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Microcircuitry of Group III Metabotropic Glutamate Receptors (mGluRs) in the Mouse Striatum

by
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Doctor of Philosophy

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Liliya Iskhakova

B.S., Biology, Oglethorpe Univeristy, 2001

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An abstract of a dissertation submitted to the Faculty of the Graduate School of Emory University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Abstract

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Liliya Iskhakova

Glutamatergic transmission is the main excitatory drive within the nervous system. Its effects are transmitted and controlled by many types of pre- and post-synaptic receptors segregated into two main categories based on sequence homology, structure, and signal transduction mechanisms. The ionotropic receptors, responsible for fast transmission, are ligand-gated ion channels. On the other hand, metabotropic receptors (mGluRs), responsible for slower, longer lasting effects, are G-protein-coupled receptors. The mGluRs are further subdivided into three groups (I, II, III) based on sequence homology, second messenger coupling and pharmacology. Availability of research tools, pharmacoagents, and an ability to selectively inhibit particular circuits has instigated an interest in group III mGluRs, because these receptors can be used as substrates for therapies alleviating symptoms in diseases exhibiting imbalances in glutamatergic transmission, such as Parkinson's disease. This study used high resolution electron microscopy immunohistochemical techniques to examine the localization and distribution of group III mGluRs in the striatum, which is the main input nucleus of the basal ganglia circuitry. In the striatum, group III mGluRs are shown to be localized mainly on presynaptic elements originating from both the cortico- and thalamo-striatal projections with significant degree of receptor co-expression in individual terminals. The group III mGluR-immunopositive terminals target, to a variable degree, postsynaptic elements of both the direct and indirect pathways, thus allowing for potential regulation of both striatofugal output systems. However, while the mGluR4-positive terminals seem to have a preference for the indirect pathway, the mGluR8-positive terminals are mainly associated with the direct pathway. On the other hand, the mGluR7-immunoreactive terminals are evenly distributed between the two output pathways. These data provide a map of the group III mGluRs localization along the main glutamatregic input pathways to the striatum, thereby offer a greater understanding of the mechanisms by which these receptors could be mediating their regulatory effects on excitatory transmission in the striatum and the basal ganglia. The anatomical schematic of receptor localization will further guide the discovery and development of pharmacoagents that can selectively activate group III mGluRs, regulate specific neuronal circuits**,** and hopefully address symptoms of numerous neurological disorders.

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1. Introduction

Glutamate was discovered in 1866 german chemist Karl Heinrich Leopold Ritthausen when he isolated isolated it from wheat germ. Glutamic acid is one of the 20 proteinogenic amino acids and its codons are GAA and GAG. It is a non-essential amino acid. The carboxylate anions and salts of glutamic acid are known as glutamates. Glutamate is a key molecule in cellular metabolism, but in the mammalian nervous system it is the most abundant excitatory neurotransmitter. The excitatory action of glutamate in the mammalian brain and spinal cord has been known since the 1950s (Curtis et al., 1960; Hayashi, 1952). However, it was in the 1970s that glutamate was recognized as the principal excitatory transmitter within the vertebrate nervous system (Meldrum, 2000). Needless to say, glutamate plays a pivotal role in many normal and pathological neurological processes such as learning and memory, neurodegenerative diseases, seizures, drug addiction etc. Thus, pharmacotherapies that target the glutamatergic systems have a great potential in addressing symptoms of numerous neurological disorders.

Glutamate acts through many types of receptors which are divided into two classes, based on transduction mechanisms: ionotropic, which are ligand-gated ion channels (Dingledine et al., 1999) and metabotropic, which are G-protein-coupled receptors. The many specific subtypes of glutamate receptors are referred to by a chemical which was originally identified to activate them selectively. The ionotropic glutamate receptors are multimeric assemblies of four or five subunits and are subdivided into three groups: *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate (Meldrum, 2000) receptors. These receptors all incorporate ion

channels that are permeable to cations, although the relative permeability to Na^{+} and Ca^{++} varies according to the family and the subunit composition of the receptor.

In the late 1980s, it was proposed that there are also metabotropic glutamate receptors (mGluRs) that are linked to G proteins and operate either by releasing second messengers in the cytoplasm or by influencing ion channels through release of G protein subunits within the membrane (Sladeczek et al., 1985); (Conn and Pin, 1997; Pin and Duvoisin, 1995; Schoepp and Conn, 1993). Eight mGluRs have been identified so far and have been classified into three groups. Group I receptors, including mGluR1 and 5, activate phospholipase C (PLC), producing diacylglycerol and inositol triphosphate as second messengers (Conn and Pin, 1997; Sladeczek et al., 1985; Sugiyama et al., 1987). Group II, including mGluR2 and 3, and group III, including mGluR4, 6, 7, and 8, are negatively coupled to adenylyl cyclase. Each mGluR has a distinct distribution, subcellular localization, and marked difference in the sensitivity to glutamate (Conn and Pin, 1997).

1.1. Glutamate Receptors

1.1.1. Ionotropic Glutamate Receptor Classification

There are three major types of ionotropic glutamate receptors (iGluRs), which are named after the agonists that were originally identified to activate them selectively, and are therefore called N-methyl-D-aspartate (NMDA), α-amino- 3-hydroxy-5-methyl-4-isoazolepropionic acid (AMPA) and 2-carboxy-3-carboxymethyl-4-isopropenyl-pyrrolidine (kainate) receptors. The receptors appear to be tetrameric (Laube et al., 1998) or pentameric and the subunits that comprise these are specific for each of the three

families (Dingledine and Conn, 2000). The subunit composition determines the biophysical properties of the receptor and to a variable extent its pharmacology. Even though iGluRs share common structural features, the overall amino acid identity across the three families is only in the 20–30% range (Kew and Kemp, 2005). Ionotropic glutamate receptor subunits possess an extracellular amino terminal domain, which exhibits homology to the metabotropic glutamate receptor (mGluR) bi-lobed agonist binding domain, followed by a first transmembrane domain and then a pore forming membrane-residing domain that does not cross the membrane but forms a reentrant loop entering from and exiting to the cytoplasm. The second and third transmembrane domains are linked by a large extracelluar loop and the third transmembrane domain is followed by an intracellular carboxy terminus (Dingledine et al., 1999; Kew and Kemp, 2005; Mayer and Armstrong, 2004).

The NMDA receptor family is composed of seven subunits, NR1, NR2A–D and NR3A and B, which are all products of separate genes. The NMDA receptor is unique amongst ligand-gated ion channels in its requirement for two obligatory co-agonists, binding at the glycine and glutamate binding sites localized on the NR1 (Kuryatov et al., 1994); (Hirai et al., 1996; Kew et al., 2000; Wafford et al., 1995) and NR2 (Anson et al., 1998; Laube et al., 1997) subunits, respectively. NMDA receptors are of tetrameric structure and are likely composed of pairs of dimers (Kew and Kemp, 2005)

AMPA receptors are composed of a four-subunit family (GluR1–4) that are products of separate genes and are thought to assemble as either pentamers or tetramers (Rosenmund et al., 1998). Like NMDA receptors, native AMPA receptors are likely heteromeric in composition. The GluR2 subunit plays a critical role in determination of

the permeability of heteromeric receptors to Ca2+. Thus, AMPA receptors that do not contain GluR2 are permeable to Ca+2 and show marked inward rectification, which is the result of a voltage-dependent block of the ion channel by intracellular polyamines (Bowie and Mayer, 1995; Kew and Kemp, 2005).

Kainate receptors are composed of two related subunit families, GluR5–7 and KA-1 and 2. Once again, native receptors are likely tetrameric combinations, possibly of both homomeric and heteromeric combinations. KA-1 and KA-2 subunits do not form functional homomeric receptors but do form high-affinity kainate binding sites and can, thus, bind agonist ligands. KA-1 and-2 combine in heteromeric assemblies with members of the GluR5–7 subfamily to form functional receptors resembling native kainate receptors (Bleakman et al., 2002). Evidence suggests that in the absence of GluR5–7 subunits, KA-2 cannot achieve cell surface expression, but is retained in the endoplasmic reticulum (Gallyas et al., 2003). Unlike the KA subunits, the GluR5, 6 and 7 subunits can form functional homomeric receptors as well as combine with KA-1 and 2 to form heteromeric receptors with distinct pharmacological properties (Alt et al., 2004).

The ionotropic receptors mediate the postsynaptic responses of target neurons to glutamate activation by opening cation channels, thereby depolarizing the neuron at resting potential, but also by allowing the entry of Ca^{++} , which has intracellular function other than charge transfer, including the release of intracellular Ca^{++} stores and the activation of Ca^{++}–dependent ion channels. Ionotropic receptors have functional properties beyond that of opening ion channels. These are provided by the capacity of the intracellular carboxy terminal to interact with a variety of intracellular proteins. These include scaffolding proteins involved in the spatial and functional organization of

postsynaptic densities, but also include proteins involved in signal transduction (Hayashi et al., 1999).

1.1.2. Metabotropic Glutamate Receptors Classification

To date, eight members of the G-protein-coupled metabotropic glutamate receptor family have been identified (mGluR1–8) which have been divided on the basis of sequence homology, second messenger coupling and pharmacology into three groups: group I (mGluR1 and 5), group II (mGluR2 and 3) and group III (mGluR4, 6, 7, 8). Compared to the effect of ionotropic receptors, the activation of mGluRs leads to a slower and longer lasting effect on cellular excitability. Thus, mGluRs have the capacity to provide a mechanism by which glutamate can modulate or fine-tune activity at the same synapses at which it elicits fast synaptic responses through ionotropic receptors. The mGluRs have seven trans-membrane domains with an extracellular N-terminal and intracellular COOH terminal. They display little sequence homology with other metabotropic receptors, except for a modest resemblance to GABAB receptors. However, mGluRs of the same group show about 70% sequence identity, whereas between groups this percentage decreases to about 45% (Conn and Pin, 1997). This classification is also supported by the respective transduction mechanisms of the different mGluR subtypes.

The group I mGluRs predominantly couple via Gq/G_{11} to phospholipase C, whereas the group II and group III receptors couple with Gi/Go to inhibit adenylyl cyclase activity. Although mGluR family members can mediate synaptic transmission via activation of slow excitatory postsynaptic potentials, they generally exert a more modulatory role, regulating neuronal excitability, synaptic transmission and plasticity.

mGluR-mediated signalling is achieved both via the activation of intracellular second messenger pathways and subsequent regulation of downstream effectors, and through the direct action of the βγ subunits of the heterotrimeric G protein (Anwyl, 1999).

Distribution studies have shown mGluR mRNA and protein expression in many cortical and subcortical regions of the CNS. The striatum is enriched in all mGluR subtypes, except for mGluR6 which is solely expressed in the retina (Duvoisin et al., 2005).

1.1.3. Structure and Pharmacology of Group III mGluRs

1.1.3.1. Group III mGluR Structure and Composition

The group III mGluRs include mGluR4, 6, 7, and 8 and are composed of an N-terminal end that binds to the neurotransmitter, the 7-transmembrane domains that bind the receptor to the membrane, and the C-terminal end that couples with the Gi/Go class of G-proteins and interacts with the intracellular signaling and scaffolding machinery. The extracellular N-terminal tails comprise two lobes separated by a hinge region, and several studies including X-ray crystallography indicated that these two lobes close like a Venus' flytrap upon binding of the ligand. Like in other G-protein coupled receptors (GPCR), the seven transmembrane helices (TM-I through -VII) compose the central core domain of the receptor where each transmembrane α-helix (Unger et al., 1997) is connected to one another by three intracellular (i1, i2 and i3) and three extracellular (e1, e2 and e3) loops (Baldwin, 1993). Two cysteine residues (one in e1 and one in e2), which are conserved in most GPCRs, form a disulfide bond which could play a role in the packing and stabilization of a restricted number of conformations of these seven TMs (Bockaert and

Pin, 1999). A conformational shift of this domain is probably responsible for receptor activation (Bockaert and Pin, 1999). An alteration in the relative orientation of TM-III and TMVI (with a rotation of TM-VI and a separation from TMIII) unmasks G protein-binding sites (Bourne, 1997; Farrens et al., 1996; Javitch et al., 1997) and switches the receptor from the inactive to the active conformation.

The mGluRs are dimeric proteins, and functional mGluRs are thought to comprise homodimers stabilized not only by an intersubunit disulfide bond, but also by hydrophobic interactions. The dimeric ligand-binding region of mGluR adopts multiple conformations, where the activated forms of the dimer are in equilibrium with other states (Kunishima et al., 2000). In the inactive state, the bi-lobed extracellular domains of the mGluR homodimer exist far from each other, and comprise an open conformation. Binding of an agonist shifts the dimer orientation and causes it to adopt a closed conformation. Full receptor activation requires agonist binding to both partners of the homodimer, but binding of an agonist to just one of the homodimers would only result in partial activatation of the receptor (Kniazeff et al., 2004). Glutamate has also been demonstrated to have a negative co-operativity binding role in the dimeric mGluRs (Suzuki et al., 2004). More specifically, in a receptor dimer complex, ligand binding can cause the conformational changes of one receptor to inhibit the activation of the second receptor in that dimer through the suppression of the closing motion (Suzuki et al., 2004).

There are 4 members of the group III mGluRs family, but splice variants further increase the multiplicity of these receptors. Alternative pre-mRNA splicing results in 2 splice variants for mGluR4 and mGluR6, 5 splice variants for mGluR7, and 3 splice variants for mGluR8 (Ferraguti and Shigemoto, 2006). Group III mGluRs range from

501-924 amino acids and show about 70% sequence identitybetween corresponding regions of each receptor subtype (Conn and Pin, 1997; Ferraguti and Shigemoto, 2006). The receptors with 501 and 508 amino acids are the short splice variants of mGluR8(c) and mGluR6(b) which only contain the extracellular N-terminal and lack the transmembrane domains and the intracellular C-terminal (Malherbe et al., 1999; Valerio et al., 2001). The rank of sensitivity of group III mGluRs to glutamate is mGluR8 (EC_{50}=0.02μM)> mGluR4 (EC_{50}=3-20 μM) ≈ mGluR6 (EC_{50}=16 μM)>>>mGluR7 (EC_{50}=1000 μM). The rank order of potency of known group III mGluR agonists is L-amino- 4-phosphonobutyrate (L-AP4) > L-serine-O-phosphate (L-SOP) > glutamate > L-CCG-I (Conn and Pin, 1997). The primary and secondary structures of the binding pocket and the surrounding loops located in the N-terminal domain are responsible for receptor sensitivity. The major contributor to agonist affinity in the group III mGluRs is the single position occupied by lysine 74 in mGluR4 and lysine 71 in mGluR8, which forms a high-energy ion bond between the receptor and the agonist, while glutamine 58 in mGluR6 and asparagine 74 in mGluR7 establish a lower-energy hydrogen bond (Rosemond et al., 2004).

1.1.3.2. Group III mGluR Function and Physiology

Group III mGluR activation has been shown to reduce the release of vesicular and non-vesicular glutamate. Previous studies have shown that local perfusion of L-AP4, a non-selective group III mGluR agonist, lowered extracellular glutamate levels in many brain regions, including the nucleus accumbens (NAc) (Anwyl, 1999; Cartmell and Schoepp, 2000; Hu et al., 1999; Kogo et al., 2004; Panatier et al., 2004; Xi et al., 2003).

Additionally, mGluR4b and/or mGluR7 were shown to regulate in vivo extracellular glutamate in the nucleus accumbens by inhibiting non-vesicular glutamate release via the PKA-dependent modulation of cysteine-glutamate exchange (Xi et al., 2003). This is very important because the basal levels of *in vivo* extracellular glutamate are largely independent of vesicular release (Timmerman et al., 2003; Xi et al., 2003). Group III mGluRs are therefore able to modulate glutamate locally at each synapse via regulation of voltage-gated Ca^{+} channels and on larger scale via the glial cysteine-glutamate transporter (Xi et al., 2003).

Agonist binding to group III mGluRs causes an inhibition of adenylate cyclase (Fig.1.1C) via G_o/G_i proteins, thereby reduces cAMP levels. This mechanism is pertussis toxin (PTX) sensitive, thus linking it to the Gα subunit. Tanabe showed a 50% inhibition of forskolin-induced cAMP accumulation after L-glutamate was added to mGluR4-expressing Chinese Hamster Ovary **(**CHO) cells (Tanabe et al., 1993). Application of a group III mGluR agonist L-AP4 resulted in a greater decrease (90%) in forskolin-stimulated cAMP (Wu et al., 1998) Nevertheless, the weak inhibition of cAMP by glutamate compared to L-AP4 suggests that this might not be the preferred transduction pathway of these receptors (Conn and Pin, 1997). The effects of group III mGluRs are also mediated via the dissociation of the Gβγ subunits. As was seen in hippocampal neurons, once Gβγ is released it can then inhibit voltage-dependent Ca^{2+} channels by binding to their second intracellular loop (Fig.1.1B) (Bushell et al., 1996; Herlitze et al., 1997; Ikeda et al., 1996). In the retina and bone marrow stromal cells, mGluR6 negatively regulates La^{3+}-sensitive Ca^{2+}-permeable cation channel causing membrane hyperpolarization and reduction of Ca^{2+} ion (Foreman et al., 2005; Nawy, 2000). The

mGluR8 has been shown to decrease the intracellular Ca^{2+} ion concentration in isolated photoreceptors from rodent retinas. This activity could be responsible for the negative feedback mechanism modulating the fine adjustment of light-regulating glutamate release from photoreceptors and may serve as a precautionary mechanism to prevent excitotoxic levels of release at this tonic synapse (Koulen et al., 1999). Additionally, mGluR8 is also capable of eliciting voltage dependent N-type Ca^{2+} channel inhibition, but the presence of the C-terminal tail is not required for mGluR8 coupling to N-type Ca^{2+} channels. This suggests that different types of group III mGluRs may use different mechanisms to mediate their regulatory functions (Guo and Ikeda, 2005).

In dissociated pyramidal neurons of adult rats, group III mGluRs showed a regulatory activity over high voltage-activated Ca^{2+} currents. Calcium channel blockers, -omega-agkistrodotoxin (ω-AgTx) and, to a lesser extent, omega-conotoxin (ω-CgTx), prevented group III mGluR modulation, arguing in favor of a strong involvement of N-type and P- or Q-type channels (Luebke et al., 1993; Martin et al., 2007; Takahashi and Momiyama, 1993; Wheeler et al., 1994). Activation of group III mGluRs was only partially voltage-dependent because the experiments on facilitated currents showed that only one-third of the mGluR-induced inhibition was removed by a preceding depolarization (Stefani et al., 1998). The involvement of group III mGluRs with Ca^{2+} concentration is an essential component to the receptor-mediated inhibition of neurotransmitter release; increased Ca^{2+} concentration is required to activate the Ca^{2+} -sensing, vesicle-docking proteins that will then fuse the vesicle with the plasma membrane and result in transmitter release into the synaptic cleft. By inhibiting Ca^{2+}

channels, group III mGluRs down-regulate the release of transmitter from the presynaptic terminal (Murthy and Stevens, 1998; Sudhof and Jahn, 1991; Takei et al., 1996).

In the corticostriatal and hippocampal synapses, PKC-activating phorbol esters and activation of A_3 adenosine receptors lead to the inhibition of group III mGluR activation of downstream pathways by PKC-induced phosphorylation and a subsequent prevention of coupling of the receptor to the GTP-binding protein: Gβγ (Fig.1.1D) (Airas et al., 2001; Macek et al., 1998). Additionally, PKC activation causes the desensitization and internalization of these mGluRs (Mathiesen and Ramirez, 2006). The inhibition of group III mGluRs can also be achieved by a cyclic AMP-dependent PKA phosphorylation of serine residues located in the KRXXS motif on the C-terminal tails (Cai et al., 2001). PKA can be activated by the Gα subunit, which is associated with slow change of synaptic transmission. PKA is then recruited to the mGluR signalosome via binding to dimeric PICK1 (protein interacting with C kinase 1), and thereby might facilitate mGluR and PICK1 phosphorylation (Fig.1.1D) (El Far and Betz, 2002) This data provides evidence for a negative feedback loop that could potentially allow the receptors to self-regulate their activity via phosphorylation. As in the case of mGluR7, it is possible that both PKA and PKC regulate mGluR activity through direct receptor phosphorylation at the same conserved site (Ser862) in their C-terminal tails (Airas et al., 2001; Cai et al., 2001). Interestingly, Ser862 lies within a region of mGluR7a C-terminal domain known to mediate Ca^{2+}/calmodulin binding (O'Connor et al., 1999) and phosphorylation of the C-terminal has been found to inhibit the interaction with Gβγ and calmodulin (Fig.1.1A) (Airas et al., 2001; Nakajima et al., 1999). Ca^{2+}/calmodulin

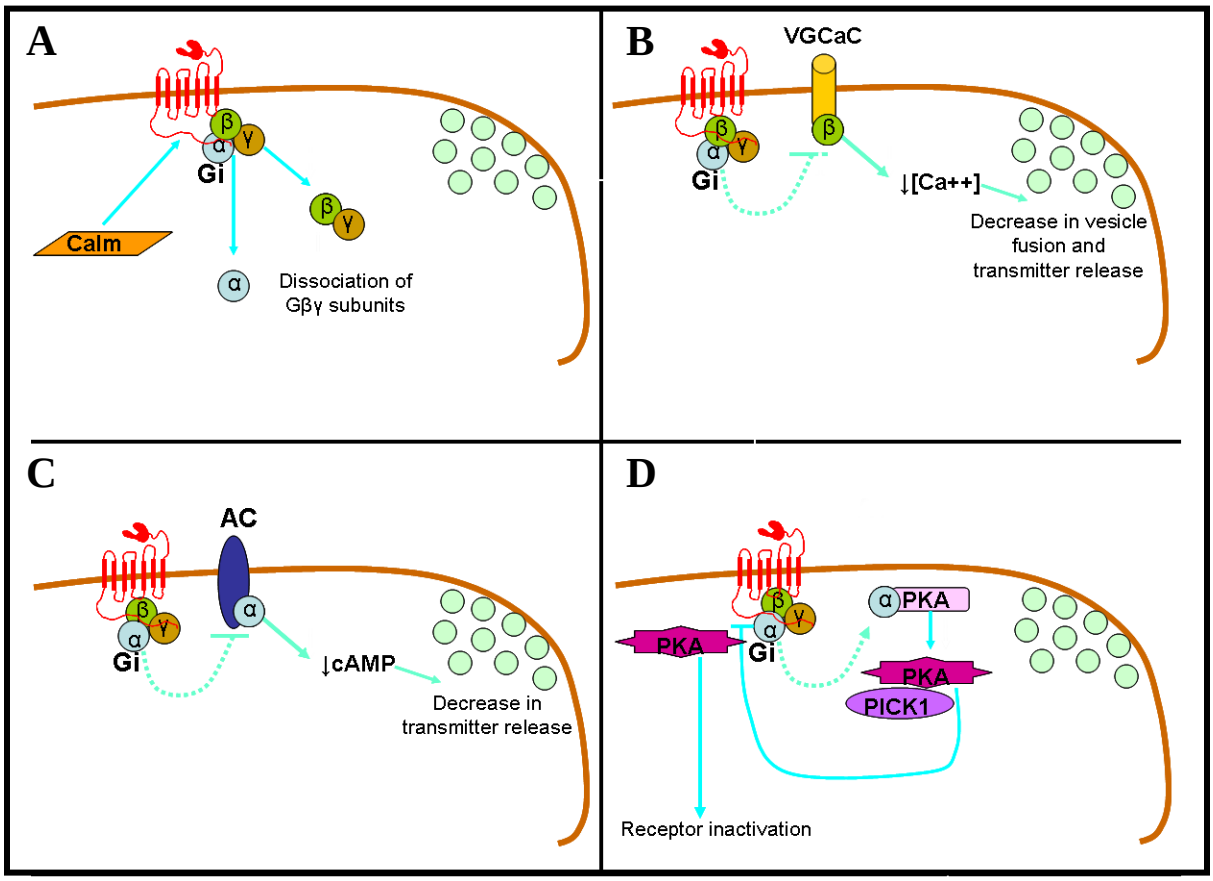


Figure 1.1: Downstream activation pathways following glutamate binding to group III mGluRs. A: Calmodulin binding to mGluR is necessary for dissociation of the Gβγ from the receptor. B: Dissociated β subunit binds to and inhibits voltage gated calcium channels (VGCaC), which results in the decrease in the intracellular calcium concentration and consequently inhibition of vesicle fusion and transmitter release. C: The dissociated α subunit inhibits adenylyl cyclase (AC), which decreases cAMP production and inhibits transmitter release. D: The dissociated α subunit activates PKA, which is then recruited by PICK1 to the mGluR. PKA phosphorylates the receptor and inactivates it.

binding is essential to group III mGluR function because it promotes the dissociation of Gβγ from presynaptic mGluR C-tails.

Activation of group III mGluRs protects cerebellar granule cells against apoptosis through the simultaneous activation of PI-3-K and MAPK pathways via αi and βγ of the Gi protein (Iacovelli et al., 2002). PI-3-K activation led to phosphorylation of AKT and translocation of β-catenin from the cytoplasm into the nucleus (Iacovelli et al., 2002). Activation of the MAPK pathway through a mechanism requiring β-arrestin2, dynamin, and src (Jiang et al., 2006) resulted in phosphorylation and increased enzymatic activity of ERK1 and ERK2, which contribute to intracellular signaling by phosphorylating a largely common subset of substrates, both in the cytosol and in the nucleus (Wiegert et al., 2007)**.** Thus, group III mGluR-mediated MAP kinase activation proceeds following arrestin recruitment and subsequent clathrin/dynamin dependent endocytosis (Lefkowitz and Shenoy, 2005; Pelkey et al., 2007), these findings are consistent with agonist-induced group III mGluR internalization via a classical arrestin-, dynamin-, and clathrin-dependent pathway.

Members of group III mGluRs display internalization, but the conditions and mechanisms of internalization likely differ between the different types of receptors. Sumoylation of the receptor at a region N-terminal to a consensus sumoylation motif by Pias1, an E3 ligase, can mediate the receptor expression, ubiquitination, and subsequent degradation (Nishida and Yasuda, 2002; Tang et al., 2005). Overexpressed GFP-tagged mGluR4 was found to undergo agonist-induced redistribution in HEK cells in a fashion consistent with receptor internalization (Iacovelli et al., 2004). Internalization of mGluR4 and mGluR7 occurred within 5 min of agonist treatment (Iacovelli et al., 2004; Pelkey et

al., 2007), however, mGluR4 fluorescence returned to the surface membrane within 10 min of continued agonist exposure (Iacovelli et al., 2004). Group III mGluR agonists L-AP4 or AMN082 induced internalization of mGluR7 fluorescence that did not recover during or after agonist application (Pelkey et al., 2007). Exposure (60 minute) of enteric neurons to mGluR8 agonists (RS)PPG and DCPG resulted in significant internalization of endogenous mGluR8 that could only be prevented by the application of a group III antagonist CPPG (Tong and Kirchgessner, 2003). The difference between the internalization mechanisms between the group III mGluRs may be due to the variability in the primary, secondary, or tertiary structures of the receptors or to the differences between trafficking in neurons and a heterologous expression system (Pelkey et al., 2007).

The protein sequence and primary structure of the group III mGluRs brought about unexpected splice variants. The short forms of mGluR6(b) and mGluR8(c) only possess the extracellular N-terminal domains of these mGluRs. mGluR6b was identified in the rodent retina (Valerio et al., 2001) and mGluR8c was identified in the rodent striatum (Malherbe et al., 1999). These truncated isoforms lack the 7-transmembrane domains and the intracellular tails, which prevent them from attaching to the plasma membrane. Though the function of these receptors is still unclear, these truncated proteins are thought to be excreted into the extracellular space, where they can bind to glutamate and other pharmaco-agents with the same specificity and sensitivity as their long versions (Peltekova et al., 2000). Thus, these proteins can act as binding, non-signaling molecules also referred as “decoy receptors” (Colotta and Mantovani, 1994; Valerio et al., 2001). On the other hand, these short isofirms similar to those of other

receptors like the soluble interleukin-6 receptor (Peters et al., 1998) can possess agonistic properties. Theoretically, the soluble extracellular domains of mGlu6 receptor could bind glutamate, and consequently act to activate or inhibit ligand of the core domain of a long isoform of mGluR6 (Bockaert and Pin, 1999; Suzuki et al., 2004).

1.1.4. Regional Localization of Group III mGluRs

Group III mGluR are widely expressed in the CNS. Multiple mRNA and peptide analyses have located these mGluRs in many cortical and subcortical nuclei, as well as in the spinal cord. In situ hybridization study of group III mGluRs in the CNS showed highest levels of expression in thalamus, moderate levels in striatum, nucleus accumbens, cortex and subthalamic nucleus (STN) and lower levels elsewhere (Table 1.1) (Kerner et al., 1997; Lourenco Neto et al., 2000; Testa et al., 1994).

1.1.4.1. Hippocampus

Immunoblot analysis of a crude membrane fraction prepared from rat hippocampus showed a strong immunoreactivity for mGluR7a//b and a much weaker reactivity for mGluR4 and 8 (Shigemoto et al., 1997). Immunohistochemical analysis of tissue revealed mGluR7a signal in all dendritic layers of the hippocampus, while mGluR7b labeling is restricted to the mossy fiber terminal zone and that for mGluR8 to the lateral perforant path terminal zone, mGluR4a immunoreactivity is weak but prominent in the inner third of the molecular layer and mGluR6 is hardly detected (Shigemoto et al., 1997). In the CA1 area, strong neuropil labeling was observed only for mGluR7a, being strongest in the strata oriens and radiatum, followed by the stratum

lacunosum moleculare. Very strong mGluR7a labeling was also detected in CA3 stratum lacunosum moleculare with the inner layer adjacent to the dentate molecular layer showing a distinct labeling pattern (Shigemoto et al., 1997). The medial perforant path terminal zone in the dentate gyrus, especially the middle third of the molecular layer also displayed prominent immunolabeling for mGluR7a (Shigemoto et al., 1997). Strong mGluR4a and mGluR8 immunorectivity was detected in the neuropil of the inner and outer thirds of the dentate molecular layer, respectively (Corti et al., 2002; Ferraguti et al., 2005; Phillips et al., 1997; Shigemoto et al., 1997). Further examination of hippocampus revealed mGluR4a localization within the stratum lucidum, stratum pyramidale and weak immunoreactivity in the closely packed pyramidal cell bodies (Blumcke et al., 1996; Phillips et al., 1997). The mGluR8 immunolabeling showed stronger immunoreactivity in the inner than in the outer edge of the dentate gyrus, resulting in a pattern that is in agreement with the previously shown preferential projections of the lateral perforant path to the dentate gyrus (Wyss, 1981; Shigemoto et al.,1997; Zhai et al., 2002; Ferraguti et al., 2005). The mgluR8 immunoreactivity was also detected in the superficial portion of the lateral perforant path terminal zone in CA3 stratum lacunosum moleculare (Shigemoto, et al., 1997). Together, these data suggest that group III mGluRs are located to subserve regulation of glutamatergic transmission.

In the hippocampus, mGluR4 is localized in terminals that form asymmetric synapses, where it probably operates as an autoreceptor. Large dendritic elements in stratum oriens/alveus interneurones were abundantly immunolabeled with mGluR4-labelled axon terminals making either asymmetric or symmetric synapses (Corti et al., 2002). Similarly, boutons expressing mGluR8 form asymmetric and symmetric synapses

with postsynaptic targets expressing mGluR1 or targets co-expressing calretinin and muscarinic receptor M2 (Ferraguti et al., 2005). This difference in localization could function as a substrate for the regulation of synaptic transmission via a target cell-dependent synaptic segregation of mGluR4 (Corti, et al, 2002).

Immunoreactivity for group III mGluRs in the hippocampus was predominantly localized to presynaptic elements (Ferraguti et al., 2005; Shigemoto et al., 1997); however there was also a significant postsynaptic component in the labeled material. The immunoreactivity for mGluR4a was detected in postsynaptic elements including cell bodies and apical dendrites of pyramidal cells, cell bodies of dentate granule cells, and cell bodies and dendrites of interneurons in both the hippocampus proper and dentate gyrus (Bradley et al., 1996). Sparse postsynaptic mGluR7 immunoreactivity was also observed (Bradley et al., 1996). It is possible that the source of the mGluR7 immunoreactive spiny dendrites are the mGluR7- immunopositive interneurons, suggesting a possible, but as of yet unknown, postsynaptic role for the mGluR7 (Bradley et al., 1996). The specific physiological properties of group III mGluR postsynaptic activity are still unidentified, however with the availability of specific pharmacoagents additional physiological properties of group III mGluRs will be investigated and uncovered.

1.1.4.2. Retina

The retina displays the expression of mGluR6 that is not found anywhere else in the CNS. In the retina mGluR6 expression was limited to the outer plexiform layer. mGluR 4, 7, and 8 exhibited discrete, punctate immunolabeling that was found

exclusively in the inner plexiform layer (IPL). mGluR4 immunostaining was most abundant in IPL sublamina 1, mGluR7 immunoreactivity was organized in four bands, corresponding to sublaminae 1-4, and mGluR8 was localized in two broad bands, one each in the OFF and ON layers of the IPL. Localization of mGluR8 selectively on the OFF cholinergic amacrine plexus and not on the ONcholinergic processes implies that this receptor could play a role in directional-selective processing of light stimuli (Quiraishi et al., 2007). Group-III mGluR immunolabeling showed little colocalization between the different subtypes and also did not co-express significantly with the vesicular glutamate transporter VGLUT1, localizing instead adjacent to bipolar cell ribbons (Quiraishi et al., 2007). The pattern of expression that group III mGluR exhibit in the retina indicates that they have of distinct functions in the retina: each receptor modulating a specific group of cells. In the case of mGluR8 the asymmetric distribution in the ON and OFF layers of the IPL suggests discrete mechanisms in modulation neurotransmission that results from the presence and absencof light (Quraishi et al., 2007). In fact , APB stimulation of group-III mGluRs was shown to depress OFF-bipolar cell output (Awatramani and Slaughter, 2001; Higgs et al., 2002).

1.1.4.3. Cerebral Cortex

Cortical expression of group III mGluR mRNA has been detected by in situ hybridization and Northern blot analyses. While group III mGluRs are widely distributed among many cortical regions, there are some cortical areas where mGluR7 mRNA is intensely or moderately expressed without any significant expression of mGluR4 mRNA. These include the neocortical regions, the cingulate, retrosplenial, piriform and perirhinal

cortices, superficial layers of the subicular cortex, deep layers of the entorhinal, parasubicular, and presubicular cortices (Bradley et al., 1996; Corti et al., 2002; Kinzie et al., 1995; Ohishi et al., 1995; Okamoto et al., 1994). In the cerebral cortex, both the mGluR4 mRNA and protein has been detected using in situ, immunoblotting and mGluR4 immunoreactivity was observed in a clear laminar pattern in all deep cortical areas (layer IV/V interface) from the frontal to entorhinal cortex inclusive (Bradley et al., 1996; Iversen et al., 1994; Phillips et al., 1997). The morphology and density of immunopositive cortical cells suggests that they are not all pyramidal output cells, but may represent a subpopulation cortical interneurons or stellate cells (Ohishi et al., 1995; Phillips et al., 1997). Shortly after, immunohistochemical analysis by Berger et al. (2001) identified mGluR4a-positive corticostriatal projection neurons in deep cortical layers. In situ hybridization with the mGluR7 cRNA probe produced a pattern of hybridization signal identical to the distribution found in previous studies (Kinzie et al., 1995; Ohishi et al., 1995). In cerebral cortex, the labeling was most intense in the occipital cortex and slightly less intense in frontal cortical areas. Immunohistohemical analysis of cortical tissue confirmed the localization of mGluR7 in the rodent cortex (Kosinski et al., 1999). Thus it is possible that group III mGluRs are expressed in cortical interneurons and projection neurons.

1.1.4.4. Thalamus

Localization of group III mGluRs in the thalamus has only been studied at the mRNA level. The parafasicular (Pf), ventral lateral and ventral medial (VL/VM), and anteroventral (AV) thalamic nuclei revealed moderate levels of group III mGluR mRNA

(Messenger et al., 2002; Thomsen and Hampson, 1999); (Ohishi et al., 1995; Okamoto et al., 1994). A detailed mGluR mRNA expression in rodent thalamus confirmed mGluR4 and mGluR7 expression in many principal relay nuclei, as well as the intralaminar and reticular thalamic nuclei. The mGluR4 signal was stronger than that of mGluR7, which suggests a possible higher level of expression of mGluR4 in thalamic nuclei. Thalamic expression of mGluR8 has not been studied in great detail. However mGluR8 mRNA was found in anterior, dorsomedial, lateral dorsal, central medial, reticular, and the nuclei of the ventral tier (Richardson-Burns et al., 2000; Saugstad et al., 1997). The expression of group III mGluRs in the thalamus will need to be followed up with experiments probing the subcellular localization and levels of protein expression of these receptors.

1.1.4.5. Basal Ganglia

Expression of group III mRNA was found in all regions of the basal ganglia motor loop. The mGluR7 immunoreactivity was reported in the striatum, GP, and sunstantia nigra pars reticulata (SNr) of rodent basal ganglia. Resembling mGluR7 localization, mGluR4 was also found on presynaptic striatal and GP elements (Bradley et al., 1999; Corti et al., 2002). The mGluR8 showed the strongest expression in the caudate and the putamen and moderate levels in nucleus accumbens, STN, GP and substantia nigra pars compacta (SNc) (Messenger et al., 2002; Ohishi et al., 1995; Thomsen and Hampson, 1999). The available neuroanatomic data suggests that group III mGluRs are localized presynaptically in the active zones of glutamatergic and non-glutamatergic terminals, implying both an auto- and hetero-receptor function for this group of receptors.

Both the striatonigral and striatopallidal projection neurons showed immunolabelled terminals forming mostly type II synapses on dendritic shafts. The localization of mGluR4 on GABAergic terminals of striatal projection neurons suggests a role as a presynaptic heteroreceptor (Corti et al., 2002). Additionally, in the caudate nucleus and putamen, the density of labeled cells indicates that they are the medium spiny cells (Makoff et al., 1996). Immunoblotting of the rodent brain showed striking immunoreactivity for mGluR4a in the GP, and a weaker signal for the striatum, SNpr and entopeduncular nucleus (ET) (Bradley et al., 1999). Thus, it is possible that mGluR4 is manufactured in striatal projection neurons and targeted to presynaptic terminals targeting the other BG nuclei (Bradley et al., 1999). Not surprisingly, quinolinic acid lesions of the striatum abolished virtually all mGluR4a immunoreactivity in the GP (Bradley et al., 1999). Striatonigral/striato-entopeduncular MSNs are likely to be the largest source of mGluR4-immunopositive axon terminals in the neostriatum (Kuramoto et al., 2007). Choline acetyltransferase (ChAT)-immunopositive dendrites were contacted by mGluR4-immunoreactive varicosities via symmetric synapses, suggesting that the mGluR4-positive terminals of GABAergic MSN axon collaterals selectively target striatal ChAT-positive neurons (Kuramoto et al., 2007).

1.1.5. Subcellular Localization of group III mGluRs

Group III mGluRs have been localized on the membrane in the active zones of symmetric and asymmetric terminals forming synapses with either spines, dendrites, or soma (Kinoshita et al., 1998). Electron microscopic analysis of rodent CNS showed that both isoforms of mGluR7(a/b) are expressed not only in as autoreceptor in axon terminals of mitral and tufted cells in the olfactory bulb, retinal ganglion neurons, and

Region	Receptor	mRNA/ Protein	Method	Author
Hippocampus	mGluR4	Protein	IB, IHC, AR	Corti, 2002; Thomsen, 1999; Bradley, 1996
	mGluR7	mRNA	RT-PCR, IS	Corti, 1998; Simonyi, 2000; Kinzie, 1995
		Protein	IB, IHC	Bradley, 1998; Bradley, 1996; Shigemoto, 1996; Kosinski, 1999
	mGluR8	mRNA	RT-PCR, IS	Corti, 1998; Saugstad, 1997
		Protein	IHC	Ferraguti, 2005
Olfactory Bulb	mGluR4	mRNA	IS	Ohishi, 1995
		Protein	IB, IHC	Bradley, 1996; Corti, 2002
	mGluR7	mRNA	RT-PCR, IS	Corti, 1998; Ohishi, 1995; Kinzie, 1995
		Protein	IHC	Bradley, 1996
	mGluR8	mRNA	RT-PCR, IS	Corti, 1998; Saugstad, 1997
Cerebellum	mGluR4	mRNA	IS	Ohishi, 1995
		Protein	IB, IHC, AR	Corti, 2002; Tomsen, 1999; Mateos, 1999; Bradley, 1996
	mGluR7	mRNA	RT-PCR, IS	Corti, 1998; Ohishi, 1995; Kinzie, 1995
		Protein	IB, IHC	Bradley, 1998; Bradley, 1996
	mGluR8	mRNA	RT-PCR, IS	Corti, 1998; Saugstad, 1997
Striatum	mGluR4	mRNA	IS	Ohishi, 1995
		Protein	IB, IHC	Corti, 2002; Kuramoto, 2007; Bradley, 1996
	mGluR7	mRNA	RT-PCR, IS	Kosinski, 1999; Corti, 1998; Ohishi, 1995; Simonyi, 2000; Kinzie, 1995
		Protein	IB, IHC	Bradley, 1996; Kosinski, 1999
	mGluR8	mRNA	IS	Saugstad, 1997
Cortex	mGluR4	mRNA	IS	Ohishi, 1995
		Protein	IB, IHC	Bradley, 1996; Corti, 2002; Kristensen, 1993; Iversen, 1994
	mGluR7	mRNA	RT-PCR, IS	Corti, 1998; Ohishi, 1995; Simonyi, 2000; Kinzie, 1995
		Protein	IB, IHC	Kosinski, 1999; Dalezios, 2002; Bradley, 1996
	mGluR8	mRNA	RT-PR	Corti, 1998
Thalamus	mGluR4	mRNA	IS	Ohishi, 1995, Neto, 2000a; Neto, 2000b
		Protein	IB, AR	Thomsen, 1999; Corti, 2002
	mGluR7	mRNA	IS	Ohishi, 1995; Simonyi, 2000; Kinzie, 1995; Neto, 2000a; Neto, 2000b
	mGluR8	mRNA	IS	Saugstad et al 1997; Richardson-Burns, 2000
Hypothalamus	mGluR4	mRNA	IS	Ohishi, 1995
		Protein	IB	Corti, 2002
	mGluR7	mRNA	IS	Ohishi, 1995, Kinzie, 1995
GP	mGluR4	Protein	IHC	Corti, 2002
	mGluR7	mRNA	IS	Kosinski, 1999; Ohishi, 1995
		Protein	IHC	Kosinski, 1999
SN	mGluR4	Protein	IB, IHC, AR	Thomsen, 1999; Corti, 2002
Entopeduncular Nucleus	mGluR4	Protein	IHC	Corti, 2002
STN	mGluR4	mRNA	IS	Ohishi, 1995
	mGluR7	mRNA	IS	Ohishi, 1995; Kosinski, 1999
		Protein	IHC	Kosinski, 1999
Amygdala	mGluR4	mRNA	IS	Ohishi, 1995
		Protein	IHC, IB	Bradley, 1996
	mGluR7	mRNA	IS	Ohishi, 1995; Kinzie, 1995
		Protein	IHC, IB	Bradley, 1996
Brainstem	mGluR4	mRNA	IS	Ohishi, 1995
	mGluR7	mRNA	IS	Ohishi, 1995; Kinzie, 1995
Retina	mGluR4, 7, 8	Protein	IHC	Quraishi, 2007
Superior Colliculus	mGluR4	Protein	AR	Thomsen, 1999
Nucleus Basalis	mGluR4	Protein	AR	Thomsen, 1999
Septum	mGluR4	mRNA	IS	Ohishi, 1995
	mGluR7	mRNA	IS	Ohishi, 1995; Kinzie, 1995
Pons	mGluR4	Protein	IB, IHC	Bradley, 1996
	mGluR7	mRNA	RT-PCR	Corti, 1998
		Protein	IB, IHC	Bradley, 1996
	mGluR8	mRNA	RT-PCR	Corti, 1998

Table 1.1: Group III mGluR mRNA and protein expression various CNS nuclei.

somatic sensory ganglion neurons, but these receptors are also playing the role of heteroreceptors GABAergic terminals of striatal medium spiny neurons (MSNs) and

Purkinje cells, and in noradrenergic neurons in the locus coeruleus (Kinoshita et al., 1998). Double labeling of hippocampal tissue using fluorescent dyes has shown colocalization of mGluR7a and mGluR8a in the nerve terminals located in stratum oriens (Ferraguti et al., 2005). The colocalization of mGluR7a and GAD immunoreactivity was observed in axon terminals in the islands of Calleja (Kinoshita et al., 1998). In the cortex, mGluR7 has been localized in the presynaptic grids of active zones at symmetric and asymmetric synapses targeting GABAergic interneurons (Dalezios et al., 2002). Electron microscopic examination of group III mGluR localization in the hippocampus revealed peroxidase and immunogold labeling for mGluR4, 7, and 8 in presynaptic membrane of asymmetrical and symmetrical synapses (Ferraguti et al., 2005; Kogo et al., 2004; Shigemoto et al., 1997). Additional information from the parallel fiber Purkinje cell synapses shows that mGluR4 is distributed in clusters along the presynaptic membranes of parallel fiber boutons (Mateos et al., 1999). It is possible that scaffolding proteins play a role in clustering these receptors on the membrane to aid in receptor signal transduction. Electron-microscopic analysis demonstrated that in hippocampus all terminals that made asymmetric synapses with dendrites expressing mGluRl had a much higher density of the mGluR7 labeling than terminals making synapses with mGluRl-immunonegative dendritic shafts or pyramidal-cell spines (Shigemoto et al., 1996). In the cortex, the soma and dendrites immunopositive for mGluR1α were also shown to be targets of mGluR7-positive glutamatergic and GABA-ergic terminals (Dalezios et al., 2002). These results raise the possibility that presynaptic terminals could regulate the

probability of transmitter release at individual synapses according to the postsynaptic target.

1.1.6. Electrophysiology of Group III mGluRs

Group III mGluRs are thought to have an inhibitory effect on cellular excitability and synaptic transmitter release. While their subcellular presynaptic localization is very similar, the differential sensitivity to glutamate and pharmacoagents provide a wide range for threshold of activation (Conn and Pin, 1997). Due to a lack of specific compounds, researchers have used available agonists and antagonists at variable concentrations to target receptors at different sensitivity levels in order to elucidate the electrophysiological properties of group III mGluRs. The availability of knockout animals proved to be a helpful tool in the field of electrophysiology because it allowed for the testing of receptor types with similar pharmacologic properties.

Group III mGluRs are consistently described as presynaptic auto- or hetero-receptors (Burke and Hablitz, 1994; Calabresi et al., 1996; Cotman and Monaghan, 1986; Forsythe and Clements, 1990; Manzoni and Bockaert, 1995; Rainnie and Shinnick-Gallagher, 1992). At the corticostriatal synapse, application of group III mGluR agonists significantly decreased the amplitude of excitatory postsynaptic potentials (EPSPs), evoked by cortical stimulation (Pisani et al., 1997).

Pharmacologic evidence has implied that mGluR4 could be responsible for the negative modulation of the striatopallidal synapse via a presynaptic mechanism (Valenti et al., 2003). In the SN, either mGluR4 or mGluR8 inhibit excitatory neurotransmission via a presynaptic mechanism (Valenti et al., 2005). Knock-out animals have shown to

have significant behavioral and physiological deficits, proposing a role for the group III mGluRs in the modulation of certain neurocircuits. Increased measures of anxiety and weight gain accompanied by performance deficits in learning tasks has been noted in mGluR8 knock-out animals (Duvoisin et al., 2005; Gerlai et al., 2002; Linden et al., 2002). Increased seizure susceptibility has been recorded in mGluR7 knock-out mice.

1.2. Functional Circuitry of the Basal Ganglia

1.2.1. General Organization of the Basal Ganglia

The basal ganglia are a group of interconnected subcortical nuclei consisting of the striatum, the internal and external segments of the GP, STN, and the SN. The striatum, the largest of the basal ganglia nuclei (Yelnik, 2002), is the main entrance for excitatory transmission from the cerebral cortex and thalamus to the basal ganglia circuitry (Fig. 1.1) (Smith et al., 1998). Interestingly, while both cortico-striatal and thalamo-striatal projections are glutamatergic, they express different types of vesicular glutamate transporters: vGluT1 and vGluT2, respectively. The striatum is often divided into dorsal and ventral regions because of differences in neuronal connectivity and function. The ventral striatum contains ventral parts of the caudate nucleus and putamen and the nucleus accumbens (Groenewegen et al., 1993; Haber, 2003). In addition to receiving excitatory input from the cortex and the thalamus, the striatum also receives inhibitory projections from the globus pallidus, and a modulatory dopaminergic innervation from the substantia nigra pars compacta (SNc) (Charara et al., 2002). Both cortex and thalamus contribute to glutamatergic innervation of striatum. Corticostriatal afferents from layers III and V form asymmetric synapses onto dendritic spines of

medium spiny neurons. Unlike cortical inputs, thalamostriatal afferents, which arise predominantly from the intralaminar nuclei, display a more heterogeneous pattern of synaptic connectivity between dendrites and spines(Charara et al., 2002; Dube et al., 1988). The centromedian (CM) and parafasicular (Pf) nuclei of the thalamus give rise to thalamostriatal terminals forming axo-dendritic synapses (Raju et al., 2006), while all other thalamic projections form axospinous synapses resembling those produced by the corticostriatal projection. The STN also receives input from the cortex (Nambu et al., 2002). The GPi and SNr are the output nuclei of the basal ganglia. Projections from the SNr and GPi innervate thalamic targets. However, in addition to the thalamus these output nuclei make other specific connections (Parent and Hazrati, 1995). The SNr neurons project to the superior colliculus (SC), pedunculopontine nuclei (PPN), and the medullary reticular formation (RF). Nigrotectal fibers converge mainly on the tectospinal neurons in the intermediate layer of the SC and play a critical role in the control of visual saccades (Wurtz and Hikosaka, 1986). The nigroreticular projection terminates in the parvicellular RF, which is directly connected with orofacial motor nuclei (Chandler et al., 1990; Mogoseanu et al., 1994).

On the other hand, the GPi projection neurons are anatomically segregated: neurons from the core of the nucleus project to PPN and arborize heavily in the ventral motor and caudal intralaminal thalamic nuclei. The projection neurons from the periphery of GPi project to the lateral habenular nucleus (lHB) (Parent et al., 2001); (Arecchi-Bouchhioua et al., 1997; Hazrati and Parent, 1991; Parent et al., 1999).

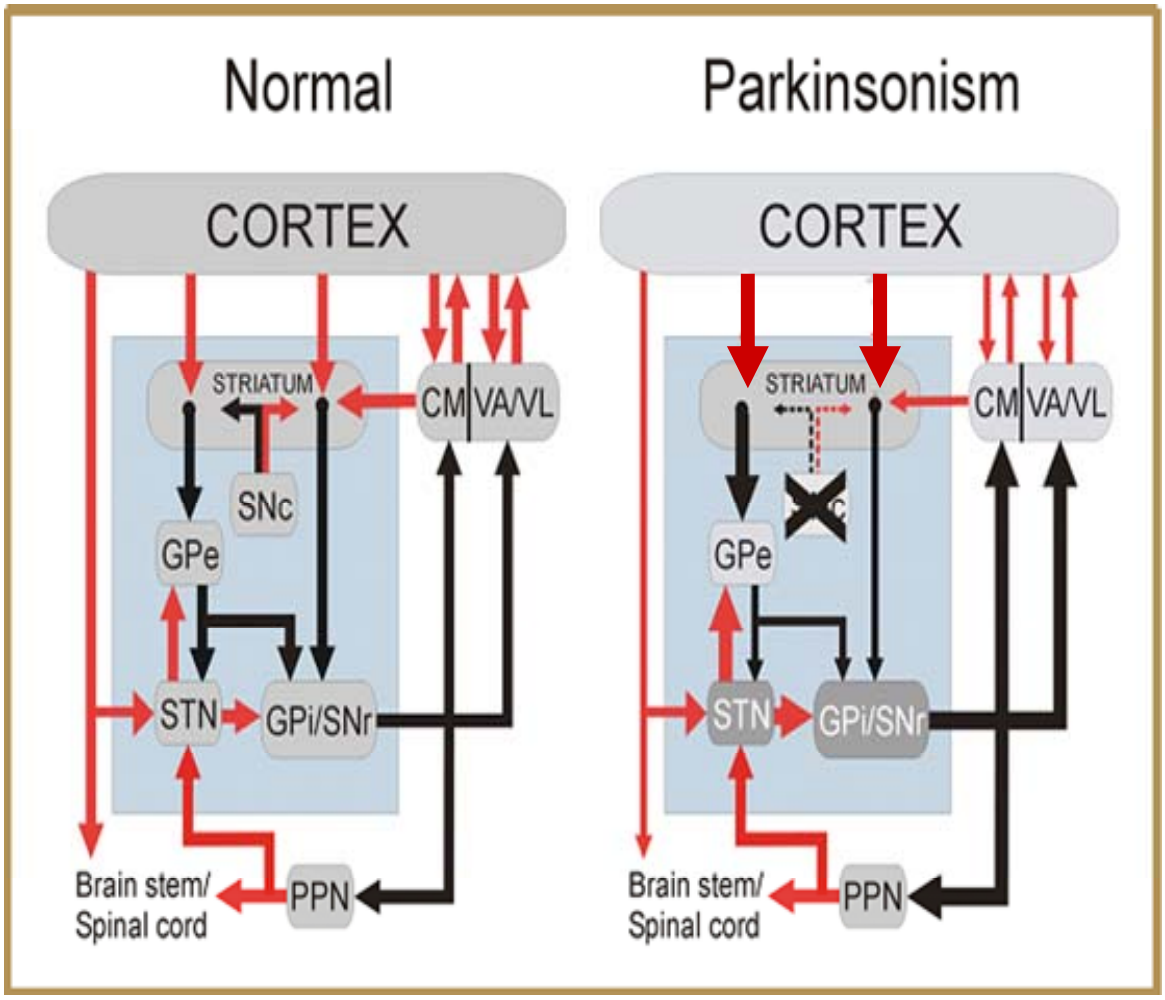


Figure 1. 2: Diagram of the functional circuitry of basal ganglia in normal and Parkinsonian conditions taken directly from Wichmann et al. (2007). The diagram illustrates the connectivity of the basal ganglia circuitry and the changes in output activity that occur in Parkinson's disease along with the loss of dopaminergic innervation of striatum by the SNc. Striatonigral degeneration leads to hypoactivity of the direct pathway and hyperactivity of the indirect pathway, leading to overinhibition of thalamocortical neurons in CM and VA/VL and disfacilitation of movement.Excitatory projections are labeled with red arrows and inhibitory projections with black arrows.

1.2.2. Intrinsic Organization of the Striatum

The striatum consists of three functionally different components that can be segregated based on the sources of cortical innervation. The caudate connects with associative cortical areas; the putamen receives its main cortical innervation from motor cortices, while the accumbens is mainly connected with limbic cortical regions (Parent et al., 1996). These territories process cognitive, motor, and emotional or motivational information, respectively (Yelnik, 2002). Additionally, dorsolateral caudate and a strip of the caudal putamen receive inputs from the higher visual and oculomotor-related areas of the cortex (Harting et.al, 2001). Based on neurochemical and hodological differences, the striatum has been divided into two distinct compartments, the patch/striosome and the matrix (Graybiel et al., 1981; Herkenham and Pert, 1981). The patch areas receive projections from the limbic and prefrontal association cortices, and thalamic paraventricular (PV) and rhomboid nuclei, while the matrix is innervated by the projections from the sensorimotor cortices and many thalamic nuclei (Berendse and Groenewegen, 1990; Gerfen and Sawchenko, 1984; Gerfen, 1989) Wang et al, 1998; (Eblen and Graybiel, 1995). Projection neurons of the striatum can be further segregated into two main output pathways so-called the direct and indirect pathways. The direct pathway projects to the substantia nigra pars reticulata (SNr) and internal globus pallidus (GPi), the two basal ganglia output nuclei, while the indirect pathway projects to the external segment of the globus pallidus (GPe) and the STN. Ultimately, both pathways innervate thalamocortical projections and balanced activity of these pathways engages thalamocortical circuits to generate fluid movement. The two pathways have functionally

opposite roles with the direct pathway facilitating movement and the indirect pathway inhibiting it. Further, the activity of both pathways is mediated by nigro-striatal dopamine via facilitation of the direct pathway and inhibition of the indirect pathway through activation of dopamine receptors.

The establishment of the theory of "direct" and "indirect" pathways of the basal ganglia (Albin et al., 1989) have provided a key concept in striatal circuitry. The MSNs are thought to comprise the direct and indirect pathways by projecting to SNr either monosynaptically or via GP and STN. The MSNs in the direct pathway express D1 dopamine receptors and contain substance P and dynorphin, while the MSNs in the indirect pathway express D2 dopamine receptors and enkephalin (Gerfen et al., 1990). Through these two classes of receptors, dopamine modulates these pathways by facilitating the activity in the direct pathway and reducing the activity in the indirect pathway. Thus dopamine innervation of the striatum plays an important part in balancing the two striatal output pathways. The GABAergic projection form the GPe targets the STN and SNr/Gpi. The STN sends excitatory glutamatergic signals to the GPe and SNc, the main output nuclei of the basal ganglia. The GPi/SNr receive an inhibitory GABAergic projection from the striatal direct pathway and an excitatory glutamatergic projection from the STN, a nucleus in the indirect pathway. These pathways require a flawless balance in order to allow for proper basal ganglia function (Wichmann and DeLong, 1996; 2003). The output signals are further projected to the pedunculopontine nucleus and thalamus, mainly the ventral anterior (VA), ventral lateral (VL), and the centromedian/parafascicular complex (CMPf) nuclei. The SNr also projects to the SC, and the GPi projects to the lHB.

1.2.3. Subtypes of Striatal Neurons

The striatum is a network of numerous morphologically and chemically distinct neuron subtypes. Inputs to the striatum converge on MSN, which are also the sole projection neurons of the striatum. The MSNs have a 10-20 μm diameter soma from which extend 7-10 moderately branched dendrites that are densely populated with spines. They express GABA as their neurotransmitter and are the major striatal cell type making up about 96% of striatal neurons (Aihara et al., 2001; Cicchetti et al., 2000; Kawaguchi, 1997). The rest ~4% of the striatal cell population is composed of interneurons: parvalbumin (PV) positive leptodendritic GABAergic neurons (2%), large cholinergic interneurons (1%), GABAergic calretinin-positive (1%), and medium-sized neurons containing somatostatin (SS), neuropeptide Y (NPY), NADPH-diaphorase (or nitric oxide synthase, NOS) and possibly GABA (1%) (Albin et al., 1989; Cicchetti et al., 1998; Cossette et al., 2005). PV-containing interneurons are categorized as fast-spiking cells (FS-cells) that receive strong excitatory inputs from the cerebral cortex (Lapper et al., 1992; Ramanathan et al., 2002) and thalamus (Sidibe and Smith, 1999) and primarily innervate the somata and dendrites of MSNs, forming asymmetric synapses, and producing substantial inhibition of MSNs (Bennett and Bolam, 1994; Kita, 1993; Kubota and Kawaguchi, 2000; Tepper and Bolam, 2004). Striatal cholinergic cells are large (20-50 μm diameter) aspiny interneurons that produces acetylcholine (ACh) as their neurotransmitter (Cicchetti et al., 2000). Cholinergic cells are classified as long-lasting afterhyperpolarization cells or tonically active neurons (TANs); this property provides a long afterhyperpolarization that is followed by a firing of single spikes. *In vivo*, these

neurones fire tonically at a frequency of 2–10Hz. The majority of excitatory inputs to cholinergic interneurons are derived from the thalamus, particularly the parafascicular nucleus (Bennett and Wilson, 1998; Lapper et al., 1992; Wilson et al., 1990). Activation of cholinergic interneurons by thalamic afferents results in a pause and rebound facilitation in tonic activity of TANs. Thalamic Pf afferents can also indirectly inhibit TANs by innervating striatal MSNs, which, in turn inhibit cholinergic interneurons via GABA release (Bolam et al., 1986; Zackheim and Abercrombie, 2005). During Pf activation, striatal acetylcholine levels decrease via inhibition of interneurons by the inhibitory MSN. However if MSNs activity is blocked striatal acetylcholine levels increase through direct activation from the thalamus. (Zackheim and Abercrombie, 2005).

1.2.4. Corticostriatal Projections

The massive glutamatergic projection from the cortex into the striatum terminates on the heads of MSN spines. These projections are organized in a highly topographic manner in the striatum. Corticostriatal innervation is further organized into a parallel functioning architecture (Alexander et al., 1990). Corticostriatal projections are segregated into circuits that process different types of information such as skeletomotor, oculomotor, associative, and limbic. The motor loop comprises information from the primary motor cortex (MC), the arcuate premotor area (APA), premotor cortex (PMC), primary somatosensory cortex (SI), and supplementary motor are (SMA). The oculomotor loop encodes the input from the frontal and supplementary eye fields, the associative loop encompasses the signals from the prefrontal and lateral orbitofrontal cortex, and limbic loop involves the anterior cingulated and medial orbitofrontal cortex

areas (Alexander et al., 1990). The cortical pyramidal neurons projecting to the striatum are located in layers II-VI, but the most projections come from layer V (Rosell and Gimenez-Amaya, 1999). There is evidence for convergence and segregation among the corticostriatal information. All these projections target the heads of spines of MSN in both the direct and indirect pathway.

1.2.5. Thalamostriatal Projections

The thalamostriatal projection, like the corticostriatal projection, provides a great gluatamatergic innervation of the basal ganglia. Numerous thalamic nuclei send projections to the striatum. The primary source of thalamostriatal projection is thought to originate from the intralaminar and midline nuclei (Beckstead, 1984; Elena Erro et al., 2002; Jayaraman, 1985; Smith and Parent, 1986), however, minor projections from relay and associative thalamic nuclei have also been described (Beckstead, 1984; Elena Erro et al., 2002; Jayaraman, 1985; Smith and Parent, 1986). Unlike the corticostriatal projection, the projection from the thalamus targets both the dendritic shafts and the spines of the MSN. Interestingly, it is the CM/Pf projection that selectively terminates on the dendritic shafts of the striatal MSN (Dube et al., 1988; Sadikot et al., 1992; Sidibe and Smith, 1996; Smith et al., 1994; Raju et al., 2006). Additionally, the different thalamic nuclei innervate functionally different areas of striatum. For example, the medial part of CM innervates the postcommisural sensorimotor putamen. The Pf innervates the associative part of the caudate and the accumbens, while the dorsolateral Pf innervates the precommissural putamen (Nakano et al., 1990; Sadikot et al., 1992; Sidibe et al., 2002; Smith and Parent, 1986; Smith et al., 2004). The thalamostriatal

projection targets striatal projection neurons in both direct and indirect pathways, as well as ChAT, PV, and somatostatin containing striatal interneurons.

1.2.6. vGluT expression in striatal afferents

Both corticostriatal and thalamostriatal projections express excitatory glutamatergic signals. Both form asymmetric synapses with striatal targets. Although some physiological differences have been suggested between the two glutamatergic projections (Ding et al., 2008) the most consistent and reliable tool in differentiating between the two pathways is the expression patterns for the vesicular glutamate transporters (vGluTs). The family of vGluTs is comprised of highly homologous proteins: vGluT1-3. Corticostriatal and thalamostriatal neurons express different vGluT proteins: vGluT1 and vGluT2, respectively (Fremeau et al., 2004a; Kaneko et al., 2002). The vGluTs reside on neurotransmitter-containing vesicles in the terminals and transport cytoplasmic glutamate into the vesicle by using a proton gradient that is constructed by hydrolyzing ATP. This creates an acidic environment within the vesicle and generates a pH gradient across the vesicle membrane.

vGluT expression has been reported in mostly glutamatergic neurons and complimentary expression across CNS with limited overlap, suggesting that each isoform subserves particular regions of the central nervous system. vGluT1 mRNA demonstrate strong signals in all layers of the cortex, hippocampal formation, lateral and basolateral amygdala, and the granule layer of the cerebellum (Fremeau et al., 2001; Herzog et al., 2001; Hisano et al., 2000; Ni et al., 1995). In contrast, vGluT2 is abundant in layer IV of the cortex, medial and central amygdala, and deep cerebellar nuclei (Fremeau et al.,

2001; Herzog et al., 2001; Hisano et al., 2000). vGluT3 is much less abundant and more restricted in its distribution. Strong signals for vGluT3 transcript have been localized in the hippocampal formation, layers II, IV and V of the neocortex, and granule layer of the cerebellum (Schafer et al., 2002). Interestingly, only vGluT3 mRNA is detected in the striatum (Fremeau et al., 2002; Schafer et al., 2002).

1.2.7. Dopaminergic Projections

In addition to the excitatory glutamatergic input from the cortex and the thalamus, the striatum receives a modulatory dopaminergic innervation from the SNc. Dopaminergic terminals synapse on the necks of spines of MSN and mediate their effects via two groups of dopamine receptors. The D1-like group includes receptors D_1 and D_5 and is thought to be localized to the MSN in the direct output pathway; while the D2-like group includes receptors D_2, D_3 and D_4 and is found on the MSN that project in the indirect pathway (Gerfen et al., 1990). These receptors are coupled to G-protein and through the association with different G-proteins the two groups of receptors have opposite effects on cellular excitability. D1-like receptors activate adenylyl cyclase, increasing the intracellular concentration of the second messenger cyclic adenosine monophosphate (cAMP) and producing an excitatory influence on the target. D2-like receptors directly inhibit the formation of cAMP by inhibiting the enzyme adenylate cyclase and thus produce an inhibitory effect. Dopamine facilitates cortical activation of the direct pathway and impedes activation of the indirect pathway.

1.2.8. Dopamine Depletion and Parkinson's Disease pathophysiology

The dopaminergic innervation of striatum is lost during Parkinson's disease (PD) leading to a lack of modulation of the direct and indirect pathways (Fig. 1.2). Without the presence of dopamine the indirect striatum-GPe pathway is no longer inhibited and exhibits increased activity, while the direct striatum-GPi/SNr connection displays a decrease in firing rate. The increased inhibition of GPe by striatal projection neurons causes a disinhibition of the STN and thereby increases the neuronal activity of STN. The hyperactivity in STN could be alleviated by group III mGluR agonists or potentiators and thus providing another potential target for group III mGluR therapy. The changes in activity of direct and indirect pathways sum up to an increased activity of GPi/SNr, leading to increased basal ganglia output to the thalamus and excessive inhibition of thalamocortical neurons.

1.2.9. Glutamate Receptors and Parkinson's Disease Therapeutics

Dopamine denervation of striatal neurons is the hallmark of PD, however equally relevant is the transformation of the glutamatergic innervation of the striatum. This system undergoes very interesting changes as part of PD pathology. There is an increased glutamatergic drive from the corticostriatal projection. However there is also a significantly decreased number of spines (Stephens et al., 2005) in conjunction with a reduction in the size of the dendritic trees (Stephens et al., 2005). Since the main input nucleus of the basal ganglia receives most of its input from glutamatergic sources and this input suffers a perturbation during PD, drugs that can target receptors in this system and restore some form of balance to the network would be of great value. Glutamate receptors

are expressed both pre- and post-synaptically and serve as auto- and hetero-receptors in the basal ganglia. The iGluRs could serve as potential targets for mediation of the glutamatergic drive into the striatum and the effects looked promising in animal models. However, the activation of these receptors would lead to outcomes that were too strong and ubiquitous. In humans iGluR drugs caused sedative effects, impairment of learning, and psychotomimetic effects (Kristensen et al., 1992; Luby et al., 1959; Muir et al., 1994). The mGluRs, on the other hand, exhibit wide diversity and heterogeneous distribution of their subtypes. Thus the opportunity exists for developing highly selective drugs that affect a limited number of CNS functions. The mGluRs therefore provide a target for development of therapeutic agents that could impact our ability to treat a variety of psychiatric and neurologic disorders. Importantly, drugs acting at mGluRs would have more subtle effects on transmission at glutamatergic synapses than would iGluR agonists and antagonists and would probably have fewer side effects (Conn and Pin, 1997). Recent data have provided strong evidence for the potential antiparkinsonian effects of group III mGluR agonists and allosteric modulators in acute rodent models of parkinsonism. A good example are drugs acting on mGluR4. These drugs appear to selectively modulate striatopallidal transmission which raises the interesting possibility that activation of this receptor could decrease the excessive inhibition of the GP that has been postulated to occur in Parkinson's disease.

Consistent with this, intracerebroventricular injections of L-AP4, an mGluR III agonist, produces therapeutic benefit in both acute and chronic rodent models of Parkinson's disease (Valenti et al., 2003). PHCCC, a selective allosteric potentiator of mGluR4, also produced a marked reversal of reserpine-induced akinesia in rats,

suggesting that potentiation of mGluR4 may be a useful therapeutic approach to the treatment of PD (Marino et al., 2003a; Marino et al., 2003b). Further discovery and identification of selective allosteric modulators of mGluR would add another level of utility to the use of mGluRs as targets of PD therapy. Discovery of mGluR pharmacological agents that are specific, able to cross the blood brain barrier, and are tolerated when administered systemically would make these drugs practically appealing and more likely to be tested in humans.

1.3. Study Objectives

The striatum is the main entrance of thalamic and cortical glutamatergic information into the basal ganglia circuitry. Fine-tuning modulation of glutamatergic transmission is essential to normal striatal activity. Glutamate transmission is mediated by various groups of ionotropic and metabotropic receptors. Because of their modulatory nature, diversity and association with specific brain regions, the mGluRs are recognized as highly promising targets for the development of novel drug therapies for various brain diseases (Conn et al., 2005; Marino and Conn, 2006; Swanson et al., 2005). Recent data have provided strong evidence for the potential antiparkinsonian effects of group III mGluR agonists and allosteric modulators in acute rodent models of parkinsonism (Marino et al., 2003a; Marino et al., 2003b; Valenti et al., 2003).

In dopamine-depleted basal ganglia, group III mGluR agonists could mediate their antiparkinsonian effects via regulation of the corticostriatal or the striatopallidal synapses. Previously published work has shown that group III mGluRs activation inhibits GABAergic transmission at the striatopallidal synapse in the rat GP, which is in line with

electron microscopic studies showing abundance of mGluR4 and mGluR7 immunoreactivity in striatopallidal GABAergic terminals (see above). Although there is evidence for group III mGluRs regulation of glutamatergic transmission in the striatum, the degree of mGluRs expression in thalamic versus cortical synapses, and the preferential relationships between specific group III mGluR-containing terminals and the two main populations of striatofugal neurons, need to be further examined to better understand the functional integration of group III mGluRs in the striatal microcircuitry.

Thus, in order to better understand the differential role and potential targets for specific group III mGluRs-related compounds in the striatum, a rigorous comparative analysis of the localization of mGluR4a, mGluR7 and mGluR8 in the rodent striatum using a combination of quantitative high resolution single and double immunocytochemical methods at the electron microscopic level was conducted. Specifically, to understand the potential functional consequence of group III expression in the striatum, the receptor distribution and subcellular localization analysis was performed. To accertain the ability of group III mGluRs to modulate the excitatory input into the striatum, vGluTs were used to localize these receptors in the terminals of either the cortio- or thalamostriatal projections. And finally, to elucidate a postsynaptic target preference of group III mGluR-positive terminals, the dopamine receptor (D1) immunoreactivity was used to identify spines on the MSNs of the direct output pathway. Overall, the aims of this thesis were to provide an anatomical mapping of the ultrastructural localization of group III mGluRs in the striatum, and determine if these receptors were associated preferentially with extrinsic glutamatergic inputs that target preferentially the direct or indirect striatal output neurons.

1.3.1. Specific Aim 1**:** To compare the subcellular localization of mGluR4, mGluR7 and mGluR8 in the rodent striatum.

Tissue from normal mouse striatum was immunostained for mGluR4a, 7 or 8. Quantitative assessments of the relative abundance of labeled pre-and post-synaptic elements were made. The chemical phenotype of pre-synaptic terminals was determined using double immunoperoxidase methods for vGluT1 and vGluT2. The degree of colocalization of different group III mGluR types in individual terminals was quantified.

1.3.2. Specific Aim 2**:** To compare the proportion of group III mGluR containing synapses innervating direct or indirect striatofugal neurons.

Tissue from normal mouse striatum was co-immunostained with an mGluR and D_1 receptor antibodies. Quantitative assessments of the relative frequency of synaptic contacts between group III mGluR-containing terminals and D_1-immunoreactive and D_1-immunonegative postsynaptic striatal elements were conducted.

2. Microcircuitry of mGluR4 and mGluR7 in the mouse striatum

2.1. Introduction

The striatum is the largest basal ganglia nucleus and the main entrance for excitatory transmission from the cerebral cortex and thalamus to the basal ganglia circuitry. The excitatory glutamatergic innervation terminates massively on spines and dendrites of GABAergic MSNs which make up about 96% of the total neuronal population in the striatum. These projection neurons are segregated into two functionally opposite output pathways, and a balanced activity of these pathways engages thalamocortical circuits to generate fluid movement.

Fine-tuning modulation of glutamatergic transmission is essential to normal striatal activity. Glutamate transmission is mediated by various groups of ionotropic and metabotropic receptors. The metabotropic glutamate receptors (mGluRs) are linked to several intracellular signal transduction mechanisms via G proteins and mediate a wide variety of modulatory effects on excitatory and inhibitory transmission. The mGluR family includes eight different subtypes pooled into three major groups (groups I, II and III) based on their sequence homology, pharmacological properties and transduction mechanisms. The group I mGluRs (mGluR1 and mGluR5) are usually postsynaptic and induce slow depolarization, whereas group II (mGluR2 and mGluR3) and III mGluRs (mGluR4, 6, 7, 8) are mainly presynaptic and negatively modulate glutamate and GABA release. Because of their modulatory nature, diversity and association with specific brain regions, the mGluRs are recognized as highly promising targets for the development of novel drug therapies for various brain diseases (Conn et al., 2005; Marino and Conn, 2006; Swanson et al., 2005).

Electrophysiologic and pharmacologic evidence suggests that group III mGluRs play important roles in regulating glutamatergic transmission and long term synaptic plasticity in the striatum (Cuomo et al., 2009). In rodents, there is anatomical evidence for the expression of mGluR7 and mGluR4 on glutamatergic terminals, some of those likely originating from cortical afferents (Kosinski et al., 1999). However, because the thalamus is another prime source of glutamatergic inputs to the striatum (Raju et al., 2006; Smith et al., 2009; Smith et al., 2004), one of the goals of this study was to further elucidate the exact source of group III mGluR-containing glutamatergic terminals in the striatum using vesicular glutamate transporters 1 and 2 (vGluT) as selective markers of corticostriatal or thalamostriatal terminals. Considering striking differences in sensitivity to glutamate between mGluR4 and mGluR7, another aim of this work was to assess the degree of mGluR4/mGluR7 co-localization at the level of individual striatal glutamatergic boutons.

Although, it has long been established that spines represent the main targets of cortical or thalamic inputs to the striatum (Kemp and Powell, 1971; Raju et al., 2006), the relative degree of innervation of direct versus indirect pathway neurons by specific neurochemically characterized inputs to the striatum remain poorly understood, though some anatomical studies have suggested that subsets of cortical or thalamic terminals may differentially innervate these two populations of neurons (Lei et al., 2004; Sidibe and Smith, 1996). To further address this issue, the final goal of this study was to examine the possibility that mGluR7- and mGluR4-containing glutamatergic terminals display a preferential synaptic innervation of D_1-containing direct striatofugal neurons in mouse.

2.2. Materials and Methods

2.2.1. Animal and tissue preparation

Five adult (4-5week old), male Swiss colony derived FVB mice (Charles River Laboratories), weighing 18-20 grams were completely anesthetized with a cocktail of ketamine (60-100mg/kg) and dormitor (0.1mg/kg) and transcardially perfused with cold Ringer's solution, followed by fixative consisting of 4% paraformaldehyde and 0.1% glutaraldehyde in phosphate buffer (0.1M;pH 7.4). After perfusion brains were removed from the skull and postfixed in 4% paraformaldehyde for 5-24 hours. Following post-fixation, coronal brain sections were cut with a vibrating microtome into 60um-thick coronal sections. Brain sections were stored in phosphate-buffered saline (PBS) at 4°C. Selected sections were put in a 1.0% sodium borohydride solution, dissolved in PBS, for 20 minutes and thereafter rinsed in PBS before being processed for immunocytochemistry. 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.

2.2.2. Antibodies:

To localize mGluR7, Protein A chromatography purified rabbit polyclonal antibody raised against peptide (C-NSPAAKKKYVSYNN) corresponding to amino acids 899-912 of human mGluR7 was used at concentration 1:500 (Upstate/Millipore, Catalog # 07-239). Immunoblotting studies by the manufacturer on rat brain microsomal preparation probed with anti-mGluR7 (0.5 micrograms/ml) showed a band at 97kD. The

rabbit polyclonal anti-mGluR4a antibody (Zymed/Invitrogen, 1:200, Catalog #513100, Lot #60103066A2) was raised against a synthetic peptide derived from the C-terminal 200 amino acids of the rat mGluR4 protein specific for the mGluR4a splice variant. Studies by the manufacturer confirmed the reactivity on Western blots (Mr=93,000-110,000) using rat brain cell lysate. Tissue from mGluR4 knock out mice is completely devoid of immunostaining when probed with this antibody (Smith, Conn et al., unpublished data).

Two antibodies, rabbit anti-VGluT1 and guinea pig anti-VGluT2, were used as specific markers of terminals from either the VGluT1-positive corticostriatal or VGluT2-positive thalamostriatal glutamatergic projections. To localize VGlut1, we used commercially available Rabbit anti-VGluT1 (MaB Technologies, Cat # VGT1-3, Lot # GA062B, 1:5000). To generate this VGluT1 antibody a peptide from the COOH terminus of the rat vesicular glutamate transporter 1 (rvGluT1), corresponding to amino acids 543–560 (cATHST*V*QPPRPPPPVRDY) was synthesized. A cysteine was added to the peptide to aid its conjugation to the protein carrier keyhole limpet hemocyanin (KLH; Pierce, Rockford, IL). Antisera were obtained from rabbits (Covance) immunized with the conjugated peptide, and the IgG fraction was recovered (Raju, et.al. 2006). Previous studies from our laboratory and others using immunoblotting of brain lysate revealed single bands at ~60 kDa. Preadsorbtion of primary antibody with synthetic peptide (0.2–0.4 μg/ml) overnight at 4°C abolished immunoreactivity, whereas preadsorbtion with a similar but nonidentical peptide preserved immunoreactivity(Montana et al., 2004; Raju et al., 2006). Immunohistochemical experiments on striatal tissue using the VGluT1 antibody in control tissue and tissue preadsorbed with vGluT1, but not vGluT2 peptide

further confirmed the specificity of the antibody (Raju, et.al. 2006). The guinea pig anti-VGluT2 was used to localize VGluT2 (Chemicon, 1:5000, Catalog # AB5907, Lot #0611046131). The manufacturer stated that the antiserum has been tested on tissue sections from the rat central nervous system (CNS) using immunofluorescence histochemistry. The antiserum mainly labeled nerve fibers and terminals. The staining pattern for the labeling obtained with the VGluT2 antiserum corresponds to the pattern described using other antisera to VGluT2 (Fremeau et al., 2001; Fujiyama et al., 2001; Kaneko et al., 2002; Tong et al., 2001; Varoqui et al., 2002). Preabsorption of the VGluT2 antiserum with immunogen peptide eliminates all immunostaining. Further, the specificity of the vGluT2 antiserum was carefully tested by immunoblotting and immunocytochemistry in a previous study (Montana et al., 2004; Raju et al., 2008).

Spines containing the D_1 receptor were identified using the Sigma Aldrich monoclonal rat anti- D_1 dopamine receptor antibody (Catalog #D187-250uL; clone 1-1-F11 s.E6; immunoglobulin purified). According to the information provided by the manufacturer, the antibody was produced by immunizing rats with a 97 amino acid synthetic peptide corresponding to the C-terminus of the human D_1 dopamine receptor. The D187 antibody is specific for human (DRD1 Gene ID 1812), monkey, and rat (Drd1a Gene ID 24316) D_1 dopamine receptors. The antibody is supplied as a solution in 20 mM sodium phosphate, pH 7.2, containing 150 mM sodium chloride and 0.02% sodium azide. The antiserum mainly labeled striatal spines and some dendrites. Tests for the specificity of this antibody have been published in previous studies (Hersch et al., 1995; Levey et al., 1993). The D_1 staining pattern seen in our study with this antibody corresponds to the pattern described using this and other D_1 antisera in previous publications (Hersch, S.M.,

et al., 1995; Levey, A.L., 1993; (Smiley et al., 1994; Villalba et al., 2009; Yung et al., 1995).

2.2.3. Single Labeling Immunoperoxidase for Light Microscopy

After sodium borohydride treatment, selected coronal tissue sections were incubated for 1 hour at RT in PBS containing 10% normal goat serum (NGS), 1% BSA, and 0.3% Triton X-100, followed by the primary antibody solution containing 1% NGS, 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 IgGs at a concentration of 1:200 (Vector Laboratories, Burlingame, CA) for 90 minutes. The sections were rinsed again in PBS and then incubated for another 90 minutes with the avidin-biotin peroxidase complex (ABC) at a dilution of 1:100 (Vector Laboratories). Finally, the sections were washed twice in PBS and once in Tris buffer (50 mM; pH 7.6) and transferred to a solution containing 0.025% 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma), 10 mM imidazole, and 0.005% hydrogen peroxide in Tris buffer for 10 minutes producing a diffusible reaction product to identify the immunoreactivity for the receptors. 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 with a CCD camera (Leica DC500) controlled by Leica IM50 software.

2.2.4. Single and Double Pre-Embedding Immunoperoxidase for Electron Microscopy

After sodium borohydride treatment, selected tissue sections were transferred to a cryoprotectant solution (PB, 0.05M, pH 7.4, containing 25% sucrose and 10% glycerol)

for 20 minutes and then frozen in the –80°C freezer for 20 minutes. The tissue was returned to the cryoprotectant solution and then, via a decreasing gradient of cryoprotectant solutions (100%, 70%, 50%, 30%) the tissue was returned to PBS. Brain tissue sections were incubated for 1 hour at RT in PBS containing 10% normal goat serum (NGS), 1% BSA, followed by a 48 hour incubation at 4 degrees with primary antibodies diluted in a solution of normal serum (1%), bovine serum albumin (BSA, 1%), 0.01% Triton X-100 and PBS. The rest of the protocol was the same as for the LM procedure. Following the DAB reaction, the sections were transferred to PB (0.1 M, pH 7.4) for 10 min and exposed to 1% osmium tetroxide diluted in PB for 20 min. They were then rinsed with PB and dehydrated in an increasing gradient of ethanol (50%, 70%, 90%, 100%). Uranyl acetate (1%) was added to the 70% alcohol to increase contrast at the electron microscope. The sections were then treated with propylene oxide before being embedded in epoxy resin (Durcupan, ACM; Fluka, Buchs, Switzerland) for 12 hours, mounted on microscope slides, and placed in a 60°C oven for 48 hours.

Blocks of tissue were cut into 60 nm ultrathin sections with an ultramicrotome (Ultracut T2; Leica, Nussloch, Germany) and serially collected on single-slot Pioloform-coated copper grids. The sections were stained with lead citrate for 5 min (Reynolds, 1963) and examined with a Zeiss EM-10C electron microscope (Thornwood, NY). 50 micrograph images of the labeling captured with a camera at 20,000X. Neuronal elements were identified based on ultrastructural criteria as described by Peters et.al. (1991). Labeled and unlabeled element were counted and analyzed.

2.2.5. Pre-embedding Immunoperoxidase and Immunogold for Electron Microscopy

After cryoprotectant exposure, sections were preincubated for 1 hr in a solution containing 10% NGS and 10% NHS in PBS-BSA (0.005% BSA, 0.05% Tween 20, and 0.001% gelatin in PBS) before being transferred to a 1% NHS and 1% NGS in PBS-BSA solution containing the primary antibodies: rabbit anti-vGluT1 and guinea pig anti-vGluT2 for 24 hr at room temperature. Next, the tissue was rinsed in PBS-BSA and incubated for 2 hr in the secondary 1.4 nm gold-conjugated goat anti-rabbit IgGs (Nanogold; Nanoprobes, Stonybrook, NY) at a concentration of 1:100 and a secondary biotinylated goat anti-guinea pig IgGs (Vector Laboratories, Burlingame, CA) at a concentration of 1:200 in 1% NGS and 1% NHS in PBS-BSA. The sections were then rinsed with PBS followed by 2% Acetate Buffer rinses. Tissue then underwent silver intensification of gold particles for 5-10 min using the HQ silver kit (Nanoprobes). After the intensification, the tissue was rinsed in Acetate buffer and the sections were put in a solution containing 1:100 avidin-biotin-peroxidase complex (Vector). The tissue was washed in PBS and then with TRIS buffer before being transferred into a solution containing 0.01% imidazole 0.0005% hydrogen peroxide, and .025% 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma, St. Louis, MO) for 10 minutes. The remainder of the procedure, involving dehydration, osmification and embedding was the same as that described above for the immunoperoxidase material.

2.2.6. Control experiments.

Control incubations for all experiments included omission of both primary antibodies to test for nonspecific secondary antibody binding.

2.2.7. Analysis of material

2.2.7.1. Immunoperoxidase material.

Five blocks taken from the striatum of 5 mice, mounted on resin blocks, and glued into place using cyanoacrylate glue. Ultrathin sections were collected and scanned for the presence of immunoperoxidase-labeled structures that were easily distinguishable from unlabeled elements by their electron-dense reaction product. For each animal, 50 photographs of randomly selected immunoreactive elements collected from superficial sections of the blocks were digitized at 20,000x. This resulted in a total surface of 7,200 μm^2 of striatal tissue to be examined for each mGluR experiment. Immunoreactive elements were categorized as presynaptic (axons, terminals) or postsynaptic (dendrites, spines) neuronal structures on the basis of ultrastructural features.

The relative abundance of each neuronal element for each receptor subtype in the striatum was calculated by dividing the number of labeled elements by the number of total labeled elements. To obtain the percent of labeled elements in a specific category, the number of labeled elements in this group was divided by the number of total number of identified elements of that type. Significant differences were assessed by using the t-test (n=5). Data presented as average of percent labeled elements ± SEM.

2.2.7.2. Double-Immunoperoxidase.

Data collection was the same as for the single immunoperoxidase experiment. For each animal, 50 photographs of randomly selected immunoreactive elements from the surface of the blocks were digitized at 20,000x. This resulted in a total surface of 7,200μm^2 of

striatal tissue to be examined for each experiment. To determine the percentage of terminals that displayed co-localization of the two markers of interest, the following formula was used: % elements labeled by Marker#1 + % elements labeled by Marker#2 - % elements labeled by Cocktail of Marker1+2 = % elements that co-express both Marker1&2. Thus, if both markers labeled 50% of terminals individually, and 50% of terminals were also labeled in tissue incubated with the cocktail of both primary antibodies, then it will be concluded that the two markers are completely colocalized. In contrast, if the cocktail method labeled 100% of terminals, then we will conclude that the two markers are completely segregated. This method has been successfully used in previous studies to demonstrate segregation of D1 and D2 receptors in striatal spines (Hersch et al., 1995; Lei et al., 2004) as well as co-localization of mGluR1 and mGluR5 in the striatum (Mitrano and Smith, 2007; Muly et al., 2001; Bordelon-Glausier et al., 2008).

2.2.7.3. Immunogold material

Five blocks of sriatal tissue from 5 animals were cut out from slides, mounted on resin blocks, and glued into place using cyanoacrylate glue. Blocks were collected in the same regions of striatum as those taken for immunoperoxidase observations. Ultrathin sections from the surface of the blocks were examined under the electron microscope, and randomly selected elements containing gold particles were digitized at high (25,000×) magnifications.

The immunogold method was paired with the immunoperoxidase technique in order to immunolabel two different antigens. This double labeling technique was applied

to the vGluT1 and vGluT2 colocalization experiment as well as the group III mGluR and D1 receptor assay.

2.2.7.4. Co-localization of mGluR4 and mGluR7 with D_1 receptor using the double immunoperoxidase antibodies cocktail method or immunoperoxidase and immunogold .

Data collection was the same as for the single immunoperoxidase experiment. For each animal, 50 photographs of randomly selected immunoreactive elements from the surface of the blocks were digitized at 20,000x. This resulted in a total surface of 7,200μm^2 of striatal tissue to be examined for each experiment. To determine the percentage of D_1-immunoreactive spines that form synapses with mGluR4- or 7-positive terminals, the immunolabeled terminals forming an identifiable asymmetric synapse(s) were first identified and the postsynaptic elements were then counted and examined for the presence of immuolabeling. This type of analysis was possible because of two premises: first, based on our previous analysis of mGluR4 and mGluR7 localization and distribution, only about 1% of immunolabeled terminals synapse with an immunolabeled spine (data not shown), thus making the error of detecting an mGluR-positive terminal synapsing with a D_1-positive spine very low. Second, it has been previously established that while striatum does possess D_1-positive terminals, these terminals do not form asymmetric synapses because they are cholinergic and GABAergic in nature (Wu et al., 2006), and thus would not be included in our analysis. The double immunoperoxidase method was possible because the D_1 and mGluR antigens are anatomically segregated into post- and pre-synaptic elements, respectively. Consequently, the data obtained

illustrated the percent of D_1-positive spines that were forming asymmetric synapses with mGluR4- or mGluR7-immunolabeled terminals.

2.3. Results

2.3.1. Subcellular localization of Group III mGluRs in the mouse striatum.

At the light microscopic level, the mGluR4 and mGluR7 antibodies labeled the striatal neuropil in a homogenous pattern similar to that described in previous studies (Ohishi et al., 1995; Corti et al., 2002; Kuramoto et al., 2007; Bradley et al., 1996; Kosinski et al., 1999; Corti et al., 1998; Simonyi et al., 2000; Kinzie et al., 1995). At the electron microscopic level, a similar pattern of pre-synaptic labeling of putative glutamatergic terminals was seen for the two mGluRs, i.e. the immunoreactivity was found to be selectively aggregated in the pre-synaptic grids of immunoreactive terminals (Fig. 2.1A-C); which represents the typical pattern of group III mGluR immunoreactivity throughout the CNS (Shigemoto et al., 1997; Ferragutti et al., 2005). A quantitative analysis of labeled elements randomly collected from our material revealed that mGluR4 and mGluR7 immunoreactivity is localized predominantly (82.6% ±2.0, 86.7% ±1.4, respectively) in presynaptic elements, including unmyelinated axons and terminals (Fig. 2.1D).

Analysis of the population of striatal terminals showed that about half of all terminals forming asymmetric synapses in the striatum were immunolabeled for mGluR4 or mGluR7 (47.0% ±2.98, 45.9% ±4.0, respectively) (Fig. 2.1E). A subset of labeled

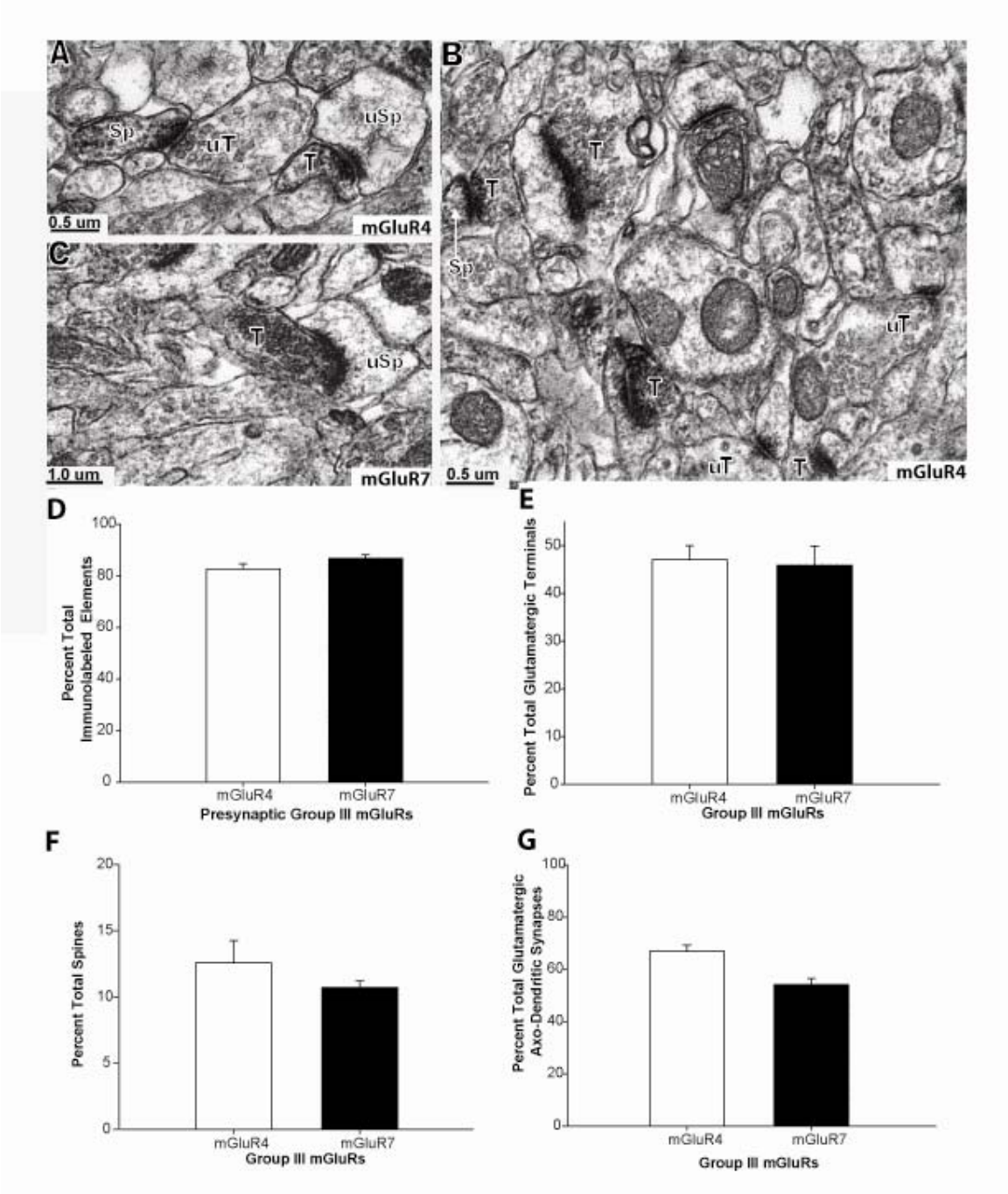


Figure 2.1: mGluR4 and mGluR7 are mainly localized in presynaptic striatal elements (axons and terminals) (82.6% ±2.0 SEM, 86.7% ±1.4, respectively) (D) originating from cortico- and thalamo-striatal projections. Immunoreactive terminals can be seen in mGluR4 (A-B) and mGluR7 (C) labeled tissue. E- mGluR4 and mGluR7 immunoreactivity in about half of terminals forming asymmetric synapses (47.0% ±2.98, 45.9% ±4.0, respectively). F- mGluR4 and mGluR7 immunoreactivity was found in a small population of spines (12.6% ±1.7-mGluR4, 10.7% ± 0.5-mGluR7). G- Many terminals from the CM/Pf thalamic projection, which were identified by the presence of an axodendritic asymmetric synapse, showed immunoreativity for mGluR4 (66.87% ±2.3) and mGluR7 (54.2% ±2.4). Data are expressed as percent of labeled elements (±SEM). T-terminal, Sp-spine, u-unlabeled

terminals formed asymmetric axo-dendritic synapses. Based on previous tracing studies in rats and monkeys, these terminals likely originate from the caudal intralaminar thalamic nuclei (Smith et al., 2004, 2009; Raju et al., 2006). A large proportion (66.87% ±2.3 for mGluR4, 54.2% ±2.4 for mGluR7) of these boutons displayed group III mGluRs immunoreactivity (Fig. 2.1G). While the majority of mGluR4 and mGluR7 labeling was found in presynaptic elements, a small amount of immunoreactivity was also found postsynaptically in dendrites and spines (Fig.2.1F). About 10% of all striatal spines were immunoreactive for either of the two group III mGluRs (12.6% ±1.7-mGluR4, 10.7% ± 0.5-mGluR7) (Fig. 2.1F).

2.3.2. Group III mGluR colocalization in striatal glutamatergic terminals.

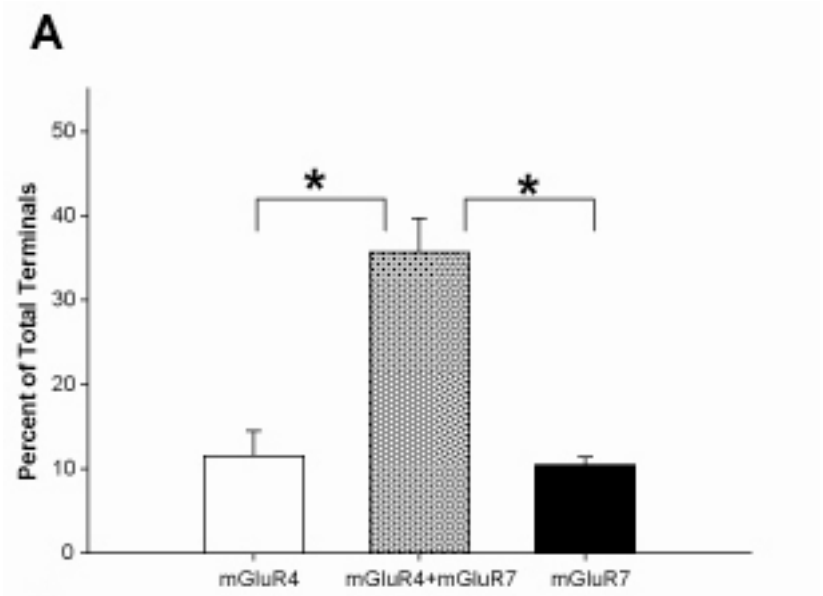
Since both antibodies labeled a significant proportion of glutamatergic terminals, we performed a series of experiments to determine the degree of mGluR4/mGluR7 co-localization at the level of single glutamatergic terminals in the mouse striatum using the double immunoperoxidase cocktail experiment method described above. The analysis of data collected from tissue immunostained only for mGluR4 or mGluR7 or double immunostained with a cocktail of mGluR4 and mGluR7 antibodies confirmed a significant degree of mGluR4/mGluR7 colocalization in glutamatergic terminals in the mouse striatum (Fig. 2.2A-B). Tissue immunostained for mGluR4 only contained 47.0% ±2.98 labeled boutons, while 45.9% ±4.0 labeled terminals were found in tissue singly labeled with the mGluR7 antibodies. Striatal tissue immunostained with the cocktail of antibodies contained 57.3% ±0.92 labeled terminals. Based on these quantitative observations, the application of the formula: % elements labeled by mGluR4 antibodies

(47%) + % elements labeled by mGluR7 antibodies (45.9%) - % elements labeled by Cocktail of mGluR4 and mGluR7 antibodies (57.3%) = % elements that co-express mGluR4 and mGluR7 (35.7%) revealed that about a third of striatal glutamatergic terminals labeled for mGluR4 or mGluR7 co-express both receptors immunoreactivity (Fig. 2.2B).

2.3.3. Group III mGluR colocalization with vGluT1 and vGluT2.

To evaluate the degree of preference of group III mGluRs to the corticostriatal or the thalamostriatal projections, vGluT1 and vGluT2 were used as specific markers of either pathway. (Rajuet al., 2006). The antibody cocktail approach was once again used to assess the degree of co-localization between each of the group III mGluR subtypes and vGluT1 or vGluT2. In singly stained tissue, the pattern of labeling for the mGluRs and the vGluTs was distinctly different, the vGluT labeling was dense and filled the entire terminal, whereas the mGluR immunoreactivity was confined to the pre-synaptic active zones of labeled terminals, as described above. In line with recent quantitative data from our laboratory and others, the vGluT1 antibodies labeled 63.97% ±4.68 of total terminals forming asymmetric synapses, while vGluT2 was found in 38.94% ±2.4 of striatal glutamatergic boutons.

The cocktail of antibodies experiments yielded to 66.73% ±3.82 of labeled terminals when co-immunostained for vGluT1 and mGluR4 or 63.16% ±3.34 when co-immunostained for vGluT1+mGluR7. On the other hand, 55.07% ±2.54 terminals were



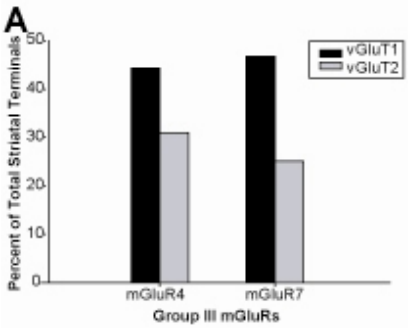
B

	mGluR4+mGluR7			
	mGluR4	mGluR7	mGluR4+ mGluR7	Co-Loc
%	47.06	45.92	57.30	35.68
SEM	2.98	4.00	.92	X

Figure 2.2: mGluR4 and mGluR7 display a significant degree of colocalization. A – Degree of colocalization between mGluR4 and mGluR7 showing percent of terminals with expressing only mGluR4 (white bar), only mGluR7 (black bar) and percent of terminals coexpression both mGluRs (gray bar). B – Table showing raw values that were used to obtain the degree of colocalization using formula listed in Material and Methods.

labeled in tissue co-immunostained with vGluT2 and mGluR4, while 59.84% ±1.45 terminals were immunoreactive in tissue co-labeled for vGluT2 and mGluR7. Based on these measurements, the degree of vGluT1/ mGluR4 co-localization is 44.32%; 30.08% for vGluT1/mGluR7, 46.74% for vGluT2/mGluR4and 25.03% for vGluT2/mGluR7.

In light of these findings, it appears that vGluT1 and vGluT2 may co-localize in a significant number of glutamatergic terminals in the mouse striatum. Thus, if about half of striatal terminals display mGluR4 immunoreactivity (Fig. 2.1), while about 45% colocalize with vGluT1 or vGluT2, then there may be some degree of colocalization between vGluT1 and vGluT2. To directly address this issue, an additional co-localization study was performed using the antibody cocktail approach to determine the degree of vGluT1/vGluT2 co-localization in the mouse striatum. As shown in figure 2.4, the



B

	mGluR4	vGluT1	mGluR4+ vGluT1	Co-Loc	vGluT2	mGluR4+ vGluT2	Co-Loc
%	47.08	63.97	66.73	44.32	38.94	55.07	30.08
SEM	2.98	4.68	3.82	X	2.4	2.54	X
	mGluR7	vGluT1	mGluR7+ vGluT1	Co-Loc	vGluT2	mGluR7+ vGluT2	Co-Loc
%	45.93	63.97	63.16	46.74	38.94	59.84	25.03
SEM	4.00	4.68	3.7	X	2.4	1.45	X

Figure 2.3: Expression of mGluR4 and mGluR7 in vGluT-positive terminals. A- Colocalization of mGluR4 and mGluR7 with vGluT1 and vGluT2. mGluR4 colocalized with vGluT1 in 44.32% of asymmetric terminals and with vGluT2 in 30.08% percent of terminals; mGluR7 and vGluT1 colocalized in 46.74% of terminals and with vGluT2 in 25.03%. B- Raw values and degree of colocalization values for colocalization of group III mGluRs and vGluTs.

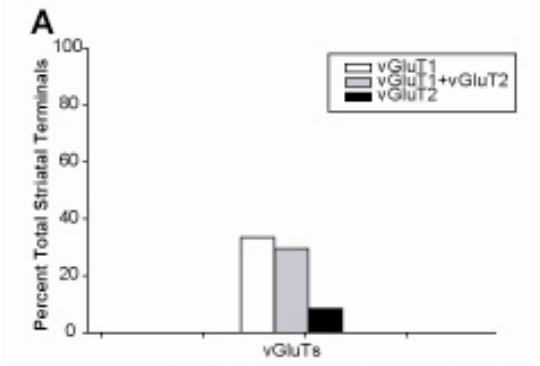
percent of striatal terminals labeled with the cocktail of vGluT1 and vGluT2 antibodies was 71.5% ±1.2. Thus, taking into consideration the percent of terminals labeled in singly immunostained sections for each vGluT subtypes and the value obtained with the cocktail of antibodies, the resulting percent of striatal terminals that colocalize vGluT1 and vGluT2 was calculated to be 31.43%, a value closely similar to the degree of vGluT1 and vGluT2 colocalization suggested by the mGluR and vGluT cocktail experiments (~30%).

To further confirm these findings, a double-labeling experiment involving immunoperoxidase and immunogold method to differentiate the two vGluTs labeling on single sections was performed using peroxidase to label vGluT1 and immunogold to label vGluT2. In line with the results of the antibody cocktail experiments, a significant number of glutamatergic terminals forming asymmetric axo-spinous synapses contained both reaction products indicating a significant level of vGluT1 and vGluT2 co-localization in the mouse striatum (Fig.2.4). Together these findings demonstrate differences between mice and other species regarding the degree of segregation of vGluT1 and vGluT2 in corticostriatal and thalamostriatal terminals, respectively. The use of vGluTs can not reliably identify the thalamostriatal and corticostriatal terminals, we can still conclude that group III mGluRs are strategically localized to mediate excitatory neurotransmission at glutamatergic terminals from both the cortex and thalamus. Future tracing studies would need to be performed to label the thalamostriatal terminals and examine group III mGluR localization at these synapses.

2.3.4. Group III mGluR innervation of striatal direct and indirect pathway neurons

To assess the degree of innervation of direct and indirect output pathway neurons by mGluR4- or mGluR7-containing terminals, we combined the D_1 receptor immunostaining as marker of the direct pathway neurons with either mGluR4 or mGluR7 antibodies to label group III mGluR-containing glutamatergic boutons. The D_1 receptor antibody labeled exclusively dendrites and spines. In line with previous findings from other studies (Hersch et al.1995; Lei et al., 2004), the labeled spines made up about 50% of all spines in the striatum (Fig. 5A). Based on that information, we assumed that the spines that were not immunolabeled with the D_1 antibody were most likely D_2-positive in the striatal tissue used for the double labeling experiments.

First, a double-labeling experiment involving immunoperoxidase and immunogold was performed using immunogold particles to label the mGluRs and peroxidase to identify the D_1 receptor immunoreactivity. From this material, a total of 142 gold labeled mGluR4 and 126 mGluR7 terminals were identified in tissue areas that also contained D_1 receptor immunoreactivity. Quantitative analysis of this material revealed that 27.3%±1.9 of mGluR4-positive terminals and 51.8%±2.6 mGluR7-positive terminals contacted D_1-positive spines, thereby suggesting that mGluR7-containing terminals have no preference between the D_1-immunoreactive and D_1- nonimmunoreactive spines, while mGluR4-positive boutons innervate preferentially D_1-negative spines, presumably of the indirect pathway neurons (Fig. 2.5B, D, G, H).



	vGluT1	vGluT2	vGluT1+vGluT2	Co-Loc
%	63.97	38.94	71.48	31.43
SEM	4.68	2.4	1.1	X

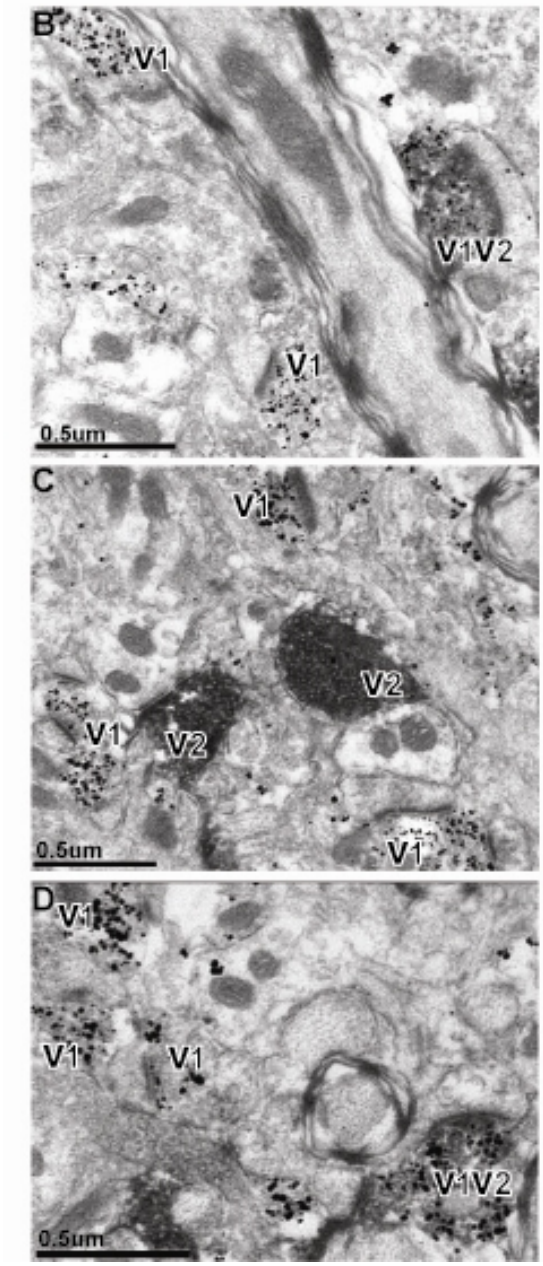


Figure 2.4: vGluT1- and vGluT2-immunoreactive terminals in the striatum. (A) – data obtained for this graph was collected from the double immunoperoxidase experiment. 32.54% of terminals express vGluT1 only (white bar), 7.51% of terminals express vGluT2 only (black bar) and 31.43% of striatal asymmetric terminals coexpress both vGluT1 and vGluT2. (B-D) – gold and peroxidase experiment was conducted to confirm the double immunoperoxidase data. vGluT1 is labeled with gold particles, vGluT2 with peroxiase deposit. Qualitative assessment of double labeled tissue confirmed colocalization of vGluT1 and vGluT2 in striatal glutamatergic terminals.

To further confirm this finding, we employed a double immunoperoxidase technique which resulted in peroxidase deposit in both group III mGluRs- and D_1 receptor-immunoreactive structures. Because the immunoreactivity for either antigens is almost completely separate (ie postsynaptic D_1 labeling and presynaptic mGluRs labeling) and that very few terminals in the striatum display D_1 immunoreactivity, we assumed that terminal labeling in this material was indicative of either mGluR4 or mGluR7 immunoreactivity, while postsynaptic labeling was mainly associated with D_1 receptor labeling. The double immunoperoxidase method, indeed, revealed closely similar results to those found with the immunoperoxidase and immunogold method. In this material, 32.2%± 1.5 mGluR4-immunoreactive terminals and 49.6%±1.2 of mGluR7-immunoreactive terminals formed synapses with D_1-positive spines (Fig. 5C, E, F), thereby confirming the differential targeting of direct and indirect pathway neurons by mGluR7 or mGluR4 glutamatergic terminals in the mouse striatum.

2.4. Discussion

Despite previous qualitative descriptions of presynaptic mGluR4 and mGluR7 immunoreactivity in the caudate-putamen, this study is the first attempt at a detailed quantitative assessment of group III mGluRs localization in the rodent striatum. Previously reported studies have shown strong expression of group III mGluRs in the rodent striatum (Ohishi et al., 1995; Corti et al., 2002; Kuramoto et al., 2007; Bradley et al., 1996; Kosinski et al., 1999; Corti et al., 1998; Simonyi et al., 2000; Kinzie et al.,

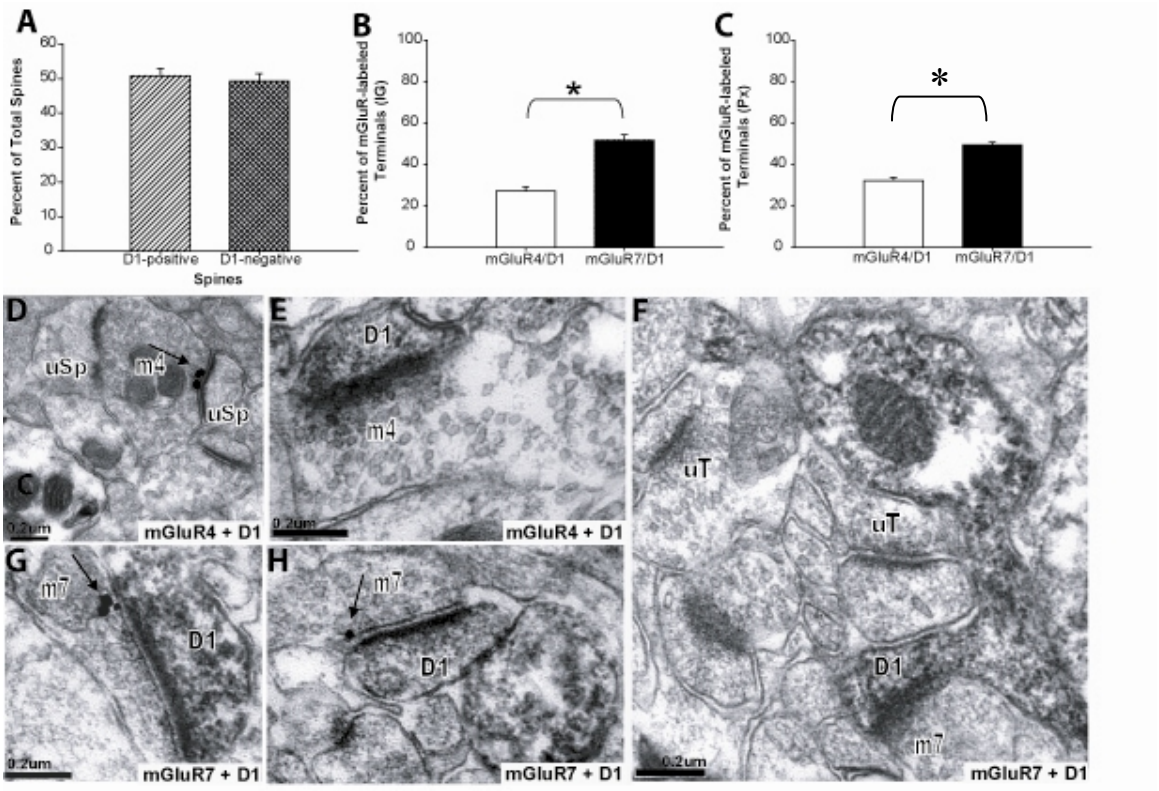


Figure 2.5: Preferential targeting of D_1-positive and D_1 negative spines by mGluR labeled terminals (A) – analysis of D_1 immunolabeling in the mouse striatum shows an equal distribution of D_1-positive and D_1-negative spines. (B) – mGluR4 and mGluR7 show differential preference for D_1-positive postsynaptic elements when examined with double immunoperoxidase method. (C) – similar examination with peroxidase and gold double labeling method confirmed the preferential targeting of mGluR4 and mGluR7 terminals of D_1-positive elements. (D) - mGluR4/gold and D_1/peroxidase labeling. (E) – double immunoperoxidase labeling of an mGluR4-positive terminal and a D_1-positive spine. (F) - double immunoperoxidase labeling of mGluR7-positive terminal and a D_1-positive spine. (G-H) – mGluR7/gold and D_1/peroxidase labeling. IG – immunogold, Px - immunoperoxidase

1995; Saugstad et al., 1997). These studies further suggested a presynaptic subcellular localization for mGluR4 and mGluR7 in the active zones of glutamatergic and non-glutamatergic terminals (Kosinski et al., 1999, Corti et al., 2002; Bradley et al., 1999). Additional important conclusions can be drawn from our study. First, our findings demonstrate that the two receptor subtypes are significantly co-expressed pre-synaptically in both cortico- and thalamo-striatal glutamatergic terminals. Considering the strikingly different sensitivity of each receptor subtype to glutamate, their co-expression at the active zones of glutamatergic terminals provides a wide range of regulatory mechanisms by which group III mGluRs may influence glutamatergic transmission in the striatum. Additionally, colocalization of mGluR4 and mGluR7 may endow synaptic terminals with distinct neurotransmitter release and firing properties and reveal the existence of a high degree of presynaptic heterogeneity.

2.4.1. Technical considerations

In this study, we utilized two different immunolabeling methods to test for colocalization. Because the antibody cocktail approach is not as widely used as the traditional double labeling method with gold and peroxidase to assess ultrastructural colocalization, a few technical issues must be discussed about the rationale for the use of this method in the present study, and the advantages or limitations of such an approach in the present material. The design of this method is based on the premise that each antibody used in a co-localization study labels a specific population of elements that selectively express the antigen of interest, and that the cocktail of both antibodies reveals all immunoreactive elements recognized by each individual antiserum. Thus, if the

epitopes for these antibodies are not colocalized, the percent of labeled elements in the cocktail experiment would equal the sum of labeled elements recognized in each single labeling experiment. However, if there is some degree of overlap between the two antigens of interest, then the sum of labeled elements from each of the single antibody experiments will be greater than that obtained with the cocktail of antibodies. The benefit of using this approach is that the sensitivity of the marker used to localize each antigen (i.e. peroxidase) is the same for the different antibodies used in the co-localization study, thereby reducing the likelihood of false negative data due to the reduced sensitivity and poor tissue penetration of gold-conjugated antibodies. However, an important technical issue to consider is that the quantitative assessment of the total number of labeled elements in each experiment is based on the relative abundance of labeled and unlabeled structures encountered in the tissue under analysis, thereby assuming that unlabeled elements are genuinely devoid of significant immunoreactivity for the other antigen under study. To make sure this is the case, we sampled tissue taken only from the most superficial sections of the blocks where both antibodies have full access to their antigens. Taking into consideration the little inter-individual variability between animals used in the same group, we believe that our sample strategy was accurate and, most importantly, consistent across experiments. Another important advantage of the cocktail method is the fact that primary antibodies can be raised in the same species, i.e. the choice of antibodies to be used can be based solely on their sensitivity and reliability instead of animal species they have been raised in. Therefore, we believe that, when applicable, the antibody cocktail method is probably a more precise, sensitive and reliable approach to be used to assess antigen co-localization in single elements at the electron microscopic level. In the

present study, we also performed additional experiments using the conventional gold/peroxidase double labeling technique to provide a qualitative confirmation of some of our quantitative data.

2.4.2. Group III mGluRs are expressed in both corticostriatal and thalamostriatal glutamatergic afferents

There is strong evidence that group III mGluRs play an important modulatory role on glutamatergic transmission in slices of rodent striatum (Pisani et al., 1997; Gubbellini et al, 2004, Cuomo et al., 2009). The group III mGluR agonist, L-SOP (10 μM) significantly reduced the EPSP amplitude at corticostriatal synapses, in a reversible manner without modifying the postsynaptic sensitivity to glutamate (Pisani et al., 1997). L-AP4, another group III agonist, reversibly and dose-dependently decreased the amplitude of EPSPs at corticostriatal (Pisani et al., 1997, Calabresi et al., 1996, Calabresi et al., 1993). Recently, mGluR4 and mGluR7 activation with specific agonists (LSP1-3081 and L-AP4) in rat brain slices reduced glutamate- and GABA-mediated postsynaptic potentials, and decreased the frequency, but not the amplitude, of glutamate and GABA spontaneous and miniature postsynaptic currents. Additionally, intrastriatal LSP1-3081 or L-AP4 injections improved akinesia in 6-OHDA-lesioned rats (Cuomo, 2009). The electrophysiologocal data suggests that group III mGluRs could be mediating excitatory and inhibitory neurotransmission in the striatum.

Evidence for group III mGluR localization in the striatum was further provided by qualitative analysis of protein and mRNA. Strong mGluR7 hybridization signals and immunolabeling are found in presynaptic elements of the striatum (Kosinski et al., 1999).

Decortication experiments showed a drastic decrease (66.2%) of striatal mGluR7 immunoreactivity and colocalization with SV2, a presynaptic terminal marker (Kosinski et al., 1999), thus showing that cortical projections are a major source of presynaptic striatal mGluR7. Glutamatergic terminals in the striatum can be identified as either cortical or thalamic based on the expression of a specific vGluT protein. In rats and monkeys, the vGluT1 and vGluT2 are completely segregated between corticostriatal and thalamostriatal boutons, respectively, thereby making vGluTs ideal markers to identify the sources of glutamatergic terminals in these species (Herzog et al., 2006, Fremeau et al., 2004, Raju et al., 2006). Our data showed greater group III mGluR colocalization with vGluT1 than vGluT2 in the mouse brain, suggesting that group III mGluRs have a preferential modulatory influence on the corticostriatal versus thalamostriatal system. This degree of colocalization is not surprising since there are many more vGluT1 terminals in the striatun than vGluT2 and previous studies have shown group III mGluR localization in the corticostriatal projection elements.

It is important to note that the degree of vGluT1/vGluT2 co-localization in individual glutamatergic terminals in the mouse striatum is significantly higher than what was found in other species (Herzog et al., 2006, Fremeau et al., 2004, Raju et al., 2006), thereby limiting the use of these vGluTs as specific markers of the corticostriatal and thalamostriatal projections in mouse. However, this higher degree of vGluT1-vGluT2 colocalization does not affect the conclusion that group III mGluRs have the ability to regulate both the cortico- and the thalamo-striatal glutamatergic pathways. This hypothesis is further supported by the expression of group III mGluRs in more than half of terminals forming asymmetric axo-dendritic synapses in the striatum. Because

axodendritic asymmetric striatal synapses likely involve pre-synaptic terminals from the parafascicular nucleus of the thalamus (Smith et al., 1990, Sadikot et al., 1992, Smith et al., 1994, Hersch et al., 1995, Sidibe and Smith, 1996; Raju et al., 2006); our findings provide strong evidence that thalamic inputs from the caudal intralaminar nuclei are endowed with pre-synaptic group III mGluRs in mice. However, there are only a few studies that have provided evidence for the localization of group III mGluRs mRNA in CM/Pf neurons per se (Neto et al., 2000a, 2000b). Future studies that address this issue in more detail and directly measure the relative degree of expression of the different group III mGluR subtypes in the CM/Pf are warranted.

Group III mGluRs could potentially play an important role in modulating the activity of the thalamostriatal pathway. The thalamus provides a major source of glutamatergic afferents to the striatum, but the role and modulatory functions of mGluR4 and 7 upon this system remain poorly understood (Smith et al., 2004). Although the data on the group III mGluR expression in the thalamus is limited, a small number of studies demonstrated mGluR4 and mGluR7 mRNA expression in the rodent thalamus (Saugstad et al., 1997, Ohishi et al., 1995, Corti et al., 1998, Kinoshita et al., 1998, Tanabe et al., 1993). Although our findings show that vGluT2-containing terminals express the two subtypes of group III mGluRs, we cannot confidently conclude that these vGluT2 terminals originate from the thalamus because of the significant degree of vGluT1/vGluT2 co-localization our immunocytochemical data revealed in mice. Thus, future tracing studies need to be conducted to clearly assess the degree of expression of group III mGluRs in thalamostriatal terminals in mice. Coexpression of group III mGluRs with the vGluTs still supports the assumption that mGluRs are ideally positioned

to modulate glutamatergic transmission at both corticostriatal and thalamostriatal synapses.

2.4.3. Co-localization of Group III mGluRs in Glutamatergic Terminals

Our data show a significant degree of co-localization between mGluR4 and mGluR7 in individual glutamatergic terminals. The presence of multiple group III mGluRs in the active zones of synaptic terminals could potentially provide mechanisms whereby modulation of synaptic transmitter release can occur over a wide range of extracellular glutamate concentration. Group III mGluR mRNA and protein expression has been shown to overlap in many brain nuclei, such as the hippocampus, olfactory tubercle, many cortical regions, septofimbrial nucleus, intercalated nuclei of the amygdala, medial mammillary nucleus, many thalamic nuclei, the retina, and pontine nuclei (Table 1). Additionally, intense expression of both mGluR4 mRNA and mGluR7 mRNA was detected in the trigeminal ganglion and dorsal root ganglia (Ohishi et al., 1995, Shigemoto et al., 1997, Corti et al., 1998, Quraishi et al., 2007). There is evidence that both mGluR4 and mGluR8 modulate excitatory transmission in the SNc (Valenti et al., 2005). Because of the limited availability of specific agonists that differentiate between the 3 main group III mGluRs, specific and preferential activation of mGluR4 or mGluR8 over mGluR7 can be achieved using low concentration of the most commonly used group III mGluR agonist, L-AP4 , (Marino et al., 2003, Kogo et al., 2004, Pelkey et al., 2007, Conn & Pin et al., 1997, Cuomo et al., 2009), while the involvement of mGluR8 over mGluR4 can be sorted out using the rather specific mGluR8 antagonist DCPG. The specific role of mGluR7 still remains poorly characterized. Although

previous reports have introduced AMN082 as a specific allosteric potentiator of mGluR7 (Mitsukawa et al., 2005; Greco B et al., 2007), there is significant controversy about the reliability and specificity of that drug for mGluR7(Ayala et al., 2008; Jones et al., 2008).

In addition to receptor sensitivity, receptor activity at the synapse is dependent on the rate and probability of endocytosis and internalization. Internalization and desensitization of mGluR4 is agonist-independent unless pathways leading to the activation of PKC are induced (Mathiesen and Ramirez, 2006). AMN082, considered as a potential mGluR7 allosteric agonist (but see discussion above), increased receptor internalization to 291 ± 30% of control levels in hippocampal neurons (Pelkey et al., 2007). Considering the diverse sensitivity to glutamate and the significant variability in receptor internalization patterns, the functional significance of multiple group III mGluR subtypes in the same synapse adds a layer of complexity to group III mGluR function expanding their relative ability to regulate neurotransmitter release under a wide range of physiological and pathological conditions.

2.4.4. Group III mGluR innervations of direct and indirect pathways

Previously published studies have shown group III mGluRs in the glutamatergic projections to the striatum, however until now there has not been any information about the selectivity of mGluR4- and mGluR7-positive terminals for the direct or indirect pathway. We were limited in our choice on dopamine receptor antibody because a specific D_2 receptor antibody from a non-rabbit species was not available. For that reason the rat anti- D_1 receptor antibody was used to identify the spines of direct pathwayMSNs, and the remaining MSNs were assumed to belong to the indirect pathway. Our findings

suggest that the D_1 and D_2 receptors are segregated onto the spines of direct and indirect output pathway neurons (see also Hersch et al. 1995; Lei et al., 2004).

Investigation of a possible preference of group III mGluRs-containing glutamatergic terminals in targeting the D_1-positive or D_1-negative striatal output pathways provides an important clue in the selective preference of these receptors to regulate glutamatergic transmission to direct or indirect pathway neurons, which could have significant implications in drug development and testing. The data from this set of experiments suggests that both mGluR4 and mGluR7 could be involved in the presynaptic regulation of glutamatergic transmission to both D_1 and D_2 receptor-positive MSNs. However, while mGluR7 has no significant preference for the D_1-positive or D_1-negative MSN, mGluR4 seems to have a slight preference for the the D_1-negative postsynaptic elements. Considering the high degree of colocalization between mGluR4 and mGluR7, this data proposes that group III mGluRs could potentially modulate both the direct and indirect striatal output pathways. However the higher preference of mGluR4-positive terminals for the D_1-negative spines, suggests that mGluR4 could have a greater access to and ability to modulate the MSNs giving rise to the indirect pathway. Whether these receptors have equal function and activation probability in terminals targeting the direct and indirect pathways is yet to be examined. However, the anatomical data demonstrates the receptor localization and suggest a potential functional consequence of this pattern of expression.

2.4.5. Group III mGluRs in the Striatum-Potential Targets for Parkinson's disease

Recent data have provided strong evidence for the potential antiparkinsonian effects of group III mGluR agonists and allosteric modulators in acute rodent models of parkinsonism. The activation of mGluR4 may preferentially modulate striatopallidal transmission raising the interesting possibility that activation of mGluR4 could decrease the excessive inhibition of GP neurons by the overactive stratopallidal GABAergic synapse that has been postulated to occur in Parkinson's disease (Valenti at al., 2003; Marino et al., 2003). Consistent with this, intracerebroventricular or intrapallidal injections of L-AP4, a group III mGluR agonist, produces therapeutic benefit in both acute and chronic rodent models of Parkinson's disease (Lopez et al., 2007; Valenti et al., 2003). Intracerebroventricular injection of PHCCC, a selective allosteric potentiator of mGluR4, produced a marked reversal of reserpine-induced akinesia in rats, suggesting that potentiation of mGluR4 may be a useful therapeutic approach to the treatment of PD (Marino, 2003a; Marino, 2003b). Group III mGluR agonists may exert their antiparkinsonian effects via auto- and hetero-receptors at corticostriatal and striatopallidal synapses.

We believe that group III mGluRs in striatal glutamatergic synapses represent another target for PD therapy. Activation or potentiation of striatal group III mGluRs could, indeed, reduce the overactive corticostriatal system, known as a major pathophysiological hallmark of PD (Papa and Chase, 1996; Picconi et al., 2005; Raju et al., 2008). However, the development of novel therapeutic strategies that rely on group III mGluRs activation is highly dependent on the introduction of compounds that display

good bioavailability, significant access to the brain and differentiation between the three major receptor subtypes. Allosteric modulators, instead of orthosteric receptor agonists, appear to be better suited to serve this role and become promising new agents to treat brain diseases (Marino and Conn et al., 2006; Marino et al., 2003). Additionally, because group III mGluRs are expressed in other brain areas, central administration of group III mGluR specific drugs might activate the group III mGluRs in extrastriatal brain areas and cause side effects.

2.4.6. Concluding Remarks

Based on their pharmacological profiles and localization, group III mGluRs could potentially play a significant role in maintenance of homeostasis in the CNS via the regulation of various types of neurotransmission. These receptors have the capacity to regulate both excitatory and inhibitory neurotransmission as well as both synaptic (Rodriguez-Moreno and Lerma, 1998) and non-synaptic release of glutamate, contribution to the maintenance of extracellular glutamate levels (Bruno et al., 2000). The functional capabilities of this group of receptors are only at their early stages of discovery. Their abundance in the striatum is especially significant due to their ability to modulate the massive glutamatergic drive form the cerebral cortex and the thalamus. The abilty of group III mGluRs to inhibit the glutamatergic input into the striatum would have downstream effects on basal ganglia activity. Due to greater expression of the more sensitive mGluR4 at terminals targeting the D_1-negative MSN, activation of mGluRs should have a greater inhibitory effect on the indirect pathway. Assuming that receptor expression does not change in the Parkinsonan brain, this inhibition could be responsible

for re-establishing a more balanced striatal output by reducing the activity in the indirect pathway, disinhibiting the GPe, and reducing the inhibition of the thalamus by the GPi/SNr. In addition to the potentially relevant therapeutic benefit of these receptors activation in PD, they are also located to potertially regulate long term plasticity at striatal glutamatergic synapses.

3. Microcircuitry of metabotropic glutamate receptor 8 (mGluR8) in the mouse striatum

3.1. Introduction

Metabotropic glutamate receptors may play a role in the modulation of excitatory glutamatergic transmission in the CNS. The mGluRs are linked to several intracellular signal transduction mechanisms via G-proteins and mediate a wide variety of modulatory effects on excitatory and inhibitory synaptic transmission. The mGluR family includes eight different receptor subtypes pooled into three major groups (groups I, II and III) based on their sequence homology, pharmacological properties and transduction mechanisms. The group I mGluRs (mGluR1 and mGluR5) are usually postsynaptic and induce slow depolarization, whereas group II (mGluR2 and mGluR3) and III mGluRs (mGluR4, 6, 7, 8) are mainly presynaptic and inhibit glutamate and GABA release. Because of their modulatory nature, diversity and association with specific brain regions, the mGluRs are recognized as promising targets for the development of drug therapies for various brain diseases (Conn et al., 2005; Swanson et al., 2005; Marino and Conn et al, 2006). The modulatory properties of the mGluRs are subject to differential regulation due to diverse sensitivity levels to glutamate and various pharmacological agents, with EC-50 values ranging from 0.02 -1000 (Conn & Pin, 1997).

The mGluR8 is the group III mGluR that displays the greatest sensitivity to both glutamate and group III mGluR agonists. Recently, electrophysiological and qualitative anatomical studies have confirmed the expression of mGluR8 at excitatory and inhibitory synapses in the hippocampus and the SN (Ferraguti et al., 2005; Valenti et al., 2005;

Wyss, 1981; Zhai et al., 2002). Although the mGluR8 protein and mRNA expression has been reported in the rodent striatum, little is known about the ultrastructural localization of this receptor (Corti et al., 2001; Parelkar et al., 2008; Zhang et al., 2009).

The striatum is the largest basal ganglia nuclei and serves as the main input station of the basal ganglia circuitry, receiving major glutamatergic projections from the cerebral cortex and the thalamus. Group III mGluRs have been shown to regulate the excitatory and inhibitory synaptic transmission in striatum. An understanding of the distribution and subcellular localization of mGluR8 will provide a clue to potential functional roles that this receptor can play in the regulation of striatal neurotransmission. To accomplish this objective, we used immunostaining for the vesicular glutamate transporters 1 and 2 (vGluT) as selective markers of either corticostriatal or thalamostriatal terminals, allowing us to examine the extent of mGluR8 immunolabeling on each of these pathways. Additionally, this study also aimed at determining whether mGluR8-containing pre-synaptic terminals displayed a preferential relationship with either of the two main populations of striatofugal neurons, the so-called “direct and indirect pathway” neurons. Knowing that a balanced activity of these two pathways is essential for normal basal ganglia functioning (DeLong et al., 1990; Smith et al., 1998), a detailed understanding of the various receptor subtypes involved in the regulation of afferents to these two populations of neurons is a prerequisite to the development of novel pharmacotherapeutics that allow targeting of specific populations of striatal output neurons.

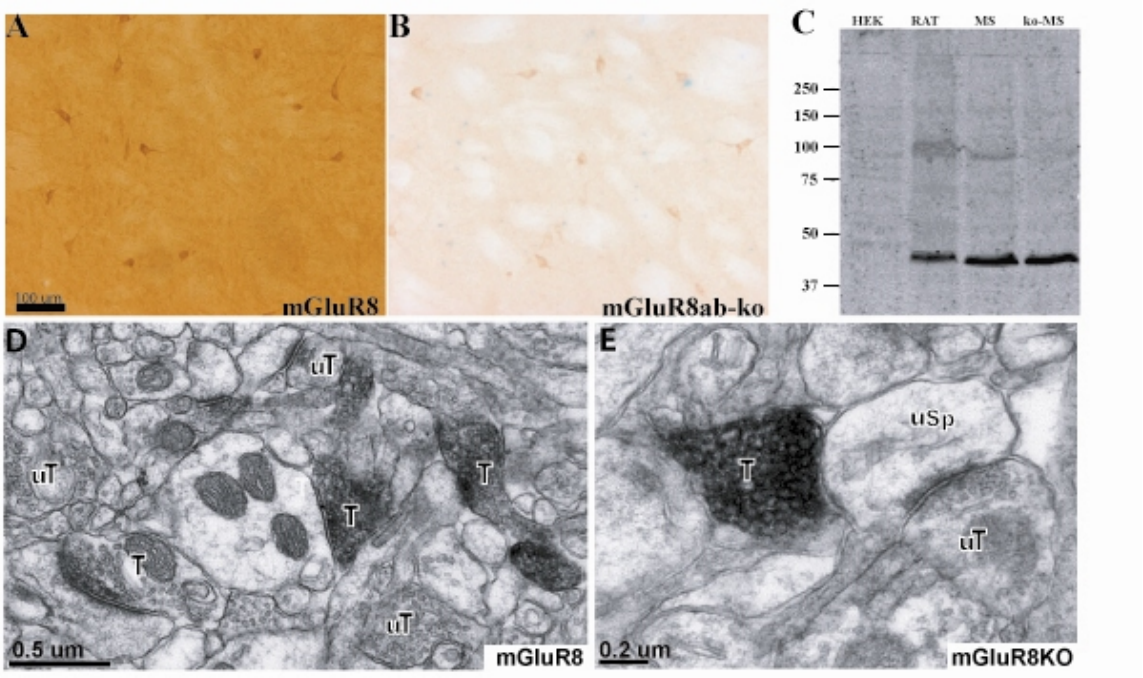


Figure 3.1: mGluR8 immunoreactivity in wildtype and knockout mouse striatum . (A) – mGluR8 immunoreactivity in wild type mouse brain tissue. mGluR8 labeled striatal neuropil and a subpopulations of large interneurons (B) – mGluR8 immunoreactivity in mGluR8a/b knockout tissue. Immunoreactivity due to the mGluR8 antibody seen only in subpopulation of striatal interneurons. The interneurons are most likely expressing the ~50kD protein that is expressed in the mGluR8a/b knock out animals. (C) – immunoblot shoing no reactive band in the human embryonic kidney (HEK) cell lane. The wild type rat and mouse brain lysate lanes show 2 bands immunoreactive at 100kD and ~50kD. The mGluR8a/b knockout mouse brain lysate is devoid of the 100kD band, with the 50kD band still present. (D) – mGluR8 labeling in wildtype brain tissue showing two patterns of immunoreactivity: along the active zone of the presynaptic terminal forming an asymmetric synapse and a dense packing of a terminal forming a symmetric synapse. (E) – mGluR8 immunoreactivity in the mGluR8a/b knockout tissue. Only

3.2. Materials and Methods

3.2.1. Animal and tissue preparation

Five adult (4-5week old), male Swiss colony derived FVB mice (Charles River Laboratories), weighing 18-20 grams were completely anesthetized with a cocktail of ketamine (60-100mg/kg) and dormitor (0.1mg/kg) and transcardially perfused with cold Ringer's solution, followed by fixative consisting of 4% paraformaldehyde and 0.1% glutaraldehyde in phosphate buffer (0.1M;pH 7.4). After perfusion, brains were removed from the skull and postfixed in 4% paraformalderhyde for 5-24 hours. Following post-fixation, coronal brain sections were cut with a vibrating microtome into 60μm-thick coronal sections. Brain sections were stored in phosphate-buffered saline (PBS) at 4°C. Selected sections were put in a 1.0% sodium borohydride solution, dissolved in PBS, for 20 minutes and thereafter rinsed in PBS before being processed for immunocytochemistry. 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.

3.2.2. Antibodies:

To localize mGluR8 we used a commercially available affinity-purified polyclonal antibody raised in rabbit against the C-terminal domain of human mGluR8 conjugated to KLH (1:750, LifeSpan BioSciences/MBL International Corporporation, Catalog #LS-A925, Lot #5715/16 AP10-1). A simple dot blot examination confirmed that the antibody was in fact specific to the N-terminal peptide against which it was created (data not shown). Additional studies from our laboratory have used striatal tissue from mGluR8a,b knockout mice (Duvoisin et al., 2005; generously given by Dr Robert

Duvoisin) to determine the specificity of this mGluR8 antiserum. The immunoblotting and immunohistochemical analyses have shown that the mGluR8 antibody recognizes the long forms of mGluR8 in the striatal neuropil and another unidentified protein which seems to be solely localized in a subset of striatal interneurons (Fig. 1).

Two antibodies, rabbit anti-VGluT1 and guinea pig anti-VGluT2, were used as specific markers of terminals from either the VGluT1-positive corticostriatal or VGluT2-positive thalamostriatal glutamateric projections (but see pg. 47). To localize VGlut1 we used commercially available Rabbit anti-VGluT1 antibodies (MaB Technologies, Cat # VGT1-3, Lot # GA062B, 1:5000). To generate these antibodies, a peptide from the COOH terminus of the rat vesicular glutamate transporter 1 (rvGluT1), corresponding to amino acids 543–560 (cATHST*V*QPPRPPPPVRDY) was synthesized. A cysteine was added to the peptide to aid its conjugation to the protein carrier keyhole limpet hemocyanin (KLH; Pierce, Rockford, IL). Antisera were obtained from rabbits (Covance) immunized with the conjugated peptide, and the IgG fraction was recovered (Raju, et.al. 2006). Previous studies from our laboratory and other laboratories using immunoblotting of brain lysate revealed single bands at ~60 kDa. Preadsorbtion of primary antibody with synthetic peptide (0.2– 0.4 ug/ml) overnight at 4°C abolished immunoreactivity, whereas preadsorbtion with a similar but nonidentical peptide preserved immunoreactivity (Montana et al., 2004; Raju and Smith, 2005). Immunohistochemical experiments on striatal tissue using the VGluT1 antibody in control tissue and tissue preadsorbed with vGluT1, but not vGluT2, peptide further confirmed the specificity of the antibody (Raju, et.al. 2006).

A polyclonal guinea pig anti-VGluT2 antiserum was used to localize VGluT2 (Chemicon, 1:5000, Catalog # AB5907, Lot #0611046131). The manufacturer stated that the antiserum has been tested on tissue sections from the rat central nervous system (CNS) using immunofluorescence histochemistry. The antiserum mainly labeled nerve fibers and terminals. The staining pattern for the labeling obtained with the VGluT2 antiserum corresponds to the pattern described using other antisera to VGluT2 (Fremeau et al., 2001; Fujiyama et al., 2001; Sakata-Haga et al., 2001; Tong et al., 2001; Kaneko et al., 2002; Varoqui et al., 2002). Preabsorption of the VGluT2 antiserum with immunogen peptide eliminates all immunostaining. Further, the specificity of the vGluT2 antiserum was carefully tested by immunoblotting and immunocytochemistry in previous studies (Montana et al., 2004; Raju et al., 2006).

Spines containing the D1 receptor were identified using the Sigma Aldrich monoclonal rat anti-D1 dopamine receptor antibody (Catalog #D187-250uL; clone 1-1-F11 s.E6; immunoglobulin purified). According to the information provided by the manufacturer, the antibody was produced by immunizing rats with a 97 amino acid synthetic peptide corresponding to the C-terminus of the human D1 dopamine receptor. The D187 antibody is specific for human (DRD1 Gene ID 1812), monkey, and rat (Drd1a Gene ID 24316) D1 dopamine receptors. The antibody is supplied as a solution in 20 mM sodium phosphate, pH 7.2, containing 150 mM sodium chloride and 0.02% sodium azide. The antiserum mainly labeled striatal spines and some dendrites. The staining pattern for the labeling obtained with the D1 antiserum corresponds to the pattern described using this and other D1 antisera in previous publications (Hersch, S.M., et al., 1995; Levey, A.L., 1993; Smiley, J.F., 1994; Yung, K.K.L., 1995; Mitrano, D.A., 2007).

3.2.3. Immunoblotting:

The immunoblotting methods were performed as described in previous studies (Betarbet et al., 2006; Orr et al., 2008; Pettus et al., 2000). Briefly, fresh tissue samples were homogenized in buffer H (210 m*M* mannitol, 70 m*M* sucrose, 1 m*M* EGTA, 5 m*M* HEPES) with protease inhibitors (1 mg/ml apoprotinin, leupeptin, and pepstatin A) and assayed for protein concentration using Bio-Rad protein assay (BioRad, Hercules, CA) according to manufacturer's protocol. The cell lysate was obtained from cultured cells that were grown on 100 mm plates, washed two times in PBS, pH 7.4, and incubated in 400 µl of cell lysis buffer (Promega, Madison, WI) containing protease inhibitors (Roche, Mannheim, Germany), 700 U/ml DNAse I (Gibco, MD), and 1% β-mercaptoethanol (Sigma, St. Louis, MO) for 15 min at room temperature. Cells were scraped and lysate was centrifuged at 10,000×g for 10 min. Supernatant was collected as the soluble fraction. Protein content was assayed using Bio-Rad protein assay (BioRad, Hercules, CA) according to manufacturer's protocol. Tissue samples were prepared at 4°C. The fresh tissue was completely homogenized with a sonicator in an ice-cold buffer solution containing protease inhibitor cocktail (1 tablet per 50 ml; Roche Diagnostics GmbH, Mannheim, Germany), 1.0% Triton X-100, 20 mM HEPES, 10 mM EDTA, and 2 mM Na3VO4. The homogenate was then centrifuged for 5 minutes at 2,000 rpm to remove tissue debris. The lysates were eluted with 6× SDS-PAGE sample buffer. Samples were loaded on a 12% acrylamide mini gel and run at 130 V in running buffer (0.1% SDS, 125 m*M* Tris base, 1 *M* glycine) on a Bio-Rad minigel apparatus. Gels were rinsed in transfer buffer (10% methanol, 25 m*M* Tris, 192 m*M* glycine) for 10 min, apposed to polyvinylidene difluoride (PVDF) membrane (Millipore, Billercia, MA U.S.A.), and

transferred overnight at 35 V under cooling conditions. PVDF membranes were incubated in KPL blocker for 3 h on a shaker at room temperature and then incubated in blocker containing primary antibody raised against mGluR8 (1:750) overnight at 4°C on a shaker. Membranes were thoroughly rinsed in PBS/Tween, incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature, and rinsed thoroughly in PBS/Tween. The blots were detected using Super-signal West Dura extended duration substrate (Pierce, Rochford, IL). The blots were developed using Eastman Kodak (Rochester, NY).

3.2.4. Single Labeling Immunoperoxidase for Light Microscopy:

After sodium borohydride treatment, selected coronal tissue sections were incubated for 1 hour at RT in PBS containing 10% normal goat serum (NGS), 1% BSA, and 0.3% Triton X-100, followed by the primary antibody solution containing 1% NGS, 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 IgGs at a concentration of 1:200 (Vector Laboratories, Burlingame, CA) for 90 minutes. The sections were rinsed again in PBS and then incubated for another 90 minutes with the avidin-biotin peroxidase complex (ABC) at a dilution of 1:100 (Vector Laboratories). Finally, the sections were washed twice in PBS and once in Tris buffer (50 mM; pH 7.6) and transferred to a solution containing 0.025% 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma), 10 mM imidazole, and 0.005% hydrogen peroxide in Tris buffer for 10 minutes producing a diffusible reaction product to identify the immunoreactivity for the receptors. 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 with a CCD camera (Leica DC500) controlled by Leica IM50 software.

3.2.5. Single Pre-Embedding Immunoperoxidase for Electron Microscopy:

After sodium borohydride treatment, selected tissue sections were transferred to a cryoprotectant solution (PB, 0.05M, pH 7.4, containing 25% sucrose and 10% glycerol) for 20 minutes and then frozen in the –80^{o}C freezer for 20 minutes. The tissue was returned to the cryoprotectant solution and then, via a decreasing gradient of cryoprotectant solutions (100%, 70%, 50%, 30%) the tissue was returned to PBS. Brain tissue sections were incubated for 1 hour at RT in PBS containing 10% normal goat serum (NGS), 1% BSA, followed by a 48 hour incubation at 4 degrees with primary antibodies diluted in a solution of normal serum (1%), bovine serum albumin (BSA, 1%), 0.01% Triton X-100 and PBS. The rest of the protocol was the same as for the LM procedure. Following the DAB reaction, the sections were transferred to PB (0.1 M, pH 7.4) for 10 min and exposed to 1% osmium tetroxide diluted in PB for 20 min. They were then rinsed with PB and dehydrated in an increasing gradient of ethanol. Uranyl acetate (1%) was added to the 70% alcohol to increase contrast at the electron microscope. The sections were then treated with propylene oxide before being embedded in epoxy resin (Durcupan, ACM; Fluka, Buchs, Switzerland) for 12 hours, mounted on microscope slides, and placed in a 60°C oven for 48 hours.

Blocks of tissue were cut into 60 nm ultrathin sections with an ultramicrotome (Ultracut T2; Leica, Nussloch, Germany) and serially collected on single-slot Pioloform-coated copper grids. The sections were stained with lead citrate for 5 min (Reynolds,

1963) and examined with a Zeiss EM-10C electron microscope (Thornwood, NY). Fifty micrographs of randomly encountered labeled elements were captured with a GATAN camera at 20,000X from the most superficial sections of blocks of tissue from each of the five animals used in these studies. Labeled and unlabeled neuronal elements were identified and counted.

3.2.6. Double immunoperoxidase labeling methods for mGluR8 and vGluT colocalization

This method was used to co-immunostain the striatal tissue for mGluR8 with vGluT1, vGluT2 in single glutamatergic terminals. The steps of the double immunoperoxidase methods were similar to those in the single immunoperoxidase method, except that the tissue was incubated with both primary antibodies simultaneously. Accordingly, in the mGluR8/vGluT1 or mGluR8/vGluT2 co-localization experiments, the tissue was co-incubated with a cocktail of both the mGluR8 and one of the vGluT primary antibodies. Likewise, the secondary antibodies were also introduced to the tissue simultaneously. Both mGluR8 and vGluT1 antibodies were raised in rabbits, so only one secondary (goat anti-rabbit) antibody was necessary to bind to the primary antibodies. The vGluT2 antibody, however, was raised in guinea pig and accordingly a different secondary (goat anti-guinea pig) antibody used was to bind to it. It is important to note that the secondary antibodies were biotinylated and after exposure to DAB, a diffuse reaction product was produced and it identified the immunoreactivity. The rest of the protocol was the same as for the single pre-embedding immunoperoxidase for EM procedure. At the LM level the distribution and the intensity of labeling resembled the

sum of labeling produced by the two antibodies individually. At the EM level labeling was seen along the active zone of presynaptic terminals, as was previously demonstrated with single mGluR8 labeling. Some terminals however were densely labeled by DAB, as was previously reported with vGluT-type labeling (Raju et al., 2006).

3.2.7. Double immunoperoxidase labeling methods for mGluR8 and D1

This method was used to co-immunostain the striatal tissue for mGluR8 with D1 in order to assess the synaptic relationships between striatal mGluR8-positive terminals and D1-positive or D1-negative spines. The steps of the double immunoperoxidase methods were similar to those in the single immunoperoxidase method and to the mGluR8/vGluT methods mentioned above. Because the D1 antibody was raised in rat, two different secondary antibodies were used to bind the primary antibodies. As mentioned before, the secondary antibodies were biotynylated and were introduced to the tissue simultaneously. After exposure to DAB, a diffuse reaction product was produced and it identified the immunoreactivity. The rest of the protocol was the same as for the single pre-embedding immunoperoxidase for EM procedure. At the LM level the distribution and the intensity of labeling resembled the sum of labeling produced by the two antibodies individually. At the EM level labeling was seen along the active zone of presynaptic terminals, as was previously demonstrated with single mGluR8 labeling. Additionally, to the presynaptic labeling, there were labeled spines and dendrites, which were clearly due to D_1 receptor immunoreactivity.

3.2.8. Co-localization Studies: Pre-embedding Immunoperoxidase and Immunogold:

This procedure was used to asses the degree of vGluT1/vGluT2 co-localization in striatal glutamatergic terminals. After cryoprotectant, sections were preincubated for 1 hr in a solution containing 10% NGS and 10% NHS in PBS-BSA (0.005% BSA, 0.05% Tween 20, and 0.001% gelatin in PBS) before being transferred to a 1% NHS and 1% NGS in PBS-BSA solution containing the primary antibodies: rabbit anti-vGluT1 and Guinea pig anti-vGluT2 for 24 hr at room temperature. Next, the tissue was rinsed in PBS-BSA and incubated for 2 hr in the secondary 1.4 nm gold-conjugated goat anti-rabbit IgGs (Nanogold; Nanoprobes, Stonybrook, NY) at a concentration of 1:100 and a secondary biotinylated goat anti-guinea pig IgGs (Vector Laboratories, Burlingame, CA) at a concentration of 1:200 in 1% NGS and 1% NHS in PBS-BSA. The sections were then rinsed with PBS and followed by 2% acetate buffer rinses. Tissue then underwent silver intensification of gold particles for 5-10 min using the HQ silver kit (Nanoprobes). After the intensification, the tissue was rinsed in Acetate buffer and the sections were put in a solution containing 1:100 avidin-biotin-peroxidase complex (Vector). The tissue was washed in PBS and then with TRIS buffer before being transferred into a solution containing 0.01% imidazole, 0.0005% hydrogen peroxide, and .025% 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma, St. Louis, MO) for 10 minutes. The remainder of the procedure, involving dehydration, osmification and embedding was the same as that described above for the immunoperoxidase material.

3.2.9. Control experiments.

Control incubations for all experiments included omission of both primary antibodies to test for nonspecific secondary antibody binding. In addition, incubations with only one primary antibody but both secondary antibodies and/or tyramide intensification were carried out to demonstrate the lack of cross-reactivity.

3.2.10. Analysis of material

3.2.10.1. Overall distribution of mGluR8-labeled elements from single immunoperoxidase-stained material.

Five blocks of striatal tissue from 5 animals were cut out, mounted on resin blocks, and glued into place using cyanoacrylate glue. Ultrathin sections from the surface of these blocks were collected and scanned for the presence of immunoperoxidase-labeled structures that were easily distinguishable from unlabeled elements by their electron-dense reaction product. For each animal, 50 photographs of randomly selected immunoreactive elements were digitized at 20,000x. This resulted in a total surface of 7,200 μm^2 of striatal tissue to be examined for each mGluR experiment. Immunoreactive elements were categorized as presynaptic (axons, terminals) or postsynaptic (dendrites, spines) neuronal structures on the basis of ultrastructural features. The relative abundance of each neuronal element for each receptor subtype in the striatum was calculated by dividing the number of labeled elements by the number of total labeled elements. To obtain the percent of labeled elements, the number of each type of labeled elements was divided by the number of total identified elements of that type. Significant differences

were assessed by using a t-test (n=5). Data presented as average of percent labeled elements ± SEM.

3.2.10.2. Co-localization of mGluR8 with vGluT1 or vGluT2 using the double immunoperoxidase antibodies cocktail method.

Data collection was the same as for the single immunoperoxidase experiment. For each animal, 50 photographs of randomly selected immunoreactive elements from the surface of the blocks were digitized at 20,000x. This resulted in a total surface of 7,200μm^2 of striatal tissue to be examined for each experiment. To determine the percentage of terminals that displayed co-localization of the two markers of interest, the same formula as was described in chapter 2.2.7.3 was used: % elements labeled by Marker#1 + % elements labeled by Marker#2 - % elements labeled by Cocktail of Marker1+2 = % elements that co-express both Marker1&2. This method has been successfully used in previous studies to demonstrate segregation of D1 and D2 receptors in striatal spines (Hersch et al., 1995; Lei et al., 2004) as well as co-localization of mGluR1 and mGluR5 in the striatum (Mitrano and Smith, 2007).

3.2.10.3. Co-localization of mGluR8 with D1 using the double immunoperoxidase antibodies cocktail method or immunoperoxidase and immunogold .

Data collection was the same as for the single immunoperoxidase experiment. For each animal, 50 photographs of randomly selected immunoreactive elements from the surface of the blocks were digitized at 20,000x. This resulted in a total surface of

7,200μm^2 of striatal tissue to be examined for each experiment. To determine the percentage of D_1-immunoreactive spines that form synapses with mGluR8-positive terminals, the immunolabeled terminals forming an identifiable asymmetric synapse(s) were first identified and the postsynaptic elements were then counted and examined for the presence of immunolabeling. This type of analysis was possible because of two premises: first, based on our previous analysis of mGluR8 localization and distribution, only about 1% of immunolabeled terminals synapse with an immunolabeled spine, thus making the error of detecting an mGluR8-positive terminal synapsing with a D_1-positive spine very low. Second, it has been previously established that while striatum does possess D_1-positive terminals, these terminals do not form asymmetric synapses because they are cholinergic or GABAergic in nature (Wu et al., 2006), and thus would not be included in our analysis.

3.3. Results

3.3.1. Specificity tests of mGluR8 Antibody.

At the light microscopic level, the mGluR8 antibody labeled striatal neuropil and a subset of interneurons in the wild type mouse (Fig. 3.1A). However, the knockout mouse tissue lacked the neuropil immunoreactivity, but the darkly stained interneurons still remained (Fig. 3.1B). When the labeled tissue was examined in the electron microscope (EM), two types of labeled terminals were seen in wild type tissue; (1) Terminals forming asymmetric synapses that displayed an electron-dense deposit of immunoreactivity confined at the presynaptic active zone of labeled terminals (Fig. 3.1D) and (2) Terminals forming symmetric synapses that were almost completely packed with

the DAB deposit (Fig. 3.1D, E). In the knockout tissue, the labeling was detected only in the type of darkly stained terminals forming symmetric synapses and sparsely distributed postsynaptic dendrites and large cell bodies (Fig. 3.1E). Together, these observations suggest that the type of immunolabeled terminals that remain labeled in the knockout tissue likely belongs to the immunoreactive cell bodies found in this material. Additional immunoblotting experiments showed that the mGluR8 antibody recognized two bands at molecular weight of 100kD and 50kD in rat and mouse brain homogenate. Only the 50 kD band was seen in the mGluR8a/b knock out mouse brain homogenate, confirming that the antibody recognized the long isoforms of the mGluR8 receptor: mGluR8a and mGluR8b. No bands were detected in the HEK cell lysate, which does not express any of mGluR8 isoforms and also do not express the ~50kD protein (Fig. 3.1C).

Together, these data suggest that the mGluR8 antibody used in this study recognized the long isoforms of mGluR8 (a and b) and another unidentified protein, expressed selectively in a subpopulation of striatal interneurons. Since this labeling remained in the knock out tissue, we considered it non-specific and unrelated to the localization of mGluR8a,b. In contrast, because the presynaptic labeling in the active zones of glutamatergic terminals was lost in the knock out tissue, we interpreted it as the genuine indication of mGluR8a,b immunoreactivity. Thus, only this subpopulation of immunoreactive terminals was considered in the remaining of the study.

3.3.2. Quantitative Assessment of the Relative Abundance of Presynaptic mGluR8 Immunoreactivity in the Mouse Striatum

In this first set of experiments, we determined the frequency of mGluR8-immunoreactive glutamatergic terminals over the whole population of striatal glutamatergic boutons. To do so, immunostained tissue from the surface of striatal blocks collected from five mice was scanned for the presence of labeled and unlabeled terminals forming axo-spinous and axo-dendritic asymmetric synapses. This analysis revealed that about half (45.8% ±2.7) of all terminals forming asymmetric synapses in the striatum displayed mGluR8 immunoreactivity. More than 90% of these terminals formed axo-spinous synapses while 7.0%±1.2 were involved in axo-dendritic synapses. Based on previous tracing studies from our laboratory and others, we assume that most of these terminals in contact with dendritic shafts originate from the caudal intralaminar nuclei in the thalamus, while other boutons arise from either the cerebral cortex or other thalamic nuclei (see Smith et al., 2004, 2009 for reviews). Because the use of vGluTs cannot reliably differentiate thalamostriatal from corticostriatal terminals in the mice used in these studies (see chapter 2), future tracing studies must be performed to label the thalamostriatal terminals and examine mGluR8 localization at these synapses.

3.3.3. mGluR8 Innervation of Direct and Indirect Pathway Neurons

To assess the degree of innervation of direct and indirect output pathway neurons by mGluR8-containing glutamatergic terminals, we used a D1 receptor antibody as marker of the direct pathway. The D1 receptor antibody labeled dendrites and spines. In line with previous studies using the same antibody (Hersch et al., 1995; Lei et al., 2004), D1-

containing spines made up about 50% of all spines in the striatum. Taking into consideration data from previous reports showing that about 50% striatal spines are immunoreactive for D1 receptor and 50% are immunoreative for the D2 receptor (Hersch et al., 1995; Lei et al., 2004), we assumed that a majority of spines that were not immunolabeled with the D1 antibody in our material most likely expressed D2.

A first double-labeling experiment was performed in which immunoperoxidase was used to label D1 and immunogold served as marker of mGluR8. Analysis of the postsynaptic target of mGluR8-containing terminals in this tissue revealed that 7.1%±1.2 of immunoreactive terminals formed synapses with D1-immunoreactive spines. The immunoreativity of postsynaptic elements of each labeled terminal were evaluated and showed that mGluR8 has a larger preference for the D1-immunoreactive postsynaptic elements (62.7%±3.4) (Fig. 2A,C). To further confirm this finding we employed the double immunoperoxidase technique, which, due to increased sensitivity, was able to identify a larger number of mGluR8-labeled terminals. The double immunoperoxidase method resulted in similar findings, showing 61.4%±1.01 of mGluR8 terminals forming synapses with D1-positive postsynaptic elements (Fig. 2B, D, E). Therefore, these observations suggest that while mGluR8 may be involved in regulating glutamatergic transmission from both cortex and thalamus, due to greatr co-expression with vGluT1, they could have a greater impact on the corticostriatal input pathway. Additionally, mGluR8 may be preferentially involved in regulating glutamatergic transmission that impinges upon direct pathway neurons in the striatum, and consqequently would affect the activity of not only striatal neurons but of those inother basal ganglia nuclei.

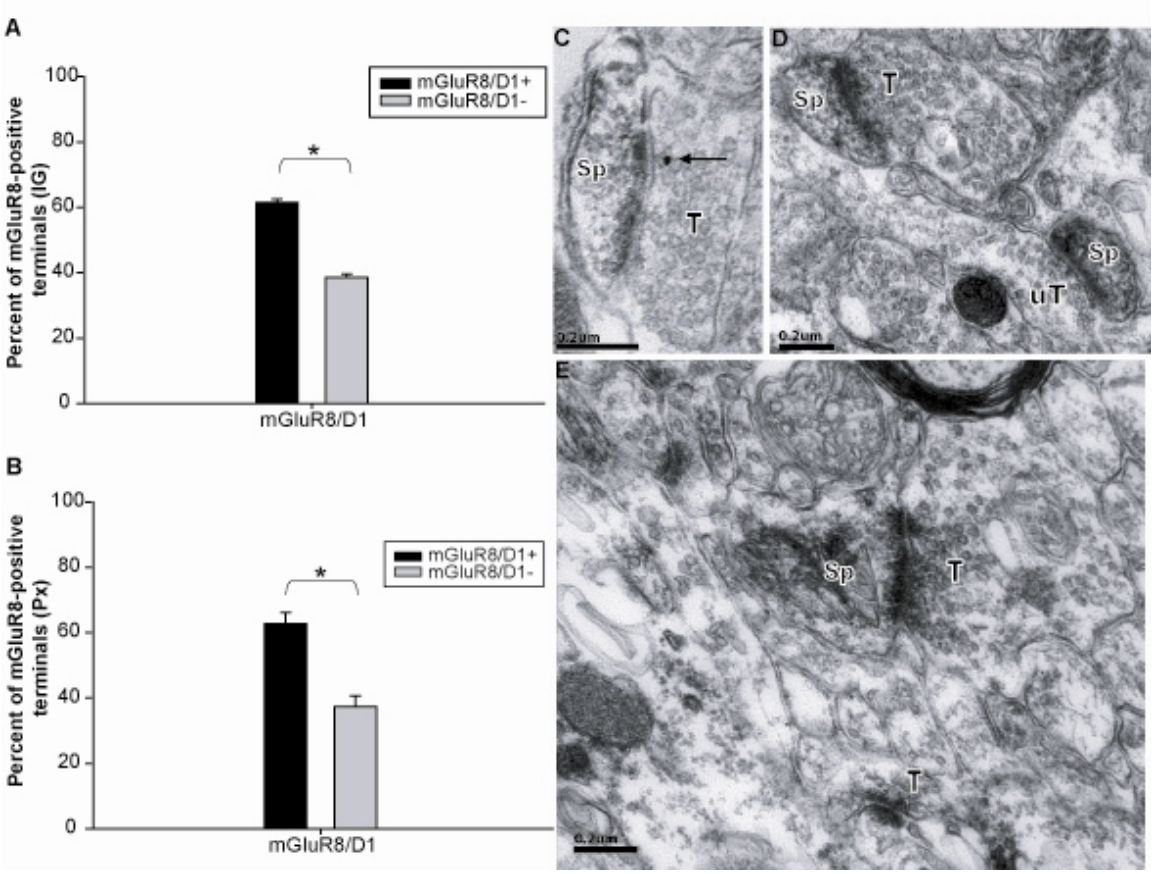


Figure 3.2: Preferential targeting of D1-positive and D1 negative spines by mGluR8 labeled terminals. (A) – mGluR shows preference for D1-positive postsynaptic elements when examined with double immunoperoxidase method. (B) – similar examination with peroxidase and gold double labeling method confirmed the preferential targeting of mGluR8 terminals of D1-positive elements. (C) - mGluR8/gold and D1/peroxidase labeling. (D-E) – double immunoperoxidase labeling of mGluR8-positive terminals and a D1-positive spine. IG – immunogold, Px – immunoperoxidase.

3.4. Discussion

Unlike the other group III mGluRs, mGluR8 has not been extensively studied due to the limited availability of commercial antibodies specific for this receptor subtype, but the expression of mGluR8 protein and mRNA in the rodent cortex, thalamus, and striatum has been previously reported (Saugstad et al., 1997; Corti et al., 1998; Richardson-Burns et al., 2000; Corti et al., 2001; Parelkar et al., 2008; Zhang et al., 2009). However, no detailed analysis of the expression of mGluR8 at the cellular and subcellular level has ever been conducted in the basal ganglia. In this study, we demonstrate that mGluR8 is predominantly expressed in presynaptic glutamatergic terminals from both the cerebral cortex and thalamus in the mouse striatum. Additionally, our findings indicate that terminals expressing mGluR8 preferentially target spines of D1-immunoreactive direct striatal output pathway neurons. Considering the high sensitivity of this receptor to glutamate compared to other group III mGluRs (Conn and Pin 1997), mGluR8 activation and the resulting downstream effects could be the first response to glutamate release in the striatum in both normal and pathologic conditions. The high sensitivity of this receptor to glutamate could also allow mGluR8 to be activated by non-synaptically released glutamate, i.e. ambient glutamate in the extracellular space. This function would allow mGluR8 to regulate cellular activity in response to global changes in neuronal and non-neuronal glutamatergic transmission.

3.4.1. Technical considerations

In this study we utilized two immunolabeling methods to assess colocalization: a double antibody cocktail immunoperoxidase method and a double immunolocalization

approach using immunoperoxidase combined with immunogold techniques. Both methods have their own the advantages and limitations, as previously discussed in chapter 2.4. The design of the double immunoperoxidase is based on the premise that individually antibodies would label a specific population of elements that selectively express the antigen of interest, and that the cocktail of both antibodies reveals all immunoreactive elements recognized by each individual antiserum. This type of technique grants the same sensitivity to the marker used to localize each antigen (i.e. peroxidase), thus reducing the likelihood of false negative data due to the reduced sensitivity and poor tissue penetration of gold-conjugated antibodies. For quantitative measurements, the antibody cocktail method is probably a more precise, sensitive and reliable approach to be used to assess antigen co-localization in single elements at the electron microscopic level.

To provide a qualitative confirmation of some of our quantitative data, the gold and peroxidase double labeling method was used. One limitation of the immunogold technique is that it is not as sensitive and that gold-conjugated secondary antibodies do not penetrate the tissue as well as biotinylated antibodies and ABC complex do, thereby resulting in a more superficial localization of gold-labeled elements than peroxidase-stained structures. When used in double labeling experiments, this difference in tissue penetration and sensitivity of gold versus peroxidase deposits can result in false negative results regarding gold-labeled structures. For these reasons, the gold and peroxidase methods must be used with caution for quantitative analysis of co-localization studies. An important aspect of data collection from the immunogold and peroxidase experiment examining the preference of mGluR8-containing terminals for D1-positive vs D1-

negative spines, is that the gold-labeled terminals were identified first and then the presence or absence of peroxidase deposit was determined in postsynaptic elements. Since the peroxidase method is more sensitive than the gold method, this approach reduced the possibility of a false negative.

The specificity of the mGluR8 antibody used in this study was meticulously tested with immunoblotting and immunohistochemical techniques in wild and knockout tissue. These tests confirmed that the antibody recognized the mGluR8a/b isoforms; but they also showed significant cross-reactivity with another unknown antigen. Using immunoblotting methods, the molecular weight of the unknown antigen was determined to be about 50kD (Fig. 3.1C). Preabsorbtion experiments using the C-terminal peptide against which the antibody was made abolished the immunosignal, thus confirming the specificity of the antibody. Preabsorbtion of the antibody with an N-terminal peptide of mGluR8 maintained immunoreactivity (data not shown).

In the wild type mouse brains, the antibody immunolabeled neuropil and some cell bodies in the striatum, whereas in the mGluR8a/b knockout mouse brains the neuropil labeling was significantly reduced, while labeled cell bodies remained. At the EM level the pattern of labeling found in terminals forming asymmetric synapses was indistinguishable from that previously reported for mGluR8 and other group III mGluRs in the hippocampus (Shiemoto et al., 1997; Ferraguti et al., 2005; Ferraguti et al., 2006) and other brain regions. The pattern of labeling was also similar to that described in chapter 2.0 of this thesis for mGluR4 and mGluR7 suggesting that the three group III mGluR subtypes are expressed presynaptically in glutamatergic terminals in the mouse striatum. These labeled boutons were not found in mGluR8a,b knock out tissue,

indicating that the immunoreativity associated with these terminals likely represent genuine presynaptic mGluR8a,b labeling, while the immunoreactivity associated with cell bodies and postsynaptic elements is nonspecific and due to crossreactivity with an unknown antigen expressed in a subset of striatal interneurons. Because we could not determine the exact significance of this labeling, we decided not to consider any postsynaptic labeling seen in this material as being indicative of mGluR8a,b.

3.4.2. Physiological Effects of mGluR8 in Basal Ganglia

Electrophysiological analysis of group III mGluR activity showed that activation of receptors by specific agonists L-AP4 and LSP1-3081 reduced glutamate- and GABA-mediated postsynaptic potentials and decreased the frequency of glutamate and GABA spontaneous and miniature postsynaptic currents in slices of rat striatum. Intrastriatal injection of LSP1-3081 or L-AP4 injection improved akinesia in 6-OHDA treated animals (Cuomo, 2009). In the rat SNc and hippocampus, mGluR8 was shown to mediate excitatory transmission via a presynaptic mechanism (Valenti, 2005; Kral, 2003; Ayala, 2008; Macek, 1996; Ci, 2001).

3.4.3. mGluR8-containing Glutamatergic Terminals Preferentially Target Direct Pathway Neurons.

Previous studies have shown group III mGluRs expression in glutamatergic projections to the striatum (Kosinski et al., 1999), but until now there has not been any information about the selectivity of mGluR8-positive terminals for direct or indirect pathway striatofugal neurons. Presuming that a balance of activity between direct and

indirect pathway neurons is essential for normal basal ganglia functioning (DeLong, 1990) combined with the fact that glutamatergic inputs are the main sources of excitatory drive to striatal projection neurons, provide a solid rationale to further understand the possible mechanisms by which synaptic transmission at glutamatergic terminals on direct and indirect striatofugal neurons is regulated. Such information could provide guidance in the development and design of pharmacoagents that could regulate receptor activity and glutamate release at synapses that impinge preferentially upon direct vs indirect pathway neurons.

To achieve this series of experiments, we used mGluR8 antibody with the D_1 receptor antibody. Because a specific D2 receptor antibodies from a non-rabbit species was not available, the rat anti-D1 dopamine receptor antibody was used to identify spines of direct pathway MSNs. D1-imunoreactive spines represented approximately 50% of total spine population in the striatum, ie the proportion of spines previously reported in studies that co-labeled striatal tissue with D1 or D2 antibodies (Hersch et al., 1995, Lei et al., 2004). Consequently we cautiously assumed that unlabeled spines in our material most likely belong to D2-containing indirect pathway neurons.

Our findings revelead that about 60% of mGluR8-positive terminals target D1-containing spines while the remaining 40% labeled boutons are in contact with unlabeled striatal elements. The fact that similar results were obtained using both the immunoperoxidase/immunogold or the double immunoperoxidase approach make us confident that mGluR8-containing glutamatergic terminals target preferentially direct striatofugal neurons in mice. This data further implies that even a slight increase in extracellular glutamate concentration, whether it is due to synaptic or non-synaptic

release, could activate this receptor and potentially cause an inhibition in striatal glutamatergic transmission. Because there may be more mGluR8 terminals on direct than indirect pathway neurons, it is reasonable to suggest that glutamatergic terminals on direct pathway neurons may be preferentially affected by slight changes in striatal glutamate release. Because mGluR8 shows the highest degree of sensitivity to glutamate relative to other mGluRs, the activation of mGluR8 should precede the stimulation of not only other group III mGluR, but also group I and II mGluRs. This fast activation of a pre-synaptic receptor may have a strong impact on consequent neurotransmission activity via inhibitory intracellular second messenger cascades.

The abundance of mGluR8 in the striatum is especially significant due to the ability to modulate the massive glutamatergic drive form the cerebral cortex and the thalamus. On the other hand, recently published data examining the role of group III mGluRs in the regulation of GABAergic and glutamatergic transmission in the striatum identified mGluR4 as the receptor largely responsible for this activity (Cuomo et al., 2009). Treatment with an mGluR8 agonist (*S*)-DCPG (1-3 μM) did not significantly affect the amplitude of evoked EPSC and IPSC in young rats. Although there is no clear explanation for the discrepancy between our anatomical data and those physiological studies, a few issues must be considered. First, the electrophysiology data were gathered from striatal slices of young rats (4-6 weeks), while we used adult mice. As previously reported, there may be significant changes in the level of protein expression and subcellular localization of mGluR subtypes during postnatal development (Hubert and Smith, 2004; Renden et al., 2005; Valenti et al., 2005). In the medial nucleus of the trapezoidal body (MNTB) group III mGluR activity seems to be developmentally

regulated during the postnatal period; inhibition due to activation of group III mGluRs at P5-7 is 92.22 ±1.07% (p<0.01), P8-10 is 88.50±1.14% (p<0.01), P12-14 is 60.68±8.79% (p<0.01), P16-18 is 26.35±6.76% (p<0.05) (Renden et al.,2005). Renden also reports that in the calyx of Held of young animals mGluR8 is localized on the extrasynaptic face of the calyx and mGluR4 in the synaptic lumen, which is surprising because group III mGluR localization in adult animals is thought to be in the active zone of presynaptic terminals. It seems that expression and localization of group III mGluRs in development is on an "as-needed basis" – i.e. the needs of a developing brain are different from that of an adult, thus mGluR expression and localization would be regulated to complement those needs and therefore might deviate from what has been found in adult brain.

Even in adult brain, mGluR8 expression and localization tends to be very sensitive to high glutamate levels and overall activity levels in a given circuit, resulting in internalization and reduced expression (Kral et al., 2003; Zhang et al., 2009). Increased glutamatergic transmission due to pilocarpine-induced status epilepticus can depresses mGluR8 expression from 60 minutes to 30 days following the seizure (Kral et al., 2003; Tang et al. 2001). More detailed analysis of mGluR8 function in adult mice needs to be conducted to supplement the anatomical evidence.

Based on its pharmacological properties and localization, mGluR8 could potentially play a significant role in the maintenance of glutamate homeostasis in the CNS. Due to its sensitivity and potential ability to respond to slight increases in ambient levels of glutamate, it is likely that this receptor has the capacity to restrain excitatory neurotransmission associated with both synaptic (Rodriguez-Moreno et al., 1998) and

non-synaptic release of glutamate. However, the functional capabilities of mGluR8 are not yet completely understood.

4. Conclusions and Future Directions

The striatum is the largest of all basal ganglia nuclei and serves as the main input hub for the excitatory projections originating in the cortex and thalamus and modulatory projections from the SN. Glutamatergic transmission in the striatum is tightly regulated by strategically expressed glutamatergic receptors. Having a clear understanding of the distribution and subcellular localization of receptors provides researchers a map with potential functional relevance. Using specific antibodies against mGluR4, mGluR7, and mGluR8 we examined the distribution, subcellular localization, preferred input source and output innervation for these receptors. Several key findings are presented in this thesis. First, group III mGluRs are preferentially locate on presynaptic elements in the mouse striatum. Second, group III mGluRs show significant degree of colocalization with the vGluT1 and vGluT2. vGluT1 and vGluT2, surprisingly, show a significant degree of colocalization, which prevented us from identifying mGluR-positive terminals as corticostriatal or thalamostriatal. Further examination, possibly including tracing studies, identifying the thalamostriatal projection and assessing group III mGluR localization on these terminals will need to be conducted. However the data still implies that group III mGluRs are strategically located to regulate glutamatergic transmission from both the cortex and the thalamus. Third, mGluR4 and mGluR7 show a significant degree of colocalization in terminals forming asymmetric synapses. Finally, group III mGluRs have

a differential preference for the D1-positive and D1-negative spines. Together, these findings pave the way for novel avenues of study of group III mGluRs in the CNS.

4.1. Group III mGluRs preferentially localize on presynaptic elements in mouse striatum.

Group III mGluRs may modulate glutamatergic transmission in slices of rodent striatum (Gubellini et al., 2004; Pisani et al., 1997). The group III mGluR agonist, L-SOP (10 μM) significantly reduced the amplitude of stimulation-evoked EPSPs at corticostriatal synapses in a reversible manner without modifying the postsynaptic sensitivity to glutamate (Pisani et al., 1997). L-AP4, another group III receptor agonist reversibly and dose-dependently decreased the amplitude of corticostriatal EPSPs (Calabresi et al., 1993; Calabresi et al., 1996; Pisani et al., 1997). The mGluR subtypes involved in these effects are not fully characterized, so these effects can be mediated by any of the three group III mGluRs. Evidence for group III mGluR localization in the striatum was further provided by qualitative analyses of protein and mRNA. Strong mGluR7 hybridization signals and immunolabeling are found in presynaptic elements of the striatum (Kosinski et al., 1999). Decortication experiment showed a decrease (66.2%) of striatal mGluR7 immunoreactivity and colocalization with SV2, a presynaptic terminal marker (Kosinski et al., 1999), thus showing that cortical projections are a major source of presynaptic striatal mGluR7. In other species, glutamatergic terminals in the striatum can be identified as either originating in cortex or thalamus based on the expression of vGluT1 or vGluT2, respectively. vGluT1 and vGluT2 have previously been shown to be completely segregated in glutamatergic terminals in the striatum. (Fremeau et al., 2004b;

Herzog et al., 2006, Raju et al., 2006). Our data showed greater group III mGluR colocalization with vGluT1 than vGluT2, suggesting that group III mGluRs have a preferential modulatory influence on the vGluT1-positive terminals than vGluT2 positive terminals. Our data also presented us with an unexpected finding of colocalization of vGluT1 and vGluT2 in individual glutamatergic terminals in the striatum. Until now if has been assumed that vGluT1 and GluT2 were completely segregated in striatal terminals forming asymmetric synapses, but our data deviated form previously reported localization of vGluTs in rat and monkey striatum. The presence of colocalization prevents us from confidently identifying the source of these mGluR-positive glutamatergic terminals, however the data still demonstrates that group III mGluRs are located on glutamatergic terminals from both cortex and thalamus and these mGluRs can mediate glutamatergic transmission via a presynaptic mechanism.

Group III mGluRs localized on thalamostriatal terminals may modulate the activity of that pathway. The thalamus provides a major source of glutamatergic afferents to the striatum, but the role and modulatory functions of group III mGluRs upon this system remain poorly understood (Smith et al., 2004). A small number of studies demonstrate group III mGluR mRNA expression in the rodent thalamus (Corti et al., 1998; Kinoshita et al., 1998; Ohishi et al., 1995; Saugstad et al., 1997; Tanabe et al., 1993). Our findings demonstrate that vGluT2-containing terminals express the three main subtypes of group III mGluRs. Thus group III mGluRs possess ideal localization to modulate glutamatergic transmission in the striatum from its two major input sources. Though, a more significant degree of vGluT1/vGluT2 co-localization was found in individual glutamatergic terminals in the mouse striatum than in other species (Fremeau

et al., 2004b; Herzog et al., 2006); (Raju et al., 2006). The vGluT1-vGluT2 colocalization does not alter the conclusion that group III mGluRs have the ability to regulate both the cortico- and the thalamo-striatal pathways. This hypothesis is further supported by the expression of group III mGluRs in the more than half of striatal axo-dendritic terminals. Axodendritic striatal terminals have been shown to originate in the parafascicular nucleus of the thalamus (Hersch et al., 1995; Sadikot et al., 1992; Sidibe and Smith, 1996; Smith and Bolam, 1990; Smith et al., 1994). Unfortunately, so far no studies have assessed group III mGluR localization in the CM/Pf. Future studies that directly measure group III mGluR expression in the CM/Pf nucleus of the thalamus would need to be conducted.

4.2. Co-localization of Group III mGluRs in Glutamatergic Terminals

Our data show a significant degree of co-localization between group III mGluRs in individual glutamatergic terminals. Presence of multiple group III mGluRs in the active zones of synaptic terminals could provide a modulation of synapses across many levels of transmitter concentration. Group III mGluR mRNA and protein expression has been shown to overlap in many brain nuclei, such as olfactory tubercle, superficial layers of the entorhinal cortex, CA4, septofimbrial nucleus, intercalated nuclei of the amygdala, medial mammillary nucleus, many thalamic nuclei, the retina, and pontine nuclei. Additionally, intense expression of both mGluR4 mRNA and mGluR7 mRNA was detected in the trigeminal ganglion and dorsal root ganglia (Corti et al., 1998; Ohishi et al., 1995; Quraishi et al., 2007; Shigemoto et al., 1997). There is evidence that both mGluR4 and mGluR8 modulate excitatory transmission in the SNc (Valenti et al., 2005). A comparison of [^{3}H]L-AP4 autoradiography between wild-type and mGluR4 knockout

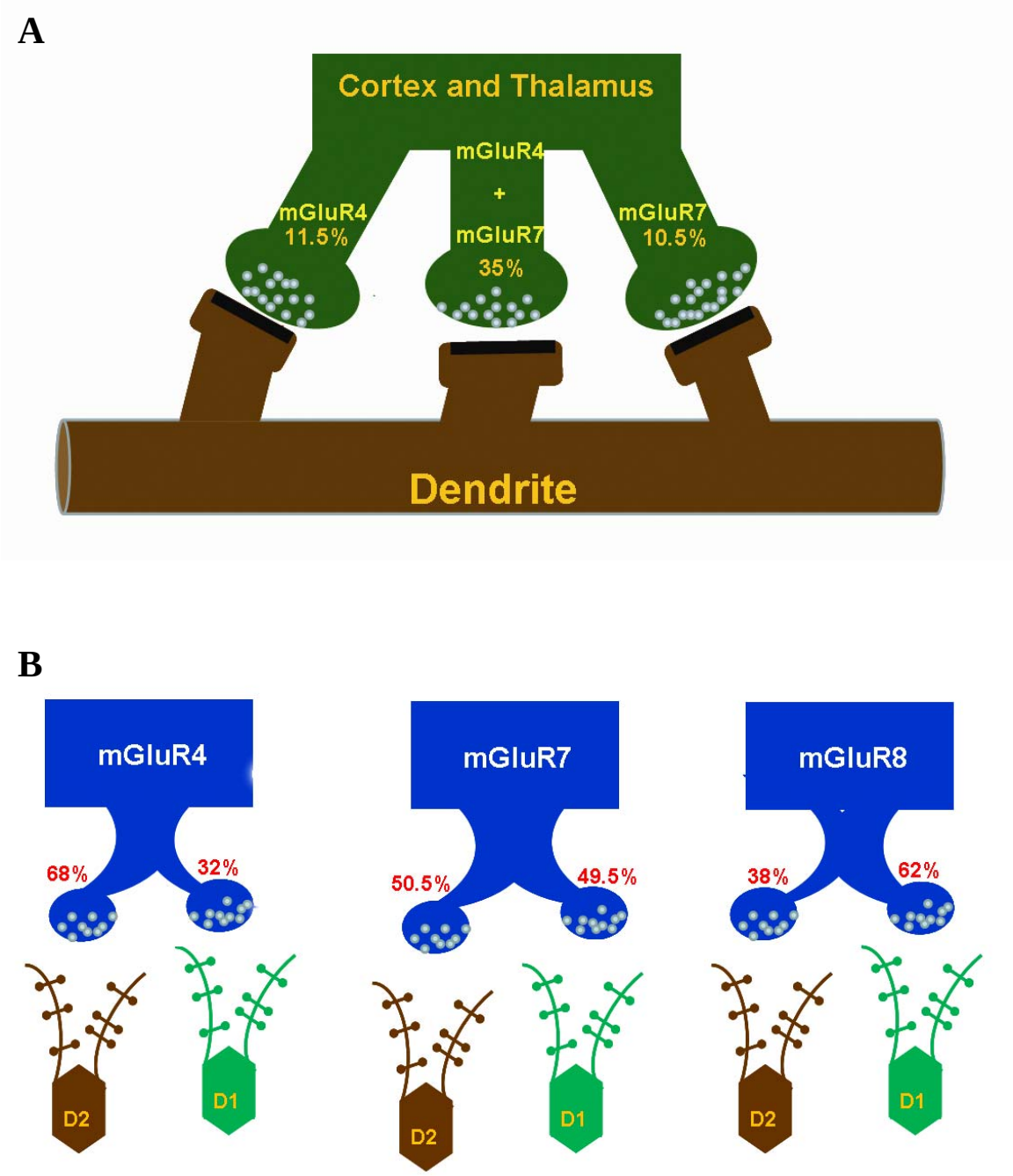


Figure 4.1: Summary figure showing mGluR4 and mGluR7 presynaptically localized on terminals from the corticostriatal and thalamostriatal projections. A: mGluR4 and mGluR7 colocalize at individual glutamatergic terminals. B: Group III mGluRs differentially innervate the direct and indirect striatal output pathways.

mice revealed significant specific binding remaining in the cerebral cortex, caudate putamen, and globus pallidus of mGluR4-deficient mice (Corti et al., 2002). This high-affinity specific binding is very likely due to the presence of mGluR8 and not mGluR7.

This is due to difference in affinities between the receptors and the antagonist: mGluR8 binds to L-AP4 with the similar affinity as mgluR4 and mGluR7 binds to L-AP4 with a much lower affinity than the other group III mGluRs (Thomsen and Hampson, 1999). Lastly, mGluR7 and mGluR8 are co-expressed in GABAergic boutons in rodent stratum oriens/alveus (Ferraguti et al., 2005) To avoid activation of multiple types of group III mGluRs researchers utilize the different receptor sensitivities and dose-dependent pharmaco-agent application to selectively affect the group III receptors (Conn and Pin, 1997; Kogo et al., 2004; Marino et al., 2003b; Pelkey et al., 2007). In addition to receptor sensitivity, receptor activity at the synapse is dependent on the rate and probability of endocytosis and internalization. In rodent colonic segments mGluR8 showed a 40% increase in cytoplasmic localization after stimulation with an agonist (Tong and Kirchgessner, 2003). Unlike mGluR8, mGluR4 internalization and desensitization is agonist-independent unless pathways leading to the activation of PKC are induced (Mathiesen and Ramirez, 2006). AMN082, an mGluR7 allosteric agonist, increased receptor internalization to 291 ± 30% of control levels in hippocampal neurons overexpressing mGluR7 (Pelkey et al., 2007). Considering the diverse sensitivity to glutamate and various pharmacologic agents and great variability in internalization patterns, receptor colocalization in individual synapses add a layer of complexity to group III mGluR function.

4.3. Group III mGluRs have a differential preference for the D1-positive and D1-negative spines.

Previously published studies have shown group III mGluRs in the gluatamatergic projections to the striatum, however until now there has not been any information about the selectivity of group III mGluR-positive terminals for the direct or indirect pathway. The rat anti-D_1 receptor antibody was used to identify the spines of the MSNs forming the direct pathway. The D_1 immunonegative spines were assumed to belong to the MSN's forming the indirect striatal output pathway. The D_1 and D_2 receptor are thought to be segregated onto the MSNs in the direct and indirect output pathways and expressed in about a 50/50 ratio in the striatum. Since the D_1 antibody labeled about 50% of spines, a reasonable assumption was made that the unlabeled spines were probably those of the MSNs positive for the D_2 receptor and projecting in the indirect pathway. The data from our experiments suggests that group III mGluRs may modulate the corticostriatal transmission at both the D_1- and D_2-receptor positive output pathways. However while mGluR7 has no significant preference for the D1-positive or D1-negative MSN, mGluR4 seems to preferentially target the D1-negative postsynaptic elements and mGluR8-positive terminals preferentially target the D1-positive spines.

4.4. Technical Limitations

To address the aims of this study, several anatomic techniques were utilized. Each technique has its benefits and limitations, however when skillfully manipulated and properly analyzed the limitations of each technique can be significantly reduced.

4.4.1. Light Microscopy(LM):

LM is a useful method to determine the gross distribution of immunoreactive (IR) elements. It allows for basic observation of IR elements. One could detect whether the labeling has targeted the neuropil or the cell bodies, but to get any more specific information about the subcellular and sybsynaptic localization EM studies must be conducted.

4.4.2. Pre-Embedding Immunoperoxidase:

The pre-embedding immunoperoxidase method is a highly sensitive technique that allows for qualitative and quantitative analysis of subcellular localization of receptors. It can be used to determine the distribution of immunoreactivity among the various neuronal elements and glia. Because the DAB product forms a diffuse deposit, in most cases it can not be used to determine the exact location of the antigen; other methods like pre- and post-embedding immunogold are used to determine the specific subsynaptic localization of receptors. However in the case of group III mGluRs, the DAB product was often deposited right along the active zone of a terminal, thus displaying a limited localization area of localization for these receptors. Such pattern for group III mGluR labeling has been previously illustrated in hippocampus (Bradley et al., 1996;

Bradley et al., 1998; Bradley et al., 1999; Corti et al., 2002; Ferraguti et al., 2005; Shigemoto et al., 1997).

4.4.3. Pre-Embedding Immunogold: This technique is not as sensitive as pre-embedding immunoperoxidase, but because it identifies the IR target with a small black particle it can help determine the exact subcellular and subsynaptic localization of the antigen, allowing for assessment of receptors as intracellular versus plasma-membrane bound, and this technique can also provide the exact location of the receptor on the plasma membrane: intrasynaptic, parasynaptic, or extrasynaptic. Altough these criteria were not used for the analysis, localization of group III mGluRs in the terminals and the active zone confirmed previously reported subsynaptic localization of group III mGluRs. The immunogold particle does not penetrate the tissue as well as the immunoperoxidase label and can be responsible for a greater degree of false negative data during analysis.

4.4.4. Double labeling using Pre-Embedding Immunoperoxidase and Immunogold:

This is a more advanced immunohistochemical method and it can help identify two different antigens for EM analysis. To ensure adequate access of the antibodies to the antigens, the tissue is incubated with both the primary antibodies simultaneously, following with the incubation of both secondary antibodies. To prevent cross reactivity the antibodies have to be from different species. The immunoperoxidase DAB-revelation step follows the immunogold silver-amplification process to ensure proper access of the silver agents to the gold particles.

A limitation of the double immunoperoxidase and immunogold techniques is the penetration of the antibodies and thus availability of the IR signal throughout the thickness of the tissue. This difference in accessibility of targets within the tissue could result in false negative data during the analysis of double labeled material. To overcome this limitation, data was carefully and consistently sampled from tissue regions that displayed both legible ultrastructure and good immunoreaction component. Additionally, the experiment that addressed mGluR preference for D1-positive spines was designed to label the mGluR-positive terminals with immunogold and D1-positive spines with DAB. Therefore when postsynaptic targets of immunogold labeled terminals were examined it was assumed that the likelihood of false-negative data was low since DAB is the more sensitive method and would have adequate access to the postsynaptic target.

4.4.5. Concluding remarks

The results presented in this thesis show the location of group III mGluRs in the mouse striatum. These findings provide a framework for understanding the role that group III mGluRs could play in striatal glutamatergic transmission. Group III mGluR (4, 7, and 8) are preferentially located on presynaptic elements in the striatum (Fig.4.1A). About half of all terminals forming asymmetric synapses contain group III mGluR immunoreativity. These glutamatergic terminals originate from the cortico- and thalamostriatal projections. Surprisingly, terminals forming asymmetric synapses showed a significant degree of colocalization between vGluT1 and vGluT2. Previously reported localization studies of vGluT1 and vGluT2 in rat and monkey showed a very slight colocalization between these transporters, suggesting that the two vGluTs are almost

completely segregated into cortico- and thalamo-striatal projections. This segregation of vGluTs might be species specific or mouse strain specific. The functional relevance of this finding should be further investigated with electrophysiological and microdialysis experiments.

Group III mGluRs showed a differential preference for the innervation of D1-positive and D1-negative spines (Fig.4.1B). This data suggests that group III mGluRs could exhibit a differential preference for the direct and indirect striatal output pathways. The direct pathway seems to have a greater influence from mGluR8, the indirect from mGluR4, and mGluR7 displaying no preference between the two output pathways. Due to variability of these receptors in sensitivity to glutamate and pharmacoagents this pattern of expression could be utilized in development of treatments for diseases with a dysfunction between the direct and indirect pathways, such as Parkinson's disease.

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