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Subcellular & Subsynaptic Localization of Group I Metabotropic Glutamate Receptors in the Nucleus Accumbens

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Subcellular & Subsynaptic Localization of Group I Metabotropic Glutamate Receptors in the Nucleus Accumbens

By

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B.S., Psychology, Washington & Lee University, 2002

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Abstract

Subcellular & Subsynaptic Localization of Group I Metabotropic Glutamate Receptors in the Nucleus Accumbens

By Darlene A. Mitrano

Changes in glutamate neurotransmission in the nucleus accumbens (NAc), a key structure in the reward pathway, have emerged as an important neuroadaptive mechanism in response to cocaine. There is evidence that group I metabotropic glutamate receptors (mGluR1 and mGluR5) in the NAc play an important role in the neurochemical and pathophysiological mechanisms that underlie addiction to psychostimulants. In order to understand the substrate by which group I mGluRs could mediate their effects, we undertook a detailed analysis, using immunocytochemical electron microscopy (EM), of the ultrastructural localization of mGluR1a and mGluR5 in the NAc of normal and cocaine-treated animals.

We first examined the subcellular and subsynaptic localization of mGluR1a and mGluR5 in the shell and core of the NAc in normal monkey and rat. In both species, group I mGluRs are mainly postsynaptic in dendrites and spines, with rare presynaptic labeling in unmyelinated axons. At the subsynaptic level, these elements had a significantly larger proportion of plasma membrane-bound mGluR5 in rats compared to monkeys. Conversely, mGluR1a displayed the same pattern of labeling in the two species.

Next, using a combination of anterograde tract-tracing and EM immunocytochemistry, we found that limbic prefrontal cortical and midline thalamic terminals contacted mGluR1a- and mGluR5-immunoreactive dendrites and spines in both the shell and core, but to varying degrees. In addition, mGluR5 was found to be more

frequently expressed perisynaptically, and closer extrasynaptically, at both types of synapses.

Finally, we looked at group I mGluR distribution in the accumbens of rats treated either acutely or chronically with cocaine. Surprisingly, only minor changes were seen in the subsynaptic localization of mGluR1a following acute and chronic cocaine exposures, while none were seen for mGluR5. In contrast, a significant reduction of plasma membrane-bound mGluR1a and mGluR5 was induced by local administration of the group I mGluR agonist, DHPG.

Overall, this work provides a detailed map of the localization of the group I mGluRs, including their relationship to two glutamatergic afferents, and how these receptors may be trafficked in response to cocaine or agonist stimulation, providing a foundation for the interpretation of future functional studies of these receptors in the NAc.

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Chapter 1:

Introduction

Preface

This dissertation, "Subcellular & Subsynaptic Localization of Group I Metabotropic Glutamate Receptors (mGluRs) in the Nucleus Accumbens", is presented in five chapters. The first chapter entitled *Introduction* gives a detailed background of information deemed necessary for the reader to understand the subsequent chapters. The introduction begins with describing the brain region this work is focused around, the nucleus accumbens, its circuitry and functions, and then how glutamate affects this region and how both glutamate and the nucleus accumbens have been implicated in drug abuse. This chapter will then go on to discuss glutamate receptors, and more specifically, mGluRs, providing a solid rationale for why the three specific aims presented at the end of the introduction were completed.

Chapters two through four give detailed descriptions of the data collected for each of the specific aims. Chapters two and three examine the localization of the group I metabotropic glutamate receptors in the nucleus accumbens of normal, untreated animals and these receptors' relationship with various glutamatergic inputs to the accumbens. Chapter four goes on to describe changes in localization of these receptors following various cocaine treatments, as well as to the agonist for the group I mGluRs, DHPG. Chapters two and four have been published in peer-reviewed journals, while chapter three is still in preparation for publication.

Chapter five (*Discussion and Future Directions*) discusses what can be concluded from the findings of this thesis and what significance and contributions this work has given to the field of mGluRs, drug abuse and neuroanatomy.

1. Introduction

1.1 Rationale behind this project

Changes in glutamate neurotransmission in the brain have emerged in recent years as a focus in psychostimulant abuse and addiction. Specifically, the group I metabotropic glutamate receptors, mGluR1a and mGluR5, have been shown, through the use of transgenic animals and newly developed specific drugs, to possibly play a role in neuroadaptations that occur following both acute and chronic cocaine exposure in various animal models. These neuroadaptations could be one of the many underlying factors that lead to cocaine dependence and relapse following withdrawal. Up until this project, the exact subcellular and subsynaptic localization of these receptors within the nucleus accumbens, an essential brain structure in the reward pathway, had not been determined. While the group I mGluRs' localization and function have been explored in other regions throughout the central nervous system (CNS), mGluR1a and mGluR5 display properties that are unique to the specific area in question. Therefore, determining the localization of these receptors in normal and cocaine-treated animals in the nucleus accumbens could provide valuable information in determining how these receptors function, how they may be activated in the accumbens, and if their change in localization following various cocaine administrations could be a factor in the neuroadaptations seen following cocaine administration. Using immunocytochemistry, tract-tracing, and electron microscopy, a detailed analysis of the subcellular and subsynaptic localization of the group I mGluRs in the core and shell of the nucleus accumbens in normal and cocaine-treated animals was determined, as well as these receptors' localization in relation to specific glutamatergic afferents. The results of these studies provide a framework for the interpretation of future

physiological experiments examining the function of group I mGluRs and their role in drug addiction.

1.2 The Nucleus Accumbens

1.2.1 General information about the nucleus accumbens and its organization

The nucleus accumbens, originally called the nucleus accumbens septi, derived its name from its location, literally meaning "a nucleus against the septum" (Swanson & Cowan, 1975). Since there are no clear boundaries of this nucleus, it was originally debated whether the nucleus accumbens should be considered an extension of the dorsal striatum, more specifically of the caudate, or whether it should be considered as part of the septum and olfactory tubercule. Following numerous autoradiographic tract-tracing studies using tritiated amino acids into the accumbens to examine the efferent projections of this nucleus, it was determined that it was more closely related to the caudate and putamen of the dorsal striatum than the septal complex and, therefore, is now also referred to as the ventral striatum (Swanson & Cowan, 1975, Powell & Leman, 1976).

The nucleus accumbens can be divided into sub-regions based on numerous properties, such as responses to drug administration, neural connections and neurochemical staining features. Most would confer that the nucleus accumbens is divided into the core, which seems to be an extension of the dorsal striatum, and the shell, which surrounds the core on its medial, ventral and lateral sides. Early studies attempted to distinguish the possible compartments of the rodent accumbens using immunocytochemistry and staining with antibodies against neurotensin, substance P, and cholecystokinin (Zahm & Heimer, 1988). Further studies went on to use other markers such as acetylcholinesterase (AChE), choline acetyltransferase, and enkephalin. For

example, intense immunoreactivity for AChE defines the properties of the nucleus accumbens shell; whereas the core is lightly labeled for AChE (Meredith et al., 1989). It was determined by Jongen-Relo and colleagues (1994) through the staining of the nucleus accumbens in its entire extent, that the calcium binding protein, calbindin-D_{28k}, in comparison to substance P and acetylcholinesterase, is the best marker to define the boundaries between the core and shell (see chapter 2, figure 2.1 for visualization of core-shell boundaries in both monkey and rat brain using calbindin-D_{28k}). In contrast to AChE immunoreactivity, calbindin antibodies stain the core very densely, while the shell appears very lightly labeled (Jongen-Relo et al., 1994). These previous studies all examined the shell/core dichotomy in the rat brain, however the same holds true in the primate as well as the human brain, with the core being more intensely stained than the shell with antibodies against calbindin-D_{28k} (Meredith et al., 1996).

In addition to various staining properties, the core and shell have specific morphological differences. The striatum, including the ventral striatum, is mainly comprised of medium spiny projection neurons (specific projections of the core and shell are discussed below). The morphological characteristics of these neurons within either compartment of the accumbens have been examined using various retrograde tracers and Golgi impregnation. The nucleus accumbens medial shell, compared to the core, is made up of neurons that tend to have a smaller dendritic arbor, dendrites that branch less, and a smaller amount of terminal segments (Meredith et al., 1992). It was also observed that the neurons in the shell have a lower spine density than the neurons of the core, indicating that the cells in the core likely receive a stronger glutamatergic synaptic innervation than neurons in the shell (Meredith et al., 1992).

As stated above, like the dorsal striatum, the ventral striatum is composed mainly of GABAergic medium spiny projection neurons (approximately 95% of the neurons in the rat and approximately 70% in the primate, Heimer et al., 1997). Based on electrophysiological studies, these medium spiny neurons are typically characterized in one of three categories; 1) silent; 2) firing, at low, constant rate; or 3) displaying two distinct states at resting membrane potential (O'Donnell & Grace, 1995). In the dorsal striatum, it has been shown that the medium spiny neurons can be further subdivided based on receptor expression and specific projection areas. Dopamine D1-receptors are found in about fifty percent of the medium spiny neurons and are considered to be part of the "direct" striatofugal pathway that projects preferentially to the substantia nigra (Gerfen et al., 1990; Yung et al., 1995). The other half of medium spiny neurons express D2-like dopamine receptors and have been categorized as part of the "indirect" striatofugal pathway that projects mainly to the globus pallidus (Gerfen et al., 1990; Yung et al., 1995).

The rest of the cells within the nucleus accumbens are larger, typically aspiny interneurons characterized by their chemical phenotype. Also based on work in the dorsal striatum, it has been determined that four major classes of interneurons are present in the nucleus accumbens. They are: 1) cholinergic interneurons identifiable by their ChAT (choline acetyltransferase) content; 2) GABAergic interneurons containing PV (parvalbumin); 3) GABAergic interneurons containing calretinin; and 4) the last class of interneurons have been shown to contain somatostatin, nitric oxide synthase and NADPH-diaphorase (Emson et al., 1993; Kawaguchi et al., 1995). The interneurons have

the ability to modulate the glutamatergic inputs and GABAergic outputs of the nucleus accumbens which, in turn, can affect many other brain regions.

1.2.2 Major Afferents and Efferents of the Nucleus Accumbens

The nucleus accumbens receives numerous neurotransmitter inputs, including serotonin projections from the raphe nucleus (Conrad et al., 1974; Parent et al., 1981), norepinephrine form the locus coeruleus and solitary tract region (Russell et al., 1989; Wang et al., 1992), and GABA from the ventral pallidum (Churchill & Kalivas, 1994).

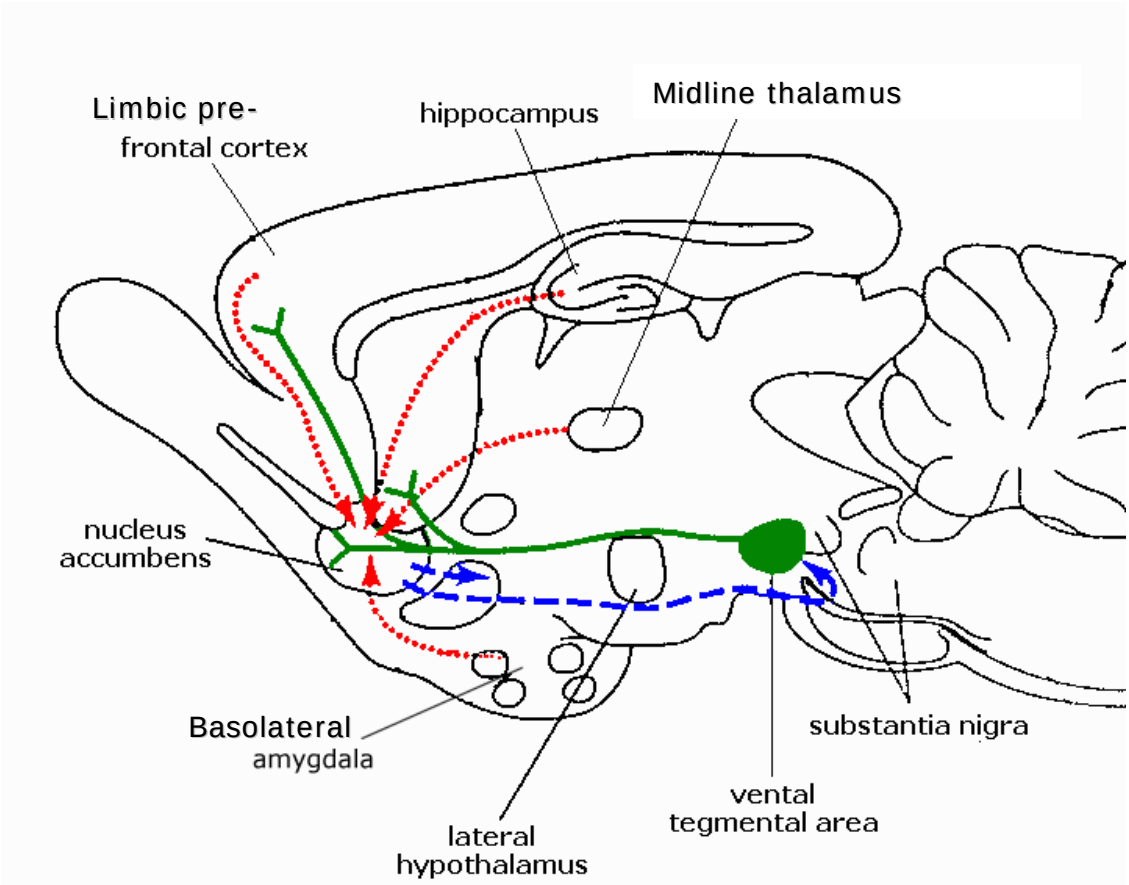

Figure 1.1: Schematic of some of the major afferent and efferent connections of the nucleus accumbens. Green indicates dopamine; red indicates glutamate; and blue indicates GABA.

However, since this thesis is focused on glutamate receptors and cocaine, only glutamatergic and dopaminergic projections to the accumbens will be discussed in detail in this section. As shown in the simplified diagram of the rat brain in figure 1.1, the nucleus accumbens receives numerous dopaminergic and glutamatergic afferents, and gives rise to various GABAergic efferents. Glutamate, more specifically, L-glutamate, the major excitatory neurotransmitter in the CNS, innervates the accumbens from the limbic prefrontal cortex, the midline thalamus, subiculum of the hippocampus and basolateral amygdala (Groenewegen et al., 1987; Berendse & Groenewegen, 1990; McDonald, 1991, Berendse et al., 1992; French & Totterdell, 2003). However, specific portions of each of the mentioned nuclei innervate the core versus the shell of the accumbens. For example, the dorsal region of the limbic prefrontal cortex (PFC) projects to the core of the accumbens, while the ventral region projects to the shell (Berendse et al, 1992). The intermediodorsal (IMD) and central medial (CM) nuclei of the midline thalamus project to the core, while the paraventricular nucleus (PVN) targets the shell (Berendse & Groenewegen, 1990). Finally, similar divisions hold true for the amygdala and hippocampus, with the anterior basolateral amygdala sending afferents to the core, while the posterior targets the shell (McDonald, 1991) and the dorsal subiculum of the hippocampus projecting to the core, while the ventral region projects to the shell (Groenewegen et al., 1987).

In general, the glutamatergic innervation into the nucleus accumbens has been shown to depolarize accumbal neurons, interact with dopamine, and regulate various behaviors such as motor output, exploratory behaviors, learning, and importantly, reward-mediated behaviors (Taber & Fibiger 1995, You et al., 1998; Young & Deutch, 1998;

Pinto et al., 2003; Baker et al., 2003; Schmidt et al., 2005; Parsons et al., 2007). There are also interactions amongst the glutamatergic afferents. One of the most cited studies are by O'Donnell & Grace (1995) which examined the properties of the projections from the hippocampus and cortex. Using in vivo electrophysiology, they found that hippocampal stimulation depolarizes neurons in the accumbens, which makes them enter an "active state". In contrast, spike firing in the accumbens induced by cortical afferents is only possible when the neurons are in this "active state" created by the hippocampus. This led the authors to suggest that the hippocampus has the ability to "gate" prefrontal cortical inputs to accumbal neurons (O'Donnell & Grace, 1995). Additional specific behavioral functions of these projections are discussed more in the following sections.

Since this thesis is mainly focused on glutamate and glutamate receptors, dopamine will not be discussed in detail. However, it is important to briefly discuss the major sources of dopamine that innervate the accumbens as there are many interactions between dopamine and glutamate. In addition, when discussing the effects of cocaine, it is important to understand how this drug affects dopamine in the brain, especially the accumbens. Dopamine is mainly supplied to the nucleus accumbens by the midbrain dopamine neurons of the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc). The cells in the midbrain can be divided into layers based on the similar phenotypes to help explain their segregation in projections. The dorsal tier includes the dorsal substantia nigra pars compacta and the contiguous VTA, while the ventral tier is comprised of a densocellular layer of cells in the ventral SNc and cell columns that invade the substantia nigra pars reticulata (SNr) (Heimer et al., 1997). It is

the dorsal tier that sends projections to both the core and shell of the accumbens (Heimer et al., 1997).

The major efferents of the accumbens include the substantia nigra, ventral pallidum, VTA, and hypothalamus. The efferents of the accumbens were originally studied using the anterograde degeneration method and the anterograde transport of tritiated amino acids (Powell & Leman, 1976; Williams et al., 1977). However, this was done before the accumbens was divided into the shell and core. Therefore, the following studies discussed below are more recent investigations of accumbens connections that acknowledge these divisions.

As with the afferents to the accumbens, there is some specificity in the efferents coming from either the core or shell as well. It has been shown that the core of the accumbens projects to the dorsolateral part of the ventral pallidum, while the medial shell projects to the dorsomedial and ventromedial areas of the ventral pallidum. On the other hand, the lateral shell projects to the ventrolateral part of the ventral pallidum (Usuda et al., 1998; Groenewegen et al., 1999). While both the shell and core innervate the VTA, the projection from the medial shell is stronger than that from the core (Usuda et al., 1998). In relation to the VTA, the accumbens core and shell project to the medial SNr and the densocellular layers of the SNc, which open up the possibility for GABA outputs from the nucleus accumbens to modulate some of the dopaminergic nigral neurons that innervate the dorsal striatum (Heimer et al., 1997). Finally, both the core and shell project equally to the anterior and tuberal part of the lateral hypothalamus (Usuda et al., 1998).

1.2.3 General Functions of the Nucleus Accumbens

The nucleus accumbens is a key structure in experiencing the rewarding effects of psychostimulants, namely cocaine; however since there is a lot of information on this topic, it will be separately discussed in the following section. There are, indeed, other functions the nucleus accumbens subserves in the brain and CNS. Being technically part of the striatum and basal ganglia, some authors suggested that the nucleus accumbens may play a role in motor control and movement, even being termed a neural substrate for limbic-motor interactions (Mogenson et al., 1980; Groenewegen et al., 1999). Therefore, many studies have addressed the role of the accumbens in locomotion and learning using lesions, electrophysiology, and local pharmacological manipulation (for review, see Pennartz et al., 1994). Initial studies found that injecting dopamine directly into the nucleus accumbens increased locomotor activity in rodents (Pijnenberg & Van Rossum, 1973; Costall & Naylor, 1975). This increase is locomotion was replicated using various dopamine agonists, including apomorphine, amphetamine, and specific D1-receptor agonists, SKF38393 and SKF89626 (Kelly et al., 1975; Freedman et al., 1979; Kelley et al., 1989). This increase in locomotor behavior elicited by drugs such as amphetamine could then be abolished using 6-hydroxydopamine lesions of the dopaminergic projections to the accumbens (Kelly et al, 1975). 6-OHDA lesions of the accumbens have also shown that the accumbens may play a role in ingestive behaviors such as chewing and swallowing (Lund & Dellow, 1971; Hockman et al., 1980) and muscle tone (Ellenbroek et al., 1988).

Studies have also been done looking at various other neurotransmitter systems in the nucleus accumbens by injecting the neurotransmitter or its agonist/antagonist locally. For instance, high doses of GABA decrease locomotor behavior, while low doses

increase it; AMPA and NMDA (glutamate receptor agonists) both increase locomotor behavior, while kynurenic acid (glutamate receptor antagonist) decreases it. Finally carbachol, a cholinergic receptor agonist, increases locomotor behavior (Pennartz et al., 1994).

In addition to locomotion, the accumbens seems to play a role in mediating learning behaviors, especially when related to reward and survival. Pennartz and colleagues (1994) proposed a hypothesis on how the accumbens processes reward-dependent learning based on numerous lesioning and pharmacological manipulation studies. In summary, an animal's exploratory behavior identifies a primary reward in its surroundings, which activates cortical, thalamic, and amygdala areas in the brain. These brain areas all project to the accumbens and activate it to lead to an action in obtaining the reward. Two usual outcomes of attempting to obtain the perceived reward are 1) punishment, such as pain or stress, or 2) the actual obtaining of a reward, which may involve consumption or sex. Hence, the animal will either learn avoidance for the punishment (conditioned avoidance), or learn that this goal-directed behavior worked (paradigms such as conditioned place preference, conditioned reinforcement, operant conditioning, and even spatial learning). The results of the animal's actions then feed back to the neural circuits of the cortex, thalamus, and amygdala, which in theory are then modified for future actions of the animal (Pennartz et al., 1994).

1.2.4 Cocaine and the Nucleus Accumbens

The nucleus accumbens has long been established as playing a key role in the rewarding effects of psychostimulants, especially cocaine. This was determined through the use of 6-OHDA lesions of the nucleus accumbens and VTA, which results in

depletion of dopamine in these brain regions, as well as injecting the excitotoxin kainic acid into the accumbens. It was shown that intravenous self-administration of cocaine was significantly disrupted or abolished in rodents following these lesions (Roberts et al., 1977; Roberts et al., 1980; Roberts & Koob, 1982; Pettit et al., 1984; Zito et al., 1985). In addition, electrophysiological studies examining the neural activity of the nucleus accumbens during cocaine self administration have shown that this nucleus plays a role in initiating and maintaining self administration (Chang et al., 1996).

Once it was established where cocaine's rewarding properties were mediated, the next step was to determine the mechanism of action of this drug at the cellular level. Through the use of in vivo microdialysis and various radioactive binding assays, it was determined that when cocaine is administered, it blocks dopamine transporters throughout the brain, increasing levels of extracellular dopamine and therefore enhancing the effects of dopamine throughout the brain (Ritz et al., 1987; Kuhar et al., 1988; Ritz et al., 1988; Pettit & Justice, 1989). As mentioned above, dopamine is released into the accumbens from terminals arising from the VTA. When cocaine is administered, there is a subsequent increase in extracellular dopamine in the accumbens because of the blockade of dopamine transporters (Ritz et al., 1987; Ritz et al., 1988; Pettit & Justice, 1989). In addition, the VTA also projects to the prefrontal cortex, so there are also enhanced levels of dopamine in this nucleus as well. The increase of dopamine in the PFC enhances the firing rate of the glutamatergic neurons which project to the accumbens. Therefore, there is an increase in the amount of extracellular glutamate in the nucleus accumbens following cocaine administration, which has been measured by in vivo microdialysis after a single cocaine injection (Smith et el., 1995; Reid et al., 1997).

There is good evidence that increased synaptic release of neurotransmitter from prefrontal cortical afferents may be the main source of extracellular glutamate buildup following acute cocaine administration and reinstatement of self-administration after withdrawal (McFarland et al., 2003).

In comparison, chronic cocaine administration has the opposite effects of glutamate in the accumbens. Using the behavioral sensitization paradigm developed by Kalivas and colleagues (1988), which employs 7 days of experimenter administered cocaine followed by three weeks withdrawal, it was found that extracellular glutamate was decreased in the nucleus accumbens, measured by in vivo microdialysis (Pierce et al., 1996; Hotsenpiller et al., 2001; Baker et al., 2003). It has been hypothesized that behavioral sensitization to chronic cocaine exposure decreases the activity of the cystine/glutamate exchanger, thereby, lowering extracellular glutamate levels in sensitized rats (Baker et al., 2003). The cystine/glutamate exchanger is comprised of two proteins (4F2hc and xCT) and is a sodium independent anionic amino transport system that exchanges cystine and glutamate in a 1:1 ratio (Sato et al., 1999). This exchanger system seems to be a major contributor to the regulation of nonsynaptic basal extracellular glutamate levels, at least in the rat striatum, which could impact the activation of the mGluRs known to be mainly extrasynaptic in this brain region (Baker et al., 2002; Paquet & Smith, 2003; Mitrano & Smith, 2007).

Cocaine has also been shown to induce morphological changes in the nucleus accumbens. Using various methods of cocaine administration, including experimenter administered cocaine (to induce behavioral sensitization) and self administration, it has been shown that cocaine induces neural plasticity in the both the core and shell of the

accumbens. For example, rats that were administered cocaine by the experimenter or through self administration for at least four weeks followed by a minimum of 3 weeks no-drug period (Robinson & Kolb, 1999; Norrholm et al., 2003; Li et al., 2004; Ferrario et al., 2005) showed increases in the density of dendritic spines on medium spiny neurons in the core or shell of the accumbens and pyramidal neurons in the prefrontal cortex (Robinson & Kolb, 1999; Norrholm et al., 2003; Li et al., 2004; Ferrario et al., 2005). This could indicate long term neural changes in complex behaviors such as learning and memory, pending the fact that these newly developed spines are functional and have the receptors needed for long-term synaptic plasticity (Segal, 2005).

1.3 Glutamate Receptors

1.3.1 Ionotropic Glutamate Receptors

Glutamate receptors are divided into two main categories, ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs). The iGluRs are ligand-gated ion channels that mediate fast neurotransmission in the CNS and are subdivided into three main categories based on the original agonists used to activate them: 1) N-methyl-D-aspartate (NMDA); 2) α-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid (AMPA); and 3) 2-carboxy-3-carboxymethyl-4-isopropenylpyrroldine (Kainate). Each of these receptor subtypes are made up of various subunit combinations that result in differential physiological effects in target cells. These receptors are ubiquitous throughout the brain, including the nucleus accumbens (Albin et al., 1992; Gracy & Pickel, 1997; Tarazi et al., 1998).

Until recently, most of the work involving glutamate neurotransmission and its modulation in the nucleus accumbens has focused on the various classes of iGluRs.

Through various pharmacological manipulations, it has been shown that these various iGluRs, especially NMDA and AMPA, play roles in cocaine abuse and addiction (Pierce et al., 1996; Bespalov et al., 2000; Famous et al., 2007; Schmidt et al., 2005). In addition, other properties of the NMDA and AMPA receptors have been altered following cocaine exposure. For example, it has been shown following chronic cocaine administration and withdrawal that specific AMPA receptors subunits redistribute in the plasma membrane which is in contrast to an acute cocaine administration that results in significant internalization of the same AMPA receptor subunits (Boudreau & Wolf, 2005; Boudreau et al., 2007). If iGluRs can mediate the effects of cocaine and change functions or localization in response to cocaine administration, it is reasonable to hypothesize that the functional localization of mGluRs may also be affected by cocaine.

1.3.2 Overview of Metabotropic Glutamate Receptors

In the mid 1980's, a new class of glutamate receptors was discovered which, instead of being ligand-gated cation channels, were actually linked to G-proteins and second messenger systems. These first mGluRs, as they are now termed, were shown to increase levels of inositol phosphate in oocytes as well as cultured striatal cells (Sugiyama, et al., 1987; Sladeczek et al., 1985). This was a significant finding in the field because up until this point it was believed that glutamate could only induce fast synaptic responses that could only be modulated by other neurotransmitter systems, such as dopamine, serotonin or acetylcholine. With the emergence of mGluRs, it was determined that glutamate could slowly modulate and fine-tune the same synapses that also contain iGluRs.

The mGluRs, all G-protein coupled receptors (GPCRs), were classified into Class 3 or C of the GPCR superfamily of receptors. The mGluRs are divided into three classes, based on sequence homology, second messengers and pharmacology (for review see Conn & Pin, 1997). They are: 1) Group I mGluRs (mGluR1 and mGluR5); 2) Group II mGluRs (mGluR2 and mGluR3); and 3) Group III mGluRs (mGluR4, mGluR6, mGluR7, mGluR8). Group II and III mGluRs are linked to Gi/Go which, in turn, inhibit adenylyl cyclase activity (see Figure 2 for the structure). The group II and III mGluRs are mainly presynaptic in most brain regions (Shigemoto et al., 1997), where they have been implicated in controlling glutamate and GABA release from axon terminals (Cartmell & Schoepp, 2000). The group II and III mGluRs have also been localized in the nucleus accumbens (Testa et al., 1998). Through the use of in vitro and in vivo electrophysiology, they have been shown to decrease extracellular glutamate levels (Manzoni et al., 1997; Xi et al., 2003). In relation to psychostimulants, the group II/III mGluRs have been implicated in modulating some of the effects of cocaine in the nucleus accumbens. Although it has been hard to distinguish between the members of the group II mGluRs due to the lack of specific agonists and antagonists, it has been shown that modulating these receptors, along with the cystine-glutamate exchanger (discussed above in Cocaine and the Nucleus Accumbens), may have an impact on reducing the rewarding effects of cocaine and reduce cocaine intake in animal models (Baker et al., 2003; for reviews see, Kalivas et al., 2003; Kenny & Markou, 2004; Kalivas, 2007).

1.3.3 Group I mGluRs

Since this project is focused on the group I mGluRs, subsequent discussion of mGluRs will only focus on mGluR1 and mGluR5. Group I mGluRs are linked to the Gα-

protein, Gq, and have been shown to activate phospholipase-C, which through two different pathways can either 1) increase inositol triphosphate (IP_3) production, bind to IP3 receptors, and increase intracellular calcium levels, or 2) increase diacylglycerol (DAG) formation and protein kinase C. In transfected cell systems it has been shown that mGluR1a activation induces a single peak in calcium levels, while mGluR5 causes oscillations in calcium concentrations in the cell (Kawabata et al., 1996; O'Malley et al., 2003). It also has been shown, though not studied as extensively as the Gq pathway, that the group I mGluRs can also be linked to Gs and increase levels of cAMP (Tateyama & Kubo, 2006; Tateyama & Kubo, 2007). Since most work has focused on the Gq pathway, subsequent discussion will focus on that pathway.

As shown in figure 2 below, Group I mGluRs have an extracellular NH_2-glutamate binding site, characterized by two globular domains or bi-lobed with a hinge region (also know as a venus-fly trap module). This is followed by a 7-transmembrane domain, ending intracellularly with a C-terminal domain (Conn & Pin, 1997; Kew & Kemp, 2005).

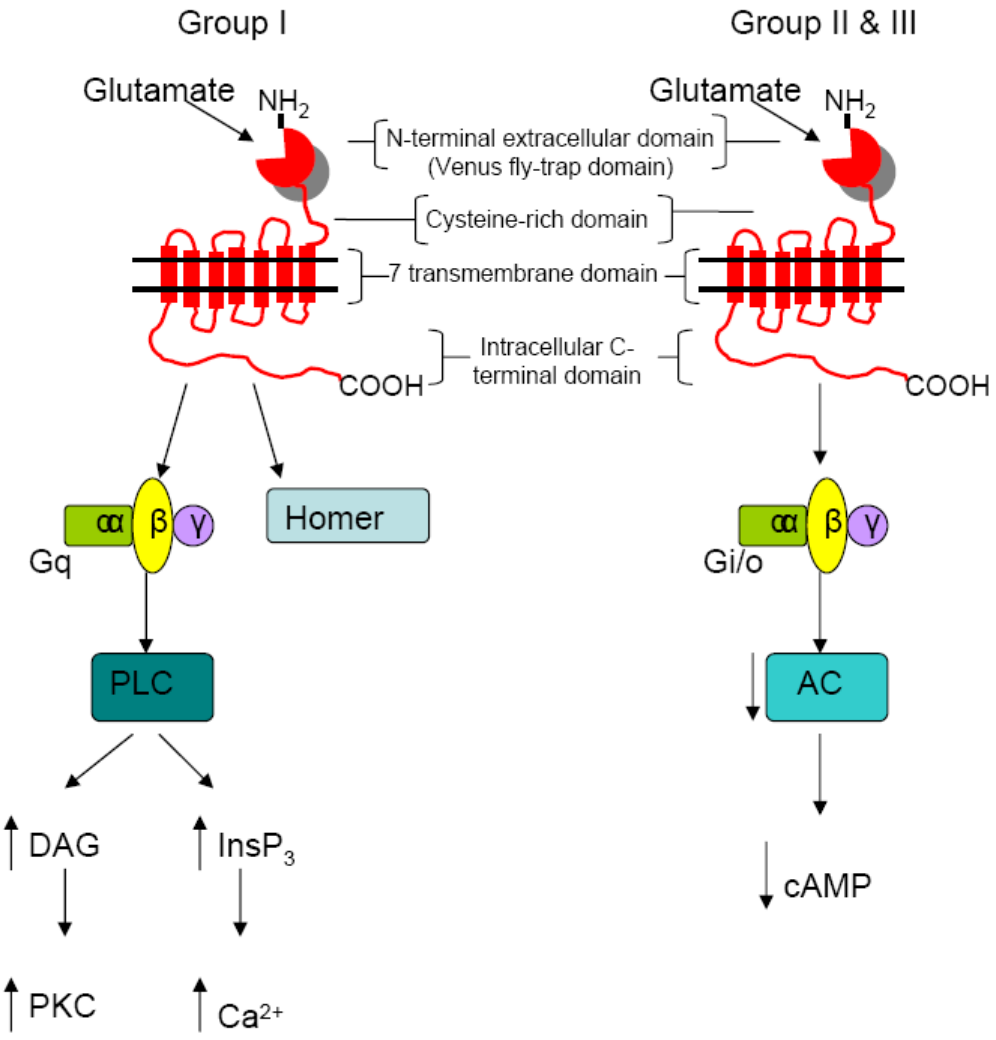

Figure 1.2: Schematic illustration of the structure of the mGluRs.

Various splice variants of both mGluR1 and mGluR5 exist. The primary sequence of mGluR1a-d has been cloned, as well as mGluR5a-b (Conn & Pin, 1997). Both classes of mGluR1 and mGluR5 show about 70% sequence homology and mainly differ in the amino acid sequence of the C-terminal regions (Conn & Pin, 1997). The commercially available antibodies currently recognize the mGluR1a splice variant and mGluR5 (not distinguishing between mGluR5a and b). Therefore, the experiments presented in this thesis will focus on mGluR1a and mGluR5.

Besides linking to G-proteins, the group I mGluRs have various scaffolding proteins that may be intermediaries in the intracellular signaling cascade or cross-link to other receptors within the neuron. Two of these scaffolding proteins that have been studied in detail are Homer and Shank. Homer proteins are members of a family of scaffolding proteins encoded by three genes, Homer 1-3. All of the Homer proteins are constitutively expressed in the brain, except Homer1a, which is an immediate early gene product induced following neuronal stimulation, such as cocaine (Brakeman et al., 1997; Xiao et al., 2000). In vitro, as well as some recent in vivo data has indicated that Homer has a role in the trafficking, synaptic targeting and intracellular signaling of the group I mGluRs (Xiao et al., 1998; Roche et al., 1999; Ango et al., 2000; Thomas, 2002; Kuwajima et al., 2007). The coiled-coiled domain present on the long forms of Homer enables the protein to dimerize as well as to link it to the mGluRs, IP_3 receptors, and NMDA receptors (Xiao et al., 2000; Fagni et al., 2002). Increased levels of the long forms of Homer (i.e., Homer 1b/c, 2, and 3) have been shown to internalize the group I mGluRs (Roche et al., 1999), while the short form (Homer 1a) causes movement of these receptors to the plasma membrane (Xiao et al., 1998; Ango et al., 2000). Shank proteins

have also been shown to link the group I mGluRs and Homer to the postsynaptic density as well as NMDA receptors (Tu et al., 1999; Naisbitt et al., 1999; Lim et al., 1999). In addition, the group I mGluRs display receptor-receptor interactions with adenosine, dopamine, and GABA receptors in various brain regions (Agnati et al., 2003; Tabata et al., 2004; Ciruela et al., 2005; Voulalas et al., 2005). Studies have also shown that increased levels of both Shank and Homer induce morphological and functional neuronal changes. Using cultured hippocampal neurons, it has been observed that overexpression of Shank and the long forms of Homer induces spine growth and maturation (Sala et al., 2001). In contrast, overexpression of Homer 1a causes a reduction in the number and size of dendritic spines (Sala et al., 2003). In addition, cells that overexpress Homer 1a show a reduction in AMPA and NMDA postsynaptic currents, which could also effect the functioning of group I mGluRs (Sala et al., 2003). Together, these studies indicate that, while we know some information about how mGluR1 and mGluR5 are controlled in the cell, further in vivo work is needed to gain a better understanding of all the proteins that may play a role in the movement and downstream effects of these receptors.

1.3.4 Plasticity of GPCRs and the Group I mGluRs

Besides being under the control of scaffolding proteins, such as Homer, in the trafficking of group I mGluRs described above, there are other characteristics of these receptors that control their placement on the plasma membrane or in the intracellular space. There are many properties of GPCRs in general that need to be understood in order to gain a full perception of how mGluR1a and mGluR5 work in the cell. For example, a general property of GPCRs is the ability for the agonist to bind to the receptor, inducing the exchange of GDP to GTP on the Gα subunit (for the group I

mGluRs, Gq), which allows the dissociation of Gα and Gβγ subunits to regulate and activate second messengers within the cell (Hamm, 1998; Dale et al., 2002). Following continuous agonist stimulation, GPCRs desensitize and are not able to respond for an allotted period of time. There are two types of GPCR desensitization, homologous (agonist-dependent) and heterologous (agonist-independent) (Dhami & Ferguson, 2006). Homologous desensitization occurs when a GRK (G-protein coupled receptor kinase) phosphorylates an agonist-activated receptor. This promotes the binding of arrestin proteins, which prevents further activation of the receptor by blocking the exchange of GTP for GDP on the Gα subunit. In addition, the clathrin adaptor protein, AP2, as well as clathrin, bind to arrestin to start the formation of clathrin-coated pits, which induce endocytosis of the receptor (Lefkowitz, 1998; Dale et al., 2002; Gainetdinov et al., 2004; Dhami & Ferguson, 2006). The receptor has multiple fates; it can be brought to the lysosome for degradation which would down regulate the receptor; or it can be recycled and brought back to the plasma membrane, also known as resensitization (Lefkowitz, 1998; Dale et al., 2002; Gainetdinov et al., 2004; Dhami & Ferguson, 2006). Heterologous desensitization can occur in the absence of agonist binding through second messenger-dependent kinases such as PKC or PKA. For example, PKA is activated by Gs and PKC is activated by Gq, either of which will phosphorylate a receptor at a serine or threonine residue, rendering it unable to interact with its G-protein (Lefkowitz, 1998). This has been considered heterologous because any stimulant that induces an increase in cAMP (which increases PKA concentrations) or DAG (which increases PKC concentrations) has the ability to phosphorylate and desensitize any GPCR that contains the right PKA/PKC phosphorylation site (Lefkowitz, 1998).

In the case of group I mGluRs, the use of in vitro cell culture studies has given us insight into desensitization mechanisms for these receptors. Since mGluR1a and mGluR5 are linked to Gq, the group I mGluRs can be heterologously desensitized by PKC at various serine/theronine residues (Alaluf et al., 1995; Ciruela et al., 1999; Gereau & Heinemann, 1998). In addition, numerous studies have looked at the specific GRK and arrestin molecules involved in the agonist-stimulated desensitization and endocytosis of the group I mGluRs. GRK2, GRK4, GRK5 and GRK6 have all been implicated in the phosphorylation and desensitization of mGluR1a in HEK-293 cells (Dale et al., 2000; Sallese et al., 2000). However, when examined in cerebellar Purkinje cells, it was observed that only GRK4 was required for mGluR1a desensitization (Sallese et al., 2000). As for arrestin molecules, β-arrestin 1 seems to be the most involved in the endocytosis of the group I mGluRs (Dale et al., 2001). Overall, the mechanisms underlying group I mGluR desensitization and internalization still need further study. Up until our work looking at the trafficking of the group I mGluRs following cocaine and agonist application (see Chapter 4), no in vivo studies in the brain have been completed examining the internalization properties of mGluR1a and mGluR5.

In vivo examination of the movement of other GPCRs, however, has been completed at the electron microscopic level. It has been shown that in rodents, D1-dopamine receptors, somatostatin sst2A, and muscarinic m2 and m4 receptors all internalize following acute administration of the specific agonist for the receptor (Bernard et al., 1998; Dumartin et al., 1998; Bernard et al., 1999; Csaba et al., 2001). In relation to psychostimulants, a study examining the localization of the D1-dopamine receptors showed that prenatal cocaine exposure reduced the plasma membrane-bound

D1 receptors expression, which could lead to behavioral and cognitive deficits (Stanwood & Levitt, 2007). In addition, one study has been completed looking at the subsynaptic localization of group I mGluRs following deletion of various Homer scaffolding proteins and MPTP treatment (Kuwajima et al., 2007). It was found that dopamine depletion and Homer gene KO lead to an increase in mGluR1a expression presynaptically in axons and axon terminals as well as at putative GABAergic synapses in the globus pallidus and subthalamic nucleus (Kuwajima et al., 2007). Overall, these studies lay a foundation to further examine the in vivo physiological and pathological mechanisms that may regulate or hamper the trafficking and plasticity of group I mGluRs in the CNS (see chapter 4).

1.3.5 Group I mGluRs as Therapeutic Drug Targets

Based on the fact that group I mGluRs are GPCRs and can modulate glutamate neurotransmission at a slower pace than the iGluRs, these receptors have become a target for drug development for various disorders. The group I mGluR specific agonist, (*RS*)-3,5-dihydroxyphenylglycine, or DHPG, is a competitive ligand at the glutamate binding site of the receptor. DHPG was discovered in the mid-1990's (Schoepp et al., 1994) but to date, no agonist has been found to completely distinguish between mGluR1a and mGluR5. Recent studies using cultured hippocampal neurons have shown that DHPG-induced activation of the group I mGluRs, but mainly of mGluR1, is neuroprotective against stroke and ischemia (Blaabjerg et al., 2003; Baskys et al., 2005). Using an NMDA-induced model of excitotoxicity, it was shown that application of DHPG suppressed NMDA-currents and enhanced GABA receptor-mediated currents which, in turn, protected the cell against death (Blaabjerg et al., 2003; Baskys et al., 2005). Competitive antagonists for the group I mGluRs have been researched extensively and

include (*R*,*S*)-1-aminoindan-1-5-dicarboxylic acid or AIDA and (*S*)-2-methyl-4-carboxyphenylglycine or LY367385, both specific for mGluR1 (Pellicciari et al., 1995; Moroni et al., 1997; Clark et al., 1997).

More recently, the development of allosteric modulators has shown even more promise in drug development. An allosteric modulator is a molecule that binds to a site, other than the orthosteric binding site, or normal ligand binding site of the receptor, and can either enhance or inhibit the actions of the endogenous ligand. In the case of the group I mGluRs, an allosteric modulator is a non-competitive ligand that binds to the seven-transmembrane domain (see Figure 2) of the receptor. The most common, specific and effective negative allosteric modulators, or antagonists, of the group I mGluRs are either CPCCOEt for mGluR1 and MPEP (2-methyl-6-(phenylethynyl)-pyridine) (Gasparini et al., 1999; Litchig et al., 1999) for mGluR5. Further investigation shows that 3-[2-methyl-1,3-thiazol-4yl)ethynyl]pyridine or MTEP is even more selective for mGluR5 and has increased bioavailability compared to MPEP (Cosford et al., 2003). The first positive allosteric modulator of mGluR5 has recently been discovered and has been termed DFB (3,3'-difluorobenzaldazine; O'Brien et al., 2003). These compounds have been shown to be neuroprotective as well as helpful in relieving some of the symptoms of certain neurodegenerative diseases. For example, using rat animal models of Parkinson's disease, MPEP has been shown to reduce akinesias (Breysse et al., 2002, 2003) and in combination with adenosine A2A receptors antagonists, was even more effective in reducing the motor symptoms associated with Parkinson's (Coccurello et al., 2004). Antagonizing both mGluR1 and mGluR5 have also been shown to be neuroprotective against Parkinson's, Huntington's, and show potential for therapy in depression and

anxiety (for reviews see Conn et al., 2005; Kew & Kemp, 2005). However, due to side effects of these compounds, such as memory and motor impairment and learning deficits (Genkova-Papazova et al., 2007; Simonyi et al., 2007) further investigation in targeting these receptors for therapeutic purposes is needed.

1.3.6 Group I mGluRs' function and localization in other brain regions

Since these receptors are targets for therapeutic drug development, understanding the localization and function of these receptors throughout the brain is vitally important in understanding how systemically administered drugs that target these receptors may effect general brain functions and in turn lead to a better understanding of how the group I mGluRs can be activated and modulate glutamate transmission. The localization of the group I mGluRs has been extensively examined in various brain regions. In both the hippocampus and cerebellum, it has been shown that group I mGluRs are located primarily postsynaptically (in dendrites and spines), and perisynaptic (touching or within a 20 nm range of the edges of postsynaptic specializations) to asymmetric synapses (putative glutamatergic) (Baude et al., 1993; Lujan et al., 1996). In basal ganglia nuclei, the receptors are also found mainly postsynaptically, but display a little more variability at the subsynaptic level (Hanson & Smith, 1999; Hubert et al., 2001; Paquet & Smith, 2003; Kuwajima et al., 2004). In the dorsal striatum of primates, for example, both receptors are mainly found in dendrites and spines, with most of the labeling for the group I mGluRs on the plasma membrane of spines and with slightly more intracellular labeling in dendrites (Paquet & Smith, 2003). However, mGluR1a, but not mGluR5, is occasionally found presynaptically in axon terminals arising mainly from the thalamus (Paquet & Smith, 2003). In the globus pallidus, interestingly, a good proportion of

postsynaptic labeling for both mGluR1a and mGluR5 was found in the main body of symmetric synapses formed by striatal GABAergic terminals (Hanson & Smith, 1999). Finally, in the substantia nigra, differences were seen between mGluR1a and mGluR5, where mGluR1a was found mainly on the plasma membrane of dendrites, while over 80% of mGluR5 immunoreactivity was found intracellularly (Hubert et al., 2001). So, based on these studies, and others, it is evident that the pattern of subsynaptic localization of group I mGluRs is nuclei and cellular specific in the brain, which could be a substrate for functional differences of mGluR1 and mGluR5 in different brain regions. These findings also provide a strong rationale to examine the localization and plasticity of these receptors in the nucleus accumbens.

1.3.7 Functional Colocalization of mGluR1 and mGluR5

The group I mGluRs have a high degree of colocalization at the cellular level. In the subthalamic nucleus, for example, almost all the cells examined displayed immunoreactivity for both mGluR1a and mGluR5 (Kuwajima et al., 2004). In the globus pallidus, the same held true with almost all the neurons being examined containing labeling for both of the group I mGluRs (Poisik et al., 2003). Despite this high degree of colocalization and same second messenger systems, the function of the receptors varies and depends on the brain region in which they are found (for review see Valenti et al., 2002). Electrophysiological slice recordings, along with the specific agonists and antagonists for the group I mGluRs, have provided a great deal of information about how these receptors function in neurons. In the dorsal striatum, activation of mGluR5, but not mGluR1, potentiates NMDA-induced membrane depolarization (Pisani et al., 2001). In comparison, in the globus pallidus, activation of mGluR1 causes depolarization, while

mGluR5 potentiates the response by preventing desensitization of mGluR1 (Poisik et al., 2003). Finally, in the substantia nigra, mGluR1, not mGluR5, causes depolarization, while the opposite was found in the subthalamic nucleus (Awad et al., 2000; Marino et al., 2001). It is clear from these studies that both the localization and function of these receptors is nuclei specific and that complex interactions between the two receptors may play an important role in the physiological mechanisms by which these receptors regulate synaptic transmission in individual cell types of specific brain regions. Therefore, examining the general localization and degree of mGluR1 and mGluR5 co-localization in the nucleus accumbens will provide valuable insights into these receptors function. It will also provide an essential substrate to determine if their localization can change following drug administration.

1.3.8 Group I mGluRs Localization and Function in the Nucleus Accumbens

As discussed in the previous section, the localization and function of group I mGluRs vary depending on the brain region examined. Therefore, knowing the localization of mGluR1 and mGluR5 in the nucleus accumbens could provide new insight about these receptors. Prior to the work presented in this thesis, the group I mGluRs had been localized in the nucleus accumbens only at the cellular level. Initially, using in situ hybridization in rats, mGluR1 and mGluR5 mRNA were found throughout the striatum, with mGluR5 being more prominent in both the dorsal and ventral striatum (Fotuhi et al., 1993; Testa et al., 1994). Further double in situ hybridization studies showed that mGluR5 mRNA is present in medium-spiny neurons that express enkephalin or substance P in the rat striatum, (Testa et al., 1995). Using retrograde tract-tracing in combination with in situ hybridization, some authors found that over 80% of the neurons projecting to

the ventral pallidum from the accumbens contain mGluR5, while only 50% of the neurons projecting from the accumbens to the VTA contain mGluR5 (Lu et al., 1999).

Upon the development of specific antibodies to mGluR1a and mGluR5, the next set of studies was able to observe these receptors using immunocytochemistry at the light microscopic level. Using rat brain, it was found that the protein for both of these receptors is found throughout the brain including the dorsal striatum and nucleus accumbens (Martin et al., 1992; Romano et al., 1995). The first objective of studies presented in this thesis was to extend these findings to the ultrastructural level using specific antibodies against mGluR1a and mGluR5 and high resolution electron microscopy.

To date, very few electrophysiological studies have studied the function of group I mGluRs in the nucleus accumbens. The first study showing functional mGluRs in the accumbens was done by Manzoni and colleagues (1997). Using whole cell patch clamp recordings in slices of rat brain containing the accumbens, it was shown that application of the group I mGluR agonist, DHPG, inhibited the postsynaptic afterhyperpolarization current (Manzoni et al., 1997). This study was completed prior to the development of specific antagonists for mGluR1 and mGluR5; therefore, it was not determined which member of the group I mGluRs was responsible for these effects.

It has also been shown that the group I mGluRs play a role in plasticity in the nucleus accumbens. Activation of postsynaptic mGluR5 at cortical-accumbal synapses is needed for endocannabinoid-mediated long term depression (LTD) in the nucleus accumbens of mice (Robbe et al., 2002; Fourgeard et al., 2004). It is hypothesized that this occurs through a negative feedback loop; when glutamate is released from a cortical

terminal, postsynaptic mGluR5 is activated, which increases intracellular calcium stores. This, in turn, causes the release of endocannabinoids from accumbens neurons which activate presynaptic CB1 receptors, reduce glutamatergic transmission, thereby causes LTD (Robbe et al., 2002; Fourgeard et al., 2004). Another study examining long term potentiation (LTP) in the nucleus accumbens found that stimulation of cortical-accumbens afferents in mouse brain slices induces LTP that requires dopamine D1 receptors and group I mGluRs (Schotanus & Chergui, 2008a). Specific antagonists for either mGluR1 (LY367385) or mGluR5 (MPEP) inhibited the induction of LTP in these slices (Schotanus & Chergui, 2008a). It is postulated that the receptor interactions the group I mGluRs have with NMDA receptors (which are necessary for the induction of LTP in the accumbens) and with intracellular calcium concentrations are factors driving these results (Kombian & Malenka, 1994; Schotanus & Chergui, 2008a; Schotanus & Chergui, 2008b). Although a basic understanding of the physiology of these receptors in the nucleus accumbens has yet to be achieved, these studies provide some insights into the level of complexity of group I mGluRs-mediated regulation of synaptic plasticity in the accumbens. Further studies are obviously needed, including in vivo recording experiments (in various species) that distinguish between the shell and core, as well as studies of physiological effects of other glutamatergic pathways on accumbal neurons

1.3.9 Group I mGluRs Role in Glia in the Nucleus Accumbens

In relation to glia and astrocytes in mouse accumbal slice preparations, it was shown that activation of mGluR5 causes calcium oscillations in astrocytes. This in turn, causes glutamate release from glial stores which activates NMDA receptors on medium spiny neurons (D'Ascenzo et al., 2007). This study provided evidence for two different

mechanisms of medium spiny neuron activation in the accumbens; i.e. direct synaptic release of glutamate or indirect activation of mGluR5 by glial glutamate release. These results were rather surprising, considering that we did not find significant glial labeling for mGluR1a or mGluR5 in either the monkey or rat (see Chapter 2, Figure 2.2 and 2.3). However, there could be species differences in the amount of glia present in the accumbens between mouse, rat and monkey to account for the lack of group I mGluRs in glia in our study. Another point to consider is the age of the animals used in this study, which was anywhere from 2-6 weeks of age. A study examining age related differences in group I mGluR expression in the SNr showed that mGluR5-containing glial processes were present in young rats (P14-P18) but absent in adult rats that were at least 2 months old (Hubert & Smith, 2004). Therefore, caution should be taken when extrapolating the functions of the group I mGluRs in glia (D'Ascenzo et al., 2007) to other species and age groups.

1.3.10 Group I mGluRs and Cocaine

Various studies have examined the role the group I mGluRs in psychostimulant abuse. Studies looking at mGluR5 knockout mice showed that these animals have reduced locomotor responsiveness to cocaine and do not self administer the drug either (Chiamulera et al., 2001). This lack of responsiveness to cocaine was seen despite increases in dopamine in the nucleus accumbens (Chiamulera et al., 2001). In line with these observations, systemic administration of mGluR5 antagonist, MPEP, reduces cocaine self administration in both rats and monkeys and attenuates the rewarding effects of cocaine in mice using the conditioned place preference paradigm (McGeehan and Olive, 2003; Kenny et al., 2005; Lee et al., 2005). On the other hand, pretreatment with

mGluR1 antagonist, EMQMCM, reduces behavioral sensitization to chronic cocaine administration in rats (Dravolina et al., 2006).

In addition, studies have examined the protein, mRNA and function of the group I mGluRs following chronic and acute cocaine exposure. A single injection of cocaine in mice induces alterations in the functions of mGluR5 (Fourgeard et al., 2004). Twenty-four hours post injection caused a reduction in endocannabinoid mGluR5-mediated retrograde LTD and decreases the surface expression of mGluR5 by half in the accumbens, indicating internalization of these receptors (Fourgeard et al., 2004). Chronic cocaine administration for seven days, which can cause behavioral sensitization (an increase responsiveness to cocaine, as compared to tolerance to a drug), has been shown to increase mRNA levels of mGluR5 (Ghasemzadeh et al., 1999) but decreases in mGluR5 protein (Swanson et al., 2001). Overall, these studies indicate that the group I mGluRs play a role in the neural adaptations (especially in the accumbens) that occur following acute and chronic cocaine exposure. However, further studies are needed to understand the mechanisms in which this occurs. In Chapter 4, we present the results of an extensive analysis of the cellular, subcellular and subsynaptic localization of group I mGluRs following acute and chronic cocaine treatments in rats.

1.4 Summary of Research

In order to gain a better understanding of the localization of the group I mGluRs in the nucleus accumbens and how this may relate to their function, my thesis addresses the three following specific aims.

1.4.1 Specific Aim 1: To compare the subcellular and subsynaptic localization of mGluR1a and mGluR5 in the shell and core of the nucleus accumbens in normal rats and monkeys.

Although known in other brain regions, the subcellular and subsynaptic localization of group I mGluRs in the core and shell of the nucleus accumbens has not been investigated at the electron microscopic level. Using both immunoperoxidase and immunogold immunocytochemistry techniques at the electron microscope, we were able to determine and compare the group I mGluRs' localization in the core and shell of the accumbens in both rats and monkeys. Knowing the subcellular and subsynaptic localization of mGluR1a and mGluR5 in the accumbens, their degree of colocalization, and the phenotype of the neurons they are associated with will provide a foundation to understand their function, physiological properties, and mechanisms of activation. The detailed results for this aim are presented in Chapter 2.

1.4.2 Specific Aim 2: To analyze the subcellular and subsynaptic localization of mGluR1a and mGluR5 in neurons contacted by glutamatergic afferents from the prefrontal cortex and midline thalamus.

Given the fact that the group I mGluRs are mainly postsynaptic on dendrites and spines, we next wanted to get an idea about the sources of terminals that formed synapse on these mGluR1a- or mGluR5-immunoreactive dendrites and spines. As discussed in Section 1.2.2, there are numerous glutamatergic projections to the accumbens, including the PFC and thalamus. Therefore, we wanted to determine the localization of mGluR1a and mGluR5 in relation to these terminals in order to gain a better understanding of how

these receptors may modulate glutamatergic transmission from these two nuclei. We divided Specific Aim 2 into the following two parts:

Aim 2A: To identify the proportion of PFC and thalamic terminals in contact with mGluR1a- or mGluR5-labeled spines in the core and shell of the nucleus accumbens.

Using a double immunoperoxidase immunocytochemistry method at the electron microscopic level, we determined the proportion of anterogradely labeled terminals arising from the PFC, PVN, or CM/IMD that made axo-spinous or axo-dendritic synapses with mGluR1a- or mGluR5-immunoreactive elements.

Aim 2B: To analyze the pattern of subsynaptic localization of mGluR1a and mGluR5 in elements contacted by glutamatergic afferents from the PFC and thalamus.

For this part of Aim 2, double labeling using immunoperoxidase and immunogold immunocytochemistry for the electron microscope was employed to determine the subsynaptic localization of the group I mGluRs in relation to positively labeled terminals from the PFC, PVN and CM/IMD. Since results in Specific Aim 1 indicated that the group I mGluRs are mainly extrasynaptic on the plasma membrane of both dendrites and spines, we wanted to compare the amount of perisynaptic labeling and the distance of extrasynaptic labeling (from the postsynaptic density) between mGluR1a and mGluR5 in relation to afferents from the PFC and thalamus.

The results of this aim provide insight into how the group I mGluRs may modulate glutamate transmission from these specific afferents. In addition, we will gain a better understanding of the relationship of peri- and extrasynaptic mGluR1a and mGluR5 immunoreactivity at specific axospinous synapses in the accumbens,

information that has not be examined in detail. The detailed results of Specific Aim 2 are presented in Chapter 3.

1.4.3 Specific Aim 3: To examine the subcellular and subsynaptic localization of mGluR1a and mGluR5 in the shell and core of the nucleus accumbens following acute or chronic cocaine treatment in rats.

In the previous aims, we addressed the localization of the group I mGluRs in normal, untreated animals. We next wanted to determine whether various cocaine treatments could be used to change the localization of these receptors. Based on the fact that GPCRs change localization in response to changes in neurotransmitter levels as well as agonist and lack of agonist stimulation (see Section 1.3.4 above), we formulated the hypothesis presented in figure 1.3 below. In addition to gaining a better understanding of the plasticity of group I mGluRs in an in vivo system, we wanted to determine whether changes in localization in the accumbens could be one of the underlying factors in the neuroadaptations previous studies have shown for both mGluR1a and mGluR5 (see Section 1.3.8 above). In short, three groups of rats were used: Saline-treated animals used as controls, animals that received an acute injection of cocaine (sacrificed at 45 minutes or 24 hours post-injection) and animals treated chronically with cocaine (using the behavioral sensitization paradigm described in Kalivas et al., 1988) followed by three weeks withdrawal. Using both immunoperoxidase and immunogold immunocytochemistry at the electron microscopic level, we compared the subcellular and subsynaptic localization of mGluR1a and mGluR5 in the core and shell of the accumbens in saline- versus cocaine-treated animals. The results of this aim are presented in Chapter 4.

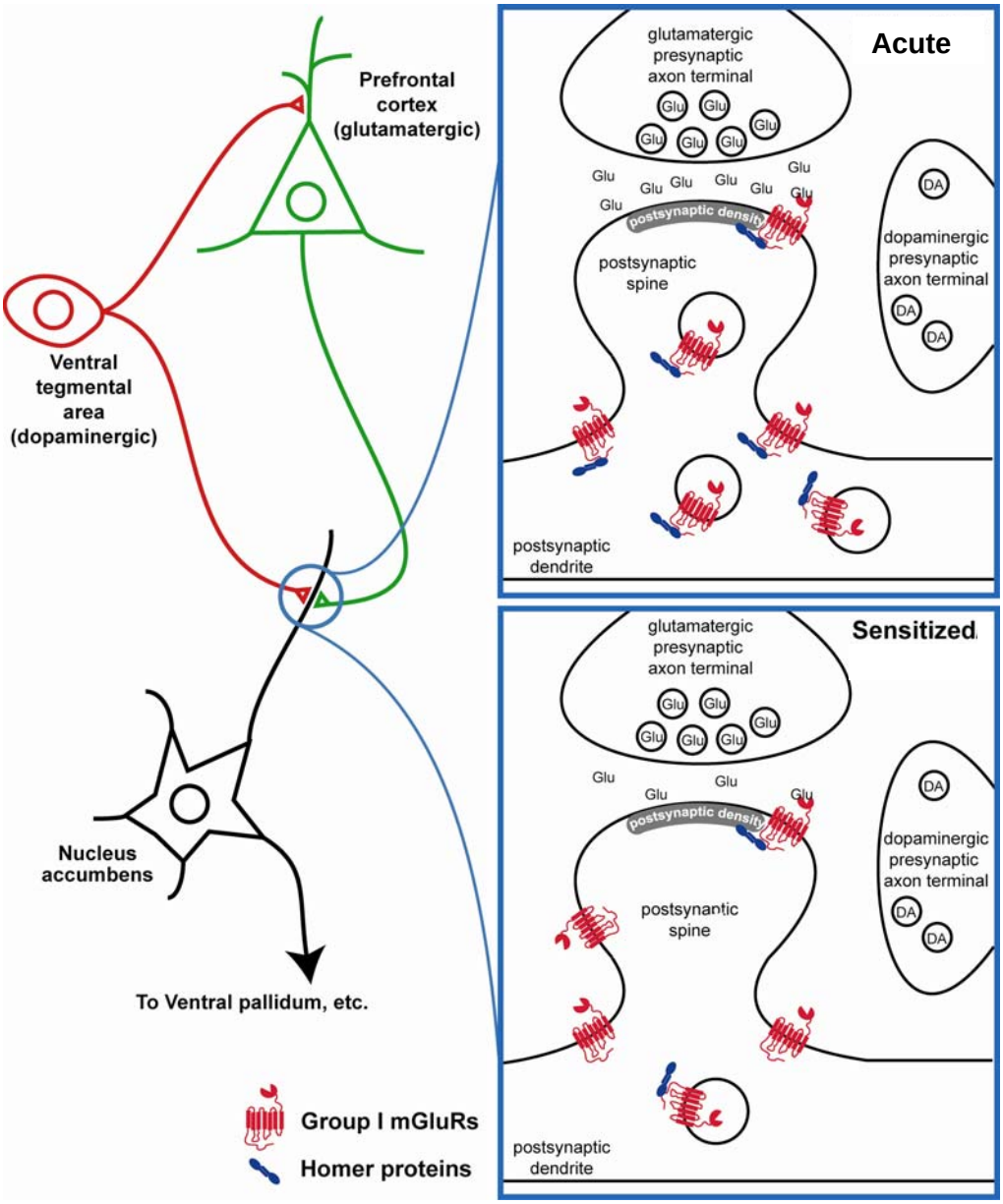

Figure 1.3: Model of hypothesized results for Specific Aim 3. When a single injection of cocaine is administered (Acute, top box), there is an increase in extracellular glutamate in the accumbens, which we hypothesize would internalize the group I mGluRs. Following chronic cocaine administration and behavioral sensitization (Sensitized, bottom box), there is a decrease in extracellular glutamate and Homer proteins in the accumbens, which would lead to an increase of group I mGluRs on the plasma membrane.

Chapter 2:

Specific Aim 1:

To compare the subcellular and subsynaptic localization of mGluR1a and mGluR5 in the shell and core of the nucleus accumbens in normal rats and monkeys.

Mitrano D.A. & Smith Y. (2007) Comparative analysis of the subcellular & subsynaptic localization of mGluR1a and mGluR5 metabotropic glutamate receptors in the shell and core of the nucleus accumbens in rat & monkey. The Journal of Comparative Neurology, 500:788-806.

2.1 Abstract

Group I metabotropic glutamate receptors (mGluRs) play critical roles in synaptic plasticity and drug addiction. To characterize potential sites whereby these receptors mediate their effects in the ventral striatum, we studied the subcellular and subsynaptic localization of mGluR1a and mGluR5 in the shell and core of the nucleus accumbens in rat and monkey. In both species, group I mGluRs are mainly postsynaptic in dendrites and spines, with rare presynaptic labeling in unmyelinated axons. Minor, yet significant, differences in proportions of specific immunoreactive elements were found between the accumbens shell and core in monkey. At the subsynaptic level, significant differences were found in the proportion of plasma membrane-bound mGluR5 labeling between species. In dendrites, spines and unmyelinated axons, a significantly larger proportion of mGluR5 labeling was bound to the plasma membrane in rats (50-70%) than monkeys (30-50%). Conversely, mGluR1a displayed the same pattern of immunogold labeling in the two species. Electron microscopic colocalization studies revealed 30% colocalization of mGluR1a and mGluR5 in dendrites and as much as 50-65% in spines in both compartments of the rat accumbens. Both group I mGluRs were significantly expressed in D1-immunoreactive dendritic processes (60-75% colocalization) and spines (30-50%) of striatal projection neurons as well as dendrites of cholinergic (30-70%) and parvalbumin (70-85%)-containing interneurons. These findings highlight the widespread expression of group I mGluRs in projection neurons and interneurons of the shell and core of the nucleus accumbens, providing a solid foundation for regulatory and therapeutic functions of group I mGluRs in reward-related behaviors and drug addiction.

2.2 Introduction

Metabotropic glutamate receptors (mGluRs) are divided into three classes based on pharmacological and structural properties. Group I mGluRs (mGluR1 and 5) are coupled to Gq and activate phospholipase C, increasing intracellular calcium and activating protein kinase C, while Group II (mGluR2 and 3) and Group III (mGluRs 4, 6, 7, and 8) mGluRs are coupled to Gi and inhibit cAMP formation (for review see (Conn and Pin, 1997).

The nucleus accumbens is divided into two main compartments, the shell and the core, based on differential neural connections, neurochemical content (Zaborszky et al., 1985; Meredith et al., 1989; Zahm and Brog, 1992; Meredith et al., 1996) and responses to drug administration (Zahm, 2000). Group I mGluRs have been localized throughout the basal ganglia, including the nucleus accumbens, using in situ hybridization (Testa et al., 1994) and light microscopic immunocytochemistry (Martin et al., 1992). Despite their common signaling pathways, electrophysiological studies have shown that mGluR1 and mGluR5 have specific functions in different basal ganglia nuclei (Pisani et al., 2001; Valenti et al., 2002; Poisik et al., 2003). Similarly, the two receptors seem to play different roles in mediating the behavioral effects of psychostimulants in the rat nucleus accumbens (Ghasemzadeh et al., 1999; Chiamulera et al., 2001; Swanson et al., 2001; Szumlinski et al., 2004).

Initial studies addressing the localization of group I mGluRs showed these receptors to be mainly perisynaptic to asymmetric synapses in the hippocampus and cerebellum (Baude et al., 1993; Lujan et al., 1996). Although this perisynaptic labeling appears to be a general pattern throughout the CNS, recent studies of various basal

ganglia nuclei have demonstrated differential and specific patterns of subcellular and subsynaptic localization for the two group I mGluRs in different structures (Hanson and Smith, 1999; Hubert et al., 2001; Paquet and Smith, 2003; Kuwajima et al., 2004). In addition, electrophysiological data showed that mGluR1 and mGluR5 play different roles whenever they co-exist in basal ganglia neurons (Valenti et al., 2002). Therefore, the localization and function of mGluR1a and mGluR5 seem to be highly specific and dependent on the neuronal population and/or brain regions they are expressed in.

The rat striatum is comprised mainly of medium spiny projection neurons (Kemp and Powell, 1971; Somogyi et al., 1979; Wilson and Groves, 1980), and about 4-5% aspiny interneurons (Bolam et al., 1981). Various neurochemical markers have been used to distinguish the different types of striatal neurons (Emson et al., 1993; Hersch et al., 1995; Lei et al., 2004). The phenotype of mGluR1a- and mGluR5-containing neurons in the dorsal striatum has been characterized at the cellular level (Tallaksen-Greene et al., 1998), but a detailed analysis of the cellular and subcellular localization of these receptors in the shell and core of the nucleus accumbens has not yet been achieved.

Therefore, in order to gain a deeper understanding of the potential sites whereby group I mGluRs mediate their effects in the nucleus accumbens, we undertook a detailed analysis of the cellular, subcellular and subsynaptic localization of mGluR1a and mGluR5 in the shell and core of the nucleus accumbens in rats and monkeys using high resolution electron microscopic immunocytochemistry.

Some of the data shown in this paper have been previously presented in abstract form (Mitrano et al., 2003; Mitrano and Smith, 2004; Mitrano and Smith, 2005).

2.3 Materials & Methods

2.3.1 Animals and tissue preparation

Fourteen male Sprague Dawley rats and six adult Rhesus monkeys were used for this study. All procedures were approved by the animal care and use committee of Emory University and conform to the U.S. National Institutes of Health guidelines. All animals were deeply anesthetized with pentobarbital (100mg/kg i.v.) for monkeys or a cocktail of ketamine (60-100mg/kg, i.p.) and dormitor (0.1mg/kg, i.p.) for rats. The animals were then transcardially perfused with cold oxygenated Ringer's solution followed by a fixative containing 4% paraformaldehyde and 0.1% glutaraldehyde in phosphate buffer (0.1M; pH 7.4). Following perfusions, brains were removed from the skull, post-fixed in 4% paraformaldehyde for 2 – 24 hours, cut into 60-µm-thick sections using a vibrating microtome and stored in PBS at 4˚C until processed for immunocytochemistry. Prior to immunocytochemical processing, all sections were put into a 1% sodium borohydride solution for 20 minutes and then washed with PBS.

2.3.2 Primary Antibodies

A commercially available monoclonal antibody against calbindin-D_{28k} (Sigma, St. Louis, MO; Cat# C-9848, Lot# 082k4879) was used at a concentration of 1:5000 to distinguish between the accumbens shell and core. The calbindin-D_{28k} antibody is derived from CB-955 hybridomas produced by fusion of mouse myeloma cells and splenocytes from BALB/c mice that were immunized with purified bovine kidney calbindin-D_{28k}. The specificity of this antibody has been demonstrated through preadsorption immunohistochemical assays which abolish calbindin labeling (Celio, 1990), through Western blot analysis of rat brain tissue which shows a distinct band at

28kD (Miyata et al., 2000) and through immunohistochemistry which shows calbindin immunostaining in brain regions known to express a significant level of calbindin-D_{28k} mRNA (Winsky et al., 1989; Celio et al., 1990; Miyata et al., 2000).

To localize mGluR1a, an affinity-purified rabbit polyclonal antibody against the C-terminus of rat mGluR1a (PNVTYASVILRDYKQSSSTL) conjugated to KLH with glutaraldehyde was used at a concentration of 1:1000 (Chemicon, Temecula, CA; Cat# AB1551, Lot# 21100471). In Western blot analysis by the manufacturer, this antibody labels a single band of ~140kD. Previous studies from our lab and others have used a combination of knock-out mice, transfected HEK-293 cells, and preadsorption to determine the specificity of this mGluR1a antiserum. These studies showed that brain tissue from mGluR1a knockout mice did not display any specific mGluR1a labeling compared to wild-type. In addition, immunoblotting of cells transfected with mGluR1a, but not mGluR5, labeled a band of 140kD (Kuwajima et al., 2004). Preadsorption studies in rat retina cells abolished mGluR1a labeling (Koulen et al., 1997).

An affinity-purified synthetic rabbit polyclonal antibody against the C-terminus of mGluR5 with a lysine added to the N-terminus (KSSPKYDTLIIRDYTNSSSSL) in a concentration of 1:5000 (Upstate Biotechnology, Lake Placid, NY; Cat# 06-451, Lot# 27884) was used to label mGluR5. According to the manufacturer's immunoblot analysis, the mGluR5 antibody labels a band of ~130kD. Specificity of the mGluR5 antibody has been shown in previous studies using knockout mice, transfected cells and homogenates of rat brain. These studies showed that brain tissue from mGluR5 knockout mice do not stain for mGluR5; HEK-293 cells transfected with mGluR5 label a band of the correct molecular weight (Kuwajima et al., 2004); and immunoblot analysis on

proteins isolated from various brain regions labels a band that corresponds to the size of mGluR5 in regions known to express mGluR5 protein and mRNA (Marino et al., 2001).

Three antibodies were used as specific markers of different populations of striatal neurons containing either mGluR1a or mGluR5. First, to label a subset of medium spiny striatofugal neurons, a monoclonal antibody against the D1 receptor was used at a concentration of 1:250 (a kind gift of Dr. Allan Levey, Emory University). This antibody is raised against the human dopamine receptor gene $D1_C$ at the 97 amino acid C-terminus, fused to GST fusion proteins and then derived from spleen cells of rats that contained the hybridoma cell line reactive with D1 (Levey et al., 1993; Hersch et al., 1995). The specificity of the D1 antibody has been demonstrated through the use of cloned D1 receptors in transfected COS-7 cells with Western blots showing reactivity for only the D1 receptor with corresponding bands at 40-45 kDa and 65-75 kDa (Levey et al., 1993; Hersch et al., 1995). The specificity was also demonstrated in isolated rat membrane from the striatum which showed a band at 65-75 kDa, similar mobility to the cloned receptor (Hersch et al., 1995). Two antibodies were used to distinguish interneurons. For GABAergic interneurons, a monoclonal antibody against the calcium-binding protein parvalbumin (PV) was used at a concentration of 1:5000 (Swant, Bellinzona, Switzerland, Cat# 235, Lot# 10-11(F)). This antibody, produced by hybridization of mouse myeloma cells with spleen cells from mice immunized with PV purified from carp muscles, labels a 12 kDa band in immunoblots (Manufacturer's information). The specificity of the PV antibody was demonstrated through the use of immunoblots and radioimmunoassay (RIA) (Celio, 1986; Celio et al., 1988). RIA showed inhibition of binding of immunoreactive PV by unlabelled rat-muscle PV with no cross-reaction to

other calcium-binding proteins (Celio et al., 1988). Nitrocellulose paper treated with numerous amino acids and calcium binding proteins showed that the PV antibody only reacts with PV and immunoreactivity; displaced by a PV conjugate (Celio, 1986). Finally to label cholinergic interneurons, a monoclonal antibody against choline acetyltransferase (ChAT) was used at a concentration of 1:50 (Calbiochem, Darmstadt, Germany, Cat# NB05L, Lot#D32295). The ChAT antibody is raised against the entire bovine enzyme and is obtained from rat spleen cells. For testing the specificity of the ChAT antibody, purified bovine ChAT was isolated using SDS-PAGE and blotted on nitrocellulose paper (Levey et al., 1983). Western blot analysis revealed that the ChAT antibody only labels bands at 68kDa and 70kDa consistent with other studies of the ChAT protein. Immunohistochemical labeling of brain tissue showed ChAT-positive neurons in areas known to be cholinergic (Levey et al., 1983).

2.3.3 Single Immunoperoxidase labeling for light microscopy

Following sodium borohydride treatment, sections were incubated for 1 hour at RT in PBS containing 10% normal goat serum (NGS) for group I mGluRs, or normal horse serum (NHS) for calbindin-D_{28k}, 1% BSA, and 0.3% Triton X-100, followed by the primary antibody solution containing 1% NGS or NHS, 1% BSA, and 0.3% Triton X-100 in PBS for 24 hours at RT. After three rinses in PBS, sections were incubated in secondary biotinylated goat anti-rabbit or horse anti-mouse IgGs at a concentration of 1:200 (Vector Laboratories, Burlingame, CA) for 90 minutes. The sections were rinsed again in PBS and then incubated another 90 minutes with the avidin-biotin peroxidase complex (ABC) at a dilution of 1:100 (Vector Laboratories). Finally, the sections were washed in PBS and Tris buffer (50mM; pH 7.6) and transferred to a solution containing

0.025% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma, St Louis, MO), 10mM imidazole, and 0.005% hydrogen peroxide in Tris buffer for 10 minutes. Sections were rinsed in PBS, mounted onto gelatin-coated slides, dehydrated and then coverslipped with Permount. Tissue was examined with a Leica DMRB microscope (Leica Microsystems, Inc., Bannockburn, IL) and images were taken using a CCD camera (Leica DC500) which was controlled by Leica IM50 software.

2.3.4 Single Preembedding Immunoperoxidase labeling for electron microscopy

Following sodium borohydride treatment, sections were placed in a cryoprotectant solution for 20 minutes (PB 0.05M, pH 7.4, 25% sucrose, and 10% glycerol), frozen at -80°C for 20 minutes, returned to a decreasing gradient of cryoprotectant solutions, and rinsed in PBS. Sections were then incubated in primary and secondary antibody solutions, identical to those used for light microscopy; with two exceptions: 1) the omission of Triton X-100 and 2) incubation in primary antibody for 48 hours at 4°C.

After the DAB reaction, the tissue was rinsed in PB (0.1M, pH 7.4) and treated with 1% OsO_4 for 20 minutes. It was then returned to PB and dehydrated with increasing concentrations of ethanol. When exposed to 70% ETOH, 1% uranyl acetate was added to the solution for 35 minutes to increase the contrast of the tissue at the electron microscope. Following dehydration, sections were treated with propylene oxide and embedded in epoxy resin for 12 hours (Durcupan ACM, Fluka, Buchs, Switzerland), mounted onto slides and placed in a 60°C oven for 48 hours. Separate samples of the nucleus accumbens core and shell were cut out of the larger sections, mounted onto resin blocks and cut into 60-nm sections using an ultramicrotome (Leica Ultracut T2). The 60-nm sections were collected on Pioloform-coated copper grids, stained with lead citrate for

5 minutes to enhance tissue contrast and examined on the Zeiss EM-10C electron microscope. Electron micrographs were taken and saved with a CCD camera (DualView 300W; Gatan, Inc., Pleasanton, CA) controlled by DigitalMicrograph software (version 3.10.1, Gatan, Inc.). Some of the digitally acquired electron micrographs were adjusted only for brightness or contrast using either the DigitalMicrograph software or Adobe Photoshop software (version 8.0, Adobe Systems Inc.). Micrographs were then compiled into figures using Adobe Illustrator (version 11.0, Adobe Systems Inc.).

2.3.5 Single Preembedding Immunogold labeling for electron microscopy

Following sodium borohydride and cryoprotectant treatments, sections were incubated for 30 minutes in PBS containing 5% dry milk at RT and then rinsed in TBS-gelatin buffer. Sections were then transferred to primary antibody solutions with 1% dry milk in TBS-gelatin buffer for 24 hours at room temperature and then rinsed again in TBS-gelatin. After rinses, sections were treated for 2 hours at RT with secondary goat anti-rabbit IgGs conjugated with 1.4nm gold particles at a concentration of 1:100 (Nanoprobes, Yaphank, NY) diluted with 1% dry milk in TBS-gelatin. Sections were rinsed in TBS-gelatin and 2% sodium acetate buffer before gold particles were silver intensified to 30-50nm (upon electron microscopic examination) using the HQ silver kit (Nanoprobes) for approximately 10 minutes. The sections were then treated according to the same protocol of osmification, dehydration, embedding, and tissue selection as used for the tissue processed according to the preembedding immunoperoxidase procedure including the following changes: 1) the tissue was kept in 0.5% OsO_4 for 10 minutes instead of 20 and 2) the tissue was stained with 1% uranyl acetate for 10 minutes instead of 35.

2.3.6 Double Preembedding Immunoperoxidase labeling for electron microscopy colocalization

The tissue was treated using the same methods as for the single electron microscopic immunoperoxidase labeling, but antibodies for mGluR1a and mGluR5 were pooled for the 48 hours incubation. The concentrations of secondary antibodies, ABC and DAB as well as the embedding procedures were the same as those used for the single immunoperoxidase labeling.

2.3.7 Double Preembedding Immunoperoxidase and Immunogold labeling for electron microscopy colocalization of mGluR1a or mGluR5 with Neuronal Markers

Following sodium borohydride and cryoprotectant treatments, sections were incubated for 30 minutes in PBS containing 5% dry milk at RT and then rinsed in TBS-gelatin buffer. Sections were then transferred to primary antibody solutions that contained a mixture of the primary antibody for either receptor subtype and one of the neuronal markers (D1, PV or ChAT antibody) with 1% dry milk in TBS-gelatin buffer for 24 hours at room temperature and then rinsed again in TBS-gelatin. After rinses, sections were treated for 2 hours at RT with a mixture of secondary goat anti-rabbit IgGs for mGluR1a or mGluR5 conjugated with 1.4nm gold particles at a concentration of 1:100 (Nanoprobes, Yaphank, NY) and either horse anti-mouse (PV) or goat anti-rat (D1 or ChAT) biotinylated IgGs at a concentration of 1:200 (Vector Laboratories, Burlingame, CA) with 1% dry milk in TBS-gelatin. Sections were rinsed in TBS-gelatin and 2% sodium acetate buffer before gold particles were silver intensified to 30-50nm (upon electron microscopic examination) using the HQ silver kit (Nanoprobes) for approximately 10 minutes. Following silver intensification, the ABC and DAB

procedures were the same as those used in single immunoperoxidase labeling for light microscopy. Immediately following the DAB reaction, the sections were rinsed with PB (0.1M, pH 7.4) and subjected to the same osmification and dehydration protocol described in the single preembedding immunogold procedure. In additional experiments, the secondary antibodies for the interneuron markers were reversed so that the markers were revealed with gold-conjugated IgGs and the receptors were revealed with biotinylated IgGs to ensure the reliability of the double labeling procedure.

2.3.8 Analysis of Material

2.3.8.1 Antibody Penetration in Tissue

To make sure the EM analysis was performed in tissue with optimal antibody penetration, we examined the degree of penetration of mGluR1a and mGluR5 antibodies in 60μm-thick accumbens sections. To do so, blocks of tissue were glued on the top of resin blocks in the vertical plane and 60nm ultrathin sections perpendicular to the surface of the tissue were collected on pioloform grids and examined on the electron microscope. It was found that mGluR1a immunoreactivity reached, on average, 10.7μm depth of penetration, with a range of 7.3-13.8 μm, while mGluR5 labeling was found on average 13.0μm deep in tissue, with a range of 10.9-14.4 μm. The means of penetration depth for the two antibodies were not significantly different using a t-test ($t=1.31$, $p=0.24$). Therefore, quantitative data presented in this study were collected from the first 5-10μm of tissue sections where both mGluR1a and mGluR5 have optimal access to antigenic sites.

2.3.8.2 Single immunoperoxidase labeling for group I mGluRs

Data for single immunoperoxidase labeling were collected from a total of 28 blocks of tissue, 1 block/animal in the medial shell (referred to as shell in the following sections) and 1 block/animal in core immunostained for either mGluR1a and mGluR5 in 3 monkeys and 4 or 5 rats. Serial ultrathin sections taken from each of the blocks were examined and 50-100 electron micrographs of randomly selected immunoreactive elements were digitized at 25,000X. This resulted in a total surface of 4,810 μm^2 of accumbens tissue to be examined for mGluR1a in the monkey, 5,344μm^2 for mGluR1a in the rat, 7,271μm^2 for mGluR5 in the monkey and 4,275μm^2 for mGluR5 in the rat. Labeled elements were categorized as dendrites, spines, unmyelinated axons, myelinated axons, and axon terminals on the basis of ultrastructural features described by (Peters et al., 1991). The density of labeled elements for each receptor subtype was calculated in shell and core by dividing the number of elements labeled by the area of tissue examined. Significant differences were assessed using Sigma stat software for two-way ANOVAs and Tukey's post-hoc test. The density of labeled elements was compared across each neuronal element within each species, with the two factors being receptor subtype and brain region (shell vs. core). In addition, the density of labeled elements was compared between species within each receptor subtype using the same statistical test, with the two factors being animal type (monkey or rat) and brain region (shell vs. core). The same tissue was used to calculate the relative abundance of labeled elements (see results section for detail).

2.3.8.3 Single immunogold labeling for group I mGluRs

Immunogold labeling data were also collected from 28 blocks of mGluR1a- and mGluR5-immunostained medial shell and core tissue as described above. Serial ultrathin

sections from the surface of the blocks were collected and 50-100 electron micrographs of randomly selected immunoreactive elements were taken at 25,000X, for a total tissue surface area of 4,810μm^2 for mGluR1a in the monkey, 4,275μm^2 for mGluR1a in the rat, 6,412μm^2 for mGluR5 in the monkey and 4,275μm^2 for mGluR5 in the rat to be examined. Labeled elements were classified as described above (Peters et al., 1991). Gold particles were classified as either intracellular or plasma membrane-bound depending on their localization relative to the plasma membrane. To be categorized as plasma membrane-bound, gold particles had to be in contact with the membrane; all other particles were considered intracellular. The percent of total gold particles in the two groups were then calculated for each animal and the mean was calculated across the number of animals and presented as a bar histogram (see Fig. 2.5). Data were analyzed for significant differences, using Sigma Stat software, by two-way repeated measures ANOVAs and Tukey's post-hoc test. The percent of plasma membrane-bound gold particles was compared across each neuronal element within each species, with the two factors being receptor subtype and brain region (shell vs. core). In addition, the percent of plasma membrane-bound gold particles was compared between species within each receptor subtype using the same statistical test, with the two factors being animal type (monkey or rat) and brain region (shell vs. core). Plasma membrane-bound gold particles were further classified into three categories; perisynaptic (touching or within a 20nm range of the edges of postsynaptic specializations); synaptic (in contact with the main body of postsynaptic specializations); or extrasynaptic (on the plasma membrane but not associated with synapses).

2.3.8.4 Double immunoperoxidase labeling for group I mGluRs colocalization

Data for double immunoperoxidase labeling were taken from 26 blocks of tissue collected from 4 sets of accumbens sections immunostained for mGluR5, 5 sets of sections stained for mGluR1a, and 4 sets of sections stained for both receptor subtypes. The single labeled tissue used in these colocalization experiments was the same as used for single immunoperoxidase labeling studies. Twenty-five electron micrographs were taken at 12,500X from each block for a total surface area of 10,219μm^2 for mGluR1a alone, 8,175μm^2 for mGluR5 alone and 8,175μm^2 for double labeled tissue. Electron micrographs were purposefully not taken from serial sections to avoid sampling and counting the same labeled elements twice. In addition, we made sure to collect micrographs of labeled elements for the same receptors in different regions on the surface of adjacent ultrathin sections. The total number of labeled or unlabeled dendrites and spines was counted and the percentage of labeled elements was calculated. The mean proportion of labeled elements was calculated across animals for each receptor subtype and then for the pooled receptors. The estimated degree of colocalization was calculated as follows:

$\%\ mGluR1a_{labeled} + \%\ mGluR5_{labeled} - (\%\ mGluR1a + mGluR5)_{labeled} = \%$ of colocalization

2.3.8.5 Double Immunoperoxidase and Immunogold Labeling for Group I mGluRs and Neuronal Markers

Data for double immunoperoxidase and immunogold labeling were collected from 44 blocks of rat accumbens tissue stained for either mGluR1a or mGluR5 with D1, PV or ChAT as described above. Serial ultrathin sections were collected and 30-40 electron micrographs were taken at 25,000X, for a total tissue surface area of 3,726μm^2 for

mGluR1a and D1, 3,715μm^2 for mGluR1a and PV, 2,830μm^2 for mGluR1a and ChAT, 3,726μm^2 for mGluR5 and D1, 3,598μm^2 for mGluR5 and PV, and 2,469μm^2 for mGluR5 and ChAT.

In the double immunostained tissue for either the medium spiny neuron marker (D1) or the interneuron markers (PV and ChAT) with mGluR1a or mGluR5, electron micrographs of elements containing immunoperoxidase labeling (i.e. D1, PV or ChAT immunoreactivity) were taken from superficial areas that contained both reaction products and then the percentage of those that also contained immunogold labeling (i.e. mGluR1a or mGluR5 immunoreactivity) was calculated and averaged across 3 (ChAT-immunostained) or 4 (D1- or PV-immunostained) animals for both the core and shell. The distribution of gold particles in double-labeled elements was categorized as described in the single immunogold labeling procedure (see above). Statistical analysis was performed to assess differences amongst percentages of double labeled elements and the proportions of plasma membrane-bound labeling in medium spiny neurons and interneurons.

2.4 Results

2.4.1 Light Microscopic Observations

Monkey and rat tissue containing the nucleus accumbens was stained separately for calbindin D_{28k}, mGluR1a or mGluR5. As shown in Figure 2.1, the distribution of calbindin immunoreactivity clearly delineated the boundaries of the shell and core of the nucleus accumbens. These sections served as a guide for the selection of blocks of tissue in subsequent experiments. Both mGluR1a and mGluR5 labeling was homogeneously distributed in the neuropil of the shell and core of the accumbens, with very light labeling

in cell bodies (Fig, 2.1B, D). No obvious difference in the gross distribution of mGluR1a and mGluR5 immunoreactivity was found between the monkey and rat accumbens (Fig. 2.1).

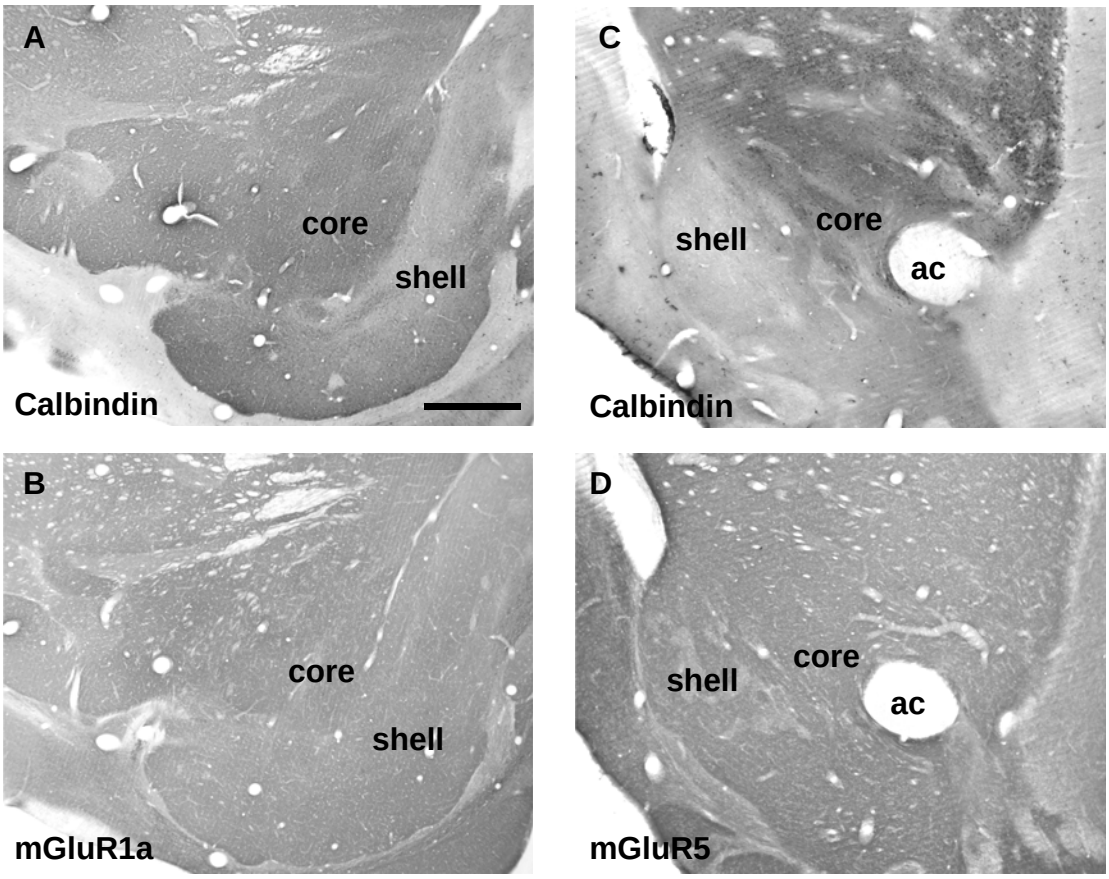

Figure 2.1: Calbindin and Group I mGluRs immunoreactivity in the Nucleus Accumbens. (A, C) Light micrographs of calbindin-D_{28k} immunoreactivity in the monkey (A) and rat (C) nucleus accumbens. Note the dense labeling in the nucleus accumbens core, while the shell exhibits a much lighter labeling. (B, D) Light micrographs of mGluR1a (B, monkey) and mGluR5 (D, rat) labeling in the nucleus accumbens. Both receptors show a heavy neuropil labeling, but very light neuronal cell body staining. Scale bar: 0.25mm.

2.4.2 Electron Microscopic Observations

2.4.2.1 Single Immunoperoxidase Labeling

To further characterize and compare the cellular and subcellular localization of mGluR1a and mGluR5 in shell and core the rat and monkey accumbens, we used immunoperoxidase labeling at the electron microscopic level. The bulk of immunoreactivity for the two receptor subtypes was found postsynaptically in dendrites and spines of both accumbens compartments in rats and monkeys (Figs. 2.2, 2.3). However, significant differences were found in the relative distribution of labeling between different neuronal elements in the same compartment or between the two compartments. First, immunoreactive dendrites (15-20/100 μm^2) for both receptor subtypes were significantly more abundant than labeled spines (5-8/100 μm^2) in both shell and core of monkeys (two-way ANOVA and Tukey's post-hoc tests, $n=3$, $p<0.001$). In addition, two-way ANOVA and Tukey's post-hoc tests revealed a significantly larger density of mGluR5-labeled spines in the core (6.5 ± 0.6/100 μm^2) than the shell (2.6 ± 0.9/100 μm^2; $n=3$, $p<0.01$). There was also a significantly larger density of labeled spines for mGluR5 than mGluR1a in the core (6.5 ± 0.6/100 μm^2 vs. 3.6 ± 0.5/100 μm^2, $n=3$, $p<0.05$). On the other hand, mGluR1a-immunoreactive unmyelinated axons were significantly more abundant than mGluR5-labeled axons in both shell (5.5 ± 1.7/100 μm^2 vs. 1.4 ± 0.6/100 μm^2, $n=3$; $p<0.01$) and core (5.9 ± 0.5/100 μm^2 vs. 2.0 ± 0.6/100 μm^2; $n=3$, $p<0.01$).

Because immunoreactive dendrites were 2-3 times more abundant than spines throughout the monkey accumbens, we assessed whether this significant difference in relative distribution of labeled elements indicates a genuine preferential expression of

mGluR1a or mGluR5 in dendrites over spines or merely reflects a random pattern of labeling consistent with the overall abundance of dendrites and spines in the neuropil composition of the shell and core of the monkey accumbens. To address this issue, we counted the total number of labeled and unlabeled dendrites and spines in the immunoperoxidase-stained material of three monkeys. These values were then converted into relative percentages of spines versus dendrites and compared with the proportion of mGluR1a- and mGluR5-labeled dendrites and spines in the monkey accumbens. Unmyelinated axons were not included in these counts because of the limited ultrastructural characteristics available to genuinely identify these elements in unlabeled tissue. Because there was no significant difference in proportion of spines vs. dendrites between mGluR1a- and mGluR5-immunostained tissue, data from both sets of sections were pooled. On average, dendrites accounted for 76% of postsynaptic elements in the shell (n=2259) while this proportion dropped to 67% in the core (n=2206). On the other hand, 24% and 33% of spines accounted for the total of postsynaptic elements in the shell (n=697) and core (n=1099), respectively. Comparative data between these percentages and those of labeled elements are shown in figure 2.3A'. In these diagrams, the relative percentages of labeled dendrites or spines in the shell or core of the accumbens were divided by the percentages of neuronal elements mentioned above; which led to the "relative abundance" values. Therefore, a relative abundance value of 1 indicates that the pattern of labeling is probabilistically random, while values under or above 1 suggest a preferential decreased or increased expression of labeling in these specific compartments. Our findings demonstrate that mGluR1a and mGluR5 are randomly expressed in dendrites, whereas the proportions of spines labeled for either receptor subtypes are lower

than what would be expected through random distribution in both compartments of the monkey accumbens (Fig. 2.3A').

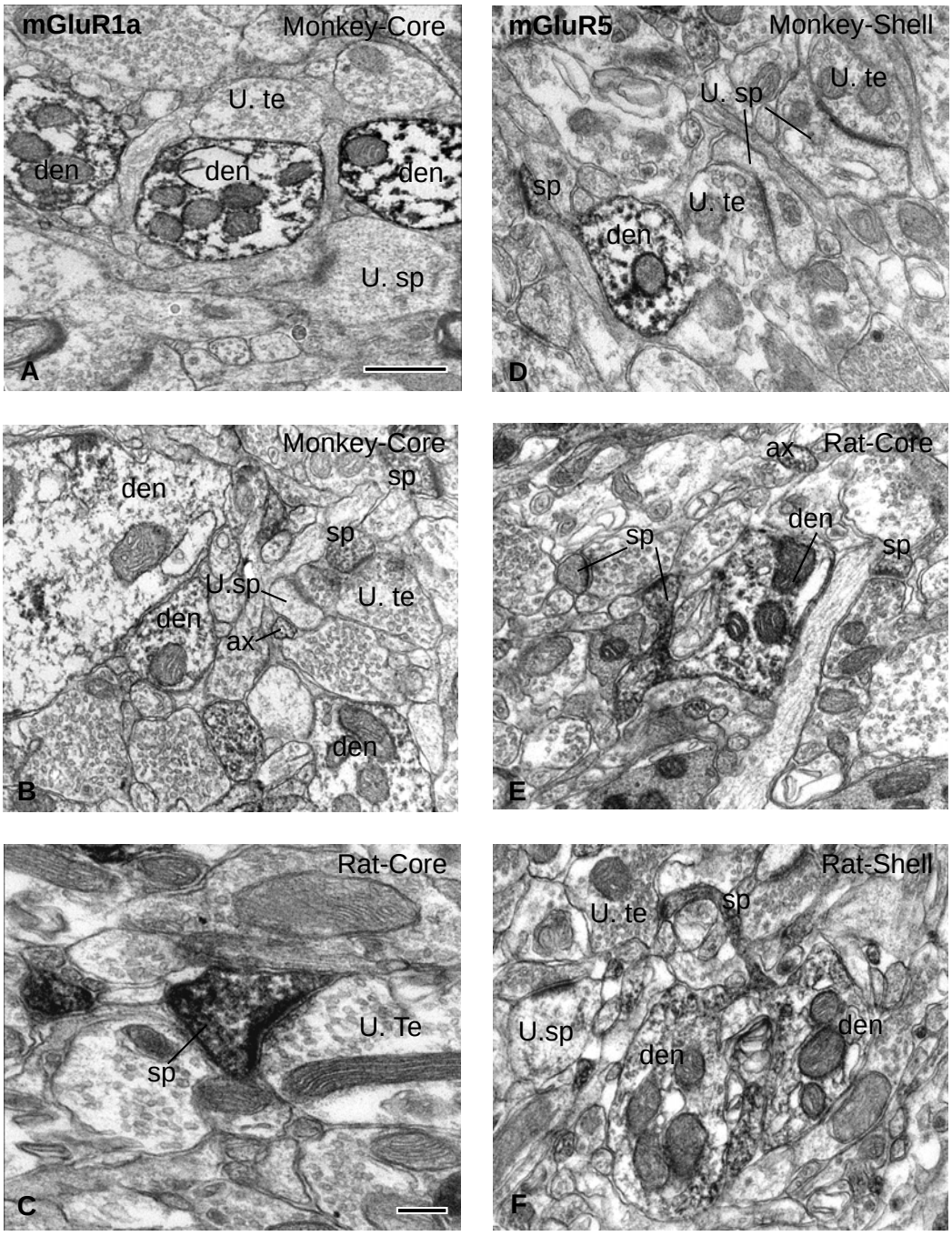

Figure 2.2: Immunoperoxidase labeling of Group I mGluRs in the monkey and rat nucleus accumbens. (A-C) mGluR1a-labeled elements in the nucleus accumbens core of monkey (A-B) and rat (C). Note that the majority of immunoreactive elements are dendrites (den) and spines (sp). Many unlabeled spines (U.sp) are seen as well. (D-F) mGluR5-labeled elements in the nucleus accumbens core of rat (E) and shell of monkey (D) and rat (F). Abbreviations: U. te = unlabeled terminal; ax = unmyelinated axon. Scale bars: 0.5μm.

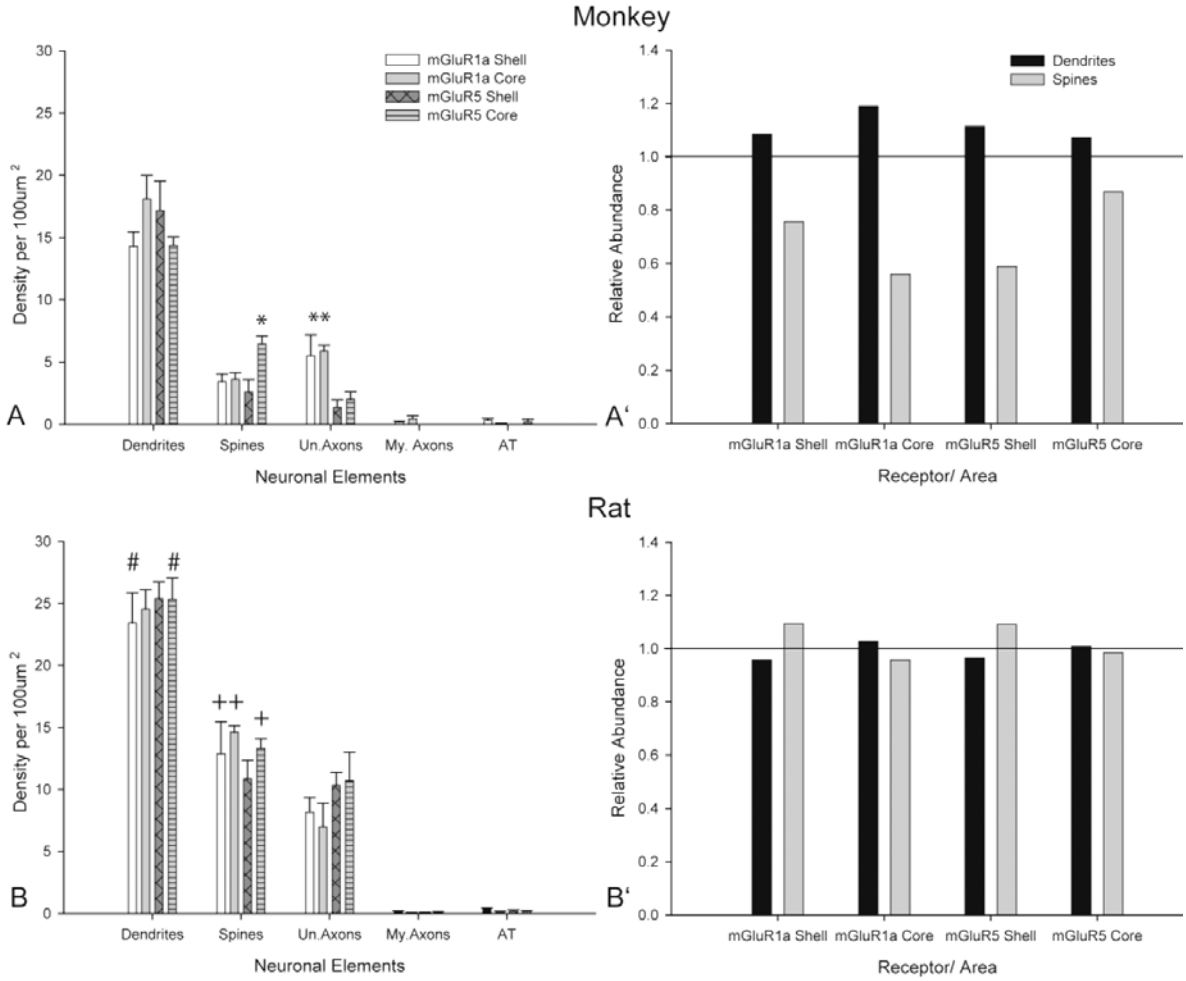

Figure 2.3: Histogram showing the distribution of Group I mGluRs immunoperoxidase labeling in the monkey (A) and rat (B) nucleus accumbens. (A) mGluR1a and mGluR5 immunoperoxidase labeling in the shell and core of the monkey nucleus accumbens. Data are expressed as mean density per 100 square microns (±SEM) of labeled elements from three monkeys. Single asterisks indicate that two-way ANOVA with Tukey's post-hoc revealed a higher density of mGluR5-labeled spines in the core than the shell ($p<0.01$) and of mGluR1a labeled spines in the core ($p<0.05$), while double asterisks indicate a higher density of mGluR1a-labeled unmyelinated axons in both the shell and core as compared to mGluR5-

labeled unmyelinated axons ($p<0.01$). Total number of labeled elements: 562-mGluR1a shell; 691-mGluR1a core; 722-mGluR5 shell; 887-mGluR5 core. (A') Relative abundance of mGluR1a- and mGluR5-labeled dendrites and spines in the shell and core of the monkey accumbens. Note that the relative abundance value for spines is lower than 1 suggesting a lower density of mGluR1a- and mGluR5-labeled spines than would be expected through a random distribution of immunoreactivity. (B) mGluR1a and mGluR5 immunoperoxidase labeling in the shell and core of the rat nucleus accumbens. Data are expressed as mean density per 100 square microns ($\pm$SEM) of labeled elements from 4 (mGluR5) and 5 (mGluR1a) rats, respectively. Total labeled elements: 1,202-mGluR1a shell; 1,283-mGluR1a core; 1,001-mGluR5 shell; 1,061-mGluR5 core. Single number signs indicate a higher density of mGluR1a- and mGluR5-labeled dendrites in the shell of the rat compared to monkey ($p<0.05$); double + indicates higher density of mGluR1a-labeled dendrites in the shell and core versus the monkey shell and core ($p<0.05$); single + indicates a higher density of mGluR5-labeled spines in the core of the rat compared with the core of the monkey ($p<0.001$). (B') Relative abundance of mGluR1a- and mGluR5-labeled dendrites and spines in the shell and core of the nucleus accumbens in rats. Note that the relative abundance value is about 1 for dendrites and spines in shell and core, indicating that the distribution of labeled elements follows a pattern that would be expected through random distribution of immunoreactivity. Abbreviations: Den.= dendrites; Sp.=spines; U.Ax= unmyelinated axons; M.Ax= myelinated axons; AT= axon terminals.

In the rat, the pattern of labeling for either receptor subtype was very similar to that described in the monkey (Figs. 2.2C, E, F & 3B). Immunoreactive dendrites (23-25/100 μm^2) for both receptor subtypes were significantly more abundant than labeled spines (10-15/100 μm^2) in both shell and core (two-way ANOVA and Tukey's post-hoc tests, n=4 mGluR5 or 5 mGluR1a, $p<0.001$). Two-way ANOVAs revealed no significant difference in the density of labeled elements for both receptor subtypes between the two compartments. The relative abundance of immunoreactive dendrites and spines in the two accumbens compartments is shown in figure 3B'. The index is close to 1 for each receptor subtype in both shell and core, indicating that mGluR1a and mGluR5 immunoreactivity follows a pattern consistent with the overall distribution and relative abundance of dendritic shafts and spines in the rat accumbens (Fig. 2.3B'). The total number of elements counted is shown in parentheses in Table 1.

The density of labeled dendrites and spines for mGluR1a and mGluR5 was compared between rat and monkey using a series of one-way ANOVAs and Tukey's post-hoc tests. A significantly larger density of mGluR1a-containing dendrites was found in the nucleus accumbens shell of rats than monkeys (23.4 ± 2.4 vs. 14.3 ± 1.2; n=5 rats, 3 monkeys; $p<0.05$). Similarly, mGluR1a-labeled spines were also significantly more abundant in both shell and core compartments of rats than primates, (rat: 12.9 ± 2.6 in shell, 14.6 ± 0.5 in core vs. monkey: 3.4 ± 0.6 in shell, 3.6 ± 0.5 in core; n=5 rats, 3 monkeys, $p<0.05$). With respect to mGluR5 labeling, a significantly larger density of labeled dendrites and spines was found in the core of the rat accumbens compared to the monkey (rat: 25.4 ± 1.7 dendrites, 13.3 ± 0.8 spines vs. monkey:14.4 ± 0.7 dendrites, 6.5 ± 0.7 spines; n=4 rats, 3 monkeys; $p<0.05$ for dendrites, $p<0.001$ for spines).

2.4.2.2 Single Immunogold Labeling

The preembedding immunogold method was used to examine the distribution of group I mGluRs immunoreactivity at the subsynaptic level because the immunoperoxidase reaction results in an amorphous and diffuse deposit that is not suitable for this type of analysis. In contrast, gold particle labeling offers a much higher level of spatial resolution at the electron microscope level.

Figure 2.4 shows micrographs of immunogold labeling for mGluR1a and mGluR5 in the shell and core of the nucleus accumbens in rat and monkey. Figure 2.5A and D convey the percentages of plasma membrane-bound gold particle labeling for either receptor subtypes in dendrites, spines, unmyelinated axons and axon terminals in rat and monkey. In the monkey, approximately 60% of mGluR1a labeling and 30-40% of mGluR5 was associated with the plasma membrane in dendrites, spines and unmyelinated axons. In axon terminals, almost 85% of mGluR1a and mGluR5 labeling was intracellular. Two-way repeated measures ANOVA revealed a main effect of receptor subtype in dendrites and unmyelinated axons. Tukey's post-hoc tests showed that the plasma membrane-bound labeling in mGluR1a-containing dendrites (58.0$\pm$1.5% shell; 55.4$\pm$1.8% core) and unmyelinated axons (68.2$\pm$3.6% shell; 63.7$\pm$3.5% core) was significantly higher than in mGluR5-labeled dendrites (29.4$\pm$4.8% shell; 28.1$\pm$5.2% core; $n=3$, $p<0.01$) and unmyelinated axons (46.9$\pm$4.6% shell; 50.8$\pm$4.2% core; $n=3$, $p<0.05$), with no difference between the shell and core (Figs 2.4A-B,D & 2.5A).

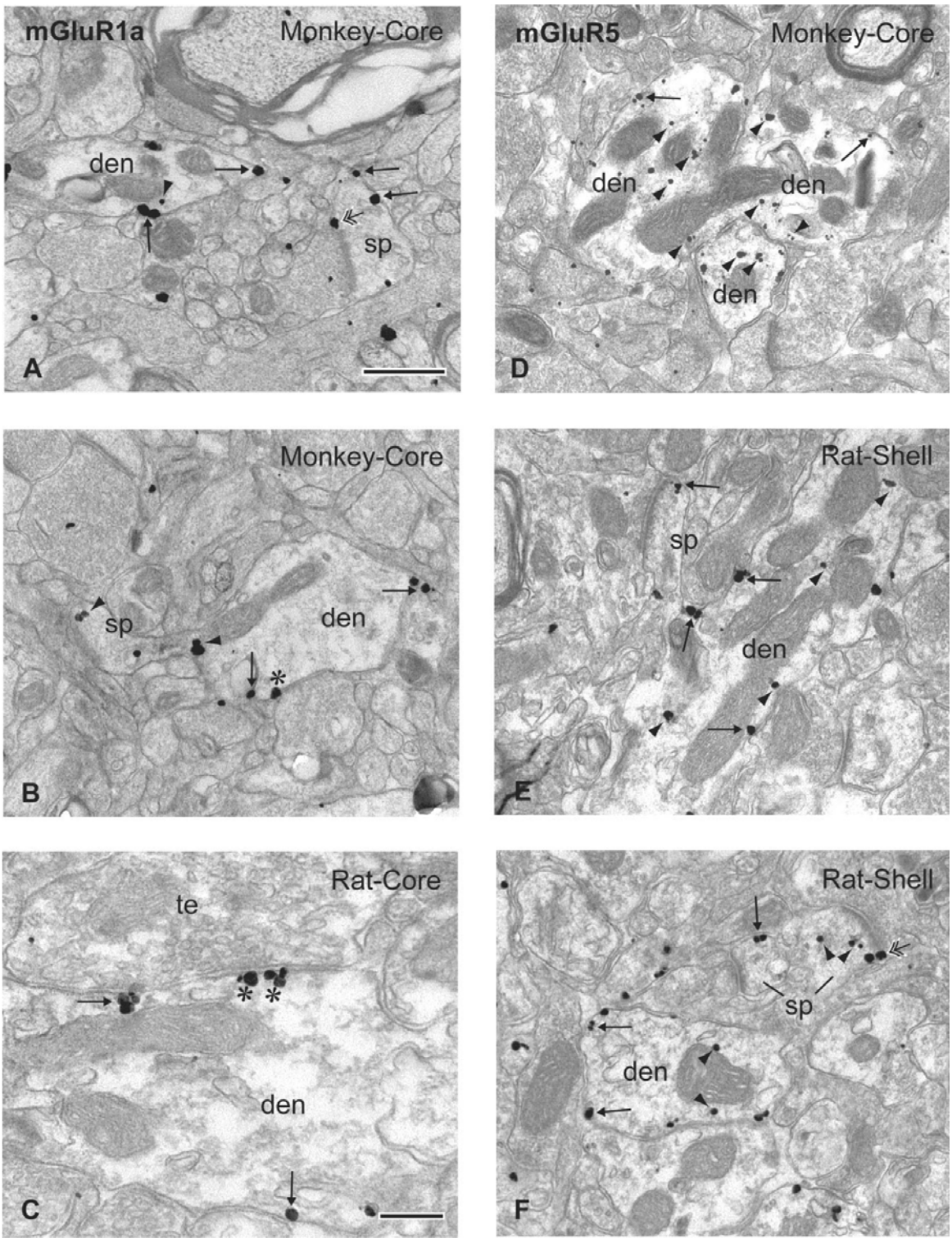

Figure 2.4: Immunogold labeling for Group I mGluRs in the monkey and rat nucleus accumbens. (A-C) mGluR1a immunogold labeling in the nucleus accumbens

core of monkey (A-B) and rat (C). Note that the plasma membrane-bound mGluR1a labeling is predominantly extrasynaptic (single arrows) in dendrites and spines or perisynaptic to asymmetric synapses (A, double arrowhead). It is also occasionally found at the edges or in the main body of symmetric synapses (B, C, asterisks). (D-F) mGluR5 immunogold labeling in the nucleus accumbens core of monkey (D) and shell of rat (E-F). Note the large amount of mGluR5 intracellular labeling in dendrites of the monkey (D, arrowheads). MGluR5 labeling in the rat resembles that of mGluR1a, with predominant extrasynaptic labeling (E, F single arrows) and some perisynaptic labeling to asymmetric synapses (F, double arrowhead). Scale bars: 0.5μm.

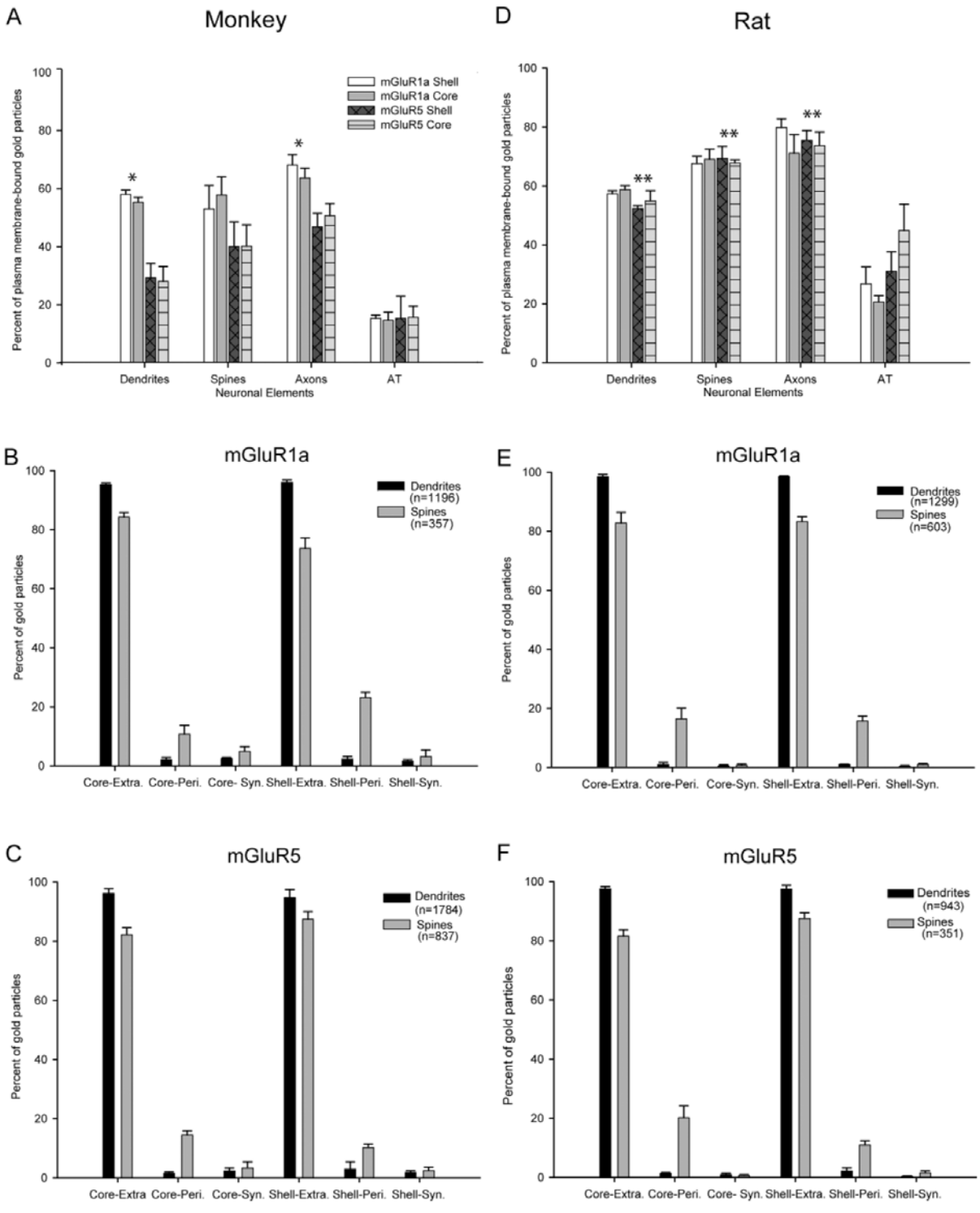

Figure 2.5: Histograms showing the percent of plasma membrane labeling of Group I mGluRs in the nucleus accumbens of monkey and rat. (A, D) the mean

percentage (± SEM) of gold particles found on the plasma membrane in the shell and core of nucleus accumbens in three monkeys (A) and four rats (D). Two-way RM ANOVA with Tukey's post-hoc revealed a higher proportion of mGluR1a than mGluR5 labeling on the plasma membrane of dendrites and unmyelinated axons in monkeys ($p<0.05$) (single asterisks). Significant differences were also found between rat and monkey in mGluR5 plasma membrane-bound labeling on dendrites, spines, and unmyelinated axons using two-way RM ANOVA and Tukey's post-hoc test, $p<0.05$ (double asterisks). No significant difference was found between the core and shell in both species. Total gold particles counted: mGluR1a shell=3,799 (m) 5,370 (r) mGluR1a core= 4,349 (m) 5,013 (r); mGluR5 shell= 5,789 (m) 3,840 (r); mGluR5 core= 5,993 (m) 4,774 (r). (B-C, E-F) Subsynaptic localization of mGluR1a and mGluR5 immunoreactivity on the plasma membrane of dendrites and spines in monkey (B-C) and rat (E-F). More than 80% of either receptors immunoreactivity is extrasynaptic ("n" indicates the number of neuronal elements from which the gold particles were counted).

Figures 2.5B-C shows the quantification of mGluR1a and mGluR5 labeling on the plasma membrane of dendrites and spines in monkey. In dendrites labeled for either mGluR1a or mGluR5, over 90% of plasma membrane-bound gold particles were extrasynaptic, 1-3% was perisynaptic or synaptic. In immunoreactive spines, 70-90% of plasma membrane-bound gold particles were extrasynaptic, 10- 20% were perisynaptic and 2 – 5% were synaptic. Using one-way ANOVAs, no significant difference was found between the shell and core.

On the other hand, no significant difference in the pattern of subsynaptic localization of mGluR1a and mGluR5 was found in rats. On average, 50-60% of mGluR1a and mGluR5 labeling was found on the plasma membrane in dendrites, while about 70% of labeling was plasma membrane-bound in spines and unmyelinated axons (Figs 2.4C, E-F; 2.5D). In axon terminals, 20– 45% of mGluR1a and mGluR5 labeling was categorized as being bound to the plasma membrane. Overall, the pattern of subsynaptic gold labeling distribution for mGluR1a and mGluR5 in rat accumbens neurons was the same as in monkey, with the majority of labeling being extrasynaptic in both dendrites and spines (Fig. 2.5E-F).

When comparing the subsynaptic localization of group I mGluRs between rat and monkey, a two-way repeated measures ANOVA revealed a significantly larger percentage of mGluR5 plasma membrane-bound labeling in rats (dendrites: 52.4$\pm$1.0% shell, 55.0$\pm$3.3% core; spines: 69.4$\pm$4.1% shell, 67.8$\pm$1.1% core; unmyelinated axons: 75.6$\pm$3.2% shell, 73.8$\pm$4.5% core) than in monkeys (dendrites: 29.4$\pm$4.8% shell, 28.1$\pm$5.2% core; spines: 40.1$\pm$8.5% shell, 40.2$\pm$7.4% core; unmyelinated axons: 46.9$\pm$4.6% shell, 50.8$\pm$4.2% core; n=3 monkey, 4 in rat; $p<0.01$ for dendrites,

unmyelinated axons; $p<0.05$ for spines). However, no species difference was found between shell and core for either receptor subtypes or in the distribution of mGluR1a labeling.

2.4.2.3 Double Immunoperoxidase Labeling for mGluR1a and mGluR5

Knowing that mGluR1a and mGluR5 are frequently co-expressed and play differential roles in basal ganglia nuclei (Conn et al., 2005), we assessed the degree of colocalization of the two group I mGluRs in the shell and core of the rat nucleus accumbens using a double immunoperoxidase labeling method at the electron microscopic level. Results are summarized in Figure 2.6 and Table 1. On average, about 50-60% of dendrites and spines expressed mGluR1a or mGluR5 in singly immunostained tissue; whereas 65-75% of elements were immunoreactive in the double stained sections. The estimated degree of colocalization was calculated as follows: % $mGluR1a_{labeled}$ + % $mGluR5_{labeled}$ – (% mGluR1a + mGluR5) $_{labeled}$ = estimated % of colocalization. Using this approach, about 30% colocalization of mGluR1a and mGluR5 was found in dendrites, while 50-55% of spines coexpressed both receptors, with no significant difference between the shell and core (see methods for more detail).

Table 1: Quantification of double immunoperoxidase labeling for mGluR1a and mGluR5 in the rat nucleus accumbens. Key: [n] = number of animals; number in parentheses= total number of elements (labeled and unlabeled) counted for that area from all animals examined; %=mean percent of labeled elements $\pm$SEM. Note the difference in the percent of colocalization between spines and dendrites; no obvious difference was found between the shell and core for either receptor subtype.

TABLE 1. Quantification of Double Immunoperoxidase Labeling for mGluR1a and mGluR5 in the Rat Nucleus Accumbens[1]

	mGluR1a [n = 5] (%)	mGluR5 [n = 4] (%)	mGluR1a + mGluR5 [n = 4] (%)	Percentage of colocalization
Shell (dendrites)	47.7 ± 1.7 (2,167)	45.6 ± 2.4 (1,905)	64.8 ± 3.0 (1,316)	28.6
Shell (spines)	65.5 ± 6.4 (989)	59.6 ± 7.2 (707)	72.4 ± 2.9 (673)	52.8
Core (dendrites)	50.8 ± 2.5 (1,662)	44.2 ± 2.7 (1,993)	65.0 ± 3.5 (1,700)	30.0
Core (spines)	66.8 ± 3.8 (1,072)	57.8 ± 4.3 (1,077)	68.8 ± 2.6 (986)	55.9

n, Number of animals; number in parentheses, total number of elements (labeled and unlabeled) counted for that area from all animals examined; %, mean percentage of labeled elements ± SEM. Note the difference in the percentage of colocalization between spines and dendrites; no obvious difference was found between the shell and the core for either receptor subtype.

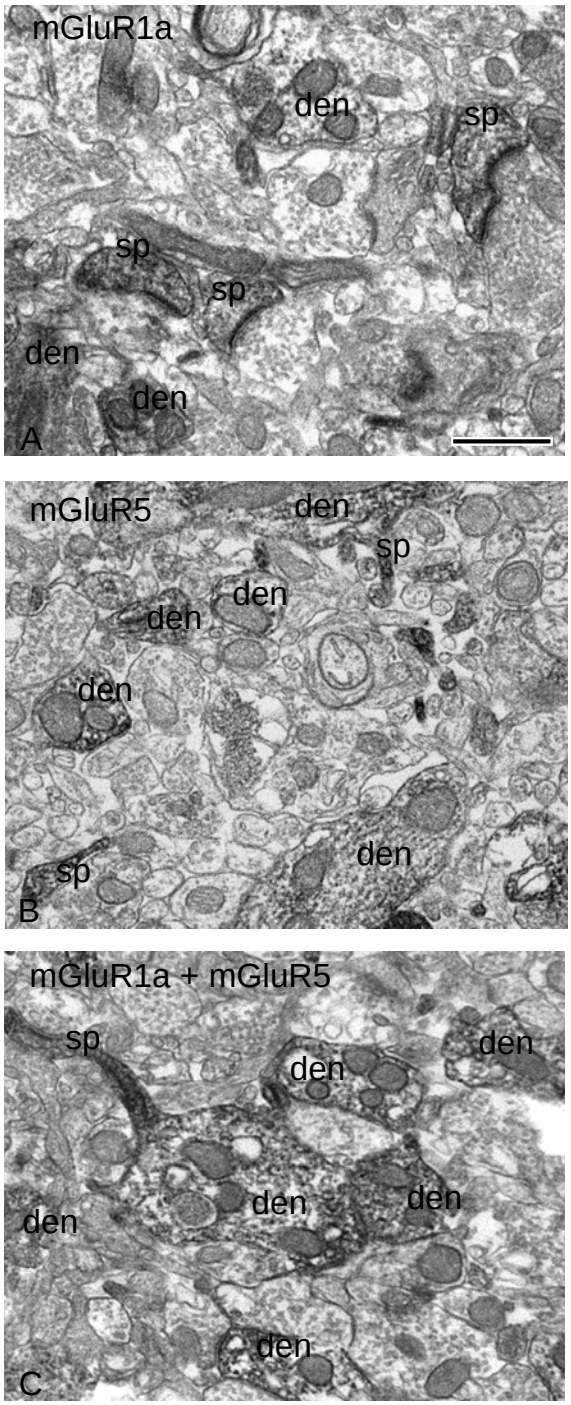

Figure 2.6: Double Immunoperoxidase labeling in rat. (A) mGluR1a labeling the nucleus accumbens core of the rat, (B) mGluR5 labeling in the nucleus accumbens shell and (C) shows a sample of tissue labeled with mGluR1a and mGluR5 in the nucleus accumbens core. The total number of labeled and unlabeled dendrites and spines was counted and expressed as a percentage of labeled elements in table 1. Labeled elements only are indicated in the figure (den=dendrites; sp=spines). Scale bar=1.0μm.

2.4.2.4 Double Immunoperoxidase and Immunogold Labeling for Group I mGluRs and Neuronal Markers

Knowing the striatum is made up of two main populations of projection neurons and various groups of interneurons that play different roles in striatal processing (Smith and Bolam, 1990; Tepper and Bolam, 2004) and display specific physiological responses to group I mGluR ligands (Conn et al., 2005), we determined the chemical phenotype of accumbens neurons that express mGluR1a and mGluR5. Three markers were chosen to distinguish between medium spiny projection neurons and interneurons. In a first series of experiments, we used an antibody against D1 dopamine receptors as a marker for a subpopulation of striatofugal neurons (Hersch et al., 1995; Yung et al., 1995) in conjunction with either mGluR1a or mGluR5 antibodies. Since both mGluR subtypes are mainly postsynaptic, the degree of colocalization was assessed in dendrites and spines. As shown in figures 2.7A-B and Table 2, both group I mGluRs and D1 receptors frequently colocalized in the core and shell of the rat accumbens, but a significantly higher degree of co-expression was found in mGluR5-containing dendrites than in mGluR1a-immunoreactive elements in both compartments (Two-way ANOVA with Tukey's post-hoc test; $n= 4$; $p<0.01$). Similarly, the accumbens shell contained a larger proportion of mGluR5/D1-labeled spines than mGluR1a/D1-labeled spines ($n=4$; $p<0.001$). On the other hand, more spines were labeled for mGluR1a/D1 in the nucleus accumbens core than in the shell ($p<0.05$).

To determine whether the subsynaptic localization of mGluR1a and mGluR5 in D1-containing elements was representative of the overall pattern of subsynaptic distribution of group I mGluRs labeling described in single immunostained tissue (see Fig. 2.5), we quantified the proportion of plasma membrane-bound gold labeling for both

receptor subtypes in D1-immunoreactive dendrites and spines (Table 2). Using two-way ANOVAs, no significant difference was found between the proportions of plasma membrane-bound gold particles for mGluR1a and mGluR5 in double-labeled dendrites or spines compared to single labeled elements in both shell and core. The overall subsynaptic distribution of plasma membrane-bound gold particles labeling for mGluR1a or mGluR5 in D1-containing dendrites and spines was also the same as shown in single labeled structures, e.g. more than 90% of either receptor type was extrasynaptic on the plasma membrane of dendrites and spines, followed by less than 10% perisynaptic to asymmetric synapses (Fig. 2.5E-F).

In a second series of colocalization studies, we assessed the expression of mGluR1a and mGluR5 in specific populations of interneurons. For this purpose, parvalbumin was used as the marker of a subpopulation of GABAergic interneurons, while ChAT antibodies labeled cholinergic interneurons. Data from the dorsal striatum demonstrated a high level of cellular co-localization of mGluR1a or mGluR5 in these two neuronal populations (Tallaksen-Greene et al., 1998). Complex functional interactions between mGluR1a and mGluR5 have been reported in cholinergic neurons, but not parvalbumin-containing neurons (Pisani et al., 2001; Bonsi et al., 2005). The degree to which the group I mGluRs colocalized with PV and ChAT varied (figures 2.7C-F, Table 2). No significant difference was found between the proportions of PV- double labeled dendrites for either receptor subtype in the shell or core of the accumbens (see Table 2). The dendritic gold particles labeling for either mGluR1a or mGluR5 in PV-immunoreactive dendrites was categorized as described previously. As shown in Table 2, there was no significant difference between the percentages of plasma membrane-bound

gold particles for both mGluR1a and mGluR5 in double labeled dendrites. However, the proportions of plasma membrane-bound mGluR5 differed in single versus double labeled dendrites. Using two-way ANOVA and Tukey's post-hoc tests, double labeled dendrites were found to contain a lower proportion of plasma membrane-bound mGluR5 than single labeled dendrites ($n=4$ for each, $p< 0.01$). On the other hand, the subsynaptic distribution of plasma membrane-bound gold particles labeling for mGluR1a and mGluR5 in PV-immunoreactive dendrites was nearly identical to that described in single labeled dendrites (Fig. 2.5E-F), i.e. over 95% of group I mGluRs labeling was extrasynaptic.

In accumbens tissue double immunostained with ChAT and either group I mGluR antibodies, one-way ANOVAs revealed no significant difference between the proportion of double labeled dendrites for either receptor subtype in shell and core (Table 2). However, there were significantly more ChAT-immunoreactive dendrites that expressed mGluR1a labeling than mGluR5 immunoreactivity in the core of the nucleus accumbens (one-way ANOVA; $n=3$, $p<0.01$).

The subsynaptic distribution of plasma membrane-bound gold particles labeling for mGluR1a and mGluR5 in double labeled dendrites was nearly identical to that shown in figure 2.5E-F for single labeled tissue. Statistical analysis revealed no significant difference in the percent of plasma membrane-bound labeling for either receptor subtype between the accumbens shell and core in ChAT-immunoreactive dendrites. However, a significantly larger proportion of plasma membrane-bound mGluR1a labeling was found in single mGluR1a-labeled dendrites compared with mGluR1a/ChAT double labeled

structures in both the shell and core (two-way ANOVA, Tukey's post-hoc, n=3 double, 4 for single, $p<0.05$).

Finally, the percentages of colocalization between group I mGluRs and the two interneuron markers were compared. In the core of the accumbens, a larger proportion of PV-immunoreactive dendrites coexpressed mGluR1a or mGluR5 than ChAT-positive dendrites (one-way ANOVA, $p<0.01$). In the shell of the accumbens, the frequency of PV/mGluR5 colocalization was larger than the occurrence of ChAT/mGluR5 double labeling ($p<0.001$). No significant difference was found between the percentages of plasma membrane-bound gold labeling for either receptor subtype in PV- or ChAT-immunoreactive dendrites.

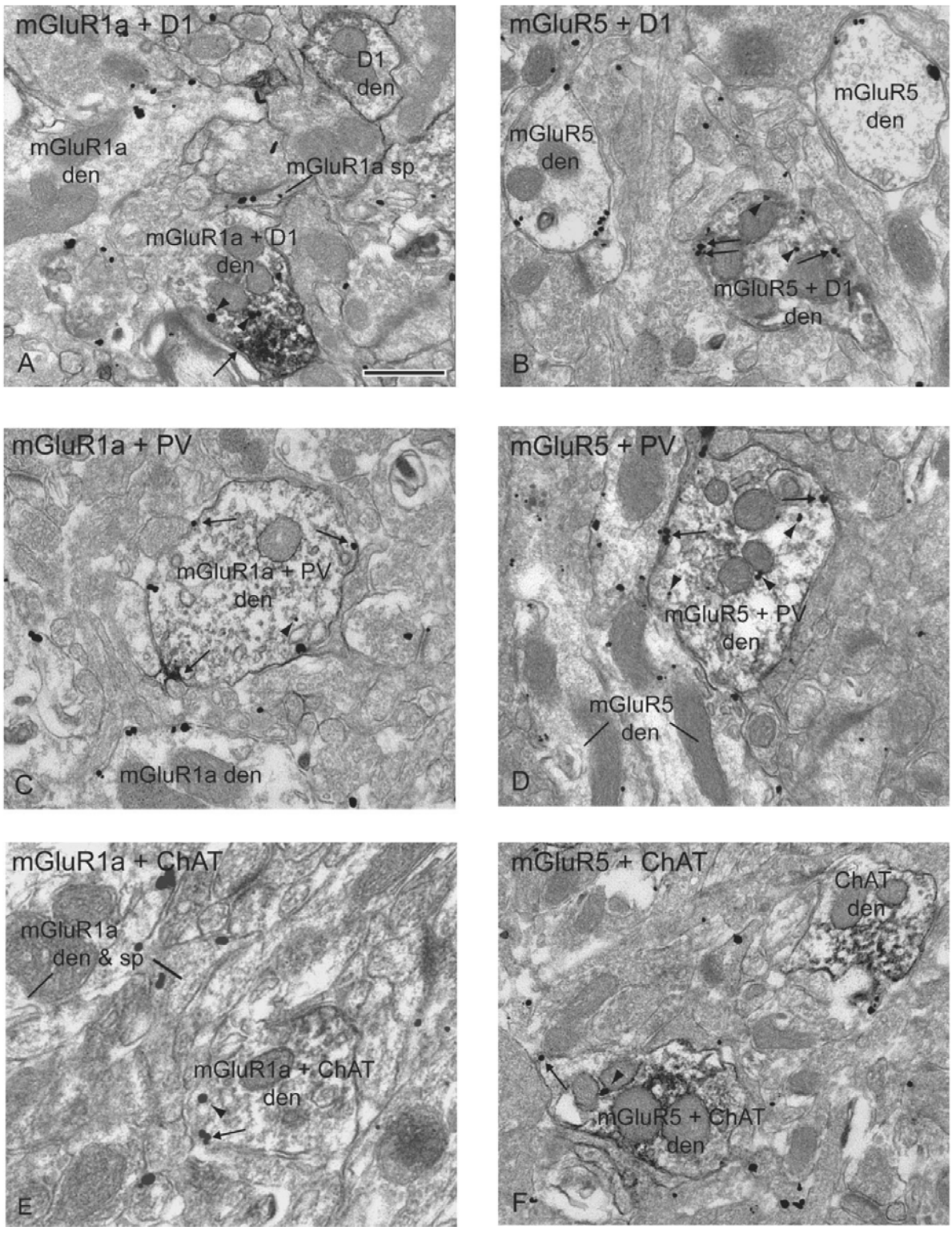
mGluR1a + D1
D1
den
mGluR1a
den
mGluR1a sp
mGluR1a + D1
den
A
mGluR5 + D1
mGluR5
den
mGluR5
den
mGluR5 + D1
den
B
mGluR1a + PV
mGluR1a + PV
den
C
mGluR1a den
mGluR5 + PV
mGluR5 + PV
den
mGluR5
den
D
mGluR1a + ChAT
mGluR1a
den & sp
mGluR1a + ChAT
den
E
mGluR5 + ChAT
ChAT
den
mGluR5 + ChAT
den
F

Figure 2.7: Double Immunoperoxidase and Immunogold labeling in the rat nucleus accumbens. (A-B) shows dual labeling for mGluR1a or mGluR5 and D1 dopamine receptors as a marker of medium spiny striatofugal neurons in the core of the rat accumbens. Receptor labeling was mainly found intracellularly (arrowheads) or extrasynaptic (single arrows) on the plasma membrane. (C-D) shows labeling of either mGluR1a or mGluR5 and PV in the core of the accumbens. (E-F) shows labeling of mGluR1a or mGluR5 and ChAT in the core (E) and shell (F) of the rat accumbens. (den=dendrite, sp=spine). Scale bar = 0.5μm.

Table 2: Colocalization studies of group I mGluRs and markers of striatal neurons. (A) Mean percents (±SEM) of colocalization of D1-, PV- or ChAT-containing dendrites or spines with mGluR1a or mGluR5 labeling in the shell and core of the rat nucleus accumbens (NA). Total number of dendrites examined: D1/mGluR1a= 489; D1/mGluR5= 447; PV/mGluR1a= 357; PV/mGluR5= 363; ChAT/mGluR1a= 184; ChAT/mGluR5= 242. Total number of spines: D1/mGluR1a= 168; D1/mGluR5= 157. (B) The mean percentages (±SEM) of plasma membrane-bound (PMB) gold particles labeling for mGluR1a or mGluR5 immunoreactivity (IR) in double labeled elements. Total number of gold particles counted in double labeled D1-IR dendrites and spines: mGluR1a core= 682(d) 99(s); mGluR1a shell= 424(d) 57(s); mGluR5 core 987(d) 110(s); mGluR5 shell 703(d) 80(s). For PV-IR dendrites: mGluR1a core = 914; mGluR1a shell = 714; mGluR5 core = 967; mGluR5 shell = 1,110. For double labeled ChAT-positive

dendrites: mGluR1a core = 223; mGluR1a shell = 214; mGluR5 core = 130; mGluR5 shell = 228.

TABLE 2. Colocalization Studies of Group I mGluRs and Markers of Striatal Neurons[1]

	NA Shell-Dendrites	NA Core-Dendrites	NA Shell-Spines	NA Core-Spines
A. Colocalization (%)				
D1/mGluR1a	57.5 ± 4.1	63.8 ± 2.9	31.4 ± 1.8	43.0 ± 2.95[c]
D1/mGluR5	67.6 ± 4.6[a]	76.2 ± 1.9[a]	49.7 ± 3.0[b]	44.0 ± 4.2
PV/mGluR1a	71.6 ± 6.9	84.3 ± 1.5[g]	—	—
PV/mGluR5	77.1 ± 2.5[h]	77.4 ± 4.8[g]	—	—
ChAT/mGluR1a	55.8 ± 11.8	67.3 ± 4.3	—	—
ChAT/mGluR5	39.2 ± 1.4	31.6 ± 2.5	—	—
B. Subsynaptic localization (% of PMB mGluR IR)				
D1/mGluR1a	54.6 ± 5.5	53.1 ± 3.0	53.0 ± 5.8	63.9 ± 6.1
D1/mGluR5	50.3 ± 6.0	52.3 ± 2.0	65.2 ± 5.5	69.3 ± 3.5
PV/mGluR1a	50.5 ± 3.1	53.6 ± 5.4	—	—
PV/mGluR5	45.6 ± 5.4[d]	39.2 ± 3.4[d]	—	—
ChAT/mGluR1a	53.8 ± 6.7[f]	44.4 ± 2.4[f]	—	—
ChAT/mGluR5	44.1 ± 9.4	51.4 ± 5.3	—	—

[1]A: Mean percentage (±SEM) of colocalization of D1-, PV-, or ChAT-containing dendrites or spines with mGluR1a or mGluR5 labeling in the shell and core of the rat nucleus accumbens (NA). Total number of dendrites examined: D1/mGluR1a = 489, D1/mGluR5 = 447, PV/mGluR1a = 357, PV/mGluR5 = 363, ChAT/mGluR1a = 184, ChAT/mGluR5 = 242. Total number of spines: D1/mGluR1a = 168, D1/mGluR5 = 157. B. The mean percentage (±SEM) of plasma membrane-bound (PMB) gold particles labeling for mGluR1a or mGluR5 immunoreactivity (IR) in double-labeled elements. Total number of gold particles counted in double-labeled D1-IR dendrites and spines: mGluR1a core = 682 (d) 99 (s), mGluR1a shell = 424 (d) 57 (s), mGluR5 core 987 (d) 110 (s), mGluR5 shell 703 (d) 80 (s). For PV-IR dendrites: mGluR1a core = 914, mGluR1a shell = 714, mGluR5 core = 967, mGluR5 shell = 1,110. For double-labeled ChAT- positive dendrites: mGluR1a core = 223, mGluR1a shell = 214, mGluR5 core = 130, mGluR5 shell = 228.
[a]Greater percentage of D1/mGluR5 den in shell and core than D1/mGluR1a den ($P < 0.01$).
[b]Greater percentage of D1/mGluR5 sp in shell and D1/mGluR1a sp in shell ($P < 0.001$).
[c]Greater percentage of D1/mGluR1a sp in core than shell ($P < 0.05$).
[d]Lower percentage of PMB mGluR5 in core and shell in double- vs. single-labeled den ($P < 0.01$).
[e]Greater percentage of ChAT/mGluR1a den than ChAT/mGluR5 den in core ($P < 0.01$).
[f]Lower percentage of PMB mGluR1a in core and shell of double- vs. single-labeled den ($P < 0.05$).
[g]Greater percentage of PV/mGluR1a or PV/mGluR5 den than ChAT/mGluR1a or ChAT/mGluR5 den in core ($P < 0.05$).
[h]Greater percentage of PV/mGluR5 than ChAT/mGluR5 den in shell ($P < 0.001$).

2.5 Discussion

In summary, four main features characterize the localization of group I mGluRs in the nucleus accumbens. First, the two group I mGluR subtypes display a similar pattern of subcellular distribution in the rat and monkey nucleus accumbens. Both receptors are preferentially expressed postsynaptically in dendrites and spines and less frequently in unmyelinated axons, with minimal difference between the shell and core. Secondly, at the subsynaptic level, mGluR5 immunoreactivity is more frequently expressed intracellularly in dendrites and spines than mGluR1a in the monkey, while in the rat, both mGluR1a and mGluR5 are mainly bound to the plasma membrane. In both species, plasma membrane-bound mGluR1a and mGluR5 labeling is primarily extrasynaptic in dendrites and spines. Third, the two group I mGluRs are frequently co-expressed in the same dendrites and spines in the shell and core of the rat accumbens. Fourth, both group I mGluRs are significantly expressed in D1-containing medium spiny striatofugal neurons and PV-positive GABAergic interneurons but less frequently in ChAT-positive cholinergic interneurons. Together, these results provide multiple target sites whereby group I mGluRs may modulate neuronal activity and regulate glutamatergic transmission in the nucleus accumbens.

2.5.1 Plasma membrane-bound Group I mGluRs

Consistent with data obtained in the monkey dorsal striatum (Paquet and Smith, 2003), both group I mGluRs in the nucleus accumbens of rats and monkeys were largely found in postsynaptic structures. On average, immunoreactive dendrites were more frequently encountered than labeled spines in both species. However, although this difference was merely representative of the overall neuropil composition of the shell and

core in the rat accumbens, it reveals a genuine decreased density of mGluR1a- and mGluR5-labeled spines in monkeys. Knowing the large variety of cortical and subcortical sources of axo-spinous glutamatergic synapses in the nucleus accumbens (Walaas, 1981; Sadikot et al., 1992; Meredith, 1999; Friedman et al., 2002), our findings suggest that most of these inputs are likely targeting group I mGluRs-containing spines in rats, whereas subsets of afferents may preferentially innervate group I mGluRs-negative spines in monkeys. However, since we did not attempt to label synaptic inputs, our findings do not provide much information on the relationships between group I mGluRs and specific glutamatergic afferents that impinge upon shell and core accumbal neurons. Tract tracing/immunogold studies are needed to directly address this issue. Such studies combined with thorough electrophysiological analysis of the differential roles of the two group I mGluRs in regulating glutamatergic transmission in the shell or core of the accumbens should help further characterize the structure/function relationships of group I mGluRs in the two accumbal compartments.

As previously described in other brain regions, more than 80% of plasma membrane-bound mGluR1a and mGluR5 labeling was extrasynaptic on dendrites and spines of the rat and monkey nucleus accumbens. It is noteworthy that such extrasynaptic localization is a common feature for most G-protein coupled receptors (GPCRs) and, especially, for group I mGluRs (Baude et al., 1993; Hubert et al., 2001; Paquet and Smith, 2003; Kuwajima et al., 2004) in the CNS. Extrasynaptic group I mGluRs are likely to be activated by synaptically released glutamate spillover in the extracellular space or glial release mediated by the cysteine-glutamate exchanger and/or reverse of glutamate transporters in astrocytes (Cho and Bannai, 1990; Rothstein et al.,

1996; Danbolt, 2001; Baker et al., 2002; Patel et al., 2004). There is, indeed, good evidence that the degree of activation of extrasynaptic group I mGluRs is controlled by neuronal and glial glutamate transporter activity in various brain regions (Brasnjo and Otis, 2001; Heinbockel et al., 2004; Melendez et al., 2005). The fact that most GPCRs are non-synaptic suggests common features regarding their mechanisms of activation and function in the CNS. One potential explanation for such a similar distribution is the fact that GPCRs can interact through direct heterodimerization and/or common signaling pathways to regulate synaptic transmission. These protein-protein interactions, referred to as receptor mosaics, represent a complex mechanism by which various transmitter systems can interact and modulate each other's responses to specific synaptic inputs (Agnati et al., 2005). Although not yet examined in accumbal neurons, direct receptor-receptor interactions between group I mGluRs and adenosine, dopamine and GABA-B receptors have been demonstrated in neuronal cultures from other brain regions (Agnati et al., 2003; Tabata et al., 2004; Ciruela et al., 2005; Kubo and Tateyama, 2005; Voulalas et al., 2005). Knowing that each of these receptor families are significantly expressed in the nucleus accumbens, our findings lay the foundation for complex receptor-receptor interactions yet to be found. It is noteworthy that group I mGluRs can also link to IP3 and NMDA receptors through the scaffolding proteins Homer and Shank (Shiraishi et al., 2003; Yuan et al., 2003). In vitro data have highlighted the importance of Homer, Shank and other synaptic proteins in the trafficking, synaptic targeting and intracellular signaling of group I mGluRs (Xiao et al., 1998; Lim et al., 1999; Xiao et al., 2000; Thomas, 2002).

2.5.2 Intracellular Group I mGluRs

Our results demonstrate that a substantial proportion of group I mGluRs immunoreactivity is found intracellularly in rat and monkey accumbens neurons. As stated previously, mGluR1a and mGluR5 display a high degree of inter-nuclei variability in the proportion of intracellular versus plasma membrane-bound labeling. For example, in the hippocampus and cerebellum, group I mGluRs are almost exclusively found on the plasma membrane with a high concentration of receptor labeling perisynaptic to asymmetric synapses (Baude et al., 1993; Lujan et al., 1996). In the substantia nigra, on the other hand, mGluR1a is evenly expressed between the plasma membrane and the intracellular compartment, whereas mGluR5 is rarely found on the plasma membrane, but displays a high level of intracellular expression (Hubert et al., 2001). The proportions of intracellular/plasma membrane-bound group I mGluRs in the shell and core of the nucleus accumbens fall in between these two brain regions with some differences between rats and monkeys. It is likely that such a heterogeneity in receptor distribution may partly account for the differential electrophysiological effects of mGluR1 and mGluR5 observed in different brain regions (Awad et al., 2000; Mannaioni et al., 2001; Marino et al., 2001; Pisani et al., 2001; Valenti et al., 2002; Poisik et al., 2003). Various roles have been proposed for intracellular group I mGluRs. In vitro, mGluR1a regulates peak calcium increases in transfected cells, while mGluR5 controls oscillatory escalations in calcium, both through the IP_3 receptor pathway (Kawabata et al., 1996). It has also been shown that nuclear mGluR5, when stimulated with glutamate, produces oscillations in calcium levels (O'Malley et al., 2003). Other reasons to explain the large amount of intracellular mGluR1a and mGluR5 is the dynamic regulation of GPCR turnover and trafficking. For example, group I mGluRs immunoreactivity in the intracellular

compartment could be the result of prolonged desensitization and endocytosis of receptors in a GRK or β-arrestin-dependent manner, or representative of receptors trafficking to the membrane following synthesis or resensitization (Dale et al., 2002; Gainetdinov et al., 2004). Based on our immunogold data showing a significantly larger percentage of intracellular mGluR5 in monkeys than rats, one may suggest that intracellular mGluR5 may play a more prominent role in primates than non-primates. These issues are currently being addressed in our laboratory using animal models characterized by an imbalance in glutamatergic transmission and transgenic animals devoid of Homer gene expression.

2.5.3 Co-localization of Group I mGluRs

Our study provides the first evidence for mGluR1a/mGluR5 colocalization in dendrites and spines of the rat accumbens. Previous studies that examined the cellular colocalization of mGluR1a and mGluR5 in other brain regions have been achieved at the light and confocal microscopic levels. In this study we used a double peroxidase cocktail method to address this issue. Since both group I mGluR antibodies were raised in the same species, we could not apply a double electron microscopic procedure on the same section using two different antigenic markers. In fact, the double peroxidase approach offers the advantage of using the same marker to label both antigens, which avoids the interpretation problem one faces when using double labeling technique on the same section due to a differential degree of marker penetration in the tissue. On the other hand, the main drawback of the cocktail method relies in the interpretation of the significance of unlabeled elements. Because antibodies do not penetrate the full thickness of sections, unlabeled structures may indicate a genuine lack of antigen or may be the result of sub-

optimal antibodies penetration. To overcome this problem, we made sure to clearly assess the degree of penetration of both mGluR1a and mGluR5 antibodies (see methods), which allowed to determine more reliably the extent of the tissue section that could be used for this analysis. A similar method has been successfully used by various groups to assess co-localization of different markers including dopamine receptors in the striatum (Hersch et al., 1995; Lei et al., 2004).

In the subthalamic nucleus, substantia nigra, and globus pallidus, light microscopic double fluorescent data showed that mGluR1a and mGluR5 colocalize in 60-95% of immunoreactive cells (Marino et al., 2002; Poisik et al., 2003; Kuwajima et al., 2004). Our findings reveal 30% dendritic co-localization and as much as 50% co-localization in spines in the rat accumbens. These observations provide evidence that mGluR1a and mGluR5 are not only co-expressed in individual neuronal cell bodies, but trafficked to similar neuronal compartments in a subset of neurons in the rat accumbens, which opens up the possibility for functional interactions between the two receptors at distal glutamatergic axo-dendritic or axo-spinous synapses. Since these two receptors are part of the same family and use the same signaling pathways, one may wonder as to why they are so frequently co-expressed in the CNS. Although very little is known about the specific roles of mGluR1a and mGluR5 in the nucleus accumbens (Manzoni et al., 1997), this issue has been addressed in other basal ganglia nuclei. Despite significant co-localization and common patterns of distribution, distinct functions have been disclosed for the two group I mGluRs whenever they co-exist in individual neurons (Valenti et al., 2002 ; Conn et al., 2005). For example, mGluR5, but not mGluR1, potentiates NMDA-induced membrane depolarization in the dorsal striatum (Pisani et al., 2001). In the

globus pallidus, activation of mGluR1 causes depolarization, while mGluR5 potentiates this response by preventing mGluR1 desensitization (Poisik et al., 2003). In the SNr, mGluR1, but not mGluR5, mediates depolarization, whereas the opposite was found in the STN (Awad et al., 2000; Marino et al., 2001). Further evidence along the same lines have also been gathered from the cholinergic striatal interneurons and CA1 hippocampal pyramidal neurons (Awad et al., 2000; Mannaioni et al., 2001; Bonsi et al., 2005). Therefore, despite their apparent widespread colocalization, it is likely that these two receptors display unique electrophysiological properties in the nucleus accumbens depending on their cellular and subcellular localization.

2.5.4 Group I mGluRs are expressed in both projection neurons and interneurons

D1 receptors are a marker of the so-called "direct" striatofugal neurons that project to the substantia nigra (Gerfen et al., 1990; Gerfen, 2006). The high degree of colocalization of group I mGluRs and D1 receptor immunoreactivity suggests important regulatory functions of mGluR1a and mGluR5 on "direct" striatofugal neurons (Yung et al., 1995). Based on previous cellular colocalization studies (Tallaksen-Greene et al., 1992; Tallaksen-Greene et al., 1998), one could estimate that about the same proportion of group I mGluRs could colocalize within D2 receptor-containing neurons, the "indirect" striatofugal neurons that project preferentially to the globus pallidus (Gerfen et al., 1990; Gerfen, 2006). Due to technical problems in using commercially available D2 receptor antibodies in the double immunocytochemical electron microscopic procedure performed in this study, we could not directly assess the degree of group I mGluRs co-localization in "indirect" striatofugal neurons. However, the fact that many D1-negative spines displayed mGluR1a and mGluR5 in the double immunostained tissue strongly

suggests that both populations of striatofugal neurons are endowed with group I mGluRs in the nucleus accumbens. So far, evidence for functional interactions between group I mGluRs and dopamine receptors in the nucleus accumbens have been mainly gathered through behavioral pharmacology studies of psychostimulants (Vezina and Kim, 1999; Swanson and Kalivas, 2000; Chiamulera et al., 2001; David and Abraini, 2001). Although these studies provide compelling evidence that group I mGluRs tightly interact with the dopaminergic system at the level of the ventral striatum to mediate behavioral effects of psychostimulants, the exact molecular mechanisms that underlie these interactions remain poorly understood. However, based on recent in vitro molecular data, direct receptor-receptor interactions and/or convergence of dopamine receptors and mGluRs signaling onto common intracellular pathways represent two likely targets whereby dopamine receptors and group I mGluRs could functionally interact (Ferre et al., 1999; David and Abraini, 2001; Voulalas et al., 2005).

In line with previous cellular studies in the dorsal striatum, our findings demonstrate that a large proportion of PV-labeled dendrites express either mGluR1a or mGluR5 in the rat accumbens, suggesting that both group I mGluRs regulate activity of this important population of intrinsic striatal neurons (Tepper and Bolam, 2004). However, although a similar degree of co-localization was recently reported in the dorsal striatum, in vitro electrophysiological recordings showed that mGluR1, but not mGluR5, mediates depolarization of fast-spiking PV-immunoreactive cells in the rat caudate-putamen (Bonsi et al., 2007). These observations extend those made in other basal ganglia nuclei and the hippocampus showing the differential roles played by mGluR1 and mGluR5 whenever they coexist in individual neurons (Valenti et al., 2002). It is

noteworthy that mGluR5, but not mGluR1a, colocalizes with PV in GABAergic interneurons of the cerebral cortex (Kerner et al., 1997; Stinehelfer et al., 2000), which emphasize the high degree of nuclear specificity in the cellular expression of group I mGluRs in the CNS.

Previous studies have shown that mGluR1a and mGluR5 colocalizes in 89% and 65% of ChAT-positive cholinergic interneurons, respectively, in the rat dorsal striatum (Tallaksen-Greene et al., 1998). Our electron microscopic data are consistent with these findings showing a higher degree of ChAT-positive dendrites expressing mGluR1a compared to mGluR5 immunoreactivity in the accumbens. In the dorsal striatum, mGluR1 activation in cholinergic neurons leads to depolarization, while mGluR5 contributes to the desensitization of mGluR1 following repeated agonist application, a phenomenon similar to that seen in the rat globus pallidus (Poisik et al., 2003). The high degree of mGluR1a and mGluR5 colocalization in ChAT-containing dendrites of the nucleus accumbens strongly suggests that the two group I mGluRs may also heteroregulate each other to modulate acetylcholine release and cholinergic functions in the ventral striatum.

2.5.5 Group I mGluRs in the Accumbens and Addiction to Psychostimulants

Glutamatergic transmission and group I mGluRs function and expression levels in the nucleus accumbens are changed following acute and chronic cocaine exposure in rats (Smith et al., 1995; Ghasemzadeh et al., 1999; Swanson et al., 2001; Baker et al., 2003; Fourgeaud et al., 2004). For instance, rats treated with cocaine according to a regimen that elicits behavioral sensitization and enduring alterations in dopamine and glutamate transmission, show a significant decrease in mGluR5, but not mGluR1a, protein levels in

the nucleus accumbens (Pierce and Kalivas, 1997; Swanson et al., 2001). The mechanisms that mediate these effects are likely to be very complex and possibly differ between the accumbens core and shell (Ghasemzadeh et al., 1999; Swanson et al., 2001). On the other hand, mGluR5 null mutant mice do not self administer cocaine, nor display any sign of increased locomotion following cocaine treatment, despite showing cocaine-induced increases in nucleus accumbens dopamine levels similar to wild type mice (Chiamulera et al., 2001). These observations suggest that mGluR5 is essential to mediate the reinforcing and locomotor stimulant effects of cocaine in rodents. In line with these observations, mGluR5 antagonists attenuate significantly cue-induced reinstatement of cocaine self-administration in rats and monkeys (Backstrom and Hyytia, 2006; Iso et al., 2006). Together, these observations highlight the importance of group I mGluRs in the development of behavioral effects of cocaine related to its abuse. Because of their modulatory functions, the development of novel drug therapies aimed at targeting mGluRs has generated tremendous interest in recent years (Swanson et al., 2005; Marino and Conn, 2006). Findings of our study provide a comprehensive map of the various targets in the nucleus accumbens whereby group I mGluR antagonists may mediate their beneficial effects in relieving behavioral symptoms and dependence associated with addiction to drugs of abuse.

Chapter 3:

Specific Aim 2:

To analyze the subcellular and subsynaptic localization of mGluR1a and mGluR5 in neurons contacted by glutamatergic afferents from the prefrontal cortex and midline thalamus.

3.1 Abstract

Glutamatergic transmission in the nucleus accumbens has been implicated in reward-mediated behaviors. The nucleus accumbens receives glutamatergic afferents from numerous sources, including the limbic prefrontal cortex and midline thalamus. The group I metabotropic glutamate receptors, mGluR1a and mGluR5, are found throughout the core and shell of the nucleus accumbens, but their localization and relationship to specific glutamatergic afferents remains unknown. Therefore, we used a combination of anterograde tract-tracing and electron microscopic immunocytochemistry to study the relationships between cortical or thalamic terminals and mGluR1a- or mGluR5-containing neurons in the rat nucleus accumbens. Overall, limbic prefrontal cortical terminals and midline thalamic terminals contacted both mGluR1a- and mGluR5-immunoreactive dendrites and spines in both the shell and core, but to varying degrees. Although cortical and thalamic terminals contacted mGluR1a-containing spines about 30% of the time, thalamic terminals formed synapses twice more frequently (~60% of the time) with mGluR5-IR spines than cortical terminals (~30% of the time). Using immunogold analysis to examine the subsynaptic localization of the group I mGluRs in serial sections of anterogradely labeled terminals, mGluR5 was found to be more frequently expressed perisynaptic at both cortical and thalamic synapses than mGluR1a. In addition, extrasynaptic plasma membrane-bound mGluR5 labeling was closer to the edges of cortical and thalamic asymmetric synapses than mGluR1a labeling, suggesting a differential degree of activation of the two group I mGluRs by glutamate spillover at cortical and thalamic synapses in the rat accumbens. The findings of this study provide a substrate by which glutamate may induce group I mGluRs-mediated effects in accumbens

neurons following cortical and thalamic stimulation. They also lay a solid foundation for the interpretation of physiological studies examining the role of these receptors in accumbal-related functions.

3.2 Introduction

Glutamate is a key neurotransmitter of accumbens-mediated functions in reward and addictive behaviors (Robinson & Berridge, 2003). The nucleus accumbens, which is divided into the shell and core, receives glutamatergic innervation from various brain nuclei, including the prefrontal cortex, thalamus, hippocampus and amygdala (Groenewegen et al., 1987; Berendse & Groenewegen, 1990; McDonald, 1991, Berendse et al., 1992; French & Totterdell, 2004). It has been shown that specific regions of these glutamatergic nuclei have segregated projections to the different areas of the accumbens. For example, the dorsal region of the limbic prefrontal cortex (PFC) projects to the core of the accumbens, while the ventral region projects to the shell (Berendse et al, 1992). The intermediodorsal (IMD) and central medial (CM) nuclei of the midline thalamus project to the core, while the paraventricular nucleus (PVN) targets the shell (Berendse & Groenewegen, 1990). Finally, the same divisions hold true for the amygdala and hippocampus, with the anterior basolateral amygdala sending afferents to the core, while the posterior targets the shell (McDonald, 1991) and the dorsal subiculum nuclei of the hippocampus projecting to the core while the ventral region projects to the shell (Groenewegen et al., 1987).

In general, glutamatergic inputs depolarize accumbal neurons, interact with dopamine, and regulate various behaviors such as motor output, exploratory behaviors, learning, and most importantly, reward-mediated behaviors (Taber & Fibiger 1995, You et al., 1998; Young & Deutch, 1998; Pinto et al., 2003; Baker et al., 2003; Schmidt et al., 2005; Parsons et al., 2007). The group I metabotropic glutamate receptors are widely expressed G-protein coupled receptors (GPCRs) in the core and shell of the accumbens

(Testa et al, 1994; Mitrano & Smith, 2007) where they play an important role in the neural mechanisms involved in addiction to psychostimulants (Swanson et al., 2001; Chiamulera et al., 2001; McGeehan & Olive, 2003). However, their localization in relation to the major glutamatergic afferents to the accumbens remains unknown. The first goal of this study was, therefore, to determine whether group I mGluRs are expressed in neurons contacted by cortical or thalamic afferents. This could provide insight into how the group I mGluRs may modulate glutamate in the accumbens following either cortical or thalamic stimulation, which is important in furthering our understanding of their function in reward mediated behaviors since both the cortico- and thalamo-striatal pathways have been implicated in the effects of psychostimulants (Deutch et al., 1998; Young & Deutch, 1998; McFarland et al., 2003).

In the rat nucleus accumbens, mGluR1a and mGluR5 are mainly extrasynaptic on the plasma membrane of both dendrites and spines (Mitrano & Smith, 2007a). These receptors are likely to be activated by spillover of vesicular glutamate out of the synaptic cleft or from glial glutamate release into the extracellular space (Rothstein et al., 1996; Danbolt, 2001; Baker et al., 2002). Therefore, the degree of activation of these receptors is highly dependent on their relative proximity to the sites of glutamate release. To further characterize the substrate that may underlie such functional relationships at cortical or thalamic synapses, another goal of this study was to determine the spatial relationships between extrasynaptic mGluR1a and mGluR5 immunoreactivity and cortical or thalamic axospinous synapses.

To achieve these goals, we combined the anterograde labeling of projections from the limbic PFC or midline thalamus with electron microscopic immunocytochemistry for

mGluR1a and mGluR5 in the shell and core of the rat nucleus accumbens. Some of these data have been previously presented in abstract form (Mitrano & Smith, 2007b).

3.3 Methods

3.3.1 Animals & Treatments: A total of 17 male, adult Sprague-Dawley rats (weighing between 200-300g) were used in this study. Rats were anesthetized with isoflurane, fixed in a stereotaxic frame (Kopf, Tujunga, California), and a glass micropipette containing the anterograde tracer, biotinylated dextran amine (BDA, 10 000MW, Invitrogen, Carlsbad, California) was placed in the limbic PFC, PVN or CM/IMD of the thalamus. The coordinates used were as follows: PFC: +3.2 A-P, +0.5 M-L, -4.0 D-V; CM/IMD: -2.8 A-P, -1.6 M-L, -6.4 D-V, 15° lateral angle; PVN: -1.6 A-P, -1.2 M-L, -5.43 D-V, 13° lateral angle, all based on coordinates from the stereotaxic atlas of Paxinos & Watson (1998). Because the CM and IMD are in close proximity to each other and both project to the accumbens core, injections into these two nuclei were grouped together. The iontophoretic delivery of BDA was performed with a 7μA positive current for 20 minutes via a 7-sec on/7-sec off cycle. After a 7-day survival period, the animals were transcardially perfused with cold oxygenated Ringer's solution followed by a fixative containing 4% paraformaldehyde and 0.1% glutaraldehyde in phosphate buffer (0.1M; pH 7.4). Following perfusion, brains were removed from the skull and post-fixed in 4% paraformaldehyde for 24 hours.

3.3.2 Tissue Processing: Following fixation, all brains were cut at 60μm on a vibrating microtome and processed for light and electron microscopy immunoperoxidase or immunogold localization of BDA, Calbindin-D28k, mGluR1a and mGluR5. Prior to

immunocytochemical processing, all sections were put into a 1% sodium borohydride solution for 20 minutes and then washed with PBS.

3.3.3 Primary Antibodies: A commercially available monoclonal antibody against calbindin-D_{28k} (Sigma, St. Louis, MO; Cat# C-9848, Lot# 082k4879) was used at a concentration of 1:5000 to distinguish between the accumbens shell and core. The calbindin-D_{28k} antibody is derived from CB-955 hybridomas produced by fusion of mouse myeloma cells and splenocytes from BALB/c mice that were immunized with purified bovine kidney calbindin-D_{28k}. The specificity of this antibody has been demonstrated through preadsorption immunohistochemical assays that abolish calbindin labeling (Celio, 1990), through Western blot analysis of rat brain tissue which shows a distinct band at 28kD (Miyata et al., 2000) and through immunohistochemistry which shows calbindin immunostaining in brain regions known to express a significant level of calbindin-D_{28k} mRNA (Celio et al., 1990; Miyata et al., 2000; Winsky et al., 1989).

To localize mGluR1a, an affinity-purified rabbit polyclonal antibody against the C-terminus of rat mGluR1a (PNVTYASVILRDYKQSSSTL) conjugated to KLH with glutaraldehyde was used at a concentration of 1:1000 (Millipore, Temecula, CA; Cat# AB1551). In Western blot analysis by the manufacturer, this antibody labels a single band of ~140kD. Previous studies from our lab and others have used a combination of knock-out mice, transfected HEK-293 cells, and preadsorption to determine the specificity of this mGluR1a antiserum. These studies showed that brain tissue from mGluR1a knockout mice did not display any specific mGluR1a labeling compared to wild-type. In addition, immunoblotting of cells transfected with mGluR1a, but not

mGluR5, labeled a band of 140kD (Kuwajima et al., 2004). Preadsorption studies in rat retina cells abolished mGluR1a labeling (Koulen et al., 1997).

An affinity-purified synthetic rabbit polyclonal antibody against the C-terminus of mGluR5 with a lysine added to the N-terminus (KSSPKYDTLIIRDYTNSSSSL) in a concentration of 1:5000 (Millipore, Temecula, CA; Cat# 06-451) was used to label mGluR5. According to the manufacturer's immunoblot analysis, the mGluR5 antibody labels a band of ~130kD. Specificity of the mGluR5 antibody has been shown in previous studies from our laboratory using knockout mice, transfected cells and homogenates of rat brain. These studies showed that brain tissue from mGluR5 knockout mice do not stain for mGluR5 and HEK-293 cells transfected with mGluR5 label a band of the correct molecular weight (Kuwajima et al., 2004). Furthermore, immunoblot analysis on proteins isolated from various brain regions labels a band that corresponds to the size of mGluR5 in regions known to express mGluR5 protein and mRNA (Mannaioni et al., 2001).

3.3.4 Light Microscopic Observations

3.3.4.1 Single Immunoperoxidase for Light Microscopy: To reveal the injection site and anterograde tracer labeling in the accumbens, 1 out of every 6 sections of the entire brain were rinsed in PBS and then incubated 90 minutes with the avidin-biotin peroxidase complex (ABC) at a dilution of 1:100 (Vector Laboratories). The sections were then washed in PBS and Tris buffer (50mM; pH 7.6) and transferred to a solution containing 0.025% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma, St Louis, MO), 10mM imidazole, and 0.005% hydrogen peroxide in Tris buffer for 10 minutes. Sections were rinsed in PBS, mounted onto gelatin-coated slides, dehydrated and then coverslipped with

Permount. The tissue was examined with a Leica DMRB microscope (Leica Microsystems, Inc., Bannockburn, IL) and images were taken using a CCD camera (Leica DC500) which was controlled by Leica IM50 software.

In the striatum, 1 out of every 6 sections was stained for calbindin-D_{28k} to distinguish the core-shell boundaries for subsequent EM studies. Following sodium borohydride treatment, sections were incubated for 1 hour at RT in PBS containing normal horse serum (NHS), 1% BSA and 0.3% Triton X-100, followed by the primary antibody solution containing 1% NHS, 1% BSA, and 0.3% Triton X-100 in PBS for 24 hours at RT. After three rinses in PBS, sections were incubated in secondary biotinylated horse anti-mouse IgGs at a concentration of 1:200 (Vector Laboratories, Burlingame, CA) for 90 minutes. The sections were then rinsed in PBS and exposed to the ABC complex as described above.

3.3.4.2 Nissl Staining for CM/IMD Thalamic Injections: Due to the difficulty in distinguishing limits between thalamic nuclei, we used Nissl stain to help delineate thalamic nuclei borders and determine the exact location of injection sites aimed at CM/IMD. The BDA-stained tissue was then exposed to a series of alcohol dilutions from 100% down to 50%. Following the 50% alcohol bath, the tissue was soaked in distilled water for 2 minutes, and then placed in 0.1% thionin for 1-2 minutes followed by 1% acetic acid in distilled water until the white matter returned to white. The sections were transferred to a 1% acetic acid bath in 70% alcohol to remove any remaining blue, and then were exposed to 95% and 100% alcohol baths (5 minutes each) and finally toluene for 5 minutes before coverslipping.

3.3.5 Electron Microscopic Observations

3.3.5.1 Double Pre-embedding Peroxidase labeling for BDA, mGluR1a and mGluR5:

Following sodium borohydride treatment, sections were placed in a cryoprotectant solution for 20 minutes (PB 0.05M, pH 7.4, 25% sucrose, and 10% glycerol), frozen at -80°C for 20 minutes, returned to a decreasing gradient of cryoprotectant solutions, and rinsed in PBS. Sections were then incubated for 1 hour at RT in PBS containing 10% normal goat serum (NGS), and 1% BSA, followed by the primary antibody solution containing 1% NGS, and 1% BSA in PBS for 48 hours at 4°C. After three rinses in PBS, sections were incubated in secondary biotinylated goat anti-rabbit IgGs at a concentration of 1:200 (Vector Laboratories, Burlingame, CA) for 90 minutes, rinsed in PBS and then exposed to ABC and DAB as described above. After the DAB reaction, the tissue was rinsed in PB (0.1M, pH 7.4) and treated with 1% OsO_4 for 20 minutes. It was then returned to PB and dehydrated with increasing concentrations of ethanol. When exposed to 70% ETOH, 1% uranyl acetate was added to the solution for 35 minutes to increase the contrast of the tissue at the electron microscope. Following dehydration, sections were treated with propylene oxide and embedded in epoxy resin for 12 hours (Durcupan ACM, Fluka, Buchs, Switzerland), mounted onto slides and placed in a 60°C oven for 48 hours. Separate samples of the nucleus accumbens core and medial shell (depending on the initial injection site) were cut out of the larger sections, mounted onto resin blocks and cut into 60-nm sections using an ultramicrotome (Leica Ultracut T2). The 60-nm sections were collected on Pioloform-coated copper grids, stained with lead citrate for 5 minutes to enhance tissue contrast and examined on the Zeiss EM-10C electron microscope. Electron micrographs were taken and saved with a CCD camera (DualView 300W; Gatan, Inc., Pleasanton, CA) controlled by Digital Micrograph software (version

3.10.1, Gatan, Inc., Pleasanton, CA). Some of the digitally acquired electron micrographs were adjusted only for brightness or contrast using either the Digital Micrograph software or Adobe Photoshop software (version 8.0, Adobe Systems Inc., San Jose, CA). Micrographs were then compiled into figures using Adobe Illustrator (version 11.0, Adobe Systems Inc., San Jose, CA).

3.3.5.2 Double Pre-embedding immunoperoxidase (BDA) & immunogold (mGluR1a & mGluR5): Following sodium borohydride and cryoprotectant treatments, sections were incubated for 30 minutes in PBS containing 5% dry milk at RT and then rinsed in TBS-gelatin buffer (0.02M and pH 7.6). Sections were then transferred to primary antibody solutions with 1% dry milk in TBS-gelatin buffer for 24 hours at room temperature and then rinsed again in TBS-gelatin. After rinses, sections were treated for 2 hours at RT with secondary goat anti-rabbit IgGs conjugated with 1.4nm gold particles at a concentration of 1:100 (Nanoprobes, Yaphank, NY) diluted with 1% dry milk in TBS-gelatin. Sections were rinsed in TBS-gelatin and 2% sodium acetate buffer before gold particles were silver intensified to 30-50nm using the HQ silver kit (Nanoprobes) for approximately 10 minutes. Following silver intensification, the ABC and DAB procedures were the same as those used in single immunoperoxidase for light microscopy. Immediately following the DAB reaction, the sections were rinsed with PB (0.1M, pH 7.4) and then treated according to the same protocol of osmification, dehydration, embedding, and tissue selection described above for the immunoperoxidase procedure including the following changes: 1) the tissue was kept in 0.5% OsO_4 for 10 minutes instead of 20 and 2) the tissue was stained with 1% uranyl acetate for 10 minutes instead of 35.

3.3.6 Analysis of Material

3.3.6.1 Double Immunoperoxidase Labeling for BDA and Group I mGluRs

Data was collected from 4 animals (each) injected in the PFC, or PVN, and 3 animals injected in the CM/IMD. This yielded a total of 8 blocks taken from the core and 8 blocks from shell of the accumbens from animals injected in the PFC, 8 from the nucleus accumbens shell of animals injected in the PVN, and 6 blocks from the nucleus accumbens core of animals injected in the CM/IMD. Serial ultrathin sections from the surface of each block were taken and examined on the electron microscope for positively labeled axon terminals (based on ultrastructural features described by Peters et al., 1991). Electron micrographs of labeled terminals (regardless of labeling in the postsynaptic element) were digitized at 31,500X. Approximately 20-30 labeled terminals were photographed per animal and the proportion of these terminals in contact with mGluR1a- or mGluR5-immunoreactive (IR) spines and dendrites was calculated for each animal, and the mean percentage of terminals forming synapses on either population of mGluR-containing elements was averaged across the number of animals and compared between injection sites and receptors using a series of t-tests in Sigma Stat software (version 2.03, Systat Software, San Jose, CA).

3.3.6.2 Double Pre-embedding Immunoperoxidase for BDA & Immunogold for mGluR1a or mGluR5

In order to assess the spatial relationships between mGluR1a and mGluR5 and synapses formed by the different thalamic and cortical inputs, the receptors were revealed with immunogold, while the tracer was localized with immunoperoxidase. Positively labeled terminals in contact with either mGluR1a- or mGluR5-IR spines or dendrites

were followed through a series of 3-6 serial sections in order to examine the pattern of immunogold labeling at individual synapses. Serial sections were used in this part of the study to ensure that the bulk of immunogold labeling was detected as its abundance and location can vary from section to section, even in 60 nm-thick sections. Data were collected from 3 animals (each) injected in the PFC, PVN, or CM/IMD. This yielded a total of 12 blocks taken from both the core and shell of the accumbens from animals injected in the PFC, 6 from the nucleus accumbens shell of animals injected in the PVN, and 6 blocks from the nucleus accumbens core of animals injected in the CM/IMD. Serial ultrathin sections were taken from each of the blocks and examined on the electron microscope for positively labeled axon terminals. Electron micrographs of labeled terminals in contact with gold-containing postsynaptic elements were digitized at 31,500X. Approximately 20-30 terminals were photographed per animal and followed through 3-6 serial sections. The pattern of gold labeling was classified as intracellular or plasma membrane-bound based on criteria defined in previous studies (Galvan et al., 2006; Mitrano & Smith, 2007; Mitrano et al., 2008). The plasma membrane-bound gold particles were further classified into three categories: perisynaptic (touching or within a 20 nm range of the edges of postsynaptic specializations); synaptic (in contact with the main body of postsynaptic specializations); or extrasynaptic (on the plasma membrane, but not associated with synapses). The proportion of spines that expressed perisynaptic labeling when contacted by a labeled terminal was determined and averaged across the number of animals and compared between input nuclei and receptors using one-way ANOVAs in Sigma Stat software (version 2.03, Systat Software, San Jose, CA). In addition, to assess potential differences in the localization of extrasynaptic receptors in

relation to cortical or thalamic inputs, the distance between the closest extrasynaptic gold particles and the edges of the postsynaptic density was measured using Image J software (National Institutes of Health) and then averaged across the number of animals and compared between regions and receptors as described for the perisynaptic labeling.

3.4 Results

3.4.1 Light Microscopic Observations

In order to determine that the injection sites were properly located, we first examined tissue from every 6th section through the rostrocaudal extent of the injected site and compared the pattern of anterograde labeling in the cortical and striatal regions with that described in previous studies using the same or other anterograde tracers (Berendse & Groenewegen, 1990; Berendse et al., 1992). The accumbens tissue was only used for further study if it was determined that the injection site was in the targeted nuclei and the corresponding area of the accumbens contained anterograde labeling. Figure 3.1 shows examples of cortical and thalamic injection sites and resulting anterograde labeling in the nucleus accumbens.

3.4.2 Electron Microscopic Observations

3.4.2.1 Double Pre-embedding Immunoperoxidase Labeling for BDA and Group I mGluRs

In order to determine the proportion of cortical and thalamic terminals in contact with positively labeled mGluR1a- or mGluR5-IR postsynaptic elements we used a double immunoperoxidase method. Examples of tissue used in these studies are depicted in figure 3.2. The majority of positively labeled terminals from the PFC, PVN or CM/IMD formed axospinous synapses, while axodendritic synapses were less commonly seen.

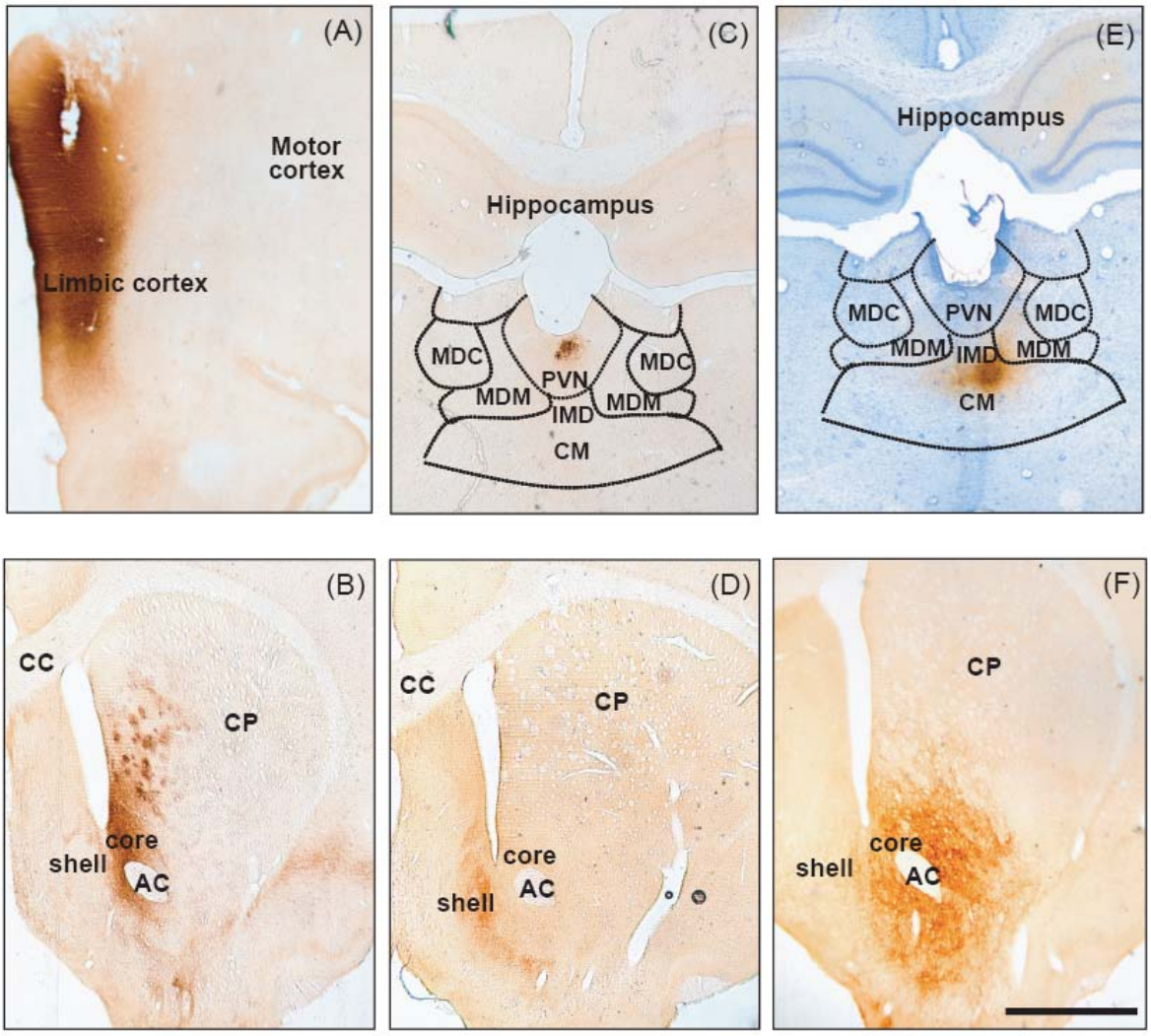

Figure 3.1: BDA injection sites in the PFC (A) and thalamus (C, E) with resulting anterograde labeling in the shell and core of the nucleus accumbens (B, D, F). (A) BDA injection site in the dorsal and ventral limbic prefrontal cortex. (B) Anterograde labeling in the core of the nucleus accumbens and the ventral medial caudate-putamen after PFC injection shown in (A). (C) BDA injection site in the PVN of the thalamus. The limit of surrounding thalamic nuclei is schematically drawn based on adjacent Nissl stained sections to better illustrate the specificity of the PVN injection. (D) shows the anterograde labeling confined to the shell of the accumbens following the PVN injection shown in (C). (E) shows an injection site in the CM/IMD while (F) illustrates the anterograde labeling in the core of the nucleus accumbens that resulted from the injection. Abbreviations: CP: caudate-putamen, CC: corpus callosum, AC: anterior commissure, MDC: mediodorsal thalamic nuclei, central part; MDM: mediodorsal thalamic nuclei, medial part. Scale bar= 0.5mm.

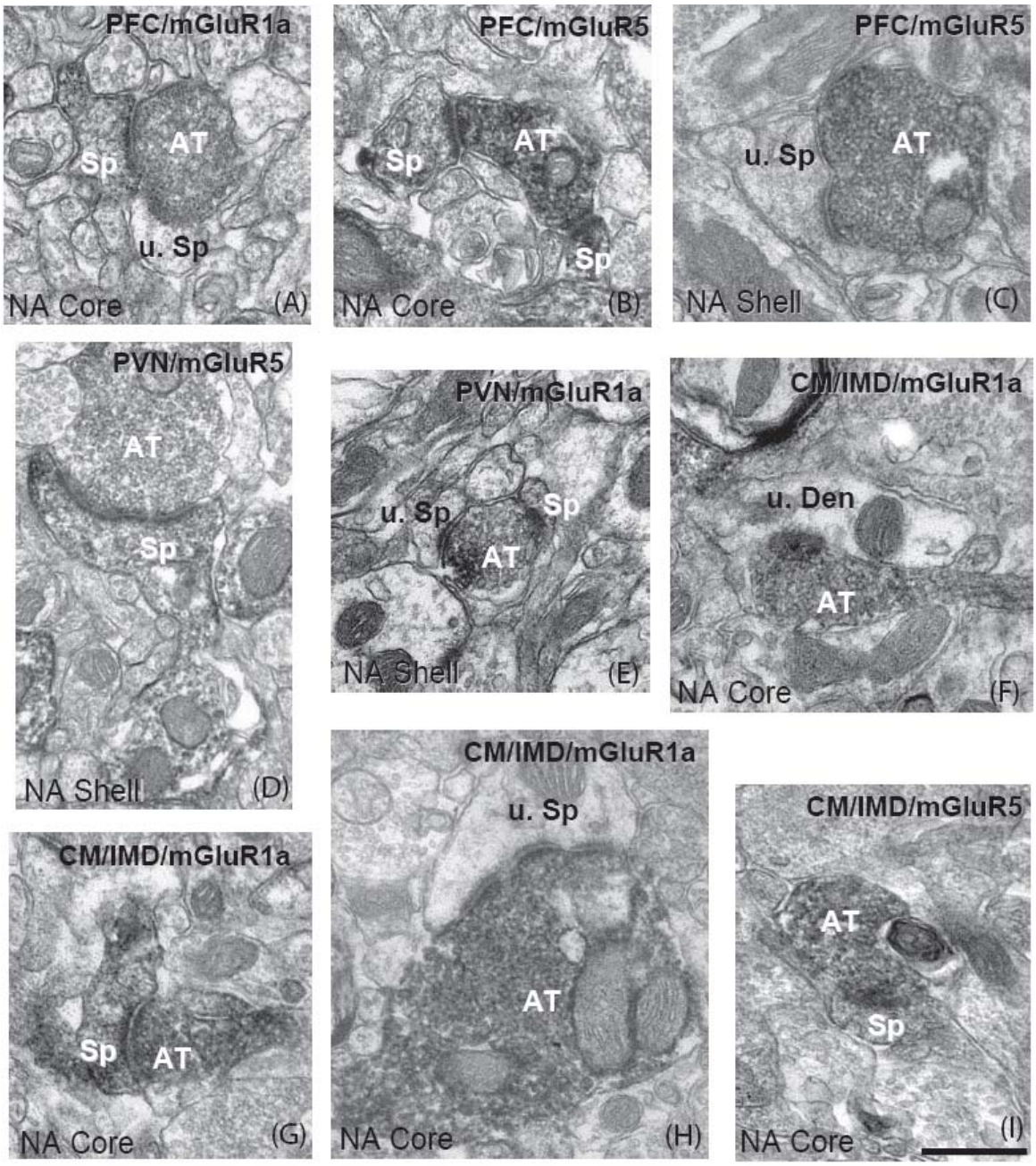

Figure 3.2: Double Immunoperoxidase for BDA and Group I mGluRs. (A-B) Electron micrographs of labeled cortical terminals from the dorsal limbic PFC in the core of the nucleus accumbens, while (C) shows a cortical terminal from the ventral limbic PFC in the shell. The location of injection site and the receptor subtype the tissue is immunostained for are indicated in the upper right of each micrograph. (D-E)

Micrographs of labeled terminals from the PVN with labeling for both group I mGluRs in spines in the shell of the nucleus accumbens. (F) Shows an example of a terminal arising from the CM/IMD in contact with an unlabeled dendrite in the core of the accumbens. (G-I) Shows CM/IMD terminals in contact with labeled or unlabeled spines in mGluR1a- and mGluR5-immunostained accumbens tissue. Abbreviations: AT: labeled axon terminal; Sp: labeled spine for receptor indicated; u. Sp: unlabeled spine for receptor indicated; u. Den: unlabeled dendrite for receptor indicated. Scale bar=0.2μm.

The quantification of these data is shown in figure 3.3. Figures 3.3A-B show the average percentage of labeled terminals (±SEM) contacted by either (A) mGluR1a- or (B) mGluR5-IR spines. Terminals from the PFC were in contact with mGluR1a-IR spines in the accumbens core 32.5±8.7% of the time and 35.7±6.3% of the time in the accumbens shell. A similar pattern was found for terminals arising from the CM/IMD or PVN in contact with mGluR1a-IR spines (31.5±1.2% and 34.1±1.2%, respectively). This is in comparison to approximately 30-40% of cortical terminals contacted by mGluR5-IR spines and about 60% of thalamic terminals synapsing on mGluR5-IR spines. In comparison, 44.7±4.7% of cortical terminals were in contact with mGluR5-IR spines in the core and 35.1±7.0% in the shell. On the other hand, 57.3±8.5% of CM/IMD terminals were in contact with mGluR5-labeled spines in the core, while 60.1±4.5% of the terminals from the PVN were in contact with mGluR5-labeled spines in the shell.

Statistical analysis revealed a significant difference in the percentage of labeled cortical terminals in contact with mGluR5-labeled spines (35.1±7.0%) compared with the percentage of thalamic terminals in contact with mGluR5-IR spines (60.1±4.5%; $t=-3.0$, $p<0.05$; Figure 3.3B) in the accumbens shell. To better illustrate the comparison between receptor types, data from 3.3A and 3.3B were shown again in figure 3.3E. As shown, there are significantly more CM/IMD thalamic terminals in contact with mGluR5-IR spines (57.3±8.5%) than mGluR1a-IR spines (31.5±1.2%; $t=3.0$, $p<0.05$). In the accumbens shell, the same pattern was observed; i.e. significantly more PVN thalamic terminals contacted mGluR5-IR spines (60.1±4.5%) than mGluR1a-IR spines (34.1±1.2%, $t=5.5$, $p=0.001$). While an initial thought could be that this result was just a technical oversight due to the fact that there are more mGluR5-containing spines than

mGluR1a-containing spines in the accumbens we decided to address this issue further. We counted the number of labeled and unlabeled spines in a series of micrographs (30 micrographs/animal/injection site) from accumbal tissue labeled with either of the two group I mGluR antibodies, and found that approximately 50% or about 20 labeled spines/100μm^2 were present in both the shell and core of accumbens tissue immunostained for mGluR1a or mGluR5.

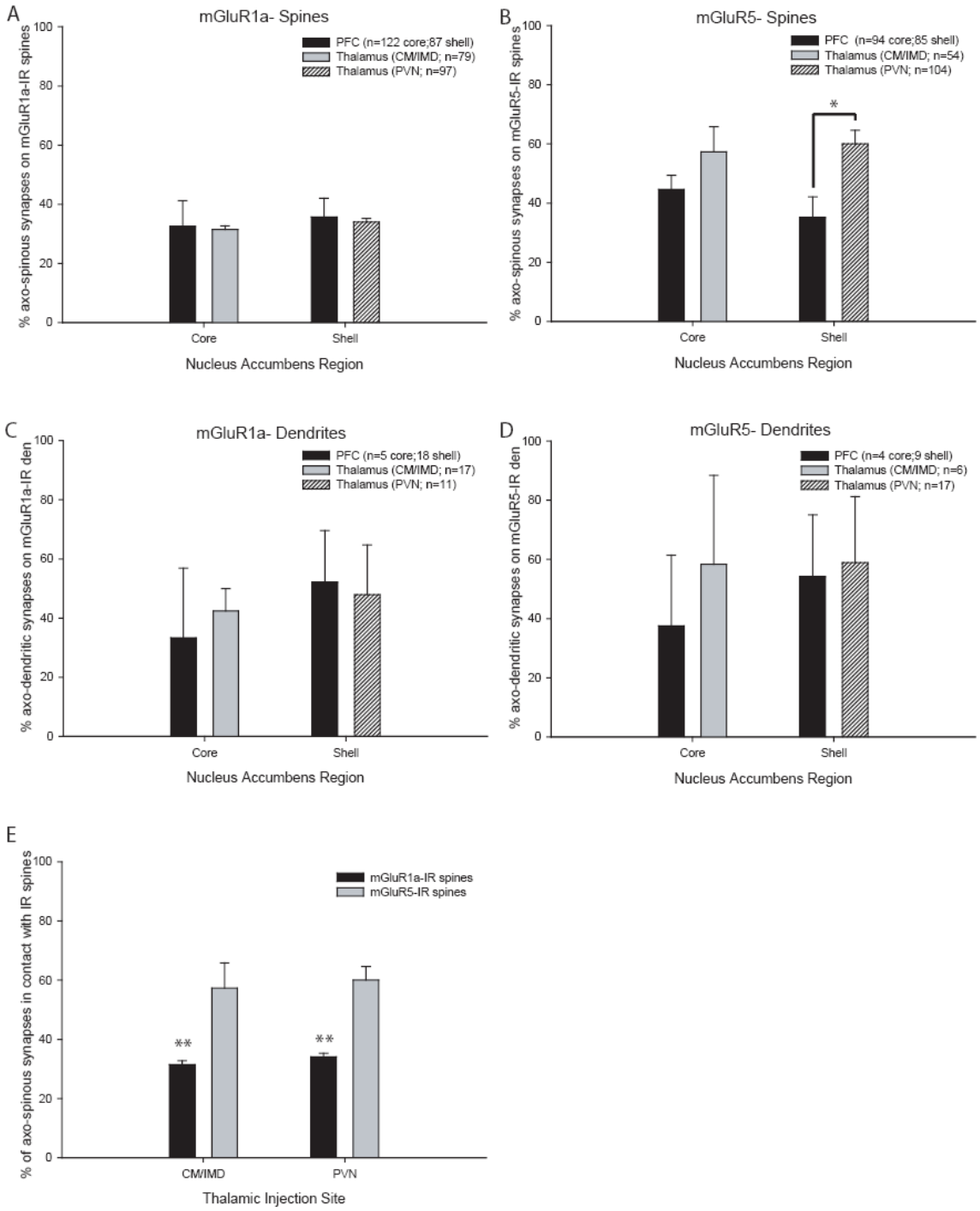

Figure 3.3: Histograms showing the percentage of labeled terminals in contact with mGluR1a- or mGluR5-IR spines and dendrites. (A-B) summarize the mean percentage (±SEM) of labeled terminals from the cerebral cortex or the different thalamic nuclei in

contact with mGluR1a-IR (A) or mGluR5-IR (B) spines in the core and shell of the nucleus accumbens. In parentheses the "n" represents the total number of terminals in the accumbens examined from 4 animals injected in the PFC, 4 in the PVN and 3 in the CM/IMD. In B, the single asterisk indicates a significantly higher percentage of positive terminals from the PVN than the PFC in contact with mGluR5-IR spines in the shell of the accumbens ($p<0.05$, $t=-3.0$). (C-D) represent the percent of labeled terminals in contact with mGluR1a-IR (C) and mGluR5-IR (D) dendrites in the core and shell of the accumbens. Note the small number of terminals examined that were in contact with dendrites (n in parentheses) compared to spines. There was no significant difference between regions or receptor types for axodendritic synapses. (E) shows a comparison of the relative percentages of mGluR1a- and mGluR5-immunoreactive spines contacted by different populations of thalamic terminals in the core and shell of the nucleus accumbens. The double asterisks indicate a significantly lower percentage of thalamic terminals from the CM/IMD ($p<0.05$, $t=3.0$) and PVN ($p=0.001$, $t=5.5$) in contact with mGluR1a-IR spines compared with mGluR5-IR spines in the accumbens core and shell, respectively.

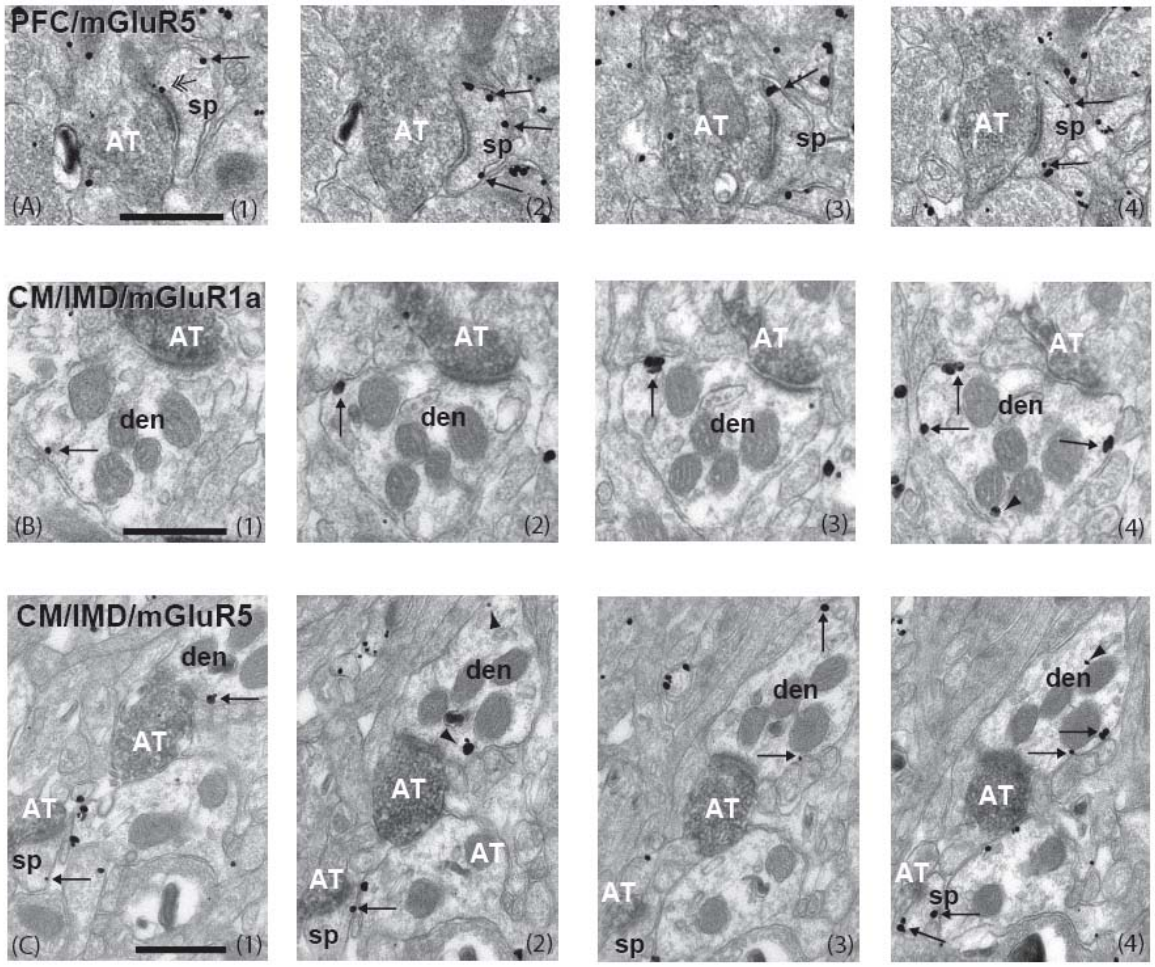

Figure 3.4: Serial sections of double immunostained sections for BDA (immunoperoxidase) and group I mGluRs (immunogold). (A1-4) show a series of micrographs of a positively labeled axon terminal (AT) from the PFC forming an asymmetric synapse with a mGluR5-labeled spine (sp) in the accumbens core. Note that the majority of plasma membrane-bound labeling is extrasynaptic (single arrows); except for A1 that shows an example of perisynaptic mGluR5 labeling (double arrowhead). (B1-4) shows four micrographs of extrasynaptic mGluR1a-labeling on a dendrite (den) contacted by an AT from the CM/IMD. (C1-4) shows an example of a mGluR5-IR dendrite and spine contacted by positively labeled AT from the CM/IMD with all of the plasma membrane-bound labeling being extrasynaptic. There is also intracellular labeling in the dendrite (arrowheads). Scale bar=0.2μm.

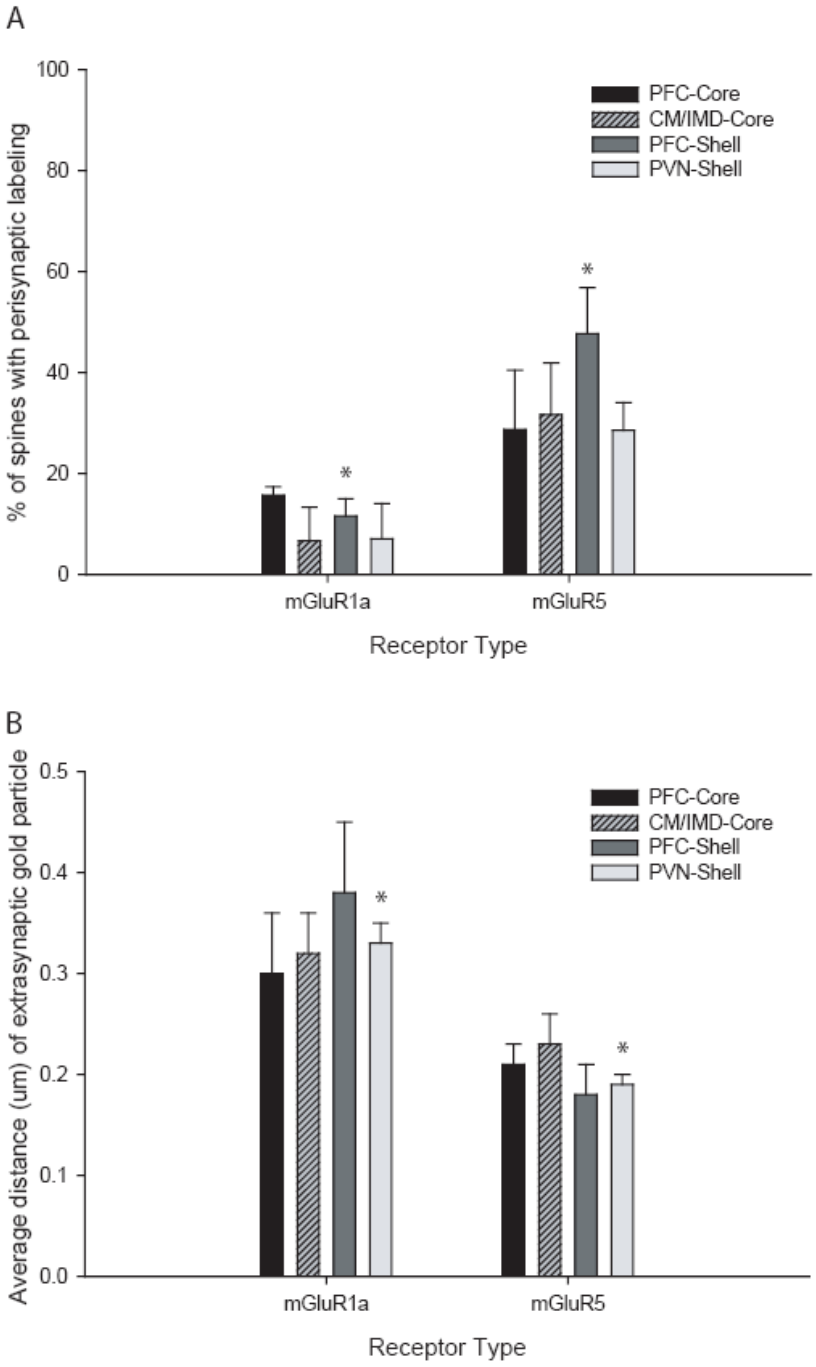

Figure 3.5: Histograms showing the subsynaptic distribution of group I mGluRs in relation to asymmetric postsynaptic specialization of cortical or thalamic axospinous synapses. (A) displays the mean percent of spines (±SEM) that showed perisynaptic labeling for either mGluR1a or mGluR5 in the core and shell when contacted by a

positively labeled terminal from the PFC or thalamus. The asterisk indicates that perisynaptic mGluR5 labeling at PFC synapses is significantly more frequent than mGluR1a in the shell of the nucleus accumbens (one-way ANOVA and Tukey's post hoc, $p<0.05$). Total number of terminals followed through 3-6 serial sections of 3 animals each: PFC/mGluR1a Core=64; PFC/mGluR1a Shell= 60; PFC/mGluR5 Core=73; PFC/mGluR5 Shell=75; CM/IMD/mGluR1a Core=64; PVN/mGluR1a Shell= 58; CM/IMD/mGluR5 Core=63; PVN/mGluR5 Shell=61. (B) illustrates the average distance of the closest extrasynaptic gold particle (μm$\pm$SEM) from the edge of the postsynaptic density (PSD) in mGluR1a- or mGluR5-labeled spines in asymmetric contact with an anterogradely labeled terminal from the PFC or thalamus. The asterisks indicate that mGluR5 is closer to the edges of PVN synapses compared to mGluR1a in the shell of the accumbens (one-way ANOVA and Tukey's post hoc test; $p<0.05$).

While the bulk of glutamatergic afferents from both the cortex and thalamus contacted spines, some positively labeled terminals formed asymmetric axo-dendritic synapses (example, Figure 3.2F). Figures 3.3C-D shows the percentage (±SEM) of positively labeled terminals contacted by (C) mGluR1a- or (D) mGluR5-IR dendrites. On average, 33.3±23.6% of terminals arising from the limbic PFC contacted mGluR1a-IR dendrites in the core and 52.1±17.5% in the shell. In the case of thalamic projections, 42.4±7.6% of CM/IMD terminals and 47.9±16.8% of terminals from the PVN were in contact with mGluR1a-IR dendrites.

As for mGluR5 labeling, 37.5±23.9% of cortical terminals were in contact with mGluR5-IR dendrites in the accumbens core and 54.2±20.8% in the shell. Terminals from the CM/IMD were in contact with mGluR5-labeled dendrites 58.5±30.1% of the time, while terminals from the PVN were in contact with mGluR5-labeled dendrites 58.9±22.3% of the time. Due to the small number of axo-dendritic synapses examined, there was a large degree of inter-individual variability, thereby, no significant differences were found between projections or receptor type.

3.4.2.2 Double Pre-embedding Immunoperoxidase for BDA & Immunogold for mGluR1a or mGluR5

Since a significant proportion of terminals originating from the limbic PFC, PVN and CM/IMD contact mGluR1a- and mGluR5-containing neurons in the core and shell of the accumbens, a second series of experiments was achieved to determine the pattern of subsynaptic labeling for mGluR1a and mGluR5 in relation to synapses formed by anterogradely labeled terminals in contact with dendrites and spines. For this part of the study, the receptors immunoreactivity was revealed with immunogold, which yields a

higher level of spatial resolution compared to the immunoperoxidase method. Figure 3.4 shows examples of labeling through serial sections in the core and shell of the accumbens.

The histograms in Figure 3.5 show the patterns of perisynaptic (A) or extrasynaptic (B) labeling in spines contacted by positively labeled terminals. The percent of synaptic labeling was also quantified, but due to the very low incidence of synaptic group I mGluRs immunoreactivity, data are not presented in graph format here. Overall, less than 10% of spines contacted by cortical or thalamic terminals displayed synaptic labeling in asymmetric synapses for both group I mGluRs. In addition, the pattern of subsynaptic labeling in dendrites contacted by positive terminals was calculated as well, but the significance of these data is limited by the small sampling of dendrites (as shown in figure 3.3), which make these findings hard to interpret.. In general, dendrites displayed a lower percentage of perisynaptic and synaptic labeling compared to positively labeled spines, but a similar extrasynaptic pattern of labeling to the data for spines presented in figure 3.5B.

As is shown in figure 3.5A, mGluR5-IR spines generally had a higher degree of perisynaptic labeling compared to mGluR1a regardless of the afferent. mGluR1a was perisynaptic on spines 15.7$\pm$1.7% of the time in the core and 11.6$\pm$3.4% of the time in the shell when in contact with a PFC terminal. A similar pattern was seen in relation to labeled thalamic terminals, with 6.7$\pm$6.7% of spines in contact with CM/IMD terminals displaying perisynaptic mGluR1a labeling and 7.0$\pm$7.0% of spines containing perisynaptic labeling when in contact with a PVN terminal. mGluR5 was perisynaptic on spines 28.7$\pm$11.7% of the time in the core and 47.7$\pm$9.1% of the time in the shell when in

contact with a terminal from the PFC. In relation to thalamic terminals, 31.6±10.3% of spines expressed perisynaptic mGluR5 labeling when in contact with a terminal from the CM/IMD and 28.5±5.5% of the spines in contact with PVN terminals. Although there was a general trend of mGluR5 having a greater percentage of perisynaptic labeling, using a one-way ANOVA and Tukey's post hoc tests, there was only a significantly higher percentage of mGluR5 perisynaptic labeling (47.7±9.1%) compared to mGluR1a perisynaptic labeling (11.6±3.4%) in the shell of the accumbens when the spines were in contact with positive cortical terminals ($F(3, 11) = 4.392$, $n=3$, $p<0.05$).

Figure 3.5B summarizes the data examining the distance of the closest plasma membrane-bound extrasynaptic gold particle in the series of sections. Overall, mGluR5 showed a trend of being closer to the edges of asymmetric synapses on spines when contacted by either cortical or thalamic terminals in both the shell and core of the accumbens. The average distance of extrasynaptic mGluR1a labeling from the PSD on spines in contact with PFC terminals in the core was 0.30±0.06μm and 0.38±0.07μm in the shell. The average distance of extrasynaptic mGluR1a labeling on spines in contact with CM/IMD terminals was 0.32±0.04μm and 0.33±0.02μm on spines in contact with PVN terminals. The average distance of mGluR5 extrasynaptic labeling from the PSD on spines in contact with PFC terminals was 0.21±0.02μm in the core and 0.18±0.03μm in the shell. The average distance of extrasynaptic mGluR5 from the PSD on spines in contact with CM/IMD terminals was 0.23±0.03μm and 0.19±0.01μm on spines in contact with PVN terminals. Using a one-way ANOVA and Tukey's post hoc tests, a significant difference was found between the average distance of mGluR5 (0.19μm±0.01) versus

mGluR1a (0.33μm±0.02) extrasynaptic labeling in relation to asymmetric synapses formed by thalamic PVN terminals ($F(3,11)=5.3$, $n=3$, $p<0.05$).

3.5 Discussion

In summary, the major findings of this study are that terminals from both the limbic prefrontal cortex and midline thalamus form synapses on mGluR1a- and mGluR5-containing spines and, to a lesser degree, dendrites of accumbens projection neurons. Approximately a third of cortical and thalamic terminals were in contact with mGluR1a-labeled spines, while there was more heterogeneity amongst the projections in contact with mGluR5-labeled spines. Although about a third of cortical terminals were also in contact with mGluR5-IR spines, almost two thirds of thalamic terminals from PVN and CM/IMD did contact mGluR5-IR spines the accumbens shell and core, respectively.. In addition, it was found that regardless of the glutamatergic afferents, mGluR5 was found more often perisynaptically and closer extrasynaptically to the active zones of thalamic or cortical synapses compared to mGluR1a. These data provide the first evidence for differences in group I mGluR localization in relation to specific glutamatergic afferents in the nucleus accumbens and lay a solid foundation for the interpretation of group I mGluR function and mechanisms of activation in the nucleus accumbens.

3.5.1 Rationale

Initial studies at the light microscopic level found both of the group I mGluRs throughout the rat brain, including the dorsal striatum and nucleus accumbens (Martin et al., 1992; Romano et al., 1995). Further in-depth analysis of the ventral striatum in both monkey and rat tissue, using light microscopy, revealed that both mGluR1a and mGluR5 labeling was homogeneously distributed in the neuropil of the shell and core of the

accumbens, with very light labeling in cell bodies (Mitrano & Smith, 2007a). Due to this widespread, homogenous distribution of both receptor types, electron microscopy was needed in order to gain a more detailed understanding of the exact subcellular and subsynaptic localization of the group I mGluRs in the accumbens. We found that the mGluR1a and mGluR5 are widely distributed postsynaptically in dendrites and spines of various cell types in both the core and shell of the nucleus accumbens in rats and monkeys (Mitrano & Smith, 2007a). While only minor differences were seen in the subcellular and subsynaptic localization between the core and shell, some differences were seen in the subsynaptic localization of mGluR5 between rat and monkey, with mGluR5 being found more often on the plasma membrane of dendrites and spines in the rat accumbens, compared to the monkey. Overall, though, in both species, the group I mGluRs, when found on the plasma membrane, were mainly extrasynaptic on dendrites and spines, with spines displaying slightly more perisynaptic labeling at asymmetric synapses (Mitrano & Smith, 2007a). Based on this information, the next step was to determine what glutamatergic afferents were in contact with mGluR1a- or mGluR5-containing neurons and what these receptors' subsynaptic localization was in relation to the various inputs. Based on the analysis done in this study, we can get a better understanding of how the group I mGluRs may be activated and by what nuclei in the brain.

As stated previously, the nucleus accumbens receives numerous glutamatergic afferents, including those arising from the cerebral cortex, hippocampus, thalamus and amygdala (Groenewegen et al., 1987; Berendse & Groenewegen, 1990; McDonald, 1991, Berendse et al., 1992; French & Totterdell, 2004). However, the localization of the group

I mGluRs in relation to the various glutamatergic afferents was unknown prior to this study. Ergo, it was not known whether these receptors had the potential to modulate accumbal glutamatergic transmission coming from these various afferents. Earlier studies that combined in vitro electrophysiology and microdialysis showed that glutamate and dopamine release are increased in the accumbens following stimulation of the cortex or the thalamic PVN (Taber & Fibiger, 1995; Pinto et al., 2003; Parsons et al., 2007). Using the glutamate analogue ACPD (trans (1S, 3R)-1-aminocyclopentane-1, 3-dicarboxylic acid), it was shown that metabotropic glutamate receptors modulate the levels and behavioral effects of these neurotransmitters in the nucleus accumbens (Taber & Fibiger, 1995). However, these studies were unable to demonstrate whether subsets of mGluR1a or mGluR5, located at specific glutamatergic synapses, mediated these effects due to the lack of specific agonists and antagonists at that time. A recent study of the electrophysiological properties of cortico- and thalamo-striatal synapses of the dorsal striatum indicated that each of these projections codes information to medium spiny neurons in temporally distinct fashions (Ding et al., 2008); yet another reason for looking at the localization of glutamate receptors at specific synapses. Therefore, our series of anatomical experiments mapping and comparing the localization of the group I mGluRs when in contact with a terminal arising from either the PFC or thalamus provides valuable information in the interpretation of these past studies. We now know that both group I mGluRs are present on a subset of postsynaptic elements in contact with terminals from the PFC and thalamus to varying degrees, and they have the potential to mediate glutamatergic neurotransmission from both of these nuclei.

As stated in the introduction, the group I mGluRs are mainly extrasynaptic on the plasma membrane of dendrites and spines of the various cell types found in the nucleus accumbens (Mitrano & Smith, 2007a). Besides determining whether or not the group I mGluRs were present at cortico- and thalamo-striatal synapses in the accumbens, we also wanted to examine the subsynaptic localization of the group I mGluRs at these synapses using the pre-embedding immunogold method. Because of their extrasynaptic localization, group I mGluRs are likely activated by glutamate spillover out of the synaptic cleft or from glial release (Rothstein et al., 1996; Danbolt et al., 2001; Baker et al., 2002). Therefore, by determining if either extrasynaptic mGluR1a or mGluR5 is found in closer proximity than the other to a cortical or thalamic terminal, or if one of the group I mGluRs displays a higher degree of perisynaptic labeling to a certain afferent could provide additional information on the mechanism(s) of activation of group I mGluRs by each glutamatergic afferent.

3.5.2 Psychostimulant Addiction and Group I mGluRs

The group I mGluRs have been shown to play a role in the rewarding effects of psychostimulants (Ghasemzadeh et al., 1999; Swanson et al., 2001; Chiamulera et al., 2001). Specifically, there have been some studies that have examined the use of group I mGluR antagonists as a means to reduce cocaine intake. The data presented in this study provide a substrate where these drugs may mediate their effects. For example, systemic administration of the mGluR5 antagonist, MPEP, reduces cocaine self administration in both rats and monkeys and attenuates the rewarding effects of cocaine in mice through the use of the conditioned place preference paradigm (McGeehan and Olive, 2003; Kenny et al., 2005; Lee et al., 2005). On the other hand, pretreatment with the mGluR1

antagonist, EMQMCM, reduces behavioral sensitization to chronic cocaine administration in rats (Dravolina et al., 2006). However, due to side effects of some of these compounds, such as memory and motor impairment and learning deficits (Genkova-Papazova et al., 2007; Simonyi et al., 2007) further investigation in targeting these receptors as a possible treatment for cocaine addiction is needed. The results presented here lay a foundation for future drug development, knowing from previous studies what pathways are activated and from our current studies the exact localization of the group I mGluRs in relation to each afferent.

While the prefrontal cortical connection to the accumbens has been most studied and more extensively associated with cocaine and psychostimulant addiction, very little is known about the role of the thalamostriatal system in drug addiction. For instance, the prefrontal cortical projection to the nucleus accumbens core has been implicated in cocaine- and stress-primed reinstatement of cocaine self-administration; a rodent behavior analogous to relapse in humans (Xi et al., 2002; Baker et al., 2003; McFarland et al., 2003; Kalivas et al., 2003). Using animal models of drug self administration followed by withdrawal, it has been found that there is an increase in extracellular glutamate when the animal once again commences drug-seeking behavior. However, if the activity of the prefrontal cortex is blocked with GABA agonists, glutamate levels do not increase in the accumbens core and the animals do not show reinstatement of self administration (McFarland & Kalivas, 2001; McFarland et al., 2003). It has also been shown that even a single cocaine exposure has the potential to alter the electrophysiological properties of accumbal neurons when stimulating the cortical-accumbal projection (Fourgeard et al., 2004).

On the other hand, a few studies examining the thalamic-accumbal circuit, namely the projection from the PVN to the shell of the accumbens, suggested that this system may be involved in the reinforcing effects of psychostimulants. It has been shown that a single cocaine injection, as well as exposure to the cocaine-paired environment, induces Fos expression in the PVN of the thalamus (Brown et al., 1992). Another study found a dose-dependent increase in Fos protein in the PVN following administration of both cocaine and amphetamine (Deutch et al., 1998). Finally, following lesions of the PVN, it was found that animals had an enhanced locomotor response to an acute exposure to cocaine (Young & Deutch, 1998). These studies indicate that the PVN-nucleus accumbens shell connection is involved in some properties of psychostimulant-induced neural changes, and therefore, study of the receptors present at these synapses is important to gain a better understanding of how neurotransmission may be altered following cocaine exposure which may lead to addiction. It also indicates that further study of this projection is warranted.

3.5.3 Physiology of Group I mGluRs in the Accumbens

The first electrophysiological study of mGluRs in the accumbens was done by Manzoni and colleagues (1997). Using whole cell patch clamp recordings in slices of rat brain containing the accumbens, it was shown that application of the group I mGluR agonist, DHPG, inhibited the postsynaptic afterhyperpolarization current (Manzoni et al., 1997). This study was completed prior to the development of specific antagonists for mGluR1 and mGluR5; therefore, it was not determined which member of the group I mGluRs was responsible for these effects.

More recent studies examining the physiology of group I mGluRs in the accumbens used in vitro slices in which electrical stimulation of the prefrontal cortex induced glutamate release in the nucleus accumbens (Robbe et al., 2002; Fourgeard et al., 2004; Schotanus & Chergui, 2008). These studies showed that Group I mGluRs are involved in both long-term potentiation (LTP) and long-term depression (LTD) of glutamatergic synapses in the ventral striatum (Robbe et al., 2002; Fourgeard et al., 2004; Schotanus & Chergui, 2008). It was specifically shown that mGluR5 mediates endocannabinoid-induced LTD in the accumbens following stimulation of the prelimbic cortex in slices of mouse brain (Robbe et al., 2002; Fourgeard et al., 2004). In comparison, another study found that high frequency stimulation of glutamatergic cortical afferents in mouse accumbal slices induced LTP that can be impaired by inhibition of either mGluR1 or mGluR5 (Schotanus & Chergui, 2008).

In relation to our findings, we observed differences in the subsynaptic localization between mGluR1a and mGluR5 when in contact with cortical and thalamic afferents, which could help explain some of these recent physiological findings. We found a trend for mGluR5 to be more perisynaptic, as well as closer extrasynaptically on the plasma membrane of spines making contact with cortical and thalamic terminals. This may explain the findings that mGluR5, but not mGluR1, is involved in LTD in the accumbens, due to its closer proximity to glutamatergic synapses (Robbe et al., 2002; Fourgeard et al., 2004). It is also important to consider the possibility that extrasynaptic group I mGluRs could mediate their physiological effects in the accumbens through receptor-receptor interactions with various receptor subtypes including glutamate NMDA (Martin et al., 1998), dopamine D1 (Schotanus & Chergui, 2008), and endocannabinoid CB1

receptors (Robbe et al., 2002; Fourgeard et al., 2004). Future studies looking at the colocalization of mGluR1a and mGluR5 with these various receptors could also add to our understanding of group I mGluRs function in the nucleus accumbens.

While the aforementioned studies provide some insight into the functions of group I mGluRs in relation to cortical inputs, they do not address the function of these receptors in relation to thalamic afferents. It is technically challenging to maintain the integrity of the thalamostriatal system in brain slices, thereby making physiological studies of mGluR1a- and mGluR5-mediated effects following midline thalamic stimulation rather difficult. Based on the ultrastructural evidence from our study showing that both mGluR1a, and especially mGluR5, are present at specific thalamostriatal synapses in the core and shell of the accumbens, it is reasonable to hypothesize that group I mGluRs likely mediate some of the thalamic-induced excitatory effects on accumbens neurons and that these effects may differ depending on the exact source of the thalamic afferents. For example, we showed that terminals arising from the CM/IMD and PVN are in contact more frequently with mGluR5-labeled spines compared to mGluR1a-labeled spines. While an initial thought could be that this result was just a technical oversight due to the fact that there are more mGluR5-containing spines than mGluR1a-containing spines in the accumbens, this is not the case (see results section, 3.4.2.1 Double Pre-embedding Immunoperoxidase Labeling for BDA and Group I mGluRs). Based on these observations, we can speculate that mGluR5 may be the predominant subtype of group I mGluRs involved in thalamostriatal excitatory effects. However, until proper electrophysiological studies are achieved in which specific thalamic nuclei are stimulated and the function of each group I mGluRs subtype is characterized using specific

antagonists, the exact role(s) of mGluR1a or mGluR5 at thalamostriatal synapses remains unknown.

3.5.4 Future Directions

In order to gain a complete understanding of how group I mGluRs modulate glutamatergic transmission from all afferents, further studies must be completed. First, additional tract-tracing studies combined with immunocytochemistry to examine the glutamatergic projections from the basolateral amygdala and hippocampus and the relationship to mGluR1a and mGluR5 must be achieved. On the other hand, physiological data obtained so far have been largely focused on the corticostriatal system using broad mGluR-related compounds in brain slices (Taber & Fibiger 1995; Martin et al., 1998; Robbe et al., 2002; Fourgeard et al., 2004; Schotanus & Chergui, 2008). Therefore, the proposed ultrastructural studies must be complemented with in vivo electrophysiological experiments using specific group I mGluRs agonists and antagonists following stimulation of the various glutamatergic afferents to the nucleus accumbens core and shell.

Chapter 4:

Specific Aim 3:

To examine the subcellular and subsynaptic localization of mGluR1a and mGluR5 in the shell and core of the nucleus accumbens following acute or chronic cocaine treatment in rats.

Mitrano D.A., Arnold C., Smith Y. (2008) Subcellular and subsynaptic localization of group I metabotropic glutamate receptors in the nucleus accumbens of cocaine-treated rats. Neuroscience, 154: 653-666.

4.1 Abstract

There is significant pharmacological and behavioral evidence that group I metabotropic glutamate receptors (mGluR1a and mGluR5) in the nucleus accumbens play an important role in the neurochemical and pathophysiological mechanisms that underlie addiction to psychostimulants. To further address this issue, we undertook a detailed ultrastructural analysis to characterize changes in the subcellular and subsynaptic localization of mGluR1a and mGluR5 in the core and shell of nucleus accumbens following acute or chronic cocaine administration in rats. After a single cocaine injection (30mg/kg) and 45 minutes withdrawal, there was a significant decrease in the proportion of plasma membrane-bound mGluR1a in accumbens shell dendrites. Similarly, the proportion of plasma membrane-bound mGluR1a was decreased in large dendrites of accumbens core neurons following chronic cocaine exposure (i.e. 1 week treatment followed by three weeks withdrawal). However, neither acute nor chronic cocaine treatments induced significant change in the localization of mGluR5 in accumbens core and shell, which is in contrast with the significant reduction of plasma membrane-bound mGluR1a and mGluR5 induced by local intra-accumbens administration of the group I mGluR agonist, DHPG. In conclusion, these findings demonstrate that cocaine-induced glutamate imbalance (Smith et al., 1995; Pierce et al., 1996; Reid et al., 1997) has modest effects on the trafficking of group I mGluRs in the nucleus accumbens. These results provide valuable information on the neuroadaptive mechanisms of accumbens group I mGluRs in response to cocaine administration.

4.2 Introduction

Changes in glutamate neurotransmission in the nucleus accumbens is a key neuroadaptive mechanism in response to acute or chronic cocaine exposure (Robinson and Berridge, 2003). *In vivo* microdialysis experiments have shown a significant increase in extracellular glutamate levels that peaks approximately 40 minutes following acute systemic cocaine injections in rats (Smith et al., 1995; Reid et al., 1997). In contrast, one week of chronic cocaine exposure, which leads to behavioral sensitization, followed by three weeks withdrawal, reduces basal extracellular glutamate levels by half compared to saline-treated animals (Pierce et al., 1996; Baker et al., 2003). These cocaine-induced effects on extracellular glutamate release lead to rapid α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor subunit internalization after acute cocaine and in contrast, increased AMPA receptor surface expression following chronic cocaine-induced behavioral sensitization (Boudreau and Wolf, 2005; Boudreau et al., 2007).

Metabotropic glutamate receptors (mGluRs) are divided into three classes based on pharmacological and structural properties. Group I mGluRs (mGluR1 and 5) are coupled to Gq and activate phospholipase C, increasing intracellular calcium and activating protein kinase C, while Group II (mGluR2 and 3) and Group III (mGluRs 4, 6, 7, and 8) mGluRs are coupled to Gi and inhibit cAMP formation (for review see (Conn and Pin, 1997)). Both group I mGluRs are widely distributed and partly co-localized in the nucleus accumbens (Mitrano and Smith, 2007a) where they likely mediate some of the neuroadaptive changes associated with repeated cocaine administration. First and foremost is the fact that mGluR5 knockout mice do not self-administer cocaine and have

decreased locomotor activity in response to cocaine administration despite a significant increase in dopamine release in the nucleus accumbens (Chiamulera et al., 2001). In line with these observations, systemic administration of mGluR5 antagonist reduces cocaine self administration in both rats and monkeys and attenuates the rewarding effects of cocaine in mice (McGeehan and Olive, 2003; Kenny et al., 2005; Lee et al., 2005). On the other hand, pretreatment with mGluR1 antagonist reduces behavioral sensitization to chronic cocaine administration in rats (Dravolina et al., 2006).

At the cellular level, modest, but significant and opposite, changes in mGluR5 protein and mRNA expression have been reported in the rat accumbens after chronic cocaine exposure and three weeks withdrawal (Ghasemzadeh et al., 1999; Swanson et al., 2001). A single in vivo exposure to cocaine abolishes endocannabinoid mGluR5-mediated retrograde long-term depression (LTD) and decreases the surface expression of mGluR5 in the mouse accumbens (Fourgeaud et al., 2004).

Most G-protein coupled receptors (GPCRs), including group I mGluRs, have the ability to travel to and from the plasma membrane in response to changes in extracellular levels of receptor agonists (see Gainetdinov et al., 2004 for review). In cell cultures and mice brain slices, mGluR1a and mGluR5 undergo agonist-stimulated internalization and endocytosis (Dale et al., 2001; Mundell et al., 2001) which, in some cases, was correlated with decreased group I mGluR-mediated physiological effects (Fourgeaud et al., 2004). However, there has been no *in vivo* study looking at changes in the trafficking of group I mGluRs following glutamate or receptor agonist stimulation in the mammalian brain.

Therefore, to address this issue, we undertook an in-depth ultrastructural analysis of changes in the subcellular and subsynaptic localization of mGluR1a and mGluR5 in

the core and shell of the nucleus accumbens in rats acutely or chronically treated with cocaine and in animals that received local intracerebral injection of group I mGluR agonist.

4.3 Materials & Methods

4.3.1 Animals and cocaine treatments

Thirty-three male Sprague Dawley rats weighing 225-250 grams upon arrival were used for the cocaine treatment experiments in this study. All procedures were approved by the animal care and use committee of Emory University and conform to the U.S. National Institutes of Health guidelines. The chronic cocaine administration regimen used in this study was developed by Kalivas and colleagues (Kalivas et al., 1988) and is routinely used to induce behavioral sensitization to psychostimulants in rats. In brief, chronically treated rats were given either an i.p. injection of 0.9% saline or 15mg/kg cocaine on days 1 and 7 of the treatment period and locomotor activity was measured every 5 minutes for 2 hours by an IBM computer using Digipro software in photocell cages (Omnitech Electronics) equipped with 32 photobeams 5 cm above the floor. On days 2-6 of the treatment period, rats were given an i.p. injection of either 0.9% saline or 30mg/kg cocaine. Following the one-week treatment period, rats were left in their home cages for three weeks after the last injection, as a withdrawal period, and then sacrificed. Only rats that showed behavioral sensitization (i.e. animals that displayed a statistically significant increase in total locomotor activity on day 7 compared to day 1, data not shown) were used in the chronically cocaine-treated group.

Two groups of acutely treated rats were given a single i.p. injection of either 0.9% saline or 30mg/kg cocaine, put back in their home cages and then sacrificed either 45 minutes or 24 hours later. These time points were chosen based on previous studies that showed glutamate levels peak at approximately 40 minutes following acute cocaine

(Smith et al., 1995; Reid et al., 1997) and changes in mGluR5 expression 24 hours following a cocaine exposure (Fourgeaud et al., 2004).

4.3.2 Tissue preparation

For perfusion, all animals were deeply anesthetized with a cocktail of ketamine (60-100mg/kg, i.p.) and dormitor (0.1mg/kg, i.p.). The animals were then transcardially perfused with cold oxygenated Ringer's solution followed by a fixative containing 4% paraformaldehyde and 0.1% glutaraldehyde in phosphate buffer (0.1M; pH 7.4). Following perfusion, brains were removed from the skull, post-fixed in 4% paraformaldehyde for 24 hours, cut into 60-µm-thick sections using a vibrating microtome and stored in phosphate-buffered saline (PBS; 0.01M, pH 7.4) at 4˚C until further processing for immunocytochemistry. Prior to the immunocytochemical reactions, all sections were put into a 1% sodium borohydride solution for 20 minutes and then washed with PBS.

4.3.3 Primary Antibodies

A commercially available monoclonal antibody against calbindin-D_{28k} (Sigma, St. Louis, MO; Cat# C-9848, Lot# 082k4879) was used at a concentration of 1:5000 to distinguish between the accumbens shell and core. The calbindin-D_{28k} antibody is derived from CB-955 hybridomas produced by fusion of mouse myeloma cells and splenocytes from BALB/c mice that were immunized with purified bovine kidney calbindin-D_{28k}. The specificity of this antibody has been demonstrated through preadsorption immunohistochemical assays that abolish calbindin labeling (Celio, 1990), through Western blot analysis of rat brain tissue which shows a distinct band at 28kD (Miyata et al., 2000) and through immunohistochemistry which shows calbindin

immunostaining in brain regions known to express a significant level of calbindin-D_{28k} mRNA (Celio et al., 1990; Miyata et al., 2000; Winsky et al., 1989).

To localize mGluR1a, an affinity-purified rabbit polyclonal antibody against the C-terminus of rat mGluR1a (PNVTYASVILRDYKQSSSTL) conjugated to KLH with glutaraldehyde was used at a concentration of 1:1000 (Chemicon, Temecula, CA; Cat# AB1551, Lot# 21100471). In Western blot analysis by the manufacturer, this antibody labels a single band of ~140kD. Previous studies from our lab and others have used a combination of knock-out mice, transfected HEK-293 cells, and preadsorption to determine the specificity of this mGluR1a antiserum. These studies showed that brain tissue from mGluR1a knockout mice did not display any specific mGluR1a labeling compared to wild-type. In addition, immunoblotting of cells transfected with mGluR1a, but not mGluR5, labeled a band of 140kD (Kuwajima et al., 2004). Preadsorption studies in rat retina cells abolished mGluR1a labeling (Koulen et al., 1997).

An affinity-purified synthetic rabbit polyclonal antibody against the C-terminus of mGluR5 with a lysine added to the N-terminus (KSSPKYDTLIIRDYTNSSSSL) in a concentration of 1:5000 (Upstate Biotechnology, Lake Placid, NY; Cat# 06-451, Lot# 27884) was used to label mGluR5. According to the manufacturer's immunoblot analysis, the mGluR5 antibody labels a band of ~130kD. Specificity of the mGluR5 antibody has been shown in previous studies from our laboratory using knockout mice, transfected cells and homogenates of rat brain. These studies showed that brain tissue from mGluR5 knockout mice do not stain for mGluR5 and HEK-293 cells transfected with mGluR5 label a band of the correct molecular weight (Kuwajima et al., 2004). Furthermore, immunoblot analysis on proteins isolated from various brain regions labels

a band that corresponds to the size of mGluR5 in regions known to express mGluR5 protein and mRNA (Mannaioni et al., 2001).

4.3.4 Immunoperoxidase labeling for light microscopy

Following sodium borohydride treatment, sections were incubated for 1 hour at room temperature (RT) in PBS containing either 10% normal goat serum (NGS; for group I mGluRs), or normal horse serum (NHS; for calbindin-D_{28k}), 1% BSA, and 0.3% Triton X-100, followed by the primary antibody solution containing 1% NGS or NHS, 1% BSA, and 0.3% Triton X-100 in PBS for 24 hours at RT. After three rinses in PBS, sections were incubated in secondary biotinylated goat anti-rabbit or horse anti-mouse IgGs at a concentration of 1:200 (Vector Laboratories, Burlingame, CA) for 90 minutes. The sections were rinsed again in PBS and then incubated another 90 minutes with the avidin-biotin peroxidase complex (ABC) at a dilution of 1:100 (Vector Laboratories). Finally, the sections were washed in PBS and TRIS buffer (50mM; pH 7.6) and transferred to a solution containing 0.025% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma, St Louis, MO), 10mM imidazole, and 0.005% hydrogen peroxide in TRIS buffer for 10 minutes. Sections were rinsed in PBS, mounted onto gelatin-coated slides, dehydrated and then coverslipped with Permount. Tissue was examined with a Leica DMRB microscope (Leica Microsystems, Inc., Bannockburn, IL) and images were taken using a CCD camera (Leica DC500) which was controlled by Leica IM50 software.

4.3.5 Preembedding Immunoperoxidase labeling for electron microscopy

Following sodium borohydride treatment, sections were placed in a cryoprotectant solution for 20 minutes (PB 0.05M, pH 7.4, 25% sucrose, and 10% glycerol), frozen at -80°C for 20 minutes, returned to a decreasing gradient of cryoprotectant solutions, and

rinsed in PBS. Sections were then incubated in primary and secondary antibody solutions, identical to those used for light microscopy; with two exceptions: 1) the omission of Triton X-100 and 2) incubation in primary antibody for 48 hours at 4°C.

After the DAB reaction, the tissue was rinsed in PB (0.1M, pH 7.4) and treated with 1% OsO_4 for 20 minutes. It was then returned to PB and dehydrated with increasing concentrations of ethanol. When exposed to 70% ETOH, 1% uranyl acetate was added to the solution for 35 minutes to increase the contrast of the tissue at the electron microscope. Following dehydration, sections were treated with propylene oxide and embedded in epoxy resin for 12 hours (Durcupan ACM, Fluka, Buchs, Switzerland), mounted onto slides and placed in a 60°C oven for 48 hours. Separate samples of the nucleus accumbens core and medial shell were cut out of the larger sections, mounted onto resin blocks and cut into 60-nm sections using an ultramicrotome (Leica Ultracut T2). The 60-nm sections were collected on Pioloform-coated copper grids, stained with lead citrate for 5 minutes to enhance tissue contrast and examined on the Zeiss EM-10C electron microscope. Electron micrographs were taken and saved with a CCD camera (DualView 300W; Gatan, Inc., Pleasanton, CA) controlled by DigitalMicrograph software (version 3.10.1, Gatan, Inc.). Some of the digitally acquired electron micrographs were adjusted only for brightness or contrast using either the DigitalMicrograph software or Adobe Photoshop software (version 8.0, Adobe Systems Inc.). Micrographs were then compiled into figures using Adobe Illustrator (version 11.0, Adobe Systems Inc.).

4.3.6 Preembedding Immunogold labeling for electron microscopy

Following sodium borohydride and cryoprotectant treatments, sections were incubated for 30 minutes in PBS containing 5% dry milk at RT and then rinsed in TBS-gelatin buffer (0.02M and pH 7.6). Sections were then transferred to primary antibody solutions with 1% dry milk in TBS-gelatin buffer for 24 hours at room temperature and then rinsed again in TBS-gelatin. After rinses, sections were treated for 2 hours at RT with secondary goat anti-rabbit IgGs conjugated with 1.4nm gold particles at a concentration of 1:100 (Nanoprobes, Yaphank, NY) diluted with 1% dry milk in TBS-gelatin. Sections were rinsed in TBS-gelatin and 2% sodium acetate buffer before gold particles were silver intensified to 30-50nm using the HQ silver kit (Nanoprobes) for approximately 10 minutes. The sections were then treated according to the same protocol of osmification, dehydration, embedding, and tissue selection described above for the immunoperoxidase procedure including the following changes: 1) the tissue was kept in 0.5% OsO_4 for 10 minutes instead of 20 and 2) the tissue was stained with 1% uranyl acetate for 10 minutes instead of 35.

4.3.7 DHPG Injections

In order to ensure that the immunogold method used in our study was sensitive enough to detect changes in plasma membrane-bound group I mGluRs immunoreactivity in response to direct receptor agonist exposure in vivo, we examined changes in the subcellular localization of mGluR1a and mGluR5 in the accumbens of rats following local administration of group I mGluRs agonist. Six male Sprague-Dawley rats were anesthetized with isoflurane and fixed in a stereotaxic frame (Kopf; Tugunga, CA). Using a 10µl Hamilton syringe (Hamilton; Reno, NV), 2µl of either 1µmol (*RS*)-3,5-dihydroxyphenylglycine (DHPG; Tocris, Ballwin, MO) dissolved in 0.9% saline or 0.9%

saline was injected over a 5 minute period into the nucleus accumbens core and medial shell based on coordinates from Paxinos and Watson (1998; +1.7mm AP, +1.3mm ML, -7.4mm DV). Rats were sacrificed 45 minutes after injections. This time point was chosen based on previous internalization studies of other GPCRs in response to agonist stimulation (Bernard et al., 1998; Dumartin et al., 1998; Bernard et al., 1999; Csaba et al., 2001). To assess the accuracy of the DHPG injection, the striatal tissue from these animals was serially cut, and 1 out of 4 sections were Nissl-stained to reveal the exact placement of the syringe. Tissue from animals in which the injection site was in the core and/or medial shell of the accumbens, was prepared for single preembedding immunogold labeling as described above.

4.3.8 Analysis of Material

4.3.8.1 Immunoperoxidase labeling for group I mGluRs

Data for single immunoperoxidase labeling were collected from a total of 132 blocks of tissue, 1 block/animal in the medial shell (referred to as shell in the following sections) and 1 block/animal in core immunostained for either mGluR1a and mGluR5 from 7 rats chronically treated with cocaine, 7 rats chronically treated with saline, 10 rats acutely treated with cocaine, and 10 rats acutely treated with saline. Serial ultrathin sections taken from each of the blocks were examined and 35-40 electron micrographs of randomly selected immunoreactive elements were digitized at 25,000X. This resulted in a total surface of 18,027μm^2 of accumbens tissue to be examined for mGluR1a in saline-treated rats and 15,700μm^2 in cocaine-treated rats; 14,305μm^2 for mGluR5 in saline-treated rats and 15,235μm^2 in cocaine-treated rats. Labeled elements were categorized as dendrites, spines, unmyelinated axons, myelinated axons, and axon terminals on the basis

of ultrastructural features described by Peters et al.(1991). The density of labeled elements for each receptor subtype was calculated in the shell and core by dividing the number of labeled elements by the area of tissue examined. The means +/- SEM of labeled elements for the different animal groups were calculated and presented as bar histograms. Significant differences were assessed using Sigma stat software (version 2.03, SPSS Inc.) for one-way ANOVAs and Tukey's post-hoc test. The density of labeled elements was compared across each neuronal element between the cocaine- and saline-treated animals within each receptor subtype.

4.3.8.2 Preembedding immunogold labeling for group I mGluRs

Immunogold data were collected from 125 blocks of mGluR1a- and mGluR5-immunostained shell and core tissue as described above. Serial ultrathin sections from the surface of the blocks were collected and 35-40 electron micrographs of randomly selected immunoreactive elements were taken at 25,000X, for a total tissue surface area of 13,316μm^2 of accumbens tissue to be examined for mGluR1a in saline-treated rats and 13,840μm^2 in cocaine-treated rats; 12,502μm^2 for mGluR5 in saline-treated rats and 14,247μm^2 in cocaine-treated rats. Labeled elements were classified as described above with the addition that dendrites were further categorized arbitrarily as large (cross-sectional diameter greater than 0.75μm) or small (cross-sectional diameter equal to or less than 0.75μm). Gold particles were classified as either intracellular or plasma membrane-bound depending on their localization relative to the plasma membrane. To be categorized as plasma membrane-bound, gold particles had to be in contact with the membrane; all other particles were considered intracellular. The total number (i.e. 100%) of gold particles for each animal is equal to the number of intracellular + plasma

membrane-bound gold particles examined. The mean (+/-SEM) percent of plasma membrane-bound gold particles was then calculated across all animals and presented as a bar histogram; the remaining gold particles were found in the intracellular compartment. Data were analyzed for significant differences between saline- and cocaine-treated animals using Sigma Stat software by one-way ANOVAs and Tukey's post-hoc test. Plasma membrane-bound gold particles were further classified into three categories; perisynaptic (touching or within a 20 nm range of the edges of postsynaptic specializations); synaptic (in contact with the main body of postsynaptic specializations); or extrasynaptic (on the plasma membrane but not associated with synapses).

4.3.8.3 Preembedding immunogold labeling for group I mGluRs following DHPG Injections

Immunogold labeling data were collected from 24 blocks of mGluR1a- and mGluR5-immunostained shell and core tissue as described above. To make sure the tissue examined had been exposed to DHPG, all blocks were taken within a range of 0.2 to 0.5mm from the tip of the injection syringe and from sections no more than 120μm away in the rostral/caudal plane. Serial ultrathin sections from the surface of the blocks were collected and 35 electron micrographs of randomly selected immunoreactive elements were taken at 25,000X, for a total tissue surface area of 2,440μm^2 of accumbens tissue to be examined for mGluR1a in saline-treated rats and 2,440μm^2 in DHPG-treated rats; 2,440μm^2 for mGluR5 in saline-treated rats and 2,440μm^2 in DHPG-treated rats. The quantitative analysis of gold labeling distribution was the same as described in the previous section.

4.4 RESULTS

4.4.1 Light Microscopic Observations

Tissue containing the nucleus accumbens was stained separately for calbindin D_{28k}, mGluR1a or mGluR5. As shown in our previous study (Mitrano and Smith, 2007a) and others (Meredith et al., 1996), calbindin immunoreactivity clearly delineated the boundaries of the shell and core of the nucleus accumbens, hence serving as a guide for the selection of blocks of tissue in subsequent electron microscopic experiments. Tissue from all three cocaine treatment groups and saline-treated animals were immunostained for the two group I mGluRs. In line with our previous study, the immunoreactivity for mGluR1a and mGluR5 was distributed heavily in the neuropil of the shell and core of the accumbens, with very light labeling in cell bodies (Mitrano and Smith, 2007a). No obvious difference in the overall distribution of mGluR1a and mGluR5 immunoreactivity was found in the nucleus accumbens between cocaine- and saline-treated rats (data not shown).

4.4.2 Electron Microscopic Observations:

4.4.2.1 Cellular and Subcellular Localization of mGluR1a and mGluR5 in Cocaine-treated Groups

Because both mGluR1a and mGluR5 immunoreactivity is found in the neuropil of accumbens tissue, high resolution electron microscopy is essential to assess potential changes in the cellular, subcellular and subsynaptic localization of these receptors between normal and cocaine-treated rats. First, we looked at the subcellular localization of both group I mGluRs in the three groups of cocaine-treated rats and control saline-treated animals using immunoperoxidase labeling for mGluR1a or mGluR5. The majority

of labeling for both receptor subtypes in each experimental group was found in dendrites and spines, and less frequently in unmyelinated axons. Electron micrographs of immunoreactive elements from each treatment group are shown in figure 4.1, while quantitative data for these observations are depicted in figure 4.2 as the average density of labeled elements per 100 square microns (±SEM) of accumbens shell or core tissue. Overall, there was no significant difference in the distribution of mGluR1a- or mGluR5-immunoreactive neuronal elements between the different cocaine treatment groups and controls, regardless of receptor type, time point, or region of the accumbens examined using a series of one-way ANOVAs (Fig. 4.2).

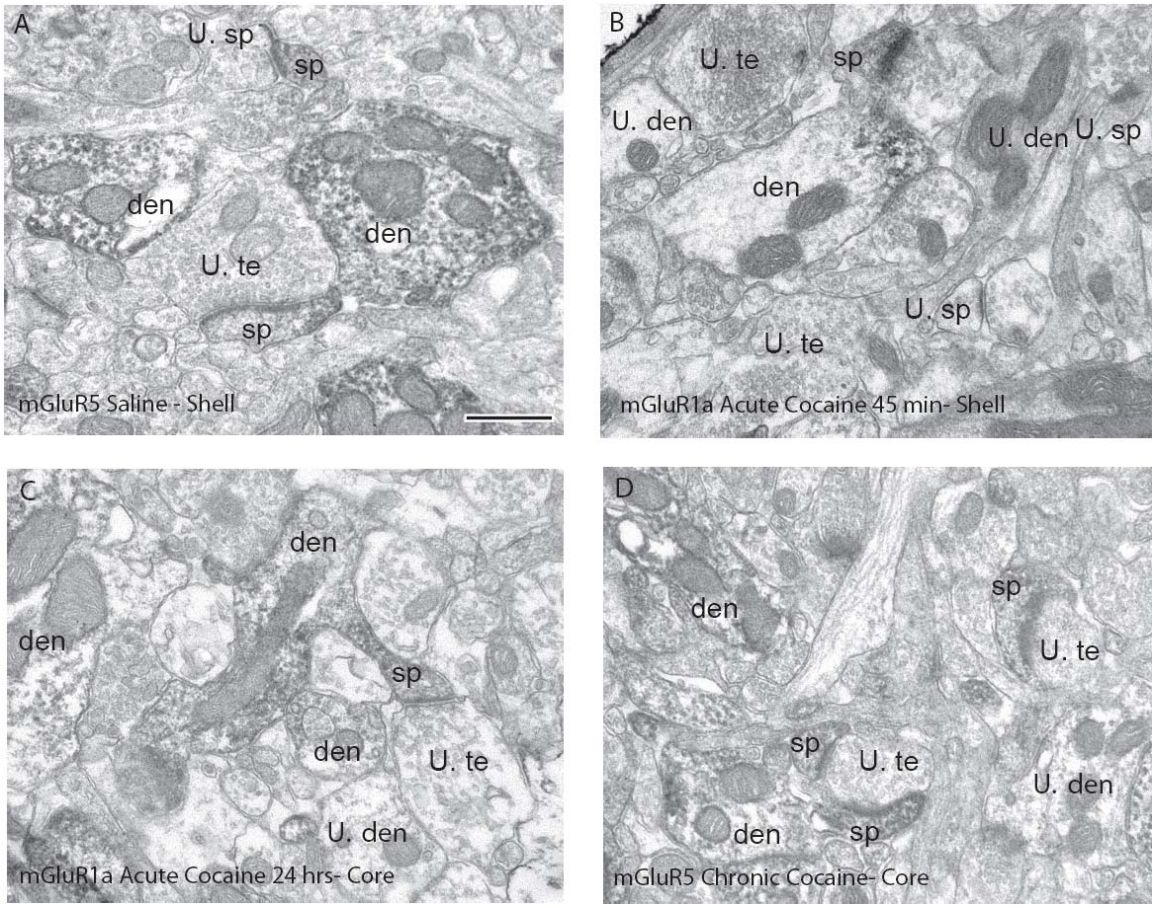

Figure 4.1: Immunoperoxidase labeling of mGluR1a and mGluR5 in saline- and cocaine-treated rats. (A) mGluR5-labeled elements in the nucleus accumbens shell of a saline-treated rat. (B) mGluR1a-labeled elements in the nucleus accumbens shell of a rat treated with cocaine, 45 minutes withdrawal. (C) mGluR1a-labeled elements in the nucleus accumbens core of a rat treated with cocaine, 24 hours withdrawal. (D) mGluR5-labeled elements in the nucleus accumbens core of animals treated chronically with cocaine for one week. Note the bulk of postsynaptic labeling in dendrites (den) and spines (sp). Abbreviations: u. te, unlabeled terminal; u. den, unlabeled dendrite; u. sp, unlabeled spine. Scale bar = 0.5 μm.

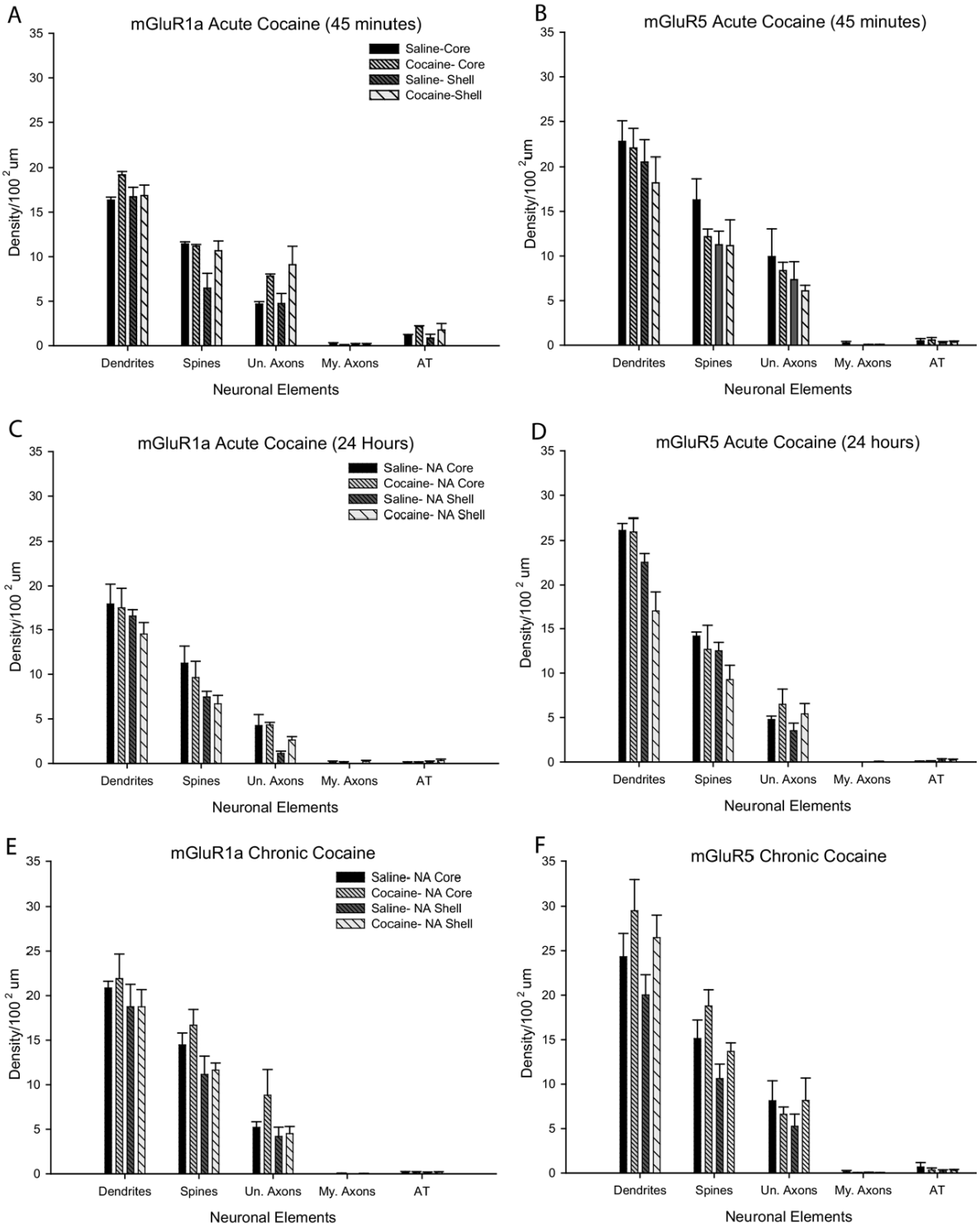

Figure 4.2: Histograms summarizing the distribution of mGluR1a and mGluR5 immunoperoxidase labeling in saline- and cocaine-treated rats, expressed as mean density of labeled elements per 100μm^2 (±SEM). (A-B) Immunoperoxidase

labeling for mGluR1a and mGluR5 in the core and shell of the nucleus accumbens 45 minutes following a saline or cocaine injection. Total number of animals and labeled elements: mGluR1a: saline, core=4, 552; saline, shell=4, 470; cocaine, core=5, 822; cocaine, shell=5, 784. mGluR5: saline, core=5, 1012; saline, shell=5, 807; cocaine, core=5, 882; cocaine, shell=5, 729. (C-D) Immunoperoxidase labeling for mGluR1a and mGluR5 24 hours following saline or cocaine. Total number of animals and labeled elements: mGluR1a: saline, core=5, 786; saline, shell=4, 473; cocaine, core=5, 672; cocaine, shell=5, 643. mGluR5: saline, core=4, 831; saline, shell=4,710; cocaine, core=5, 1053; cocaine, shell=5, 694. (E-F) Immunoperoxidase labeling for mGluR1a and mGluR5 3 weeks following one-week chronic saline or cocaine. Total number of animals and labeled elements: mGluR1a: saline, core=7, 1682; saline, shell=7, 1381; cocaine, core=7, 1770; cocaine, shell=7, 1365. mGluR5: saline, core=7, 1897; saline, shell=7, 1471; cocaine, core=7, 1939; cocaine, shell=7, 1882. Abbreviations: Un. Axons: Unmyelinated axons; My. Axons: Myelinated axons; AT: Axon terminals.

4.4.2.2 Subsynaptic Localization of mGluR1a and mGluR5 in Cocaine-treated Groups

Because the immunoperoxidase method results in an amorphous diffuse reaction product within labeled elements, this method is not suitable to determine the exact subsynaptic localization of group I mGluRs in accumbens neurons. We therefore used the preembedding immunogold method that allows for a higher level of spatial resolution to detect changes in the subsynaptic localization of mGluR1a and mGluR5 immunoreactivity in response to chronic and acute cocaine treatment regimens. Examples of immunogold-labeled elements analyzed for this part of the study are illustrated in figure 4.3. The quantitative data, which provide the percentage of plasma membrane-bound gold particles (±SEM) labeling for mGluR1a or mGluR5 on dendrites and spines for the different treatment groups are presented in figure 4.4. Because the majority of gold labeling for mGluR1a and mGluR5 was found postsynaptically in dendrites and spines, the quantitative analysis of immunoreactivity was focused on these elements.

In the 45 minute acute treatment group, a significant decrease in the percent of plasma membrane-bound mGluR1a in large (30.9±3.0% saline vs. 21.3±1.9% cocaine, $F(3,15)=3.3$, n=5, $p<0.05$) and small (47.6±1.0% saline vs. 35.2±2.6% cocaine, $F(3,15)=4.9$, n=5, $p<0.05$) dendrites in the accumbens shell was found in cocaine-treated rats compared to controls, but no noticeable change in mGluR5 localization was found in these animals (Figs 4.3A,B; 4.4 A,B). In the 24 hours post-treatment group, neither mGluR1a or mGluR5 displayed any significant change in their pattern of subsynaptic localization compared to controls (Figs 4.3C; 4.4 C,D). Finally, a significant decrease in the percent of plasma membrane-bound mGluR1a immunoreactivity was found in large

dendrites in the accumbens core of chronically cocaine-treated rats (48.0±1.8%, n=7) compared to controls (56.7±2.3%, $F(3,21)=3.1$, n=6; $p<0.05$), but no significant change in the subsynaptic distribution of either group I mGluR subtypes was found in spines or small dendrites in these animals.

The pattern of subsynaptic labeling on the plasma membrane was further classified into extrasynaptic, perisynaptic and synaptic. As shown in tables 3 and 4, there was no significant difference in the pattern of subsynaptic labeling between all treatment groups. In each group, more than 80% plasma membrane labeling in spines and 96-98% labeling in dendrites was extrasynaptic. A slightly larger incidence of perisynaptic labeling to asymmetric synapses was seen in spines compared to dendrites.

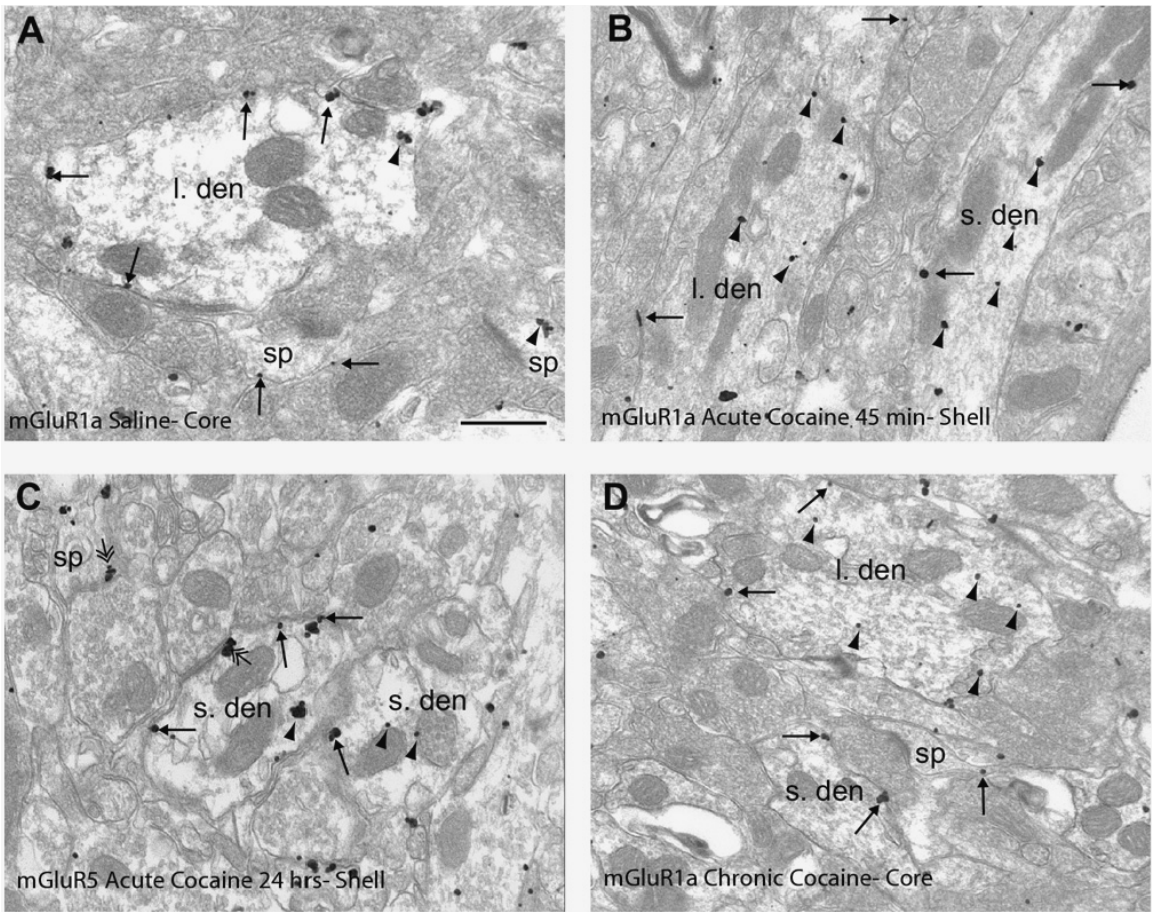

Figure 4.3: mGluR1a and mGluR5 immunogold labeling in saline- and cocaine-treated rats. (A) mGluR1a immunogold labeling in the nucleus accumbens core of a saline-treated rat. Note the extrasynaptic (single-headed arrows) labeling on the plasma membrane of the large dendrite (l. den) and spine (sp). (B) mGluR1a immunogold labeling in nucleus accumbens shell 45 minutes following cocaine treatment. Note the increased intracellular labeling (arrowheads) in large and small dendrites (s. den). (C) mGluR5 immunogold labeling in the nucleus accumbens shell 24 hours following cocaine injection. mGluR5 is distributed extrasynaptically (single-headed arrows) and intracellularly (arrowheads) in the dendrites. Also note the perisynaptic labeling to an asymmetric axodendritic and axospinous synapse (double-headed arrow). (D) mGluR1a immunogold labeling in the nucleus accumbens core of a rat chronically treated with cocaine followed by 3 weeks withdrawal. Note the larger amount of intracellular labeling in the large dendrite and extrasynaptic plasma membrane labeling in the small dendrite and spine. Scale bar=0.5µm.

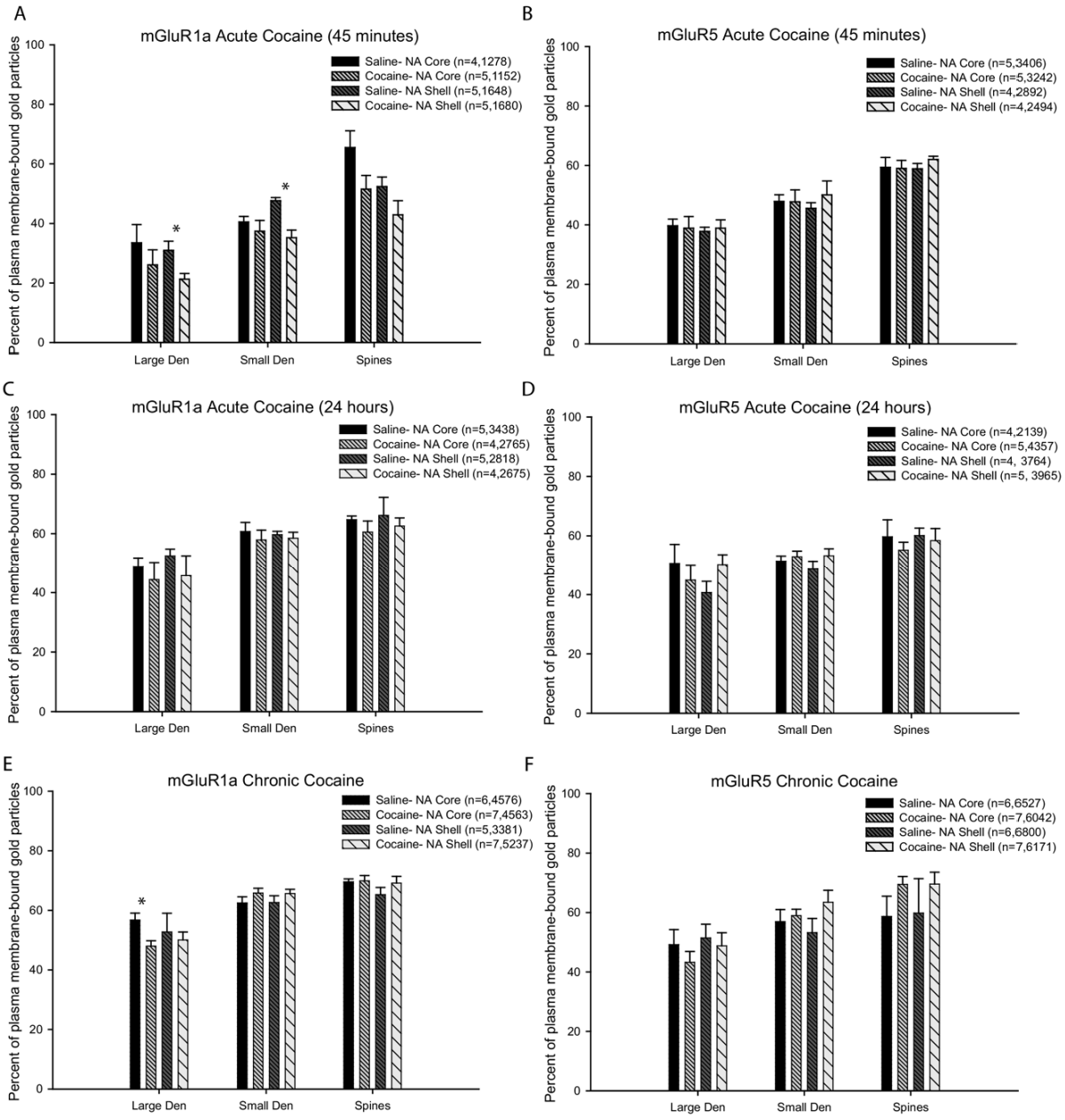

Figure 4.4: Histograms showing the percentage of plasma membrane labeling of mGluR1a and mGluR5 in the core and shell of the nucleus accumbens of saline- and cocaine-treated rats. Data is presented as the mean percentage (±SEM) of

gold particles on the plasma membrane of large or small dendrites and spines; in parentheses n= number of animals used followed by the total number of gold particles counted in each experimental group. (A-B) Mean percentage of plasma membrane-bound mGluR1a (A) and mGluR5 (B) labeling in rats sacrificed 45 minutes following cocaine treatment. One-way ANOVA with Tukey's post-hoc test revealed a lower proportion of plasma membrane-bound mGluR1a in large and small dendrites in the shell of cocaine-treated rats ($p<0.05$, single asterisks). Total number of elements examined: mGluR1a: Saline, Core: large den=38, small den=233, spines=117; Cocaine, Core: large den=55, small den=198, spines=114; Saline, Shell: large den=64, small den=236, spines=116; Cocaine, Shell: large den=52, small den=260, spines=140. mGluR5: Saline core: large den=64, small den=365, spines=248; Cocaine, Core: large den=44, small den=365, spines=248; Saline, Shell: large den=65, small den=260, spines=171; Cocaine, Shell: large den=47, small den=292, spines=170. (C-D) Percentage of plasma membrane-bound mGluR1a (C) and mGluR5 (D) in rats sacrificed 24 hours following cocaine treatment. No significant difference was found between the saline- and cocaine-treated animals. Total number of elements examined: mGluR1a: Saline, Core: large den=53, small den=432, spines=263; Cocaine, Core: large den=19, small den=342, spines=210; Saline, Shell: large den=34, small den=368, spines=216; Cocaine, Shell: large den=36, small den=311, spines=179. mGluR5: Saline, core: large den=20, small den=270, spines=165; Cocaine, Core: large den=40, small den=464, spines=315; Saline, Shell: large den=34, small den=416, spines=256; Cocaine, Shell: large den=39, small den=446, spines=263. (E-F)

Percentage of plasma membrane-bound mGluR1a (E) and mGluR5 (F) in rats chronically treated with cocaine and sacrificed 3 weeks later. One-way ANOVA and Tukey's post-hoc tests revealed that the there is significantly lower percentage of plasma membrane-bound mGluR1a in the accumbens core of rats treated chronically with cocaine compared to saline ($p<0.05$, single asterisk). Total number of elements examined: mGluR1a: Saline, Core: large den=53, small den=575, spines=380; Cocaine, Core: large den=76, small den=667, spines=531; Saline, Shell: large den=53, small den=427, spines=264; Cocaine, Shell: large den=83, small den=732, spines=427. mGluR5: Saline, core: large den=78, small den=717, spines=430; Cocaine, Core: large den=85, small den=748, spines=471; Saline, Shell: large den=104, small den=678, spines=372; Cocaine, Shell: large den=71, small den=783, spines=427.

Table 3: Subsynaptic Group I mGluRs labeling after Cocaine Treatments; Accumbens Core

mGluR1a *mGluR5*	Acute cocaine, 45 min		Acute cocaine, 24 h		Chronic cocaine	
	Saline-Core	Cocaine-Core	Saline-Core	Cocaine-Core	Saline-Core	Cocaine-Core
SPINES Extra	**78.7±4.5**	**79.8±6.1**	**85.3±2.3**	**81.0±1.2**	**82.6±1.3**	**86.8±1.5**
	84.3±3.5	83.8±2.1	82.7±7.4	83.2±2.2	83.1±1.3	82.2±1.3
Peri-Asym	**18.3±3.4**	**20.2±6.1**	**14.5±2.1**	**18.2±1.4**	**14.8±0.7**	**12.5±1.4**
	14.9±1.7	16.0±2.1	18.9±5.5	14.8±2.3	14.6±1.9	16.2±1.7
Syn-Asym	**2.9±1.4**	**0**	**0.3±0.3**	**0.2±0.2**	**1.6±0.7**	**0.7±0.3**
	0.8±0.6	0.2±0.2	1.4±0.7	1.7±0.5	1.1±0.3	1.1±0.4
Peri-Symm	**0**	**0**	**0**	**0.6±0.6**	**1.3±0.6**	**0**
	0	0	0.4±0.4	0.3±0.3	1.1±0.4	0
Syn-Symm	**0**	**0**	**0**	**0**	**0**	**0**
	0	0	0	0	0.2±0.2	0.3±0.3
SM. DEN Extra	**98.1±1.2**	**96.3±1.7**	**97.6±0.8**	**96.2±0.7**	**97.3±0.9**	**98.5±1.0**
	98.5±0.5	97.3±1.4	97.9±0.7	98.3±0.6	98.5±0.6	98.0±0.4
Peri-Asym	**0.3±0.3**	**1.8±1.2**	**1.1±0.5**	**0.7±0.3**	**0.9±0.2**	**1.5±0.2**
	0.4±0.2	0.7±0.3	1.3±0.7	0.4±0.2	0.4±0.2	0.9±0.4
Syn-Asym	**0.3±0.3**	**0**	**0.2±0.1**	**0.3±0.2**	**0.1±0.1**	**0±0.1**
	0.1±0.1	0.3±0.3	0.4±0.4	0	0.2±0.2	0.1±0.1
Peri-Symm	**1.4±0.8**	**1.9±1.1**	**0.7±0.3**	**1.3±0.4**	**1.7±0.9**	**0±0.6**
	0.8±0.3	1.3±1.0	0.2±0.1	0.7±0.3	0.7±0.2	1.0±0.3
Syn-Symm	**0**	**0**	**0.5±0.2**	**1.6±0.6**	**0.1±0.1**	**1.0±0.5**
	0.2±0.1	0.5±0.5	0.1±0.1	0.7±0.3	0.1±0.1	0.1±0.0
LG. DEN Extra	**97.5±1.6**	**96.1±2.9**	**96.4±1.5**	**94.9±3.4**	**96.0±1.5**	**96.5±1.7**
	94.2±3.3	97.3±1.7	100±0	96.4±2.3	96.7±2.3	96.1±1.5
Peri-Asym	**0**	**0**	**1.9±1.2**	**2.4±2.4**	**0**	**0**
	1.4±1.4	0.8±0.8	0	1.4±0.9	0.9±0.9	2.2±1.3
Syn-Asym	**0**	**0**	**0.3±0.3**	**0**	**0**	**0**
	0	0	0	0	0	0
Peri-Symm	**2.4±1.5**	**2.2±2.2**	**0.8±0.8**	**1.6±1.6**	**4.0±1.5**	**3.0±1.8**
	1.8±1.1	0.8±0.8	0	0.4±0.4	2.3±2.3	1.8±1.0
Syn-Symm	**0**	**1.7±1.0**	**0.7±0.4**	**1.2±1.2**	**0**	**0.5±0.5**
	1.8±1.4	1.2±1.2	0	1.9±1.9	0	0

Table 4: Subsynaptic Group I mGluRs labeling after Cocaine Treatments; Accumbens Shell

mGluR1a *mGluR5*	Acute cocaine, 45 min		Acute cocaine, 24 h		Chronic cocaine	
	Saline-Shell	Cocaine-Shell	Saline-Shell	Cocaine-Shell	Saline-Shell	Cocaine-Shell
SPINES Extra	**79.5±4.1**	**73.8±1.7**	**83.7±2.8**	**85.6±1.9**	**82.2±3.3**	**85.5±2.4**
	82.6±3.5	85.4±3.6	88.0±1.7	85.3±2.5	80.6±1.3	84.3±1.4
Peri-Asym	**16.6±4.5**	**21.0±1.2**	**13.3±1.3**	**13.5±1.5**	**14.7±2.4**	**13.0±2.2**
	17.1±3.4	13.6±3.2	10.9±1.4	13.0±2.0	17.7±1.5	13.6±1.6
Syn-Asym	**3.9±1.1**	**5.2±2.6**	**2.0±1.1**	**0.5±0.5**	**1.0±0.6**	**0.9±0.3**
	0.3±0.3	1.0±0.6	1.1±0.7	1.3±0.8	1.3±0.5	1.6±0.6
Peri-Symm	**0**	**0**	**0.8±0.8**	**0.5±0.5**	**1.7±1.4**	**0.6±0.5**
	0	0	0	0.2±0.2	0.4±0.4	0.6±0.4
Syn-Symm	**0**	**0**	**0.3±0.3**	**0**	**0.5±0.3**	**0**
	0	0	0	0.2±0.2	0	0
SM. DEN Extra	**94.7±1.7**	**93.0±1.5**	**96.8±0.5**	**96.3±0.4**	**96.4±1.2**	**95.4±0.9**
	97.0±1.0	98.0±1.0	98.3±0.5	97.9±0.5	97.9±0.6	95.9±0.9
Peri-Asym	**2.3±1.0**	**2.6±0.9**	**1.6±0.5**	**0.7±0.4**	**1.2±0.5**	**2.0±0.6**
	1.9±0.5	0.6±0.4	0.5±0.3	0.6±0.2	0.6±0.2	1.1±0.4
Syn-Asym	**0.4±0.4**	**0**	**0.2±0.2**	**0.1±0.1**	**0**	**0**
	0.1±0.1	0.3±0.2	0	0.2±0.1	0.1±0.1	0
Peri-Symm	**1.4±0.6**	**2.1±0.9**	**0.1±0.1**	**1.9±0.4**	**1.5±0.8**	**2.3±0.6**
	0.7±0.4	0.5±0.5	0.9±0.4	0.9±0.4	1.0±0.6	2.1±0.6
Syn-Symm	**1.2±0.5**	**2.2±1.0**	**1.4±0.5**	**1.1±0.3**	**0.8±0.2**	**0.3±0.2**
	0.2±0.2	0.7±0.4	0.3±0.1	0.5±0.2	0.4±0.1	0.7±0.1
LG. DEN Extra	**100±0**	**100±0**	**98.2±1.1**	**93.2±1.9**	**88.8±4.4**	**96.9±1.8**
	98.0±1.2	99.5±0.5	97.4±2.6	98.5±1.5	98.4±1.0	93.6±2.1
Peri-Asym	**0**	**0**	**0**	**1.6±1.6**	**4.9±1.8**	**0.1±0.1**
	0.6±0.6	0	0	0	0.8±0.6	0.3±0.3
Syn-Asym	**0**	**0**	**0.9±0.9**	**0**	**2.2±2.2**	**0.3±0.3**
	0	0	0.5±0.5	0	0	0
Peri-Symm	**0**	**0**	**0.6±0.6**	**5.3±0.5**	**2.5±1.6**	**2.4±1.6**
	1.3±0.8	0	1.6±1.6	1.0±1.0	0.7±0.7	2.7±1.4
Syn-Symm	**0**	**0**	**0.3±0.3**	**0**	**1.6±1.6**	**0.2±0.2**
	0	0.5±0.5	0.5±0.5	0.5±0.5	0.1±0.1	3.4±2.4

Table 3: Subsynaptic group I mGluRs labeling after cocaine treatment in the accumbens core. Data are presented as percent of gold particles ±SEM for each subsynaptic domain in Spines, Sm. Den (small dendrites) and Lg. Den (large dendrites). The different categories of subsynaptic domains are abbreviated as follows: Extra= extrasynaptic; Peri-Asym=perisynaptic labeling to asymmetric synapses; Syn-Asym=synaptic labeling of asymmetric synapses; Peri-Symm=perisynaptic labeling at symmetric synapses; Syn-Symm=synaptic labeling at symmetric synapses. Data for mGluR1a are in the top row of each box, while mGluR5 data are in the bottom row of each box. The number of animals used, elements examined and gold particles counted is presented in the legend of figure 4.

Table 4: Subsynaptic group I mGluRs labeling after cocaine treatment in the accumbens shell. Data are presented as percent of gold particles ±SEM for each subsynaptic domain in Spines, Sm. Den (small dendrites) and Lg. Den (large dendrites). The different categories of subsynaptic domains are abbreviated as follows: Extra= extrasynaptic; Peri-Asym=perisynaptic labeling to asymmetric synapses; Syn-Asym=synaptic labeling of asymmetric synapses; Peri-Symm=perisynaptic labeling at symmetric synapses; Syn-Symm=synaptic labeling at symmetric synapses. Data for mGluR1a are in the top row of each box, while mGluR5 data are in the bottom row of each box. The number of animals used, elements examined and gold particles counted is presented in the legend of figure 4.

4.4.2.3 Agonist-induced changes in the subsynaptic localization of group I mGluRs in the nucleus accumbens

Despite the well-established increases or decreases in extracellular glutamate in the nucleus accumbens (Smith et al., 1995; Pierce et al., 1996; Reid et al., 1997; Baker et al., 2003), only modest changes in group I mGluRs distribution were induced following acute or chronic cocaine treatment (see above). A potential interpretation for these observations might be that the pre-embedding immunogold method is not sensitive enough to detect changes in the subcellular and subsynaptic localization of group I mGluRs induced by changes in extracellular glutamate in cocaine-treated rats. In order to validate our approach, we assessed changes in the distribution of the two group I mGluRs in the rat accumbens forty-five minutes after intra-accumbal injection of the group I mGluR agonist, DHPG (Fig. 4.5C). Consistent with previous studies that showed internalization of various GPCRs following agonist stimulation (Bernard et al., 1998; Dumartin et al., 1998; Bernard et al., 1999; Csaba et al., 2001), local DHPG application induced significant decreases in the percentage of plasma membrane-bound labeling for mGluR1a or mGluR5 in specific neural elements in the shell or core of the rat accumbens (Fig. 4.5). In the shell, a significant decrease in plasma membrane-bound mGluR1a immunoreactivity was found in small dendrites (61.0$\pm$4.1% saline vs. 40.3$\pm$2.8% DHPG, $F(3,8)=11.2$, n=3, $p<0.05$) and spines (83.3$\pm$1.6% saline vs. 40.9$\pm$2.4% DHPG, $F(3,8)=96.5$, n=3, $p<0.001$), while plasma membrane-bound labeling for mGluR5 was significantly reduced in all neural compartments examined (large dendrites: 53.5$\pm$8.2% saline vs. 26.3$\pm$4.9% DHPG, $F(3,8)=7.0$, n=3, $p<0.05$; small dendrites: 58.5$\pm$4.1% saline vs. 43.4$\pm$0.5% DHPG, $F(3,8)=12.0$, n=3, $p<0.05$; spines: 77.2$\pm$3.4% saline vs.

54.9±3.8% DHPG, $F(3,8)=12.4$, n=3, $p<0.01$). The pattern was quite different in the accumbens core where a significant decrease in the percent of plasma membrane-bound mGluR1a was found in large dendrites (55.0±7.5% saline vs. 32.3±2.1% DHPG, $F(3,8)=5.8$, n=3, $p<0.05$) and spines (78.7±1.7% saline vs. 55.4±2.2% DHPG, $F(3,8)=96.5$, n=3, $p<0.001$), but not in small dendrites. As for mGluR5, no significant change in the percentage of plasma membrane-bound labeling was found in the core.

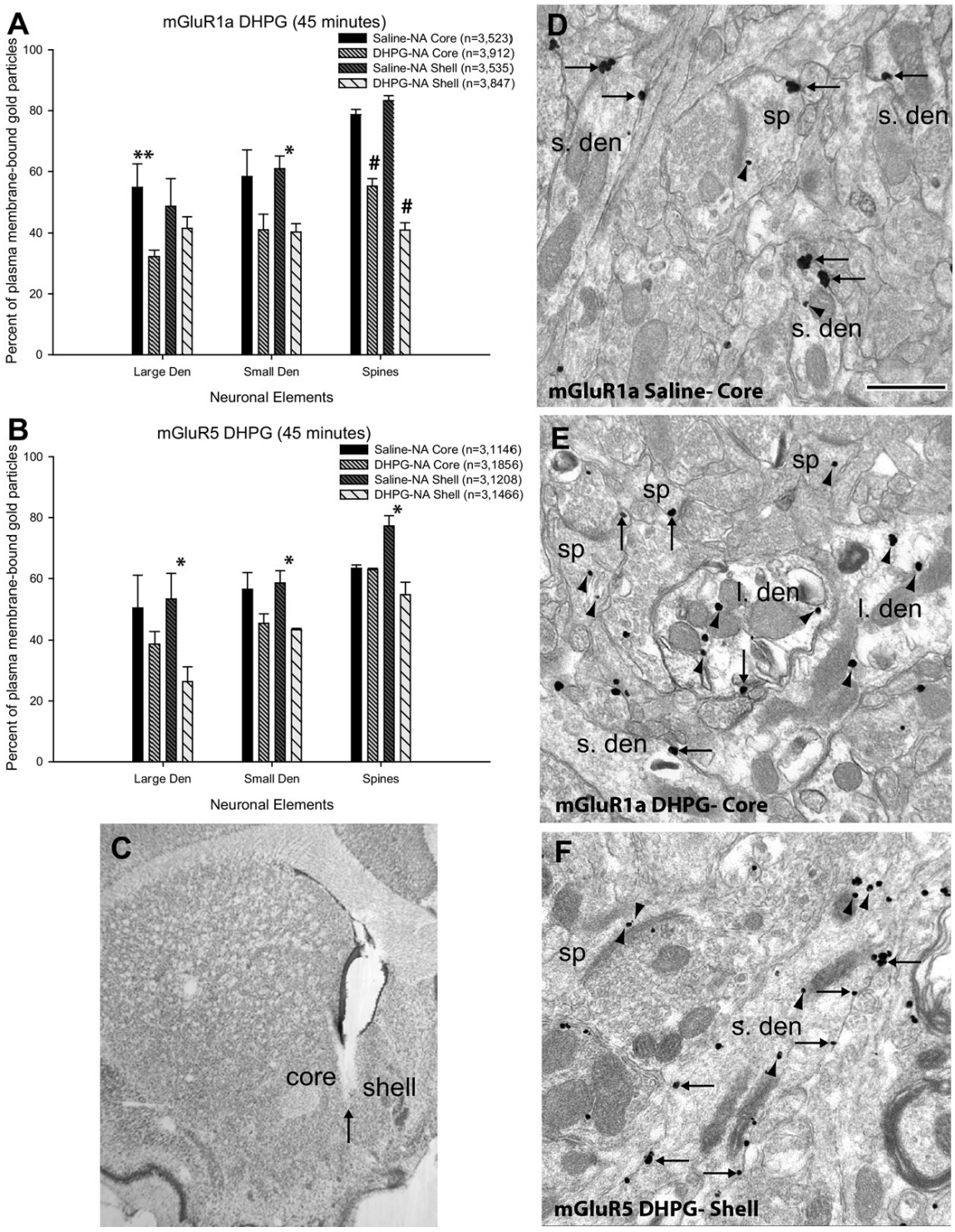

Figure 4.5: Histograms and immunogold labeling for mGluR1a and mGluR5 in saline- and DHPG-treated rats. (A) and (B) are summary histograms showing the

percentage of plasma membrane-bound labeling for mGluR1a (A) and mGluR5 (B) in the core and shell of the nucleus accumbens of saline- and DHPG-treated rats. Data are presented as mean percentages (±SEM) of gold particles on the plasma membrane of large or small dendrites and spines; in parentheses n= number of animals used, followed by the total number of gold particles counted in each experimental group. One-way ANOVAs and Tukey's post-hoc tests reveal that there is a lower percentage of plasma membrane-bound mGluR1a in large dendrites of the core ($p<0.05$, double asterisk), in small dendrites of the shell ($p<0.05$, single asterisk), and in spines of both shell and core of the accumbens ($p<0.001$, number signs) in DHPG-treated animals. For mGluR5, there is a significantly lower percentage of labeling on the plasma membrane in the shell of the accumbens on large and small dendrites ($p<0.05$, single asterisk) and spines ($p<0.01$, number sign). (C) Light micrograph of a sample DHPG injection into the core and medial shell of the nucleus accumbens. The arrow indicates the tip of the syringe. (D-F) show examples of labeled elements from saline- treated (D) and DHPG-treated animals (E-F). (D) mGluR1a labeling in the core of the accumbens of a saline-treated animal. Note the majority of extrasynaptic labeling (single-headed arrows) on the plasma membrane of small dendrites (s.den) and spines (sp). (E) mGluR1a labeling in the core of the accumbens of a DHPG-treated animal. Notice the increase in intracellular (arrowheads) labeling in both dendrites and spines. (F) mGluR5 labeling in the shell of the accumbens of a DHPG-treated animal. Note the large pool of intracellular labeling in dendrites and spines. Scale bar=0.5µm. Total number of elements examined: mGluR1a:

Saline, Core: large den=15, small den=137, spines=72; DHPG, Core: large den=57, small den=170, spines=91; Saline, Shell: large den=24, small den=94, spines=40; DHPG, Shell: large den=50, small den=134, spines=60. mGluR5: Saline core: large den=36, small den=115, spines=111; DHPG, Core: large den=38, small den=190, spines=105; Saline, Shell: large den=34, small den=147, spines=47; DHPG, Shell: large den=32, small den=157, spines=57.

4.5 Discussion

Two main conclusions can be drawn from this study. First, in contrast to the strong response of ionotropic AMPA receptors (Boudreau and Wolf, 2005; Boudreau et al., 2007), group I mGluRs in the nucleus accumbens display modest changes in their subcellular and subsynaptic localization after chronic or acute cocaine administration. Second, both mGluR1a and mGluR5 show a significant degree of internalization following local extracellular application of the group I mGluR agonist DHPG in both the core and shell of the nucleus accumbens. Together, these observations suggest that the trafficking of AMPA and group I mGluRs is differently affected by acute or chronic cocaine-induced changes in extracellular glutamate in the nucleus accumbens (Smith et al., 1995; Pierce et al., 1996; Reid et al., 1997). Furthermore, in line with other GPCRs (Bernard et al., 1998; Dumartin et al., 1998; Bernard et al., 1999; Csaba et al., 2001), our data provide the first in vivo evidence, in the brain, that group I mGluRs in the nucleus accumbens are endowed with trafficking properties that allow for rapid internalization in response to strong agonist stimulation.

One explanation for the minimal effect of cocaine administration on group I mGluRs trafficking could be that the cocaine administration regimen used in our study did not induce changes in extracellular glutamate in the nucleus accumbens. However, this is unlikely based on many previous studies showing rapid changes in glutamate release in the rat accumbens following administration regimens of acute or chronic cocaine similar to those used in the present study. For instance, extracellular glutamate levels in the accumbens are increased by as much as 300% over baseline approximately 40 minutes after a single injection of 30 mg/kg cocaine (Smith et al., 1995; Reid et al.,

1997). In contrast, the behavioral sensitization paradigm of 7 days of chronic cocaine exposure followed by three weeks withdrawal results in almost 50% reduction in extracellular glutamate compared to controls (Pierce et al., 1996; Hotsenpiller et al., 2001; Baker et al., 2003). Although not fully understood, there is good evidence that increased synaptic release of neurotransmitter from prefrontal cortical afferents may be the main source of extracellular glutamate buildup following acute cocaine administration (McFarland et al., 2003), while behavioral sensitization to chronic cocaine exposure decreases the activity of the cystine/glutamate exchanger, thereby, lowering extracellular glutamate levels in sensitized rats (Baker et al., 2003). In light of these findings, we can, therefore, assume that the levels of glutamate in the nucleus accumbens of rats used in the present study have been altered in the same manner by acute and chronic cocaine exposure.

A significant 15-20% increase in mGluR5 mRNA, but 10% decrease in mGluR5 protein expression along with blunted functioning of mGluR1 in the medial accumbens was reported in chronically cocaine-treated rats (Ghasemzadeh et al., 1999, Swanson 2001). On the other hand, 24 hours following a single cocaine injection, endocannabinoid-mediated LTD, regulated by mGluR5, is abolished and accompanied by a 50% reduction in surface expression of mGluR5 in mice accumbal medium spiny neurons (Fourgeaud et al., 2004). Furthermore, Homer 1b/c, one of the scaffolding proteins that regulates the trafficking and plasma membrane expression of group I mGluRs in vitro (Roche et al., 1999; Ango et al., 2002), is decreased by about 20% in the accumbens of behaviorally sensitized rats three weeks following chronic cocaine exposure (Swanson et al., 2001).

Apart from minor changes of dendritic mGluR1a distribution in the 45 minutes acutely cocaine-treated group and the chronically cocaine-treated animals, our electron microscopic study did not reveal any significant change in the overall pattern of subcellular and subsynaptic localization of mGluR1a or mGluR5 in response to either regimens of cocaine administration. Because the regulation of extracellular glutamate that reaches extrasynaptic group I mGluRs may be very complex and rely on many factors including the amount of glutamate released from axon terminals, amount of glutamate released by astrocytes and amount of glutamate reuptake by glial and neuronal glutamate transporters (Brasnjo and Otis, 2001; Oliet et al., 2001; Reichelt and Knopfel, 2002; Rusakov and Lehre, 2002), it is hard to predict how much glutamate, indeed, reaches these mGluRs. As shown in the cerebellum, there could be very subtle subsynaptic regulation of glutamate by neuronal transporters that strongly modulate the activity of group I mGluRs (Brasnjo and Otis, 2001). If such regulatory processes are in place in the nucleus accumbens, changes in extracellular glutamate levels measured with microdialysis do not provide an accurate index of the exact concentration of transmitter individual group I mGluRs may be exposed to following cocaine administration.

Our results are different from those of Fourgeaud et al. (2004) showing a dramatic reduction in mGluR5 plasma membrane expression 24 hours post-cocaine injection in mouse accumbal neurons. The use of different species, approaches and experimental conditions between this study and ours may account for these divergent results. Although the limited sensitivity of the immunogold method to localize small amount of protein cannot be ruled out (Galvan et al., 2006), it is noteworthy that the same method was sensitive enough to detect changes in both group I mGluRs plasma membrane expression

following intra-accumbens DHPG administration, and was successfully used by different groups to demonstrate internalization of various GPCRs in response to systemic or local agonist application (Bernard et al., 1998; Dumartin et al., 1998; Bernard et al., 1999; Csaba et al., 2001).

In striking contrast with group I mGluRs, AMPA receptor subunits display significant and opposite changes in their subsynaptic distribution after acute or chronic cocaine exposures (Boudreau and Wolf, 2005; Boudreau et al., 2007). Following the chronic behavioral sensitization paradigm, known to decrease extracellular glutamate (Pierce et al., 1996; Baker et al., 2003), an increased surface expression of synaptic AMPA receptors was found in the rat accumbens compared to saline-treated animals (Boudreau and Wolf, 2005). In contrast, acute cocaine exposure, which results in a significant raise in accumbens glutamate (Smith et al., 1995; Reid et al., 1997), lead to the internalization of AMPA GluR1- and GluR2-containing receptors (Boudreau and Wolf, 2005; Boudreau et al., 2007). Although these findings likely illustrate genuine differences in the trafficking of AMPA versus group I mGluRs in response to cocaine-induced changes in extracellular glutamate, the use of different techniques measure the surface expression of the receptors, may also account for these differential responses between ionotropic and metabotropic receptors after cocaine challenges. An important issue to consider is the exact localization of these glutamate receptors in relation to the release sites of glutamate. In contrast to AMPA receptor subunits, which are mainly concentrated in the main body of asymmetric glutamatergic synapses (Petralia et al., 1992; Baude et al., 1995), both mGluR1a and mGluR5 are largely extrasynaptic or perisynaptic to glutamatergic synapses (Baude et al., 1993; Ottersen and Landsend, 1997;

Galvan et al., 2006). In light of data showing that increased glutamatergic transmission at cortical synapses may account for the raise in extracellular glutamate measured in the accumbens after acute cocaine administration (McFarland et al., 2003), the rapid internalization of AMPA receptors following such treatment is predictable because of the increase of glutamate in the synaptic cleft. On the other hand, group I mGluRs, located further away from these synapses may be less affected by this synaptically regulated change in glutamate release. Although this may partly account for the differential responses of these receptors after acute cocaine, it is unlikely to be the case in chronically treated animals which display a significant reduction in extracellular glutamate mainly due to functional changes in the cystine/glutamate exchangers predominantly located on glial cells (Baker et al., 2003). Overall, these findings indicate that cocaine can induce significant changes in AMPA receptor subsynaptic localization, but not as much in group I mGluRs. The mechanisms underlying these differential effects may be complex and involve a multitude of factors including the localization, trafficking as well as the pharmacological and physiological properties of these receptors.

A few in vivo studies demonstrated significant changes in the ultrastructural localization of GPCRs in response to modulation of extracellular activating neurotransmitter (Dumartin et al., 1998; Dumartin et al., 2000; Bernard et al., 2003; Toda et al., 2003; Stanwood and Levitt, 2007). However, in MPTP-treated mice, an experimental condition characterized by a significant increase in glutamatergic transmission in the subthalamic nucleus and globus pallidus (Mally et al., 1997; Fedele et al., 2001; Wichmann and DeLong, 2003), very little, if any, changes were induced in the subsynaptic and subcellular distribution of group I mGluRs in these nuclei (Kuwajima et

al., 2007). Together, these findings highlight the unique intrinsic properties of group I mGluRs compared to other GPCRs in their response to changes in ambient activating transmitter levels. Receptor phosphorylation, lateral trafficking and receptor-receptor interactions are additional mechanisms, not examined in this study, that should be considered as playing major roles in regulating group I mGluRs responses to agonist exposure (Bernard et al., 2006; Dhami et al., 2006).

In contrast to systemic cocaine, intracerebral injections of group I mGluR agonist significantly decreased the percentage of plasma membrane-bound mGluR1a and mGluR5 in the accumbens. These observations are consistent with previous studies of other GPCRs known to internalize following direct agonist application (Bernard et al., 1998; ; Dumartin et al., 1998; Bernard et al., 1999; Csaba et al., 2001). Together, these in vivo data demonstrate that group I mGluRs share common internalization properties with other GPCRs when directly exposed to high dose of receptor agonists, but not in response to physiological or pathological changes in the level of ambient activating neurotransmitter. One possible explanation might be that the magnitude of cocaine-induced changes in glutamate release is not high enough to affect group I mGluRs trafficking. Another possibility is that the mechanism underlying the internalization of group I mGluRs after DHPG is different from that following glutamate-mediated receptor activation. For instance, in vitro data have shown that application of glutamate versus the agonist quisqualate attract different arrestin proteins that aid in internalization of mGluR1a (Dale et al., 2001; Mundell et al., 2001). Thus, the mechanisms underlying group I mGluRs internalization appear to be quite complex and highly dependent on

various factors including the source and pharmacological properties of the activating agonist.

Chapter 5:

Discussion & Future Directions

5.1 Summary of Findings

Prior to these studies, very little was known about the exact localization of mGluR1a and mGluR5 in the nucleus accumbens. Through the use of electron microscopic immunocytochemistry, tract-tracing method and behavior, findings presented in this thesis highlight various features of the subcellular and subsynaptic localization of group I mGluRs in the nucleus accumbens in normal animals and a rat model of cocaine addiction. The major findings of this dissertation are as follows (and are summarized in figure 5.1): 1) The group I mGluRs are widely distributed postsynaptically in dendrites and spines of various cell types in both the core and shell of the nucleus accumbens in rats and monkeys. While only minor differences were seen in the subcellular and subsynaptic localization between the core and shell, some differences were seen in the subsynaptic localization of mGluR5 between rat and monkey, with mGluR5 being found more often on the plasma membrane of dendrites and spines in the rat accumbens, compared to the monkey. 2) Limbic prefrontal cortical terminals and midline thalamic terminals contact both mGluR1a- and mGluR5-immunoreactive dendrites and spines in both the shell and core, but to varying degrees. In addition, mGluR5 was found to be more frequently expressed perisynaptically at both cortical and thalamic synapses than mGluR1a. Extrasynaptic mGluR5 labeling was also closer to the edges of cortical and thalamic asymmetric synapses than mGluR1a. 3) Only minor changes were seen in the subsynaptic localization of mGluR1a following acute and chronic cocaine exposure. Neither acute nor chronic cocaine treatment induced a significant change in the localization of mGluR5 in the accumbens core and shell, which

is different from the significant reduction of plasma membrane-bound mGluR1a and mGluR5 induced by local DHPG administration.

Overall, these results provide a solid framework whereby mGluR1a and mGluR5 could mediate some of their physiological and behavioral effects in the core and shell of the nucleus accumbens. This brings us one step closer to understanding possible functions, mechanisms of activation, level of plasticity and trafficking properties of group I mGluRs in the nucleus accumbens.

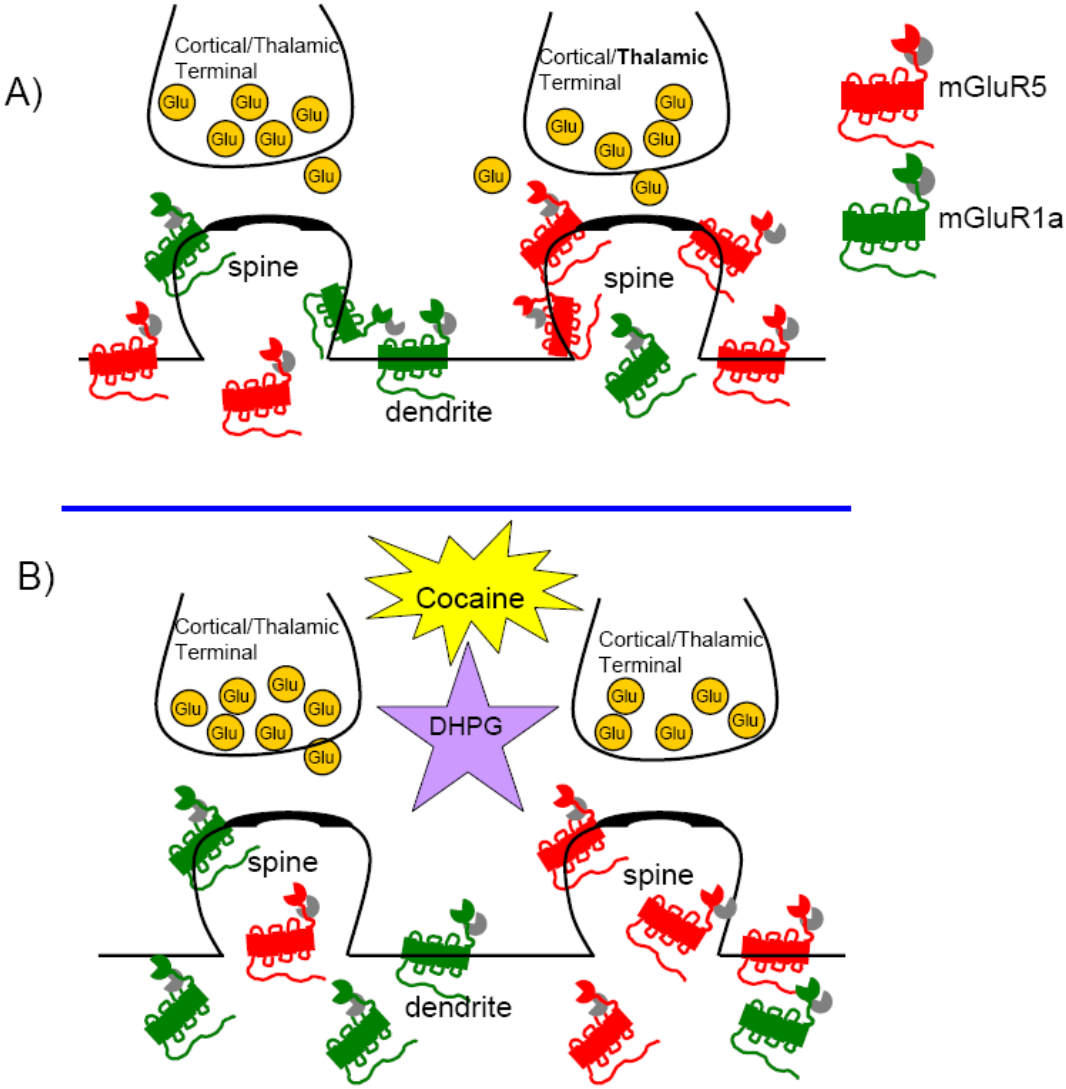

Figure 5.1: Results Summary Diagram. A) Shows the localization of mGluR1a and mGluR5 in dendrites and spines, mainly extrasynaptic on the plasma membrane in normal animals. In addition, both PFC and midline thalamic terminals are in contact with mGluR1a- and mGluR5-containing spines, but to varying degrees as indicated by thalamic being in bold. B) In the presence of cocaine, mGluR1a shows modest internalization, while both receptors have decreased plasma membrane-bound expression following DHPG.

5.2 Implications for drug abuse

Cocaine remains one of the most commonly abused drugs in the United States, with estimates of about 34 million people having tried the drug at least once (NSDUH, 2004). It has also been estimated that approximately 2 million cocaine addicts exist in the United States today (NSDUH, 2004). Based on these numbers, it is evident that there still remains a lot of work to be done in the area of cocaine abuse and addiction. Currently, there is no commercially available pharmacotherapy to reduce the rewarding effects of and alleviate the addictive properties of cocaine. Therefore, studies like those presented in this thesis help us get a better idea of the neural substrates that may mediate the transition from drug abuse to addiction. Previous studies have shown that the group I mGluRs seem to be involved in psychostimulant abuse and have the potential to be a target for pharmacotherapies aimed at reducing cocaine intake. As mentioned previously, mGluR5 knockout mice do not self-administer cocaine; following behavioral sensitization to cocaine, mGluR5 mRNA and protein levels are altered; and finally antagonists for both of the group I mGluRs have been shown to alter the effects and intake of cocaine (Ghasemzadeh et al., 1999; Chiamulera et al., 2001; Swanson et al.,

2001; McGeehan and Olive, 2003; Kenny et al., 2005; Lee et al., 2005; Dravolina et al., 2006). While only minor changes were seen in the localization of group I mGluRs in the accumbens following acute or chronic cocaine administration, other properties of mGluR1a or mGluR5 could have been altered by these treatments that were not addressed in this study. The results of this thesis, however, in addition to emerging evidence about the exact roles that the core and shell play in abuse and addiction, could provide the basis for newer more efficient drug development. For example, the shell has been implicated in the initial rewarding effects of cocaine (Pontieri et al., 1995), while the core is involved in drug-associated cue-induced cocaine seeking (Fuchs et al., 2004). Furthermore, perhaps by combining what we know about the localization of various neurotransmitter systems in brain, such as glutamate and dopamine, with the function and subcellular distribution of their receptors, one could achieve the development of better pharmacotherapy to aid those seeking help for their addictions.

5.3 Other clinical applications of this work

As stated in the introduction in chapter 1, the nucleus accumbens is involved in other behaviors besides drug abuse, such as the rewarding feelings experienced from natural rewards like food or sex, and certain motor skills. It actually has been shown that there are different neurons in the nucleus accumbens that respond to drug reward compared to natural reward, in this case food and water (Carelli et al., 2000; Carelli & Wondolowski, 2003). It would be interesting to anatomically explore the different cell types to determine if they possess the same receptor types or distinct populations of receptors and whether group I mGluRs play a role in natural rewards such as water consumption.

Although the nucleus accumbens is not as affected as the dorsal striatum in neurodegenerative diseases like Parkinson's and Huntington's diseases, group I mGluRs have been implicated in the pathogenesis of these diseases. As was mentioned also in the introduction, antagonists for both mGluR1a and mGluR5 have been shown to alleviate some of the symptoms of, as well as being neuroprotective against, both Parkinson's and Huntington's diseases (Breysse et al., 2002, 2003; Coccurello et al., 2004; Conn et al., 2005). In contrast, the group I mGluR agonist, DHPG, mediated by mGluR1a, has been shown to be protective against ischemia and stroke (Blaabjerg et al., 2003; Baskys et al., 2005). The findings about the localization and trafficking of the group I mGluRs in an in vivo system provide valuable information for future studies targeting these receptors for drug development for neurodegenerative diseases. Gaining an understanding of the localization of mGluR1a and mGluR5, especially in relation to certain afferents, sheds light on possible mechanisms of activation of these receptors, which could be crucial to explore in other brain regions, so more efficient drug therapies may be developed.

5.4 A Critical Look at Methodology

Although the techniques used in this thesis, including immunocytochemistry, tract tracing and electron microscopy, have been used for numerous decades, it is important to consider possible limitations of these methods. The advantages and disadvantages of the main techniques used throughout this dissertation and how these apply to the interpretation of the work presented will be examined below.

5.4.1 Tissue Fixation, Antibodies

As was described in the methods section of chapters 2-4, animals were fixed using a combination of paraformaldehyde and glutaraldehyde. Both are excellent fixatives that

preserve the ultrastructure of membranes and harden the tissue to allow for sectioning. These fixatives react with the proteins in the tissue to stabilize them through cross-linkage. However, if the concentrations of the fixatives are too high, this could stop the antibody from being able to react with the receptor in question. In addition, the fixative could denature the antigenic site and stop the penetration of other reagents into the tissue (Bolam, 1992). Therefore it is important, after sectioning the tissue to rinse it in an agent such as sodium borohydride to aid in breaking the cross-linkage of the fixatives to the proteins in the brain sections (Totterdell et al., 1992). Based on this, it is important to realize that although the tissue is clearly labeled in my experiments, there is the possibility that not all receptors were recognized by the antibodies, which could lead to false negatives in our results.

The ultrastructure of the tissue is also an important point to consider. The fixatives aid in the preservation of ultrastructural features of brain tissue but, in addition, it is important not to freeze and thaw too often the brain tissue (hence why it is cut on a vibrating microtome) if it has to be used for electron microscopy because frozen tissue usually yields poorer ultrastructure, which is a very important trait to preserve if proper EM analysis is to occur. This is extremely important especially when examining immunogold labeled tissue because most of the data analysis is dependent on the visualization of plasma membranes in the EM. In our studies, if the ultrastructure from a certain animal was not up to par, it was not used for further analysis.

As for the antibody itself, it is important to know that it is specific for the receptor that we were localizing. An antibody must be highly specific and have a high affinity for the antigen, or receptor in question, and should not cross-react with other proteins in the

tissue. As explained in the Primary Antibodies section in Chapters 2-4, there have been various studies examining the specificity of mGluR1a, mGluR5 and Calbindin-D_{28k} antibodies using transfected cells, Western blot analysis, and knockout tissue. Using these methods, immunoreactivity for the proteins in question using specific antibodies are expected to: (1) be absent from knockout tissue and (2) labels a band at the correct molecular weight on a Western blot. Antibodies used in our work have been characterized as fairly specific using these methods, thereby, provide an accurate location of group I mGluRs immunostaining in the nucleus accumbens (Koulen et al., 1997; Marino et al., 2001; Kuwajima et al., 2004).

5.4.2 Pre-embedding Immunogold vs. Pre-embedding Immunoperoxidase

The two major methods used to reveal the primary antibodies for both mGluR1a and mGluR5 throughout my experiments are the pre-embedding immunoperoxidase (species specific secondary biotinylated IgGs bind to the avidin-biotin complex (ABC), and in the presence of DAB and H_20_2 forms an amorphous dark deposit) and pre-embedding immunogold (the species specific secondary IgGs conjugated with 1.4nm gold particles are silver-intensified to be seen as 30-50nm dark, round particles on the EM). There are advantages and disadvantages that characterize the sensitivity and spatial resolution of the reaction products that result from the immunoperoxidase or immunogold methods. The avidin and biotin that form the ABC complex have an extremely high affinity for each other, almost stronger than the affinity for the primary antibody and its antigenic site. This allows for a high degree of specificity of labeling in the tissue being examined, however it does not provide as much spatial resolution as the pre-embedding immunogold method. As can be seen in the results sections of chapters 2-4,

immunoperoxidase allows us to detect which neuronal elements express mGluR1a and mGluR5. This is in comparison to the immunogold method which allows us to first determine whether the receptor was found on the plasma membrane or in the intracellular space. We can then also detect where on the plasma membrane the receptors are found, i.e. extrasynaptic, synaptic or perisynaptic. While the immunogold method does provide this additional degree of spatial resolution, the gold-conjugated secondary antibodies do not penetrate the tissue as well as reagents used for the immunoperoxidase method. This forces one to only examine tissue towards the surface of the section, which increases chances for background, false positive labeling, and poorer ultrastructure of the tissue being examined. Therefore, when examining immunogold labeled tissue at the EM, one should not take micrographs of tissue along the edges or too deep to try to prevent false negatives and false positives. These are important methodological considerations to keep in mind when analyzing the results from pre-embedding immunogold experiments. It is possible that some of the gold particles I counted in my results were possibly background or non-specific labeling. If the antibody in question is known to be specific, this background labeling could be a result of the gold particles getting stuck in the tissue. As mentioned previously, the gold particles have difficulty penetrating the tissue; therefore, following exposure to the gold-conjugated secondary antibodies, the gold particles do have the potential to stay bound to the tissue even with the proper rinses. This could have been a factor explaining why I did not find much significant differences between plasma membrane-bound vs. intracellular labeling for the group I mGluRs following different cocaine treatments in chapter 4. However, because we did see major changes in gold labeling distribution after local DHPG administration using the same approach, we

concluded that the pre-embedding immunogold method is sensitive and specific enough across immunoreactions and experimental cases to provide an accurate view of the reorganization of gold labeling following an experimental treatment. Besides trying to analyze the best possible tissue, controls are always run alongside the experimental tissue that has not been exposed to primary, but only the secondary antibodies to ensure that the tissue was labeled correctly.

One method that helps eliminate some of the limitations of the pre-embedding immunogold technique is the post-embedding immunogold method. Post-embedding staining occurs once the tissue has been processed for EM analysis (as described in the method sections of chapters 2-4 and includes osmification, dehydration and embedding the tissue in resin); cut into ultrathin 60nm thick sections and placed on grids. This enables all areas of the tissue to be exposed to the antibody and allows for a more quantitative measurement of immunoreactivity. In addition, it offers a maximum level of resolution, for example, the ability to measure the density of receptors at a certain synapse. However, while the post-embedding immunogold method has proven valuable for analyzing the localization of ionotropic glutamate receptors, it does not work as well for metabotropic glutamate receptors and, therefore, was not used in this series of studies examining the localization of mGluR1a and mGluR5.

5.4.3 Tracers

The anterograde tracer used in the tract tracing experiments in Chapter 3 was biotinylated dextran amine or BDA. The use of this tracer to study neuronal pathways started in the early 1990's as an alternative to the use of other tract tracing methods, such as neuronal degeneration and tritiated amino acids. These older methods lacked

sensitivity and resolution, so newer tracers like PHA-L (*Phaseolus vulgaris*-leucoagglutinin) and BDA were introduced, making tract tracing easier and more reliable (Gerfen & Sawchenko, 1984; Veenman et al., 1992; Rajakumar et al., 1993). BDA can be injected either iontophoretically through a glass micropipette (as we did in our study) or by pressure. Although easier and less time consuming, pressure injections yield a larger injection site and when trying to inject small nuclei in brain areas, such as the thalamus, iontophoretic injections are more useful. The mechanism by which BDA is taken up by neurons is still unclear. It possible that the tracer enters the neuron through endocytosis for intact neurons (Reiner et al., 2000) or through damaged axons, and then transported by diffusion both in the anterograde or retrograde directions through the neurons (Fritzsch & Wilm, 1990; Fritzsch, 1993). One of the major considerations when using BDA is the possibility of retrograde cell body labeling and, most importantly, labeling of axon collaterals of retrogradely labeled cells, when the anterograde labeling is the only feature of interest. However, BDA comes in two different molecular weights, 3000MW and 10000MW, where the 3000MW is primarily used for retrograde tracing, while the heavier BDA is transported primarily in the anterograde direction (Veenman et al., 1992; Fritzsch, 1993). Because the systems I examined are interconnected, we serially sectioned the entire brain of the rats injected and revealed the BDA tracer in every 6th section. There was no obvious retrograde labeling from any of the structures injected that could, in turn, complicate the interpretation of axonal and terminal labeling seen in the nucleus accumbens. We are, therefore, confident what we saw in the accumbens was a result of primarily anterograde labeling from the targeted injected structures.

5.4.4 Double Pre-embedding Immunocytochemistry

Double labeling at the EM is a rather valuable tool to visualize two receptors, proteins, etc., at the same time in the same tissue. In my thesis, this has allowed us to determine whether the two group I mGluRs colocalize, characterize the cell types that express group I mGluRs and finally study the spatial relationships between postsynaptic group I mGluRs and various glutamatergic afferents to the accumbens. There were various instances where double labeling was used in this dissertation: 1) Chapter 2: *Double immunoperoxidase labeling for group I mGluRs colocalization;* 2) Chapter 2: *Double Immunoperoxidase and Immunogold Labeling for Group I mGluRs and Neuronal Markers*; 3) Chapter 3: *Double Pre-embedding Immunoperoxidase Labeling for BDA and Group I mGluRs;* and 4) *Double Pre-embedding Immunoperoxidase for BDA & Immunogold for mGluR1a or mGluR5.*

The double pre-embedding immunoperoxidase method was utilized in both chapters 2 and 3 for various reasons. First off, in chapter 2, in order to colocalize mGluR1a and mGluR5, we could not use a double immunocytochemical procedure using two different antigenic markers due to the fact that the antibodies for mGluR1a and mGluR5 were raised in the same species, rabbit. The double immunoperoxidase technique offers an advantage over the combination of peroxidase and immunogold. Labeling both receptors with the same marker avoids interpretation problems induced by the use of two markers that do not penetrate brain tissue to the same depth, as explained in previous sections, immunoperoxidase penetrates the tissue more so than immunogold. The main disadvantage in using this method is the interpretation of unlabeled elements in the tissue. Are these elements truly devoid of one of the two receptor subtypes or is the

lack of labeling for a specific antigen the result of poor antibody penetration into the tissue? This is an important limitation one should keep in mind when interpreting the results of colocalization studies using double pre-embedding methods with different markers.

The double immunoperoxidase method was also used in the first set of experiments in Chapter 3 in order to get an accurate assessment of the proportion of mGluR1a- and mGluR5-containing dendrites or spines contacted by terminals from the various glutamatergic afferents for 2 reasons: 1) in previous studies it has been shown that the group I mGluRs are rarely found in axon terminals in both the shell and core of the rat accumbens (Mitrano & Smith, 2007a), therefore all labeling that was seen in axon terminals was almost definitely from the BDA tracer; and 2) as stated in the previous paragraph, double labeling with immunogold as a marker for the receptors may yield false negatives because immunogold is not as specific as immunoperoxidase and does not penetrate the tissue as well. There are disadvantages to the double immunoperoxidase labeling in this experiment as well. This once again has to do with the interpretation of non-labeled dendrites or spines that were in contact with positively-labeled axon terminals arising from the cortex and thalamus. The primary antibodies obviously do not penetrate the tissue as well as the tracer which is throughout the tissue, therefore false negative labeling of the postsynaptic target is something to keep in mind when interpreting these results. We did, however, try to keep observation of the tissue in the accumbens to areas where both receptor and terminal labeling was found.

In both chapters 2 and 3, double labeling methods using both immunogold and immunoperoxidase techniques were used. In chapter 2, it was used as a mean to

determine the presence of group I mGluRs in various cell types in the nucleus accumbens. In chapter 3 it was used to determine the subsynaptic localization of mGluR1a or mGluR5 (revealed with immunogold) in relation to the cortical and thalamic afferents.

In chapter 3, we only observed spines or dendrites that were in contact with a positively labeled axon terminal and that had immunogold labeling for either mGluR1a or mGluR5. Therefore, false negatives would not impact my results as much in this section. In order to ensure that all gold labeling was accounted for, we followed positively labeled terminals in contact with positively labeled spines or dendrites through a series of approximately 3-6 serial sections. As shown in chapter 3, figure 3.4, the pattern of gold labeling does vary from section to section, suggesting a differential distribution of receptor proteins along synapses.

Despite these limitations, each of these methods offers their specific strength to the present study, thereby; provide unique tools to address issues raised in this thesis when used in combination.

5.5 Future Directions

While this dissertation provides a framework for the localization of the group I mGluRs in the core and shell of the nucleus accumbens of monkeys and rats, further studies are needed to gain a better understanding of the exact function of these receptors in this brain region. First, since our overall goal is to understand the function of mGluR1a and mGluR5 in the human brain, further analysis of the localization of group I mGluRs in the primate nucleus accumbens is needed, such as the colocalization of group I mGluRs with each other and the various markers of striatal neurons, as well as the tract

tracing studies that were completed only in the rodent brain. Although there are many similarities in the localization of group I mGluRs between primates and non-human primates, it has been shown, even in our studies in chapter 2, that there are species differences in the localization of the group I mGluRs. Additionally, since the size of the nucleus accumbens is relatively smaller in the primate brain, it would be interesting to continue examining differences in receptor localization as well as function of this brain region between the rat and monkey. Furthermore, it would be interesting to examine the subsynaptic localization of group I mGluRs in relation to other glutamatergic inputs, such as those from the hippocampus and amygdala. This would then provide a comparison whereby all glutamatergic pathways could mediate their effects through activation of mGluR1a and mGluR5 in the core and shell of the accumbens.

Because the electrophysiological features of group I mGluRs studied using the latest pharmacological agents for group I mGluRs have been largely characterized in vitro using brain slices from young rats or mice (Robbe et al., 2002; Fourgeard et al., 2004; Schotanus & Chergui, 2008), there could be species- and age-dependent differences in the localization and function of mGluR1a and mGluR5 (Hubert & Smith, 2004). Furthermore, these studies only claimed to stimulate afferents from the cortex to the nucleus accumbens, which leaves the numerous other glutamatergic afferents (from the thalamus, hippocampus, and amygdala), unstudied. In order to gain a better understanding of the mechanisms by which group I mGluRs may modulate glutamatergic transmission from these other nuclei, in vivo electrophysiology is needed in adult rats, and even possibly primates.

It would also be beneficial to continue studying mGluR1a and mGluR5 in relation to drug abuse. Despite the fact that we did not see any changes in the localization of the group I mGluRs following cocaine administration, antagonists for these receptors alter the effects of cocaine (McGeehan and Olive, 2003; Kenny et al., 2005; Lee et al., 2005; Dravolina et al., 2006), and mGluR5 knockout mice do not self administer cocaine and have altered locomotor responses to cocaine administration (Chiamulera et al., 2001). Therefore, some property of this family of receptors must be altered in relation to cocaine. Perhaps various phosphorylation states of the group I mGluRs could be altered by cocaine or receptor-receptor interactions, for example with dopamine receptors, may be disrupted, which could lead to various downstream effects we are yet to understand. Additionally, it would be interesting to examine whether different results would be obtained if the cocaine was self-administered by the animal rather than experimenter administered, as was done in this series of studies. Finally, since the prefrontal cortex has been implicated so extensively in the glutamatergic neurotransmission changes involved in cocaine use, one could examine the changes in localization of the group I mGluRs only in dendrites and spines that are directly in contact with PFC afferents. This would require a combination of cocaine administration, tract tracing, and immunocytochemistry at the electron microscopic level, but would provide some further valuable information about the neural changes that could underlie cocaine addiction.

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