INSIGHTS INTO THE EFFECTS OF NOREPINEPHRINE ON MEMORY:
STUDIES OF NORADRENERGIC MODULATION OF SYNAPTIC PLASTICITY IN THE DENTATE GYRUS OF THE RAT

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INSIGHTS INTO THE EFFECTS OF NOREPINEPHRINE ON MEMORY:

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PLASTICITY IN THE DENTATE GYRUS OF THE RAT

by

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Abstract

Norepinephrine (NE) is known to increase memory for emotional events. This catacholamine, applied exogenously or through natural release mechanisms, increases memory in rats and humans, and increases cell excitability in areas of the rat brain (NE-induced potentiation), in a manner congruent with other models of long-lasting memory support. The purpose of this dissertation is to investigate the effects of NE on synaptic efficacy in rat dentate gyrus, a component of the memory structure, the hippocampus. Three chapters are presented, each utilizing a different method of intensifying the synaptic levels of NE in the dentate gyrus, and investigating the ensuing effects on the perforant path-dentate gyrus evoked potential.

In the first chapter, exogenous NE and noradrenergic agents were applied in the lateral ventricle of the awake rat, a technique used to meld *in vitro* bath application of drugs, with *in vivo* whole animal recording in the absence of anesthetic. Here it was found that NE, infused ventricularly reliably increased the synaptic contribution of the evoked potential (EPSP slope), a result characteristic of bath application of NE in the hippocampal slice. NE also increased dentate granule cell firing as indexed by the population spike amplitude of the evoked response. Though these initial increases returned to baseline within 30 min, a subset of animals monitored 24 hr after NE infusion, demonstrated long-term potentiation of the EPSP

slope and population spike amplitude. Both the short-term and long-term potentiation were dependent on β-adrenergic receptor activation. The data suggest NE can mediate multiple phases of synaptic plasticity and long-term potentiation may not reflect an uninterrupted progression from short-term potentiation, a widely held theory of how long-term memories are formed.

In the second chapter, the evoked population activity in the dentate gyrus of the anesthetized rat was monitored while the recently discovered neuropeptide orexin-A (OREX-A) was infused directly into the LC as a method of discretely activating LC neurons to evoke NE release in the hippocampus. Application of OREX-A at 3 concentrations (1,10, and 100 nM) produced a robust potentiation of the population spike amplitude lasting for greater than 3 hr. This potentiation was reduced by intradentate application of the β-adrenergic receptor antagonist propranolol. Infusion of vehicle into the LC failed to produce changes in the evoked activity. LC infusion of the α_2-adrenergic receptor agonist clonidine prior to OREX-A infusion, a method of pharmacologically inactivating LC neurons, blocked the effect of OREX-A. Lastly, hippocampal levels of NE were monitored to confirm that infusion of OREX-A into the LC produced a transient elevation of NE levels in the hippocampus. This is the first study to investigate hippocampal synaptic effects of orexinergic activation of LC neurons and the first to suggest that OREX-A can produce changes in synaptic activity reminiscent of memory formation.

The third study takes advantage of a technique developed in the anesthetized preparation whereby endogenous release of NE is initiated by the application of the excitatory amino acid transmitter glutamate to the noradrenergic neurons of the LC. As anesthetized preparations are limited by the duration over which effects can be monitored, this technique was used in the awake rat to assess the effects of NE on the dentate gyrus evoked potential at periods 24 hr after LC activation. Glutamatergic activation of the LC neurons produced a robust facilitation of the dentate gyrus population spike and EPSP slope, an increase more sizable than that seen within the first 3 hr following LC activation. This effect is unusual in that short-term potentiation of the evoked potential was not a necessary requirement for the NE-induced long-term potentiation observed at 24 hr. Though this effect is novel in the mammalian nervous system, there are behavioral studies that are in keeping with this finding and similar synaptic effects have been seen in an invertebrate. These effects were dependent on the activation of β-adrenergic receptors and on the synthesis of *de novo* protein.

Taken together these studies suggest locus coeruleus activation has a special role in the initiation of long-term increases in synaptic strength and cell responses in circuitry known to be critical for memory formation and, further, that short-term and long-term synaptic plasticity may be supported by distinct and independent processes.

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I dedicate this work to my parents David and Kathy Walling for their love, and for their never ending support. And most of all, I would like to thank them for allowing me to share in their lives.

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5-HT	- Serotonin
6-OHDA	- 6-hydroxydopamine
ACh	- Acetylcholine
Aniso	- anisomycin, protein synthesis inhibitor
CA1	- Ammon's horn regio superior
CA3	- Ammon's horn regio inferior
cAMP	- Cyclic adenosine 3',5'-monophosphate
CLON	- Clonidine: α_2-adrenergic receptor agonist
CREB	- cAMP-response element binding protein
CRF	- Corticotrophin Releasing Factor
DA	- Dopamine
DBH	- Dopamine beta-hydroxylase
DG	- Dentate gyrus
EPSP	- field excitatory postsynaptic potential (unless specified)
E-S coupling	- EPSP/population spike ratio
GLUT-LC	- Glutamatergic activation of the locus coeruleus
GPCR	- G-protein coupled receptor
HPLC	- high performance liquid chromatography
ISO	- Isoproterenol: β-adrenergic receptor agonist
LC	- Locus coeruleus
LTP	- Frequency (tetanus) induced long-term potentiation
MAPK	- Mitogen-activated protein kinase (ERK)
MHPG	- Methoxy-4-hydroxyphenylglycol: metabolite of NE in brain
NE	- Norepinephrine/noradrenaline
NE-LTP	- Norepinephrine-induced long-term potentiation
NMDA	- *N*-methyl-D-aspartate
ORX	- Orexin (hypocretin)
ORX-A	- Orexin-A subtype (hypocretin-1)
pCREB	- phosphorylated cAMP-response element binding protein
PHENT	- Phentolamine: α-adrenergic antagonist
PKA	- cAMP-dependent protein kinase A
PROP	- Propranolol: β-adrenergic receptor antagonist
TH	- Tyrosine hydroxylase, precursor of DA, NE and epinephrine
TTX	- Tetrodatoxin, voltage-gated sodium channel blocker

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Introduction

...it is a fact that there are some movements, by a single experience of which persons take the impress of custom more deeply than they do by experiencing others many times; hence upon seeing some things but once we remember them better than others which we may have seen frequently.

(Aristotle, 350 BC)

The complex puzzle of how we retain tacit representations of past events and circumstances in our minds has bewildered philosophers and scientists, most likely since the human brain evolved enough to recognize the complexity of understanding its own self. If you think back to your most memorable moments, you will probably find that the memories that are most clear are about events that carried with them some emotional relevance. This memory mechanism is remarkably conserved across species, allowing each a method of forming and retaining impressions of events that are significant and perhaps adaptive.

The present work examines the hypothesis that the neuromodulator norepinephrine (NE), a substance known to have an important role in emotion and attentional processes, contributes to the formation of memory for events that hold some emotional significance. More specifically, the present thesis

investigates the effects of NE and NE release on the modification of brain connections in the dentate gyrus, a region of the mammalian brain previously implicated in memory acquisition and a region that provided the first physiological evidence of long-lasting synaptic plasticity in the adult mammalian brain. Three sets of experiments set out to address these issues. The first set of experiments explores the effects of intraventricular application of NE into the central nervous system of the awake rat via the lateral ventricles in order to assess alterations in the perforant path-dentate gyrus evoked potential in a preparation that is free from anesthetic constraints. This technique allows long-term assessment (>24 hr) of NE induced plasticity and investigation into the pharmacology behind the effects. The second set of experiments examines alterations in the dentate gyrus evoked potential of the anesthetized rat after endogenous release of NE evoked by application of the excitatory neuropeptide orexin-A (ORX-A) in the locus coeruleus (LC), the primary supplier of NE to forebrain structures. This experiment is the first to detail physiological responses in the hippocampus, reminiscent of memory, after orexinergic activation of the LC which provides a method for selectively activating noradrenergic neurons in the LC. The last set of experiments explores the NE-induced long-term changes in dentate gyrus plasticity of the awake rat. In this study NE release was evoked by glutamatergic activation of the LC, and the resultant physiological response in the dentate gyrus could be followed for long-term periods, producing increased support for the role of NE

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in memory as NE-induced potentiation in the dentate gyrus has not been examined at long-term periods.

This introduction considers modern ideas about the biological underpinnings of memory, introduces evidence for the role of NE in memory, briefly reviews the anatomy of the hippocampus and dentate gyrus and then focuses on the characteristics of the NE input to the dentate gyrus and prior evidence for its role in synaptic plasticity in that structure.

1.1 HEBB'S POSTULATE

Existing memory or memory traces, as many thousands or more there may be in a single organism (be it human, monkey, cat, rat, slug or fruit fly), must somehow be represented within what constitutes the physiological attributes of the organism's "brain". How these traces are made into long-lasting, stable representations of prior events has been under meticulous investigation for more than a century.

Ramon y Cajal was the first to propose that alterations in the connections between cells in the brain are the mechanism by which memories are stored (y Cajal, 1894) as referenced by (Bailey et al., 2000). Some fifty years later Donald O. Hebb furthered this theory by formulating what he referred to as "A Neurophysiological Postulate". In his work, "Organization of Behavior", published in 1949 (Hebb, 1949), Hebb put to paper a comprehensive conjecture underlying the principle of memory by

taking into account integration of sensory input and the resultant neural activity and reverberation that is now believed to correspond to the process in which memories are laid down. It reads:

> *When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased.*

> Donald O. Hebb (Hebb, 1949)

Widely known and accepted today as "Hebb's Postulate", this ground-breaking theory describing "homosynaptic" plasticity proposed that reverberating activity of cell assemblies would initiate activity in a cell complex and could recruit, maintain and perpetuate activity of other cells as a mode of laying down the basis of neural memory traces.

In addition to the homosynaptic principle, Hebb deduced that the firing of the presynaptic neuron and consequent firing of the post-synaptic neuron would become associated in that the next time the presynaptic neuron fired it would be more likely to successfully initiate a response in the postsynaptic cell (associativity). This would also be selective in that synapses not receiving input would not be altered (input specificity). Theories of mammalian memory

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formation use Hebb's basic premises, homosynaptic plasticity, associativity, and input specificity strengthen neural circuits associated with significant stimuli to produce a long-term representation within the cellular circuitry.

1.2 KETY'S HYPOTHESIS

Twenty years after Hebb proposed his theory of reverberating cell assemblies noted psychiatrist Seymour S. Kety proposed a theory based upon the observation that an organism's ability to learn was heightened when it was in an aroused state (for examples see: Davidson et al., 2001; Kazui et al., 2003; Yoder and Elias, 1987). Kety proposed that norepinephrine (NE), "… released in affective states may regulate trophic processes occurring at recently activated synapses to promote selectively the persistence of adaptive neuronal patterns or states" (Kety, 1972). Perhaps proposed independently of Hebb's work (perhaps not), Kety credited his ideas to the work of physicists Widrow and Angell (Widrow and Angell, 1962) who developed a system of "memistors" (conductive units immersed in salt solutions). When connected together into complex circuits, the conductivity of a network could become permanently enhanced when a current was applied to just one of the units (as described in (Kety, 1970a).

Noticing noradrenergic metabolism increased during behavioral manipulations, Kety further contemplated that activation of the second messenger cyclic adenosine 3',5'-monophosphate (cAMP) by NE may initiate

a cascade that leads to synthesis of new proteins "probably at synapses contingent on neuronal activity wherever it occurs throughout the brain" (Kety, 1970b).

1.3   NOREPINEPHRINE

1.3.1   Norepinephrine or noradrenaline? You say tomato...

The history surrounding the discovery of NE reads much like a modern day soap opera of Nobel Laureates. In favor of brevity a short synopsis is presented here. For a more expansive rendition see (Valenstein, 2002). The story begins in 1899 when the study of chemical transmission, beginning in the visceral system, was in its infancy. John Jacob Abel, a biochemist, isolated what he referred to as "epinephrine" from the adrenal medulla (epinephrine derived from *epi-*"above", *nephros-* "kidney" though a major pharmaceutical would later patent it as "adrenaline"). Sometime during the period of the First World War Sir Henry Dale (Nobel Laureate 1970) described a sympathomimetic substance synthesized by his colleague George Barger that was similar in composition, though more powerful than epinephrine. It would be another 25 years before Swedish physiologist Ulf von Euler (Nobel Laureate, 1970) isolated and defined a compound in sympathetic tissue related to adrenaline (von Euler, 1946). Up until 1954 it was not known that

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NE was also present in the CNS when Marthe Vogt discovered that "sympathin" (a name coined earlier by Walter Cannon to describe this undetermined substance related to epinephrine) was present in various areas of the brain.

1.3.2. NE Synthesis

NE belongs to the catacholamine family and is classified as a small molecule neurotransmitter, with a molecular weight of 187.7. It is synthesized from its precusor dopamine (DA- synthesized from tyrosine→DOPA by the enzymes tyrosine hydroxylase and dopa-decarboxylase respectively) taken up into synaptic vesicles by the enzyme dopamine-β-hydroxylase (DBH). Following the chain of catacholamine synthesis to completion, epinephrine (adrenaline) can then be synthesized in neurons from NE by the enzyme phenylethanolamine *N*-methyltransferase).

There exists two adrenergic receptor classes to which NE binds, the α- and β-adrenergic receptors (to be discussed further below). After its release from nerve terminals and receptor activation, NE action is terminated by a number of processes such as enzymatic breakdown by monoamine oxidase (to dihydroxymandelic acid) and catachol-O-methyltransferase (to normetanephrine) and re-uptake into both neural and glial cells by a variety of NE-transporters.

1.4 NOREPINEPHRINE AND MEMORY

Noradrenergic transmission in the central nervous system has been implicated in numerous behavioral and physiological processes. Pathophysiologically, disruption of noradrenergic transmission has been implicated in depression (e.g. Tejani-Butt et al., 1994), seizure activity (e.g. Carre and Harley, 1986; Clough et al., 1994), and Alzheimer's disease (e.g. Kalaria and Andorn, 1991; Powers et al., 1988).

1.4.1. Human Models

As many before have iterated, human behavioral experience indicates that we recall events involving states of heightened arousal more vividly than non-emotional or monotonous events. There is a large body of literature implicating activation of the noradrenergic system in models of human memory supporting the hypothesis that Kety put forth over thirty years ago. Many studies have utilized emotionally arousing stimuli to investigate memory during periods of heightened arousal. An example of an emotional story comes from Cahill et al. (1994) about a small boy who is going to visit his father who works in a hospital. On his way to the hospital the boy is involved in a serious accident and the doctors have to work to save his leg from being amputated. The neutral presentation of this story would be the boy visiting his father at work at the hospital and witnessing an emergency drill while he was

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there. In this study, subjects given the β-adrenergic receptor antagonist prior to the emotional story recalled less than subjects given a placebo drug.

Noradrenergic stimulants (O'Carroll et al., 1999) or behavioral tasks (Nielson and Jensen, 1994) have also been used to increase levels of NE and facilitate memory formation. Again, under these conditions subjects given β-adrenergic receptor antagonists inhibit recall for memories tested (Nielson and Jensen, 1994; O'Carroll et al., 1999).

A recent study investigating noradrenergic metabolism and memory scores for subjects one week after viewing an emotionally charged story found that recall scores were highly correlated with blood levels of methoxy-4-hydroxyphenylglycol (MHPG), a major metabolite of NE in central nervous tissue, at the time of viewing the story (Southwick et al., 2002). An important progression from these studies occurred when it was further determined that peripheral enhancement of noradrenergic activity was not necessary or sufficient to produce the effect on memory and thus enhancement of NE in the CNS was critical to increase memory formation (van Stegeren et al., 1998).

1.4.2. Animal Models

The emphasis of NE involvement in behavioral modulation has evolved over the last 50 years. Early investigation associated NE with reward pathways in the brain. For example, in a preparation developed by Olds and

Milner (1954) in which rats were chronically implanted with stimulating electrodes in the medial forebrain bundle (MFB), central administration of NE facilitated self-stimulation responses (Wise and Stein, 1969). Some years later, using the same principles of MFB stimulation it was found that self-stimulation of the locus coeruleus (LC), the primary noradrenergic nucleus located in the brain stem, was found also to produce rewarding effects (Crow et al., 1972) and can initiate place preference learning in the rat (Duvauchelle et al., 1992). The focus of studies soon turned to investigations of noradrenergic modulation of learning and memory due to increasing evidence that NE was released during learning procedures and that manipulation of NE levels or synaptic effects could alter learning (see below).

Investigations into the role of NE in memory formation in rodents have allowed researchers the liberty of manipulating experimental variables and of course, afford a higher level of control over experimental conditions. Accordingly, there exists a large amount of data, encompassing a variety of experimental procedures that implicates NE in learning and memory. Examples of such are as follows:

1.) Measurement of NE during learning:

The most obvious test of the hypothesis that NE is involved in the formation of long-term memory would be to investigate whether increases in synaptic levels, or levels of activity of the catacholamine neurons, occur

during the learning. Evidence supporting this premise has been presented by numerous investigations. One of the first behavioral studies investigating the effects of NE on learning was done by Fuxe and Hanson (1967) in which they determined by a histofluorescent technique, that NE release in the brain increased during acquisition of a conditioned avoidance response. More notably, this effect was higher in the septal-hippocampal area, implicating the limbic structures in NE-influenced learning.

In the amygdala, levels of NE after training sessions for a passive avoidance task increased to over 300% of pretraining levels with initial NE responses being highly correlated with successful learned responses at the 24 hr retention test (McIntyre et al., 2002).

2.) Exogenous application of NE and noradrenergic agonists:

If NE is involved in memory formation then memories for certain events should be strengthened if it is exogenously applied to structures in which neural assemblies are active during acquisition. In line with this reasoning Lee et al. (1993) demonstrated that application of exogenous NE prior to a one-way passive avoidance task increases retention of the task in a dose-dependent manner when placed directly into the dentate gyrus, a structure of the hippocampus known to be involved in memory (Jeltsch et al., 2001). Similar effects have been seen when NE is infused into area CA1 of the

hippocampus (Bevilaqua et al., 1997; Izquierdo et al., 1998) and entorhinal cortex (Izquierdo et al., 1998).

3.) Disruption of NE transmission:

Pretreatment of rats with the selective noradrenergic neurotoxins DSP-4 or 6-hydroxydopamine (6-OHDA) prior to behavioral training indicates that disruption of noradrenergic modulation disturbs active avoidance (Bennett et al., 1990) and one-trial passive avoidance memory (Rainbow et al., 1976). Central DSP-4 lesions have also been shown to inhibit appetitive memory tasks in addition to aversive tasks (Kumar and Karanth, 1994). Using a method to restrict NE synthesis, inhibitors of the NE synthesizing enzyme DBH have shown similar effects (Izquierdo et al., 1979). In another study administration of NE reverses the effects of DSP-4 lesions (Stein et al., 1975). Likewise, genetic manipulation of noradrenergic function to decrease NE levels in the CNS decreases memory for a variety of behavioral tasks (Kobayashi et al., 2000; Low et al., 1984).

One particularly well-characterized model of noradrenergic modulation of memory is the olfactory learning model in rat pups. This model provides evidence of the necessity of noradrenergic activation, particularly that of the β-adrenergic receptor, for the formation of long-term olfactory memory. When presented together with an odor (conditioned stimulus- CS), the tactile

stimulation used to mimic maternal contact (unconditioned stimulus- UCS) increases bulbar levels of NE (Rangel and Leon, 1995) and results in a conditioned preference for the particular odor presented. This olfactory preference produced by tactile stimulation (UCS) can also be produced by direct β-adrenergic receptor activation (Langdon et al., 1997) or by stimulation of the locus coeruleus (Sullivan et al., 2000) and can be prevented by pretraining administration of propranolol (PROP), a β-adrenergic receptor antagonist (Sullivan et al., 2000). β-adrenergic receptor activation also leads to an increase in intrabulbar levels of cAMP (Yuan et al., 2003a) while phosphorylation of the cAMP response-element binding protein (CREB), the "molecular switch" of memory, is also necessary for the formation of the odor preference. At this time, the role of noradrenergic modulation of memory involving the hippocampus is not as well characterized.

1.5 THE HIPPOCAMPUS

The hippocampus is an "allocortical" structure (a cortical structure with less than six layers), and has classically been defined as a part of the "limbic system", an aggregate arrangement of cortical, and sub-cortical structures, involved in emotion and memory formation. There have been many theories proposed to explain the role of the hippocampus in memory formation. In 1978, O'Keefe and Nadel presented The Cognitive Map Theory (O'Keefe and

Nadel, 1978), which stated that the hippocampus was a structure that was involved in the formation of memories that are specifically spatial. Countless studies investigating the effects of hippocampal lesions have substantiated the role of the hippocampus in spatial learning. In turn, noticing that hippocampal damage often disrupted memories that were not spatial in nature, Rudy and Sutherland proposed the *Configural Association Theory* (Rudy and Sutherland, 1989). They theorized that the hippocampus plays a role in assessing the association among cues, not in remembering specific objects or events without context.

1.5.1. Anatomy

The simplest definition of the "hippocampal formation" defines a largely unidirectional circuit involving the entorhinal cortex, dentate gyrus and the three fields of Ammon's Horn (CA1, CA2 and CA3; See Figs. 1-1 and 1-2). The major input into the dentate gyrus arises from the axons of the medial and lateral entorhinal cortex via what is termed the perforant pathway (also known as the angular bundle). From the granule cells of the dentate gyrus, information is passed to area CA3 by way of the granule cell axons, the mossy fibers. From here, CA3 pyramidal cell axons collateralize and either project within CA3, or they project to area CA1 through the Schaffer collaterals (Schaffer collateral pathway). The dentate gyrus-CA3-CA1 projections have typically been the most studied and are often referred to as

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the "trisynaptic pathway". CA1 sends output to entorhinal cortex through the subiculum and back to the deep layers of the entorhinal cortex. An overview of the structures of the hippocampal formation will be presented in brief (with particular emphasis on the anatomy of the dentate gyrus).

1.5.1.1. *Entorhinal Cortex*

The entorhinal cortex is comprised of six cortical layers which are highly laminated in function. Afferent projections to the first four layers of the entorhinal cortex arise from the following areas: Layer I: olfactory, perirhinal cortex and subiculum; Layer II: parasubiculum; Layer III: amygdala, presubiculum and perirhinal cortex; Layer IV: septum, subiculum and limbic cortex (Amaral and Witter, 1995). See Fig. 1-1. Superficial layers (I-III) project extrinsically to the hippocampus with Layer II being the primary input into the dentate gyrus via the perforant pathway, and Layer III projecting to area CA1 and the subiculum (Steward and Scoville, 1977;Witter and Groenewegen, 1990). These projections are glutamatergic (White et al., 1977) although GABA-ergic perforant path projections have also been observed (Germroth et al., 1989). The deep layers (IV-VI) of the entorhinal cortex receive relatively little intrinsic innervation from the superficial layers of the entorhinal cortex, instead these layers primarily receive output from area CA1 and the subiculum (Kloosterman et al., 2003; Naber et al., 2001).

Fig. 1-1   Diagrammatic representation of the hippocampal formation

Diagram detailing major projections of the hippocampal formation with extrinsic input arising from perirhinal (PR) and frontal cortices. See text for details. Abbrev: Sub: subiculum; PreSub: presubiculum; ParaSub: parasubiculum. Adapted from (Amaral and Witter, 1995).
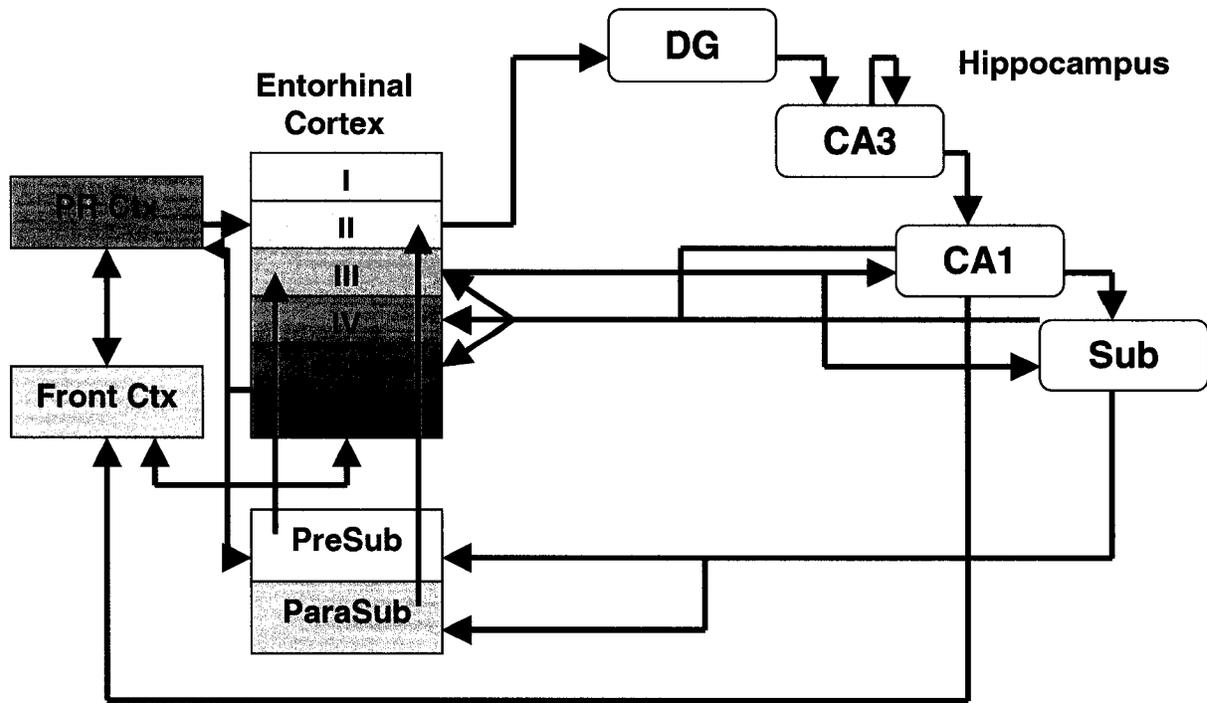
Fig. 1-1   Diagrammatic representation of the hippocampal formation

1.5.1.2. Dentate Gyrus and Hilus

The dentate gyrus, or fascia dentata, consists of three cortical layers. The principle cell layer consists of densely packed granule cells; the molecular layer, primarily consisting of a complex arborization of granule cell dendrites; and a polymorphic layer, historically referred to as CA4, or the polymorphic layer, but now more commonly designated as hilus or the hilar region (Fig.1-2). Granule cells are characterized by a spiny dendritic arborization that projects unidirectionally into the molecular layer. Information originating in the entorhinal cortex projects to the dentate gyrus where it synapses on the granule cell dendrites exclusively in a highly typified manner. Glutamatergic projections arising from the lateral entorhinal cortex synapse on the distal 1/3 of the molecular layer while afferents of the dentate gyrus originating from the medial entorhinal cortex synapse on the middle 1/3 of the granule cell dendritic tree (Steward, 1977). The inner 1/3 of the molecular layer also receives intrinsic, presumably excitatory input from both ipsilateral and contralateral hilar regions (Blackstad, 1956; Zimmer, 1971). Electrical stimulation of the medial and lateral entorhinal cortex produces two identifiably different excitatory post-synaptic potential (EPSP) profiles in the dentate gyrus, with lateral perforant path stimulation evoking an EPSP and population spike of longer latency than that seen with medial perforant path stimulation (Abraham and McNaughton, 1984; McNaughton and Barnes, 1977).

Fig. 1-2 The hippocampus and the hippocampal formation

Diagrammatic representation of the anatomy of the hippocampal formation and the hippocampus. Abbrev: EC, entorhinal cortex; PrS, presubiculum; PaS, parasubiculum; F, fornix.
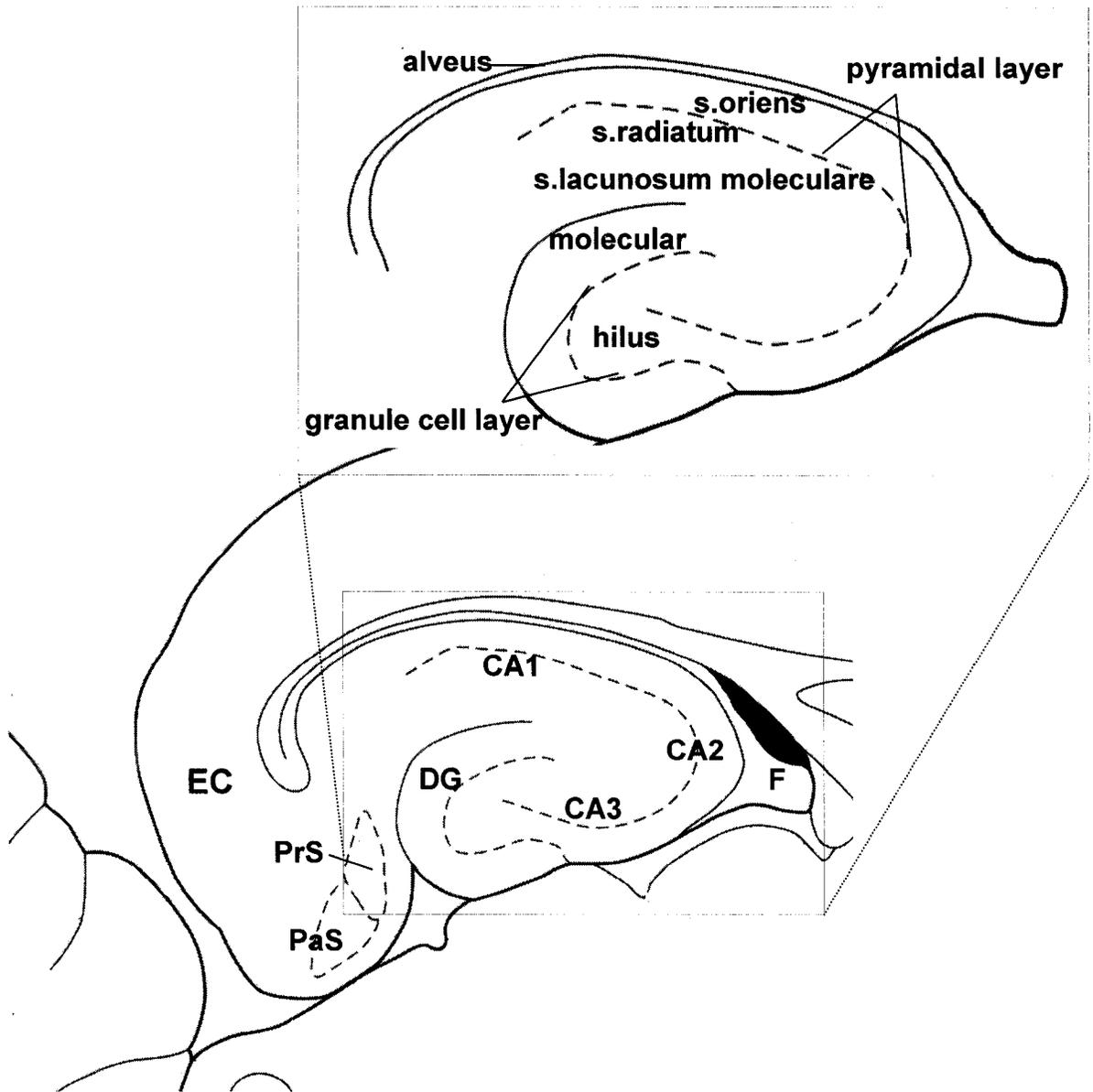
Fig. 1-2 The hippocampus and the hippocampal formation

The hilar region possesses numerous cell types including "aspiny" interneurons and the most prevalent cell in the hilar region, the "spiny" mossy cells. Dendrites of mossy cells most often extend only within the polymorphic region but can often penetrate the granule cell layer and terminate in regions as far as the outer molecular layer (Scharfman, 1991). Numerous types of inhibitory interneurons have been identified in the hilus. Many of these cells are immunoreactive to substances such as GABA, parvalbumin, calbindin, somatostatin, and substance P (Boyett and Buckmaster, 2001; Sik et al., 1997; Sloviter et al., 2001). Though the role of the dentate gyrus-CA3 connection has typically been classified as excitatory, inhibitory GABAergic cells in the hilus receive direct excitatory input from the granule cells which may serve to suppress activity in area CA3 (Penttonen et al., 1998). The granule cells also receive a GABA-ergic projection from terminals of "basket cells" located under the granule cell layer (Kosaka et al., 1984). Other inhibitory influences on dentate granule cells arise from "chandelier cells" of the molecular layer and somatostatin-positive cells in the hilus (Morrison et al., 1982).

The only projection leaving the principle cells of the dentate gyrus is the mossy fiber projection originating from the unmyelinated axons of the granule cells synapsing with CA3. A single mossy fiber makes extensive contact with CA3 pyramidal cell dendrites in stratum lucidum and its

projections from the mossy fibers extend throughout the entire CA3 field to the point where CA3 and CA2 converge.

Although the dentate gyrus receives the largest projection from the entorhinal cortex it also receives a number of projections from subcortical structures including: the septum (Amaral and Kurz, 1985), the supramammilary area of the hypothalamus (Amaral and Witter, 1995) as well as what will be discussed at length, brain stem monoaminergic projections.

What is the role of the dentate gyrus in memory? It has been difficult to separate the dentate gyrus from the rest of the tri-synaptic pathway in order to study selectively the behavioral role of the granule cells alone. However, lesion studies using colchicine, a selective neurotoxin to dentate granule cells have been used as a method of probing the role of the dentate principle cells in memory. These studies have found that damage to granule cell layer disrupts acquisition of active avoidance (McLamb et al., 1988), and passive avoidance tasks (Nakayama.T. and Sawada, 2002) and impairs spatial memory in water maze tests (Jeltsch et al., 2001). These effects are dependent on the extent of damage to the granule cell layer.

1.5.1.3.   Ammon's horn

The principle cell of the hippocampus, or Ammon's Horn, is the pyramidal cell (Fig. 1-2). These cells have two dendritic arborizations, the basal dendrites that extend into stratum oriens and the apical dendrites which extend towards the hippocampal fissure. The principle cells of CA3 are typically larger than those found in region CA1.

1.5.1.3.1.   CA3

CA3 neurons collateralize within CA3 as well as CA2 and CA1. They also project to the same regions contralaterally and a small number also project to the hilar region (Amaral and Witter, 1995). As an example of the largely unidirectional flow of the hippocampus, CA3 neurons do not appear to project to the entorhinal cortex though they receive direct projections from this area (Fig. 1-1). CA3 neurons project to CA1 through axons known as the Schaffer collaterals. These projections are topographically organized and vary according to the transverse location of origin of the projecting CA3 neuron.

1.5.1.3.2.   CA2

The CA2 region of the hippocampus is unique in that it is difficult to delineate from CA1 and CA3 without using particular histological techniques. An interesting component of CA2 principle cells is that they appear to contain

a large amount of calcium binding proteins, particularly parvalbumin (Leranth and Ribak, 1991). Of late, there are few studies investigating the functional significance of these cells. The appearance of CA2 neurons is similar to the pyramidal cells of CA3 though they receive no direct input from dentate gyrus mossy fibers. Behaviorally, there is little evidence to determine their selective role in behavior or memory systems.

1.5.1.3.3.   CA1

As mentioned, the primary inputs into region CA1 of the hippocampus arise from the Schaffer collateral pathway terminating in the stratum oriens and stratum radiatum and those arising from layer III of the entorhinal cortex and terminating in stratum lacunosum-moleculare (Fig. 1-2). Other minor inputs exist including from the thalamus (Dolleman-Van der Weel and Witter, 1996) and reciprocal connections with the amygdala (Kemppainen et al., 2002; Saunders et al., 1988).

CA1 gives rise to two principle outputs, one to the subiculum, the second to the deep layers (V-VI) of the entorhinal cortex (Calderazzo et al., 1996). Output from CA1 appears to be topographically organized, the output targets include the retrosplenial and perirhinal cortex as well as the anterior olfactory nucleus, the olfactory bulb, the amygdala and the hypothalamus (Amaral and Witter, 1995).

1.5.2. Norepinephrine and Adrenergic Receptor Localization in Hippocampus

Since the first studies of fluorescent catecholaminergic localization in brain (Dahlström and Fuxe, 1964) improved visualization techniques have allowed for higher resolution of the localization of noradrenergic variscosities in CNS tissue. Utilizing a quantitative autoradiographic approach, Oleskevich et al. (1989) estimated the total number of noradrenergic varicosities in the rat hippocampus to be 2.1 million/mm^3. In mature rat, DBH-immunoreactivity is shown to be highest in the hilus, the inner molecular layer of the dentate gyrus, and stratum lucidum of CA3 (Moudy et al., 1993). Similar results have been documented with TH-like reactivity (Milner and Bacon, 1989).

As mentioned, investigations into the pharmacological activation of adrenergic receptors have detailed two distinct receptor classes, α and β. Of these two classes, the α-receptor has two sub-types referred to as α_1 and α_2. The α_1-adrenoceptor is found in almost all brain regions though the dentate gyrus shows comparatively high levels of α_1-receptors in contrast to other areas in the hippocampal tri-circuit (Young and Kuhar, 1980). In situ hybridization indicates that levels of the α_{2A}-adrenoceptor is as high in areas of the hippocampus (particularly dentate gyrus) as that found in cortex while the α_{2B}-receptor less than that of other cortical areas (Tavares et al., 1996).

The β-adrenergic receptor is most commonly associated with four receptor sub-types: β_1-β_4, two of which are not commonly found in brain β_3-β_4.

Autoradiographic studies have estimated the percentage of β_1 and β_2 receptors as being distributed approximately in a 3:1 ratio in dentate gyrus, respectively (Ordway et al., 1988; Rainbow et al., 1984). Though β_1/β_2 ratios are similar in other regions in the hippocampus; overall receptor binding is lowest in CA3 and highest in CA1 (Rainbow et al., 1984); although Ordway et al. (1988) demonstrate similar levels of receptor binding in CA1 and dentate gyrus. This last point is interesting because innervation by noradrenergic neurons, as detailed by autoradiographic studies, is higher in dentate gyrus (Oleskevich et al., 1989). In the only electron microscopic study of β-receptor localization in the dentate gyrus, antibodies raised against β-receptors revealed that this receptor class is largely localized to dendrites in the molecular layer (Milner et al., 2000). Closer inspection of published histology shows that the middle molecular layer shows the highest immunoreactivity followed by the inner and outer molecular layer, followed by the hilar region. The only receptors found on somata were found in the infragranular region of the hilus.

Both α- and β-adrenergic receptors are g-protein coupled receptors (GPCR), both initiating further cascades of intracellular events that alter the effects of synaptic transmission through second messenger systems. Activation of β-adrenergic receptors, coupled to the G_s-subtype of GPCR, increases levels of adenyl cyclase and cyclic adenosine monophosphate (cAMP) which in turn activates protein kinase A (PKA) which mediates a

variety of physiological effects including phosphorylation of the β-adrenergic receptors (desensitization), ion channel phosphorylation and also intranuclear translocation and phosphorylation of the cAMP response element binding protein (CREB) (Nestler and Greengard, 1999).The α_1-receptor class initiates the polyphosphoinositide turnover (McGeer et al., 1987) while activation of some sub-types of α_2-adrenergic receptors inhibits the cAMP cascade (Hancock et al., 1995) through the G_i-subtype of GPCR (Limon-Boulez et al., 2001).

1.5.3. Noradrenergic Modulation of Hippocampal Activity

The earliest reported effects of NE on hippocampal neurons were inhibitory (Segal and Bloom, 1976). However, newer techniques have confirmed that instead of possessing an inhibitory role, NE produces an increase in excitability of principle cells in the hippocampus, acting primarily as a neuromodulator (see below). It has also been observed in our lab (Brown, 2003), and by others (Segal and Bloom, 1976), that NE has a direct inhibitory effect on subsets of interneurons in the hilus. Thus NE may increase granule cell excitability in the dentate gyrus through multiple mechanisms.

In granule cells patch clamp experiments Gray and Johnston (1987) show that application of NE facilitates the activity of voltage-dependent

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calcium channels, an effect mediated by β-adrenergic receptors and mimicked by cAMP. Intracellular studies also reveal that NE increases excitability by depolarizing granule cells (Lacaille and Schwartzkroin, 1988), and reducing both accommodation of cell firing and the afterhyperpolarization (AHP) phase of the action potential (Haas and Rose, 1987). These effects are thought to be produced by β-adrenergic receptor activation (Haas and Rose, 1987; Lacaille and Schwartzkroin, 1988).

1.6 THE NUCLEUS LOCUS COERULEUS

1.6.1. Locus Coeruleus Anatomy

Discovered in 1791 by Felix Vicq d'Azyr the locus coeruleus (LC) is known for its blue pigmented appearance in human brain (rat brain is devoid of this pigmentation). The LC, is a small nucleus (compared to its extensive projections) in the pontine tegmental brainstem that sits adjacent to the fourth ventricle (see Fig. 1-3 for LC projections and LC localization in reference to other adrenergic nuclei).

Fig. 1-3 Projections of the LC and identification of adrenergic nuclei in the brain stem

The LC (yellow), the largest, and most widely projecting noradrenergic nucleus sits bilaterally, adjacent to the IVth ventricle. Major afferent projections of the LC include the olfactory bulb, the periaqueductal grey, the raphe nucleus, dorsal thalamus, the amydgala, hippocampus, septum and the frontal neocortex (see text). Other noradrenergic brain stem nuclei are illustrated in relation to LC. Projections from A1 and A2 descend to spinal cord and are the primary noradrenergic input to the hypothalmus. (Adapted from Paxinos and Watson, 1998).
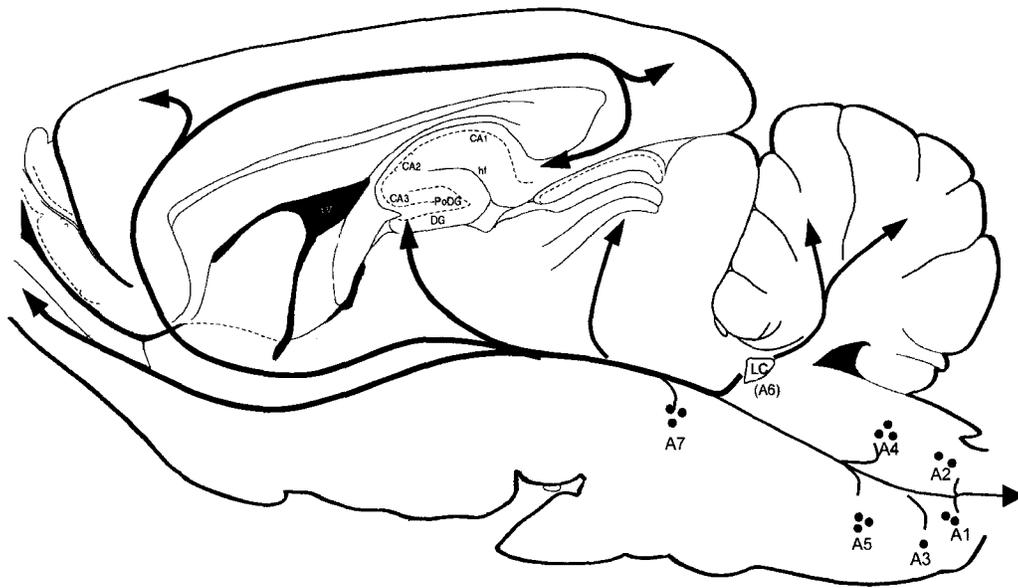
Fig. 1-3 Projections of the LC and identification of adrenergic nuclei in the brain stem

The LC contains approximately 1500 cells in rat (Swanson, 1976) and 12,000-15,000 in human (Ohm et al., 1997). Using a fluorescent technique to determine the distribution of catacholamines throughout the brain, Dahlström and Fuxe (1964) were the first to report catacholaminergic fluorescent signals in cells of the LC. They also determined that the entire nuclei were likely constituted in the majority, if not entirety, of noradrenergic neurons. In many cases the literature refers to the LC collectively as the A6 cell groups, the subcoerulear region containing an extensive arborization of dendritic processes of the LC neurons and the A4 cell groups, inclusively (Aston-Jones et al., 1995). For purposes of this work, the LC region will refer only to the densely packed nuclei of LC proper and the subcoerulear region.

A complex nucleus, the LC possesses a variety of discrete cell populations and can be divided into a number of distinct regions on the basis of cell type and areas to which the efferents project. For example using a horse-radish peroxidase labeling technique Loughlin et al. (Loughlin et al., 1986) have shown that fusiform cells of the dorsal LC project to areas of the hippocampus and cortex while the large multipolar cells of the ventral nucleus are known to project to the spinal cord and cerebellum. Other cells in the central and anterior portion project to the hypothalamus (Shipley et al., 1996). Neuroanatomically, the LC possesses receptors for many neurotransmitter and neuromodulator systems. In situ hybridization details the prevalence of α_2-receptors in the LC (Nicholas et al., 1993). While once thought that these

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receptors were located predominantly presynaptically, ultrastructural identification reveals the predominant α_{2A}-receptor subtype as autoreceptors on tyrosine hydroxylase-IR dendrites while also being present post-synaptically on neurons not receiving catacholaminergic input from LC axons (Lee et al., 1998a; Lee et al., 1998b). Examples of other receptor types include excitatory amino acids (Luque et al., 1994; Van Bockstaele and Colago, 1996), GABA (Luque et al., 1994), acetylcholine (ACh) (Ruggiero et al., 1990; Vincler and Eisenach, 2003), serotonin (5-HT) (Leger and Descarries, 1978), enkephalin (Van Bockstaele et al., 2000), substance P (Chen et al., 2000; Wolf et al., 1994), somatostatin (Dournaud et al., 1996; Gagne et al., 1990; Moyse et al., 1992), opiate (Van Bockstaele et al., 1996a; Van Bockstaele et al., 1996b), corticotrophin releasing factor (CRF) (Sauvage and Steckler, 2001), and orexin: (Greco and Shiromani, 2001; Hervieu et al., 2001; Marcus et al., 2001).

1.6.1.1.   Afferent projections to the locus coeruleus

Although the LC is diversely rich with various receptor types it remains difficult to determine from where the corresponding inputs arise. However, it is known that two major extrinsic inputs of the LC arise from the nucleus paragigantocellularis (PGi), the major glutamatergic, excitatory projection. Electron microscopy has revealed that these projections arise from both the ventral and lateral PGi and synapse monosynaptically on tyrosine

hydroxylase-positive dendrites in the LC. Another afferent projection stems from the prepositus hypoglossi, an inhibitory projection containing GABA-ergic neurons (Aston-Jones et al., 1986; Ennis and Aston-Jones, 1986). Examples of other inputs include the hypothalamus (Aston-Jones et al., 1986), dorsal periaqueductal grey, the raphe nucleus, the parabrachial nucleus and the Kolliker-Fuse nucleus (Ennis et al., 1991).

1.6.1.2.   *Efferent projections from the locus coeruleus*

The LC is the major supplier of NE in the brain, and accordingly, noradrenergic efferents originating from the LC terminate on a wide variety of structures in the brain and spinal cord. One of the major ascending projections is to the olfactory bulb which receives input from approximately 40% of LC neurons (Shipley et al., 1985). Other major targets are the periaqueductal grey, the raphe nucleus, dorsal thalamus, the amydgala, septum and the frontal neocortex (Jones and Moore, 1977). The hypothalamus is unusual in that it possesses rich NE innervation arising chiefly from the noradrenergic nuclei A1 and A2 with only minor contributions from the LC (Aston-Jones et al., 1995). Descending projections include the pons and medulla (Levitt and Moore, 1979), the cerebellum (Kimoto et al., 1978) and the spinal cord (Fritschy and Grzanna, 1990; Guyenet, 1980; Nygren and Olson, 1977).

In the hippocampus, the sole source of NE is the LC. It arises from three pathways, the ventral amygdaloid, the fornix, and the cingulum each terminating in a slightly different field of the hippocampus (Aston-Jones et al., 1995). Moreover, noradrenergic projections to the hippocampus include innervation from the LC of the contralateral hemisphere (Adèr et al., 1980;Room et al., 1981).

1.6.3. Locus Coeruleus Physiology and Pharmacology

Characteristically, LC neurons fire with broad (1-2 ms) spikes at roughly a rate of 1-5 Hz, making them relatively easy to isolate in the whole animal preparation. Besides a slower, tonic level of activity, LC cells can also elicit a "burst" firing response in which firing rates increase (up to 20 Hz) for brief periods, after which cells enter into a "quiescent" stage before returning to basal activity levels. These phasic firing properties are highly dependent on behavioral state. Rat and primate studies have shown that little, or no, activity occurs in LC neurons during slow-wave and paradoxical sleep (Aston-Jones and Bloom, 1981), however, activity increases with waking (or anticipation of waking), alertness, tasks requiring vigilance and sensory events (Grant et al., 1988). Novel sensory events, or exploration of novel objects also elicit a transient increase in firing levels of LC neurons, a response that adapts quickly to subsequent presentations (Vankov et al., 1995).

Numerous neurotransmitter substances are known to modulate LC activity directly e.g. glutamate (Harley and Sara, 1992; Ivanov and Aston-Jones, 1995; Olpe et al., 1989) and ACh or cholinergic agonists (Adams and Foote, 1988; Valentino and Aulisi, 1987)), NE (Adams and Foote, 1988; Chessell et al., 1996; Svensson et al., 1975), 5-HT (Aston-Jones et al., 1991; Chiu et al., 1995). Neuroactive peptides are also known to have potent effects on LC firing properties (e.g. Chessell et al., 1996; Page and Abercrombie, 1999). Of particular interest is the recently discovered neuropeptide, orexin, also known in the literature as hypocretin. The LC receives amongst the densest projections of orexin-A (ORX-A) neurons originating from lateral hypothalamus in the brain and the ORX-A receptor, OR_1R, is highly localized in the LC (Hagan et al., 1999; Horvath et al., 1999; Peyron et al., 1998). ORX-A has also been shown to be a potent activator of LC neurons (Hagan et al., 1999; Horvath et al., 1999; Kiyashchenko et al., 2001) making its application an excellent method of selectively activating brainstem NE release in target structures. Thus orexin may prove to be a strong modulator of attentional and learning processes (see Chapter 3).

Not surprisingly, levels of activity of LC neurons correlate with levels of NE in efferent target areas i.e., higher levels of firing result in increased levels of NE and periods of low activity, lower levels of NE. Application of ACh in the vicinity of the LC increase neuronal discharge (Adams and Foote, 1988) and induces a corresponding increase in NE in the ipsilateral prefrontal cortex

(Berridge and Abercrombie, 1999; Van Gaalen et al., 1997) and hippocampus

(Palamarchouk et al., 2000; Pudovkina et al., 2001).

1.6.3. Locus coeruleus modulation of memory

As mentioned, LC neurons respond in a highly predictive fashion during attention or conditions associated with arousal making them a likely participant in tasks of learning and memory. To this end, it has also been shown that LC neurons respond transiently when stimuli are associated with appetitive and aversive conditions, and during extinction (Sara et al., 1994). Correspondingly, studies investigating the effects of LC lesions on memory tasks have shown that animals with bilateral lesions of the LC have decreased memory for both appetitive (Anlezark et al., 1973) and aversive (Crow and Wendlandt, 1976), or spatial (Compton et al., 1995) tasks. Electrical (Velley et al., 1991) or glutamatergic stimulation of the LC afferent nuclei (PGi) (Clayton and Williams, 2000a) has been shown to contribute to the increased retention of a food-reinforced operant task and avoidance/spatial tasks, respectively. Conversely, inactivation of the PGi by intranuclei infusion of lidocaine or muscimol after training of a passive avoidance task reduced latencies to re-enter the footshock area during a retention test 48 hr post-training (Clayton and Williams, 2000b). Lastly, transplantation of LC neurons into the third ventricle also improves memory for an inhibitory avoidance task. (Collier et al., 1988).

1.7   NEURAL MEMORY MODELS: LTP and NE-LTP

1.7.1.   Long-Term Potentiation

The first definitive physiological evidence of Hebb's reverbatory neural circuit hypothesis was first obtained 20+ years after "The Organization of Behavior". In a landmark paper, Bliss and Lömo (1973) discovered that if they applied a train of high-frequency (100 Hz) stimulation to the perforant path-dentate gyrus synapse in rabbit hippocampi they produced a long-lasting potentiation of the evoked potential. This provided evidence that cortical circuits could sustain long-term changes in synaptic firing properties and supported the Hebbian hypothesis. Since the discovery of what would be known as long-term potentiation (LTP), literally thousands of studies have been executed in a vast number of brain areas e.g. auditory (Kudoh and Shibuki, 1994), and visual (Artola and Singer, 1987; Berry et al., 1989) cortices and numerous other pathways demonstrating the generalizability of the LTP model.

1.7.1.1.   Norepinephrine and LTP

In hippocampal slice preparations perforant path-dentate gyrus LTP is dependent both upon activation of the glutamatergic perforant path input (Burgard et al., 1989) and on synaptic levels of NE (Stanton and Sarvey, 1985a) activating β-adrenergic receptors (Bramham et al., 1997). Recently,

microdiasylate samples of hippocampal levels of NE in the awake rat show that NE is released upon tetanization of the perforant path input (Bronzino et al., 2001). In the anesthetized rat, NE contributions to LTP appear to be more selective in that intradentate β-adrenergic receptor blockade reduces the synaptic contribution (EPSP slope) of the evoked response after tetanization without reducing the LTP of the population spike (Munro et al., 2001). Behaviorally, appetitive and footshock reinforcement can increase the duration of "decremental" LTP (LTP lasting <8 hr). When footshock or reward is given shortly after decremental LTP, potentiation of the dentate gyrus evoked potential is facilitated at periods of 24 hr, an effect that is abolished by systemic application of a β-adrenergic receptor antagonist (Seidenbecher et al., 1997).

In passing, NE is also known to regulate LTP at other synapses in the hippocampus. In area CA1 application of NE to hippocampal slices can produce LTP in preparations normally devoid of the facilitation (Izumi and Zorumski, 1999; Katsuki et al., 1997) although Stanton and Sarvey have shown depletion of NE does not affect LTP in this area (Stanton and Sarvey, 1985a). Similarly, brief periods of NE exposure will also modulate LTP at the mossy fiber-CA3 synapse through β-adrenergic receptors (Hopkins and Johnston, 1984).

1.7.2. Norepinephrine Induced Long-Term Potentiation

Norepinephrine induced long-term potentiation, or NE-LTP, is the term coined to describe the potentiation of the dentate gyrus evoked potential when NE is either applied or endogenously released at the perforant path-dentate gyrus synapse. NE-LTP is a memory mechanism that does not require tetanic stimulation to the pathway of input making it a possibly more natural model for the formation of memory. This phenomenon appears to be specific to this synapse in the hippocampus as the same protocol applied to area CA1 does not produce the same facilitation (Stanton and Sarvey, 1985b). Beyond this, the effects on the dentate gyrus granule cell population depends upon which entorhinal input is activated when NE is present. Input arising from the lateral entorhinal cortex via the lateral perforant path which synapses on the outer 1/3 of the molecular layer depresses both the synaptic response as well as the population spike (Dahl and Sarvey, 1989). To this point it is unclear if this depression lasts for extended periods or whether it requires protein synthesis, as no studies have investigated this to date. NE facilitates the medial perforant path input which synapses on the middle 1/3 of the dendrites of the granule cells in the molecular layer. Separating the inputs is difficult with the greatest success being in hippocampal slice preparations. In most *in vivo* studies the medial population spike is studied, so it is unclear how much lateral perforant path input is also evoked. In these studies, the

resultant EPSP is likely composed of a combination of lateral and medial perforant path evoked input.

Neuman and Harley (1983) were the first to report that NE is capable of producing long-lasting potentiation in the dentate gyrus. Here NE was iontophoresed for a period of up to 8 min directly into the dentate gyrus while the evoked potential was monitored. Subsequently, many studies investigating the effects of NE in the dentate gyrus have been done using numerous techniques including hippocampal slice preparations, anesthetized preparations and in the awake animal. The outcomes of these studies will be briefly discussed below.

Iontophoretic studies: Only two studies using intra-dentate iontophoresis in the anesthetized rat have been done. The first is that mentioned above by Neuman and Harley (1983) where iontophroretic application of 0.1 M NE for 1-8 min produced a long-lasting facilitation of the population spike. Changes in EPSP slope however, were rarely seen. Winson and Dahl (1985) also reported long-term increases to the population spike after prolonged application of NE to the granule cells.

Hippocampal slice: Numerous studies have shown that addition of NE to the bath also potentiates the dentate gyrus evoked potential recorded in the hippocampal slice (Dahl and Sarvey, 1989; Haas and Rose, 1987; Stanton

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and Sarvey, 1985b). Lacaille and Harley (1985) found that a 10 min wash of 10 μM NE facilitated both the amplitude of the population spike as well as the EPSP slope. Burgard et al (1989) report similar increases in the population spike amplitude and the evoked molecular layer EPSP with bath application of 50 μM NE.

Intracerebroventricular (i.c.v.) NE: A method of studying the effects of exogenous NE in the whole animal preparation is to infuse NE or pharmacological substrates into the ventricular system while simultaneously probing the physiological effects in the hippocampus. It has been shown that ^{3}H-NE injected into the ventricles readily penetrates into cortical tissue (Reivich and Glowinski, 1967). This method provides a bath environment, much like that of *in vitro* slice preparations, but allows one to investigate the effects in an intact, *in vivo* system. Chaulk and Harley (1998) investigated the effects of NE application in the anesthetized and awake preparation showing that exogenous NE is able to facilitate both the population spike amplitude and the EPSP slope. A later study investigating extracellular hippocampal NE levels after i.c.v. application of NE determined that the observed population spike increase is not evident until levels of NE reach 30x that of basal levels (Harley et al., 1996).

Glutamatergic Activation of LC (Anesthetized Preparation):

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The above studies investigate the effects of exogenous NE modulation of hippocampal activity. In order to investigate the effects of endogenous release of NE at hippocampal synapses, one method is to directly activate the LC to promote NE release in downstream targets. The noradrenergic neurons of this nucleus can be activated either by pharmacological, in this preparation typically glutamatergic (Harley et al., 1989a; Harley and Evans, 1988; Harley and Milway, 1986), or electrical stimulation (Dahl and Winson, 1985; Washburn and Moises, 1989). The resultant effect of LC stimulation on the dentate gyrus evoked potential is best characterized as an increase in the amplitude of the population spike alone but effects on both spike amplitude and EPSP slope have been documented (Frizzell and Harley, 1994). As only one study exists investigating the short-term (30 min) effects of glutamatergic activation of the LC in the awake animal (Klukowski and Harley, 1994), exactly how long the potentiation lasts has not been determined.

1.7.2.1.   Pharmacology of NE-LTP

Examination of the pharmacology behind NE-LTP has detailed a critical role for the β-adrenergic receptor. In hippocampal slice preparations NE-LTP is blocked by application of the β-adrenoceptor antagonists propranolol (Stanton and Sarvey, 1985b), timolol (Lacaille and Harley, 1985) and the β_1-adrenoreceptor blocker metoprolol (Stanton and Sarvey, 1985b).

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Similarly, in the whole animal preparation, potentiation of the population spike after LC activation is reduced by systemic application of propranolol (Babstock and Harley, 1992; Harley and Milway, 1986) or by intra-dentate micropressure application of timolol (Harley and Evans, 1988).

The non-specific β-adrenergic agonist isoproterenol (ISO) produces powerful facilitatory effects on the dentate gyrus evoked potential. In fact this phenomenon is known as β-adrenergic potentiation, or βAP, a restricted form of NE-LTP. Low concentrations of this agonist (as low as 1 μM) produce increases in the population spike as well as the synaptic response (EPSP) when bath applied on hippocampal slices (Lacaille and Harley, 1985). Dahl and Li (1994) have also illustrated an unusual form of facilitation with sequenced application (reminiscent of spaced learning trials) of even lower concentrations of ISO. Bath applied 75 nM ISO produces a short-term facilitation of the population spike. A second application of the same concentration separated from the first by a 30 min wash produces a facilitation of the spike that is long-lasting. Corresponding with β-adrenergic receptor evidence, application of NE onto hippocampal slices increases levels of cAMP to 3X basal levels, even higher than that seen after tetanic high-frequency stimulation protocols (Stanton and Sarvey, 1985c).

Though β-adrenergic receptor activation is pivotal it appears that α-adrenergic receptors may play a lesser role in NE-LTP. Winson and Dahl (1985) iontophoretically applied the α-adrenergic receptor agonist

phenylephrine and clonidine (CLON) onto dentate granule cells and produced a facilitation of the population spike. Similar results have been found in small numbers of slices tested in the hippocampal slice preparation (Lacaille and Harley, 1985) and also when infusing α-adrenergic agonists into the lateral ventricle of the anesthetized rat (Chaulk and Harley, 1998).

1.8   PROTEIN SYNTHESIS AND MEMORY OVERVIEW

Hebb predicted that cells activated during the learning process would somehow physically alter their constitution in order to incorporate the changes and to retain information for the events or experiences. In contrast to short-term memory which does not require synthesis of new proteins, it is now known that long-lasting memory must be followed by changes in the protein constituents of cells involved in the reverbatory firing pattern. Inhibitors of protein synthesis such as anisomycin and actinomycin D have been shown to inhibit the formation of memory for behavioral tasks in rat (Meiri and Rosenblum, 1998; Vianna et al., 2001), mice (Lattal and Abel, 2001) and prevents sensitization of the gill withdrawl response in *Aplysia californicus* an invertebrate nervous system. At the cellular level, an abundance of studies have shown that LTP, a postulated model of memory formation, consists of both a short-term protein synthesis-*independent* phase and a long-term

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protein synthesis-*dependent* phase (Nguyen and Kandel, 1996a; Stanton and Sarvey, 1984).

Does NE-LTP also require protein synthesis for the maintenance of the long-lasting potentiation in the dentate gyrus? Only one study done to date suggests that it is indeed necessary. By bathing hippocampal slices with the protein synthesis inhibitor emetine, Stanton and Sarvey (1984) prevented long-term potentiation induced by application of NE. As seen in studies of LTP, they did not prevent the initial short-term potentiation observed soon after NE was applied. No studies to date have shown that NE-LTP in whole animal preparations is protein synthesis dependent.

1.9. OBJECTIVES

Hypothesis: Norepinephrine, a neuromodulator involved in the cognitive states of arousal and emotion, produces alterations in synaptic efficacy in the dentate gyrus reminiscent of memory formation.

The purpose of this thesis is to examine the effects of NE on the dentate gyrus-perforant path evoked potential, the first synapse of the tri-synaptic pathway in the hippocampus. Three chapters are presented, each exploring the effects of NE on synaptic plasticity using different techniques for increasing synaptic concentrations of NE in the dentate gyrus. Each chapter is presented in "Manuscript format", as it would be submitted for journal

publication with an abstract, a short Introduction of the relevant literature, a Material and Methods section, Results and Discussion in each. Two exceptions occur from typical manuscript format as per Memorial University of Newfoundland School of Graduate Studies Guidelines for "Manuscript" submission in that figures are presented in the body of the chapter and the bibliographic references are combined and placed *en total* in a Reference section at the end of the overall Summary section.

Co-Authorship Statement

As this thesis is submitted in Manuscript (Publication) Format a statement is necessary to address the specific contributions of intellectual and practical contribution made by the primary author.

The origins of the research proposals were conducted jointly between my Ph.D. supervisor, Dr. Carolyn Harley, and myself. All other work found within this text was conducted by myself (with one exception- see below). My contributions include inception of the experimental design, design of the chronic electrophysiological recording apparatus, data collection and analysis, and preparation of the manuscripts for publication.

The third chapter (A role for orexin-A in attention and memory functions: Norepinephrine release and norepinephrine-induced long-term potentiation in the dentate gyrus following orexinergic activation of the locus coeruleus) is a collaborative effort between myself, Dr. Harley and Dr. David Nutt and Ms. Margaret Lallies of Bristol University UK. Again, I was responsible for experimental design and implementation of all electrophysiological data, data analysis and preparation of the manuscript. Dr. Harley collected the microdialysis data in Dr. Nutt's laboratory with the help of Ms. Margaret Lallies at Bristol University.

CHAPTER 2:

Intracerebroventricular norepinephrine increases the perforant path evoked potential in the dentate gyrus of the freely moving rat.

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2.1 ABSTRACT

Norepinephrine (NE) is implicated in memory formation in the mammalian nervous system. Application of NE in hippocampal slices and in whole animal preparations shows NE produces an enduring facilitation of synaptic transmission at the perforant path-dentate gyrus synapse similar to that seen with frequency-induced long-term potentiation (LTP). NE-induced potentiation *in vivo* characteristically involves facilitation of the dentate gyrus population spike while *in vitro* investigations commonly include potentiation of both the population spike and the field EPSP slope. At this time, the differences between the preparations are not fully understood. In this study, we combine *in vitro* and *in vivo* techniques using a pharmacological bath environment in the behaving animal by applying NE into the lateral ventricles while monitoring the perforant path evoked potential. Here, bath application of NE produced facilitation of both population spike and the EPSP slope in a manner similar to that seen with *in vitro* preparations. However, unlike NE-induced potentiation *in vitro*, these responses were not long-lasting, typically returning to baseline within 60 min. Next, we explored the pharmacology behind NE-induced potentiation and found, as in both *in vitro* and *in vivo* preparations, β-adrenergic receptor activation is critical in producing population spike and EPSP slope facilitation. Application of the α-adrenergic receptor antagonist, phentolamine, prior to NE did not inhibit the potentiation observed, but increased the duration of the population spike amplitude

potentiation suggesting NE-induced potentiation in the dentate gyrus is not mediated by α-adrenergic receptors. Analysis of rats 24 hr after NE infusion indicates that though short-term increases return to baseline after NE, long-term increases may be seen 24 hr post infusion. Thus, NE may mediate separable short-term and long-term potentiation processes.

2.2 INTRODUCTION

Kety (1970a) initially proposed that the monoaminergic neurotransmitter, norepinephrine (NE), was critical for memory formation. Kety's hypothesis has support from behavioral evidence that shows NE as a potent modulator of memory in rats (Crow and Wendlandt, 1976; Izquierdo et al., 1979; Low et al., 1984; Stein et al., 1975) and humans (Cahill et al., 1994; Nielson and Jensen, 1994). Kety hypothesized that the release of NE and the resultant initiation of the cAMP-protein kinase A (PKA) cascade and protein synthesis would trigger a persistent facilitation of inputs that were active during NE release and underpin the memory for those inputs.

The dentate gyrus, a structure involved in memory formation, receives a diffuse noradrenergic projection (Oleskevich et al., 1989) originating solely from the locus coeruleus (LC). Consistent with Kety's hypothesis, NE release in the hippocampus triggered by pharmacological (e.g. Harley and Milway, 1986; Klukowski and Harley, 1994) or behavioral activation (e.g. Kitchigina et al., 1997) of the LC, produces potentiation of the glutamatergic perforant path-evoked potential in the dentate gyrus. However, specific features of NE-induced potentiation in the dentate gyrus vary between *in vitro* and in vivo preparations with consistent long-term potentiation of EPSP slope only being reported *in vitro* (Burgard et al., 1989; Stanton and Sarvey, 1985b), while NE-induced long-term potentiation of the perforant path-evoked population spike occurs both *in vitro* and *in vivo* (Haas and Rose, 1987; Harley and Milway,

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1986; Neuman and Harley, 1983). NE-induced long-term potentiation occurs more robustly and more reliably *in vitro* and has been shown to depend on protein synthesis (Stanton and Sarvey, 1987).

Both *in vivo* and *in vitro* studies have demonstrated that β-adrenergic receptor activation is pivotal in the production of NE-induced potentiation as application of the β-adrenergic receptor antagonists timolol (Harley and Evans, 1988; Lacaille and Harley, 1985), propranolol (PROP, (Harley et al., 1989; Harley and Milway, 1986; Washburn and Moises, 1989) and metoprolol (Chaulk and Harley, 1998; Stanton and Sarvey, 1987) decrease the ability of NE to induce potentiation. In the presence of the α-adrenergic receptor antagonist phentolamine (PHENT) bath application of NE on hippocampal slices initiates potentiation at a much lower concentration (1 μM) than when NE is applied alone (Dahl and Sarvey, 1989) suggesting α-adrenergic receptors counter the potentiation effects of β-adrenergic receptor activation.

Mechanical properties of *in vitro* and *in vivo* studies differ in a number of ways which may account for the divergence of results with respect to the ability of NE to induce alterations in the synaptic component of the dentate gyrus evoked potential. Bath application of NE *in vitro* is one notable difference, as *in vivo* preparations have normally used concentrated discrete NE applications (e.g., Neuman and Harley, 1983) or the endogenous release of NE (e.g., Harley and Milway, 1986; Harley and Evans, 1988). Absence of anesthesia *in vitro* is another, while anesthesia is commonly used in the

majority of *in vivo* experiments. The present study asks if a bath-like application of NE and NE agonists in unanesthetized rats would produce a pattern of NE-induced potentiation more like that seen *in vitro*.

One *in vivo* study has been carried out in which NE and NE agonists/antagonists were infused into the lateral ventricle of the anesthetized rat and a small number of awake rats were also infused with NE while simultaneously recording population activity in the dentate gyrus (Chaulk and Harley, 1998). In the Chaulk and Harley study ±NE (10-100 µg) produced reliable short-term potentiation of the population spike amplitude in the anesthetized preparation, with the larger concentrations producing reliable long-lasting (>20 min) effects. However, the EPSP slope showed less consistent potentiation, increasing in only 20% of the anesthetized rats. When NE was infused into the ventricles of awake rats, however, the percentage of animals exhibiting EPSP slope facilitation increased to 40%. Thus, it is unclear if the effects of anesthesia in rats may be responsible for the differences in the ability of NE to facilitate the synaptic component of the evoked potential.

In an effort to further elucidate the effects of exogenous NE on synaptic transmission in the dentate gyrus, we infused NE into the ipsilateral lateral ventricle of the awake rat and monitored the evoked response until responses returned to baseline levels. In a sub-group of rats, these responses were again monitored at 24 hr. Rats were also given subsequent

infusions of noradrenergic agonists and antagonists as a comparison with the effects of NE alone. Similar to what has been reported *in vitro*, both the EPSP slope and the population spike were potentiated by NE; however potentiation effects returned to baseline during the initial recording. In the subgroup observed at 24 hr, evidence was seen of long-term EPSP slope and population spike increases. Pharmacological probes suggested β-adrenergic receptors were important for perforant path-population spike potentiation and for the 24 hr increases observed. α-adrenergic receptors did not contribute to spike facilitation. Instead, as *in vitro*, an α-adrenergic receptor antagonist prior to NE enhanced the size and duration of spike potentiation. These data are consistent with Kety's hypothesis that NE can induce a facilitation of informational pathways in anaesthetized rat. The distinction between short-term and long-term effects of NE merits further exploration.

2.3   MATERIALS AND METHODS

Subjects and surgical implantation

All procedures in this investigation were reviewed and carried out in accordance with the Canadian Council on Animal Care. Twelve male Sprague-Dawley rats (Memorial University of Newfoundland) weighing 240-310 g at the time of surgery were anesthetized with chloral hydrate (400 mg/kg, i.p.) and placed in a stereotaxic instrument in the skull-flat position. A 26 gauge metal cannula (Plastics One, Inc.) was implanted in the lateral

ventricle (0.8 mm posterior to bregma, 1.5 mm lateral from midline and 3.2 mm ventral from brain surface). The cannula was secured with dental acrylic to a single stainless steel jewelers' screw and capped with a temporary stylet to prevent obstruction. A bipolar stimulating electrode constructed of Teflon-coated stainless steel wire (125 μm) was directed at the perforant path (7.2 mm posterior to bregma, 4.1 mm lateral from midline and $\cong$ 3.0 mm ventral) while a recording electrode of similar construction (using 50 μm wire) was implanted in the granule cell layer of the dentate gyrus (3.5 mm posterior, 2.0 mm lateral and approximately 2.5 mm ventral from brain surface). Electrical stimulation was delivered to the perforant path (0.2 ms, 0.1 Hz) and the negative going population spike was maximized. Both electrodes were secured with dental cement to three skull screws placed around the trephinated holes. Two of the skull screws were used as reference and ground sources with the former being placed equidistant from the two electrodes. The cemented electrodes and ground connections were then placed in a nine-hole plug, which was then also secured by dental cement. The animals were given 60,000 IU of penicillin (G-sodium: 30,000 IU x 2, i.m.), Acetaminophen was added to the water bottle (TEMPRA; 270 mg/100 ml, *ad libitum* for 3 days) and rats were allowed one week to recover before additional procedures.

Habituation and Recording Preparation

During the post-surgical recovery period the rats were handled daily and placed in the Plexiglas recording box lined with bedding for habituation. Rat chow (Purina) was supplied *ad-libitum* in the recording chamber at all times. Ten minute habituation sessions occurred on at least three occasions before recording began. Prior to each recording session, the animals were also allowed to habituate for 10 min before stimulation and recording were initiated. All habituation and recording sessions were carried out during the light cycle of the animals' 12 hour light/dark cycle.

Electrical stimulation was delivered to the perforant path at 0.1 Hz and the responses were filtered (1 Hz - 3 kHz) and digitized at 10 kHz. Waveforms were collected using DataWave software and analyzed off-line. Each recording session consisted of 1) an initial input-output current intensity curve (I/O curve), 2) a period of baseline recording (minimum of 30 min at an intensity determined by the amount of current necessary to elicit a population spike of 50% of maximum during the I/O curve) and 3) on treatment days, a drug/saline infusion into the lateral ventricle. Some animals were monitored 24 hr after drug infusion. During these sessions the intensity of stimulation used was always that of the prior session. There was a minimum of two successive days of baseline recording prior to commencement of experimental procedures to ensure stable baselines.

Drug Infusion Procedures

Drug concentrations were selected according to the results of prior studies (Chaulk and Harley, 1998). (-)Arterenol, (NE, 50 µg) and the β-adrenergic receptor agonist (±)-isoproterenol (ISO, 30 µg) were mixed in 4 µL of sterile saline and infused into the lateral ventricle ipsilateral to the recording/stimulating electrodes, at a rate of 2 µL/min using a 28 gauge insertion cannula attached to flexible tubing and microsyringe. All animals received i.c.v. NE infusion and subsets of rats that exhibited moderate to robust responses to NE infusions were chosen to receive other infusions. The α-adrenergic receptor antagonist PHENT (phentolamine mesylate, 25 µg) and the β-adrenergic receptor antagonist DL-propranolol (PROP, 30 µg) were prepared and infused as above 10 min prior to infusion of NE. All drugs were prepared immediately before injection and were obtained from Sigma with the exception of phentolamine mesylate (RBI). In most cases i.c.v. infusions were separated by a minimum of 2 days.

Histology

Animals were injected with chloral hydrate (i.p.) and cannula placements were marked by an infusion of 4 µL of a solution containing Evans Blue (20%) and methylene blue (1%). The animals were then sacrificed; the brains were removed and rapidly frozen in methyl butane (-70 °C). Alternate sections were sectioned by cryostat (30 µm) with the first set

stained with cresyl violet to confirm electrode placement and the second, unstained set was used to determine lateral ventricle placement. On two occasions the dye infusion could not be performed and cannula placements were confirmed in the Nissl stained sections.

Statistical Analysis

The field EPSP slope and population spike amplitude were extracted for each waveform as detailed in Fig. 2-1A. Briefly, the population spike amplitude was measured as the difference between the peak and valley of the downward deflecting spike (c). The EPSP slope was measured as: voltage (b-a)/time(a-b). Analysis of group data was performed on averages of 3 min means for 60 minutes post-infusion and contrasted to two baseline time points (2 x 15 min= 30 min) using repeated measures analysis of variance (ANOVA). Tests of least significant differences (LSD) were performed where applicable. No differences were found between infusion sets for the baseline measurements of EPSP slope or population spike. In cases where individual responses are reported, data were converted to means of one minute with 3 consecutive means falling outside of the 95% confidence interval for the individual rat taken as a significant increase/decrease.

2.4 RESULTS

Short-term effects and pharmacology of i.c.v. NE

The effect of bath application of NE by i.c.v. infusion was examined for an initial period of up to 60 min post-NE infusion in twelve awake rats. This was the initial infusion for 11/12 rats. Intraventricular NE increased both the dentate gyrus EPSP slope (repeated measures ANOVA; $F_{21,147}=2.51$, p<.0007) and the amplitude of the population spike ($F_{21,147}=6.00$, p<.0001) to perforant path stimulation. Analysis of the group data indicate that the increase of the EPSP slope occurred approximately 9 min after the drug infusion (mean at maximum increase = 108.5% Fig.2-1A) and continued for 12 min. The population spike amplitude increased 3 min post-NE infusion and remained increased for approximately 15 min post-infusion (mean at maximum increase = 124.6%; Table 2-1 and Fig. 2-1B). All twelve (100%) of the animals infused with NE demonstrated potentiation of the dentate gyrus evoked response either as potentiation of EPSP slope or as potentiation of the amplitude of the population spike, however, the individual profiles of EPSP slope and population spike potentiation varied for individual rats. Facilitation of the EPSP slope occurred in 8 of 12 rats (75%; see Table 2-1 and Fig. 2-1B) and potentiation of the population spike occurred in 8/12 rats (75%; Fig. 2-2B); four rats (33%) demonstrated potentiation of both the EPSP slope and population spike. One rat demonstrated potentiation of the

population spike amplitude while the synaptic response was significantly

depressed.

Table 2-1. Effects of i.c.v. NE and NE agonists/antagonists on the perforant path-dentate gyrus evoked potential in the awake rat.

i.c.v. infusion	n	Population Spike	EPSP Slope	Population Spike 24 hour	EPSP Slope 24 hour
NE	12	124.6% *	108.5% *	136.6% * (4/7)	103.8% *
Spike Increase	*8/12*	*139.7%*	*107.8%*	-	*(4/7)*
Slope Increase	*8/12*	*133.7%*	*114.6%*	-	-
PHENT+NE	5	148.1% *	107.5% *	-	-
PROP+NE	5	107.1%	101.0%	85.7% (4)	89.1% (4)
ISO	5	129.3%	94.8%	155.4% (4)	115.6% (4)
Saline	4	91.5%	94.1%	-	-

Data represent mean percentage increase at time of NE-infusion maximal response (15 min post-NE) with the exception of ISO which reached maximum potentiation 3 min post-infusion. * = minimum of $p<.05$ compared to baseline responses of the respective infusion period (repeated measures ANOVA on raw data and illustrated as percentage increase).

Fig. 2-1

Intraventricluar application of NE induces a potentiation of the field EPSP slope and population spike amplitude in the awake rat. *Inset:* Sample waveforms and parameter measurements of the dentate gyrus evoked potential before (*solid line*) and after (*dashed line*) infusion of NE into the lateral ventricle. Scale bar= 2 mV and 2.5 ms. A) i.c.v. NE (*arrow*) induced a short-term potentiation of the dentate gyrus evoked potential in the freely moving rat (n=12). B) Population spike amplitude as described in A. Intraventricular NE also produced a short-term potentiation of the evoked population spike. All animals infused with NE (12/12) exhibited short-term potentiation of either the EPSP slope or population spike amplitude (See Table 2-1). All data represent 3 min means ± s.e.m. (* = minimum $p < .05$ compared to responses prior to NE infusion, raw data).
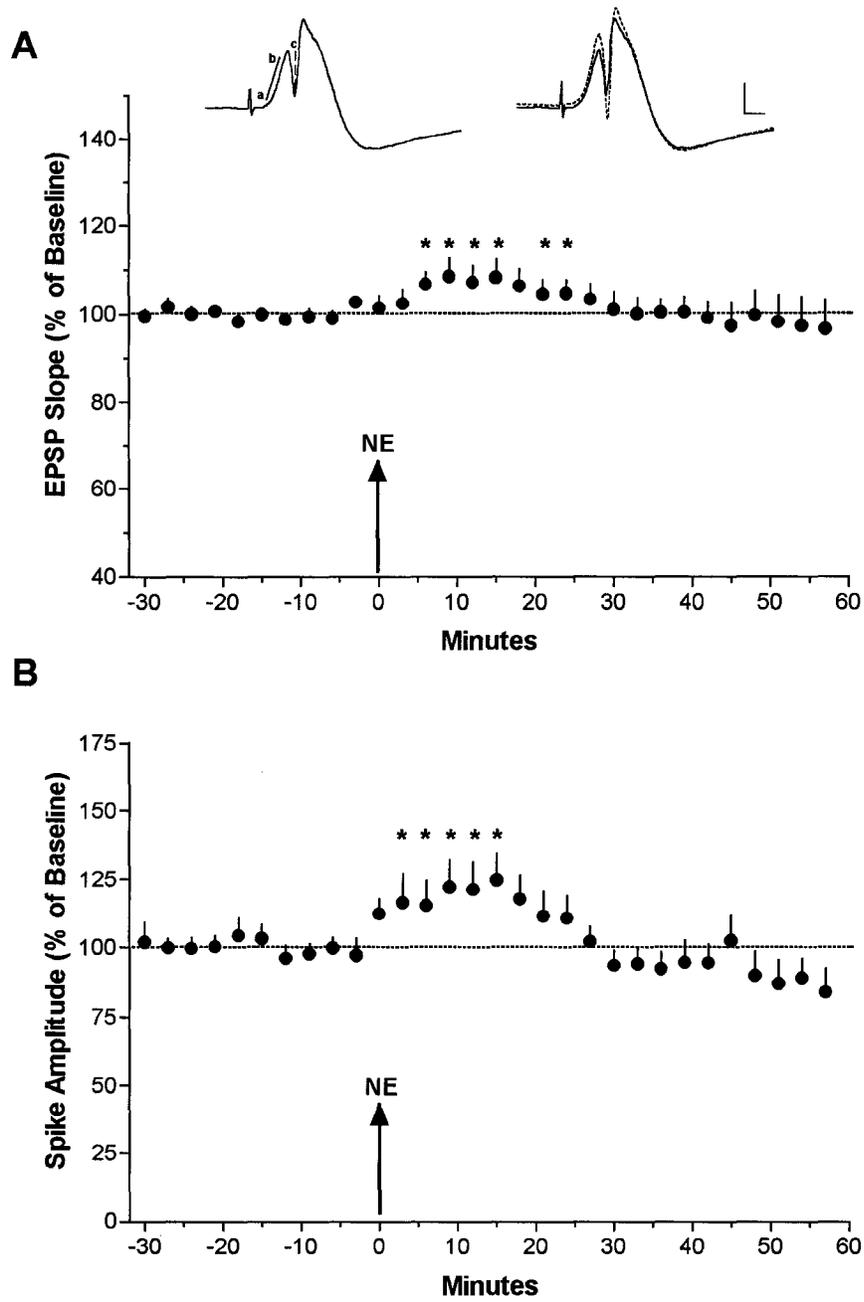
Fig. 2-1 Intraventricluar application of NE induces a potentiation of the field EPSP slope and population spike amplitude in the awake rat.

In the hippocampal slice preparation, application of the α-adrenergic receptor antagonist PHENT prior to a low concentration of NE (1 μM) produced a long-lasting potentiation of both the EPSP slope and the amplitude of the population spike (Dahl and Sarvey, 1989). To determine whether blockade of α-adrenergic receptors prior to i.c.v. infusion of NE would produce a similar response in the awake rat, the α-adrenergic receptor antagonist PHENT (25 μg), was infused into the lateral ventricle 15 min prior to NE (PHENT + NE infusion; n=5) and the results were contrasted with i.c.v. infusions of NE alone (as detailed above). Analysis of the EPSP slope indicated that there were no significant interactions between the drug and sample times (repeated measures ANOVA; drug x sample) demonstrating that infusion of the α-adrenergic receptor antagonist prior to NE did not alter the potentiation of the EPSP slope observed with NE infusion alone (see Table 2-1). However, the profile of NE-induced potentiation of the population spike was significantly altered from that observed with NE alone (drug x sample; $F_{21,231}=1.80$, $p<.02$; Table 2-1 and illustrated in Fig. 2-2). Blockade of the α-adrenergic receptors prior to NE infusion lengthened the duration of the population spike potentiation to 45 min while the mean increase at 15 min post-NE infusion (time of maximum increase when NE was applied alone) infusion was 148% of baseline responses compared to 124.6% of the NE alone infusion. In this set of infusions, all animals exhibited an increase in the population spike

Fig. 2-2

Infusion of the α-adrenergic antagonist PHENT prior to NE. Infusion NE after PHENT (P, n=5, diamonds) produced potentiation of the population spike amplitude over baseline levels (* = minimum $p<.05$). Compared to NE infusion alone (n=12, circles), blockade of the α-adrenergic receptors produced a more robust facilitation of the population spike amplitude (^ = $p<.05$) and also increased the duration of the potentiation. See also Table 2-1.
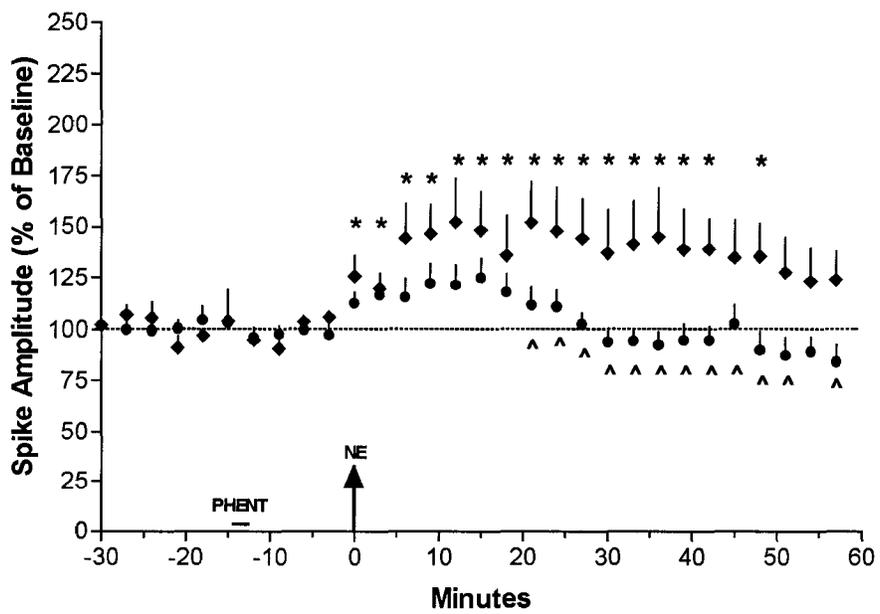
Fig. 2-2 Infusion of the α-adrenergic antagonist PHENT prior to NE.

amplitude and 4/5 rats exhibited potentiation of the EPSP slope with 1 rat exhibiting a substantial decrease of the EPSP slope.

β-adrenergic receptor activation is critical for NE-induced potentiation *in vitro* (Dahl and Li, 1994; Dahl and Sarvey, 1989) and in anesthetized *in vivo* (Harley and Evans, 1988; Harley and Milway, 1986) preparations. We examined the effects of β-adrenergic receptor blockade on i.c.v. NE-induced potentiation by infusing the β-adrenergic receptor antagonist PROP (30 μg), 15 min prior to NE in a subset of rats that had each exhibited potentiation of the population spike or EPSP slope after prior infusion of NE (PROP+NE, n=5). β-adrenergic receptor blockade prevented potentiation of both the synaptic response (101%) and population spike amplitude (107.1%) observed with prior infusion of NE (see Table 2-1). Infusion of the β-adrenergic receptor agonist isoproterenol (ISO) in 5 animals also previously shown to exhibit NE-induced potentiation, revealed a more variable profile. ISO increased the EPSP slope in 2/5 animals while 3 showed no effect (mean at maximal population spike increase 3 min post-ISO infusion- 94.8%. See Table 2-1). The population spike increased within 3 min of i.c.v. ISO (mean= 129.3%) however, these results failed to reach significance. Infusion of vehicle (saline) failed to produce facilitation of either the EPSP slope (94.5%) or population spike amplitude (91.5%). See Table 2-1.

Long Term Effects of i.c.v. NE: Profiles at 24 hr

The results of i.c.v. NE on the dentate gyrus evoked potential appears to consist of short-term potentiation (<30 min) of the synaptic response and population spike amplitude. However, it is possible that NE or β-adrenergic activation and initiation of the cAMP cascade may be producing effects that go beyond a short-term time window. To examine this, a number of the rats receiving infusions of NE, PROP+NE and ISO were monitored 24 hr after the drug infusions (NE=7, PROP+NE=5, ISO=4; short-term data presented above). This would determine whether NE or β-adrenergic receptor activation might produce long-term alterations in synaptic efficacy after initial periods of short-term potentiation of the dentate gyrus evoked response.

Examination of the evoked responses 24 hr post- i.c.v. NE revealed 6/7 rats monitored for long-term effects exhibited potentiation of the dentate gyrus evoked response. This potentiation was characterized as follows 4/7 (57%) rats that received i.c.v. NE exhibited a long-term increase of the EPSP slope and 4 of the 7 (57%) rats exhibited a potentiation of the population spike (See Table 2-1 and Fig. 2-3). The mean percentage increase of the EPSP slope and population spike in animals exhibiting 24 hr potentiation of the population spike was 103.8% and 136.5%* ($F_{1,3}$=13.685, p<.03) respectively. For the 4 rats exhibiting potentiation of the EPSP slope the mean percentage of EPSP slope was 116.7% and the mean percentage change of population spike was 133.7% (data not shown).

Fig. 2-3

Analysis of the dentate gyrus evoked potential 24 hr after NE infusion.

A subset of the rats were monitored 24 hr post-infusion in which it was found that the population spike amplitude was significantly elevated over baseline periods 24 hr prior. Rats which received PROP prior to NE infusion did not demonstrate this facilitation. Though the EPSP slope for the animals with population spike increases at 24 hr was not elevated over prior baseline periods (*shown*), the remaining four rats receiving i.c.v. NE alone did show increases in EPSP slope (not shown) while no rat receiving PROP prior to infusion demonstrated these increases. Infusion of the β-adrenergic receptor agonist ISO also produced a facilitation of the EPSP slope and population spike however, these results did not reach criteria for significance. See also Table 2-1.
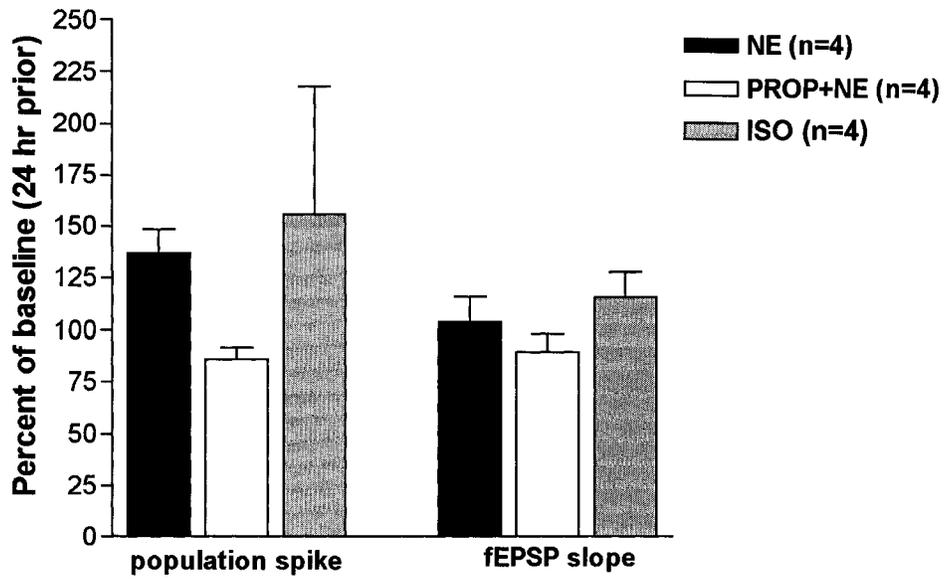
Fig. 2-3   Analysis of the dentate gyrus evoked potential 24 hr after NE infusion.

There did not appear to be a correlation between the short-term effects of i.c.v. NE and the effects seen at 24 hr.

Infusion of PROP prior to NE (PROP+NE, n=4) prevented the potentiation of the EPSP slope (89.1%) and population spike (85.7%) observed at 24 hr in 4/4 rats (Fig. 2-3). β-adrenergic receptor activation by the β-adrenergic agonist ISO, produced a larger potentiation of the population spike amplitude in 2/4 rats at 24 hr than that observed with NE (group mean=155%, n=4. See Table 2-1 and Fig. 2-3) however, two animals did not exhibit potentiation of the population spike so, again the group results of ISO infusion did differ statistically from baseline levels 24 hr prior.

2.5   Discussion

Using a technique designed to mimic the bath delivery procedures of *in vitro* preparations in freely moving animals, short-term increases in the amplitude of the dentate gyrus population spike and the EPSP slope were observed immediately after rats received intraventricular NE. Infusion of the α-adrenergic receptor antagonist PHENT prior to NE increased the duration of the NE-induced potentiation of the population spike amplitude, indicating that α-adrenergic receptor activation is not necessary for NE-induced potentiation and may actually oppose it. Monitoring the evoked responses 24 hr post-NE infusion hints that although the initial increase in the evoked

response may be short-term, there can be changes in the properties of the population cell firing and synaptic responses at longer-term periods due to prior activation of the β-adrenergic receptor. This is the first study to investigate NE-induced potentiation in the awake rat at 24 hr and suggests that NE may modulate the activity of dentate granule cells for at least 24 hr after activation.

The effects of PHENT on NE-induced potentiation found in this study support the results of Dahl and Sarvey (1989) in which bath application of PHENT prior to NE in hippocampal slices produced potentiation of the dentate gyrus evoked potential using a lower concentration of NE than normal. In the present study, α-adrenergic receptor blockade prior to i.c.v. infusion of NE increased the duration of population spike potentiation. Also, PHENT did not augment (nor did it obstruct) NE-induced potentiation of the EPSP slope suggesting that α-adrenergic receptors are not necessary for NE-induced increases in synaptic efficacy at the dentate gyrus synapse in the behaving rat. This is in contrast to the report of Chaulk and Harley (1998) which noted that the majority of NE infusions decrease or produce no change in spike amplitude when PHENT precedes NE. Differences may be due to the effects of anesthetic; urethane anesthetic has been shown to decrease peripheral and central α_2-adrenergic receptor responses in rat (Armstrong et al., 1982). Unlike the results found in the i.c.v. urethane anesthetized preparation (Chaulk and Harley, 1998), where infusion of the β-adrenergic

receptor agonist ISO produced consistent long-term potentiation of the population spike, infusion effects of ISO in the behaving rats presented here were less consistent producing long-term potentiation in less than 50% of the animals.

In the anesthetized preparation, Chaulk and Harley (1998) determined that short-term increases in spike amplitude with i.c.v. NE were not dose dependent, though the most reliable long-term (>20 min.) results were observed with higher concentrations of NE. Prior to the present experiment, Harley and colleagues (Harley et al., 1996) demonstrated, using a combined microdialysis/recording assembly in the anesthetized rat, that i.c.v. infusion of NE only produced long-lasting potentiation when hippocampal intrasynaptic levels of NE in the hippocampus were estimated to be 0.4 μM (30 times greater than basal levels). In the present study, histological evidence of ventricular diffusion in animals showing NE-induced increases of the perforant path-evoked potential was similar to that of Harley et al. (1996) suggesting a similar dispersion of drug.

Contrary to studies using both hippocampal slice and glutamatergic activation of the LC in anesthetized rats, it is possible that the early, short-term increases in the evoked potential observed here might only be present while extracellular levels of NE are increased. However, the β-adrenergic receptor mediated potentiation of the evoked potential 24 hr post-NE infusion suggests that i.c.v. NE is capable of inducing cascade events, presumably

involving cAMP-protein kinase- dependent mechanisms and likely the transcription/translation of new proteins. These processes are instrumental in the noradrenergic model of olfactory learning in rat pups (Mclean et al., 1999; Yuan et al., 2003a). Further investigation of the involvement of these mechanisms in NE-induced potentiation in the dentate gyrus is necessary.

The potentiation of the perforant path-evoked potential 24 hr after NE-infusion suggests NE or β-adrenergic receptor activation may initiate a delayed increase after the initial potentiation has returned to baseline. This late increase was not present if there had not been an initial increase in spike amplitude or EPSP slope measurements within 30 min post-NE. Although it is known that evoked potentials in the rat dentate gyrus are modulated by a circadian rhythm (Barnes et al., 1977; West and Deadwyler, 1980) recording field EPSPs at 24 hr intervals should mitigate against the increases occurring due to a circadian influence.

In summary, bath application of NE by way of intraventricular infusion alters short-term and long-term granule cell activity and synaptic transmission in the dentate gyrus of the awake rat. This alteration in synaptic activity shares characteristics with *in vitro* (e.g. enhancement of the field EPSP response) and *in vivo* (e.g. predominance of short-term potentiation after application of NE) preparations but also suggests the interesting possibility that NE is capable of modulating a long-term potentiation in the dentate gyrus that may be distinct from initial short-term processes.

CHAPTER 3:

A role for orexin-A in attention and memory functions: Norepinephrine release and norepinephrine-induced long-term potentiation in the dentate gyrus following orexinergic activation of the locus coeruleus

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3.1 ABSTRACT

The orexins (ORX-A/ORX-B) are neuroactive peptides known to have roles in feeding and sleep. Evidence of dense, excitatory projections of ORX-A neurons to the noradrenergic pontine nucleus, the locus coeruleus (LC), suggests ORX-A also participates in attention and memory. Activation of LC neurons by glutamate produces a β-adrenergic receptor mediated long-term potentiation (LTP) of the perforant path evoked potential in the dentate gyrus, a target structure of the LC that has been implicated in memory. We asked if ORX-A also activates norepinephrine (NE)-induced LTP by initiating NE release in the hippocampus. Here we show that ORX-A infusion (.25-25 femtomoles) into the LC produces a robust, β-adrenergic receptor-dependent, long-lasting potentiation of the perforant path evoked dentate gyrus population spike in the anesthetized rat. Pharmacological inactivation of the LC with an α_2-adrenergic receptor agonist prior to ORX-A infusion prevents this potentiation. Analysis of NE concentrations in the hippocampus after ORX-A infusion into the LC reveals a transient, but robust, increase in NE release. Thus, this study demonstrates that the dense orexinergic projection to the LC is likely to promote the induction of NE-LTP in the dentate gyrus. ORX-A modulation of LC activity may provide important support for the cognitive processes of attention and memory.

3.2 INTRODUCTION

The orexins (ORXs), also known as hypocretins, are a group of peptides initially implicated in the regulation of appetite and in the control of sleep (De Lecea et al., 1998;Sakurai et al., 1998). ORXs comprise two similar peptides, ORX-A and ORX-B, derived from the same precursor, prepro-orexin (Sakurai et al., 1998). Two receptors, OR_1R and OR_2R, are coupled to G-proteins, presumed to be the G_q-subtype. OR_1R is two to three times more selective for ORX-A, whereas the OR_2R binds ORX-A and ORX-B with equal affinity (Sakurai et al., 1998). Though synthesis of the ORXs is largely restricted to discrete regions of the lateral hypothalamus, ORX axons (particularly ORX-A axons) project widely in the brain and spinal cord (Cutler et al., 1999; Date et al., 1999; Nambu et al., 1999) suggesting this peptide modulates multiple brain and behavioral systems. The locus coeruleus (LC), a pontine nucleus, receives a particularly dense innervation by ORX containing fibers as revealed by immunohistochemistry (Cutler et al., 1999; Date et al., 1999; Nambu et al., 1999) and has one of the highest densities of OR_1R mRNA in the CNS (Hervieu et al., 2001; Marcus et al., 2001; Trivedi et al., 1998) making it an area of particular interest for understanding ORX function. The LC is the source of norepinephrine (NE) in forebrain areas and is involved in vigilance, attention, and memory (see Aston-Jones et al., 1999) and (for review see Harley, 1991). As such, researchers have speculated that

ORX may have a role in the modulation of these cognitive processes (Horvath et al., 1999; Jaeger et al., 2002).

Recent studies have revealed a role for ORX-A in memory formation. ORX-A infused into the lateral ventricle of rats immediately after learning produces an increase in the retention of a passive avoidance task (Telegdy and Adamik, 2002). This study has been replicated in mice with the additional finding that ORX-A also decreased the mean number of trials to reach criterion in active avoidance (Jaeger et al., 2002).

Electron microscopy has revealed tyrosine hydroxylase-positive LC neurons receive largely asymmetrical input from ORX neurons similar to Gray's type I synapses, suggesting these synapses are primarily excitatory (Horvath et al., 1999). *In vitro* application of ORX-A (Hagan et al., 1999; Van den Pol et al., 2002), ORX-B (Horvath et al., 1999; Ivanov and Aston-Jones, 2000; Van den Pol et al., 2002) or infusion of either ORX *in vivo* (Bourgin et al., 2000; Kiyashchenko et al., 2001) increases the firing of LC neurons. Intracellular recordings reveal LC neurons depolarize following the application of ORX-A (Van den Pol et al., 2002) and ORX-B (Ivanov and Aston-Jones, 2000). The depolarization is TTX resistant indicating ORXs directly activate LC neurons (Ivanov and Aston-Jones, 2000).

One target of the LC is the hippocampus, a structure long implicated in memory. The hippocampus itself has only a modest ORX input (Cutler et al., 1999; Nambu et al., 1999) and corresponding modest ORX receptor density

(Trivedi et al., 1998); however, the noradrenergic innervation that it receives from the LC is significant, particularly in the dentate gyrus (Loy et al., 1980). Numerous studies have shown that NE modulates synaptic efficacy at the glutamatergic perforant path to dentate gyrus synapse by initiating a long-lasting potentiation of the perforant path-dentate gyrus evoked potential. This potentiation effect of NE occurs in the absence of tetanization and is referred to as NE-induced long-term potentiation (NE-LTP). NE-LTP provides one mechanism for the enduring changes in neural circuitry required to support memory. NE-LTP was first reported when NE, briefly iontophoresed on the granule cells of the dentate gyrus of the anesthetized rat, produced a long-lasting potentiation of the perforant path evoked population spike (Neuman and Harley, 1983). In other *in vivo* studies, glutamate infusion in the LC has been used to induce LC firing and putative endogenous NE release. This method also produces a long-lasting, β-adrenergic receptor-dependent potentiation of the dentate gyrus population spike in anesthetized rat (Harley and Evans, 1988; Harley and Milway, 1986). However, the duration of the initial potentiation *in vivo*, with either direct application of NE or glutamatergic activation of LC, is variable with only ~50% of experiments demonstrating a potentiation of 30 min or longer. *In vitro* studies have replicated β-adrenergic receptor dependent NE-LTP with bath applied NE and possibly due to the more consistent mode of receptor activation, NE-LTP is consistently long-lasting. NE-LTP is accompanied by increases in cAMP (Stanton and Sarvey,

1985c) and requires protein synthesis (Stanton and Sarvey, 1985b), common characteristics of other memory mechanisms.

Presently, there is little evidence linking the ORX innervation of LC to a physiological action of LC in a target structure. The present study demonstrates that ORX-A infused into the LC produces robust NE release in the hippocampus and a strong and consistent long-lasting potentiation of the dentate gyrus response to its glutamatergic perforant path input. This long-lasting potentiation is dependent upon β-adrenergic receptor activation in the hippocampus and is sustained for hours following the transient elevation of NE by ORX-A.

3.3   MATERIAL AND METHODS

Surgical Procedures

Subjects were male Sprague-Dawley rats 250 to 400 g (Memorial University of Newfoundland) receiving water and regular rat chow *ab libitum* and housed under climate controlled conditions with a 12:12 hour light cycle (lights on at 08:00). All animal procedures were reviewed by the Institutional Animal Care Committee and were in conformity with the guidelines set out by the Canadian Council on Animal Care.

Rats were anesthetized with urethane (1.5 g/kg, i.p) and placed in a stereotaxic instrument in the skull flat position. Trephine holes were drilled for electrodes and cannula placement. A 22 gauge guide cannula (Plastics One),

angled 20° from the vertical, was implanted 2.3-2.5 mm above the LC (12.5-12.6 mm posterior (P), 1.3 mm lateral (L) from midline) and secured with dental acrylic to a small jeweler's screw. A concentric bipolar stimulating electrode (Kopf, NE-100) was directed at the perforant path (P 7.2 mm, L 4.1 mm to bregma and ≈ 3.0 mm ventral from brain surface). An adjustable dual recording assembly allowing independent manipulation of two glass micropipettes, one filled with saline, the other with the β-adrenergic antagonist, propranolol (PROP; 100 mM, Sigma), was aimed at the granule cell layer of the dentate gyrus (AP- 3.5 mm, L- 2.0 mm L from bregma and ≈ 2.5-2.8 mm from brain surface). The tips of the pipettes were separated by approximately 500-750 μm.

Electrophysiology

The perforant path was stimulated at .5 Hz (0.2 ms pulse). The evoked responses from the granule cell layer were filtered (1 Hz - 3 kHz) and digitized at 10 kHz. The recording pipettes (3-5 MΩ) were maneuvered to provide maximal positive going waveforms. Initially an input-output current intensity series (I/O curve) was determined (50-1000 μA, using 50 μA increments), sampling 3 evoked potentials at each current level. The current intensities used for baseline measurements were the currents producing a population spike 50% of max. Waveforms were collected using DataWave software and

analyzed off-line. Evoked responses were sampled every 30 sec for a minimum of 1 hr prior to drug infusion.

Pharmacology

ORX-A (California Peptide) was made up in sterile saline (100 pM, 1 nM, 10 nM, 100 nM) and kept frozen until used. An infusion cannula (28 ga, Plastics One) was attached to a 0.5 µL micro-syringe by autoanalyzer tubing (Fisher) and gently lowered into the LC. ORX-A or saline (200-250 nL) was infused into the LC over a period of 30-60 sec. The injection cannula was left in place for 3 min post-infusion. DL-Propranolol (100 mM, Sigma), used in the second recording pipette, was mixed fresh in sterile saline. As needed, clonidine (200 ng in 200 nL) was mixed in saline and infused into the LC 5 min prior to ORX-A (1 nM).

Microdialysis

NE diasylate in the hippocampus was obtained using a concentric dialysis probe constructed as in Harley et al. (1996), but using a 4 mm exposed membrane. The probe was directed at the hippocampus 5.3 mm posterior to bregma, 5.0 mm lateral from midline and approximately 6.5 mm from brain surface. A 22 ga guide cannula was lowered dorsal to the LC and

secured as detailed above. ORX-A (a gift from Dr. MS Harbuz) was mixed in saline and kept frozen until required.

The microdialysis probe was perfused continually (1.19 μL/min) with artificial cerebrospinal fluid (ACSF; 147 mM NaCl, 3 mM KCl, 1.3 mM CaCl$_2$, and 1 mM MgCl$_2$). Samples of 20 μL were collected every 20 min until three consecutive baseline samples were stable. Following ORX infusion into the LC, sampling continued until NE returned to baseline. Analysis of NE was performed by means of HPLC with electrochemical detection (Harley et al., 1996). In brief, the mobile phase was prepared using a stock mixture of 24 mM sodium acetate, 15 mM citric acid, and 2.3 mM octane-sulphonic acid in deionised water to which methanol (13% v/v) was added. The pH was adjusted to 5.0 with 10 mM NaOH before filtering and degassing. The mobile phase was then pumped through the system at a rate of 1.0 mL/min (Hichrom, 3 μm ODS, 12.5 cm length and 4.6 mm i.d.) maintained at a temperature of 32.5 °C. Electrochemical detection was performed via an Antec Leyden 'Intro' detector with the flowcell set at a potential of +700 mV and output to a chart recorder. NE standards were run prior to each experiment.

Histology and Statistical Analysis

Upon the conclusion of the experiment, a final injection of 200-250 nL of 1% methylene blue (electrophysiology) or polygraph ink mixed 50% with

0.25 M glutamate (microdialysis) was infused into the LC to mark the injection site. Rats were decapitated and the brains were quickly removed and frozen. Saggital sections (30 µm) were taken to verify LC cannula placement. Due to the substantial dendritic arborization of LC neurons (Aston-Jones et al., 1995), rats with LC cannulae tips within 300 µm of the nucleus were considered to have positive sites for the electrophysiological studies, whereas rats with dye touching the body of the LC were considered to have positive sites for the microdialysis studies.

Population spike amplitude and EPSP slope were measured as detailed in the inset to Fig. 3-1. Briefly, the population spike amplitude was measured as the difference between the peak and valley of the downward deflecting spike (c). EPSP slope was measured as: voltage (b-a)/time(a-b). I/O data were normalized using the largest mean population spike or EPSP slope collected using a single current level. Effects of ORX-A infusion in the LC were determined by averaging the last 15 min of the 3 hr recording period post-ORX infusion and responses were compared to the average of the 60 min baseline period. Hippocampal NE levels measured by microdialysis were also compared to baseline data. Repeated measures analyses of variance (ANOVA) were used on raw data and post-hoc comparisons using Duncan's Multiple Range Test were used where applicable (p<.05).

Fig. 3-1 ORX-A infusion into the LC produces a long-lasting potentiation of the dentate gyrus population spike

(A) *Inset*: Evoked potential parameters prior to commencement of experiment. Sample waveforms from a saline and PROP pipettes in one animal at baseline. Vertical scale= 4 mV; horizontal scale= 2 msec. *Graph*: Population spike amplitude with 100 nM ORX-A infusion in the LC, n=6. (B) Population spike amplitude with 10 nM ORX-A infusion in the LC, n=4. C. Population spike amplitude with 1 nM ORX-A infusion in the LC, n=5. All data shown are 3 min means ± s.e.m.
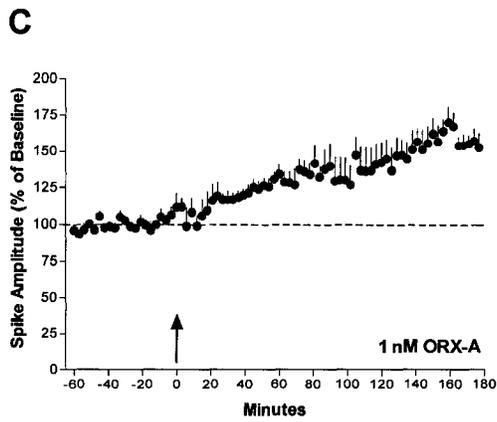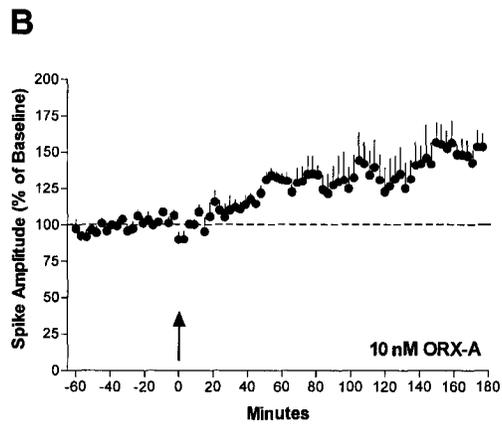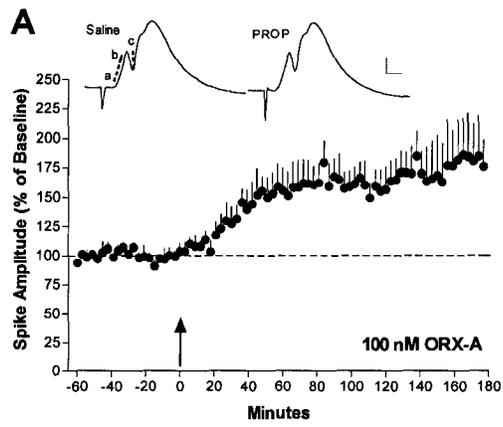
Fig. 3-1 ORX-A infusion into the LC produces a long-lasting potentiation of the dentate gyrus population spike

3.4   RESULTS

The Dentate Gyrus Evoked Potential Following ORX-A Infusion in the LC

Infusing ORX-A into the LC resulted in significant increases of the population spike response at 1 nM ($F_{1,5}$= 9.97, p<0.02) 10 nM ($F_{1,3}$= 27.38, p<0.01) and 100 nM ($F_{1,4}$= 30.612, p<0.005). See Fig. 3-1. No potentiation was observed at 100 pM (data not shown). For the three higher ORX-A concentrations a two way repeated measures ANOVA (concentration x pipette) was carried out to assess whether the β-adrenergic antagonist, PROP, or the orexin concentration affected the final level of potentiation. There was no differential effect of concentration on potentiation (mean overall potentiation = 154.9%). However, at the PROP pipette the potentiation was significantly reduced ($F_{1,12}$=23.19, p<.0004; mean overall potentiation = 123.9%; Fig. 3-2A). There was no long-term effect of ORX-A on EPSP slope at any concentration (Fig. 3-2B). ORX-A injection did commonly elicit a modest, transient increase in EPSP slope at injection that returned to baseline levels.

Infusion of vehicle (NaCl, .9%; n=5) into the LC did not produce an increase in the population spike over the first hour following infusion (Fig. 3-3A). One animal is shown receiving a vehicle injection into the LC and then a subsequent infusion of 1 nM ORX-A (Fig. 3-3B).

Fig. 3-2 **β-adrenergic receptor antagonist reduces potentiation elicited by ORX-A in LC**

(A) Population spike amplitude and EPSP slope (B) patterns summed over the 3 effective concentrations of ORX-A for both the saline and propranolol (PROP) pipettes. PROP reduced the ORX-A effect ($F_{1,12}=23.2$, $p<.0004$) implicating activation of β-adrenergic receptors in the dentate gyrus as mediators of the potentiation.
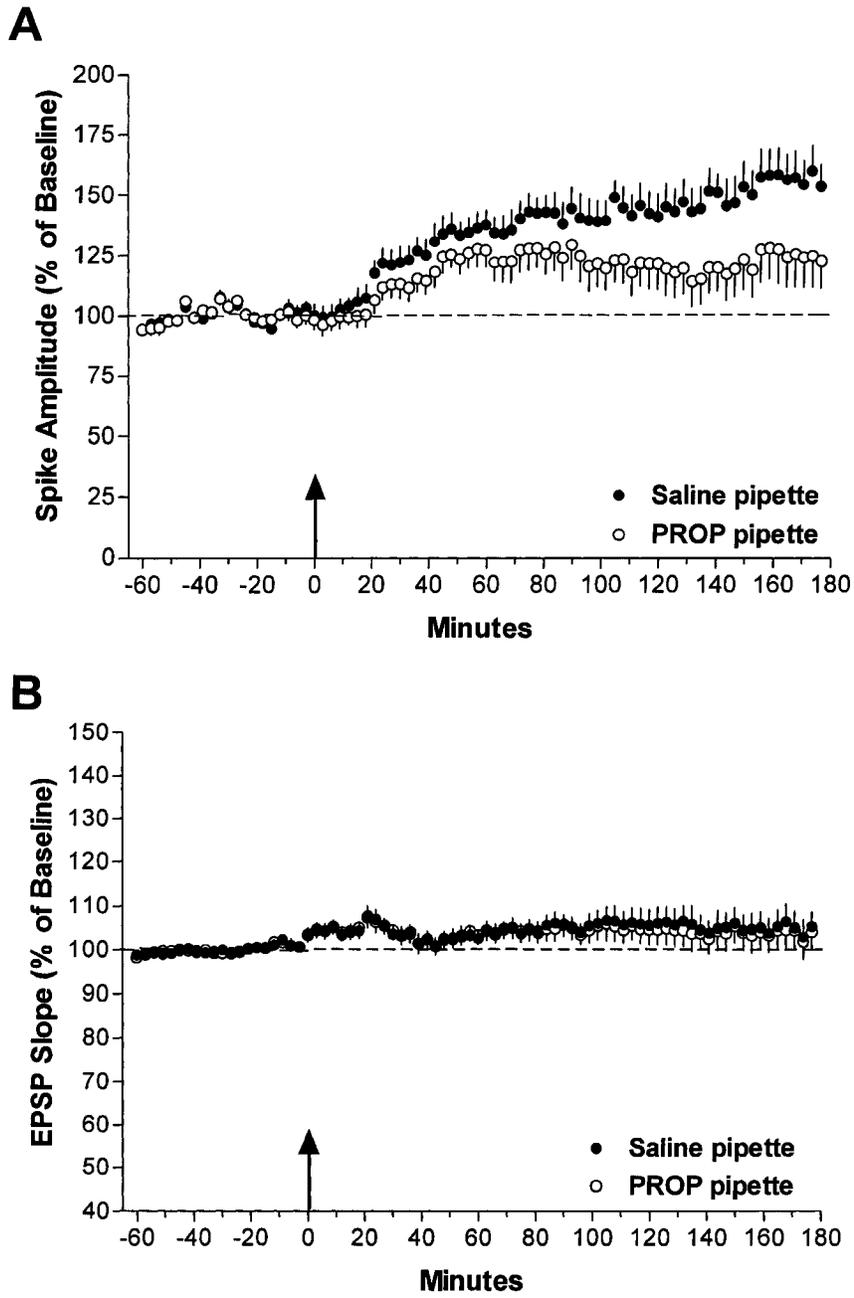
Fig. 3-2 β-adrenergic receptor antagonist reduces potentiation elicited by ORX-A in LC

Fig. 3-3 **Vehicle infusion into LC does not produce increase in evoked response**

(A) Population spike amplitude with saline infusion into the LC, n=5. (B) An example of population spike amplitude changes with saline infusion in the LC followed 75 min later by 1 nM ORX-A infusion. Data shown are means: (A) 3 min (B)10 min ± s.e.m.
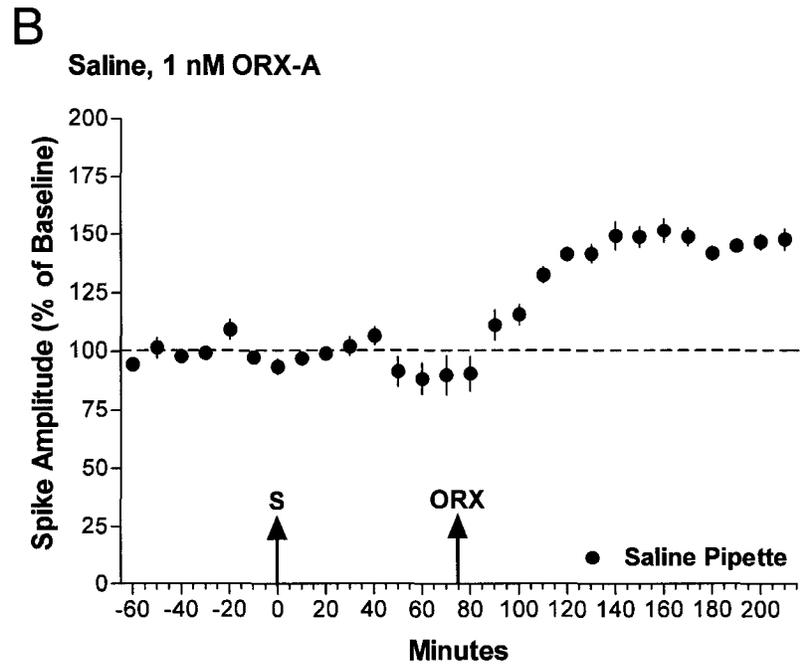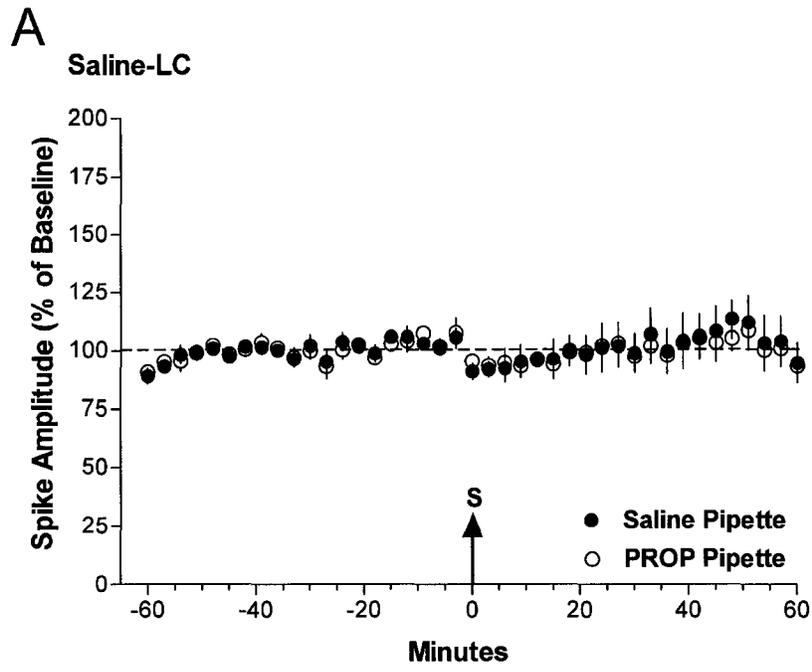
Fig. 3-3 Vehicle infusion into LC does not produce increase in evoked response

To address the specificity of LC activation we infused clonidine, an α_2-adrenoceptor agonist, into the LC to inhibit firing 5 min prior to an infusion of 1 nM ORX-A (Fig. 3-4). Infusion of clonidine prior to ORX-A prevented the facilitation of the population spike (clonidine + 1 nM ORX-A=102.9%; previous 1 nM ORX-A= 156.1%), supporting the critical role of LC activation in producing the potentiation observed with ORX-A.

Analysis of the I/O curves by a two-way repeated measures ANOVA (pipette x current) indicated no differences in the population spike amplitude or the EPSP slope between the two pipettes at any concentration prior to infusion of ORX-A into the LC (see Appendix 1).

NE Release in the Hippocampus Following ORX-A Infusion in the LC

Though application of the ORXs onto LC neurons by either bath application (Hagan et al., 1999; Van den Pol et al., 2002) or by infusion into the LC *in vivo* (Bourgin et al., 2000; Kiyashchenko et al., 2001) can increase the firing rate of LC neurons, the enduring change in response to the perforant path stimulus led us to ask if ORX-A infused into the LC was also producing an enduring change in NE levels in the hippocampus. To assess this we sampled NE levels in the hippocampus using microdialysis preceding

Fig 3-4 Agonism of α_2-adrenoceptor in LC blocks ORX-A induced increase

Population spike amplitude for rats receiving either 1 nM ORX-A (n=6) or clonidine followed by 1 nM ORX-A (n=5). Clonidine prevented the long-term population spike potentiation observed previously in animals receiving 1 nM ORX-A. Data shown are shown as 3 min means ± s.e.m.

Clonidine + ORX-A
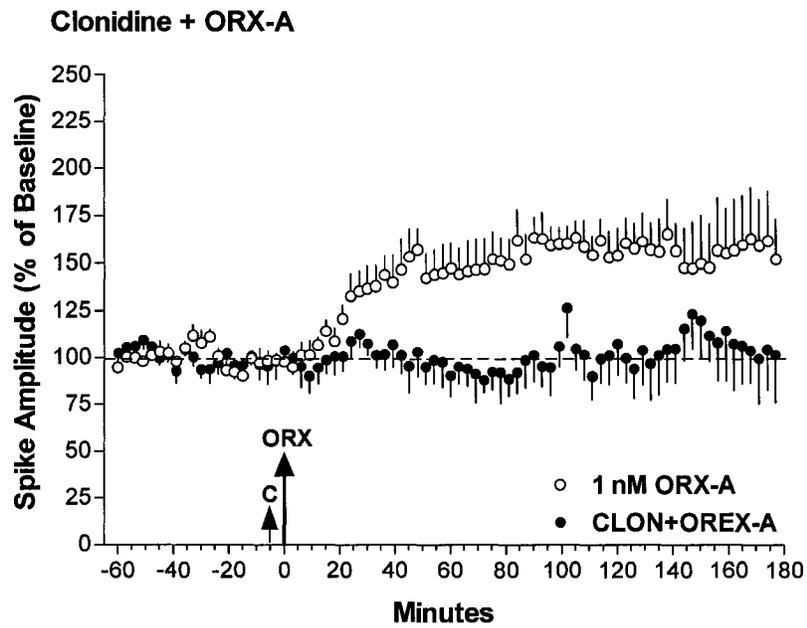
Fig. 3-4 Agonism of α_2-adrenoceptor in LC blocks ORX-A
induced increase

and following effective ORX-A (100 nM, 10 nM, 1 nM) infusion into the LC. A repeated measures ANOVA (concentration x sample) was performed on the three baseline levels of hippocampal NE to assess differences between groups (100 nM, 10 nM, 1 nM) and across samples (-3.0 to -1.0). No differences were found in basal levels of NE among the groups or among the three successive baseline NE responses and, as expected, no interactions between concentrations and baseline samples were found. The mean NE baselines across the three groups of ORX-A (100 nM, 10 nM, 1 nM) concentrations were 13.30, 13.88 and 13.24 fmol/20 µL respectively).

A second repeated measures ANOVA (concentration x sample) was carried out comparing the mean of the baseline samples to the three samples following ORX-A infusion into the LC (samples 1.0 - 3.0). There were no differential effects due to the concentration of ORX-A infused, but there was a significant effect of sample (188.4%; $F_{3,30}=16.55$, $p<0.0001$). Post hoc comparisons revealed that the first NE sample for the 20 min after the infusion of ORX-A into the LC was significantly increased above baseline for each of the three concentrations of ORX-A (Fig. 3-5A-C). Samples of NE in the hippocampus taken in the subsequent 20 min periods did not differ from baseline NE levels for any concentration of ORX-A, indicating a transient release of NE following ORX-A activation of the LC.

To compare ORX-A with glutamate effects on NE release, and to probe site effectiveness, glutamate infusions were carried out at the

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conclusion of most experiments. In 7 experiments microdialysis measurements were continued for an additional hour to evaluate time course, transient NE release was seen in the first 20 min sample (p<.007; see Fig. 3-5D).

A second infusion of ORX-A at the same site was often unsuccessful in eliciting a second increase in NE (6/11 tries). Mechanical infusion effects (vehicle or vehicle + peptide) were evaluated by comparison to a later glutamate infusion at the same site. These comparisons revealed that NE release was triggered by mechanical stimulation on 2/10 occasions.

Fig. 3-5 ORX-A infusion in LC increases hippocampal NE

Microdiasylate concentrations of NE in the hippocampus before (Samples $^-$3.0 – $^-$1.0) and after (Samples 1.0 – 3.0) infusions into the LC. Baseline levels were taken as the mean of the three samples taken prior to LC infusion. Samples of NE diasylate were taken every 20 min. The first sample after infusion of the three concentrations of ORX-A (A-C) or after glutamate (D) were significantly elevated ($p<.05$). Data represent means ± s.e.m.

A

OREX-A 1 nM (*n*=4)

B

OREX-A 10 nM (*n*=5)

C

OREX-A 100 nM (*n*=6)

D

Glutamate 250 mM (*n*=7)
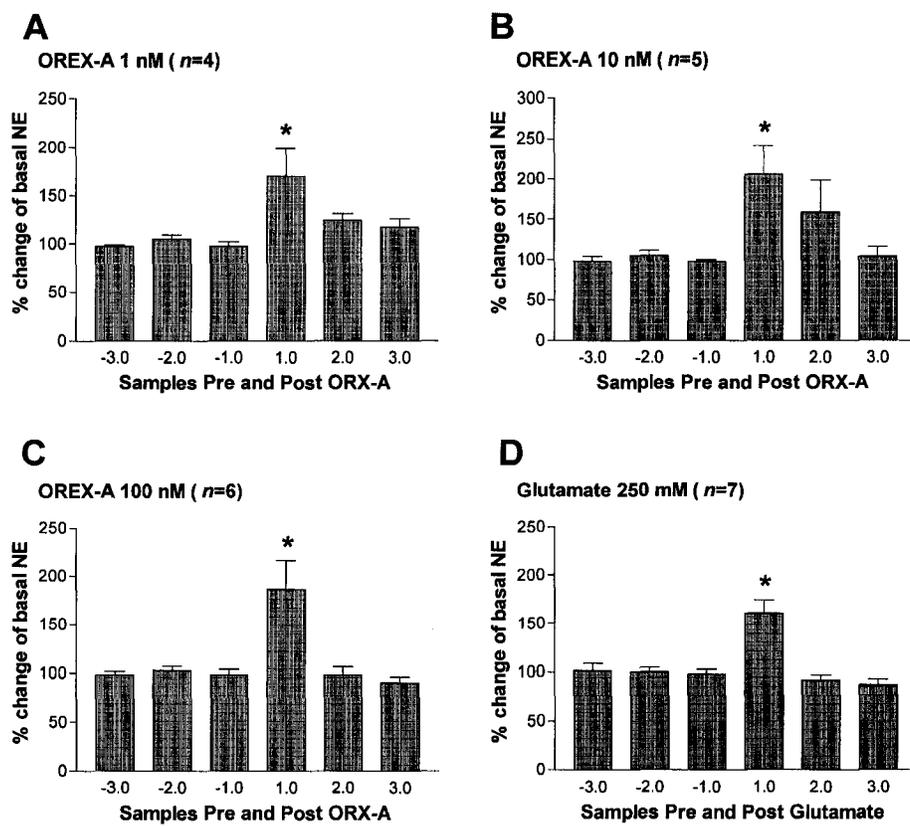
Fig. 3-5 ORX-A infusion in LC increases hippocampal NE

3.5 DISCUSSION

ORX-A application to LC neurons initiated robust, but transient, NE release in the hippocampus and produced a large and long-lasting, β-adrenergic receptor-dependent potentiation of the dentate gyrus population spike in anesthetized rat. ORX-A neurons terminating in the LC provide a pathway for orexinergic modulation of attention and memory processes by regulating NE release in target areas of the LC. The sensitivity of this system is such that less than a femtomole of orexin is sufficient to evoke NE release and facilitate the population spike. In keeping with the potent effects on the perforant path evoked potential, the orexin-LC pathway appears to play a significant role in supporting the neural plasticity that underlies learning and memory. These results may have clinical relevance. In narcolepsy, where there is an deficit in orexinergic transmission, memory impairments (Henry et al., 1993) and difficulties with vigilance and attention tasks (Rieger et al., 2003; Rogers and Rosenberg, 1990).

The long-lasting facilitation of perforant path throughput to the rest of the trisynaptic hippocampal circuit resulting from ORX-A infusion into the LC resembles that observed following the infusion of glutamate into the LC, although details of the facilitation vary. ORX-A infusion into LC produces a substantial increase in the population spike (~155% of baseline) and consistently enduring NE-LTP in contrast to the more variable effects observed following glutamate infusion (for glutamate examples see Harley et

al., 1989; Harley and Milway, 1986). The overall pattern of potentiation also varies somewhat with an earlier and more abrupt potentiation following glutamate infusion and a more gradual increase over time following ORX-A infusion. Despite these differences in the extent and time course of the facilitation, enhancement of the population spikes in both cases is dependent upon activation of β-adrenoceptors in the hippocampus (see Harley and Milway, 1986), consistent with a common final pathway underlying the facilitation (Harley and Evans, 1988).

Differences between the effects of ORX-A and glutamate on the firing pattern of LC neurons may explain the variability of their action on the perforant path population spike. The infusion of glutamate into LC results in a ~500 msec burst of spikes recorded from LC neurons followed by a silent period and then a gradual return to baseline firing (Harley and Sara, 1992). On the other hand, ORX-A infusion results in a tonic activation of LC neurons (Kiyashchenko et al., 2001), through a slower G-protein coupled receptor mechanism. When 200 nM of ORX-A is infused in the vicinity of the LC, muscle tone is modulated following a delayed onset, that parallels a transient, dose dependent, rise in LC firing (Kiyashchenko et al., 2001). Doses in the present study were lower than those evaluated for changes in muscle tone. Thus, in the present study, LC neurons were probably activated tonically, but transiently, by the infusion of ORX-A. ORX also increases LC cell synchrony (Van den Pol et al., 2002). A combination of tonic firing with enhanced

synchrony may be relevant to the robust, consistent NE-LTP induced by ORX infusion in the LC. Preliminary studies in our laboratory reveal that glutamatergic activation of the LC in the awake rat produces a late-phase, β-adrenergic receptor-dependent facilitation of both synaptic strength and population spike amplitude 24 hr after infusion into the LC (Walling and Harley, unpublished observations). Whether orexinergic activation of LC neurons can initiate a similar pattern of change at 24 hr remains to be examined.

The low does of ORX-A employed in the present study, relative to previous *in vitro* and *in vivo* studies, may account for the flat dose-response curve observed here both for potentiation and for NE release. Site variability with infusion methods also makes dose-response assessments difficult. The ineffectiveness of repeated doses at the same site corroborates observations of desensitization *in vitro* (Van den Pol et al., 2002).

Results from the microdialysis experiments demonstrate that both glutamate and ORX-A activation of LC neurons produce a transient, but robust, increase in the level of NE in the hippocampus. The long-lasting increase in the population spike (>3 hr) in the face of a transient increase in evoked NE release is in keeping with the hypothesis that elevated NE is an initiating event for the change in synaptic transmission and that elevated NE is not required for maintenance of the enhancement.

In addition to the ability of ORX-A to initiate NE release by activating the LC, ORX-A promotes NE release presynaptically when applied to cerebrocortical slices (Hirota et al., 2001). Other studies have shown i.v. infusions of ORX-A also increase glutamate release in the LC suggesting indirect, as well as, direct excitatory actions of ORX-A on LC neurons (Kodama and Kimura, 2002). Thus, multiple mechanisms are in place to reinforce the ability of ORX-A to modulate behavior through increases in forebrain NE.

In summary, the present data confirm, *in vivo*, that ORX-A is a robust activator of LC neurons and initiates NE release in an LC target structure. This ORX-A action promotes an enduring potentiation in the response of dentate gyrus neurons to their perforant path input, potentially contributing to increased attention to, and memory for, perforant path mediated events. Electrophysiological and behavioral studies in awake rats will enhance this initial characterization of the effects of the ORXs on attention and memory mechanisms and on the modulation of NE release.

CHAPTER 4:

Locus coeruleus activation initiates a persistent β-adrenergic- and protein synthesis-dependent synaptic facilitation of perforant path input to the dentate gyrus of behaving rats: A novel mammalian memory mechanism.

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4.1 ABSTRACT

Norepinephrine, acting through β-adrenergic receptors, is implicated in mammalian memory. The present study uses glutamatergic activation of the locus coeruleus to induce release of norepinephrine in the hippocampus in awake rats and examine modulation of perforant path input to the dentate gyrus. Locus coeruleus activation initiates a potentiation of synaptic perforant path input to the dentate gyrus that is observed 24 hr later. This late phase potentiation of the synaptic potential is not preceded by an early phase potentiation, although spike potentiation is seen both immediately following and 24 hr after locus coeruleus activation. Intracerebroventricular infusion of the β-adrenergic antagonist, propranolol, or the protein synthesis inhibitor, anisomycin, prior to locus coeruleus activation blocks the potentiation of perforant path input observed at 24 hr. The present data are consistent with Kety's prediction that locus coeruleus activation initiates persistent synaptic facilitation in support of memory encoding in the mammalian brain. The initiation of a late phase potentiation observed at 24 hr, but not in the 3 hr following locus coeruleus activation, parallels the observation of a cAMP- and protein synthesis- dependent long-lasting synaptic facilitation in *Aplysia* that is not preceded by a short-term synaptic facilitation. Locus coeruleus-initiated synaptic potentiation may selectively support long-term, rather than short-term, memory. The observation of selective initiation of long-term synaptic facilitation in a mammalian brain, as in invertebrates, is additional evidence

that these two forms of memory depend on separable biological underpinnings.

4.2   INTRODUCTION

In 1970 Seymour Kety (Kety, 1970b) proposed that "the state of arousal by means of adrenergic input to each (cerebral, hippocampal and cerebellar cortex) may serve to concurrently reinforce and to consolidate the significant sensory patterns, the affective associations and the motor programs necessary in the learning of a new adaptive response". This proposal suggested the widely distributed noradrenergic locus coeruleus (LC) neurons would serve the function of providing a "diffusely projecting reticular formation discharge throughout both hemispheres, a discharge conceived to be a '"Now print!' order for memory" as described earlier by Livingston (1967).

The diffuse anatomical distribution of noradrenergic fibers and older pharmacological data underpinned Kety's proposal that LC activation initiates memory storage. Kety envisaged a persistent synaptic facilitation linked to norepinephrine's elevation of cAMP and recruitment of protein synthesis. He hypothesized that novelty and affective events triggered noradrenergic activity and that inputs occurring at that time would be incorporated into memory. As he predicted, LC neurons do respond to novelty (Vankov et al., 1995) and to affective events (for review (Berridge and Waterhouse, 2003).

Pharmacological data since Kety's proposal continue to support a role for norepinephrine in memory, particularly in long-term memory, in rodents (Clayton and Williams, 2000b; Izquierdo et al., 1979) and humans (Quevedo et al., 2003; van Stegeren et al., 1998). To this point, however, physiological

studies support an attentional, rather than a memory, role for the LC. As recently reviewed, LC activation enhances the efficacy of both excitatory and inhibitory synaptic transmissions in sensory areas, increases the signal-to-noise ratio in sensory responses, promotes detection of perithreshold sensory events and the sharpening of suprathreshold events, facilitates the fidelity of sensory transmission in the thalamus and supports arousal patterns of EEG activity in cortical structures (Berridge and Waterhouse, 2003). These effects depend directly on the level of LC activity and do not persist when LC activity returns to baseline.

Although *in vitro* studies have demonstrated norepinephrine induced long-term potentiation (LTP) of perforant path synaptic strength in the dentate gyrus of the hippocampus, the potentiation is limited to the medial perforant path, as the lateral perforant path is depressed by norepinephrine (Dahl and Sarvey, 1989). In *in vivo* studies using anesthetized rats, norepinephrine, or activation of the LC, increases granule cell excitability, as indexed by larger population spikes to perforant path stimulation, without increasing synaptic strength, as indexed by EPSP slope.

In the invertebrate, *Aplysia*, a cAMP-dependent increase in synaptic strength has been demonstrated to occur 24 hr after pairing of sensory input and the monoamine serotonin (Brunelli et al., 1976; Schacher et al., 1988). This long-term synaptic facilitation does not require short-term synaptic facilitation (Emptage and Carew, 1993) and supports other evidence for

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separate short- and long-term memory processes. In mammals, a cAMP-associated plasticity that uniquely relates to long-term facilitation of synaptic strength has not been previously reported.

In the present study we monitor the dentate gyrus response to perforant path input in freely moving rats for 3 hr after glutamate infusion into the LC to induce NE release, and at 24 hr after glutamate infusion into the LC, to show that LC activation initiates a ß-adrenergic and protein synthesis-dependent long-term, but not a short term, increase in the synaptic strength of concurrently activated perforant path input to the dentate gyrus.

4.3   MATERIALS AND METHODS

Surgical Procedures

Male Sprague-Dawley rats (electrophysiology- Memorial University) and male Wistar rats (microdialysis- Bantin and Kingman, Hull, UK) weighing 250-350 g were housed singly and allowed access to food and water *ad libitum*. Experimental procedures occurred within the dark phase of the animals' cycle and were performed in accordance with the Canadian Council of Animal Care guidelines and followed a protocol approved by the Institutional Animal Care Committee.

Rats were anesthetized with chloral hydrate (80 mg/kg, i.p.) and placed in a stereotaxic instrument in the skull flat position. Trephine holes were drilled and four jewelers' screws were used to anchor the recording assembly

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with two serving as ground electrodes. A 22 gauge stainless steel cannula (Plastics One) was angled 20° from the vertical, positioned approximately 2.7 mm above the LC (12.4 mm posterior to bregma, 1.3 mm lateral from midline), and held in place with dental acrylic. Teflon-coated stainless steel wire (A-M Systems) was used to construct stimulating (bipolar, 150 μm) and recording (50 μm) electrodes. The electrodes were positioned in the perforant path (7.2 mm posterior to bregma, and 4.1 mm lateral from midline, ~3.0 mm ventral from brain surface) and the granule cell layer of the dentate gyrus (3.5 mm posterior and 2.0 mm lateral, ~ 2.5 mm ventral from brain surface), respectively. The field EPSP and the population spike were maximized by small movements of the electrodes before they were cemented into place. Electrodes were secured in a nine-hole McIntyre connector (Ginder Scientific). A second cannula was placed above the lateral ventricle (0.8 mm posterior to bregma and 1.5 mm lateral) ipsilateral to the electrodes and LC cannula in those animals receiving an i.c.v. injection. Following an injection of chloramphenicol (10 mg in 0.2 ml, s.c) animals were allowed to recover for one week.

Habituation and Recording Procedures

Animals were handled daily and allowed to habituate to the recording chamber for a minimum of 30 min on two occasions before commencement of recording. The recording chamber consisted of a Plexiglas box measuring 42

cm x 30 cm x 42 cm in which the animals could roam freely while attached to a commutator (Joseph Biela Idea Development). Bedding covered the floor and rat chow and water were provided. Recordings were made during three sessions (total ~6 hr) starting at the same time over three consecutive days with drug application occurring during the second session. Rats were allowed to habituate for 10 minutes before each recording session.

Stimulation to the perforant path consisted of a single, 0.2 msec square wave pulse (ISI 30 sec) delivered by a constant current unit (Neurodata Instruments). Evoked potentials were differentially amplified at a bandwidth of 1 Hz – 3 kHz using P511 polygraph amplifiers (Grass Instruments), digitized at 10 kHz, and stored on a PC for further analysis.

Each recording session began with the determination of an input/output current intensity relationship (I/O curve) followed by a Baseline and/or Test period. Sessions proceeded as follows: *Session 1* (24 hr pre-LC activation): I/O curve and 1 hr Baseline stimulation; *Session 2* (LC activation): I/O curve, 1 hr Baseline stimulation, glutamate injection into the LC and 3 hr Test period (Short-term Test); and *Session 3* (24 hr post-LC activation): I/O curve and 1 hr Test period (Long-term Test). See Appendix 2 for diagrammatic details. I/O stimulation of the perforant path consisted of increasing current intensities (50-1000 μA, ISI of 10 seconds, 50 μA increments) collecting three samples at each current level. Baseline and Test sessions consisted of stimulation of the perforant path (ISI 30 seconds) at the

intensity that elicited a population spike 50% of max during the I/O curve of Session 1. For diagrammatic representation of methods see Appendix 2.

Drug Application

Monosodium-L-glutamate (Sigma) was mixed fresh (250 mM) in sterile saline prior to injection into the LC. A 28 gauge internal cannula attached to a 1 μl syringe (Hamilton) by autoanalyzer tubing (Fisher) was positioned in the LC. Glutamate (200-250 nL) was infused over 30 s and the injection cannula left in place for 3 min.

DL-Propranolol (Sigma, 30 μg in 5 μl) was mixed in saline prior to each experiment and was injected into the lateral ventricle over 5 min 15 min prior to the glutamate infusion. Anisomycin (Sigma, 5 mg) was dissolved in 30 μL 1 N HCl, the pH was adjusted ($\cong$ pH 7.0) with NaOH, and the solution diluted to 100 μl with saline. Infusion of anisomycin into the lateral ventricle (5 μl, 1 μl/min) began 30 min prior to the glutamate infusion. Following the last recording session rats were anesthetized with chloral hydrate and 250 nL of methylene blue (1%) was infused in the LC, followed by decapitation and removal of the brain. Brains were frozen in chilled methylbutane and stored at -70°C for sectioning and Nissl staining.

Data Analysis

The I/O curve consisted of three successive stimulations of the perforant path at each intensity the average of which was normalized to the largest mean population spike or EPSP slope of the I/O curve taken during the second session. Repeated measures Analyses of Variance (ANOVA) were used to compare I/O data between Sessions 1 and 2 and Sessions 2 and 3. Population spike amplitude or EPSP slope did not change between the two baseline I/O curves (Sessions 1 and 2) demonstrating the I/O relationship was stable prior to the commencement of experimental procedures.

Repeated measures analyses of variance (ANOVAs) were performed on the 1 hr averages for the baseline and test periods of Sessions 1-3. Post-hoc comparisons using Duncan's multiple range test were performed where applicable.

Analysis of the baseline recording sessions of Session 1 and Session 2 prior to activation of the LC (repeated measures ANOVA; group x session) revealed there were no significant group x session interactions (EPSP slope: $F_{2,15}$=2.286, p>0.05; population spike amplitude: $F_{2,15}$=0.24, p>0.05). Also, there were no differences in the baseline population spike or EPSP slope responses of the animals among the three groups (rats receiving glutamatergic activation of the LC, GLUT-LC; rats receiving the β-adrenergic antagonist propranolol prior to LC activation, PROP and rats receiving perforant path stimulation alone) nor were there differences in the evoked

responses over the two baseline recording sessions for either the population spike amplitude ($F_{2,15}$=1.21, p>.05) or EPSP slope ($F_{2,15}$=1.04, p>.05).

4.4 RESULTS

Norepinephrine-Induced Potentiation

To initially explore the long-term effects of NE release on the perforant path-dentate gyrus evoked potential, we examined the evoked responses after glutamatergic activation of the LC in the freely moving rats. Three groups of rats were used: 1) glutamate infusion into the LC (GLUT-LC, n=7); 2) intracerebroventricular infusion of the β-adrenergic receptor antagonist, propranolol (30 µg in 5 µl), 15 min prior to LC activation (PROP, n=5); and 3) a control group that received perforant path stimulation (Control, n=7). Control animals did not receive infusions of a vehicle into the LC as the mechanical stimulation has been shown to activate LC neurons (Stone et al., 1995) presumably promoting NE release.

Glutamatergic activation of the LC (GLUT-LC) resulted in a substantive increase in the 24 hr synaptic response to perforant path input as measured by the slope of the field excitatory synaptic potential (EPSP) over prior baseline levels (126%, p<.00005; Fig. 4-1A$_1$) and by an increase in the population spike amplitude (156%, p<.001, Fig. 4-1A$_2$).

Fig. 4-1 NE-LTP is β-adrenergic receptor-dependent

(a_1) Inset: Examples of the evoked potential waveforms from a GLUT-LC rat during the Baseline period of Session 2 (solid line), 1 hr and 24 hr post-injection (dotted lines). Scales are 2 mV and 2 msec. Graph: EPSP slope for the GLUT-LC group (n=7) receiving glutamate at the *arrow* and the Control group (n=7) during Session 2. Data are normalized to the baseline of session 2 and 5 min averages with standard errors of the mean are shown for both the 3 hr Test and Long-Term Test (24 hr) periods. EPSP slope of the GLUT-LC group did not increase during the 3 hr Test period, but an increase was observed at 24 hours. (a_2) Population spike data as outlined for EPSP slope in a_1. The population spike amplitude in the GLUT-LC group increased over baseline following the glutamate injection and was elevated at 24 hr.

(b): Bar graphs of data from A representing 1 hr means for the 3 hr Test session, and the Long-Term Test period (24 hr) for GLUT-LC, Control, and PROP (n=5) groups. (b_1) The GLUT-LC group showed a significant EPSP slope increase at 24 hours. The PROP group demonstrated a small decrease in EPSP slope over the 3 hr Test period, but had returned to baseline at 24 hr. Controls, receiving stimulation of the perforant path alone, showed no change in EPSP slope at any time point. (b_2) The GLUT-LC group showed significant increases in spike amplitude in all sessions after glutamate injection. Neither the Control nor PROP group showed any change in spike amplitude. Data are normalized to the baseline taken during the second session and are shown with the standard error of the mean. Duncan's multiple range tests were carried out on raw data, but are shown as percentage change; * $p<.05$, ** = $p<.001$, *** $p<.00005$.
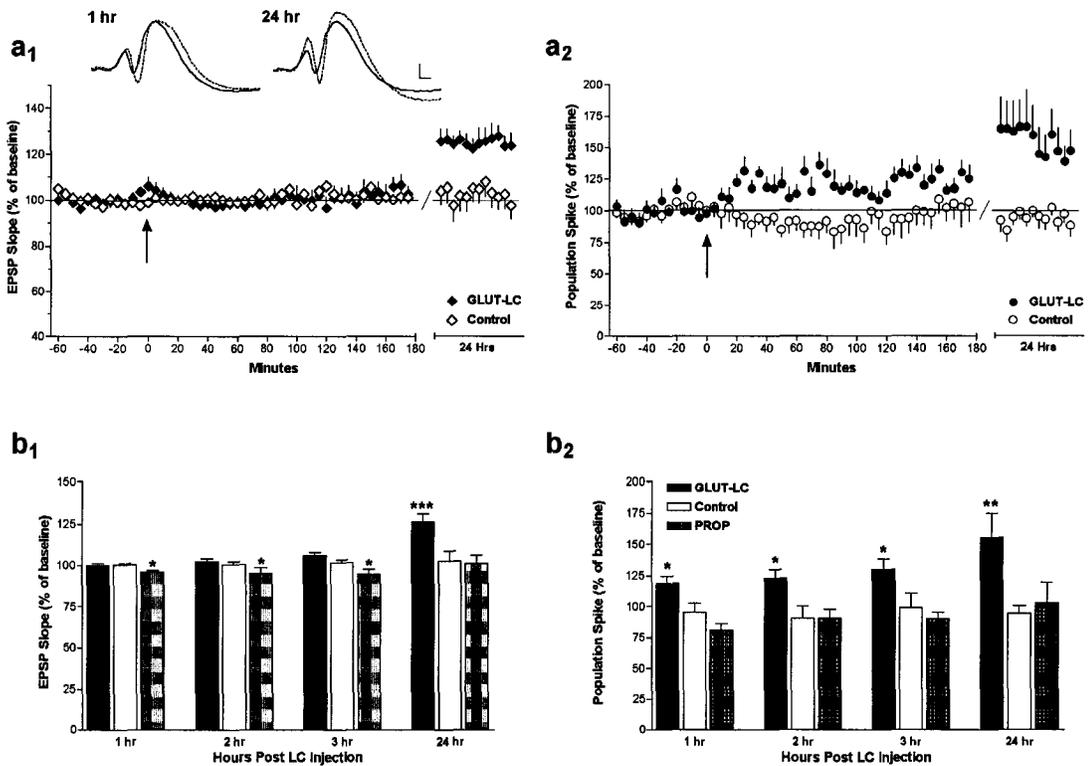
Fig. 4-1 NE-LTP is β-adrenergic receptor-dependent

All rats exhibited these increases and β-receptor blockade prevented the facilitation of both EPSP slope and population spike amplitude (PROP group, EPSP 101%; population spike amplitude 102%, Fig. 4-1B). In the absence of LC activation in Control rats there was no change in EPSP slope or population spike amplitude over the 24 hr period (Fig. 4-1).

During the immediate 3 hr period after LC activation EPSP slope did not vary, however an overall long-lasting increase in the amplitude of the population spike was observed (118%, 122% and 130%; Fig. 4-1). Propranolol prevented the increase in population spike amplitude and there was a small reduction in the synaptic component of the evoked potential lasting for the 3 hr ($p<.05$; Fig. 4-1B). Individual animals varied in their profiles of spike potentiation, 5 rats exhibited elevated spike amplitude throughout the 3 hr period, whereas spikes were elevated in the first 2 hr, but decreased at 3 hr in the other two rats.

An input/output current intensity curve (I/O curve) was carried out at the beginning of each recording session to more completely assess the changes in the evoked potential after activation of the LC. The I/O curve revealed that the EPSP slope (Fig. 4-2A) increased at all but the lowest current intensity 24 hr after LC activation. The population spike (Fig. 4-2B) was increased at higher current intensities. Control animals showed no change in the I/O curves of the EPSP slope (Fig. 4-2A) or population spike amplitude (Fig. 4-2B) over the 3 recording sessions.

Fig 4-2. Input/ouput intensity curve analysis

I/O curves for the 3 recording sessions. The mean population spike amplitude or EPSP slope for each intensity was converted to a percentage of the largest mean spike amplitude or EPSP slope obtained during the (I-O) curve of the second session. All data represent the mean across groups ± s.e.m.

(a) I/O analysis of the EPSP slope. No differences were observed in the population spike during the baseline periods in GLUT-LC and Control groups. In contrast, significant differences were observed in the GLUT-LC group 24 after the glutamate injection (repeated measures ANOVA comparing Sessions 2 and 3).

(b) I/O analysis of the population spike amplitude. No differences were observed in the population spike during the baseline periods in GLUT-LC and Control groups. As with the EPSP slope data significant differences were observed 24 after the injection of glutamate. (Repeated measures ANOVA; Duncan's range, * = minimum p<.05)

(c) EPSP slope to population spike (E-S) coupling ratio for GLUT-LC and Control groups. At 24 hours after glutamate injection there is no leftward shift in E-S coupling as occurs in short-term NE potentiation. There is also no change in the Control group.

a

GLUT-LC

CONTROL

- Session 1 (24 hr pre)
- Session 2
- Session 3 (24 hr post)

b

GLUT-LC

CONTROL

- Session 1 (24 hr pre)
- Session 2
- Session 3 (24 hr post)

c

GLUT-LC

CONTROL

- ● Baseline
- ○ 24 hrs

— Baseline
----- 24 hrs
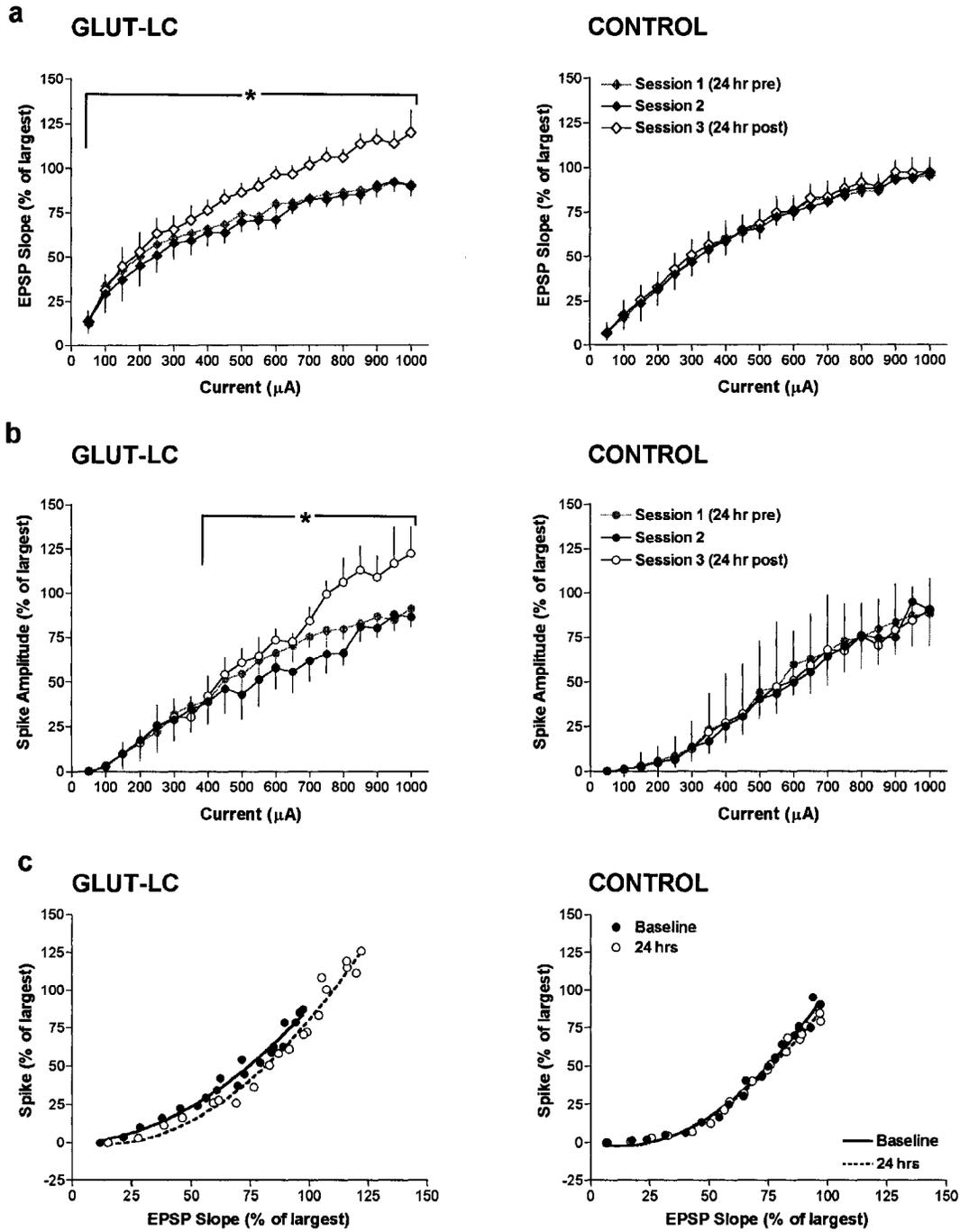
Fig. 4-2 Input/output intensity curve analysis

The effects of late phase NE-induced LTP on E-S coupling was probed by plotting the mean percentage EPSP slope vs. spike amplitude in GLUT-LC and Control animals (Fig. 4-2C). There was no leftward shift in the EPSP/population spike relationship at 24 hr suggesting EPSP slope potentiation at 24 hr accounted for population spike potentiation, a common characteristic of tetanic models of LTP.

Histological analysis of LC activation sites for both GLUT-LC and PROP+GLUT-LC animals revealed a mixture of intranuclear and pericoerulear locations, though pericoerulear locations were predominant. For LC cannulae placements see Appendix 3.

Potentiation is dependent on protein synthesis

One condition, common across memory models, is that long-term changes in neural responses are dependent on the synthesis of new proteins. Both long-term facilitation in Aplysia (Dale et al., 1987) and late phase frequency-induced long-term potentiation in the mammalian hippocampus (Dale et al., 1987;Sarvey et al., 1989) rely on the products of protein synthesis. In order to determine whether protein synthesis was necessary for the NE-induced long-term potentiation observed at 24 hr, we repeated the initial experiment infusing glutamate into the LC with (GLUT-LC+Aniso, n=7), and without (GLUT-LC$_2$, n=7) first infusing the protein synthesis inhibitor,

anisomycin (250 μg in 5 μl), into the ipsilateral lateral ventricle 30 min prior to the infusion of glutamate in the LC. A group of animals received intraventricular anisomycin only in order to assess whether inhibition of protein synthesis affects baseline responses over a 24 hr period (Aniso, n=5).

An analysis comparing the mean of 24 hr after LC activation to that of Session 2 baseline revealed that all animals receiving LC activation (GLUT-LC_2) show a significant increase in EPSP slope (110%, repeated measures ANOVA, $F_{1,6}$=19.20, p<.005) and population spike amplitude (130%, repeated measures ANOVA, $F_{1,6}$=9.31, p<.02) at 24 hr (Fig. 4-3). Animals receiving intraventricular anisomycin to inhibit protein synthesis prior to LC activation (GLUT-LC+Aniso) showed no change in EPSP slope ($F_{1,6}$=3.80, p<0.10) or the amplitude of the population spike ($F_{1,6}$=1.12, p<0.33). Animals receiving intraventricular application of anisomycin alone did not vary from baseline levels indicating inhibition of protein synthesis in the absence of LC activation does not affect baseline synaptic efficacy.

As found in the prior set of experiments a two-way repeated measures ANOVA (group x session) indicated no differences in EPSP slope ($F_{2,16}$=1.24, p<0.32) or population spike amplitude ($F_{2,16}$=1.63, p<0.22) over the two baseline recording sessions (Sessions 1 and 2). These parameters were also stable over the two baseline periods (EPSP slope $F_{1,16}$=3.14, p<0.10 and population spike: $F_{1,16}$=.0009, p<0.98).

Fig. 4-3   Late phase NE-LTP is protein synthesis-dependent.

(A) EPSP slope amplitude during the Long-Term Test (24 hr). Infusion of anisomycin, an inhibitor of protein synthesis (GLUT-LC + Aniso), blocks the increase in EPSP slope induced by injection of glutamate into the LC (GLUT-LC_2: repeated measures ANOVA; ***$p < 0.005$). Responses in animals receiving anisomycin alone (Aniso) did not vary from baseline response.

(A) Population spike amplitude during the Long-Term Test (24 hr). As with the EPSP slope, anisomycin prevented the increase in the population spike observed following glutamate injection into the LC (repeated measures ANOVA; **$p < 0.02$). Anisomycin alone did not alter the population spike.

a

EPSP Slope (% of baseline)

b

Spike Amplitude (% of baseline)

Long-Term Test (24hrs)

Long-Term Test (24hrs)

GLUT-LC$_2$
GLUT-LC + Aniso
Aniso
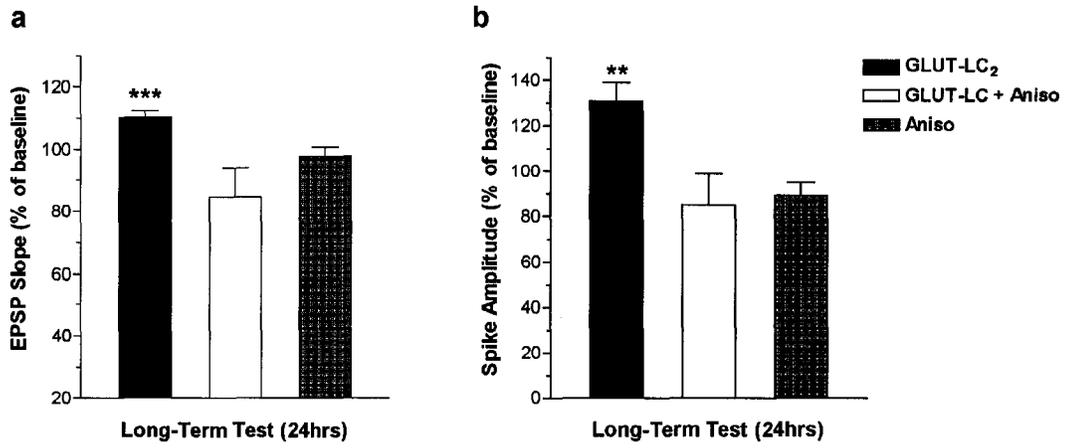
Fig. 4-3   Late phase NE-LTP is protein synthesis-dependent

During the 3 hr post-LC activation there was a 5-10 min increase in EPSP slope in 5/7 rats (data not shown), but no significant EPSP slope effect over the 3 hr blocks. There was also no overall population spike potentiation, however, individual animals varied. Two rats in the GLUT-LC group and in the LC+Aniso group exhibited spike potentiation throughout the 3 hr block, the remainder showed no change or a decrease in spike amplitude.

Location of the glutamate infusion sites were predominantly in the body of the LC as revealed by histological analysis. A separate analysis of LC activation effects in rats with pericoerulear versus direct coerulear locations of the cannula over the two sets of experiments revealed that pericoerulear sites were more often associated with early potentiation of spike amplitude and no change in EPSP slope (5/7), while coerulear sites were associated with a brief 5-10 min increase in EPSP slope without early potentiation of spike amplitude (4/7). One coerulear targeted rat showed both effects. In any case both locations produced a protein synthesis and β-adrenergic receptor dependent potentiation of EPSP slope and spike amplitude at 24 hr.

4.5   DISCUSSION

To our knowledge, this is the first study to demonstrate persistent facilitation of synaptic strength following activation of the LC, as predicted originally by Kety (1970b), confirming the hypothesis that the LC provides a mechanism for memory consolidation, a "Now print!" order. Two important

features of these observations are the occurrence of the increase in synaptic strength at 24 hr and a failure to see the increase in synaptic strength during the immediate 3 hr after LC activation. This suggests the LC mechanism supports long-term, but not short-term, memory. Thus, glutamate infusion in the LC, known to produce a burst of LC cell activity (Harley and Sara, 1992), may initially promote spike potentiation, as seen here and in previous studies (Harley et al., 1989a; Harley and Milway, 1986; Washburn and Moises, 1989), however, 24 hr later a significant increase in EPSP slope is observed. The increase in EPSP slope at 24 hr predicts the increase in population spike amplitude at that time; the immediate increase in spike amplitude after glutamate infusion in the LC appears to reflect a change in cell excitability. Both the 24 hr EPSP slope and population spike amplitude increase induced by LC activation depend on β-adrenergic receptor mediation and protein synthesis as predicted by Kety.

In *Aplysia* repeated 5-HT application produces long-term facilitation of the sensory-motor neuron synaptic response (Mauelshagen et al., 1998; Montarolo et al., 1986). This depends on activation of a receptor coupled to the cAMP cascade (Schacher et al., 1988), is blocked by anisomyocin (Montarolo et al., 1986; Schacher et al., 1988; Sherff and Carew, 1999) and can occur in the absence of short-term facilitation (Emptage and Carew, 1993). Thus, the noradrenergic promotion of long-term synaptic potentiation observed in the rat parallels serotonergic promotion of a long-term synaptic

facilitation in *Aplysia*. The ability to induce LTP, without an initial short-term potentiation, is consistent with other evidence for separate mechanisms supporting short-term and long-term memory processes (Izquierdo et al., 2002; Sullivan and Sagar, 1991; Warrington, 1979).

NE-induced LTP at 24 hr also shares important similarities with late phase tetanic-induced LTP. The latter has been examined at all synapses in the tri-synaptic hippocampal pathway (Huang et al., 1996). This form of LTP also depends on the cAMP-protein kinase A cascade (Abel et al., 1997; Nguyen and Kandel, 1996b) and protein synthesis (Barea-Rodriguez et al., 2000; Frey et al., 1988; Krug et al., 1984). β-adrenergic receptor activation is a requirement for tetanus-induced late phase LTP at the perforant path-dentate gyrus synapse (Bramham et al., 1997), at the mossy fiber CA3 synapse (Huang and Kandel, 1996), and late phase LTP in the lateral amygdala (Huang et al., 2000). In a recent study using the population spike as the index of potentiation Straube and Frey have shown LTP induced by moderate or strong protocols in the perforant path require ß-adrenergic receptor activation for late-phase maintenance but the strongest protocol was independent of ß-adrenergic receptor activation (Straube and Frey, 2003). Thus, multiple mechanisms recruit persistent facilitation at this synapse and LTP protocols synergize with ß-adrenergic receptor activation supported heterosynaptic facilitation.

While repeated presentations of serotonin and tetani are typically used to produce long-term facilitation in *Aplysia* and late phase LTP in hippocampus respectively, a 3 sec theta-burst tetanus will trigger late phase LTP in the hippocampus (Nguyen and Kandel, 1997). LC activation initiates theta rhythm in the hippocampus (Berridge and Foote, 1991). We suggest that the combination of convergent glutamatergic and noradrenergic input coincident with theta may be initiating conditions for the late phase NE-induced LTP seen here. Late phase LTP of EPSP slope can also be induced with repeated, spaced application of a low concentration of the β-adrenergic agonist, isoproterenol, *in vitro* (Dahl and Li, 1994).

The early effects of LC activation parallel those reported in other studies e.g. (Harley et al., 1989; Klukowski and Harley, 1994; Lacaille and Harley, 1985) and suggest there may be both short-term and intermediate forms of NE potentiation of the evoked potential depending on details of the NE release pattern. The early effects of LC activation are either predominantly on cell excitability or on medial perforant path input (Dahl and Sarvey, 1989). The critical conditions for their appearance, such as the differing sites of LC activation seen here, and the details of their properties, require further investigation. The early effects of spike facilitation alone are consistent with other evidence implicating activation of LC in attention (Berridge and Waterhouse, 2003) rather than memory.

The 24 hr data suggest, however, that LC also acts to selectively promote LTP, a putative mediator of long-term memory. LC activation can produce long-term memory in some paradigms (e.g., Sullivan et al., 1989). In rat pup olfactory preference learning, β-adrenoceptor stimulation in the olfactory bulb paired with glutamatergic input from olfactory neurons produces an odor preference 24 hr later (Sullivan et al., 2000). This olfactory memory depends on cAMP elevation (Yuan et al., 2003a) and the phosphorylation of cAMP response element binding protein (CREB) at the time of acquisition (Yuan et al., 2000). Both cAMP and phosphorylation of CREB are widely implicated in memory formation (Silva et al., 1998).

Though few behavioral studies have investigated the effects of NE on memory using designs that assess both short-term and long-term memory, there are data suggesting NE may have a more important role for long-term than short-term memory. Infusing NE into the hippocampus immediately post-training, Izquierdo and colleagues found no difference in the retention of an inhibitory avoidance task measured 1.5 hrs after NE infusion, but when tested 24 hrs after NE infusion, retention of the memory task was greater than that of controls (Izquierdo et al., 1998). In an active avoidance task, the level of NE release at training correlated positively with the strength of memory at 24 hr (McIntyre et al., 2002). In a taste aversion task, activation of the protein kinase A pathway is necessary for long-term, but not short-term memory (Koh et al., 2002). With genetic reduction of NE synthesis, long-term memory, but

not short-term memory, for three forms of associative learning is selectively impaired (Kobayashi et al., 2000). Other paradigms demonstrate a critical role for the cAMP cascade in long-term mammalian memory (Abel et al., 1997). Most recently, in humans, the enhancement of memory by emotional arousal, previously shown to depend on the activation of central β-receptors (van Stegeren et al., 1998), improved long-term memory tested at 1 week, but not short-term memory tested at 1 hr (Quevedo et al., 2003). The present pattern of results, following burst activation of the locus coeruleus, is consistent with a selective role for a NE and β-adrenergic receptor mediated potentiation of synaptic circuitry in long-term memory.

CHAPTER 5: SUMMARY

The emotional significance of events clearly modulates an organism's ability to recall important information regarding both their external and internal environments. This thesis has shown that putative increases in synaptic concentrations of NE, a neurotransmitter released during emotional and arousing circumstances, mediate long-term changes in the dentate gyrus, a neural structure involved in memory. Many specific comments and concerns with respect to the results of the previous chapters have been addressed in the bodies of each of the individual chapters. What follows are: a summary of the research outcomes, a model of NE-induced potentiation, a discussion of cAMP-dependent memory processes and future directions for research are presented.

5.1 RESEARCH OUTCOMES

This work investigated NE-induced synaptic plasticity in the dentate gyrus of the rat. Three separate techniques were employed to facilitate NE transmission at the perforant path-dentate gyrus synapse in the awake and anesthetized rat. First, exogenous NE was introduced through the lateral ventricle in awake rats in an effort to meld *in vitro* bath application of drug with evoked potential assessment in the absence of anesthetic. The results of this study demonstrated that bath-type application of NE in the awake rat, as in hippocampal slice models, could produce potentiation of EPSP slope, an

effect not consistently demonstrated in *in vivo* NE models, in addition to the normally observed potentiation of population spike amplitude. The potentiation effects appeared to depend on β-adrenergic receptor activation, a signature of both *in vitro* and *in vivo* models of NE-potentiation. Though the initial NE-induced potentiation in the dentate gyrus returned to baseline levels, usually within 30 min of infusion, a long-term (24 hr), β-adrenoceptor-mediated potentiation also occurred, giving the first indication that NE may produce distinct effects supporting short-term and long-term memory processes.

A second study investigated NE-induced plasticity in the dentate gyrus of the anesthetized rat after activation of LC neurons by a newly discovered neuropeptide arising from the lateral hypothalamus, ORX-A. This approach has potential benefits in that it permits potent, yet highly selective, activation of LC neurons in the brainstem. Results from this study indicate that ORX-A infusion (1-100 nM) into the LC transiently increases NE release in the hippocampus and produces a reliable, long-lasting (>3 hr) potentiation of the perforant path-dentate gyrus population spike. As in prior studies of NE-induced potentiation, *in vitro* and *in vivo*, this potentiation depended on β-adrenergic receptor activation in the dentate gyrus. Direct infusion of the α_2-adrenergic receptor agonist, clonidine into the LC prior to ORX-A also antagonized potentiation suggesting again that the effects of ORX-A were selectively mediated by noradrenergic LC neurons.

Though it was already known that NE could modulate short-term plasticity in the dentate gyrus, a pressing question still remained unanswered. Could NE, released through natural mechanisms, modulate *long-term* plasticity in the dentate gyrus? Although Kety proposed that NE did provide the basis for long-term memory in cortical structures over 30 years ago, there has been no direct physiological evidence, to this point, that it does. This was the focus of the third set of experiments. Glutamatergic activation of the LC was used to elicit NE release in the hippocampus of the awake rat and the perforant path response was monitored for long-term alterations in dentate granule cell activity. As the prior study presented in this work (Chapter 2) investigating long-term effects of i.c.v. NE suggested, glutamatergic activation of LC neurons elicited a substantial late-phase (protein synthesis dependent) potentiation of evoked activity in the dentate gyrus. This potentiation included β-adrenergic receptor-dependent facilitation of *both* the EPSP slope and the population spike, again, a characteristic not typical of acute *in vivo* preparations. Further, this potentiation was independent of short-term potentiation of either EPSP slope or population spike amplitude, a result previously described in *Aplysia* after monoaminergic activation at the sensory-motor neuron synapse in that preparation, but never described in a mammalian nervous system.

5.2 Late Phase Norepinephrine-Induced Potentiation: A Novel Mammalian Memory Model

The data in this dissertation support a role for NE in the production of long-term synaptic plasticity in the dentate gyrus of the rat. It has been shown that NE-induced long-lasting potentiation of granule cell activity to perforant path stimulation observed after either exogenous NE application or after endogenously released NE in both the anesthetized and the awake rat is strongly dependent on the activation of β-adrenergic receptors. Results in awake animals demonstrate that NE potentiation is novel in that late-phase, long-term potentiation of the dentate gyrus evoked potential does not depend on the prior appearance of short-term potentiation (though it may occur). This finding goes against much of the current literature which proposes that long-term potentiation is a protein synthesis dependent progression from protein synthesis-independent short-term potentiation (e.g. Mayford and Kandel, 1999). However, this surprising finding in a mammalian nervous system has been previously reported in a simple invertebrate nervous system (Emptage and Carew, 1993); and is supported by behavioral studies in both rats (Izquierdo et al., 1998) and humans (van Stegeren et al., 1998) where application of NE or increases of emotional arousal has been shown to selectively facilitate long-term retention of memory while short-term memory remains unaffected.

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5.2.1. The Role of the β-adrenergic Receptor

This model of NE-induced long-term potentiation is mediated by the β-adrenergic receptor and can initiate events that are either short-term (protein synthesis independent) or long-term (protein synthesis dependent). The implications of β-adrenergic dependence will be discussed further in the sections that follow. See Figure 5.1

Activation of β-adrenergic receptors by NE, or β-adrenergic receptor agonists, stimulate adenylyl cyclases which increase intracellular levels of cAMP (Atkinson and Minneman, 1991; Siggins et al., 1969). Electrical stimulation of the LC is known to increase cAMP levels in ipsilateral cerebral cortex compared to the contralateral control hemisphere (Korf et al., 1979). In turn, cAMP regulates cAMP-dependent protein kinase (PKA). PKA is known to mediate multiple events which may determine whether short-term or long-term potentiation (or both) is observed:

5.2.2. PKA Effects: Short-Term Potentiation

One short-term PKA-mediated event depends on the phosphorylation of rectifying K^+ channels (see Fig. 5.1). This phosphorylation event closes the K^+ channels and thus inhibits rectifying K^+ currents, the net effect leaves the membrane potential in a slightly depolarized (more excitable) state.

Fig. 5.1   NE-Induced Potentiation

A model of NE-induced potentiation is presented. NE, applied exogenously, or through release from noradrenergic neurons in the LC activates β-adrenergic receptors. Activation of β-adrenergic receptors (β_1-R) increases the levels of adenylyl cyclase, which in turn increase intracellular levels of cAMP and cAMP-dependent protein kinase (PKA). PKA can modulate short-term potentiation by phosphorylating (encircled P's) membrane-bound proteins, e.g., closing rectifying K^+ channels thereby depolarizing the membrane potential. PKA is also known to phosphorylate Ca^{++} channels (L-type) increasing intracellular Ca^{++} levels. Late phase, long-term processes can be initiated by the phosphorylation of intranuclear CREB by PKA, activating protein transcription. At this time, the role of α-adrenergic receptors in late-phase NE-induced potentiation in the awake rat is unclear though it has been shown that activation of α_1-adrenergic receptors (α_1-R) can also initiate CREB phosphorylation through PKC. α_2-adrenergic receptor (α_2-R) activation has been shown to inhibit adenylyl cyclase in non-hippocampal tissue (*red arrow*) decreasing intracellular levels of PKA. The effects of α_2-adrenergic receptor activation on intracellular cascades has not been directly investigated in granule cells so their role in NE-induced potentiation remains unclear.
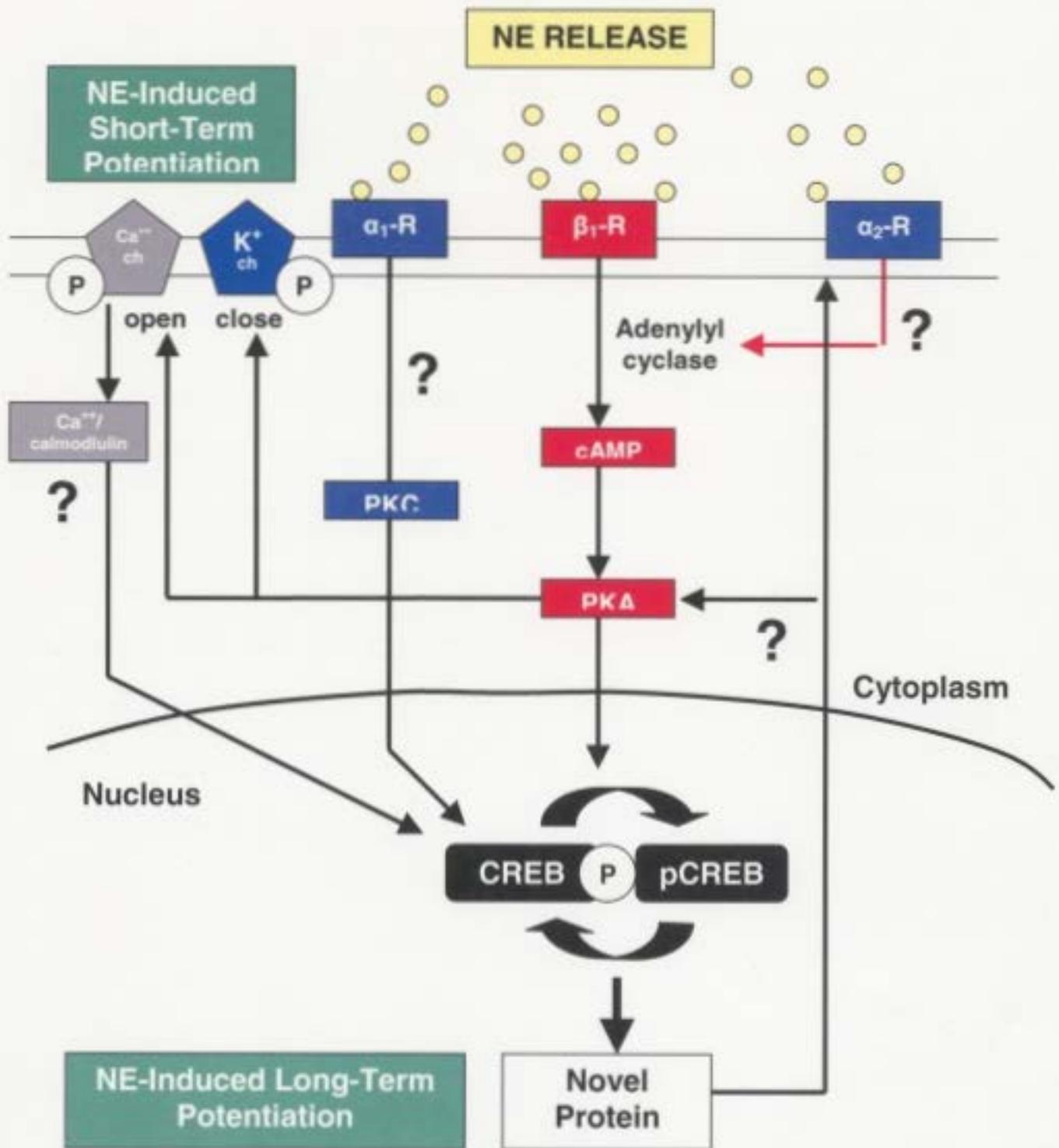
Fig. 5-1: A model of late phase NE-induced potentiation

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There is also evidence of phosphorylation of Ca^{++} (L-type) channels by β-adrenergic receptor mediated-PKA (Gao et al., 1997) activation. In keeping with this, Stanton and Heinemann (1986) report that perfusion of NE onto hippocampal slices results in decreases in extracellular concentrations of Ca^{++} in the granule cell layer but similar changes were not observed in area CA1 (Stanton and Heinemann, 1986).

5.2.3. PKA Effects: CREB Phosphorylation and Late-Phase Potentiation

NE induces phosphorylation of CREB, a nuclear regulator of protein transcription. This effect is mimicked by β-receptor activation and stimulation of adenylyl cyclase activity by forskolin (Schomerus et al., 2003). After PKA activation, PKA translocates to the nucleus from the cytoplasm where it phosphorylates CREB and initiates protein synthesis (Rehfuss et al., 1991). The role of CREB in memory has been well documented in a variety of species (see below).

5.2.4. Role of α-adrenergic receptors?

The role of the α-adrenergic receptors in late-phase NE-induced potentiation has only been touched on in this work. NE-induced potentiation of the evoked EPSP slope in granule cells after i.c.v. infusion was not impaired by the prior application of an α-adrenergic receptor antagonist and α-adrenergic receptor blockade increased the duration of population spike

potentiation. Thus, it can be surmised that α-adrenergic receptor activation is not a requirement for the short-term effects of i.c.v. NE in the awake rat. However, the effects of α-adrenergic blockade on NE-induced potentiation (elicited by either i.c.v. NE or activation of the LC) at 24 hr is not known. Though data from *in vitro* and *in vivo* studies on the role that α-adrenergic receptors play in NE-induced potentiation have largely been inconclusive, application of α-adrenergic receptor agonists have produced potentiation in both preparations (Lacaille and Harley, 1985; Winson and Dahl, 1985). There is evidence to suggest these receptors may play at least a small role in the modulation of granule cell evoked activity:

1.) α_1-**adrenergic receptors**: A recent study has shown that α_1-adrenergic receptor activation leads to phosphorylation of CREB through Ca^{++}-dependent protein kinase (PKC) (Thonberg et al., 2002), although a prior study (Roseboom and Klein, 1995) found that α_1-adrenergic receptor activation was not able to phosphorylate CREB in pineal tissue. This issue remains to be specifically investigated in dentate gyrus.

2.) α_2-**adrenergic receptors** There is evidence that α_2-adrenergic receptors in non-hippocampal tissue, antagonize the cAMP-dependent PKA cascade by inhibiting the action of adenylyl cyclase (for review see (Saunders

and Limbird, 1999). However, this effect has not been investigated in dentate gyrus.

5.2.5. Short-term and Long-Term Memory: Are They Dissociable?

A common view in learning theory is that stable long-term, protein synthesis-dependent memory requires the formation of less stable, protein synthesis-independent short-term memory prior to consolidation (De Luca and Giuditta, 1997; Mayford and Kandel, 1999). However, there is a growing body of evidence that suggests short-term and long-term memory may be distinct, and not progressive, phases (Mauelshagen et al., 1996). The two experiments in this dissertation investigating the effects of NE on long-term synaptic efficacy in the dentate gyrus (Chapter 2 and 4) found that different methods of NE application had different effects on short-term and long-term potentiation. Intraventricular application of NE produced consistent, though transient, short-term potentiation in the dentate gyrus with responses likely to be potentiated 24 hr after activation. Endogenous NE release elicited by glutamatergic activation of LC neurons was less likely to produce short-term potentiation but produced consistent and robust potentiation 24 hr after activation. These results distinctly parallel those found in *Aplysia*. As mentioned previously, short-term facilitation of the sensory-motor neuron synapse (responsible for sensitization of the gill withdrawal response in

Aplysia) is not required for expression of long-term facilitation (Emptage and Carew, 1993).

One method Emptage and Carew (1993) used to elicit long-term facilitation in the absence of short-term facilitation, was the application of 5-HT at concentrations subthreshold for the induction of short-term facilitation. The concentration of 5-HT was "sufficient to increase sensory neuron excitability but insufficient to produce short-term synaptic facilitation". It is a possibility that the differences in short-term potentiation found using i.c.v. application and evoked release of NE may be a consequence of differential synaptic concentrations of NE in the dentate gyrus; 50 μg NE infused into the lateral ventricle is likely to increase synaptic concentrations of NE sufficiently to initiate short-term potentiation while the concentration of NE released by glutamatergic activation of the LC may not be high enough to elicit short-term potentiation but is still able to initiate long-term transcription processes. While previous studies of glutamatergic LC activation have found consistent short-term potentiation, these studies have all used small infusions of 500 mM glutamate to activate the LC while the present study reduced glutamate concentration to 250 mM. The difference in frequency of observation of short-term excitability effects may relate to the strength of the LC burst effect seen with the two methods of LC activation.

5.3. cAMP-Dependent Learning

A number of studies have investigated the behavioral consequences of cAMP-dependent modulation in hippocampus. β-adrenergic receptor blockade and an inhibitor of PKA activity, given into the hippocampus immediately after training on a passive avoidance task interferes with long-term memory (Bevilaqua et al., 1997). Bernabeu et al. (Bernabeu et al., 1997) demonstrated that memory for a passive avoidance task is interrupted if the PKA inhibitor KT5720 is infused into the hippocampus immediately, or at 3 and 6 hr after training. This effect is interesting as it implies that PKA activity, at periods long after (3-6 hr) short-term memory, is necessary for long-term memory for an event to occur. This point will be addressed further in the Future Directions section below.

LTP studies have also revealed a crucial role for the β-adrenergic receptor/cAMP-dependent cascade. High frequency stimulation inducing LTP in the dentate gyrus is known to be dependent upon the activation of β-adrenergic receptors (Bramham et al., 1997; Straube and Frey, 2003). Tetanic stimulation protocols increase the level of cAMP in hippocampal slices which is blocked by prior depletion of NE by 6-OHDA (Stanton and Sarvey, 1985c). A dominant negative inhibitor of PKA decreases LTP in CA1 and decreases spatial and long-term memory for conditioned fear but leaves short-term memory intact (Abel et al., 1997).

5.3.1. cAMP-dependent CREB and Learning

It is accepted that long-term memory requires alterations of existing synapses and requires synthesis of new proteins and thus, gene expression. CREB has been dubbed the "universal memory molecule" as its initiation of transcriptional processes appears necessary for the formation of memory. Numerous biochemical techniques used in a variety of species establish the role of CREB phosphorylation in learning. Some examples follow:

5.3.1.1.   Aplysia

Studies investigating the role of CREB on memory began in *Aplysia*. The first study investigated the effects of injection of a competitive oligionucleotide into the nuclei of a sensory neuron selectively blocking long-term facilitation in *Aplysia* (Dash et al., 1990). Later it was found that two forms of CREB exist, CREB1 and CREB2. CREB2 (ApCREB2 in *Aplysia*) represses CREB1 activity and inhibits long-term facilitation in *Aplysia* sensory neurons. Injection of ApCREB2 antibodies reverses this effect and decreases threshold stimulation levels for elicitation of long-term facilitation (Bartsch et al., 1995).

5.3.1.2. Drosophila

In *Drosophila*, a paired odor/shock memory is known to initiate a g-coupled protein cascade involving cAMP. Using this model a number of

mutants have been developed involving disruption of cAMP-mediated transmission e.g. *dunce* (Dudai et al., 1976) and *rutabaga* (Duerr and Quinn, 1982). Yin et al. (1994) described a mutant CREB transgene that could selectively impair long-term rather than short-term memory retention. Soon after, Yin et al. (1995) produced another mutant with an activator isoform of CREB attached to a heat-shock promoter. This inducible CREB facilitated learning and turned one-trial learning, which normally produces only short-term memory, into long-term memory.

5.3.1.3.   Mice

The development of mutant mice models of CREB based learning allowed researchers the opportunity to investigate more complex behavior. Mice with targeted mutation of the alpha/delta isoforms of CREB (decreasing functional CREB levels) have decreased long-term memory in fear conditioning and water maze tests. In these mice short-term memory lasting 30-60 min remained undisturbed (Bourtchouladze et al., 1994). A recent study using reversible interference with CREB transcription factors in dorsal hippocampus have shown learning deficits only in hippocampus-based tasks. In these mice forskolin-induced LTP is also reduced further implicating cAMP and cAMP-dependent protein kinase in hippocampal-based learning (Pittenger et al., 2002).

5.3.1.4. Rats

Unfortunately, a mutant model technology in rats is not yet developed. However, numerous studies using immunocytochemical and viral vector techniques have done much to increase the knowledge of CREB based learning in this species. Immunocytochemical analysis of pCREB levels have revealed the time window for pCREB upregulation in the hippocampus during stressor events (Bilang-Bleuel et al., 2002), memory acquisition (Colombo et al., 2003), LTP (Schulz et al., 1999), and long-term depression (Deisseroth et al., 1996) and have correlated the increase in pCREB levels to the cAMP-PKA cascade during spatial memory tasks (Mizuno et al., 2002).

Introduction of retrovirus technology has allowed further assessment of pCREB on learning. Recently, Yuan et al. (2003b) injected herpes simplex virus expressing dominant negative CREB into the olfactory bulbs of rat pups. The dominant negative form of CREB inhibited olfactory learning while pups receiving viral CREB injection experienced a leftward shift in the dose-learning curve. Similar effects have been observed with fear conditioning when viral-CREB is injected into the amygdala (Josselyn et al., 2001).

5.4. FUTURE DIRECTIONS

The experiments presented here support the hypothesis that NE produces alterations in synaptic efficacy in the dentate gyrus, reminiscent of memory formation and that these alterations may specifically facilitate long-term memory. However, much work is still necessary to further elucidate the role of NE in memory. Here, a few ideas regarding further investigation of the role of NE in memory formation are discussed under three separate themes: Physiology, Molecular Biology and Behavior (though none are mutually exclusive).

5.4.1. Physiology

5.4.1.1.   Effects of ORX-A activation of LC in the awake rat

ORX-A activation of LC neurons produced a robust potentiation of the perforant path evoked population spike in the anesthetized rat. Although the profile of the potentiation varied somewhat from the LC-glutamate evoked event, LC-ORX-A induced potentiation shared many similarities with NE-induced potentiation by glutamatergic activation. First, potentiation of the population spike was the primary effect while effects on the field EPSP slope were inconsistent. Secondly, ORX-A activation of LC produced increases in the levels of hippocampal NE similar to those seen with glutamatergic activation. Population spike potentiation was also reduced by the β-

adrenergic receptor antagonist, PROP, suggesting that the LC-ORX-induced facilitation was mediated by the same intracellular cascade as the glutamatergic-induced facilitation. In keeping with this, it is predicted that ORX-A activation of the LC in awake rats would produce potentiation of both EPSP slope and population spike 24 hr after activation and that these responses would also be β-adrenergic receptor- and protein synthesis-dependent. Possible cautions: it has been shown that firing activity of LC neurons varies between the two methods of LC activation (glutamate is associated with transient burst firing, while ORX is associated with tonic firing) which may lead to differences at long-term time periods.

5.4.1.2. Role of α-adrenergic receptors in late-phase, NE-induced potentiation

As mentioned earlier, evidence about the exact role of α-adrenergic receptors in NE-induced potentiation using *in vitro* and acute *in vivo* preparations has not been conclusive. The role of these receptors needs to be investigated at long-term time points in the awake rat because if the α_1-adrenergic receptors are capable of phosphorylating CREB and initiating transcriptional processes these will not necessarily be uncovered by investigation of NE-induced potentiation in anesthetized preparations as transcription/translation processes are typically very lengthy.

5.4.1.3. Address the origin of the perforant path stimulation

NE-induced short-term potentiation of the dentate gyrus evoked potential is input specific. NE increases the response elicited from the medial perforant path while it depresses lateral perforant path input (Dahl and Sarvey, 1989). It has been hypothesized that the failure of NE to induce changes in the field EPSP in *in vivo* preparations is that evoked responses recorded from the granule cell layer are actually a mixed input from both medial and lateral perforant paths. In essence, this might lead to nil effects, or an absence of change if the population response is a combination of potentiation + depression. In an attempt to determine whether this is a plausible explanation a simple procedure is suggested to determine input specificity of electrical stimulation of the entorhinal cortex. The mRNA of an immediate early gene, *Arc*, has been shown to selectively target active synapses (Guzowski et al., 2001; Steward and Worley, 2001). Transcribed in the nucleus, *Arc* mRNA translocates to areas in which NMDA receptors have been activated (e.g. mid-molecular layer of the dentate gyrus after medial perforant path stimulation). *In situ hybridization* of *Arc* mRNA levels in the dentate gyrus in anesthetized or awake animals would help determine the localization of perforant path stimulation and plasticity effects.

5.4.1.4. NE-induced potentiation and hippocampal EEG

A few studies have investigated the effects of NE on hippocampal EEG. Effects of increases in synaptic concentrations of NE on theta frequency (4-11 Hz) EEG have been unclear. Heynen and Sainsbury (1991) report that intrahippocampal injection of NE decreases theta rhythm EEG, a result they found to be modulated by α_2-adrenergic receptors. Berridge and Foote (1991) report "intense theta rhythm in hippocampus" 5-30 sec after application of a cholinergic agonist in LC neurons. To this point, the effects of gamma frequency oscillations (30-80 Hz), believed to underlie cognitive and motor functions (Csicsvari et al., 2003), have not been explored.

5.4.2. Molecular Biology of Late Phase NE-Induced Potentiation

5.4.2.1. Determination of phosphorylated CREB levels

β-adrenergic receptor activation in the rat pup is required for early olfactory learning and increases bulbar levels of the phosphorylated CREB (Yuan et al., 2000). Does β-adrenergic receptor-dependent NE-induced potentiation in the dentate gyrus increase levels of the transcription factor as well? At this point, the levels of CREB and phosphorylated CREB in the dentate gyrus after NE-induced potentiation have not been directly investigated. Protein levels of both forms of CREB can be visualized using immunocytochemical techniques and quantified by Western blot analysis at various time points after NE activation. To further implicate activation of the β-

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adrenergic receptor-cAMP cascade, β-adrenergic receptor and/or cAMP antagonists could be given prior to NE activation in some animals, which would be expected to both decrease NE-induced potentiation and levels of pCREB.

5.4.2.2.   Contribution of cAMP-dependent protein kinase

Application of 5-HT, in a manner that produces facilitation at the sensory-motor synapse produces three distinctly different increases in PKA levels. The first phase (initiated by 1 pulse 5-HT) is an early increase in PKA (within 5 min) that does not rely on protein synthesis. A second increase in PKA, occurring at 3 hr after 4 pulses of 5-HT (coinciding with the 5-HT application inducing intermediate term facilitation) is dependent only on mRNA translation. The PKA increase that occurs at 20 hr (5 pulses of 5-HT) is persistent, and is dependent upon both protein transcription and translation processes as is long-term facilitation (Muller and Carew, 1998). In the rat hippocampus, PKA activity after delivery of LTP stimuli has very different temporal characteristics. In area CA1, PKA increases are immediate (at 2 min) and transient returning to within baseline levels between 10-45 min after tetanization and are below baseline levels 3-5 hr after tetanization (Roberson and Sweatt, 1996). What would the profile of PKA activity look like during the induction and expression of late-phase NE-induced potentiation? To further

investigate this question, PKA levels should be assayed before and after induction of NE-induced potentiation. As in the *Aplysia* preparation, inhibitors of transcription and translation could be employed to determine the mechanisms of change in PKA activity.

5.4.3. NE and Learning

5.4.3.1.   Activation of the LC and short-term and long-term memory

The long-term physiological evidence presented here indicates that short-term potentiation of the evoked potential is not a requirement for the induction of late-phase, long-term potentiation after NE is released in the hippocampus. Most behavioral studies have focused on long-term memory (>24 hr) after NE activation (e.g. Stein et al., 1975; Wilson et al., 1994) with very few investigating acquisition or short-term memory (e.g. Izquierdo et al., 1998; Izquierdo et al., 2002). It is predicted from the physiological evidence presented here that rats with increases in synaptic levels of NE during acquisition would not learn the task faster than control rats not receiving NE, nor would they perform better during short-term memory tests, but they would demonstrate increased recall during long-term memory probes.

To investigate the effects of NE on the three phases of memory (acquisition, short-term and long-term memory) beginning with a simple memory task which animals could learn in 2-3 trials, e.g. finding a platform in

a Morris water maze from a single entry point, rather than single trial learning, would be recommended. This would allow more flexibility in distinguishing learners and non-learners during acquisition and would avoid ceiling effects that may be found with single trial learning, e.g. passive avoidance tasks. Using this memory model many of the concepts described above could be examined in unison. Is there a behavioral distinction between orexinergic and glutamatergic activation of the LC? Many other questions about the effects of NE on learning could be explored e.g. examination of pCREB levels in learners versus non-learners, and the pharmacology behind the NE effects on learning.

In conclusion, the results of this dissertation provide physiological support for Kety's theory that NE is capable of modulating memory processes in cortical structures. Here it was shown that NE mediates potentiation of the perforant path-dentate gyrus evoked potential, via β-adrenergic activation in both anesthetized (Chapter 3) and awake rats (Chapter 2 and 4). In the awake rat, NE produces protein synthesis dependent increases in the evoked responses 24 hr after NE release (Chapter 2 and 4), independent of short-term potentiation (Chapter 4), a novel finding in the mammalian nervous system. Further investigation on the effects of NE on acquisition and short-term and long-term memory in addition to exploration of the mechanisms of NE-induced potentiation is warranted.

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Appendix 1:

Input/output current intensity curves for saline and PROP pipettes prior to ORX-A infusion into the LC (Chapter 3)

Input/output current intensity curves were taken prior to baseline recording and ORX-A infusion in the LC. Analysis demonstrates that no differences exist between the intradentate recording pipettes prior to infusion of ORX-A into the LC. Data was converted to the percentage of the largest mean evoked population spike or EPSP slope elicited at a single current intensity. Baseline stimulation was taken as the current that elicted a population spike 50% of maximum. This appendix shows that though the tips of the two electrodes were separated by 500 to 1000 μm the perforant path stimulation evoked responses similar in magnitude on the two pipettes (see data on following page).

Appendix 1: Input/output current intensity curves for saline and PROP pipettes prior to ORX-A infusion into the LC (Chapter 3)


Population Spike

EPSP Slope

100 nM
OREX-06,10,11,40,70

10 nM
OREX-13,22,23,30,54

100 pM
OREX-21,47,50,51,52
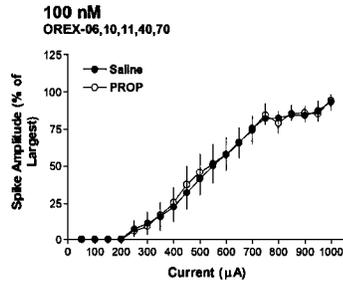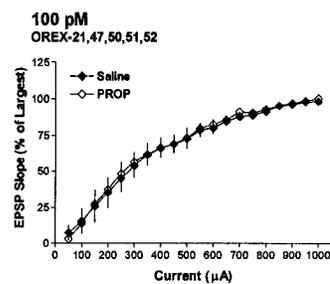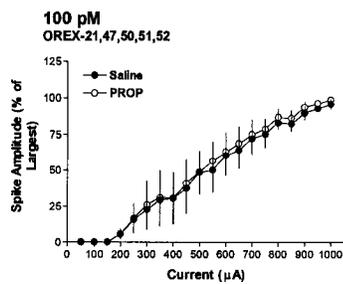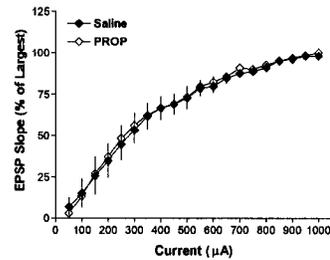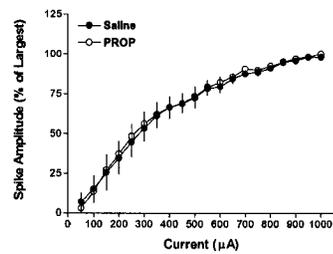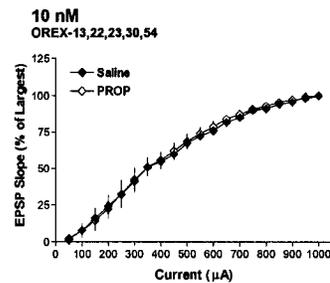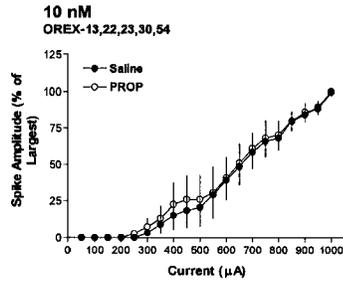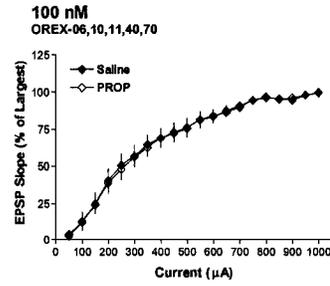
APPENDIX 2:

Diagrammatic Method for Glutamatergic Activation of the LC (Chapter 4)

SESSION 1   **I-O**   **1 hr**
24 hrs pre

Baseline

SESSION 2   **I-O**   **1 hr**   **3 hour**
LC activation

Baseline ↑   **TEST**

LC

SESSION 3   **I-O**   **1 hr**
24 hrs post

**Long Term
TEST**
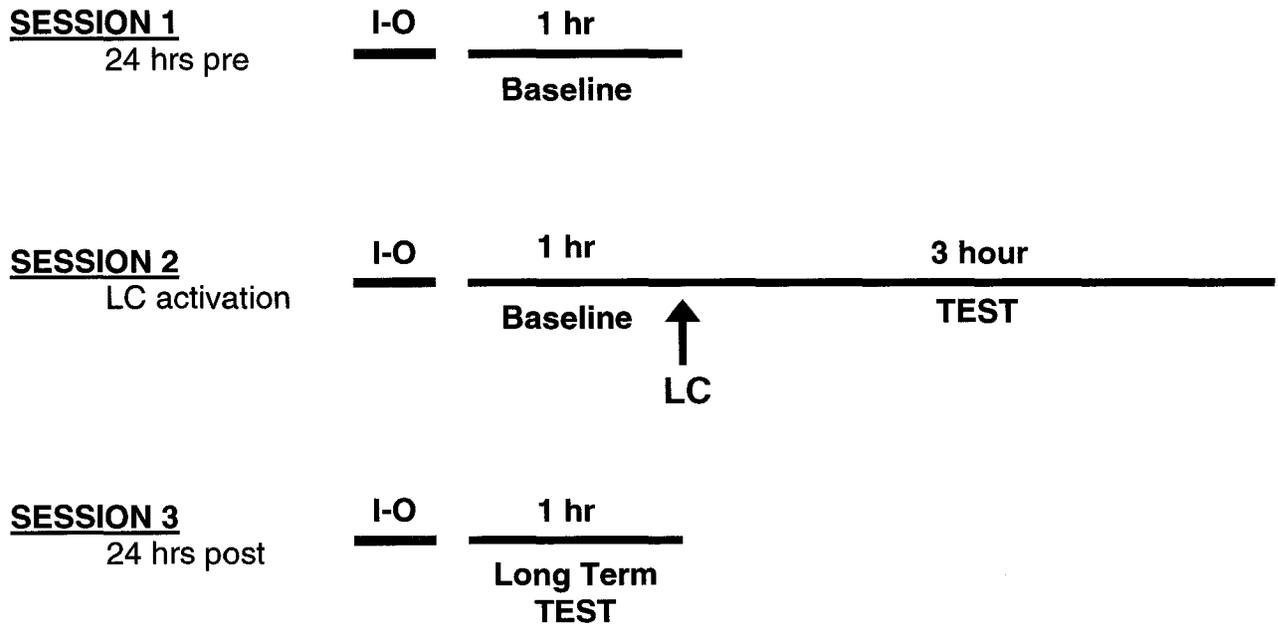
Diagrammatic representation of recording procedures for awake rats receiving glutamatergic activation of the LC (Chapter 4; see Materials and Methods). Recording sessions took place over three consecutive days. Session 1 consisted of a preliminary I/O analysis followed by a 1 hr baseline recording session using the current intensity that produced a population spike 50% of maximal. Session 2, occurring 24 hr later again began with a I/O curve followed by 1) a 1 hr baseline recording session using the same current level used for baseline in Session 1, 2) LC injection (~200 nL of 250 mM glutamate), 3) three hour test period. Session 3 occurred 24 hr after injection of glutamate in the LC and included both an I/O curve and 1 hr test period using the same stimulation levels of the prior sessions.

APPENDIX 3:

Locus Coeruleus Cannulae Placements of Glutamate Injection into the LC (Chapter 4)

A

A. β-adrenergic test groups

LC cannula placement for animals receiving glutamatergic activation of the LC (filled circles) and animals receiving i.c.v. PROP prior to glutamatergic activation of the LC (open circles). The majority of these injections were pericoerulear in localization.

Elevation of the population spike amplitude during the 3 hr test period after LC activation was typical.

B

B. Protein synthesis test groups

LC cannula placement for animals receiving glutamatergic activation of the LC (filled circles) and animals receiving the protein synthesis inhibitor anisomycin i.c.v. prior to glutamatergic activation of the LC (open circles). The majority of these injections were coerulear in localization.

Elevation of the population spike amplitude during the 3 hr test period after LC activation was *not* typical and a transient increase in EPSP slope was often seen after injection.