UNIVERSITY OF CALGARY

Noradrenaline and the strength of glutamate signals in magnocellular neurosecretory cells: An obligatory role for neuronal and glial elements

by

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Abstract

The release of noradrenaline (NA) into the paraventricular nucleus (PVN) and supraoptic nucleus (SON) is a critical step for enhanced magnocellular neuroendocrine cell (MNC) excitability *in vivo*. The adaptive responses mediated by these hypothalamic cell groups often require both rapid and sustained activation, yet clear synaptic mechanisms through which these demands might be met have not been explored. Here we tested whether NA elicited rapid and persistent changes in synaptic strength at glutamatergic synapses onto MNCs in male p21-28 Sprague-Dawley rats. Our results, based on the examination of miniature excitatory postsynaptic currents (mEPSCs), indicate that NA-induces three novel types of plasticity. First, we tested whether physiological activation of PKC via α_1-adrenoceptors could remove inhibitory feedback by inactivating presynaptic metabotropic glutamate receptors (mGluRs). The results demonstrate that NA inactivates group III mGluRs, effectively priming these synapses such that subsequent activation is more efficacious. In the second study, prompted by the observation that NA elicits an enduring increase in the amplitude of mEPSCs, and supported by previous studies showing that NA is an activator of glial cells, that ATP is a ubiquitous gliotransmitter and that MNCs express Ca^{2+} permeable ATP-gated P2X receptors, we examined the contributions of glial-derived ATP to changes in postsynaptic efficacy. The NA induced increase in mEPSC amplitude exhibited enhanced postsynaptic responsiveness and was blocked both by a P2X receptor antagonist and by the withdrawal of glial processes from synapses following physiological dehydration. The gliotransmitter ATP therefore, contributes directly to postsynaptic efficacy. Finally, we examined the mechanisms responsible for large amplitude mEPSCs in response to NA, which are impervious to postsynaptic manipulations. Here, we tested whether NA recruits the synchronous release of multiple vesicles of glutamate. Large mEPSCs exhibited a putative multimodal amplitude histogram, were sensitive to ryanodine and were associated with an enhanced glutamate cleft concentration. These data suggest large mEPSCs result from the synchronous release of multiple vesicles via Ca^{2+} expulsion from intracellular stores. The observations presented here indicate that NA-mediated processes trigger rapid, robust responses at glutamate synapses that may be critical for contributing to the long-lasting excitability of MNCs necessary to meet physiological challenges.

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Dedication

To my parents for having lovingly supported my education at an institute distant from home. To my brother for being an inexhaustible source of scientific inspiration. To Heather for continually offering her love, encouragement and understanding.

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
NA	Noradrenaline / Norepinephrine
PVN	Paraventricular Nucleus
SON	Supraoptic Nucleus
MNC	Magnocellular Neuroendocrine Cell
PAN	Parvocellular Autonomic Neuron
PNC	Parvocellular Neuroendocrine Cell
VP	Vasopressin
OT	Oxytocin
CNS	Central Nervous System
ER	Endoplasmic Reticulum
LDCV	Large Dense Core Vesicle
Ca^{2+}	Calcium
Mg^{2+}	Magnesium
Na^{+}	Sodium
K^{+}	Potassium
Cl^{-}	Chloride
CRH	Corticotropin Releasing Hormone
AMPA	-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR	AMPA receptor
NMDA	N-methyl D-aspartate
NMDAR	NMDA receptor
GABA	γ-aminobutyeric acid
ANS	Autonomic Nervous System
CVO	Circumventricular organ
ECF	Extracellular Fluid
cAMP	3'5'-cyclic adenosine mono phosphate
$V_{(X)}R$	Vasopressin type (x) Receptor
OTR	Oxytocin Receptor
PKC	Protein Kinase C
IP_3	Inositol tri-phosphate
ACTH	Adrenocorticotropin Hormone
TRPV1	Transient Receptor Potential Vanilloid type 1 receptor
DAP	Depolarizing-After-Potential
EPSP	Excitatory Postsynaptic Potential
mEPSP	Miniature Excitatory Postsynaptic Potential
EPSC	Excitatory Postsynaptic Current
mEPSC	Miniature Excitatory Postsynaptic Current
mEPP	Miniature End Plate Potential
IPSP	Inhibitory Postsynaptic Potential
mIPSC	Miniature Postsynaptic Potential
IPSC	Inhibitory Postsynaptic Current
mIPSC	Miniature Inhibitory Postsynaptic Current
$G_{q/11}$	Trimeric G-protein with a $_{q/11}$ α subunit

$G_{i/o}$	Trimeric G-protein with a $_{i/o}$ α subunit
G_s	Trimeric G-protein with a $_s$ α subunit
SMC	Smooth Muscle Cell
NTS	Nucleus Tractus Solitarius
L-DOPA	L-Dihydroxyphenylalanine
COMT	Catechol-O-Methyl Transferase
MAO	Monoamine Oxidase
DOPAC	3,4-dihydroxyphenylacetic acid
DHPG	3,4-dihydroxyphenylglycol
MHPG	3-Methoxy-4-hydroxyphenylglycol
A(X)	Numbered Noradrenergic Cell Group
ADD	Attention Deficit Disorder
ADHD	Attention Deficit and Hyperexcitability Disorder
TCA	Tri-Carboxylic Acid
PKA	cAMP dependent protein kinase
PLC-beta	Phospholipase C-β
PIP_2	Phosphotidylinositol 4,5-bisphosphate
DAG	Diacylglycerol
VLM	Ventrolateral Medulla
ICV	Intracerebroventricular
NPY	Neuropeptide Y
$P2X_{(X)}$	ATP-gated ionotropic receptor/channel
$P2Y_{(X)}$	ATP metabotropic receptor
ATP	Adenosine triphosphate
TTX	Tetrodotoxin
NMJ	Neuromuscular Junction
VGLUT	Vesicle Glutamate Transporter
$GluR_{(X)}$	AMPAR subunit
$mGluR_{(X)}$	Metabotropic Glutamate Receptor
$EAAT_{(x)}$	Excitatory Amino Acid Transporter
GLAST	$EAAT_1$
GLT-1	$EAAT_2$
LTP	Long Term Potentiation
LTD	Long Term Depression
CA1	Pyramidal Cell Region of the Hippocampus
CA3	Pyramidal Cell Region of the Hippocampus
PI3-K	Phosphotidyl Inositol 3-Kinase
CaMKII	Ca^{2+}-calmodulin dependent kinase II
P_r	Release Probability
q	Size of quantal synaptic response
N	Number of available vesicles for release
MVR	Multivesicular Release
Non-NMDA	AMPA and Kainate receptors
GAT-1	GABA transporter
OPC	oligodendrocyte precursor cell
OVLT	organum vasculosum of the lamina terminalis

OSOVH	one site one vesicle hypothesis
ACh	Acetylcholine
CICR	Ca^{2+} Induced Ca^{2+} Release
ACSF	Artificial Cerebrospinal Fluid
IR-DIC	Infrared-Differential Interference Contrast
GTP	Guanine triphosphate
$GABA_A$	Ionotropic GABA receptor/channels
PSNA	Peak Scaled Non-Stationary Noise Analysis
I	Current
V or E	Voltage or Potential
R	Resistance
g	Conductance
PBS	Phosphate buffered Saline
GFAP	Glial Fibrillary Acid Protein

Epigraph

Carl Sagan

“We have a method, and that method helps us to reach not absolute truth, only asymptotic approaches to the truth — never there, just closer and closer...”

“At the heart of science is an essential tension between two seemingly contradictory attitudes — an openness to new ideas, no matter how bizarre or counterintuitive they may be, and the most ruthless sceptical scrutiny of all ideas, old and new.”

Peter Medawar

“Science is essentially an imaginative excursion into what *might* be true.”

Grant Gordon

“The brain is very complicated.”

English Language Paradox

The following sentence is true

The previous sentence is false

The Epimenidies Paradox

Epimenidies was a Cretan who was famous for uttering these words: “All Cretans are liars.”

Zenos Paradoxes of Motion

- *"In a race, the quickest runner can never overtake the slowest, since the pursuer must first reach the point whence the pursued started, so that the slower must always hold a lead." Aristotle*
- *"That which is in locomotion must arrive at the half-way stage before it arrives at the goal." Aristotle*

Chapter One: Introduction

1.1 The Draw of MNC Study

The study of magnocellular neurosecretory cells (MNCs) of the paraventricular (PVN) and supraoptic nuclei (SON) of the hypothalamus has yielded considerable insights into broader functions of the nervous system. First, vasopressin (VP) and oxytocin (OT), which are synthesized and then secreted by MNCs directly into the blood were the first neuropeptides ever characterised and synthesized (Du Vigneaud, 1954), earning Vincent du Vigneaud the Nobel Prize in chemistry in 1955. Prior to the work of Sachs and Takabatake, there was no clear understanding of how peptides were processed and targeted for cellular secretion; their studies gave rise to the concept of a prohormone as a critical intermediate molecule during biological synthesis and trafficking (Sachs and Takabatake, 1964). Another significant progression occurred in the field of peptide pharmacology, where VP and OT had the first agonist and antagonist generated for their receptors (Manning and Sawyer, 1989). The diameter of the MNC cell body, measuring in at an impressive 30μm on average, coupled with the discrete and densely packed organization of the MNCs near the ventral surface of the brain, has made it relatively easy to use a number of diverse experimental approaches, ranging from stereotaxically guided *in vivo* electrophysiology, to micropunch for gene arrays, to ask questions that span the gamut from physiology to molecular biology. Additionally, the physical segregation between the MNC cell body and the terminals located in the neural lobe, has allowed discrete *in vivo* microdialysis measurements of dendritic peptide release in the nuclei without contamination from axons (Ludwig, 1998). Perhaps the most fruitful experimental preparation is that of the lactating rat. Suckling pups provide a real afferent stimulus to the MNC nuclei and when coupled with *in vivo* recordings from OT MNCs as well as intramammary pressure as a means for measuring the effectiveness of hormone release, lactation has provided data on a wealth of intriguing neural phenomena (see introduction section 'Lactation an E-phys Perspective'). These nuclei were also the stage for the first characterization of a novel, dynamic physical relationship between neurons and glial cells, which depends on the physiological state of the organism (Tweedle and Hatton, 1977;Theodosis et al., 1981;Hatton and Tweedle, 1982;Theodosis and Poulain,

1984). This neuropil remodelling involves the retraction of glial processes from synaptic elements, affecting homo (Oliet et al., 2001) and heterosynaptic (Piet et al., 2004) neurotransmitter release probability. Finally, VP and OT MNCs represent the final integration step for both local changes in osmolarity and afferent signals before neurohormone is secreted into the systemic circulation. This property makes these cells ideally suited for the study and interpretation of changes in synaptic strength as such alterations are easier to relate to changes in system output (neurohormone release) (Gordon and Bains, 2006).

1.2 The Introduction to the Introduction

While the above MNC history is very important and certain aspects will be covered in further detail in the pages to follow, the main focus of the introduction is not to convince the reader on the practical and often insightful uses of MNC preparations, but to review the essential concepts and literature that will equip the individual not only to grasp but to scrutinize the data presented. To start the preparation, here we will talk at length about synapses. Synapses are tiny sites of chemical communication between two neurons (or between a neuron and another cell type such as muscle, gland or glia), where the propagation of electrical signals from cell to cell relies, for a brief moment, on the release of diffusible molecules that must make a short journey (~20nm, ~1 ms) across a small gap called the synaptic cleft. Finding novel methods by which synapses alter their ability to convert electricity to diffusible chemicals and to electricity once more is the goal of this thesis.

Since synapses comprise a fundamental anatomical unit of the brain, by understanding how neurons dynamically talk to one another we can begin to provide the foundation for a greater understanding of communication between multiple neurons leading to an enhanced ability to grasp the totality of neural networks and ultimately the holistic workings of the brain and the behaviour of the organism. Achieving these goals, however, is based on one critical assumption—that all processes in the universe can be observed, measured and tested experimentally by some method and in due course understood. Electrophysiology, pharmacology and multifarious microscopy techniques

have been indispensable tools towards synaptic inquiry through sensitive utilization and/or measurement of physical synaptic properties. Though much information has been acquired, there is much yet to be learned; therefore, it remains the hope of synaptologists that these and future techniques will continue to unravel *all* elements of synaptic function so that one day this tiny comprehension will provide a foundation for greater, holistic CNS understanding. All that stands in the way is the validity of our one critical assumption.

It is generally accepted that alterations in the output of a neural circuit require alterations in the strength of the individual synapses that comprise that circuit (Bains et al., 1999;Malenka and Bear, 2004). This concept, which can involve long-term changes in synaptic signalling, forms the basis of how the brain learns and remembers (Takahashi et al., 2003;Rumpel et al., 2005;Whitlock et al., 2006). Synaptic plasticity of this type is likely ubiquitous throughout the nervous system yet its description has been limited largely to circuits responsible for motor, cognitive and behavioural processes with few descriptions of long-lasting synaptic changes in neural pathways involved in restoring homeostatic set points (e.g. osmotic balance) or pathways that respond quickly to essential physiological demands (e.g. lactation).

What would be the advantages of learning and remembering in autonomic circuits? If one considers that changes in the internal or external environment can occur swiftly and endure for prolonged periods of time, long-term alterations in synaptic strength may increase the gain and/or sensitivity of system output for the duration of the physiological challenge so that the demands of the organism are met more efficiently. For example, *in vivo* studies demonstrating augmented release of the neurohormones vasopressin (VP) and corticotropin releasing hormone (CRH) from the pituitary gland in response to repetitive hypovolemic challenges (Lilly et al., 1983;Lilly et al., 1986;Lilly et al., 1989;Thrivikraman et al., 1997) hint at underlying learning and memory processes that may reside in autonomic circuitry.

This dissertation will focus on synaptic mechanisms initiated by NA that may contribute to learning and memory at glutamate synapses on the MNCs of the hypothalamus. The specific questions we address are 1) can NA target and inactivate

functional presynaptic autoreceptors, which normally act to curtail glutamate release, so that glutamatergic transmission onto MNCs may be enhanced, 2) can NA activate glial cells to release glia-derived signalling molecules that in turn affect postsynaptic efficacy in neighbouring MNCs and 3) can NA synchronize stochastic release of neurotransmitter to yield large amplitude 'quantal' currents? The answers to these questions have broad reaching implications towards our general understanding of brain cell communication. An autoreceptor that acts as a plasticity switch, a gliotransmitter that affects long-lasting postsynaptic strength and a large miniature postsynaptic current that is actually comprised of multiple quanta have been scarcely demonstrated. Each finding has the potential to alter the way in which neuroscientists examine and interpret their electrophysiological data. Before we journey down this path though, we will first take a step backwards and review some basics. In the first half of the introduction to this thesis we will review the fundamental aspects of the MNCs. After all, what is a MNC besides a big cell with a powerful name? Where in the brain do they reside and what function(s) do they serve? Are these cells important physiologically or are they simply an easy system to study with the hope that any information obtained from their examination will transfer or provide clues towards the understanding of more important areas of the brain, or do both of these queries justify their examination? In the second half of the introduction we will review some basics about the star of our show, NA, and the noradrenergic afferent control of the MNCs. From here we will delve into the glutamate synapse and the concept of plasticity before we review the essential background for the data chapters.

1.3 The Basics

The hypothalamus is the major integration and output centre for all incoming neural and blood borne signals related to the unconscious internal workings of an organism and the conscious behaviours that help the internal environment accomplish desired goals. This encompasses fluid balance, sexual reproduction, thirst, hunger, temperature, circadian rhythms, metabolism etc, as well as the physical behaviours associated with these processes i.e. sexual posturing, putting on a coat if it's cold outside or drinking a glass of

water when parched. The hypothalamus is an aggregation of many diverse nuclei. The region is located under the thalamus in the ventral diencephalon situated bilaterally around the third ventricle in the central basal forebrain.

There are two principle nuclei in the hypothalamus containing the vast majority of the large cell bodied MNCs: the SON and the PVN. In varying proximity to the large conglomerations of MNCs, there are numerous smaller satellite groups (Caverson et al., 1987). One of the most prominent of these is the nucleus circularis, which almost perfectly divides the two principle nuclei, while the most incognito are individual MNCs that can be scattered as far as the anterior reaches of the third ventricle.

1.3.1 Neurohormone Synthesis and Secretion

Each MNC is a specialist, synthesizing and secreting either the neurohormone arginine vasopressin (VP) or the neurohormone oxytocin (OT). This holds true except for a small fraction of cells (2-3%) that possess mRNA for both peptides, which can increase dramatically in the physiological state of lactation (Mezey and Kiss, 1991;Glasgow et al., 1999). VP and OT are each nine amino acids in length (called a nonapeptide), differing in only two positions: an Isoleucene and a Leucene for OT and a Phenylalanine and an Arginine for VP at amino acid position 3 and 8 respectively when counting from C-terminus to N-terminus. VP and OT are translated in the soma as a larger (~100 amino acid) prohormone consisting of the hormone proper, neurophysin and in the case of VP a glycoprotein domain. The prohormone is inserted into the endoplasmic reticulum (ER) where it is folded before shipping to the Golgi apparatus. Both proper folding and trafficking the prohormone into a secretory pathway are thought to depend on the neurophysin component of the peptide (for review see (de Bree, 2000)). During transport the prohormone is enzymatically cleaved within large dense core vesicles (LDCVs) to produce the biologically active hormone (VP or OT) and the physiologically inactive neurophysin peptide.

Each MNC sends out a single axon and together all MNC axons course ventrally and emerge from the bottom surface of the forebrain to form the bulk of the posterior lobe of the pituitary gland, otherwise known as the neurohypophysis or neural lobe. Each

axon gives rise to thousands of neurosecretory terminals, which are packed with LDCVs containing the peptides to be secreted. When an action potential generated in the axon hillock of an MNC reaches the axon terminal and provides sufficient depolarization, Ca^{2+} dependent exocytosis occurs, releasing VP or OT into the extracellular space of the gland which then diffuses through perforations in the walls of nearby capillaries to enter the systemic circulation (Hatton, 1990;Armstrong, 1995). Magnocellular neurons have been termed 'neurosecretory' as they, for the most part, do not communicate via the axon to other neurons but instead talk to distant targets in the body by secreting non-classical transmitters, hormones, directly into the blood. The sights of action and the physiological role of VP and OT will be covered below.

1.3.2 Nucleus Heterogeneity: SON versus PVN

The MNC nuclei show different levels of cell type heterogeneity. The vast majority of cells that comprise the SON are MNCs, whereas the PVN consists of multiple, phenotypically and morphologically distinct cell types (van den Pol, 1982). In addition to the MNCs, this includes parvocellular autonomic neurons (PANs) present in the central zone of the PVN (midway along the lateral to medial axis) in dorsal and ventral 'caps' of cell bodies and parvocellular neuroendocrine cells (PNCs), which lie medial to the MNCs in closer proximity to the third ventricle. Even in the presence of many different cell types however, the MNCs can still be easily identified based on their location, soma size, soma-to-soma density (which is almost as high as it can be given their girth) and electrophysiological characteristics (Tasker and Dudek, 1991;Hirasawa et al., 2003). Of the latter VP MNCs display a linear current-voltage relationship, whereas OT MNCs display inward rectification in response to hyperpolarizing voltage steps (Hirasawa et al., 2003). Both cell types exhibit a prominent I_A conductance that delays action potential firing, a lack of spike accommodation and a lack of low threshold depolarizing potentials (Tasker and Dudek, 1991).

1.4 Vasopressin

1.4.1 The Role of VP and VP Receptors

Once secreted into the general circulation from the neurohypophysis the primary role of VP is to conserve water at the kidney, a process known as antidiuresis, which means to reduce urine volume. This helps to maintain a constant plasma osmolarity by making the extracellular fluid (ECF) more dilute and helps to increase blood pressure by adding to the total ECF volume. Osmolarity is tightly controlled, allowing fluctuations of only 3% occur under normal physiological circumstances (Leng et al., 1999). VP acts at the distal and collecting tubule of innumerable nephrons in the kidney by binding to membrane bound metabotropic V_2 G_s-coupled receptors. V_2 receptor activation increases the permeability of the distal and collecting tubules to water by increasing the number of aquaporin-2 water channels in the membrane that directly apposes the lumen of the nephron (Holmes et al., 2003;Holmes et al., 2004). As nephron filtrate travels through these tubules towards the final, large collection chamber of the Kidney (the renal pelvis), the filtrate is met with an increasing osmotic gradient in the surrounding ECF, which draws the water out thereby reabsorbing it.

In addition to its effects on the kidney VP also combats the low blood pressure resulting from hypovolemia by binding to and activating $G_{q/11}$-protein linked V_{1a} and V_{1b} (V_{1b} is also called V_3) receptors located on smooth muscle cells (SMCs) surrounding arterioles (Holmes et al., 2003;Holmes et al., 2004). $G_{q/11}$-linked intracellular cascades, among other things (more detail later), increase the free Ca^{2+} concentration within cells by generating inositol triphosphate (IP_3), a second messenger dedicated to triggering the release of Ca^{2+} from internal stores, which promotes contraction.

1.4.2 When is VP secreted?

There are three principal situations that require the release of VP from the neural lobe: high Na^+, hyperosmolarity and hypovolemia. There are many mechanisms by which MNC excitability is increased and VP is secreted under elevated Na^+ and/or hyperosmotic conditions that will not be covered thoroughly here. Briefly, two major

components are 1) afferent input from the median preoptic nucleus (MnPO)(McKinley et al., 1994;Grob et al., 2004) and the anterior CVO areas which includes the subfornical organ (SFO) and the organum vaculosum of the lamina terminalis (OVLT) (McKinley et al., 1994;Bourque et al., 1994), and 2) the intrinsic osmosensitivity of the MNCs themselves (Oliet and Bourque, 1993).

VP combats the high Na^+ and/or hyperosmotic perturbation by diluting the ECF with enhanced water reabsorption at the kidney. As an increase in ECF volume may have adverse side effects on blood pressure, ultimately Na^+ ions need to be excreted in the urine. Na^+ excretion, referred to as naturesis, is under the control of natriuretic peptides from the heart and sympathetic neural control of the kidney. Naturesis does not result in plasma hypoosmolarity as any loss in Na^+ ions in the urine is accompanied by an equivalent loss in water (Sherwood, 1997).

Insufficient water intake will result in hypovolemia if ECF volume drops, in addition to elevating plasma osmolarity. Again, hyperosmolarity will trigger VP release but this release will be facilitated further by additional excitatory drive to MNCs when baroreceptors in the aortic arch and the carotid bodies and/or stretch receptors in the atria relay information about pressure and volume respectively via cardiac afferents and several brainstem nuclei to the hypothalamus (Jordan and Spyer, 1977;Wallach and Loewy, 1980;Calaresu and Ciriello, 1980). The vasoconstrictor actions of VP will help to maintain blood pressure as ECF volume is restored. Further, any water adsorption that makes the plasma too dilute will be met with enhanced Na^+ reabsorption at the kidney due to the actions of the renin-angiotensin-aldosterone system. Chloride ions are also passively reabsorbed in tandem with active Na^+ transport (Sherwood, 1997).

Excessive salt and water loss from vomiting, diarrhoea, haemorrhage or sweating triggers the same VP mediated processes as mentioned above: water reabsorption and vasoconstriction, while other systems work to restore the electrolytes lost as a result of these processes. Emesis and diarrhoea can trigger VP release through gastric-vagal afferents but how haemorrhage influences VP release depends on the relative importance of baroreceptor activity over stretch receptor activity in the species under study. Finally, changes in physical exertion not only trigger VP release from the water loss inherent in

sweating, but alterations in physical activity also cause fluctuations in the concentration of O_2 and CO_2 in the blood that alter VP secretion via peripheral chemoreceptors and cardiac afferents (for review see (Leng et al., 1999)).

1.4.3 VP MNCs: An E-phys Perspective

VP MNCs exhibit a phasic bursting pattern of action potential discharge (Arnauld et al., 1974). VP cells burst independently of one another making the release of hormone at the neural lobe continuous rather than pulsatile as is with OT MNCs (see below) (Leng et al., 1999). Although the release of VP occurs in an uninterrupted fashion, high frequency bursts of action potentials are necessary to optimize VP exocytosis by augmenting the terminal Ca^{2+} signal. The methods by which this can occur include: 1) bursts allows for action potential broadening as the spike train progresses (Bourque and Renaud, 1991), 2) bursts can provide a higher probability that an action potential invades an axon terminal (Dyball et al., 1988) 3), phasic firing can enhance terminal depolarization from K^+ accumulation in the ECF (Leng and Shibuki, 1987) and 4) short inter-spike intervals facilitate terminal Ca^{2+} accumulation (Dayanithi et al., 1992). VP MNC bursts are slower (~5-10 Hz), longer (~20-40 s) and occur more often (~0.05-0.025 Hz) than OT MNC bursts (see below). In response to elevations in osmolarity VP MNC bursts become longer (Brimble and Dyball, 1976;Brimble and Dyball, 1977) and if the challenge persists, bursts become briefer and achieve higher peak frequencies (Wakerley et al., 1978). If VP MNCs are stimulated very strongly, continuous firing can also be induced (Dyball and Pountney, 1973).

Intrinsic membrane properties play a critical role in generating the phasic firing observed in VP MNCs (Renaud, 1994). Neither spontaneous undulation in membrane potential nor burst-like patterns of afferen tinput are thought to account for the aggregates of spikes that oscillate on and off. At burst initiation a small but long lasting depolarizing-after-potential (DAP), which succeeds every spike, triggers and works together with a persistent inward current to ramp the cells voltage from rest up to a stable plateau potential (Andrew and Dudek, 1984a). This new, elevated baseline is maintained by Ca^{2+} currents and facilitates the generation of a burst because individual excitatory

postsynaptic potentials (EPSPs) can more easily break Na^+ channel threshold. The unwavering plateau potential is terminated by the build-up of intracellular Ca^{2+} and the subsequent activation of Ca^{2+} activated K^+ channels (Andrew and Dudek, 1984b) as well as by the dendritic release and autocrine feedback of dynorphin on Kappa opioid receptors (Brown and Bourque, 2004;Brown et al., 2006).

1.5 Oxytocin

1.5.1 The Job of OT

Once liberated into the bloodstream OT has two principal jobs: 1) to trigger milk letdown from the mammary glands, a process termed lactation and 2) to aid the birthing of new young by stimulating uterine contraction, a process termed parturition. Surprisingly, males possess a similar number of OT MNCs and produce OT in similar quantities, suggesting alternate functions for this peptide that are unrelated to lactation or parturition. OT can act in conjunction with VP at the kidney to enhance salt excretion (Verbalis et al., 1991) and facilitate the release of natriuretic peptides from the heart (Haanwinckel et al., 1995). There are also many other less well-understood actions of OT in males, including sexual arousal, orgasm, monogamous pair bonding, analgesia, anxiolytic effects, positive social interaction effects and the development of trust between individuals. For females, OT plays an important role in general maternal behaviour too (see (Gimpl and Fahrenholz, 2001;Filippi et al., 2003;Bancroft, 2005) for some reviews).

1.5.2 The OT Receptor

OT binds to a single receptor, the OT receptor (OTR). Activation of this receptor requires both Mg^{2+} and cholesterol, in addition to OT. The receptor sequence is highly conserved throughout mammalian phylogeny, no doubt owing to its essential role in all placental organisms (Gimpl and Fahrenholz, 2001). The OTR is functionally expressed in the myoepithelium of the mammary glands and in the myometrium and endometrium of the uterus at the very end of gestation (Ivell et al., 2001). As with $V_{1a}R$ and $V_{1b}R$, the OTR couples to the $G_{q/11}$-protein intracellular signalling cascade, which initiates SMC

contraction in myoepithelial and myometrial cells in the same manner described for VP acting on arterioles. SMC contraction in the mammary gland increases intra-mammary pressure and leads to ejection of milk out the nipple via the mammary duct, while SMC contraction in the uterus facilitates the birthing process.

1.5.3 Afferent Control of OT Release

The afferent input required for OT secretion and the subsequent initiation of milk letdown stems from nipple stimulation that results from the actions of a suckling newborn (Leng et al., 1999). The precise afferent pathway(s) relaying sucking information to OT MNCs is not well understood. After breast afferents project to the spinal cord, some areas thought to be important in the rat include: the dorsal horn, the lateral cervical nucleus and part of the dorsolateral funiculus (Dubois-Dauphin et al., 1985a;Dubois-Dauphin et al., 1985b;Tasker et al., 1986). The afferent pathways utilized to initiate OT release during parturition begin in the uterus and cervix, where sensory neurons send electrical signals to the NTS via the vagus nerve (Bailey and Wakerley, 1997;Leng et al., 1999). Approximately 80% of the projecting axons from the NTS to the OT MNCs are noradrenergic and they facilitate the release of OT through the activation of α_1-adrenoceptors (a lot more about this below). However, a metabolic mapping study has revealed a long list of putative participating nuclei during parturition and lactation pervading the hypothalamus, midbrain and brain stem, suggesting that the neural circuits mediating the totality of these behaviours may be quite complex (Lin et al., 1998).

1.5.4 Lactation: An E-phys Perspective

Knockout mouse studies have confirmed that OT is an absolute necessity for suckling-induced lactation; sans the OT gene, neophyte suckling is unrewarded regardless of the degree to which the mammary glands are engorged with milk (Nishimori et al., 1996;Young, III et al., 1996). Direct stimulation of the infundibular stalk has revealed that OT release from the neurohypophysis requires an action potential rate of at least 40Hz (Harris et al., 1969). *In vivo* recordings indicate that OT cells transition from a basal action potential firing rate of only a few Hertz to transient, intense bursts of action

potentials at 5 to 10 minute intervals in response to suckling pups. Individual bursts are limited to 1 to 2 seconds and reach frequencies up to 100 Hz (Wakerley et al., 1973;Wakerley and Lincoln, 1973a;Wakerley and Lincoln, 1973b). Simultaneous recordings from all four MNC regions in the hypothalamus (2 SON and 2 PVN due to bilateral symmetry) show that all OT MNCs burst concomitantly within 400 ms of one another, with relatively long pauses of silence in between the rapid explosions of population activity (Belin et al., 1984;Belin and Moos, 1986). The precise mechanisms controlling this astonishing global synchronization property have not yet been fully elucidated. However, current hypotheses include the autocrine and paracrine actions of dendritically released OT (Moos et al., 1989;Moos and Richard, 1989;Richard et al., 1991), a role for brainstem nuclei in relaying suckling information (Meyer et al., 1987;Moos et al., 2004) and the possible involvement of glutamate interneurons that link the spatially separated MNC nuclei (Boudaba and Tasker, 2006).

Coordinated OT cell bursting is necessary for milk letdown for several reasons. The most important is the amount and duration of blood borne OT required to achieve maximal mammary gland pressure via myoepithelium contraction. If the concentration is insufficient or if OTRs desensitize as a result of prolonged ligand interactions, no milk is ejected. The concentration of OT therefore, must be high and only available to the receptors briefly. Transitory population bursts possessing large interburst intervals are the perfect match to OT receptor properties as they provide intermittent, highly concentrated boluses of OT to the circulation, which can maximally activate OTRs and allow for receptor recovery before the next bolus arrives (Bicknell, 1988). An intense burst of action potentials generated by an individual MNC is also necessary to maximize hormone release from its many axon terminals in the neurohypophysis. The reasons are congruent to those discussed above for VP release (Dyball et al., 1988;Leng et al., 1988;Jackson et al., 1991;Jackson, 1993). Finally, to highlight the specialized nature of the suckling-induced bursting response, the tertiary job of OT—to aid the fight of VP against rising osmolarity—does not lend way to concomitant bursts. In the presence of a salty challenge in a lactating rats OT MNCs deal with the perturbation by raising background spiking (Moos and Ingram, 1995). This clarifies why OTRs on the

mammary gland require such large and rapid OT challenges, so that OT release for the purpose of naturesis does not induce milk letdown. Fittingly, a bolus of OT resulting from pulsatile secretion is not the optimal signal to trigger naturesis at the kidney, which requires lower, more continuous concentrations of the peptide to be effective (Sjoquist et al., 1999).

1.5.5 Parturition: the abridged version

There are less *in vivo* electrophysiological studies examining the birthing process for logistical reasons. Nevertheless, we will review a small portion of the OT/parturition literature. As is now apparent from the sequence homology between VP and OT as well as the similarities between the $V_{1a}R$, $V_{1b}R$ and OTR, it should come as no surprise that OT is a potent utertonic agent (uterine contractor). In contrast to the essential role of OT in mediating the lactation reflex, knockout mouse studies have demonstrated that this peptide is not necessary for parturition, as the process continues in its absence (Nishimori et al., 1996;Young, III et al., 1996). Similar to milk ejection, pulsatile secretion of OT release is utilized during the middle to late stages of parturition (Russell and Leng, 1998). *In vivo* recordings during parturition demonstrate OT MNC excitation and OT release coincides with birth contractions, the passage of young and ejection of the placenta (Summerlee, 1981). The effect of OT-mediated constriction is made possible by a rapid rise in the expression of OTRs on the SMCs of the uterus at the end of term. Also increasing during the late stages of pregnancy is the total content of OT in MNCs, which is thought to accumulate by enhanced synthesis and suppressed secretion (see (Leng et al., 1999) for review), so that the high demands placed on the OT system during parturition and lactation are met.

1.6 Noradrenaline

1.6.1 Synthesis

The central focus of this thesis involves the actions of NA on excitatory glutamatergic synapses that make direct contact onto MNCs in the PVN. NA, along with adrenaline

and dopamine, is apart of the chemical family known as catecholamines, which are compounds derived from the amino acid tyrosine and are characterized by a 'catechol' aromatic ring that has two alcohol groups in the *meta* and *para* position. Tyrosine is an important precursor for a number of other biologically useful molecules including the pigment melanin, the thyroid hormones and in the opium poppy, morphine. In humans tyrosine is a non-essential amino acid, meaning it can be synthesized by the body from phenylalanine and thus does not need to be obtained from the diet. By expressing appropriate enzymatic machinery a given cell can turn the tyrosine into an intended molecule.

For the biological synthesis of catecholamines, tyrosine is oxidized by the enzyme tyrosine hydroxylase to become L-dihydroxyphenylalanine (L-DOPA). This step forms the catechol aromatic ring, the hallmark of the catecholamine structure. The aromatic-L-amino acid decarboxylase then removes a carboxyl group from L-DOPA, converting it to dopamine. Next, dopamine is converted to NA through a beta-oxidation step in which dopamine beta-hydroxylase places a hydroxyl group on the beta carbon atom of the molecule. NA can then be methylated to become adrenaline by the actions of phenylethanolamine-N-methyl transferase. Due to this strict sequence of enzymatic steps, one way catecholaminergic cell type can be determined is by identifying the enzymes expressed i.e. if a cell does not express phenylethanolamine-N-methyl transferase but does express the other preceding enzymes than the cell must synthesizes NA. All of these enzymes are cytoplasmic with the exception of dopamine beta-hydroxylase, which resides on the inner luminal surface of the synaptic vesicles destined to house NA. Thus, NA is synthesized from dopamine inside vesicles and is the only classical transmitter to be made in this way. A beautiful example of how evolution selects for survival and not molecular process efficiency, the synthesis of adrenaline requires that NA be extruded from the vesicle back into the cytosol to allow enzymatic access for the conversion.

1.6.2 Noradrenergic Cell Groups

NA has plethora of effects, which is a direct consequence of the vast number of tissues influenced by this molecule. NA is released as a neurotransmitter throughout the central and peripheral nervous systems and as a hormone into the general circulation. In the central nervous system NA is synthesized in a finite number of cells that reside in small, fairly discrete anatomical territories in the brainstem. The ventral noradrenergic column contains the A1 and A5 cell groups, where as the dorsal noradrenergic column holds A2, A6 and the A7 groups. A1 and A2 are relevant to this thesis and will be discussed in more detail below. The A5 and A7 noradrenergic cell groups primarily send descending projections to the spinal cord to modulate both pain and autonomic signals entering from the periphery. The largest group is A6 and is more commonly referred to as the locus ceruleus. The axons of A6 project diffusely to nearly all corners of the CNS, which includes but is not limited to cortex, hippocampus, cerebellum, amygdala, thalamus, hypothalamus, midbrain, medulla and spinal cord.

1.6.3 Adrenergic Receptors and Signalling

NA and adrenaline bind to the same membrane bound metabotropic receptors, which belong to three families, α_1, α_2 and β. The beta-adrenergic receptors can be further sub divided into β_1 to β_4 but are apart of the same family because they all couple intracellularly to G_s-trimeric proteins, which activate adenylyl cyclase to produce cAMP molecules that in turn activate PKA. Conversely, α_2-adrenoceptors inhibit adenylyl cyclase and decrease the production of cAMP by coupling to $G_{i/o}$-trimeric proteins. α_1-adrenoceptors are of interest to this thesis and will be described in more detail. The α_1-adrenoceptor is linked intracellularly to $G_{q/11}$-trimeric proteins. The α subunit of this tri-protein complex activates the membrane associated effector enzyme phospholipase C-β (PLC-β) after it dissociates from the $\beta\gamma$ subunits (the remaining subunits of the trimetric G-protein). PLC-β hydrolyzes phosphotidylinositol 4,5-bisphosphate (PIP_2) lipids contained within the cell's phospholipid membrane to generate two second messengers: inositol triphosphate (IP_3), which is freely diffusible in the cytosol and diacylglycerol (DAG), which remains in the lipid bilayer. IP_3 travels to the endoplasmic reticulum (ER

or more generally Ca^{2+} stores) where it binds to ER surface IP_3 receptors/channels, which open to allow Ca^{2+} efflux into the cytosol. Store Ca^{2+} release from a localized group of channels can spread to adjacent IP_3 receptor/channels and/or ryanodine receptor/channels to trigger further release, a process term Ca^{2+} induced Ca^{2+} release (CICR), which amplifies the Ca^{2+} signal. The increased availability of DAG in the plasma membrane attracts and activates PKC (see (Zhong and Minneman, 1999) for review). These α_1-mediated signalling cascades will be experimentally targeted and manipulated a number of times in the papers to be presented in chapters 3-5.

1.7 Noradrenergic Afferent Control of MNCs

A great deal of afferent information related to the control of VP and OT release from the neurohypophysis is relayed through noradrenergic nuclei in the brainstem. The aortic, carotid sinus and vagus nerves are the primary cardiac afferents carrying volume, pressure and chemo related information to the ventrolateral medulla (VLM) and NTS in the brainstem (Jordan and Spyer, 1977;Wallach and Loewy, 1980;Calaresu and Ciriello, 1980). The vagus nerve also contains an afferent component carrying information related to parturition, which provides input to the NTS (Bailey and Wakerley, 1997). Interposed amongst the VLM and NTS and receiving many of these visceral inputs, are the A1 and A2 noradrenergic cell groups respectively. These cell groups project to the MNCs to regulate the release of VP and OT (Sawchenko and Swanson, 1981;Cunningham, Jr. and Sawchenko, 1988). Both of these cell groups are partial to the MNCs they target; A1 preferentially innervates VP cells where as A2 favours OT cells (Shioda et al., 1992) yet the total amount of noradrenergic innervation between both cell types is approximately equal (Michaloudi et al., 1997). The majority of noradrenergic afferents input to the ipsilateral MNCs (Weiss and Hatton, 1990). The A2 group also projects to the neurohypophysis where it is thought to directly influence neurohormone release (Zhao et al., 1988a;Zhao et al., 1988b;Garten et al., 1989). The largest and most influential noradrenergic cell group, the locus ceruleus (A6) plays only a minor role in dictating MNC physiology, as its primary target in the hypothalamus is the parvocellular division of the PVN (Sawchenko and Swanson, 1982).

NA released from the A1 and A2 cell group terminals is a critical mediator of MNC responses (Sawchenko and Swanson, 1981;Day et al., 1984;Day and Renaud, 1984;Kannan et al., 1984;Day et al., 1990;Tanaka et al., 1992;Shioda et al., 1992;Shioda and Nakai, 1992;Horie et al., 1993;Buller et al., 1996). Intracerebroventricular (ICV) injection or direct injection of NA into the MNC nuclei excites OT and VP cells (Moss et al., 1971;Arnauld et al., 1983;Day et al., 1985;Brooks et al., 1986b). This effect requires α_1-adrenoceptor activation (Armstrong et al., 1986;Yamashita et al., 1987;Shioda et al., 1997;Daftary et al., 1998), which can trigger the release of OT and VP from hypothalamic explants (Randle et al., 1986a). Central blockade of adrenoceptors (Pacak et al., 1995b) or destruction of noradrenergic terminals by prior injection of the neurotoxin 6-hydroxydopamine (Day et al., 1984) completely eliminates the increase in MNC activity observed in response to physiological stressors. There are three primary methods through which α_1-adrenoceptor activation increases MNC output. First, NA alters MNC excitability by affecting glutamate release onto these cells at the intranuclear glutamate interneuron level (Daftary et al., 1998), which may enhance neuroendocrine output (Nissen et al., 1995;Jourdain et al., 1998). Second, NA depolarizes MNC membrane potential (Randle et al., 1986a) and third, NA decreases postsynaptic K^+ conductances to facilitate phasic firing (Dudek et al., 1989). However, the effects of NA at the level of the synapse have not been investigated. Three specific types of glutamatergic plasticity will be introduced below and then each will be tested experimentally in a designated data chapter.

To a lesser extent compared to excitation, NA has been found to have inhibitory influence over MNC behaviour in some studies. During lactation, microelectrophoretic application of NA around OT MNCs inhibited the majority of these cells (Honda et al., 1985). In anaesthetized dogs ICV injection of NA did not excite but instead depressed VP release, an effect mediated by α_2-adrenoceptor activation (Kimura et al., 1984). Others observing reduced VP MNC activity in response to NA have either backed the involvement of the α_2 receptor (Brooks et al., 1986a;Yamashita et al., 1988;Khanna et al., 1993) or the β-adrenergic receptor in mediating this effect (Day et al., 1985). One study examining the effect of NA on inhibitory PSCs (IPSCs) has reported an α_2-mediated

attenuation (Wang et al., 1998), an observation that also conflicts with the general inhibitory action of α_2-adrenoceptor activation on MNC output. There is clear consensus on the overall excitatory role of α_1-adrenoceptor activation yet numerous inconsistencies persist regarding the putative nature of and conditions under which adrenergic receptors may mediate inhibition.

1.7.1 NA and Co-Transmitters?

The A1 and A2 noradrenergic cells are not homogenous populations. In the A1 group several other peptides are co-expressed with NA including, substance P (Bittencourt et al., 1991), galanin (Levin et al., 1987) and neuropeptide Y (NPY) (Sawchenko et al., 1985). In addition, there is indirect evidence that ATP is co-released with NA in response to A1 activation (Day et al., 1993;Buller et al., 1996)(more on this finding below). A2 NA cells do not show the same degree of co-expression with other molecules as A1 but the A2 nucleus does harbour other non-noradrenergic cell types including neurons that express inhibin B, somatostatin and enkaphalin (Sawchenko et al., 1990). Due to this heterogeneity, there have been a number of studies examining the potential interplay between NA and different factors on hormone release and on the electrophysiological properties of MNCs. One interaction worth noting is between NA and NPY. Most of the A1 neurons co-expressing NA and NPY impinge on the VP MNCs, similar to other A1 cells. The NPY Y_1 receptor agonist excites VP and OT release and NPY can potentiate the excitatory effects of NA on VP (Kapoor and Sladek, 2001) and OT release (Sibbald et al., 1989) *in vitro* as well as OT release in lactating rats *in vivo* (Parker and Crowley, 1993). Another interaction worth examining in more detail, as it is unequivocally pertinent to the thesis at hand, is the relationship between NA and ATP.

While there is overwhelming evidence for NA as a prominent mediator of MNC responses, there is also support for the transmitter ATP in eliciting excitatory effects on these cells as well as evidence for cooperative action between both signalling molecules. P_2-purinoceptors are present in MNCs (Xiang et al., 1998;Loesch et al., 1999;Loesch and Burnstock, 2001;Xiang et al., 2006) and there are multiple types of P2X receptors that are

functionally expressed in the SON (Shibuya et al., 1999). Injection of ATP into the PVN or SON increases plasma VP concentration through P2- rather than P1-receptors (Mori et al., 1992;Mori et al., 1994). In the SON, direct application of ATP excites VP cells (Day et al., 1993) and P2X receptors mediate TTX-insensitive depolarization of both MNC types (Hiruma and Bourque, 1995). The first evidence in support of the combined role of NA and ATP came from a study showing an increase in the extracellular concentrations of both NA and the purine metabolite uric acid in the SON in response to haemorrhage (Kendrick and Leng, 1988). Sladek's group has demonstrated synergistic action of NA and ATP in the hypothalamic explant preparation by showing VP and OT release is greater and more sustained when both molecules are given simultaneously compared to the cumulative effect of each molecule delivered in isolation (Kapoor and Sladek, 2000). While a precise locus of action still needs to be defined (Song and Sladek, 2006) their evidence thus far suggests that P2X receptors, PKC activation, gene transcription (Kapoor and Sladek, 2000) and sustained postsynaptic Ca^{2+} rises in MNC somas that result from both external Ca^{2+} influx and Ca^{2+} release from intracellular stores (Song et al., 2006) are involved. The fact that the P2X antagonist PPADS blocked synergism as well as some of the isolated NA effect (Kapoor and Sladek, 2000) is supported by earlier *in vivo* experiments demonstrating MNC excitation by direct A1 stimulation (Day et al., 1993) or A1 activation via haemorrhage (Buller et al., 1996) can also be blocked by ATP receptor antagonists, indicating that this effect may result from ATP rather than NA itself. Originally these data were interpreted as co-transmission but other explanations can be supported such as NA triggering the release of ATP, which then acts as the final affecter.

ATP is thought to be co-transmitted with NA from terminals found across the peripheral sympathetic nervous system (Burnstock, 2004). In the vas deferens ATP is responsible for the fast component of the excitatory junctional potential and subsequent SMC twitches resulting from sympathetic stimulation, while co-transmitted NA is responsible for the slow component eliciting longer SMC contractions (Burnstock, 1995). An inherent problem with these studies comes in ascertaining whether or not an additional, surreptitious party member may be contributing one of the supposed 'co-

transmitters' instead of both signalling molecules arising from the same neuronal source (Vizi and Burnstock, 1988). This difficulty was first made clear by the demonstration that SMCs—or so they hypothesized—released ATP in response to NA (Vizi et al., 1992), arguing against the ATP-NA co-transmitter hypothesis. Since this time however, evidence in support co-transmission from the same neuronal population has been provided by detecting simultaneous NA and ATP release from pure sympathetic neuron cultures (von, I and Starke, 1991). If we return to the MNCs for a moment and look again at the Kapoor and Day observations, that ATP-NA synergism and the effects of A1 noradrenergic activation on MNCs respectively, can be blocked with P2X receptor antagonism, does this mean co-transmission? While this is a reasonable hypothesis, other explanations can be supported as we shall see in the second of three papers to be presented here entitled: *Norepinephrine triggers the release of glial derived ATP to increase postsynaptic efficacy*, which provides evidence not for co-transmission but for NA interactions with neighbouring glial cell.

1.8 The Glutamate Synapse

Glutamate synapses are ubiquitous throughout the CNS and have now surpassed the neuromuscular junction (NMJ) as the most well described neuronal communication point. Glutamate is a non essential amino made locally from α-ketoglutarate from the tricarboxylic acid cycle by the enzyme glutamate dehydrogenase or from astrocytic glutamine by the enzyme glutaminase. Free glutamate is loaded into synaptic vesicles by the transporter VGLUT, a process that requires an acidic vesicle lumen from the actions of proton pumps. Presynaptic varicosities house a variable number of synaptic vesicles, each containing a finite amount of glutamate. Upon sufficient depolarization and Ca^{2+} influx resulting from action potential invasion, a designated release site called an active zone allows one glutamate filled vesicle to fuse with the apposing plasma membrane thereby allowing the neurotransmitter access to the synaptic cleft. The liberated transmitter diffuses across this small gap (approximately 10nm wide) and binds to postsynaptic receptors on the receiving neuron, which, once activated, elicit postsynaptic changes constituting information transfer (see (Kandel and Schwartz, 2001) for review).

1.8.1 Ionotropic Glutamate Receptors

There are two classes of ionotropic glutamate receptors that are directly responsible for the chemical-to-electrical glutamate signal, N-methyl D-aspartate (NMDA) receptors and Non-NMDA receptors. Ionotropic NMDA receptors (NMDARs) are generally located at postsynaptic sites in close proximity to presynaptic release machinery. The current response measured in response to channel opening has a relative fast rise time (~5 ms) and a slower exponential decay time (20 ms). These receptors are permeable to Ca^{2+} and display a voltage sensitive Mg^{2+} block at negative membrane potentials. These features make NMDARs ideally suited for detecting coincident signals and initiating plastic changes because both ligand binding and sufficient depolarization are necessary for channel opening (Malenka and Bear, 2004). Although NMDARs are a cornerstone of most forms of long-lasting synaptic plasticity described to date in the vertebrate CNS, they are not involved in any of the three types of plasticity to be presented in the data chapters. Only control experiments examining their lack of influence on plasticity with the use pharmacological antagonists will be presented.

Non-NMDA receptors include α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, and kainate receptors. The measurement of synaptic responses mediated by AMPA receptors constitute the central methodology of this thesis, while the role of kainate receptors at glutamate synapses on MNCs is not investigated and will not be mentioned further. AMPA receptors (AMPARs) are located in the postsynaptic membrane directly apposed to presynaptic release sites. They are rapidly gated, cation permeable channels that mediate fast excitatory synaptic currents/potentials in neurons. A typical AMPAR-mediated current possess a rapid rising phase (~0.5 ms) from the fast opening of several to many channels after glutamate binding and a slower, exponential decay (~2.5 ms) that results from transmitter unbinding or receptor desensitization if glutamate remains in the presence of the receptors for prolonged periods (Colquhoun et al., 1992).

AMPARs are composed of four different subunits, $GluR_1$ to $GluR_4$, which are assembled in a tetrameric structure. In the mature brain the dominant forms are

heteromeric combinations that combine $GluR_1$ with $GluR_2$ and $GluR_2$ with $GluR_3$. Each subunit has three full transmembrane domains (1, 3 and 4) and one domain (2) that is kinked from the intracellular side, providing an ion selectivity filter and a structure that allows the N and C-terminus to be on opposing sides of the plasma membrane. The $GluR_2$ subunit determines Ca^{2+} permeability; if present in the tetra-subunit complex, negligible Ca^{2+} passes when the channel is open (see (Malenka, 2003) for review).

AMPA-mediated synaptic currents can be readily observed in nearly all neurons of the CNS via an electrophysiological technique called voltage clamping. They can be evoked experimentally by providing a sufficient voltage stimulus to trigger action potentials in afferent axons projecting to a cell of interest to initiate glutamate release, or AMPA-mediated currents can be observed after glutamate is released stochastically, resulting in 'miniature' events (see just below). Changes in AMPA-mediated synaptic currents will provide the readout for plasticity in this dissertation.

1.8.2 Miniatures

Miniatures, or 'minis' as they are colloquially labelled, are current or voltage responses (depending on whether they are recorded in voltage clamp or current clamp respectively) measured postsynaptically, which result from the stochastic release of individual neurotransmitter filled vesicles. They do not depend on action potential triggered exocytosis or, in some synapses in the brain, extracellular Ca^{2+} influx through voltage gated Ca^{2+} channels (Llano and Gerschenfeld, 1993;Glitsch, 2006). In particular, mEPSCs recorded from MNCs are insensitive to complete extracellular Ca^{2+} removal (Inenaga et al., 1998).

1.8.3 Why We Examine Minis

The randomly occurring nature of mEPSCs has been traditionally perceived as an artefact of synapse design; a pure consequence of how a synapse must be built for spike-triggered exocytosis. Due to this, 'noise' has been a popular way to describe these events. Even in this light however (which may be erroneous), they have been regarded as a useful tool for studying synaptic transmission. In this dissertation we measure glutamate-mediated

miniatures, rather than synaptic responses measured as a result of glutamatergic fibre stimulation, for several reasons: 1) enhanced frequency and amplitude of mEPSCs has been shown to elicit action potentials in the postsynaptic cell thereby conveying meaningful information transfer and dispelling the notion that miniatures are just noise, 2) mEPSCs are relatively large (~25 pA on average) and frequent (2-8 Hz) in MNCs under our recording conditions, making them an easy and robust assay of synaptic strength, 3) evoked glutamate release onto MNCs in the PVN is highly asynchronous (Boudaba et al., 1997), suggesting both that the careful analysis and correct interpretation of evoked release data is difficult and that action potential triggered release is essentially a borage of miniatures, and 4) recent data from the Pittman laboratory suggests that miniatures and evoked synaptic responses are generated by the same synapses, suggesting that changes in miniatures may reflect changes in evoked release. Specifically, in this thesis we examine the effects of NA on the frequency and amplitude of mEPSCs, which provides us with a useful method for delineating how this catecholamine changes the efficacy of glutamate synapses on MNCs.

1.8.4 A Miniature Warning

There are several ideas to consider when examining the frequency and amplitude of miniature currents. A mEPSCs putatively represent the release of a single vesicle, acting on a single postsynaptic receptor density; thus, under classical thinking if the frequency of stochastic release events increases this can be interpreted as a presynaptic locus of action, as P_r must be higher. Conversely, if the amplitude of synaptic currents generated by single vesicles becomes larger this is interpreted as a postsynaptic locus of action, as postsynaptic receptor sensitivity must have improved in some way. However, there are a number of caveats associated with classical thinking. First, to claim definitively that a change in mEPSC frequency is presynaptic, one has to believe that an entire postsynaptic densities cannot come 'online' or off-line' rapidly enough to account for the effect. Second, to believe with certainty that a change in mEPSC amplitude is postsynaptic several other possibilities have to be discounted. For instance, to what degree postsynaptic receptors are saturated by a single packet of neurotransmitter plays an

important role. Simply recruiting larger vesicles with more glutamate or increasing the concentration of glutamate within a single vesicle could result in larger the mEPSCs by a purely presynaptic mechanism if postsynaptic receptors are under saturated. Alternatively, if two vesicles of glutamate are allowed to fuse simultaneously, approximately doubling the transmitter concentration in the cleft, larger mEPSCs could be observed if there are plenty of receptors available to sense it. Therefore, when using frequency and amplitude measurements of minis classical thinking should not be followed blindly. An experimentalist should take the observed mini change at 'face value' and stringently test the meaning of such a change with a mind open to numerous possibilities.

The final caveat when trying to interpret data from recorded miniatures is the potentially for heterogeneity between randomly occurring synaptic responses. The heterogeneity can be broadly dividing into two categories: 1) intersite variability, in which differences between synaptic currents arise because each synapse on a given neuron possesses its own unique presynaptic and postsynaptic profiles as well as distances of electrotonic travel and 2) intrasite variability, in which variable synaptic responses arise from stochastic processes within an single synapse. Understanding synaptic variability becomes critical when trying to assess the root cause for alterations in size, shape, or frequency of measured synaptic currents. In this thesis both locus of action and synaptic heterogeneity are carefully considered.

1.9 The 'How To' on Synapse Strengthening

From our outline on the essential geometry and machinery of the glutamatergic synapses it is very easy to see the many ways in which the strength of the glutamate signal can be enhanced or attenuated. Two classical concepts for changing synaptic strength are: 1) to vary the amount of neurotransmitter released and 2) vary the effectiveness with which the postsynaptic receptors respond to the released transmitter. Within these simple concepts lay a multitude of mechanistic possibilities. With respect to the former, the amount of glutamate released could be varied by changing the probability of vesicle release, altering the concentration of transmitter within the vesicle, changing vesicle size, changing the

number vesicles allowed to fuse simultaneously from a single stimulus just to name a few. With respect to the latter, the postsynaptic responsiveness could be varied by changing the number of receptors available to bind transmitter, altering the amount of current passed by a single receptor when activated, changing the affinity of the receptor for the ligand and so on. There are also many alternative ways to change synaptic efficacy that do not fall within the boundary of these two classical ideas, which include but are not limited to changing the geometry of the synapse, altering the diffusion of glutamate, changing the degree of transporter reuptake etc.

While all of these are potentially important, some mechanisms have garnered more favour than others at glutamate synapses. Three mechanisms of interest to this dissertation are: 1) turning off the inhibitory effect of presynaptic autoreceptors to increase P_r, 2) triggering the release of multiple vesicles of transmitter simultaneously so that the concentration of glutamate in the synaptic cleft can be enhanced and 3) Augmenting the number of AMPARs to enhance postsynaptic responsiveness. The first two mechanisms will be covered below in designated sections entitled 'mGluRs: Presynaptic Control and Plasticity' and 'Multivesicular Release and Miniatures' whereas the postsynaptic details of the later plasticity will be described directly below because the events initiating AMPAR insertion are of greater interest and require a separate and focused section to be described properly. These initiating events involve a novel type of neuron-glial signalling and will be covered in 'Glial Neuronal Communication'.

1.9.1 AMPAR Insertion: A Ubiquitous Mechanism

The most established form of long term synaptic strengthening, properly referred to as long term potentiation (LTP), is found in the excitatory connections between the CA3 pyramidal neurons to the CA1 pyramidal neurons (Schaffer collateral synapses). The principle mechanism responsible for this long-term strengthening relies on the insertion of additional AMPARs into the postsynaptic membrane, which, once exposed to the cleft, are able to sense more glutamate each time it is released (Shi et al., 1999). The recruitment of additional AMPARs to a working synapse has now been demonstrated in several brain regions and may represent a ubiquitous mechanism by which postsynaptic

responsiveness is improved and long term memories are formed (Shi et al., 1999;Takahashi et al., 2003;Rumpel et al., 2005). There are two central processes involved in AMPAR insertion: 1) AMPARs are continuously recycled in a manner that maintains a certain number of receptors in the postsynaptic density and 2) AMPARs are recruited to augment the total number of receptors in response to changes in synaptic activity. If more AMPARs are added, the recycling process maintains this new augmented number (Shi et al., 2001). Specific tetrameric subunit arrangements dictate which AMPARs participate in a given process, as different subunit C-termini sequences endow a receptor with trafficking information pertinent to a particular task. In the former, AMPARs composed of $GluR_2$ and $GluR_3$ subunits are thought to be constitutively recycled, while in the latter AMPARs built from $GluR_1$ and $GluR_2$ subunits are thought to be incorporated into the postsynaptic density in response to elevated intracellular Ca^{2+} from NMDAR activation (Hayashi et al., 2000;Shi et al., 2001). The mechanisms responsible for AMPAR incorporation into the postsynaptic density are still being elucidated but likely involve an internal pool of vesicles containing AMPARs that fuses with the plasma membrane (Shi et al., 1999) and lateral diffusion of AMPARs along the plasma membrane to designated sites (Borgdorff and Choquet, 2002;Adesnik et al., 2005). Though Ca^{2+} is necessary, the activation of two kinases has been labelled critical for the expression of plasticity, Ca^{2+}-calmodulin dependent kinase II (CaMKII) (Malinow et al., 1989) and phosphotidyl inositol 3-kinase (PI3-K). The activation of PI3-K is important for LTP in the hippocampus (Sanna et al., 2002;Raymond et al., 2002;Opazo et al., 2003;Man et al., 2003), the potentiation of mEPSCs (Baxter and Wyllie, 2006), fear conditioning in the amygdala (Lin et al., 2001) and the growth and branching of dendrites (Kumar et al., 2005;Jaworski et al., 2005). In chapter 4 we demonstrated that MNCs too insert AMPARs to increase postsynaptic signal strength for prolonged periods. Though the intracellular process appears similar, with a reliance on postsynaptic Ca^{2+} and PI3-K, the method by which AMPAR insertion is initiated requires the release of glial derived ATP and the subsequent activation of postsynaptic Ca^{2+} permeable P2X receptors.

1.10 mGluRs: Presynaptic Control and Plasticity

Not all glutamate signalling relies on ionotropic receptors. Metabotropic glutamate receptors (mGluRs) are a heterogeneous family of G-protein-coupled receptors, consisting of eight types (mGluRs1-8) that are categorized into three subfamilies (Conn and Pin, 1997). Unlike the ionotropic glutamate receptors, which are responsible for fast excitatory neurotransmission, metabotropic glutamate receptors initiate slower modulatory effects on the synapse through intracellular signal transduction (Schoepp and Conn, 2002). Group I ($mGluR_1$ and $_5$) are found on glial cells (Pearce et al., 1986) and postsynaptically, both away from membrane specializations (Baude et al., 1993) and near them (Shigemoto et al., 1997). Group II ($mGluR_2$ and $_3$) and group III ($mGluR_4$, and $_{6\text{-}8}$) are located presynaptically and function as autoreceptors—short time course, negative feedback system to mitigate the release of neurotransmitter. Group II and III mGluRs are coupled to the $G_{i/o}$-protein and therefore inhibit the effector enzyme adenylyl cyclase, decreasing the production of cAMP and PKA activity. Generally, these mGluR subtypes inhibit excitatory (Schrader and Tasker, 1997b;Panatier et al., 2004;Billups et al., 2005) and inhibitory (Schrader and Tasker, 1997b;Piet et al., 2004) neurotransmitter release when activated. $mGluR_7$ has been localized to the presynaptic grid at the site of vesicle fusion (Shigemoto et al., 1996). Other subtypes including $mGlu_2$ and $mGlu_8$ are located at extopic sites away from the active zone (Schoepp, 2001), while $mGluR_4$ and $mGlu_8$ have been localized to symmetric synapses (non-glutamatergic) (Bradley et al., 1996;Ferraguti et al., 2005). These regional differences correspond to their relative affinities for glutamate; centrally localized is low and ectopically/heterosynaptically localized is high. The differences in receptor expression may depend on the type of negative feedback required at each synapse. mGluRs poised ectopically or at symmetrical synapses may only regulate glutamate release under periods of high demand when transmitter spills out. This would stand in contrast to more discreet feedback mechanisms where centralized mGluRs may be involved in tonic control.

1.10.1 Glutamate autoreceptors in MNC nuclei

Membrane binding assays in the hypothalamus have established a wide distribution of mGluRs, comparable to both NMDA and non-NMDARs in pre and postsynaptic locations (Meeker et al., 1994). Group I mGluRs have been shown to elicit excitatory effects in MNCs by inhibiting potassium channels (Schrader and Tasker, 1997a) and glutamate release onto MNCs is controlled by group III mGluRs (Schrader and Tasker, 1997b;Panatier et al., 2004). In the SON, there is some discrepancy over whether high (Panatier et al., 2004) or low (Schrader and Tasker, 1997b) affinity mGluRs are present. Supporting low affinity autoreceptors, single synaptic responses in the SON are tonically regulated by mGluRs (Schrader and Tasker, 1997b), suggesting sensitive, centralized control of release. Tonic mGluR activity is further enhanced during dehydration (Boudaba et al., 2003b) and lactation (Oliet et al., 2001) when the retraction of glial processes from synaptic elements (Tweedle and Hatton, 1977;Theodosis et al., 1981;Hatton and Tweedle, 1982;Theodosis and Poulain, 1984) increase the accessibility of synaptic glutamate to the presynaptic autoreceptors. While lowering glutamate P_r by increased mGluR activation during these periods of high demand seems counterintuitive, such a mechanism may serve as a high frequency filter so that only afferent signals of sufficient frequency will be able to overcome enhanced mGluR-mediated inhibition (Oliet, 2002).

1.10.2 Turning Autoreceptors Off

In addition to varying levels of tonic activity that are dictated by glutamate availability, mGluR activity can also be modified directly through intracellular mechanisms. Previous evidence attributes an important role for PKC in regulating mGluR function. PKC-mediated inhibition of $mGluR_7$ uncouples the receptors G-protein cascade, as the ability of the $G\alpha_{i/o}$ protein to bind [^{35}S]-GTPγS is markedly reduced after kinase activation (Macek et al., 1998). Recent studies have demonstrated a direct PKC-$mGluR_7$ interaction via phosphorylation of Ser^{862} on the intracellular carboxy terminus of the receptor (Sorensen et al., 2002). Since this time, prolonged application of mGluR agonist been shown to trigger internalization of the receptor, suggesting that complete mGluR removal

from the plasma membrane, thereby denying its access to synaptic glutamate, may be a principle mechanism for reduced mGluR function (Pelkey et al., 2005;Pelkey et al., 2006).

Notably, PKC is activated by metabotropic $G_{q/11}$-protein signalling. In the PVN, NA elicits a robust increase in spontaneous (Daftary et al., 1998) frequency that requires $G_{q/11}$-linked α_1-adrenoceptors, suggesting a possible link between an endogenous neuromodulator and the intracellular processes required to affect mGluR activity. With the notable exception of one study which utilized adenosine analogs to increase PKC activity and demonstrate a subsequent decrease in mGluR function (Macek et al., 1998), most studies have relied on the non-physiological activation of PKC using phorbol esters. Therefore, it remains unclear whether or not mGluRs can be regulated by the actions of local neurotransmitters. The physiological integration of PKC mediated inhibition of mGluRs, initiated by the endogenous ligand NA, would add an additional level of sophistication to signal processing at these synapses. In the first manuscript presented in chapter 3 **we test the hypothesis that 'physiological' activation of PKC via α1-adrenoceptors influences mEPSCs by inhibiting mGluR activity**. Specifically, we will test the effects of successive NA treatments on mEPSC frequency and determine if any response differences can be attributed to changes in mGluR function. We predict the inactivation of presynaptic group III mGluRs would remove autoinhibition at these synapses and effectively 'prime' them so that additional treatments with NA are potentiated.

1.11 Glial Neuronal Communication

There are three main types of glial cells in the central nervous system. The microglia, which are involved in CNS immunity and repair, the oligodendrocytes, which are responsible for wrapping numerous axon fibres with myelin and the astrocytes, named for their star-like appearance, which have been traditionally thought of as support tissue by playing a role in transmitter uptake and recycling, extracellular ion balance and forming a portion of the blood brain barrier by end-feet coverings of capillaries. Recent discoveries have expanded our current view of the capabilities of astrocytes. Here we will review

their integral role in modulating neural excitability and synaptic transmission. This field came to fruition by the demonstration that astrocytes not only possess transporters such as GAT-1 (Gomeza et al., 1994), and GLT-1 and GLAST (Rauen and Kanner, 1994) for the uptake of GABA and glutamate respectively, but also a wide variety of membrane bound receptors for classical neurotransmitters like glutamate (Pearce et al., 1986), NA (Duffy and MacVicar, 1995), dopamine (Hertz et al., 1984) and acetylcholine (Sharma and Vijayaraghavan, 2001). This led to the idea that these 'support cells' may play a more complex role in neural signalling than previously thought (Barres, 1989). Over the past decade and a half, numerous studies have suggested that glial cells respond to classical neurotransmitters and in turn release glial-derived signalling molecules (gliotransmitters) back onto neurons to modulate synaptic efficacy and cell spiking. The two-sided synapse dogma has now changed to reflect these exciting findings with the now accepted term *tripartite synapse*, which incorporates presynaptic, postsynaptic and glial elements.

1.11.1 In the Beginning there was Glutamate...and then Others Joined

The first experiments to test an expanded role for astrocytes were performed in cultured hippocampal astrocytes loaded with the Ca^{2+} indicator. In response to exogenous glutamate oscillating increases in internal free Ca^{2+} were observed resulting from the activity of intracellular Ca^{2+} stores (Cornell-Bell et al., 1990). The oscillations propagated as a wave in individual cells and continued in adjacent astrocytes in the same manner. These data suggested that astrocytes can respond to glutamate in a manner inconsistent to the prescribed actions of transporters and instead indicated the involvement of astrocytic glutamate receptors. Glial glutamate receptors could elicit novel, long-range signalling cascades incorporating networks of astrocytes. Further studies demonstrated a similar effect using more physiological preparations, synaptically released glutamate or both (Dani et al., 1992;Porter and McCarthy, 1995;Porter and McCarthy, 1996). These results had profound implications on our understanding of signalling in the central nervous system, as there was now an entirely new player to consider.

Further studies have expanded these findings to include other neurotransmitters in the induction of Ca^{2+} waves in astrocytes. These include the release of acetylcholine in the hippocampus from cholinergic septal afferents (Araque et al., 2002), the exogenous effects of NA (Duffy and MacVicar, 1995) and GABA (Kang et al., 1998;Serrano et al., 2006) in the hippocampus, as well as the actions of the non-classical transmitter nitric oxide on the Bergmann glial cells of the cerebellum (Matyash et al., 2001) and on astrocytes of the cortex (Bal-Price et al., 2002). These data indicate that neuron to glial cell communication is not confined to a specific transmitter or a specialized cell group in the CNS, but alternatively may represent a novel and ubiquitous signalling method.

1.11.2 ATP release from astrocytes

Long-range Ca^{2+} signals in astrocytes are made possible by two principle mechanisms: 1) the intracellular diffusion of IP_3 through gap junctional channels (Sanderson et al., 1994;Venance et al., 1997), which allow electrical and fluid connectivity between adjacent astrocytes and 2) the extracellular paracrine actions of glial-derived ATP (Guthrie et al., 1999). The latter is important for our purpose, as the second manuscript to be discussed in chapter 4 deals with the release of ATP from glial cells in response to the application of NA. That ATP was released from glial cells to act on neighbouring cells in a paracrine fashion was shown by the detection ATP in the surrounding medium as a result of astrocytic Ca^{2+} wave propagation. Collecting this medium and then applying it back onto astrocytes generated Ca^{2+} waves, an effect that could be blocked with purinergic antagonists (Guthrie et al., 1999). The spatial characteristics of ATP release from astrocytes remain controversial. In one study, ATP release was found to occur in continuous waves coinciding with Ca^{2+} wave propagation (Wang et al., 2000), while in another ATP release occurred in isolated bursts that were highly localized and separated by large distances (Arcuino et al., 2002). The mechanisms of ATP release from glial cells is also controversial (see discussion section 'Glial ATP Release'), but there is strong support for metabotropic receptor $G_{q/11}$ activation including purinergic P2Y receptors (James and Butt, 2002) and group I mGluRs (Cai and Kimelberg, 1997;Cai et al., 2000). α_1-adrenoceptors are now an additional candidate and the

evidence for this assertion will be presented in the second of three manuscripts in chapter 4.

1.11.3 ATP: The Gliotransmitter

The three primary gliotransmitters are glutamate, ATP, and D-serine (the endogenous cofactor for NMDAR activation). Until very recently glutamate was labelled as the gliotransmitter exclusively involved in neuronal communication (Parpura et al., 1994;Hassinger et al., 1995;Bezzi et al., 1998), while ATP alone signalled to neighbouring glia (Guthrie et al., 1999;Wang et al., 2000;Arcuino et al., 2002). The latter view has changed significantly over the last 3 years, as glial-derived ATP is now thought to influence synaptic transmission and neuronal excitability. The initial idea to test the action of glial ATP on neuronal function was made from a simple link between numerous studies showing an abundance of ATP release from astrocytes and that ATP can be co-packaged with classical transmitters to have direct effects on postsynaptic targets (Edwards et al., 1992;Burnstock, 1995). In 2003 two papers demonstrated that ATP released from glial cells can inhibit neighbouring neurons. In the first demonstration performed in the retina, glial cell activation hyperpolarized nearby ganglion cells (Newman, 2003). The effect was blocked by adenosine receptor antagonists and lessened by ectoATPase inhibitors, suggesting glial-derived ATP was broken down by enzymes into adenosine to have this effect. The second manuscript performed in the CA1 region of the hippocampus utilized culture and slice preparations (Zhang et al., 2003). In culture, excitatory transmission was tonically inhibited by the activation of presynaptic P2Y receptors, an effect that depended on the presence of glia. In culture and slice, glutamate release from afferent stimulation triggered homo and heterosynaptic depression, which relied on non-NMDA glutamate receptors, gap junctional channels and adenosine receptors. These data suggested 1) endogenous glutamate initiates ATP release from glia, 2) glia to glia signalling may be involved and 3) ATP is broken down into adenosine to decrease P_r presynaptically (Zhang et al., 2003). Since these experiments others have demonstrated that glial ATP can activate inhibitory interneurons (Bowser and Khakh, 2004), which in turn trigger glial cells to release ATP resulting in

heterosynaptic suppression after ATP degradation (Serrano et al., 2006). Finally, evidence from transgenic mice suggest that the glial-derived ATP mediating heterosynaptic suppression is released in vesicles, as this process depends of astrocytic snare proteins (Pascual et al., 2005). As there is accumulating evidence for the presence of purinergic receptors on neurons (Anderson and Nedergaard, 2006), another question is whether or not ATP itself can affect synaptic efficacy in slices without being degraded into adenosine? Very recently, postsynaptic $P2X_4$ channels are critical for the full expression of Schaffer collateral LTP (Sim et al., 2006). As P2X receptors are Ca^{2+} permeable (North, 2002) and may be linked to PI3-K (Jacques-Silva et al., 2004), a critical kinase involved in AMPAR insertion, P2X receptors may serve as an aid or alternative to NMDAR-mediated plasticity. An important piece of the P2X-neural plasticity puzzle is offered by the current thesis, where the release of ATP from glial cells has direct actions on postsynaptic $P2X_7$ receptors (possibly $P2X_2$ or $P2X_4$) on MNCs, causing a Ca^{2+} dependent enhancement of mEPSC amplitude and acting as a surrogate to the NMDAR (see chapter 4).

1.11.4 Glial-Neuronal Interactions in the MNC nuclei

In addition to active glial-neuronal signalling, the MNC nuclei of the hypothalamus provide an excellent example of a unique glial-neuronal interaction that is dynamic and purely physical. This structural plasticity is observed during the physiological states of dehydration, lactation and parturition, and has direct consequences on synaptic strength. During these physiological challenges, the glial cell processes that extend between MNC cell bodies and around synapses retract, increasing soma appositions and removing glial cell synapse barriers respectively (Tweedle and Hatton, 1977;Theodosis et al., 1981;Hatton and Tweedle, 1982;Theodosis and Poulain, 1984). In a series of electrophysiological experiments in virgin and lactating rats, the concentration of cleft glutamate and its diffusion in the extracellular space was found to depend on synaptic glial cell coverage. In the absence of glial cells, glutamate can access presynaptic mGluRs lowering P_r (Oliet et al., 2001). This observation was not seen in virgin rats, indicating the anatomy of the neuropil plays a critical role in regulating synaptic efficacy.

In light of neuropil remodelling, the MNCs have become an excellent place to undertake study of neuronal glial cell interactions because one can test the properties of a given plasticity in the presence or absence of glial cells by using a simple experimental manipulation, dehydration or lactation. Recently this model has shown that glial cell diffusion barriers govern heterosynaptic inhibition of GABAergic terminals by glutamate spillover (Piet et al., 2004) and the threshold for classical synaptic strengthening (Panatier et al., 2006b). With respect to the latter, glutamate afferents, and in particular those originating in the OVLT, exhibit NMDAR dependent LTP and LTD (Panatier et al., 2006a). Interestingly, the induction of plasticity is precisely controlled by the astrocytes that ensheath the synaptic contacts. In the SON, astrocytes serve as the sole source of D-serine (Panatier et al., 2006b), an amino acid which is the endogenous ligand for the glycine binding site on the NMDAR. These experiments reveal that when the concentration of D-serine is relatively high there is robust NMDA activation resulting in reproducible LTP in response to high frequency stimulation of afferents. Compromising the availability of d-serine, either by inhibiting its synthesis or by decreasing the physical interposition between glial cells and synapses, causes an LTD of synapses in response to the same stimulation parameters. The ability to induce LTP in the absence of glial cells could be recovered by applying a more intense stimulation protocol, suggesting that glial cells use D-serine to not only activate NMDARs, but also to set the threshold for plasticity in this system (Panatier et al., 2006b).

In chapter 4, a long-lasting increase in the amplitude of mEPSCs induced from the actions of NA is examined. The sustained augmentation in synaptic current amplitude is reminiscent of classical LTP in CA1 pyramidal neurons of the hippocampus, in that the same postsynaptic second messengers and the insertion of more AMPARs into the receiving side of the synapse are mechanistically involved. However, the effect occurs without afferent stimulation or the activation of NMDARs. When considering the known cooperative interactions between NA and ATP in the hypothalamus, **we test the hypothesis that ATP is released as an intermediate transmitter and is responsible for the NA-induced enhancement of synaptic strength**. Further, the anatomical

remodelling properties of the nucleus are utilized **to test the hypothesis that the source of ATP comes from glial cells**.

1.12 Multivesicular Release and Miniatures

For a larger glutamate transient to contribute to synaptic current amplitude one must forgo the notion that changes in the amplitude of synaptic events are determined by changes in postsynaptic efficacy (Frerking and Wilson, 1996). This idea is based on the assumption that the neurotransmitter released by one vesicle is sufficient to saturate postsynaptic receptors completely (Frerking et al., 1995;Hanse and Gustafsson, 2001;Karunanithi et al., 2002;Van der et al., 2002). Recent demonstrations arguing against this precept (Liu et al., 1999;Ishikawa et al., 2002;Oertner et al., 2002), suggest that the amplitude of postsynaptic currents can be regulated by altering the amount of neurotransmitter released at a single synapse (Tong and Jahr, 1994;Frerking et al., 1995;Prange and Murphy, 1999;Wadiche and Jahr, 2001;Sharma and Vijayaraghavan, 2003).

The synchronous release of multiple vesicles is one method by which the amount glutamate in the synaptic cleft can be increased. One of the central tenets arguing against this possibility is the one site one vesicle hypothesis (OSOVH), where upon presynaptic depolarization a single vesicle can be released per active zone in a probabilistic manner. The OSOVH came to fruition by the strong correlation between the binomial parameter *N* and the number of anatomically determined presynaptic terminals (Korn et al., 1981). Since this time studies have supported these findings (Korn et al., 1982;Redman, 1990;Stevens and Wang, 1995;Auger and Marty, 2000;Hanse and Gustafsson, 2001). However, the mechanism(s) reducing P_r to < 1 at a single active zone remain unidentified, allowing for the possibility that under certain circumstances and/or at specific synapses in the CNS, this precept may not hold. This view has been supported by groups backing MVR, a process that results in an augmented concentration of cleft glutamate (Tong and Jahr, 1994;Auger et al., 1998;Oertner et al., 2002). A view synthesizing aspects from both synaptic ideologies is the synchronization of single vesicles across multiple active zones, which still upholds the OSOVH and may result in

an elevated glutamate cleft concentration due to the accumulation of glutamate at a single postsynaptic sites via transmitter crosstalk (Wadiche and Jahr, 2001).

1.12.1 Multivesicular Minis: A Semantics Problem

Although there is a growing support for the occurrence of MVR in response to electrical stimulation of axons in certain synapses, this process has been scarcely demonstrated for miniatures. Perhaps one reason for this is a psychological problem due to a semantical contradiction: a MVR miniature goes directly against the definition of mini. A mPSC is defined as the measured current resulting from the gating of postsynaptic receptor/channels in response to the stochastic release of neurotransmitter from the presynaptic fusion of an individual vesicle. However, there is growing evidence that a miniature does not always result from a single vesicle fusion and that the simultaneous fusion of multiple vesicles may comprise the current.

Amplitude histograms of mEPSCs in the CNS do not exhibit a Gaussian distribution but instead exhibit a characteristic rightward skew towards larger currents that is best fit by a log normal function (Van der, 1987). This result would not be anticipated from the release of a single vesicle onto a single postsynaptic receptor field under favourable recording conditions (Forti et al., 1997). 'Favourable' means that one can directly record from a single or at least a uniform population of synapses, a reality that exists mostly outside CNS synapses (but see (Forti et al., 1997)) at the neuromuscular junction (NMJ) in which the amplitude histogram observed for miniature endplate potentials (mEPPs) displays a single peak that is normally distributed (Del Castillo and Katz, 1954). In contrast to the NMJ preparation, central neurons offer a complex array of synapses, possessing differences in synaptic electrotonic decaying distance, intrasite-variance (Bekkers et al., 1990;Frerking et al., 1995), and intersite variance (Forti et al., 1997) in the quantal parameter *q,* which provide a reasonable explanation for the amplitude histogram skew. Another possibility is that central synapses can, under some circumstances, release multiple vesicles simultaneously generating large amplitude mEPSCs, which is responsible for the skew (Raghavachari and Lisman, 2004). If MVR is a prominent contributor, multiple equidistant modes

should be observed in the mEPSC amplitude histogram, where each successive mode represents the release of an additional quantum (Del Castillo and Katz, 1954). However, if multivesicular miniatures do occur at CNS synapses, it is likely they coexist with inter and intra-synaptic sources of variability and that both processes contribute to the rightward skew in the mEPSC amplitude histogram (Llano et al., 2000). Synaptic variability may be the primary reason for equidistant mode obfuscation and without these confounding variables their appearance may be clearly seen.

The single most convincing demonstration of multivesicular miniatures comes from cerebellar interneuron-Purkinje cell synapses. With the use of electrophysiology and two photon Ca^{2+} imaging, this study demonstrates that extremely rapid rises in presynaptic terminal Ca^{2+}, resulting from the activity of intracellular stores, is capable of triggering MVR (Llano et al., 2000). Although originally believed to be involved in a slower, more modulatory action (Finch and Augustine, 1998), intracellular Ca^{2+} stores are shown to be the principle mediator of fast vesicle synchronization. This effect has been demonstrated to depend on extracellular Ca^{2+} influx through voltage gated Ca^{2+} channels (Llano et al., 2000) or through presynaptic ionotropic ACh receptors (Sharma and Vijayaraghavan, 2003) to trigger CICR from presynaptic stores. As the α_1-adrenoceptor couples to the $G_{q/11}$-protein, leading to the production of the Ca^{2+} store signalling messenger IP_3, the actions of NA may also be capable of initiating a MVR process. The demonstration that gonadotropin releasing hormone can trigger Ca^{2+} release from stores via IP_3 generation in non-excitable pituitary cells to increase the efficacy of vesicle fusion a hundred fold (Tse et al., 1997), supports this idea. That excitatory synapses onto MNCs may be capable of MVR was first demonstrated by brief, high frequency stimulation of afferents (Kombian et al., 2000). The amplitude distribution of mEPSCs produced as a result of this protocol appeared multimodal, suggesting the concerted release of several vesicles at one time.

In the final manuscript to be presented a robust and transient enhancement of mEPSC amplitude is observed in response to NA. Because the effect is impervious to postsynaptic manipulations and does not appear to result from the release or activation of a quiescent vesicle or synapse respectively, **we test the hypothesis NA synchronizes the**

release of multiple vesicles at glutamate terminals synapsing onto MNC's in the PVN. From the results of many experiments the effect is found to be sensitive to lipid permeable Ca^{2+} buffering, a high dose of ryanodine and large amplitude mEPSCs are associated with a greater concentration of glutamate in the synaptic cleft, consistent with the idea that NA triggers multivesicular release.

Chapter Two: Materials and Methods

2.1 Electrophysiology

Hypothalamic coronal slices (300 μm) containing the PVN were prepared from male Sprague-Dawley rats (p 21-27). Rats that underwent dehydration were subjected to 7-10 days of a 2% NaCl water source upon arrival until electrophysiological experimentation. Care and use of animals was according to approved guidelines set forth by the University of Calgary Animal Care and Use Committee. Animals were anaesthetized (sodium pentobarbital 0.1mL/100g of body weight), decapitated, and the brains removed into ice-cold slicing solution for 3 min containing (in mM): NaCl 87; KCl, 2.5; $NaHCO_3$, 25; $CaCl_2$, 0.5; $MgCl_2$, 7; NaH_2PO_4, 1.25; glucose, 25; sucrose, 75; saturated with 95% O_2/5% CO_2. The brain was then blocked and mounted on a vibrating slicer (Leica Instruments) submerged in ice-cold slicing solution. Slices were incubated at 32.5°C in artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl, 126; KCl, 2.5; $NaHCO_3$, 26; $CaCl_2$, 2; $MgCl_2$, 2; NaH_2PO_4, 1.25; glucose, 10; saturated with 95% O_2/ 5%CO_2, for a minimum of sixty minutes. Using an upright microscope (Zeiss AxioskopII FS Plus) fitted with infrared differential interference contrast (IR-DIC), whole-cell recordings were obtained from magnocellular PVN neurons confirmed by their morphology and distinct electrophysiological characteristics (Tasker and Dudek, 1991). All experimental recordings were obtained at 32.5°C in voltage clamp mode at –60 mV and were accepted barring changes in access resistance of >15%. Neither the liquid junction potential nor the access resistance was corrected. The intracellular solution contained (in mM): potassium gluconate 123, $MgCl_2$ 2, NaCl 8, potassium EGTA 1, potassium ATP 4, and sodium GTP 0.3 buffered with 16 mM $KHCO_3$. For experiments in which postsynaptic G-protein signalling was blocked, GTP was excluded from the internal solution and GTP-β-S was added. For experiments in which enhanced postsynaptic Ca^{2+} buffering was desired to block postsynaptic plasticity, the concentration of EGTA was raised to 10 mM in the intracellular solution. The perfusate always contained picrotoxin (100 μM) to block $GABA_A$ channels and TTX (1 μM), to block voltage-gated Na^+ channels. In

experiments in which drugs were introduced to the postsynaptic cell through the patch electrode, a minimum of 20 min was allowed for intracellular diffusion. For experiments with postsynaptic thapsigargin, an extra 10 min was given to allow for the drug to take action. When GDP-βs was included in the internal solution, GTP was removed. In experiments utilizing the focal administration of AMPA (3 ms pulse, 100 μM, 25 psi), puffs were delivered once every minute throughout the experiment using a General Valve Corporation Picospritzer 2. The spritzing tips (resistance 3-6 MΩ) were placed directly over the soma of the patched cell. The response elicited was validated with 5 μM DNQX, which completely blocked the response (n = 3).

2.2 Data Collection and Statistics

Signals were amplified with the Multiclamp 700A amplifier (Axon Instruments), low pass filtered at 1 kHz and digitized at 10 kHz using the Digidata 1322 (Axon Instruments). Data were collected (pClamp, Axon Instruments) and stored on computer for off-line analysis using software designed to detect miniature synaptic events using a variable threshold (MiniAnalysis, Synaptosoft). The amplitude of mEPSCs were obtained during control and either at the peak of mEPSC amplitude increase when measuring the immediate and robust increase in mEPSC amplitude (usually occurring a few minutes after NA application) or thirty minutes after the cessation of NA (or BzATP) when measuring the long-lasting increase in mEPSC amplitude. A minimum of 5 min (up to 10 min) of recording was taken for analysis. In each experiment, the change in mEPSC amplitude was assessed as a fraction of one. Experimental values are presented as the mean ± SEM and statistical analyses were performed using Student's t-test when comparing two groups and analysis of variance (ANOVA) with a post-hoc Newman-Keuls test for comparisons across multiple groups. $p<0.05$ was accepted as statistically significant (* = $p<0.05$, **= $p<0.01$).

2.3 Analysis

2.3.1 Gaussian Fits

Amplitude histograms were plotted with 2 pA bins and appeared multimodal. Up to 4 modes could be fit with Gaussian functions as larger currents were too infrequent to fit curves accurately. Gaussian curves and a sum of Gaussian curve was fit using Graph pad Prism software (version 3.0). The size of a single quantum, q, was calculated from the mean separation between Gaussian curves by the expression:

$$q = \sum(M_k/k)/n$$

where M equals the mean pA value of each mode, k equals the mode number and n equals the total number of fitted modes in the distribution.

2.3.2 Inter-event Interval Fits

The time interval between events was best fit with a mono-exponential equation, indicative of a stochastic Poisson release process underlying the mEPSCs in both control and NA conditions. The probability of two mEPSC temporally summing to confer a larger amplitude mEPSC can be calculated using:

$$p_{(\text{time interval} \leq t)} = 1\text{-}e^{-t/\tau}$$

where *t* is the average event half-width and τ is equal to 1 / the decay constant (λ) of the mono-exponential equation.

We also estimated an upper limit of event summation probability using:

$$p = t/avg)t.$$

Utilizing the putative multimodal amplitude distribution and assuming that every event in a given mode is actually the sum of individual events (equal to the mode number), we approximated the total number of individual events in a given amplitude distribution by deconstructing the modes. After deconstruction, the number of observations in a mode (X) was multiplied by the mode number, k. The number of quanta in each mode was then summed to achieve the maximum number of quantal events (X_{max}) that could occur over the time period in which the original data were collected by:

$$X_{max} = \sum [(X_k)(k)]$$

This produces an avg)t value that is as small as possible and a summation probability that is as high as possible.

2.3.3 Peak-Scaled Non-Stationary Noise Analysis (PSNA)

The stochastic gating properties of AMPARs were assessed with PSNA using Synaptosoft Minianalysis software. In brief, 100-200 mEPSCs were selected with rise-times < 1 ms, aligned at steepest rise and then averaged. The mean mEPSC waveform was scaled to each individual mEPSC and subtracted to obtain the difference decay current. The variance was calculated between all difference currents, and a correction for post mEPSC baseline variance was made. A parabolic relationship was obtained from the decay variance versus mean decay current plot and a regression line was fit to the first 50% of the data points back calculated from the end of decay baseline. The slope of this relationship represents the weighted mean single channel current and the conductance is calculated from Ohm's law: $g = I/E\text{-}E_x$ where I = the unitary current and $E\text{-}E_x$ is the driving force = 60 mV.

2.4 Immunohistochemistry

Male Sprague-Dawley rats (p21-28)(Charles River) were anesthetized with sodium pentobarbital and perfused transcardially with cold phosphate buffered saline (PBS) followed by 4% paraformaldehyde. The brains were harvested and post-fixed in 4% paraformaldehyde overnight and then cryoprotected in 30% sucrose for another 24 hours. Sections were cut on a cryostat to 30 μM and floated in PBS. The sections were washed in 0.2% Tween-20 in PBS for 10 min, followed by 30 min in 1% hydrogen peroxide to clear excess peroxidases. Sections were then washed three times prior to blocking (3% donkey serum, 0.2% Tween-20 and 2% dimethylsulfoxide (DMSO) in PBS) for 1 hour. Subsequently, sections were incubated at room temperature for 24 hours with an affinity purified rabbit polyclonal antibody against the α_{1a}-adrenoceptor (Sigma, 1:250 in blocking buffer) followed by three washes (0.2% Tween-20, 2% DMSO in PBS) before incubation for two hours with Alexa Fluor 488 donkey anti rabbit (Molecular Probes,

1:250 in blocking buffer). Sections were washed again three times (0.2% Tween-20, 2% DMSO in PBS), followed by a second 24 hour incubation with a mouse monoclonal GFAP antibody conjugated with Cy3 (Sigma, 1:500 in blocking buffer). Sections were subsequently washed and mounted onto Chrom Alum coated slides, coverslipped with Vectashield (Vector Labs) and visualized on a confocal microscope (Olympus, BX-51). Each image in figure 4.6 is comprised of 12 sequential 1 μm plane images stacked together. Co-localization of GFAP and α_1-adrenoceptor was also verified on a single plane occurring within the upper-most section of the glial process.

2.5 Glial cell culture

Brains of two days old neonatal Wistar rats were removed after decapitation and carefully dissected using a dissecting microscope to separate hippocampus and cortex. These were separately mashed through an 80 μm Nitex mesh using a metal rod under aseptic conditions, as described (Bekar et al., 2005). All steps were carried out on ice. The collected cell suspension was cultured in 35-mm culture dishes in DMEM medium (Invitrogen Corporation) containing 10% fetal calf serum and maintained at 37°C in a humidified atmosphere containing 5% CO_2. The medium was changed every 3 - 4 days. The cultures were used for the ATP assay after 10 days. For the pituicyte culture, posterior pituitaries were separated from decapitated adult Long-Evans rats using a dissecting microscope. The tissue was cut into eight pieces, each of which was attached to the bottom of a 35-mm culture dish in a fibrin clot and cultured under the conditions described above. The cells were used for the ATP assay after 14 days. For the ATP assay, the cell media was replaced with physiological saline containing (in mM): NaCl, 120; KCl, 4; $MgCl_2$, 1.2; glucose 10; $CaCl_2$, 2; HEPES, 10; pH 7.35. During the experiment, the saline was replaced every 2 min and an aliquot was used to measure ATP using an ATP Bioluminescent Assay Kit (Sigma) and a 1251 Luminometer (LKB Wallac) at room temperature. The light output was measured 1 min after the beginning of the luciferase reaction. During the two exchanges indicated, the saline also contained 10-50 μM NA.

2.6 Drugs

Noradrenaline (1-[3,4-dihydroxyphenyl]-2-aminoethanol), phenylephrine ((R)-(-)-1-(3-hydroxyphenyl)-2-methylaminoethanol hydrochloride), prazosin (1-[4-amino-6,7-dimethoxy-2-quinazolinyl]-4-[2-furanylcarbonyl]-piperazine), calphostin C, PMA (1-[3,4-dihydroxyphenyl]-2-aminoethanol), DNQX (6,7-dinitroquinoxaline-2,3(1H,4H)-dione), AMPA, LY294002 (2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride), wortmannin, BBG, BzATP (2'-3'-o-(4-benzoylbenzoyl)adenosine 5'-triphosphate triethylammonium salt), Sucrose (b-D-Fructofuranosyl-a-D-glucopyranoside), BAPTA-AM (1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester)), EGTA-AM (EGTA-tetra(acetoxymethyl ester), ATP (adenosine triphosphate), picrotoxin, GDP-βs (guanosine 5'-[b-thio]diphosphate trilithium salt), MCPG (α-methyl-(4-carboxyphenyl)glycine)), fluorocitric acid (DL-fluorocitric acid, barium salt), baclofen ((±)-b-(aminomethyl)-4-chlorobenzenepropanoic acid), thapsigargin and ryanodine (Ryanodol 3-(1H-pyrrole-2-carboxylate) Ryania speciosa) were purchased from Sigma. Tetrodotoxin (TTX) was purchased from Alomone Labs Ltd. Botulinum toxin C was purchased from Calbiochem. L-AP4, (L-(+)-2-amino-4-phosphonobutyric acid), MAP4 ((S)-2-amino-2-methyl-4-phosphonobutanoic acid) and γDGG (γ-D-glutamylglycine) were purchased from Tocris Cookson (Ballwin, MO). Caffeine was a gift from Dr. Ray Turner. Picrotoxin, calphostin C, wortmannin and LY294002 were dissolved in DMSO (final concentration of DMSO <0.1%). Prazosin was dissolved in methanol (final bath concentration of methanol <0.05%). MCPG was dissolved in 0.1M NaOH. Fluorocitric acid was dissolved in ACSF with fifteen minutes of sonication. All other reagents were dissolved in ACSF.

Chapter Three: Priming of Excitatory Synapses by α_1 Adrenoceptor-Mediated Inhibition of Group III Metabotropic Glutamate Receptors

3.1 Summary

Adaptive responses mediated by the hypothalamus require sustained activation until homeostasis is achieved. Increases in excitatory drive to the magnocellular neuroendocrine cells that mediate these responses, however, result in the activation of a presynaptic mGluRs that curtails synaptic excitability. Recent evidence that group III mGluRs can be inhibited by PKC prompted us to test the hypothesis that activation of PKC by NA inhibits group III mGluRs and increases excitatory synaptic input to these cells. To examine the effects of NA on mEPSCs, we obtained whole-cell recordings from magnocellular vasopressin and oxytocin neurons in the paraventricular nucleus of the hypothalamus. All of the neurons tested in the current study displayed an α_1 adrenoceptor-mediated increase in mEPSC frequency in response to NA (1–200 μM). The excitatory effects of NA were mimicked by the phorbol ester PMA and blocked by the PKC inhibitor calphostin C. The activation of PKC inhibits the efficacy of group III mGluRs, resulting in an increase in mEPSC frequency in response to a subsequent exposure to NA. By removing feedback inhibition, this mechanism effectively primes the synapses such that subsequent activation is more efficacious. The novel form of synaptic rescaling afforded by this cross-talk between distinct metabotropic receptors provides a means by which ascending catecholamine inputs can facilitate the control of homeostasis by hypothalamic networks.

3.2 Introduction

In the CNS, the release of neurotransmitter from the nerve terminal is regulated by a number of inhibitory feedback mechanisms (Zucker and Regehr, 2002). These mechanisms include, but are not limited to, the activation of high-affinity presynaptic receptors by neurotransmitters such as acetylcholine (MacDermott et al., 1999), adenosine (Oliet and Poulain, 1999;Dunwiddie and Masino, 2001), or GABA (Mouginot

et al., 1998). At the majority of excitatory synapses, however, this feedback is initiated by the binding of glutamate to presynaptic, G-protein-coupled autoreceptors (for review, see (Schoepp, 2001)). Activation of these metabotropic glutamate receptors (mGluRs) results in a decrease in the release of neurotransmitter (Baskys and Malenka, 1991;Schoppa and Westbrook, 1997), thereby providing a local, short-term mechanism through which synaptic strength (Anwyl, 1991;Scanziani et al., 1996;Oliet et al., 2001) and hyperexcitability (Sansig et al., 2001) may be regulated.

In certain physiological situations, however, neuronal output must be sustained for longer durations. Under these conditions, a process that limits the continuous release of glutamate may be unfavourable. In particular, adaptive responses mediated by the hypothalamus generally require the sustained activation of neuronal populations until homeostasis is achieved. In the SON and PVN, the MNCs cells exhibit prolonged discharges in response to specific physiological perturbations (Wakerley et al., 1978). This increase in neuronal activity may be achieved by changes in intrinsic conductances (Bourque and Renaud, 1984;Legendre and Poulain, 1992;Hatton and Li, 1998;Shibuya et al., 2000) or, alternatively, by a persistent increase in excitatory synaptic drive (Nissen et al., 1995;Moos et al., 1997;Jourdain et al., 1998;Shibuya et al., 2000) to these neurons. Because this glutamatergic input to magnocellular neuroendocrine cells is regulated by presynaptic mGluRs (Schrader and Tasker, 1997b;Panatier et al., 2004), this raises the possibility that targeting mGluR autoreceptor activity may be an attractive means for potentiating this input and ultimately augmenting the output of these neurons.

Recent evidence demonstrates that an increase in PKC activity functionally inhibits mGluRs (Macek et al., 1998;Nakajima et al., 1999). Thus, substrates that activate signalling pathways linked to PKC may exert long-lasting changes in synaptic efficacy by decreasing mGluR activity. In the hypothalamus, the physiological trigger for this override of mGluR feedback may be the activation of presynaptic, G-protein-coupled α_1 adrenoceptors by NA, which is released from ascending afferents in response to a number of physiological challenges (Crowley et al., 1987;Leibowitz et al., 1990). Because α_1 adrenoceptors are positively coupled to PKC, we hypothesize that the functional

inactivation of mGluRs by NA primes excitatory glutamatergic synapses terminating on MNCs.

Using whole-cell, voltage-clamp recordings from MNCs in the PVN of the hypothalamus, we demonstrate that α_1-adrenoceptor activation increases glutamate release and inactivates presynaptic mGluRs. This PKC-mediated inhibition of negative feedback is long lasting, as evidenced by an amplification of transmitter release in response to subsequent application of NA. The rescaling of synaptic input that results from cross-talk between distinct metabotropic receptors provides a means by which ascending catecholamine inputs can facilitate excitatory drive to MNCs.

3.3 Results

In contrast to neurons in most cortical regions in which the spontaneous, action potential-independent release of neurotransmitter is relatively infrequent (Staley, 1999), the MNCs of the hypothalamus are subjected to relatively high rates of quantal input. In the cells tested in the current study, the rate of stochastic release under basal conditions ranged from 0.46 to 8 Hz (mean, 2.74 ± 0.35 Hz). This high frequency of events combined with the demonstration that in MNCs, increases in mEPSC frequency lead to increases in firing (Kombian et al., 2000) suggests that quantal glutamatergic release conveys important signalling information to these neurons.

NA exerts predominantly excitatory effects on MNCs (Day et al., 1984;Armstrong et al., 1986;Randle et al., 1986b). These are partially attributable to an α_1-mediated depolarization of the postsynaptic membrane (Randle et al., 1986a) and an increase in TTX-sensitive glutamate release (Daftary et al., 1998). The effects of NA on quantal release at the presynaptic nerve terminal, however, have been less well defined. To clarify a role for NA at afferent excitatory terminals synapsing on MNCs, we examined the effects of this compound on TTX-insensitive mEPSCs.

3.3.1 NA increases the frequency and amplitude of mEPSCs

In response to a brief application of NA (2 min, 100 μM), all of the neurons tested exhibited an increase in mEPSC frequency (354.2 ± 34.9% of control; $n = 24$; $p < 0.01$)

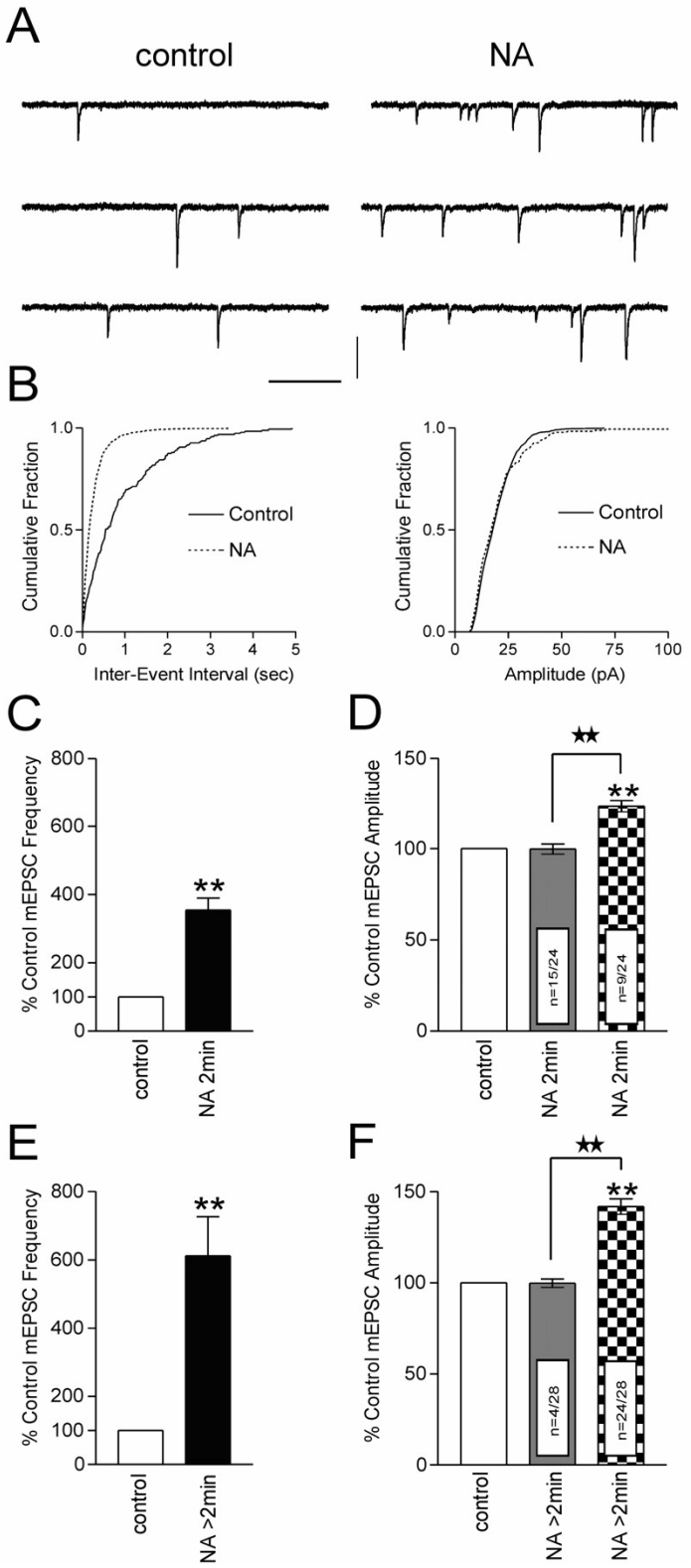
(figure 3.1 A-C). Although in the majority of cells tested (63%, n = 15 of 24) NA had no effect on mEPSC amplitude (99.8 ± 2.8% of control; $p > 0.05$) (figure 3.1 B, D), an increase in amplitude was observed in the remaining cells (123.3 ± 3.0% of control; $p < 0.01$) (figure 3.1 D). We also noted a mean inward current of 22.1 ± 2.2 pA in cells tested at 100 μM ($p < 0.05$; data not shown), consistent with a direct postsynaptic effect of NA on MNCs (Randle et al., 1986b;Daftary et al., 1998). We next tested the effects of longer NA applications on mEPSC frequency and amplitude. In response to NA applications that ranged from 4 to 10 min, the mEPSC frequency increased to 612.2 ± 114.2% of control ($p < 0.01$; n = 28) (figure 3.1 E). Interestingly, in these longer applications, the majority of cells (86%; n = 24 of 28) exhibited an increase in mEPSC amplitude (141.8 ± 4.2% of control; $p < 0.01$) (figure 3.1 F). The remaining cells (14%; n = 4 of 28) displayed no change in mEPSC amplitude (99.7 ± 2.3% of control; $p > 0.05$) (figure 3.1 F). This discrepancy in response profiles for short versus long applications of NA suggests a time-dependent component to the excitation induced by NA. The effects of NA were also dose dependent. Cells tested with brief applications of 1, 10, and 200 μM NA exhibited increases in mEPSC frequency of 104.6 ± 8.6% (n = 2), 188.4 ± 14.9% (n = 6), and 877.6 ± 37.8% (n = 2), respectively (data not shown). No inward current was observed at 1 μM NA, whereas 10 μM NA elicited significantly less inward current compared with 100 μM treatments (9.7 ± 2.4 pA; $p < 0.05$; n = 3; data not shown). Because of the robust increase in mEPSC frequency, we were unable to accurately assess changes in holding current for 200 μM NA.

3.3.2 NA primes glutamatergic synapses

Sustained increases in neuronal activity and hormonal output are reliably observed in magnocellular neuroendocrine cells in response to acute physiological stressors such as dehydration or haemorrhage. Although a single hemorrhagic stimulus elicits a sustained

Figure 3.1 NA increases frequency and amplitude of mEPSCs.

A, Representative voltage-clamp traces from a cell tested with a 2 min application of NA (100 μM). **B**, In the cumulative fraction plots, NA decreases the inter-event interval (left, $p < 0.01$) with no change in mEPSC amplitude (right, $p > 0.05$). **C–F**, Summary bar graphs of NA data. *C*, A 2 min application of NA increase smEPSC frequency to 354.2 ± 34.9% of control ($p < 0.01$; $n = 24$). **D**, In 63% of these cells ($n = 15$ of 24), there is no change in mEPSC amplitude (99.8 ± 2.8% of control1; $p > 0.05$). The remaining 37% of cells tested ($n = 9$ of 24) exhibit an increase in mEPSC amplitude (123.3 ± 3.0% of control; $p < 0.01$). **E**, When the NA application exceeds 2 min, the mEPSC frequency increases to 612.2 ± 114.2% of control ($p < 0.01$; $n = 28$). **F**, In these cells, 86% ($n = 24$ of 28) exhibited an increase in mEPSC amplitude (141.8 ± 4.2% of control; $p < 0.01$). No change in mEPSC amplitude (99.7 ± 2.3% of control; $p > 0.05$) was observed in the remaining 14% ($n = 4$ of 28). Calibration (in *A*): 15 pA, 250 msec. Stars indicate comparing treatment with treatment. Asterisks indicate comparing treatment with control.


A
control
NA
B
Cumulative Fraction
1.0
0.5
0.0
0 1 2 3 4 5
Inter-Event Interval (sec)
Control
NA
Cumulative Fraction
1.0
0.5
0.0
0 25 50 75 100
Amplitude (pA)
Control
NA
C
% Control mEPSC Frequency
800
600
400
200
100
0
**
control
NA 2min
D
% Control mEPSC Amplitude
150
100
50
0
**
**
n=15/24
n=9/24
control
NA 2min
NA 2min
E
% Control mEPSC Frequency
800
600
400
200
100
0
**
control
NA >2min
F
% Control mEPSC Amplitude
150
100
50
0
**
**
n=4/28
n=24/28
control
NA >2min
NA >2min

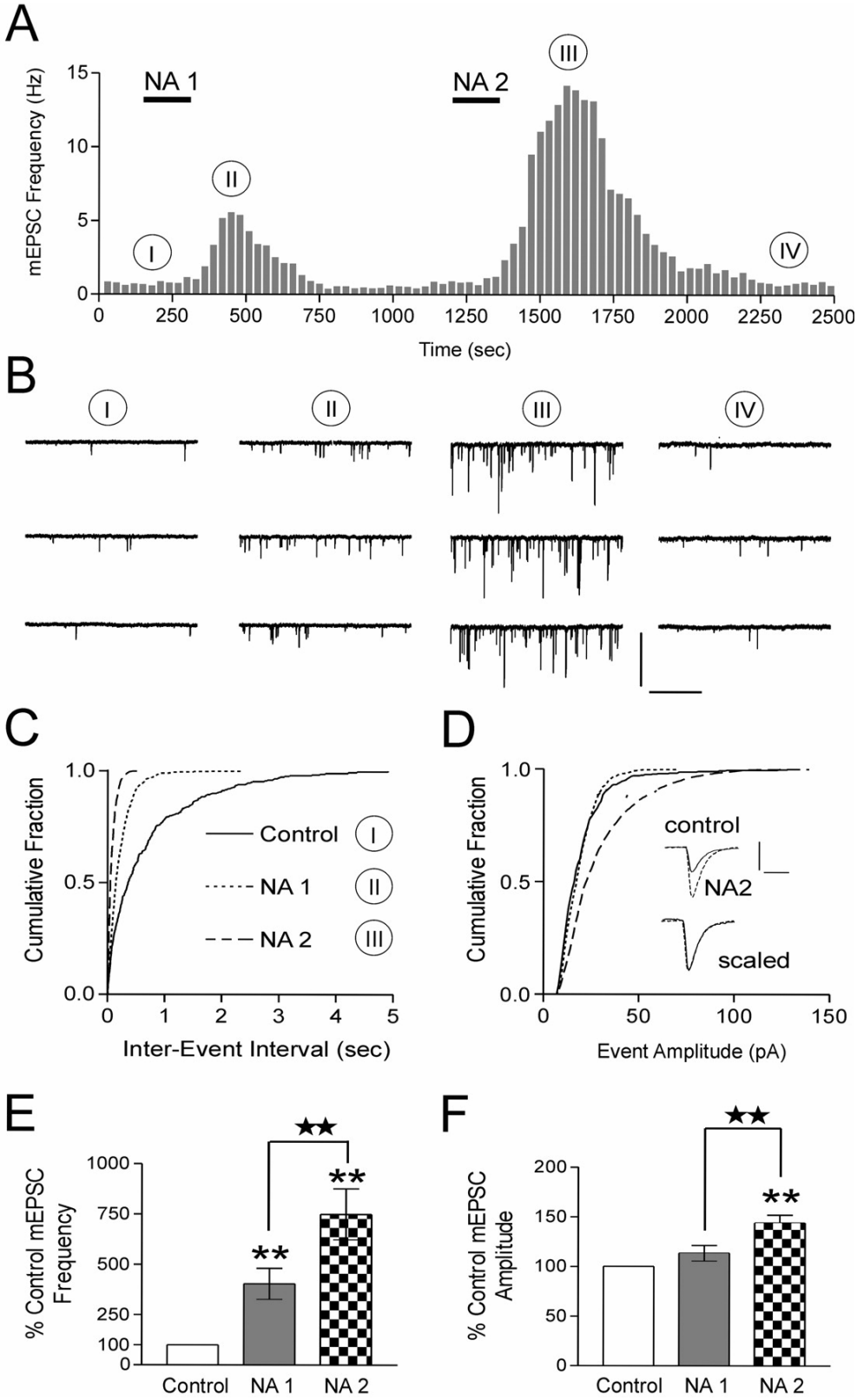
increase in hormone release from these cells, repeated haemorrhages result in an amplification of hormone release (Lilly et al., 1983;Lilly et al., 1986;Lilly et al., 1989). We examined whether repeated applications of NA would mimic these observations and provide an explanation at the synaptic level for the amplification of hormone release. We conducted experiments that used brief (2 min) repetitive applications of NA. The initial application of NA elicited an increase (figure 3.2 A-C) in the frequency of mEPSCs (403.8 ± 77.2% of control; $n = 6$; $p < 0.01$) (figure 3.2 E). After the recovery of mEPSC frequency to control levels, the synapses were rechallenged for 2 min with the same dose of NA. This application (15 min after the initial test) resulted in an additional amplification (figure 3.2 A-C) in the mEPSC frequency (749.8 ± 126.4% of control; $n = 6$; $p < 0.01$) (figure 3.2 E). We also observed an increase in mEPSC amplitude in response to the second application of NA only (figure 3.2 B, D). We did not observe any changes in the kinetics of the mEPSCs after application of NA (figure 3.2 D, inset scaled traces). The mEPSC amplitude increased from 113.4 ± 7.8% ($p > 0.05$) of control to 143.9 ± 7.8% ($p < 0.05$) (figure 3.2 F) of control for NA1 and NA2, respectively. Additional applications of NA failed to increase the frequency of mEPSCs further ($n = 3$; data not shown). Our analysis failed to reveal any consistent changes in amplitude for subsequent applications. These results indicate that NA primes excitatory synapses for subsequent exposure to the agonist.

3.3.3 Increase in mEPSC frequency is α_1 adrenoceptor mediated and PKC dependent

The excitatory effects of NA on MNCs involve the activation of the α_1 adrenoceptor (Armstrong et al., 1986;Yamashita et al., 1987;Shioda et al., 1997;Daftary et al., 1998;Boudaba et al., 2003a). These effects have been characterized as a direct α_1-mediated depolarization of the postsynaptic membrane and an α_1-mediated increase in TTX-sensitive glutamate release (Randle et al., 1986b;Daftary et al., 1998). On the basis of these findings, we tested whether activation of the α_1 adrenoceptor was necessary for the NA-induced increase in mEPSC frequency. The α_1 adrenoceptor antagonist prazosin (10 μM) completely abolished the excitatory effects of NA, uncovering an inhibitory

Figure 3.2 NA primes excitatory synapses.

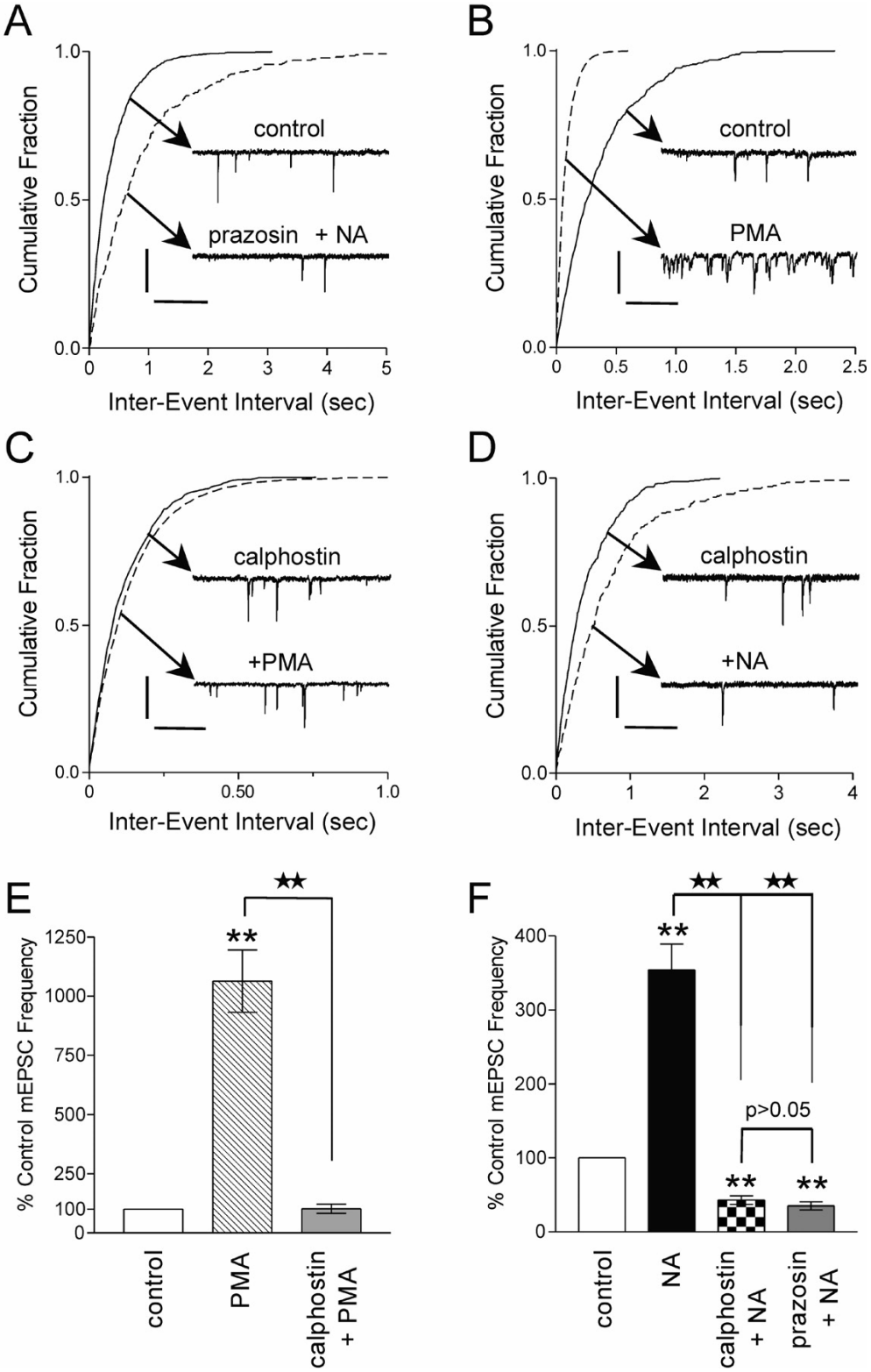
A, Two 2 min applications of NA (100 μM) separated by 15 min demonstrate an enhancement of the mEPSC frequency from NA1 to NA2. **B**, Representative voltage-clamp traces from the same neuron as *A*. The roman numerals in *B* correspond to the same time point in *A*. **C**, Cumulative fraction of mEPSC inter-event intervals before and during each NA application in this neuron. NA2 elicits an additional decrease in mEPSC inter-event interval ($p < 0.01$). **D**, Cumulative fraction of the amplitude of mEPSCs before and during each NA application. Here, only NA2 results in a significant increase in mEPSC amplitude ($p < 0.05$). Inset traces are averages of 40 spontaneous events in control and NA2. Control and NA2 traces are overlaid below to demonstrate that no change in the kinetics of the mEPSCs is observed. **E**, Summary bar graph showing the increase in mEPSC frequency after each successive NA application (NA1, 403.8 ± 77.2% of control; $p < 0.01$; NA2, 749.8 ± 126.4% of control; $p < 0.01$; $n = 6$). The mEPSC frequencies from NA1 and NA2 are significantly different ($p < 0.05$). **F**, Summary bar graph of increases in mEPSC amplitude (NA1, 113.4 ± 7.8% of control; $p > 0.05$; NA2, 143.9 ± 7.8% of control; $p < 0.01$, $n = 6$). The mEPSC amplitudes from NA1 and NA2 are significantly different ($p < 0.05$). Calibration: *B*, 50 pA, 1 sec; *D*, 20 pA, 5 msec. Stars indicate comparing treatment with treatment. Asterisks indicate comparing treatment with control.


A
mEPSC Frequency (Hz)
NA 1
NA 2
I
II
III
IV
Time (sec)
B
C
Cumulative Fraction
Control
NA 1
NA 2
Inter-Event Interval (sec)
D
control
NA2
scaled
Event Amplitude (pA)
E
% Control mEPSC Frequency
Control
NA 1
NA 2
F
% Control mEPSC Amplitude

action (mEPSC frequency decreased to 35.2 ± 5.2% of control values; $p < 0.01$; $n = 12$) (figure 3.3 A, F). These data are consistent with previous results demonstrating an α_1-mediated increase in spontaneous IPSCs in PVN neurons that masks a weaker inhibition of GABA release via α_2 adrenoceptor (Han et al., 2002). Our results suggest a similar role for NA at excitatory synapses. These findings demonstrate that NA acts predominantly via presynaptic α_1 adrenoceptors to increase glutamate release from afferent nerve terminals. This increase in mEPSC frequency likely acts in concert with the previously defined actions of this catecholamine in sustaining the elevated activity of these cells. The α_1 adrenoceptor activates phospholipase C through the Gq family of G-proteins, resulting in phosphatidylinositol metabolism, the release of stored Ca^{2+}, and activation of PKC (for review, see (Zhong and Minneman, 1999)). PKC activation mobilizes the reserve pool of vesicles (Gillis et al., 1996;Stevens and Sullivan, 1998), increases the sensitivity of the release process for Ca^{2+} (Brager et al., 2002), and facilitates the fusion of vesicles (Scepek et al., 1998;Yawo, 1999). These mechanisms may act individually or in concert to increase neurotransmitter release. Phorbol esters, such as PMA, activate PKC by binding to the C1 domain in an ATP-dependent manner (Liu and Heckman, 1998). The nonphysiological activation of PKC by lipid-soluble phorbol esters has been used widely for discerning the effects of the kinase in a number of neuronal and neuroendocrine preparations (Hilfiker and Augustine, 1999). To test the hypothesis that phorbol esters mimic the effects of NA, we examined mEPSC frequency in response to PMA (1 μM) (figure 3.3 B). mEPSC frequency dramatically increased (1063.5 ± 131.4% of control; $n = 5$; $p < 0.01$)(figure 3.3 E) in response to a 10 min PMA application. We observed no change in mEPSC amplitude (data not shown). To confirm that this increase in release was a result of the activation of PKC, the slice was incubated for 30 min with the broad-spectrum PKC inhibitor calphostin C (100 nM) (Hasuo et al., 2002), and the PMA application was repeated (figure 3.3 C). In the presence of calphostin C, PMA treatment had no effect on mEPSC frequency (102.1 ± 19.5% of control; $n = 5$; $p > 0.05$) (figure 3.3 E). These data suggest that PMA acts presynaptically to activate PKC and increase the release of glutamate.

Figure 3.3 Increase in mEPSC frequency is α1-adrenoceptor mediated and PKC dependent.

A, Cumulative fraction of mEPSC inter-event intervals before and during prazosin (10 μM) plus NA (100 μM) application. Inset are voltage-clamp traces depicting a decrease in the mEPSC frequency in response to NA when the α_1-adrenoceptor is blocked ($p < 0.01$). **B**, Cumulative fraction of mEPSC inter-event intervals before and during PMA (1 μM) application. Inset are voltage-clamp traces depicting an increase in the mEPSC frequency in response to PMA ($p < 0.01$). **C***,* Cumulative fraction of mEPSC inter-event intervals in response to PMA (1 μM) in the presence of calphostin C (100 nM). Inset are voltage-clamp traces depicting that no change in mEPSC frequency in response to PMA in the presence of calphostin C ($p > 0.05$) is observed. **D**, Cumulative fraction of mEPSC inter-event interval in response to NA (100 μM), in the presence of calphostin C (100 nM). Inset are voltage-clamp traces depicting a decrease in mEPSC frequency in response to NA in calphostin C ($p < 0.05$). **E**, Summary bar graph showing that PMA alone increases mEPSC frequency (1063.5 ± 131.4% of control; $p < 0.01$; $n = 5$) but fails to increase mEPSC frequency in the presence of calphostin C (102.1 ± 19.5% of control; $n = 5$; $p > 0.05$). **F**, Summary bar graph showing that NA decreases the frequency of mEPSCs in the presence of calphostin C (43.0 ± 5.7% of control; $p < 0.01$; $n = 5$) or prazosin (35.2 ± 5.2% of control; $p < 0.01$; $n = 12$). Calibration: *A,* 20 pA, 250 msec; *B,* 25 pA, 100 msec; *C,* 20 pA, 250 msec; *D,* 15 pA, 250 msec. Stars indicate comparing treatment with treatment. Asterisks indicate comparing treatment with control.


A
Cumulative Fraction
control
prazosin + NA
Inter-Event Interval (sec)
B
Cumulative Fraction
control
PMA
Inter-Event Interval (sec)
C
Cumulative Fraction
calphostin
+PMA
Inter-Event Interval (sec)
D
Cumulative Fraction
calphostin
+NA
Inter-Event Interval (sec)
E
% Control mEPSC Frequency
control
PMA
calphostin + PMA
F
% Control mEPSC Frequency
p>0.05
control
NA
calphostin + NA
prazosin + NA

Experiments were conducted to test whether PKC activation was necessary for the NA-mediated increase in glutamate release. Slices were preincubated for 30 min with calphostin C (100 nM), and NA was applied at a dose (100 μM) that normally elicits a robust increase in mEPSC frequency (figure 3.3 D). Calphostin C completely abolished the NA-induced increase in mEPSC frequency and unmasked an inhibitory effect (mEPSC frequency decreased to 43.0 ± 5.7% of control; $p < 0.01$; $n = 5$)(figure 3.3 F).

3.3.4 Phorbol ester and NA-induced PKC activation inhibits mGluR activity

Membrane-binding assays have established a robust distribution of mGluRs in the hypothalamus (Meeker et al., 1994). In particular, electrophysiological evidence demonstrates an important role for group III mGluRs in regulating glutamate release at magnocellular synapses (Schrader and Tasker, 1997b;Oliet et al., 2001). We confirmed this finding with the demonstration that bath application of the group III mGluR agonist L-AP4 (25 μM) decreased the frequency of mEPSCs in MNCs (36.9 ± 8.5%; $n = 3$; $p < 0.05$) (figure 3.4 A, C). No apparent postsynaptic effects of the agonist were observed (data not shown). mGluRs themselves are also subject to regulation by intracellular messengers. Specifically, some studies propose the inhibition of group III mGluRs by PKC (Macek et al., 1998;Sorensen et al., 2002). To test the hypothesis that mGluR function would be compromised after the activation of PKC, we applied PMA (1 μM) and then L-AP4 (25 μM) 15 min later. As demonstrated above, PMA application elicited a robust increase in mEPSC frequency. In the presence of PMA, the effect of L-AP4 on mEPSC frequency was blunted (87.5 ± 5.8% of control; $n = 5$; $p < 0.05$)(figure 3.4 B, C). This response was significantly different from L-AP4 alone ($p < 0.05$). These data suggest that PKC activation attenuates the decrease in glutamate release normally observed in response to activation of group III mGluRs on the presynaptic terminal.

We next examined whether activation of PKC by NA would also attenuate mGluR function. After a return to baseline of the NA-induced increase in mEPSC frequency, L-AP4 was applied at a dose that had previously decreased mEPSC frequency (25 μM) (figure 3.5 A). Under these conditions, the effect of L-AP4 on mEPSC frequency was significantly attenuated (L-AP4 after NA; 82.1 ± 3.7 % of control; $p < 0.01$; $n = 5$)

Figure 3.4 PKC activation attenuates mGluR-mediated inhibition.

A, Cumulative fraction of mEPSC inter-event interval before and during L-AP4 (25 μM) application. Inset are voltage-clamp traces depicting a decrease in the mEPSC frequency in response to L-AP4 ($p < 0.01$). **B**, Cumulative fraction of mEPSC inter-event interval in response to L-AP4 (25 μM) in the presence of PMA (1 μM). Inset are voltage-clamp traces depicting a small decrease in mEPSC frequency in response to L-AP4 in PMA ($p > 0.05$). **C**, Summary bar graph demonstrates that L-AP4 decreases mEPSC frequency (36.9 ± 8.5% of control; $p < 0.05$; $n = 3$). In the presence of PMA, L-AP4 exhibits a small decrease in mEPSC frequency (87.5 ± 5.8% of control; $p < 0.05$; $n = 5$). The decrease in mEPSC frequency in L-AP4 is significantly different from that observed in PMA plus L-AP4 ($p < 0.05$). Calibration: *A,* 20 pA, 250 msec; *B,* 25 pA, 100 msec. Stars indicate comparing treatment with treatment. Asterisks indicate comparing treatment with control.

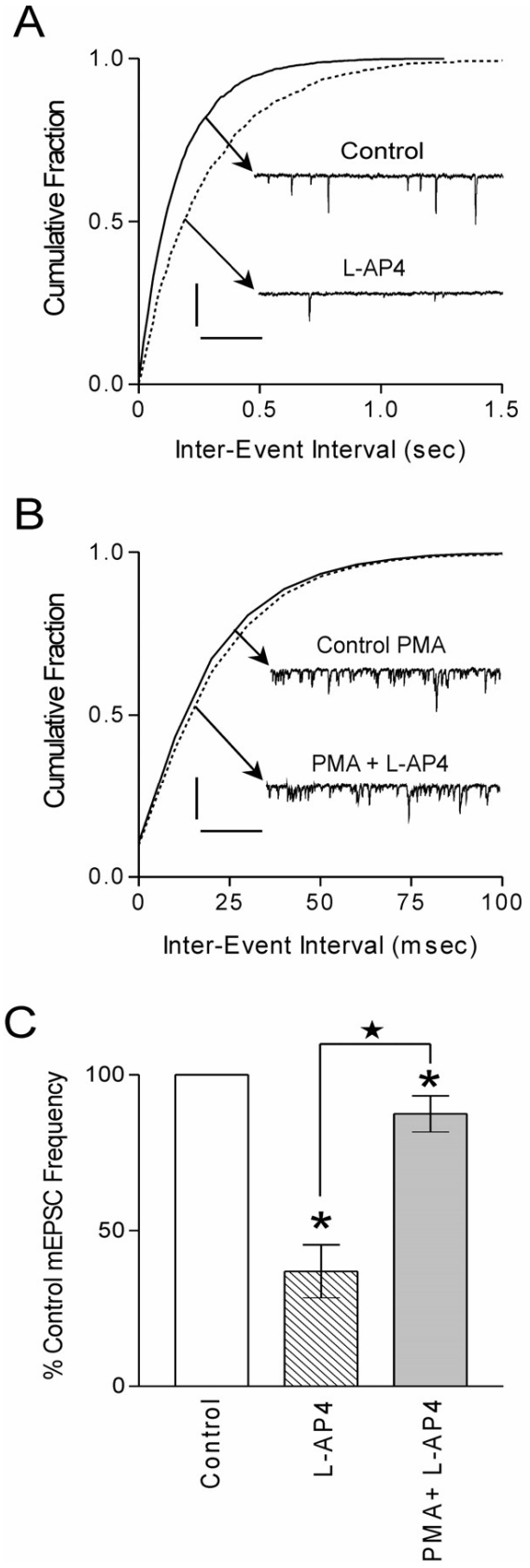
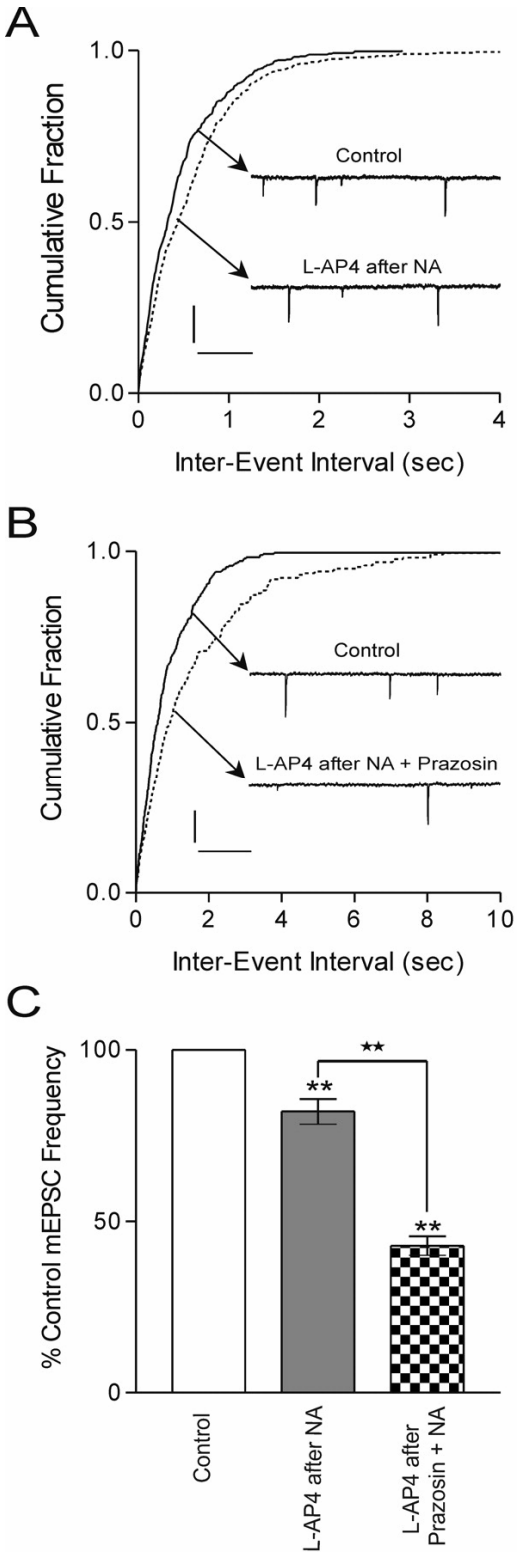

A
1.0
0.5
0.0
Cumulative Fraction
Control
L-AP4
0
0.5
1.0
1.5
Inter-Event Interval (sec)
B
1.0
0.5
0.0
Cumulative Fraction
Control PMA
PMA + L-AP4
0
25
50
75
100
Inter-Event Interval (msec)
C
100
50
0
% Control mEPSC Frequency
*
*
★
Control
L-AP4
PMA+ L-AP4

Figure 3.5 α_1-adrenoceptor activation attenuates mGluR-mediated inhibition.

A, Cumulative fraction of mEPSC inter-event interval before and during L-AP4 (25 μM) after the application of NA (100 μM). Inset are voltage-clamp traces depicting a small decrease in mEPSC frequency in response to L-AP4 after NA ($p < 0.05$). **B**, Cumulative fraction of mEPSC inter-event interval before and during L-AP4 (25 μM) after application of prazosin (10 μM) plus NA (100 μM). Inset are voltage-clamp traces depicting a clear decrease in mEPSC frequency in response to L-AP4 after NA when the α_1 adrenoceptor is blocked ($p < 0.01$). **C**, Summary bar graph showing a decrease in the frequency of mEPSCs with L-AP4 after NA (82.1 ± 3.7% of control; $p < 0.01$; $n = 5$) and with L-AP4 after prazosin plus NA (42.9 ± 2.8% of control; $p < 0.01$; $n = 7$). The decrease in mEPSC frequency after L-AP4 after NA is significantly different from that observed in L-AP4 after prazosin plus NA ($p < 0.01$). Calibration: *A,* 20 pA, 250 msec; *B,* 25 pA, 200 msec. Stars indicate comparing treatment with treatment. Asterisks indicate comparing treatment with control.


A
1.0
0.5
0.0
Cumulative Fraction
Control
L-AP4 after NA
0
1
2
3
4
Inter-Event Interval (sec)
B
1.0
0.5
0.0
Cumulative Fraction
Control
L-AP4 after NA + Prazosin
0
2
4
6
8
10
Inter-Event Interval (sec)
C
100
50
0
% Control mEPSC Frequency
**
**
**
Control
L-AP4 after NA
L-AP4 after Prazosin + NA

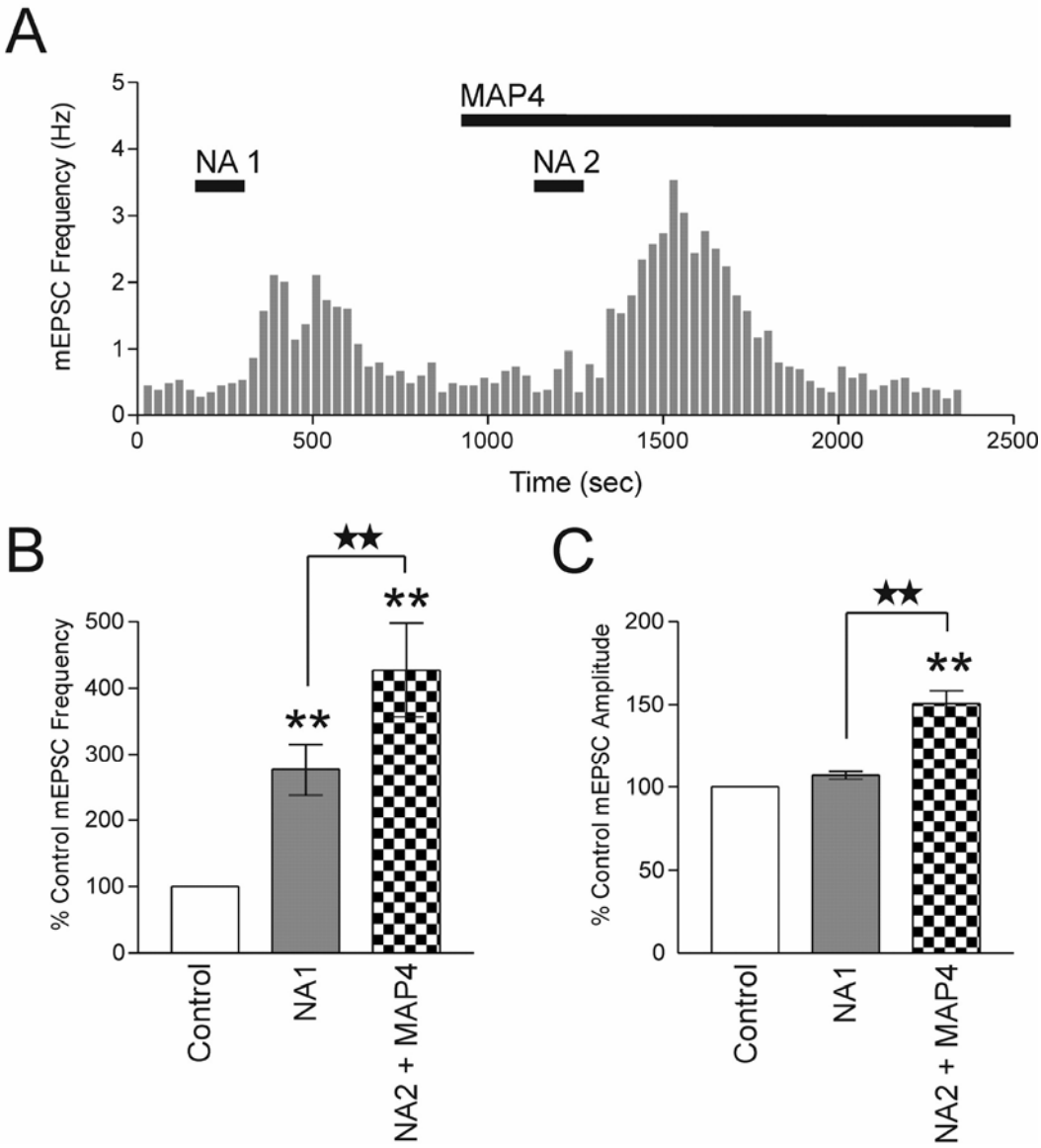
(figure 3.5 C) compared with L-AP4 alone ($p < 0.01$) (Fig 3.4 A, C, comparison not shown). Finally, we tested whether activation of the α_1 adrenoceptor by NA was necessary for the inhibition of group III mGluRs. After the application of NA in the presence of prazosin (to block α_1 adrenoceptors), L-AP4 clearly decreased the frequency of mEPSCs (42.9 ± 2.8% of control; $p < 0.01$; $n = 7$)(figure 3.5 B, C). This was significantly different from the effects of L-AP4 after NA alone ($p < 0.01$) but was not different from L-AP4 alone ($p > 0.05$) (figure 3.4 C, comparison not shown).

3.3.5 Blockade of group III mGluRs after NA1 does not affect priming

We next tested whether pharmacological inhibition of group III mGluRs during the second NA application would alter the priming response. If synaptic priming occurs because of different activation states of the mGluR (where less glutamate release during NA1 is attributed to a functional mGluR and more glutamate release during NA2 is attributed to mGluR inactivation), then inhibiting the mGluR during NA2 should have little effect on priming. The priming experiment was repeated, but instead, the group III mGluR antagonist MAP4 (250 μM) was bath applied after NA1 (figure 3.6 A). The initial application of NA increased the frequency of mEPSCs to 276.3 ± 38.9% of control ($p < 0.01$; $n = 7$)(figure 3.6 A, B). After the recovery of mEPSC frequency to control levels, the synapses were rechallenged with NA in the presence of MAP4. This resulted in an additional amplification in the mEPSC frequency to 427.0 ± 70.5% of control ($p < 0.01$; $n = 7$)(figure 3.6 A, B). The responses elicited by NA1 and NA2 are significantly different ($p < 0.01$)(figure 3.6 B). Consistent with the results from earlier experiments, we observed an increase in mEPSC amplitude in response to the second application of NA (figure 3.6 C). The mEPSC amplitude increased from 107.0 ± 2.4% ($p > 0.05$) of control to 150.6 ± 7.9% ($p < 0.01$; $n = 5$)(figure 3.6 C) of control for NA1 and NA2, respectively. These data collectively suggest that mGluRs are inactivated after NA application. Consequently, they are unavailable to curtail the release of glutamate when the synapses are rechallenged with NA.

Figure 3.6 Blockade of group III mGluRs after NA1 does not affect priming.

A, The priming effect of NA is still observed when NA2 is applied in the presence of MAP4 (250 μM). NA2 (2 min, 100 μM) shows a greater increase in mEPSC frequency than NA1 (2 min, 100 μM). **B,** Summary bar graph of increases in mEPSC frequency (NA1, 276.3 ± 38.9% of control; $p < 0.01$; NA2, 427.0 ± 70.5% of control; $p < 0.01$; $n = 7$). The mEPSC frequencies from NA1 and NA2 are significantly different ($p < 0.01$). **C**, Summary bar graph showing effects of NA on mEPSC amplitude (NA1, 107.0 ± 2.4% of control; $p > 0.05$; NA2, 150.6 ± 7.9%; $p < 0.01$; $n = 5$). The mEPSC amplitudes from NA1 and NA2 are significantly different ($p < 0.01$). Stars indicate comparing treatment with treatment. Asterisks indicate comparing treatment with control.


A
MAP4
NA 1
NA 2
mEPSC Frequency (Hz)
Time (sec)
B
% Control mEPSC Frequency
Control
NA1
NA2 + MAP4
C
% Control mEPSC Amplitude
Control
NA1
NA2 + MAP4

3.3.6 Group III mGluRs mediate priming

Finally, we tested the inhibitory contribution of group III mGluRs during the initial response to NA. If synaptic priming is caused by differences in mGluR activity during NA1 and NA2, then inhibiting the mGluR for the duration of NA1 and NA2 application should potentiate release under both conditions, effectively eliminating the observation of "priming." In the presence of MAP4, NA elicited a robust increase in mEPSC frequency (353.5 ± 61.1%; $n = 4$; $p < 0.01$)(figure 3.7 A, B) but had no effect on mEPSC amplitude (95.4 ± 4.4%; $n = 4$; $p > 0.05$)(figure 3.7 C). We failed to see any more potentiation when NA was applied a second time in the presence of MAP4. The effect on mEPSC frequency (376.5 ± 57%; $p < 0.01$)(figure 3.7 A, B) and amplitude (93.5 ± 4.5%; $p > 0.05$)(figure 3.7 C) by NA2 was not different from the response to the first application ($p > 0.05$) (figure 3.7 B, C). The absence of any change in amplitude may be because of the continuous application of MAP4 for the duration of the experiment. The demonstration that inhibition of presynaptic mGluRs increases the amplitude of spontaneous EPSCs (Bandrowski et al., 2003) is consistent with this idea. We tested this possibility in a separate set of experiments by applying MAP4 in the presence of TTX. Under these conditions, MAP4 significantly increased the amplitude of mEPSCs (121.7 ± 1.9% of control; $p < 0.01$; $n = 6$; data not shown). In light of this evidence, it is plausible that the priming of both mEPSC frequency and amplitude results from the functional inactivation of presynaptic autoreceptors. Together, these data suggest that activation of α_1 adrenoceptors by NA curtails the efficacy of group III mGluRs via a PKC-dependent mechanism to prime the glutamatergic synapse for subsequent exposure of the agonist (figure 3.8).

Figure 3.7 Blockade of group III mGluRs abolishes NA-induced priming.

A, In the presence of MAP4 (250 μM), two 2 min applications of NA (100 μM) separated by 15 min exhibit similar increases in mEPSC frequency. **B**, Summary bar graph of increases in mEPSC frequency (NA1, 353.5 ± 61.1% of control; $p < 0.01$; NA2, 376.5 ± 57.0% of control; $p < 0.01$; $n = 4$). The mEPSC frequencies from NA1 and NA2 are not significantly different ($p > 0.05$). **C**, Summary bar graph showing effects of NA on mEPSC amplitude (NA1, 95.4 ± 4.4% of control; $p > 0.05$; NA2, 93.4 ± 4.5%; $p > 0.05$; $n = 4$). The mEPSC amplitudes from NA1 and NA2 are not significantly different ($p > 0.05$). Asterisks indicate comparing treatment with control.

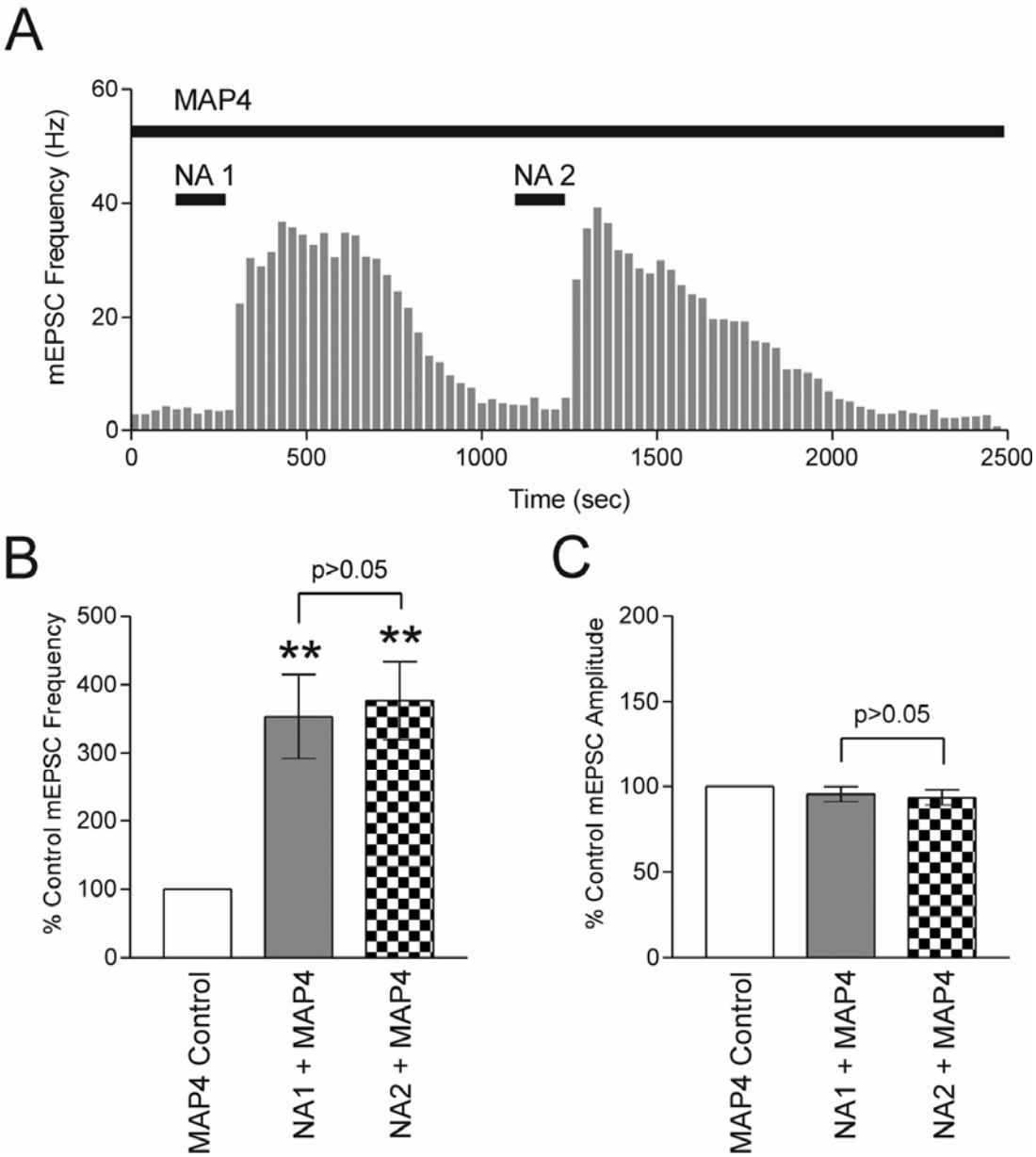
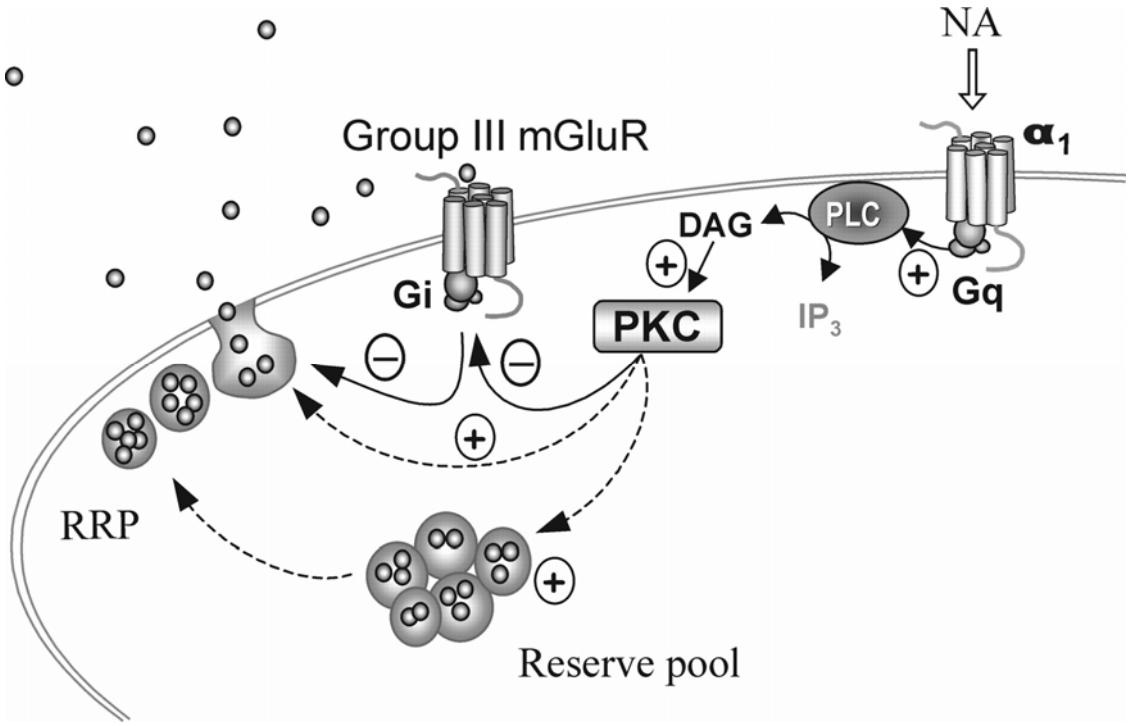

A
MAP4
NA 1
NA 2
mEPSC Frequency (Hz)
60
40
20
0
0
500
1000
1500
2000
2500
Time (sec)
B
p>0.05
**
**
% Control mEPSC Frequency
500
400
300
200
100
0
MAP4 Control
NA1 + MAP4
NA2 + MAP4
C
% Control mEPSC Amplitude
200
150
100
50
0
p>0.05
MAP4 Control
NA1 + MAP4
NA2 + MAP4

Figure 3.8 Cross-talk between metabotropic receptor signalling pathways.
Schematic illustrates the putative interaction between the presynaptic α_1 adrenoceptor and group III mGluRs. α_1 Adrenoceptor; PLC, phospholipase C; IP_3, inositol triphosphate; DAG, diacylglycerol; PKC, protein kinase C; RRP, readily releasable pool; NA, noradrenaline.


NA
Group III mGluR
α1
DAG
PLC
Gi
PKC
IP3
Gq
RRP
Reserve pool

3.4 Discussion

Our findings demonstrate that the activation of PKC via α_1 adrenoceptors not only increases the frequency of mEPSCs but also functionally attenuates presynaptic group III mGluRs. Moreover, by removing mGluR-mediated feedback inhibition, α_1 adrenoceptor activation effectively primes excitatory synapses that terminate on the magnocellular neuroendocrine cells of the PVN. Physiologically, this interaction between two distinct classes of presynaptic metabotropic receptors (α_1 adrenoceptor and group III mGluR) may provide a means by which hormone output can be sustained or potentiated to meet homeostatic demands.

3.4.1 PKC inhibition of mGluR activity

In addition to the short-term increases in neurotransmitter release, increasing evidence suggests that protein kinases may also serve as molecular substrates that regulate the initiation and maintenance of longer-term changes in synaptic efficacy. Our findings demonstrate that PKC serves as a molecular link between α_1 adrenoceptors and group III mGluRs, leading to a long-lasting inhibition of mGluRs. Because these receptors play a pivotal role in gating excitatory input to MNCs (Schrader and Tasker, 1997b;Oliet et al., 2001), their functional inactivation by PKC results in an amplification of glutamate release when the synapses are rechallenged by a second application of NA. Our findings are supported by the demonstration that PKC interacts with the group II and group III mGluRs to inhibit their function (Macek et al., 1998;Sorensen et al., 2002). The inhibition by PKC of group III mGluRs may result from an uncoupling of the receptor from its G-protein cascade (Macek et al., 1998) or by a direct phosphorylation of Ser^{862} on the intracellular C terminus by PKC (Sorensen et al., 2002). The demonstration that the endogenous ligand NA can inhibit group III mGluR function is consistent with previous findings from the hippocampus that adenosine analogs, which also increase PKC activity (Macek et al., 1998), inhibit mGluR function. Importantly, we now demonstrate that the inhibition of mGluRs conferred by PKC is long lasting, extending well beyond the time

frame of α_1 adrenoceptor activation. Exposing synapses to NA once increases the capacity of the terminals to release glutamate in response to subsequent exposure to the agonist. Essentially, the synapses have been primed.

3.4.2 Mechanism of mGluR inhibition of mEPSC release

Group III mGluRs can decrease neurotransmitter release by inhibiting presynaptic voltage-dependent Ca^{2+} channels (Perroy et al., 2001). Because the TTX-insensitive release of glutamate in MNCs does not depend on extracellular Ca^{2+} (Inenaga et al., 1998), an interaction between mGluRs and presynaptic Ca^{2+} channels is unlikely to be responsible for the effects observed here. Our findings, when coupled with the demonstration that glutamate release at mitral bulb synapses is also regulated by group III mGluRs in a manner that is downstream of Ca^{2+} influx (Schoppa and Westbrook, 1997), provide evidence for an alternative mechanism through which mGluRs may inhibit transmitter release. The demonstration that a subset of group III mGluRs is located at the site of vesicle fusion (Shigemoto et al., 1996) suggests that the effect we described may be attributable to a direct protein–protein interaction between the mGluR complex and a part of the vesicle release machinery (Scanziani et al., 1996).

The role of mEPSCs in conveying information at CNS synapses has been hotly debated (for review, see (Staley, 1999). Findings from a number of central nuclei suggest that these stochastic, action potential-independent events may not be mere noise. Increasing the frequency of mEPSCs has functional consequences for action potential generation in magnocellular neuroendocrine cells (Kombian et al., 2000) and cerebellar interneurons (Carter and Regehr, 2002). Additionally, a decrease in miniature GABAergic IPSCs has been linked in the hippocampus to the development of epilepsy (Hirsch et al., 1999). This increase in the background "noise" resulting from the activation of α_1 adrenoceptors may in fact lead to an increase in the general excitability of these cells.

3.4.3 Physiological significance

Adaptive responses mediated by the hypothalamus generally require sustained activation until homeostasis is achieved. In an attempt to restore fluid homeostasis in response to acute challenges such as dehydration or hemorrhage, the magnocellular neuroendocrine cells exhibit a prolonged increase in firing rate (Wakerley et al., 1978). This increase in activity, in response to a single acute stimulus, may outlast the physiological perturbation by >24 hr in some cases (Wakerley et al., 1978). Furthermore, the second stimulus in a repeated hemorrhagic protocol elicits an increase in vasopressin release compared with the first stimulus (DeMaria et al., 1987;Lilly et al., 1989). Our findings that NA primes the excitatory synaptic input to magnocellular neuroendocrine cells offer a potential explanation for this observation. The synaptic mechanisms described here would act in concert with previously described changes in activity of these cells in response to changes in plasma osmolarity (Oliet and Bourque, 1993) to increase spiking activity.

Because mGluRs are vital in regulating the synaptic excitability of these cells (Schrader and Tasker, 1997b;Panatier et al., 2004), it stands to reason that activating intracellular pathways that inhibit the activity of these receptors would provide an ideal solution for sustaining or potentiating glutamatergic input. That this switch may be initiated by NA, which is released in the hypothalamus during the response to physiological stressors, only serves to highlight the exceedingly clever design of neuronal circuitry that restores homeostasis.

Chapter Four: Noradrenaline triggers release of glial ATP to increase postsynaptic efficacy

4.1 Summary

Glial cells actively participate in synaptic transmission. They clear molecules from the synaptic cleft, receive signals from neurons and, in turn, release molecules that can modulate signalling between neuronal elements. Whether glial-derived transmitters can contribute to enduring changes in postsynaptic efficacy, however, remains to be established. Analysis of mEPSCs in brain slices of the rat hypothalamic PVN reveals an enduring increase in the amplitude of these events in response to NA that requires the release of ATP from glial cells. The increase in quantal efficacy, likely resulting from an insertion of AMPARs, is secondary to the activation of $P2X_7$ receptors, an increase in postsynaptic Ca^{2+} and the activation of phosphoinositide 3-kinase (PI3-K). The gliotransmitter ATP, therefore, contributes directly to the regulation of postsynaptic efficacy at glutamatergic synapses in the central nervous system.

4.2 Introduction

Astrocytes (Parpura et al., 1994;Kang et al., 1998;Beattie et al., 2002;Zhang et al., 2003) and Schwann cells (Robitaille, 1998) can respond to and influence neuronal signals by releasing glial-derived substances (gliotransmitters). It is somewhat surprising then that, until very recently (Zhang et al., 2003), the most ubiquitous gliotransmitter, ATP, had been categorized almost exclusively as a paracrine messenger responsible for inter-glial propagation of Ca^{2+} waves (Guthrie et al., 1999;Wang et al., 2000;Cotrina et al., 2000). There is now growing support for the idea that activation of neuronal purinergic receptors can affect synaptic plasticity (Khakh, 2001;Armstrong et al., 2002;Shigetomi and Kato, 2004). To date, the majority of the characterized actions of ATP on excitatory synaptic transmission however, have focused on short-term, presynaptic changes in neurotransmitter release, with little evidence for enduring, postsynaptic changes. Given that ATP-gated P2X channels are Ca^{2+} permeable (Shibuya et al., 1999;North, 2002) and that the $P2X_7$ receptor is linked directly to PI3-K (Jacques-Silva et al., 2004), an

intracellular signalling molecule that is crucial for the insertion of AMPARs (Man et al., 2003) and the expression of LTP in the hippocampus (Sanna et al., 2002;Raymond et al., 2002) and amygdala (Lin et al., 2001), it seems plausible to hypothesize that ATP can induce enduring changes in postsynaptic efficacy.

To study the putative contributions of glial-derived ATP to postsynaptic changes in synaptic strength, we examined glutamatergic synapses onto MNCs in the PVN. The glial cells that surround the MNCs and their synaptic contacts exhibit a remarkable degree of anatomical plasticity. In response to physiological challenges, they undergo a remodelling process that results in a decrease in the astrocytic coverage of synaptic contacts (Tweedle and Hatton, 1977;Theodosis et al., 1981;Hatton and Tweedle, 1982;Theodosis and Poulain, 1984). By taking advantage of this propensity of glial cells to retract when the animal is physiologically challenged, we can study synaptic function during conditions in which there is either a relative paucity or relative abundance of glial cells surrounding the MNCs. This allows us to address the question of glia directly and, by extension, gliotransmitters in the regulation of synaptic function. Important for the study of ATP and long-term synaptic plasticity in this system is the demonstration that the MNCs possess several subtypes of P2X receptors that are permeable to Ca^{2+} (Shibuya et al., 1999), the critical trigger for enduring changes in postsynaptic efficacy (Malinow and Malenka, 2002). Furthermore, ATP receptors are crucial for the increases in activity observed in MNCs in response to physiological perturbations that selectively recruit noradrenergic fibres (Day et al., 1993) and for facilitating hormone release in response to NA (Kapoor and Sladek, 2000). Since a large fraction of catecholaminergic (and more specifically noradrenergic) terminals in the brain lack postsynaptic contacts (Sawyer and Clifton, 1980), this raises the intriguing possibility that signalling in this vital homeostatic circuit may require dynamic glial-neuronal interactions.

Here, we demonstrate that NA increases the amplitude of mEPSCs in MNCs through the release of ATP from glial cells. ATP acts at postsynaptic $P2X_7$ receptors to promote the insertion of AMPARs through a mechanism requiring the Ca^{2+}-dependent activation of PI3-K. These data uncover a new mechanism through which glial cells can impact synaptic strength at excitatory synapses.

4.3 Results

We examined changes in mEPSC amplitude as a measure of synaptic strength because of the compelling evidence that mEPSCs are important for information transfer in a number of vertebrate preparations (Carter and Regehr, 2002;Sharma and Vijayaraghavan, 2003;Zucker, 2003). Changes in mEPSC amplitude directly translate into changes in the firing patterns of CNS neurons, and, in particular, the MNCs (Kombian et al., 2000).

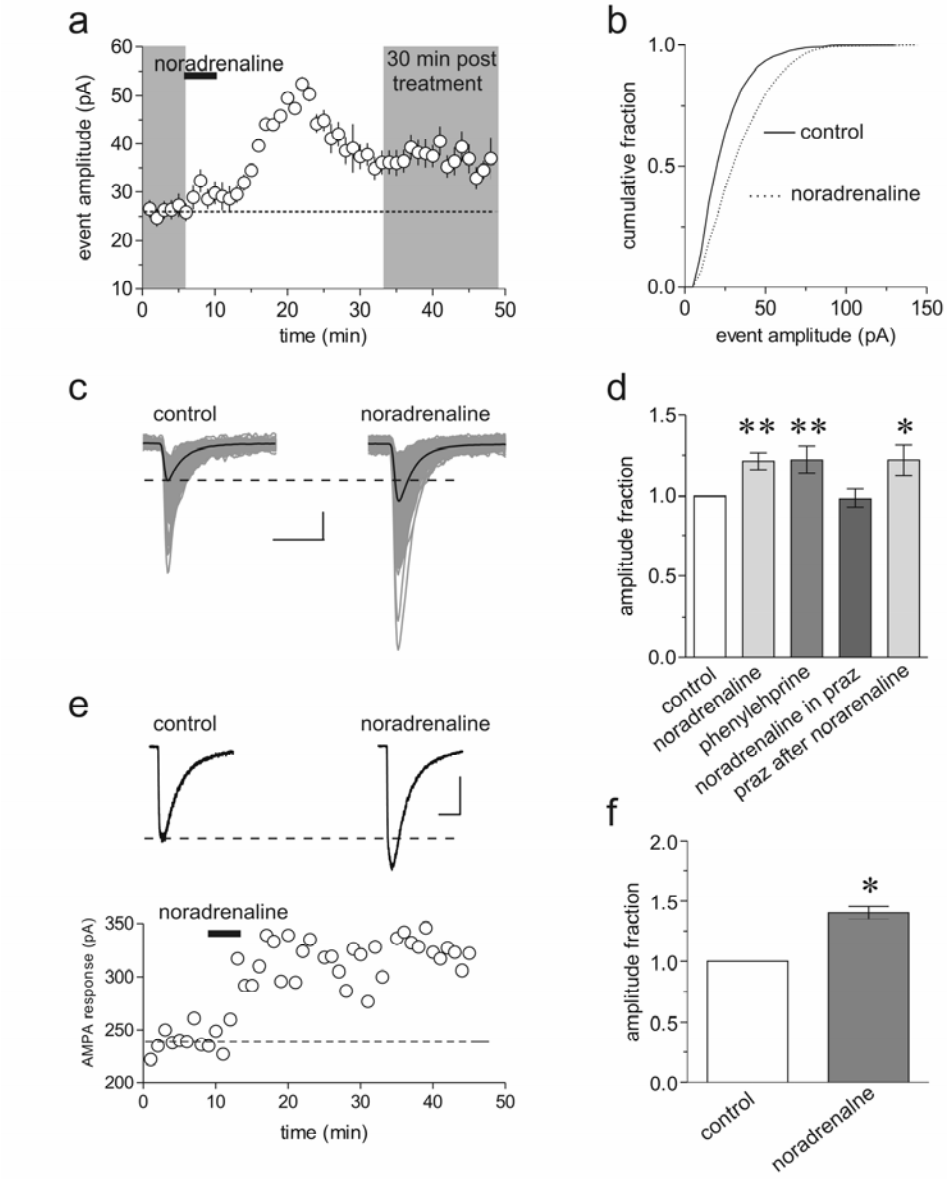
4.3.1 NA permanently increases mEPSC amplitude

Bath application of NA (5 min, 200 μM) elicited three sequential changes in mEPSC amplitude. In temporal order, these include a small enhancement followed by a robust increase lasting approximately 15 min, which in turn relaxes to a new mean and remains elevated even during our longest whole cell recordings (exceeding 90 min in length). This manuscript focuses on the enduring increase in mEPSC amplitude and not the initial increases during agonist application. In each cell, mEPSC amplitudes were compared between control and 30 min post treatment, as indicated by the grey demarcations in figure 4.1 A. A minimum 5 min recording segment was utilized for the analysis. The enduring increase in mEPSC amplitude was 1.22 ± 0.05, P <0.01, n = 20, figure 4.1 A-D. All values are expressed as a fraction of control. For the remainder of the manuscript, we often use the terms 'increase in synaptic strength' or 'synaptic potentiation' interchangeably with 'enduring increase in mEPSC amplitude'.

The α_1 adrenoceptor mediates the excitatory effects of NA on MNCs (Armstrong et al., 1986;Randle et al., 1986a;Daftary et al., 1998;Gordon and Bains, 2003). Here, the α_1-adrenoceptor agonist, phenylephrine (5 min, 200 μM), effectively mimicked (1.23 ± 0.08, P < 0.01, n = 5, figure 4.1 d), while the α_1-adrenoceptor antagonist, prazosin (10 μM), attenuated (0.99 ± 0.05, P > 0.05, n = 6, figure 4.1 d) the effects of NA. To rule out the possibility that the enduring increase in synaptic strength resulted from a slow washout of the agonist, prazosin was added to the bath immediately after NA application

Figure 4.1 NA induces an enduring increase in mEPSC amplitude that is accompanied by an increase in postsynaptic efficacy.

(**a**) Running average of mEPSC amplitudes in 1-min bins. NA was bath applied for 5 min (200 μM). Amplitude values were assessed at the time points indicated by gray bands. (**b**) Cumulative fraction plot of mEPSC amplitudes from the control condition and 30 min after NA treatment ($P < 0.01$). (**c**) Average (black) and raw (gray) mEPSC traces from the same time points as described in a and b. (**d**) Summary showing the enduring increase in mEPSC amplitude observed after application of NA (1.22 ± 0.05, $P < 0.01$, $n = 20$), phenylephrine (1.23 ± 0.08, $P < 0.01$, $n = 5$), NA in prazosin (0.99 ± 0.05, $P > 0.05$, $n = 6$) and prazosin after NA (1.22 ± 0.09, $P < 0.05$, $n = 4$). (**e**) Postsynaptic responses to focal AMPA puff; average traces from control condition and 30 min post-NA shown above graph. (**f**) Summary showing an increase in postsynaptic responsiveness to AMPA puff 30 min post-NA (1.41 ± 0.09, $P < 0.05$, $n = 5$). Scale bars, a: 20 pA, 5 ms; e: 100 pA, 250 ms. Error bars: s.e.m. *, $P < 0.05$; **, $P < 0.01$, here and in all figures.


a
noradrenaline
30 min post treatment
event amplitude (pA)
time (min)
b
cumulative fraction
control
noradrenaline
event amplitude (pA)
c
control
noradrenaline
d
amplitude fraction
control
noradrenaline
phenylehprine
noradrenaline in praz
praz after norarenaline
e
control
noradrenaline
AMPA response (pA)
time (min)
f
amplitude fraction
control
noradrenalne

was terminated. This failed to attenuate the enduring increase in synaptic strength (1.22 ± 0.09, $P < 0.05$, $n = 4$, figure 4.1 d). These data collectively suggest that transient activation of α_1-adrenoceptors triggers a robust and enduring enhancement of mEPSC amplitude.

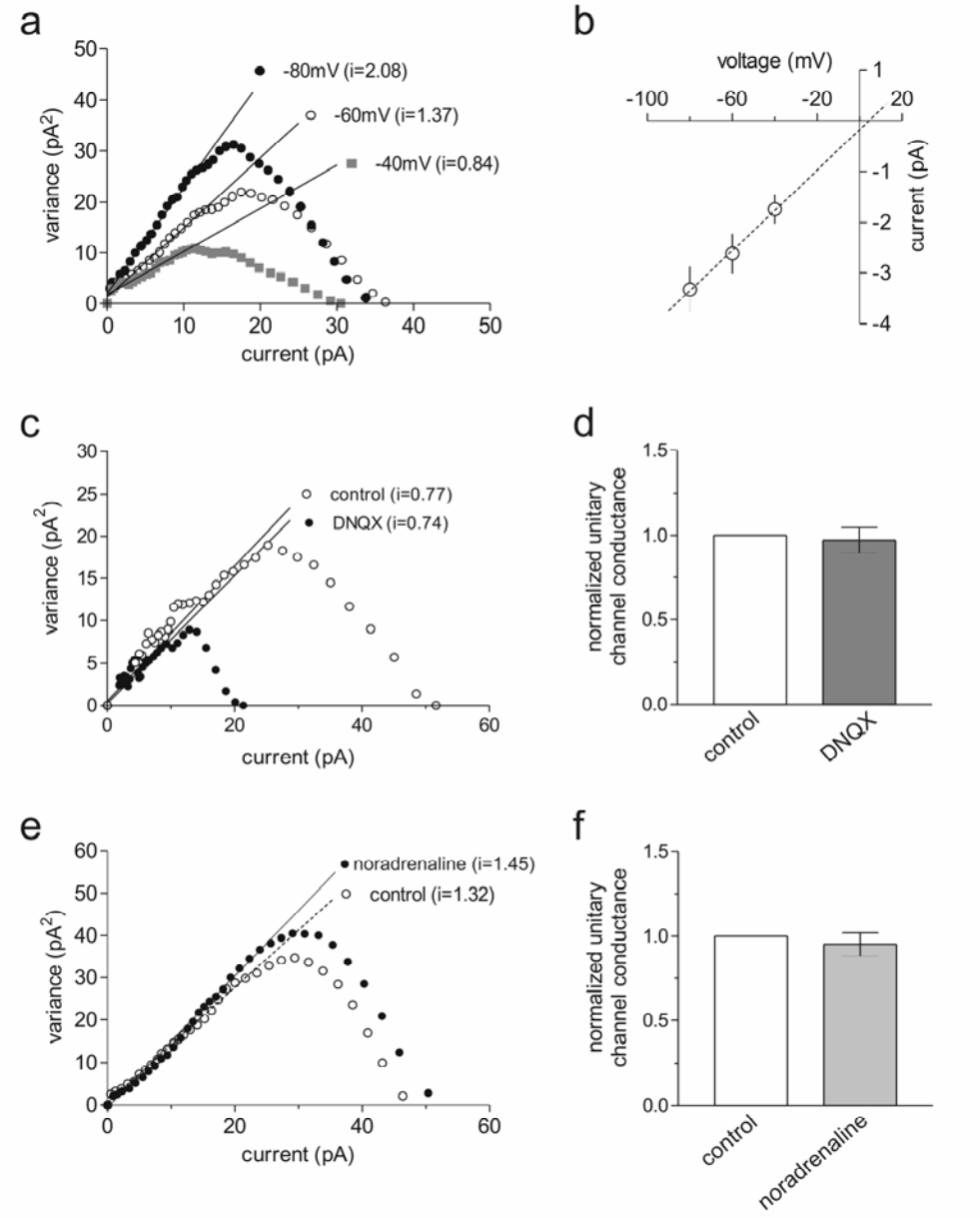
This increase in synaptic strength is reminiscent of activity-dependent changes in postsynaptic receptor function responsible for LTP at cortical synapses (Malinow and Malenka, 2002). Consequently, we investigated whether NA increased the postsynaptic response to exogenous AMPA application. The response to focal application of AMPA (see methods) directly onto MNCs (mean: 197.4 ± 28.9 pA) was significantly increased after NA (1.41 ± 0.09, $P < 0.05$, $n = 5$, figure 4.1 e, f).

4.3.2 NA does not change AMPA channel conductance

We used peak-scaled non-stationary noise analysis (PSNA) (Traynelis et al., 1993) to investigate whether the increase in synaptic strength was associated with an increase in AMPA channel conductance (Benke et al., 1998). To ensure the fluctuation in mEPSC decay arises mostly from stochastic channel properties, we examined mEPSCs in control and after NA treatment and confirmed there was no strong relationship between the rise time and decay time of the mEPSCs (Traynelis et al., 1993) (control: 10-90 rise-time *versus* decay-time r^2: 0.15 ± 0.02; 30 min post NA treatment: 10-90 rise-time *versus* decay-time r^2: 0.11 ± 0.03, $P > 0.05$, $n = 6$). To establish that PSNA yielded unitary currents that were linearly related to voltage and predicted the reversal potential of the channel (approximately 0 mV), cells were voltage clamped at different potentials and unitary current values satisfying these criteria were obtained (-40 mV: -1.73 ± 0.29 pA, -60 mV: -2.61 ± 0.39 pA, -80 mV: -3.32 ± 0.44 pA, channel reversal: 4.4 mV, $n = 5$, figure 4.2 a, b). Next, we determined that there was no change in the unitary AMPA conductance upon application of a low dose of the AMPAR blocker DNQX (500 nM); this decreases mEPSC amplitude by completely blocking a fraction of AMPARs (control: 16.82 ± 2.85 pS; DNQX: 16.35 ± 2.81 pS, $P > 0.05$, $n = 6$, figure 4.2 c, d).

Figure 4.2 The enduring increase in mEPSC amplitude is not associated with an increase in AMPA channel conductance.

(**a**) Variance versus current for mEPSCs when the postsynaptic cell is held at different potentials. The slope, *i*, represents the unitary channel current. (**b**) The unitary AMPA channel current changes linearly with voltage and estimates the reversal potential of the channel (-40 mV: -1.73 ± 0.29 pA, -60 mV: -2.61 ± 0.39 pA, -80 mV: -3.32 ± 0.44 pA, channel reversal: 4.4 mV, $n = 5$). (**c**) There is no change in the unitary AMPA channel current between control mEPSCs and mEPSCs treated with DNQX (500 nM). (**d**) Summary of effects of DNQX on channel conductance (0.97 ± 0.07, $P > 0.05$, $n = 6$) (**e**) There is no change in the unitary AMPA channel current between control mEPSCs and mEPSCs post-norepinephrine. (**f**) Summary of effects of norepinephrine on channel conductance (0.95 ± 0.06, $P > 0.05$, $n = 7$). Error bars: s.e.m.


a
-80mV (i=2.08)
-60mV (i=1.37)
-40mV (i=0.84)
variance (pA²)
current (pA)
b
voltage (mV)
current (pA)
c
control (i=0.77)
DNQX (i=0.74)
variance (pA²)
current (pA)
d
normalized unitary channel conductance
control
DNQX
e
noradrenaline (i=1.45)
control (i=1.32)
variance (pA²)
current (pA)
f
normalized unitary channel conductance
control
noradrenaline

The mEPSC amplitude in DNQX was 0.73 ± 0.08, $P < 0.05$, $n = 7$. The unitary conductance of mEPSCs in control was not different from that after NA (control: 20.02 ± 3.63 pS; post NA: 18.22 ± 2.69 pS, $P > 0.05$, $n = 7$, figure 4.2 e, f). These values are consistent with those reported previously for excitatory synapses onto MNCs in the supraoptic nucleus (SON) of the hypothalamus (Stern et al., 1999).

4.3.3 NA triggers AMPA receptor insertion

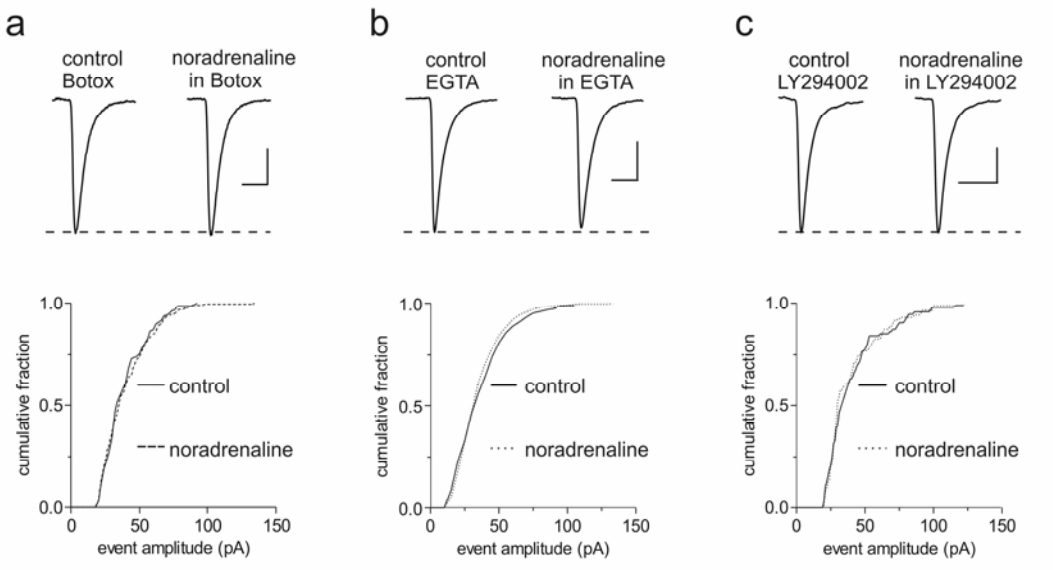
The insertion of postsynaptic AMPARs is a widely accepted mechanism by which synapses increase their strength (Malinow and Malenka, 2002). We tested for this possibility by targeting SNARE-dependent vesicle exocytosis. Inclusion in our patch pipette of botulinum toxin C (5 μg/ml), which proteolytically cleaves the tSNARE syntaxin (O'Connor et al., 1997), completely blocked the enduring increase in quantal amplitude caused by NA (0.96 ± 0.04, $P > 0.05$, $n = 7$; figure 4.3 a). Consistent with previous observations (Malinow and Malenka, 2002), we found that this exocytotic process requires increases in postsynaptic Ca^{2+}, since the inclusion of EGTA (10 mM) in our internal solution completely blocked the effects of NA (1.03 ± 0.02, $P > 0.05$, $n = 6$, figure 4.3 b). Finally, we examined the cellular signals required for the insertion of AMPARs by targeting PI3-K, which is important in the expression of LTP in the hippocampus (Sanna et al., 2002;Raymond et al., 2002;Man et al., 2003). Inclusion of either of two different PI3-K inhibitors in the patch pipette (wortmannin (100 nM) or LY294002 (10 μM)) blocked the effects of NA on mEPSC amplitude (wortmannin: 1.02 ± 0.02, $P > 0.05$, $n = 4$; LY294002: 1.01 ± 0.02, $P > 0.05$, $n = 5$, figure 4.3 c). Collectively, these data suggest that NA promotes the Ca^{2+}-dependent, postsynaptic insertion of AMPARs through the activation of PI3-K.

4.3.4 NA does not utilize postsynaptic receptors

To examine the possibility that activation of the postsynaptic G_q-coupled α_1-adrenoceptor directly increases mEPSC amplitude, we targeted two separate points on the α_1-adrenoceptor intracellular cascade. First, we introduced thapsigargin (5 μM), which

Figure 4.3 The enduring increase in mEPSC amplitude requires SNARE-dependent exocytosis, an increase in postsynaptic Ca^{2+} and activation of PI3-K.

(**a**) Top: average mEPSCs before and after norepinephrine application with postsynaptic botulinum toxin C (5 μg/ml) to block SNARE-dependent exocytosis. Bottom: cumulative probability plot of mEPSC amplitudes ($P > 0.05$) from the same cell. (**b**) Top: average mEPSCs before and after norepinephrine with postsynaptic EGTA (10 mM) to chelate Ca^{2+}. Bottom: cumulative fraction plot ($P > 0.05$) from the same cell. (**c**) Top: average mEPSCs before and after norepinephrine with postsynaptic LY294002 (10 μM) to block PI3-K activation. Bottom: cumulative fraction plot ($P > 0.05$) from the same cell. Scale bars 10 pA, 5 ms.

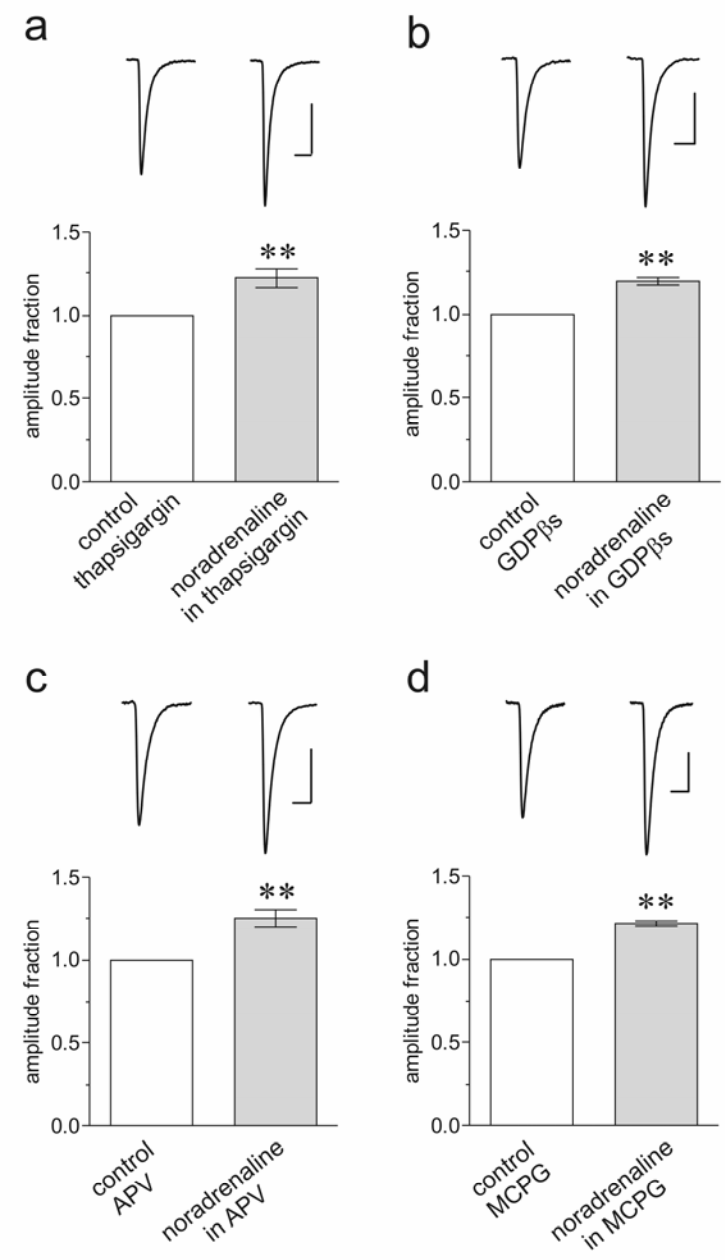

a
control
Botox
noradrenaline
in Botox
cumulative fraction
1.0
0.5
0.0
0
50
100
150
event amplitude (pA)
control
noradrenaline
b
control
EGTA
noradrenaline
in EGTA
cumulative fraction
1.0
0.5
0.0
0
50
100
150
event amplitude (pA)
control
noradrenaline
c
control
LY294002
noradrenaline
in LY294002
cumulative fraction
1.0
0.5
0.0
0
50
100
150
event amplitude (pA)
control
noradrenaline

depletes Ca^{2+} stores, into our patch electrode. This treatment failed to block the NA-mediated increase in quantal amplitude (1.22 ± 0.05, $P < 0.01$, $n = 8$, figure 4.4 a). Second, we targeted the G_q-protein directly with intracellular perfusion of GDP-βs (1 mM). This experimental manipulation had no impact on the enduring increase in mEPSC amplitude following NA treatment (1.19 ± 0.02, $P < 0.01$, $n = 6$, figure 4.4 b). To confirm the validity of our negative result with GDP-βs, we conducted an experiment in which inclusion of this compound in the patch pipette blocked an established action on synaptic function - the inhibition of glutamate release by postsynaptic activation of glucocorticoids receptors (Di et al., 2003). Consistent with this report, dexamethasone (50 μM, 3 min) elicited a significant decrease in mEPSC frequency (0.49 ± 0.04, $P < 0.01$ in comparison to control, $n = 3$). This was attenuated when GDP-βs was included in the internal solution (0.86 ± 0.13, $P < 0.05$ in comparison to dexamethasone alone, $n = 3$). These data suggest that postsynaptic α_1-adrenoceptors are not responsible for the enduring increase in mEPSC amplitude.

We have previously shown that NA transiently increases the frequency of glutamate release through a presynaptic α_1-adrenoceptor mediated mechanism (Gordon and Bains, 2003). This enhanced period of release may activate NMDA and/or mGluRs leading to a postsynaptic increase in synaptic strength. Although activation of NMDARs is crucial for hippocampal plasticity (Malinow and Malenka, 2002), blockade of these receptors (100 μM D-L-APV) failed to affect the ability of NA to increase quantal amplitude (1.25 ± 0.05, $P < 0.01$, $n = 5$, figure 4.4 c). While the experiments in which postsynaptic inclusion of GDP-βs and thapsigargin (above) would argue against a role for postsynaptic mGluRs, we directly tested for their contribution by applying NA in the presence of the group I mGluR antagonist MCPG (200 μM). Under these conditions, NA still increased synaptic strength (1.21 ± 0.01, $P < 0.01$, $n = 5$, figure 4.4 d). When combined, these observations argue against a role for postsynaptic α_1-adrenoceptors, NMDARs or group I mGluRs in mediating the enduring increase in mEPSC amplitude.

Figure 4.4. The enduring increase in mEPSC amplitude does not involve postsynaptic α_1-adrenoceptors or glutamate signalling.

Top: average mEPSCs before and after norepinephrine application with postsynaptic thapsigargin (5 μM) to deplete Ca^{2+} stores. Bottom: summary of effects of thapsigargin (1.22 ± 0.05, $P < 0.01$, $n = 8$). (**b**) Top: average mEPSCs before and after norepinephrine application with postsynaptic GDP-βs (1 mM) to block G protein signalling. Bottom: summary of effects of GDP-βs (1.19 ± 0.02, $P < 0.01$, $n = 6$). (**c**) Top: average mEPSCs before and after norepinephrine application with the NMDAR antagonist AP5 (100 μM). Bottom: summary of effects of AP5 (1.25 ± 0.05, $P < 0.01$, $n = 5$). (**d**) Top: average mEPSCs before and after norepinephrine with the mGluR antagonist MCPG (200 μM). Bottom: summary of effects of MCPG (1.21 ± 0.01, $P < 0.01$, $n = 5$). Scale bars: 10 pA, 5 ms. Error bars: s.e.m.


a
b
c
d
amplitude fraction
1.5
1.0
0.5
0.0
**
control
thapsigargin
noradrenaline
in thapsigargin
control
GDPβs
noradrenaline
in GDPβs
control
APV
noradrenaline
in APV
control
MCPG
noradrenaline
in MCPG

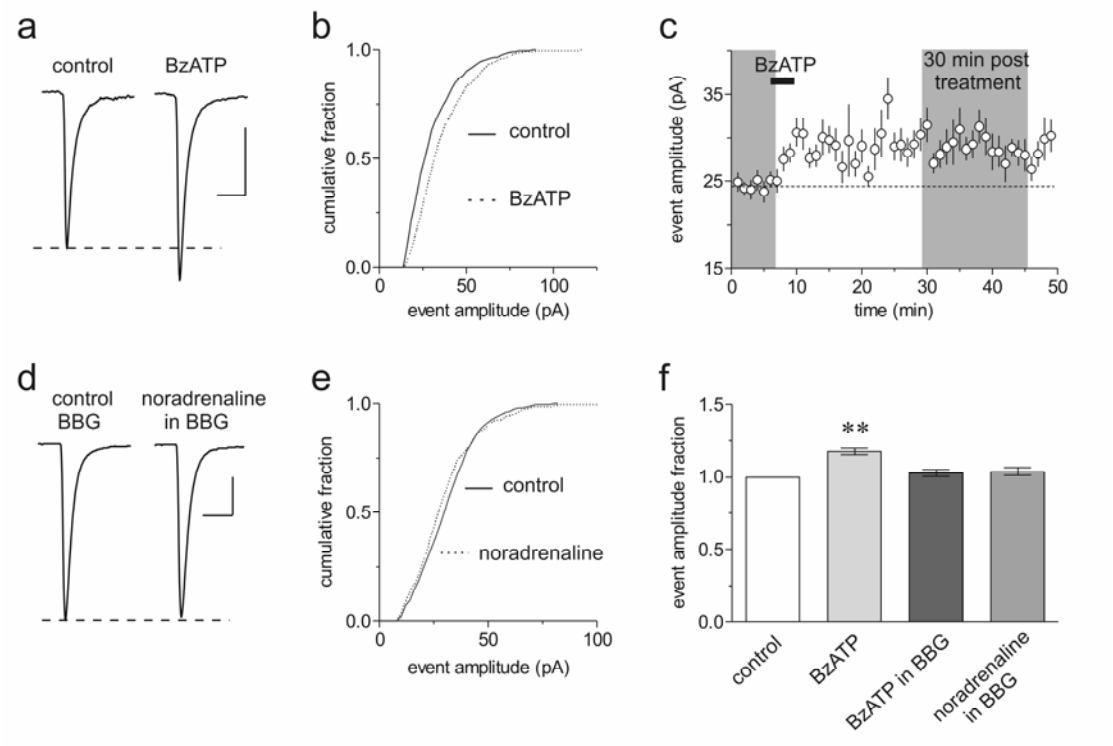
4.3.5 $P2X_7$ receptor activation is necessary and sufficient

The excitatory effects of NA on MNC firing observed *in vivo* are attenuated by blockade of purinergic receptors (Day et al., 1993). We tested the hypothesis that direct activation of ATP-gated P2X receptors in our slice preparation would effectively mimic the effects of NA on synaptic strength. While our experiments targeting postsynaptic G-protein signalling argue against the metabotropic P2Y receptors, MNCs possess a variety of functional P2X receptors capable of passing Ca^{2+} (Shibuya et al., 1999). We first tested ATP directly. Bath application of 30 μM ATP elicited a sustained increase in mEPSC amplitude (1.15 ± 0.02, $P < 0.05$, $n = 7$). Since ATP can be rapidly broken down by ectonucleotidases, this effect may be an underestimation. We repeated the experiment with BzATP, an analogue that is not readily degraded and can also activate several P2X channels, including $P2X_1$, $P2X_3$ and $P2X_7$ (North, 2002). BzATP (30 μM, 3 min) increased mEPSC amplitude (1.17 ± 0.02, $P < 0.01$, $n = 8$, figure 4.5 a-c, f). This increase in synaptic strength was blocked by Brilliant Blue G (BBG, 1 μM)(1.03 ± 0.03, $P > 0.05$, $n = 5$, figure 4.5 f), a P2X receptor antagonist that blocks $P2X_7$ at the concentration used here (North, 2002).

We next tested the hypothesis that activation of $P2X_7$ receptors was necessary for the NA-induced increase in synaptic strength. In the continuous presence of BBG, NA failed to cause an enduring increase in mEPSC amplitude (1.04 ± 0.02, $P > 0.05$, $n = 5$, figure 4.5 d-f). These data indicate that ATP-gated $P2X_7$ receptor activation is both necessary and sufficient for increasing mEPSC amplitude.

Figure 4.5 Activation of $P2X_7$ receptors is necessary and sufficient for the expression of the enduring increase in mEPSC amplitude.

(**a**) Average mEPSC traces from control condition and 30 min post-BzATP (30 μM, 3 min). (**b**) Cumulative fraction plot of mEPSC amplitudes ($P < 0.01$); same cell as a. (**c**) Running average of mEPSC amplitude in 1-min bins. (**d**) Average mEPSC traces from control condition and post-norepinephrine in the presence of the P2X antagonist BBG (1 μM). (**e**) Cumulative fraction plot ($P > 0.05$), same cell as d. (**f**) Summary: BzATP induces an enduring increase in mEPSC amplitude (1.17 ± 0.02, $P < 0.01$, $n = 8$), this effect is blocked by BBG (1.03 ± 0.03, $P > 0.05$, $n = 5$), and the enduring increase in mEPSC amplitude observed after norepinephrine is blocked with BBG (1.04 ± 0.02, $P > 0.05$, $n = 5$). Scale bars: 10 pA, 5 ms. Error bars: s.e.m.


a
control
BzATP
b
cumulative fraction
1.0
0.5
0.0
0
50
100
event amplitude (pA)
control
BzATP
c
BzATP
30 min post treatment
event amplitude (pA)
35
25
15
0
10
20
30
40
50
time (min)
d
control
BBG
noradrenaline in BBG
e
cumulative fraction
1.0
0.5
0.0
0
50
100
event amplitude (pA)
control
noradrenaline
f
event amplitude fraction
1.5
1.0
0.5
0.0
**
control
BzATP
BzATP in BBG
noradrenaline in BBG

4.3.6 α_1-adrenoceptors are co-localized with a glial marker

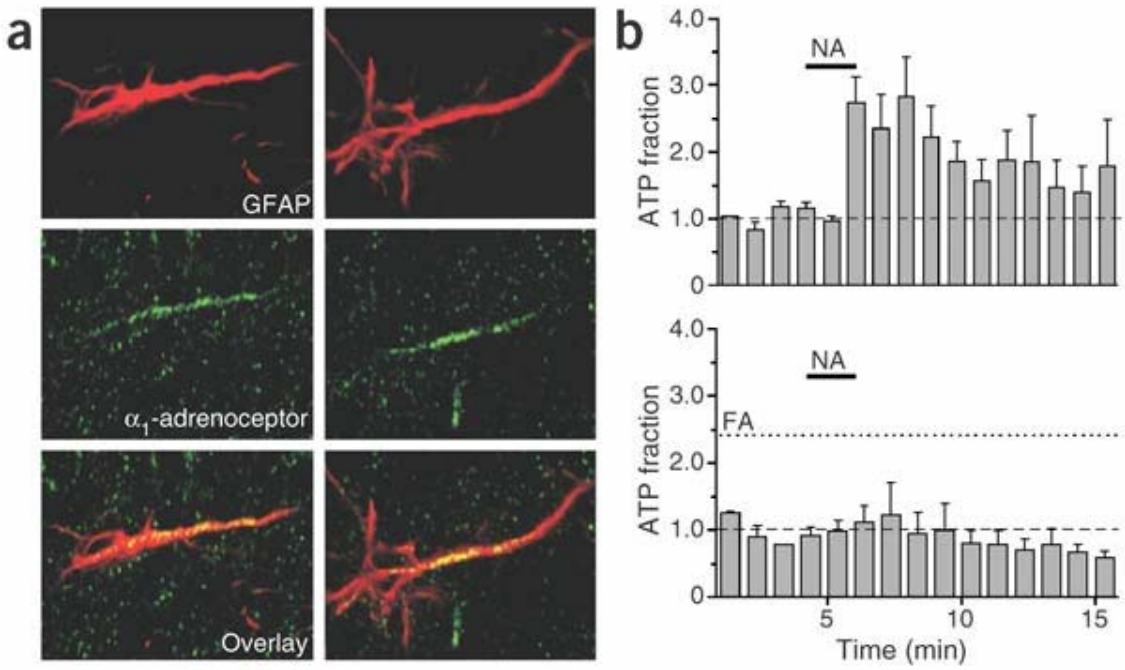
Glial cells possess receptors for NA (Duffy and MacVicar, 1995) and, in the PVN, a large fraction of noradrenergic terminals lack postsynaptic contacts (Sawyer and Clifton, 1980), leaving NA free to activate these cells. As the enduring enhancement of quantal amplitude is independent of glutamate receptors and postsynaptic G-protein signalling, yet requires the activation of α_1-adrenoceptors, we tested the hypothesis that the observed effect in response to NA was secondary to the activation of glial cells. We first tested for the presence of α_1-adrenoceptors on glial cells in PVN using immunohistochemical techniques. Astrocytes were identified with glial fibrillary acidic protein (GFAP) staining and long astrocytic processes were clearly labelled in the PVN (figure 4.6 a, top). In addition, a primary antibody directed against the α_{1a}-adrenoceptor subunit resulted in both punctate staining and staining characterized by long thin sections (figure 4.6 a, middle). Although it is clear that α_1-adrenoceptors are not localized exclusively to glial cells, confocal images revealed a strong co-localization between the signal for the α_{1a}-antibody and GFAP in the glial processes (figure 4.6 a, bottom).

4.3.7 NA releases ATP from cultured glial cells

We next investigated whether NA would elicit the release of ATP from cultured glial cells. To test this directly we employed a system of cultured pituicytes. These glial cells engulf the neuropeptide secreting terminals of the MNCs in the posterior pituitary and undergo structural reorganization similar to that seen with SON and PVN glia in response to dehydration, parturition and lactation (Tweedle and Hatton, 1987). Using an ATP bioluminescence assay, we observed an increase in the ATP concentration in the surround saline in response to NA (10–50 μM). We then confirmed the ubiquitous nature of this phenomenon in three separate culture systems in which glial cells were isolated from the neonatal hypothalamus, hippocampus or the neo-cortex. Again, in each of these preparations, NA robustly increased the concentration of ATP in the surround media.

Figure 4.6 NA targets glial cells to release ATP.

(**a**) Slice immunohistochemistry for the glial marker GFAP (top) and for the α_1-adrenoceptor (middle). Overlaid confocal images show colocalization (bottom). Each picture comprises a series of stacked images, and each column is an example from a different rat. (**b**) Top: summary of relative increase in ATP release in response to norepinephrine (10−50 μM) from cultured glial cells (peak ATP release: 2.39 ± 0.17, $P < 0.01$, $n = 18$). Bottom: summary demonstrating that norepinephrine-induced release of ATP is blocked in the presence of the fluorocitric acid (1.02 ± 0.07, $P > 0.05$, $n = 7$). Data represent the pooled responses from four separate glial culture systems: pituicytes, neonatal hypothalamus, hippocampus and neocortex. Error bars: s.e.m.


a
GFAP
α1-adrenoceptor
Overlay
b
NA
ATP fraction
4.0
3.0
2.0
1.0
0
NA
FA
ATP fraction
4.0
3.0
2.0
1.0
0
5
10
15
Time (min)

As the increases in extracellular ATP were qualitatively similar across cultures, the data were pooled. During the first five minutes after NA, ATP levels increased 2.39 ± 0.17 fold over control ($P < 0.01$, $n = 18$, figure 4.6 b, top).

We then utilized a pharmacological tool, fluorocitric acid (100 μM), to examine whether the NA effect could be abated when the glial Krebs cycle was blocked (Clarke, 1991). Cultures were incubated for a minimum of two hours in fluorocitric acid before NA was applied. Under these conditions, NA failed to trigger the release of ATP (1.02 ± 0.07, $P > 0.05$, $n = 7$, figure 4.6 b, bottom).

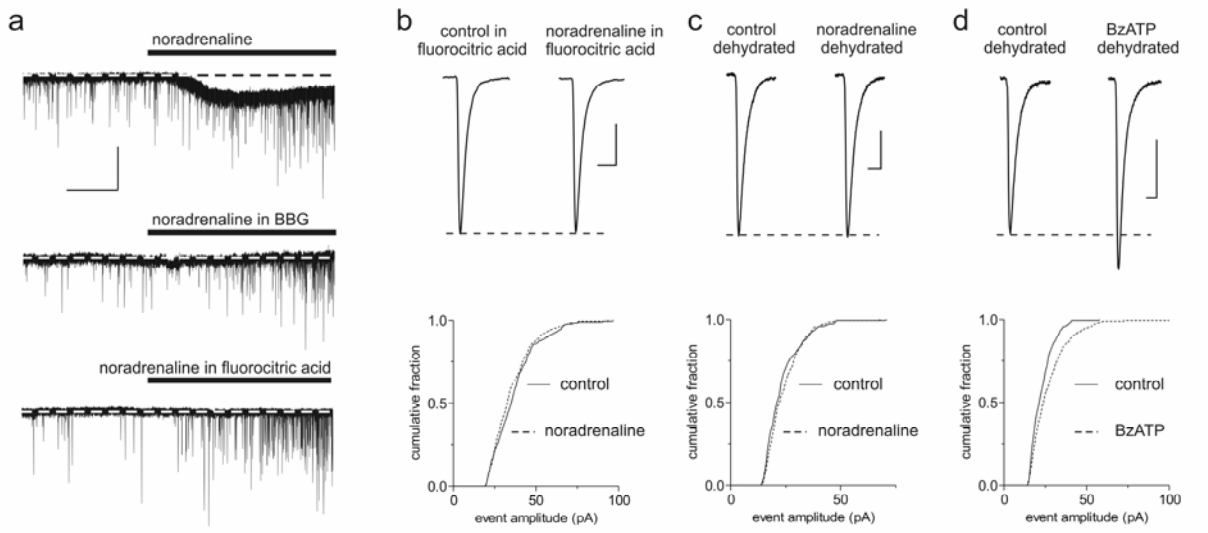
4.3.8 Glial cells are necessary for the NA effect

We next employed several different tactics to determine whether the ATP responsible for the NA-induced plasticity in brain slices was glial derived. First, we tested for the possibility that ATP, released from nerve terminals in our preparation, gates fast purinoceptor-mediated synaptic transmission (Edwards et al., 1992). To maximize the likelihood of detecting ATP-mediated synaptic events, AMPARs were blocked (DNQX, 10 μM) and NA was applied. We failed to detect any currents with kinetic features consistent with the rapid activation of ligand-gated ionotropic channels in response to synaptically released ATP (Edwards et al., 1992)(data not shown). We next examined our data for a slower, more prolonged inward current that might be indicative of a neuronal response to diffuse activation of membrane receptors. Both NA and BzATP elicited a slow inward current that preceded the increase in mEPSC amplitude (NA: 18.6 ± 3.4 pA, $n = 8$, figure 4.7 a, top; BzATP: 8.8 ± 1.5 pA, $n = 8$). Furthermore, the inward current induced by NA was abolished by BBG (2.3 ± 3.6 pA, $n = 6$, figure 4.7 a, middle), suggesting this response is $P2X_7$ mediated. Importantly, both the *transient* increases in frequency and amplitude elicited by NA were unaffected by BBG (figure 4.7 a).

To test the hypothesis that ATP in the slice is derived from glial cells, we again utilized fluorocitric acid to inhibit the glial Krebs cycle (Clarke, 1991). Similar to the culture systems, slices were incubated for a minimum of two hours in fluorocitric acid before NA treatment. Under these conditions, NA failed to increase the amplitude of mEPSCs (0.98 ± 0.02, $P > 0.05$, $n = 6$, figure 4.7 b). Notably, the basal amplitude of

Figure 4.7 Glial cells are necessary for the enduring increase in mEPSC amplitude triggered by α1-adrenoceptor activation.

(**a**) Top: inward current at the onset of the norepinephrine effect (18.6 ± 3.4 pA, $P < 0.01$, $n = 8$). The inward current was blocked when P2X channels were blocked with BBG (2.3 ± 3.6 pA, $P > 0.05$, $n = 6$; middle) and when slices were incubated for >2 h with fluorocitric acid (3.6 ± 1.0 pA, $P > 0.05$, $n = 6$; bottom). (**b**) Top: average mEPSC traces from control condition and post−NA treatment after fluorocitric acid incubation. Bottom: cumulative fraction plot of mEPSC amplitudes ($P > 0.05$) from the same cell. (**c**) Top: average mEPSC traces from control condition and post−NA treatment after a 7- to 10-d dehydration protocol that elicits reduced glial coverage of synaptic contacts. Bottom: cumulative fraction plot of mEPSC amplitudes ($P > 0.05$) from the same cell. (**d**) Top: average mEPSC traces from control condition and post−BzATP treatment in the dehydrated state. Bottom: cumulative fraction plot of mEPSC amplitudes ($P < 0.01$) from the same cell. Scale bars, **a**: 40 pA, 30 s; **b**,**c**,**d**: 10 pA, 5 ms.


a
noradrenaline
noradrenaline in BBG
noradrenaline in fluorocitric acid
b
control in fluorocitric acid
noradrenaline in fluorocitric acid
cumulative fraction
1.0
0.5
0.0
0
50
100
event amplitude (pA)
control
noradrenaline
c
control dehydrated
noradrenaline dehydrated
cumulative fraction
1.0
0.5
0.0
0
50
event amplitude (pA)
control
noradrenaline
d
control dehydrated
BzATP dehydrated
cumulative fraction
1.0
0.5
0.0
0
50
100
event amplitude (pA)
control
BzATP

mEPSCs from slices incubated in fluorocitric acid was not different from that in slices incubated in control solution (control: 28.15 ± 2.25 pA; fluorocitric acid: 27.10 ± 2.40 pA, $P > 0.05$, $n = 10$). We also failed to observe an inward current in response to NA (3.6 ± 1.0 pA, $P > 0.05$, $n = 6$, figure 4.7 a, bottom), yet the transient increases in mEPSC frequency and amplitude at the commencement of NA application were unaltered.

Finally, we utilized a physiological tool to assess the putative contribution of glial cells to the NA-induced synaptic potentiation. Glial coverage of synapses in the magnocellular neurosecretory nuclei can be decreased dramatically by physiological challenges, such as parturition, lactation and dehydration (Tweedle and Hatton, 1977;Theodosis and Poulain, 1993). Such dynamic changes in the physical relationship between neurons and glial cells increase glutamate spillover and increase presynaptic auto-inhibition (Oliet et al., 2001). To test the hypothesis that synaptic glial processes are necessary for the NA-induced synaptic strengthening, we utilized an established dehydration protocol to cause glial retraction from synaptic contacts (Miyata et al., 1994). In these experiments, NA failed to cause an enduring increase in the mEPSC amplitude (1.04 ± 0.02, $P > 0.05$, $n = 12$, figure 4.7 c). Finally, to confirm that this effect is due to the absence of glial investiture (and hence availability of ATP) and not due to changes in postsynaptic P2X receptor expression or signalling, we applied BzATP directly to these slices and observed that the enduring increase in mEPSC amplitude could still be induced (1.16 ± 0.02, $P < 0.01$, $n = 6$, figure 4.7 d). It is important to note that in six additional cells, BzATP had no effect on amplitude. Taken together, these findings support the assertion that the NA-induced enduring increase in synaptic strength requires the release of ATP from surrounding glial cells.

Although the precise mechanism of ATP release from glial cells remains controversial, there is increasing support for the idea that the process may be Ca^{2+} independent (Leybaert et al., 1998;Wang et al., 2000;Anderson et al., 2004). To examine whether the release of ATP from hypothalamic glial cells is consistent with these observations, we employed several strategies to increase Ca^{2+} in glia and assessed their impact on mEPSC amplitude. First, we bath applied 50 mM K^+ in iso-osmotic ACSF (2 min) (Duffy and MacVicar, 1995). This resulted in a pronounced inward current as well

as an increase in mEPSC frequency that washed completely (data not shown) but did not elevate mEPSC amplitude (0.97 ± 0.01, $P > 0.05$, $n = 5$, figure 4.8 a). Second, we applied the $GABA_B$ receptor agonist baclofen (20 μM, 3 min) (Kang et al., 1998) which also failed to increase mEPSC amplitude (0.99 ± 0.01, $P > 0.05$, $n = 7$, figure 4.8 b). A decrease in the frequency of mEPSCs, consistent with the activation of $GABA_B$ receptors on glutamatergic terminals was always observed, indicating that baclofen was reaching its target (data not shown). Although mGluRs can also increase Ca^{2+} in glia (Fellin et al., 2004), this receptor, like the α_1-adrenoceptor, is G_q-coupled and thus would not allow us to discriminate between the contribution of Ca^{2+} from intracellular stores and that of other second messengers (i.e. DAG or IP_3/IP_4) in promoting the release of ATP. Instead, we applied caffeine (5 μM, 3 min), which liberates Ca^{2+} from intracellular stores. Caffeine, however, did not increase mEPSC amplitude (1.01 ± 0.06, $P > 0.05$, $n = 5$, figure 4.8 c). Finally, we either depleted intracellular Ca^{2+} stores by incubating slices in thapsigargin (5 μM, 45 min) or blocked Ca^{2+} release with a high dose of ryanodine (100 μM, 20 min) prior to testing with NA. These experimental manipulations failed to block the NA-induced increase in mEPSC amplitude (thapsigargin + NA: 1.22 ± 0.06, $P < 0.01$, $n = 8$; ryanodine + NA: 1.21 ± 0.04, $P < 0.01$, $n = 5$). These results are consistent with previous demonstrations suggesting that ATP release from glial cells occurs through a Ca^{2+} independent mechanism (Leybaert et al., 1998;Wang et al., 2000;Anderson et al., 2004). A schematic of the possible mechanism(s) involved in glial-mediated synaptic strengthening, as well as an artistic representation of neuropil remodelling and the subsequent block of the effect are presented in figure 4.9.

Figure 4.8 A general rise in Ca^{2+} in glial cells does not mimic the enduring increase in mEPSC amplitude observed in response to norepinephrine.

(**a**) Left: average mEPSC traces showing no change in current amplitude after the bath application of 50 mM K^+ in osmotically normal ACSF (2 min). Right: cumulative fraction plot of mEPSC amplitudes ($P > 0.05$) from the same cell. (**b**) Left: average mEPSC traces showing that the $GABA_B$ receptor agonist baclofen (20 μM, 3 min) does not increase mEPSC amplitude. Right: cumulative fraction plot of mEPSC amplitudes ($P > 0.05$) from the same cell. (**c**) Left: average mEPSC traces showing no change in current amplitude after bath application of caffeine (5 μM, 3 min). Right: cumulative fraction plot of mEPSC amplitudes ($P > 0.05$) from the same cell. Scale bars: 10 pA, 5 ms.

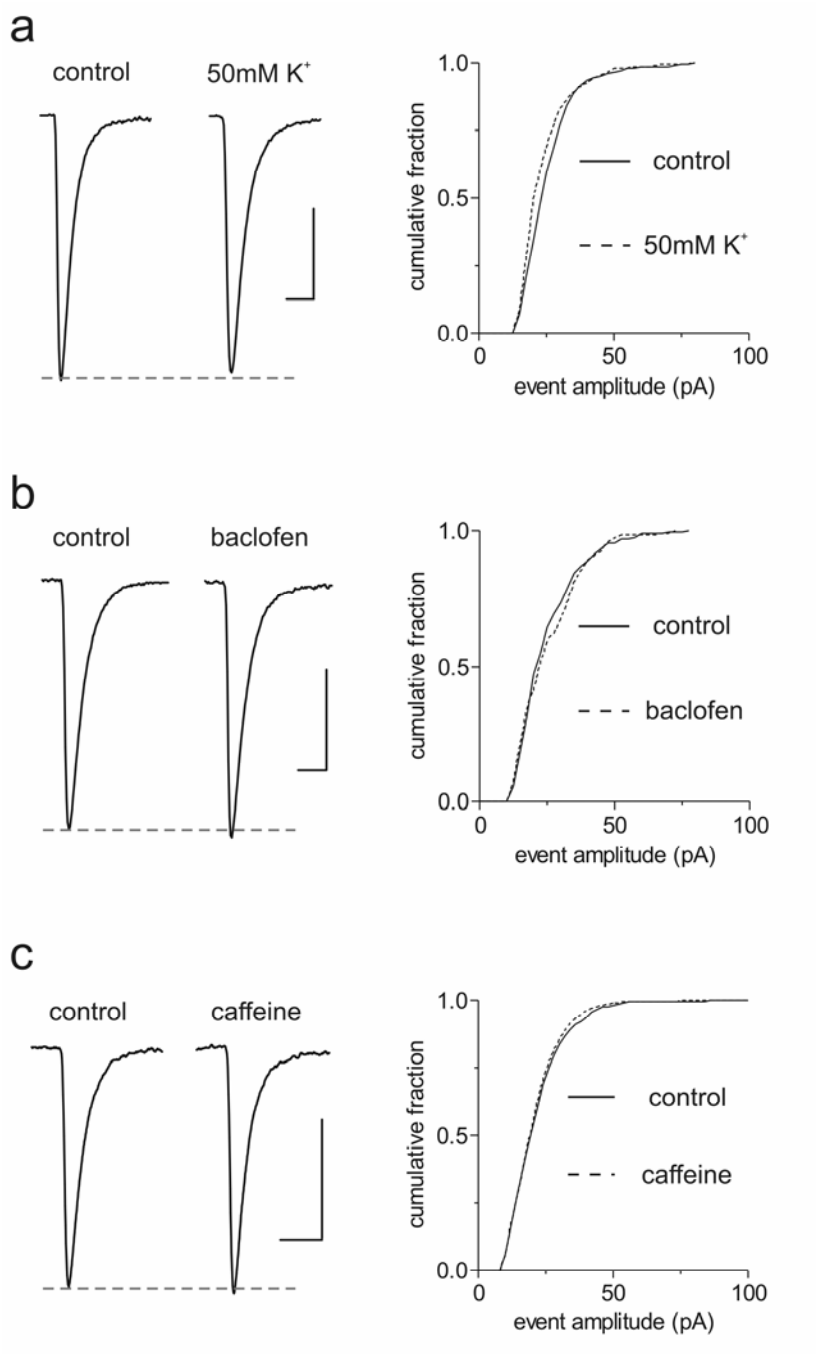
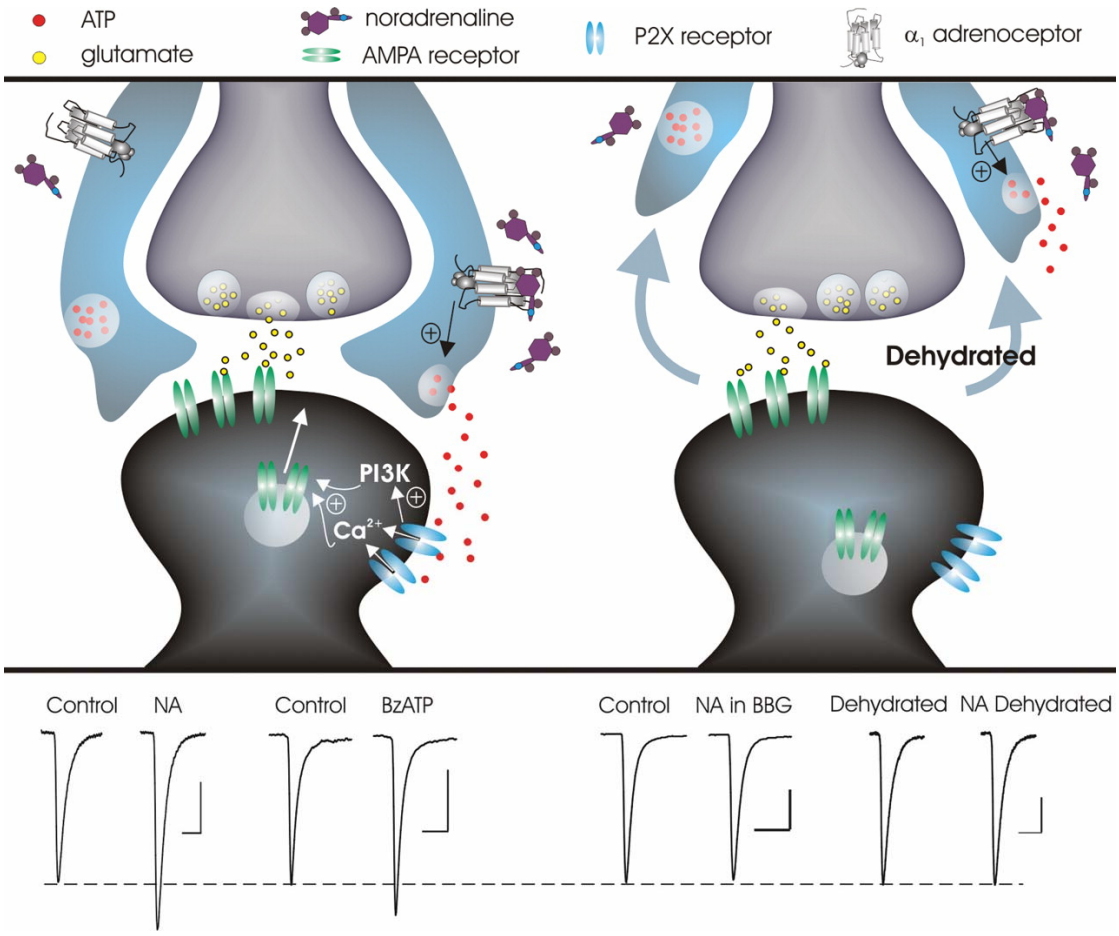

a
control
50mM K+
cumulative fraction
1.0
0.5
0.0
0
50
100
event amplitude (pA)
control
50mM K+
b
control
baclofen
cumulative fraction
1.0
0.5
0.0
0
50
100
event amplitude (pA)
control
baclofen
c
control
caffeine
cumulative fraction
1.0
0.5
0.0
0
50
100
event amplitude (pA)
control
caffeine

Figure 4.9 Induction of long-term synaptic strengthening by NA-mediated release of glial ATP depends on the physical neuro-glia relationship.

Upper panel, **left**: in the control state, where there is a relative abundance of glial processes surrounding synaptic elements, glial cell α_1-adrenoceptor activation triggers the release of ATP which can then activate postsynaptic P2X channels on MNCs. P2X channel activation results in Ca^{2+} influx and the activation of phosphotidyl inositol 3-kinase (PI3-K) leading to the insertion of AMPARs which is manifested as a long lasting increase in mEPSC amplitude. **Upper panel, right**: during states of chronic dehydration or lactation where there is a withdrawal of glial processes from around synaptic elements, NA fails to elicit changes in mEPSC amplitude. **Lower panel**: average mEPSC traces taken during control and 30 min after treatment. **Left**: NA causes a long-lasting increase in mEPSC amplitude, an effect that is mimicked by the P2X receptor agonist BzATP. **Right**: the long-lasting enhancement of mEPSC amplitude caused by NA is blocked either by the $P2X_7$ antagonist Brilliant Blue G (BBG) or by withdrawal of glial processes. Scale bars: 10pA, 5 ms.


ATP
glutamate
noradrenaline
AMPA receptor
P2X receptor
α1 adrenoceptor
PI3K
Ca2+
Dehydrated
Control
NA
Control
BzATP
Control
NA in BBG
Dehydrated
NA Dehydrated

4.4 Discussion

The data presented here support the conclusion that ATP, released from glial cells in response to activation of α_1-adrenoceptors, increases synaptic strength at glutamatergic synapses by promoting the postsynaptic insertion of AMPARs. Our findings further expand upon the role of glial cells in regulating the efficacy at excitatory synapses in the CNS (Parpura et al., 1994;Araque et al., 1998;Oliet et al., 2001;Bezzi and Volterra, 2001;Beattie et al., 2002;Zhang et al., 2003).

The assertion that glia are the source of the ATP is consistent with both anatomical findings describing catecholaminergic terminals that lack direct postsynaptic contacts (Sawyer and Clifton, 1980), as well as physiological findings that NA can directly activate astrocytes (Duffy and MacVicar, 1995). Although it is clear that α_1-adrenoceptors, in addition to being present on glial processes, are also present on non-GFAP positive elements throughout the PVN, our inability to affect the expression of synaptic potentiation by either interfering with postsynaptic G-protein function or depleting postsynaptic intracellular Ca^{2+} stores argues against a direct postsynaptic action of NA. ATP may also be released in a vesicular fashion from presynaptic terminals in PVN. ATP-mediated synaptic currents have been reported in the CNS (Edwards et al., 1992) but, in our hands, we did not see any evidence for fast synaptic currents in the absence of glutamatergic signalling. We did, however, observe a slow, inward $P2X_7$-mediated current in response to either NA or Bz-ATP, which may be indicative of a slow increase in the concentration of a transmitter in the extracellular space, as might be predicted if ATP were released some distance from the postsynaptic cell. Although not on the same time scale, slow inward currents mediated by the activation of extrasynaptic NMDARs in response to glial glutamate were recently described in hippocampal CA1 pyramidal neurons (Fellin et al., 2004). We were also able to block the increase in synaptic strength in response to NA by either pharmacologically compromising glial cells (Clarke, 1991), or by using physiological manipulations that decrease the astrocytic coverage of synapses in the magnocellular neurosecretory system (Tweedle and Hatton, 1977;Miyata et al., 1994). Finally, we demonstrate that application of NA directly onto

neuron-free glial cultures increases the release of ATP. The NA driven ATP release from glial cells provides a mechanism by which glia can respond to, and exert influence over, synaptic activity. Glia are known to respond to NA with increases in intracellular Ca^{2+} (Duffy and MacVicar, 1995). Whether an increase in intraglial Ca^{2+} can stimulate, or is even necessary for, ATP release is contentious. Mechanisms exhibiting both Ca^{2+}-dependence and independence (Leybaert et al., 1998;Wang et al., 2000;Anderson et al., 2004) have been described. Our findings, demonstrating that, increasing Ca^{2+} either non-specifically (high K^{+}), by activating a membrane-bound G-protein coupled receptor (baclofen), or by promoting release from intracellular stores (caffeine) does not mimic the effects of NA on synaptic strength, are most consistent with the idea that ATP release is not intimately linked to an increase in intraglial Ca^{2+}.

Our results also indicate that the key intracellular signalling molecules, postsynaptic Ca^{2+} and PI3-K (Malinow and Malenka, 2002;Man et al., 2003), that have been linked to the insertion of new AMPARs in other brain regions, are conserved during the ATP-mediated increase in postsynaptic efficacy described here. Although the initial trigger for activation of this cascade in MNCs is different than in a region such as the hippocampus, the ATP-gated $P2X_7$ receptor essentially serves as a surrogate for the NMDAR. The increase in mEPSC amplitude was blocked by buffering postsynaptic Ca^{2+} with EGTA, interfering with PI3-K signalling or by preventing vesicular fusion, all critical requirements for the insertion of new AMPARs into the postsynaptic membrane (Malinow and Malenka, 2002;Man et al., 2003). Our findings also support previous reports of $P2X_7$ coupling to the PI3-K/Akt pathway (Jacques-Silva et al., 2004). Although we cannot explicitly rule out the involvement of heteromeric P2X receptors or a contribution of P2Y receptors, our observations indicate that activation of the $P2X_7$ receptor alone is sufficient for the Ca^{2+} entry and PI3-K activation necessary for the enduring increase in mEPSC amplitude in our preparation.

In addition to advancing our understanding of the co-dependent interactions between glia and neurons, our findings also offer insights into a form of plasticity that may not be synapse specific (like LTP) (Malinow and Malenka, 2002). Rather, it may be a new mechanism through which a single point activation of interconnected glial

networks may lead to changes in the synaptic strength of spatially distributed neuronal circuits and may contribute to the synchronization of firing activity in populations of cells that are not synaptically coupled (Fellin et al., 2004).

Chapter Five: Multivesicular miniatures elicited by noradrenaline at glutamatergic synapses in the paraventricular nucleus of the hypothalamus

5.1 Summary

The origin of large amplitude mEPSCs at central synapses remains to be firmly established. Here we demonstrate that at excitatory synapses onto MNCs in the hypothalamus, NA induces a rapid and robust increase in mEPSC amplitude that requires α_1-adrenoceptor activation but is impervious to postsynaptic manipulations that block the putative insertion of AMPARs. In response to NA, mEPSCs exhibit a putative multimodal amplitude histogram distribution that is not due to either random temporal summation, the unveiling of a quiescent synapse or the release of large vesicles. Large amplitude mEPSCs are sensitive to a high dose of ryanodine and are associated with an enhanced glutamate cleft concentration. Together, these data are consistent with the hypothesis that large amplitude mEPSCs result from the synchronous release of multiple vesicles via rapid presynaptic Ca^{2+} expulsion from intracellular stores.

5.2 **Introduction**

In mammals, physiological homeostasis relies on an elegant feedback control system that is orchestrated by neural circuits in the hypothalamus. Perturbations in the 'milieu interior', sensed by visceral receptors, are relayed by a plexus of noradrenergic fibres to neurons in the PVN (Sawchenko and Swanson, 1981). MNCs in the PVN also receive glutamatergic input that exhibits a high frequency of quantal release, which is sensitive to NA (Gordon and Bains, 2003;Boudaba et al., 2003a). As synapses onto MNCs constitute the final integration step before the exocytosis of the hormones oxytocin and vasopressin, modulating the efficacy of mEPSCs provides one mechanism through which neuroendocrine output can be enhanced (Kombian et al., 2000).

Classical thinking postulates that presynaptic changes in quantal signals alter the frequency of miniature events, while postsynaptic changes alter the amplitude of the quantal current. Recent demonstrations, however, are beginning to challenge this precept. For instance, there is now compelling evidence indicating that changes in

presynaptic function can increase the amplitude of the postsynaptic current (Tong and Jahr, 1994;Frerking et al., 1995;Auger et al., 1998;Liu et al., 1999;Prange and Murphy, 1999;Llano et al., 2000;Oertner et al., 2002;Sharma and Vijayaraghavan, 2003). Among these is the demonstration that the release of Ca^{2+} from presynaptic stores can be extremely rapid, and is capable of synchronizing the release of multiple vesicles of neurotransmitter. This results in the appearance of large amplitude miniatures and has been shown to occur both endogenously (Llano et al., 2000) and in response to nicotinic acetylcholine receptor activation (Sharma and Vijayaraghavan, 2003). Other studies demonstrating that larger synaptic currents are associated with an increased glutamate cleft concentration (Liu et al., 1999;Wadiche and Jahr, 2001), suggest that quantal synchronization processes may also result in this end point.

NA may be ideally suited to initiate vesicle synchronization via presynaptic Ca^{2+} store activation in the PVN. Evidence suggests that functional α_1-adrenoceptors are present on glutamatergic terminals synapsing onto MNCs (Gordon and Bains, 2003;Boudaba et al., 2003a). Activation of these receptors elicit the release of stored Ca^{2+} through a Gq-protein linked intracellular cascade (Zhong and Minneman, 1999). At the neuromuscular junction NA facilitates the synchronization of evoked release (Bukcharaeva et al., 1999) at terminals that rely on the activation of ryanodine sensitive intracellular Ca^{2+} stores for the elevation of terminal Ca^{2+} to release of acetylcholine (Narita et al., 1998;Narita et al., 2000). Furthermore, as the impact of synchronization may be constrained by the saturation of postsynaptic receptors (Tong and Jahr, 1994;Holmes, 1995;Frerking and Wilson, 1996;Clements, 1996;Auger et al., 1998), the fact that NA can also promote the insertion of postsynaptic AMPARs in MNCs (Gordon et al., 2005) provides an ideal complementary change to fully take advantage of the enhanced presynaptic signal.

In this study, we demonstrate that NA induces a robust, but transient increase in mEPSC amplitude that cannot be blocked with postsynaptic manipulations that affect AMPAR insertion. This increase results in a skewing of the amplitude distribution displaying putative multiple peaks, which is indicative of the synchronization of vesicles. The large amplitude mEPSCs observed in NA are unlikely to occur from random

temporal summation, the unveiling of a quiescent synapse or vesicle, but are sensitive to a high dose of ryanodine. Furthermore, large amplitude mEPSCs in NA are associated with an enhanced glutamate cleft concentration, suggesting these events are likely multivesicular.

5.3 **Results**

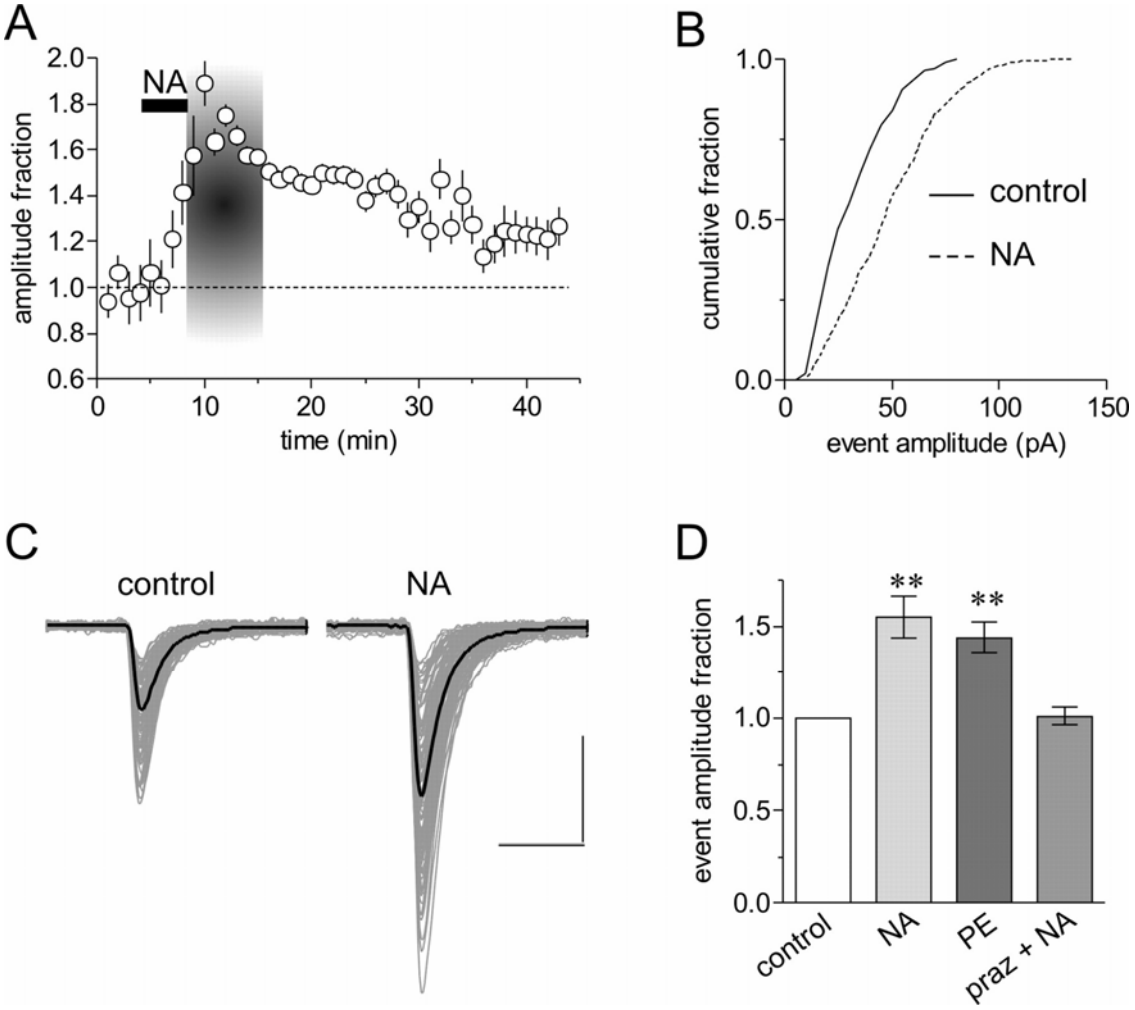
5.3.1 NA robustly increases quantal strength

Bath application of NA (3-5min, 200μM) elicited a robust increase in mEPSC amplitude (1.55 ± 0.11, $p<0.01$, n=20, figure 5.1 A-D), which was accompanied by an increase in frequency that we have characterized previously (Gordon and Bains, 2003). This is a saturating effect and while we observe increases in mEPSC amplitude in response to NA at 25 μM (1.22 ± 0.07, $p<0.05$, n=6), in order to delineate the underlying mechanism we have used a maximal dose. The robust increase in mEPSC amplitude is transient, lasting in the range of 10 to 20min, and then relaxes to a new, but elevated mean approximately 20% greater than baseline. This long-lasting enhancement of mEPSC amplitude results from the release of glial-derived ATP and the subsequent insertion of postsynaptic AMPARs (Gordon et al., 2005). The postsynaptic effect displays a mono-phasic, 'plateau-like', increase in the responsiveness to exogenous AMPA puff, and is therefore, unlike the biphasic increase in mEPSC amplitude (Gordon et al., 2005). The experiments described here will focus almost exclusively on the transient increase in mEPSC amplitude caused by NA.

Consistent with previously characterized α_1 adrenoceptor-mediated excitatory effects of NA on MNCs (Day et al., 1985;Armstrong et al., 1986;Randle et al., 1986a;Randle et al., 1986b;Daftary et al., 1998;Gordon and Bains, 2003;Boudaba et al., 2003a), we were able to mimic the observed increase in mEPSC amplitude with bath application of the α_1 adrenoceptor agonist phenylephrine (PE) (5 min, 200μM) (1.44 ± 0.08, $p<0.01$, n=7, figure 5.1 D) and block the effect with the α_1 adrenoceptor antagonist prazosin (10μM)(1.03 ± 0.06, $p>0.05$, n=8, figure 5.1 D). The transient increase in mEPSCs amplitude is entirely comprised of currents that arise from the activation of

Figure 5.1 NA induces a robust increase in mEPSC amplitude.

A, Running average of mEPSC amplitude in 2 min bins during control and in response to a 3-5 min, 200 μM bath application of NA in a single cell. The area demarcated in gray represents the time during the NA response that is compared with control values. The long-lasting increase in mEPSC amplitude caused by NA can also be observed 20 min after NA application. **B**, Cumulative fraction plot of mEPSC amplitudes taken from control and during NA ($p<0.01$); same cell as A. **C**, Raw mEPSCs (light gray) and average mEPSC traces (solid black) from control and NA; same cell as A. **D**, Summary data depicting the change in mEPSC amplitude in response to NA (1.55 ± 0.11; $^{**}p < 0.01$ compared with control; $n = 20$), the α_1-adrenoceptor agonist phenylephrine (PE) (200 μM) (1.44 ± 0.08; $^{**}p < 0.01$ compared with control; $n = 7$), and NA in the presence of the α_1-adrenoceptor antagonist prazosin (praz) (10 μM) (1.03 ± 0.06; $p > 0.05$; $n = 8$).


A
NA
amplitude fraction
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0
10
20
30
40
time (min)
B
cumulative fraction
1.0
0.5
0.0
0
50
100
150
event amplitude (pA)
control
NA
C
control
NA
D
event amplitude fraction
1.5
1.0
0.5
0.0
**
**
control
NA
PE
praz + NA

AMPA channels, as no synaptic events were observed during NA in the presence of the glutamate receptor antagonist DNQX (n=5, data not shown). Collectively, these data suggest that activation of α_1 adrenoceptors elicits a transient and robust increase in quantal glutamate signalling.

5.3.2 The increase in mEPSC amplitude is not blocked with postsynaptic manipulations

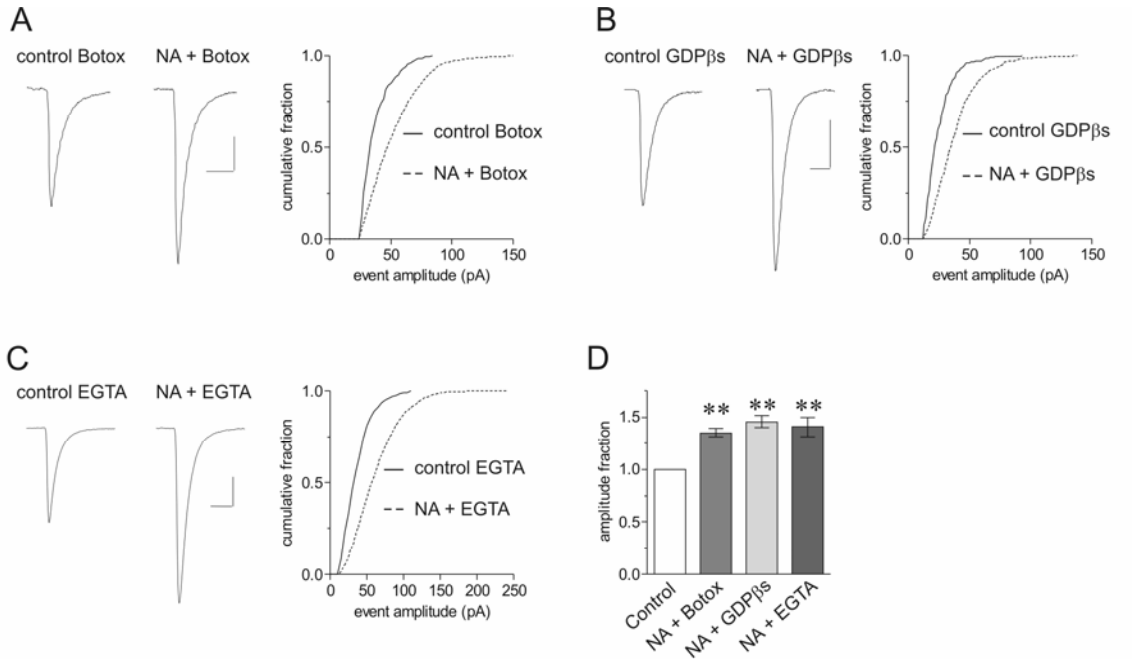
Traditionally, a change in mEPSC amplitude is thought to arise from a postsynaptic modification. More recently, the insertion of postsynaptic AMPARs has become a widely accepted mechanism by which synapses increase their strength (Malinow and Malenka, 2002). We tested for this possibility directly by targeting SNARE-dependent vesicle exocytosis in the postsynaptic cell. Inclusion of botulinum toxin C (5μg/ml) in our patch pipette, a compound that proteolytically cleaves the tSNARE syntaxin (O'Connor et al., 1997) thereby blocking vesicular fusion, failed to block the transient increase in quantal amplitude caused by NA (1.35 ± 0.04, $p<0.01$, n=7; figure 5.2 A, D). We next tested for the involvement of postsynaptic α_1-adrenoceptors by inhibiting postsynaptic G-protein signalling with the inclusion of GDP-βs (1mM) in our internal solution (Di et al., 2003;Gordon et al., 2005). This manipulation had no impact on the transient increase in mEPSC amplitude following NA treatment (1.45 ± 0.05, $p<0.01$, n=5, figure 5.2 B, D). We also found that the transient increase in mEPSC amplitude was not attenuated when the concentration of EGTA was increased 10 fold (1mM to 10mM) in our internal solution (1.41 ± 0.09, $p<0.01$, n=7, figure 5.2 C, D), a treatment that can block changes in postsynaptic strength in MNCs (Gordon et al., 2005). When combined, these observations argue against a role for the involvement of postsynaptic mechanisms in mediating the transient increase in mEPSC amplitude.

5.3.3 NA enhances the skew of mEPSC amplitude distributions

We examined the amplitude distribution of mEPSCs in control and during NA to test the hypothesis that NA acts presynaptically to synchronize the release of multiple vesicles, which would be made evident by the appearance of multiple, equidistant modes. As there

Figure 5.2 Postsynaptic manipulations fail to abolish the increase in mEPSC amplitude in response to NA.

A-C, Average mEPSC traces on the left and cumulative fraction plots of mEPSC amplitude on the right. Inclusion of botulinum toxin C (5 μg/ml) (**A**), which blocks vesicular fusion and therefore AMPAR insertion, GDP-βs (1 mM) (**B**), which blocks intercellular G-protein signalling, or EGTA (10 mM) (**C**), which buffers intracellular Ca^{2+}, in the patch pipette failed to block the increase in mEPSC amplitude in response to NA ($p < 0.01$). **D**, Summary data showing that the NA effect remains in the presence of postsynaptic botulinum toxin C (1.35 ± 0.04; $^{**}p < 0.01$ compared with control; $n = 7$), GDP-βs (1.45 ± 0.05; $^{**}p < 0.01$ compared with control; $n = 5$), and elevated EGTA (1.41 ± 0.09; $^{**}p < 0.01$ compared with control; $n = 7$). Botox, Botulinum toxin C.


A
control Botox
NA + Botox
cumulative fraction
control Botox
NA + Botox
event amplitude (pA)
B
control GDPβs
NA + GDPβs
cumulative fraction
control GDPβs
NA + GDPβs
event amplitude (pA)
C
control EGTA
NA + EGTA
cumulative fraction
control EGTA
NA + EGTA
event amplitude (pA)
D
amplitude fraction
**
**
**
Control
NA + Botox
NA + GDPβs
NA + EGTA

are numerous caveats associated with the use of amplitude distributions in the CNS (Wahl et al., 1995;Walmsley, 1995), we employed two strategies to help facilitate the detection of multiple quanta released simultaneously. First, we analyzed cells treated with botulinum toxin, PI3-K inhibitors or elevated EGTA postsynaptically, to remove any confounding effects of the NA-mediated insertion of AMPARs on the amplitude distributions (Gordon et al., 2005). While these treatments would be unlikely to interfere with, or facilitate, a presynaptic mechanism, they could provide a means for examining an isolated synchronization process. Second, we utilized a stringent selection procedure to focus our analysis on cells exhibiting a putative single population of events. Only cells exhibiting a unimodal event rise-time and decay-time histogram in control were used for analysis (figure 5.3 A), while cells exhibiting bimodal or trimodal rise-time and decay-time histograms where excluded (figure 5.3 B). We reasoned that, kinetically dissimilar mEPSCs might possess dissimilar mEPSC amplitudes, giving rise to multiple peaks in the amplitude histogram that do not result from the synchronous release of vesicles (Wahl et al., 1995). Under these conditions, amplitude histograms in control were unimodal exhibiting a characteristic rightward skew. In the presence of NA the skew was greatly enhanced with the emergence of new equidistant modes (Figure 5.4 A, B), where putatively mode 1 represents the response from a single quantum and successive modes represent the response from summed quanta. Each mode was fit by a Gaussian curve where the mean separation between modes equalled the quantal size q (13.15 ± 1.26 pA, n=10). However, the average mode standard deviation was 32.5% of the q value, much higher than values previously reported demonstrating unequivocal modes (Wall and Usowicz, 1998). The standard deviation of each mode did not increase linearly with mode number (mode 1: 4.26 ± 0.41, mode 2: 4.30± 0.78, mode 3: 4.52 ± 0.63, mode 4: 6.10 ± 0.48), a result unlike that of the neuromuscular junction (Katz, 1966) but in line with several CNS observations (Edwards et al., 1990;Larkman et al., 1991;Paulsen and Heggelund, 1994;Min and Appenteng, 1996;Wall and Usowicz, 1998). Upon wash out of NA, the multi-peaked amplitude distribution collapsed to the control form (figure 5.4 C). By contrast, in cells in which postsynaptic insertion was not blocked, NA caused a rightward spread of the amplitude distribution without the clear appearance of multiple

Figure 5.3 MNCs possess kinetically homogenous or heterogeneous mEPSCs.

A, Left, The 10-90 rise-time histogram; right, the 10-90 decay-time histogram from a single cell. The distributions possess a single mode, suggesting that the mEPSCs are kinetically homogenous and result from a putative single population of synapses. **B**, Left, The 10-90 rise-time histogram; right, the 10-90 decay-time histogram from a different cell. The distributions clearly possess more than one mode, suggesting that the mEPSCs in this cell are kinetically heterogeneous and putatively result from different populations of synapses. Cells displaying characteristics shown in *A* were chosen for analyses; cells exhibiting characteristics shown in *B* were discarded.

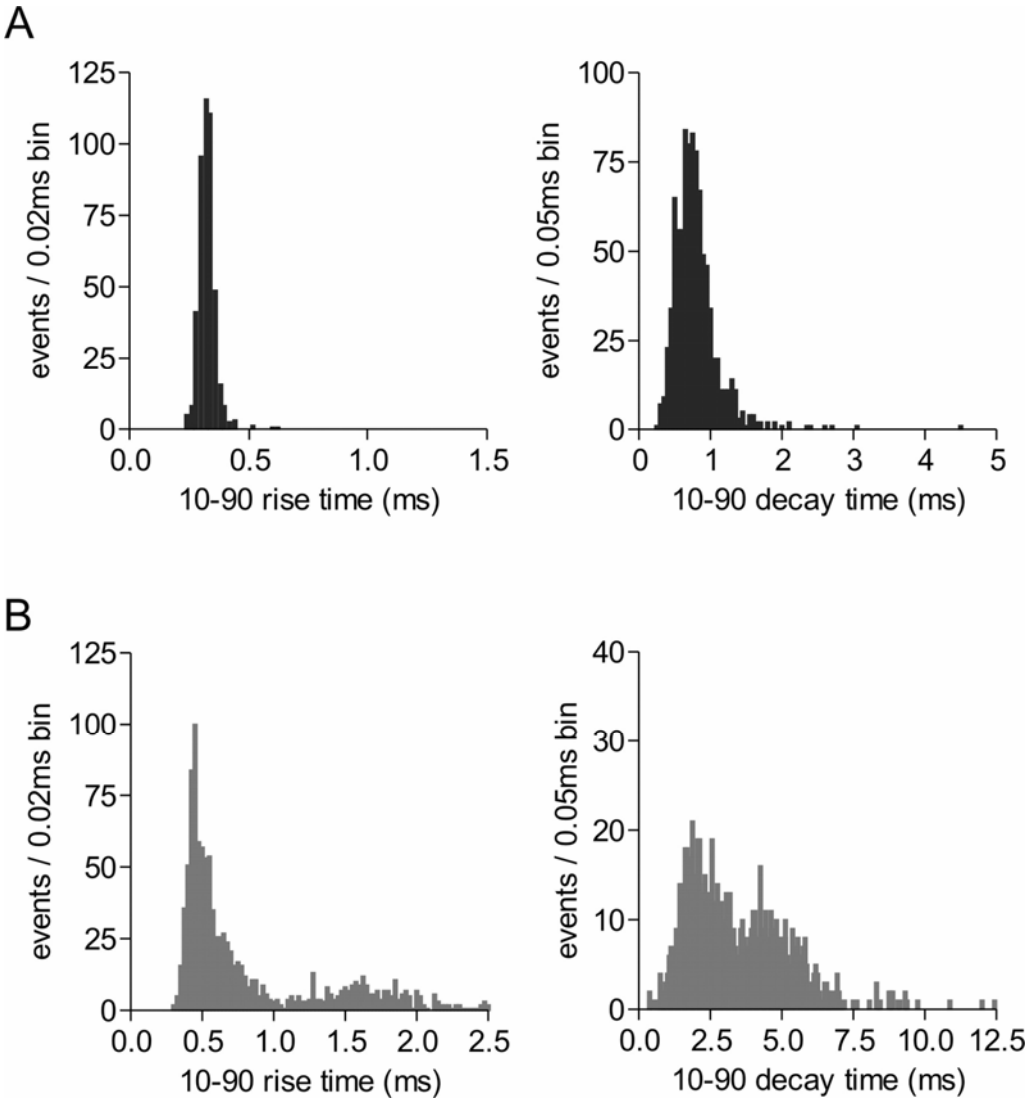
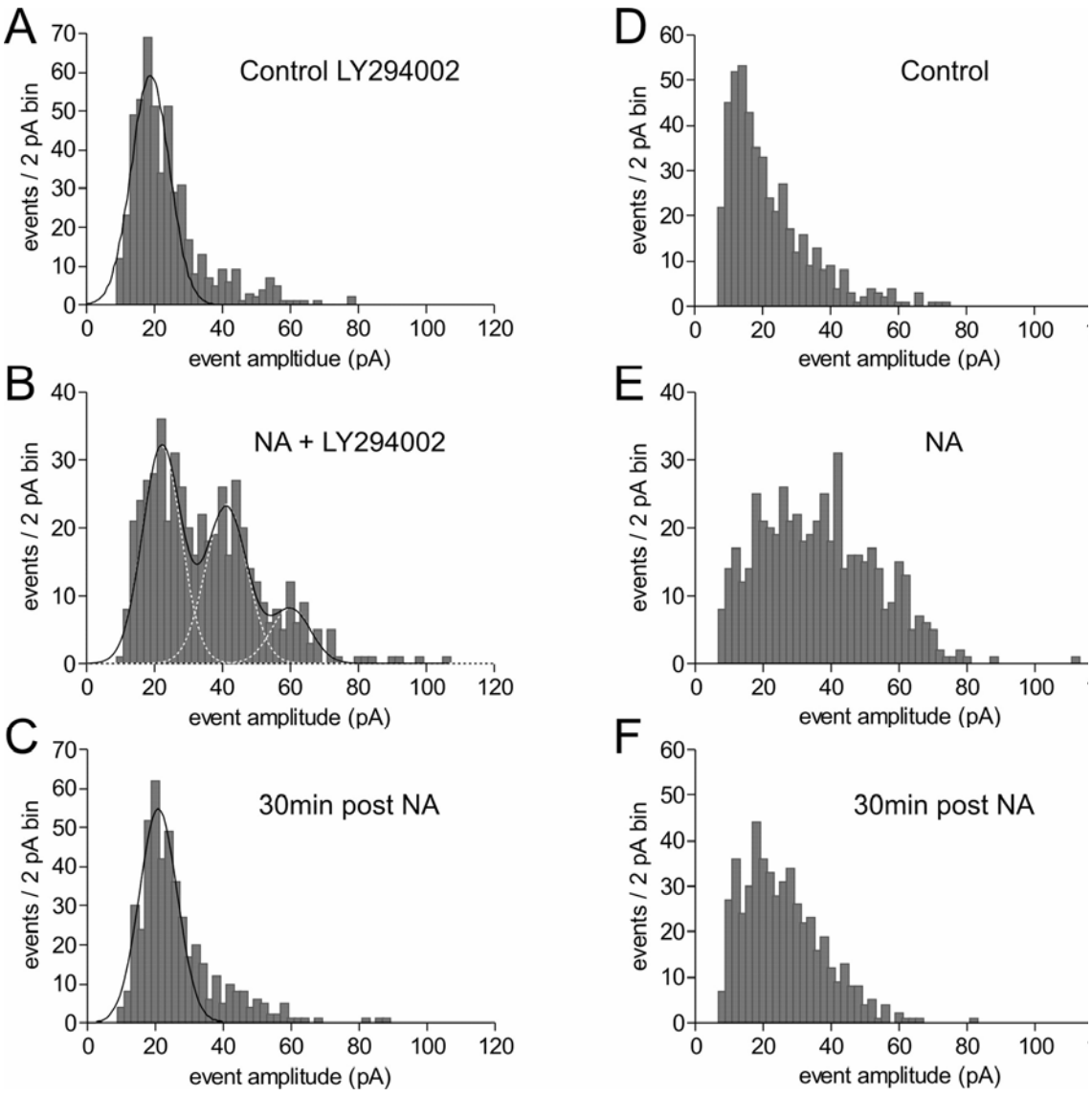

A
125
100
75
50
25
0
events / 0.02ms bin
0.0
0.5
1.0
1.5
10-90 rise time (ms)
100
75
50
25
0
events / 0.05ms bin
0
1
2
3
4
5
10-90 decay time (ms)
B
125
100
75
50
25
0
events / 0.02ms bin
0.0
0.5
1.0
1.5
2.0
2.5
10-90 rise time (ms)
40
30
20
10
0
events / 0.05ms bin
0.0
2.5
5.0
7.5
10.0
12.5
10-90 decay time (ms)

Figure 5.4 The mEPSC amplitude distribution in NA displays putative equidistant modes.

A, Control mEPSC amplitude distribution displaying a characteristic rightward skew, which cannot be fit well with a single Gaussian function. **B**, When postsynaptic AMPAR insertion is blocked with the phosphatidylinositol-3 kinase inhibitor LY294002, NA enhances the skew in the amplitude distribution by the appearance of putative peaks of equal amplitude increments; same cell as *A*. **C**, Thirty minutes after NA (30 min post NA) treatment, the amplitude distribution returns to the control state; same cell as *A*. **D**, Control mEPSC amplitude distribution. **E**, In the absence of postsynaptic blockade, NA increases the amplitude distribution skew without the clear appearance of multiple modes; same cell as *D*. **F**, Thirty minutes after NA treatment, the amplitude distribution remains slightly shifted to the right, indicative of the postsynaptic enhancement caused by NA; same cell as *D*.


A
Control LY294002
events / 2 pA bin
event ampltidue (pA)
B
NA + LY294002
events / 2 pA bin
event amplitude (pA)
C
30min post NA
events / 2 pA bin
event amplitude (pA)
D
Control
events / 2 pA bin
event amplitude (pA)
E
NA
events / 2 pA bin
event amplitude (pA)
F
30min post NA
events / 2 pA bin
event amplitude (pA)

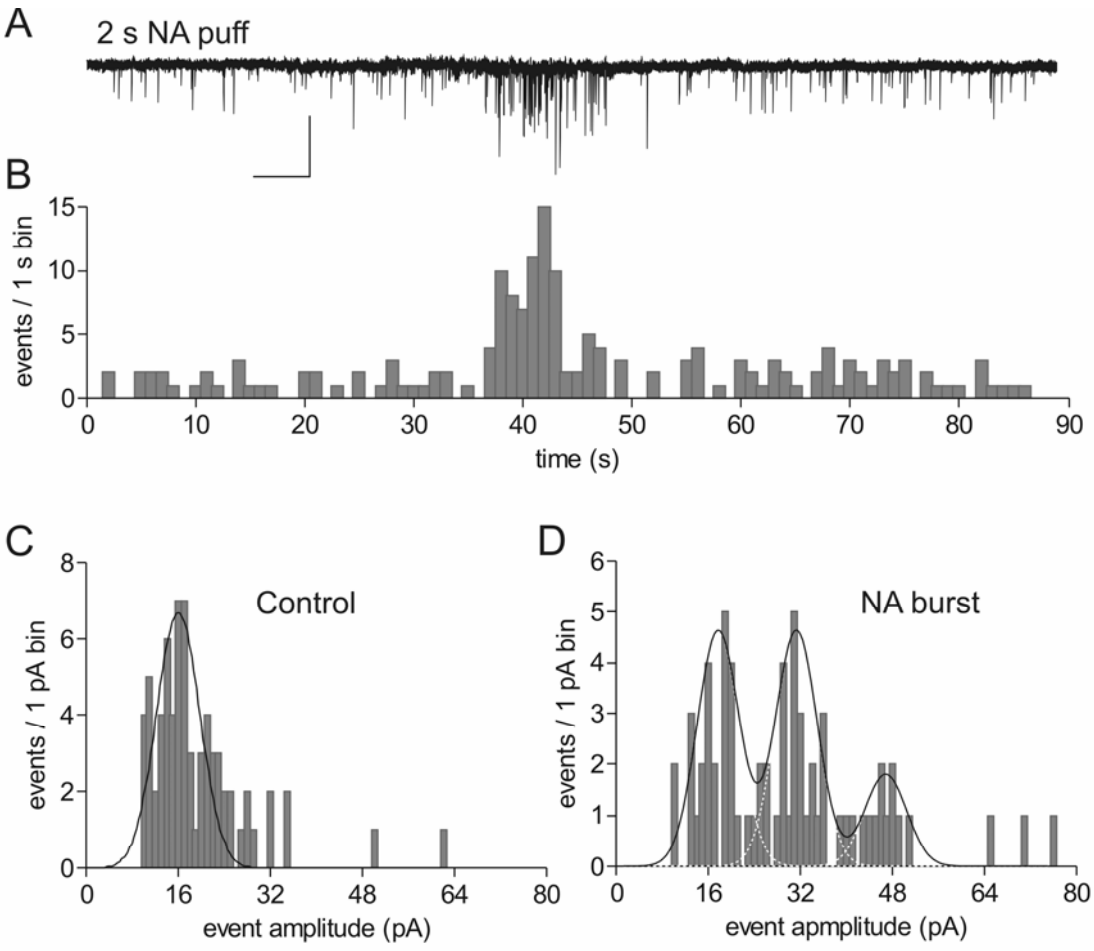
modes (figure 5.4 D, E). Furthermore, when assessed 30min post NA, the amplitude distribution remained slightly shifted towards larger mEPSCs compared to the control amplitude distribution (figure 5.4 F), indicative of the long-lasting postsynaptic enhancement caused by NA (Gordon et al., 2005).

If the action of NA on presynaptic terminals can synchronize the release of vesicles, extended application of NA may not be absolutely necessary and a more subtle technique may yield a similar result. Therefore, we hypothesized that a very brief, localized application of NA might elicit short bursts of mEPSCs, and that a single burst would yield a multi-modal amplitude distribution. To test this hypothesis, an electrode containing 100μM NA was placed near the dendritic region of the magnocellular neurons and NA was picospritzed with a slow pressure pulse for approximately 2 s. Using this technique we observed bursts of mEPSCs in 6 of 12 cells tested in this manner (figure 5.5 supplementary). The bursts were short in duration (5.37 ± 1.18 s, n=6), and showed a large fractional increase in mEPSC frequency (18.09 ± 3.9, n=6, $p<0.01$) and amplitude (1.45 ± 0.17, n=6, $p<0.01$). The control amplitude distributions displayed a single mode that was skewed to the right, while the distributions generated from mEPSCs that comprised a burst were multi-modal with an average q value closely resembling that calculated for the experiments using bath application of NA (q = 15.50 ± 1.63 pA, n=6, figure 5.5 supplementary). These data support the hypothesis that NA promotes the release of multiple vesicles and suggest that a rapid, localized action of NA can produced a result similar to that observed with bath application of the drug.

While these data are consistent with the release of multiple vesicles for the transient increase in mEPSC amplitude, the enhanced skew in the amplitude distribution during NA may be equally well explained by the unveiling of a new synapse (Kohara et al., 2001) or vesicle (Bruns et al., 2000;Henze et al., 2002) that confers large amplitude mEPSCs. The skew may also be explainedby the random temporal summation of individual events due to the enhanced mEPSC frequency (Gordon and Bains, 2003), yielding true multiple peaks that do not result fro m a concerted synchronization process. We attempted to examine each of these possibilities below.

Figure 5.5 supplemental. NA-induced bursts of mEPSCs display multimodal amplitude histograms.

A, Voltage clamp trace depicting a short burst of mEPSCs after a 2 s focal application of NA into the dendritic region of the magnocellular neurons. **B**, Frequency plot of mEPSCs from the same cell in A using the same time scale. The frequency of mEPSCs in the burst is many fold higher than the background frequency. **C**, Control amplitude distribution from the same cell above; 70 events. **D**, Amplitude distribution generated from all of the mEPSCs in the NA-induced burst; 70 events. From these two distributions one can see a single mode in the control data and the addition of multiple, equidistant modes in the NA-induced burst. Scale bars: 50pA, 5s


A
2 s NA puff
B
events / 1 s bin
time (s)
C
Control
events / 1 pA bin
event amplitude (pA)
D
NA burst
events / 1 pA bin
event apmplitude (pA)

5.3.4 The unveiling of a new synapse or vesicle does not account for the increase in mEPSC amplitude observed in NA

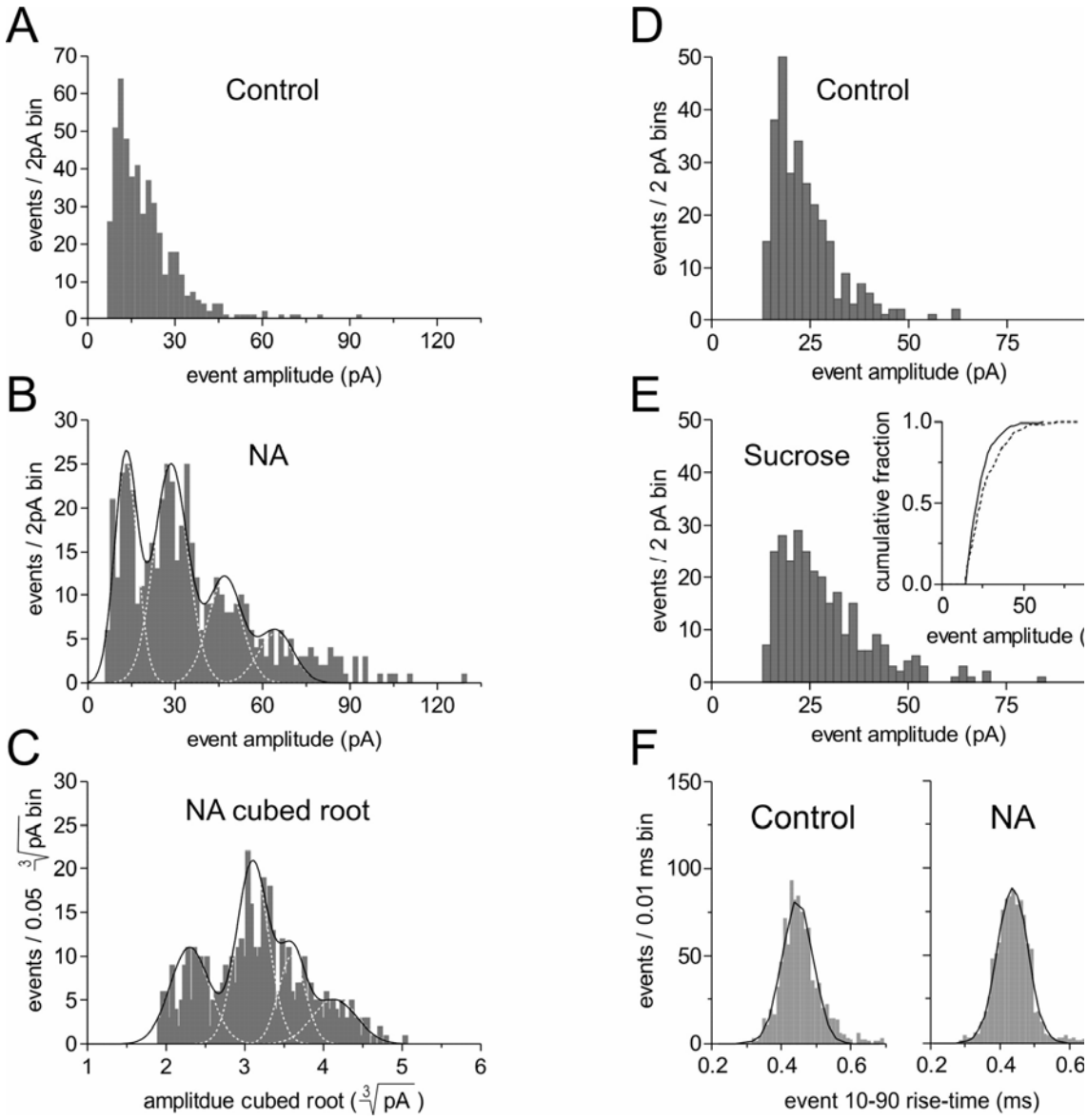
We employed an analysis to examine the possibility that NA recruits larger vesicles containing more glutamate, which was based on the demonstrations that the size of individual neurotransmitter vesicles at nerve terminals is not uniform (Karunanithi et al., 2002) and that the amplitude distribution of mEPSCs varies with the third power of vesicle radius (Bekkers et al., 1990). If the increase in mEPSC amplitude in response to NA is due to the preferential release of larger vesicles containing more glutamate, the relationship between the cubed-root of the individual mEPSC amplitudes in NA and the resulting transformed distribution should be Gaussian (Bruns et al., 2000). The cubed-root transform however, failed to collapse the highly skewed amplitude distribution to one that was Gaussian (n=5, figure 5.6 A-C).

If the transient increase in mEPSC amplitude observed during NA occurs by bringing a previously quiescent synapse 'on-line' or again by triggering the release of previously quiescent vesicle, then the action of a secretagogue should mimic the effect of NA. For this we utilized sucrose, which has been used extensively as a hyperosmotic stimulus to trigger release (Bekkers and Stevens, 1995). Bath application of sucrose (3min, 500mM) did significantly increase the amplitude of mEPSCs (1.15 ± 0.02, $p<0.01$, n=5, figure 5.6 D, E) but this effect was significantly less than that observed during NA. Moreover, we have previously reported that globally increasing release probability with phorbol esters also fails to increase mEPSC amplitude to the same extent as NA (Gordon and Bains, 2003).

Finally, if NA triggers the activation of a synapse that yields large, kinetically dissimilar mEPSCs compared to control, this might be detected by the emergence of an additional mode in the 10-90 rise-time distribution. Utilizing our selection process to focus the analysis on a putative single population of events, we failed to detect a distinct peak in the resulting 10-90 rise-time distribution following NA (n=6, figure 5.6 A). In fact, the standard deviation of the rise time histogram was reduced slightly in NA (0.87 ±

Figure 5.6 Large mEPSCs in NA do not result from a previously quiescent synapse or vesicle.

A, Control mEPSC amplitude distribution. **B**, NA amplitude distribution fit with multiple Gaussians and a sum of Gaussians function; same cell as *A*. **C**, The cubed root transform of the NA amplitude distribution in *B* remains multimodal, suggesting that the release of larger vesicles by NA cannot account for the prominent skew ($n = 5$). **D**, Control mEPSC amplitude distribution. **E**, The secretagogue sucrose fails to increase mEPSC amplitude to the same extent as NA. This is shown by a small rightward shift in the amplitude distribution and the cumulative fraction plot of mEPSC amplitudes (inset); same cell as *D*. **F**, The 10-90 rise-time histogram in control (left) remains unimodal in NA (right), suggesting that kinetically dissimilar events are not recruited during NA.


A
Control
events / 2pA bin
event amplitude (pA)
B
NA
events / 2pA bin
event amplitude (pA)
C
NA cubed root
events / 0.05 $\sqrt[3]{\text{pA}}$ bin
amplitdue cubed root ($\sqrt[3]{\text{pA}}$)
D
Control
events / 2 pA bins
event amplitude (pA)
E
Sucrose
events / 2 pA bin
cumulative fraction
event amplitude (pA)
F
Control
NA
events / 0.01 ms bin
event 10-90 rise-time (ms)

0.03, $p<0.05$, n=6). Collectively, these data suggest that the transient increase in mEPSC amplitude caused by NA does not result from the activation of a previously quiescent synapse or vesicle.

5.3.5 The increase in mEPSC amplitude does not occur via random event summation.

As NA results in an enhanced mEPSC frequency (Gordon and Bains, 2003) and multi-modal amplitude distribution, we investigated the contribution of random temporal summation as an explanation for the transient increase in mEPSC amplitude. We tested for a positive correlation between fractional increases in mEPSC frequency and fractional increases in mEPSC amplitude. In ten randomly selected cells, there was no correlation between frequency and amplitude (slope= -0.013 ± 0.018, $r^2=0.061$, figure 5.7A).

We then calculated the probability of random event overlap in two ways: by $p = 1-e^{t/\tau}$ where t is average event half-width and τ is the decay time constant from the mono-exponential equation fit to binned)t mEPSC data, ()t is the inter-event interval, figure 6B) and by $p = t/avg$)t where *avg*)t is average inter-event interval over one second (Hz in Table 1). These two methods represent the lower limit of event summation probability as each assumes that no two events have summed, making the)t values used in each calculation as large as possible (Table 1). We then estimated an upper limit of event summation probability. Utilizing the putative multimodal amplitude distribution and assuming that every event in a given mode is actually the sum of individual events, we can approximate the total number of individual events in a given amplitude distribution by deconstructing the modes to achieve the smallest *avg*)t value possible (figure 5.7 D)(see methods). Regardless of the method utilized, our analyses indicate that the probability of individual events randomly overlapping was much lower than the fraction of mEPSCs greater than the first mode in the amplitude distributions for both control and NA (Table 1), suggesting that large mEPSCs are not the result of random temporal summation.

We examined the rise-time of individual mEPSCs to see if large mEPSCs in NA were the result of summed quanta. First, we used high sampling (40kHz) and filtering

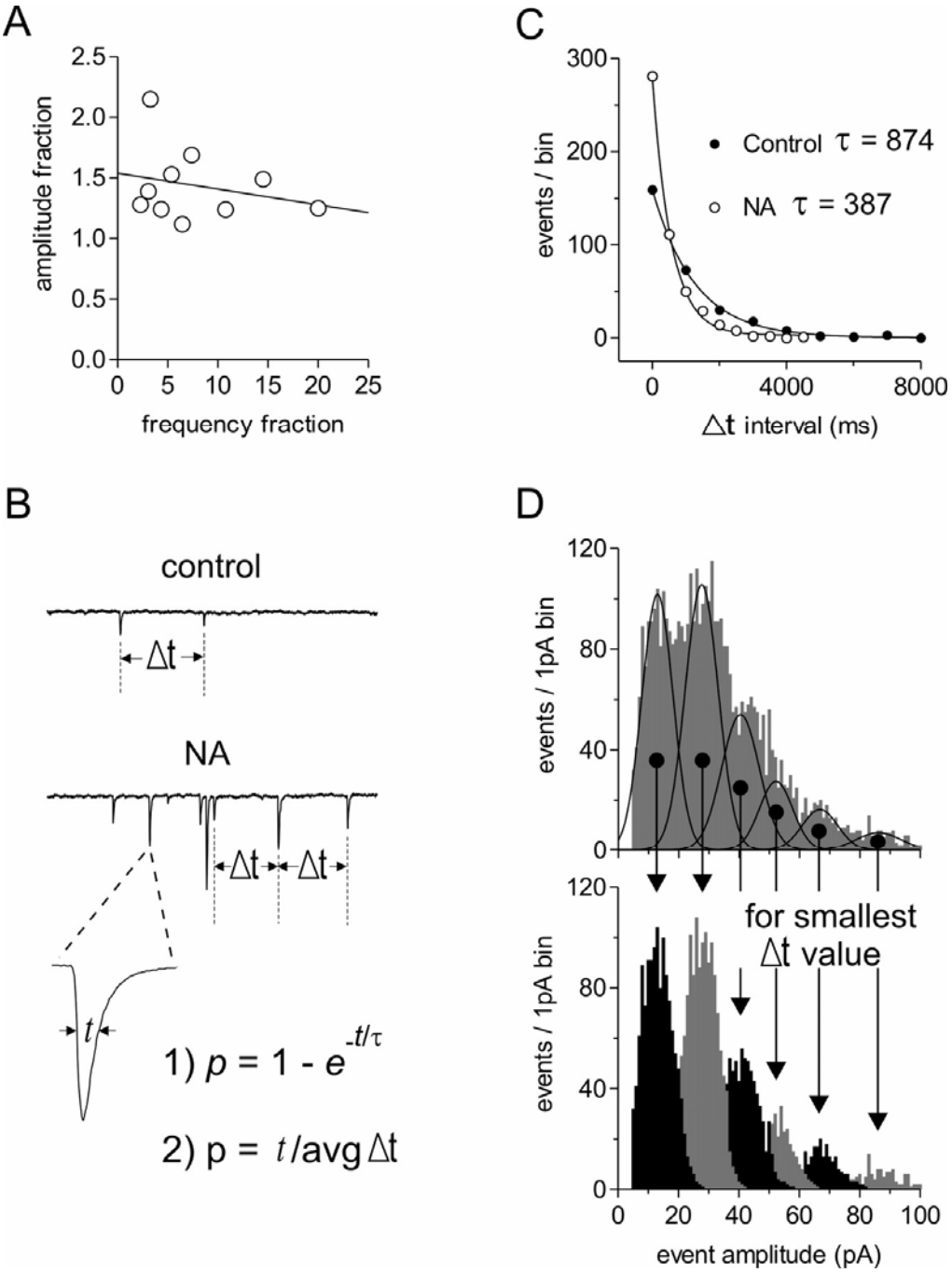
(2kHz) rates to facilitate the detection of inflections, which would be indicative of individual quanta 'stacking' on top of one another (Williams et al., 1998;Wall and Usowicz, 1998). In this analysis, however, even the largest mEPSCs displayed smooth rising phases without visible inflections (data not shown), an observation that is not consistent with summation. Second, we examined mEPSCs for a positive rise-time versus amplitude relationship. This has been interpreted as being consistent with a slight asynchrony during the release of multiple vesicles (Williams et al., 1998;Wall and Usowicz, 1998;Sharma and Vijayaraghavan, 2003). After ensuring our normal filtering and sampling rates did not produce an artificial positive rise time versus amplitude relationship to square current responses on a model cell (data not shown), we observed that the largest mEPSCs in NA were also the slowest. However, the slope of the rise-time versus amplitude relationship in control and NA was not significantly different (control slope = 0.80 ± 0.08, R^2 = 0.92; NA slope = 0.87 ± 0.09; R^2 = 0.94), suggesting that the rate-limiting step governing mEPSC kinetics at a given population of synapses occurs downstream of vesicle exocytosis (Llano et al., 2000).

5.3.6 The control amplitude distribution skew results from the release of larger vesicles

In addition to synapse geometry (Kruk et al., 1997) and postsynaptic response variability (Faber et al., 1992), there has been strong support for a presynaptic mechanism explaining the skew in the control miniature amplitude distribution at central synapses (Frerking et al., 1995;Forti et al., 1997;Liu et al., 1999;McAllister and Stevens, 2000;Hanse and Gustafsson, 2001;Franks et al., 2003). Two popular hypotheses are 1) the skew results from an endogenous synchronization process (Ulrich and Luscher, 1993;Paulsen and Heggelund, 1994;Llano et al., 2000) and 2) it results form variations in vesicle neurotransmitter content (Bruns et al., 2000;Karunanithi et al., 2002). To examine the first hypothesis we tested for endogenous ryanodine-sensitive Ca^{2+} bursts from intracellular stores, which are capable of synchronizing the release of multiple vesicles (Llano et al., 2000). For this we analyzed the amplitude of mEPSCs in response to: removal of extracellular Ca^{2+}, which should rundown the Ca^{2+} supply from intracellular stores that are continually active (Llano et al., 2000), BAPTA-AM

Figure 5.7 The increase in mEPSC amplitude by NA does not occur via random event summation.

A, The NA-induced fractional increase in mEPSC frequency is not related to the corresponding fractional increases in mEPSC amplitude (slope = -0.013 ± 0.018; R^2 = 0.061; n = 10). **B**, Schematic depicting Δt, the interevent interval, t, the average event half-width, and the two formulas used to calculate the probability of random temporal summation. **C**, Time interval between events is fit with a monoexponential equation in control (t = 874 ms) and in NA (t = 387 ms), and the probability of two mEPSCs summating was calculated with $p_{(\text{time interval} \leq t)} = 1 - e^{-t/\tau}$ in a single cell (control, p = 0.0016; NA, p = 0.0037) (Table 1). **D**, Amplitude histogram in NA fit with multiple Gaussian curves (top) showing the deconstruction of the raw data into the putative modes (bottom). The number of observations in a given mode after deconstruction is multiplied by the mode number to achieve the maximum number of quantal events that could have occurred in that time period, making the Δt value as small as possible to calculate the upper limit of event summation probability (Table 1).


A
amplitude fraction
frequency fraction
C
events / bin
Control τ = 874
NA τ = 387
Δt interval (ms)
B
control
Δt
NA
Δt
Δt
t
1) p = 1 - e^-t/τ
2) p = t/avg Δt
D
events / 1pA bin
for smallest
Δt value
event amplitude (pA)

	mEPSC Half-Width t (ms)	Calculated lower limit using $p=1-e^{-t/\tau}$ assuming no summation present		Calculated lower limit using $p=t/avg\Delta t$ assuming no summation present		Estimated upper limit using $p=t/avg\Delta t$ assuming summation present		Fraction of mEPSCs > mode 1 from amplitude distribution
		τ (ms)	Probability	$avg\Delta t$/sec (Hz)	Probability	$avg\Delta t$/sec (Hz)	Probability	
Control	1.93 +/- 0.14	582.5 +/- 180.7	**0.0056** +/- 0.0024	2.67 +/- 0.79	**0.0053** +/- 0.0016	3.77 +/- 0.78	**0.0074** +/- 0.0017	**0.339** +/- 0.086
Noradrenaline	2.21 +/- 0.14	154.8 +/- 64.14	**0.0226** +/- 0.0089	11.21 +/- 3.61	**0.0259** +/- 0.0087	25.67 +/- 8.86	**0.0595** +/- 0.0212	**0.646** +/- 0.047

Table 1. Calculating the probability of random mEPSC summation in control and NA

(50 μM), a lipid permeable Ca^{2+} chelator with fast binding kinetic, and a high-dose of ryanodine (100μM)(Llano et al., 2000;Sharma and Vijayaraghavan, 2003), which blocks these receptor/channels thereby preventing Ca^{2+} release from intracellular stores. These manipulations however, failed to attenuate mEPSC amplitude when assessed 20 minutes after the beginning of treatment (pooled data: 0.96 ± 0.02, $p>0.05$, n=14, figure 5.8 A-C).

To test the second hypothesis, we performed an analysis examining the amplitude distribution skew in control for a relationship to vesicle radius (Bruns et al., 2000). Taking the cubed root of the raw control amplitudes resulted in a transformed distribution that was fit well by a Gaussian curve (R^2=0.913 ± 0.018, n=5, figure 5.8 D). These data suggest that the skew in the mEPSC amplitude distribution observed in control is not the result of a store-mediated synchronization process but instead results from larger vesicles containing more glutamate yielding larger mEPSCs.

5.3.7 Presynaptic Ca^{2+} stores are responsible for the transient increase in mEPSC amplitude

As there was no Gaussian relationship between the cubed root of the NA mEPSC amplitudes (figure 5.6), we asked whether the recruitment of a store-mediated synchronization process might account for the large increase in mEPSC amplitude observed during NA. We first tested for a rise in presynaptic Ca^{2+} levels. In the presence of BAPTA-AM (50μM), the increase in mEPSC amplitude caused by NA was completely abolished (1.04 ± 0.04, $p>0.05$, n=4 figure 5.9 A, E). We could not mimic this effect with EGTA-AM (50μM)(data not shown), although we cannot determine if this negative outcome is the result of slower Ca^{2+} binding kinetics or unsuccessful loading of the EGTA-AM. To test for the presence of presynaptic Ca^{2+} stores, we applied caffeine (5mM) to trigger Ca^{2+} release (Sharma and Vijayaraghavan, 2003). If stores were present, this treatment could result in an elevated mEPSC frequency without necessarily mimicking rapid Ca^{2+} bursts and the subsequent increase in mEPSC amplitude observed with NA (Llano et al., 2000). In accordance with this hypothesis, caffeine increased mEPSC frequency (4.05 ± 1.35, $p < 0.01$, $n = 5$, figure 5.9 B, F) but failed to increase mEPSC amplitude (1.01 ± 0.06, $p > 0.05$, $n = 5$, figure 5.9 E).

Figure 5.8 The control distribution skew does not result from endogenous synchronization but likely results from the release of larger vesicles containing more glutamate.

Bath application of Ca^{2+}-free extracellular solution (**A**) BAPTA-AM (50 μM) (**B**), a lipid permeable Ca^{2+} chelator with fast binding kinetics, or a high dose of ryanodine (100 μM) (**C**), which blocks ryanodine receptor-channels, failed to attenuate mEPSC amplitude when assessed 20 min after the beginning of treatment (pooled data; 0.96 ± 0.02; $p > 0.05$; $n = 14$) (**A-C**). **D**, The cubed root transform (right) of the control amplitude distribution (left) is Gaussian ($n = 5$), suggesting that vesicles containing more glutamate underlie the control amplitude distribution skew.

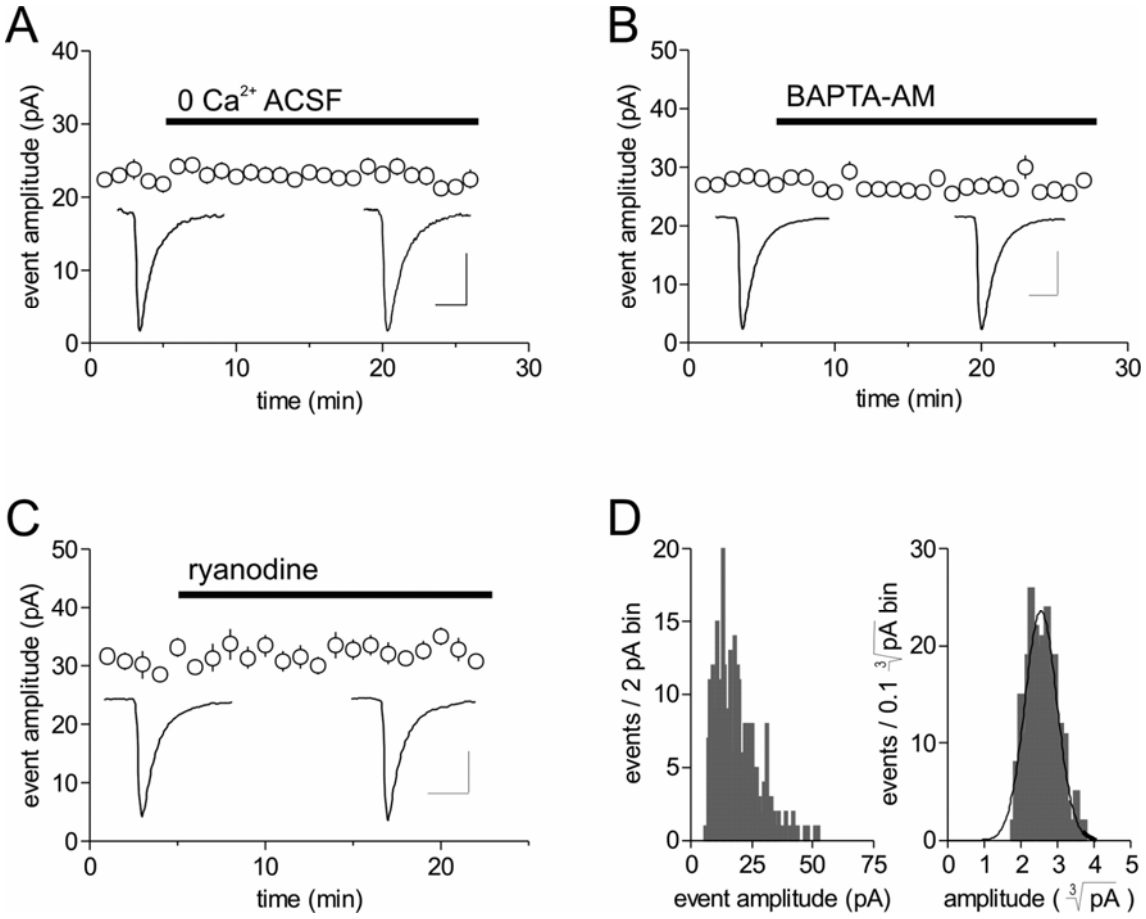
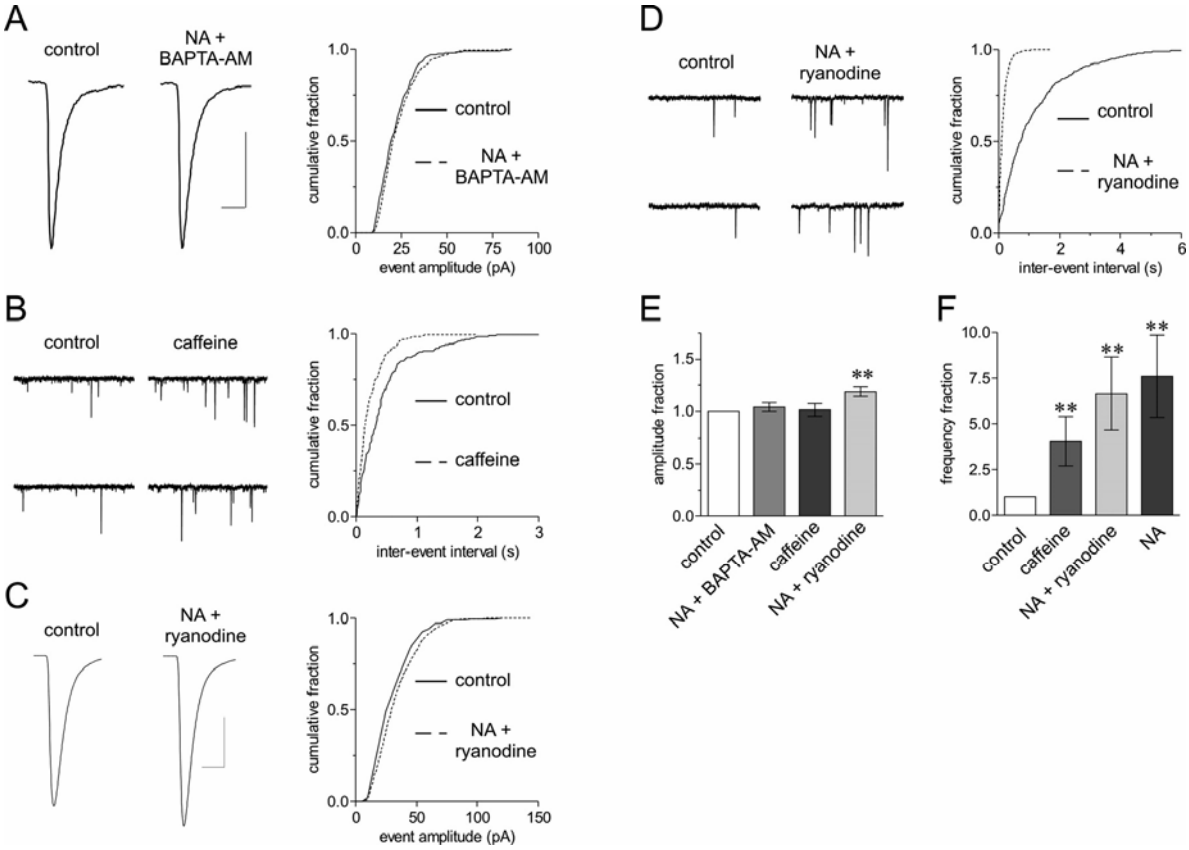

A
0 Ca2+ ACSF
event amplitude (pA)
time (min)
B
BAPTA-AM
event amplitude (pA)
time (min)
C
ryanodine
event amplitude (pA)
time (min)
D
events / 2 pA bin
event amplitude (pA)
events / 0.1 ∛pA bin
amplitude (∛pA)

Figure 5.9 Presynaptic Ca^{2+} stores mediate NA-induced vesicle synchronization.
A, An incubation period of >20 min with BAPTA-AM (50 μM) completely blocked the increase in mEPSC amplitude observed in NA, shown by average mEPSC traces (right) and an amplitude cumulative fraction plot (left). **B**, Caffeine treatment (5 mM) increases the frequency of mEPSCs as shown by representative voltage-clamp traces (left) and an interevent interval cumulative fraction plot (right). **C**, A high dose of ryanodine (100 μM) significantly reduces the amplitude increase of mEPSCs in response to NA shown by average mEPSC traces (left) and an amplitude cumulative fraction plot (right). **D**, Ryanodine does not block the increase in mEPSC frequency caused by NA, shown by representative voltage-clamp traces (left) and an interevent interval cumulative faction plot (right). **E**, Summary data showing the effects of BAPTA-AM plus NA (1.04 ± 0.04; $p > 0.05$; $n = 4$), caffeine (1.01 ± 0.06; $p > 0.05$; $n = 5$), and ryanodine plus NA (1.18 ± 0.04; $^{**}p < 0.01$ versus control; $n = 6$) on mEPSC amplitude. **F**, Summary data showing the effects of caffeine (4.05 ± 1.35; $^{**}p < 0.01$ versus control; $n = 5$), ryanodine plus NA (6.65 ± 2.00; $^{**}p < 0.01$ versus control; $n = 6$), and NA alone (7.63 ± 2.25; $^{**}p < 0.01$ versus control; $n = 20$) on mEPSC frequency.


A
control
NA + BAPTA-AM
cumulative fraction
control
NA + BAPTA-AM
event amplitude (pA)
B
control
caffeine
cumulative fraction
control
caffeine
inter-event interval (s)
C
control
NA + ryanodine
cumulative fraction
control
NA + ryanodine
event amplitude (pA)
D
control
NA + ryanodine
cumulative fraction
control
NA + ryanodine
inter-event interval (s)
E
amplitude fraction
control
NA + BAPTA-AM
caffeine
NA + ryanodine
F
frequency fraction
control
caffeine
NA + ryanodine
NA

We then tested for the involvement of intracellular Ca^{2+} stores in the NA-induced increase in mEPSC amplitude by blocking Ca^{2+}-releasing ryanodine receptor/channels with a high dose of ryanodine (100μM) (Llano et al., 2000;Sharma and Vijayaraghavan, 2003). In the presence of ryanodine the increase in mEPSC amplitude seen during NA was attenuated (1.18 ± 0.04, $p<0.01$ compared to control, n=6, figure 5.9 C, E)($p<0.01$ when compared to NA alone), suggesting that Ca^{2+} release from stores is critical for the NA effect. To examine the specificity of the ryanodine block, we analysed the change in mEPSC frequency during NA, which occurs through a separate α_1-adrenoceptor mediated mechanism and therefore, should not be greatly affected by this compound (Gordon and Bains, 2003). Consistent with this idea, the increase in mEPSC frequency observed during NA in ryanodine (6.65 ± 2.00, $p<0.01$, n=6, figure 5.9 D, F) was not different ($p>0.05$) from NA alone (7.63 ± 2.25, $p<0.01$, n=20) These data support the hypothesis that rapid release of Ca^{2+} from intracellular stores is necessary for the transient increase in mEPSC amplitude observed during NA.

5.3.8 Larger mEPSCs in NA are associated with an increase in glutamate cleft concentration

While our experiments and the aforementioned analyses favour the fusion of multiple vesicles, they fail to demonstrate whether there is more neurotransmitter available in the synaptic cleft to bind to postsynaptic receptors. To determine whether activation of the metabotropic, presynaptic α_1 adrenoceptor can increase glutamate in the cleft, we compared the effects of a high affinity AMPAR antagonist, DNQX, with the effects of a low affinity antagonist, γDGG. In contrast to DNQX, γDGG has a transient receptor 'bound-time' resulting in a less effective block as glutamate concentration increases as predicted by the laws of mass action (Liu et al., 1999). A low dose of DNQX (250nM) uniformly blocked mEPSCs representing the median and the 95th percentile in NA (figure 5.10 A), as well as the entire amplitude distribution for both control ($p>0.05$) and NA ($p>0.05$, figure 5.10 C). In contrast, γDGG (500μM) blocked smaller mEPSCs more effectively than larger mEPSCs. This is depicted in the traces (figure 5.10 B) and the normalized cumulative graphs (control: $p<0.05$, NA: $p<0.01$, figure 5.10 E), in which the

greatest disparity is observed in the presence of NA. This idea is further substantiated by the demonstration that the fractional mEPSC amplitude during γDGG application was larger in the presence of NA (0.81 ± 0.03, n=6) than in either control (0.65 ± 0.03, n=5, $p<0.01$) or post NA (0.62 ± 0.02, $p<0.01$, n=5, figure 5.10 F, see also supplementary figure 5.11). In the latter case, the increase in mEPSCs amplitude is the result of a postsynaptic enhancement of AMPARs (Gordon et al., 2005), and as such would not be expected to be less sensitive to (DGG. Importantly, these differential effects were not observed during low dose DNQX (NA: 0.77 ± 0.03, n=5; control: 0.85 ± 0.03, n=5, $p>0.05$, figure 5.10 D). These data demonstrate that increases in the concentration of glutamate available to bind postsynaptic AMPARs contribute to the presence of large mEPSCs observed in NA.

Figure 5.10 The increase in mEPSC amplitude is accompanied by a greater concentration of glutamate in the synaptic cleft.

A, Voltage-clamp traces from the median and 95th percentile of amplitude distributions in NA and NA plus DNQX (250 nM), a high-affinity AMPAR antagonist. DNQX uniformly attenuates mEPSCs from these percentiles. **B**, Same as *A* for NA plus γDGG (500 μM), a low-affinity AMPAR antagonist. γDGG attenuates the median mEPSCs more effectively. **C**, Cumulative amplitude distribution normalized to the largest mEPSC for NA and NA plus DNQX (left), as well as control and control plus DNQX (right). DNQX uniformly attenuates all mEPSC amplitudes ($p > 0.05$). **D**, In DNQX, the fractional mEPSC amplitude was the same in NA (0.77 ± 0.03; $n = 5$) and control (0.85 ± 0.03; $p > 0.05$; $n = 5$). **E**, Cumulative amplitude distribution normalized to the largest mEPSC for NA and NA plus γDGG (left) as well as control and control plus γDGG (right). γDGG preferentially attenuated small mEPSCs ($p < 0.01$), with the greatest disparity seen in NA. **F**, In γDGG, the fractional mEPSC amplitude was larger during NA (0.81 ± 0.03; $n = 6$) than either control (0.65 ± 0.03; $p < 0.01$; $n = 5$) or after NA (0.62 ± 0.02; $p < 0.01$; $n = 5$). Calibration: 20 pA, 5 ms.

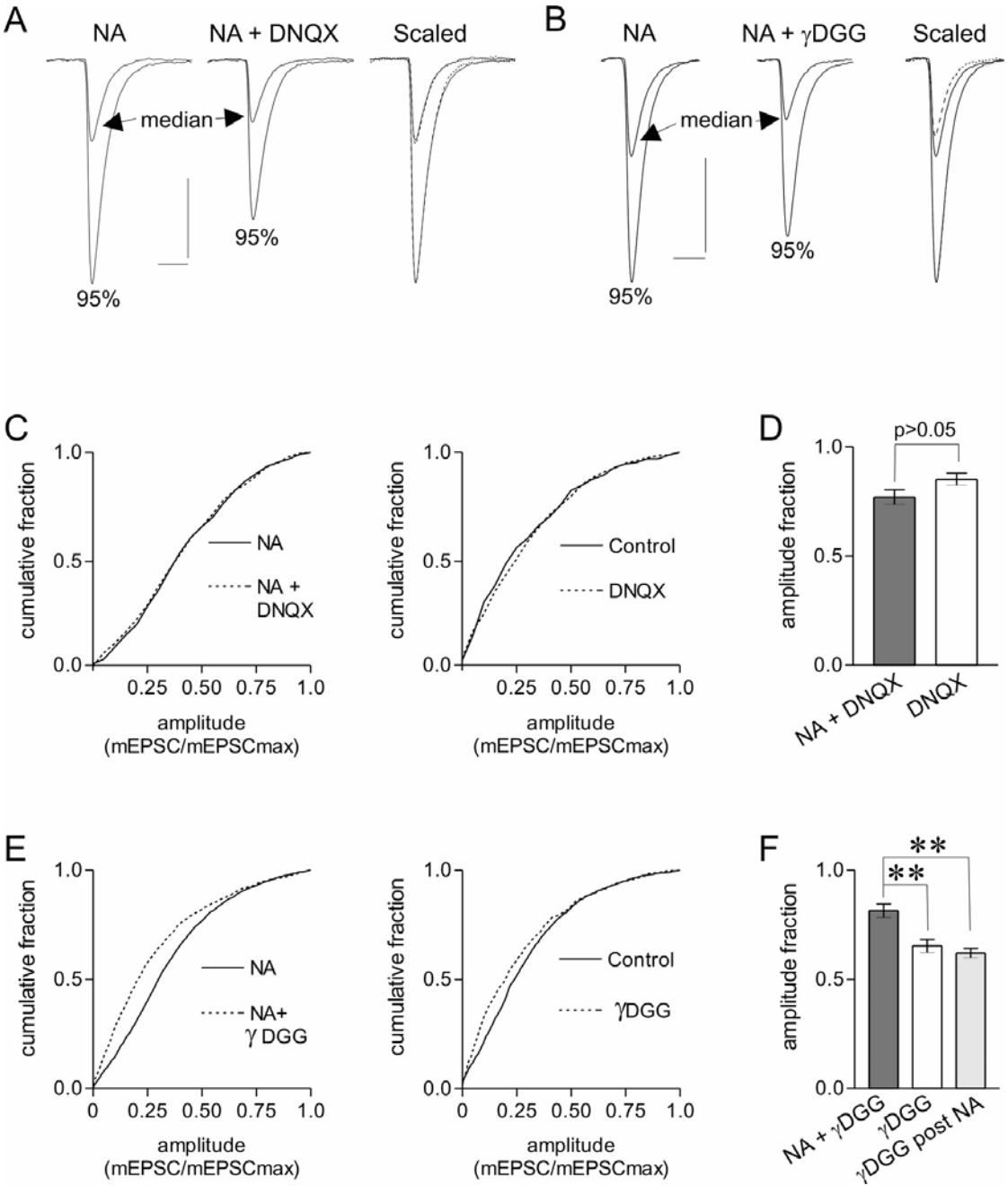
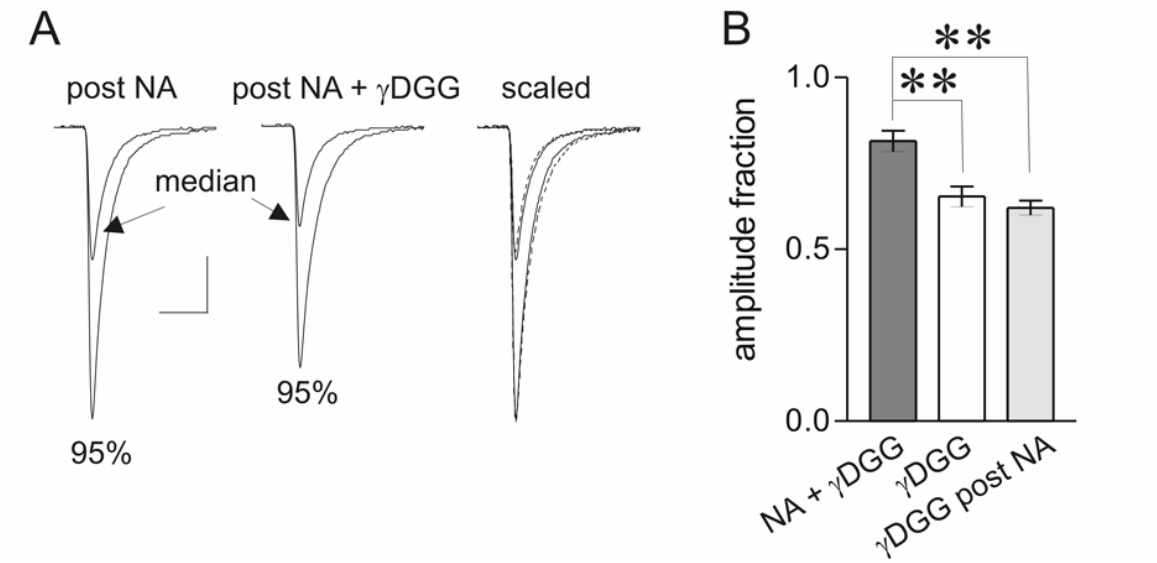

A
NA
NA + DNQX
Scaled
median
95%
95%
B
NA
NA + γDGG
Scaled
median
95%
95%
C
cumulative fraction
1.0
0.5
0.0
NA
NA + DNQX
0.25 0.50 0.75 1.0
amplitude
(mEPSC/mEPSCmax)
cumulative fraction
Control
DNQX
amplitude
(mEPSC/mEPSCmax)
D
p>0.05
amplitude fraction
NA + DNQX
DNQX
E
cumulative fraction
NA
NA+
γ DGG
0 0.25 0.50 0.75 1.0
amplitude
(mEPSC/mEPSCmax)
cumulative fraction
Control
γDGG
amplitude
(mEPSC/mEPSCmax)
F
**
**
amplitude fraction
NA + γDGG
γDGG
γDGG post NA

Figure 5.11 supplemental. Small versus large events do not display preferential attenuation by γDGG when an increase in mEPSC amplitude is conferred postsynaptically.

A, Post NA mEPSCs taken from the 50th percentile and the 95th percentile of the raw amplitudes (left). Post NA mEPSCs taken from the 50th percentile and the 95th percentile of the raw amplitudes in the presence of γDGG (500μM) (middle). Scaling the γDGG events demonstrates that (DGG shows no preferential effects on either percentile group (right). **B**, Summary bar graph from manuscript figure 9 showing the fractional amplitude of mEPSCs remaining in the presence of (DGG during NA (0.81 ± 0.03, n=6), control (0.65 ± 0.03, n=5, $p<0.01$) and post NA (0.62 ± 0.02, $p<0.01$, n=5). Scale bars: 10pA, 5ms


A
post NA
post NA + γDGG
scaled
median
95%
95%
B
**
**
1.0
0.5
0.0
amplitude fraction
NA + γDGG
γDGG
γDGG post NA

5.4 Discussion

From the data collected in our study we conclude that activation of α_1 adrenoceptors elicits a rapid and robust increase in the amplitude of mEPSCs, which is consistent with the synchronous release of multiple vesicles. This is based on several independent observations: 1) The effect was not blocked by postsynaptic treatments designed to target either α_1-adrenoceptors, intracellular Ca^{2+} handling or the insertion of AMPARs; 2) the modes in the mEPSC amplitude distributions exhibited putative equidistant modes in the presence of NA; 3) large amplitude mEPSCs do not result from the unveiling a previously quiescent vesicle or synapse as the amplitude distributions could not be collapsed by a cubed-root transform, the effects of NA were not mimicked by a secretagogue and that the emergence of a kinetically dissimilar population of events in NA was not observed 4) large amplitude mEPSCs were sensitive to fast lipid permeable Ca^{2+} buffering, as well as a high dose of ryanodine and 5) the block of large mEPSCs by γ-DGG was less effective in the presence of NA.

While both the transient and long-lasting increase in mEPSC amplitude depend on α_1-adrenoceptor activation, application of the α_1-antagonist prazosin in the presence of NA blocked only the transient effect, suggesting that synchronization relies on continual activation of the adrenergic receptor while insertion only requires brief activation. By contrast, the long-lasting, postsynaptic change, once induced by adrenoceptor activation is impervious to subsequent pharmacological block (Gordon et al., 2005). We currently have little evidence these pre and postsynaptic processes interact, only that they occur simultaneously. Without postsynaptic treatment the transient increase in mEPSC amplitude appears to be the sum of both the presynaptic and postsynaptic effects of NA. This idea is supported by the observation that the average fractional increase in mEPSC amplitude of the transient effect (~1.55) is a sum of the long-lasting effect (~1.20) and the transient effect when the long-lasting effect is blocked (~1.35).

Although the release of multiple quanta at the neuromuscular junction is thought to be responsible for the rightward skew in the amplitude distributions (Del Castillo and

Katz, 1954), it has been difficult to determine if this principle is valid at central synapses. Additional factors such as non-uniform postsynaptic responses due to inter-(Forti et al., 1997) or intra-site variance (Bekkers et al., 1990;Frerking et al., 1995) may also contribute to amplitude distributions that are skewed or exhibit multiple peaks. As we detected kinetically disparate populations of mEPSCs in control, we could minimize inter-site sources of variability with kinetic selection to examine cells exhibiting unimodal rise-time and decay-time histograms. Under these parameters, the amplitude distributions in control were still skewed, which was not the result of an endogenous synchronization process, demonstrated by an inability to affect mEPSC amplitude with extracellular Ca^{2+} removal, BAPTA-AM or a high dose of ryanodine. When we examined the control amplitude distribution for a relationship to vesicle radius (Bekkers et al., 1990;Bruns et al., 2000;Karunanithi et al., 2002), we found that the control histogram, when subjected to a cubed-root transformation, could be collapsed to a single Gaussian curve, consistent with larger vesicles containing more glutamate being responsible for this observation. While obviously not an exhaustive search for explanation of the control amplitude histogram skew, the cubed root data is also supported by the small but significant shift in the normalized cumulative fraction plot observed when control mEPSC were treated with (DGG (figure 5.10 E, right panel). This suggests that larger mEPSC in control can also possess a greater glutamate cleft concentration.

Both precautions (Wahl et al., 1995;Walmsley, 1995) and validations (Larkman et al., 1997;Stratford et al., 1997) of the use amplitude histograms for detecting the release of multiple quanta have been reported. There may be several reasons why putative equidistant modes were detected in our preparation. MNCs in the PVN and SON possess simple dendritic trees (van den Pol, 1982) and a high input resistance (1GΩ) (Tasker and Dudek, 1991;Bains and Ferguson, 1997), minimizing inter-site variability by lessening the effects of neuronal filtering on event amplitude. We also found that blocking postsynaptic AMPAR insertion helped reduce the blurring of amplitude histograms. Our data demonstrate that large amplitude mEPSCs rely on rapid Ca^{2+} release from presynaptic ryanodine-sensitive receptor/channels, a process previously demonstrated to

synchronize vesicular fusion (Llano et al., 2000). Furthermore, we observed that the percent block of mEPSC in NA by γDGG is less than the percent block of mEPSCs during control, indicating that larger mEPSCs are associated with a greater concentration of cleft glutamate (Liu et al., 1999). Collectively, these results support the observation of multi-modal histograms and are consistent with the explanation that large mEPSCs triggered by NA are the result of multivesicular release.

Experimental manipulations that increase the probability of release directly can also increase the concentration of cleft glutamate (Wadiche and Jahr, 2001). This classical experiment, which entails altering the Ca^{2+}/Mg^{2+} ratio, is not well suited for our preparation since mEPSCs in MNCs are insensitive to changes in extracellular Ca^{2+} concentration (Inenaga et al., 1998). This is further supported by our data showing that mEPSC amplitude remains unaltered 20min after extracellular Ca^{2+} removal or in the presence of BAPTA-AM. We attempted to circumvent this limitation by globally increasing release probability with hypertonic sucrose, a commonly used secretagogue (Bekkers and Stevens, 1995). This manipulation, however, failed to increase the amplitude of mEPSCs to the same extent as NA, suggesting that merely increasing the probability of a single release event has no impact on the probability of synchronizing minis, and that the unveiling of previously quiescent synapse or the preferential release of vesicles conferring large amplitude mEPSCs are also unlikely possibilities. The increase in mEPSC amplitude observed with sucrose treatment may reflect the fusion of postsynaptic vesicles containing AMPARs, as the degree of the sucrose-induced enhancement is consistent with this process in MNCs (Gordon et al., 2005). The NA-triggered insertion of new AMPARs is also be reflected in our inability to completely block the transient increase in mEPSC amplitude caused by NA in the presence of a high dose of ryanodine. This is because ryanodine should have little effect on the long-lasting enhancement of mEPSC amplitude that requires Ca^{2+} influx through ATP-gated P2X receptors postsynaptically (Gordon et al., 2005).

The dogma, that changes in the amplitude of synaptic events are determined by changes in postsynaptic efficacy alone, is based on the assumption that the neurotransmitter released by one vesicle is sufficient to saturate a single postsynaptic

receptor field completely (Frerking and Wilson, 1996;Atwood and Karunanithi, 2002). Recent demonstrations, however, argue against this precept (Liu et al., 1999;Oertner et al., 2002), suggesting that the amplitude of postsynaptic currents at a given site can be regulated by altering the amount of neurotransmitter released (Tong and Jahr, 1994;Wall and Usowicz, 1998;Prange and Murphy, 1999;Wadiche and Jahr, 2001;Sharma and Vijayaraghavan, 2003). Whether our data demonstrating that the release of multiple vesicles of glutamate onto MNCs occurs at a single release site (Tong and Jahr, 1994;Auger et al., 1998;Wadiche and Jahr, 2001;Oertner et al., 2002) or involves the synchronization of release at multiple sites (Auger and Marty, 2000;Sharma and Vijayaraghavan, 2003) with accompanying spillover and pooling of transmitter at adjacent sites, cannot be distinguished. In either case, the concentration of glutamate at a single postsynaptic receptor field will be elevated. One way these two ideas may be teased apart is to try and take advantage of glutamate spillover. If spillover contributes to larger amplitude mEPSC, their rising time would reflect a longer glutamate diffusion (DiGregorio et al., 2002), which could be protracted by blocking glutamate uptake. However, our data suggests that the kinetics of mEPSCs in MNCs are governed by elements downstream of vesicle exocytosis and transmitter diffusion properties. A lack of an effect when glutamate transport is inhibited may not be sufficient evidence to back true multivesicular release as there is insufficient data on the location and sensitivity of glutamate transporters to what might be very small amounts of glutamate crosstalk. We do know that there are no reported kinetic changes in mEPSCs when re-uptake is blocked at excitatory synapses onto MNCs in the SON (Oliet et al., 2001) but large amplitude mEPSC triggered by NA have not been tested.

The cellular mechanisms responsible for vesicle synchronization may involve the production of inositol 1,4,5-triphosphate (IP_3) in response to α_1 adrenoceptor activation and the subsequent liberation of Ca^{2+} from ryanodine-sensitive intracellular stores. Immunolocalization of both IP_3 receptors in the deep cerebellar nuclei and in the retina (Nakanishi et al., 1991;Peng et al., 1991), as well as ryanodine receptors within basket cell terminals in the cerebellum (Llano et al., 2000) both support a role for presynaptic Ca^{2+} stores at central synapses. Their functional role in terminals has been demonstrated

by observing elevations in presynaptic Ca^{2+} levels in photoreceptors (Krizaj et al., 1999) and in autonomic ganglia (Peng, 1996;Smith and Cunnane, 1996) in response to caffeine and/or ryanodine treatment, while similar manipulations have demonstrated an increase in the frequency (Savic and Sciancalepore, 1998) and amplitude (Sharma and Vijayaraghavan, 2003) of miniatures in hippocampal pyramidal cells. Furthermore, the release of Ca^{2+} from intracellular stores can be extremely fast, resulting in the synchronization of up to 15 vesicles of neurotransmitter in a few milliseconds (Llano et al., 2000). The dose of ryanodine utilized here to block ryanodine receptor/channels is high, but is consistent with that used for previous studies examining store-mediated vesicle synchronization (Llano et al., 2000;Sharma and Vijayaraghavan, 2003). This may be necessitated by a lower affinity of ryanodine for high molecular weight isoforms in the presynaptic terminal involved in mediating the synchronization process (Llano et al., 2000).

5.4.1 Physiological Significance

In response to numerous physiological challenges including dehydration, hemorrhage, stress, lactation and parturition, NA is released in the PVN (Pacak et al., 1995a;Pacak et al., 1995b) to increase neuroendocrine and autonomic output (Day et al., 1984;Pacak et al., 1995a;Pacak et al., 1995b;Cole and Sawchenko, 2002). High frequency stimulation of glutamatergic fibres onto MNCs in the supraoptic nucleus has also been shown to increase the amplitude of mEPSCs and increase postsynaptic spiking (Kombian et al., 2000). Our data offer a physiological context for the rapid amplification of hormone output from the posterior pituitary during periods of high demand. Such a mechanism would work in conjunction with changes in the gating of postsynaptic osmosensitive channels (Oliet and Bourque, 1993) or membrane depolarizations resulting from the direct actions of neuromodulators such as angiotensin II on postsynaptic ion channels (Li and Hatton, 1996), such that large amplitude quantal events would be more successful at generating postsynaptic firing when MNCs idle closer to sodium spike threshold.

Chapter Six: General Discussion

A single physiological stress (or homeostatic challenge) increases the efficiency with which the brain responds to subsequent challenges (Lilly et al., 1983;Lilly et al., 1986;Thrivikraman et al., 1997). This priming represents one clear example of learning in adaptive circuitry. Effectively, the initial challenge 'teaches' the circuitry to meet ensuing challenges in a more robust fashion. It is generally accepted that permanent alterations in the output of a neural circuit (memory) require permanent alterations in the strength of the individual synapses that comprise that circuit (Bains et al., 1999;Malenka and Bear, 2004). Synaptic plasticity of this type is likely ubiquitous throughout the nervous system yet its description has been limited largely to circuits responsible for motor, cognitive and behavioural processes with few descriptions of long-lasting synaptic changes in neural pathways involved in restoring homeostatic set points (e.g. osmotic balance) or pathways that respond quickly to essential physiological demands (e.g. lactation). Here we have demonstrated that NA induces several types of short and long-lasting forms of synaptic strengthening in excitatory glutamatergic synapses on MNCs in the PVN, which may participate in *in vivo* autonomic learning events.

The types of short-term plasticity described here include an increase in the frequency of mEPSCs and a robust enhancement of mEPSC amplitude resulting from the spontaneous release of multiple vesicles of glutamate simultaneously. In the latter, NA-induced multivesicular miniatures represents one of few descriptions showing a synchronization process that does not rely on action potentials in any brain system. Along with recent studies showing NMDA-mediated LTP with an induction threshold that critically depends on glial-derived D-serine (Panatier et al., 2006b), the enduring types of NA plasticity constitute the first demonstration of long-lasting changes in synaptic efficacy in these important autonomic synapses. The types of long-lasting plasticity described here include a persistent enhancement of mEPSC amplitude and mEPSCs frequency priming when NA is applied for a second time. In the former, NA triggers the release of glial derived ATP, which upon binding to postsynaptic ATP-gated P2X receptors initiates AMPAR insertion to strengthen glutamatergic synapses for prolonged periods of time. In the latter, NA targets presynaptic glutamate autoreceptors

through intracellular mechanisms to inactivate these receptors; thus priming mEPSC frequency for potentiated release when NA acts again.

6.1 Frequency Priming by mGluR inactivation

6.1.1 Some conflicting observations

The first type of short-term plasticity described is a straightforward enhancement of mEPSC frequency. This data conflicts with an earlier description of a positive NA effect on the frequency of spontaneous EPSCs, which could be totally abolished with TTX, suggesting no role for NA-induced enhancement of miniatures (Daftary et al., 1998). However, the same group corroborates our finding by later demonstrating an α_1-adrenoceptor-mediated enhancement of mEPSC frequency (Boudaba et al., 2003a). Some differences still exist between our findings and the Boudaba paper including a minor contribution of α_2-adrenoceptors to the mini frequency enhancement, mEPSC frequency priming is not observed when NA is applied a second time at the same dose and duration and mEPSC amplitude does not increase in the Boudaba findings. The excitatory influence of α_2-adrenoceptor activation on mEPSC frequency is interesting because there have been few demonstrations of α_2 induced excitation in MNCs (Kimura et al., 1984). Most studies examining α_2-adrenoceptor contributions have shown inhibition (Brooks et al., 1986a;Yamashita et al., 1988;Khanna et al., 1993). In our hands α_2-adrenoceptor involvement has not been tested thoroughly. Our effect on mEPSC frequency is completely blocked by the α_1-adrenoceptor antagonist prazosin at a dose that should not affect α_2 receptors. In fact we observed a decrease in the frequency of mEPSCs in response to NA in the presence of prazosin. That this decrease is mediated by α_2-adrenoceptor activation is supported by similar actions of the α_2-adrenoceptor agonist clonidine. However, an α_2-adrenoceptor antagonist such as Yohimbine was not tested to try and block or decrease the NA effect. That both mEPSC frequency priming and an increase in mEPSC were not observed in the Boudaba paper may be explained by the large experimental temperature difference (room temperature for their work, versus

32.5 degrees centigrade in the experiments reported here), which can have profound effects on synaptic physiology (Klyachko and Stevens, 2006).

6.1.2 Importance of PKC and Phorbol Esters

From the evidence presented in chapter 3 the intracellular signalling molecule thought to be responsible for mGluR inactivation subsequent to α_1-adrenoceptor activation is PKC. PKC encompasses an entire family of related serine-threonine kinases that carry out vital cellular responses mediated by the second messenger DAG. Acting as a surrogate to DAG for experimental activation of PKC are phorbol esters, which includes phorbol 12-myristate 13-acetate (PMA), the molecule utilized in our experiments. Many studies have demonstrated phorbol ester enhancement of neurotransmitter release through the presynaptic activation of PKC (Stevens and Sullivan, 1998;Yawo, 1999;Lou et al., 2005). Lipid soluble phorbol esters activate PKC by internalizing and binding to the regulatory C1 domain on the PKC molecule (Kazanietz, 2000). However, in addition to this well-characterized interaction with PKC isoforms, phorbol esters can bind to other receptors lacking kinase activity including the vesicle exocytosis protein Munc13, the cytoskeletal organizing proteins chimaerins and Ras guanyl-releasing proteins which can activate MAPK pathway (Kazanietz, 2000). Similar to PKC, all of these proteins possess a single copy of the C1 domain that is involved in the binding of phorbol esters and DAG. Thus, preventing the presynaptic effects of NA by blocking PKC in our experiments does not preclude the involvement of other proteins with the same C1 regulatory domain because our PKC antagonist of choice, calphostin C, cannot differentiate between these molecules. In support of true PKC activation and PKC-mediated effects in our system, there are numerous examples of inhibiting the presynaptic actions of PKC with bisinadoylemalimide (Shoji-Kasai et al., 2002;Berglund et al., 2002), which does not inhibit via the C1 domain. The credibility of our use of phorbol esters to discern the effects of PKC is further backed by the direct interaction between PKC and metabotropic glutamate receptors (Macek et al., 1998;Sorensen et al., 2002) in which mGluR inactivation would not be expected if PMA was activating other proteins with C1 domains. A recent a study demonstrating $mGLuR_4$ internalization through a $G_{q/11}$ and

PKC dependent mechanism also supports the correct interpretation of our data (Mathiesen and Ramirez, 2006).

6.1.3 mGluR Inactivation In Vivo?

The inactivation of mGluRs persists for at least one hour in the brain slice (unpublished observation) but the occurrence and persistence of this effect in the whole animal remain to be established. There are a few lines of evidence arguing against persistent mGluR inactivation in chronic dehydration (Boudaba et al., 2003b) and lactation (Oliet et al., 2001), physiological process which use NA as a key mediator. As we shall see below, these studies do not unequivocally rule out the functional attenuation of autoreceptors and in fact other observations in dehydration fit nicely with the idea that mGluR efficacy is reduced (Di and Tasker, 2004). Finally, the possibility exists that the experimental manipulations of dehydration and lactation are not optimal for observing mGluR inactivation and a more intense physiological challenge such as haemorrhage might be better suited to reveal this effect in the intact animal.

Performing experiments on *in vitro* brain slices taken from control animals we made two important observations. First, on the second of two NA applications the increase in mEPSC frequency is greater, an effect that can be blocked if a group III antagonist applied before the first NA treatment. Second, an agonist of group III mGluRs is less effective after a single treatment of NA than when applied in isolation. Given this information, for experiments performed on *in vitro* slices taken from animals that have been physiologically challenged, one might predict that: 1) a single dose of NA should elicit a greater increase in mEPSC frequency and that 2) a group III mGluR agonist should be less effective in curtailing glutamate release, if mGluR inactivation occurs and persists in the intact animals. In fact, an augmented response to NA in the dehydrated state has been observed (Di and Tasker, 2004) and the effects of the mGluR agonist L-AP4 are diminished in dehydration (Boudaba et al., 2003b), when compared to the efficacy of this drug at the same or at a lower dose in the control condition (Panatier et al., 2004).

The enhanced increase in mEPSC frequency in slices taken from a dehydrated rat compared to naive animals (Di and Tasker, 2004), is consistent with mGluR activation. However, there are many other possible explanations. One might be the presence of new glutamatergic terminals that formed during dehydration (El et al., 1996), an observation supported by a higher mEPSC frequency with no corresponding change in paired pulse ratio compared to naive rats (Di and Tasker, 2004). However, with no other changes except terminal number, one might expect that the absolute baseline and increase in mEPSC frequency is greater in dehydration but the relative change would be equivalent to control. As this is not the case, mGluR inactivation becomes a candidate for explaining this effect, as the potentiated increase in mEPSCs frequency seen in dehydration appears more or less equivalent to the priming effect observed with successive bouts of NA in naïve rats. In fairness, there are other changes in P_r unrelated to mGluRs function that cannot be ruled out such as the efficacy of α_1-adrenoceptor coupling to the release process, but we do not have data to either support or refute this possibility.

The study showing a blunted effect of a group III mGluR agonist in dehydration did not interpret this finding as being the result of mGluR inactivation (Boudaba et al., 2003b). This was because a group III antagonist had a greater effect at potentiating mEPSC frequency in dehydration than control, suggesting there was a greater occupancy of functional mGluRs by synaptically released glutamate and that this occupancy did not allow for proper activation of autoreceptors by the agonist. This is a fairly sound conclusion based on a lack of glial diffusion barriers in this physiological condition (Tweedle and Hatton, 1977;Miyata et al., 1994) and that a similar observation was made in lactating rats (Oliet et al., 2001). These data argue against mGluR inactivation during dehydration. However, the result is inconsistent when compared with data obtained by the same group showing no change in paired pulse ratio in dehydration (Di and Tasker, 2004). Even if true these data do not explicitly rule out mGluR inactivation, as the exact level of total mGluR that is functional remains undetermined during these physiological periods. Anatomical studies suggest that accompanying the neuropil remodelling is a large increase in glutamate synapse number (El et al., 1996). If pre-existing mGluRs

became inactivated at the initiation of physiological perturbations from the actions of NA and nascent glutamatergic synapse with 'fresh' mGluRs came online, the group III antagonisms data can be reconciled. As long as there is still enhanced mGluR activation by endogenous glutamate at a given synapse due to actions of enhanced glutamate spillover, the percent increase in mEPSC frequency will be larger with a group III antagonist in dehydration compared to control. The one caveat arguing against this possibility is an increased number of noradrenergic terminals in the PVN and SON during remodelling (Michaloudi et al., 1997), which could potentially inactivate the 'fresh' mGluRs on newly formed glutamate terminals.

Alternatively, regardless of the addition of new glutamate terminals with functional mGluRs, all that is required to reconcile the group III antagonist data is that the total inhibition of glutamate release by functioning mGluRs in dehydration is greater than the total mGluR-mediated inhibition of glutamate release in the control state. If some fraction of mGluRs become inactivated, this situation can be achieved by having the remaining mGluRs work better than all mGluRs in the control condition. In other words, instead of having all mGluRs work in a moderate range of effectiveness, one could have some mGluRs work really well, so much so that the sum total of their inhibition is greater than the effects of the many. While this may seem unlikely, when considering that a neuron may want to receive greater or lesser influence from a particular afferent pathway the notion becomes more than plausible. Inconsistent data, which tends confuse and bewilder us may just be the result of our own imprecise experimental techniques that cannot differentiate between something as simple as two separately regulated afferent inputs. A clear resolution of these issues will require further experimentation.

Knowing the exact contribution from putative populations of autoreceptors, which could encompass receptors that are segregated based on location or regulation, may become very important for delineating mGluR inactivation *in vivo*. Even immediately after NA treatment in control brain slices mGluRs are partially functional (Gordon and Bains, 2003). From the data obtained in lactating or dehydrated animals, mGluR activity has at least partially, if not fully recovered. Even though mGluR function seems

counterintuitive when enhanced MNC output is called for, reducing release probability at excitatory synapses may in fact lead to larger amounts of glutamate release by enabling synaptic responses to facilitate instead of depress when trains of action potentials invade presynaptic terminals (Zucker and Regehr, 2002). Alternatively, the presence of mGluRs may serve as a high frequency filter so that only afferent signals of sufficient frequency will be able to overcome enhanced mGluR-mediated inhibition (Oliet, 2002).

6.1.4 mGluR Inactivation: The unknowns

How the amplification of quantal release resulting from autoreceptor inactivation impacts afferent, action potential driven signals is not known. As evoked release in the SON is also under the influence of presynaptic mGluR activity (Schrader and Tasker, 1997b;Oliet et al., 2001), it is likely that priming occurs with spike-triggered release as well. The demonstration that *in vivo* recordings from PVN neurons show that forebrain inputs to PVN neurons can be potentiated by co-activation of noradrenergic cell bodies in the brain stem also supports this notion, although other reasons can of course be exercised. However, the possibility exists that miniature and evoked signals are regulated differently (Sara et al., 2005).

Another unknown is whether dormant plasticity resides exclusively in local glutamate synapses, or whether ascending noradrenergic inputs can alter GABAergic synapses possessing presynaptic mGluRs. NA can influence inhibitory transmission in the SON (Wang et al., 1998). High affinity mGluRs have been physically localized to inhibitory terminals in other systems (Bradley et al., 1996;Ferraguti et al., 2005) and in the SON they play an important functional role in lowering GABA P_r after neuropil remodelling when glutamate spillover can reach GABAergic synapses (Piet et al., 2004).

6.1.5 mGluR inactivation today

Thus far we have used the term ‘inactivation’ but our precise meaning when using this word is unclear. From our experiments all that can be concluded with certainty is that mGluRs do not work as well after NA treatment or PKC activation. As previously

mentioned, the reason for the reduced efficacy of group III mGluRs may include a concerted inactivation mechanism that result from intracellular actions of PKC-mediated phosphorylation (Sorensen et al., 2002;Mathiesen and Ramirez, 2006) and a decoupling of its` G-protein cascade (Macek et al., 1998). More recent studies though are garnering support for internalization of the receptor due to intracellular signals (Mathiesen and Ramirez, 2006) or the direct actions of the agonist (Pelkey et al., 2005;Pelkey et al., 2006), which has been demonstrated for $mGluR_4$ and $mGLuR_7$ respectively. Notably, in the former case, PKC is thought to be an important signal mediating the internalization. The mechanisms underlying mGluR internalization has been best worked out for postsynaptic group I mGluRs ($mGluR_1$ and $_5$). These studies have identified many participating elements. The ones pertinent to our study concerning an α_1-adrenoceptor mediated reduction in autoreceptor efficacy are the involvement of $G_{q/11}$-protein activation and of course PKC (Mundell et al., 2003;Mundell et al., 2004a). Other studies have shown that dynamin and chlathrin dependent endocytosis (Mundell et al., 2001;Pula et al., 2004) and that PKA can inhibit internalization (Mundell et al., 2004b). Whether or not these latter signals govern autoreceptor internalization remains to be established.

6.2 Glial-Neuronal Plasticity in MNCs

6.2.1 NA Activates Glia

That NA can robustly activate glial cells has been circulating for some time (Agullo and Garcia, 1991;Duffy and MacVicar, 1995), but to our knowledge there are few demonstrations of specific NA effects in glia. While there is no ultrastructural evidence for direct noradrenergic terminal-glial cell communication, in the PVN and other brain nuclei, there are reports that some noradrenergic varicosities lack postsynaptic specializations (Sawyer and Clifton, 1980), leaving released NA free to activate other cell types such as glial cells. Our experiments corroborate these data by showing immunolocalization of α_1-adrenoceptors on astrocytes in PVN, an observation made in other brain regions as well (Hertz et al., 1984;Nakadate et al., 2006).

6.2.2 Glial-mediated AMPAR insertion

AMPAR insertion is a commonly accepted means for increasing signal strength postsynaptically. This process is thought to occur via Spike timing dependent plasticity (STDP) where coincident presynaptic release and postsynaptic depolarization provide a sufficient stimulus to relieve the magnesium block on NMDARs. The Ca^{2+} influx through NMDA channels can promote the insertion of AMPARs into the postsynaptic side of the synapse if the Ca^{2+} signal is large or can promote the removal of AMPARs if the Ca^{2+} signal is small (Malenka and Bear, 2004). The activation of kinases such as CaMKII and PI3-K are necessary for AMPAR incorporation into the postsynaptic density (Sanna et al., 2002;Raymond et al., 2002;Opazo et al., 2003;Man et al., 2003;Baxter and Wyllie, 2006).

NMDAR independent forms of this plasticity have also been shown to co-exist at CA1 Schaffer collateral glutamate synapses with the recruitment of postsynaptic group I mGluRs (Oliet et al., 1997). This observation has also been made in parallel fibre Purkinje cell synapses in the cerebellum (Linden et al., 1991), relying on intracellular Ca^{2+} and alterations in AMPAR trafficking (Wang and Linden, 2000). Even though both NMDAR-dependent and independent forms of plasticity occur through different membrane bound receptors, they both couple to the same general end effector, AMPAR trafficking, suggesting that other receptors able to promote Ca^{2+} changes and activate critical kinases may target the same end process. Our results showing the effects of NA on glutamate terminals synapsing onto MNCs in the PVN have provided an additional mechanism through which postsynaptic signal strength can be augmented. By acting at α_1-adrenoceptors NA triggers the release of glial derived ATP. Liberated ATP binds to postsynaptic ATP-gated P2X channels on MNCs. The ionotropic, Ca^{2+} permeable P2X channel activates PI3-K and leads to the insertion of AMPARs through snare dependent mechanism; thus recruiting a similar molecular pathway as NMDARs. This represents the first demonstration of a gliotransmitter, ATP, and purinergic P2X receptors linking to AMPAR trafficking. This raises several interesting questions as to why glial cells would be involved in this form of synaptic strengthening, why NA does not directly signal to the

postsynaptic neuron to promote the insertion of AMPARs and why NA utilizes P2X channels as opposed to NMDARs by way of enhanced evoked signalling.

One possibility for the utilization of glial cells is that α_1-adrenergic receptors cannot link to the appropriate molecular pathways postsynaptically targeting AMPAR insertion. This idea is supported by several accounts of postsynaptic mGluR-mediated LTD in the hippocampus (Oliet et al., 1997) and cerebellum (Linden et al., 1991), a metabotropic receptor that is similarly coupled to $G_{q/11}$ proteins. Even though $G_{q/11}$-protein linked metabotropic receptors can lead to an elevation in postsynaptic Ca^{2+} by activating intracellular stores and can activate PKC, a kinase believed to be central to LTP (Malinow et al., 1989;Boehm et al., 2006) our experiments reveal that neither PKC activation nor Ca^{2+} stores are involved in long-term strengthening of excitatory synapses on MNCs. The fact that PKC activation alone fails to initiate long-term changes is likely due to a requirement on the activation of other essential proteins such as CaMKII and PI3-K.

Another possibility is that NMDARs in glutamatergic synapses on MNCs do not serve the same purpose as they do in other areas of the brain and thus NA would have to, by default, make use of a differ receptor (i.e. ATP-gated P2X channels) to initiate synaptic changes in MNCs. However, NMDA currents are present in these cells and a recent demonstration in the SON clearly shows that NMDA dependent LTP can be evoked in glutamate afferents that input onto MNCs (Panatier et al., 2006a). A more likely scenario is that glial cells and P2X channels are utilized, not because of an insufficiency or an inability of α_1-adrenoceptors and NMDARs to elicit plasticity in MNCs, but because of glial cell location, structure, physiology and the biophysical properties wielded by P2X receptors.

6.2.3 To NMDAR or Not to NMDAR, that is the Question?

That postsynaptic AMPAR insertion can be triggered by the activation of P2X receptor channels and that NMDARs are not utilized, there are interesting consequences to the induction of synaptic plasticity onto MNCs. NMDAR depended plasticity stipulates that sufficient depolarization must occur in a localized region to relieve NMDAR Mg^{2+} block

so that a given synapse can be strengthened (Malinow and Malenka, 2002). P2X channels however, are not regulated by Mg^{2+} in a voltage sensitive manner and thus do not require the same local depolarization to become activated. All that is needed is an adequate concentration of ATP (North, 2002), suggesting that synapses can undergo strengthening independent of the voltage state of the postsynaptic neuron and the activity of afferent inputs. This type of plasticity may be beneficial so that MNCs do not have to rely on coincident detection to augment hormone output in the face of physiological challenges. If the goal is to dramatically augment hormone secretion, why rely on the concerted efforts of many afferent inputs so that numerous synapses can be potentiated to meet this end? A simpler solution would be to enhance excitability regardless of what other inputs may be saying by using a diffuse and likely broad reaching signal such as glial derived ATP (see also section 1.2.5 below for a similar topic). This may represent a method by which hormone always gets secreted, a lovely thought seeing that a hormone like VP is vital to the organisms survival.

6.2.4 Abstaining from Plasticity?

Astrocytic processes interdigitate synaptic elements and in the PVN and SON, glial processes can retract away from synapses during dehydration and lactation. As glial cells have been implicated in many brain regions to modulate synaptic transmission (Volterra and Meldolesi, 2005), one reason glial-neuronal plasticity may be utilized in the MNC nuclei is because of the unique property of neuropil remodelling. This could represent an excellent method for selectively inducing or abstaining from changes in synaptic efficacy depending on the relative presence or absence of glial cells respectively. In the retracted state afferent signals coming from the medulla or other hypothalamic nuclei would be unable to influence MNC activity if these inputs recruited the release of gliotransmitters from local glia. Thus glial-neuronal plasticity described here may be important for MNC system behaviour by constituting a novel method in selecting for or against plasticity, which would be governed by how abundant or vacant glial cell processes are surrounding MNC synapses. Long-term strengthening of excitatory synapses by glial ATP may be utilized at the onset of dehydration or lactation to combat the ensuing challenge and only

after they persist will astrocytic processes retract to 'lock' synapses in the potentiated state. It follows then that after retraction, plasticity requiring glial cells will not be induced as easily while other types of plasticity not requiring glial cells are permissible. These ideas are supported by 1) the observation dehydrated animal in which mEPSCs display larger amplitudes (Di and Tasker, 2004), which might have resulted from earlier actions of NA and ATP or D-serine, 2) the demonstration that NA is incapable of inducing further postsynaptic strengthening (Gordon et al., 2005), while the D-serine effect requires greater afferent activation (Panatier et al., 2006b) when glial cell processes have withdrawn and 3) that NA can still elicit MVR, which does not rely on glial cells (unpublished observation).

6.2.5 Glial Global Influence Over MNCs

In addition to 'sampling' the synapse, glial cell processes can also form physical communication points with adjacent glia via gap junctional hexameric channels. This results in a lattice of interconnected cells capable of propagating short and long-range Ca^{2+} signals (Charles et al., 1991) that occur in response to various transmitters and other stimuli. The long range Ca^{2+} signal (normally referred to as a Ca^{2+} wave) is not the result of passive Ca^{2+} diffusion or active Ca^{2+} propagation through the astrocytic network but is instead the result of two separate mechanisms that trigger increases in the free Ca^{2+} concentration sequentially within individual astrocytes as the 'wave' propagates: 1) the diffusion of IP_3 through gap junctions coupling adjacent astrocytes (Venance et al., 1997) and 2) by the release of ATP which acts as an extracellular paracrine messenger to activate neighboring cells (Guthrie et al., 1999). By acting on either metabotropic P2Y receptors or ionotropic P2X on adjacent glial, ATP causes an intracellular Ca^{2+} increase. Cultured astrocytes express all cloned purinergic receptors with the exception of $P2X_6$, including the ionotropic $P2X_{(1\text{-}5,7)}$ receptors and the metabotropic $P2Y_{(1,2,4,6,12,14)}$ receptors, where $P2X_7$ and $P2Y_1$ were predominantly responsible for Ca^{2+} waves (Fumagalli et al., 2003). The large scaffolding and signalling capabilities of astrocytic networks present a unique opportunity for incoming afferent signals to influence spatial neuronal 'domains'. Where classical thinking stipulates that the strengthening or

weakening of the synapse depends on the specific activity of that synapse, glial networks may provide a means to change the efficacy of groups of synapses at one time, an effect that may be independent of the previous activity of the affected synapses. This concept is supported by: 1) the appearance of Ca^{2+} changes in a large number of neurons neighboring activated glial cells (Charles, 1994), 2) astrocyte excitation in the intact retina can increase or decrease firing in adjacent neurons, where the magnitude of the firing change correlates to the astrocytic Ca^{2+} signal (Newman and Zahs, 1998) 3) the demonstration that glial derived glutamate can activate extrasynaptic NMDARs on multiple neurons simultaneously (Fellin et al., 2004) and 4) that ATP released from glia can trigger heterosynaptic depression after it has been broken down into adenosine (Zhang et al., 2003). That NA also utilizes glial-ATP for the induction of long-term strengthening of excitatory synapses, a molecule central to the propagation of long range glial signals (Anderson et al., 2004), suggests that NA may also target glial cell networks to control synaptic efficacy globally.

Further, these effects may go beyond changes in synaptic strength. Propagating ATP/Ca^{2+} waves may affect different neuronal regions by influencing action potential generation at the axon hillock and gene transcription at the cell body. Long-range signalling by astrocytes may affect populations of neurons and synapses that were previously quiescent (Charles, 1994), bridging a gap between different neuronal populations, separate neural pathways and the concept that the circuits themselves require activity changes to alter their efficacy. Glial cell signalling may also be highly adaptable, given that long-range communication occurs in response to stronger neural activity compared to short-range astrocytic signalling, and that Ca^{2+} wave propagation becomes more efficacious when stimulated repeatedly (Pasti et al., 1997). The possibilities for increasing the complexity of brain signalling are enormous.

6.2.6 ATP versus Adenosine

The initiating step for both heterosynaptic suppression in hippocampal pyramidal neurons and synaptic strengthening in MNCs is the release of ATP from glial cells, but an interesting disparity exists between these two types of plasticity. In the former glial

derived ATP is broken down into adenosine by the action of ectoATPases whereas in the latter glial ATP must remain intact to bind postsynaptic P2X receptor channels. One reason the difference may exist is the location and or potency of ectoATPases around MNC synapses versus synapses in cortical regions. The presence of adenosine in the hippocampus does depend on ectoATPase activity (Zhang et al., 2003). However, a regional distribution of ectoATPases in the hippocampus and cerebellum suggests that the relative presence or absence of these enzymes may dictate to what degree adenosine is utilized as a transmitter over ATP respectively (Zinchuk et al., 1999). That ectoATPase activity can change over development (Banjac et al., 2001) and become altered after epileptic discharge (Bonan et al., 2000) and stress (Fontella et al., 2004), suggests these enzymes are not static and may change depending on the age and physiological state of the organism. One caveat with these studies is that some measure of the transmitter-ATP needs to coincide with measured levels ectoATPase in order to confirm that a lack of enzyme means more extracellular ATP or just that no ATP is utilized as a transmitter in this region. In the hippocampus a very recent demonstration points to a critical link between CO_2 levels (and corresponding changes in pH) and the functioning of ectoATPases, which ultimately dictates the amount of extracellular adenosine and the size of synaptic currents (Dulla et al., 2005). This study suggests that cell activity and the degree of blood flow may determine the relative amounts of ATP and adenosine. Thus, there is no golden rule stating all extracellular ATP must be broken down to adenosine, an idea easily digested due to functional expression of many P2X and P_{2Y} receptors on a variety of cell types.

The presence and/or function of ectoATPases in the PVN is as of yet unknown, but the enzymes are present in the neural lobe (Thirion et al., 1996). We do know that prolonged afferent stimulation in the SON results in the release of endogenous adenosine, which can reduce the appearance of both mIPSCs and mEPSCs (Oliet and Poulain, 1999). The source of adenosine remains unknown. Both neuronal and/or glial elements could be involved. If from glial cells, our data showing an action of glial ATP over glial adenosine (after glial ATP is release and broken down) can be reconciled by entertaining a few ideas. First, a specific type of ATP release from glial cells may be controlled by

enzymatic breakdown whereas another type may not be. For example, synaptic glutamate acting on glial glutamate receptors may trigger ATP release from specific glial cell locations that are subject to ectoATPase activity (Zhang et al., 2003), whereas NA-induced glial release of ATP may occur into extracellular areas that are not influenced by enzymatic breakdown to the same degree. Second, there may be something specific about NA induced ATP release, which allows this gliotransmitter to remain intact in the presence of the enzyme, possibly by releasing such copious amounts of ATP ectoATPases become saturated and overwhelmed or through a concerted mechanism whereby NA drastically reduces ectoATPase activity. This is an interesting idea and is amenable to experimentation.

6.2.7 Glial ATP Release

The mechanism(s) responsible for ATP release from glial cell is contentious. Several different possibilities have been proposed and consensus on Ca^{2+} dependence is lacking. There are many studies backing a Ca^{2+} dependent process (Queiroz et al., 1999;Arcuino et al., 2002;Bal-Price et al., 2002;Coco et al., 2003) and many supporting Ca^{2+} independence (Queiroz et al., 1999;Wang et al., 2000;Anderson et al., 2004). Much of the disparity comes from different preparations and the method used to stimulate ATP release, which in the latter includes, direct electrical stimulation, mechanical stimulation, application of ATP and different purinergic agonists, application of glutamate and different glutamate receptor agonists, lowering extracellular Ca^{2+}, stimulating afferents, uncaging Ca^{2+}, uncaging intracellular IP_3 and so forth. For instance, glial cell AMPAR activation has been shown to trigger ATP release in a Ca^{2+}-independent manner, while ATP release after NMDA activation requires Ca^{2+} (Queiroz et al., 1999).

For Ca^{2+} dependent ATP release, vesicular release is believed to be important. Application of the nitric oxide donor DETE-NONOate causes an increase in astrocytic free Ca^{2+} and the release of glutamate and ATP (Bal-Price et al., 2002). Both were blocked by the fast Ca^{2+} chelator BAPTA AM and botulinum toxin C, suggesting that ATP release requires vesicles and the associated SNARE proteins. Incubating astrocytes in bafilomycin A1, a vesicle transport inhibitor, also generated evidence for the release of

ATP in vesicles as this treatment attenuated ATP-induced Ca^{2+} waves (Coco et al., 2003). These results have been further supported by an inducible knock in mouse study, in which only astrocytes express a dominant-negative SNARE protein domain that blocks exocytosis. In the absence of vesicle fusion glial-ATP-mediated heterosynaptic suppression in the hippocampus is drastically reduced (Pascual et al., 2005).

Other studies have shown that neither bafilomycin nor the application of tetanus toxin, a molecule that cleaves release proteins, completely blocked ATP release, suggesting ATP may be secreted through a separate pathway. Indeed this idea has been supported in a number of other studies, placing an important role for semi connexons or hemichannels in the membrane of glial cells that do not appose another cell and thus do not assemble to form complete gap junctions (Cotrina et al., 1998;Braet et al., 2003;Leybaert et al., 2003). In addition to hemichannels, non-selective anion channels (Anderson et al., 2004) and P2X channels (Duan and Neary, 2006) have been shown to be potential candidates for ATP release.

As our data demonstrates that ATP release from glial cells is PKC and intracellular store independent, we are tempted to speculate that either IP_3 itself (Leybaert et al., 1998) or other unorthodox intracellular signalling cascades may be involved in response to α_1-adrenoceptor activation. The latter has been described in a number of different tissues. These intracellular signalling molecules include the mitogen-activated protein kinase (MAPK) pathway, a coupling to cAMP metabolism, phospholipase D activation as well as phospholipase A_2 for the production of arachidonic acid (Zhong and Minneman, 1999;Michelotti et al., 2000;Piascik and Perez, 2001). Whether or not these pathways and messengers can cause ATP release from glia by targeting vesicles, hemichannels, P2X channels or anion channels remains to be determined. Nevertheless, they will likely represent the starting point for delineating all of the molecular processes involved. Finally, when examining both roles for glial derived ATP: 1) primary paracrine messenger responsible of long-range Ca^{2+} signals and 2) to affect synaptic efficacy and neuronal excitability, it remains unclear if these two roles are the same process, different processes that always occur simultaneously or if they are completely separate processes that can be independently regulated and controlled. As interest in

glial-ATP acting as a plasticity molecule tends to increase and as the separate fields of glial signalling and glial-mediated synaptic plasticity become intertwined, this issue will likely be resolved in the next five to ten years. Some evidence suggests that glial-mediated heterosynaptic suppression relies of gap junctional coupling (Zhang et al., 2003) however, the effects of gap junction antagonists used to generate this conclusion cannot distinguish between long range glial signals that require gap junctional coupling between astrocytes and the release of ATP directly through hemichannels with no requirement for distant communication.

6.3 Multivesicular Miniatures Triggered by NA

In addition to the effect of priming mEPSCs frequency, our data shows that the presynaptic actions of NA can synchronize the release of multiple vesicles at glutamate terminals synapsing onto MNCs in the PVN. Notably, this effect occurs in the absence of spike driven release of neurotransmitter. The robust enhancement of mEPSC amplitude is impervious to postsynaptic manipulations, is not the result of the release or activation of a quiescent vesicle or synapse respectively, but is sensitive to lipid permeable Ca^{2+} buffering, a high dose of ryanodine, and large amplitude mEPSCs are associated with a greater concentration of glutamate in the synaptic cleft.

6.3.1 Bursts of mEPSCs

As vesicle synchronization was observed with bath application of NA some questions arose as to whether the effect could be observed more subtly and whether the observed multimodal amplitude distribution was an artefact of global synapse activation. In addition to our experiments with sucrose, PMA and a cubed root transform analysis to eliminate the possibility that inter and intrasynaptic variability accounted for our multimodal amplitude distribution, the effect could also be elicited with a brief and localized application of NA into the dendritic region of the MNCs. This treatment resulted in randomly occurring intense bursts of mEPSCs that were transient and clearly distinguishable from the background mEPSC frequency. This result is worth discussing, as it is similar to an observation made with a low concentration of the black widow spider

toxin α-latrotoxin in cerebellar interneurons (Auger and Marty, 1997). α-latrotoxin is a potent promoter of rapid vesicle fusion and when applied in very small doses the time in between when the toxin interacts with individual synapses is long enough to discern its effect on single release sites. In other words α-latrotoxin generates intense bursts of mEPSCs at random intervals in which each burst corresponds to the action at a given synapse. The activation of individual active zones by α-latrotoxin is supported by the uniform characteristics of the mEPSCs from a single burst, which possess a Gaussian amplitude distribution, an observation dissimilar to histograms generated when recording all miniatures from the cell body of a CNS neuron and similar to the mEPPs at the NMJ. The frequency characteristics of mEPSC bursts generated by NA were qualitatively similar to that observed in α-latrotoxin, suggesting that NA too can activate individual synapses or at the very least a single group of spatially confined synapses. What is very interesting about the mEPSC amplitude distributions generated from a single NA-induced burst is they appeared to have multi-equidistant modes, standing in stark contrast to the single normally distributed mode seen with spider toxin bursts. These data are highly suggestive that NA synchronizes release at a single site or at a highly localized group of active zones and that multimodal amplitude histograms are not an artefact of bath application or result from inter and intrasite variance. Our findings that NA triggers MVR in the absence of action potential depolarization challenges four central tenets of synaptic transmission in the CNS: 1) the complete saturation of postsynaptic receptors by the release of single vesicles of transmitter, 2) the origin of large amplitude mEPSCs, 3) the one site one vesicle hypothesis (OSOVH) and 4) the importance of miniatures versus evoked release.

6.3.2 Aiding Spike Generation in MNCs

Large amplitude mEPSCs in MNCs, which result from a concerted synchronization process, may play a prominent role in spike timing. Rapid deflections in voltage that occur from a single event would present a faithful way of eliciting action potentials postsynaptically at desired times. Although the output of MNCs is not to code information to receiving cells, bursts of action potentials in both cell types are necessary

to maximize hormone release at the neural lobe (Leng and Shibuki, 1987;Dyball et al., 1988;Bourque and Renaud, 1991;Dayanithi et al., 1992). During a burst of action potentials, a persistent inward current helps to ramp the cells voltage up to a stable plateau potential. During this new, elevated baseline, individual EPSPs are believed to generate the spikes comprising the burst because they can more easily break Na^+ channel threshold (Brown et al., 2004a). One can see that a borage of large amplitude mEPSPs induced by NA could facilitate action potential bursting, a process that has been shown to rely on glutamatergic transmission (Nissen et al., 1995;Brown et al., 2004a). By targeting glutamate terminals on MNCs to generate large, spontaneous potentials that occur at high frequency, the actions of NA might be an easy method for eliciting desirable spiking patterns, rather than relying on the convergence of many afferent pathways to provide enough action potential-generated EPSPs during the specific moment of the burst. By the same general reasoning, large amplitude mEPSPs could also increase the number of spontaneous action potentials during a period when MNCs are not bursting. Such spontaneous activity in OT MNCs is thought to be important for the 'tertiary' roles of blood borne OT, to aid naturesis at the kidney (Moos and Ingram, 1995;Sjoquist et al., 1999). Supporting the facilitation of bursting and spontaneous background activity by NA, ICV injection of the α_1-adrenoceptor antagonist phentolamine dramatically reduces both of these firing properties in OT MNCs during suckling (Moos and Richard, 1980).

6.3.3 Miniature MVR and the Consequence for Evoked Signals

As the NA induced enhancement of mEPSC amplitude likely results from the rapid expulsion of Ca^{2+} from presynaptic intracellular stores, interesting questions arise as to how this impacts evoked transmitter release. When combined with a drastically elevated mEPSC frequency also observed in NA, one tends to wonder which signal will be selected for: miniatures or evoked? This would depend on many factors including whether miniatures and evoked share the same or separate pools of vesicles, whether or not vesicles are in short supply versus their abundance and whether or not there are

intrinsic mechanisms that select for a preferred signal i.e. action potential driven release or miniatures.

If vesicles are in short supply and evoked and spontaneous release use the same available pool, one possibility is that these different types of signalling exist in a 'push-pull' equilibrium, where the more active signal is passively selected. Alternatively, if the synapse can actively 'switchgears' to prefer signalling in one method over the other, this would be distinct from the push-pull system because the synapse would, in this scenario, possess intrinsic mechanisms that either inhibit the undesirable method, augment the desirable method of signalling or do both simultaneously. If vesicles are not in short supply, an idea evinced by the ability of glutamate synapses onto MNCs to release at rates up to 60 Hz (Gordon and Bains, 2003), then processes that augment evoked and spontaneous release could act cooperatively to generate spikes postsynaptically. This could only occur if the synapse is not actively switching gears to inhibit or augment one signalling method over another. However, as stated above, spontaneous and evoked transmitter pools might be separate and regulated independently of one another. While in some regions changes in mini frequency strongly correlate to changes in evoked release probability at single synaptic sites in cortical neurons (Prange and Murphy, 1999), suggesting a reciprocity between mini and evoked transmitter pools, there have been other demonstrations illuminating the opposite, that miniature and evoked pools of transmitter are completely separate (Sara et al., 2005). These ideas are only now beginning to be tested. Our unpublished data showing that during NA application evoked P_r can decrease when mEPSC frequency and amplitude increase dramatically, suggests that one form of the above processes occurs at glutamate synapses on MNCs, where a specific signal is observed more than another. Whether or not there is a passive shift in the push-pull equilibrium of vesicles, there is a concerted mechanism to switch signalling gears, or miniature and evoked vesicles pools are completely separate and can be regulated independently of one another remains to be established.

6.3.4 Multivesicular Miniatures in Other Autonomic Regions

At excitatory synapses in the NTS, ATP has been shown to elicit large amplitude mEPSCs by acting on ATP-gated P2X channels located on presynaptic terminals. Ionotropic P2X channels allow for Ca^{2+} influx, a process that here is speculated to trigger the synchronization of multiple vesicles (Shigetomi and Kato, 2004). When examined with in the context of the NA effect on glutamate synapses in MNCs, the fact that ATP shows elicits a similar change on glutamatergic transmission is interesting because there have been many document synergistic and/or collaborative actions between NA and ATP (Burnstock, 1995). Our finding, that an increase in the number of postsynaptic AMPARs by NA, which requires the release of ATP from glial cells onto MNCs to activate P2X channels, constitutes a new collaborative action. As of yet though, we have little evidence that NA mediated MVR acts cooperatively with ATP. Although the P2X antagonist brilliant blue G blocks the insertion of AMPARs postsynaptically, effectively eliminating the long-lasting enhancement of mEPSC amplitude, this antagonists does not block the transient increase in mEPSC amplitude resulting from MVR. Further, in the chronically dehydrated state, where glial cell processes have withdrawn away from synaptic elements taking with them the source of ATP, the transient increase in mEPSC amplitude remains. When coupled to our demonstration that in the presence of NA fast, purinergic synaptic transmission is not detected, it is unlikely that the synchronous release of multiple vesicles also depends on ATP. However, other sources of ATP, such as the MNC cell body remain a possibility; therefore the potential cooperative and/or synergistic effects between these two molecules that could mediate MVR should still be examined in future studies.

6.4 Combining the three types of plasticity

NA induces three different types of synaptic plasticity that when combined dramatically potentiate the excitatory effects of glutamatergic transmission onto MNCs. To summarize they are: 1) priming of excitatory synapses by the inactivation of presynaptic mGluRs, 2) MVR release by activating the release of Ca^{2+} from presynaptic intracellular Ca^{2+} stores and 3) insertion of postsynaptic AMPARs triggered by the release of glial

ATP. Presynaptic α_1-adrenoceptor activation results in increased PKC activity and the production of IP_3. While each of these messengers act to induce mGluR inactivation and MVR respectively, the actions of PKC may also aid in the full expression of MVR, as this kinase has been shown to increase the ready releasable pool (Gillis et al., 1996) and prime the release machinery to be more sensitive to Ca^{2+} (Lou et al., 2005). With more vesicles docked that are more sensitive to Ca^{2+} released from intracellular stores the number of vesicles able to release at one time is likely enhanced after PKC activation. This may also represent one method by amplitude priming occurs (figure 6.1). Many PKC isoforms require Ca^{2+}, which can be supplied from intracellular stores, suggesting that PKC and IP_3 act cooperatively to induce both priming and MVR instead of one messenger being exclusively associated with a single mechanism. Although, in the presence of a high dose of ryanodine the increase in mEPSC frequency, which occurs through a PKC dependent process, was similar to NA alone, suggesting that either the PKC isoform involved is atypical i.e. Ca^{2+} independent, or that there are separate PKC isoforms regulating the increase in mEPSC frequency, mGluR inactivation and MVR. Because MVR occurs in the presence of a dramatically elevated glutamate release rate, there is also a possibility of separate pools regulating quantal miniatures and multiquantal miniatures. For the synchronous release of multiple vesicles to confer larger amplitude currents, this enhanced presynaptic signal should not be prohibited by a limited number of AMPARs postsynaptically. That NA causes the insertion of AMPARs postsynaptically is an ideal alteration so that the elevated concentration of glutamate in the synaptic cleft is not wasted. However, more experiments are required to see if these mechanisms occur at the same synapse or if they occur simultaneously at separate synapses. The long-lasting increase in mEPSC amplitude, which is the result of AMPAR insertion, is still detected after MVR has stopped, suggesting that the new AMPARs can sense single packets of glutamate. But how is this possible if the postsynaptic receptor field is greatly under saturated as to detect MVR? To account for this discrepancy we have adopted a model where NA synchronizes the release of single vesicles across multiple active zones that are closely apposed. The elevated concentration of cleft

Figure 6.1 Priming of mEPSC frequency and amplitude by NA

Left panel: in control conditions, synaptic glutamate activates presynaptic mGluRs keeping mEPSC frequency low. Middle panel: initial α_1-adrenoceptor activation increases PKC activity causing an enhancement of mEPSC frequency without changing mEPSC amplitude. Although the facilitating effect on mEPSC frequency is still limited by the activity of functional mGluRs, during this time PKC is working to inactivate these autoreceptors. Right panel: the consequences of mGluR inactivation become apparent when an additional NA challenge is administered in which an increase in mEPSC frequency is observed that is substantially larger than the first. Successive α_1-adrenoceptor activation also results in dramatically larger mEPSCs that arise from the rapid release of stored Ca^{2+}. The mechanism underlying amplitude priming, however, has not been elucidated. The voltage clamp traces of mEPSCs are adapted from Gordon & Bains (2003). Scale bars, 50 pA and 1 s.

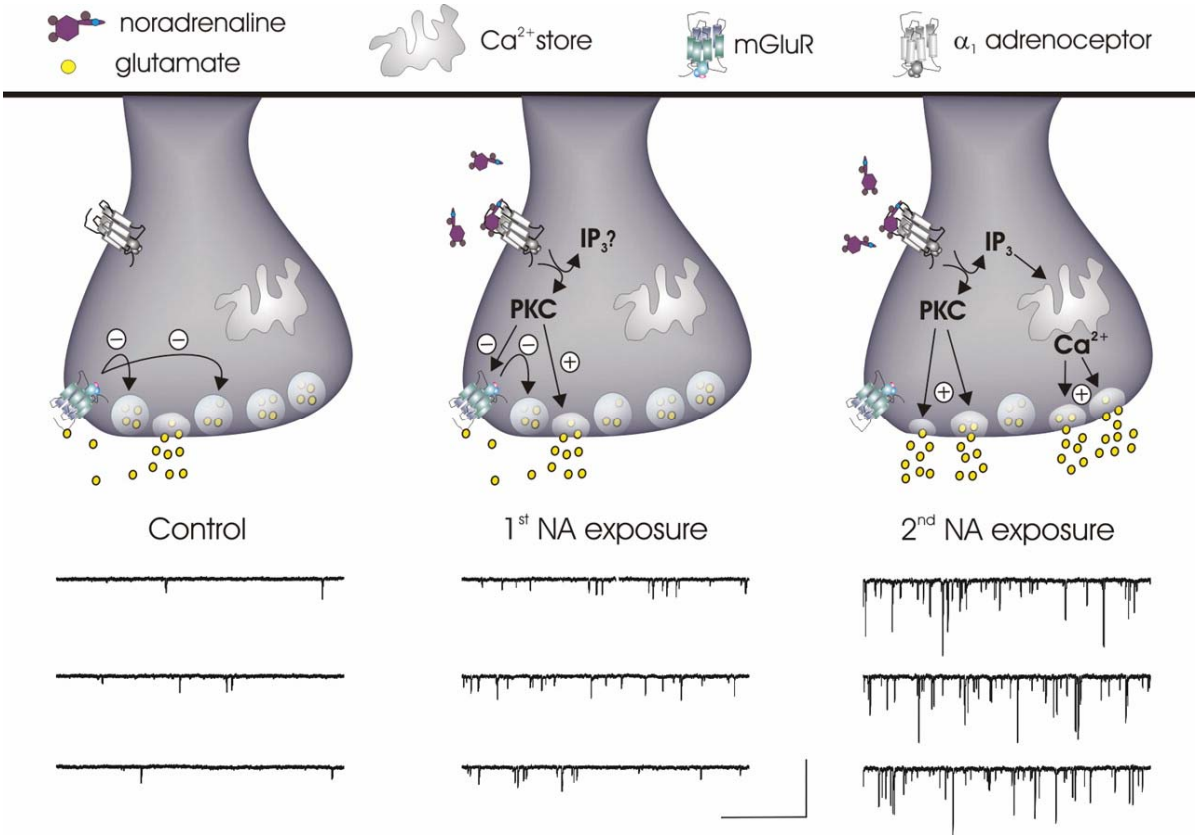
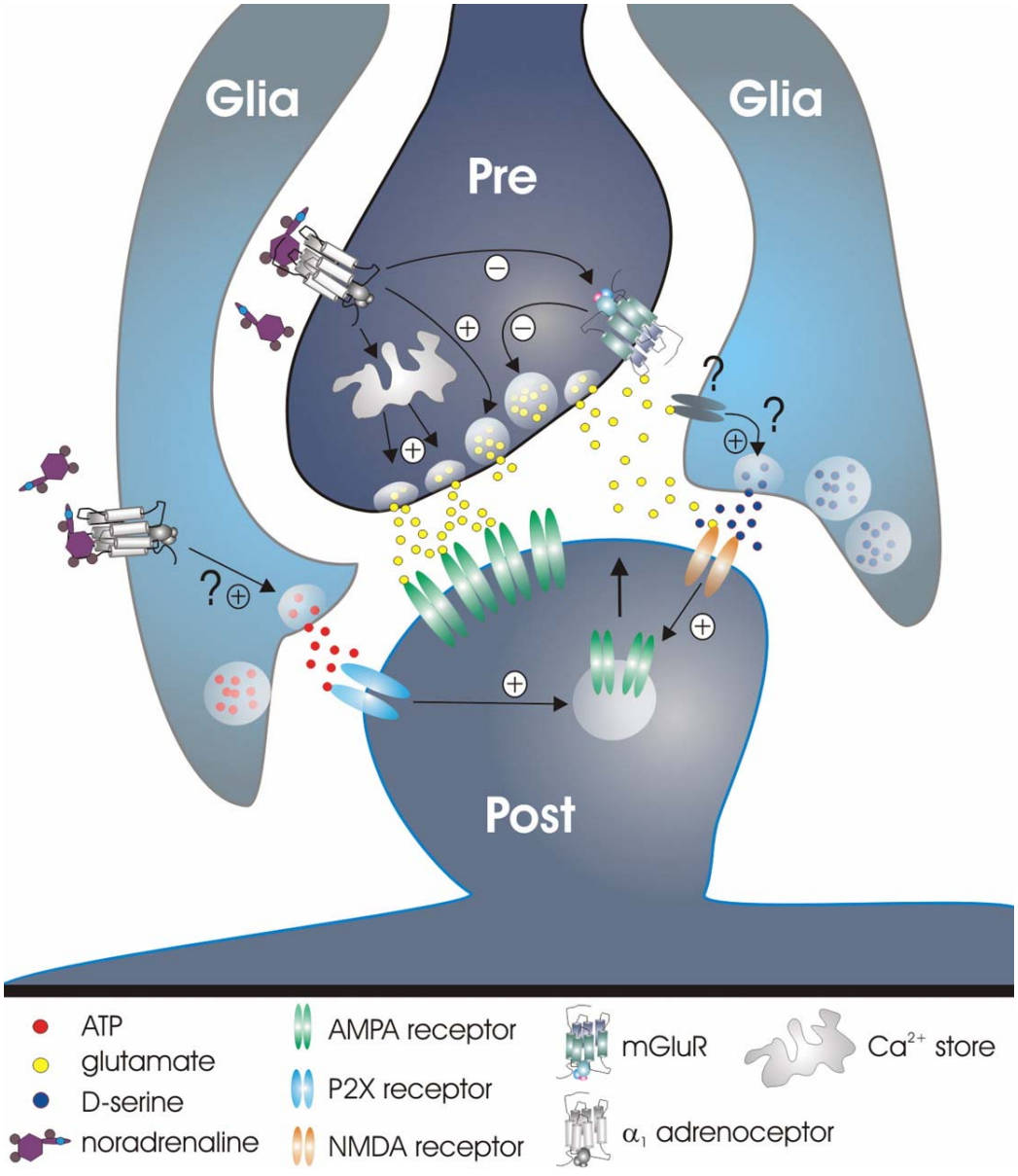

noradrenaline
glutamate
Ca2+store
mGluR
α1 adrenoceptor
IP3?
PKC
IP3
PKC
Ca2+
Control
1st NA exposure
2nd NA exposure

Figure 6.2 Long-lasting plasticity at glutamatergic synapses on MNCs

Summary of different types of enduring plasticity at glutamatergic synapses on MNCs, which incorporate presynaptic, postsynaptic and glial signalling to enhance excitatory drive for extended periods of time. NA utilizes the presynaptic terminal to prime glutamate release by inactivating mGluRs and to trigger MVR by recruiting Ca^{2+} release from internal stores. The two remaining elements of the tripartite synapse are utilized concurrently to elicit AMPA receptor insertion postsynaptically and thus a long-lasting change in synaptic strength. NA acts on glial cells to trigger the release of ATP which subsequently acts on postsynaptic P2X channels to induce activity-independent changes in synapse function. Finally, glia-derived D-serine acts as an essential co-transmitter with synaptically released glutamate to activate postsynaptic NMDA receptors in the induction of classical activity-dependent plasticity.


Glia
Pre
Glia
Post
ATP
glutamate
D-serine
noradrenaline
AMPA receptor
P2X receptor
NMDA receptor
mGluR
α1 adrenoceptor
Ca2+ store

glutamate observed during NA would then result from spillover of glutamate between postsynaptic receptor fields. Under this schematic a single vesicle would largely saturate a receptor field and new AMPARs could sense and amplify this signal. An equally plausible scenario, and perhaps simpler, is that entirely separate and structurally unique set of synapses are utilized for MVR. A simplified schematic showing all three types of NA-induced plasticity can be seen in figure 6.2. In addition, glial derived D-serine and classical NMDA dependent long-term strengthening is included in the diagram.

6.5 Physiological Significance

We have described novel ways in which excitatory synapses store information in the hypothalamus, but what would be the advantages of learning and remembering in autonomic circuits? If one considers that changes in the internal or external environment can occur swiftly and endure for prolonged periods of time, long-term alterations in synaptic strength may increase the gain and/or sensitivity of system output for the duration of the physiological challenge so that the demands of the organism are met more efficiently. For example, *in vivo* studies demonstrating augmented release of the neurohormones vasopressin (VP) and corticotropin releasing hormone (CRH) from the pituitary gland in response to repetitive hypovolemic challenges (Lilly et al., 1983;Lilly et al., 1986;Thrivikraman et al., 1997) hints at underlying learning and memory processes that may reside in autonomic circuitry. Sadly though, how these types of plasticity 'fit' into the behaviour of the MNC system requires further clarification. This is an unfortunate but realistic outcome that is due to the type of *in vitro* experiments conducted and the limitations of the preparation and techniques used in our studies. For instance, a critical aspect of MNC physiology is their ability to undergo distinct patterns of action potential discharge *in vivo* to elevate neurohormone secretion. Although the mechanisms responsible for these firing phenomena have remained largely elusive, two of the central hypotheses includes the autocrine and paracrine actions of dendritically released peptide (Moos et al., 1989;Moos and Richard, 1989;Richard et al., 1991;Brown et al., 2004b;Brown et al., 2006) and intrinsic membrane properties (Renaud, 1994). Notably, NA has been shown to enhance the somatodendritic release of these peptides (Armstrong

et al., 1986), which in turn can facilitate further NA release (Ludwig et al., 2000), and NA has been shown to facilitate MNC firing by altering K^+ conductances (Dudek et al., 1989). Whether the types of aforementioned plasticity recruit the dendritic release of hormone and potentially regulate the firing behaviour of VP and OT cells requires further investigation. However, both MVR and the glial-ATP effect occur without the involvement of postsynaptic α_1-adrenoceptors, given by our experiments targeting G-protein coupling, PKC and intracellular Ca^{2+} stores (Gordon et al., 2005;Gordon and Bains, 2005). Further, the priming effect appears entirely presynaptic, suggesting that the dendritic release of VP and OT is not directly involved in any of these forms of NA-mediated plasticity and that dendritic neurohormone release will likely only facilitate these processes. However, we have not used OT or VP receptor antagonists to directly address this issue. It is likely that NA and ATP, while important for augmenting system excitability and, along with D-serine, putatively important for learning and memory in the MNCs, are likely part of a more complex network of signals that generates the totality of MNC behaviour.

6.6 Conclusion

The success of science lay with in its collective operation. The work of an individual lab never holds the truth but instead truth resides in the work of innumerable laboratories that takes place over decades, generations and centuries. The pure accumulation of all work does not constitute the truth, but it is acceptance and selection of certain pieces of work that over time gradually refines truth. The concept that many independently operating entities can generate something that is greater than the sum of their parts is referred to as collective intelligence or swarm intelligence. The term is commonly used to describe the workings of gregarious insects and as such the process of science is analogous to this. Swarm intelligence occurs when there is no centralized control structure dictating how simple, independent agents, such as laboratories or insects, interact with one another and with their surrounding environment. The interactions result in the emergence of a 'global' behaviour that cannot be produced from any one agent. For example, an individual insect, say a termite, with its simple reflexive nervous system cannot possibly

know how to construct the elaborate tunnels, ventilation shafts and storage facilities common to a termite mound, but if fifty thousand termites work together, this wonderful and complicated structure 'emerges' without the guidance of a floor plan or a wise authority figure. Individual mistakes are smoothed over by the collective effort, similar to incorrect interpretations of scientific data; the science collective will carry on with accepted ideas and ignore unpalatable findings. The best way to determine if the actions of a single agent are inline with or accepted by the collective effort is to look into the future and see if the agent's actions helped, hindered or were of no consequence. This I am afraid is an unfortunate impossibility. The second best thing to do is to see if other agents are doing something similar, if they are changing their current course to do what you are doing or if they are largely ignoring your actions.

Several results from other 'agents' in the field of synaptic plasticity have, at least for the mean time, offered support for our contributions to the collective effort of neuroscience. One recent study shows that presynaptic mGluRs are important targets for plasticity in which the presence or absence of autoreceptors act as a functional switch dictating the polarity of plasticity. If autoreceptors are present, high frequency stimulation induces LTD and if absent the same stimulation induces LTP (Pelkey et al., 2005). While we did not test the impact of activity dependent plasticity before and after NA, the basic results are similar i.e. the same NA treatment produces a different outcome on synaptic efficacy when mGluRs no longer actively decrease glutamate P_r. This study and another have also suggested that the reduction in autoreceptor efficacy is due to receptor internalization (Pelkey et al., 2005;Mathiesen and Ramirez, 2006) and that PKC is an important mediator of internalization (Mathiesen and Ramirez, 2006). Both ideas can easily be incorporated with our data.

A recent transgenic mouse study has presented some very encouraging data in support of our novel result linking ATP and P2X receptor activation to AMPAR insertion and subsequent long-term enhancement of synaptic strength. Even though all studies concerned with glial ATP and synaptic plasticity in the hippocampus have exclusively examined the role of adenosine and heterosynaptic suppression, when postsynaptic $P2X_4$ receptors are knocked out of CA1 pyramidal neurons, stimulation protocols that normally

produce robust LTP result in approximately half of the normal synaptic potentiation (Sim et al., 2006), in spite of the fact this process has been shown time and time again to rely entirely on NMDAR activation (Malinow and Malenka, 2002). We anticipate there will be many more studies to follow examining a role for postsynaptic P2X receptors in mediating LTP like processes and under what conditions extracellular ectoATPase select for ATP versus adenosine (Dulla et al., 2005), which may represent an excellent method for favouring either postsynaptic enhancement or presynaptic inhibition respectively.

While there have been no publications after our most recent paper demonstrating multivesicular release that support our findings, there is one manuscript reporting a similar result that was published at roughly the same time, suggesting that presynaptic P2X receptor can synchronize the release of multiple vesicles (Shigetomi and Kato, 2004)(see above in 'Multivesicular Miniatures in Other Autonomic Regions'). However, the studies most closely resembling our NA-mediated MVR story are the Llano paper demonstrating tonic multivesicular miniatures that result presynaptic intracellular stores Ca^{2+} sparks and a recent paper demonstrating that quantal size at the NMJ depends on presynaptic ryanodine receptors (Liu et al., 2005). The occurrence of multivesicular miniatures is an interesting and controversial topic that will undoubtedly yield many future papers arguing both for and against this process. Currently, additional studies are required to settle the debate of its existence.

Whether or not our interpretations will continue to be supported in the synaptic neuroscience literate remains to be seen. Even if they do not persist, new ideas based on hard evidence that perturb scientific dogmas, like the origin of miniatures and a critical role for glial derived ATP in long-term synaptic strengthening, are absolutely essential for the complete comprehension of observed phenomena as well as the 'health' of the scientific process as a whole. Correct interpretation of data is the ultimate goal of science in our pursuit for 'truth'. However, truth is an elusive and intangible concept, as it incessantly evolves when new data and new interpretations of old data change our understanding. The ideological view that there exists an absolute truth is thus debatable, as we are uncertain when or if the process will stop. While having confidence in ones data can be a powerful force, in attempting to convince others of the validity of your

research and to have others see the data from your perspective, it is almost certain that the interpretation of our data will be subject to this relentless force of an ever changing truth. Our interpretation may become altered, disproved or simply refined as more knowledge is gained concerning how neurons and glial cells contribute to changes in synaptic strength. Whatever the change, big or small, one can be confident that the collective intelligence of science will appropriately utilize those ideas backed by substantial evidence and ignore those distasteful findings that do not fit the current mould of understanding. At the same time the collective must be vigilant and retain the notion that one day the insipid may become palatable when taken under a new and evolved context.

6.7 Speculation: the Importance of Glia in Intelligence

Only in the last century has mankind begun to unravel the mysteries regarding the foundation for intelligence. For the mind to produce the behaviours that to us have become the quintessence of humanity, i.e. thought, reasoning and the complex usage of symbols in mathematics and language, the brain must possess enormous computational power. Let us perform a very rough (and possibly very ignorant) estimate of this power by looking at the enormous number of basic signalling elements. The neuron is the unitary signalling device of the central nervous system, with approximately 10^{11} number of cells. If we then simplify a neuron to a binary signalling unit, like the transistor in a computer, which can either be on or off and we consider the number of connections a neuron makes (A single pyramidal neuron in the neocortex can project to 10,000 others, while a Purkinje cell of the cerebellum can receive up to 120,000 inputs), the combinatorial possibilities become gargantuan, in the range of multiplying 10 billion by itself 10,000 times. The incredulity of this number, however, is not the final story. The signal used to communicate with other neurons (the action potential) and the point of communication (the synapse) cannot be reduced to a binary code. Their strength and duration can be modulated in innumerable ways, increasing the subtlety of brain computation far beyond calculability.

Higher order mammals including humans and non-human primates possess brains with these properties, with a similar number of neurons per body mass and similar

connectionism, indicating it is not these qualities making humans laureate. What then gives humans singularity? Because our total brain mass to body mass ratio* is the highest of any organism, *a priori* something else in the *homo sapien* physiology enables a higher level of processing than can be achieved by any other species. As is likely all too clear from the writings presented thus far, the glial cell, that surreptitious party member, which has been largely ignored since the beginnings of 'neural' science, may be responsible for human ascendancy. Glial cells are proportionally more numerous in the human brain compared to other higher order mammals (Nedergaard et al., 2003), potentially explaining our larger brain to body mass ratio. In this final section I will explore some of interesting data on our current understanding of glial cells and intelligence from the perspective of the prefrontal cortex and evolutionary brain development.

*The brain mass to body mass ratio can be used as a measure of intelligence and marker for evolutionary ranking. The theory is simple; every organism requires a minimal amount of neural tissue to sense and to act. As organisms increase in size this minimum also increases because more neural tissue is required to service the body. Therefore any extra neural tissue above this minimum can be used for more complex signal integration and higher order functions. By normalizing for body size, the brain mass to body mass ratio can estimate where an organism is positioned on the hierarchical evolutionary scale of brain development. This technique is not without fault (Harvey and Krebs, 1990).

Individuals often think that intelligence involves quick mindedness and the ability to generate insightful thoughts. Recent human and non-human fMRI studies are suggesting these qualities, although important, may not constitute the root of intelligence. Instead, its basis may lie in ones ability to keep track of, and manipulate multiple items over a time period for solving a problem. These items can be words, objects, concepts or spatial relationships. This process is commonly referred to as working memory and is the erasable blackboard of the mind that allows one to store information briefly for the

purpose of comprehension, reasoning, and planning (Wickelgren, 1997). Ones ability to do this is an excellent predictor of intelligence, including a specific form called fluid intelligence. This is a type of intelligence not derived from acquired knowledge of ones environment or culture and is considered by some to be true intelligence. It is essentially ones ability to solve spatial problems involving shapes and patterns. Thus, in working memory, ones ability hold ideas and select ideas have been deemed essential. In humans, the areas of the prefrontal cortex that participate in these two processes are the prefrontal area 8 and the prefrontal area 46 of the dorsolateral cortex (Rowe et al., 2000). What is fascinating about these and other areas of the prefrontal cortex is the emergence of a unique glial cell architecture in both density and connectionism seen in human and non-human primates compared to lower order rodent species (Colombo, 1996). Like those studied above, rodents contain networks of interconnected cortical stellate astrocytes that are referred to as a panglial syncytium, which reside within a single laminae of the cortex. Evolutionary pressures have selected for a different cortical astrocyte organization in higher order primate species. Astrocytes are aligned vertically in a columnar distribution possessing long processes that span multiple laminae. In evolutionary brain development, this appears in the neocortex for the first time in old world monkeys such as *Macaca mulatta* and becomes progressively more prominent from new world monkeys such as *Microcebus murinus* to *homo sapiens* where in the latter, astrocytic processes span the greatest lamina distances (Colombo et al., 2000). The columnar organization is thought to optimize information processing into spatially discrete units while minimizing neural wiring. The syncytium architecture of lower order organisms might compromise signal specificity and lower computational power compared to that of primates. The emergence of interlaminar glial projections in the neocortex is a recent evolutionary event that has seen the largest growth in mammalian brain development (Colombo and Reisin, 2004). In particular, as prefrontal areas also display these advances, speculation on their role in complex information processing and the emergence of intelligence is tempting. One human study has compared the cytoarchitectonic differences in area 44 and 45 of the frontal cortex between ‘normal’ individuals and gifted individuals with creative professional talents (Bogolepova, 1994).

A larger pyramidal neuron glial cell index and a peculiar neuronal grouping in lamina III and V were demonstrated in the gifted individuals. Yet our current understandings of how glial cells modulate neural excitability and synaptic transmission has been done almost exclusively in rodents, using astrocyte cultures and slice preparations and only very recently in vivo, a reality that places the understanding of a complex human behaviour such as intelligence in the distant future. Furthermore, for logistical reasons there is a paucity of primate *in vivo* studies that are concerned with prefrontal cortex and the basis for fluid intelligence. These studies fail to examine a role for glial cells and instead correlate action potential firing with behaviour. In lieu of this, understanding the basis for our ability to reason, comprehend and deduct must progress beyond our current techniques. In the future, methods such as genetically encoded Ca^{2+} sensors, genetically targeted activation and inactivation of population of cells, combined with multiunit recordings and fMRI in non-human primates may aid in our search. As complex human behaviours mask the elementary components that comprise them, once delineated, comprehending the root of our own intelligence will represent one of the greatest feats of scientific inquiry.

References

1. Adesnik H, Nicoll RA, England PM (2005) Photoinactivation of native AMPA receptors reveals their real-time trafficking. Neuron 48:977-985.

2. Agullo L, Garcia A (1991) Norepinephrine increases cyclic GMP in astrocytes by a mechanism dependent on nitric oxide synthesis. Eur J Pharmacol 206:343-346.

3. Anderson CM, Bergher JP, Swanson RA (2004) ATP-induced ATP release from astrocytes. J Neurochem 88:246-256.

4. Anderson CM, Nedergaard M (2006) Emerging challenges of assigning P2X7 receptor function and immunoreactivity in neurons. Trends Neurosci 29:257-262.

5. Andrew RD, Dudek FE (1984a) Analysis of intracellularly recorded phasic bursting by mammalian neuroendocrine cells. J Neurophysiol 51:552-566.

6. Andrew RD, Dudek FE (1984b) Intrinsic inhibition in magnocellular neuroendocrine cells of rat hypothalamus. J Physiol 353:171-185.

7. Anwyl R (1991) The role of the metabotropic receptor in synaptic plasticity. Trends Pharmacol Sci 12:324-326.

8. Araque A, Martin ED, Perea G, Arellano JI, Buno W (2002) Synaptically released acetylcholine evokes Ca2+ elevations in astrocytes in hippocampal slices. J Neurosci 22:2443-2450.

9. Araque A, Parpura V, Sanzgiri RP, Haydon PG (1998) Glutamate-dependent astrocyte modulation of synaptic transmission between cultured hippocampal neurons. Eur J Neurosci 10:2129-2142.

10. Arcuino G, Lin JH, Takano T, Liu C, Jiang L, Gao Q, Kang J, Nedergaard M (2002) Intercellular calcium signaling mediated by point-source burst release of ATP. Proc Natl Acad Sci U S A 99:9840-9845.

11. Armstrong JN, Brust TB, Lewis RG, MacVicar BA (2002) Activation of presynaptic P2X7-like receptors depresses mossy fiber-CA3 synaptic transmission through p38 mitogen-activated protein kinase. J Neurosci 22:5938-5945.

12. Armstrong WE (1995) Morphological and electrophysiological classification of hypothalamic supraoptic neurons. Prog Neurobiol 47:291-339.

13. Armstrong WE, Gallagher MJ, Sladek CD (1986) Noradrenergic stimulation of supraoptic neuronal activity and vasopressin release in vitro: mediation by an alpha 1-receptor. Brain Res 365:192-197.

14. Arnauld E, Cirino M, Layton BS, Renaud LP (1983) Contrasting actions of amino acids, acetylcholine, noradrenaline and leucine enkephalin on the excitability of supraoptic vasopressin-secreting neurons. A microiontophoretic study in the rat. Neuroendocrinology 36:187-196.

15. Arnauld E, Vincent JD, Dreifuss JJ (1974) Firing patterns of hypothalamic supraoptic neurons during water deprivation in monkeys. Science 185:535-537.

16. Atwood HL, Karunanithi S (2002) Diversification of synaptic strength: presynaptic elements. Nat Rev Neurosci 3:497-516.

17. Auger C, Kondo S, Marty A (1998) Multivesicular release at single functional synaptic sites in cerebellar stellate and basket cells. J Neurosci 18:4532-4547.

18. Auger C, Marty A (1997) Heterogeneity of functional synaptic parameters among single release sites. Neuron 19:139-150.

19. Auger C, Marty A (2000) Quantal currents at single-site central synapses. J Physiol 526 Pt 1:3-11.

20. Bailey AR, Wakerley JB (1997) Electrophysiological responses of neurones in the rat nucleus tractus solitarius to oxytocin-releasing stimuli. Neurosci Lett 236:163-166.

21. Bains JS, Ferguson AV (1997) Nitric oxide regulates NMDA-driven GABAergic inputs to type I neurones of the rat paraventricular nucleus. J Physiol 499 (Pt 3):733-746.

22. Bains JS, Longacher JM, Staley KJ (1999) Reciprocal interactions between CA3 network activity and strength of recurrent collateral synapses. Nat Neurosci 2:720-726.

23. Bal-Price A, Moneer Z, Brown GC (2002) Nitric oxide induces rapid, calcium-dependent release of vesicular glutamate and ATP from cultured rat astrocytes. Glia 40:312-323.

24. Bancroft J (2005) The endocrinology of sexual arousal. J Endocrinol 186:411-427.

25. Bandrowski AE, Huguenard JR, Prince DA (2003) Baseline glutamate levels affect group I and II mGluRs in layer V pyramidal neurons of rat sensorimotor cortex. J Neurophysiol 89:1308-1316.

26. Banjac A, Nedeljkovic N, Horvat A, Kanazir D, Nikezic G (2001) Ontogenetic profile of ecto-ATPase activity in rat hippocampal and caudate nucleus synaptic plasma membrane fractions. Physiol Res 50:411-417.

27. Barres BA (1989) Neuronal-glial interactions. A new form of transmission? Nature 339:343-344.

28. Baskys A, Malenka RC (1991) Agonists at metabotropic glutamate receptors presynaptically inhibit EPSCs in neonatal rat hippocampus. J Physiol 444:687-701.

29. Baude A, Nusser Z, Roberts JD, Mulvihill E, McIlhinney RA, Somogyi P (1993) The metabotropic glutamate receptor (mGluR1 alpha) is concentrated at perisynaptic membrane of neuronal subpopulations as detected by immunogold reaction. Neuron 11:771-787.

30. Baxter AW, Wyllie DJ (2006) Phosphatidylinositol 3 kinase activation and AMPA receptor subunit trafficking underlie the potentiation of miniature EPSC amplitudes triggered by the activation of L-type calcium channels. J Neurosci 26:5456-5469.

31. Beattie EC, Stellwagen D, Morishita W, Bresnahan JC, Ha BK, Von ZM, Beattie MS, Malenka RC (2002) Control of synaptic strength by glial TNFalpha. Science 295:2282-2285.

32. Bekar LK, Loewen ME, Cao K, Sun X, Leis J, Wang R, Forsyth GW, Walz W (2005) Complex expression and localization of inactivating Kv channels in cultured hippocampal astrocytes. J Neurophysiol 93:1699-1709.

33. Bekkers JM, Richerson GB, Stevens CF (1990) Origin of variability in quantal size in cultured hippocampal neurons and hippocampal slices. Proc Natl Acad Sci U S A 87:5359-5362.

34. Bekkers JM, Stevens CF (1995) Quantal analysis of EPSCs recorded from small numbers of synapses in hippocampal cultures. J Neurophysiol 73:1145-1156.

35. Belin V, Moos F (1986) Paired recordings from supraoptic and paraventricular oxytocin cells in suckled rats: recruitment and synchronization. J Physiol 377:369-390.

36. Belin V, Moos F, Richard P (1984) Synchronization of oxytocin cells in the hypothalamic paraventricular and supraoptic nuclei in suckled rats: direct proof with paired extracellular recordings. Exp Brain Res 57:201-203.

37. Benke TA, Luthi A, Isaac JT, Collingridge GL (1998) Modulation of AMPA receptor unitary conductance by synaptic activity. Nature 393:793-797.

38. Berglund K, Midorikawa M, Tachibana M (2002) Increase in the pool size of releasable synaptic vesicles by the activation of protein kinase C in goldfish retinal bipolar cells. J Neurosci 22:4776-4785.

39. Bezzi P, Carmignoto G, Pasti L, Vesce S, Rossi D, Rizzini BL, Pozzan T, Volterra A (1998) Prostaglandins stimulate calcium-dependent glutamate release in astrocytes. Nature 391:281-285.

40. Bezzi P, Volterra A (2001) A neuron-glia signalling network in the active brain. Curr Opin Neurobiol 11:387-394.

41. Bicknell RJ (1988) Optimizing release from peptide hormone secretory nerve terminals. J Exp Biol 139:51-65.

42. Billups B, Graham BP, Wong AY, Forsythe ID (2005) Unmasking group III metabotropic glutamate autoreceptor function at excitatory synapses in the rat CNS. J Physiol 565:885-896.

43. Bittencourt JC, Benoit R, Sawchenko PE (1991) Distribution and origins of substance P-immunoreactive projections to the paraventricular and supraoptic nuclei: partial overlap with ascending catecholaminergic projections. J Chem Neuroanat 4:63-78.

44. Boehm J, Kang MG, Johnson RC, Esteban J, Huganir RL, Malinow R (2006) Synaptic incorporation of AMPA receptors during LTP is controlled by a PKC phosphorylation site on GluR1. Neuron 51:213-225.

45. Bogolepova IN (1994) [The cytoarchitectonic characteristics of the speech center of the brain in gifted people in the plan to study individual variability of human brain structure]. Morfologiia 106:31-38.

46. Bonan CD, Walz R, Pereira GS, Worm PV, Battastini AM, Cavalheiro EA, Izquierdo I, Sarkis JJ (2000) Changes in synaptosomal ectonucleotidase activities in two rat models of temporal lobe epilepsy. Epilepsy Res 39:229-238.

47. Borgdorff AJ, Choquet D (2002) Regulation of AMPA receptor lateral movements. Nature 417:649-653.

48. Boudaba C, Di S, Tasker JG (2003a) Presynaptic noradrenergic regulation of glutamate inputs to hypothalamic magnocellular neurones. J Neuroendocrinol 15:803-810.

49. Boudaba C, Linn DM, Halmos KC, Tasker JG (2003b) Increased tonic activation of presynaptic metabotropic glutamate receptors in the rat supraoptic nucleus following chronic dehydration. J Physiol 551:815-823.

50. Boudaba C, Schrader LA, Tasker JG (1997) Physiological evidence for local excitatory synaptic circuits in the rat hypothalamus. J Neurophysiol 77:3396-3400.

51. Boudaba C, Tasker JG (2006) Intranuclear coupling of hypothalamic magnocellular nuclei by glutamate synaptic circuits. Am J Physiol Regul Integr Comp Physiol 291:R102-R111.

52. Bourque CW, Oliet SH, Richard D (1994) Osmoreceptors, osmoreception, and osmoregulation. Front Neuroendocrinol 15:231-274.

53. Bourque CW, Renaud LP (1984) Activity patterns and osmosensitivity of rat supraoptic neurones in perfused hypothalamic explants. J Physiol 349:631-642.

54. Bourque CW, Renaud LP (1991) Membrane properties of rat magnocellular neuroendocrine cells in vivo. Brain Res 540:349-352.

55. Bowser DN, Khakh BS (2004) ATP excites interneurons and astrocytes to increase synaptic inhibition in neuronal networks. J Neurosci 24:8606-8620.

56. Bradley SR, Levey AI, Hersch SM, Conn PJ (1996) Immunocytochemical localization of group III metabotropic glutamate receptors in the hippocampus with subtype-specific antibodies. J Neurosci 16:2044-2056.

57. Braet K, Vandamme W, Martin PE, Evans WH, Leybaert L (2003) Photoliberating inositol-1,4,5-trisphosphate triggers ATP release that is blocked by the connexin mimetic peptide gap 26. Cell Calcium 33:37-48.

58. Brager DH, Capogna M, Thompson SM (2002) Short-term synaptic plasticity, simulation of nerve terminal dynamics, and the effects of protein kinase C activation in rat hippocampus. J Physiol 541:545-559.

59. Brimble MJ, Dyball RE (1976) Contrasting pattern changes in the firing of vasopressin and oxytocin secreting neurones during osmotic stimulation [proceedings]. J Physiol 263:189P-190P.

60. Brimble MJ, Dyball RE (1977) Characterization of the responses of oxytocin- and vasopressin-secreting neurones in the supraoptic nucleus to osmotic stimulation. J Physiol 271:253-271.

61. Brooks DP, Share L, Crofton JT (1986a) Central adrenergic control of vasopressin release. Neuroendocrinology 42:416-420.

62. Brooks DP, Share L, Crofton JT (1986b) Central adrenergic mechanisms in hemorrhage-induced vasopressin secretion. Am J Physiol 251:H1158-H1162.

63. Brown CH, Bourque CW (2004) Autocrine feedback inhibition of plateau potentials terminates phasic bursts in magnocellular neurosecretory cells of the rat supraoptic nucleus. J Physiol 557:949-960.

64. Brown CH, Bull PM, Bourque CW (2004a) Phasic bursts in rat magnocellular neurosecretory cells are not intrinsically regenerative in vivo. Eur J Neurosci 19:2977-2983.

65. Brown CH, Leng G, Ludwig M, Bourque CW (2006) Endogenous activation of supraoptic nucleus kappa-opioid receptors terminates spontaneous phasic bursts in rat magnocellular neurosecretory cells. J Neurophysiol 95:3235-3244.

66. Brown CH, Ludwig M, Leng G (2004b) Temporal dissociation of the feedback effects of dendritically co-released peptides on rhythmogenesis in vasopressin cells. Neuroscience 124:105-111.

67. Bruns D, Riedel D, Klingauf J, Jahn R (2000) Quantal release of serotonin. Neuron 28:205-220.

68. Bukcharaeva EA, Kim KC, Moravec J, Nikolsky EE, Vyskocil F (1999) Noradrenaline synchronizes evoked quantal release at frog neuromuscular junctions. J Physiol 517 (Pt 3):879-888.

69. Buller KM, Khanna S, Sibbald JR, Day TA (1996) Central noradrenergic neurons signal via ATP to elicit vasopressin responses to haemorrhage. Neuroscience 73:637-642.

70. Burnstock G (1995) Noradrenaline and ATP: cotransmitters and neuromodulators. J Physiol Pharmacol 46:365-384.

71. Burnstock G (2004) Cotransmission. Curr Opin Pharmacol 4:47-52.

72. Cai Z, Kimelberg HK (1997) Glutamate receptor-mediated calcium responses in acutely isolated hippocampal astrocytes. Glia 21:380-389.

73. Cai Z, Schools GP, Kimelberg HK (2000) Metabotropic glutamate receptors in acutely isolated hippocampal astrocytes: developmental changes of mGluR5 mRNA and functional expression. Glia 29:70-80.

74. Calaresu FR, Ciriello J (1980) Projections to the hypothalamus from buffer nerves and nucleus tractus solitarius in the cat. Am J Physiol 239:R130-R136.

75. Carter AG, Regehr WG (2002) Quantal events shape cerebellar interneuron firing. Nat Neurosci 5:1309-1318.

76. Caverson MM, Ciriello J, Calaresu FR, Krukoff TL (1987) Distribution and morphology of vasopressin-, neurophysin II-, and oxytocin-immunoreactive cell bodies in the forebrain of the cat. J Comp Neurol 259:211-236.

77. Charles AC (1994) Glia-neuron intercellular calcium signaling. Dev Neurosci 16:196-206.

78. Charles AC, Merrill JE, Dirksen ER, Sanderson MJ (1991) Intercellular signaling in glial cells: calcium waves and oscillations in response to mechanical stimulation and glutamate. Neuron 6:983-992.

79. Clarke DD (1991) Fluoroacetate and fluorocitrate: mechanism of action. Neurochem Res 16:1055-1058.

80. Clements JD (1996) Transmitter timecourse in the synaptic cleft: its role in central synaptic function. Trends Neurosci 19:163-171.

81. Coco S, Calegari F, Pravettoni E, Pozzi D, Taverna E, Rosa P, Matteoli M, Verderio C (2003) Storage and release of ATP from astrocytes in culture. J Biol Chem 278:1354-62.

82. Cole RL, Sawchenko PE (2002) Neurotransmitter regulation of cellular activation and neuropeptide gene expression in the paraventricular nucleus of the hypothalamus. J Neurosci 22:959-969.

83. Colombo JA (1996) Interlaminar astroglial processes in the cerebral cortex of adult monkeys but not of adult rats. Acta Anat (Basel) 155:57-62.

84. Colombo JA, Fuchs E, Hartig W, Marotte LR, Puissant V (2000) "Rodent-like" and "primate-like" types of astroglial architecture in the adult cerebral cortex of mammals: a comparative study. Anat Embryol (Berl) 201:111-120.

85. Colombo JA, Reisin HD (2004) Interlaminar astroglia of the cerebral cortex: a marker of the primate brain. Brain Res 1006:126-131.

86. Colquhoun D, Jonas P, Sakmann B (1992) Action of brief pulses of glutamate on AMPA/kainate receptors in patches from different neurones of rat hippocampal slices. J Physiol 458:261-287.

87. Conn PJ, Pin JP (1997) Pharmacology and functions of metabotropic glutamate receptors. Annu Rev Pharmacol Toxicol 37:205-237.

88. Cornell-Bell AH, Finkbeiner SM, Cooper MS, Smith SJ (1990) Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling. Science 247:470-473.

89. Cotrina ML, Lin JH, Alves-Rodrigues A, Liu S, Li J, Azmi-Ghadimi H, Kang J, Naus CC, Nedergaard M (1998) Connexins regulate calcium signaling by controlling ATP release. Proc Natl Acad Sci U S A 95:15735-15740.

90. Cotrina ML, Lin JH, Lopez-Garcia JC, Naus CC, Nedergaard M (2000) ATP-mediated glia signaling. J Neurosci 20:2835-2844.

91. Crowley WR, Shyr SW, Kacsoh B, Grosvenor CE (1987) Evidence for stimulatory noradrenergic and inhibitory dopaminergic regulation of oxytocin release in the lactating rat. Endocrinology 121:14-20.

92. Cunningham ET, Jr., Sawchenko PE (1988) Anatomical specificity of noradrenergic inputs to the paraventricular and supraoptic nuclei of the rat hypothalamus. J Comp Neurol 274:60-76.

93. Daftary SS, Boudaba C, Szabo K, Tasker JG (1998) Noradrenergic excitation of magnocellular neurons in the rat hypothalamic paraventricular nucleus via intranuclear glutamatergic circuits. J Neurosci 18:10619-10628.

94. Dani JW, Chernjavsky A, Smith SJ (1992) Neuronal activity triggers calcium waves in hippocampal astrocyte networks. Neuron 8:429-440.

95. Day TA, Ferguson AV, Renaud LP (1984) Facilitatory influence of noradrenergic afferents on the excitability of rat paraventricular nucleus neurosecretory cells. J Physiol 355:237-249.

96. Day TA, Randle JC, Renaud LP (1985) Opposing alpha- and beta-adrenergic mechanisms mediate dose-dependent actions of noradrenaline on supraoptic vasopressin neurones in vivo. Brain Res 358:171-179.

97. Day TA, Renaud LP (1984) Electrophysiological evidence that noradrenergic afferents selectively facilitate the activity of supraoptic vasopressin neurons. Brain Res 303:233-240.

98. Day TA, Renaud LP, Sibbald JR (1990) Excitation of supraoptic vasopressin cells by stimulation of the A1 noradrenaline cell group: failure to demonstrate role for established adrenergic or amino acid receptors. Brain Res 516:91-98.

99. Day TA, Sibbald JR, Khanna S (1993) ATP mediates an excitatory noradrenergic neuron input to supraoptic vasopressin cells. Brain Res 607:341-344.

100. Dayanithi G, Stuenkel EL, Nordmann JJ (1992) Intracellular calcium and hormone release from nerve endings of the neurohypophysis in the presence of opioid agonists and antagonists. Exp Brain Res 90:539-545.

101. de Bree FM (2000) Trafficking of the vasopressin and oxytocin prohormone through the regulated secretory pathway. J Neuroendocrinol 12:589-594.

102. Del Castillo J., Katz B (1954) Quantal components of the end-plate potential. J Physiol 124:560-573.

103. DeMaria EJ, Lilly MP, Gann DS (1987) Potentiated hormonal responses in a model of traumatic injury. J Surg Res 43:45-51.

104. Di S, Malcher-Lopes R, Halmos KC, Tasker JG (2003) Nongenomic glucocorticoid inhibition via endocannabinoid release in the hypothalamus: a fast feedback mechanism. J Neurosci 23:4850-4857.

105. Di S, Tasker JG (2004) Dehydration-induced synaptic plasticity in magnocellular neurons of the hypothalamic supraoptic nucleus. Endocrinology 145:5141-5149.

106. DiGregorio DA, Nusser Z, Silver RA (2002) Spillover of glutamate onto synaptic AMPA receptors enhances fast transmission at a cerebellar synapse. Neuron 35:521-533.

107. Du Vigneaud, V (1954) Hormones of the posterior pituitary gland: oxytocin and vasopressin. Harvey Lect 50:1-26.

108. Duan S, Neary JT (2006) P2X(7) receptors: Properties and relevance to CNS function. Glia 54:738-746.

109. Dubois-Dauphin M, Armstrong WE, Tribollet E, Dreifuss JJ (1985a) Somatosensory systems and the milk-ejection reflex in the rat. I. Lesions of the mesencephalic lateral tegmentum disrupt the reflex and damage mesencephalic somatosensory connections. Neuroscience 15:1111-1129.

110. Dubois-Dauphin M, Armstrong WE, Tribollet E, Dreifuss JJ (1985b) Somatosensory systems and the milk-ejection reflex in the rat. II. The effects of lesions in the ventroposterior thalamic complex, dorsal columns and lateral cervical nucleus-dorsolateral funiculus. Neuroscience 15:1131-1140.

111. Dudek FE, Tasker JG, Wuarin JP (1989) Intrinsic and synaptic mechanisms of hypothalamic neurons studied with slice and explant preparations. J Neurosci Methods 28:59-69.

112. Duffy S, MacVicar BA (1995) Adrenergic calcium signaling in astrocyte networks within the hippocampal slice. J Neurosci 15:5535-5550.

113. Dulla CG, Dobelis P, Pearson T, Frenguelli BG, Staley KJ, Masino SA (2005) Adenosine and ATP link PCO2 to cortical excitability via pH. Neuron 48:1011-1023.

114. Dunwiddie TV, Masino SA (2001) The role and regulation of adenosine in the central nervous system. Annu Rev Neurosci 24:31-55.

115. Dyball RE, Grossmann R, Leng G, Shibuki K (1988) Spike propagation and conduction failure in the rat neural lobe. J Physiol 401:241-256.

116. Dyball RE, Pountney PS (1973) Discharge patterns of supraoptic and paraventricular neurones in rats given a 2 per cent NaCl solution instead of drinking water. J Endocrinol 56:91-98.

117. Edwards FA, Gibb AJ, Colquhoun D (1992) ATP receptor-mediated synaptic currents in the central nervous system. Nature 359:144-147.

118. Edwards FA, Konnerth A, Sakmann B (1990) Quantal analysis of inhibitory synaptic transmission in the dentate gyrus of rat hippocampal slices: a patch-clamp study. J Physiol 430:213-249.

119. El MM, Poulain DA, Theodosis DT (1996) The glutamatergic innervation of oxytocin- and vasopressin-secreting neurons in the rat supraoptic nucleus and its contribution to lactation-induced synaptic plasticity. Eur J Neurosci 8:1377-1389.

120. Faber DS, Young WS, Legendre P, Korn H (1992) Intrinsic quantal variability due to stochastic properties of receptor-transmitter interactions. Science 258:1494-1498.

121. Fellin T, Pascual O, Gobbo S, Pozzan T, Haydon PG, Carmignoto G (2004) Neuronal synchrony mediated by astrocytic glutamate through activation of extrasynaptic NMDA receptors. Neuron 43:729-743.

122. Ferraguti F, Klausberger T, Cobden P, Baude A, Roberts JD, Szucs P, Kinoshita A, Shigemoto R, Somogyi P, Dalezios Y (2005) Metabotropic glutamate receptor 8-expressing nerve terminals target subsets of GABAergic neurons in the hippocampus. J Neurosci 25:10520-10536.

123. Filippi S, Vignozzi L, Vannelli GB, Ledda F, Forti G, Maggi M (2003) Role of oxytocin in the ejaculatory process. J Endocrinol Invest 26:82-86.

124. Finch EA, Augustine GJ (1998) Local calcium signalling by inositol-1,4,5-trisphosphate in Purkinje cell dendrites. Nature 396:753-756.

125. Fisher TE, Bourque CW (1995) Voltage-gated calcium currents in the magnocellular neurosecretory cells of the rat supraoptic nucleus. J Physiol 486 (Pt 3):571-580.

126. Fontella FU, Bruno AN, Crema LM, Battastini AM, Sarkis JJ, Netto CA, Dalmaz C (2004) Acute and chronic stress alter ecto-nucleotidase activities in synaptosomes from the rat hippocampus. Pharmacol Biochem Behav 78:341-347.

127. Forti L, Bossi M, Bergamaschi A, Villa A, Malgaroli A (1997) Loose-patch recordings of single quanta at individual hippocampal synapses. Nature 388:874-878.

128. Franks KM, Stevens CF, Sejnowski TJ (2003) Independent sources of quantal variability at single glutamatergic synapses. J Neurosci 23:3186-3195.

129. Frerking M, Borges S, Wilson M (1995) Variation in GABA mini amplitude is the consequence of variation in transmitter concentration. Neuron 15:885-895.

130. Frerking M, Wilson M (1996) Saturation of postsynaptic receptors at central synapses? Curr Opin Neurobiol 6:395-403.

131. Fumagalli M, Brambilla R, D'Ambrosi N, Volonte C, Matteoli M, Verderio C, Abbracchio MP (2003) Nucleotide-mediated calcium signaling in rat cortical astrocytes: Role of P2X and P2Y receptors. Glia 43:218-03.

132. Garten LL, Sofroniew MV, Dyball RE (1989) A direct catecholaminergic projection from the brainstem to the neurohypophysis of the rat. Neuroscience 33:149-155.

133. Gillis KD, Mossner R, Neher E (1996) Protein kinase C enhances exocytosis from chromaffin cells by increasing the size of the readily releasable pool of secretory granules. Neuron 16:1209-1220.

134. Gimpl G, Fahrenholz F (2001) The oxytocin receptor system: structure, function, and regulation. Physiol Rev 81:629-683.

135. Glasgow E, Kusano K, Chin H, Mezey E, Young WS, III, Gainer H (1999) Single cell reverse transcription-polymerase chain reaction analysis of rat supraoptic magnocellular neurons: neuropeptide phenotypes and high voltage-gated calcium channel subtypes. Endocrinology 140:5391-5401.

136. Glitsch M (2006) Selective inhibition of spontaneous but not Ca2+ -dependent release machinery by presynaptic group II mGluRs in rat cerebellar slices. J Neurophysiol 96:86-96.

137. Gomeza J, Gimenez C, Zafra F (1994) Cellular distribution and regulation by cAMP of the GABA transporter (GAT-1) mRNA. Brain Res Mol Brain Res 21:150-156.

138. Gordon GR, Baimoukhametova DV, Hewitt SA, Rajapaksha WR, Fisher TE, Bains JS (2005) Norepinephrine triggers release of glial ATP to increase postsynaptic efficacy. Nat Neurosci 8:1078-1086.

139. Gordon GR, Bains JS (2003) Priming of excitatory synapses by alpha1 adrenoceptor-mediated inhibition of group III metabotropic glutamate receptors. J Neurosci 23:6223-6231.

140. Gordon GR, Bains JS (2005) Noradrenaline triggers multivesicular release at glutamatergic synapses in the hypothalamus. J Neurosci 25:11385-11395.

141. Gordon GR, Bains JS (2006) Can homeostatic circuits learn and remember? J Physiol.

142. Grob M, Drolet G, Mouginot D (2004) Specific Na+ sensors are functionally expressed in a neuronal population of the median preoptic nucleus of the rat. J Neurosci 24:3974-3984.

143. Guthrie PB, Knappenberger J, Segal M, Bennett MV, Charles AC, Kater SB (1999) ATP released from astrocytes mediates glial calcium waves. J Neurosci 19:520-528.

144. Haanwinckel MA, Elias LK, Favaretto AL, Gutkowska J, McCann SM, ntunes-Rodrigues J (1995) Oxytocin mediates atrial natriuretic peptide release and natriuresis after volume expansion in the rat. Proc Natl Acad Sci U S A 92:7902-7906.

145. Han SK, Chong W, Li LH, Lee IS, Murase K, Ryu PD (2002) Noradrenaline excites and inhibits GABAergic transmission in parvocellular neurons of rat hypothalamic paraventricular nucleus. J Neurophysiol 87:2287-2296.

146. Hanse E, Gustafsson B (2001) Quantal variability at glutamatergic synapses in area CA1 of the rat neonatal hippocampus. J Physiol 531:467-480.

147. Harris GW, Manabe Y, Ruf KB (1969) A study of the parameters of electrical stimulation of unmyelinated fibres in the pituitary stalk. J Physiol 203:67-81.

148. Harvey PH, Krebs JR (1990) Comparing brains. Science 249:140-146.

149. Hassinger TD, Atkinson PB, Strecker GJ, Whalen LR, Dudek FE, Kossel AH, Kater SB (1995) Evidence for glutamate-mediated activation of hippocampal neurons by glial calcium waves. J Neurobiol 28:159-170.

150. Hasuo H, Matsuoka T, Akasu T (2002) Activation of presynaptic 5-hydroxytryptamine 2A receptors facilitates excitatory synaptic transmission via protein kinase C in the dorsolateral septal nucleus. J Neurosci 22:7509-7517.

151. Hatton GI (1990) Emerging concepts of structure-function dynamics in adult brain: the hypothalamo-neurohypophysial system. Prog Neurobiol 34:437-504.

152. Hatton GI, Li Z (1998) Mechanisms of neuroendocrine cell excitability. Adv Exp Med Biol 449:79-95.

153. Hatton GI, Tweedle CD (1982) Magnocellular neuropeptidergic neurons in hypothalamus: increases in membrane apposition and number of specialized synapses from pregnancy to lactation. Brain Res Bull 8:197-204.

154. Hayashi Y, Shi SH, Esteban JA, Piccini A, Poncer JC, Malinow R (2000) Driving AMPA receptors into synapses by LTP and CaMKII: requirement for GluR1 and PDZ domain interaction. Science 287:2262-2267.

155. Henze DA, McMahon DB, Harris KM, Barrionuevo G (2002) Giant miniature EPSCs at the hippocampal mossy fiber to CA3 pyramidal cell synapse are monoquantal. J Neurophysiol 87:15-29.

156. Hertz L, Schousboe I, Hertz L, Schousboe A (1984) Receptor expression in primary cultures of neurons or astrocytes. Prog Neuropsychopharmacol Biol Psychiatry 8:521-527.

157. Hilfiker S, Augustine GJ (1999) Regulation of synaptic vesicle fusion by protein kinase C. J Physiol 515 (Pt 1):1.

158. Hirasawa M, Mouginot D, Kozoriz MG, Kombian SB, Pittman QJ (2003) Vasopressin differentially modulates non-NMDA receptors in vasopressin and oxytocin neurons in the supraoptic nucleus. J Neurosci 23:4270-4277.

159. Hirsch JC, Agassandian C, Merchan-Perez A, Ben-Ari Y, DeFelipe J, Esclapez M, Bernard C (1999) Deficit of quantal release of GABA in experimental models of temporal lobe epilepsy. Nat Neurosci 2:499-500.

160. Hiruma H, Bourque CW (1995) P2 purinoceptor-mediated depolarization of rat supraoptic neurosecretory cells in vitro. J Physiol 489 (Pt 3):805-811.

161. Holmes CL, Landry DW, Granton JT (2003) Science review: Vasopressin and the cardiovascular system part 1--receptor physiology. Crit Care 7:427-434.

162. Holmes CL, Landry DW, Granton JT (2004) Science Review: Vasopressin and the cardiovascular system part 2 - clinical physiology. Crit Care 8:15-23.

163. Holmes WR (1995) Modeling the effect of glutamate diffusion and uptake on NMDA and non-NMDA receptor saturation. Biophys J 69:1734-1747.

164. Honda K, Negoro H, Fukuoka T, Higuchi T, Uchide K (1985) Effect of microelectrophoretically applied acetylcholine, noradrenaline, dopamine and serotonin on the discharge of paraventricular oxytocinergic neurones in the rat. Endocrinol Jpn 32:127-133.

165. Horie S, Shioda S, Nakai Y (1993) Catecholaminergic innervation of oxytocin neurons in the paraventricular nucleus of the rat hypothalamus as revealed by double-labeling immunoelectron microscopy. Acta Anat (Basel) 147:184-192.

166. Inenaga K, Honda E, Hirakawa T, Nakamura S, Yamashita H (1998) Glutamatergic synaptic inputs to mouse supraoptic neurons in calcium-free medium in vitro. J Neuroendocrinol 10:1-7.

167. Ishikawa T, Sahara Y, Takahashi T (2002) A single packet of transmitter does not saturate postsynaptic glutamate receptors. Neuron 34:613-621.

168. Ivell R, Kimura T, Muller D, Augustin K, Abend N, Bathgate R, Telgmann R, Balvers M, Tillmann G, Fuchs AR (2001) The structure and regulation of the oxytocin receptor. Exp Physiol 86:289-296.

169. Jackson MB (1993) Passive current flow and morphology in the terminal arborizations of the posterior pituitary. J Neurophysiol 69:692-702.

170. Jackson MB, Konnerth A, Augustine GJ (1991) Action potential broadening and frequency-dependent facilitation of calcium signals in pituitary nerve terminals. Proc Natl Acad Sci U S A 88:380-384.

171. Jacques-Silva MC, Rodnight R, Lenz G, Liao Z, Kong Q, Tran M, Kang Y, Gonzalez FA, Weisman GA, Neary JT (2004) P2X7 receptors stimulate AKT phosphorylation in astrocytes. Br J Pharmacol 141:1106-1117.

172. James G, Butt AM (2002) P2Y and P2X purinoceptor mediated Ca2+ signalling in glial cell pathology in the central nervous system. Eur J Pharmacol 447:247-260.

173. Jaworski J, Spangler S, Seeburg DP, Hoogenraad CC, Sheng M (2005) Control of dendritic arborization by the phosphoinositide-3'-kinase-Akt-mammalian target of rapamycin pathway. J Neurosci 25:11300-11312.

174. Jordan D, Spyer KM (1977) Studies on the termination of sinus nerve afferents. Pflugers Arch 369:65-73.

175. Jourdain P, Israel JM, Dupouy B, Oliet SH, Allard M, Vitiello S, Theodosis DT, Poulain DA (1998) Evidence for a hypothalamic oxytocin-sensitive pattern-generating network governing oxytocin neurons in vitro. J Neurosci 18:6641-6649.

176. Kang J, Jiang L, Goldman SA, Nedergaard M (1998) Astrocyte-mediated potentiation of inhibitory synaptic transmission. Nat Neurosci 1:683-692.

177. Kannan H, Yamashita H, Osaka T (1984) Paraventricular neurosecretory neurons: synaptic inputs from the ventrolateral medulla in rats. Neurosci Lett 51:183-188.

178. Kapoor JR, Sladek CD (2000) Purinergic and adrenergic agonists synergize in stimulating vasopressin and oxytocin release. J Neurosci 20:8868-8875.

179. Kapoor JR, Sladek CD (2001) Substance P and NPY differentially potentiate ATP and adrenergic stimulated vasopressin and oxytocin release. Am J Physiol Regul Integr Comp Physiol 280:R69-R78.

180. Karunanithi S, Marin L, Wong K, Atwood HL (2002) Quantal size and variation determined by vesicle size in normal and mutant Drosophila glutamatergic synapses. J Neurosci 22:10267-10276.

181. Katz B (1966) In: Nerve, Muscle and Synapse McGraw-Hill, New York.

182. Kazanietz MG (2000) Eyes wide shut: protein kinase C isozymes are not the only receptors for the phorbol ester tumor promoters. Mol Carcinog 28:5-11.

183. Kendrick KM, Leng G (1988) Haemorrhage-induced release of noradrenaline, 5-hydroxytryptamine and uric acid in the supraoptic nucleus of the rat, measured by microdialysis. Brain Res 440:402-406.

184. Khakh BS (2001) Molecular physiology of P2X receptors and ATP signalling at synapses. Nat Rev Neurosci 2:165-174.

185. Khanna S, Sibbald JR, Day TA (1993) Alpha 2-adrenoceptor modulation of A1 noradrenergic neuron input to supraoptic vasopressin cells. Brain Res 613:164-167.

186. Kimura T, Shoji M, Iitake K, Ota K, Matsui K, Yoshinaga K (1984) The role of central alpha 1- and alpha 2-adrenoceptors in the regulation of vasopressin release and the cardiovascular system. Endocrinology 114:1426-1432.

187. Klyachko VA, Stevens CF (2006) Temperature-dependent shift of balance among the components of short-term plasticity in hippocampal synapses. J Neurosci 26:6945-6957.

188. Kohara K, Ogura A, Akagawa K, Yamaguchi K (2001) Increase in number of functional release sites by cyclic AMP-dependent protein kinase in cultured neurons isolated from hippocampal dentate gyrus. Neurosci Res 41:79-88.

189. Kombian SB, Hirasawa M, Mouginot D, Chen X, Pittman QJ (2000) Short-term potentiation of miniature excitatory synaptic currents causes excitation of supraoptic neurons. J Neurophysiol 83:2542-2553.

190. Korn H, Mallet A, Triller A, Faber DS (1982) Transmission at a central inhibitory synapse. II. Quantal description of release, with a physical correlate for binomial n. J Neurophysiol 48:679-707.

191. Korn H, Triller A, Mallet A, Faber DS (1981) Fluctuating responses at a central synapse: n of binomial fit predicts number of stained presynaptic boutons. Science 213:898-901.

192. Krizaj D, Bao JX, Schmitz Y, Witkovsky P, Copenhagen DR (1999) Caffeine-sensitive calcium stores regulate synaptic transmission from retinal rod photoreceptors. J Neurosci 19:7249-7261.

193. Kruk PJ, Korn H, Faber DS (1997) The effects of geometrical parameters on synaptic transmission: a Monte Carlo simulation study. Biophys J 73:2874-2890.

194. Kumar V, Zhang MX, Swank MW, Kunz J, Wu GY (2005) Regulation of dendritic morphogenesis by Ras-PI3K-Akt-mTOR and Ras-MAPK signaling pathways. J Neurosci 25:11288-11299.

195. Larkman A, Stratford K, Jack J (1991) Quantal analysis of excitatory synaptic action and depression in hippocampal slices. Nature 350:344-347.

196. Larkman AU, Jack JJ, Stratford KJ (1997) Assessment of the reliability or amplitude histograms from excitatory synapses in rat hippocampal CA1 in vitro. J Physiol 505 (Pt 2):443-456.

197. Legendre P, Poulain DA (1992) Intrinsic mechanisms involved in the electrophysiological properties of the vasopressin-releasing neurons of the hypothalamus. Prog Neurobiol 38:1-17.

198. Leibowitz SF, Eidelman D, Suh JS, Diaz S, Sladek CD (1990) Mapping study of noradrenergic stimulation of vasopressin release. Exp Neurol 110:298-305.

199. Leng G, Brown CH, Russell JA (1999) Physiological pathways regulating the activity of magnocellular neurosecretory cells. Prog Neurobiol 57:625-655.

200. Leng G, Shibuki K (1987) Extracellular potassium changes in the rat neurohypophysis during activation of the magnocellular neurosecretory system. J Physiol 392:97-111.

201. Leng G, Shibuki K, Way SA (1988) Effects of raised extracellular potassium on the excitability of, and hormone release from, the isolated rat neurohypophysis. J Physiol 399:591-605.

202. Levin MC, Sawchenko PE, Howe PR, Bloom SR, Polak JM (1987) Organization of galanin-immunoreactive inputs to the paraventricular nucleus with special reference to their relationship to catecholaminergic afferents. J Comp Neurol 261:562-582.

203. Leybaert L, Braet K, Vandamme W, Cabooter L, Martin PE, Evans WH (2003) Connexin channels, connexin mimetic peptides and ATP release. Cell Commun Adhes 10:251-7.

204. Leybaert L, Paemeleire K, Strahonja A, Sanderson MJ (1998) Inositol-trisphosphate-dependent intercellular calcium signaling in and between astrocytes and endothelial cells. Glia 24:398-407.

205. Li Z, Hatton GI (1996) Oscillatory bursting of phasically firing rat supraoptic neurones in low-Ca2+ medium: Na+ influx, cytosolic Ca2+ and gap junctions. J Physiol 496 (Pt 2):379-394.

206. Lilly MP, DeMaria EJ, Bruhn TO, Gann DS (1989) Potentiated cortisol response to paired hemorrhage: role of angiotensin and vasopressin. Am J Physiol 257:R118-R126.

207. Lilly MP, Engeland WC, Gann DS (1983) Responses of cortisol secretion to repeated hemorrhage in the anesthetized dog. Endocrinology 112:681-688.

208. Lilly MP, Engeland WC, Gann DS (1986) Pituitary-adrenal responses to repeated small hemorrhage in conscious dogs. Am J Physiol 251:R1200-R1207.

209. Lin CH, Yeh SH, Lin CH, Lu KT, Leu TH, Chang WC, Gean PW (2001) A role for the PI-3 kinase signaling pathway in fear conditioning and synaptic plasticity in the amygdala. Neuron 31:841-851.

210. Lin SH, Miyata S, Matsunaga W, Kawarabayashi T, Nakashima T, Kiyohara T (1998) Metabolic mapping of the brain in pregnant, parturient and lactating rats using fos immunohistochemistry. Brain Res 787:226-236.

211. Linden DJ, Dickinson MH, Smeyne M, Connor JA (1991) A long-term depression of AMPA currents in cultured cerebellar Purkinje neurons. Neuron 7:81-89.

212. Liu G, Choi S, Tsien RW (1999) Variability of neurotransmitter concentration and nonsaturation of postsynaptic AMPA receptors at synapses in hippocampal cultures and slices. Neuron 22:395-409.

213. Liu Q, Chen B, Yankova M, Morest DK, Maryon E, Hand AR, Nonet ML, Wang ZW (2005) Presynaptic ryanodine receptors are required for normal quantal size at the Caenorhabditis elegans neuromuscular junction. J Neurosci 25:6745-6754.

214. Liu WS, Heckman CA (1998) The sevenfold way of PKC regulation. Cell Signal 10:529-542.

215. Llano I, Gerschenfeld HM (1993) Inhibitory synaptic currents in stellate cells of rat cerebellar slices. J Physiol 468:177-200.

216. Llano I, Gonzalez J, Caputo C, Lai FA, Blayney LM, Tan YP, Marty A (2000) Presynaptic calcium stores underlie large-amplitude miniature IPSCs and spontaneous calcium transients. Nat Neurosci 3:1256-1265.

217. Loesch A, Burnstock G (2001) Immunoreactivity to P2X(6) receptors in the rat hypothalamo-neurohypophysial system: an ultrastructural study with extravidin and colloidal gold-silver labelling. Neuroscience 106:621-631.

218. Loesch A, Miah S, Burnstock G (1999) Ultrastructural localisation of ATP-gated P2X2 receptor immunoreactivity in the rat hypothalamo-neurohypophysial system. J Neurocytol 28:495-504.

219. Lou X, Scheuss V, Schneggenburger R (2005) Allosteric modulation of the presynaptic Ca2+ sensor for vesicle fusion. Nature 435:497-501.

220. Ludwig M (1998) Dendritic release of vasopressin and oxytocin. J Neuroendocrinol 10:881-895.

221. Ludwig M, Onaka T, Yagi K (2000) Vasopressin regulation of noradrenaline release within the supraoptic nucleus. J Neuroendocrinol 12:477-479.

222. MacDermott AB, Role LW, Siegelbaum SA (1999) Presynaptic ionotropic receptors and the control of transmitter release. Annu Rev Neurosci 22:443-485.

223. Macek TA, Schaffhauser H, Conn PJ (1998) Protein kinase C and A3 adenosine receptor activation inhibit presynaptic metabotropic glutamate receptor (mGluR) function and uncouple mGluRs from GTP-binding proteins. J Neurosci 18:6138-6146.

224. Malenka RC (2003) Synaptic plasticity and AMPA receptor trafficking. Ann N Y Acad Sci 1003:1-11.

225. Malenka RC, Bear MF (2004) LTP and LTD: an embarrassment of riches. Neuron 44:5-21.

226. Malinow R, Malenka RC (2002) AMPA receptor trafficking and synaptic plasticity. Annu Rev Neurosci 25:103-126.

227. Malinow R, Schulman H, Tsien RW (1989) Inhibition of postsynaptic PKC or CaMKII blocks induction but not expression of LTP. Science 245:862-866.

228. Man HY, Wang Q, Lu WY, Ju W, Ahmadian G, Liu L, D'Souza S, Wong TP, Taghibiglou C, Lu J, Becker LE, Pei L, Liu F, Wymann MP, MacDonald JF, Wang YT (2003) Activation of PI3-kinase is required for AMPA receptor insertion during LTP of mEPSCs in cultured hippocampal neurons. Neuron 38:611-624.

229. Manning M, Sawyer WH (1989) Discovery, development, and some uses of vasopressin and oxytocin antagonists. J Lab Clin Med 114:617-632.

230. Mathiesen JM, Ramirez MT (2006) The metabotropic glutamate receptor 4 is internalized and desensitized upon protein kinase C activation. Br J Pharmacol 148:279-290.

231. Matyash V, Filippov V, Mohrhagen K, Kettenmann H (2001) Nitric oxide signals parallel fiber activity to Bergmann glial cells in the mouse cerebellar slice. Mol Cell Neurosci 18:664-670.

232. McAllister AK, Stevens CF (2000) Nonsaturation of AMPA and NMDA receptors at hippocampal synapses. Proc Natl Acad Sci U S A 97:6173-6178.

233. McKinley MJ, Hards DK, Oldfield BJ (1994) Identification of neural pathways activated in dehydrated rats by means of Fos-immunohistochemistry and neural tracing. Brain Res 653:305-314.

234. Meeker RB, Greenwood RS, Hayward JN (1994) Glutamate receptors in the rat hypothalamus and pituitary. Endocrinology 134:621-629.

235. Meyer C, Freund-Mercier MJ, Richard P (1987) Facilitatory effect of oxytocin on oxytocin cell background activity in the rat is suckling-dependent. Neurosci Lett 75:80-84.

236. Mezey E, Kiss JZ (1991) Coexpression of vasopressin and oxytocin in hypothalamic supraoptic neurons of lactating rats. Endocrinology 129:1814-1820.

237. Michaloudi HC, El MM, Poulain DA, Papadopoulos GC, Theodosis DT (1997) The noradrenergic innervation of identified hypothalamic magnocellular somata and its contribution to lactation-induced synaptic plasticity. J Neuroendocrinol 9:17-23.

238. Michelotti GA, Price DT, Schwinn DA (2000) Alpha 1-adrenergic receptor regulation: basic science and clinical implications. Pharmacol Ther 88:281-309.

239. Min MY, Appenteng K (1996) Multimodal distribution of amplitudes of miniature and spontaneous EPSPs recorded in rat trigeminal motoneurones. J Physiol 494 (Pt 1):171-182.

240. Miyata S, Nakashima T, Kiyohara T (1994) Structural dynamics of neural plasticity in the supraoptic nucleus of the rat hypothalamus during dehydration and rehydration. Brain Res Bull 34:169-175.

241. Moos F, Marganiec A, Fontanaud P, Guillou-Duvoid A, Alonso G (2004) Synchronization of oxytocin neurons in suckled rats: possible role of bilateral

innervation of hypothalamic supraoptic nuclei by single medullary neurons. Eur J Neurosci 20:66-78.

242. Moos F, Poulain DA, Rodriguez F, Guerne Y, Vincent JD, Richard P (1989) Release of oxytocin within the supraoptic nucleus during the milk ejection reflex in rats. Exp Brain Res 76:593-602.

243. Moos F, Richard P (1980) [Dual noradrenergic control of oxytocin release during the milk ejection reflex in the rat]. C R Seances Acad Sci D 290:1261-1264.

244. Moos F, Richard P (1989) Paraventricular and supraoptic bursting oxytocin cells in rat are locally regulated by oxytocin and functionally related. J Physiol 408:1-18.

245. Moos FC, Ingram CD (1995) Electrical recordings of magnocellular supraoptic and paraventricular neurons displaying both oxytocin- and vasopressin-related activity. Brain Res 669:309-314.

246. Moos FC, Rossi K, Richard P (1997) Activation of N-methyl-D-aspartate receptors regulates basal electrical activity of oxytocin and vasopressin neurons in lactating rats. Neuroscience 77:993-1002.

247. Mori M, Tsushima H, Matsuda T (1992) Antidiuretic effects of purinoceptor agonists injected into the hypothalamic paraventricular nucleus of water-loaded, ethanol-anesthetized rats. Neuropharmacology 31:585-592.

248. Mori M, Tsushima H, Matsuda T (1994) Antidiuretic effects of ATP induced by microinjection into the hypothalamic supraoptic nucleus in water-loaded and ethanol-anesthetized rats. Jpn J Pharmacol 66:445-450.

249. Moss RL, Dyball RE, Cross BA (1971) Responses of antidromically identified supraoptic and paraventricular units to acetylcholine, noradrenaline and glutamate applied iontophoretically. Brain Res 35:573-575.

250. Mouginot D, Kombian SB, Pittman QJ (1998) Activation of presynaptic GABAB receptors inhibits evoked IPSCs in rat magnocellular neurons in vitro. J Neurophysiol 79:1508-1517.

251. Mundell SJ, Matharu AL, Pula G, Roberts PJ, Kelly E (2001) Agonist-induced internalization of the metabotropic glutamate receptor 1a is arrestin- and dynamin-dependent. J Neurochem 78:546-551.

252. Mundell SJ, Pula G, Carswell K, Roberts PJ, Kelly E (2003) Agonist-induced internalization of metabotropic glutamate receptor 1A: structural determinants for protein kinase C- and G protein-coupled receptor kinase-mediated internalization. J Neurochem 84:294-304.

253. Mundell SJ, Pula G, McIlhinney RA, Roberts PJ, Kelly E (2004a) Desensitization and internalization of metabotropic glutamate receptor 1a following activation of heterologous Gq/11-coupled receptors. Biochemistry 43:7541-7551.

254. Mundell SJ, Pula G, More JC, Jane DE, Roberts PJ, Kelly E (2004b) Activation of cyclic AMP-dependent protein kinase inhibits the desensitization and internalization of metabotropic glutamate receptors 1a and 1b. Mol Pharmacol 65:1507-1516.

255. Nakadate K, Imamura K, Watanabe Y (2006) Cellular and subcellular localization of alpha-1 adrenoceptors in the rat visual cortex. Neuroscience 141:1783-1792.

256. Nakajima Y, Yamamoto T, Nakayama T, Nakanishi S (1999) A relationship between protein kinase C phosphorylation and calmodulin binding to the metabotropic glutamate receptor subtype 7. J Biol Chem 274:27573-27577.

257. Nakanishi S, Maeda N, Mikoshiba K (1991) Immunohistochemical localization of an inositol 1,4,5-trisphosphate receptor, P400, in neural tissue: studies in developing and adult mouse brain. J Neurosci 11:2075-2086.

258. Narita K, Akita T, Hachisuka J, Huang S, Ochi K, Kuba K (2000) Functional coupling of Ca(2+) channels to ryanodine receptors at presynaptic terminals. Amplification of exocytosis and plasticity. J Gen Physiol 115:519-532.

259. Narita K, Akita T, Osanai M, Shirasaki T, Kijima H, Kuba K (1998) A Ca2+-induced Ca2+ release mechanism involved in asynchronous exocytosis at frog motor nerve terminals. J Gen Physiol 112:593-609.

260. Nedergaard M, Ransom B, Goldman SA (2003) New roles for astrocytes: redefining the functional architecture of the brain. Trends Neurosci 26:523-530.

261. Newman EA (2003) Glial cell inhibition of neurons by release of ATP. J Neurosci 23:1659-1666.

262. Newman EA, Zahs KR (1998) Modulation of neuronal activity by glial cells in the retina. J Neurosci 18:4022-4028.

263. Nishimori K, Young LJ, Guo Q, Wang Z, Insel TR, Matzuk MM (1996) Oxytocin is required for nursing but is not essential for parturition or reproductive behavior. Proc Natl Acad Sci U S A 93:11699-11704.

264. Nissen R, Hu B, Renaud LP (1995) Regulation of spontaneous phasic firing of rat supraoptic vasopressin neurones in vivo by glutamate receptors. J Physiol 484 (Pt 2):415-424.

265. North RA (2002) Molecular physiology of P2X receptors. Physiol Rev 82:1013-1067.

266. O'Connor V, Heuss C, De Bello WM, Dresbach T, Charlton MP, Hunt JH, Pellegrini LL, Hodel A, Burger MM, Betz H, Augustine GJ, Schafer T (1997) Disruption of syntaxin-mediated protein interactions blocks neurotransmitter secretion. Proc Natl Acad Sci U S A 94:12186-12191.

267. Oertner TG, Sabatini BL, Nimchinsky EA, Svoboda K (2002) Facilitation at single synapses probed with optical quantal analysis. Nat Neurosci 5:657-664.

268. Oliet SH (2002) Functional consequences of morphological neuroglial changes in the magnocellular nuclei of the hypothalamus. J Neuroendocrinol 14:241-246.

269. Oliet SH, Bourque CW (1993) Mechanosensitive channels transduce osmosensitivity in supraoptic neurons. Nature 364:341-343.

270. Oliet SH, Malenka RC, Nicoll RA (1997) Two distinct forms of long-term depression coexist in CA1 hippocampal pyramidal cells. Neuron 18:969-982.

271. Oliet SH, Piet R, Poulain DA (2001) Control of glutamate clearance and synaptic efficacy by glial coverage of neurons. Science 292:923-926.

272. Oliet SH, Poulain DA (1999) Adenosine-induced presynaptic inhibition of IPSCs and EPSCs in rat hypothalamic supraoptic nucleus neurones. J Physiol 520 Pt 3:815-825.

273. Opazo P, Watabe AM, Grant SG, O'Dell TJ (2003) Phosphatidylinositol 3-kinase regulates the induction of long-term potentiation through extracellular signal-related kinase-independent mechanisms. J Neurosci 23:3679-3688.

274. Pacak K, Palkovits M, Kopin IJ, Goldstein DS (1995a) Stress-induced norepinephrine release in the hypothalamic paraventricular nucleus and pituitary-adrenocortical and sympathoadrenal activity: in vivo microdialysis studies. Front Neuroendocrinol 16:89-150.

275. Pacak K, Palkovits M, Kvetnansky R, Yadid G, Kopin IJ, Goldstein DS (1995b) Effects of various stressors on in vivo norepinephrine release in the hypothalamic paraventricular nucleus and on the pituitary-adrenocortical axis. Ann N Y Acad Sci 771:115-130.

276. Panatier A, Gentles SJ, Bourque CW, Oliet SH (2006a) Activity-dependent synaptic plasticity in the supraoptic nucleus of the rat hypothalamus. J Physiol.

277. Panatier A, Poulain DA, Oliet SH (2004) Regulation of transmitter release by high-affinity group III mGluRs in the supraoptic nucleus of the rat hypothalamus. Neuropharmacology 47:333-341.

278. Panatier A, Theodosis DT, Mothet JP, Touquet B, Pollegioni L, Poulain DA, Oliet SH (2006b) Glia-derived D-serine controls NMDA receptor activity and synaptic memory. Cell 125:775-784.

279. Parker SL, Crowley WR (1993) Central stimulation of oxytocin release in the lactating rat: interaction of neuropeptide Y with alpha-1-adrenergic mechanisms. Endocrinology 132:658-666.

280. Parpura V, Basarsky TA, Liu F, Jeftinija K, Jeftinija S, Haydon PG (1994) Glutamate-mediated astrocyte-neuron signalling. Nature 369:744-747.

281. Pascual O, Casper KB, Kubera C, Zhang J, Revilla-Sanchez R, Sul JY, Takano H, Moss SJ, McCarthy K, Haydon PG (2005) Astrocytic purinergic signaling coordinates synaptic networks. Science 310:113-116.

282. Pasti L, Volterra A, Pozzan T, Carmignoto G (1997) Intracellular calcium oscillations in astrocytes: a highly plastic, bidirectional form of communication between neurons and astrocytes in situ. J Neurosci 17:7817-7830.

283. Paulsen O, Heggelund P (1994) The quantal size at retinogeniculate synapses determined from spontaneous and evoked EPSCs in guinea-pig thalamic slices. J Physiol 480 (Pt 3):505-511.

284. Pearce B, Albrecht J, Morrow C, Murphy S (1986) Astrocyte glutamate receptor activation promotes inositol phospholipid turnover and calcium flux. Neurosci Lett 72:335-340.

285. Pelkey KA, Lavezzari G, Racca C, Roche KW, McBain CJ (2005) mGluR7 is a metaplastic switch controlling bidirectional plasticity of feedforward inhibition. Neuron 46:89-102.

286. Pelkey KA, Yuan X, Lavezzari G, Roche KW, McBain CJ (2006) mGluR7 undergoes rapid internalization in response to activation by the allosteric agonist AMN082. Neuropharmacology.

287. Peng Y (1996) Ryanodine-sensitive component of calcium transients evoked by nerve firing at presynaptic nerve terminals. J Neurosci 16:6703-6712.

288. Peng YW, Sharp AH, Snyder SH, Yau KW (1991) Localization of the inositol 1,4,5-trisphosphate receptor in synaptic terminals in the vertebrate retina. Neuron 6:525-531.

289. Perroy J, Gutierrez GJ, Coulon V, Bockaert J, Pin JP, Fagni L (2001) The C terminus of the metabotropic glutamate receptor subtypes 2 and 7 specifies the receptor signaling pathways. J Biol Chem 276:45800-45805.

290. Piascik MT, Perez DM (2001) Alpha1-adrenergic receptors: new insights and directions. J Pharmacol Exp Ther 298:403-410.

291. Piet R, Vargova L, Sykova E, Poulain DA, Oliet SH (2004) Physiological contribution of the astrocytic environment of neurons to intersynaptic crosstalk. Proc Natl Acad Sci U S A 101:2151-2155.

292. Porter JT, McCarthy KD (1995) GFAP-positive hippocampal astrocytes in situ respond to glutamatergic neuroligands with increases in [Ca2+]i. Glia 13:101-112.

293. Porter JT, McCarthy KD (1996) Hippocampal astrocytes in situ respond to glutamate released from synaptic terminals. J Neurosci 16:5073-5081.

294. Prange O, Murphy TH (1999) Analysis of multiquantal transmitter release from single cultured cortical neuron terminals. J Neurophysiol 81:1810-1817.

295. Pula G, Mundell SJ, Roberts PJ, Kelly E (2004) Agonist-independent internalization of metabotropic glutamate receptor 1a is arrestin- and clathrin-dependent and is suppressed by receptor inverse agonists. J Neurochem 89:1009-1020.

296. Queiroz G, Meyer DK, Meyer A, Starke K, von K, I (1999) A study of the mechanism of the release of ATP from rat cortical astroglial cells evoked by activation of glutamate receptors. Neuroscience 91:1171-1181.

297. Raghavachari S, Lisman JE (2004) Properties of quantal transmission at CA1 synapses. J Neurophysiol 92:2456-2467.

298. Randle JC, Bourque CW, Renaud LP (1986a) Alpha 1-adrenergic receptor activation depolarizes rat supraoptic neurosecretory neurons in vitro. Am J Physiol 251:R569-R574.

299. Randle JC, Day TA, Jhamandas JH, Bourque CW, Renaud LP (1986b) Neuropharmacology of supraoptic nucleus neurons: norepinephrine and gamma-aminobutyric acid receptors. Fed Proc 45:2312-2317.

300. Rauen T, Kanner BI (1994) Localization of the glutamate transporter GLT-1 in rat and macaque monkey retinae. Neurosci Lett 169:137-140.

301. Raymond CR, Redman SJ, Crouch MF (2002) The phosphoinositide 3-kinase and p70 S6 kinase regulate long-term potentiation in hippocampal neurons. Neuroscience 109:531-536.

302. Redman S (1990) Quantal analysis of synaptic potentials in neurons of the central nervous system. Physiol Rev 70:165-198.

303. Renaud LP (1994) Hypothalamic magnocellular neurosecretory neurons: intrinsic membrane properties and synaptic connections. Prog Brain Res 100:133-137.

304. Richard P, Moos F, Freund-Mercier MJ (1991) Central effects of oxytocin. Physiol Rev 71:331-370.

305. Robitaille R (1998) Modulation of synaptic efficacy and synaptic depression by glial cells at the frog neuromuscular junction. Neuron 21:847-855.

306. Rowe JB, Toni I, Josephs O, Frackowiak RS, Passingham RE (2000) The prefrontal cortex: response selection or maintenance within working memory? Science 288:1656-1660.

307. Rumpel S, LeDoux J, Zador A, Malinow R (2005) Postsynaptic receptor trafficking underlying a form of associative learning. Science 308:83-88.

308. Russell JA, Leng G (1998) Sex, parturition and motherhood without oxytocin? J Endocrinol 157:343-359.

309. Sachs H, Takabatake Y (1964) Evidence for a precursor in vasopressin biosynthesis. Endocrinology 75:943-948.

310. Sanderson MJ, Charles AC, Boitano S, Dirksen ER (1994) Mechanisms and function of intercellular calcium signaling. Mol Cell Endocrinol 98:173-187.

311. Sanna PP, Cammalleri M, Berton F, Simpson C, Lutjens R, Bloom FE, Francesconi W (2002) Phosphatidylinositol 3-kinase is required for the expression but not for the induction or the maintenance of long-term potentiation in the hippocampal CA1 region. J Neurosci 22:3359-3365.

312. Sansig G, Bushell TJ, Clarke VR, Rozov A, Burnashev N, Portet C, Gasparini F, Schmutz M, Klebs K, Shigemoto R, Flor PJ, Kuhn R, Knoepfel T, Schroeder M, Hampson DR, Collett VJ, Zhang C, Duvoisin RM, Collingridge GL, van Der PH (2001) Increased seizure susceptibility in mice lacking metabotropic glutamate receptor 7. J Neurosci 21:8734-8745.

313. Sara Y, Virmani T, Deak F, Liu X, Kavalali ET (2005) An isolated pool of vesicles recycles at rest and drives spontaneous neurotransmission. Neuron 45:563-573.

314. Savic N, Sciancalepore M (1998) Intracellular calcium stores modulate miniature GABA-mediated synaptic currents in neonatal rat hippocampal neurons. Eur J Neurosci 10:3379-3386.

315. Sawchenko PE, Arias C, Bittencourt JC (1990) Inhibin beta, somatostatin, and enkephalin immunoreactivities coexist in caudal medullary neurons that project to the paraventricular nucleus of the hypothalamus. J Comp Neurol 291:269-280.

316. Sawchenko PE, Swanson LW (1981) Central noradrenergic pathways for the integration of hypothalamic neuroendocrine and autonomic responses. Science 214:685-687.

317. Sawchenko PE, Swanson LW (1982) The organization of noradrenergic pathways from the brainstem to the paraventricular and supraoptic nuclei in the rat. Brain Res 257:275-325.

318. Sawchenko PE, Swanson LW, Grzanna R, Howe PR, Bloom SR, Polak JM (1985) Colocalization of neuropeptide Y immunoreactivity in brainstem catecholaminergic neurons that project to the paraventricular nucleus of the hypothalamus. J Comp Neurol 241:138-153.

319. Sawyer CH, Clifton DK (1980) Aminergic innervation of the hypothalamus. Fed Proc 39:2889-2895.

320. Scanziani M, Nicoll RA, Malenka RC (1996) Heterosynaptic long-term depression in the hippocampus. J Physiol Paris 90:165-166.

321. Scepek S, Coorssen JR, Lindau M (1998) Fusion pore expansion in horse eosinophils is modulated by Ca2+ and protein kinase C via distinct mechanisms. EMBO J 17:4340-4345.

322. Schoepp DD (2001) Unveiling the functions of presynaptic metabotropic glutamate receptors in the central nervous system. J Pharmacol Exp Ther 299:12-20.

323. Schoepp DD, Conn PJ (2002) Metabotropic glutamate receptors. Pharmacol Biochem Behav 74:255-256.

324. Schoppa NE, Westbrook GL (1997) Modulation of mEPSCs in olfactory bulb mitral cells by metabotropic glutamate receptors. J Neurophysiol 78:1468-1475.

325. Schrader LA, Tasker JG (1997a) Modulation of multiple potassium currents by metabotropic glutamate receptors in neurons of the hypothalamic supraoptic nucleus. J Neurophysiol 78:3428-3437.

326. Schrader LA, Tasker JG (1997b) Presynaptic modulation by metabotropic glutamate receptors of excitatory and inhibitory synaptic inputs to hypothalamic magnocellular neurons. J Neurophysiol 77:527-536.

327. Serrano A, Haddjeri N, Lacaille JC, Robitaille R (2006) GABAergic network activation of glial cells underlies hippocampal heterosynaptic depression. J Neurosci 26:5370-5382.

328. Sharma G, Vijayaraghavan S (2001) Nicotinic cholinergic signaling in hippocampal astrocytes involves calcium-induced calcium release from intracellular stores. Proc Natl Acad Sci U S A 98:4148-4153.

329. Sharma G, Vijayaraghavan S (2003) Modulation of presynaptic store calcium induces release of glutamate and postsynaptic firing. Neuron 38:929-939.

330. Shi S, Hayashi Y, Esteban JA, Malinow R (2001) Subunit-specific rules governing AMPA receptor trafficking to synapses in hippocampal pyramidal neurons. Cell 105:331-343.

331. Shi SH, Hayashi Y, Petralia RS, Zaman SH, Wenthold RJ, Svoboda K, Malinow R (1999) Rapid spine delivery and redistribution of AMPA receptors after synaptic NMDA receptor activation. Science 284:1811-1816.

332. Shibuya I, Kabashima N, Ibrahim N, Setiadji SV, Ueta Y, Yamashita H (2000) Pre- and postsynaptic modulation of the electrical activity of rat supraoptic neurones. Exp Physiol 85 Spec No:145S-151S.

333. Shibuya I, Tanaka K, Hattori Y, Uezono Y, Harayama N, Noguchi J, Ueta Y, Izumi F, Yamashita H (1999) Evidence that multiple P2X purinoceptors are functionally expressed in rat supraoptic neurones. J Physiol 514 (Pt 2):351-367.

334. Shigemoto R, Kinoshita A, Wada E, Nomura S, Ohishi H, Takada M, Flor PJ, Neki A, Abe T, Nakanishi S, Mizuno N (1997) Differential presynaptic localization of metabotropic glutamate receptor subtypes in the rat hippocampus. J Neurosci 17:7503-7522.

335. Shigemoto R, Kulik A, Roberts JD, Ohishi H, Nusser Z, Kaneko T, Somogyi P (1996) Target-cell-specific concentration of a metabotropic glutamate receptor in the presynaptic active zone. Nature 381:523-525.

336. Shigetomi E, Kato F (2004) Action potential-independent release of glutamate by Ca2+ entry through presynaptic P2X receptors elicits postsynaptic firing in the brainstem autonomic network. J Neurosci 24:3125-3135.

337. Shioda S, Nakai Y (1992) Noradrenergic innervation of vasopressin-containing neurons in the rat hypothalamic supraoptic nucleus. Neurosci Lett 140:215-218.

338. Shioda S, Shimoda Y, Nakai Y (1992) Ultrastructural studies of medullary synaptic inputs to vasopressin-immunoreactive neurons in the supraoptic nucleus of the rat hypothalamus. Neurosci Lett 148:155-158.

339. Shioda S, Yada T, Muroya S, Takigawa M, Nakai Y (1997) Noradrenaline activates vasopressin neurons via alpha1-receptor-mediated Ca2+ signaling pathway. Neurosci Lett 226:210-212.

340. Shoji-Kasai Y, Itakura M, Kataoka M, Yamamori S, Takahashi M (2002) Protein kinase C-mediated translocation of secretory vesicles to plasma membrane and enhancement of neurotransmitter release from PC12 cells. Eur J Neurosci 15:1390-1394.

341. Sibbald JR, Wilson BK, Day TA (1989) Neuropeptide Y potentiates excitation of supraoptic neurosecretory cells by noradrenaline. Brain Res 499:164-168.

342. Sim JA, Chaumont S, Jo J, Ulmann L, Young MT, Cho K, Buell G, North RA, Rassendren F (2006) Altered hippocampal synaptic potentiation in P2X4 knock-out mice. J Neurosci 26:9006-9009.

343. Sjoquist M, Huang W, Jacobsson E, Skott O, Stricker EM, Sved AF (1999) Sodium excretion and renin secretion after continuous versus pulsatile infusion of oxytocin in rats. Endocrinology 140:2814-2818.

344. Smith AB, Cunnane TC (1996) Ryanodine-sensitive calcium stores involved in neurotransmitter release from sympathetic nerve terminals of the guinea-pig. J Physiol 497 (Pt 3):657-664.

345. Song Z, Sladek CD (2006) Site of ATP and phenylephrine synergistic stimulation of vasopressin release from the hypothalamo-neurohypophyseal system. J Neuroendocrinol 18:266-272.

346. Song Z, Vijayaraghavan S, Sladek CD (2006) Simultaneous exposure to ATP and phenylephrine induces a sustained elevation in the intracellular calcium concentration in supraoptic neurons. Am J Physiol Regul Integr Comp Physiol 291:R37-R45.

347. Sorensen SD, Macek TA, Cai Z, Saugstad JA, Conn PJ (2002) Dissociation of protein kinase-mediated regulation of metabotropic glutamate receptor 7 (mGluR7) interactions with calmodulin and regulation of mGluR7 function. Mol Pharmacol 61:1303-1312.

348. Staley KJ (1999) Quantal GABA release: noise or not? Nat Neurosci 2:494-495.

349. Stern JE, Galarreta M, Foehring RC, Hestrin S, Armstrong WE (1999) Differences in the properties of ionotropic glutamate synaptic currents in oxytocin and vasopressin neuroendocrine neurons. J Neurosci 19:3367-3375.

350. Stevens CF, Sullivan JM (1998) Regulation of the readily releasable vesicle pool by protein kinase C. Neuron 21:885-893.

351. Stevens CF, Wang Y (1995) Facilitation and depression at single central synapses. Neuron 14:795-802.

352. Stratford KJ, Jack JJ, Larkman AU (1997) Calibration of an autocorrelation-based method for determining amplitude histogram reliability and quantal size. J Physiol 505 (Pt 2):425-442.

353. Summerlee AJ (1981) Extracellular recordings from oxytocin neurones during the expulsive phase of birth in unanaesthetized rats. J Physiol 321:1-9.

354. Takahashi T, Svoboda K, Malinow R (2003) Experience strengthening transmission by driving AMPA receptors into synapses. Science 299:1585-1588.

355. Tanaka J, Nomura M, Saito H (1992) Noradrenergic inputs enhance the response of neurosecretory cells to osmotic stimulation. Neuroreport 3:256-258.

356. Tasker JG, Dudek FE (1991) Electrophysiological properties of neurones in the region of the paraventricular nucleus in slices of rat hypothalamus. J Physiol 434:271-293.

357. Tasker JG, Theodosis DT, Poulain DA (1986) Afferent projections from the mammary glands to the spinal cord in the lactating rat--I. A neuroanatomical study using the transganglionic transport of horseradish peroxidase-wheatgerm agglutinin. Neuroscience 19:495-509.

358. Theodosis DT, Poulain DA (1984) Evidence for structural plasticity in the supraoptic nucleus of the rat hypothalamus in relation to gestation and lactation. Neuroscience 11:183-193.

359. Theodosis DT, Poulain DA (1993) Activity-dependent neuronal-glial and synaptic plasticity in the adult mammalian hypothalamus. Neuroscience 57:501-535.

360. Theodosis DT, Poulain DA, Vincent JD (1981) Possible morphological bases for synchronisation of neuronal firing in the rat supraoptic nucleus during lactation. Neuroscience 6:919-929.

361. Thirion S, Troadec JD, Nicaise G (1996) Cytochemical localization of ecto-ATPases in rat neurohypophysis. J Histochem Cytochem 44:103-111.

362. Thrivikraman KV, Su Y, Plotsky PM (1997) Patterns of Fos-Immunoreactivity in the CNS Induced by Repeated Hemorrhage in Conscious Rats: Correlations with Pituitary-Adrenal Axis Activity. Stress 2:145-158.

363. Tong G, Jahr CE (1994) Multivesicular release from excitatory synapses of cultured hippocampal neurons. Neuron 12:51-59.

364. Traynelis SF, Silver RA, Cull-Candy SG (1993) Estimated conductance of glutamate receptor channels activated during EPSCs at the cerebellar mossy fiber-granule cell synapse. Neuron 11:279-289.

365. Tse FW, Tse A, Hille B, Horstmann H, Almers W (1997) Local Ca2+ release from internal stores controls exocytosis in pituitary gonadotrophs. Neuron 18:121-132.

366. Tweedle CD, Hatton GI (1977) Ultrastructural changes in rat hypothalamic neurosecretory cells and their associated glia during minimal dehydration and rehydration. Cell Tissue Res 181:59-72.

367. Tweedle CD, Hatton GI (1987) Morphological adaptability at neurosecretory axonal endings on the neurovascular contact zone of the rat neurohypophysis. Neuroscience 20:241-246.

368. Ulrich D, Luscher HR (1993) Miniature excitatory synaptic currents corrected for dendritic cable properties reveal quantal size and variance. J Neurophysiol 69:1769-1773.

369. van den Pol AN (1982) The magnocellular and parvocellular paraventricular nucleus of rat: intrinsic organization. J Comp Neurol 206:317-345.

370. Van der KW (1987) Pretreatment with hypertonic solutions increases quantal size at the frog neuromuscular junction. J Neurophysiol 57:1536-1554.

371. Van der KW, Molgo J, Cameron R, Colasante C (2002) Vesicle size and transmitter release at the frog neuromuscular junction when quantal acetylcholine content is increased or decreased. J Physiol 541:385-393.

372. Venance L, Stella N, Glowinski J, Giaume C (1997) Mechanism involved in initiation and propagation of receptor-induced intercellular calcium signaling in cultured rat astrocytes. J Neurosci 17:1981-1992.

373. Verbalis JG, Mangione MP, Stricker EM (1991) Oxytocin produces natriuresis in rats at physiological plasma concentrations. Endocrinology 128:1317-1322.

374. Vizi ES, Burnstock G (1988) Origin of ATP release in the rat vas deferens: concomitant measurement of [3H]noradrenaline and [14C]ATP. Eur J Pharmacol 158:69-77.

375. Vizi ES, Sperlagh B, Baranyi M (1992) Evidence that ATP released from the postsynaptic site by noradrenaline, is involved in mechanical responses of guinea-pig vas deferens: cascade transmission. Neuroscience 50:455-465.

376. Volterra A, Meldolesi J (2005) Astrocytes, from brain glue to communication elements: the revolution continues. Nat Rev Neurosci 6:626-640.

377. von K, I, Starke K (1991) Release of noradrenaline and ATP by electrical stimulation and nicotine in guinea-pig vas deferens. Naunyn Schmiedebergs Arch Pharmacol 344:419-429.

378. Wadiche JI, Jahr CE (2001) Multivesicular release at climbing fiber-Purkinje cell synapses. Neuron 32:301-313.

379. Wahl LM, Stratford KJ, Larkman AU, Jack JJ (1995) The variance of successive peaks in synaptic amplitude histograms: effects of inter-site differences in quantal size. Proc Biol Sci 262:77-85.

380. Wakerley JB, Dyball RE, Lincoln DW (1973) Milk ejection in the rat: the result of a selective release of oxytocin. J Endocrinol 57:557-558.

381. Wakerley JB, Lincoln DW (1973a) Proceedings: Unit activity in the supra-optic nucleus during reflex milk ejection. J Endocrinol 59:xlvi-xlvii.

382. Wakerley JB, Lincoln DW (1973b) The milk-ejection reflex of the rat: a 20- to 40-fold acceleration in the firing of paraventricular neurones during oxytocin release. J Endocrinol 57:477-493.

383. Wakerley JB, Poulain DA, Brown D (1978) Comparison of firing patterns in oxytocin- and vasopressin-releasing neurones during progressive dehydration. Brain Res 148:425-440.

384. Wall MJ, Usowicz MM (1998) Development of the quantal properties of evoked and spontaneous synaptic currents at a brain synapse. Nat Neurosci 1:675-682.

385. Wallach JH, Loewy AD (1980) Projections of the aortic nerve to the nucleus tractus solitarius in the rabbit. Brain Res 188:247-251.

386. Walmsley B (1995) Interpretation of 'quantal' peaks in distributions of evoked synaptic transmission at central synapses. Proc Biol Sci 261:245-250.

387. Wang YF, Shibuya I, Kabashima N, Setiadji VS, Isse T, Ueta Y, Yamashita H (1998) Inhibition of spontaneous inhibitory postsynaptic currents (IPSC) by noradrenaline in rat supraoptic neurons through presynaptic alpha2-adrenoceptors. Brain Res 807:61-69.

388. Wang YT, Linden DJ (2000) Expression of cerebellar long-term depression requires postsynaptic clathrin-mediated endocytosis. Neuron 25:635-647.

389. Wang Z, Haydon PG, Yeung ES (2000) Direct observation of calcium-independent intercellular ATP signaling in astrocytes. Anal Chem 72:2001-2007.

390. Weiss ML, Hatton GI (1990) Collateral input to the paraventricular and supraoptic nuclei in rat. II. Afferents from the ventral lateral medulla and nucleus tractus solitarius. Brain Res Bull 25:561-567.

391. Whitlock JR, Heynen AJ, Shuler MG, Bear MF (2006) Learning induces long-term potentiation in the hippocampus. Science 313:1093-1097.

392. Wickelgren I (1997) Getting a grasp on working memory. Science 275:1580-1582.

393. Williams SR, Buhl EH, Mody I, I (1998) The dynamics of synchronized neurotransmitter release determined from compound spontaneous IPSCs in rat dentate granule neurones in vitro. J Physiol (Lond) 510:477-497.

394. Xiang Z, Bo X, Oglesby I, Ford A, Burnstock G (1998) Localization of ATP-gated P2X2 receptor immunoreactivity in the rat hypothalamus. Brain Res 813:390-397.

395. Xiang Z, He C, Burnstock G (2006) P2X5 receptors are expressed on neurons containing arginine vasopressin and nitric oxide synthase in the rat hypothalamus. Brain Res 1099:56-63.

396. Yamashita H, Inenaga K, Dyball RE (1988) Thermal, osmotic and chemical modulation of neural activity in the paraventricular nucleus: in vitro studies. Brain Res Bull 20:825-829.

397. Yamashita H, Inenaga K, Kannan H (1987) Depolarizing effect of noradrenaline on neurons of the rat supraoptic nucleus in vitro. Brain Res 405:348-352.

398. Yawo H (1999) Protein kinase C potentiates transmitter release from the chick ciliary presynaptic terminal by increasing the exocytotic fusion probability. J Physiol 515 (Pt 1):169-180.

399. Young WS, III, Shepard E, Amico J, Hennighausen L, Wagner KU, LaMarca ME, McKinney C, Ginns EI (1996) Deficiency in mouse oxytocin prevents milk ejection, but not fertility or parturition. J Neuroendocrinol 8:847-853.

400. Zhang JM, Wang HK, Ye CQ, Ge W, Chen Y, Jiang ZL, Wu CP, Poo MM, Duan S (2003) ATP released by astrocytes mediates glutamatergic activity-dependent heterosynaptic suppression. Neuron 40:971-982.

401. Zhao BG, Chapman C, Bicknell RJ (1988a) Opioid-noradrenergic interactions in the neurohypophysis. I. Differential opioid receptor regulation of oxytocin, vasopressin, and noradrenaline release. Neuroendocrinology 48:16-24.

402. Zhao BG, Chapman C, Brown D, Bicknell RJ (1988b) Opioid-noradrenergic interactions in the neurohypophysis. II. Does noradrenaline mediate the actions of endogenous opioids on oxytocin and vasopressin release? Neuroendocrinology 48:25-31.

403. Zhong H, Minneman KP (1999) Alpha1-adrenoceptor subtypes. Eur J Pharmacol 375:261-276.

404. Zinchuk VS, Okada T, Kobayashi T (1999) Detection of ecto-ATPase activity in synaptic plasma membranes for studying extracellular ATP-induced signal transduction. Brain Res Brain Res Protoc 4:258-265.

405. Zucker RS (2003) Can a synaptic signal arise from noise? Neuron 38:845-846.

406. Zucker RS, Regehr WG (2002) Short-term synaptic plasticity. Annu Rev Physiol 64:355-405.