AN ASSOCIATIVITY REQUIREMENT FOR NORADRENERGIC DENTATE GYRUS LONG-TERM POTENTIATION IN THE URETHANE-ANESTHETIZED RAT

ANDREW THOMAS REID

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by

Andrew Thomas Reid

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Abstract

Glutamatergic activation of the locus coeruleus results in long-term potentiation of dentate gyrus perforant path-evoked population spike amplitude, when paired with perforant path stimulation (.25 Hz). *In vitro* evidence, however, suggests that this β-adrenoceptor-mediated effect can also occur in the absence of perforant path activity. The present study seeks to determine whether pairing of perforant path stimulation and locus coeruleus activation is necessary for norepinephrine-induced population spike potentiation to occur *in vivo* in urethane-anesthetized rats. Glutamatergic stimulation of the locus coeruleus in the absence of perforant path stimulation resulted in a transient spike and slope increase which returned to baseline by 10 min. In contrast, locus coeruleus activation concurrent with perforant path stimulation resulted in long-lasting potentiation of both spike and slope. These results suggest that, under the conditions of the present study, a temporal pairing of noradrenergic activity with perforant path activity in the dentate gyrus is necessary for the induction of long-term population spike and EPSP slope potentiation.

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List of Abbreviations

6-OHDA	*6-hydroxydopamine*
A/D	*analog-to-digital*
AC	*adenylate cyclase*
AMPAR	*alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor*
ANOVA	*analysis of variance*
AP	*anteroposterior*
BLA	*basolateral amygdala*
CA1	*cornu ammonis area 1 (region of hippocampus)*
CA3	*cornu ammonis area 3 (region of hippocampus)*
CaM	*calcium/calmodulin complex*
CaMKII	*calcium/calmodulin-dependent protein kinase II*
cAMP	*cyclic adenosine monophosphate*
CNS	*central nervous system*
COMT	*catechol-O-methyltransferase*
CPP	*3-((+/-)2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid*
CREB	*cAMP response element binding protein*
D(-)APV	*D(-)-2-amino-5-phosphonovaleric acid*
DA	*dopamine*
DBH	*dopamine beta-hydroxylase*
DG	*dentate gyrus*
EC	*entorhinal cortex*

EEG	*electroencephalogram*
EFP	*evoked field potential*
EPSP	*excitatory postsynaptic potential*
E-S	*EPSP-to-spike (as in E-S potentiation)*
fEPSP	*field excitatory postsynaptic potential*
GluR1	*AMPAR subunit*
HFS-LTP	*high-frequency stimulation-induced long term potentiation*
I/O	*input/output*
I-1	*phosphatase inhibitor 1*
IP3	*inositol 1,4,5-triphosphate*
LC	*locus coeruleus*
L-DOPS	*L-threo-3,4-dihydroxyphenylserine*
LEC	*lateral entorhinal cortex*
LOT	*lateral olfactory tract*
LPP	*lateral perforant pathway*
LSD	*least significant difference*
LTP	*long term potentiation*
MAO	*monoamine oxidase*
MAPK	*mitogen-associated protein kinase*
MEC	*medial entorhinal cortex*
ML	*mediolateral*
MPP	*medial perforant pathway*
NE	*norepinephrine*

NE-LTP	*norepinephrine-induced long term potentiation*
NMDAR	*N-methyl-D-aspartate receptor*
NR1, NR2C	*NMDAR subunits*
pCREB	*phosphorylated CREB*
pCaMKII	*autophosphorylated CaMKII*
PGI	*nucleus paragigantocellularis*
PKA	*cAMP-dependent protein kinase*
PNMT	*phenylethanolamine* N*-methyltransferase*
PP	*perforant pathway*
PP1, PP2	*protein phosphatases 1, 2*
PTSD	*post-traumatic stress disorder*
REM	*rapid eye movement*
Rp-CAMPS	*adenosine- 3', 5'- cyclic monophosphorothioate, Rp-isomer*
Ser831, Ser845	*GluR1 phosphorylation sites*
TH	*tyrosine hydroxylase*

1. Introduction

1.1 Overview

This thesis outlines an investigation into the neurophysiological bases of arousal-associated memory, by observation of the electrical behaviour of a discrete brain structure, the hippocampal dentate gyrus. The effects upon dentate gyrus activity of pharmacological activation of the locus coeruleus, a brainstem nucleus whose function is linked to emotion and arousal, are considered. Specifically, I address the question of whether a noradrenergic signal, originating in the locus coeruleus, must be temporally paired with electrical stimulation of the perforant path input to dentate gyrus in order to induce long-term potentiation, a phenomenon which may serve as the neurobiological basis of some forms of memory. What follows in the Introduction is a description of the theoretical origins of memory research, with a particular focus upon the scientific dialogue leading to the present paradigm. In subsequent sections of the Introduction I outline the anatomical organization of the hippocampus, focusing upon the dentate gyrus, as well as the locus coeruleus. Finally, evidence regarding the intracellular signalling cascades proposed to underlie specific long-lasting neuronal changes related to dentate gyrus plasticity is discussed.

1.2 What is memory?

This question, at a glance, is deceptively simple. Webster's Dictionary offers the following definition: "the mental capacity or faculty of retaining and reviving facts, events, impressions, etc., or of recalling or recognizing previous experiences" (Random House Webster's Unabridged Dictionary, 1999). A computer scientist, on the other hand,

might take a more formal approach: memory is the persistence of some pattern of data or, in different terms, it is the encoding and storage of a previous state of activation which, upon later recall, can be re-established in a system. This latter definition works as much as a description of human memory as it does for computer memory, the brain being essentially a rather complex computer. The important difference between these two contexts, however, is that a computer scientist can give you a precise physical explanation of how a personal computer obtains, encodes, stores, retrieves, and utilizes its data. Whereas the brain scientist can offer a good deal of experimentally obtained evidence pertaining to the brain's capacity for these functions (as indeed the present thesis will attempt), much of how memory works in this particular computer remains a mystery. Accordingly, a broad range of thinkers, including philosophers, psychologists, and neuroscientists, has for centuries bent its collective will towards the question: "what is memory?". Neuroscience, in particular, has focused upon a refined version of the question: "how does memory work?".

It is often the case with a given area of research that it can trace its origins clearly to some seminal insight or discovery. Two such origins are evident in the present study. The first, long-term potentiation of neuronal activity, will be discussed in detail below. The second, the case of patient H.M., has had an enormous impact upon memory research in general, and deserves some mention here. In 1957, Brenda Milner and William Scoville published a paper describing their observations of various human patients having received specific brain lesions (Scoville & Milner, 1957). The most famous of these cases was of a patient referred to as H.M., who had received surgery to treat severe epilepsy, effectively

removing, bilaterally, a section of temporal lobe including the hippocampal structure and amygdala. These lesions resulted in a severe loss of the ability to form new explicit memory, a condition termed *anterograde amnesia*, as well as a milder loss of memory for up to eleven years prior to the surgery, termed *retrograde* amnesia. Despite this, H.M. displayed no loss of intelligence, as measured on I.Q. tests, and also demonstrated an intact working memory for events as they were occurring.

The findings of Milner and Scoville, and those of later research involving H.M., have very important implications for the understanding of human explicit memory. Firstly, they implicate the hippocampus as a structure critical for the conversion of short-term explicit memory into long-term memory, termed *memory consolidation*. Secondly, they shed some light on the rules of memory formation. The extent of retrograde amnesia implies a time course for the consolidation process; long-term memories can take months or even years to fully consolidate, and in the meantime they require an intact hippocampus to be maintained and/or recalled. Working memory, moreover, must be mediated by mechanisms distinct from the type of memory mediated by the hippocampus. When given a three digit number and asked to remember it, H.M. was able to do so up to 15 min, if left uninterrupted; any interruption, however, could annihilate the memory (Milner, 1966). Not all memory formation is disrupted in H.M., however, demonstrating the existence of distinct, differentially mediated forms of memory. When trained on a task involving tracing an object reflected in a mirror, for instance, H.M. exhibited a normal learning curve, and retained this skill despite having no explicit knowledge of having learned it (Milner, 1966).

While the case of H.M. was certainly not the only important discovery in the pursuit of an answer to the question "how does memory work?", it has certainly had an enormous impact on the direction of this pursuit. When investigating the machinery of human memory, the hippocampus more than any other distinct brain structure has been the focus of researchers. Neuroscience in particular has turned to animal models of memory to better elucidate the role of this structure in the encoding, consolidation, and recall of certain types of memory. Often such research involves evaluating behavioural performance on certain learning and memory tasks, and the ways in which various environmental, physical, electrical, pharmacological, or genetic manipulations affect this performance. Another approach is to evaluate neural processing through various imaging techniques to better understand the regions, cell types, or neurotransmitters involved in the processing of memory. The present study utilizes a third approach, termed *electrophysiology*, which monitors the electrical behaviour of cells or cell populations, and evaluates the ways in which various manipulations affect this electrical behaviour. In particular, the ability of neurons, in the hippocampus and elsewhere, to display long-lasting *plasticity* (i.e., changes in their electrical response to stimulation) has implications for how memory machinery is organized at the level of the brain's basic processing units.

1.3 Neuronal plasticity and Hebb's Postulate

It has long been understood that neurons communicate by firing action potentials, and that action potentials are induced largely through synaptic connections between axons and dendrites. What is less understood, however, is how the neuronal networks built from

these connections give rise to the higher-level phenomena of, for instance, perception, emotion, motor activity, or memory. For the case of memory in particular, it seems probable that lasting alterations to neuronal networks are the chief underlying mechanism, but making the leap from this general theory to a full understanding of how these alterations are formed, how memory is regulated, and how it is retrieved, is a quite daunting prospect. Nonetheless, much progress has been made in this direction. In 1949, Canadian neuroscientist Donald O. Hebb proposed an activity-dependent mechanism of synaptic plasticity, an idea that came to be known as Hebb's Postulate (Hebb, 1949). The basic idea of this mechanism is that the activity of some presynaptic cell can determine the effect of subsequent firing of this cell upon some postsynaptic cell. In other words, the more active a synaptic connection is, the stronger it becomes. The postulate has the particular requirement that a postsynaptic cell must be active in order for its synaptic inputs to be strengthened. In this way, "traces" of neuronal activation can develop within a neuronal network based upon experience, and these traces could conceivably be the storage medium for memory.

Hebb's original theory has been substantiated both by experimental and theoretical findings. The discovery of long-term potentiation (LTP) by Bliss and Lømo (1973) was pivotal in this respect. LTP is a long-lasting increase in the response of cells to specific inputs, induced by applying a high-frequency stimulation train to the presynaptic cell. Importantly, LTP satisfies the requirements of Hebb's Postulate, demonstrating both *associativity* (i.e., all synapses activated coincident to LTP induction are potentiated), and *input selectivity* (i.e., synapses which are not active during LTP induction are not

potentiated). Hebb's Postulate has also been instrumental in the inception of *neural network theory*, which builds upon the notion of biological neuronal networks to create artificial computational network models. These so-called artificial neural networks have been used both as artificial intelligence tools, and as models of real neuronal function. One instance of the latter is the use of a neural network system to simulate visual processing in area MT of the visual cortex (Nowlan & Sejnowski, 1995). Remarkably, the behaviour of this network and its units corresponds nicely to observations of neuronal behaviour in primate cortex.

1.4 The neuromodulation of memory

Hebb's model of neuronal plasticity is referred to as *homosynaptic*, as it is mediated by a single synapse. A second type of plasticity has been postulated, however, involving modulation from synapses other than the one being altered. This *heterosynaptic* plasticity is thought to arise from the actions of neuromodulatory transmitters, such as catecholamines, opioids, or cannabinoids. Such a process was proposed as early as 1970 by Seymour Kety, who suggested a role for "biogenic amines", including catecholamines and serotonin (5-HT), in assigning an adaptive value to environmental stimuli, such that some are encoded in memory much more robustly than others (Kety, 1970). One's state of arousal, in other words, can influence the precision and strength of his or her memory formation. This idea has the advantages of corresponding with popular experience, as well as having a quite logical evolutionary basis. Moreover, a number of psychiatric conditions provide a very stark portrayal of the robustness of neuromodulation upon

memory formation (see Section 1.5). The present study concentrates on the catecholamine norepinephrine (NE), and its role in heterosynaptic neuronal plasticity.

1.5 Norepinephrine and memory

Research into the involvement of norepinephrine in memory followed from the ideas of Kety and others, and this relationship continues to be widely investigated. NE release in the nervous system is associated with heightened arousal, and there is much evidence, both systematic and anecdotal, that heightened arousal results in the strengthening of memory for sensations experienced during the period of arousal (see Cahill & McGaugh, 1998, for review). An extreme example of this relationship can be observed in cases of post-traumatic stress disorder (PTSD), in which people who have experienced some traumatic event or series of events develop very strong, seemingly indelible memories of the trauma, which can be quite intrusive and are often very difficult to alleviate. NE has been implicated in this syndrome, and indeed CNS levels of NE in male combat veterans with chronic PTSD were found to be significantly higher than those of healthy controls, and levels of NE correlated strongly with the severity of PTSD symptoms in this study, demonstrating a clear role for this transmitter in this disorder (Geracioti et al., 2001).

The relationship of NE to memory in humans has been studied more directly by James McGaugh and colleagues. Subjects initially demonstrated an enhanced long-term memory for emotionally-charged stories over emotionally-neutral ones. This enhancement, however, was blocked by systemic application of the β-adrenoceptor antagonist propranolol prior to presentation, which had no effect on memory for the

neutral story (Cahill et al., 1994). The β-adrenoceptor antagonist effect was later demonstrated to be CNS-specific, as it was not reproduced by the β-adrenoceptor blocker nadolol, which crosses the blood-brain barrier much less readily (van Stegeren et al., 1998).

To further investigate these β-adrenoceptor-dependent phenomena, animal models of memory have been utilized. The basolateral amygdala (BLA) has been of particular interest to McGaugh and colleagues, and intra-BLA infusions of NE and other β-adrenoceptor agonists have been observed to enhance memory retention on several tests of memory in the rat, including inhibitory avoidance, the Morris water maze, and contextual fear conditioning (Ferry and McGaugh, 1999, LaLumiere et al., 2003). This and other evidence led researchers to suggest that the BLA plays a modulatory role in memory formation, but it appears unlikely to be the site of memory storage. Lesions of the stria terminalis, the major efferent pathway of the amygdalar complex, block the memory enhancement of BLA β-adrenoceptor agonism on an inhibitory avoidance task, suggesting that the BLA is modulating memory function in structures downstream of this pathway (Liang et al., 1990). Further research has focused on two particular structures which receive amygdalar input, the hippocampus and the caudate nucleus. Infusion of *d*-amphetamine, which effectively blocks reuptake of NE and dopamine (DA), into the amygdala facilitated memory for both a spatial and a visually cued water maze task. Infusions of *d*-amphetamine into either the hippocampus or caudate nucleus selectively enhanced the spatial and cued versions of the task, respectively (Packard et al., 1994).

Importantly, deactivation of the amygdala with lidocaine did not disrupt these enhancements.

Another novel method of examining the noradrenergic modulation of memory is the study of dopamine β-hydroxylase (DBH; see below) knockout mice. Mice lacking this enzyme are effectively unable to produce NE, and thus are naturally deprived of its functionality. While this wholesale disruption of an important neurotransmitter carries a number of caveats, the power of this genetic model remains evident. By introduction of L-threo-3,4-dihydroxyphenylserine (L-DOPS), a synthetic amino acid precursor to NE, levels of NE can be quickly restored (Thomas et al., 1998) and thus the behaviour of mice studied in the presence and absence of a putatively functional noradrenergic system. DBH knockout mice were trained on both a cued and a contextual version of a fear conditioning paradigm, and exhibited deficits on day two tests for the contextual, but not the cued task (Murchison et al., 2004). Importantly, mice receiving L-DOPS before *testing*, but not before training, demonstrated intact conditioning, suggesting that NE is critical for retrieval, but not for inception or consolidation of hippocampal-dependent memory. Propranolol administration before testing, but not before training, resulted in a disruption of conditioning similar to that of DBH knockouts, demonstrating a β-adrenoceptor dependence. These recent discoveries are somewhat puzzling in the face of a substantial collection of literature suggesting an apparent role for NE in the formation and consolidation of memory (e.g., McGaugh & Roozendaal, 2002), but they carry promising implications for the eventual elucidation of this role.

1.6 Norepinephrine

1.6.1 Biosynthesis, storage, and release

All catecholamines are synthesized in a chain of reactions originating with the amino acid tyrosine (Siegel et al., 1999; Figure 1.1). The rate-limiting factor for this synthesis is the enzyme tyrosine hydroxylase (TH), which converts L-tyrosine to L-DOPA. Dopamine (DA) is synthesized from L-DOPA in a reaction catalyzed by the enzyme DOPA-decarboxylase (DDC). NE, in turn, is synthesized from DA in a further reaction catalyzed by the enzyme DBH. Finally, the last step in the chain produces epinephrine from NE, by a process requiring phenylethanolamine *N*-methyltransferase (PNMT). Catecholamines, including NE, are stored at high densities in terminally-located vesicles. Catecholamines which are not protected by vesicular storage can be quickly degraded by the enzymes monoamine oxidase (MAO) and catechol-*O*-methyltransferase (COMT). Synaptic NE release is rapidly deactivated by reuptake. NE is degraded intracellularly by MAO and extracellularly by COMT or transported back into neurons by the NE-specific membrane transporter and re-stored in terminal vesicles, its chief means of deactivation.

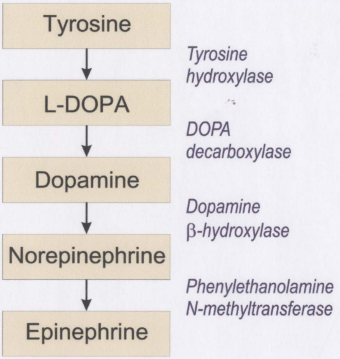

Figure 1.1 – *Biosynthesis of norepinephrine*. Schematic representation of catecholamine biosynthesis, including norephinephrine (NE), shown in order of precursors. The enzymes listed to the right are catalytic enzymes facilitating each step in the process. Tyrosine hydroxylase (TH) is the rate limiting enzyme in this process (Siegel et al., 1999).

1.6.2 Noradrenergic receptors

Noradrenergic receptors are commonly referred to as adrenoceptors. There are two main classes of adrenoceptor, α and β. α-adrenoceptors have three subtypes, α_1, α_2, and α_3, all of which are G-protein coupled. α_2 adrenoceptors can be either presynaptic autoreceptors, which can regulate the release of NE (Van Gaalen et al., 1997), or postsynaptic receptors, which couple to G_i proteins, and ultimately function to deactivate adenylate cyclase (AC). α_2-adrenoceptors expressed in the LC itself also act to inhibit firing of LC neurons, as application of the α_2-adrenoceptor antagonist idazoxan enhances firing (Linner et al., 1999). α_1 receptors are found primarily postsynaptically, where they are G_q-coupled. β-adrenoceptors have three subtypes, β_1, β_2, and β_3. Both β_1 and β_2 receptors are present in the CNS. All three subtypes are coupled to G_s proteins, which act to activate AC (Siegel et al., 1999; see also Section 1.10).

1.7 Hippocampus

1.7.1 Gross anatomy

The hippocampus derives its name from the Greek *hippokampos*, which refers to its putative resemblance to a seahorse, in coronal slices of the human brain. Generally speaking, the hippocampus consists of a trisynaptic loop involving the dentate gyrus (DG), CA3, and CA1, respectively (Figure 1.2). "CA" represents the Latin *cornu amonis*, meaning "Amon's horn", in reference to its hornlike shape in humans (derived from *Amun*, an Egyptian deity with the head of a ram). The main pathways involved in this system are: the perforant path projecting primarily from entorhinal cortex to DG (but see

below), the mossy fibre pathway projecting from DG granule cells to CA3, and the Schaffer collateral pathway connecting CA3 to CA1.

All three main subregions of the hippocampus have a consistent sheet-like structure, with distinct laminar organization, and are typically described in terms of the principal cells which comprise them. The three sheets are folded and interlaced in a fashion that persists along its anteroposterior axis, as is illustrated in Figure 1.2 (Amaral and Witter, 1989). The principal cells of the dentate gyrus are granule cells, described in more detail below. Both CA1 and CA3 have large pyramidal neurons as their principal cells, which are organized such that they give rise to distinctive somatic, proximal dendritic, and distal dendritic strata. All three regions also host a wealth of inhibitory interneurons.

The major source of cortical afferents to the hippocampus is the perforant pathway, which originates in the overlying entorhinal cortex (EC). The perforant pathway is so-called due to the manner in which it perforates subicular space in its projection to hippocampus. Its input is most substantial from layer II EC to dentate gyrus, but layer II also projects to CA3, and layer III EC has projections to CA1 and the subiculum (Steward & Scoville, 1976). CA1 is considered to be the main source of output from the hippocampal system, and its main target is the subiculum, which relays this output to EC, such that the entire network represents a feedback loop originating and terminating in the EC. CA1 also has direct projections to the EC (Witter et al., 2000).

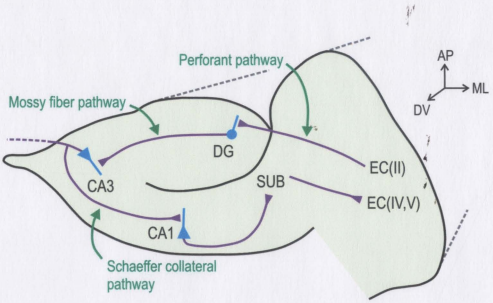

Figure 1.2 – *Diagram of the hippocampal trisynaptic loop in horizontal cross-section.* The dentate gyrus (DG) receives most of its cortical input from layer II of the entorhinal cortex (EC) via perforant pathway fibres. DG granule cells project mainly to CA3 via the mossy fibre pathway, and CA3 pyramidal cells project to CA1 pyramidal cells via the Schaffer collateral pathway. The major output of CA1 is to the subiculum (SUB), which relays to layers IV and V of EC. Adapted from Witter et al. (2000).

1.7.2 Anatomy of dentate gyrus and hilar region

The dentate gyrus is generally considered to consist of three layers: the granule cell body, or somatic layer, the dendritic, or molecular layer, and the polymorphic layer, or hilar region (Figure 1.3; Lorente de No, 1934; Ramon Y Cajal, 1911). The soma of a granule cell is small and somewhat elongated in shape, with an average width of 10.3 μm and length of 18.6 μm (Claiborne et al., 1990). Granule cell dendrites are spiny, and extend approximately 300 μm from the soma, determining the cross-sectional extent of the structure, which is delineated by the hippocampal fissure. Somata in this structure are very densely packed, with an estimated 0.6 to 1.0 x 10^6 cells/mm^3 (West et al., 1988).

The hilar region is host to a large variety of cell types, including bipolar fusiform spiny cells, multipolar "mossy" cells, and aspiny cells of various morphologies (Scharfman, 1991). It is notable that the threshold for firing in the hilar region, for the majority of spiny and aspiny neurons, is much lower than that of granule cells. This is particularly true of cells whose dendrites extend into the molecular layer; hilar cells with thresholds similar to those of granule cells have little or no visible dendritic extension into the molecular layer (Scharfman, 1991). A substantial number of hilar cell types are inhibitory interneurons, and may provide feedback or feedforward inhibition to dentate granule cells (Sloviter et al., 1999).

1.7.3 Afferents to dentate gyrus

Perforant path input to the dentate gyrus terminates primarily in the outer two-thirds of the molecular layer (McNaughton & Barnes, 1977), whereas the inner third is innervated

by excitatory monosynaptic connections from hilar mossy cells, through associational and commissural pathways (Scharfman, 1995; Lysetskiy et al., 2005). The perforant path itself can be separated into lateral and medial components, and the subset of these fibres projecting to dentate gyrus originate in cytoarchitectonically distinct lateral and medial regions of layer II entorhinal cortex, respectively. Perforant path fibres innervate the molecular layer in an *en passage* fashion, such that a single axon will make synaptic contact with multiple granule cells as it projects through the dendritic space. Some of these fibres continue on to contact cells in CA3 as well. This termination pattern is further organized such that the lateral perforant path projects to the distal third of the molecular layer, while the medial pathway innervates the middle third (Witter et al., 2000).

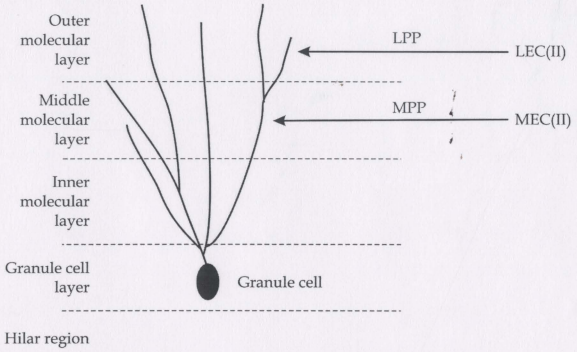

Figure 1.3 – *Laminar organization of the dentate gyrus.* The lateral perforant pathway (LPP) projects from layer II lateral entorhinal cortex (LEC) to the outer third of the molecular layer. The medial perforant pathway (MPP) projects from layer II medial entorhinal cortex (MEC) to the middle third of the molecular layer.

1.8 Locus coeruleus

1.8.1 Gross anatomy

The nucleus locus coeruleus (LC), so-called because of its blue appearance in primates, is located within the pontine brainstem, proximal to the fourth ventricle (see Aston-Jones et al., 1995, for review). In the rat, the nucleus extends 1.2 mm along the edge of the ventricle, and is comprised of approximately 1500 cells (10,000 to 15,000 in the human). The axons of LC neurons are highly ramified and the efferent projections of this structure are quite diffuse within the CNS, innervating forebrain, cerebellar, and spinal cord targets (Foote et al., 1983). The vast majority of LC neurons have been shown to contain DBH, the enzyme critical for the synthesis of NE from DA (Grzanna et al., 1991).

1.8.2 Afferent projections to the LC

Early studies identified two brainstem structures, the nucleus paragigantocellularis (PGI) and the prepositis hypoglossus, as being the probable primary sources of afferent regulation of LC activity in rats (Aston-Jones et al., 1986). The PGI, in particular, is associated with the regulation of sympathetic arousal, and as such, this connection represents a possible relay signal of sympathetic arousal to the forebrain (Berridge & Waterhouse, 2003). Further sources of input, albeit less robust, have been described more recently, including prefrontal cortex (Jodo & Aston-Jones, 1997) and the central nucleus of the amygdala (Luppi et al., 1995). While minor, these inputs present possible pathways for higher-level cognitive and affective modulation of LC activity, respectively. In primates, whose prefrontal cortical region in particular is more highly developed and much more similar to humans than rodent brains, a series of tracing studies has revealed

prominent connections from both anterior cingulate and orbital prefrontal cortices to LC (Aston-Jones & Cohen, 2005).

1.8.3 Efferent projections of the LC to the hippocampus

LC fibres innervate all major substructures of the hippocampus. Within the dentate gyrus, these afferents are densest in the hilar region. LC projects bilaterally, with 80% of inputs being ipsilateral in CA1, and as low as 70% in the dentate gyrus (Loy et al., 1980).

1.8.4 Firing patterns & NE release

Noradrenergic LC neurons exhibit both tonic and phasic firing patterns. Tonic firing consists of slow, spontaneous discharges (<5 Hz) at highly regular intervals, with wide action potentials (1-2 ms), whereas phasic-type firing consists of a short burst of spikes followed by a period of suppressed activity (200-500 ms; Berridge & Waterhouse, 2003). In adult rats, LC neurons tend to fire together in synchronous bursts, and this behaviour appears to be mediated through gap-junction interactions between dendrites (Ishimatsu et al., 1996). Tonic rates of cell firing have been shown to correlate with arousal state; they are virtually quiescent during REM sleep, fire at <1 Hz during slow-wave sleep, <2 Hz during quiet wakefulness, and >2 Hz during active wakefulness (Foote et al., 1980; Hobson et al., 1975). Phasic discharge appears in response to novel or salient stimuli in an animal's environment, and tends to habituate upon repeated exposure to the same stimulus (Aston-Jones & Bloom, 1981).

The rate of tonic firing of LC neurons correlates positively with extrasynaptic levels of NE in rat neocortex, measured by microdialysis (Berridge & Abercrombie, 1999). It is difficult, given the limited temporal resolution of this method, to measure the effects of phasic firing on extrasynaptic levels of NE; its effects upon intrasynaptic release are also an open question. Tonic firing appears to stimulate release of NE from non-synaptic axon terminal varicosities, and it has been speculated to facilitate volume transmission of NE signals, whereas the more rapid phasic firing may have a stronger effect upon synaptic transmission (see Berridge & Waterhouse, 2003).

1.8.5 Effects of glutamate on locus coeruleus firing patterns

Glutamate, pressure-ejected into the LC elicits a robust bursting pattern of firing, as determined from single-unit recording (Harley & Sara, 1992). This burst firing lasts 250-400 ms and is followed by a prolonged suppression of firing lasting approximately 4.6 min, after which cells return to normal tonic discharge rates. Glutamatergic activation of LC cells also produces potentiation of the perforant path-evoked field potential population spike in the dentate gyrus. The duration of this effect is variable and appears dependent upon the volume and/or targeting of glutamate ejected.

1.8.6 LC response patterns and behavioural correlates

Both tonic and phasic LC activity appear to be driven by *salient* stimuli, whether the valence of these stimuli is appetitive or aversive (Aston-Jones & Bloom, 1981). LC phasic firing can also be induced by novelty. Single cell recordings from rats placed in a hole-board apparatus showed a phasic LC response to novel objects when they were

observed by the rat, which did not reoccur upon subsequent visitations of that hole (Vankov et al., 1995). Notably, when objects were moved within the apparatus, an encounter with a previously observed object in a new location induced phasic firing, indicating that *change* to an environment can also activate LC. A closer examination of this relationship, as it occurs in primates, indicates that phasic activity in particular can be induced by the introduction of task-relevant cues. The salience of these cue stimuli is derived from the nature of the particular task; the same cues fail to activate LC when they are not relevant to a task (Aston-Jones & Cohen, 2005).

Phasic LC activity has been further hypothesized to facilitate the behavioural response in decision-based tasks; for instance, in a two-alternative forced choice paradigm, phasic firing was observed to be temporally linked to the behavioural response, rather than the task-relevant cue preceding it (Aston-Jones et al., 1994). Increasing tonic firing rates, conversely, can have the effect of decreasing an animal's ability to discriminate a salient stimulus from a distractor, as well as its threshold for responding to a stimulus (Aston-Jones et al., 1994). Moreover, direct enhancement of LC tonic firing by the muscarinic agonist pilocarpine decreased phasic firing in response to stimuli, and interfered with task performance, whereas reduction of the tonic firing rate with the α_2-adrenoceptor antagonist clonidine increased phasic responses, and facilitated performance (Ivanova et al., 1997). Aston-Jones et al. have proposed an "adaptive gain" theory of LC function, such that phasic LC firing, determined to a large extent by high-level cortical modulation, can enhance the gain of neuronal responses to activity occurring within a discrete time window following an LC burst (Aston-Jones et al., 2005). Consistent with this idea,

novelty-elicited LC bursts have been shown to transiently enhance the population spike response of dentate gyrus neurons to perforant path stimulation, an indication of granule cell firing rates (see Section 1.9), and this facilitation can be blocked by propranolol (Kitchigina et al., 1997).

1.9 Long-term potentiation

1.9.1 Tetanic long-term potentiation

The phenomenon of long-term potentiation (LTP) was first reported *in vivo* in the rabbit hippocampus over 30 years ago (Bliss and Lømo, 1973; Bliss and Gardner-Medwin, 1973). This discovery pioneered a field of research that is still a very active frontier of neurobiological science. In the dentate gyrus, single-pulse stimulation of the perforant path (PP) evokes a characteristic field potential waveform consisting, when recorded at the somatic level, of a positive-going excitatory post-synaptic potential (EPSP), punctuated by a negative-going population spike (Figure 1.4). This waveform is thought to arise from an electric dipole induced by the entire field of synchronously firing granule cells. Its shape and polarity is dependent upon its laminar position in the DG. A high-frequency (*tetanic*; 100 Hz) pulse train results in an increase in both EPSP slope (signifying increased postsynaptic *sensitivity and/or release of glutamate*), and population spike amplitude (signifying an increased number of cells firing, or *excitability*, and possibly increased synchrony of this firing), in response to the same single-pulse stimulation (Figure 1.4). Since this potentiation can last for months *in vivo*, it has been termed "long-term potentiation". Dentate gyrus LTP has subsequently been demonstrated *in vitro* using brain slice preparations (e.g., Schwartzkroin and Wester, 1975).

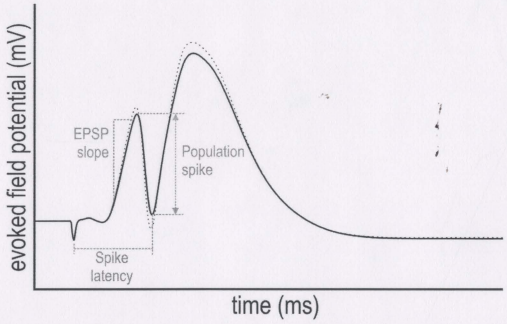

Figure 1.4 – *Perforant path-evoked field potential.* Typical perforant path-evoked field potential (EFP) waveform recorded under urethane anaesthesia from the granule cell layer of rat dentate gyrus. The solid black line shows pre-potentiation and the dashed black line shows a potentiated waveform. Potentiation is measured in this study by extracting three parameters, as shown in grey: EPSP slope, population spike, and spike latency. Initial bump in the EFP is an artifact of perforant path stimulation.

1.9.2 Norepinephrine-induced long-term potentiation

Population spike potentiation, recorded at the granule cell layer, can also be produced by application of norepinephrine to both anaesthetized rats (Neuman & Harley, 1983) and brain slice preparations (Lacaille & Harley, 1985; Stanton & Sarvey, 1985a). In both cases, this potentiation was long-lasting (>30 min and often lasted hours or until termination of recording) and was thus called long-lasting potentiation, or LLP, to distinguish it from tetanic LTP. For the purposes of the present study, NE-induced LLP will be referred to as NE-LTP, and tetanic LTP as HFS-LTP. *In vitro*, NE-LTP has been shown to be β_1 adrenoceptor dependent, as both propranolol (β-adrenoceptor antagonist) and metoprolol (β_1-adrenoceptor-specific antagonist) block its induction, and isoproterenol (β-adrenoceptor agonist) mimics it (Stanton & Sarvey, 1985a; Lacaille & Harley, 1985). Agents acting upon α adrenoceptors have not been observed to have substantial effects upon potentiation. HFS-LTP in dentate gyrus can be blocked by propranolol, but this effect is dependent upon the induction strength of the LTP train (Straube & Frey, 2003), indicating an important, but not essential, role for NE in this form of potentiation as well.

As the dentate gyrus receives its noradrenergic input exclusively from the locus coeruleus, further investigations have focused upon LC stimulation as a more physiologically relevant means of observing NE-LTP. Electrical stimulation of the LC, direct cannula infusion of either glutamate or orexin, or electrical stimulation of PGI produce population spike LTP when paired with perforant path stimulation, a phenomenon that has been demonstrated in both anaesthetized and freely moving rats

(Harley & Milway, 1986; Harley et al., 1989; Washburn & Moises, 1989; Babstock & Harley, 1993; Walling et al.., 2004; Walling & Harley, 2004). This potentiation is also β-adrenoceptor dependent, as it is blocked by propranolol. When the LC is activated by glutamate in freely-moving animals, both EPSP slope and population spike are substantially potentiated as recorded 24 hours later. This effect is both β-adrenoceptor and protein synthesis dependent, as it can be blocked by intracerebroventricular infusion of either propranolol or anisomycin prior to LC activation (Walling & Harley, 2004).

1.9.3 E-S potentiation

In the original Bliss and Lømo article, the authors note that the observed increase in population spike amplitude is not completely accounted for by the increase in EPSP (Bliss & Lømo, 1973). This phenomenon, termed EPSP-spike potentiation (E-S potentiation) has been observed in subsequent LTP studies, including studies of NE-LTP. Harley and Neuman report an increase in spike with no significant increase in EPSP slope (Neuman & Harley, 1983). Using *in vitro* preparations, a potentiation of both components is typically observed (Stanton & Sarvey, 1985a; Lacaille & Harley, 1985); Lacaille and Harley reported, however, that EPSP increases only account for 47% of population spike increases. E-S potentiation suggests that some aspect of the population spike amplitude, representing neuronal *excitability*, is being potentiated apart from what might be explained by an increase in *synaptic sensitivity* (as would be inferred from an increased EPSP slope). The nature of this dissociation, however, remains an open question.

1.9.4 Plasticity of lateral vs. medial perforant path

Analysis of the field potential evoked by stimulating different components of the perforant path reveals that the obtained waveforms are noticeably different. In particular, lateral perforant path (LPP) stimulation evokes a waveform with a longer peak latency, smaller EPSP slope, and greater EPSP width than that evoked by medial perforant path (MPP; McNaughton & Barnes, 1977). Examining these pathway differences in detail, Abraham and McNaughton (1984) observed an abrupt change in the evoked waveform while recording stepwise from increasingly proximal positions in the molecular layer. This abrupt difference indicated that differential pathway responses to perforant path stimulation were only minimally (20%) attributable to signal decay over an increasing synapse-to-soma distance; if this were the only explanation, a continuous decay of evoked potential would be expected over increasingly distal recording sites. These findings suggest instead that differences in synaptic transmission have a stronger role in determining specific pathway responses.

Norepinephrine effects upon the dentate gyrus evoked field potential are also pathway specific, as was first demonstrated by Dahl and Sarvey (1989). Specifically, NE applied in the presence of an α_1-antagonist resulted in LTP of MPP and long-term depression (LTD) of LPP. Both effects were also shown to be β-adrenoceptor dependent, as they were reproduced by isoproterenol and blocked by propranolol. This same relationship has subsequently been demonstrated *in vivo* using electrical stimulation of the nucleus paragigantocellularis (PGI) input to LC. Such stimulation results in LTP of the MPP-evoked population spike. It also results in a transient depression of the dentate gyrus

fEPSP (on several parameters including amplitude and slope) induced by stimulation of the lateral olfactory tract (LOT), which selectively activates LPP (Babstock & Harley, 1993). It is notable that LOT stimulation is not sufficient to produce a population spike in the dentate gyrus; these results are, nonetheless, an interesting parallel to the *in vitro* findings.

1.9.5 Associativity of NE-LTP

NE-LTP has been described as a heterosynaptic process, since it involves both noradrenergic and glutamatergic signals. How these signals interact temporally, however, and in particular whether they must be simultaneously active to produce LTP, has been unclear. *In vitro*, at least two separate NE studies have observed a lasting slope and spike potentiation in dentate gyrus in the absence of perforant path stimulation (Lacaille & Harley, 1985; Dahl & Sarvey, 1990). LTD of the LPP-evoked field potential was also observed to be activity independent in the Dahl study. These findings are somewhat confusing within the theory that norepinephrine is providing an arousal-related modulation of memory formation. This theory predicts that NE is interacting with sensory inputs in an associative fashion; lacking a specific pattern of cortical input to modulate (i.e., as is mediated by perforant path fibres), the function of a generalized potentiation or depression of synapses and/or cell excitability by norepinephrine is unclear. Fortunately, a few lines of evidence exist to better address this paradox.

In vitro, the glutamatergic N-methyl-D-aspartate receptor (NMDAR) has a necessary role in the formation of both NE-LTP and HFS-LTP, as both are blocked by the NMDAR

antagonist D(-)-2-amino-5-phosphonovaleric acid (D(-)APV; Burgard et al., 1989). In the same study that demonstrated activity independence of NE-mediated granule cell plasticity, Dahl and Sarvey also demonstrated an NMDAR requirement for both LLP and LLD, as both are blocked by D(-)APV applied concurrently with isoproterenol (Dahl & Sarvey, 1990). Since NMDARs normally require glutamatergic stimulation to function, it seems to follow that perforant path activity has a critical role in the production of NE-induced plasticity. One possible explanation for the lack of an associative requirement, as demonstrated *in vitro*, is that the associative component has a prolonged time-course, exceeding the washout periods in these experiments. The prolonged presence of elevated neurotransmitter (or agonist) characteristic of the slice technique may also serve to compound this effect; such levels of continuous β-receptor activation would not be expected under normal physiological conditions (i.e., *in vivo*), and may serve to saturate whatever intracellular processes are underlying the plasticity.

1.10 Intracellular signalling in NE-LTP

1.10.1 Actions of cAMP-dependent protein kinase

6-hydroxydopamine (6-OHDA) depletes NE, and when applied to hippocampal slices can significantly decrease HFS-LTP in the dentate gyrus, implicating a modulatory role for β-receptors in this potentiation (Stanton & Sarvey, 1985c). This finding also demonstrates a common factor in the two distinct forms of LTP discussed above. The 6-OHDA effect can be reversed by concurrent application of low concentrations of forskolin, a non-selective AC activator, implicating AC as an important component in the production of LTP. β-receptors act through a G_s-protein-coupled process, by activating AC, which is

critical in the production of the ubiquitous second messenger protein cyclic adenosine monophosphate (cAMP; Siegel et al., 1999). Accordingly, Stanton and Sarvey measured a 3-fold increase in cAMP levels in dentate gyrus at 1 min, but not at 30 min, following HFS application, and this increase was blocked by 6-OHDA. NE application also increased cAMP levels 3- to 4-fold, and this increase was sustained through washout (Stanton & Sarvey, 1985c). More recent characterization of the cAMP response to NE *in vivo*, using microdialysis measurement of extracellular cAMP in frontal cortex, shows a rapid increase which reaches a peak at 6 min, even in the continued presence of NE, and gradually declines to baseline by 30 min (Stone and John, 1990).

Prolonged elevation of cAMP levels could be one mechanism by which associativity between NE and PP signals arises, even over the prolonged period of silence (30 min) in which NE-LTP was observed in the *in vitro* associativity studies. The major function of cAMP appears to be its activation of cAMP-dependent protein kinase (PKA), which has a multitude of intracellular targets, including the GluR1 subunit of AMPAR (Banke et al., 2000), NR1 and NR2C subunits of NMDAR (Tingley et al., 1997; Chen et al., 2006), phosphatase inhibitor 1 (I-1; Ingebritsen & Cohen, 1983) and cAMP response element binding protein (CREB; Hagiwara et al., 1993). Each of these targets may play a critical role in the formation of LTP (see Figure 1.5). Phosphorylation of GluR1 by PKA increases channel open probability (Banke et al., 2000) and can increase the surface expression of GluR1 (Mangiavacchi & Wolf, 2004), effectively sensitizing the synapse to glutamate. PKA phosphorylation of GluR1 is also necessary for NMDA-dependent HFS-LTP (Esteban et al., 2003). Similarly, PKA phosphorylation of NR2C results in an

alteration of NMDAR kinetics, effectively lowering both its rise and decay time constants (Chen et al., 2006). There is further evidence that PKA is involved in synaptic NR1 insertion (Crump et al., 2001).

1.10.2 Importance of Ca^{2+} influx

These actions of PKA predict an amplification, however transient, of glutamatergic effects on a postsynaptic cell. Indeed, both AMPAR and NMDAR amplification is seen in hippocampal slices undergoing LTP, with the AMPAR component being two-fold greater than the NMDAR (Dozmorov et al., 2006). In an olfactory memory model, increases in both AMPAR and NMDAR components of the olfactory nerve-evoked field potential have also been observed (Yuan et al., 2000). The critical signal that is being amplified by these actions may be Ca^{2+} influx, which has a multitude of intracellular effects. HFS-LTP in CA1 requires an increase in intracellular Ca^{2+} (English & Sweatt, 1997). Intracellular Ca^{2+} levels can be elevated through a number of means, including: release of intracellular Ca^{2+} stores, transmission through NMDARs, and transmission through non-NMDAR Ca^{2+} channels.

Intracellular Ca^{2+} is stored primarily in endoplasmic reticulum and mitochondria. It can be released from endoplasmic reticulum via channels regulated by inositol 1,4,5-triphosphate (IP3). IP3 channels have three sites which are phosphorylated by PKA (Soulsby and Wojcikiewicz, 2005), but the functional significance of this phosphorylation is still unclear. Ca^{2+} transmission through NMDARs requires a voltage-dependent removal of a Mg^{+} blockade, and influx through L-type Ca^{2+} channels is also

voltage-dependent. Thus it appears that some level of excitatory glutamatergic activity is necessary to activate these channels. There is also evidence of β receptor-dependent modulation of both pathways. NE applied to a patch-clamped dentate granule cell results in an enhancement of voltage-dependent Ca^{2+} channels, and this effect is mimicked by cAMP (Gray & Johnston, 1987).

Ca^{2+} influx has a number of important functions with respect to LTP. Intracellularly, Ca^{2+} interacts with the protein calmodulin, to form an active Ca^{2+}/calmodulin (CaM) complex. One important function of CaM is its facilitation of the autophosphorylation of CaM kinase II (CaMKII), which is densely distributed in postsynaptic densities (Petersen et al., 2003) and has been widely implicated in learning and memory (Yamauchi, 2005; Lisman & Goldring, 1988-89, for review). CaMKII also appears important for LTP induction, as its inhibition by the selective inhibitor CaMKII(273-302) (Malinow et al., 1989) or knockout mutation (Silva et al., 1992) interferes with HFS-LTP in CA1. Moreover, direct infusion of constitutively active CaMKII enhances synaptic transmission as well as the postsynaptic response to AMPA, and this effect occludes HFS-LTP (Lledo et al., 1995). This may be partly due to CaMKII phosphorylation of the Ser831 site of GluR1, which has been shown to enhance AMPA channel conductance (Derkach et al., 1999). Ca^{2+} can also activate Ca^{2+}-sensitive adenylate cyclases, and thereby recruit cAMP production and activate PKA (Chetkovich et al., 1991). Thus, two kinase pathways can be activated by Ca^{2+} influx, both of which are important for the induction of LTP.

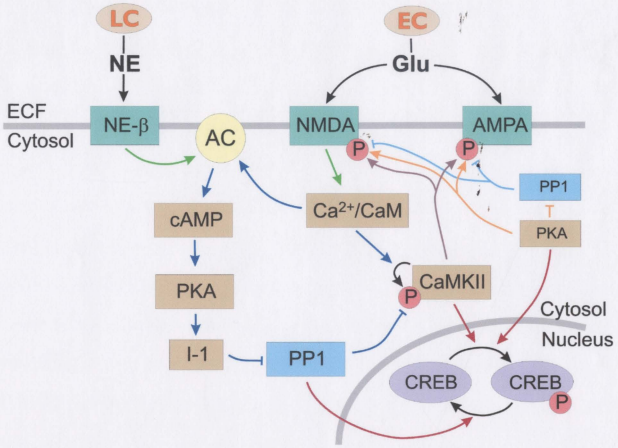

Figure 1.5 – *NE- and glutamate-related signalling cascades.* Intracellular signalling cascades induced postsynaptically by noradrenergic β activation and glutamatergic NMDAR and AMPAR activation. While this model is not comprehensive, it serves to illustrate the major intracellular agents implicated in both HFS- and NE-LTP production and activated by the two major signals observed in the phenomenon of NE-LTP. Arrows indicate a positive action (activation or phosphorylation) and lines indicate a negative action (deactivation or dephosphorylation). "P" indicates a phosphate group. Note that CaMKII is able to autophosphorylate in the presence of Ca^{2+}/CaM.

1.10.3 β-adrenoceptor and NMDAR-induced phosphorylation of GluR1

β-adrenoceptor activity may have differential effects upon kinase activity with respect to NMDAR activity. In CA1, despite a substantial NMDAR-induced cAMP production via Ca^{2+}-sensitive ACs, NMDAR activation actually dephosphorylates GluR1 at its PKA-specific Ser845 site (Vanhoose & Winder, 2003). NMDARs recruit phosphatase as well as kinase activation; however, while inhibition of PP1 and PP2 by calyculin A prevented this dephosphorylation, it did not result in PKA phosphorylation of Ser845. β-adrenoceptor activation, while it produced around a third of the cAMP levels produced by NMDAR activation, resulted in a significant and long-lasting phosphorylation of Ser845. Interestingly, when the two signals were paired, order was critical in determining the extent of phosphorylation. β adrenoceptor activation prior to NMDAR activation resulted in an additive phosphorylation of Ser845; conversely, NMDAR activation prior to β activation prevented phosphorylation. While the machinery underlying this phenomenon remains somewhat of a mystery, it does offer some insight into a precisely ordered pattern of signal integration between these two pathways, both of which have been implicated in NE-LTP.

β-adrenoceptor activity appears to phosphorylate Ser845 even in the absence of an NMDAR signal; however, NMDAR robustly phosphorylates the Ser831 site of GluR1, which is specifically targeted by CaMKII and PKC, while β-adrenoceptor activity has no effect on this site (Vanhoose & Winder, 2003). Also in CA1, PKA appears to play a permissive role in LTP induction. Application of both Rp-CAMPS, an inhibitor of cAMP, and the regulatory subunit of PKA, which deactivates the kinase, block HFS-LTP

induction in CA1 (Blitzer et al., 1995). However, LTP is recovered by protein phosphatase inhibitors microcystin LR and okadaic acid (Blitzer et al., 1995) as well as thiophosphorylated I-1 (Blitzer et al., 1998). PP1 acts to dephosphorylate CaMKII, among other substrates, and the actions of PKA in this case could be to prevent this dephosphorylation, thus increasing the levels of autophosphorylated CaMKII. Indeed, HFS-LTP results in increased levels of Thr286-phosphorylated CaMKII, and this increase is blocked by Rp-CAMPS. Application of a CaMKII inhibitor, moreover, completely blocks LTP in this system, and thiophosphorylated I-1 results in only a very modest recovery of potentiation (Blitzer et al., 1998). Thus, in CA1 at least, PKA may be playing a primarily permissive role in facilitation of a prolonged CaMKII signal that is more critical for LTP generation. In dentate gyrus, however, the story may be slightly different. Inhibition of either PKA, CaMKII, or mitogen-associated protein kinase (MAPK) individually does not block HFS-LTP (Wu et al., 2006). Inhibiting CaMKII concurrently with either PKA or MAPK *does* block HFS-LTP, suggesting the existence of two separate signal cascades that can independently support potentiation.

1.11 Objectives and predictions

In all, the findings illustrated above present a complex model of noradrenergic modulation of memory, and include evidence drawn from a number of fields of research – including human and animal behaviour, lesion studies, electrophysiology, genetics, and molecular neuroscience. An integration of these findings into a larger theory of memory requires that models conceived at one level reliably predict the behaviour of a system observed at another level, and vice versa. Accordingly, the present study seeks to

examine the associativity of NE-LTP in DG *in vivo*. If this phenomenon actually does, to some extent, subserve the higher-level phenomenon of arousal-modulated memory, this relationship predicts that NE-LTP itself will be associative, and thus require a pairing of noradrenergic and glutamatergic signals to be produced. Evidence from *in vitro* studies of NE-LTP, however, have not found such a requirement, and cell signalling findings do not suggest an unambiguous hypothesis with respect to NE-LTP. The present study, therefore, attempts to test the associativity of NE-LTP *in vivo* in the urethane-anaesthetized rat, using pharmacological stimulation of the LC. It is expected that pairing of LC noradrenergic and PP glutamatergic signals is necessary for NE-LTP to be produced, thus demonstrating an associative requirement for this form of potentiation. It is likely, based upon molecular research findings discussed above, that these signals converge on a Ca^{2+} transient that is critical for the intracellular events which mediate lasting synaptic and cellular changes and underlie plasticity.

2. Procedure

2.1 Apparatus

This procedure used glass micropipettes to record field potentials from the dentate gyrus. These micropipettes were pulled using a pipette puller (Kopf model 700C), and had an impedance ranging from 1 to 2 MΩ, measured immediately prior to a given experiment. Micropipettes were filled with 1M NaCl solution. Potential recordings were enabled by grounding the circuit to scalp tissue, and amplifying the separate signals using three Grass amplifiers (P511K series). The amplified signal was converted from analog to digital using a Data Translation DT 2821 A/D board. Perforant path stimulation was provided using a concentric bipolar stimulating electrode (Kopf NE-100, 0.5mm) connected to an adjustable stimulator (NeuroData SIU 90). Temporal control of stimulation events and sampling and storage of digital signals were provided using DataWave SciWorks software (version 3.0). Delivery of glutamate to the locus coeruleus was provided using a guide and internal cannula (Plastics1, 30ga). Figure 2.1 illustrates the basic experimental setup utilized in this study.

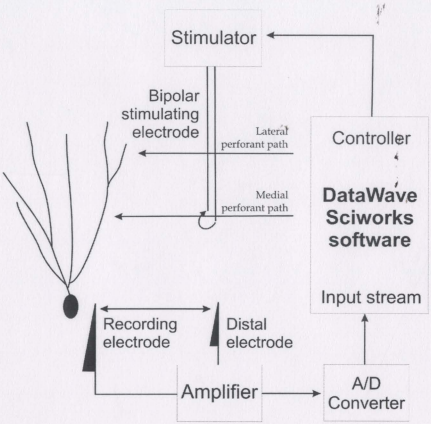

Figure 2.1 – *Basic experimental setup for recording of DG evoked field potentials.*

2.2 Subjects

Subjects were adult male Sprague Dawley rats obtained from the Vivarium breeding facility, St. John's, Newfoundland. Their weight ranged from 300 to 400g, and their age ranged from 53 to 78 days. Subjects were assigned to two groups, Paired and Non-paired. A third condition, the Control condition, was comprised of animals from either the Paired or Non-paired groups, and data were collected from these animals prior to any experimental manipulation (see Section 2.7).

2.3 Preparation

Rats were anaesthetized using 1ml/100g of a 15% urethane solution and their state of anaesthesia was determined by a lack of toe pinch response. Once anaesthetized, the rats' heads were shaved and the animals were secured in a stereotaxic instrument with skull flat orientation. Temperature was regulated with a rectal thermostat (FHC Brunswick, ME 04011) to ~36.5°C. 0.5M glutamate was prepared and used to fill a length of polypropylene tubing (Fisherbrand, 0.38mm) into which the internal locus coeruleus cannula was inserted. A 2 µl Hamilton microsyringe was filled with distilled water and inserted into the end of the tube opposite the cannula, for later infusion of glutamate and dye solutions.

2.4 Surgery

Once the head was secured, scalp tissue was reflected with a midline incision and held aside using weighted clamps. The location of bregma was identified and marked with a felt pen. For later reference, the anteroposterior (AP) and mediolateral (ML) coordinates

of bregma were determined for each of the three probe arms with respect to the stereotaxic apparatus, and the skull elevation at bregma was also determined for the locus coeruleus cannula. Three holes were subsequently drilled in the skull, one to accommodate each of: dentate recording micropipette (-3.5 mm AP, +1.8 mm ML), perforant path stimulating electrode (-7.2 mm AP, +3.9 mm ML), and locus coeruleus cannula (-12.5 mm AP, +1.1 mm ML). Care was taken to ensure that dura overlying the cortical tissue was also removed. Once these holes were completed, probe arms were secured in place at the above coordinates, with the exception of the locus coeruleus cannula arm. The elevation of cortical surface for each probe was determined, and the probes were lowered to the following depths below this elevation: dentate recording micropipette (2.5 mm) and perforant path stimulating electrode (2.5 mm). Wires were attached and the circuit was grounded to scalp tissue using an alligator clamp.

2.5 Recording

To record the dentate gyrus evoked field potential, SciWorks was programmed to sample a 40 ms duration following a 0.2 ms perforant path stimulation pulse, at a rate of 20,000 Hz, for a total of 800 data points per sweep. Waveforms were plotted onscreen as they were obtained. Using this display, the depths of the dentate recording and/or perforant path stimulating electrodes could be adjusted to obtain an optimal population spike.

2.6 Input/output relation

In order to calculate an input/output (I/O) relation before and after experimental manipulation, field potentials were recorded at differing stimulation currents. Typically

this involved starting at a current below the threshold for producing a spike, and increasing this current by increments of 100 μA until the spike stabilized (i.e., did not substantially increase with added current). Four samples per current level were obtained to get an average value for spike and slope at each.

2.7 Experiments

Based upon I/O observations, an optimal current level was selected based on the criteria that population spike amplitude was sufficiently high for measurement, but sufficiently below its maximum level to prevent a ceiling effect. Prior to experimental runs, field potentials were obtained for a period of approximately 30 min to ensure a stable baseline. For all conditions, field potentials were collected every 15s (0.25 Hz). The experimental procedure differed for each of the three conditions, Control, Non-paired, and Paired, as follows. For the purposes of this study, the term "silence" will be used to refer to an absence of perforant path stimulation.

Control

A 30 min baseline was obtained, followed by 20 min of silence. Stimulation resumed and was recorded for a further 30 min to 1 hr.

Non-paired

A 30 min baseline was obtained, followed by 20 min of silence. At ~9.5 min into this period of silence, an LC guide cannula was lowered to a depth of 9.7mm below the previously obtained bregma elevation, at a forward sloping angle of 20°. Once the guide

cannula was lowered, the internal cannula, attached to glutamate-filled tubing and a microsyringe, was inserted into it. 0.2 µl of the glutamate solution was immediately ejected, over a period of ~30 s, and the precise ejection time was noted. Perforant path stimulation was resumed following 20 min of silence, for a period of 1.5 to 2 hr.

Paired

A 30 min baseline was obtained. At ~29.5 min, the guide cannula was lowered as described for the Non-paired condition. Glutamate was also delivered in the same manner as the Non-paired condition. Stimulation continued uninterrupted at 0.25 Hz throughout this condition, for a further 1.5-3 hr.

2.8 Locus coeruleus cannula placement

To approximate the extent of diffusion of the glutamate ejection, methylene blue dye was ejected through the same cannula immediately following termination of the recording session, for both Non-paired and Paired groups. The animal was then decapitated and the brain extracted and submerged, intact, in methylbutane that was stored in a –70°C freezer. The frozen tissue was then preserved in the –70°C freezer until it was sectioned. Sectioning was performed using a Leica (Jung Frigocut 2800E) cryostat. 30 µm sections were obtained sagittally in two alternating sets, one of which was used to localize dye ejection, and the other of which was stained with a metachromatic cresyl violet stain to localize LC. A criterion of no greater than 300 µm from the somatic extent of LC, as seen in cresyl violet stained sections, was utilized to ascertain the accuracy of the glutamate ejection. This value is based upon anatomical evidence of LC dendritic field extent

(Aston-Jones et al., 1995). Any animal in which this criterion was not met was excluded from the analysis.

2.9 Parameter extraction

To analyze evoked field potentials, three parameters were extracted from the waveforms. Population spike amplitude was calculated using the "leading peak" method, by subtracting the minimum value of the negative-going spike from the maximum value of the positive leading peak. EPSP slope was obtained by calculating the slope of the initial rising phase of the EPSP. Spike latency was measured as the difference between the time of perforant path stimulation to that of the negative-most point of the population spike (see Figure 1.4).

3. Results

3.1 Animals included in study on the basis of dye ejection proximal to the locus coeruleus

All animals included as subjects in this study had dye ejection sites within the 300 μm criterion range of the LC specified in the procedure. Proximity to LC was measured from 30 μm sagittal slices of brain tissue, compared to adjacent slices stained with metachromatic cresyl violet to visualize the extent of the relevant brainstem nuclei (Figure 3.1). 6 animals were accepted in the Paired condition, and 7 in the Non-paired condition. 7 animals were used in the Control condition.

3.2 No baseline differences in spike between Paired and Non-paired conditions, but significant time effect for slope

To ensure that both the Paired and Non-paired groups were similar with respect to evoked potentials prior to any manipulation, a repeated measures ANOVA was applied to the 30 min baseline raw (unnormalized) population spike and EPSP slope values, averaged over 3 min intervals. Means and standard deviations for the two groups are shown in Table 3.1. For population spike, there was no significant effect of group ($p = 0.33$), or interaction effect ($p = 0.20$), but there was an apparent effect of time that approached significance ($p = 0.067$). For EPSP slope, there were no group ($p = 0.86$) or interaction ($p = 0.68$) effects, but there was a significant time effect ($p < 0.05$). A least significant difference (LSD) test of this time effect did not reveal a systematic trend of significant differences for either spike or slope over time that would be indicative of a differential baseline trajectory for the two conditions.

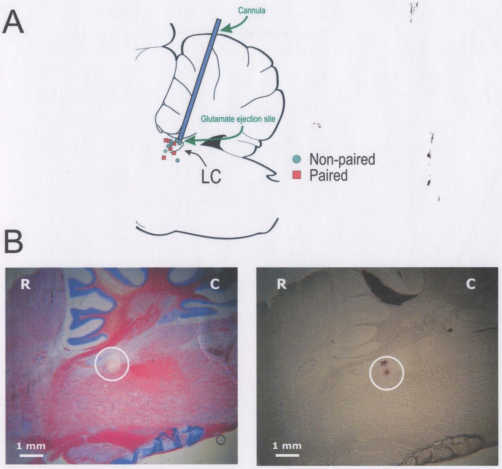

Figure 3.1 – *Activation of locus coeruleus.* **A.** Illustration of anatomical location of locus coeruleus (LC), cannula path, and glutamate ejection site. Dye ejection sites for individual subjects are represented by squares (Paired) and circles (Non-paired). **B.** Microphotographs of adjacent 30 μm sagittal sections of one animal for whom glutamate ejection met placement criteria (white circles). *Left:* section stained with metachromatic cresyl violet to differentiate structures. *Right:* unstained section showing dye ejection. R: rostral; C: caudal.

Table 3.1 – *Means and standard deviations for spike and slope from unnormalized data*

Group	n	Population Spike		EPSP Slope	
		Mean (mV)	St. Dev.	Mean (mV)	St. Dev.
Paired	6	3.82	1.98	7.92	1.35
Non-paired	7	4.99	2.32	7.47	1.81

3.3 Both spike and slope are potentiated in the Paired but not Non-paired conditions

A repeated measures ANOVA was applied to the population spike data, normalized to per-animal baseline averages, including the 30 min baseline period and excluding the interval from 30 to 50 min, during which stimulation was absent in the Non-paired condition. This test revealed significant group ($p < 0.05$), time ($p < 0.01$) and interaction ($p < 0.01$) effects (Figure 3.2). A least significant difference (LSD) test showed a significant increase in spike in the Paired group for all time-points following 50 min, compared to baseline values. Spike increases were also found in the Non-paired group, but only over a 3-6 min period following the resumption of stimulation, after which values were not significantly different from baseline. Spike in the Paired condition was significantly greater than that in the Non-paired condition for all but the first 3-6 min after 50 min.

A repeated measures ANOVA applied to normalized slope data, over the same period, also yielded significant group ($p < 0.05$), time ($p < 0.01$), and interaction ($p < 0.01$) effects (Figure 3.3). An LSD analysis of the interaction showed a significant increase in slope in the Paired condition, for all time-points following 50 min, compared to baseline values. Slope in the Non-paired condition showed a slight depression of slope for later

time-points, compared to baseline values, but this effect was not consistent over any period. Slope in the Paired condition was significantly higher than that in the Non-paired condition for all time-points after 50 min.

3.4 Spike and slope are potentiated in the first 20 min after LC injection for the Paired condition

A repeated measures ANOVA applied to normalized spike data, averaged over 3 min intervals, for the 30-50 min period following LC activation in the Paired condition showed a significant effect of time ($p < 0.01$). An LSD analysis of these data showed a significant increase of post-activation time-points, compared to baseline values. The same tests applied to slope data showed a significant time effect ($p < 0.01$), and post-activation time-points were significantly greater than baseline values.

3.5 Slope displays a lasting increase in the Control condition

A repeated measures ANOVA applied to normalized spike data, averaged over 3min intervals, for the Control condition, showed no significant time effect ($p = 0.08$; see Figure 3.4). However, the same test applied to slope data did reveal a significant effect of time ($p < 0.01$). An LSD test applied to these data showed a significant increase in all time-points following 50 min, compared to baseline values. One study on urethane-anaesthetized rats reports a rising baseline effect of both slope for 1-2 hr, and spike for 2-4 hr, following urethane injection, when recording from dentate gyrus (Gilbert & Mack, 1999). Since Control group data was obtained prior to either Paired or Non-paired data in many of the animals observed in the present study, one explanation for the observed slope

increase could be the proximity of the data collection to the original urethane injection, which would introduce this rising baseline effect. Since absolute times were unfortunately not recorded, time elapsed from the start of data collection was evaluated instead, with the assumption that each experiment had a similar latency to recording. A simple t-test was performed on these times, revealing a significantly later data collection period for Paired and Non-paired data, compared to that of Control data ($p < 0.01$).

3.6 Significant correlations of spike & slope for Paired and Non-paired conditions

To assess the relationship of EPSP slope to population spike amplitude, which would be expected to diverge in the presence of E-S potentiation, correlational analyses were applied case-by-case to animals in the Paired and Non-paired conditions. In all cases in both groups, spike significantly correlated with slope ($p < 0.01$ in all cases; see Table 3.1). Correlation coefficients did not significantly differ across groups ($p = 0.488$). Correlations were also obtained for pre- and post-LC values for both groups (Table 3.1). A univariate ANOVA applied to these data found no significant effect of Group ($p = 0.300$) or of Pre-post ($p = 0.0820$), although the latter appears to be approaching significance.

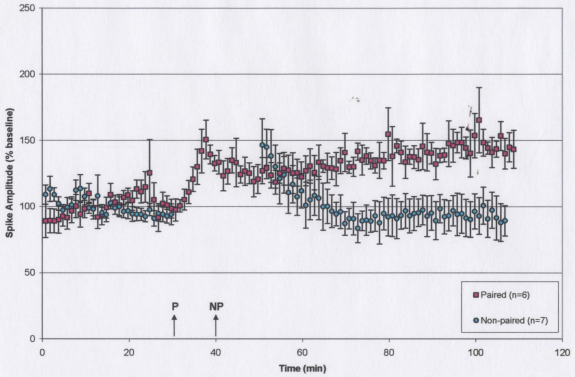

Figure 3.2 – *Population spike time-course*. Population spike averaged over 1 min intervals for the Paired (square) and Non-paired (circle) conditions. LC glutamate ejection times are indicated by arrows (P = paired, NP = Non-paired). Population spike rises over the first ten minutes following LC activation and remains elevated for the duration of the experiment. While a transient spike increase is observed immediately following resumption of stimulation in the Non-paired condition, spike values returns to baseline by 10 min. Error bars represent standard error.

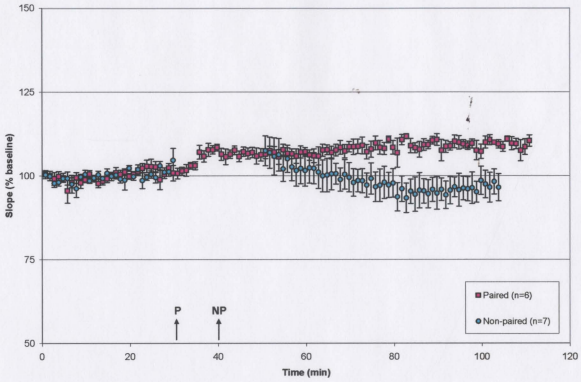

Figure 3.3 – *EPSP slope time-course*. EPSP slope average over 1 min intervals for the Paired (square) and Non-paired (circle) conditions. LC glutamate ejection times indicated by arrows. EPSP slope follows a similar time-course to that of population spike, increasing in the Paired condition over the first 10 min and remaining elevated for the duration of the experiment. EPSP is also similar to spike for the Non-paired condition, rising immediately after resumption of stimulation, but returning to baseline by 10 min. Error bars are standard error.

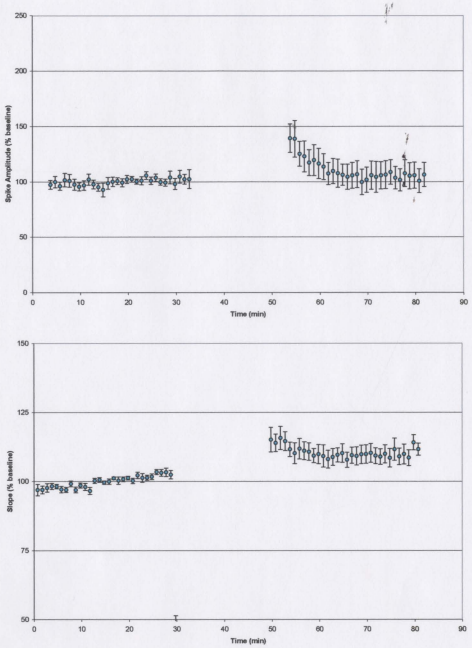

Figure 3.4 – *Spike and slope time-courses for the Control condition.* Population spike and EPSP slope averaged over 1 min intervals for the Control condition (n = 7). Spike increases immediately following resumption of stimulation in a manner similar to the Non-paired condition, returning to baseline after 10 min. Slope shows a significant increase following resumption of stimulation that persists throughout the experiment. Error bars are standard error.

Table 3.2 – *Spike-slope correlations for the Paired and Non-paired conditions*

Paired				Non-paired			
Animal	**Overall**	**Pre-LC**	**Post-LC**	**Animal**	**Overall**	**Pre-LC**	**Post-LC**
05/11/05	0.828	0.454	0.672	04/01/05	0.727	0.660	0.756
06/13/05	0.813	0.205	0.719	04/05/05	0.643	0.403	0.681
09/12/05	0.502	0.274	0.387	04/20/05	0.725	0.770	0.716
10/10/05	0.609	0.569	0.560	05/25/05	0.446	0.505	0.705
01/24/06	0.509	0.388	NS	02/13/06	0.632	NS	0.494
01/31/06	0.822	0.645	0.762	03/01/06	0.494	0.366	0.649
				03/14/06	0.780	0.477	0.613
Mean	**0.681**	**0.423**	**0.620**	**Mean**	**0.635**	**0.530**	**0.659**

3.7 No leftward shift apparent in the spike/slope curve obtained from I/O data

A plot of the spike vs. slope values obtained from the Paired group I/O data does not illustrate any leftward shift in the post-LC curve, with respect to the pre-LC curve (Figure 3.5). Quadratic regression curves plotted for both sets of data are practically indistinguishable.

3.8 Significant effect of time for spike latency in the Paired condition

A repeated measures ANOVA applied to spike latency data, averaged over 3 min intervals, revealed no significant effect of Time ($p = 0.173$) or Group ($p = 0.737$), but did find a significant Time x Group interaction ($p < 0.01$; see Figure 3.6). An LSD test applied to these data demonstrated a significant difference ($p < 0.05$) in the Paired condition for all time-points following 50 min, over time-points in the preceding 30 min. Post-50 min time-points in this group also differed significantly from most time-points in the Non-paired condition. There were no significant pre-post differences within the Non-paired group. Latencies for the Paired condition also showed a significant decrease for time-points from 40 to 50 min, over preceding time-points.

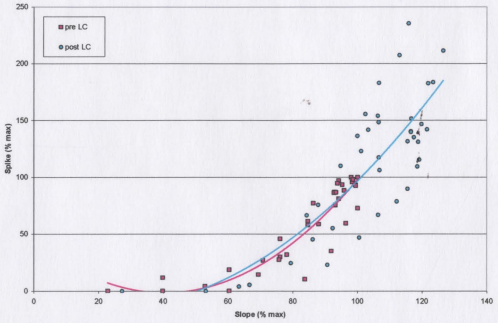

Figure 3.5 – *Spike vs. slope scatterplot of data obtained from I/O recordings.* While an E-S potentiation effect would be demonstrated by a leftward shift in I/O data obtained pre-LC (squares) compared with those obtained post-LC (circles), no such shift is observed here, for the Paired condition. Regression curves plotted for both sets of data are very similar.

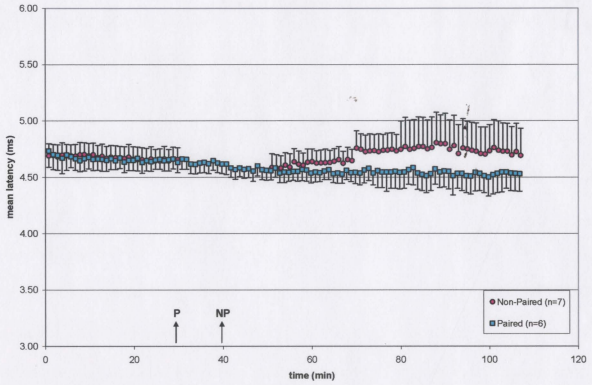

Figure 3.6 – *Population spike latency time-course*. Population spike latency values averaged over 1 min intervals for the Paired and Non-paired conditions. In the Paired condition, latencies measured at time-points 50 min and later are significantly decreased, compared to time-points 30 min or earlier. There were no significant differences in latency for the Non-paired condition.

4. Discussion

4.1 Pairing is required for long-lasting norepinephrine-induced potentiation of dentate population spike and EPSP slope under the present conditions

In animals receiving locus coeruleus activation paired with perforant path stimulation (Paired condition), potentiation of the population spike was long-lasting, persisting until termination of recording at 1.5 to 3 hr. This result is consistent with past findings using this paradigm and was therefore expected. None of the animals in the Non-paired condition, however, showed persistent potentiation of population spike. A transient increase in spike was observed immediately following resumption of stimulation in this group, but a similar increase was observed in the Control condition (see Figure 3.3). This evidence leads us to conclude an associative action of norepinephrine-induced LTP under the conditions present here, such that it requires concurrent perforant path stimulation, at least within a 10 minute window, to occur. This relationship has not been observed *in vitro*; in at least two independent slice studies, norepinephrine or isoproterenol application results in lasting potentiation of population spike and EPSP slope even in the absence of stimulation, which is resumed immediately after a washout of 10 min (Lacaille & Harley, 1985) or 30 min (Dahl & Sarvey, 1990).

Differences in conditions *in vitro* versus *in vivo* may help explain this discrepancy. Notably, cAMP levels measured in the slice paradigm increase 3- to 4-fold in the presence of NE and persist even during washout (Stanton & Sarvey, 1985c). This increased level of cAMP conceivably provides a persistent co-activation signal when stimulation is resumed in these studies. Such robust cAMP activation may not occur

under physiological conditions *in vivo*; NE activity would certainly not be as consistent and prolonged. In the presence of iontophoretically applied norepinephrine or isoproterenol *in vivo*, extracellular levels of cAMP in frontal cortex have been observed to peak at 6 min and gradually return to baseline, an effect which is blocked by the β antagonist timolol (Stone & John, 1990). While this study suggests that cAMP would remain significantly elevated at 10 min, the time at which stimulation was resumed in the present study, it is notable that iontophoretic application of isoproterenol ensures a persistent and prolonged activation of β receptors, similar to that seen *in vitro*. This would not be expected under the more phasic robust release of NE observed with glutamatergic LC activation (Harley & Sara, 1992), although the time-course of cAMP activation under these conditions remains to be evaluated. Interestingly, in the Dahl and Sarvey study, no stimulation was present for either a 30 min application of isoproterenol or a 30 min washout period. This hour-long period seems sufficient to strongly suggest against a pairing requirement. The effect might be explained, however, by the fact that agonist was introduced immediately following stimulation. Any residual dentate gyrus activity could conceivably provide a sufficient pairing requirement in this situation. For the present experiment, to avoid this possibility, 10 min of silence was allowed prior to locus coeruleus activation.

4.2 Lasting EPSP slope potentiation accounts for population spike potentiation

In previous findings using this *in vivo* paradigm, EPSP slope has not consistently been observed to potentiate, despite lasting potentiation of the population spike amplitude (e.g., Harley & Neuman, 1983). The original HFS-LTP effect reported by Bliss & Lømo

included an EPSP potentiation, but one which was insufficient to account for the change in population spike in all experiments (Bliss & Lømo, 1973). The differential potentiation observed in both cases has been interpreted by some as an increase in neuronal excitability without a corresponding increase in synaptic sensitivity, a phenomenon referred to as EPSP-to-spike (E-S) potentiation. One hypothesis explaining E-S potentiation is an increase in *intrinsic excitability*, possibly mediated through an alteration in specific ion channels, particularly A-type K^+ channels (Frick et al., 2004; Daoudal & Debanne, 2003; Zhang and Linden, 2003). In the present study, however, no such E-S potentiation effect was observed. There was a general long-lasting potentiation of EPSP slope in the Paired condition, corresponding to a lasting potentiation in population spike. There was also no observable shift in the slope vs. spike curve for post-manipulation I/O data, as would be expected if E-S potentiation were present (see Figure 3.4). The decrease in spike latency observed in the Paired condition suggests a synaptically-driven spike increase, rather than one attributable to excitability changes alone; spike latencies are not typically observed to change in cases where E-S potentiation is observed (unpublished observations). This is supported by the observation that spike and slope were also highly correlated for most animals (Table 3.1), both before and after LC activation, suggesting a tight coupling of slope to spike in these experiments.

An alternative explanation for the E-S potentiation effect seen in previous experiments is the differential (and antagonistic) effects of LPP versus MPP stimulation. *In vitro*, NE application results in potentiation of the MPP- and depression of the LPP-evoked EPSP

(Dahl & Sarvey, 1990). *In vivo*, stimulation of the PGI input to LC depresses the LPP-evoked EPSP, obtained by stimulation of the lateral olfactory tract, an effect which is blocked by propranolol (Babstock & Harley, 1993). If both pathways are stimulated simultaneously, it is possible that EPSP slope potentiation in MPP is masked by slope depression in LPP. As population spike remains potentiated in these cases, input from these separate pathways may have differential effects upon the response of the postsynaptic granule cell. Indeed, an abrupt change in synaptic transmission properties has been observed between the regions of the DG molecular layer receiving input from either pathway (Abraham & McNaughton, 1984). In the present experiment it might be speculated that the MPP was preferentially activated, and thus the LPP depression effect on EPSP slope potentiation was minimal, although this speculation remains to be systematically examined. It is notable that the pre-manipulation spike latencies were relatively short in the Paired condition (mean = 4.66 ms), and shorter latencies are characteristic of a medially-driven spike (Abraham and McNaughton, 1984). Note that this possibility does not rule out a role for intrinsic excitability changes, particularly at LPP synapses.

It is also prudent to acknowledge a possible caveat to the idea that perforant path electrode placement might account for the present lack of E-S potentiation. In awake rats, Walling and Harley report a population spike potentiation without a concomitant slope increase over three hours following glutamatergic LC activation (Walling & Harley, 2004). Despite this early E-S potentiation, both spike and slope were observably potentiated 24 h following LC activation. Moreover, the authors report that the increase

in slope accounted for the increase in spike in this delayed potentiation, indicating that the early E-S potentiation was no longer present. This is a somewhat puzzling result if differential perforant pathway effects are indeed causing E-S potentiation, as the pattern of potentiation might be expected to persist. It is quite possible, however, that the potentiation effects of the separate pathways *do* follow distinct timelines, such that the depressing effect of the LPP may not have persisted until 24 h in the awake animal study. This possibility remains to be systematically investigated.

4.3 Lasting increase of EPSP slope in the Control condition

The lasting increase of slope observed in the Control condition is a puzzling result, and one that requires some discussion. Both population spike and EPSP slope have been observed to have rising baselines in rats under urethane anaesthesia (Gilbert & Mack, 1999). Slope increases stabilize within 1-2 hr while spike stabilizes within 2-4 hr. In the present study, data obtained in the Control condition was collected significantly earlier than either experimental condition, so it is quite possible that the increase observed in the this condition was due to such rising baseline effects. These data were generally collected within the 2 hr rising baseline period reported by Gilbert and Mack. The failure to observe any lasting increase in slope in the Non-paired condition argues that the effect found in the Control condition is not a necessary consequence of a stimulation rest period.

4.4 Extent of LC activation and synaptic NE levels may be important for long-lasting potentiation

Each animal which showed a potentiation in the Paired condition did so for the duration of the recording period (1.5 to 3 hr). This is in contrast with previous *in vivo* studies reporting spike potentiation in response to glutamatergic LC activation. Harley and Milway (1986), for instance, report a potentiation lasting more than 20 min in only 37% of subjects. This experiment used an identical concentration of glutamate (0.5 M), as well as a similar volume (100-150 nL compared to 200 nL here); however, it utilized a micropipette injection which may have resulted in a more localized ejection area. This possibility suggests that the longevity of the potentiating effect of LC activation may be graded, dependent upon the extent of LC activation. This idea is substantiated by evidence that spike potentiation depends upon a critical level of LC firing in response to glutamate ejection, and the duration of changes are related to the volume of glutamate ejected (Harley and Sara, 1992). These findings appear to demonstrate a role for phasic LC firing rates in facilitating the long-term effects of spike potentiation. Phasic firing of LC has been proposed to give rise to rapid increases in synaptic levels of the neurotransmitter, and thus act as a means of synaptic transmission (Berridge & Abercrombie, 1999), whereas tonic firing has been more associated with volume transmission (Berridge & Waterhouse, 2003). Examining dentate gyrus NE levels using microdialysis while infusing NE intracerebroventricularly, Harley and colleagues report that relatively high levels of NE (30 times basal levels) are necessary to induce lasting potentiation in the dentate gyrus (Harley et al., 1996). It is possible that NE modulates the

longevity of the potentiation effect in a concentration-dependent manner, although more systematic investigation of this possibility remains to be undertaken.

4.5 Possible postsynaptic mechanisms of associative NE-LTP

The associativity requirement observed in this study has important implications for the understanding of the NE-LTP model. As a mechanism underlying emotionally modulated memory, associativity is an expected property of this phenomenon. Despite the lack of associativity in previous *in vitro* studies, it is evident from the present data that, with NE release through pharmacological activation of LC *in vivo*, this requirement is present. The precise nature of this requirement, however, remains to be investigated. It would be of particular interest to evaluate the temporal limits of associativity. Since it is possible to produce LTP under bath conditions *in vitro*, in the absence of stimulation until after washout, it is plausible to speculate that some transient intracellular response to NE is acting as a modulator for any glutamatergic signals that impinge upon the cell within a discrete time window. This modulation is, in effect, an amplification of the signal cascade leading to long-lasting potentiation.

The nature of this signalling cascade continues to be elucidated through various experimental approaches, and a better description of the temporal pattern of NE-LTP may facilitate an informative comparison with the temporal patterns of known signalling cascades. One interesting early study in particular provides some insight into this temporal pattern. Characterizing the electrical behaviour of NE-containing LC neurons in spider monkeys, Aston-Jones and colleagues demonstrated an increased conduction

velocity in a large subset of these cells, compared with values previously obtained in rats (Aston-Jones et al., 1985). These enhanced velocities corresponded to similar signal conduction latencies to target areas between the two species, suggesting a conservation of these latencies across species. This finding ultimately argues for an important role of LC signal latency in the functioning of this structure, and suggests a requirement for temporal precision between events activating the LC and its subsequent effects upon target cells. Further investigation is necessary to better elucidate the nature of this temporal relationship.

4.5.1 Kinase activity

The major route through which the β-adrenoceptor mediates potentiation is presumably its activation of PKA. PKA has numerous intracellular functions, which are well documented. Of particular importance to the present topic is its ability to enhance both AMPAR (Banke et al., 2000; Mangiavacchi & Wolf, 2004) and NMDAR (Chen et al., 2006; Crump et al., 2001) currents. Enhanced AMPAR currents effectively sensitize a cell to further excitatory events, and NMDAR enhancement can conceivably result in an increased Ca^{2+} signal, which is a critical component of LTP induction (Yamauchi, 2005, for review). PKA can also act to phosphorylate CREB, which serves to regulate the transcription of genes and has been implicated as critical to both LTP and long-term memory (Bourtchuladze et al., 1994; Bozon et al., 2003). β-adrenoceptor activation has also been linked to CREB phosphorylation in an rat pup olfactory memory paradigm. Significant CREB phosphorylation is seen only at learning-effective doses of isoproterenol in this paradigm (Yuan et al., 2000), and pCREB is critical for long-term

memory formation to occur (Yuan et al., 2003). In addition, PKA can activate I-1, which has the effect of inhibiting protein phosphatases and effectively prolongs kinase activity. Through this means it has also been proposed as a gate for CaMKII effects upon LTP (Blitzer et al., 1995). CaMKII, which is activated by a Ca^{2+}/CaM signal, can act as a prolonged kinase signal due to its ability to autophosphorylate and thus remain constitutively active for relatively long periods of time. In HFS-LTP recorded *in vitro* from CA1, for instance, pCaMKII was observed to peak at 8 h (Ahmed & Frey, 2005). The downstream targets of CaMKII include AMPAR (Derkach et al., 1999) and CREB (Ahmed & Frey, 2005).

4.5.2 NMDA receptors

It is tempting to conclude that the pairing requirement demonstrated here implicates the NMDAR as a critical component for this phenomenon. This possibility is consistent with the findings of several *in vitro* studies. For instance, application of either D(-)APV, or 3-((+/-)2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP), both NMDAR antagonists, prevents both HFS- and NE-LTP in dentate gyrus slices (Burgard et al., 1989; Dahl & Sarvey, 1990). β-adrenoceptor mediated enhancements of Ca^{2+} influx and K^+ efflux are also antagonized by D(-)APV (Stanton et al., 1989). Moreover, intracellular recordings from dentate gyrus granule cells, *in vitro*, reveal both cell depolarization and increased input resistance which persists at least 90 min after washout, an effect which is β-adrenoceptor dependent. While application of D(-)APV does not prevent these effects, it does attenuate their persistence, resulting in a return to baseline values by approximately 16 min (Stanton et al., 1989).

Since NMDARs require both a glutamatergic signal and a pre-existing depolarization, they fit nicely into the requirements imposed by Hebb's Postulate, namely that a cell must be active in order for an input to effect a lasting change to its synaptic response. β-adrenoceptor activation may serve to provide an amplification of NMDAR effects when occurring prior to these effects. The findings of Vanhoose and Winder (2003) provide a quite interesting insight into this possibility. Specifically, the authors report a long-lasting and preferential phosphorylation of the Ser845 site of the AMPAR subunit GluR1, which has a number of links to LTP induction (Vanhoose & Winder, 2003) in response to β-adrenoceptor activation. Conversely, NMDAR activation actually dephosphorylated this site, and failed to phosphorylate it even in the presence of multiple phosphatase blockers, despite elevated cAMP levels threefold above those produced by β-adrenoceptor activation. Moreover, β-adrenoceptor activation prior to that of NMDAR resulted in an additive phosphorylation of Ser845, whereas NMDAR activity prior to that of β-adrenoceptor prevented any phosphorylation. The temporal interaction of these two receptor effects is especially interesting in light of the present pairing effect, and further examination of such interactions upon the cell signalling cascades underlying LTP may prove enlightening.

The evidence above presents a strong argument for the role of NMDARs in associative NE-LTP. However, some lines of evidence suggest the issue may be more complex. *In vivo* micropipette application of the NMDA antagonist ketamine does not block, and in fact enhances NE mediated potentiation of population spike in the rat dentate gyrus

(Frizzell & Harley, 1994). While the enhancement was attributed to the ability of ketamine to increase presynaptic norepinephrine release, the finding demonstrates that at least some component of NE-potentiation is NMDA-independent. It is noteworthy, however, that this study had an exceptionally small success rate for longer spike potentiation (3 of 20 experiments), which prevents drawing a clear conclusion with respect to the involvement of NMDARs in this phenomenon. Further examination of this effect is warranted.

4.5.3 L-type Ca^{2+} channels

An alternative means of Ca^{2+} influx is the L-type Ca^{2+} channel, and since this channel is voltage-dependent, it would also require a glutamate signal for activation. Indeed, inactivation of L-type channels has been shown to disrupt both LTP in CA1 and hippocampal-dependent spatial memory, as well as resulting in decreased activation of the MAPK pathway – important for LTP – in CA1 (Moosmang et al., 2005). In a BLA-induced dentate gyrus LTP paradigm, blockade of L-type channels significantly impairs induction of NMDA-independent HFS-LTP (Niikura et al., 2004). Moreover, in an *in vitro* preparation of rat visual cortex, HFS-LTP is induced only in the presence of isoproterenol (Kato, 1993). Blocking either NMDARs or L-type channels prevents this isoproterenol gated potentiation and, notably, the L-type channel agonist BAY K8644 can substitute for isoproterenol in facilitating its induction. Additionally, using a patch clamp technique, Gray and Johnston (1987) showed that application of NE to dentate granule cells enhances the activity of voltage-dependent Ca^{2+} channels, and this effect was mimicked by cAMP application. Taken together, these findings suggest a possible role

for these non-NMDAR channels in the pairing effect observed in the present study, which is worthy of further investigation. As a caveat, however, it must be remarked that, while the NMDAR, which requires a local glutamate signal, provides the important component of input specificity, L-type Ca^{2+} channels appear to lack this requirement, as they are simply voltage-dependent and can therefore be activated by depolarization alone.

4.6 Conclusions

The present findings are consistent with a general model of arousal-modulated memory, as proposed by Kety (1970) and others. An associative role for norepinephrine in the induction of NE-LTP in rat dentate gyrus fits with the idea that this phenomenon is a mechanism underlying emotional or arousal associated memory, since it provides a link between the brainstem and cortical signals associated with arousal and the sensory patterns that are encoded in memory. Much remains to be investigated with respect to this relationship, however, not the least of which is the functioning of this system in the alert, freely-moving organism, whose cortical activation and LC firing patterns are more subtle than the robust, wholesale activation obtained under the present paradigm. One such avenue of inquiry that is currently being investigated is the effect of LC activation on electroelcephalographic (EEG) patterns in discrete brain structures such as hippocampus and cortex. In the hippocampus in particular, LC activation has been reported to increase power in the theta frequency of the EEG signal, and suppress power in the beta and gamma frequencies (Brown et al., 2005). The importance of theta phase in determining the dynamics of signalling patterns in memory formation (Hasselmo, 2005) is also an interesting line of investigation, and may prove informative with respect to both the

present findings and the broader pursuit of an answer to the question "how does memory work?"

5. References

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