likely possibility that BDNF has an active role in the induction and maintenance of LTP.

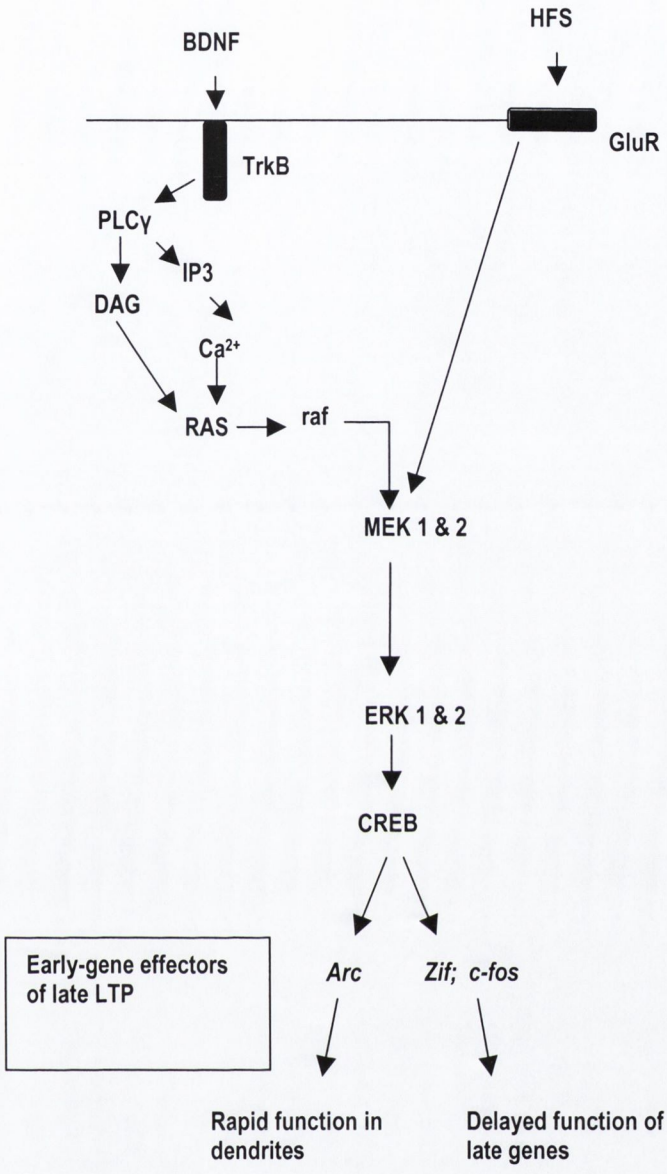



Figure 9: Proposed ERK-BDNF pathway

## Exercise and BDNF

Exercise has been shown to increase levels of BDNF (Neeper *et al.*, 1995; Neeper *et al.*, 1996; Oliff *et al.*, 1998; Russo-Neustadt *et al.*, 1999). Rats seem to find running rewarding,most will voluntarily run during the night; once used to running rats tend to maintain a similar distance each night. Neeper *et al.*, (1995) allowed rats to run for either 2, 4 or 7 nights and found a significant positive correlation between distance run and hippocampal BDNF mRNA. Areas CA1

and CA4 showed the greatest upregulation whereas CA3 and the dentate gyrus had smaller levels of BDNF mRNA. One rat did not run during the experiment and had BDNF mRNA levels close to the control group. Oliff *et al.* (1998) found that running upregulated BDNF exon III mRNA expression in the hippocampal formation. Similarly, kindling upregulates exon III mRNA. Other brain regions also show an increase in BDNF (Neeper *et al.*, 1995; Neeper *et al.*, 1996) but the greatest effects of running on BDNF expression occurred in the hippocampus, a highly plastic area of the brain, supporting the electrophysiological and behavioural data indicating that BDNF is involved in synaptic plasticity. Clinical studies show that physical fitness may be related to cognitive and mnemonic function (Clarkson-Smith & Hartley, 1989; Laurin *et al.*, 2001).

## Hippocampal-Dependent Learning and Stress

Repeated stress can produce dendritic atrophy in the CA3 region of the hippocampus (Lupien *et al.*, 1998; Fuchs *et al.,* 1997) and can impair hippocampal-dependent learning (Luine *et al.,* 1994). Recent studies have shown that people with chronically high cortisol levels show reduced hippocampal volume and associated cognitive problems (Lupien *et al.*, 1998). Elderly people (mean age 76.5) with chronically elevated cortisol had a 14% reduction in hippocampal volume, as measured by an MRI scan, and were also impaired on hippocampal-dependent memory tasks compared to age-matched controls (Lupien *et al.*, 1998). Chronic psychosocial stress in tree shrews results in more substantial hippocampal damage than a single acute stressful episode; the dentate gyrus in chronically stressed tress shrews was 30% smaller than unstressed controls (Fuchs *et al.*, 1997). Rats who underwent 21 days of restraint stress were impaired in performance on a 8-arm radial maze when they were trained 1 day after the end of stress but not when trained 18 days later (Luine *et al.,* 1994). A subsequent study showed that 21 days of repeated restraint stress impaired short-term retention (4h) of a spatial recognition memory in a hippocampal-dependent Y-maze task (Conrad *et al.*, 1996).

Long-term stress also accelerates a number of biological markers of aging in rats, including increasing the excitability of CA1 pyramidal neurons via a calcium-dependent mechanism and causing loss of these CA1 neurons (Kerr *et al.*, 1991). An important factor may be the enhancement of calcium currents by glucocorticoids in the hippocampal formation (Kerr *et al.*, 1992); the hippocampus may also be more vulnerable because of the persistence of excitatory amino acid release after the termination of a stressful experience (Lowry *et al.*, 1995) - which has

been collectively termed the glucocorticoid hypothesis of cognitive decline in ageing by Porter & Landfield (1998).

Behavioural stress has multiple and differential effects on memory consolidation, such that, stress can both impair (reviewed above) and enhance learning in animals (Oitzl *et al.*, 1998; de Kloet *et al.*, 1999; Luine *et al.*, 1996). A brief increase in corticosterone can aid in learning the watermaze task whereas higher levels of stress can impair the learning of this task (Sandi *et al.*, 1997; Oitzl & de Kloet, 1995). Using water temperature as the stressor, Sandi *et al.* (1997) showed that cold water (19°C) accelerated learning in the watermaze compared to animals trained in warmer water (25°C). The animals trained in the colder water also had significantly higher corticosterone levels than the rats trained in the 25°C water (Sandi *et al.,* 1997). Thus, variations in circulating corticosterone have an important role in determining the extent of learning and memory that occurs in the watermaze. The opposing effects of stress have been attributed to the two corticosterone-receptor types: mineralcorticoid receptor (MR) and glucocorticoid receptor (GR). MRs are activated by low levels of circulating corticosterone while GRs are activated by higher corticosterone levels suggesting that mild stress (MR activation only) facilitates memory formation while higher levels of stress (GR activation) impairs memory formation (de Kloet, 1999). Oitzl and de Kloet (1992) found that infusions of glucocorticoids impaired learning in the watermaze, suggesting that GRs are important in regulating glucocorticoid effects on memory consolidation.

## LTP and Stress

LTP is also differentially affected by MRs and GRs. LTP is facilitated in the hippocampal formation when there is some MR activation yet cannot be induced when there is GR activation (Lupien & McEwen, 1997; Diamond *et al*, 1994; Pavlides *et al*, 1996). Additionally, moderate behavioural stress facilitates the induction of long-term depression in the rat hippocampal formation at DG synapses (Xu *et al.*, 1997) and CA1-subicular synapses (Commins & O'Mara, 2000). The mechanisms underlying tissue loss resulting from stress are currently unknown. One popular model suggests that stress promotes hippocampal aging (Porter & Landfield, 1998). Another possibility is that chronically-elevated corticosterone levels affect either the expression or alteration of trophic factors in the brain: disruption of the supply of trophic factors, required to sustain neurons in a healthy state, may result in compromised tissue function.

## Neurotrophins and Stress

Corticosterones regulate the expression and production of neurotrophins (Wion *et al.*, 1986, Nitta *et al.*, 1999; Smith *et al.*, 1995; Viadya, 1999). Wion *et al.* (1986) injected mouse fibroblast cells with the synthetic glucocorticoid dexamthasone and found a dose-dependent decrease in NGF mRNA, this same decrease was also found with endogenous corticosterones (Siminoski *et al.*, 1987). It has been suggested that cortiocsterone reduces NGF mRNA levels by inhibiting NGF gene transcription and not by affecting NGF mRNA stability (Barbany, 1996). Nitta *et al.* (1997) investigated the effect of glucocorticoids on BDNF synthesis and metabolism. In the immunohistochemical part of their study, they found that BDNF-like immunoreactivity (LI) was mostly situated in the nuclei of hippocampal and cortical neurons in control rats. In corticosterone-treated rats BDNF-LI was reduced in the nuclei and increased in the cytoplasm. BDNF-LI is a technique that stains only part of the BDNF gene (amino acids 92-99 of the mature BDNF sequence) and has to potential of binding to other genes not making it as specific as the current immunohistostaining techniques used today. In this experiment, Nitta *et al.* (1997) suggest an inhibition of BDNF nuclear transportation by corticosterone, since a high concentration of corticosterone inhibited the accumulation of BDNF-LI in the nuclei. In a more detailed experiment, Schaaf et *al.* (1998) found that adrenalectomised-corticosterone treated rats showed a dose-dependent decrease in the production of BDNF mRNA and protein (ELISA) in the hippocampus. BDNF mRNA levels recovered at 12 and 24 hours post-injection. BDNF mRNA down regulation was mostly found in DG, suggesting that this hippocampal subfield is the main contributor to the observeddown-regulation at the protein level. This experiment reveals that BDNF downregulation may be mediated by the glucocortisterone receptor (GR) as high levels of corticosterone activate GRs. GRs may downregulate BDNF expression via protein-protein interaction with other transcription factors e.g.: The actions of AP-1 and NFk-B are repressed by interaction with GR. The BDNF gene contains several AP-1 sites (Hayes *et al.*, 1997).

Behavioural stress can reduce the synthesis of BDNF mRNA in the hippocampus (Smith *et al.*, 1995; Nitta *et al.*, 1999; Hansson *et al.*, 2000). Smith *et al.* (1995) found that acute or repeated immobilization stress reduced BDNF mRNA in the hippocampus. Nibuya *et al* (1999) also found that repeated immobilization stress (10 days), and not acute (45 minutes), decreased hippocampal BDNF mRNA; they also found that repeated stress upregulated hippocampal TrkB mRNA. This upregulation of TrkB mRNA may be a compensatory mechanism to chronic stress. By increased

the number of TrkB receptors the neurons become more responsive BDNF which have been depleted due to stress.

Several explanations have been put forward to account for how corticosterone regulates BDNF. Schaaf *et al* (2000) suggested that BDNF downregulation was mediated by glucocorticoid receptors (GR) and these receptors might downregulate BDNF expression via protein-protein interaction with other transcription factors. Glucocorticoids diffuse through the plasma membrane, where it then binds with its receptor entering the nucleus and influencing the transcription of RNA. This decrease in the BDNF mRNA then results in a reduced BDNF protein concentration in the neuron, as we have found. A different explanation suggests that corticosterone disrupts the transportation of BDNF to the nucleus (Nitta *et al.*, 1997) or corticosterone might block the uptake of BDNF.

## Lipopolysaccharide and learning

Lipopolysaccharide (LPS), a potent bacterial endotoxin, produces neural and endocrine responses that are similar to responses produced by behavioural stress (see Figure 10; Oitzl *et al.*, 1993).

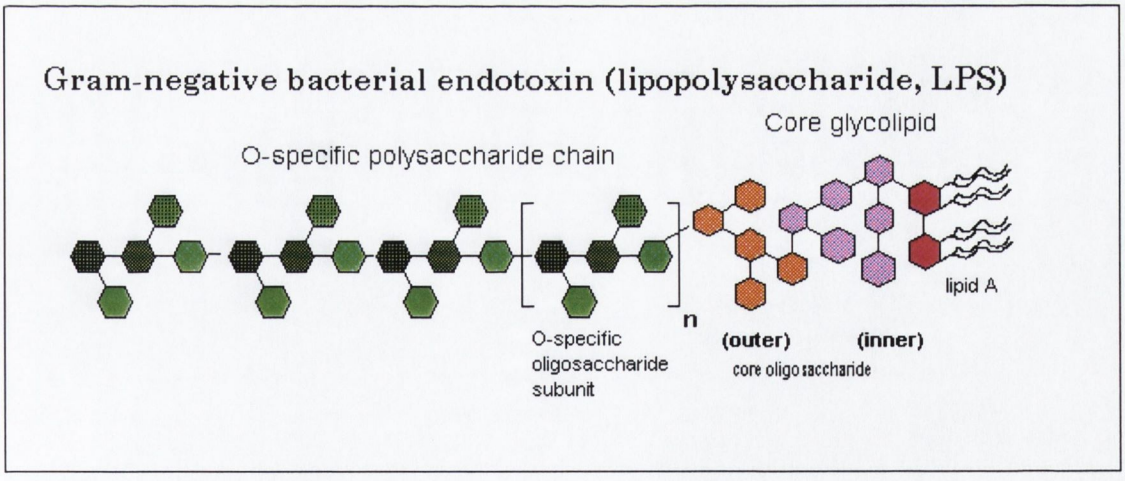



Figure 10: Molecular structure of Lipopolysaccharide.

LPS produces a wide range of non-specific behavioural effects collectively termed 'sickness behaviours' (Klein & Nelson, 1999). These behaviours include a reduction in activity, a reduction in

exploration, decreased social interaction, fever, a reduction in consumption of food and drink, hypersomnia, activation of the hypothalamic-pituitary-adrenal axis and increased sympathetic activation (Hart, 1988; Kent *et al.*, 1992; Pugh *et al.*, 1998). The exact mechanism of action by LPS in the CNS remains unknown; it is thought to act primarily through the release of cytokines (Drum & Oppemtom, 1989; Rivier *et al.*, 1989). Interleukin-1 (IL-1) and tumour necrosis factor (TNF), for example, are thought to play an important role in sustaining the 'sickness behaviours' induced by LPS. IL-1 and TNF are pro-inflammatoryare synthesised and produced during times of injury and pathological events (see Rothwell & Hopkins, 1995). Cytokines stimulate the HPA-axis resulting in the activation of a chain of immune and endocrine responses (Dunn & Wang, 1995) including an increased release of corticosterone. Cytokines also stimulate the inflammatory pathway, which results in the production of prostaglandins via the arachadonic- cyclooxygenase-prostaglandin pathway (see below for more discussion of this pathway) as administration of a non-steroidal anti-inflammatory drug (NSAID) blocks the inflammatory actions of cytokines (Dinarello *et al.*, 1991). A direct action by LPS within the CNS is an important possibility (Elmquist *et al.*, 1997): it is possible that LPS can access the brain directly via peripheral nerve transduction (Hansen *et al.*, 2000), the circumventricular organ (Blatteis, 1992), area postrema (Ericsson *et al.*, 1994) or even at the level of the hypothalamus (Yasuda & Greer, 1978; Zhang *et al.*, 2000).

## Cytokines, LPS and hippocampal-dependent learning

The hippocampus contains cytokine receptors and the IL-1β gene and protein are also expressed in hippocampal neurons. That the hippocampus is rich in cytokine receptors suggests that cytokines and associated pro-inflammatory bacterium (e.g. LPS) may influence memory processes, such as LTP, mediated by the hippocampus (see Pugh *et al*, 2001 for review). Several studies report that cytokines impair hippocampal-dependent learning. Oitzl *et al* (1993) for example, found that ICV administration of IL-1β 60 minutes prior to watermaze training impaired performance of rats in the task. LPS seems to have more selective effects on learning. Acute administration of LPS prior to training impairs contextual-cue fear conditioning, a hippocampal-dependent learning paradigm but this impairment is dose-dependent. Rats injected with 0.125mg/kg (i.p.) of LPS showed more learning impairment than rats given 0.5 mg/kg. A possible explanation of this is that a higher dose of LPS results in a longer expression of corticosterone, which then facilitates memory consolidation (Pugh *et al.*, 1998). An alternative theory is that higher levels of cytokines stimulate the release of nitric oxide (NO) and arachidonic acid (AA), two

messengers required for LTP (Mehler *et al.*, 1993) whereas lower levels of cytokines blocks LTP (see below). Chronic infusion of LPS for 37 and 74 days into the fourth ventricle of rats impaired learning the watermaze task; waiting 37 days after the cessation of the LPS infusion did not result in normal learning (Hauss-Wegrzyniak *et al*, 2000a; Hauss-Wegrzyniak *et al*, 2000b). These studies suggest that specific levels of cytokines and LPS may disrupt the consolidation of certain memory processes.

## Cytokines, LPS and synaptic plasticity

Similar to cytokine affects on hippocampal-dependent learning, cytokines have been reported to affect synaptic plasticity in hippocampal neurons. Slices of dentate gyrus pre-treated with either IL-1β, TNF, or LPS prevented the induction of LTP (Cunningham *et al.*, 1996). It has also been recently shown that LPS also blocks LTP in thesubiculum in vivo (Commins *et al.*, 2001). A possible explanation for the inhibitory action of cytokines is that IL-1 inhibits the required influx of $Ca^{2+}$ into either the presynaptic or postsynaptic neuron (Cunningham *et al.*, 1996).

## LPS and NSAIDs

The expression of cyclooxygenase 2 (COX2), a proinflammatory enzyme, is also stimulated by LPS (Needleman, 1990) at the site of inflammation and leads to the production of prostaglandins. Whether this effect of LPS is direct or indirect via the release of cytokines (Cunningham *et al.*, 1996) remains unresolved. LPS may therefore be considered a possible target for anti-inflammatory drugs. Rats injected with LPS (100 micrograms/kg, i.p.) showed reduced behavioural activity but increased extracellular concentrations of serotonin, 5-hydroxyindoleacetic acid, noradrenaline and 3-methoxy-4-hydroxyphenylglycol in hippocampal neurons. Intraperitoneal pre-treatment of rats with the cyclooxygenase inhibitor indomethacin attenuated, but did not completely abolish, theendotoxin-induced increases in hippocampal extracellular levels of serotonin, noradrenaline and their metabolites (Linthorst *et al.*, 1996). From these results it was concluded that the hippocampal serotonin and noradrenaline neurotransmitter systems are part of the brain circuitry responsive to an endotoxic challenge. Moreover, arachidonic acid metabolites seem to represent important, but not the sole, mediators of the endotoxin-induced changes in hippocampal neurotransmission (Linthorst *et al.*, 1996). In another experiment bilateral infusion of LPS into the hippocampus (1 µg/ hemisphere), resulted in a significant impairment both in acquisition and retention of one-way active avoidance in a shuttle box task. Indomethacin (10

mg/kg daily for 7 days, i.p.) improved the LPS-induced amnesia (Ma & Zhu, 1997). In an *in vivo* microdialysis study in anesthetized rats, intrahippocampal infusion of 80ng interleukin-6 (IL-6) markedly increased prostaglandin E2 (PGE2) release but perfusion of indomethacin (0.3 mol/l) into the hippocampus for 1 h suppressed the IL-6-induced PGE2 response (Ma & Zhu, 1997). LPS and cyclooxygenase, may have roles both in inflammation and in learning and memory.

## LPS and BDNF

Little research has been done on the effects of LPS on BDNF expression and the existing research is conflicting. Elkabes *et al.* (1998) found BDNF expression was unaltered in the rat brain in the presence of LPS, whereas Miwa *et al.* (1997) found that LPS stimulated the synthesis of BDNF. Interleukin-1 induces NGF mRNA expression in non-neuronal cells and in hippocampal neurons (Friedman *et al.*, 1990; Lindholm *et al.*, 1987). Cytokine, IL-1β, and LPS have been found to reduce BDNF mRNA in CA2, CA3 and DG of the hippocampal formation (Lapchak *et al.*, 1993). In this experiment they harvested the rat brains 4 hours post-systemic injection of LPS (500 μg/kg); and IL-1β (50 μg/kg and 500 μg/kg) and then used in situ hybridisation to assess BDNF mRNA levels in the hippocampus. The decrease in BDNF mRNA suggests that the inflammatory pathway (AA-COX2-PG) in some way, regulates or affects the expression of BDNF.

## Cyclooxygenase

Cyclooxygenase (COX) is the enzyme involved in the biosynthesis of prostaglandins(PGs). Prostaglandins have diverse biological functions including the maintenance of vascular and kidney homeostasis, relaxation and contraction of smooth muscle, regulation of gastrointestinal secretion and motility, and induction of sleep, pain and inflammation (Narumiya *et al.*, 1999). Arachidonate is metabolised to PG by COX after which stable PGs are formed by a group of synthases (see Figure 11). It was found that the particular PG produced by a given cell type is determined by the PG present in that cell (Smith, 1992). The conversion of AA to prostaglandin $H_2$ ($PGH_2$) is a two step conversion process, the first part takes place in the cyclooxygenase active site when two molecules of oxygen are added to AA to form the bicyclic peroxide intermediate, prostaglandin $G_2$ (see Figure 12). This intermediate must then diffuse to the peroxidase active site located on the opposite side of the enzyme, where it is reduced to prostaglandin $H_2$.

Prostaglandins have a protective effect on the lining of the gastro-intestinal tract by stimulating the secretion of mucus and bicarbonate and also by increasing the blood flow to mucus cells. However, it is the reversible role of PG in inflammation that has attracted considerable

attention over recent years. Both COX and PG are potential targets for anti-inflammatory drugs. It is now known that two forms of the COX enzyme exist: COX1 and COX2 (Yamagata *et al.*, 1993). Both enzymes are very similar in structure and both have the same ability to convert arachadonic acid to prostaglandins. There are however important differences between them. COX1 is expressed in all tissues, including the gastro-intestinal tract, platelets and endothelial cells, whereas COX2 is constitutively expressed in some tissues and is rapidly increased at the site of inflammation. COX1 is present continually and increases 2-4 fold following stimulation, whereas COX is increased 10-80 fold upon stimulation (Golden & Abramson, 1999). Such stimulation may take the form of injury, endotoxin challenge or cytokines. COX2 expression is restricted under basal conditions with the highest levels found in the brain, vas deferens, and renal cortex (DuBios *et al.*, 1998). There is considerable variability in the specific transcription factors that mediate increased COX2 expression and it is dependent on cell type and species. Ristimaki *et al.* (1994) found that IL-1 increased COX2 transcription and mRNA half-life. The synthesis of PGs is regulated by the activation of phospholipases and the release of arachidonate but the net level of prostaglandin production is determined by COX.

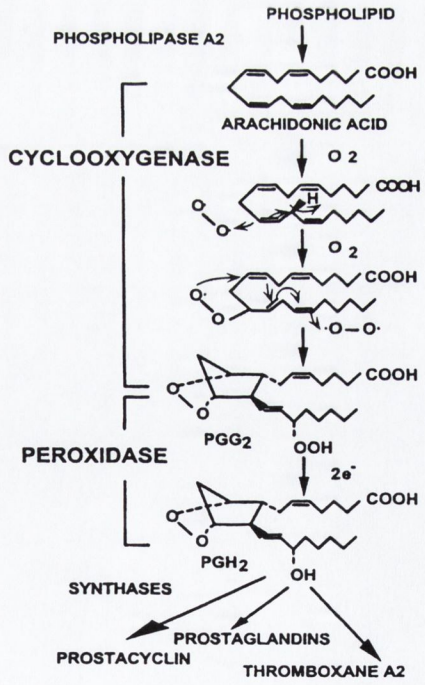



Figure 11: The biosynthetic pathway for the formation of prostanoids derived from arachidonic acid. Reprinted from Smith et al. (2000) Cyclooxygenase: Structural, cellular, and molecular biology. Annual Review of Biochemistry, 69, 145-82.

Control of COX isoform levels is critical since the COX enzyme is inactivated during catalysis. COX is highly regulated at both the transcription and posttranscriptional level. Many agents that stimulate arachidonate release also stimulate the transcription of COX2 (DeWitt, 1991). COX2 but not COX1 is inhibited by glucocorticoids, but the mechanisms by which glucocorticoids inhibit COX2 are unclear. Glucocorticoids have an anti-inflammatory action and may block the production of PGs by inhibiting COX2 expression/action. Masferrer et al. (1990) found that COX1 and COX2 increased when mice are exposed to bacteria and COX1 and COX2 is decreased when mice are exposed to glucocorticoids suggesting that COX might be encoded by differently regulated genes.

Presently a number of non-steroidal anti-inflammatory drugs (NSAIDs) exist which target the COX enzyme. NSAIDs are very well tolerated, widely used and are often the initial therapy for common inflammation such as that which follows a muscles strain. They can also be used to treat a high fever (DeWitt, 1999; Seibert et al., 1999). The action of COX was discovered by Sir John Vane in 1971; he found that NSAIDS inhibit production of PGs and concluded that the action of aspirin is to block the formation of PGs (Crofford, 2000). The majority of these drugs, for example, aspirin and ibuprofen, have about equal inhibition affinities for both COX1 and COX2. As a result these can lead to serious side effects such as gastric ulcers and renal complications. NSAIDs are widely used in the treatment of rheumatic disease due to their anti-inflammatory and analgesic properties. The NSAIDs inhibit the production of PGs that play a role in inflammation. Inhibition of the COX pathway of AA metabolism has been the accepted explanation for the anti-inflammatory, analgesic, and antipyretic properties of NSAIDs. All NSAIDs bind only on the cyclooxygenase active site and do not affect peroxidase activity (DeWitt, 1999). The result of this inhibition is to decrease the generation of two unstable cyclic endoperoxidases, $PGG_2$ and $PGH_2$, which are then usually metabolised into stable prostaglandins ($PGE_2$, $PGF_2$ and $PGD_2$), thromboxanes, prostacyclin ($PGI_2$), and toxic oxygen radicals. Inhibition of PG synthesis occurs via irreversible acetylation of COX by NSAIDs thus, the action of NSAIDs is to add an acetyl group (CH3CO-) into the COX compound. Only a limited number of COX specific inhibitors are currently available (such as celecoxib which is COX2 specific) and these are speculated to have the anti-inflammatory effects but without the intestinal problems associated with current NSAIDs.

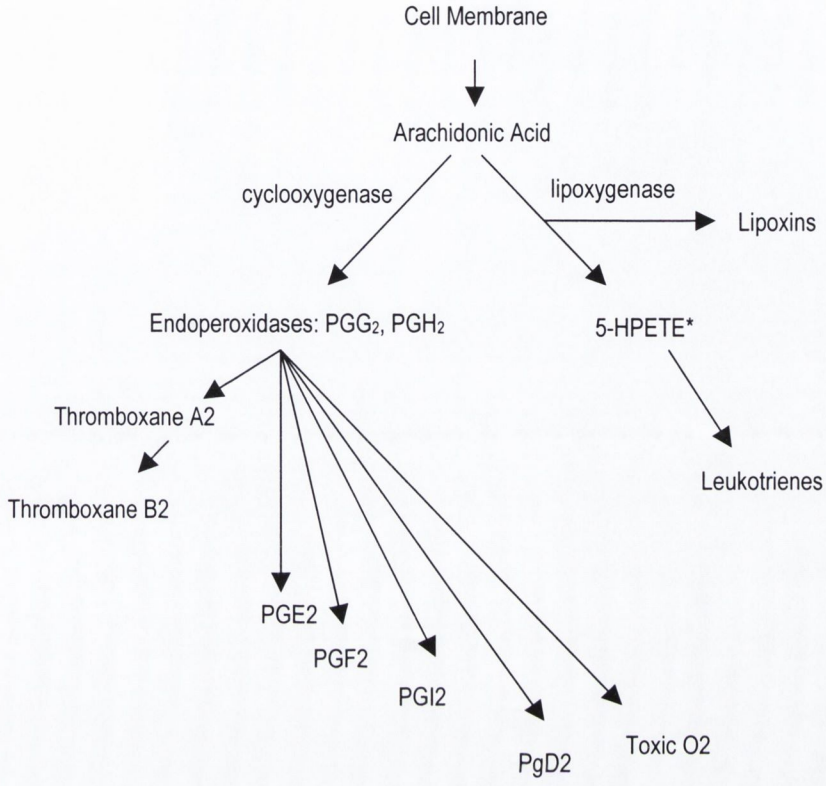



Figure 12: Inflammatory pathway.

*5-HPETE = Hydroperoxyeicosatetraenoic Acid

## COX and the Brain

Both COX 1 and 2 are expressed in neurons (Breder et al., 1995; Yamagata et al., 1993), and especially in the hippocampal formation, amygdala, some periventricular regions and the brain stem (Kaufmann et al., 1997; Yamagata et al., 1993). There are few studies of COX induction or inhibition on synaptic transmission in the brain; the molecular mechanisms underlying COX activity in the brain have not been described in any detail (Hoffmann, 2000). Cyclooxygenase activity may be important in synaptic plastic changes related to memory consolidation; it is induced by neuronal activity during learning and its activity is essential for the consolidation of memories (Holscher, 1995). According to Holscher (1995), COX2 is induced by neuronal activity during learning and its activity is essential for the consolidation of memories. Holscher (1995) found that COX inhibitors (ibuprofen, indomethacin, and naproxen) caused amnesia of a previously learned passive avoidance task; more specifically, he found that both ibuprofen and SC58125, a selective COX2 inhibitor, produced amnesic effects 2 hours after learning and PG production was also suppressed

up to 3 hours after training (Holscher, 1995b). These studies suggest that COX2 and PG are important in memory processes.

It has been suggested that inhibition of cyclooxygenases prevents the release of glutamate that is associated with long-term depression *in vitro* (Dickie *et al.*, 1994). If COX2 is responsible for memory deficits, a selective inhibitor should inhibit a learning-related up regulation of PG and cause mnemonic deficits in a learning task. In one of the few electrophysiological studies of the effects of COX inhibition, Dickie *et al.* (1994) found that the broad spectrum COX inhibitor flurbiprofen significantly increased stimulated glutamate release in cerebellar slices; the NMDA receptor antagonist CPP abolished this effect, and the metabotropic glutamate receptor antagonist, L-AP3, had a stimulation-dependent effect. Linthorst *et al.* (1996) report that pre-treatment with a COX inhibitor, indomethacin, attenuates the increases in hippocampal levels of noradrenaline and serotonin induced by endotoxic stress.

## COX and Neurodegenerative Disease

Hippocampal COX2 is up regulated in the brain (post-mortem) of Alzheimer's Disease patients, particularly in areas CA3 and CA2; the COX2 increase is proportionate to the severity of the disease (Ho, 2001). Halliday *et al.* (2000) reviewed the literature on the inflammatory events and treatments related to AD. It is known that COX2 is constitutively expressed in neurons and is unregulated in degenerative brain regions in AD and it is believed that NSAIDs may help to reduce this expression. There have been several epidemiological studies that have found a reduced risk on AD in patients chronically treated with anti-inflammatory drugs especially in younger patients (less than 75 years old). Stimulation of glutamate receptors by kainic acid affects the brain expression of COX2. Three hours after injection of kainic acid (i.p.) COX2 levels were increased in rat hippocampus. A COX1 increase was not found. This suggests that hippocampal expression of COX2 increases quickly when glutamate receptors are stimulated by kainite. Involvement of the excitatory neurotransmitter glutamate has been suggested in age related neurodegeneration (Manev *et al.*, 2000).

Some studies have investigated the protective and therapeutic action of NSAIDs in relation to AD (Dash *et al*, 2000; Halliday *et al.*, 2000). These drugs appear to be neuroprotective in the context of an underlying neurodegenerative disorder, perhaps by reducing the over expression of COX that is either the result of or consequent on the initiation of cellular death (Kadoyama *et al.*, 2001; Ho *et al.*, 2001). However, the effects of NSAIDs on healthy tissue are unknown. AA is also

required for learning (McGahon *et al.*, 1996) and LTP (Izumi *et al.*, 2000) and many agents that stimulate arachidonate release also stimulate the transcription of COX2 (DeWitt, 1991). It is entirely possible that the actions of NSAIDs on healthy tissue can affect learning and memory.

## AIMS OF THESIS

The primary aim of this thesis is to investigate the role played by BDNF during the processes associated with the biological consolidation of memory. We have conducted experiments that focus on the regulation of endogenous BDNF in learning and LTP and the parallels between the intracellular signal transduction pathways in learning and after LTP. Exercise and various types of stress are used as means of manipulating the BDNF level endogenously. This approach is considered important for establishing roles for BDNF signalling and BDNF levels in learning and memory. The watermaze is used as the learning paradigm and tetanic stimulation (LTP) as the model of activity-dependent synaptic change. We have used a variety of conditions that challenge hippocampal-dependent learning and investigate the resulting behaviour in relation to BDNF concentration and mRNA levels. BDNF protein concentration was measured using an ELISA kit and BDNF mRNA was assessed by *in situ* hybridisation.

### Behavioural Stress

We hypothesise that behavioural stressors would impair spatial learning in the watermaze; specifically, animals repeatedly exposed to moderate levels of behavioural stress would perform worse than those exposed to lesser levels of stress. We expect BDNF levels to correlate with learning, such that poor performance in learning the watermaze task would result in diminished BDNF levels. We also expect that levels of blood corticosterone would be increased in animals exposed to a moderate levels of stress compared with controls or mildly stressed animals. Finally, we examine the effects of learning and behavioural stress on BDNF mRNA using *in situ* hybridisation analyses in order to determine the locus of any effect of corticosterone on BDNF itself.

### Endotoxic Stress (LPS)

We hypothesise that LPS will impair spatial learning in the water maze in a dose-dependent manner and that animals repeatedly injected with LPS would perform worse than the single injected animals. We expect BDNF levels to correlate with learning and LTP, such that poor performance in learning the water maze task and the inability to induce LTP would result in diminished BDNF levels. We also wish to investigate if LPS has any effect on memory once it has already been formed.

We also hypothesise that ibuprofen, a broad spectrum COX inhibitor, will attenuate the LPS-induced learning deficit in both the watermaze and LTP. We expect that by blocking the LPS induced COX expression the LPS produced learning deficit and BDNF levels should be reversed. We also wish to examine the effects of ibuprofen on hippocampal-dependent learning and on BNDF expression in the dentate gyrus in normal animals. We also aim to investigate the effects of ibuprofen on baseline recordings and LTP in the dentate gyrus. We hypothesise that ibuprofen will impair learning and LTP due to a reduction in BDNF, we expect the BDNF levels to correlated with learning and LTP. We also hypothesise that by increasing endogenous BDNF levels via exercise we will attenuate the learning and LTP deficits caused by ibuprofen. We propose a model to explain the role of COX2 in learning and memory.

The ultimate aim of this thesis is to describe the role that BDNF plays in synaptic plasticity and in learning, and we will manipulate endogenous BDNF levels via stress, learning, LTP induction, pharmacological agents and by exercise. We intend to make use of several different paradigms that challenge the endogenous production of BDNF in a dose-dependent manner (outlined above). We propose that by naturally decreasing BDNF in the dentate gyrus, via behavioural or endotoxic stress or via neuropharmacological agents, learning (indexed by performance in the water maze) will be impaired. We also aim to show that the non-steroidal anti-inflammatory drug, ibuprofen, attenuates the learning and LTP deficits caused by endotoxic stress. We predict this recovery of learning and LTP is by means of a possible interaction between BDNF and cyclooxygenase. In conclusion, we hope to devise a possible regulatory interaction between BDNF and cyclooxygenase via intracellular cascades required for learning associated synaptic plasticity (i.e. ERK and PGE2).

# CHAPTER 2

# Spatial Learning and BDNF

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## Introduction

We sought to elucidate the relationship between hippocampal-dependent spatial learning and hippocampal expression of BDNF. If there is a relationship between BDNF expression and learning and memory, then we would predict that learning a hippocampal-dependent spatial task would cause an increase in the expression of BDNF in the structures required for the learning of the task. We therefore examined the effect of training in the watermaze, a hippocampal-dependent task, and hippocampal BDNF concentration, as measured by ELISA. Previous experiments indicate that such a relationship might exist. Spatial learning, in both the radial arm maze and watermaze have been reported to induce an increase in BDNF mRNA in hippocampus compared to non-learning controls (Mizuno *et al.*, 2000; Kesslak *et al.*, 1998). Moreover, Linnarsson *et al.* (1997) found that young BDNF gene knock-out mice (6-9 weeks) were significantly impaired on learning the watermaze task compared with normal young mice. The BDNF mutants required twice the number of days to reach full performance (4 vs. 8 days) compared to the controls. In addition to this, previous training for 27 days had no residual effect on BDNF mRNA levels, suggesting that BDNF mRNA levels may be turned over between training trials and increased on subsequent trials. Similarly, Mizuno *et al.* (2000) found that animals trained on the radial arm maze had significantly more BDNF mRNA in the hippocampus; when these animals were continuously infused with antisense BDNF oligonucleotide (a BDNF scavenger) the rats showed a spatial learning impairment. Their findings suggest that learning is associated with an elevation of BDNF mRNA in the hippocampus.

To date, many experiments have sought to measure learning induced BDNF mRNA changes in hippocampal neurons (Kesslak *et al.*,1998; Mizuno *et al.*,2000; Castren *et al.*, 1993; Patterson *et al.*, 1992) while very few studies have sought to investigate BDNF protein expression in relation to learning (Croll *et al.*, 1998). In this experiment we investigated if hippocampal-dependent learning results in an increase in hippocampal BDNF protein concentration. We expect that rats trained in the watermaze will show higher BDNF protein levels in the hippocampus compared to untrained motor controls as indicated by previous BDNF mRNA studies.

## Materials and Methods

### Animals

12 experimentally naïve male Wistar rats were used; 6 were trained in the Morris water maze task (trained group) and 6 motor controls who experienced the water maze without a hidden platform (untrained group) The animals were 3-4 months old when they were tested for spatial learning in the Morris water maze.

Rats were pair-housed and maintained on a 12:12 - light/dark cycle with free access to food and water. The watermaze was a black circular pool (2 m diameter; 35 cm deep) filled to 31 cm with water 20 ± 1°C. Blood corticosterone levels from animals exposed to the watermaze at this temperature fall with in the normal range (100-200 μg/ml; see subsequent chapters for results); this indicates that our Wistar rats do not find this water temperature stressful. Rats could escape the water by climbing on to a hidden platform (29 cm x 9 cm). The hidden platform was placed in the Northwestern quadrant of the pool and submerged 2 cm below waster surface so it was invisible at water level; the platform's location did not change during the experiment. Distal cues were standard room objects (e.g. doors, shelving, and curtains). A computerised digital tracking system (EthoVision) recorded escape latencies and swim paths during each trial (Noldus Information Technology, Wageningen, Netherlands).

### Watermaze training

Rats were trained on the watermaze for 8 days: 5 days learning (acquisition phase) with 3 days retention testing after a three-day rest period. The trained rats received 5 trials/day for 5 days where they had to search for the platform hidden in the Northwestern quadrant of the pool. The rat was allowed 60 seconds to find the platform, if the rat did not find the platform within 60 sec the rat was led to the platform by the researcher. The rat remained on the platform for 15 seconds. The inter-trial interval was 5 seconds. A computerised digital tracking system (EthoVision) recorded escape latencies and swim paths during each trail. Motor controls swam in the pool for the same average length of time as the trained group (5 trials/day). All rats entered the maze at a fixed start position.

We use a fixed start position rather than a random, variable start position as suggested by Stewart and Morris (1993). Experiments in our lab showed that rate of acquisition in the watermaze from either a fixed start position or random start positions were not significantly

different. This was also true when the platform was moved to a new location and the rats had to learn this new location.

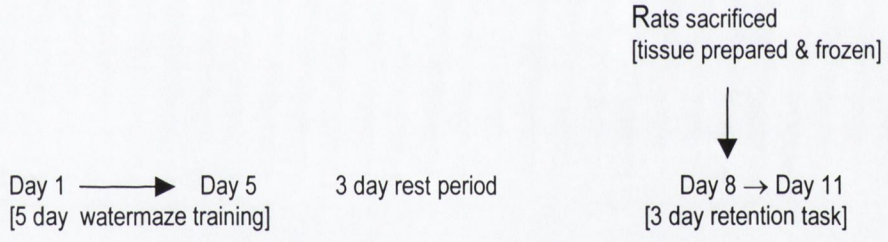
### Motor Controls

Rats followed the same procedure as outlined in the watermaze training except there was no spatial learning: These animals were therefore a "yoked control" group. The platform was removed from the pool and the rats swam each trial for the same average length of time, as did the spatial learning group. The researcher then removed the rats.

### Probe Test

On the last day of the retention task a probe trial was conducted. During this trial, the platform was removed and the rats swam freely for 60 sec in the pool; persistent swimming in the platform quadrant is commonly interpreted as an indication of spatial learning (Stewart & Morris, 1993).

### Behavioural Timeline




### BDNF Elisa procedure
**(BDNF Emax ™ ImmunoAssay System obtained from Promega UK LTD)**

### Tissue Preparation

All rats were killed by cervical dislocation on the last day of the retention phase, their brains removed, put on ice where tissue from the dentate gyrus was taken. The tissue was sliced to 250 ng thickness using an electric tissue chopper. The slices of dentate gyrus were preincubated in 250 µl Krebs solution containing 2mM CaCl 2 for 3 minutes and the supernatant was removed and discarded. This step was repeated in a volume of 100 µl, and at the end of the 3 minutes incubation samples were centrifuged at 1000g for 3 minutes and the supernatant was retained. Both samples of supernatant were stored at -80 °C for later analysis of BDNF by two-site immunoassay. BDNF concentration was determined in the supernatant. To prepare the samples, dentate gyri were homogenised in ice-cold Krebs 25 times. Samples

were centrifuged and supernatant retained. Protein was assessed and samples were diluted to give equal protein concentrations and stored at -80°C.

## BDNF Elisa Protocol

### Plate Coating

In a 50ml polypropylene tube, 10µl of the Anti-BDNF mAb was added to 10ml of carbonate coating buffer; this prepared enough reagent for each full 96 well plate; 100µl of the solution was added to each well of the ELISA plate. The wells were sealed with a plate sealer and incubated overnight at 4°C.

### Preparation of Block & Sample 1X Buffer

Block & Sample 1X Buffer was prepared by mixing 42.4ml deionized water with 10.6ml of Block & Sample 5X Buffer.

### Blocking the Plate

The coated plates were removed from the refrigerator and the contents of the wells were flicked out and each plate was slapped upside down three times on a paper towel to help clear the wells. All wells were vigorously washed with TBST wash buffer (see appendix for composition), 3 times. 200µl of Block & Sample 1X Buffer was carefully added to each well so not to scratch the surface of the wells where antibody is bound to the plate. The plates were incubated at room temperature for one hour without shaking. Following plate blocking the contents of the wells were removed and washed once with TBST wash buffer.

### Addition of BDNF Standards and Samples

BDNF standards (ranging from 0.0078 to 1ng/ml) were added to 16 wells (in dupicate); 100µl of the samples was added to each of the remaining wells and the wells were sealed with a plate sealer and incubated for two hours at room temperature with shaking (400 ± 100rpm). The plate was then washed five times with TBST wash buffer.

### Addition of Anti-Human BDNF pAb

100µl of the diluted Anti-Human BDNF pAb (diluted 1:500) was added to each well; the plates were then sealed with a plate sealer and incubated for two hours at room temperature with shaking (400 ± 100rpm). The plates were then washed five times with TBST wash buffer as described above.

### Addition of Anti-IgY HRP Conjugate

100µl of diluted Anti-IgY HRP Conjugate (1:2000 dilution) was added to each well, being careful not to disturb the bottom or sides of the wells. The plates were then incubated for 1 hour at room temperature with shaking (400 ± 100rpm). Following this, the plates were washed five times with TBST wash buffer as described above

### Colour Development

100µl TMB One Solution was added to each well using a multichannel pipettor. The wells were incubated at room temperature with shaking for 10 minutes. A blue colour formed in the wells. The reaction was stopped by adding 100µl of 1N hydrochloric acid to the wells in the same order in which substrate was added. The blue colour changed to yellow upon acidification. The absorbance was read at 450nm on a plate reader within 30 minutes of stopping the reaction.

A standard curve was performed on each plate and BDNF concentrations were estimated from the standard curve and expressed as ng/mg protein (see appendix for more details).

## Quantitation of protein using the Bradford assay

The protein concentration of tissue samples was calculated according to the method of Bradford (1976). The standard curve was run using concentrations ranging from 2µg/ml to 100µg/ml prepared in a final volume of 160µl, from a stock solution of 200µg/ml bovine serum albumin (BSA). Tissue samples (5µl) were diluted 1:40 in distilled water (155µl) and Bio-Rad dye reagent (40µl), which was added to all samples including the standards. Absorbances were read in a 96-well plate reader at a wavelength of 630nm, following gentle agitation. The concentration of protein in samples was calculated with reference to the standard curve plotted from the absorbance of the BSA standards. Protein concentrations were expressed as mg protein/ml tissue homogenate.

## Statistical Analysis

The behavioural data was analysed using an one-way ANOVA; a value of $P< 0.05$ was considered to be significant. Post hoc comparisons were assessed using Tukey's method.

## Results

### Watermaze training

The rats in this group learned the task evidenced by the decreasing daily escape latencies from days 1 to 5 (see Figure 1). This group also showed retention of the task after the 3 day break as indicated by the low escape latencies (ANOVA: $F(7,232) = 20.31$, $p < 0.001$). The rats also showed persistent swimming in the quadrant where the platform had been placed on the day of the probe test (Figure 1B), indicating that they were engaged in a localized search for the hidden platform and that they had learned its position. The post-hoc comparisons indicated that rats swam progressively shorter distances across days with significant differences between the distances swam on the first and last days of investigation ($p < 0.05$). In the case of motor controls, no learning was involved but the time spent in the water maze was the same as the experimental group; the data are therefore not shown.

Motor Controls: This was a motor task where no spatial learning was involved. The time the rats spent in the watermaze was yoked to the learning group and thus, is not plotted on the learning graph, as the rats simply swam for the time they were in the pool. There were differences in speed between the groups.

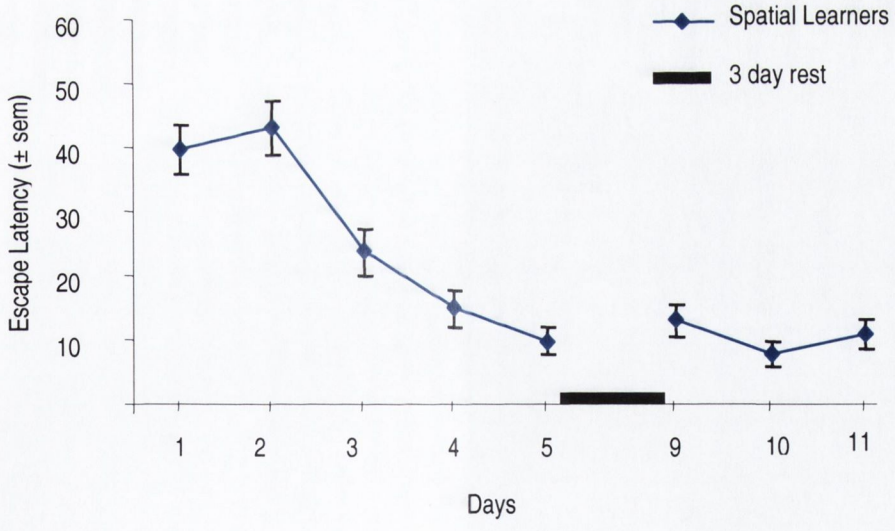



Figure1: Escape latencies for spatially trained rats. The graph reflects a normal learning curve.

The *post- hoc* comparisons show that rats swam progressively shorter distances across days. On day 1 the rats swam significantly slower compared to days 3,4,5,9,10, and 11 ($p < 0.05$). On day 2 the rats swam significantly slower compared to days 3,4,5,9,10, and 11 ($p < 0.05$).

On day 3 the rats swam significantly faster compared to days 1 and 2 yet they were significantly slower than days 4, 5, and 10 ($p < 0.05$). On day 4 the rats swam significantly faster compared to days 1 and 2 ($p < 0.05$). On days 5 and 7 the rats swam significantly faster compared to days 1, 2, and 3($p < 0.05$). On day 9 and 11 the rats swam significantly faster compared to days 1 and 2 ($p < 0.05$).

Figure 2 shows the representative swim paths of the trained rats on selected days. The probe test shows a persistence of swimming in the platform quadrant, an indication that the rats have learned the location of the hidden platform.

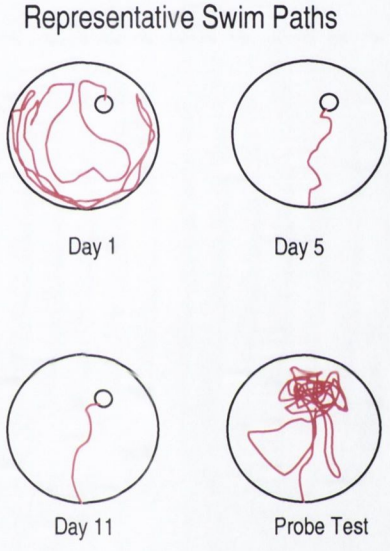



Figure 2: Representative swim paths for spatially trained rats.

BDNF was assessed in homogenate prepared from dentate gyrus of the trained and untrained motor controls. Figure 3 shows that BDNF concentration was significantly increased in the spatially trained animals compared to the untrained motor controls ($p < 0.05$; student's t-test for unpaired values).

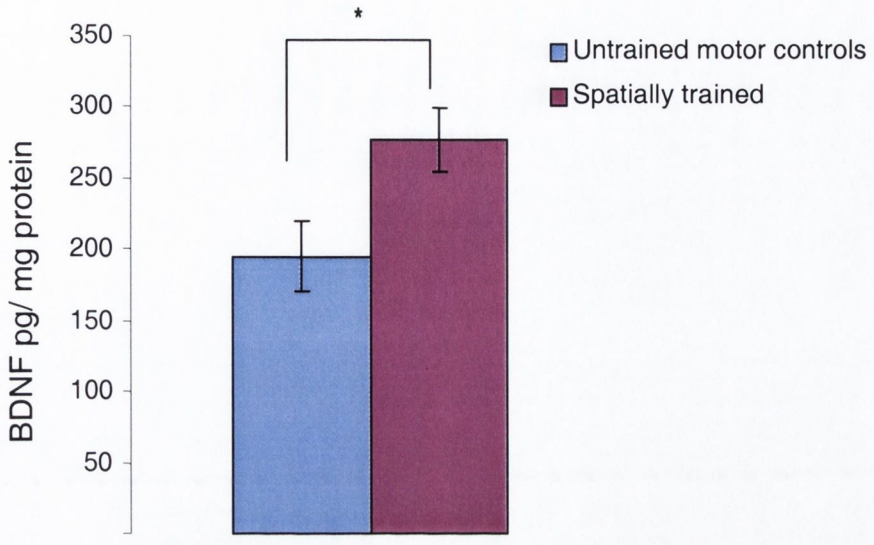



Figure 3 shows the BDNF concentrations in dentate gyrus for both groups ($p < 0.05$ *)

## Discussion

We trained animals on the watermaze task and then assessed the total BDNF protein concentration in the dentate gyrus at the end of training. The BDNF concentration from the spatially trained group was compared to the motor controls. The motor controls were used to control for the possible effects of exercise and novelty increases of BDNF expression (Neeper et al., 1995; Neeper et al., 1996; Oliff et al., 1998; Russo-Neustadt et al., 1999). We found that spatial learning was associated with an increase in BDNF protein in the dentate gyrus.

Most studies have investigated the effects of learning on BNDF mRNA levels in hippocampal tissue and have found that there is an upregulation of BDNF mRNA (Kesslak et al., 1998; Mizuno et al., 2000). Measuring BDNF protein concentration is important in establishing the successful expression of the gene and we have shown that this learning-mediated BDNF mRNA increase is synthesised into protein after spatial learning. The increased levels of BDNF mRNA in previous studies suggested that BDNF may promote the structural modifications associated with the formation of new synaptic connections for the storage of the memory for the watermaze task (Barde, 1994). Our experiment provides further support for this hypothesis.

BDNF enhances spontaneous synaptic activity and synaptic transmission in hippocampal neurons (Lessmann et al., 1994; Kang & Schuman, 1995) and BDNF mRNA is

upregulated in hippocampal tissue following LTP (Castren et al., 1993). All of these experiments suggest an interaction between BDNF expression and synaptic plasticity. The consolidation of short-term plasticity to long-term changes seems to require a change at the level of gene expression and neuronal morphology and/or connectivity via an upregulation of protein. The protein may also then cause longer-term changes in neuronal form and connectivity. Engert & Bonhoeffer (1999) evoked EPSPs in hippocampal neurons and found that spines grow 20-40 minutes after LTP. It was not known if the new spines contained synapses or if neurotrophins played a role in this new growth but it is entirely possible that BDNF mRNA was required for the short-term growth and protein is required for the development and maintenance of the spines. It is therefore possible that training related increases in BDNF expression might have similar effects.

# CHAPTER 3

## Stress: How it affects spatial learning and BDNF

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## Introduction

Damage to the hippocampal formation causes deficits in spatial and mnemonic processing in humans (Eichenbaum, 2001), monkeys (Zola-Morgan & Squire, 1990; Zola et al., 2000), and rats (Morris et al., 1982). It is particularly sensitive to stress; cumulative exposure to high levels of glucocorticoids has negative functional and structural effects on the hippocampus (Issa et al., 1990; McNaught & Jenner, 2000). Recent human studies have shown that people with chronically high cortisol levels show a reduction in hippocampal volume and deficits in hippocampal-dependent memory tasks (Lupien et al., 1998). This result mirrors previous work demonstrating that stressed rats show cognitive deficits on hippocampal-dependent tasks such as the watermaze (de Quervain, et al., 1998). The induction of LTP is inhibited by behavioural stress (McNaught & Jenner, 2000), and induction of long-term depression in the hippocampus is facilitated by exposure to acute, moderate stress (Xu et al., 1997). Paired-pulse facilitation in the hippocampal area CA1–subiculum projection is also markedly reduced after exposure to behavioural stress (Commins & O'Mara, 2000). Behavioural stress also induces atrophy of hippocampal dendrites in primates and rodents (Magarinos et al., 1997; Fuchs & Flugge, 1998) suggesting that stress alters both the electrophysiological activity and morphology of hippocampal neurons.

The mechanisms underlying tissue loss resulting from stress are currently unknown. One possibility is that chronically-elevated corticosterone levels affect either the expression or concentration of trophic factors in the brain (Hauterform et al., 1998; Smith et al., 1995; Choo et al., 1998): disruption of the supply of trophic factors, required to sustain neurons in a healthy state, may result in compromised tissue function or tissue loss and growth of neurons (Oppenheim et al, 1992; Yan et al, 1992; Freidman et al., 1993

The experiments presented here examine the effects of differing types of behavioural stressors on hippocampal-dependent spatial learning and on BNDF expression in the dentate gyrus of the hippocampal formation. We hypothesise that behavioural stressor would impair spatial learning in the water maze; specifically, animals repeatedly exposed to moderate levels of behavioural stress would perform worse than those exposed to lesser levels of stress. We expect BDNF levels to correlate with learning, such that poor performance in learning the watermaze task would result in diminished BDNF levels. We also expect that levels of blood corticosterone would be increased in animals exposed to a moderate levels of stress compared with controls or mildly stressed animals. Finally, we examine the effects of learning and behavioural stress on BDNF

mRNA using *in situ* hybridisation analyses in order to determine the locus of any effect of corticosterone on BDNF itself.

## Methods and Materials

### Animals

18 male Wistar rats (mean weight 250 g) maintained on a 12:12 - light/dark cycle with free access to food and water were used in this experiment.

### Behavioural Equipment and Procedure

### Watermaze training

The watermaze was a black circular pool (2 m diameter; 35 cm deep; water 20 ± 1°C) filled to 31 cm. Rats could escape the water by climbing on to a hidden platform (29 cm x 9 cm). The hidden platform was placed in the northwestern quadrant of the pool and submerged 2 cm below water surface so it was invisible at water level; the platform's location did not change during the experiment. Distal cues were standard room objects (e.g. doors, shelving, and curtains). A computerised digital tracking system (EthoVision) recorded escape latencies and swim paths during each trial (Noldus Information Technology, Wageningen, Netherlands). Rats were trained on the watermaze for 8 days: 5 days learning (acquisition phase) with 3 days retention testing after a three day rest period. The trained rats received 5 trials/day for 5 days where they had to search for the platform hidden in the North- western quadrant of the pool. The rat was allowed 60 seconds to find the platform; if the rat did not find the platform within 60 sec the rat was led to the platform by the researcher. The rat remained on the platform for 15 seconds. The inter-trial interval was 5 seconds. All rats entered the maze at a fixed start position.

### Behavioural stress protocol

### Repeated moderate stress protocol

The protocol was similar to that used by Xu et al., (1997) and Commins and O'Mara (2000). Rats were individually exposed to an inescapable bright light for 30 minutes prior to watermaze training (total days = 11). After testing rats were pair-housed with free access to food and water.

## Continual mild stress protocol

Rats were housed singly in cages 5 days prior to watermaze training and for the duration of the watermaze training (total days = 16). The cages were designed to prevent any visual contact with other rats.

The rats were randomly assigned to one of 3 groups (n=6/group):

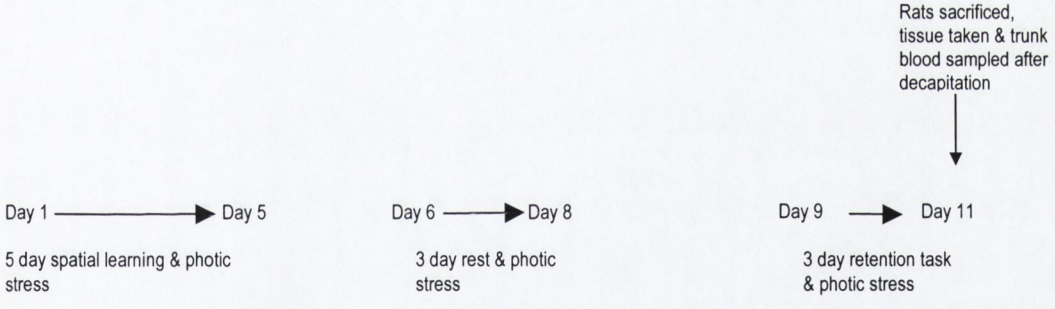
Group 1: Repeated moderate stress.

Group 2: Continual mild stress.

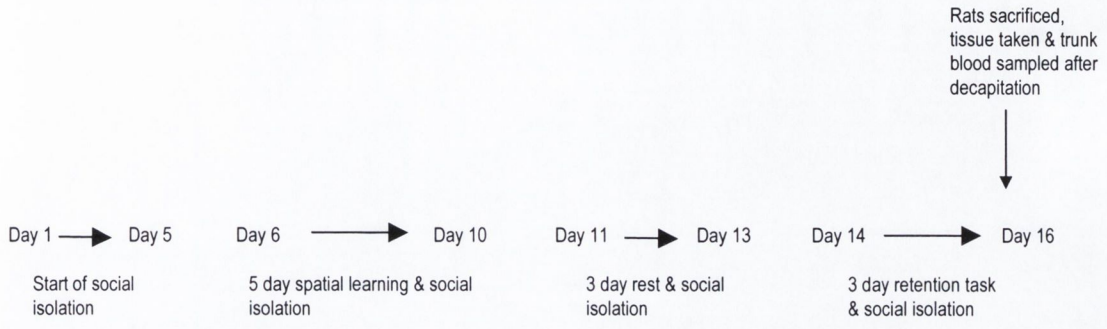
Group 3: Controls: Normal pair housing.

On the last day of the retention task a probe trial was conducted. During this trial, the platform was removed and the rats swam freely for 60 sec in the pool; persistent swimming in the platform quadrant is commonly interpreted as an indication of spatial learning. All rats were killed on the last day of the retention phase. Blood was taken and their brains were removed; tissue was taken from the dentate gyrus.

## Behavioural Time Lines:
## Repeated Moderate Stress Condition



## Continual Mild Stress Condition




## BDNF Elisa procedure

See previous chapter

## Corticosterone radioimmunoassay

All rats were killed on the last day of the retention phase; blood was taken after decapitation and stored at -80 °C for later analysis. Corticosterone was measured by radioimmunoassay (Coat-a-Count, Diagnostic Products Corporation, CA, USA). With this procedure serum samples are assayed directly, without preparation. Samples were thawed to room temperature and 50 μL of either serum or calibrator (ranging from 0 – 2 000 ng/mL corticosterone) was added to the rat corticosterone AB-Coated test tubes. These tubes were pre-coated with antibodies to rat corticosterone. 100 mL of $^{125}$I Rat Corticosterone was added to every tube and incubated for 2 hours at room temperature. The tubes were then decanted and counted for 1 minute in a gamma counter. Concentrations were estimated from the calibrator standard curve and expressed as ng/mL of corticosterone.

## *In situ* hybridisation histochemistry.

## Animals

31 male Wistar rats (mean weight 250 g) were used in this experiment. Rats maintained on a 12:12 - light/dark cycle with free access to food and water.

The rats were randomly assigned to one of 4 groups:

Group 1: Repeated moderate stress (n=10).

Group 2: Controls: Normal pair housing (n=10)

Group 3: Motor controls: Normal pair housing (n=10).

Group 4: Naïve: Normal pair housing (n=1).

## Behavioural Procedure

Watermaze training was the same as outlined above. Yoked motor controls had the same experiences with respect to time in the maze as the control group but since there was no platform, there was no learning. The naïve rat was never exposed to the watermaze. Two rats from the control, motor control and moderately stressed groups were sacrificed 30 minutes after watermaze training on days 1, 3, 5, 8 and 11. Their brains were taken and slowly frozen by placing it on a sheet of aluminum foil placed on top of dry ice pellets. Once the brains were frozen, they were stored in an -80°C freezer until hybridisation.

## In situ Hybridization Histochemistry Procedure

Hybridisations were performed essentially as described by Wisden and Morris (1994). 15 5m-cryostat sections were mounted on polysine (BDH/Merck) coated slides, dehydrated and stored in 95% ethanol. Sections to undergo ISH were air dried for 1-2 hours. 35S radiolabelled oligonucleotide probe was hybridised to the sections in a sealed humid chamber at 42 °C for 14 B 18 hours. The sections then underwent 3 washes of decreasing stringency (2 washes of 1 x SSC 55°C 30mins; 1 wash of 0.1 x SSC RT 1 min). Sections were dehydrated: air-dried and then opposed to Eastman Kodak (Rochester NY) Biomax MR X-ray film for 1-2 weeks. To define non-specific hybridisation, adjacent slide-mounted sections were incubated with radiolabelled oligonucleotide in the presence of an excess (100x) concentration of unlabelled oligonucleotide probe.

Antisense oligonucleotides (45mers) were synthesised (Oswel DNA Service) complementary to BDNF nucleotides 602 - 646, (Maisonpierre *et al.*). Oligonucleotides were 3' end-labelled with [-35S] dATP (1200Ci/mmol; New England Nuclear) in a 30:1 ratio of radiolabelled ATP:oligonucleotide using terminal deoxynucleotidyl transferase (Promega) as described by Wisden and Morris (1994). Specific activity of the 35S-labelled probe was between 100,000 and 300,000 cpm/5l probe. The BDNF sequence was 5' catgggattacacttggtctcgtagaaatattgcttcagttggc 3'.

Quantitative densitometry was performed by calibrating to a set of concentration standards before reading density values in the unit of calibration. The quantitative densitometry used to obtain the values was isotope autoradiography, using radioisotope standards (Amersham microstandards) exposed with each film to establish a standard curve. The area of interest is then highlighted and a value on the standard curve is obtained. The system used for the quantification is called MCiD Elite (Imaging Research Inc), this system allows for precise calibration to radiolabel standards, with curve fitting, extrapolation and algorithms that make data comparable across detection technologies.

## Statistics

The behavioural data were analysed using a repeated measures ANOVA for both the acquisition phase (days 1-5) and the retention phase (days 9-11); the biochemical and in situ data were analysed using an one-way ANOVA; a value of $p< 0.05$ was considered to be significant. Between Group post hoc comparisons were assessed using Tukey's method ($p< 0.05$ was significant) and within group post hoc comparisons were assessed using a paired t-test Bonferonni conversion where $p < 0.005$ was considered to be significant.

## Results

### Spatial Learning Analysis

Rats repeatedly exposed to moderate inescapable stress showed spatial learning impairment compared to the mildly stressed and control rats (see Figure 1a). An overall effect was found between and within groups but no interaction was found between group and day, as the shape of the escape latency curves suggest. All groups learned the task albeit as different rates (Repeated Measures ANOVA: Days 1-5 $F(4, 348) = 28.65$, $p < 0.001$); as indicated by the within group analysis. The within group data show that the controls differed from day to day, in that they had significantly decreasing escape latencies on days 3, 4, 5 compared with day 1 and day 6 compared with day 5 ($p < 0.005$). Similarly the mildly-stressed group had significantly decreasing escape latencies on days 3, 4, 5 compared with day 1 and days 2 and 3 compared with day 5 ($p < 0.005$). The moderately stressed group only differed across days on day 1 compared with days 3 and 4 and on days 2 and 5 ($p < 0.005$). More interestingly the between group data showed that the moderately stressed group had significantly longer escape latencies than both the control and mildly-stressed groups on days 2,4,5, and 11 ($p < 0.01$); they had significantly longer escape

latencies than the mildly-stressed animals on the first day of the retention task ($p < 0.01$) and all groups differed on the last day of the retention task, with the moderately-stressed group performing the worst ($p < 0.01$).

There were no significant differences between groups during the probe test ($p < 0.05$) although all groups spent longer in the platform quadrant than chance (25%). The mildly-stressed group spent 55% of their time in the platform quadrant, the controls 40% and the moderately stressed 37%.

## BDNF Concentration

Rats repeatedly exposed to moderate stress had significantly lower concentrations of BDNF in the dentate gyrus on the last day of watermaze training compared to both control and mildly- stressed animals (ANOVA: $F(2, 15) = 3.388$ $p < 0.05$). Figure 1b shows roughly a 60% difference in BDNF concentration between the moderately stressed animals and the controls; there was no significant difference in BDNF levels between the mildly stressed group and the controls.

## Plasma Corticosterone

Significantly higher concentrations of corticosterone were found in the serum of rats repeatedly exposed to moderate stress compared to both control and mildly- stressed animals (ANOVA: $F(2, 15) = 82.768$, $p < 0.001$; see Figure 1c). When BDNF concentration is plotted against corticosterone, the differing groups are clearly separable (see Figure 1d).

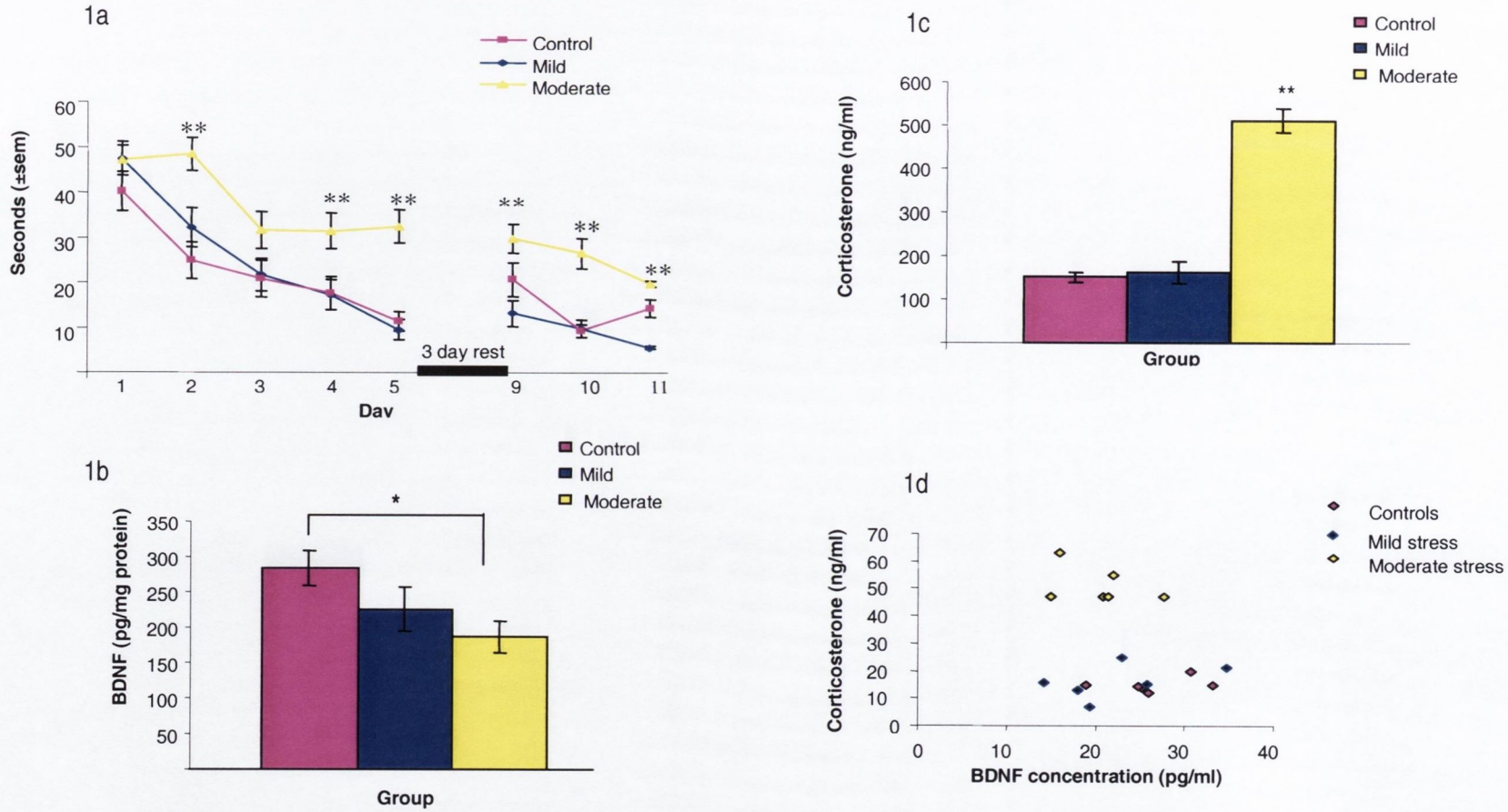


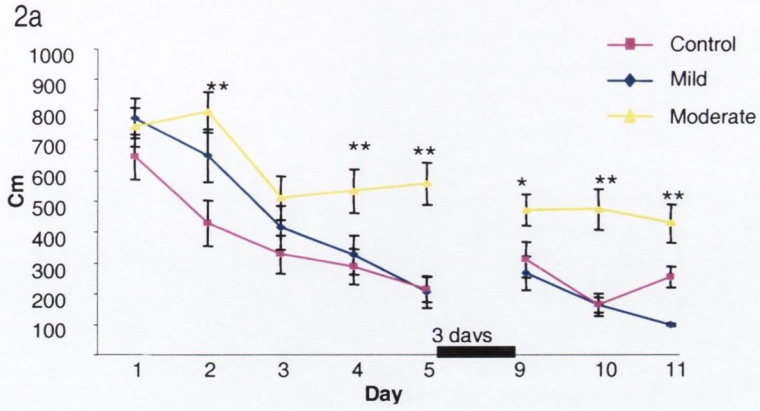

**Figure 1a** shows the escape latencies for all groups; 1b shows the total BDNF concentration in the dentate gyrus from all groups; 1c shows the corticosterone levels for all groups, and 1d is a scatter plot showing the relationship between BDNF concentration and corticosterone levels ($p < 0.05$ *; $p < 0.01$ **)

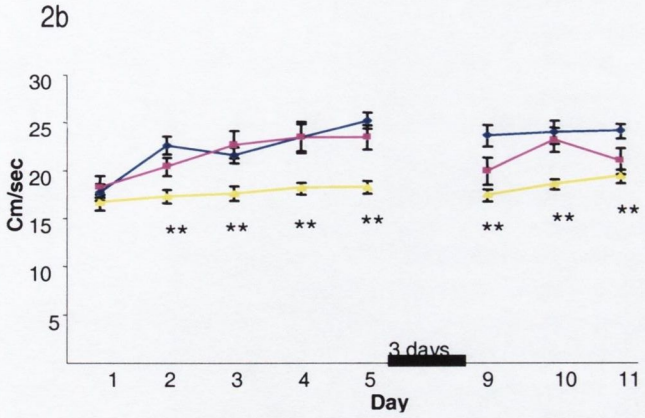
## Swim Path Length

The moderately stressed rats swam further than both the control and mildly stressed rats during acquisition and retention task (Figure 2a). A repeated measures ANOVA showed there was no interaction between group and day although there was a main effect for group ($F(2,15)$ 63.86, $p < 0.0001$) and day (Days 1-5: $F(3,40) = 8.817$, $p < 0.001$; Days 9-11: $F(1,20) = 6.5$, $p < 0.05$). Post hoc tests for group revealed that the moderately stressed group were significantly different from the controls on days 2, 4, 5, 10, and 11 ($p < 0.05$). The moderately stressed group swam significantly further than the mildly stressed group on days 5, 9, and 11 ($p<0.05$) and the mildly stressed group swam a significantly shorter distance than the controls on day 11 ($p < 0.01$). Within group comparisons found that both the mildly-stressed and control groups differed across days ($p < 0.005$) and the moderately stressed group only differed on days 2 and 3 ($p < 0.005$).

## Swim Velocity

Repeated measures ANOVA showed that there was an interaction between group and day during both the acquisition and retention phases (Days 1-5: $F(8,60) = 4.034$, $p = 0.001$; Days 9-11: $F(4,30) = 3.8$, $p < 0.05$), suggesting that the effect of stress on swim speed is dependent on day. Overall main effects were also found between groups (Days 1-5: $F(2,15) = 1530$, $p = 0.0001$; Days 9-11: $F(2,15) = 927.3$, $p < 0.0001$) and within groups (Days 1-5: $F(84,60) = 10.56$, $p < 0.001$). Post hoc tests for the group differences showed that the moderately stressed rats swam significantly more slowly than both the control and mildly stressed groups on days 2, 3, 4, 5 and 10and significantly slower than the mildly stressed rats on days 9 and 11 ($p < 0.05$; see Figure 2b). Representative swim paths illustrate that the control and mildly stressed groups swam shorter distances to the platform than the moderately stressed group (see Figure 2c).



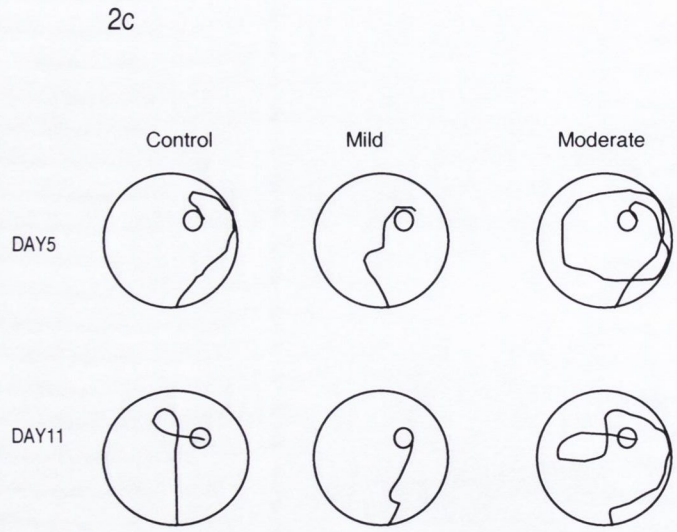




Figure 2a shows the total distance swam for each group across days; Figure 2b shows the velocity for each group; and Figure 2c shows the representative swim paths for each group on days 5 and 11 of the water maze task ($p < 0.05$ *, $p < 0.001$ **)

### *In situ* hybridisation

The effects of learning and stress on BDNF mRNA expression in the dentate gyrus were assessed by densitometric analysis of the autoradiographs; Figure 3 shows the difference between the groups. The spatially trained controls show significantly more BDNF mRNA in the dentate gyrus than the spatially trained stress and motor control groups on day 1 (Day 1 ANOVA: $F(2,19) = 28.406$ $p < 0.001$). The spatially trained controls also had more BDNF mRNA compared to the motor controls on day 9 (Day 9 ANOVA: $F(2, 15) = 4.308$ $p < 0.05$). The stressed group showed more BDNF mRNA on day 11 than the both the spatially trained controls and motor controls (ANOVA: $F(2, 19) = 10.403$ $p < 0.01$). It is important to note here that even though approximately 5 slices were used from each animal, the group size was only 2 and so the statistical quantification of the data must be viewed with caution. Figure 4a shows BDNF mRNA *in situ* hybridisation in the hippocampus only for each group. We chose to show only the hippocampus as there was little to no cortical BDNF mRNA activation; Figure 4b shows an example of whole brain BDNF mRNA for a spatial learner and motor control on day 1.

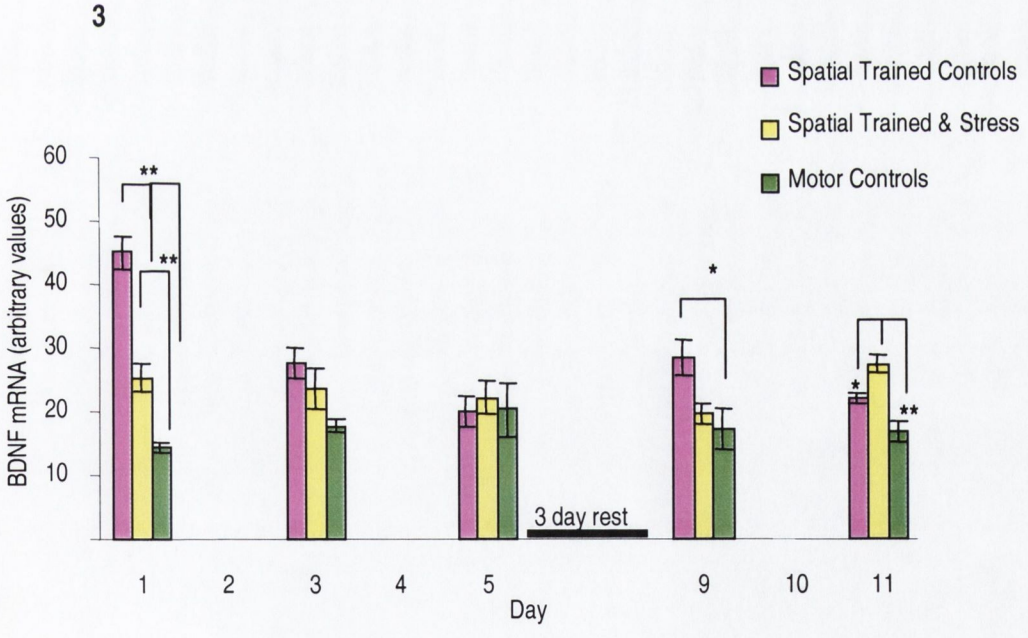



Figure 3 shows the quantitative BDNF mRNA levels in the hippocampal formation ($p < 0.05$ *. $p < 0.01$ **).

4a

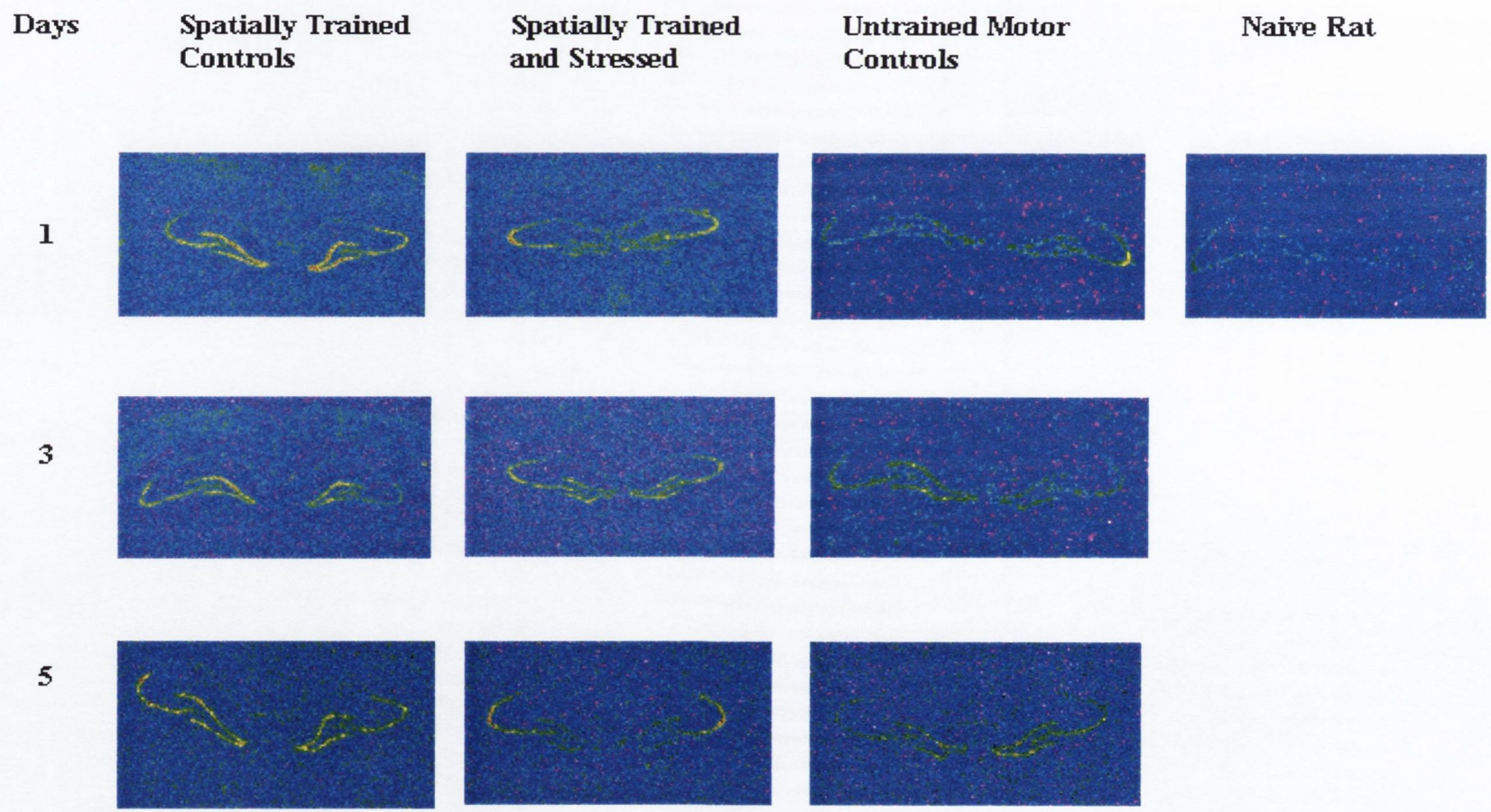



Figure 4a shows the *in situ* BDNF mRNA in the hippocampal formation of the spatially trained controls, spatially trained stress group and untrained motor controls for days 1, 3 and 5.

**Spatially Trained Control** **Untrained Motor Control**

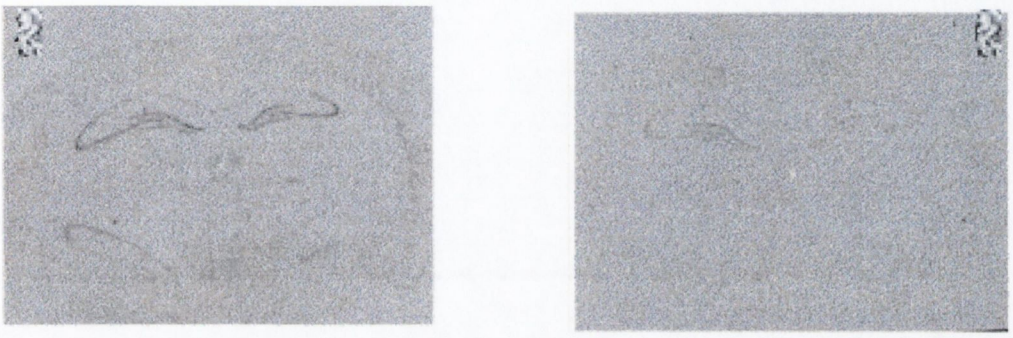


Figure 4b shows the *in situ* BDNF mRNA in the whole brain of the spatially trained controls and untrained motor controls for day 1.

## Discussion

We investigated the effects of differing degrees of behavioural stress on watermaze performance and BDNF expression in the dentate gyrus. We found that moderate inescapable stress impaired spatial learning in the watermaze whereas mild stress had no effect on spatial learning, compared with controls. Furthermore, this moderate inescapable stressor elevated circulating plasma corticosterone levels approximately 4.5 fold over that of both the continual mild stress (social isolation) and control groups. There were no differences in circulating plasma corticosterone levels between the mildly stressed and control groups. While a stress response is usually observed shortly after isolation in the adult rat it can be reversed upon social rehousing (Hall, 1998). In our experiments, the socially isolated rats were housed in an animal unit that allowed for auditory and olfactory contact with other rats, and this may have provided enough contact to alleviate the isolation. In addition to this, the animals were socially isolated for 16 days and may have habituated to the isolation. The moderately stressed group showed impairments in the overall escape latency; they also swam the slowest and the farthest, compared to the other two groups. The moderately stressed group performed significantly worse in both the acquisition and retention phases of the watermaze task indicating a both an encoding and consolidation deficit resulting from behavioural stress.

We examined the effects of stress and learning on BDNF concentration in the dentate gyrus on the last day of training. We found a learning-dependent BDNF increase in the control group; no increase was found in the moderately stressed group. The mildly stressed group was not significantly different from either the control or moderately stressed group. The animals in this group were socially isolated for 16 days and while social isolation may have been stressful at the onset, the corticosterone levels of the animals in this group had returned to basal levels by day 16. The effect of the initial behavioural stress on BDNF may have been enduring and by day 16 we see only a partial recovery of the protein in the dentate gyrus. Variations in circulating corticosterone have an important role in determining the extent of learning and memory that occurs in the watermaze. A brief increase in corticosterone can aid in learning the watermaze task whereas higher levels of stress can impair the learning of this task (Sandi et al, 1997; Oitzl & de Kloet, 1995). The opposing effects of stress have been attributed to the two corticosterone-receptor types: mineralcorticoid receptor (MR) and glucocorticoid receptor (GR). MRs are activated by low levels of circulating corticosterone while GRs are activated by higher corticosterone levels suggesting that mild stress (MR activation only) facilitates memory formation while higher levels of stress (GR activation) impairs memory formation (de Kloet, 1999). Our data suggest that glucocorticoid activation alters or degrades the expression of BDNF in such a way that it interferes with learning.

We used in situ hybridisation to assess levels of dentate gyrus BDNF mRNA in spatially trained control rats, untrained yoked-motor control rats and spatially trained stressed rats. Rats from each group were sacrificed after watermaze training on days 1, 3, 5, 9 and 11. We found an elevation in levels of dentate gyrus BDNF mRNA in trained animals compared to both the untrained, yoked-motor controls and spatially-trained stressed rats. In the spatially trained control group; this elevation of BDNF mRNA occurred in a time and training-dependent fashion, peaking on the first day of training and diminishing thereafter. The spatially trained stressed rats did not show any training-dependent BDNF mRNA increase and the BDNF mRNA levels of this group remained low for the duration of the experiment. On the first day of the retention task the spatially trained control groups had significantly higher levels of BDNF mRNA compared to the motor controls; this increase may be due to retrieval after the 3 day rest period. On day 11, the BDNF mRNA levels of the spatially trained control rats were significantly lower then the spatially trained stressed. This reduction in BDNF mRNA in the spatially trained control rats on the last day of the experiment may indicate that BDNF mRNA is upregulated during memory consolidation and not retrieval.

This result mirrors previous work in rodents that reports stress reduces BDNF levels (Nitta *et al.*, 1999; Smith *et al.*, 1995; Viadya, 1999), but we here have extended the result to show how stress regulates both hippocampal-dependent learning and hippocampal BDNF. Several explanations have been put forward to account for how corticosterone regulates BDNF. Schaaf *et al.* (2000) suggested that BDNF downregulation was mediated by glucocorticoid receptors (GR) and these receptors might downregulate BDNF expression via protein-protein interaction with other transcription factors. Glucocorticoids diffuse through the plasma membrane, where it then binds with its receptor entering the nucleus and influencing the transcription of RNA. This decrease in the BDNF mRNA might result in a reduced BDNF concentration in the neuron as we have found. A different explanation suggests that corticosterone disrupts the transportation of BDNF to the nucleus (Nitta *et al.*, 1997) or corticosterone might block the uptake of BDNF. We found an interaction between corticosterone and decreased BDNF mRNA levels in the dentate gyrus; while learning increased BDNF mRNA and protein; corticosterone diminished this learning-related increase.

A number of computational models suggest that the hippocampal formation acts as an intermediate term memory, which holds memories for a period of time, pending their transfer to cortex (e.g., O'Mara *et al.*, 2000; Rolls, 1996; 2000; Sutherland and McNaughton, 2000; O'Reilly & Rudy, 2000). This view suggests that the hippocampal formation stores memories for a time-limited period and the rate of transfer to cortex is suggested to be dependent on the rate of acquisition of new memories (Rolls, 2000) or the number of times that the hippocampus reinstates representations in cortex each time a memory is recalled (O'Reilly & Rudy; 2000). Our results do not lend support to this view; BDNF levels are at a maximum only on the first day of training and decrease rapidly to control levels thereafter. Thus if memories are retained in the hippocampus for an extended period, it is because of a BDNF-independent mechanism. An alternative suggestion to explain the pattern of observed data is that the hippocampal formation plays a privileged role only in the encoding or consolidation of memories in cortical stores but that memories per se are not stored in the hippocampal formation, pending their transfer to their eventual cortical home. On this view, we might expect there to be only transient changes in the cortex, which in turn facilitate such mnemonic consolidation at downstream cortical locations. The diagram (Figure 5) below elegantly shows how our proposed function of the hippocampus differs from the current hypotheses of Rolls (2000) and O'Reilly and Rudy (2000).

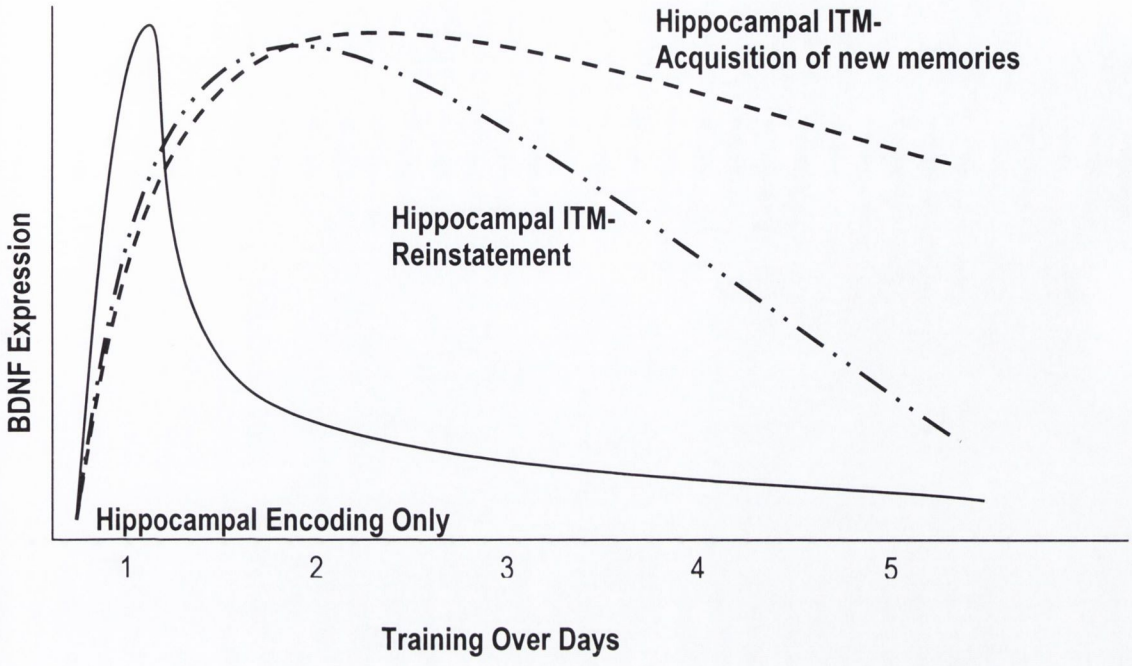



Figure 5: A schematic representation of the role of the hippocampus during learning.

# CHAPTER 4

## LPS: A dose-dependent effect on spatial learning and BDNF

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Introduction

It has been well demonstrated that acute or chronic stress leads to reduction of learning ability (Issa et al., 1990; McNaught & Jenner, 2000; Oitzl *et al.*, 1998; de Kloet *et al.*, 1999; Luine *et al.*, 1996). Lipopolysaccharide (LPS), a component of the outer membrane of gram-negative bacteria, induces profound physiological and behavioural changes, including fever, decrease in food motivation, and decrease in social behaviour (Hart, 1988; Kent *et al.*, 1992; Pugh *et al.*, 1998). These changes might be interpreted as an acute stress reaction to the LPS as LPS activates the hypothalamic-pituitary-adrenal axis resulting in increased sympathetic activation.

It has been shown that LPS treatment impairs spatial learning performances: acute administration of LPS before training impairs contextual-cue fear conditioning, a hippocampal-dependent learning paradigm (Pugh et *al.*, 1998), watermaze performance and decreased the percent of correct choices in the Y-maze test (Arai et *al.*, 2001), while a chronic infusion of LPS has been found to impair spatial memory (Hauss-Wegrzyniak et al., 1998; 2000). This LPS-induced disruption of the consolidation of certain memory processes may be dose dependent. Pugh et *al.* (1998) found that 0.125 mg/kg LPS impaired contextual-cue fear conditioning more than a dose of 0.250 mg/kg and 0.500 mg/kg had no effect on learning. Conversely, Arai *et al.* (2001) found that a dose of 0.600 mg/kg (i.p.) LPS resulted in increased escape latencies in the watermaze and 0.800 mg/kg (i.p.) LPS decreased the percent of correct choices in the Y-maze test. These results suggest that LPS affects learning in the inverted U shape manner that is indicative of stress. Furthermore, LPS inhibits LTP in the rat dentate gyrus *in vitro* (Cunnigham et al., 1996), and LPS also blocks LTP in the CA1-subiculum projection *in vivo* (Commins et al., 2001

Studies of the effects of LPS on BDNF expression conflict; Elkabes *et al.* (1998) found BDNF expression was unaltered in the rat brain in the presence of LPS, whereas Miwa *et al.* (1997) found that LPS stimulated the synthesis of BDNF. The experiments presented here examine the effects of LPS on hippocampal-dependent spatial learning, and BNDF expression in the dentate gyrus of the hippocampal formation. We hypothesised that LPS would impair spatial learning in the water maze in a dose-dependent manner, and that animals repeatedly injected (over days) with LPS would perform worse than the single injected animals. Furthermore, we expect BDNF levels to correlate with learning, such that poor performance in learning the water maze task would result in diminished BDNF levels. We also wished to investigate if LPS has any effect on memory once it has already been formed.

## Materials and Methods
## Experiment 1: LPS 100 µg/kg administered pre-training

### Animals

18 male Wistar rats (mean weight 250 g) were used in this experiment. Rats were pair-housed and maintained on a 12:12 - light/dark cycle with free access to food and water.

### Behavioural Equipment and Procedures

### The Watermaze

As described in Chapter 1

### Watermaze Training

As described in Chapter 1.

### LPS Protocol

LPS (100 µg/kg was obtained from *Escherichia Coli*; (L-2630 100 mg): Sigma Chemical Co., St. Louis, MO, USA). The rats were injected intraperitoneally with either 100 µg/kg of LPS at 1ml volume or saline (0.9% NaCl; 1 ml volume). Our LPS dose was based on previous work by Commins *et al.* (2001) who injected rats with 100 µg/kg of LPS before attempting to induce LTP in anaesthetised rats.

The rats were randomly assigned to one of 3 groups (n=6/group):

1. Single LPS: LPS (i.p. 100 µg/kg; 1 ml volume) injected once on the first day of the acquisition phase 4 hours before training.
2. Daily LPS: LPS (i.p. 100 µg/kg; 1 ml volume) injected 4 hours before each daily test of the training phase (5 days).
3. Saline Control: Saline (i.p. 0.9%; 1 ml volume) injected 4 hours before each daily test of the training phase (5 days).

## Experiment 2:

### LPS 250 µg/kg administered pre-training

Due to the findings from the first experiment we decided to increase the LSP dose from 100 µg/kg to 250 µg/kg to see if LPS has a dose-dependent affect on learning. Pugh *et al.* (1998) injected 250 µg/kg (i.p.) of LSP into rats and found this dose disrupted learning.

### Animals

18 male Wistar rats (mean weight 250 g) were used in this experiment. Rats were pair-housed and maintained on a 12:12 - light/dark cycle with free access to food and water.

### Behavioural Equipment and Procedures

### The Watermaze

As above.

### Watermaze Training

As above.

### LPS Protocol

LPS (250 µg/kg was obtained from *Escherichia Coli*; (L-2630 100 mg): Sigma Chemical Co., St. Louis, MO, USA). The rats were injected intraperitoneally with either 250 µg/kg of LPS or saline (0.9% NaCl).

The rats were randomly assigned to one of 3 groups (n=6/group):

1. Single LPS: LPS (i.p. 250 µg/kg; 1 ml volume) injected once on the first day of the acquisition phase 4 hours before training.
2. Daily LPS: LPS (i.p. 250 µg/kg; 1 ml volume) injected 4 hours before each daily test of the training phase (5 days).
3. Saline Control: Saline (i.p. 0.9%; 1 ml volume) injected 4 hours before each daily test of the training phase (5 days).

### BDNF Elisa protocol

(BDNF Emax ™ ImmunoAssay System obtained from Promega UK LTD).

See previous chapter.

### Corticosterone radioimmunoassay

See chapter 3.

### Experiment 3: LPS 500 µg/kg

Pugh *et al* (1998) injected rats with 500 µg/kg of LPS and assessed its impact on learning; in this experiment we chose to investigate the effect of a high dose of LPS (500 µg/kg) on retention in the watermaze.

### Animals

12 male Wistar rats (mean weight 250 g) were used in this experiment. Rats were pair-housed and maintained on a 12:12 - light/dark cycle with free access to food and water.

### Behavioural Equipment and Procedures

### The Watermaze

As above.

### Watermaze Training

As above except there was only 1 day of retention testing after the 3 day rest period.

### LPS Protocol

LPS (500 µg/kg was obtained from *Escherichia Coli*; (L-2630 100 mg): Sigma Chemical Co., St. Louis, MO, USA).

The rats were randomly assigned to one of 2 groups (n=6/group):

1. Single LPS: LPS (i.p. 500 µg/kg; 1 ml volume) injected once on the first day of the retention phase 4 hours before training.

2. Saline Control: Saline (i.p. 0.9%; 1 ml volume) injected once on the first day of the retention phase 4 hours before training.

## Statistics

The behavioural data was analyses using a repeated measure ANOVA; BDNF ELISA values and corticosterone levels were analysed using a one-way ANOVA; a value of $P< 0.05$ was considered to be significant. Between group *post-hoc* comparisons were made using Tukey's method ($p < 0.05$) and dependent 2-tailed Bonferonni t-tests were used to determine within group *post-hoc* comparisons ($p < 0.005$).

## Results

## Experiment 1: LPS 100 µg/kg administered pre-training

### Direct Swim analysis

A direct swim to the hidden platform was defined as a swim path that remained inside a corridor 30cm wide from the start position to the platform. Repeated measures ANOVA showed that there was an interaction between group and day during the acquisition phase of the watermaze task (Days 1-5: $F(7,297) = 2.02$, $p = 0.05$), suggesting that the effect of LPS on performance was due to day. There was main effect for group for both the acquisition and retention phases (Days 1-5: $F(2,81) = 1200.97$, $p = 0.0001$; Days 9-11: $F(2,81) = 57$, $p < 0.01$). Figure 1a shows the direct routes for each group. *Post-hoc* tests showed the single LPS group performed worse than the other two groups on day 5 and on day 9 the single LPS group performed worse than just the saline controls ($p < 0.05$). An overall main effect was also found within groups (Days 1-5: $F(4,297) = 34.732$, $p < 0.001$; Days 9-11: $F(1, 162) = 4.78$; $p < 0.01$). *Post hoc* tests for the within group differences showed that the single LPS spent more time in the direct swim corridor on days 3, 4, and 5 compared with day 1 ($p < 0.005$). Both of the controls and daily LPS groups spent significantly more time in the direct route on days 3, 4,and 5 compared to day 1 and on days 4 and 5 when compared with day 2 ($p < 0.005$). Figure 1b shows representative swim paths taken from each group on days 5 and 11.

### Overall Escape Latency Analysis

Figure 2a demonstrates that escape latencies for all groups decreased during the training period with the saline control group finding the platform faster than the LPS groups. A repeated measures ANOVA found no interaction between group and day for both the acquisition and retention phases of the task. There was an overall main effect within group (Days 1-5: $F(3,299) = 55.545$, $p = 0.001$; Days 9-11: $F(2,155) = 8.72$, $p < 0.01$). A between group difference was only found during the acquisition phase (Days 1-5: $F(3,87) = 408$, $p < 0.001$). *Post-hoc* tests revealed the single LPS group was slower than both the daily LPS group and the saline controls on day 4 of the acquisition task ($p < 0.05$). *Post hoc* within group differences found that the control group had significantly shorter escape latencies on days 3, 4, and 5 compared with day 1 and also on days 4 and 5 compared with day 2 ($p < 0.005$). Both the daily LPS and single LPS groups had

significantly shorter escape latencies on days 2, 3, 4, and 5 when compared with day 1 and also on day 5 when compared with day 2 ($p < 0.005$).

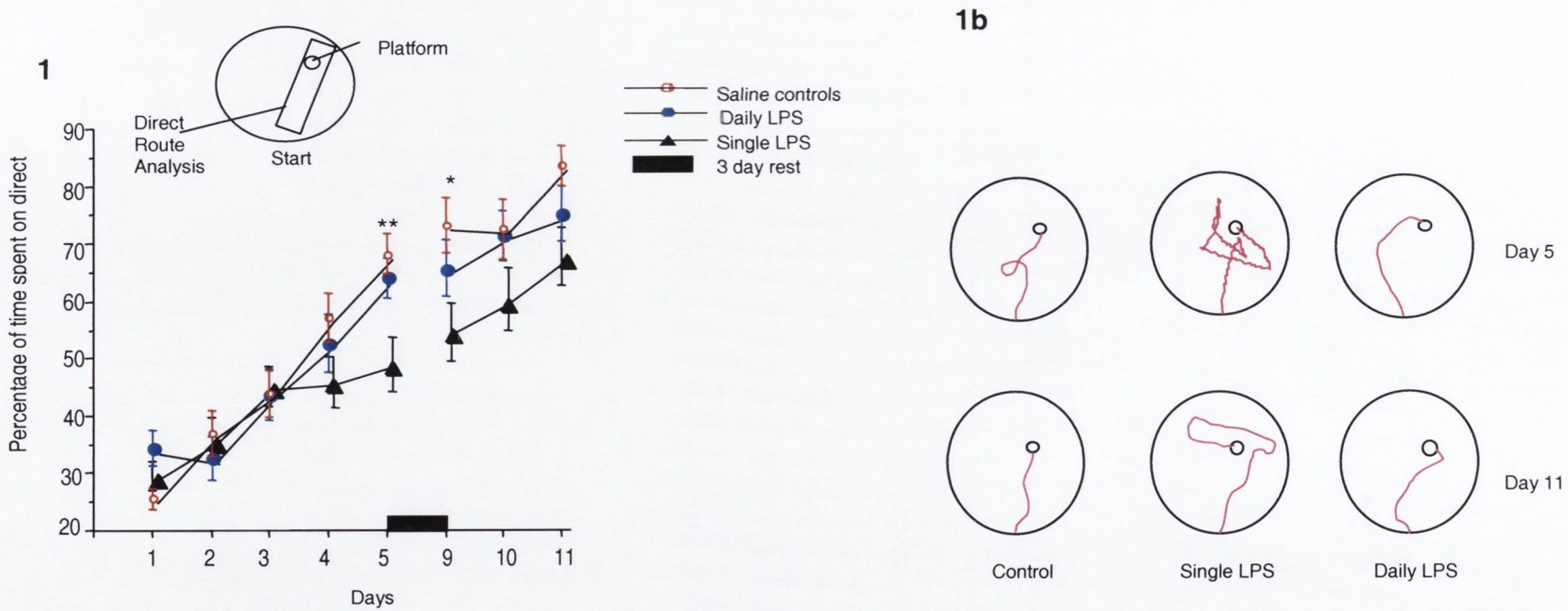



**Figure 1(a):** Percentage time spent in direct route to platform (see inset) for all groups across the acquisition and retention periods. **1(b):** Representative swim paths taken from the final day of acquisition (day 5) and the final day of retention (day 11) for each group (n= 6/group; *p<0.05; **p<0.01).

## Cumulative Distance analysis

The cumulative mean distance per day for each group was calculated. Figure 2b shows that the daily LPS group swam the shortest distance to reach the platform while the rats in the single LPS group swam the furthest. An overall group effect was found for group for both the acquisition and retention phases (Days 1-5: $F(2,80) = 3.37$, $p = 0.05$ Days 9-11: $F(2,78) = 4.9$, $p < 0.01$). *Post-hoc* tests revealed the daily LPS group swam a significantly shorter distance than the saline control group on day 1 and the single LPS group on day 5 of training and on days 9, 10, and 11 the rats in the single LPS group swam further than the daily LPS-injected group ($p < 0.05$). There was also a significant overall effect for performance within group during the acquisition phase (Days 1-5: $F(3, 267) = 37.792$, $p < 0.001$). The within *post-hoc* analysis showed that the distance swam by the controls decreased over days (Days 2, 3, 4, and 5 were significantly shorter compared with day; $p < 0.005$), this was similar for the daily LPS group who swam less on days 4 and 5 compared to both day 1 and 2. The single LPS only differed significantly on days 3 and 5 compared to day 1. An interaction was found during the acquisition phase indicating that decreasing distance across days is due to drug effect ($F(7, 267) = 2.98$, $p < 0.01$).

## Velocity analysis

The mean speed per day for each group was calculated. Figure 2c shows that the daily LPS group swam the slowest during the training period while the single LPS group swam the fastest. A repeated measures ANOVA showed that there was main effect for group during the acquisition phase of the watermaze (Days 1-5: $F(2,80) = 10.52$ $p < 0.001$) this was not so for the retention phase. On days 2, 3, and 5 the single LPS group swam significantly faster than the daily LPS group. There was a within main effect for both the acquisition and retention phases (Days 1-5: $F(4,320) = 24.58$, $p = 0.001$; Days 9-11: $F(2,129) = 8.889$, $p < 0.01$). *Post-hoc* tests confirmed that the speed differed significantly for both the daily and single LPS group on days 2, 3, 4,and 5 compared with day 1 and on day 6 compared with 7 ($p < 0.005$). There were no differences for velocity across days for the controls.

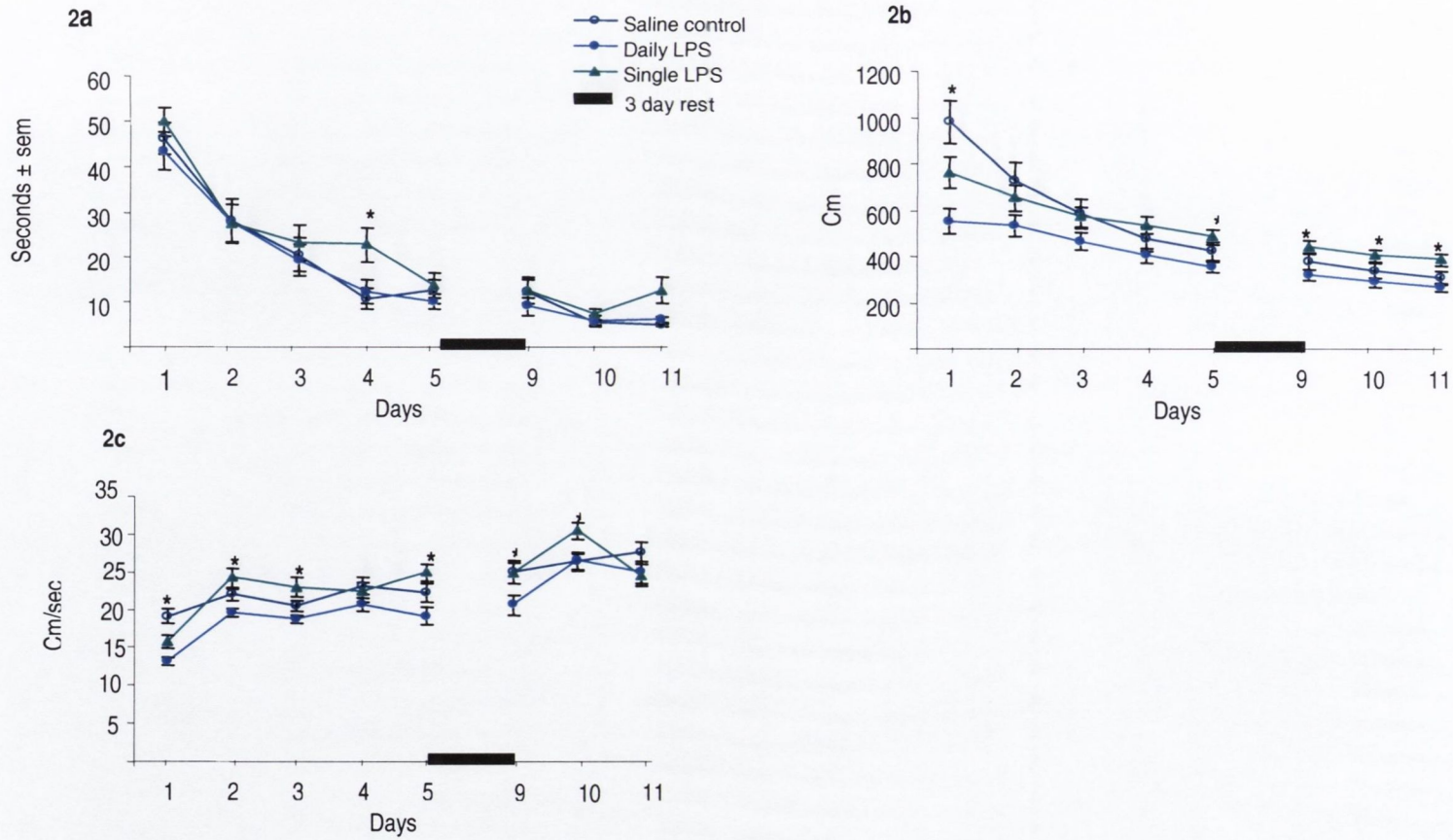



**Figure 2(a):** Mean escape latencies (seconds ± sem) for the three groups across the acquisition and retention periods. **2(b):** Total distance swam within 60 seconds allowed for each group across the acquisition and retention periods. **2(c):** Total velocity of each group within 60 seconds allowed across the acquisition and retention period (n= 6/group; *p<0.05; **p<0.01).

## Watermaze Probe Trial Analysis

### Platform Quadrant analysis

No difference was found between groups for the amount of time spent in the platform quadrant during the 60 second probe trial (ANOVA (2, 14) = 1.744, $p > 0.05$).

### Velocity analysis

Figure 3a shows that the groups swam at significantly different speeds during the probe trial (ANOVA F (2,14) = 4.059, $p < 0.05$). The *post-hoc* test revealed that the daily LPS group swam slower than the single LPS group ($p < 0.05$). This finding is consistent with the training and retention phases (see above).

### Distance analysis

An overall difference in total distance swam during the probe trial was found (ANOVA F (2,14) = 3.929, $p < 0.05$). The *post-hoc* test revealed that the daily LPS group swam the shortest distance than the single LPS group (see Figure 3b). This finding is also consistent with the training and retention phases (see above).

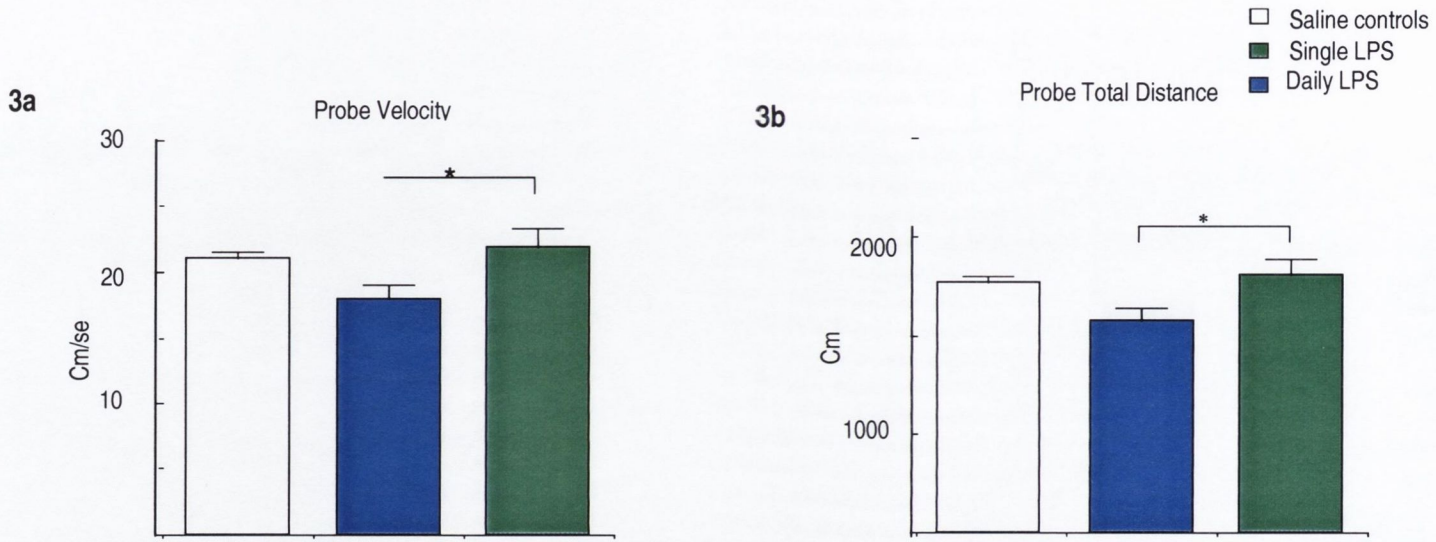



**Figure 3(a):** Total velocity of each group during the probe test. **3(b):** Total distance swam during the probe test for each group (n= 6/group; * $p<0.05$; ** $p<0.01$).

## BDNF Expression

BDNF concentration was determined in the dentate gyrus from all animals in each group. No significant differences were found between groups (ANOVA: $F(2,15) = 0.479$, $p>0.05$, see Figure 4).

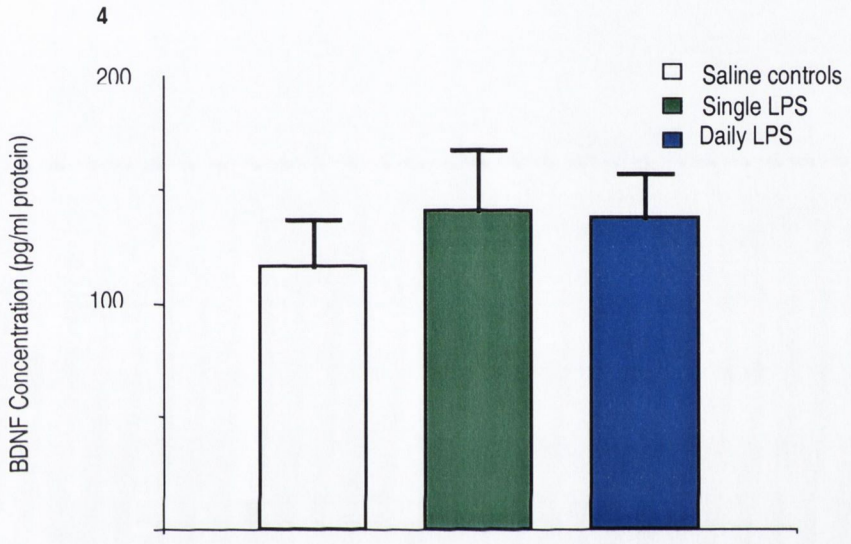



**Figure 4:** Mean concentration of BDNF in dentate gyrus for each group. (n= 6/group; *p<0.05; **p<0.01)

## Experiment 2: LPS 250 μg/kg administered pre-training

## Direct Swim analysis

Figure 5a demonstrates that there was a significant difference between groups on both the acquisition and retention phases of the task (Days 1-5: $F(2,82) = 5.67$, $p < 0.01$; Days 9-11 $F(2, 87) = 14.15$, $p < 0.001$). *Post-hoc* tests showed the controls used the most direct route compared to the other two groups on days 4, 10, and 11 and on day 9 the single LPS group performed worse than just the saline controls ($p < 0.05$). No interactions were found for either the acquisition and retention phases. There was an overall main effect for the within group variable for the acquisition phase (Days 1-5: $F(4, 348) = 40.28$, $p < 0.001$). *Post-hoc* tests showed that the single LPS group

spent more time in the direct route on days 2, 3, 4, and 5 compared with day 1 and also on day 5 compared with days 2 and 4. Both the daily LPS and control groups spent more time in the direct route corridor on days 3, 4, and 5 when compared to days 1 and 2. Figure 5b shows representative swim paths taken from each group on days 5 and 8.

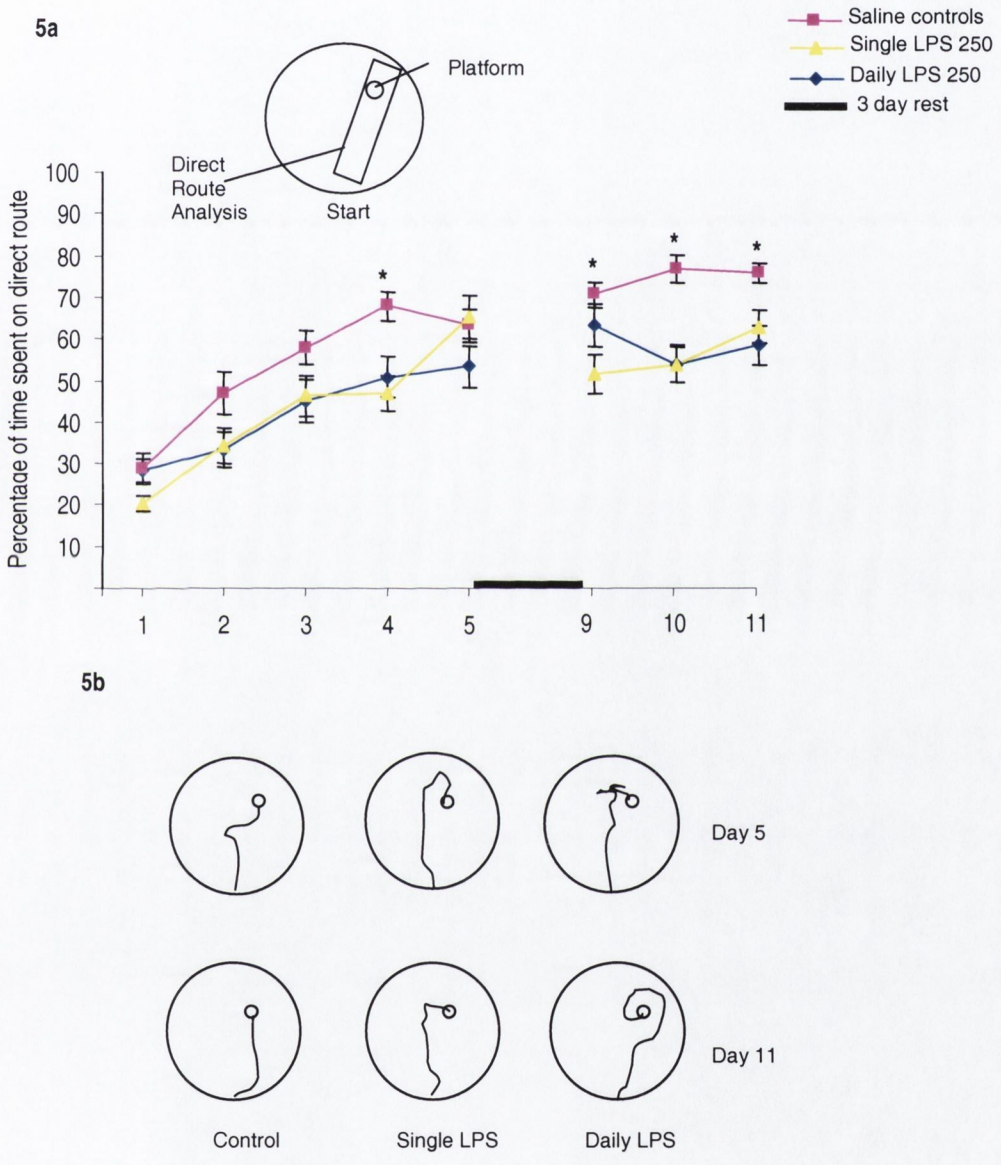



**Figure 5(a):** Percentage time spent in direct route to platform (see inset) for all groups across the acquisition and retention periods. **5(b):** Representative swim paths taken from the final day of acquisition (day 5) and the final day of retention (day 11) for each group (n= 6/group; *p<0.05; *p<0.01).

## Overall Escape Latency Analysis

Figure 6a demonstrates that escape latencies for all groups decreased during the training period with the saline control group finding the platform faster than the LPS groups. A repeated measures ANOVA revealed an overall main effect was found for group on both the acquisition and retention phases (Days 1-5: $F(2, 87) = 4.7$, $p < 0.01$; Days 9 –11: $F(2, 87) = 3.95$, $p < 0.05$). *Post-hoc* tests revealed the single LPS group was slower than both the daily LPS group and the saline controls on day 4 of the training task whereas the single LPS group was only slower than the controls on the last day of the training task (day 5) and the first day of the retention period ($p < 0.05$). A within group difference across days was also found during both the acquisition and retention tasks (Days 1-5: $F(3, 257) = 55.9$, $p < 0.001$; Days 9 –11: $F(2, 152) = 5.71$, $p < 0.01$). Controls had significantly shorter escape latencies on days 2, 3, 4, and 5 compared to day 1 and days 4 and 5 compared to day 2 ($p < 0.005$). The single LPS group also had significantly shorter escape latencies on days 2, 3, 4, and 5 compared to day 1 and also day 8 compared with day 6 ($p < 0.005$). The daily LPS group had shorter escape latencies on days 3, 4, and 5 compared with both days 1 and 2 ($p < 0.005$). No interaction was found.

## Cumulative Distance analysis

The cumulative mean distance per day for each group was calculated. An overall within group main effect was only found during the acquisition task (Days 1-5: $F(3, 244) = 62.105$, $p < 0.001$; see Figure 6b). *Post-hoc* tests revealed the single LPS group swam significantly shorter distances on days 3, 4, and 5 compared to day 1 and day 5 compared to day 2 ($p < 0.005$). The daily LPS injected group also significantly shorter distances on days 3, 4, and 5 compared with both days 1 and 2 ($p < 0.005$). The saline controls also swam a shorter distance on days 3, 4, and 5 compared with the first day and on days 4 and 5 compared with day 2 ($p < 0.005$). No between group differences or interactions were found.

## Velocity analysis

The mean speed per day for each group was calculated (see Figure 6c). A between group difference was found (Days 1-5: $F(2,92) = 64.6$, $p < 0.001$; Days 9-11: $F(2,92) = 13.93$, $p < 0.001$)

and *post-hoc* tests confirmed that the single LPS group swam slower than the other two groups ($p < 0.05$). The single LPS group swam significantly slower than both the saline controls and daily LPS group on every day of the training phase (days 1-5). On day 9 the single LPS group only swam slower than the saline controls and on days 10 and 11 the single LPS group swam significantly slower than the daily LPS group. A within group main effect was also found (Days 1-5: $F(3, 349) = 7.321$, $p < 0.001$; Days 9-11: $F(2,232) = 20.35$, $p < 0.001$) and *post-hoc* analysis showed that the daily LPS group were faster on days 7 and 8 compared to day 6; the controls were slower on days 1, 2 ,and 3 compared with day 5 ($p < 0.005$). No differences in speed were found for the single LPS group.

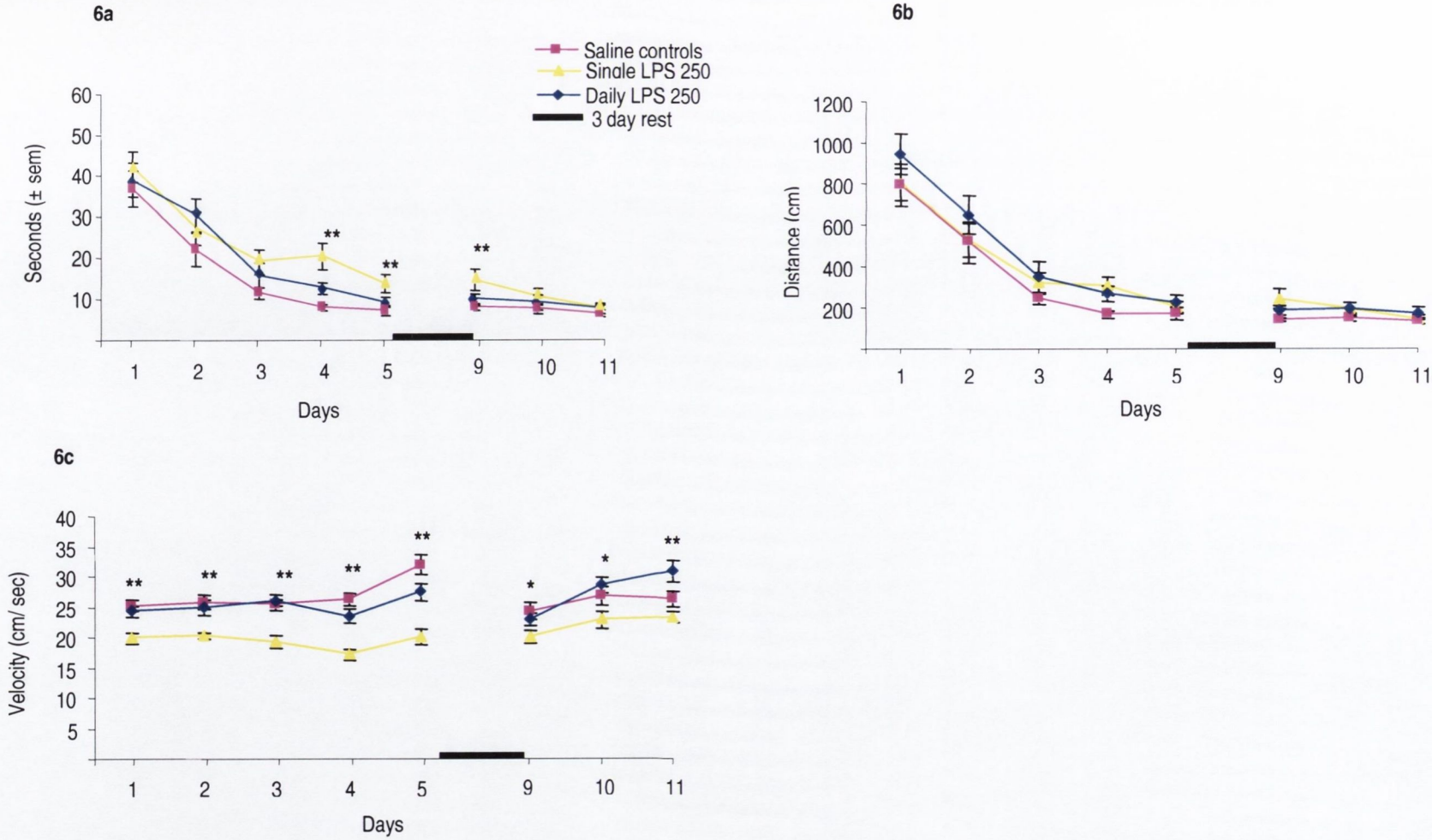



**Figure 6(a):** Mean escape latencies (seconds ± sem) for the three groups across the acquisition and retention periods. **6(b):** Total distance swam within 60 seconds allowed for each group across the acquisition and retention periods. **6(c):** Total velocity of each group within 60 seconds allowed across the acquisition and retention period (n= 6/group; * $p<0.05$; ** $p<0.01$).

### Platform Quadrant Probe Analysis

No significant differences were found between groups in the amount of the time spent in the platform quadrant during the 60 second probe trail (ANOVA: $F(2, 15) = 0.048$, $p > 0.05$).

### Body Temperature

Body temperatures were recorded from 2 days prior to LPS injections and during watermaze training. No significant differences were found between groups or within groups across days (see Figure 7a).

### BDNF Expression

BDNF concentration was determined in the dentate gyrus from all animals in each group. No significant differences were found between groups (ANOVA: $F(2,31) = 0.272$, $p > 0.05$, see Figure 7b).

### Circulating Blood Corticosterone Levels.

Corticosterone levels were determined from trunk blood taken on the last day of the experiment. No significant differences were found between the groups (ANOVA: $F(2,15) = 1.583$, $p > 0.05$, see Figure 7c).

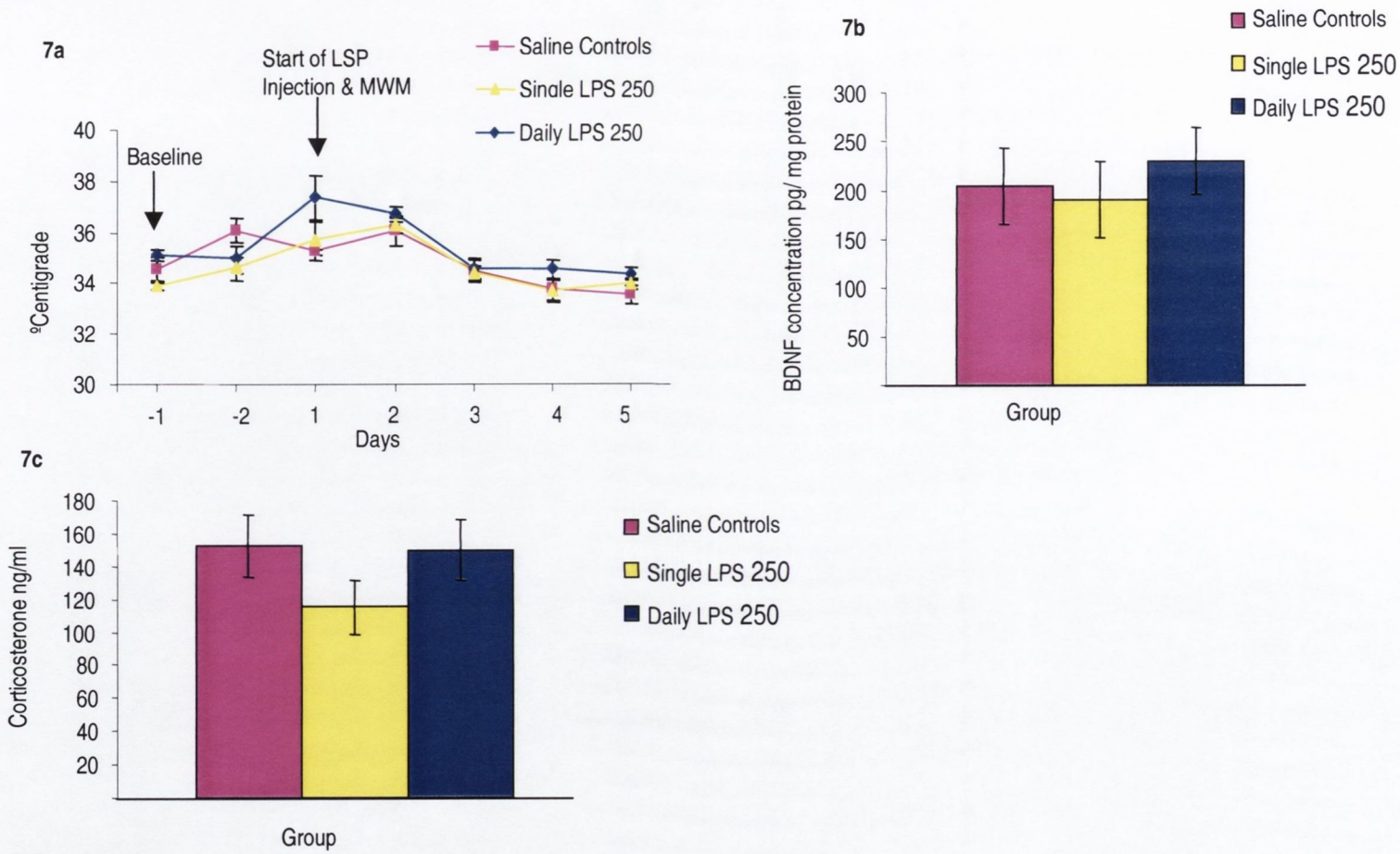



**Figure 7(a):** Body temperatures taken 2 days before watermaze testing and for the first 5 days of testing. **7(b):** Total BDNF concentration in dentate gyrus. **7(c):** Total corticosterone levels from each group (n= 6/group; * $p<0.05$;p** $<0.01$).

## Experiment 3: LPS 500 µg/kg administered post-training

### Overall Escape Latency Analysis

Figure 8a shows the escape latencies for both groups. There were no significant difference between the groups (Day 1: ANOVA $F(1,58) = .85$, $p < 0.5$; Day 2: ANOVA $F(1,58) = .028$, $p < 0.5$; Day3: ANOVA $F(1,58) = .186$, $p < 0.5$; Day 4: ANOVA $F(1,58) = .67$, $p < 0.5$; Day 5: ANOVA $F(1,58) = 3.29$, $p < 0.5$; Day 9: ANOVA $F(1,58) = .907$, $p < 0.5$).

### Velocity analysis

The mean speed on the last day of the experiment (day 9) was calculated for each group. Figure 8b shows that both groups swam at the same speed during testing that day (ANOVA: $F(1, 58) = 0.001$, $p > 0.05$).

### Cumulative Distance analysis

The cumulative mean distance on day 9, the last day of the experiment, was calculated for each group. Although there was no significant difference between the two groups (ANOVA: $F(1, 58) = 1.546$, $p > 0.05$) the saline group swam a longer distance (see Figure 8c).

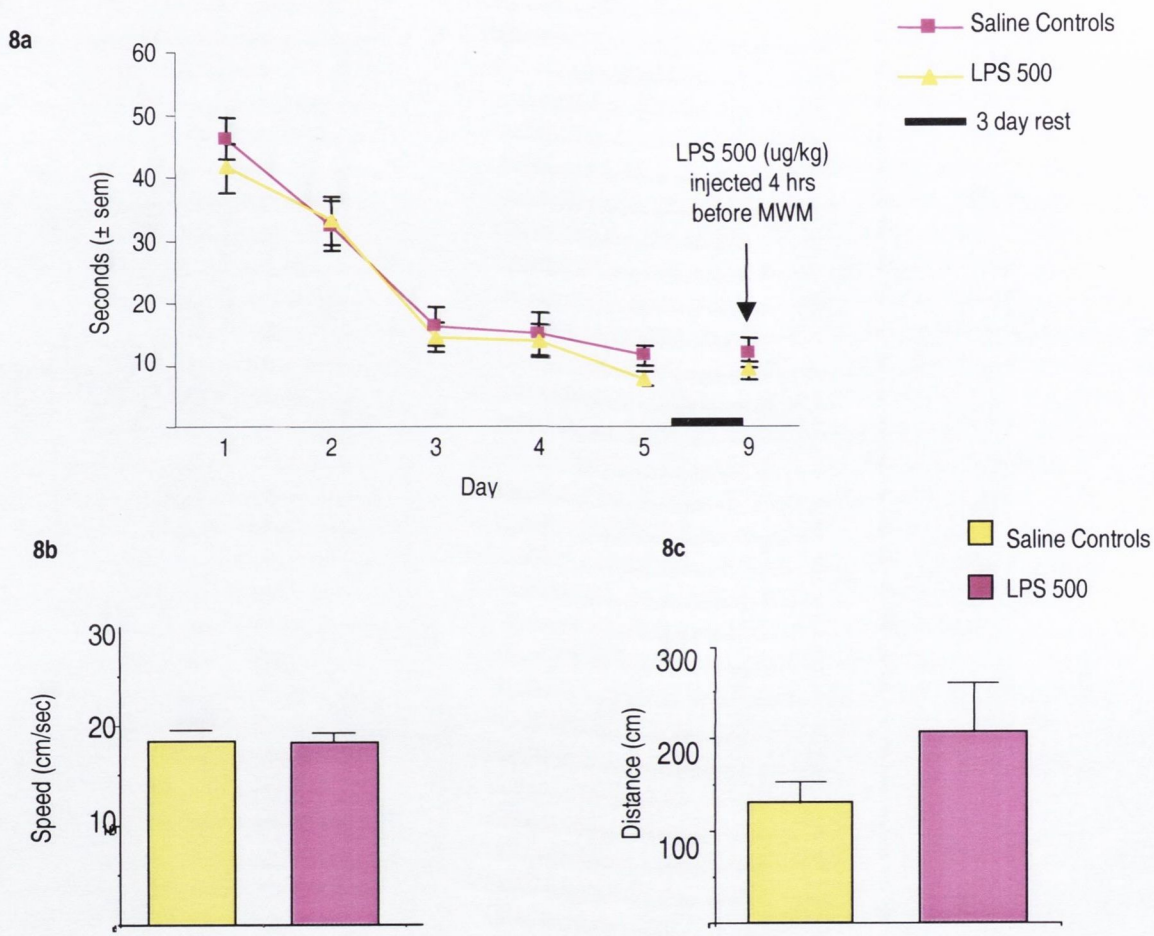



**Figure 8(a):** Mean escape latencies (seconds ± sem) for the two groups across the acquisition and retention periods. **8(b)**: Total velocity for both groups during the retention task (day 9). **8(c):** Total distance swam during the retention task (day 9).

## Discussion

We have confirmed a further possible relationship between synaptic plasticity and learning, consistent with the hypothesis outlined by Martin *et al.*, (2000) which states that synaptic plasticity underlies the biological consolidation of memories. We have also demonstrated the important regulatory impact of the neuroimmune system on cognitive processing.

## Experiment 1

We investigated if the administration of LPS affected spatial learning in the watermaze and also if LPS affected the expression of BDNF in the rat dentate gyrus. A learning deficit was apparent after a single injection of LPS 100 μg/kg compared to a daily LPS 100 μg/kg-injection or saline-injected controls. The single LPS 100 μg/kg group both swam faster and further and spent the least amount of time on direct route to the platform compared to the daily LPS 100 μg/kg group and saline control group. We also found that there were no differences in BDNF concentration between the different treated groups and controls. Here we reveal that animals that received a daily injection of LPS 100 μg/kg swam the slowest and swam the shortest distance during both the acquisition and retention phases. This may suggest a manifestation of some of these behavioural sickness symptoms, that is, a reduction in activity and a reduction in exploratory behaviour. These symptoms were supported by the probe test. The daily LPS 100 μg/kg group swam significantly shorter distances and were slower than the single LPS 100 μg/kg group within the 60 seconds allowed per trial. Although animals with a single injection of LPS swam the fastest, they also swam the furthest indicating an inability to locate the platform and thus revealing a subtle spatial learning deficit. This group also showed impairments in the overall escape latency measure and in their direct swim paths, which revealed that the single LPS 100 μg/kg group spent significantly less time in the direct route area than the controls and the daily LPS 100 μg/kg group.

## Experiment 2

We administered a single and daily dose (5 days) of 250 μg/ kg LPS to rats 4 hours prior to watermaze training. As in the first experiment we found that a single LPS injection impaired learning compared to the daily LPS-injection or saline-injected controls. The single LPS 250 μg/kg-injected group swam the slowest compared to the other two groups. Both the single and

daily LPS 250 μg/kg-injected group spent the least amount of time in the direct route to the platform compared to the saline-injected controls. We also found that there were no differences in BDNF concentration or corticosterone levels between the different treated groups and controls. We measured corticosterone levels to ensure that the learning impairment was not due to stress.

Unlike the single LPS 100 μg/kg-injected group, the single LPS 250 μg/kg-injected group showed a retention deficit. The single LPS 250 μg/kg-injected group were significantly slower on the first day after the 3 day rest period compared to the daily LPS 250 μg/kg-injected group and saline-injected controls, whereas the single LPS 100 μg/kg-injected group performed normally on this day. The single LPS 100 μg/kg-injected group swam considerably faster than the single LPS 250 μg/kg-injected group throughout the experiment, this dose-dependent difference may be due to sickness behaviour, such as a reduction in activity and a reduction in exploratory behaviour. In experiment 1, the daily LPS 100 μg/kg-injected group swam slower than the other two groups while in the second experiment the single LPS 250 μg/kg-injected group was the slowest, suggesting than an acute high dose of LPS affects swimming speed in the same way as a repeated lower LPS dose. Conversely, a repeated high dose of LPS does not impair swimming speed in the same way suggesting a dose-dependent tolerance effect.

### Experiment 3

Here we investigated if LPS has any effect on memory once it has already been formed. No differences in performance were found between the groups. It appears that LPS disrupts memory encoding but has no effect on memories that are consolidated. If this is so, memory encoding must take place within 8 days of learning of that task. It is very likely that encoding occurs within the first day, as suggested by the increase of BNDF mRNA in controls rats from the stress experiment (see Chapter 3).

## General Discussion

Cognitive processing may be disrupted following either endotoxin or cytokine administration. *Legionella pneumophila* (an endotoxin) and IL-1β impair watermaze performance in rodents (Gibertini et al., 1995, Oitzl et al., 1993). Furthermore, the disruption of learning by endotoxins appears to be dose dependent; Pugh *et al.* (1998) demonstrated that 0.125 and 0.25

mg/kg of LPS interfered with contextual fear conditioning while 0.5 mg/kg had no effect. Similarly, LPS differentially affects the hypothalamic-pituitary axis in that a higher dose of LPS stimulates the sustained release of corticosterone (Nguyen et al., 1998; Pugh et al., 1998). This appears to be only true for acute exposure to LPS; repeated injections of LPS have no such effect on corticosterone levels (Takemura et al., 1997). Repeated LPS exposure also resulted in lower Il-1β activation, this may be due to an alteration in peripheral sympathetic responsiveness (Zhang et al., 2000). It is very possible that repeated exposure to LPS produces a tolerance effect, as suggested by the lower Il-1β activation, which might account for the behavioural changes we have observed here. The notion of a differential effect from a single and repeated challenge to the neuroimmune system needs further extensive investigation.

No significant differences of BDNF concentration in the dentate gyrus were found between groups. Previous research on the effects of LPS on BDNF expression is inconsistent. Elkabes *et al.* (1998) found BDNF expression to be unaltered in the rat brain in the presence of LPS, whereas Miwa *et al.* (1997) found that LPS stimulates the synthesis of BDNF. Miwa *et al.* (1997) looked at the expression of BDNF in microglia cultured from embryonic rat brains that were exposed to LPS. They concluded that the LPS-activated microglia participate in neuronal regeneration via production of neurotrophins; thus, this increase in BDNF seems to be neuroprotective in the embryonic brain. We administered a single and daily dose of LPS and then examined its effect on BDNF concentration in the dentate gyrus on the last day of the watermaze task (day 11). We expected to find a difference between groups because BDNF is implicated in learning and memory, but no difference in BDNF concentration was apparent. A possible explanation is that our dose was too small to cause any neuronal damage that would result in the activation of BDNF synthesis. An alternative reason for this may be that by the time the tissue samples were taken, the LPS was completely metabolised. If BDNF is neuroprotective and if there were no circulating levels of LPS in the animals, then an elevation of BDNF would not be expected. Similarly, if repeated LPS exposure produces a tolerance effect then, normal levels of BDNF would be expected. It would be of interest in future experiments to determine the half-life of LPS after acute administration and to investigate the interaction between different doses of LPS and learning, and on BDNF concentration.

In summary, we have shown that a single injection of LPS impairs learning while a daily injection does not. We have also shown that LPS (either a single or daily dose) does not affect BDNF concentration in the dentate gyrus. Our findings implicate an important regulatory impact of

the neuroimmune system on cognitive processing and synaptic plasticity in the hippocampal formation.

# CHAPTER 5

## Ibuprofen rescues LPS-induced spatial learning deficits

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## Introduction

The expression of COX2 is stimulated by LPS at the point of inflammation and leads to the production of prostaglandins (Ristimaki *et al.*, 1994; Needleman, 1990). This LPS-induced increase in COX2 expression also increases the transcription of COX2 in microglial cells (Zhang & Rivest, 2001) suggesting that LPS increases COX2 expression at both the genetic and enzymatic levels. In addition to LPS increasing the production of PG via COX2 activation, LPS also increases the production of nitric oxide (NO) in rat intestinal tissue (Hori *et al.*, 2001). The inflammatory actions of LPS may result from the stimulation of cytokines (Cunningham *et al.*, 1996) making LPS-mediated inflammation a possible target for anti-inflammatory drugs, such as non-steroidal anti-inflammatories (NSAIDs).

Subsequent research has investigated the actions of NSAIDs on LPS and cytokine-treated tissues. Indomethacin, a COX inhibitor, blocked the LPS-induced expression of iNOS mRNA, but not the expression of COX-2 mRNA in muscularis macrophages (Hori *et al.*, 2001), whereas Hoozemans *et al.* (2000) found that indomethacin blocked the IL-1β induced COX2 increase in human brain tissue. These findings suggest that COX inhibitors may act differently depending on the type and site of stimulation. Matsumura *et al.* (2000) investigated if LPS administered i.p. in rats contributed to increased PGE2 in the brain via vagal afferents. They did not find a COX2 increase in the vagal afferents, but instead found an increase in COX2 in brain endothelial cells, suggesting a direct action of LPS within the CNS (Elmquist *et al.*, 1997). LPS has been suggested to disrupt certain hippocampal-dependent learning and memory tasks. Acute administration of LPS has been shown to block contextual-cue fear conditioning (Pugh *et al.*, 1998) and spatial memory (Hauss-Wegrzyniak *et al.*, 1998; 2000). In addition, LPS inhibits the induction of LTP in the dentate gyrus *in vitro* (Cunningham *et al.*, 1996) and in the subiculum *in vivo* (Commins *et al.*, 2001). We have found that LPS blocks learning (Shaw *et al.*, 2001; see Chapter 4); this blockade may be due to COX expression as COX expression is related to cognitive impairment e.g. Alzheimer's disease (Ho, 2001; Halliday *et al.*, 2000). LPS by triggering cytokine expression, it may contribute to neuropathological cell death via COX expression. It follows that by blocking the LPS-induced COX expression, there should be a reversal of the observed LPS-produced learning deficit. Activation of LPS and subsequently COX2 may have a role both in inflammation and in learning and memory. Kaufmann *et al.* (1996) reported that COX2 is upregulated in excitatory post-synaptic neurons in the cerebral cortex and hippocampus. Immunohistostaining of the hippocampus showed a high

density of COX2 in the dentate gyrus, CA3/4 and subiculum in rat brain tissue. Lerea & McNamara (1993) found that the NMDA dependent-response in the hippocampus is blocked by the COX inhibitor, indomethacin. COX2 expression and NO expression interact with each other; NO is a retrograde messenger implicated in synaptic plasticity. Salvemini *et al.* (1993) found in mouse macrophage cell cultures that both endogenous and exogenous NO released PGE2 by activating COX. LPS also stimulates the synthesis of NO (McCann, 1997) and ibuprofen reduces NO activity (Stratman et al., 1997). It is possible that COX2 needs to be present for LTP maintenance by stimulating the expression of NO as NO is important in increasing cAMP (Lerea & McNamara, 1993; Salvemini *et al.* 1993). In the same way, PGE2 also increases cAMP and it is possible that the direct enzymatic action of COX2 through the conversion of AA to PGE2 is required for LTP (Salvemini *et al.*, 1993). This second possibility is unlikely in neurons as Rettori *et al.* (1992) show convincing data that presynaptic NO stimulates the conversion of AA to PGE2 by regulating the production of COX in hypothalamic neurons.

The experiments presented here examine the effects of LPS on hippocampal-dependent spatial learning, LTP and BNDF expression in the dentate gyrus of the hippocampal formation. We also investigated the pharmaco-therapeutic effects of ibuprofen (a broad spectrum COX inhibitor; Crofford *et al.*, 2000) on LPS-treated animals. We hypothesised that ibuprofen will attenuate the LPS-induced learning deficit in both the watermaze and LTP. We expect that by blocking the LPS induced COX expression the LPS produced learning deficit should be reversed. We also expect BDNF levels to correlate with learning and LTP, such that poor performance in learning the water maze task (see previous Chapters) and no LTP would result in diminished BDNF levels.

## Methods and Materials

### Behavioural Procedures: LPS 250 µg/kg and Ibuprofen 30mg/kg

### Animals

18 male Wistar rats (mean weight 250 g) were used in this experiment. Rats were pair-housed and maintained on a 12:12 - light/dark cycle with free access to food and water.

### Watermaze Training

See chapter 3

### LPS Protocol

LPS (250 µg/kg was obtained from *Escherichia Coli*; (L-2630 100 mg): Sigma Chemical Co., St. Louis, MO, USA).

### Ibuprofen Protocol

Ibuprofen (30 mg/kg was obtained from Sigma Chemical Co., St. Louis, MO, USA). The rats were injected intraperitoneally with 30 mg/kg of ibuprofen 1 hour prior to treatment or testing. This dose of ibuprofen was chosen as it was similar to the doses used by Lim *et al.* (1999) and Nakayama *et al.* (1998) who investigated the neuroprotective properties of NSAIDs on hippocampal tissue.

The rats were randomly assigned to one of 3 groups (n=6/group):

1. Single LPS: LPS (i.p. 250 µg/kg) injected once on the first day of the acquisition phase 4 hours before training.
2. LPS + Ibuprofen: Animals were pre-treated with ibuprofen (i.p. 30 mg/kg) one hour before the LPS (i.p. 250 µg/kg) injection; animals were tested 4 hours after the LPS injection.
3. Saline Control: Saline (i.p. 0.9%) injected once on the first day of the acquisition phase 4 hours before training.

### Electrophysiological Procedures: LPS 250 µg/kg and Ibuprofen 30mg/kg

The rats were randomly assigned to one of 3 groups (n=6/group).

1. Single LPS: LPS (i.p. 250 µg/kg) injected 4 hours before anesthesia.
2. LPS + Ibuprofen: Animals were pre-treated with ibuprofen (i.p. 30 mg/kg) one hour before the LPS (i.p. 250 µg/kg) injection; animals were anaesthetised 4 hours after the LPS injection.
3. Saline Control: Saline (i.p. 0.9%) 4 hours before anesthesia.

All rats were initially anaesthetized with sagatal (sodium pentobarbitone: 60mg/kg, i.p.) and were mounted in a stereotactic holder. Further injections of urethane (ethyl carbonate: 1.5g/kg, i.p.) were given to sustain anesthesia throughout the experiments. A local anesthetic/adrenaline combination

was injected under the scalp and an incision was made to visualise the skull. Stimulating electrodes were aimed at entorhinal cortex and the recording electrodes at dentate gyrus. Electrode implantation sites were identified using stereotaxic coordinates, relative to bregma (Paxinos & Watson, 1996); recording and stimulating electrodes were 3.9mm posterior and 2.5mm lateral to the midline and 8.1 posterior and 4.4mm lateral to midline, respectively. Bipolar stimulating and monopolar recording electrodes consisted of two pieces of twisted 50mm tungsten wire, insulated to the tips.

Signals were filtered between 0.1Hz and 1Khz and then amplified (DAM-50 differential amplifier, World Precision Instruments, Hertfordshire, UK). Recordings were digitised online using a PC connected to a CED-1401 plus interface (CED, Cambridge, UK). Electrodes were slowly lowered to a depth of 2.5mm; test stimuli were administered during electrode movement at a rate of 0.05Hz. The final depths were adjusted until maximal field excitatory post-synaptic potentials (fEPSPs) were obtained; electrodes were allowed to settle for 10 minutes before baseline recordings were conducted. Initial slope was measured to assess the amount of LTP.

### BDNF Elisa protocol

(BDNF Emax ™ ImmunoAssay System obtained from Promega UK LTD).

See chapter 2.

### Corticosterone radioimmunoassay

See chapter 3.

### Statistics

The behavioural data was analyses using a repeated measure ANOVA; BDNF ELISA values and corticosterone levels were analysed using a one-way ANOVA; a value of $P< 0.05$ was considered to be significant. Between group *post-hoc* comparisons were made using Tukey's method ($p < 0.05$) and dependent 2-tailed Bonferonni t-tests were used to determine within group *post-hoc* comparisons ($p < 0.005$). Dependent t-tests were used to analyse the electrophysiological data within group and ANOVA were used to compare across groups.

## Results

### Watermaze Training and Retention Analysis

### Direct Swim analysis

A direct swim to the hidden platform was defined as a swim path that remained inside a corridor 30cm wide from the start position to the platform. A repeated measures ANOVA demonstrated that there was significant differences between groups during both the acquisition and retention phases (see Figure 1a; Days 1-5: $F(2,87) = 6.509$, $p < 0.01$; Days 9-11: $F(2,87) = 18.6$, $p < 0.001$). *Post-hoc* tests showed that the LPS group failed to use the most direct route to find the hidden platform compared to the other groups. On day 1 the LPS group was significantly worse than the LPS + ibuprofen groups; both the saline controls and the LPS + ibuprofen used the most direct route to find the platform on day 4 compared to the LPS group. The saline controls and the LPS + ibuprofen group performed better than the LPS group on day 9 and 10 and better than the LPS + ibuprofen group on day 10 ($p < 0.05$). No interaction between group and day was found for either the acquisition or retention phases. Over all main effects within groups was found (Days 1-5: $F(4,384) = 46.993$, $p < 0.001$; Days 9-11: $F(2,160) = 11.42$, $p < 0.001$) but the *post-hoc* testing did not show any significant differences across days. No interactions were found. Figure 1b shows representative swim paths taken from each group on days 5 and 8.

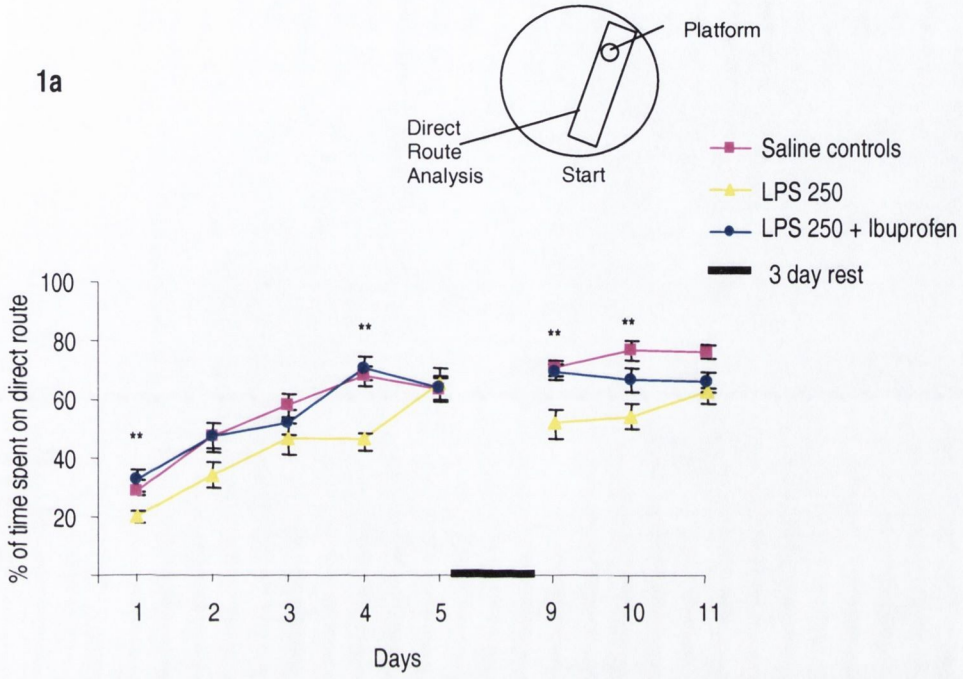



**Figure 1(a):** Percentage of time spent in the direct route to the platform for all groups across the acquisition and retention periods.

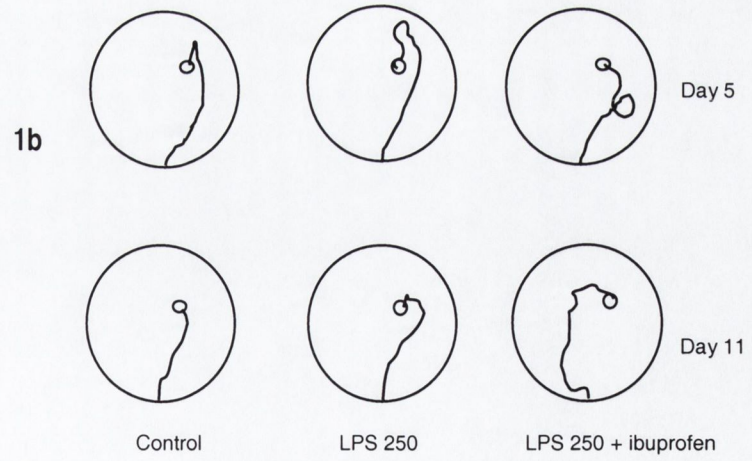



**Figure1(b):** Representative swim paths taken from the last days of acquisition (day 5) and retention (day 11) for each group (n= 6/group; * $p < 0.05$; ** $p < 0.01$).

### Overall Escape Latency Analysis

Figure 2a demonstrates that escape latencies for all groups decreased during the training period with the saline control group finding the platform the fastest. Repeated measures ANOVA showed there was a main effect for group during the acquisition phase only (Days 1-5: $F(2,87) = 5.38$; $p < 0.01$); *post-hoc* tests revealed the LPS group was slower than both the LPS + ibuprofen group and the saline controls on day 3 of the training task and the LPS group was slower than both the saline controls and the LPS + ibuprofen group on day 4. The LPS group was significantly slower than the saline controls on days 4 and 5 ($p < 0.05$). Within group differences were found in both phases (Days 1-5: $F(4, 348) = 62.726$, $p < 0.001$; Days 9–11: $F(2, 174) = 7.3$, $p < 0.001$). The *post-hoc* tests found that the controls and the LPS + ibuprofen group had shorter escape latencies on days 2, 3, 4, and 5 compared with day 1 and days 4 and 5 compared with day 2 ($p < 0.005$). The single LPS group also had shorter escape latencies on days 2, 3, 4, and 5 compared with day 1 and also on the last day of the retention task when compared with day 9 ($p < 0.005$). No interactions were found during either the acquisition or retention phases.

### Cumulative Distance Analysis

A repeated measures ANOVA was used to analyse the data; a within group main effect was found for both the acquisition and retention phases (Days 1-5: $F(3,246) = 44.32$, $p < 0.001$; Days 9–11: $F(2, 151) = 6.55$, $p < 0.001$). *Post-hoc* tests showed that the control group swam increasingly shorter distances on days 3, 4, and 5 compared to day 1 and also on days 4 and 5 compared to day 2 ($p < 0.005$). The LPS group also swam shorter distances on days 3, 4, and 5 when compared to day 1 and also on day 5 compared to day 2 ($p < 0.005$). The LSP + ibuprofen group swam shorter distances on days 4 and 5 compared to the first and second days, they also swam a significantly shorter distance on day 5 compared to day 3 and also day 11 compared to day 9 ($p< 0.005$; data not shown). No between group differences and interactions were found.

### Velocity analysis

The mean speed per day for each group was calculated. Figure 2b shows that the saline controls swam the fastest for the duration of the experiment. A significant difference was found between the groups (Days 1-5: $F(2,87) = 92.4$, $p < 0.001$; Days 9–11: $F(2,87) = 12.69$, $p < 0.001$). *Post-hoc* tests found that the saline controls swam faster than all the groups on days 1, 2,3,4, 5, and 9. An interaction between group and day was only found for the acquisition task (Days 1-5: F

(8, 306) = 3.543, $p < 0.001$). Within group effects were also found (Days 1-5: $F(3, 306) = 5.53$, $p < 0.001$; Days 9–11: $F(2, 174) = 9.285$, $p < 0.001$). *Post-hoc* tests showed that the controls swam faster on day 5 when compared to days 2 and 3 ($p < 0.005$). The LPS group swam faster on day 1 than on day 4 and the LPS + ibuprofen group were faster on days 10 and 11 compared to day 9 ($p < 0.005$).

### Probe Analysis: Time in Platform Quadrant

The ANOVA found that there were no differences between groups during the probe test (ANOVA: $F(2, 15) = 2.51$, $p > 0.05$).

### BDNF Expression

BDNF concentration was determined in the dentate gyrus from all animals in each group on the last day of the experiment (day 11). No differences were found between groups (ANOVA: $F(2, 25) = .817$, $p > 0.05$; see Figure 2c).

### Circulating Blood Corticosterone Levels.

Corticosterone levels were determined from trunk blood taken on the last day of the experiment. No significant differences were found between the groups (ANOVA: $F(2,15) = .033$, $p > 0.05$; see Figure 2d).

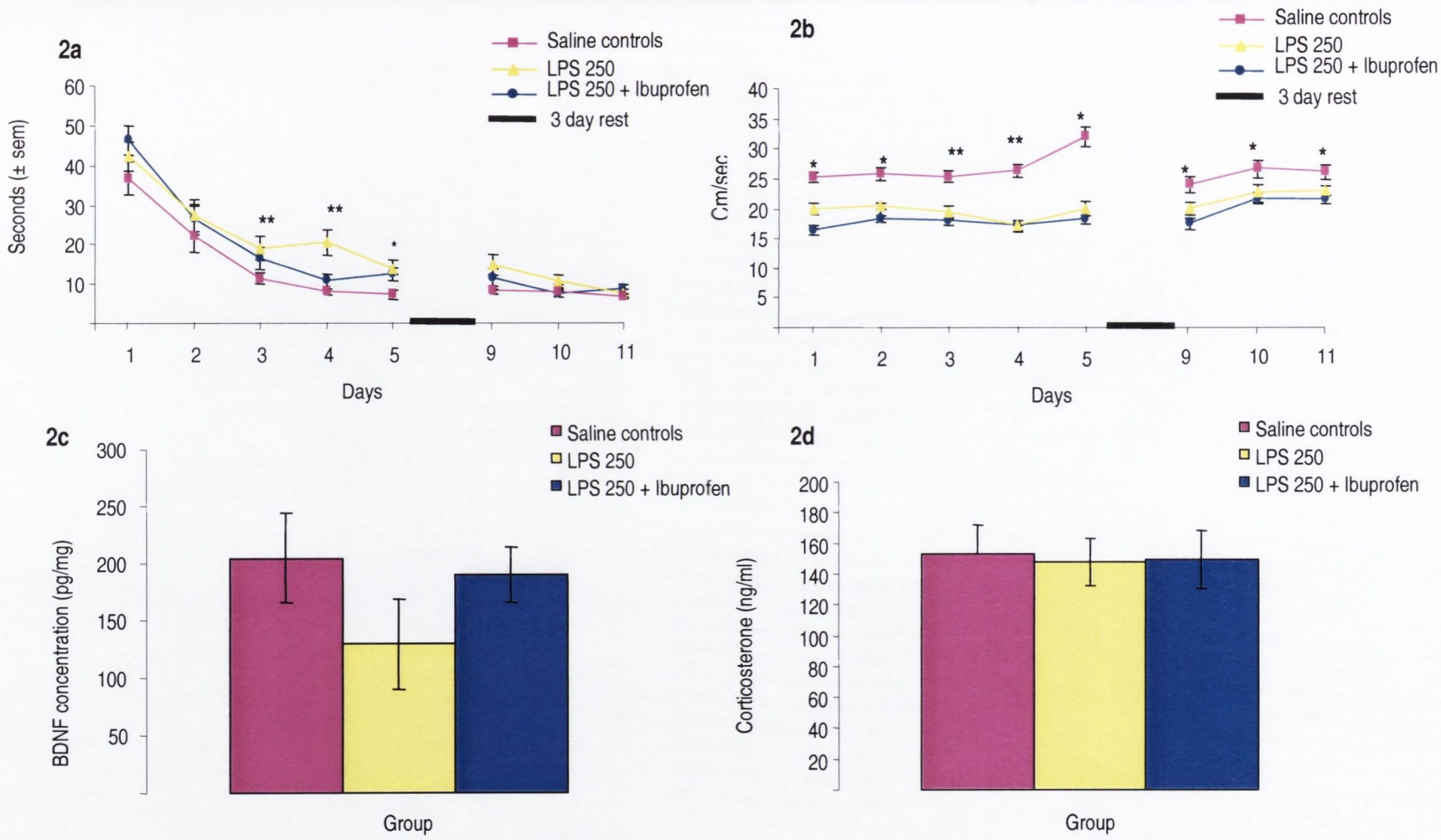



**Figure 2(a):** Mean escape latency (seconds ± sem) for the groups across the acquisition and retention periods. **2(b):** Total velocity of each group within the 60 sec allowed across the acquisition and training periods. **2(c):** Mean concentration of BDNF in dentate gyrus for each group. **2(d):** Mean corticosterone levels of each group taken on the last day of the retention period (day 8; n= 6/group; * $p < 0.05$; ** $p < 0.01$).

## Electrophysiological Results

*Effect of LPS, LPS & ibuprofen and saline on synaptic plasticity*

In saline-treated animals LTP was successfully induced and fEPSPs remained potentiated for at least 30 minutes as indicated by the change in fEPSP amplitude which stood at 139.27 ±10.8%, 146.39 ±9.3%, 150.5 ± 9.5% of baseline 5, 15 and 30 minutes post-HFS respectively (see Figure 3a). Using a paired sample t-test, the responses in the final 10 minutes of the recording period were found to have increased significantly when compared to baseline measurements for all groups (Saline: t= -17.347, df=9, $p<0.001$; LPS: t=-5.457, df=9, $p<0.001$; LSP + ibuprofen: t=-13.462, df=9, $p<0.001$).

There was an overall difference between groups for last 10 minutes of the HFS (ANOVA: $F(2,29) = 53.095$, $p < 0.001$). Post hoc tests showed that all groups were significantly different from each other, with the saline group showing the most potentiation (150.5 ±9.5%,) followed by the LPS group (113.7 ±4.7%) and finally the LPS + ibuprofen group (107.4 ±2.6%).

## BDNF Expression

BDNF concentration was determined in the dentate gyrus from all animals in each group. An overall difference was found between groups (ANOVA: $F(2,14) = 21.961$, $p < 0.001$, see Figure 3b).

## Circulating Blood Corticosterone Levels.

Corticosterone levels were determined from trunk blood taken 30 minutes after HFS; Figure 3c shows that no significant differences were found between the groups (ANOVA: $F(2,14) = 0.957$, $p > 0.05$).

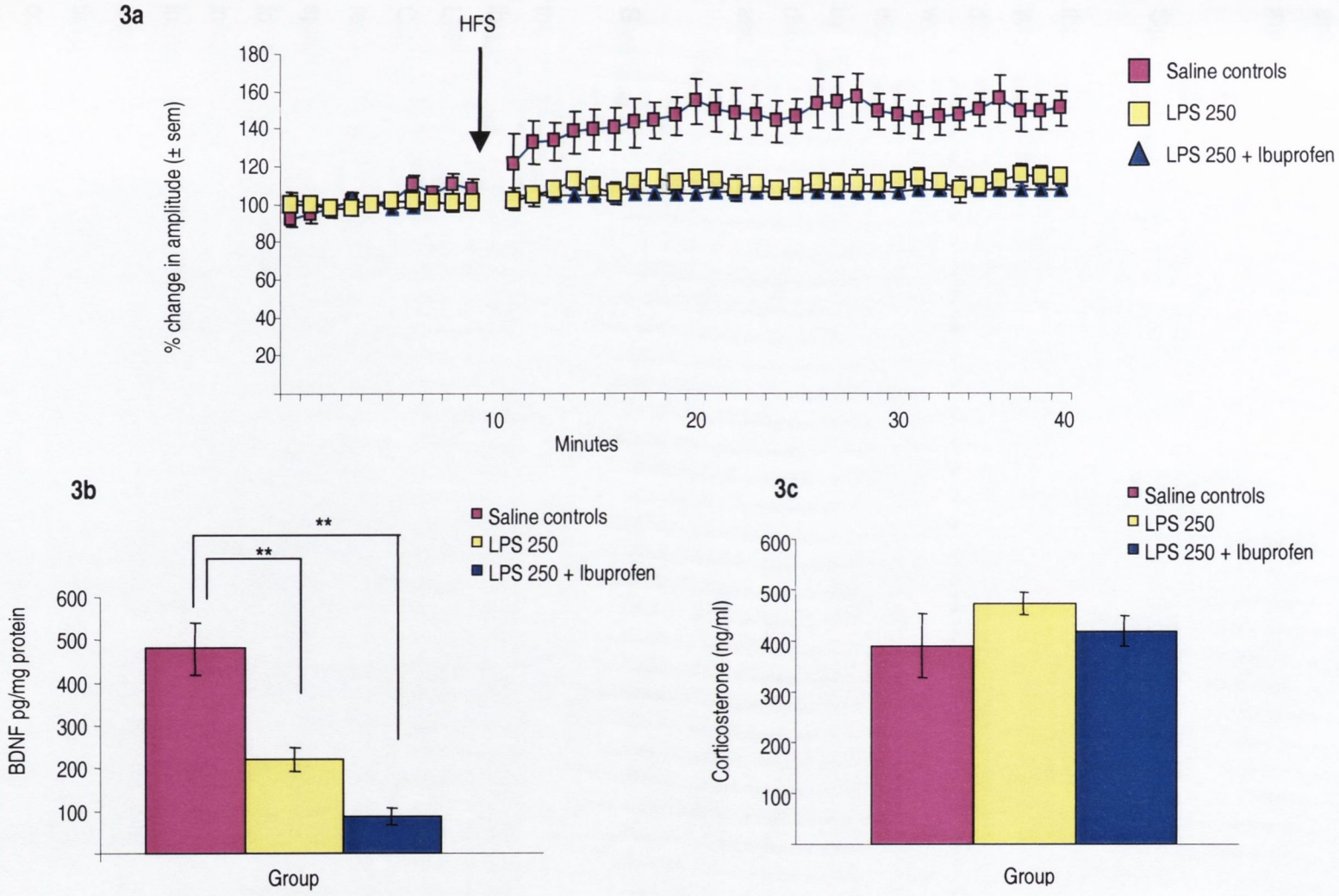



**Figure 3(a):** Percentage change in amplitude of fEPSPs after HFS in all groups; **3(b):** Mean concentration of BDNF in dentate gyrus from tetanised tissue; **3(c):** Average corticosterone levels from animals in the above experiment (n= 6/group; * $p < 0.05$; ** $p < 0.01$).

## Discussion

Here we investigated the naturally neuroprotective effects of ibuprofen on LPS-treated animals in relation to both spatial learning and LTP induction. We were particularly interested to investigate if ibuprofen attenuated the LPS-induced spatial learning and LTP blockade.

## Spatial Learning

We found that the LPS 250 μg/kg group + ibuprofen treated group learned the task better than the LPS 250 μg/kg, as shown by the direct swim route analysis and faster escape latencies. While the LPS + ibuprofen group consistently swam slower than the saline-controls, they did not differ greatly in direct swim routes or escape latency. One would have expected that this group would have swam at an intermediate or equal speed compared to the saline-controls; a possible explanation for the slow speed is that while ibuprofen prevented the inflammatory response evoked by LPS, it didn't abolish the other sickness behaviours. We also found that there were no differences in BDNF concentration or corticosterone levels between the different treated groups and controls.

## Synaptic Plasticity

After HFS, the saline-controls showed increased synaptic potentiation compared to both the LPS 250 μg/kg group and the LPS 250 μg/kg group + ibuprofen treated group. While ibuprofen attenuated the LPS- learning deficits in the watermaze, it did not have the same effect on LTP. Cunningham *et al.* (1996) found that LPS blocks LTP in vitro in the dentate gyrus and Commins *et al.* (2001) previously showed that LPS blocked LTP in the subiculum in vivo. We have replicated this finding in the dentate gyrus in vivo. This LPS-mediated inhibition of LTP mirrors our spatial learning results, lending support to the hypothesis that LTP is a possible biological model for learning and memory. No difference was found in corticosterone levels suggesting that the inhibition of LTP was not due to increased circulating glucocorticiods while the saline-controls had increased BDNF levels compared to the other groups. We have previously shown that spatial learning increased BDNF concentration and others have found an increase in BDNF in tetanised tissue (Gooney *et al.*, submitted). We thus expected to find an increase in BDNF in the saline-controls. As LTP was inhibited in the other two groups, we did not expect to find the same BDNF increase.

There was no difference in BDNF concentration between the watermaze trained groups yet there was a difference between the tetanised groups. It appears that the BDNF increase that facilitates learning and/or LTP is time dependent. The tissue analysed from the watermaze trained animals was taken 11 days after the LPS and IBU injections whereas the tetanised dentate gyri was taken only 6 hours following injection. The BDNF increase due to HFS may suggest that BDNF-dependent LTP is needed for the encoding of memory and once this process had finished BDNF levels dissipate. This would also help explain the fact that we found no BDNF difference in the spatially trained animals, by day 11 the memory had been encoded and consolidated returning BDNF levels to baseline. An alternative way to explain the behavioural BDNF data is that BDNF is increased in all groups and the reasons for this may be either due to learning or to neuroprotection. On the last day of the experiment there were no differences in performance between the groups, suggesting that the BDNF protein levels should be the same, alternatively the equal BDNF levels could be interpreted as being increased in the LPS and LPS+IBU groups is due to neuroprotection while the saline-control increase is due to synaptic plasticity. LSP stimulates the release of cytokines which can trigger cellular death; BDNF may be increased in response to the presence of increased levels of cytokines. If this is the case BDNF would be expected to be increased in pre-synaptic neurons as the neurotrophin targets the compromised region(s). Alternatively, if BDNF is increased due to learning then, one would expect to find a post-synaptic increase in order to facilitate synaptic plasticity. A study that measures BDNF in both pre-and post-synaptic neurons would elucidate the role of BDNF in LPS-treated tissue.

## General Discussion

There is very little research investigating if there is an interaction between COX regulation and neurotrophins. The stimulation of glutamate receptors by kainic acid increases COX2 levels in rat hippocampus (Manev *et al.*, 2000). Glutamate, an excitatory neurotransmitter, also stimulates NMDA receptors that permit an influx of Ca2+ into the post-synaptic cell. This Ca2+ influx stimulates a cascade of reactions required for LTP, one end-product being BDNF transcription (Gooney *et al*, in press). Perhaps the stimulation of COX2 by LPS activates the COX2-PG pathway thus reducing the available COX2 required for the stimulation of learning related pathways. By inhibiting the LPS induced production of COX with a NSAID and thus blocking the

stimulation of the AA-PG pathway, learning-dependent Ca2+ may stimulate constituent COX levels required for further learning-related changes.

Manev *et al.*, (2000) reported a link between increased glutamate, Ca2+ and COX2 activation. This finding suggests a possible relationship between the Ras and PLA2- AA pathways. The Ras pathway, via ca2+ influx, stimulates the production of BDNF while COX is important in the PLA2- AA pathway. Munir *et al.*, (1999) looked at MAP kinase activation and COX2 expression by platelet-activating factor (PAF) in human endometrial cells and found that MAP kinase is activated with PAF and that COX2 expression is stimulated through the MAP kinase pathway with PAF. Both pathways seem to be neuroprotective; IL-1 stimulates NGF in neonatal cortical astrocytes, suggesting that a possible mechanism for this IL-1 stimulated NGF secretion is by the activation of the PLA2- lipoxygenase pathway (Carman-Krzan & Wise, 1993).

# CHAPTER 6

## Cyclooxygenase inhibition negatively affects spatial learning, BDNF and PGE2

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## Introduction

Arachidonate is metabolised to prostaglandins (PG) by the biofunctional enzyme COX after which stable PGs are formed by a group of synthases. It is present in two isoforms: COX1 and COX2 (Crofford *et al.*, 2000; Yamagata *et al.*, 1993). COX1 is found constitutively in most cells and tissues (Smith & DeWitt, 1996); this enzyme has the characteristics of a housekeeping protein whose activity is dependent solely on the substrate. COX2 shares 60% of its overall identity with COX1 at the amino acid level (O'Banion *et al.*, 1991; Xie *et al.*, 1991) and is regulated at the transcription and translation level (Smith & DeWitt, 1996). The most important difference between COX1 and COX2 is their pattern of tissue expression and regulation. COX1 is constitutively expressed in virtually all tissues, most notably platelets, endothelial cells, gastrointestinal tract, renal microvasculature, glomerulus and collecting ducts. Its expression can increase 2-4 fold under stimulatory conditions and is little affected by glucocorticoids (Golden & Abramson, 1999). COX2 is typically undetected in most tissue under basal conditions but its mRNA and protein expression is increased 10-80 fold by cytokines (Masferrer *et al.*, 1994a; Seibert *et al.*, 1994). COX1 and COX2 are both expressed in neurons, especially in the hippocampal formation, amygdala, some periventricular regions and the brain stem (Kaufmann *et al.*, 1997; Breder *et al.*, 1995; Yamagata *et al.*, 1993). Both COX1 and COX2 are inhibited by non-steriodal anti-inflammatory drugs (NSAIDs). NSAIDs act by blocking the actions of both COX1 and COX2, which prevents the production of prostaglandins. NSAIDs, such as aspirin or ibuprofen, are commonly used for the occasional headache or fever or to reduce soreness and inflammation resulting from overexertion (DeWitt, 1999). People who have rheumatoid arthritis and osteoarthritis depend daily on this medication to not only reduce pain but also restore function to their joints (Abramson & Weissman, 1989; Kaufmann *et al.*, 1997).

As COX is highly expressed in the hippocampal formation, it may be involved in learning and memory. There are few studies of COX induction or inhibition on synaptic transmission in the brain and the molecular mechanisms underlying COX activity in the brain have not been described in any detail (Hoffmann, 2000), although some studies have suggested that COX activity may be important in the synaptic plastic changes related to memory consolidation. Yamagata *et al.* (1993), for example, found that COX2 expression is induced by NMDA-receptor activation and others have found increases in PGs after stimulation of hippocampal NMDA receptors (Lazarewicz & Salinska,

1995). Furthermore, according to Holscher (1995), COX2 is induced by neuronal activity during learning and its activity is essential for the consolidation of memories. In this study Holscher (1995) injected chicks with both COX and lipoxygenase inhibitors and found that only the broad-spectrum COX inhibitors (ibuprofen, indomethacin, and naproxen) caused amnesia of the previously learned passive avoidance task. Following this, Holscher (1995b) investigated the role of PG in learning and memory and found that both ibuprofen and SC58125, a selective COX2 inhibitor, produced amnesic effects 2 hours after learning and PG production (as measured by ELISA) was also suppressed up to 3 hours after training. These studies imply that both COX2 and PG are important in the biological consolidation of memory.

Few studies have investigated the role of COX in LTP; one such study found that rats stimulated with HFS showed marked increase in COX2 mRNA up to 4 hours after stimulation (Yamagata *et al.*, 1993). To examine if COX2 mRNA increases were mediated by NMDA activation, MK-801, an NMDA blocker, was given to rats before HFS. When given MK-801, COX2 mRNA was not increased suggesting that COX2 is regulated by NMDA receptor activation (Yamagata *et al.,* 1993). Following this, Yamagata *et al.* (1993) investigated the effects of COX mRNA inhibition and LTP and found that indomethacin (a COX mRNA antagonist) did not block LTP (Yamagata *et al.,* 1993). Other COX inhibitors affect the action of the enzyme rather than the mRNA (e.g. ibuprofen); more specifically ibuprofen binds to COX and prevents COX from binding to AA.

The expression of COX2 has not only been associated with the consolidation of memory but also with memory deficits. COX2 seems to play a role in Alzheimer's disease (AD); COX2 is up-regulated in the brain of people with AD; and in particular, in areas CA3 and CA2 of the hippocampus: this increase is proportionate to severity of the disease (Yasojima *et al.,* 1999; Ho, 2001). A general finding from research that has investigated the treatments related to AD is that chronic anti-inflammatory drug treatment in people under 75 years old appears to result in a reduced risk of developing AD (see Halliday *et al.,* 2000 for a review).

Very little research has investigated if there is an interaction between COX regulation and neurotrophins. Hashimoto *et al.* (1998) found that kainic acid-induced seizures stimulated both COX2 mRNA and BDNF mRNA in hippocampal neurons; they suggest that COX2 mRNA upregulation might play a role in the pathogenesis of neuronal injury and BDNF mRNA is upregulated as a trophic response to this development. It is possible therefore, that COX and

BDNF interact with each other during the intracellular cascades associated with synaptic transmission and synaptic plasticity. Manev *et al.* (2000) reported a link between increased glutamate and COX2 activation; they found that an intraperitoneal injection of kainic acid increased COX2 mRNA. Kainic acid stimulates glutamate receptors (Michaelis, 1998) and glutamate stimulated the Ras pathway (Chen *et al.*, 1998); this suggests a possible relationship between the Ras and PLA2- AA pathways. The Ras pathway stimulates the production of BDNF while COX is important in the PLA2- AA pathway; both pathways play an important role in neuroprotection. Carman-Krzan & Wise (1993) found that IL-1 stimulates NGF in neonatal cortical astrocytes and suggested that IL-1 stimulates NGF secretion by the PLA2- lipoxygenase pathway.

Exercise increases levels of BDNF in the hippocampal formation, especially areas CA1, CA3 and DG (Neeper *et al.,* 1995; Neeper *et al.,* 1996; Oliff *et al.,* 1998; Russo-Neustadt *et al.,* 1999). Other brain regions such as the retrosplenial and visual cortices, also show an increase in BDNF (Neeper *et al.,* 1995; Neeper *et al.,* 1996), but the greatest effects of exercise on BDNF expression occurred in the hippocampal formation- a highly plastic area of the brain- supporting the electrophysiological and behavioural data indicating that BDNF is involved in synaptic plasticity. Clinical studies show that physical fitness may be related to cognitive and mnemonic function (Clarkson-Smith & Hartley, 1989; Laurin *et al.,* 2001) and running in rats is associated with hippocampal theta rhythm, a rhythm that is implicated in hippocampal-dependent learning (Shin & Talnov, 1998; Seager *et al.*, 2002). It is entirely possible that as BDNF is activity –dependent and so is theta that running stimulates theta which in turn upregulates BDNF. Possible supporting evidence for this hypothesis is that exercise also decreases the neuronal loss resulting from a stroke in rats (Radak *et al.,* 2001) and reduces stress in humans (Throne *et al.,* 2000; Salmon, 2001).

Neurotrophins, especially BDNF, have also been investigated as therapeutic agents in patients with AD as there is a decrease of BDNF in surviving hippocampal neurons in people with AD (Ferrer *et al.,* 1999; Siegel & Chauhan (2000). In addition to this, the BDNF receptor, Trk B, is also reduced in the brains of AD patients further diminishing the actions of the remaining BDNF Allen *et al.* (1999). Some studies have investigated the protective and therapeutic action of NSAIDs in relation to AD (Dash *et al.*, 2000; Halliday *et al.,* 2000). The effects of these drugs appear to be neuroprotective, slowing the rate of neurodegeneration; the action of the NSAIDs is to reduce the overexpression of COX2 that is either the result of or the initiation of cellular death

(Kadoyama *et al.*, 2001; Ho *et al.*, 2001). However, the effects of NSAIDs on healthy tissue are unknown.

The experiments presented here examine the effects of ibuprofen on hippocampal-dependent learning and on BNDF expression in the dentate gyrus. We selected ibuprofen, a nonspecific COX inhibitor, for several reasons: ibuprofen is a popular, readily available drug used by millions of people world-wide each day; it has been used previously when investigating NSAID usage and AD (Lim *et al.*, 2000) and the action of COX2 inhibitors on brain tissue is still relatively unknown (Yasojima *et al.*, 1999). We also aimed to investigate the effects of ibuprofen on baseline recordings and LTP in the dentate gyrus. We hypothesise that ibuprofen would impair learning and LTP due to a reduction in BDNF, we expect the BDNF levels to correlate with learning and LTP. We also hypothesise that by increasing endogenous BDNF levels via exercise we will attenuate both the learning and LTP deficits caused by ibuprofen.

## Materials and Methods

### Animals

Male Wistar rats (mean weight 250 g) were used in this experiment. Rats were pair-housed and maintained on a 12:12 - light/dark cycle with free access to food and water.

### Behavioural Equipment and Procedures

### The Watermaze

See previous chapters

### Watermaze Training

Watermaze training was the same as in all other experiments (see previous chapters) except that rats were trained on the watermaze for 5 days using standard procedures: the trained rats received 5 trials/day for 5 days where they had to search for the hidden platform.

### Electrophysiological Procedures

See chapter 5.

### Exercise Protocol

Rats ran for 4 consecutive nights in a running wheel prior to experimental manipulation. Distance and time spent running were recorded and rats had to run at least 100 meters over the

last 2 nights to be included in the experiment. The running wheels were placed in the housing unit and animals only had access to the wheels at night. A running wheel comprised of 50, 10 cm long stainless steel rungs attached 1 cm apart to a circular plexi-glass board 35 cm in diameter; the wheel was attached to a wooden frame. Distance was calculated using a speedometer; the running wheels were designed and constructed by Eddie Bolger, a senior technician from the Dept of Psychology, Trinity College Dublin.

### Ibuprofen Protocol

Ibuprofen (30 mg/kg was obtained from Sigma Chemical Co., St. Louis, MO, USA). The rats were injected intraperitoneally with either 30 mg/kg of ibuprofen or saline (0.9% NaCl) 1 hour prior to either the first day of watermaze training or the start of electrophysiology. The dose of ibuprofen was similar to that used by Lim *et al.* (1999) and Nakayama *et al.* (1998) who investigated the neuroprotective properties of NSAIDs on hippocampal tissue.

### BDNF Elisa protocol

(BDNF Emax ™ ImmunoAssay System obtained from Promega UK LTD).

Described earlier see chapter 2.

### Corticosterone radioimmunoassay

Described earlier see chapter 3.

### PGE2 High Sensitivity Enzyme Immunoassay Kit protocol

(Assay Designs' Correlate-EIA PGE2 kit, 93001; Metachem, Nottingham, UK).

All rats were killed on the last day of the watermaze or at the end of the electrophysiology, their brains removed and tissue taken from the dentate gyrus. Slices of dentate gyrus were preincubated in 250 µl Krebs solution containing 2mM CaCl 2 for 3 minutes and the supernatant was removed and discarded. This step was repeated in a volume of 100 µl, and at the end of the 3 minutes incubation samples were centrifuged at 1000g for 3 minutes and the supernatant was retained. This step was repeated in the presence of 40mM KCL to depolarise the slices and supernatant was retained. Both samples of supernatant were stored at -80 °C for later analysis of PGE2 by immunoassay. PGE2 concentration was determined in the supernatant. To prepare the samples, dentate gyri were homogenised in ice-cold Krebs 25 times. Samples were centrifuged and supernatant retained. Protein was assessed and samples were diluted to give equal protein

concentrations and stored at -80°C. Samples of supernatant (100 µl) or PGE2 standards (100 µl; ranging from 7.81 pg/mL to 1ng/mL) were added to the ninety-six well plates. The plates were pre-coated with goat antibody specific to mouse IgG. 50 µl of PGE2 HS-EIA conjugate was then added to each well, followed by 50 µl of PGE2 HS-EIA antibody; the wells were covered, incubated overnight at 4°C and washed. 200 µl of p-Npp Substrate solution was then added to every well and the plates were incubated for 1h at 37°C. The reaction was stopped by the addition of 50µl trisodium phosphate to the wells. Plates were read a 405nm in a 96-well plate reader and PGE2 concentrations were estimated for the standard curve and expressed as pg/mg protein.

## Statistics

The behavioural data was analysed using a repeated measures ANOVA. All biochemical assays were analysed using a one-way ANOVA; a value of $p < 0.05$ was considered to be significant. Between group *post-hoc* comparisons were made using Tukey's method and the within group comparisons were made with a paired t-test, using a Bonferonni conversion to assess significance ($p < 0.005$ was considered to be significant). The electrophysiological data was analysed using independent or dependent t-tests.

## Experiment 1: The effects of broad-spectrum cyclooxygenase inhibitors on synaptic transmission and synaptic plasticity.

*Group 1:Pre-treated baseline recordings.*

Rats were injected with either saline (0.9%, n=4) or ibuprofen (30mg/kg, n=4) one hour pre-anesthesia. Following electrode settlement, fEPSPs were recorded for 60 minutes. All fEPSP values are expressed as percentage of the initial 10 minute measurements.

*Group 2: Baseline recordings*

Following electrode settlement, baseline measurements were taken for 10 minutes. Following this, rats were injected with either saline (n=5) or ibuprofen (n=5) and fEPSPs were recorded for 60 minutes

*Group 3: Long-term potentiation.*

Rats were injected with either saline (0.9%, n=13) or ibuprofen (30mg/kg, n=14) one hour pre-anesthesia. Following electrode settlement, baseline measurements were taken for 10 minutes. Induction of LTP was then attempted using high-frequency stimulation (HFS; this consisted of 10 trains of 20 stimuli at 200Hz, inter train interval of 2 seconds; this protocol identical to that used by Commins *et al*, 1998). Stimulation was resumed at baseline stimulation and fEPSPs were recorded for 30 minutes. The post-stimulation values are expressed as percentage of the pre-stimulation baseline.

## Experiment 2: The effects of broad-spectrum cyclooxygenase inhibitors spatial learning.

The rats were randomly assigned to one of 2 groups (n = 6/group):

i) Ibuprofen: Ibuprofen (i.p. 30 mg/kg) injected once on the first day of the watermaze task 1 hour before training.

ii) Saline Control: Saline (i.p. 0.9%) injected once on the first day of the watermaze task 1 hour before training.

See page 120 for watermaze protocol.

## Non-hippocampal Learning

The rats were randomly assigned to one of 2 groups (n = 6/group):

i) Ibuprofen: Ibuprofen (i.p. 30 mg/kg) injected once on the first day of the visible platform task 1 hour before training.

ii) Saline Control: Saline (i.p. 0.9%) injected once on the first day of the visible platform task 1 hour before training.

*Visible Platform Protocol*

The watermaze set-up was the same as in all other experiments (see previous chapters) except that rats had to swim to a visible platform. This is not a hippocampal dependent task as the rats do not have to rely on distal cues to find the hidden platform. The rats received 5 trials/day for 5 days where they had to swim to the visible platform. This task was used to assess and control for ibuprofen induced sickness behaviours or motor impairments.

## Experiment 3: The effects of broad-spectrum cyclooxygenase inhibitors on exercise, cyclooxygenase and synaptic plasticity.

*Effect of exercise on LTP*

Rats ran for four nights consecutively. Distance and time were recorded, and the rats had to run at least 100m over the last 2 two nights to be included in the experiment. On the fifth day rats were injected with either saline (0.9%, n=4) or ibuprofen (30mg/kg, n=5) one hour pre-anesthesia. Following electrode settlement, baseline measurements were taken for 10 minutes. Induction of LTP was then attempted using high-frequency stimulation (as above). Stimulation was resumed at baseline stimulation and fEPSPs were recorded for 30 minutes.

At the end of each experiment rats were sacrificed and their brains were removed and tissue was taken from the dentate gyrus. The stimulated DG's were used for BDNF and PGE2 analysis and the unstimulated dentate was used for COX analysis. Trunk blood samples were also taken from each animal for corticosterone analysis.

## Experiment 4: The effects of broad-spectrum cyclooxygenase inhibitors on exercise, cyclooxygenase and spatial learning.

The rats were randomly assigned to one of 4 groups (n = 6/group):

1. Ibuprofen: Ibuprofen (i.p. 30 mg/kg) injected once on the first day of the watermaze task 1 hour before training.
2. Saline: Saline (i.p. 0.9%) injected once on the first day of the watermaze task 1 hour before training.
3. Ibuprofen + Exercise: Rats ran 4 nights consecutively before watermaze training and for the duration of the watermaze task; ibuprofen (i.p. 30 mg/kg) was injected once on the first day of the watermaze task 1 hour before training.
4. Saline + Exercise: Rats ran 4 nights consecutively before watermaze training and for the duration of the watermaze task; saline (i.p. 0.9%) was injected once on the first day of the watermaze task 1 hour before training.

We also wanted to measure BDNF concentration in animals that have exercised but not learned any task. In this part of the experiment rats were randomly assigned to one of 3 groups (n=6):

1. Saline sedentary: This was an inactive control group that was injected with saline (i.p. 0.9%) 1 hour before tissue was taken.
2. Saline + Exercise: Rats ran 4 nights consecutively prior to being injected with saline (i.p. 0.9%) 1 hour before tissue was taken.
3. Ibuprofen + Exercise: Rats ran 4 nights consecutively prior to being injected with ibuprofen (i.p. 30 mg/kg) 1 hour before tissue was taken.

## Results
## Experiment 1

### Cyclooxygenase, synaptic transmission and synaptic plasticity.

*Effect of ibuprofen and saline on baseline synaptic transmission*

In saline-treated animals fEPSPs remained stable over one hour (100.7 ±1.8%, 98.04 ±1.7%, 103 ±4.8%, 102.6 ±7.0%, 98.33 ±8.2% and 102.1 ±9.2% of the mean fEPSP 10, 20, 30, 40, 50 and 60 minutes respectively). In ibuprofen-treated animals fEPSP slopes also remained stable as indicated by the percentage changes in fEPSP slope which stood at 103.7 ±2.1%, 108.8 ±4.1%, 101.7 ±7.8%, 98.5 ±5.0%, 100.6 ±6.9%, 102.3 ±6.4%, at 10, 20, 30, 40, 50 and 60 minutes post the commencement of recordings (see Figure 1a). An independent t-test ($t=1.921$, $df=118$, $p>0.05$) confirmed that there were no significant differences in fEPSP responses between the two groups over the 60 minutes. Furthermore, no significant differences in slope measurements were observed post-injection in either saline or ibuprofen-treated animals when compared to pre-injected values (data not shown). Dependent t-tests compared pre-saline injected values to measurements taken during the final 10 minutes of the recording session ($t=-1.349$, $df=9$, $p>0.05$). This was also true for the ibuprofen-treated group ($t=1.354$, $df=9$, $p>0.05$).

### BDNF Concentration

BDNF concentration was determined in the dentate gyrus from animals in each group. No significant differences were found between the saline and ibuprofen pre-treated baseline conditions (Figure 1b: $t = 2.272$, $df. = 6$, $p > 0.06$). The rats injected with ibuprofen 10 minutes following recording had significantly lower levels of BDNF compared to the saline controls (Figure 1e: $t = 2.449$, $df. = 8$, $p < 0.05$; data not shown).

## Corticosterone Levels

Corticosterone was determined from the blood of animals in the baseline recording group; no significant differences were found between groups (see Figure 1c).

## PGE2 Concentration

PGE2 concentration was determined in the dentate gyrus from animals in each group. No significant differences were found between the saline and ibuprofen pre-treated baseline conditions (Figure 1d: ANOVA: $F(1,22) = 1.568$, $p > 0.05$).

*Effect of ibuprofen and saline on synaptic plasticity.*

In saline-treated animals LTP was successfully induced and fEPSPs remained potentiated for at least 30 minutes as indicated by the change in fEPSP slope which stood at 138.68 ±9.2%, 151.18 ±9.8%, 161.4 ± 10.1% of baseline 5, 15 and 30 post-HFS respectively (see Figure 2a). Using a paired samples t-test, the responses in the final 10 minutes of the recording period were found to have increased significantly when compared to baseline measurements ($t=-47.34$; $df=9$; $p<0.001$). In ibuprofen-treated animals there was a slight increase in response; 116.06± 8% of baseline 5 minutes post-HFS, 120.65 ±10.2% at 15 minutes post-HFS and 124.8 ± 11.4% at 30 minutes post-HFS (see Figure 1b). The fEPSP measurements observed in the final 10 minutes of the recording period was significantly higher than baseline measurements (using a paired samples t-test; $t=-34.0$; $df=9$; $p<0.001$). Using an independent t-test to compare the fEPSP slopes in the final 10 minutes of the recording period of both saline-injected animals and ibuprofen-injected animals. Field EPSP slopes were significantly higher ($t=-24.7$, $df=18$, $p<0.001$) in saline-injected animals compared to the ibuprofen-injected animals.

## BDNF Concentration

A significant increase in BDNF concentration was also found in saline- compared to ibuprofen-treated animals (see Figure 2b; $t=2.929$; $df=25$; $p<0.001$).

## Corticosterone Levels

Corticosterone was determined from the blood of animals in the HFS group; no significant differences were found between groups (see Figure 2c).

## PGE2 Concentration

PGE2 concentration was determined in the dentate gyrus from animals in each group and significant differences were found between the saline and ibuprofen HFS conditions (Figure 2d: ANOVA: $F(1,18) = 6.372$, $p < 0.05$).

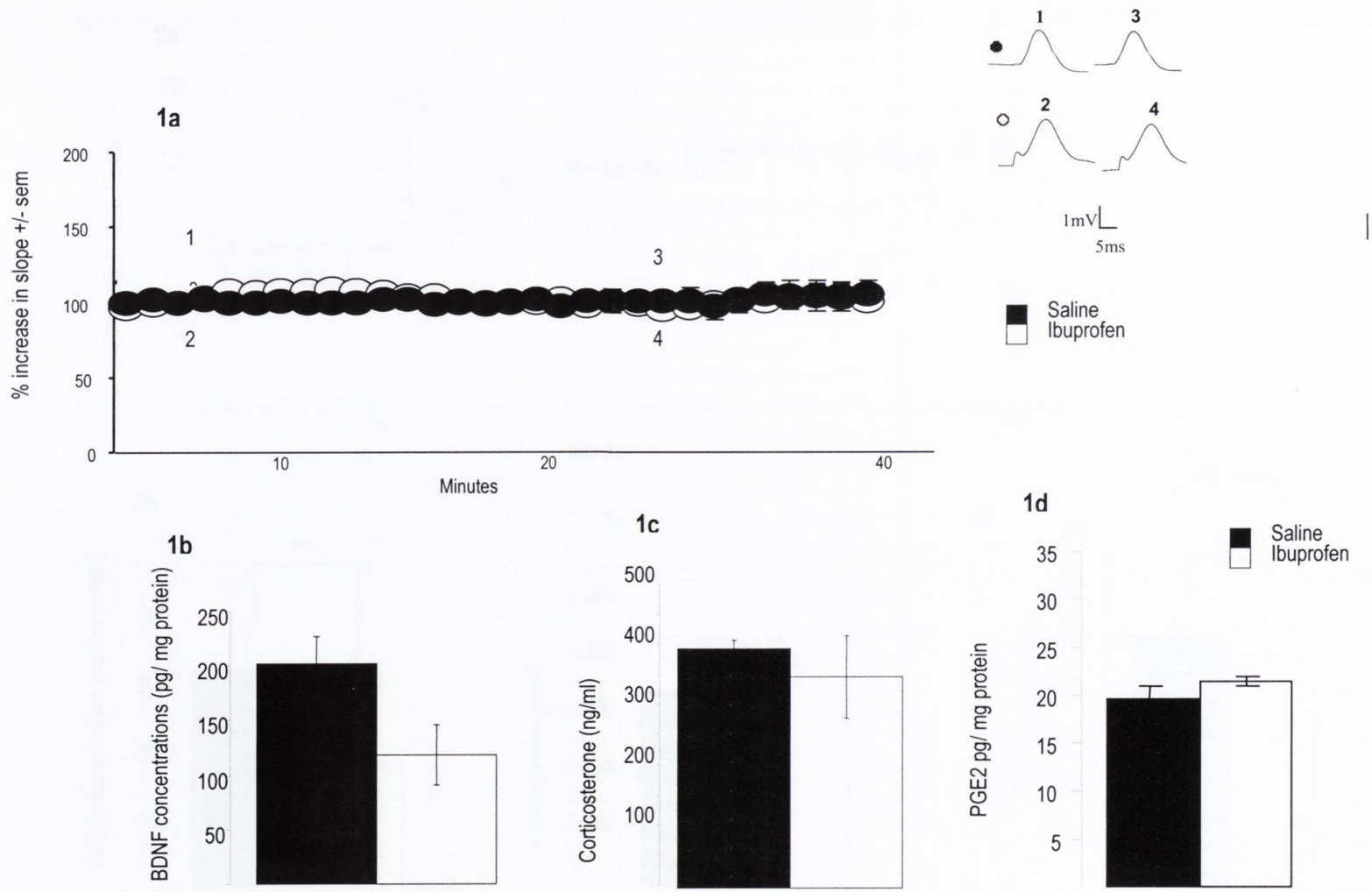



**Figure 1(a):** Percentage change in slope of fEPSPs after baseline recoding in both groups; **1(b):** Mean concentration of BDNF in dentate gyrus from stimulated tissue; **1(c):** Average corticosterone levels from animals in the above experiment; **1(d)** PGE2 levels in dentate gyrus from stimulated tissue (* $p < 0.05$; ** $p < 0.01$).

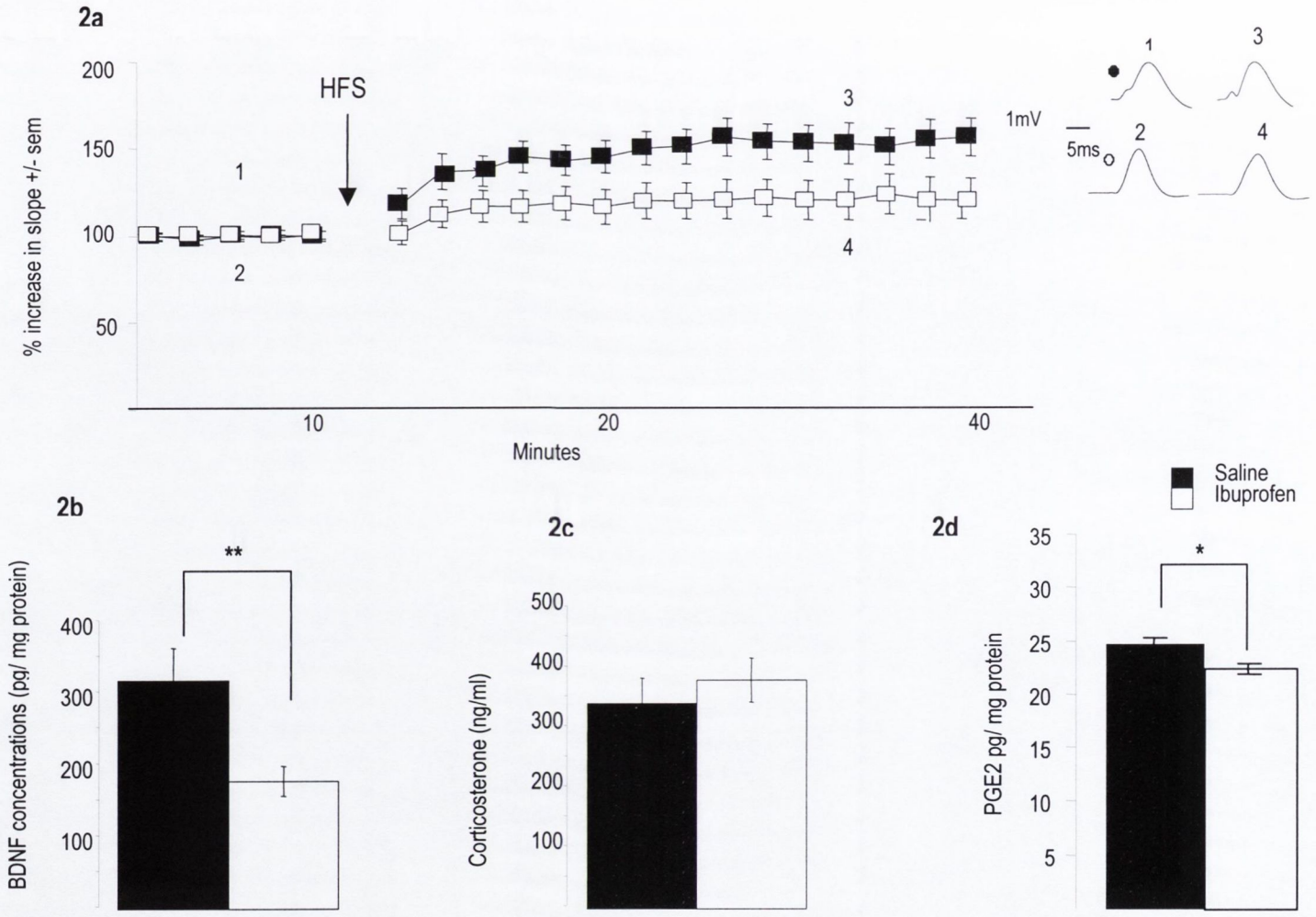



**Figure 2(a):** Percentage change in slope of fEPSPs after HFS in both groups; **2(b):** Mean concentration of BDNF in dentate gyrus from tetanised tissue; **2(c):** Average corticosterone levels from animals in the above experiment; **2(d)** PGE2 concentration in dentate gyrus from tetanised tissue (* $p < 0.05$; ** $p < 0.01$).

## Experiment 2:
## Cyclooxygenase and spatial learning.
### Overall Escape Latency

Figure 3a shows the escape latencies for both the ibuprofen and saline injected groups; the swim times decreased for both groups during the watermaze task although the ibuprofen consistently spent more time finding the platform. A repeated measures ANOVA showed no interactions; there was a within group difference ($F(3,175) = 27.882$, $p < 0.001$). The saline group had significantly shorter escape latencies on days 4 and 5 compared to days 1 and 2 and also on day 5 compared with day 3 ($p < 0.005$). The ibuprofen group also had significantly within group differences; on days 3, 4 and 5 they found the platform faster when compared with day 1 and they were also faster on days 4 and 5 compared to day 2 ($p < 0.005$). Significant between group differences were also found ($F(1,67) = 23.28$, $p < 0.001$); *post-hoc* tests show that differences were found on the last 4 days of the task ($p < 0.01$).

### BDNF Concentration

BDNF concentration was determined in the dentate gyrus from animals in each group the ibuprofen group had significantly lower levels of BDNF compared to the saline group (Figure 3b: ANOVA: $F(1,10) = 11.420$, $p < 0.001$).

### Corticosterone Levels

Figure 3c shows that no significant differences in circulating corticosterone levels were found between the groups (ANOVA: $F(1,8) = .37$, $p > 0.05$).

### PGE2 Concentration

Figure 3d shows the PGE2 levels from the dentate gyrus in the spatially trained animals; a significant difference was found (ANOVA: $F(1,140 = 6.327$, $p < 0.01$).

### Direct Swim Path Analysis

A direct swim to the hidden platform was defined as a swim path that remained inside a corridor 30 cm wide from the start position to the platform, the most direct route to the platform. An interaction between group and day was found ($F(4, 232) = 2.73$, $p < 0.05$).The saline rats increasingly used the direct route across days compared to the ibuprofen treated group ($F(1, 58) = 4.513$, $p < 0.05$).

*Post-hoc* tests showed that there was a significant difference between groups on day 4 ($p < 0.05$). An overall main effect of within group was found ($F(4, 232) = 22.712$, $p < 0.001$) but *post-hoc* tests did not show any daily differences. Figure 3f shows representative swim paths taken from each group on days 1 and 5.

### Probe Analysis: Time in Platform Quadrant

An ANOVA found that there were no differences between groups during the probe test (ANOVA: $F(2, 10) = 2.9$, $p > 0.05$).

### Non-hippocampal Learning

A repeated measures ANOVA showed that there was no interaction between group and day and no between group differences were found ($p > 0.05$). An overall main effect was found within groups ($F(2, 57) = 36.16$, $p < 0.001$) but *post-hoc* tests failed to show significant differences across days. Data not shown.

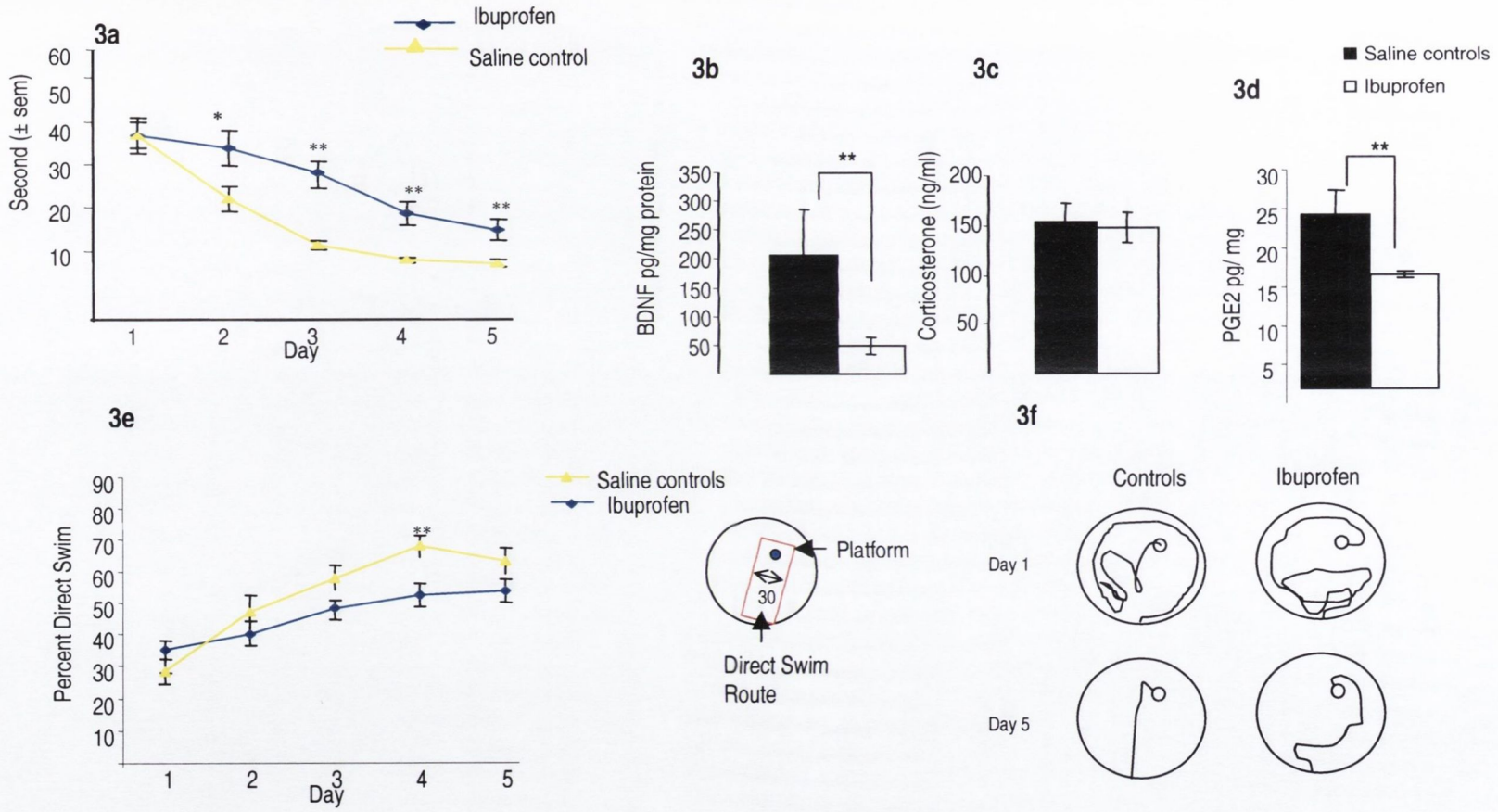



**Figure 3(a):** shows the mean escape latencies for each group across days; **3(b):** BDNF concentration in the dentate gyrus from spatially trained animals; **3(c):** Corticosterone levels from animals in the above experiment; **3(d):** PGE2 concentration in the dentate gyrus from spatially trained animals; **3(e):** Percentage of time each group spent in the direct route to the platform (see inset); **3(f):** representative swim paths for each group on days 1 and 5 of the water maze task ($p < 0.05$ *, $p < 0.001$ **).

## Experiment 3

## Exercise, cyclooxygenase, and synaptic plasticity.

*Effect of exercise, saline and ibuprofen on synaptic plasticity.*

In saline-treated animals LTP was successfully induced and fEPSPs remained potentiated for at least 30 minutes as indicated by the change in fEPSP slope which stood at 142.86 ±21.3%, 168.3 ±16.1%, 161.03 ± 23.1% of baseline 5, 15 and 30 post-HFS respectively (see Figure 3a). Using a paired sample t-test, the responses in the final 10 minutes of the recording period in the saline-treated animals were found to have increased significantly when compared to baseline measurements ($t = -30.132$; $df=9$; $p<0.001$). In ibuprofen-treated animals there was a gradual increase in response; 114.8± 15.2% of baseline 5 minutes post-HFS, 131.3 ±8.7% at 15 minutes post-HFS and 145.2 ± 9% at 30 minutes post-HFS (see Figure 4a). This we term as a 'late-developing' potentiation. The fEPSP measurements observed in the final 10 minutes of the recording period was significantly higher than baseline measurements (using a paired samples t-test; $t=-39.98$; $df=9$; $p<0.001$). Using an independent t-test to compare the fEPSP slope in the final 10 minutes of the recording period of both saline-injected animals and ibuprofen-injected animals. Field EPSP slopes were significantly higher ($t=10.045$, $df=18$, $p<0.001$) in saline-injected animals compared to the ibuprofen-injected animals.

### BDNF Concentration

No significant differences were found in BDNF concentration ($t=0.1246$, $df=6$, $p>0.05$) between saline or ibuprofen-treated groups (see Figure 4b).

### Corticosterone Levels

No significant differences in circulating corticosterone levels were found between the groups (ANOVA: $F(1,6) = .261$, $p > 0.05$; see Figure 4c).

### PGE2 Concentration

No significant differences were found in PGE2 levels (ANOVA: $F(1,22) = 0218$, $p > 0.05$) between saline or ibuprofen-treated groups (see Figure 4d).

### Distance, LTP and BDNF

No significant correlation was found when the total cumulative distance ran by all animals was compared with BDNF concentration (Pearson $r=-0.131$, $p>0.05$). Examination of distance ran on the final day was also found not to be correlated with BDNF concentration (Pearson $r=-0.096$, $p>0.05$). No significant correlations were found when groups were broken into saline- and ibuprofen-treated animals (BDNF concentration vs distance ran on final day, saline; $r=0.164$, $p>0.05$, ibuprofen; $r=-0.361$, $p>0.05$).

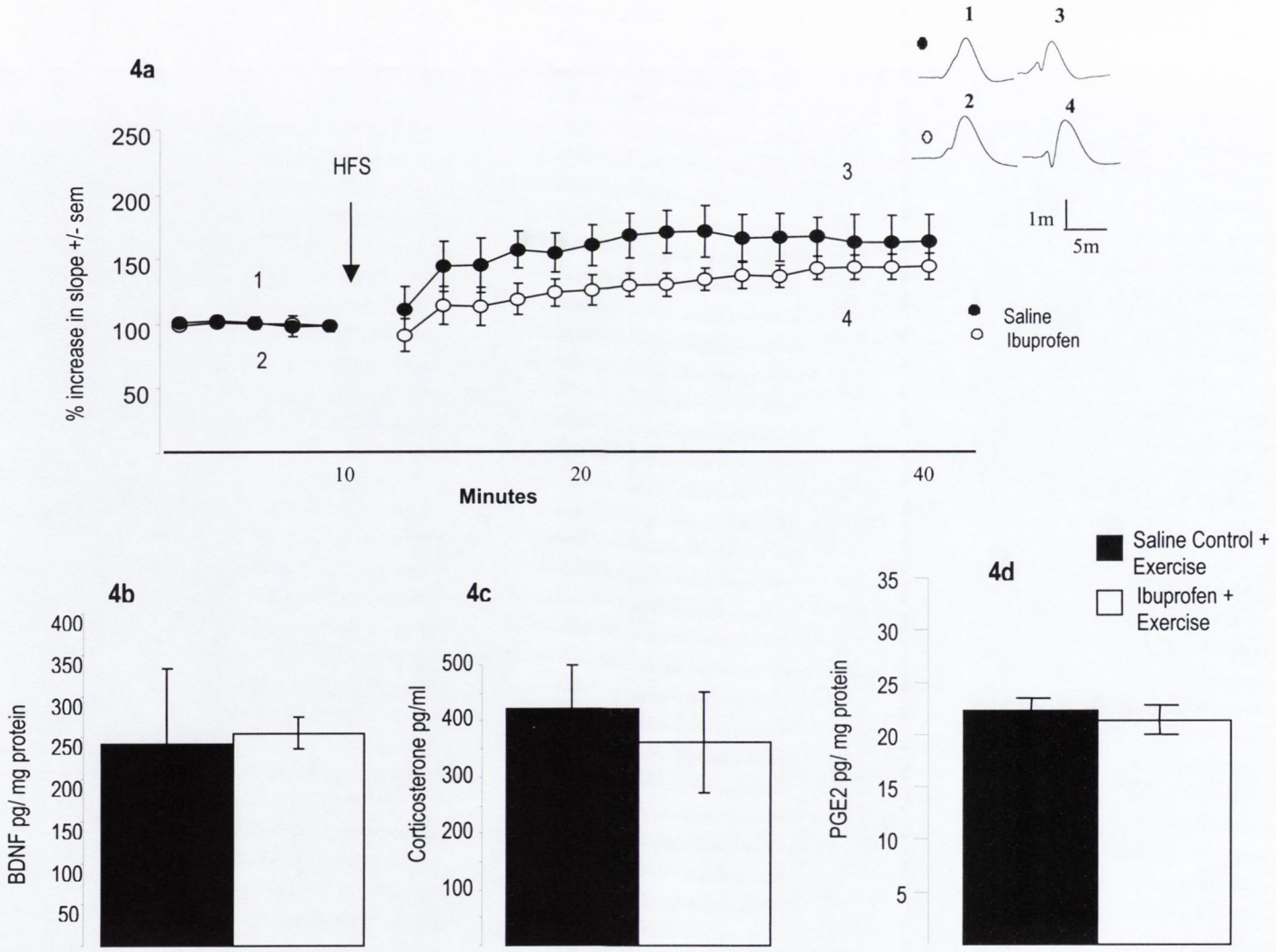



**Figure 4(a):** Percentage change in amplitude of fEPSPs after HFS in all groups; **4(b):** Mean concentration of BDNF in dentate gyrus from tetanised tissue; 4**(c):** Average corticosterone levels from animals in the above experiment; **4 (d)** Average PGE2 levels from animals in the above experiment (n= 6/group; * $p < 0.05$; ** $p < 0.01$).

## Experiment 4
## Exercise, ibuprofen and spatial learning.
### Overall Escape Latency

Figure 5a shows the escape latencies for the ibuprofen, the ibuprofen plus exercise and saline plus exercise groups; the saline only group is not shown as they were not significantly different from the saline plus exercise group. The swim times decreased for all groups during the watermaze task although the ibuprofen plus exercise group found the platform faster. A repeated measures ANOVA did not find an interaction between group and day although there was a significant difference between group ($F(1, 3) = 14.061$, $p < 0.001$). *Post-hoc* tests revealed differences between groups on all 5 days of the task ($p < 0.05$). Within group differences were also found ($F(3,386) = 31.58$). Within *post-hoc* tests found that the ibuprofen only group had shorter escape latencies on day 4 compared with day 1 and day 5 compared with days 2 and 3 ($p < 0.005$). The ibuprofen plus exercise group had significantly shorter escape latencies on days 3, 4, and 5 compared with day 1 and on days 4 and 5 compared with day 2 ($p < 0.005$). Within group differences across days were also found for the saline plus exercise groups; the rats on day 5 has significantly shorter escape latencies on day 5 when compared to days 1, 2, and 3 ($p < 0.005$). Figure 5b shows representative swim paths taken from each group on days 1 and 5.

### BDNF Concentration

BDNF concentration was determined in the dentate gyrus from animals that exercised and were spatially trained; no significant differences were found between these two groups (see Figure 5c). BDNF was also measured in the animals that exercised only and a BDNF was significantly increased in both the saline and ibuprofen treated groups who ran, compared to the saline sedentary controls (ANOVA: $F(2,15) = 8.666$, $p < 0.01$; see Figure 5e).

### PGE2 Concentration

PGE2 concentration was determined in the dentate gyrus from animals who were just exercised and who were exercised and spatially trained. No significant differences were found between the two groups (ANOVA: $F(3, 20) = .2229$, $p > 0.05$; see Figure 5d), PGE2 was also measured in the animals that exercised only (no spatial training) and no significant differences were found between the saline controls and the ibuprofen treated animals (ANOVA: $F(1,22) = .525$, $p > 0.05$, see Figure 5f).

## Corticosterone

We measured the corticosterone levels of all the rats in the various running conditions and found a significant difference between the exercised and learning groups. The exercised and learning- saline group had significantly elevated corticosterone levels compared to the exercised and learning- ibuprofen treated group (ANOVA: $F(1, 10)$ 11.33, $p < 0.01$. Data not shown). There were no differences between the groups that exercised but did not learn (ANOVA: $F(1,8) = 3.54$, $p > 0.05$; data not shown). We also measured the corticosterone levels of animals that did not exercise at all but were injected with either saline or ibuprofen; no differences were found between these groups (ANOVA: $F(1,7) = 2.82$, $p > 0.05$; data not shown).

## Probe Analysis: Time in Platform Quadrant

An ANOVA found that there were no differences between groups during the probe test (ANOVA: $F(2, 15) = 0.39$, $p > 0.05$).

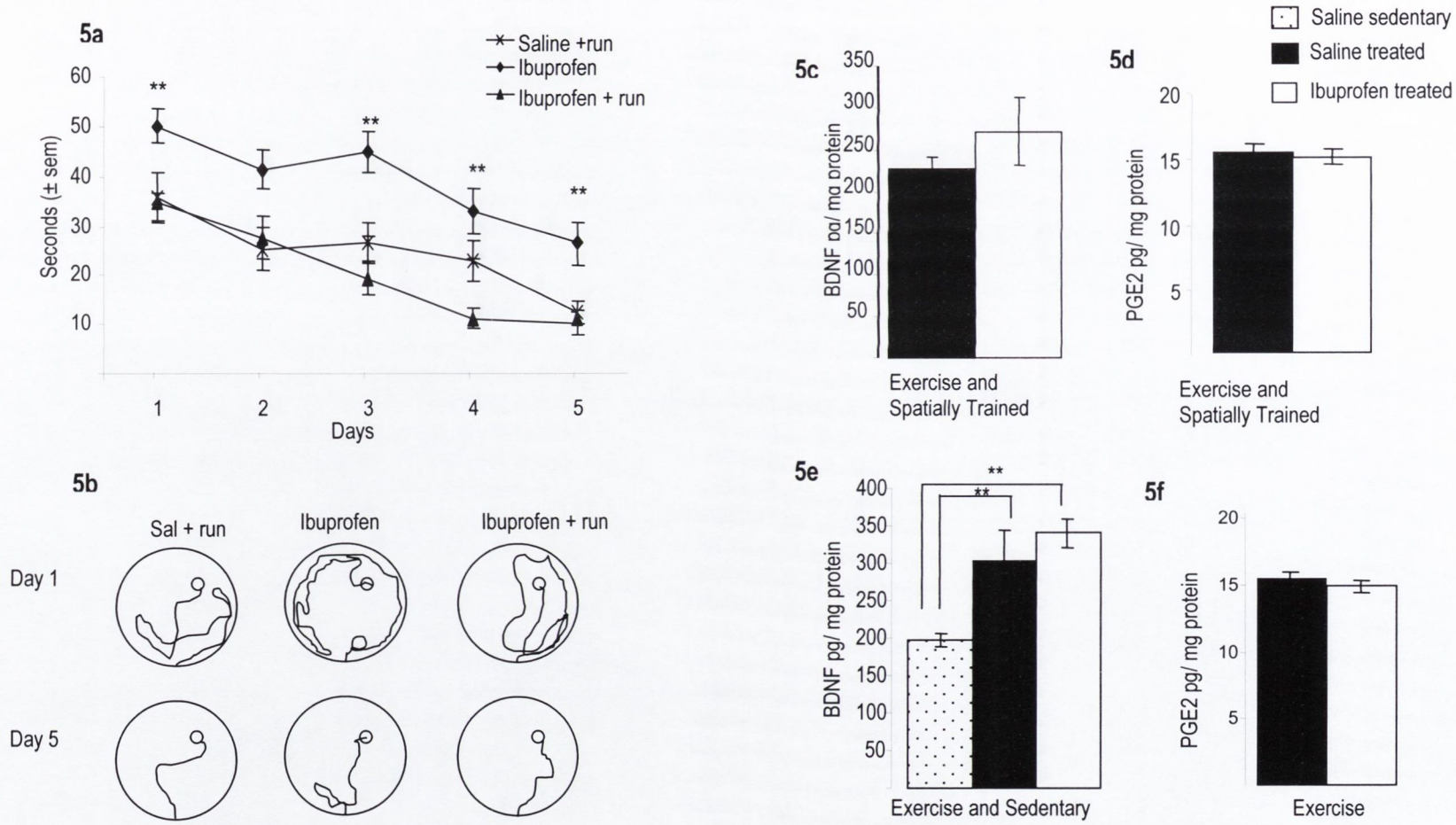



**Figure 5(a):** shows the mean escape latencies for each group across days; **5(b)** representative swim paths for each group on days 5 and 11 of the water maze task; **5(c)** BDNF concentration in the dentate gyrus from exercised and spatially trained animals; **5(d)** PGE2 concentration in the dentate gyrus from exercised and spatially trained animals; **5(e)** BDNF concentration in the dentate gyrus from exercised and sedentary animals who have not been spatially trained; **5(f)** PGE2 concentration in the dentate gyrus from exercised who have not been spatially trained ($p < 0.05$ *, $p < 0.001$ **). **Exercise groups only shown.**

## Discussion

### Experiment 1: Cyclooxygenase, synaptic transmission and synaptic plasticity.

*Effect of ibuprofen and saline on baseline synaptic transmission*

In both of the saline-treated (saline injected 1 hour prior to recording and 10 minutes post recording) and IBU-treated (ibuprofen injected 1 hour prior to recording and 10 minutes post recording) animals, fEPSPs remained stable over one hour. No significant differences in fEPSP responses between the groups over the 60 minutes. Furthermore, no significant differences in slope measurements were observed post-injection in either saline or ibuprofen-treated animals when compared to pre-injected values (data not shown). BDNF concentration was determined in the dentate gyrus from animals in each group. No significant differences were found between the saline and ibuprofen pre-treated baseline conditions. No difference in PGE2 levels were found between the pre-treated saline and ibuprofen groups suggesting that ibuprofen has no effect on basal COX levels and only blocks increases in COX arising from stimulation (e.g. synaptic plasticity).

*Effect of ibuprofen and saline on synaptic plasticity.*

In saline-treated animals LTP was successfully induced and fEPSPs remained potentiated for at least 30 minutes but in ibuprofen-treated animals there was only a slight increase in response. Field EPSP slopes were significantly higher in saline-injected animals compared to the ibuprofen-injected animals. A significant increase in BDNF concentration and PGE2 levels were found in the saline controls compared to ibuprofen-treated animals; this suggests that BDNF and PGE2 are increased by synaptic plasticity.

The above results show that pre-treatment of ibuprofen has no effect on normal neuronal transmission. More specifically when animals are pre-treated with ibuprofen 1 hour prior to recording in the absence of high-frequency stimulation, COX inhibition is without effect on either synaptic transmission, basal COX expression or BDNF expression. This is interesting, as ibuprofen does not seem to affect the constitutive and/or basal levels of COX.

When animals are pre-treated with ibuprofen and then given HFS, COX inhibition blocks the induction of LTP and blocks the LTP-induced increase in BDNF and PGE2. The influx of $ca2^{+}$ that results from HFS stimulates the learning/ LTP- related pathways. One such pathway is the AA

pathway, a pathway in which COX plays a role; ibuprofen seems to disrupt the activity of COX in this learning/ LTP- related pathway. This is supported by our data that show a decrease in PGE2 levels in the dentate gyrus of ibuprofen-treated animals compared to saline-treated controls. Another pathway is the Ras pathway, important in neurotrophic activation. This experiment suggests that BDNF acts on the COX pathway by increasing COX expression, which lends further support to a BDNF-COX interaction that we have already alluded to in the baseline recording experiment.

**Experiment 2: Cyclooxygenase and spatial learning.**

The ibuprofen-treated rats did not learn the watermaze task as quickly as the saline controls, suggesting that ibuprofen in some way impaired their ability to construct a spatial representation of the watermaze. Furthermore, the ibuprofen treated group did not use the quickest route to find the platform, as shown by the direct route analysis. In addition, the ibuprofen treated rats swam consistently slower and farther than the saline controls; this is not due to 'sickness behaviour' or motor impairment as we tested ibuprofen treated and saline control rats on a non-hippocampal dependent task in the watermaze and found no distance or velocity differences between the groups. The distance and velocity differences in the hippocampal-dependent task further suggest an inability of the ibuprofen treated rats to develop a spatial representation of their environment.

The ibuprofen-treated rats did not show the same learning-related BDNF increase as the saline controls. This may be due to several reasons, one possible explanation is that the lower BDNF levels is a result of the learning impairment, another explanation is that ibuprofen in some way blocks the expression of BDNF, or that ibuprofen inhibits the intracellular mechanisms required for learning and hence BNDF expression. This last explanation is discussed in the general discussion section of this chapter. The corticosterone levels for both groups were the same, which is an important control condition because glucocorticoid inhibits COX2 expression. We found that the PGE2 levels were significantly higher in the saline groups suggesting that learning increases PGE2; this increase might be mediated by BDNF, as the ibuprofen-treated group did not show increases in BDNF or PGE2.

## Experiment 3: Exercise, cyclooxygenase, and synaptic plasticity.

For animals to be included in the experiment, they had to run at least 100m over the last 2 nights; all animals reached criteria. LTP was successfully induced in the saline-running group and was maintained for up to 30 minutes after HFS. In the ibuprofen-running group LTP was also achieved but at a more gradual rate and by 20 minutes after HFS the fESPS measurements for this group were the same as the saline- running group. In the first experiment we found that ibuprofen blocks the induction of LTP and our results here suggest that exercise attenuates the effects of ibuprofen on LTP albeit as a late-developing potential.

In the first experiment the high frequency stimulated ibuprofen-treated group had significantly lower levels of BDNF compared to the high frequency stimulated saline-treated group. We have shown before that learning (see chapter 2) and LTP results in BDNF upregulation (Gooney *et al.*, in press) and so we expected a learning and/or LTP associated BDNF increase. BDNF levels in the ibuprofen-running group were not significantly different from the saline-running group; this was the same for PGE2. It is possible that BDNF has a direct influence on the regulation of COX expression in hippocampal neurons in that an endogenous increase in BDNF results in an increase in COX and we have shown that this endogenous increase can be either via LTP or exercise. There was no difference in corticosterone levels between the two groups; this was an expected result.

Talk *et al.* (1997) investigated the effect of indomethacin on long-term enhancement of photoreceptor excitability in the mollusc, *Hermissenda crassicornicus*. Associative pairings in the *in vitro* preparation consisted of 5 pairings of a light-statocyst stimulation. The statocyst is a balancing organ found in many invertebrates, similar to the semicircular canals in the ears of vertebrates. Indomethacin blocked the increases in excitability in photoreceptor cells that have been conditioned. This suggests that COX is important in facilitating the excitability in photoreceptor cells in *Hermissenda* possibly by the conversion of AA to PGs.

## Experiment 4: Exercise, ibuprofen and spatial learning.

Overall escape latencies show that both the saline-running and ibuprofen-running groups quickly learned the location of the hidden platform whereas, as found in experiment 2, the ibuprofen group was much slower. These results suggest that exercise attenuates the learning deficits caused by ibuprofen and we propose that this is due to an increase in BDNF. It has been well documented

that running increases BDNF in rats (Neeper *et al.,* 1995; Neeper *et al.,* 1996; Oliff *et al.,* 1998; Russo-Neustadt *et al.,* 1999) and we found a significant increase in BDNF in the exercised groups. We also found no differences in PGE2 levels. It appears that the endogenous increase of BDNF through exercise somehow increases the COX expression that ibuprofen suppresses; BDNF may be solely acting in a neuroprotective role by attending to the compromised neurons and facilitating normal neuronal functioning required for learning or, based on our findings that there was no difference in PGE2 levels, it is possible that BDNF may be directly increasing COX expression via MAP or ERK. Molina-Holgado *et al.* (2000) stimulated murine astroglial cells with IL-1b to establish if the IL-1b-induced PGE2 production is dependent on the activation of protein kinases; others have found that IL-1b increases COX2 transcription and mRNA and so increasing PGE2 production (Ristimaki *et al.,* 1994). IL-1beta treated astroglial cells led to activation of ERK kinase and p38 MAP kinases and the inhibition of ERK kinase with PD 98059, as well as the inhibition of p38 MAPK with SB 203580, prevented IL-1beta-induced PGE2 release. These results suggest key roles for ERK1/2 and p38 MAP kinase cascades in the biosynthesis of PGE2, likely by regulating the induction of COX2, in IL-1beta-stimulated astroglial cells (Molina-Holgado *et al.*, 2000). These results suggest a key role for ERK and MAP kinase cascades in the biosynthesis of PGE2 by regulating the induction of COX2 in IL-1b stimulated astroglial cells. An easy way to investigate if BDNF activates or regulates COX expression is with an *in vitro* experiment that examines the levels of COX and PGE2 in hippocampal tissue that has been treated with BDNF. If BDNF activates COX2 then, the BDNF-treated tissue should yield higher levels of COX2 and PGE2 compared to control tissue. To verify the action of BDNF on COX in hippocampal tissue the addition of K252a, a TrkB blocker, should result in a decrease of COX2 and PGE2 in the BDNF-treated hippocampal tissue. Furthermore, the addition of PD 98059 to the BDNF-treated tissue would elucidate if ERK has a mediating role in the BDNF-COX relationship. The possible pathways are discussed in the following chapter.

## General Discussion

### Suggested COX2 Activity in Neurons

The expression of COX2 in neurons is very restricted expression under basal conditions (Crofford *et al.,* 2000). COX2 expression is highly inducible, increasing 10-80 fold upon stimulation, and is actively turned over (Golden and Abramson, 1999). Basal COX2 levels are altered by either stimulation or inhibition. Stimulation such as the increased excitability that occurs

via glutamate convulsions or via cytokine activation increases COX2 expression in such a way as to trigger cellular damage/ death (Ho, 2001; Kadoyama *et al.*, 2001). When COX2 is overstimulated without inhibitory regulation (via COX inhibitors) cellular death can result in the hippocampus (Kim *et al.,* 2001). On the other hand inhibition of COX2, via NSAIDs, blocks COX2 expression. At the site of injury/ trauma it is necessary to treat the inflammation with an NSAID in order to prevent the production of PGs. When COX2 is stimulated, for example by cytokines and then inhibited by ibuprofen, it is possible that with this combination, COX2 levels return close to basal level as the increase in COX2 has been regulated by the NSAID. Thus, in stimulated tissue, COX2 expression is regulated and can returned to near basal levels by a COX inhibitor. Uninhibited, highly stimulated tissue results in neural damage (see Figure 5).

The unstimulated, basal COX2 expression may also be actively turned over but on a more constant and regular basis than stimulated or induced COX2 expression. The purpose of this unstimulated, basal COX2 expression may be to assist other processes, for example, synaptic plasticity, as COX2 interacts with AA and AA is implicated in many processes, such as LTP. It is plausible that basal COX2 is required, in some way, to transform AA during synaptic plasticity. If unstimulated tissue is treated with a COX inhibitor, the basal COX2 required for normal cellular functions (for example, synaptic plasticity) may be prevented from occurring, resulting in cognitive impairments. Furthermore, Ca2+ influx may result in moderate COX2 stimulation; this expression may aid in AA assisted synaptic plasticity. Our results suggest that by adding ibuprofen to healthy tissue we have inhibited the low constitutive COX2 levels in neurons which has resulted in the blockade of synaptic plasticity, learning, and BDNF production.

# CHAPTER 7

# Concluding remarks

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We investigated the possibility that BNDF might play a role in the modulation of synaptic function in the hippocampal formation, namely hippocampal-dependent spatial learning and hippocampal LTP. Our experiments have focused on the regulation of endogenous BDNF systems in learning and on parallels between the intracellular signal transduction pathways in learning and LTP. Stress, exercise and cyclooxygenase inhibition were used as a means of manipulating endogenous BDNF. We found correlations between decreased BDNF concentration, spatial learning deficits, a block of LTP induction and behavioural stress, endotoxic stress and cyclooxygenase inhibition. This approach was considered important for establishing roles for BDNF signaling and BDNF levels in learning and memory.

## Spatial Learning and BDNF Concentration in DG

Chapter two shows that training in the watermaze increased total BDNF concentration in DG. Several reports are consistent with these findings: for example, radial arm maze training increases BDNF mRNA in hippocampus (Mizuno et al., 2000); BDNF infusion reverses ischemia-induced impairments in cognitive function (Kiprianova et al., 1999; Almli et al., 2000); an age-related decrease in BDNF mRNA was associated with impaired memory performance (Croll et al., 1998). These data suggest a vital role for BDNF in spatial learning, although it has been reported that performance of heterozygous BDNF knockout mice in the watermaze was comparable to controls (Montkowski and Holsboer, 1997). We found that the changes in BDNF concentration induced by training, were mirrored in tissue prepared from dentate gyrus of rats that had sustained LTP (see chapters 5 & 6) and these data are consistent with previous reports indicating that BDNF mRNA is increased following induction of LTP (Castren et al., 1993; Dragunow et al., 1993; Bramham et al., 1996; Patterson et al., 1992). This experiment shows that BDNF is activity-dependent and is increased by spatial learning, but we do not know how BDNF regulates or influences learning, although it most probably facilitates intracellular mechanisms required for learning or LTP and/or for the retrieval of memory.

## Behavioural Stress: Effects on Spatial Learning and BDNF mRNA and Protein Concentration in the DG.

We investigated the effects of differing degrees of behavioural stress on watermaze performance and BDNF expression in the dentate gyrus. We found that moderate inescapable

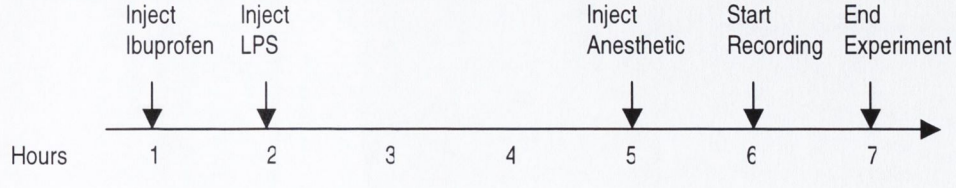
stress impaired spatial learning in the watermaze, whereas mild stress had no effect on spatial learning, compared with controls. We found a learning-dependent BDNF increase in the control group and no increase was evident in the moderately stressed group. We used *in situ* hybridisation to assess BDNF mRNA levels in the DG of spatially trained control rats, untrained yoked-motor control rats and spatially trained moderately stressed rats. We found an elevation in levels of dentate gyrus BDNF mRNA in trained animals compared to both the untrained, yoked-motor controls and spatially-trained stressed rats. In the spatially trained control group, this elevation of BDNF mRNA occurred in a time and training-dependent fashion, peaking on the first day of training and diminishing thereafter. In our results we appear to have a contradiction in that our BDNF mRNA levels do not correspond with our BDNF protein levels on the last day of the experiment. We found that spatial learning increased BDNF mRNA in the DG on the first day of the experiment and subsequently decreased but when BDNF protein concentration levels were assessed on the final day of the experiment, protein levels were high in the DG. Conversely, the stressed spatial learning group showed an increase of BDNF mRNA on the last day of the experiment and a decrease in BDNF protein concentration. This reduction in BDNF mRNA in the spatially trained control rats on the last day of the experiment may indicate that BDNF mRNA is upregulated during memory encoding and not during consolidation or retrieval. It is possible that BDNF mRNA is quickly translated and degraded but the protein remains stable and endures for a much longer time. If this is do, what is the purpose or function of this enduring BDNF protein? It may have no function and it may simply be that it takes longer for the protein to be degraded. Alternatively the protein concentration may remain high in the tissue to aid in the synaptic plasticity and morphological changes associated with learning and memory. We suggest that (i) BDNF mRNA is increased initially to aid in the encoding of memory and the lingering protein levels may act to facilitate synaptic mobilisation and docking of proteins important in plasticity; (ii) there is a temporal lag between mRNA expression and subsequent protein expression. Pozzo-Miller *et al.* (1999) suggest that the impairment of LTP in BDNF mutant slices might be due to a reduction in synaptobrevin and synaptophysin, synaptic vesicle proteins implicated in vesicle docking or fusion in the presynaptic membrane. Their study suggests that BDNF regulates synaptic transmission by facilitating synaptic mobilisation and docking of proteins important in plasticity.

## Lipopolysaccharide Impairs Spatial Learning: A Dose-Dependent Effect.

We investigated the effects of a single injection and a daily injection of LPS on spatial learning and BDNF expression in the rat dentate gyrus. Rats were injected with different doses of LPS i.p. (100 or 250 µg/kg) and trained in the water maze. The behavioural results showed a single injection of LPS at both doses impaired escape latency in both the acquisition and retention phases of the study, whereas a daily injection of LPS did not significantly impair acquisition or retention; the effects were dose-dependent. BDNF expression was analysed in the dentate gyrus of all animals. No significant differences in BDNF expression were found. We also found that LPS does not disrupt memory once it has been formed, suggesting that LPS somehow compromises either the encoding or consolidation of memory. Our findings implicate an important regulatory impact of the neuroimmune system on cognitive processing and synaptic plasticity in the hippocampal formation.

## Cyclooxygenase Inhibition Attenuates LPS-Impaired Spatial Learning but not LPS-Impaired LTP.

LPS stimulates COX production in the brain and we suggest that the broad spectrum COX inhibitor, ibuprofen, blocks the induction of COX, minimizing the neuroimmune and neurocognitive injury. We have provided the first evidence that suggests that LPS inhibits hippocampal-dependent spatial learning in a dose-dependent manner and that the administration of ibuprofen attenuates this cognitive deficit. We expected to the find that ibuprofen would also attenuate the LPS-stimulated block of LTP; we, unfortunately did not find this. This may be due to a difference in timing in that an electrophysiological experiment lasts for, at the most, about 7 hours: Ibuprofen is injected 1 hour prior to LPS administration and we wait 4 hours for the LPS to take effect (see below for a timeline).



The watermaze task lasts for 11 days, and therefore, for ibuprofen to have a beneficial effect on synaptic plasticity in LPS-compromised tissue, as seen in the behavioural experiments, the interval of more than 7 hours may be required. The first action of ibuprofen may be to target the inflammation/cellular damage by returning the high levels of COX to normal which then results in the normal interactions between COX and BDNF; this may also account for the low BDNF levels found in the tetanised tissue.

### Broad-Spectrum Cyclooxygenase Inhibition Reversibly Blocks Long-Term Potentiation and Spatial Learning Via a Brain-Derived Neurotrophic Factor-Dependent Mechanism

We have examined the effects of ibuprofen on the induction of LTP in the dentate gyrus in vivo, as well as on spatial learning (all comparisons vs. saline-treated controls). Pre-treatment with ibuprofen completely blocks the induction of LTP and causes severe deficits in spatial learning. Ibuprofen was without effect on corticosterone levels but blocks the increase in brain-derived neurotrophic factor (BDNF) that occurs after spatial learning and LTP. A period of prior exercise reverses the ibuprofen-induced block of LTP induction and of spatial learning; exercise increases endogenous BDNF levels. We therefore have provided the first evidence that COX regulates BDNF expression, LTP and spatial learning and demonstrated that this block is reversed by an increase in endogenous BDNF. We have proposed a possible model explaining the role that COX plays in hippocampal neurons (see below).

### Suggested COX2 Activity in Hippocampal Neurons

The expression of COX2 in neurons is very restricted expression under basal conditions (Crofford *et al.*, 2000). COX2 expression is highly inducible, increasing 10-80 fold upon stimulation, and is actively turned over (Golden and Abramson, 1999). Basal COX2 levels are altered by either stimulation or inhibition. Stimulation such as the increased excitability that occurs via glutamate convulsions or via cytokine activation increases COX2 expression in such a way as to trigger cellular damage/death (Ho, 2001; Kadoyama *et al.*, 2001). When COX2 is overstimulated without inhibitory regulation (via COX inhibitors) cellular death can result in the hippocampus (Kim et al., 2001). On the other hand inhibition of COX2, via NSAIDs, blocks COX2 expression. At the site of injury/trauma it is necessary to treat the inflammation with an NSAID in order to prevent the production of PGs. When COX2 is stimulated, for example by cytokines and then inhibited by

ibuprofen, it is possible that with this combination, COX2 levels return close to basal level as the increase in COX2 has been regulated by the NSAID. Thus, in stimulated tissue, COX2 expression is regulated and can returned to near basal levels by a COX inhibitor. Uninhibited, COX over production results in neural damage (Ho, 2001; Manev *et al.*, 2000).

The unstimulated, basal COX2 expression may also be actively turned over but on a more constant and regular basis than stimulated or induced COX2 expression. The purpose of this unstimulated, basal COX2 expression may be to assist other processes, for example, synaptic plasticity, as COX2 interacts with AA and AA is implicated in many processes, such as LTP. It is plausible that basal COX2 is required, in some way, to transform AA during synaptic plasticity. If unstimulated tissue is treated with a COX inhibitor, the basal COX2 required for normal cellular functions (for example, synaptic plasticity) might be prevented from occurring, resulting in cognitive impairments. Furthermore, Ca2+ influx may result in moderate COX2 stimulation; this expression may aid in AA- assisted synaptic plasticity.

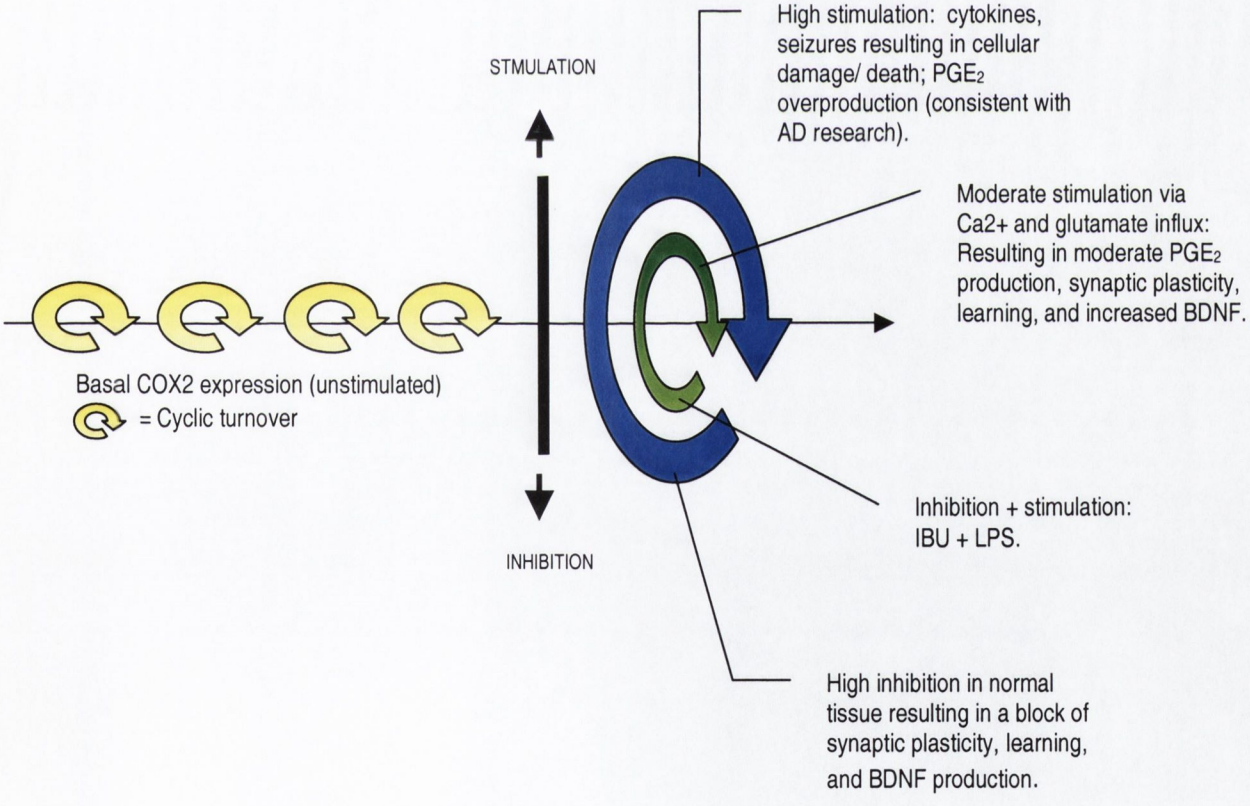



Figure 1: A hypothetical model for the regulation of COX2 during varying degrees of stimulation and inhibition.

The current data suggest that spatial learning and maintenance of LTP rely on modulation of similar cellular mechanisms and that both are associated with increased release of BDNF. BDNF stimulates phosphorylation of *TrkB* and ERK and in turn enhances glutamate release, which we propose permits strengthening of synaptic connections upon which both forms of plasticity rely. COX is also stimulated by glutamate release via NDMA activation. It is possible that COX interacts and/or regulates BDNF. There are several points of possible interactions between pathways; we have combined information from previous experiments with our own (Schulman & Hyman, 1999; Bazon & Allan, 1996; Lerea & McNamara, 1993; Kaufmann et al., 1996; Salvemini et al., 1993; Pillinger et al., 1998; Yamagata et al., 1993) and have suggested a possible pathway for this interaction. The model below is described according to pathway and is colour coded.

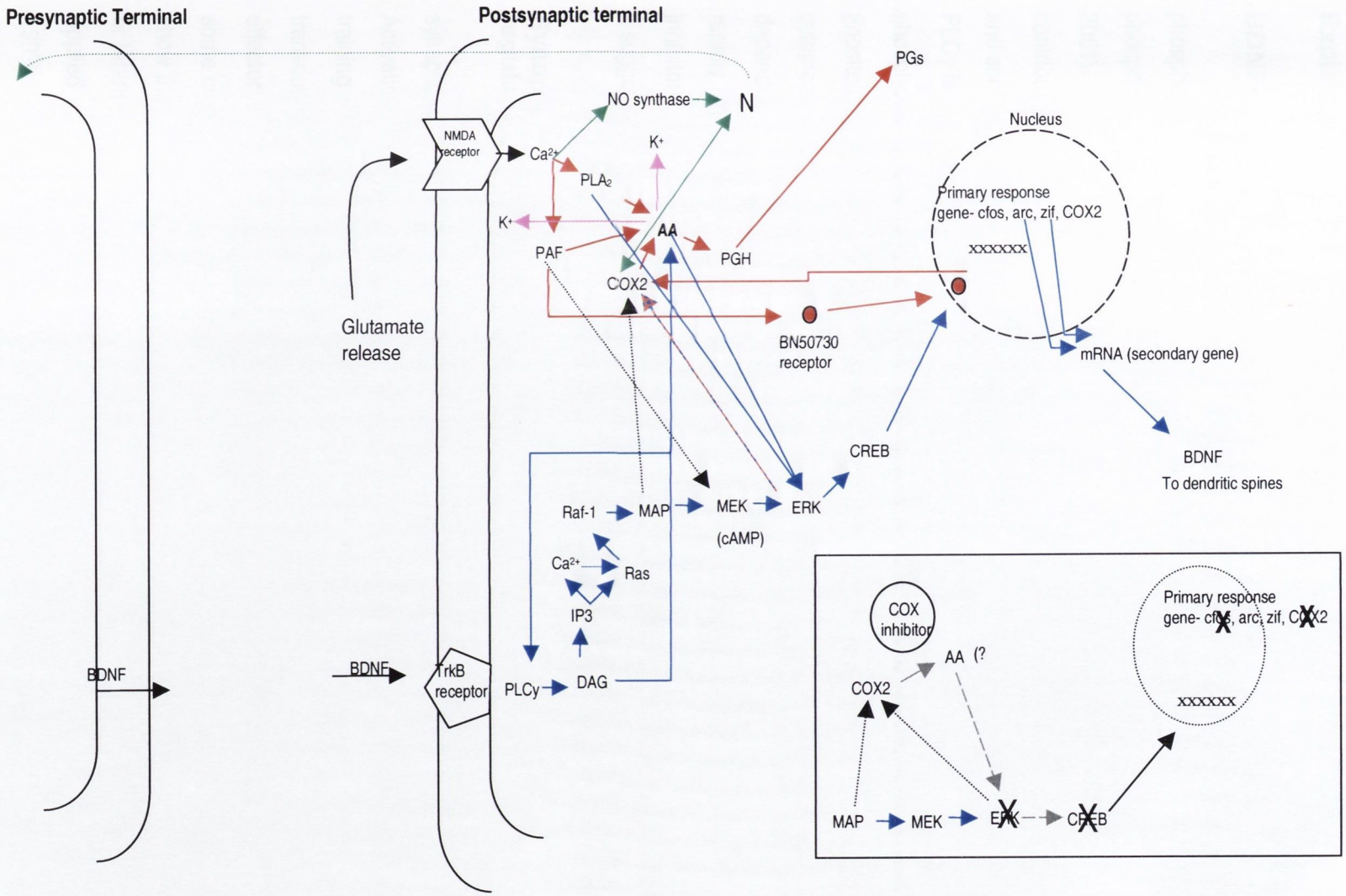



Figure 2: Proposed pathways and interaction between pathways during learning and synaptic plasticity.

## Explanations of the Pathways

### BDNF-Ras Pathway (BLUE LINES)

AA, a precursor of PGs, is cleaved from the cell membrane phospholipids by the action of phospholipase $A_2$ ($PLA_2$) and phospholipase C (PLC) (Wagner *et al.,* 1996). These phospholipases are activated by hormones, autocoids, $Ca^{2+}$ and trauma (Vanegas & Schaible, 2001). $PLA_2$ is a lipid messenger that is activated by G proteins; $PLA_2$ releases AA, which, in turn, contributes to the production of PGs and leukotrienes. AA can also modulate $K^+$ channels, PLCγ, and some forms of PKC. $PLA_2$ also activates MAP kinases in response to growth signaling factors. PLCγ is a soluble enzyme regulated by the growth factor, tyrosine kinase (Trk). The Trk receptor phosphorylates and activates PLCγ, which then activates second messengers DAG and $IP_3$ (inositoltriphosphate). $IP_3$ induces the release of $CA^{2+}$ intracellular stores which increases the cytoplasmic levels of $CA^{2+}$ thus, activating excitatory pathways controlled by $CA^{2+}$ i.e., the Ras-dependent pathway: PKC, Ras, Raf-1, MAP, MEK, ERK (Schulman & Hyman, 1999). C-fos is rapidly induced by hippocampal NMDA activation and this NMDA response is blocked by a COX inhibitor (Lerea & McNamara, 1993). Since the c-fos response must involve events in the nucleus, it suggests a COX-dependent signal linking receptor activation and gene expression.

### Synaptic plasticity, learning and memory and ERKs (extracellular-signal-regulated kinase).

It is proposed that BDNF is released pre-synaptically and acts on its receptor *TrkB* to effect synaptic changes in the post-synaptic cell (Glass & Yancopoulos, 1993; Segal & Greenberg, 1996). Activation of *TrkB* may be the first step in a cascade of reactions triggered by BDNF following training in a spatial task and following induction of LTP. The identity of the effector molecules transducing the signal following phosphorylation of *TrkB* remains to be established; one possible effector is ERK which has been shown to be activated downstream of *TrkB* phosphorylation in some cells, including hippocampal cultured neurons (Marsh et al., 1993). Gooney *et al.* (in press) show that ERK phosphorylation is increased following activation of *TrkB* induced by spatial learning or tetanic stimulation. Several LTP studies have shown that when PD98059, an ERK inhibitor, is applied to tissue, LTP is blocked in the hippocampus (English & Sweatt, 1997; McGahon et al., 1999). Behavioural data provide evidence for a role of ERK in learning (Selcher et al., 1999; Blum et al., 1999). Blum et al. (1999) found hippocampal ERK activation in rats trained on the

watermaze task, but when rats were given a MEK blocker, an upstream mechanism of ERK, they were unable to form long-term memories of the task. Similarly, Atkins et al. (1998) found that MEK inhibition resulted in fear-conditioning learning impairments and the inability to induce LTP in rats. Gooney *et al.*'s found that ERK activation is stimulated by BDNF in circumstances where glutamate receptor activation occurs; activation of ERK can be also achieved through the receptor tyrosine kinase-induced activation of the *ras/raf* cascade (Graves and Krebs, 1999). Gooney *et al.* (in press) findings are consistent with the view that the increase in ERK activation that accompanies spatial learning and LTP may be activated because of BDNF-stimulated *TrkB* phosphorylation.

**NMDA-COX Pathway (RED LINES)**

The enzymes involved in the biosynthesis of PGs include the phospholipases that release AA from phospholipid membranes, in particular, phospholipase $A_2$ ($PLA_2$). NMDA receptor stimulation by an influx of $CA^{2+}$ activates phospholipase $A_2$ ($PLA_2$), and AA. Receptor-mediated activation of $PLA_2$ also triggers PAF synthesis (Farooqui *et al.*, 1999); PAF causes a rapid and pronounced increase in $CA^{2+}$ levels in the hippocampus, through the release of $CA^{2+}$ from intracellular stores. In neurons, PAF induces COX2, an immediate early gene (IEG) (Bazon & Allan, 1996). $PLA^2$ stimulates PAF synthesis and binds to its receptor BN50730 which, in turn, triggers IEG expression (COX enzyme). PAF-induced transcription factors then bind to the promoters of secondary response genes inducing their expression. Some of these secondary genes may then encode receptors and other signal transduction proteins that eventually promote the actions of other signaling molecules and induce further waves of gene expression, which may lead to long-term changes such as plasticity (Bazan & Allan, 1996).

The two pathways implicated in inflammatory actions, the NO and the AA pathways, are also implicated in learning and synaptic plasticity (Kaufmann et al., 1996; Salvemini et al., 1993). AA is generated in response to hippocampal NMDA receptor activation needed for LTP: Kauffman et al. (1996) reported that the product of COX2, prostaglandins, diffuses to distal sites of the same neuron or to contiguous cells where it serves as a substrate for the production of other PGs. Recent studies have shown that dendritic spines play an active role in shaping neuronal activity and gene expression (Engert & Bonhoeffer, 1999; Spruston et al., 1995; Ghosh & Greenberg, 1995). In view of the many regulatory signals involved in COX2 activity and its localization in spines, Kauffman et al., (1996) suggest that COX2 may generate a diffusible signal as a function of the activity at specific synapses. C-fos is rapidly induced by hippocampal NMDA activation and

this NMDA response is blocked by a COX inhibitor (Lerea & McNamara, 1993). Since c-fos response must involve events in the nucleus, it suggests a COX-dependent signal linking receptor activation and gene expression. Others have found that COX2 is an immediate early gene that directly modifies cellular function (Yamagata et al., 1993; Yamagata et al., 1994).

### NMDA-Nitric Oxide Pathway (GREEN LINES)

Salvemini et al. (1993) found that COX2 is modulated by nitric oxide (NO), a gaseous molecule that plays a role in synaptic plasticity and cellular death. NO is thought to be a possible retrograde messenger important in LTP.

### Action of COX Inhibitors on these Pathways (INSERT)

The possible interaction between the BDNF-Ras pathway and the NDMA-COX pathway has not been studied before and the action of NSAIDs on the Ras/Raf pathway has not been studied in hippocampal neurons; most of the literature is related to other cell types, such as neutrophils (Capodici *et al.*, 1998; Pillinger et al., 1998). Capodici *et al.* (1998) found that AA stimulates ERK activity via a G-protein Raf-1 and Mek dependent pathway while aspirin, a non-selective COX inhibitor, blocks ERK activation (Pillinger *et al.*, 1998); there may be an interaction between the NDMA-Cox pathway and the BDNF-Ras pathway via ERK. The blockade of ERK by COX inhibitors might produce a downregulation of BDNF and thereby result in an impairment in the synaptic changes required for learning and LTP. A way of investigating if the pathways are related is to induce COX increases by, for example IL-1β or ischemia and to see if BDNF is also upregulated and by what mechanisms. It is well know that ischemia results in the activation of the COX pathway (Miettinen *et al.*, 1997; Dash *et al.*, 2000) and that ischemic damage is attenuated by COX2 inhibition and in COX2 mutant mice (Iadecola *et al.*, 2000). Furthermore, Han & Holtzman (2000) showed that BDNF protects the neonatal brain from hypoxic-ischemic injury *in vivo* via the ERK pathway; inhibition of ERK with U0216 abolished the neuroprotective effects of BDNF. We have shown that increases in endogenous BDNF via exercise abolishes the kainic acid-induced spatial learning impairments (Gobbo & O'Mara, unpublished). It appears then, that COX and BDNF may be regulating each other by the pathway(s) proposed above. An experiment is needed to test this hypothesis; one such study to investigate if BDNF acts on COX is to exogenously apply BDNF to BDNF depleted tissue and then measure COX, if BDNF regulates COX via ERK both COX and ERK should be increased in a dose-dependent manner.

In conclusion we have clearly shown that BDNF is increased during learning, LTP and by exercise. We challenged the endogenous production of BDNF with behavioural and endotoxic stressors and found dose-dependent decreases in BDNF and memory impairments and we clarified the role of endogenous stress and BDNF production in the dentate gyrus. We found that ibuprofen attenuates the learning deficits caused by LPS and suggested a possible interaction between BDNF and COX. Further investigation of this interaction had led us to suggest that BDNF regulates COX via ERK; we are the first to suggest such an interaction and to formulate a of model this interaction. We have shown also for the first time that PGE2 plays an regulatory role in LTP and learning.

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# APPENDICES

## APPENDIX A: Composition of Buffers and Solutions

**Carbonate Coating Buffer**
0.025M sodium bicarbonate
0.025M sodium carbonate
Adjust pH to 9.7 using 1N HCl or 1N NaOH.

**DPBS (per liter)**
0.2g KCl
8.0g NaCl
0.2g $KH_2PO_4$
1.15g $Na_2HPO_4$
133mg $CaCl_2\ 2H_2O$
100mg $MgCl_2\ 6H_2O$
Add room temperature deionized water to the KCl, NaCl, $KH_2PO_4$ and $Na_2HPO_4$ to a one liter final volume. Adjust pH to 7.35 using 1N HCl or 1N NaOH. Add the $MgCl_2\ 6H_2O$; mix thoroughly; then add the $CaCl_2\ 2H_2O$ and mix thoroughly.

**1N hydrochloric acid**
Add 82.7ml of concentrated hydrochloric acid to 917.3ml deionized water.

**TBST wash buffer**
20mM Tris-HCl (pH 7.6)
150mM NaCl
0.05% (v/v) Tween® 20

**lysis buffer**
137mM NaCl
20mM Tris-HCl (pH 8.0)
1% NP40
10% glycerol
1mM PMSF
10µg/ml aprotinin
1µg/ml leupeptin
0.5mM sodium vanadate

**KREBS-RINGER BICARBONATE BUFFER** (Sigma No. K4002)
**Components** g/L
D-Glucose 1.8
Magnesium Chloride [Anhydrous] 0.0468
Potassium Chloride 0.34
Sodium Chloride 7.0
Sodium Phosphate Dibasic [Anhydrous] 0.1
Sodium Phosphate Monobasic [Anhydrous] 0.18

**Preparation Instructions**

1. Measure out 90% of final required volume of water. Water temperature should be 15-20° C.
2. While gently stirring the water, add the powdered medium. Stir until dissolved. Do NOT heat.
3. Rinse original package with a small amount of water to remove all traces of powder. Add to solution in step 2.
4. To the solution in step 3, add 1.26 g sodium bicarbonate or 16.8 ml of sodium bicarbonate solution [7.5%w/v] for each liter of final volume of medium being prepared. Stir until dissolved.
5. While stirring, adjust the pH of the medium to 0.1-0.3 pH units below the desired pH since it may rise during filtration. The use of 1N HCl or 1N NaOH is recommended.
6. Add additional water to bring the solution to final volume.
7. Sterilize immediately by filtration using a membrane with a porosity of 0.22 microns.
8. Aseptically dispense medium into sterile container.

**BDNF ELISA Protocol**

**(BDNF Emax ™ ImmunoAssay System obtained from Promega UK LTD)**

Plate Coating
In a 15ml or 50ml polypropylene tube, add exactly 10µl of the Anti-BDNF mAb to 10ml of carbonate coating buffer to prepare enough reagent for each full 96 well plate. Mix thoroughly, but avoid creating excess bubbles. Use a multichannel pipettor to add 100µl to each well of a polystyrene ELISA plate.

2. Seal the wells with a plate sealer and incubate overnight at 4°C. Note: This assay has been optimized using the carbonate coating buffer prepared as described in Section VIII.B; other buffers may give poor results.

B. Preparing Block & Sample 1X Buffer
Each 96 well plate requires approximately 53ml of Block & Sample 1X Buffer to be used on the second day. Prepare Block & Sample 1X Buffer by mixing 42.4ml deionized water with 10.6ml of Block & Sample 5X Buffer. Use aseptic transfer techniques when using the Block & Sample 5X Buffer stock solution. Mix gently
and completely by inversion prior to use.

C. Blocking the Plate
1. Remove the coated plate from the refrigerator. Flick out the contents of the wells and slap the plate upside down three times on a paper towel to help clear the wells. Vigorously wash all wells with TBST wash buffer using an automated plate washer, wash bottle or multichannel pipettor. For manual washing, fill each well with TBST wash buffer, flick out the contents over a sink and slap the plate three times on a paper towel. Add 200µl of Block & Sample 1X Buffer to each well using a multichannel pipettor. Do not touch
or scratch the surface of the wells where antibody is bound to the plate. Note: We strongly recommend the use of an automated plate washer for consistent results.

2. Incubate at room temperature for one hour without shaking.

D. Preparing the BDNF Standard Curve
The BDNF Standard provided with this system will generate a linear standard curve from 7.8–500pg/ml. Use only values that are within the linear range to determine the BDNF concentration of the test samples. The BDNF Standard is supplied at a concentration of 1µg/ml. Accurately dilute the supplied BDNF Standard 1:2,000 in Block & Sample 1X Buffer to achieve a concentration of 500pg/ml. For example, transfer 10µl of undiluted standard into 390µl of Block & Sample 1X Buffer (1:40 dilution). Then, transfer 10µl of this
solution into 490µl of Block & Sample 1X Buffer (1:50 dilution). This stepwise protocol results in a 1:2,000 dilution of the standard.

1. Following plate blocking, flick out the contents of the wells over a sink. Slap the plate three times upside down on a paper towel to remove residual liquid and wash once with TBST wash buffer as

described in Section V.C, Step 1. Designate two columns of wells (16 wells) for the standard curve. To prepare the BDNF standard curve within the assay plate, add 100µl/well of the Block & Sample 1X Buffer to wells in rows B through H in the two columns designated for the standard curve (Figure 2). 2. Add 200µl of the diluted BDNF Standard (500pg/ml) to the first well in each column designated for the standard curve (row A).
3. Immediately perform serial 1:2 dilutions (100µl/well) down the plate in the columns designated for the standard curve. In the last set of wells for the standard curve, do not add any BDNF. The final concentrations (in duplicate) within the plate will be 0–500pg/ml

E. Addition of Sample
We recommend starting with a 1:4 dilution of each test sample and preparing 1:2 serial dilutions per column in the ELISA plate. Alternatively, you may wish to screen samples at a single concentration (perhaps in triplicate) and subsequently reassay all positive samples not on the linear portion of the curve to determine the exact BDNF concentration. Where the sample carrier solution may contribute nonspecific sources of BDNF (such as serum in culture media), we also recommend performing a series of negative control reactions containing the carrier solution alone.

1. After preparing the BDNF standard curve, add 100µl of the samples to each of the remaining wells.
Note: Add samples as quickly as possible to minimize evaporation.
2. Seal the wells with a plate sealer and incubate the plate for two hours at room temperature with shaking (400 ± 100rpm).

3. Wash the plate five times with TBST wash buffer as described in Section
V.C, Step 1.

F. Addition of Anti-Human BDNF pAb
1. In a 15ml or 50ml polypropylene tube, add 20µl of the Anti-Human BDNF pAb to 9.98ml of Block & Sample 1X Buffer (1:500 dilution) to prepare enough reagent for a full 96 well plate. Mix thoroughly, but avoid creating excess bubbles. Use a multichannel pipettor to add 100µl of the diluted Anti-Human BDNF pAb to each well, being careful not to touch or scratch the surfaces of the wells.

2. Seal the wells with a plate sealer and incubate two hours at room temperature with shaking.

3. Wash the plate five times with TBST wash buffer as described in Section V.C, Step 1.
G. Addition of Anti-IgY HRP Conjugate

1. In a 15ml or 50ml polypropylene tube, accurately add 50µl of the Anti-IgY HRP Conjugate to 9.95ml of Block & Sample 1X Buffer (1:200 dilution) to prepare enough reagent for a full 96 well plate. Mix thoroughly and avoid creating excess bubbles. Using a multichannel pipettor, add 100µl of the diluted Anti-IgY HRP Conjugate to each well, being careful not to disturb the bottom or sides of the wells.
2. Incubate for 1 hour at room temperature with shaking (400 ± 100rpm).

3. Wash the plate five times with TBST wash buffer as described in Section V.C, Step 1.
Add samples as quickly as possible to minimize evaporation.

H. Color Development

1. Add 100µl of the room temperature TMB One Solution to each well using a multichannel pipettor.
2. Incubate at room temperature with shaking for 10 minutes. A blue color will form in the wells.
3. Stop the reaction by adding 100µl of 1N hydrochloric acid to the wells in the same order in which substrate was added in Step 2. The blue color will change to yellow upon acidification. Take care to avoid the creation of bubbles.
4. Record the absorbance at 450nm on a plate reader within 30 minutes of stopping the reaction. See Figure 3 for a representative BDNF standard curve.

A standard curve must be performed on each plate.

**APPENDIX B:** Equipment Providers Names and Addresses:

**Animal Tracking System**:
EthoVision
Noldus Information Technology,
Wageningen, Netherlands

**BDNF Elisa Kit**
BDNF Emax (tm) ImmunoAssay System
Promega UK LTD

**Lipopolysacchrade**
Escherichia Coli; (L-2630 100 mg)
Sigma Chemical Co.,
St. Louis, MO, USA).

**Corticosterone radioimmunoassay Kit**
Coat-a-Count, Diagnostic Products Corporation,
CA, USA

**Ibuprofen**
Sigma Chemical Co.,
St. Louis, MO, USA

**MCID™ Elite**
**Image Analysis and Quantification**
Imaging Research Inc.,
500 Glenridge Ave.,
St. Catharines, Ontario,
Canada L2S 3A1

**Oligonucleotide synthesis**
Lab 5005
Medical and Biological Sciences Building
University of Southampton
Boldrewood,
Bassett Crescent East
Southampton
SO16 7PX

**[-35S] dATP**
PerkinElmer Life Sciences Ltd
PerkinElmer House
204 Cambridge Science Park
Cambridge CB4 0GZ, United Kingdom

PUBLICATIONS

## PUBLICATIONS

**Shaw KN**, Commins S, & O'Mara SM. (2001). Lipopolysaccharide causes deficits in spatial learning in the watermaze but not in BDNF expression in the rat dentate gyrus. *Behavioural Brain Research*, 124, 47-54.

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## CONFERENCE PRESENTATIONS

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