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The Role of Type III-Nrg1 signaling in excitatory transmission in the nucleus accumbens

A Dissertation Presented

by

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Abstract of the Dissertation

The Role of Type III-Nrg1 signaling in excitatory transmission

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Nrg1-ErbB4 signaling is important for the maintenance of synaptic plasticity, and the fine-tuning of GABAergic, dopaminergic, cholinergic and glutamatergic neurotransmission. The Type III-isoforms of Nrg1 are predominantly targeted to dendrites and axons, and are expressed by the principal neurons of the hippocampus, cortex and amygdala. These neurons all send glutamatergic inputs into the nucleus accumbens, which expresses the Nrg1 receptor ErbB4. Integration of the information encoded by these Nrg1-expressing inputs, together with midbrain dopamine inputs is crucial for normal functioning of the accumbens. Based on the expression pattern of Type III-Nrg1 and its receptor, ErbB4, I propose that presynaptic Type III-Nrg1/postsynaptic ErbB4 signaling may play an important role in modulating excitatory transmission within accumbens circuits.

Type III-Nrg1 heterozygous mice display (i) deficient functional connectivity between the ventral hippocampus and the nucleus accumbens, and (ii) alterations in behaviors in which the ventral hippocampus-accumbens circuit plays a role. Based on these findings, I hypothesize that

Type III-Nrg1/ErbB4 signaling is required for the appropriate establishment and maturation of glutamatergic transmission within accumbens circuits.

Here, I shed light on the distinct accumbens neuro-circuitry that participates in Nrg1/ErbB4 signaling, by defining the specific accumbens neuronal population that expresses ErbB4. We investigated the pattern of expression of ErbB4 in the accumbens during critical stages of development: at perinatal ages (P0-P3), during the ages when accumbens neurons undergo maturation (adolescence, P20-P23) and in adulthood (>P60). I also investigated the role of Nrg1 in the regulation of accumbens glutamatergic transmission by delineating the glutamate receptor profile of the accumbens. My major findings are that:

a. ErbB4 expression is widespread in perinatal accumbens but becomes limited to few cells in adults,
b. The subcellular distribution of ErbB4 changes during maturation from somatic at birth, to dendritic and post synaptic compartments,
c. Type III-Nrg1 genotype, but not ErbB4 genotype, modulates the flop vs flip exon usage specificity of GluA1 transcripts during maturation and
d. The alternative splicing factor Rbfox1 is selectively down-regulated in the accumbens of Type III-Nrg1 heterozygous mice.

These findings provide evidence that deficient Nrg1 signaling alters maturational changes in the accumbens. Such alterations are likely to influence the establishment and maturation of glutamatergic transmission in the accumbens.

Dedication Page

To my dearest friend and wife

Maryann Obiorah

For sharing in all the joys and pain of science, for providing the push when my 'battery' needed to recharge. You, darling, are my ever-present evidence that I am blessed!

To my family: dad (Owelle), mum (Eziafa), Okey, Arinze, Chigozie, Ify, Chukwuma Onyinye, Oge, Ekene, Ego, Chika, Nnamdi, Chidinma my baby, Ezinne, etc.

For your unwavering support for me, and for believing in me, perhaps, more than I believe in myself; I love you dearly!

To my uncles

Ken Obi Ebonwu & Emmanuel Ofulu Ndupu

For being very present as catalysts during the rate-limiting steps of my life, in my quest to start carving a niche for myself

To my teachers and mentors

David A. Talmage, Lorna W. Role, Quinn Vega

For teaching me how to answer the questions science throws my way, by allowing me to study how you answer the questions science throws at you. I hope I can make you proud when you look back tomorrow

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List of Abbreviations

Nrg1	Neuregulin 1
ErbB	v-erb-b erythroblastic leukaemia viral oncogene homolog
MSN	Gabaergic striatal medium spiny neurons
D1/D2	Dopamine receptor 1/2
PI3K	Phosphoinositide 3-kinase
nα7ChR	α7 nicotinic acetylcholine receptor
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
NAcc	nucleus accumbens
GPi	internal segment of globus pallidus
GPe	external segment of globus pallidus
STN	subthalamic nucleus
SNr	substantia nigra pars reticulata
SNc	substantia nigra pars compacta
VTA	ventral tegmental area
EPSC	Excitatory postsynaptic currents
LGE	Lateral ganaglionic eminence
MGE	Medial ganglionic eminence

Chapter 1

INTRODUCTION

The nucleus accumbens (NAcc) entered the limelight in the early 1950s when renowned psychologist James Olds showed that rats greatly relished the arousal obtained from electrically stimulating their NAcc – they would rather self-stimulate themselves as much as 2000 times per hour instead of going towards food, even after being starved (Olds and Miller, 1954; Olds J, 1958). Olds went on to propose that this could allow for the discovery of “one drug that will raise or lower thresholds in the hunger system, another for the sex-drive system, and so forth” (Olds J, 1956). From then on research into the physiology of ‘the pleasure center’ of the brain gained prominent interests and has led to the discovery of the role of the NAcc in the modulation of motivation, working memory and sensory gating (Carlezon and Thomas, 2009; Chen et al, 2008; Goto and Grace, 2008; Groenewegen H, 2007; Lenz and Lobo, 2013). Consequently, the NAcc have been implicated in various brain disorders such as schizophrenia, Tourette’s syndrome, obsessive-compulsive disorder and attention deficit hyperactivity disorder. More recently, studies on the NAcc have focused on elucidating how the NAcc functions on a molecular level, with a view to defining specific molecular cues that could be targeted for treatment of such disorders.

I have divided this introductory chapter into three parts. In the first part, the NAcc will be described in more detail, with particular attention paid to its anatomy, origin, organization, physiology and function. In the second part, I will introduce Neuregulin1 (Nrg1), one of the most promising schizophrenia susceptibility genes; its signaling and diverse functions in the brain. The last part of this chapter will describe various evidence that support a role for the Type III

isoform of Nrg1 in the normal functioning of the NAcc, thereby providing a rational for this study.

The Nucleus Accumbens

Location and anatomy of the nucleus accumbens

The nucleus accumbens (NAcc) occupies the ventral striatum and is located in the caudal base of the coronal extension of the lateral ventricle in the brain (Figure 1.1). It is indistinguishable from the dorsal striatum under a light microscope, but can be distinguished morphologically by its more tightly packed neurons (Haber and McFarland, 1999), and by the expression of a wider array of neurotransmitters and neuropeptides (Groenewegen H, 2007). In addition, even though they both have similar afferent innervation, the nucleus accumbens has a higher density of limbic-related afferent innervation than the dorsal striatum (Britt et al, 2012; Donoghue and Herkenham, 1986; Kitai et al, 1976). However, there is no clearly defined border between the NAcc and the dorsal striatum.

The NAcc is made up of “core” and “shell” regions, first named in the early 1980s. The core region has multiple dense clusters of cells within myelin-poor regions which encircle the anterior commissure (Herkenham et al, 1984), whereas the outer shell has a unique pattern of expression of tyrosine hydroxylase (Zahm D, 1992), more loosely-packed ensemble of cells and a high expression of acetylcholinesterase, calbindin-28 and cholecystokinin compared to the core NAcc or other regions of the rostral forebrain (Zaborsky et al, 1985, Meredith et al, 1996).

Similar to the dorsal striatum, two distinct compartments can also be found in the nucleus accumbens: the patch (also called striosome) and matrix compartments. Both compartments are innervated by different regions of the cortex (Chuhma et al, 2011; Donoghue and Herkenham,

1986; Gerfen C, 1992; Roberts et al, 2005), both have different neuronal birth dates (Crittenden and Graybiel, 2011; Song and Harlan, 1994; van der Kooy and Fishell, 1987), and both have different gene expression patterns (Johnston et al, 1990; Lenz and Lobo, 2013).

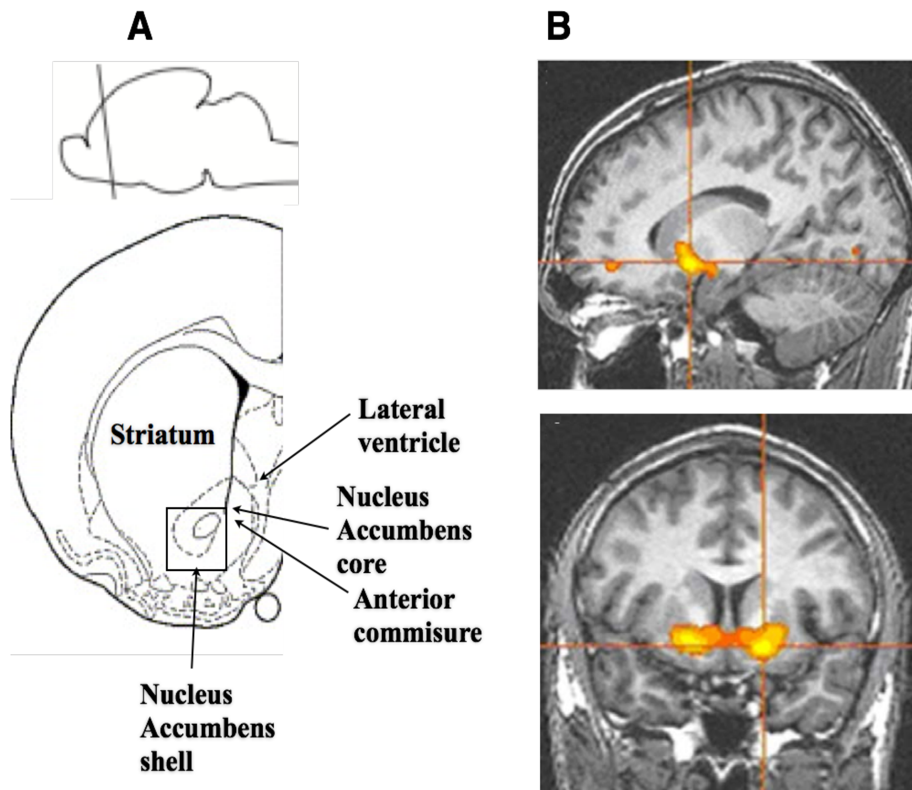

Figure 1.1. Location of the nucleus accumbens. The nucleus accumbens is located in the ventral striatum. **A. Top left picture** shows position on the brain where the coronal slice was obtained from the mouse brain. **Bottom left picture** shows half hemisphere of coronal slice, showing position of the striatum, lateral ventricle, nucleus accumbens core and shell regions. Boxed area is an estimate of the region I isolated from animals for my experiments. **B.** Picture on the right shows the location of the accumbens in human brain. Adapted from Paxinos et al, 2007; Knutson, et al, 2001).

Again, as with the dorsal striatum, 95% of NAcc neurons are GABAergic medium spiny neurons (MSNs), which express dopamine D1 or dopamine D2 receptor or both (in < 2% of MSNs), DARPP-32, and GAD-65 and/or GAD-67 (Figure 1.2). The D1 receptor-expressing MSNs co-express Substance P, whereas D2-positive MSNs co-express enkephalin. The other 5% of the accumbens neuronal population are interneurons, identified by the expression of specific protein markers as cholinergic interneurons, parvalbumin-, somatostatin- and calretinin-positive GABAergic interneurons (Kreitzer A, 2009; Matamales et al, 2009).

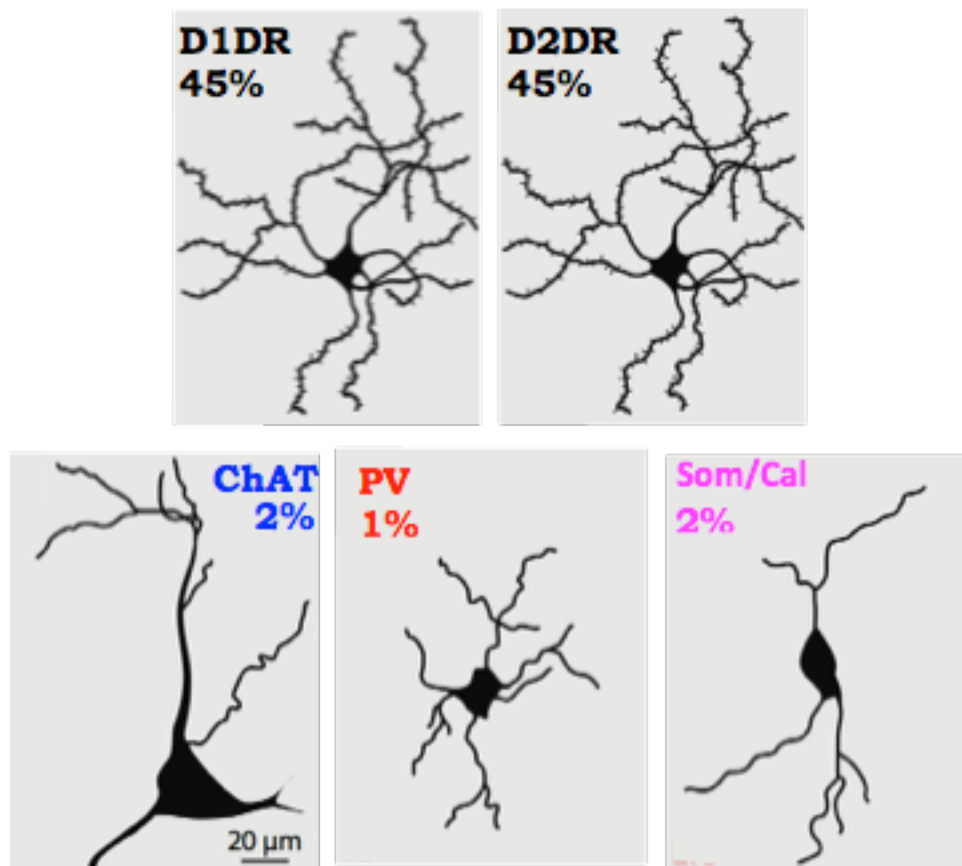

Figure 1.2 Neuronal population of the striatum. Adapted from Kreitzer A. C. *Ann. Rev. Neurosci.* **32(1):** 129. **(Top left)** dopamine D1 receptor-positive medium spiny neuron. **(Top right)** dopamine D2 receptor-positive medium spiny neuron. **(Bottom left)** Cholinergic

interneuron. **(Bottom middle)** Parvalbumin-positive interneuron. **(Bottom right)** Somatostatin/Calretinin-positive interneuron.

Embryonic and postnatal development of accumbens neurons

Striatal neurons are derived from the basal telencephalon

The birthplace of neurons usually differs from where they eventually reside. Therefore, the specific and accurate positioning of neurons and fibers requires a critical and precise regulation of birthdate, migration and maturation. In the developing brain, neurons that eventually reside in the striatum are born in the periventricular germinal regions of the telencephalon. There, neuroblasts become postmitotic and migrate towards the striatum by outward (radial), tangential or inward migration (Figure 1.3).

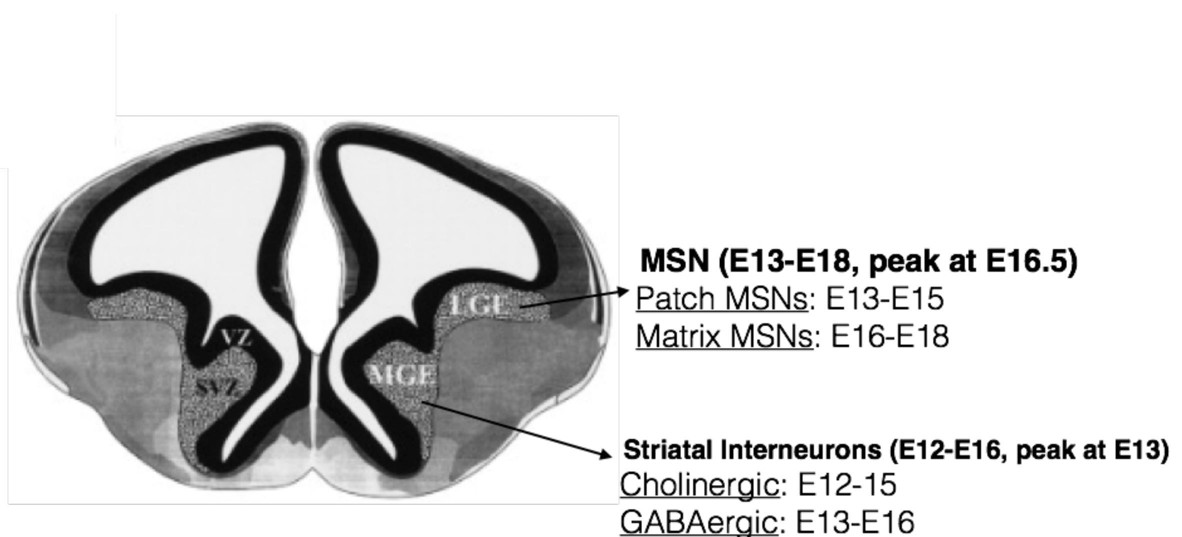

Figure 1.3. Schematic of a coronal section of E15.5 telencephalon, showing the origin of accumbens neurons. Adapted from Olsson M. *Neurosci.* **84(3):** 868. **MGE-derived** neurons give rise to striatal interneurons, while **LGE-derived** neurons give rise to striatal projection neurons (MSNs).

A subset of neuroblasts are born in the medial ganglionic eminence of the basal telencephalon between E12 and E15 (peak at E13) in rats, migrate tangentially to the developing striatum and differentiate into striatal interneurons - cholinergic-, parvalbumin- or calretinin-positive interneurons (Marin et al, 2000; Phelps et al, 1989). Striatal MSNs are born in the lateral ganglionic eminence between E13 and E18 (peak at E16.5) in rats, migrate into the striatum and differentiate into DARPP-32-positive medium spiny neurons (Hamasaki et al, 2001; Hamasaki et al, 2003; Marin and Rubeinstein, 2001; Song and Harlan, 1994; Wichterle et al, 2001).

Among neuroblasts that differentiate into striatal neurons, there is evidence that the birthdate of each subclass determines the location within the striatum that the neurons eventually occupy. In rats, early born MSNs (E13) localize preferentially in striosome compartments (patch), while the late born MSNs (E16-E18) are preferentially located in the matrix (Johnston et al, 1990; Olsson et al, 1998; van Vulpen van der Kooy, 1998). Populations of MSNs segregate into clusters which have specific afferent and efferent connections, thereby defining the organization of the patch or matrix compartments (Chuhma et al, 2011; Gerfen C, 1984; Gerfen C, 1985; Gerfen C, 1992; Kawaguchi et al, 1989). Cholinergic interneurons are the first to become post-mitotic and are thus the earliest born striatal neurons (Bayer S, 1984; Phelps et al, 1989; Sember and Fibiger, 1988). The GABAergic interneurons (parvalbumin, somatostatin and calretinin) are born later than most cholinergic interneurons but earlier than MSNs (Rymar et al, 2004; Sadikot and Sasseville, 2004).

Organization of inputs into the nucleus accumbens

Precise activation of accumbens inputs elicits distinct responses

The NAcc is a central site of convergence of information from limbic and cortical regions of the brain. It is innervated by glutamatergic inputs from the ventral hippocampus (vHIPP), basolateral amygdala (BLA), prefrontal cortex (PFC), ventral tegmental area and thalamus (Fiedman et al, 2002; Phillipson and Griffiths, 1985; Figure 1.4). vHIPP stimulation can depolarize accumbens neurons and consequently allow PFC inputs to generate an action potential in accumbens neurons (O'Donnell and Grace, 1995). Thus, precise activation of specific inputs into the accumbens produces distinct physiological and behavioral effects and also generates different outputs within accumbens (Goto and Grace, 2008).

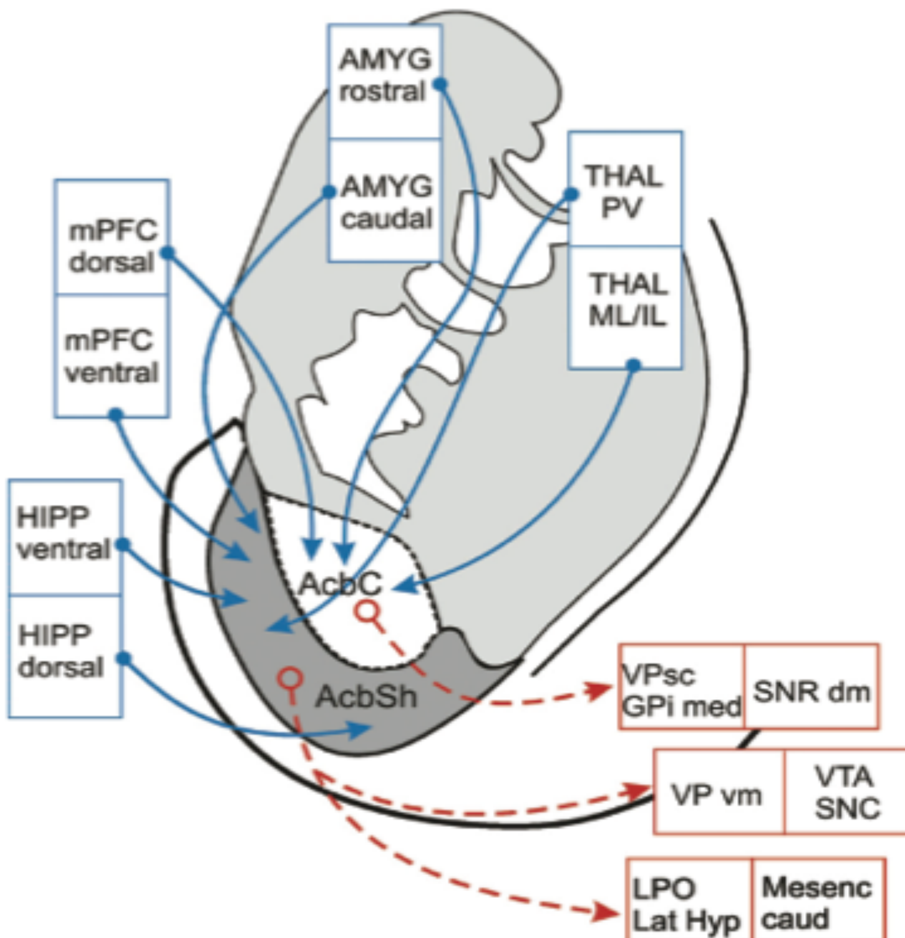

Figure 1.4. Illustration of NAcc circuitry, with emphasis on inputs. Direction of inputs in drawing corresponds to the approximate region where inputs target within the accumbens. The vHIPP preferentially target the accumbens shell, dorsal PFC targets the core whereas inputs from more ventral PFC neurons target the shell. Similarly, the inputs from more rostral parts of the amygdala target the core whereas inputs from caudal parts preferentially target the shell. Inputs from the paraventricular thalamic nucleus preferentially project to the shell whereas inputs from midline and intralaminar thalamic nuclei preferentially project to accumbens core regions. Adapted from Groenewegen H. J. *CNS Spectr.* **12(12):**889.

vHIPP projections are the major inputs into the accumbens shell

The input-specific response of accumbens neurons suggests the possibility of different innervation patterns of presynaptic projections into the accumbens. Britt *et al* recently showed that there are twice as many vHIPP inputs into the accumbens shell than BLA or PFC inputs, and that retrograde tracing of accumbens inputs showed that more vHIPP neurons project to the accumbens shell. They also showed that evoked excitatory postsynaptic currents recorded in MSNs after optical stimulation of vHIPP inputs had the largest amplitudes, whereas PFC inputs evoked the smallest amplitudes. There is evidence also that EPSCs evoked by vHIPP inputs into the striatum were weaker onto D2 MSNs compared to D1 MSNs, suggesting that vHIPP inputs to the NAcc preferentially formed synapses onto D1 MSNs. PFC and thalamic inputs formed synpases onto D1- and D2-MSNs equally (MacAskill et al, 2012).

Within the striatum, cholinergic interneurons receive inputs from MSNs and other cholinergic interneurons and in turn synapse onto MSNs and cholinergic interneurons (Chuhma et al, 2011). GABAergic interneurons (parvalbumin-, calretinin-, somatostatin-positive

interneurons) receive inputs from other interneurons and synapse directly onto MSNs. MSNs synapse directly onto other MSNs and cholinergic interneurons and are the projection neurons of the striatum.

Organization of nucleus accumbens output projections

MSNs are divided into two groups on the basis of their output targets: striatonigral and striatopallidal MSNs. Striatonigral MSNs send output to the ventral pallidum, substantia nigra, VTA and internal globus pallidus, whereas striatopallidal MSNs project to the external globus pallidus and subthalamic nucleus (Groenewegen et al, 1999; Groenewegen H, 2007; Zahm D, 1999). Striatonigral MSNs express dopamine D1-receptor, substance P and dynorphin while striatopallidal MSNs express dopamine D2-receptor and enkephalin (Lobo et al, 2006).

On the basis of their projections through basal ganglia nuclei, MSNs can also be divided into two classes: direct and indirect pathway MSNs (Figure 1.5). Direct pathway MSNs are the striatonigral, D1-positive MSNs, and are involved in reinforcement, reward seeking and initiation of movement. Indirect pathway MSNs are the striatopallidal D2-positive MSNs and are implicated in pathways that antagonize the direct pathway such as promoting punishment, inhibiting reward seeking, inhibition of movement, etc (Bolam et al, 2000; Gerfen C, 1984). Thus, the direct and indirect MSNs produce opposing effects to maintain balance in behavior.

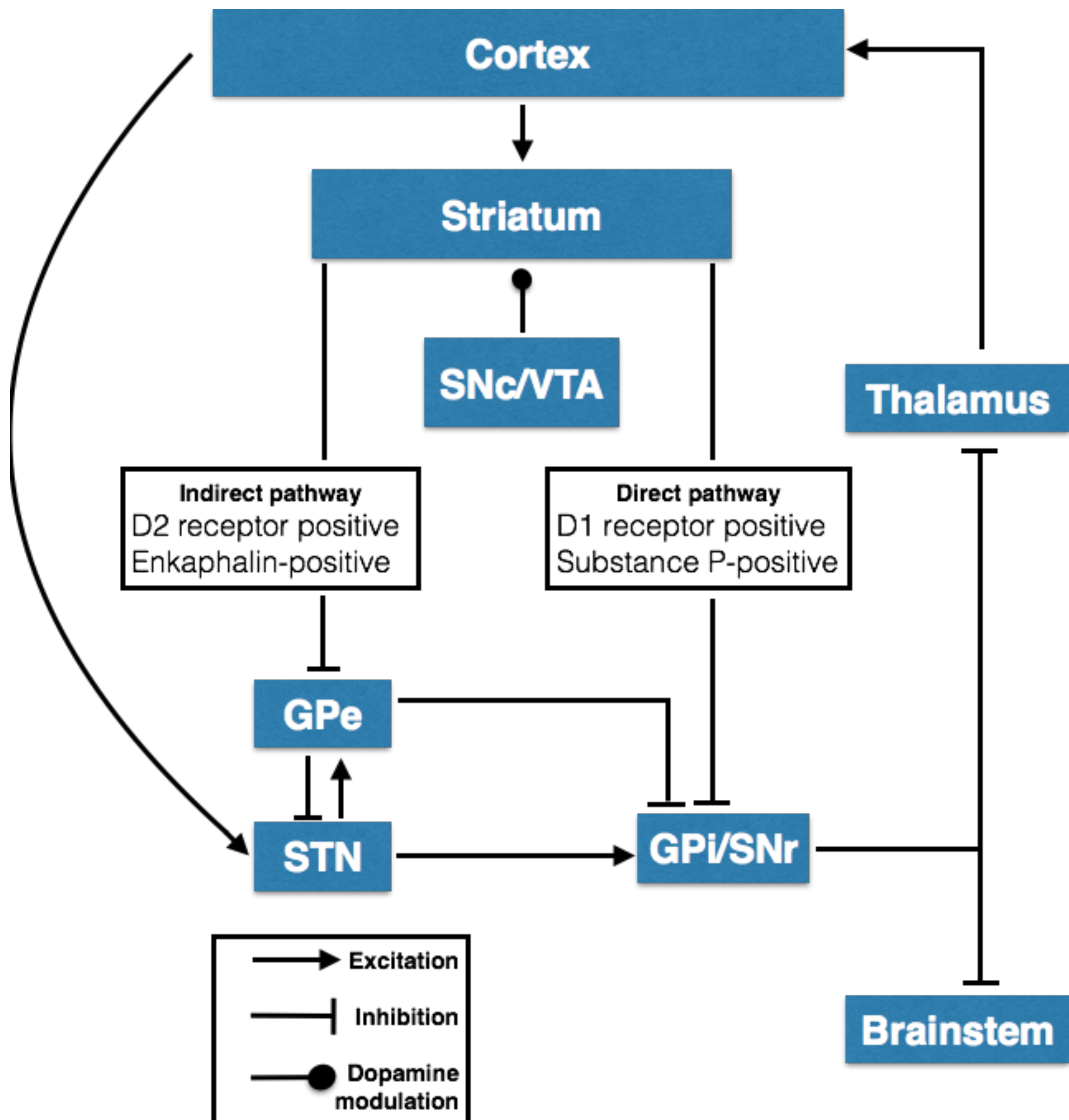

Figure 1.5. Direct vs indirect pathway NAcc output, taken from Yin H. H. *et al. Nat. Neurosci.* **7**:465. Direct pathway MSNs project to the thalamus via the internal globus pallidus (GPi). Indirect pathway project to the thalamus via the external globus pallidus, subthalamic nucleus and the and GPi.

Nucleus accumbens function and behavior

Before the development of optogenetic tools, the predominant view about behaviors mediated by the accumbens was motivation. MSNs have been implicated in drug-related behaviors; D2-MSNs have been shown to be involved in inhibition of drug reward (Durieux et al, 2009), whereas, D1-MSNs seem to enhance drug reward (Lobo et al, 2010), and are associated with a change in activity within presynaptic regions that innervate the accumbens (Thanos et al, 2013). Accumbens cholinergic interneurons have also been shown to enhance the role the inhibition of drug reward, thereby suggesting that they may preferentially synapse with D2-MSNs (Kravitz and Kreitzer, 2012; Lobo et al, 2010). It is not known if any of these behaviors are mediated directly by the accumbens core or shell.

Glutamatergic projections into the accumbens carry a diverse array of information, including signals with emotional content (amygdala), executive information (prefrontal cortex), information about arousal state (thalamus) and contextual information about novelty vs prior experience (ventral hippocampus). The accumbens also integrates dopaminergic signals with motivational significance. Accumbens MSNs project to brain regions that mediate a wide range of basic functions such as feeding behaviors (hypothalamus), motivational behaviors (dopaminergic neurons of the ventral tegmental area and the substantia nigra), movement behaviors (caudal mesencephalon) and executive functions (through the thalamic inputs to the prefrontal cortex). Thus, studies in animals have implicated the accumbens in modulation of behaviors such as feeding behaviors, learning and memory, and motivational behaviors (Cardinal et al, 2002; Kelley A, 1999; Kelley A, 2004; Maldonado-Irizarry et al, 1995; Reynolds and Berridge, 2002; Reynolds and Berridge, 2003).

Glutamatergic transmission and AMPA receptors

Glutamate is the main excitatory neurotransmitter in the CNS and binds to glutamate-gated channels to mediate neuronal excitability, formation of neuronal circuits, excitatory transmission and synaptic plasticity. AMPA receptors account for the primary depolarization during glutamate-mediated neurotransmission and are the main transducers of fast excitatory transmission in the central nervous system. Within the context of accumbens circuits, glutamatergic transmission involves the release of glutamate from presynaptic vesicles in presynaptic axons and the binding to glutamate receptors on accumbens neurons, resulting in synaptic plasticity and synaptic signaling (Palmer et al, 2005).

AMPA Channels

AMPA channels are assembled from four subunits, GluA1-GluA4, which are products of four different genes. AMPA receptor subunits combine as homotetramers or symmetric dimer of dimers to form a functional AMPA receptor. In addition to variation in subunit composition, alternative splicing of primary transcripts and RNA editing results in additional levels of variations of AMPA receptor subunits. Thus, different glutamatergic synapses could employ different tetramer combinations of AMPA receptors during development or in response to synaptic inputs. Thus, knowledge of the specific factors that may mediate the regulation of expression, splicing of primary transcripts and trafficking of AMPA receptor subunits is essential for understanding how glutamate transmission is regulated in disease states.

Flip and flop isoforms contribute to distinct channel properties of different combinations of AMPA receptor subunits. The flip form generally bestows resistance to desensitization and fast resensitization to the AMPA channel. Thus, AMPA receptors with these channels have

increased Ca^{2+} influx and longer depolarizations than their flip variants (Sommer et al, 1990). High flip isoforms of these AMPA receptor subunits have been implicated in increased vulnerability to excitotoxicity. The flip and flop isoforms of GluA1 have similar desensitization kinetics but differ in their current amplitude, thus, GluA1-containing AMPA receptors have an increased synaptic gain and are more sensitive to glutamate (Geiger et al, 1995; Jonas P, 2000, Palmer et al, 2005). Flip-flop splicing of AMPA receptor subunits involve the insertion of a mutually exclusive exon encoding a 38-amino acid cassette into the AMPA receptor subunit mRNA. The resulting AMPA channels, differ in their kinetic properties and their trafficking to dendrites and synaptic sites.

Editing at the Q/R site of GluA2 replaces Glutamine586 with arginine, consequently, the presence of GluA2 subunit in an AMPA channel renders it Ca^{2+} impermeable. In addition, editing at the R/G site results in the introduction of a glycine residue at Arginine743 in GluA2-GluA4. These alterations are developmentally regulated in the CNS and result in AMPA channels with faster desensitization and resensitization. Alternative splicing results in changes in nine residues of GluA1-GluA4 subunits. Flip-flop splicing and R/G editing contribute to the regulation of AMPA receptor assembly and endoplasmic reticulum retention time (Geiger et al, 1995; Lomeli et al, 1994).

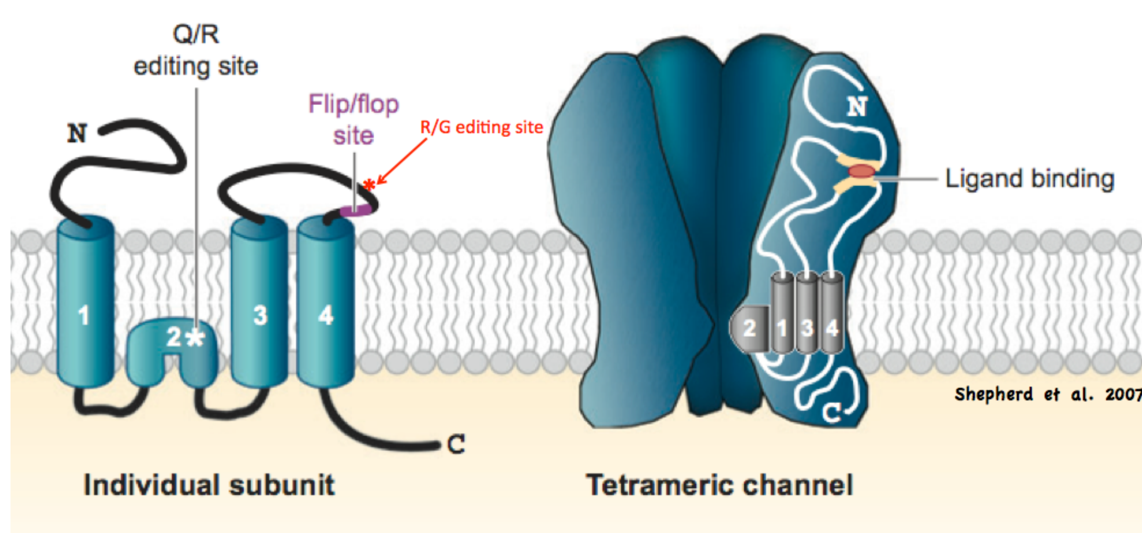

Figure 1.6. Structure of the AMPA receptor subunit. Functional receptors are formed as homotetramers or symmetric dimer of dimers, with an extracellular N-terminus and intracellular C-terminus. Alternative splicing occuring in the 'Flip/flop site' gives rise to two variants for each gene sequence. The 'Q/R editing site' is located only in the GluA2 subunit and controls Ca2+ permeability of AMPA receptors.

Role of AMPA receptors in accumbens-mediated behaviors

AMPA receptors have been implicated in the modulation of specific behaviors mediated by accumbens circuits. As a critical part of the brain's reward system, the accumbens is affected by drugs of abuse. In adults, exposure to cocaine results in alterations in glutamatergic transmission and plasticity in the accumbens. Pharmacological inhibition of AMPA receptors in the accumbens attenuates cocaine addiction (Backstrom and Hyytia, 2003; Harris and Aston-Jones, 2003). Extinction training following cocaine administration in adult rats increases the expression of GluA1 in the accumbens (Mead et al 2005), and over expression of GluA1 results in increase in response to extinction training (Sutton et al 2003). Glutamate transmission mediates cocaine-induced drug seeking, while AMPA receptor antagonists inhibited cocaine-induced

reinstatement. Taken together, results from these studies provide evidence that glutamateric transmission via AMPA receptors are critical modulators of accumbens functions.

Neuregulin 1 signaling

Signaling events mediated by the Nrg1 and ErbB family of proteins are crucial for the formation and maintenance of a variety of organs. Nrg1/ErbB signaling has been linked to cancer, heart failure and neuropsychiatric disorders (reviewed in Chen et al, 2010; Pentassuglia and Sawyer, 2009). The signaling network mediated by these proteins is complex and includes several proteolytic processing events, paracrine signaling via soluble Nrg1, juxtacrine signaling by membrane-tethered Nrg1, trans-synaptic Nrg1/ErbB forward signaling, Nrg1-back signaling via Ptdins-3-kinase and Nrg1-ICD back signaling (reviewed in Talmage DA, 2008).

Neuregulin 1 processing

The Nrg1 family of proteins are encoded by a large (1.5Mbp), complex gene that can be transcribed into six known transcription units. Each isoform of Nrg1 has a unique 5' exon and several variations in the EGF-like domain that interact with ErbB receptors with varying affinity (Figure 1.6).

Types I, II, IV and V Nrg1 isoforms contain an immunoglobulin-like domain, are expressed on the cell surface and undergo ADAMS17- or β-secretase-mediated proteolytic cleavage that results in the release of soluble Nrg1 with the EGF-domain. These isoforms are able to diffuse away from the cell of origin and activate ErbB receptors on neighboring cells (paracrine signaling). Type III-Nrg1 has a unique transmembrane domain (Figure 1.7). Consequently, Type III-Nrg1 is unique in its signaling - ADAMS17- or β-secretase cleavage of

Type III-Nrg1 results in a membrane-bound Nrg1 protein that can interact with ErbB proteins in a juxtacrine manner. Since this isoform is usually targeted to the axons of neurons (Shamir and Buonanno, 2010; Yang et al, 1998), it can signal in regions far from the soma of the neurons in which they are expressed, with accumbens circuits being a classic example, where Type III-Nrg1 is expressed in axons from presynaptic regions such as the hippocampus, cortex and amygdala.

Our lab has shown that these isoforms can also undergo γ-secretase cleavage in the transmembrane domain that generates a Nrg1-intracelluar domain (Nrg1-ICD). Binding of ErbB receptor dimers to the extracellular domain of Nrg1 can stimulate proteolytic release of Nrg1-ICD. Nrg1-ICD can translocate to the nucleus and act as a transcriptional regulator of target genes (Bao et al, 2003). Therefore, Type III-Nrg1 isoforms are bi-directional signaling proteins that can function both as a ligand for ErbB receptors and as a receptor. However, back signaling by this isoform can also be activated by neuronal depolarization, independent of ErbBs.

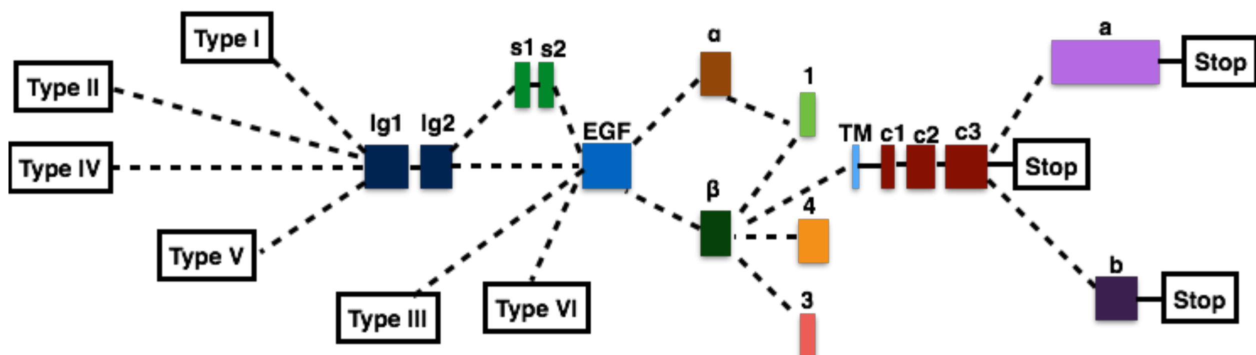

Figure 1.7 Illustration of Nrg1 isoforms. Schematic of the Nrg1 gene, showing the unique 5' N-terminal exon of each isoform, the Ig-domain of Types I, II, IV and V, the EGF-domain and the transmembrane domain.

<u>ErbB receptor tyrosine kinases - the Nrg1 receptors</u>

ErbB receptors are receptor tyrosine kinases that regulate a variety of cell processes and are transducers of Nrg1 signaling. ErbB2 (also known as HER2 or NEU), ErbB3 (also known as

HER3) and ErbB4 (also known as HER4) are expressed in several tissues including neuronal and mesenchymal tissues. ErbB receptors have a high degree of sequence identity, with a glycosylated ligand binding domain, a transmembrane domain, a juxtamembrane domain, a tyrosine kinase domain, but dissimilar intracellular domain among the ErbBs (Jones et al, 1999; Burden et al, 1997).

Ligand binding to ErbB receptors induces homo- or heterodimerization (Yarden and Sliwkowski, 2001). Of all the ErbBs, ErbB4 is unique because it is the only ErbB that can interact with Nrg1 and has a tyrosine kinase domain that is subsequently activated after Nrg1 binding (Table 1.1). ErbB2 lacks a Nrg1-binding domain and can form heterodimers with ErbB3 or ErbB4 (Tzahar et al, 1996). ErbB3 has a Nrg1 binding domain but lacks a tyrosine kinase domain and is thus, catalytically inactive (Guy et al, 1994). ErbB4, like Nrg1, has been implicated in schizophrenia. ErbB2 and ErbB3 mutant mice on the other hand, do not show schizophrenia-associated behavioral deficits (Gerlai et al, 2000).

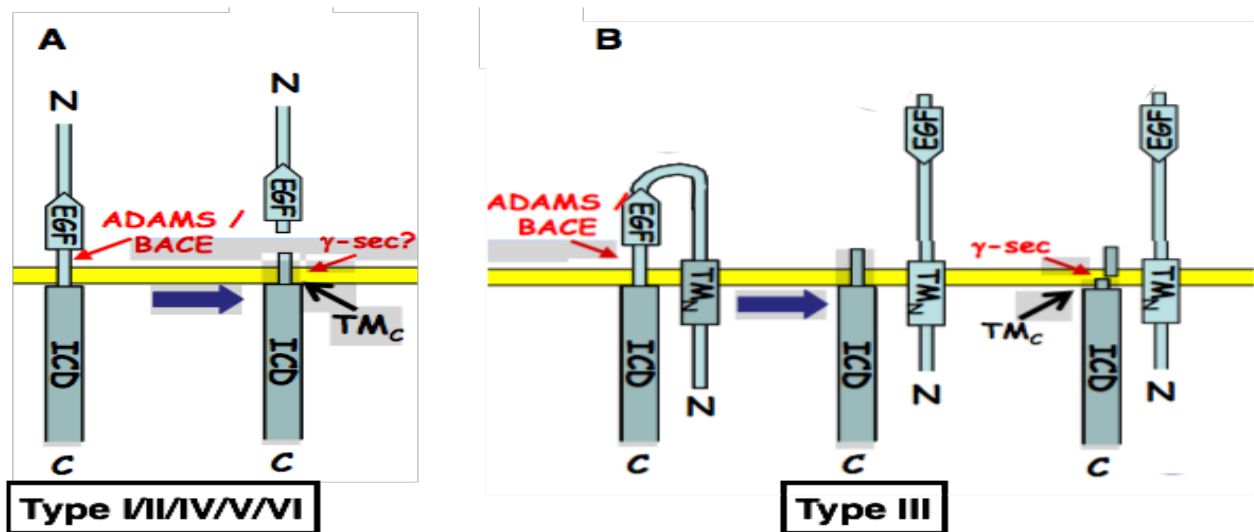

Figure 1.7 Nrg1 processing. (A) ADAMs17/β-secretase cleavage of Ig-Nrg1 containing isoforms results in release of soluble Nrg1 that participates in paracrine or autocrine signaling. It is not known if these Nrg1 isoforms can undergo γ-secretase cleavage. **(B)** Type III-Nrg1

cleavage by ADAMs17 or β-secretase results in a membrane-tethered Nrg1 protein that can participate in juxtamembrane signaling. γ-secretase cleavage of Type III-Nrg1 generates Nrg1-intracellular domain that can act as a transcription factor.

	ErbB2	ErbB3	ErbB4
Kinase Activity	Yes	No	Yes
Nrg1 Binding	No	Yes	Yes
Adaptors and effectors	Shc, Syk, Abl, RasGAP, MAPK8, IRS, Vav, PLCg, DOK, STAT1/3	Shc, Syk, RasGAP, PI 3-kinase, JAK, Nck, Crk, Grb7	Shc, Syk, Abl, PI 3-kinase

Table 1.1 ErbB receptor capability to bind Nrg1 and kinase activity. ErbB4 is the only ErbB that can bind to Nrg1 and also has kinase activity. Even though ErbB2 cannot bind Nrg1, it can recruit a variety of adaptors and effectors. Phosphorylation of ErbB3 by another ErbB can also induce the recruitment of adaptors and effectors on ErbB3, allowing ErbB3 to act as an active receptor.

Physiological and behavioral deficits of ErbB mutants.

Nrg1/ErbB signaling plays essential roles in the development of the central nervous system. ErbB4 is the only ErbB receptor that can bind to Nrg1 and also has tyrosine kinase activity, and is the most studied ErbB receptor in the CNS. ErbB4 knock out mice die at E10.5 as

a result of deficits in cardiac development (Jiang and Zhou, 2010; Lemmens et al, 2007). Nrg1 signaling through ErbB4 regulates dendritic morphology of hippocampal neurons and cerebellar granule cells, the number of parvalbumin interneurons in the hippocampus and the density of calbindin interneurons in the cortex.

Several physiological deficits have been shown to be a direct consequence of deletion of ErbB receptors. Specific deletion of ErbB2, ErbB3 or ErbB4 in mice results in abnormal development of the heart and nervous system (Erickson et al, 1997; Golding et al, 2000; Riethmacher et al, 1997). ErbB2 or ErbB4 deletion in mice results in fetal death at embryonic day 10.5 as a result of trabecular malformation in the heart. Expression of an ErbB2 receptor lacking a kinase domain results in similar phenotype as the ErbB2 null mice, suggesting that the kinase activity of ErbB2 is essential for embryonic development (Chan et al, 2002; Lee et al, 1995). ErbB3 deletion in mice results in fetal death at embryonic day 13.5 as a result of disruption of endocardial cushion during development of the heart.

In the nervous system, ErbB2 and ErbB3 deletion results in migratory deficits in neural crest cells and deficits in myelination in the peripheral nervous system (Lee et al, 1995; Meyer and Birchmeier, 1995; Pentassuglia et al, 2009). ErbB4 deletion results in axon guidance deficits in the hindbrain, myelination deficits, deficits in migration of cortical interneurons, deficits in hippocampal gamma oscillations, in glutamate receptor surface expression and in modulation of long term potentiation (Fisahn et al, 2009; Gassmann et al, 1995).

Expression of ErbB receptors

ErbB receptors are expressed throughout development in the nervous system and are regulated in a time and cell/brain region-specific manner (Fox and Kornblum, 2005). In the CNS,

Nrg1 binds to ErbB4, which alone as a homodimer or together with ErbB2 or ErbB3 as a heterodimer, results in different functions (Buonanno and Fischbach, 2001). Previous studies have detected the expression of ErbB2, ErbB3 and ErbB4 in striatal regions. ErbB2 and ErbB4 were both detected in striatal progenitor cells during embryonic stages in mice and rats, from around E13. However, ErbB4 expression was detectable throughout development whereas ErbB2 expression appeared to diminish during postnatal development and was restricted to cells lining the lateral ventricle after birth. ErbB3 expression was detectable in embryonic striatal region from around E16, but unlike ErbB2 and ErbB4, its expression appears to be very restricted and confined to very few cells in the germinal zones and striatal region (Calaora et al, 2001; Erickson S, 1997; Fox and Kornblum, 2005; Kornblum et al, 2000; Meyer D, 1997; Steiner et al, 1999; Yau et al, 2003).

Type III isoforms of Neuregulin1

Type III-Nrg1 is expressed throughout development, is expressed only in neurons and is the predominant Nrg1 isoform in postnatal cortex of mice (Bao et al, 2003; Liu et al, 2011; Wolpowitz et al, 2000). Type III-Nrg1 is predominantly targeted to axons (Shamir and Buonanno, 2010) and has an N-terminal cysteine-rich domain that forms a transmembrane domain, resulting in both N- and C-terminus of Type III-Nrg1 being within the cell. Consequently, unlike other isoforms of Nrg1 that release soluble Nrg1, Type III-Nrg1 remains membrane-bound after ADAMS/BACE cleavage. This allows it to be involved in juxtacrine signaling, interacting with ErbB4 to signal via ErbB4 receptor tyrosine kinase (forward signaling). Our lab has also shown that Type III-Nrg1 can signal bi-directionally by acting as a ligand for ErbB receptors or as a receptor (Bao et al, 2003; Canetta et al, 2011; Chen et al, 2010;

Hancock et al, 2007; Zhong et al, 2008). Back signaling by Type III-Nrg1 involves either (i) a γ-secretase cleavage event initiated by synaptic activity or by binding to ErbB that generates Nrg1-ICD, which acts as a transcriptional regulator of target genes; (ii) PI-3-kinase mediated back signaling, which is important for targeting neurotransmitter receptors to the cell surface.

Physiological and behavioral deficits of Type III-Nrg1 mutants.

Studies in Type III-Nrg1 heterozygous mice from our lab have identified several deficits that point to the role of this isoform in the regulation of neuronal networks, neuronal morphology and neurotransmission (Figure 1.8). In the embryonic brain, Type III-Nrg1-expressing cells migrate from the lateral ganglionic eminence and form a corridor within the medial ganglionic eminence (MGE). This allows thalamocortical axons to migrate through the normally intolerant environment in the MGE to the cortex (Lopez-Benditto, et al, 2006). This thalamocortical pathway is essential for the coordination of information in the cortex, and impairments within this pathway have been implicated in working memory deficits in schizophrenia (Friston K, 1997; Jones E, 1997; Parnaudeau et al, 2013); we have also observed working memory deficits in Type III-Nrg1 heterozygous mice (Chen et al, 2008). Thus, Type III-Nrg1 modulates the formation of appropriate neuronal networks by regulating the migration of neurons/axons during development.

Within the cortex, our lab has also shown that Type III-Nrg1 is important for normal corticogenesis. Back signaling by Type III-Nrg1 enhances dendritic arborization in cortical pyramidal neurons in an ErbB4-independent manner (Chen et al, 2010), thus providing more evidence that Type III-Nrg1 is important for the formation of appropriate neuronal connectivity within cortical and other brain circuits.

Type III-Nrg1 has also been implicated in the modulation of neurotransmission. Type III-Nrg1 signaling regulates the surface expression of alpha-7-nicotinic acetylcholine receptor (α7nAChR) via signaling through PI-3 kinase pathway. These events are mediated by Type III-Nrg1 back signaling, and can be triggered by synaptic activity, depolarization or by ErbB4 acting as a ligand.

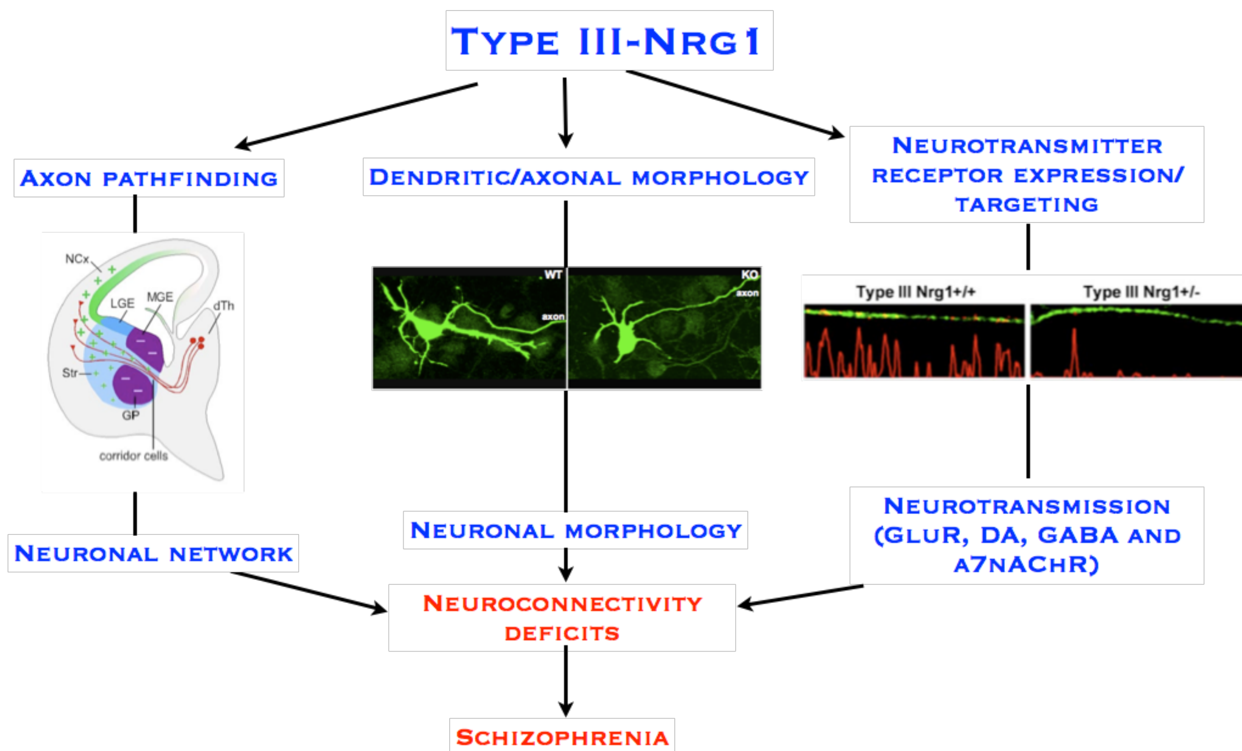

Figure 1.9. Physiological functions of Type III-Nrg1 in the central nervous system. Type III-Nrg1 regulates the formation of appropriate neuronal networks, modulates neuronal morphology of cortical pyramidal neurons and regulates the expression of neurotransmitter receptors at the surface of axons. Disruption of these processes has been implicated in schizophrenia.

Type III-Nrg1/ErbB4 signaling in the nucleus accumbens

The expression pattern of Type III-Nrg1/ErbB4 suggests that Nrg1 signaling may play a role within accumbens circuits. Nrg1 is expressed within presynaptc regions that innervate the accumbens, while ErbB4 is expressed within the accumbens. In order to define the specific functional significance of Nrg1 signaling within accumbens circuits, it is important to define the expression of ErbB4 during development and the specific cells in which it is expressed.

Type III-Nrg1 is expressed by the glutamatergic principal neurons of presynaptic regions that innervate the accumbens, while ErbB4 is expressed by a majority of cells in the perinatal accumbens and in a diffused manner in adult accumbens (Chen et al, 2008; Jiang et al, 2013; Zhong et al, 2008). Specific deficits observed within accumbens circuits of Type III-Nrg1 heterozygous mice suggest that Nrg1 signaling is important for the maintenance of excitatory inputs within the accumbens. Type III-Nrg1 heterozygote mice exhibit altered functional connectivity between the ventral hippocampus and the accumbens (Nason et al, 2011). Subsequent to alterations in glutamatergic transmission, behaviors that require appropriate functional connectivity between the hippocampus and accumbens are altered in Type III-Nrg1 heterozygous mice.

Within accumbens circuits, Type III-Nrg1 is expressed by presynaptic glutamatergic axons and ErbB4 is expressed by GABAergic neurons of the accumbens. Similar to the accumbens, Type III-Nrg1 signaling within the hippocampus and cortex involves interaction between Nrg1 expressed by glutamatergic neurons and ErbB4 expressed by GABAergic interneurons. In the hippocampus, Nrg1 signaling through ErbB4 modulates synaptic plasticity at glutamatergic synapses Nrg1 modulates synaptic plasticity by internalization if GluA1-containing AMPA receptors.

Our lab has recently shown that the nicotine-induced enhancement of glutamatergic transmission in accumbens MSNs depend on the presynaptic Type III-Nrg1 genotype of presynaptic axons that synapse onto the MSNs. Preliminary results from our lab, suggests that MSNs receiving Type III-Nrg1 heterozygous inputs show alterations in the kinetics of the channels, including a reduction in the frequency and amplitude of mini excitatory postsynaptic currents and a reduction in the fast desensitizing current in response to glutamate. Literature review of properties of functional channels formed by different AMPA receptor subunits in heterologous systems indicated that the properties of the postsynaptic glutamate receptors when innervated by Type III-Nrg1 heterozygous inputs are consistent with alterations in the AMPA receptor subunit profile of the accumbens neurons. Thus, we hypothesize that Type III-Nrg1 signaling regulates glutamatergic transmission in the accumbens by modulating AMPA receptor subunit biogenesis (expression, and splicing of primary transcripts) and trafficking of receptors to the surface.

Scope of this dissertation

Our lab has shown that Type III-Nrg1 signaling regulates various functions in the developing and adult CNS. Type III-Nrg1 signaling regulates the formation of appropriate neuronal network in the cortex, modulates dendritic density of cortical neurons, regulates neurotransmitter receptor expression at the surface of hippocampal neurons. Deficient Type III-Nrg1 signaling results in altered functional connectivity between the ventral hippocampus and the accumbens (Nason et al, 2011) and alterations in nicotine-induced enhancement of glutamatergic transmission in accumbens neurons. Our preliminary data suggests that presynaptic Type III-Nrg1 modulates postsynaptic glutamate receptor responses via AMPA receptors. Having observed multiple

physiological and behavioral deficits as a result of a partial deletion of Type III-Nrg1, this thesis set out to answer two questions that are pertinent to delineating the role of Nrg1 signaling within accumbens circuits:

1. What specific neuronal networks are involved in Type III-Nrg1/ErbB4 signaling within nucleus accumbens circuits?
2. How does Type III-Nrg1 signaling affect AMPA receptor profile in the nucleus accumbens?

I have attempted to delineate the specific neuronal networks that participate in Type III-Nrg1/ErbB4 signaling by defining the specific cells within the accumbens that express ErbB4 at different critical stages of accumbens development. I have also defined the effect of Type III-Nrg1 and ErbB4 independently in the modulation of AMPA receptor subunit expression, mRNA editing and splicing of primary transcripts.

Chapter 2

Type III-Nrg1/ErbB4 expression within accumbens circuits suggests a trans-synaptic signaling module

Abstract

Nrg1-ErbB signaling has been implicated in a variety of functions at various stages of development in the CNS. Studies in animals heterozygous for a mutation disrupting the Type III isoforms of Nrg1 indicate that Nrg1 plays critical roles in the formation of appropriate neural networks, neuronal morphology and appropriate neurotransmission within neuronal circuits. Specifically, Type III-Nrg1 heterozygous animals show alterations in the functional connectivity between the ventral hippocampus and the nucleus accumbens (Nason et al, 2011), suggesting that Type III-Nrg1 plays critical roles in the formation and maintenance of neural networks and neurotransmission within accumbens circuits. Type III-Nrg1 is predominantly targeted to dendrites and axons, and is expressed by principal neurons from presynaptic regions that innervate the nucleus accumbens; while ErbB4 is widely expressed in the perinatal accumbens.

Here, I investigated the pattern of ErbB4 expression in the accumbens during critical stages of development: at perinatal ages (P0-P3) during which accumbens neurons are settling into their niche; at adolescent ages (P21-P24) during which accumbens neurons undergo maturation; and in adulthood (>P60). I also shed light on the distinct accumbens neuro-circuitry that participates in Nrg1/ErbB4 signaling, by defining the specific accumbens neuronal population that express ErbB4.

In the perinatal accumbens, ErbB4 expression is detected in the soma of a majority of neurons. As the animals mature, ErbB4 is detectable in dispersed puncta, reminiscent of cellular processes. In the perinatal and adult accumbens, ErbB4 expression co-localizes with dendritic

and axonal markers, confirming that ErbB4 is targeted to dendrites and axons as animals mature. I observed that ErbB4 protein is visibly reduced as animals mature, but ErbB4 mRNA is expressed at all stages in the accumbens. I also employed a single cell RT-PCR technique to profile individual neurons of the accumbens obtained from acute coronal slices. Using this technique, I observed that over 70% of MSNs have detectable ErbB4 mRNA in the perinatal accumbens. ErbB4 expression is also detectable in a majority of adolescent accumbens neurons, but is undetectable in adult accumbens neurons.

I conclude that ErbB4 is expressed within MSNs in accumbens, which are the major synaptic targets of Nrg1-expressing inputs from presynaptic regions. I hypothesize that Type III-Nrg1/ErbB4 signaling may be involved in a trans-synaptic signaling module, in which presynaptic Type III-Nrg1 interacts with postsynaptic ErbB4 to influence the formation and maintenance of appropriate neural networks within the NAcc.

Introduction

Type III-Nrg1/ErbB4 signaling has been shown to be involved in a diverse array of CNS functions such as migration of neurons, axon pathfinding (Lopez-Benditto et al, 2006), and the elaboration of dendritic and axon arbors (Chen et al, 2010). Type III-Nrg1 is preferentially targeted to dendrites and axons (compared to other Nrg1 isoforms, Shamir and Buonanno, 2010) and is expressed by the principal neurons of presynaptic regions that innervate the NAcc. Type III-Nrg1 and ErbB4 are expressed in the developing and adult brain in a complemetary manner; Type III-Nrg1 is expressed by the principal neurons of presynaptic regions that innervate the NAcc and is preferentially targeted to dendrites and axons, while ErbB4 is expressed postsynaptically (Chen et al, 2008; Liu et al, 2011; Zhong et al, 2008; Chen et al, 2010; Jiang et

al, 2013). In the CNS, Nrg1 binds to ErbB4 homodimer or together with ErbB2 or ErbB3 as a heterodimer. This difference in ErbB receptor compositions results in different functions (Buonanno and Fischbach, 2001). Previous studies have detected the expression of ErbB2, ErbB3 and ErbB4 in striatal regions. ErbB2 and ErbB4 were both detected in striatal progenitor cells during embryonic stages in mice and rats, from around E13, whereas, ErbB4 expression was detectable throughout development. ErbB2 expression appeared to be diminished and restricted to cells lining the lateral ventricle after birth. ErbB3 expression was detectable in embryonic striatal region from around E16, but unlike ErbB2 and ErbB4, its expression appears to be very restricted to very few cells near the lateral ventricle in the striatal region (Fox et al, 2005; Steiner et al, 1999; Calaora et al, 2001; Yau et al, 2003; Meyer et al, 1997; Erickson et al, 1997).

ErbB receptors are made up of an ectodomain containing cysteine rich domains, a transmembrane domain, a juxtamembrane domain and a C-terminal intracellular domain (Mei and Xiong, 2008). Binding of Nrg1 to ErbB receptors results in homo- or heterodimerization and auto- or transphosphorylation of tyrosine residues in the intracellular domains and downstream signaling via second messenger proteins. Alternative splicing of ErbB4 results in isoforms that participate in Nrg1/ErbB4 signaling through distinct second messenger systems, resulting in diverse effects downstream. The functional significance of this signaling diversity in the brain is not clear (Yarden et al, 2001).

Nrg1/ErbB4 signaling provides a patterning mechanism to ensure the formation and maintenance of appropriate neuronal networks within circuits. The formation of such specific neuronal connection patterns is initiated during development, is similar between species and is crucial for normal functioning of brain circuits. Deficits in the functional connectivity of

neuronal circuits within the striatum have been observed in Type III-Nrg1 heterozygotes and are a hallmark of schizophrenia (Abi-Dargham et al, 1998; Morris et al, 2011; Nason et al, 2011; Yoon et al, 2013;). Thus, there is a pertinent need for a better understanding of the functional role of Type III-Nrg1/ErbB4 signaling within striatal circuits. To understand this, knowledge of the specific neurons that express Nrg1 and ErbB4 is needed in order to map out the precise circuits that participate in Nrg1/ErbB4 signaling. Here, I attempt to do just that; I define the pattern of expression of ErbB4 within NAcc circuits, and the specific cells that express ErbB4 during specific periods of development. Using immunohistochemistry techniques, I show that ErbB4 is expressed in the accumbens from birth to adults. ErbB4 expression is predominantly in the soma at birth but becomes targeted to dendrites in later stages of development. I also determined by single cell PCR that MSNs are the major cell types that express ErbB4 in the accumbens at birth and in adolescence.

Results

ErbB4 is expressed throughout development in the NAcc and is preferentially targeted to dendrites during postnatal development

Prior studies on ErbB4 expression in the striatum suggest that ErbB4 is expressed by MSNs in perinatal NAcc and by cholinergic neurons in adult NAcc (Chen et al, 2010; Neddens and Buonanno, 2006). To determine if ErbB4 is expressed throughout development in the NAcc, we used an ErbB4-specific antibody to examine ErbB4 immunoreactivity. ErbB4 immunoreactivity is prominent in the soma of cells in the perinatal accumbens (Fig 2.1). In P21 and in adult accumbens, ErbB4 expression is detectable in processes. We did not detect ErbB4

immunoreactivity in sections from ErbB4 knockout mice, confirming the specificity of the antibody to ErbB4.

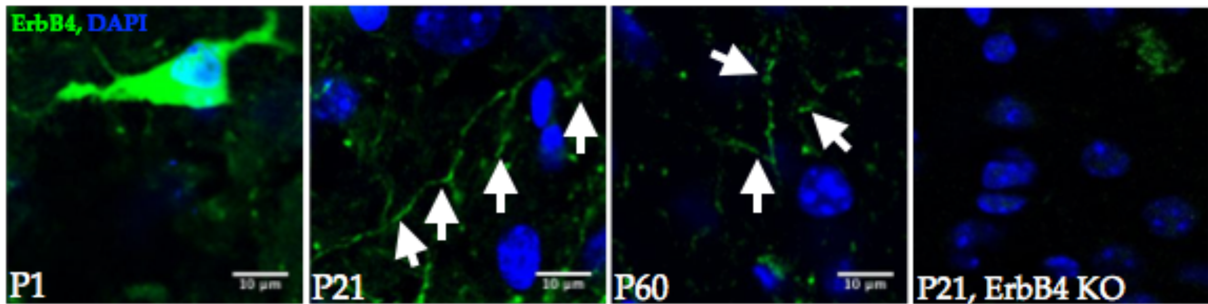

Figure 2.1. ErbB4 expression in the nucleus accumbens changes during development. Representative confocal immunofluorescence image of ErbB4 (green) expression in accumbens neurons of WT or ErbB4$^{-/-}$ mice. ErbB4 is not detected in accumbens neurons of ErbB4$^{-/-}$ but is expressed in the soma of P1 accumbens cells and in processes of P21 and P60 neurons.

Double immunostaining with anti-ErbB4 and anti-MAP2 and PSD-95 antibodies confirmed that ErbB4 is targeted to dendrites, with a subset of puncta that overlap with PSD95 in later stages of development in the NAcc (Fig 2.2).

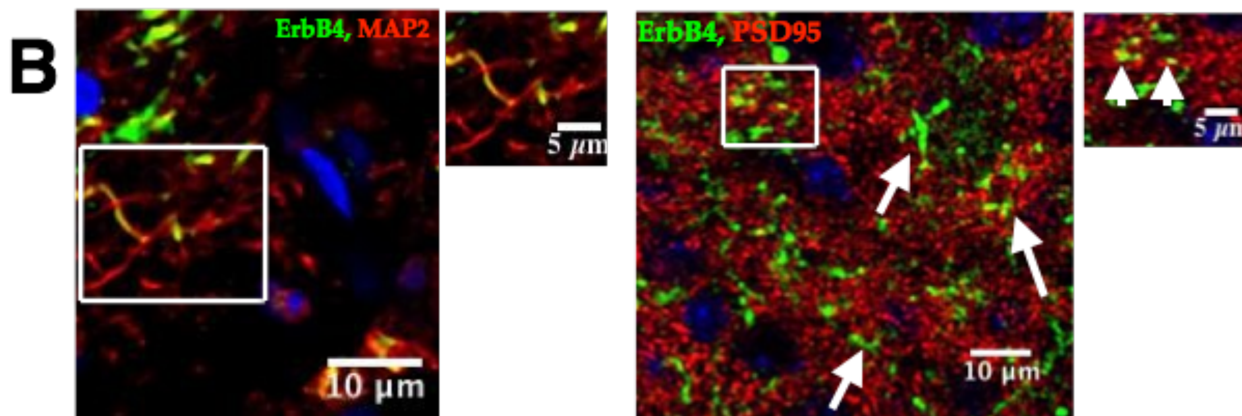

Figure 2.2. ErbB4 is targeted to dendrites of nucleus accumbens neurons as animals mature. ErbB4 expression in P21 accumbens colocalizes with dendritic marker MAP2 (left) and PSD95 (right).

ErbB4 is expressed predominantly by MSNs, parvalbumin-positive interneurons and a subset of cholinergic interneurons

Next, we investigated the types of NAcc neurons that express ErbB4. We were unable to accomplish this at adolescent and adult ages because of the diffused pattern of ErbB4 immunostaining in the NAcc. Therefore, we utilized a single cell RT-PCR technique, in which cytoplasmic contents harvested from individual NAcc neurons in an acute slice were used as template for RT-PCR (Esumi et al, 2006). Indeed, as observed in Figure 2.1 and 2.2, and consistent with prior in situ hybridization results (Chen et al, 2008), ErbB4 mRNA expression is detectable in a majority of individual NAcc neurons at perinatal ages (>70%). In the adolescent NAcc, ErbB4 mRNA is detectable in a majority of D1- and D2-positive MSNs (73.2% +/- 6.7). In the adult accumbens, ErbB4 expression is detectable in only a few MSNs (2.2% +/- 0.021, Fig 2.3). The vast majority of NAcc neurons are D1- or D2-positive MSNs (>94%). Therefore, random sampling is unlikely to adequately capture the expression pattern in the interneuronal population of the NAcc.

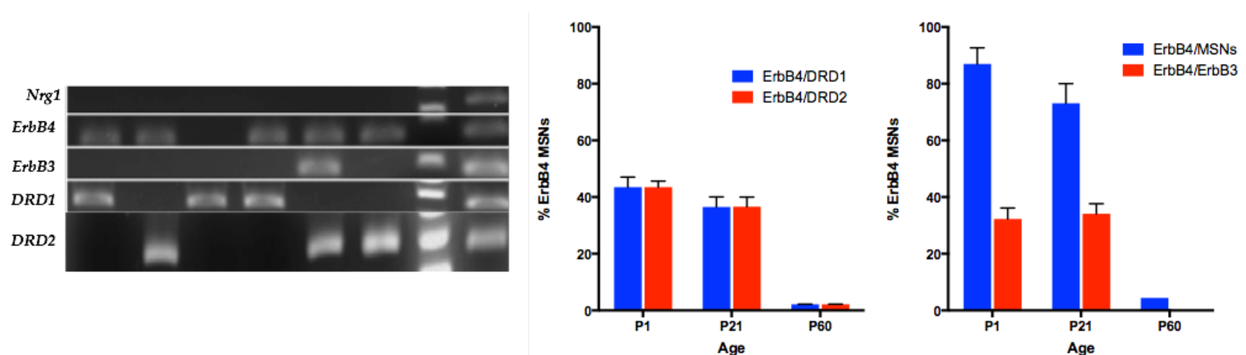

Figure 2.3. Single cell RT-PCR analysis of individual cells isolated from acute slice of P1 accumbens. ErbB4, ErbB3 dopamine D1 and D2 receptor, but not Type III-Nrg1 mRNA can be detected from cytoplasmic extract of single cells from an acute accumbens slice. Each column

represents a cell. Lane 1-6 are cells obtained from P21 accumbens, lane 8 is positive control amplified using whole brain cDNA as template.

I also determined if individual NAcc neurons express ErbB3. Using single cell RT-PCR, I detected ErbB3 mRNA in 32% of MSNs sampled at P1 and 36% of MSNs sampled at P21 (Fig 2.3). ErbB3 was not detected in the 46 neurons sampled from adult NAcc.

Discussion

My major findings are: (i) that ErbB4 is expressed in the accumbens throughout development, (ii) the pattern of expression of ErbB4 changes during development, from mainly somatic at birth, to predominantly dendritic in adulthood, (iii) ErbB4 is expressed by the MSNs of the accumbens at birth and in adolescence. ErbB4 is not detectable in adult accumbens and (v) ErbB3 is also expressed by accumbens neurons. ErbB3 expression is detectable only in cells that also express ErbB4. Based on my findings, I predict that presynaptic Nrg1/postsynaptic ErbB4 signaling may be involved in a trans-synaptic signaling module within the nucleus accumbens.

My findings on ErbB4 expression in perinatal NAcc are consistent with prior studies from our lab (Chen et al, 2008) and provide a more in depth analysis of ErbB4 expression that is critical to understanding the specific neural networks that participate in Nrg1/ErbB4 signaling. We utilized a well-characterized ErbB4-specific antibody to demonstrate that ErbB4 is expressed throughout development in the accumbens. My studies agree with previous studies that show that ErbB4 expression in adult striatum has a dispersed pattern (Neddens et al, 2011). We show that ErbB4 expression pattern changes during development, from predominantly somatic at birth, to mainly dendritic in adolescent and adult NAcc.

ErbB4 is important for the tangential migration of cortical interneurons from the MGE and the migration of cortical pyramidal neurons along the radial glia during development (Anton et al, 1997; Flames et al, 2004; Lopez-Benditto, 2006). We predict that the subcellular localization of ErbB4 at different developmental stages may be correlated to the kind of synapses the neurons receive at each stage; ErbB4 may be important for migration of NAcc neurons during development whereas it may be important for the formation and maintenance of accumbens circuits in adolescent NAcc.

At birth (P0-P3) NAcc neurons are just finishing migration from the telencephalon, are settling within the NAcc regions, and are forming symmetric synapses which are predominantly axodendritic, and presumably inhibitory (Tepper et al, 1998). I observed that neurons in the perinatal NAcc express ErbB4 predominantly in the soma. I speculate that the somatic expression of ErbB4 in perinatal NAcc neurons is important for its role in neuronal migration of NAcc neurons from the basal telencephalon.

In the adolescent NAcc (around P21), synaptic connections are maturing and MSNs are receiving glutamatergic inputs from several regions and also projecting to target regions. During these later stages, the majority of synapses formed onto MSNs are asymmetric synapses, predominantly axospinous, and presumably from glutamatergic inputs (Hassler and Chung, 1976; Sharpe and Tepper, 1998; Tepper et al, 1998). ErbB4 has also been shown to be important during the period of synaptic connections in the cortex and hippocampus, affecting both the number and strength of glutamatergic synapses onto parvalbumin-positive interneurons, spine formation, etc. The redistribution of ErbB4 during maturation of NAcc neurons might be related to a similar role of ErbB4 in the NAcc.

Results from several studies have provided evidence that Nrg1 signaling via ErbB4 regulates LTP at glutamatergic hippocampal synapses (Fisahn et al, 2009, Neddens et al, 2009). The reversal of LTP by Nrg1-ErbB4 signaling is dependent on dopamine receptor activation. In the hippocampus, ErbB4 is expressed in GABAergic neurons and interacts with Nrg1 in pyramidal neurons, which results in the reversal of LTP. Thus, Nrg1/ErbB4 signaling simultaneously modulates both glutamatergic and dopaminergic signaling via GABAergic neurons. Similar to the hippocampus, I show here that ErbB4 is expressed by GABAergic MSNs in the accumbens that receive inputs from glutamatergic projections of pyramidal neurons. Type III-Nrg1/ErbB4 signaling is therefore positioned within accumbens circuits, to modulate both glutamatergic and dopaminergic transmission within the accumbens. In the next chapter, I investigate the effect of Nrg1/ErbB4 signaling on glutamate receptors in the NAcc.

Chapter 3

Type III-Nrg1 signaling modulates glutamate receptor responses in the accumbens

Abstract

The schizophrenia susceptibility gene, *NRG1* (Neuregulin 1) encodes a family of proteins that are critical for development of the nervous system. Deficient Nrg1/ErbB4 signaling results in alterations in neuronal network activity, neuronal morphology, neuronal migration and survival, and altered GABAergic, dopaminergic, cholinergic and glutamatergic neuro-transmission. Type III-Nrg1 isoforms are targeted to both dendrites and axons, and are expressed in the principal neurons of the hippocampus, cortex, thalamus and amygdala which send glutamate-encoded information to the accumbens. Integration of the information encoded by these Nrg1-expressing inputs, together with mid-brain dopamine inputs, is crucial for normal functioning of the accumbens. In perinatal and adolescent accumbens, the Nrg1 receptor, ErbB4 is expressed by a majority of medium spiny neurons in the nucleus accumbens, but is undetectable in adult MSNs. I hypothesize that presynaptic Type III Nrg1/postsynaptic ErbB4 signaling plays an important role in the integration and modulation of glutamatergic transmission in the nucleus accumbens. Regulation of glutamatergic transmission by AMPA receptor subunits could result from (i) change in subunit expression and composition in AMPA channels, (ii) change in the flip-flop splice variants, (iii) change in trafficking to cell surface. To determine the role of Nrg1 signaling on glutamatergic transmission and AMPA receptor biology, I quantified the pattern of expression of AMPA-type glutamate receptor subunits during critical stages of development in the accumbens in vivo, in mice deficient in either Type III NRG1 or ErbB4. I found that alternative splicing of GluA1 transcripts changes during development. In the perinatal accumbens, 92% of GluA1 transcripts contain the flip exon, at adolescence 65% of GluA1

transcripts contain the flip exon and in adults 39% do. The developmental profile of GluA1 splice variants in the accumbens of Type III Nrg1 heterozygotes is altered. The transition from all flip to a mix of flip and flop isoforms is accelerated in Type III-Nrg1 heterozygotes, reaching adult levels (22% GluA1flip) in adolescence. I show that the developmental profile of GluA1 splice variants in ErbB4-null mice is similar to that of WT animals. Thus, although expression of ErbB4 in MSNs and expression of the GluA1flip exon changes in parallel during maturation, the regulation of GluA1 splicing by Type III-Nrg1 is independent of ErbB4.

Our findings provide evidence that (i) splicing of GluA1 transcripts changes during development, suggesting that changes in glutamate receptor splicing may be a mechanism by which glutamate-encoded information is integrated within accumbens circuits, (ii) presynaptic Type III-Nrg1 regulates postsynaptic glutamate receptor mRNA splicing within accumbens circuits during development, suggesting that physiological and behavioral deficits seen in Type III-Nrg1 heterozygotes may be due, at least in part, to alterations in glutamate receptor profile and (iii) Type III-Nrg1 regulation of glutamate receptor mRNA splicing is independent of ErbB4.

Introduction

Our lab has shown that deficits in Type III-Nrg1/ErbB4 signaling underlie a variety of cellular and molecular dysfunctions including those involving prefrontal (Chen et al, 2010), hippocampal (Zhong et al, 2008), amygdalal (Jiang et al, 2013) and striatal circuits (Nason et al, 2011). Reduced levels of Type III-Nrg1 result in myelination deficits (Taveggia et al, 2005), deficits in migration of cortical interneurons (Lopez-Benditto et al, 2006), deficits in dendritic morphology of cortical neurons (Chen et al, 2010), deficits in neurotransmitter expression on the

surface of hippocampal neurons and deficits in glutamatergic neurotransmission (Zhong et al, 2008; Jiang et al, 2013).

Several studies have demonstrated that Nrg1 signaling is important for glutamate transmission. Nrg1 reverses LTP in vivo in hippocampal CA1 area through activation of dopamine D4 receptor and internalization of GluA1-containing AMPA receptors. Increased Nrg1 signaling by ErbB4 also enhances AMPA receptor synaptic currents and regulates dendritic spine density in hippocampal slices (Fisahn et al, 2009, Neddens et al, 2009). The properties of AMPA channels can be regulated by changing the expression levels of receptor subunits or their mRNA splicing (Jonas P, 2000). Recently, our lab demonstrated that reduced Nrg1 signaling reduces the NMDA/AMPA ratio (Jiang et al, 2013) and alters the amplitude and kinetics of EPSCs (Zhong et al, 2008).

Type III-Nrg1 signaling is important for glutamatergic transmission and functional connectivity within accumbens circuits. The accumbens is an important site where glutamatergic inputs together with dopaminergic inputs are integrated to mediate accumbens functions (Chuhma et al, 2011; Lenz and Lobo, 2013). Nrg1/ErbB4 signaling modulates AMPA synaptic currents (Li et al, 2007) and links dopaminergic with glutamatergic transmission in the hippocampus. In preliminary studies, our lab demonstrated that presynaptic Type III-Nrg1 regulates the profile of postsynaptic glutamate receptors in accumbens neurons (C. Du, unpublished). I hypothesize that presynaptic Type III-Nrg1 is required for the appropriate establishment and maturation of glutamatergic transmission within accumbens circuits. Here I investigate how reduced presynaptic expression of Type III-Nrg1 or postsynaptic deletion of ErbB4 affects the expression and mRNA splicing of glutamate receptors in the accumbens. I

demonstrate that reduced Type III-Nrg1 signaling alters the splicing of GluA1 transcripts in an ErbB4-independent manner.

Results

Increased expression of AMPA receptor subunits in the accumbens during development

To investigate whether Nrg1 signaling regulates the expression levels of AMPA receptor subunits in accumbens in vivo, I quantified the expression levels of GluA1, GluA2, GluA3 and GluA4 AMPA receptor subunit mRNA in the accumbens of WT, Type III-Nrg1 heterozygotes and ErbB4 knock out animals. The expression levels of AMPA receptor subunits increase with age in all three genotypes. Analysis of GluA subunit expression between genotypes revealed no significant difference in GluA1-4 levels between WT and Type III-Nrg1 HET at P1 and at P21 (Fig 3.1). Analysis of GluA levels at P60 revealed a statistically significant difference between WT and Type III-Nrg1 HET (n = 7 per genotype, GluA1 $p<0.0001$, GluA2 $p=0.0033$, GluA4 $p<0.0001$, Tukey's multiple comparisons test.

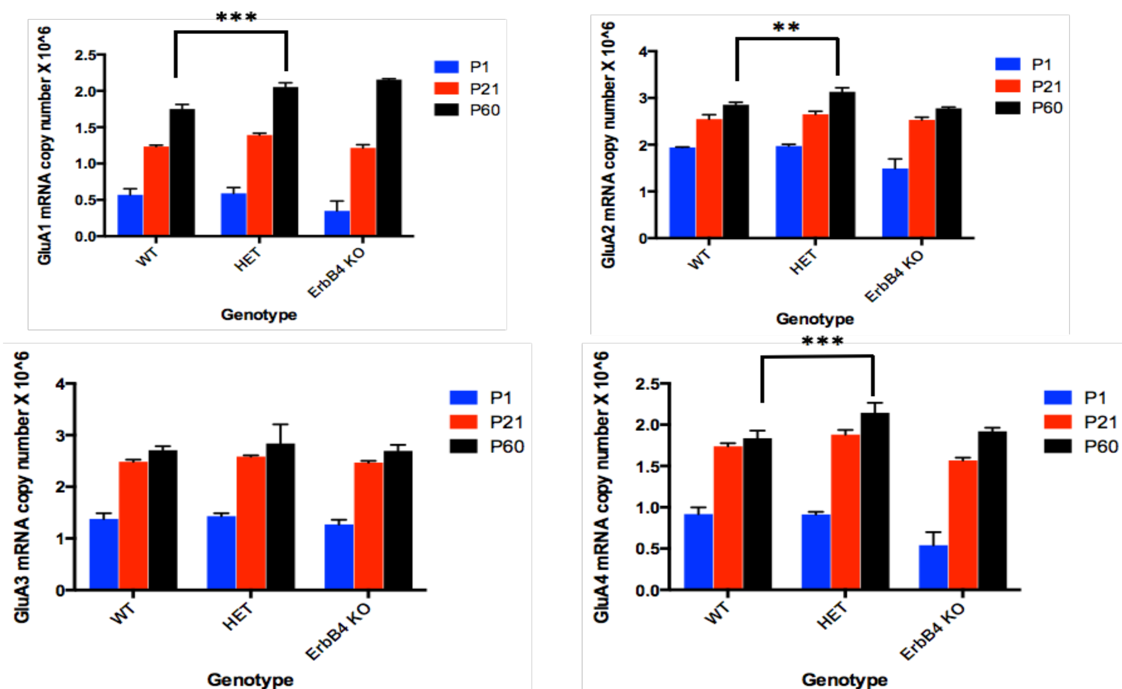

Figure 3.1 mRNA levels of GluA1-4 subunits of AMPAR in the accumbens. Accumbens from WT, Type III-Nrg1 heterozygotes and ErbB4 KO at P1, P21 and P60 were dissected and total RNA was extracted and used to synthesize cDNA. qPCR was carried out using Roche LightCycler 480. N=7 each.

I also investigated the expression levels of GluA1 and GluA2 by immunoblotting and analyzed expression levels between WT and Type III HET. Similar to my mRNA data, I observed an increase in GluA1 and GluA2 levels with age but did not observe any significant difference between genotypes (Fig 3.2). Therefore, we conclude that AMPA receptor subunit levels increase during development with similar time courses in WT, Type III-Nrg1 heterozygote and ErbB4 knock out animals.

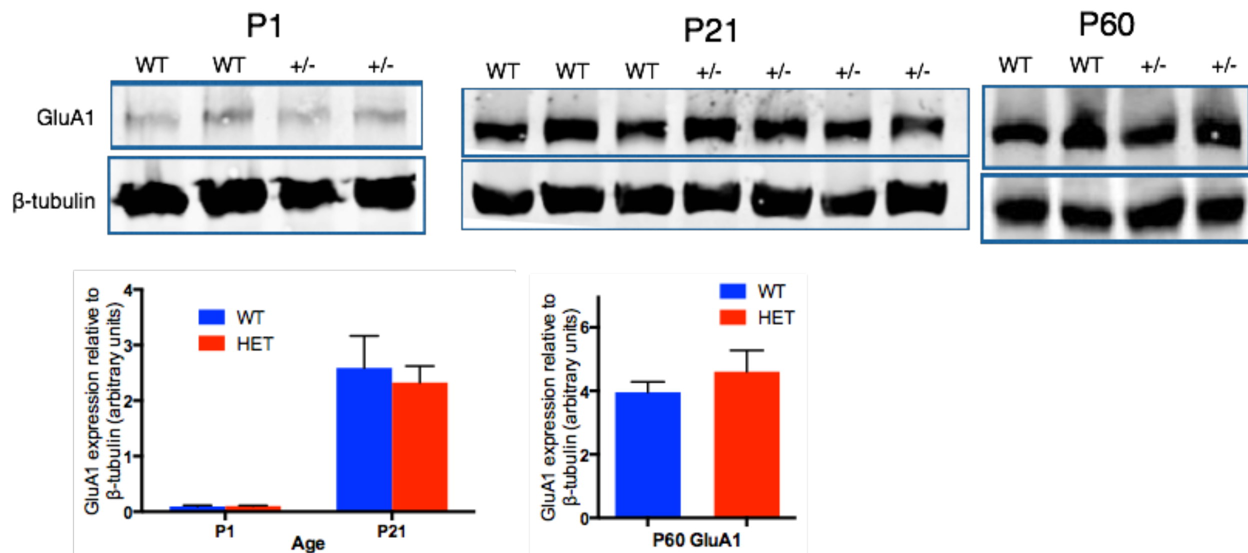

Figure 3.2 Immunoblot analysis of GluA1 receptor expression levels during maturation. Accumbens tissue was extracted from P1 and P21 mice and total protein was resolved on a SDS-PAGE gel and probed with GluA1 antibody. N=5 for each genotype at P1, N=6 for P21 WT, N=

7 for P21 Nrg1 Het, N=5 for each genotype at P60. The intensity of GluA1 bands were quantified and normalized to β-tubulin.

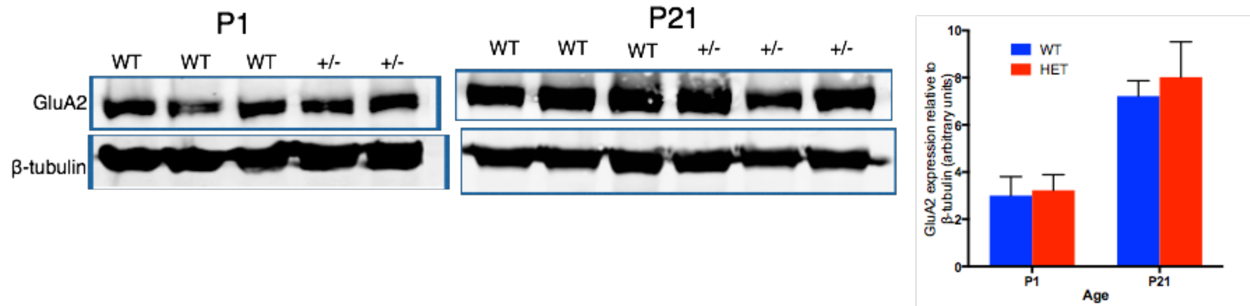

Figure 3.3. Immunoblot analysis of GluA2 receptor expression levels in accumbens increases at P1 and P21. Accumbens tissue was extracted from P1 and P21 animals and total protein was resolved on a SDS-PAGE gel and probed with GluA2 antibody. N= 5 for each genotype at P21, N=6 for P1 WT, N= 5 for P1 Nrg1 Het.

Presynaptic Type III-Nrg1 alters the developmental profile of GluA1 splicing in the accumbens

I investigated the effect of altered Nrg1 signaling on AMPA receptor splicing in the accumbens. I determined the proportion of flip or flop isoforms of GluA subunits during development in Type III-Nrg1 HET and their WT littermates. I observed a developmental switch from GluA1flip to GluA1flop during development within the accumbens. At birth, 92% of GluA1 transcripts are the GluA1flip isoform. This decreases to about 65% GluA1flip at P21 and about 40% in adult accumbens. The developmental profile of GluA1 transcripts in Type III-Nrg1 HET is significantly different from their WT littermates. For Type III-Nrg1 HET, at P1 81% of

GluA1 transcripts contain the flip exon and at P21 and in adults, about 25% of GluA1 transcripts contain the flip exon (Fig 3.4).

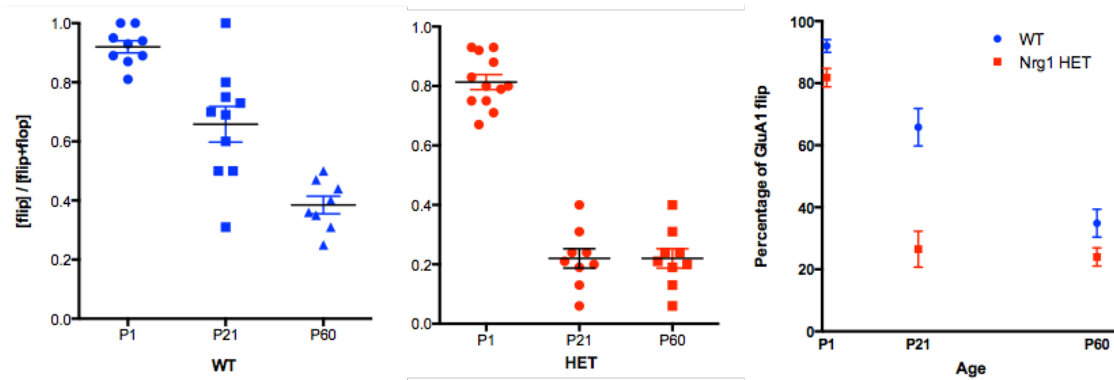

Figure 3.4. Type III-Nrg1 regulates the developmental progression of GluA1 splicing in the accumbens. GluA1 flip/flop ratio in the accumbens of WT (left) and Type III-Nrg1 +/- (middle) mice during development; (right) pooled data showing developmental progression of GluA1 flip/flop ratio in the accumbens

I also determined the GluA1 transcript profile in ErbB4 knockout animals compared to their WT littermates. At birth, 87% of GluA1 transcripts in ErbB4-null mice consist of the GluA1flip isoform. This decreases to about 48% GluA1flip at P21 and about 42.75% in adult accumbens. Therefore, Type III-Nrg1 genotype, but not ErbB4 genotype modulates the flip vs flop exon usage specificity of GluA1 transcripts during maturation.

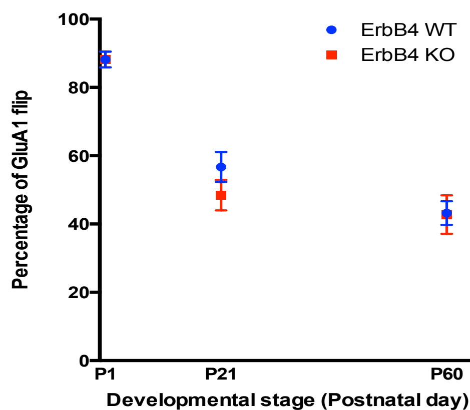

Figure 3.5. Expression of GluA1 splice variant in ErbB4 knockout animals is similar to WT. Pooled data showing developmental progression of GluA1 flip/flop ratio in the accumbens of ErbB4 -/- mice and their WT littermates.

Next, using the flip/flop ratio values, and the values obtained from absolute qPCR, I extrapolated the amount of flip or flop variants of GluA1 expressed at the different timepoints. The utilization of the GluA1flop splice site increases in the NAcc during maturation (P21 and P60) and accounts for the increased expression of GluA1 splice transcripts during maturation (Fig. 3.6). In P21 and adult Nrg1 heterozygotes, GluA1 flip exon usage is reduced and the flop exon usage is increased compared with both WT and ErbB4 KO animals.

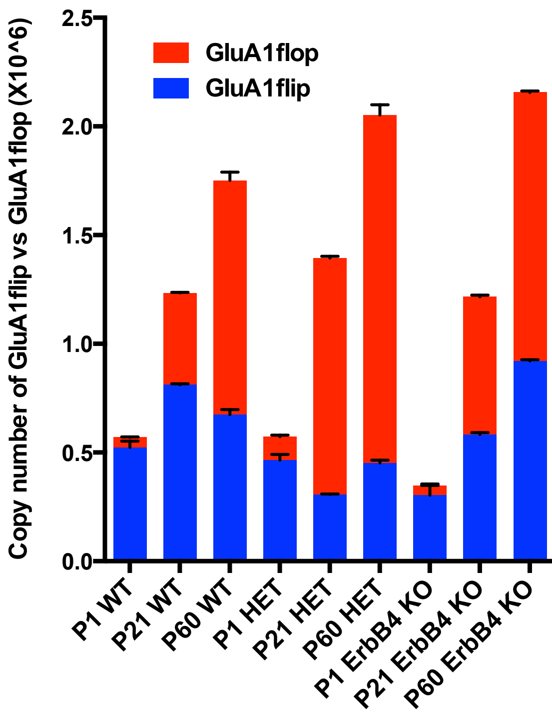

Figure 3.6. Pooled data of GluA1 flip/flop expression during accumbens maturation. Data was extrapolated from absolute qPCR values and flip/flop ratio of GluA1 (copy number from figure 3.1 × fraction of total flip or flop from figure 3.4).

I did not observe any significant difference in GluA2 mRNA splicing and GluA2 R-to-G editing between Type III-Nrg1 HET and their WT littermates at P1 and P21 (Fig 3.7). At birth, 60.8% (N=5) of GluA2 transcripts are the GluA2flip isoform. This decreases to about 45% (N=7) GluA2flip at P21. For the Type III-Nrg1 heterozygotes, at birth 64.8% (N=5) of GluA2 transcripts contain the flip exon and at P21 about 37.6% (N=5) of GluA2 transcripts contain the

flip exon. 43.4% (N=5) of GluA2 transcripts are in the edited G form in WT, while 38.6% (N=5) of Type III-Nrg1 heterozygotes are in the edited G form.

I did not observe significant differences in GluA3 mRNA splicing and GluA3 R-to-G editing between Type III-Nrg1 HET and their WT littermates at P21 (Fig 3.7). At P21, 97.17% (N=6) of GluA3 transcripts are the GluA3flip isoform in WT, and 97% are GluA3flip in Type III-Nrg1 heterozygotes. Similarly, 91.25% of GluA3 transcripts are in the edited G form in WT, while 93.5% (N=5) of Type III-Nrg1 heterozygotes are in the edited G form at P21.

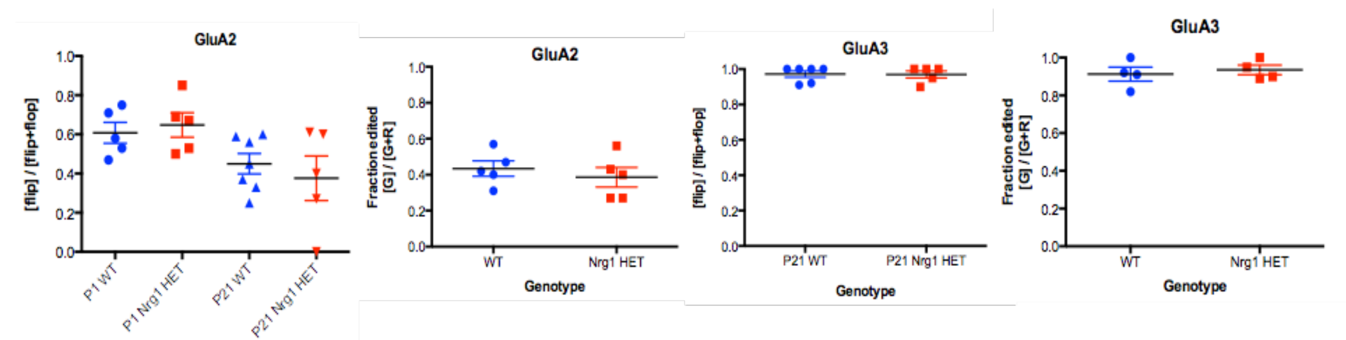

Figure 3.7. GluA2 and GluA3 splicing and A-to-I (R-to-G) editing is unaffected by presynaptic Nrg1 genotype. Accumbens from WT and Type III-Nrg1 heterozygotes at P21 were dissected and total RNA was extracted and used to synthesize cDNA. Flip/flop ratio and ratio of edited form of GluA2 and GluA3 was determined for each animal. Each dot represents the flip/flop ratio of 1 animal.

I investigated if the developmental change in expression of GluA1 splice variants is specific to a subpopulation of accumbens neurons by determining the GluA1 flip/flop ratio in individual accumbens neurons. At P21, D1- and D2 MSNs expressed both variants of GluA1 (average [flip]/[total] for ErbB4-positive MSNs: WT D1 = 48%; D2 = 44%; HET D1 = 25%, D2 = 39%; Fig 4). These findings suggest that during normal maturation, the increase in flop exon

results from the relaxation in the flip exon usage in individual nAc neurons, and this increase is most prominent in D1-positive MSNs.

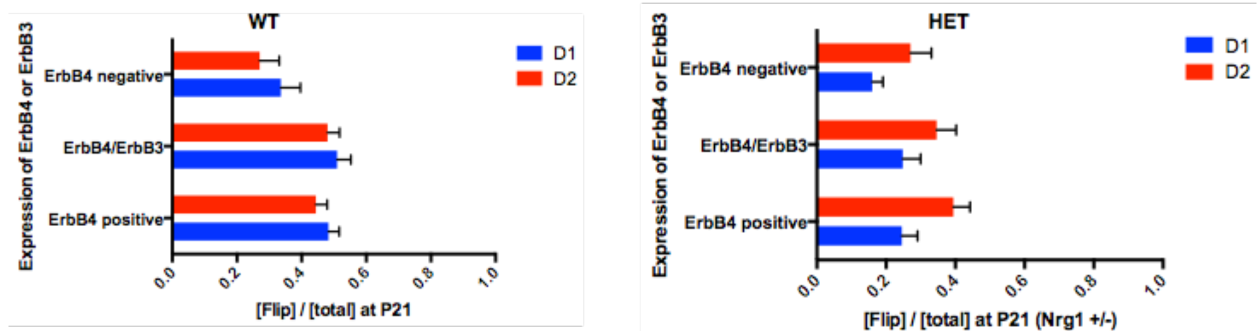

Figure 3.8. Preference for flop exon during maturation is most prominent in D1-MSNs. GluA1 [flip]/[total] ratio in individual accumbens MSNs from WT (left) and Type III-Nrg1 +/- mice (right) at P21

Alterations in splicing of GluA1 transcripts reported here are directly associated with reduced Type III-Nrg1 expression, suggesting that Nrg1 signaling could be a modulator of alternative splicing. This could be as a result of Nrg1 acting as an RNA binding protein to directly control splicing of GluA1 transcripts. This is highly unlikely, as I did not detect Type III-Nrg1 mRNA in NAcc MSNs (Fig 2.3).

I tested the hypothesis that Nrg1 might alter GluA1 splicing through its effect on the expression of known splicing regulators. Several pre-mRNA splicing factors have been implicated in the regulation of alternative splicing in the nervous system. The SR-protein (serine/arginine-rich splicing factor) SF2 mediates flop exon inclusion (Crovato and Egebjerg, 2005). Reduced expression of the SR-protein SRp38 results in flop exon inclusion (Feng et al, 2008). Down-regulation of Rbfox1 (a member of the fox family of splicing regulators) can act as a splicing enhancer or repressor and has been shown to alter expression levels of AMPAR subunit mRNA (Pistoni et al, 2013). There was no difference in NAcc mRNA levels of SF2 and

SRp38 between WT, Nrg1 +/- and ErbB4-/- at P21 and P60 (Fig 3.9). Rbfox1 mRNA expression levels was decreased in Type III-Nrg1 +/- at P21 and P60, but was similar between WT and ErbB4 KO. This suggests that Rbfox1 may be involved in the regulation of GluA mRNA processing in the nAc.

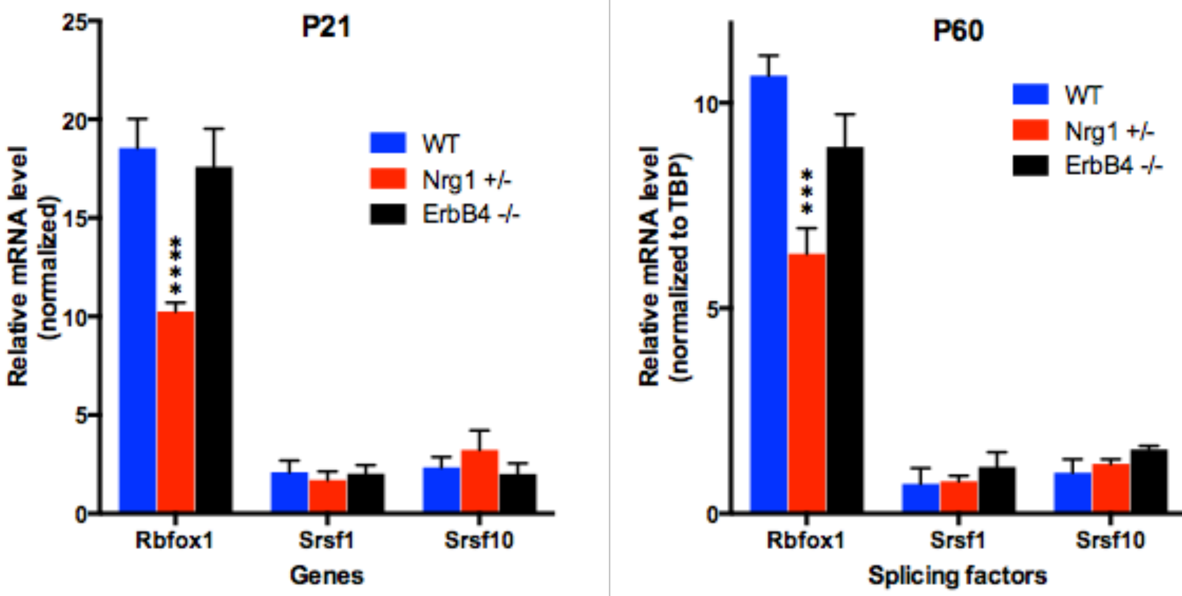

Figure 3.9. Rbfox1 is selectively down-regulated in the NAcc of Type III-Nrg1 heterozygotes. mRNA levels of Rbfox1, SF1 (Srsf1) and SRp38 (Srsf10) in the NAcc at P21 and P60.

Discussion

My major findings are that: (i) the expression of AMPA receptor subunits in the NAcc increase during maturation, (ii) the expression levels of GluA1, A2 and A4 mRNA is higher in Nrg1 +/- but not in ErbB4 -/-, (iii) expression of GluA1 flop variant increases in the NAcc during maturation and accounts for the increase in GluA1 expression, (iv) the transition from GluA1 flip to flop is accelerated in Nrg1 +/-, but not ErbB4 -/- during maturation, (v) the increase in

GluA1flop expression occurs in individual NAcc neurons and more prominent in D1-positive MSNs, (vi) GluA1flop usage is increased most in D1-positive MSNs in Type III-Nrg1 +/-, independent of ErbB4 or ErbB3 expression and (vii) expression of the alternative splicing protein, Rbfox1 is selectively reduced in Nrg1 +/-.

By comparing the levels of expression of AMPA receptor subunits and splice variants in the accumbens, we have determined a modulatory role for Nrg1 signaling that might result in altered synaptic transmission in the accumbens. I show here that the expression of AMPA receptor subunits increases in the NAcc during development, and that GluA1, GluA2 and GluA4 expression levels are altered in adult Type III-Nrg1 heterozygotes. I also show that Type III-Nrg1 regulates GluA1 splicing in the NAcc independently of ErbB4. This effect appears to be specific to GluA1 as GluA2 and GluA3 subunit levels and mRNA editing is unaltered.

Increase in AMPA receptor subunit expression at P21 may be due to the formation of new synapses during a period when innervation by glutamatergic inputs and synaptic activity is occurring. I propose that Nrg1 signaling may provide a fine-tuning mechanism in the regulation of glutamatergic transmission, by modulating the expression and flip vs flop exon usage of AMPA receptors during maturation. It has been shown that as neurons mature or as neuronal activity increases, expression levels of all four AMPA receptor subunits and mRNA editing changes (Orlandi et al, 2011). Alterations in subunit or flip vs flop variant expressed may affect AMPA channel properties by changing desensitization and resensitization time course of functional receptors (Jonas P, 2000; Sommer et al, 1990). Neurons that predominantly express the flop variant of AMPA receptors have faster densensitization kinetics whereas cells with mainly the flip isoforms exhibit slower desensitization kinetics (Lomeli et al, 1994). For example, AMPA receptors expressed in pyramidal neurons of the hippocampus and cortex have

a slow deactivation and desensitization time while inhibitory neurons express AMPA receptors with faster gating (Colquhon et al, 1992; Geiger et al, 1995; Hetrin S, 1993). In addition, developmental changes in splicing of GluA subunits occur in several other brain regions (Jonas P, 2000; Penn et al, 2012; Sommer et al, 1990).

The molecular mechanisms that modulate changes in splicing and coordinate these changes with synaptogenesis are not known. Several protein splicing regulators, such as the SR protein family and the RNA-binding fox family of proteins have been implicated in the regulation of splicing of target pre-mRNAs (Feng et al, 2008; Pistoni et al, 2013). These proteins have been shown to regulate the expression of splice variants of their target pre-mRNA. Here, I show that Rbfox1 expression is altered in the NAcc of Nrg1 +/- mice, suggesting that Rbfox1 as a downstream target of Nrg1 signaling. Change in Rbfox1 concentration has been shown to affect splicing of exons (Lee et al, 2009), especially those of transcripts that regulate synaptic function, neuronal excitation and calcium homeostasis including Grin1 (NMDA receptor 1) and Atp2b1 (calcium ATPase).

Our findings contribute significantly to advance our understanding of the modulatory role of Type III-Nrg1 in the nucleus accumbens. I show here that Nrg1 signaling within the NAcc modulates AMPA receptor subunit expression and splicing, thereby defining a specific role for Nrg1 signaling in the modulation of AMPA receptor biology within these circuits.

Chapter 4

General Discussion and Future Directions

The current view about ErbB4 expression in the accumbens comes from analysis with ErbB4-specific antibodies. As I show here, the pattern of expression of ErbB4 in the adolescent and adult accumbens explains why it has been a challenge to characterize ErbB4-positive cells in the accumbens. I found that ErbB4 expression is detectable in MSNs in later stages of development. ErbB4 is expressed in the soma of accumbens neurons at birth, and in dendrites of of accumbens neurons in adults. I observed that over 70% of ErbB4-positive neurons in the accumbens at birth are MSNs and over 70% in adolescent accumbens are MSNs. ErbB4 expression appears to be absent from MSNs in adult accumbens.

I found that reduced Type III-Nrg1 results in incease in AMPA receptor subunit expression levels and specifically alters the developmental profile of GluA1 splicing. GluA1, GluA2 and GluA4 expression levels are increased in the adult accumbens of Type III-Nrg1 heterozygous animals. Expression of splice isoforms of GluA1 transcripts changes during development, from over 90% GluA1 flip at birth, to about 65% GluA1 flip in adolescent accumbens, down to about 40% GluA1 flip in adult accumbens. In Type III-Nrg1 heterozygotes, the developmental profile of GluA1 splice isoforms is altered, such that the GluA1 flip/flop ratio of adolescent accumbens is similar to that of adults (about 25%). The developmental profile of GluA1 splice isoforms in the accumbens of ErbB4 knock out animals is similar to that of WT animals. Thus, the Type III-Nrg1 genotype, not ErbB4 genotype, modulates the flip vs flop exon expression of GluA1 during maturation.

ErbB4 expression in perinatal accumbens neurons

Striatal interneurons migrate from the MGE between E12 and E15, while the MSNs migrate from the LGE between E13 and E18 (Hamasaki et al, 2001; Marin et al, 2000; Phelps et al, 1989; Song and Harlan, 1994; Wichterle et al, 2001 and reviewed in Marin and Rubenstein, 2001). Nrg1/ErbB4 signaling has been shown to play critical roles in the migration of neurons (Anton et al, 1997; Flames et al, 2004; Lopez-Benditto, 2006). ErbB4 is expressed in a subset of interneurons that migrate to the cortex by passing through a permissive corridor formed by the expression of Type III-Nrg1. Indeed, alteration in the number of GABAergic interneurons in the cortex has been observed in ErbB4 knock out animals (Flames et al, 2004). I found that ErbB4 is expressed in the soma of NAcc cells at birth, a period immediately after accumbens neurons have migrated from the LGE. I speculate that ErbB4 may play important roles in the migration of neurons from the MGE and LGE towards the accumbens.

ErbB4 expression in the accumbens during development

Nrg1/ErbB4 signaling has been shown to be involved in several developmental processes in the CNS, including in synaptogenesis (Li et al, 2007; Yang et al, 1998). Specifically, Nrg1/ErbB4 signaling has been implicated in the activity-dependent maturation of excitatory synapses, and the activity-driven development of glutamatergic synapses involves the recruitment of ErbB4 to synaptic sites. Loss of Nrg1/ErbB4 signaling results in decreases in dendritic spines, depotentiation of synaptic AMPA currents and impairments in the normal activity-dependent development of glutamatergic synapses. Overexpression of ErbB4 results in increase in dendritic spines and enhancement of synaptic AMPA currents. I found that ErbB4 is expressed in the soma of accumbens neurons at birth, but targeted to the dendrites and

postsynaptic sites in adolescent and adult accumbens. During the development of the accumbens, synaptic activity increases during adolescence as a result of inputs from presynaptic glutamatergic projections into the accumbens. We speculate that a similar event may be occurring in the accumbens, whereby ErbB4 is recruited to synaptic sites during development. This may explain the subcellular localization of ErbB4 in later stages of development of the accumbens.

ErbB4 expression in accumbens neurons

I found that ErbB4 is expressed by a majority of MSNs at birth and in adolescent accumbens. ErbB4 is expressed by GABAergic interneurons in the cortex and hippocampus (Fox and Kornblum, 2005; Kornblum et al, 2000, Yau et al, 2003). In the hippocampus, Nrg1 interacts with ErbB4 expressed on GABAergic neurons to regulate network activity by modulating the integration of dopaminergic and glutamatergic transmission (Kwon et al, 2008). Nrg1/ErbB4 signaling in the hippocampus and cortex have also been shown to increase gamma oscillations, which are important for maintenance of appropriate activity within neuronal networks and proper structuring of information processing within brain circuits (Fisahn et al, 2009). Gamma oscillations have been observed within striatal cirtcuits (Block et al, 2007; DeCoteau et al, 2007; Gruber et al, 2009; Tort et al, 2008; van der Meer, 2009; van der Meer, 2010) and Type III-Nrg1 heterozygotes have altered connectivity between the ventral hippocampus and ventral striatum (Nason et al, 2011). I propose that Type III-Nrg1 is important for the maintenance of gamma oscillations and the integration of functional glutamatergic inputs onto ErbB4-expressing neurons in the accumbens.

The properties of AMPA-type glutamate receptors are regulated during development

The kinetic properties, calcium ion permeability and synaptic targeting of AMPA receptors are regulated in a developmental and tissue-specific manner in the CNS (Geiger et al, 1995; Sommer et al, 1990). Specific factors that regulate these developmental changes include change in AMPA receptor subunit expression levels (GluA1-GluA4), change in the flip vs flop splice variant of AMPA receptor subunit expressed, change in transcriptional processing that replaces an adenosine with inosine (A to I editing) resulting in a glutamine residue being replaced by an arginine residue in GluA2 (Q to R), and another A-to-I editing that replaces an arginine residue with glycine (R to G) in GluA2 to GluA4. The Q to R editing event is essential in postnatal animals and results in GluA2-containing AMPA channels that are impermeable to Ca^{2+} ions. These changes, together with change in expression of flip-flop splice variants result in AMPA channels with different desensitization and resensitization rates, and alterations in trafficking of AMPA receptors.

My findings here indicate that reduced Type III-Nrg1 or deletion of ErbB4 alters the expression levels of AMPA receptor subunits in the accumbens. Reduced Type III-Nrg1 alters the developmental profile of GluA1 splice variants, independently of ErbB4. I found that ErbB3 is expressed by a subset of accumbens MSNs. Since ErbB3 lack a kinase domain, it is possible that ErbB3 expressed by accumbens neurons could act as a ligand for Type III-Nrg1 expressed in presynaptic axons in the accumbens to activate back signaling. It has been shown that synaptic activity can activate back signaling by Type III-Nrg1 and can also target ErbB4 to synaptic sites in glutamatergic synapses. Indeed, the presence of ErbB receptors may not be essential to activate Type III-Nrg1 signaling in adolescent accumbens, a period of synaptic maturation in the accumbens.

Future directions

AMPA receptors are the mediators of fast excitatory synaptic transmission in the CNS and are important for synaptic plasticity and a variety of brain functions. Therefore, understanding how AMPA receptor function is regulated in the CNS is critical to defining their role in CNS disorders.

It has been shown that activity can induce AMPA receptor subunit mRNA transport to dendrites, suggesting that sub-cellular localization of AMPA receptors could be a mechanism for regulation of glutamatergic neurotransmission in response to specific stimuli (Grooms et al, 2006; La Via et al, 2012). RNA editing, splicing and trafficking may all be coordinated events that provide a mechanism for regulation of AMPA receptor channel properties and function.

I have found that reduced Type III-Nrg1 signaling alters the developmental profile of GluA1 subunit of AMPA receptors in the accumbens. GluA2 and GluA3 flip-flop profile are unaffected by reduced Type III-Nrg1 signaling, suggesting that Nrg1 effect on AMPA receptor flip-flop profile in the accumbens is specific to GluA1. The flip-flop profile of ErbB4 knock out animals is similar to that of WT animals, suggesting that Type III-Nrg1 regulates the developmental profile of GluA1 isoforms independently of ErbB4. Since the effect of flip or flop isoforms on AMPA receptor trafficking has been documented, I attempted to ask if reduced presynaptic expression of Type III-Nrg1 had any effect on surface expression of GluA1 in dispersed accumbens neurons. In order to do this, I employed a specialized preparation of hippocampal-accumbens circuits in vitro to study the presynaptic and/or postsynaptic contributions of Type III-Nrg1/ErbB4 binding partners on accumbens synapses. This technique involved plating of ventral hippocampal (vHIPP) microexplants in minimal medium to allow for spreading of the tissue, after which dispersed accumbens neurons are added to the preparation [13].

The technique is ideal for this study because (i) presynaptic and postsynaptic components come from different mice, thereby allowing me to specifically alter either the presynaptic or postsynaptic genotype; (ii) I can combine brain regions that are normally connected in vivo by fiber paths that cannot be maintained or readily identified in acute slices and study the physiological or morphological effects of presynaptic or postsynaptic factors at the cellular or molecular level. Using this technique, our lab has studied the presynaptic effects of reduced Type III-Nrg1 signaling in vHIPP-NAcc co-cultures (Zhong et al, 2008; Zhong et al, 2013).

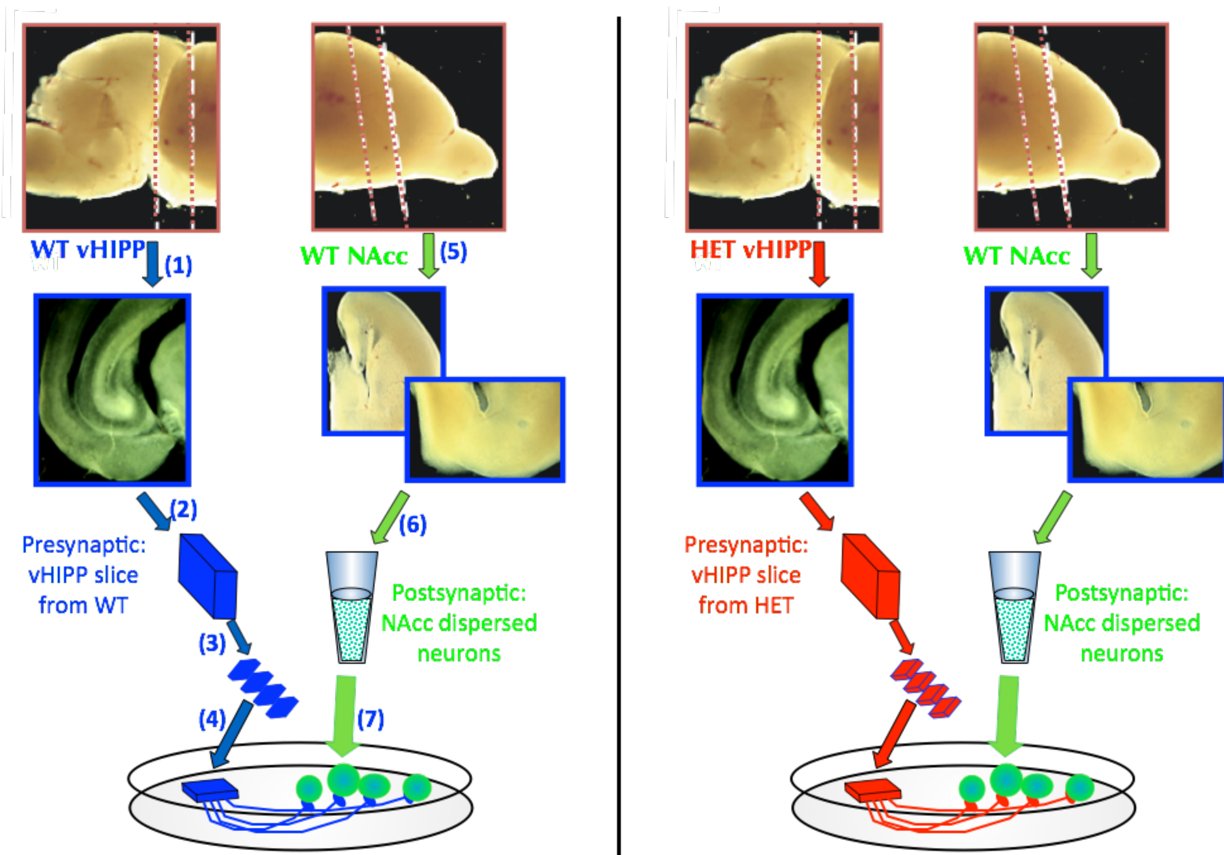

Figure 4.1. Heterogenotypic hippocampal-accumbens circuits in vitro. vHIPP microslices from WT or Type III-Nrg1 Heterozygote mice are plated and allowed to spread. Steps in making vHIPP-NAcc co-culture are shown in the illustration: (1) Slice containing ventral hippocampal region is obtained from WT brain (left) or Type III-Nrg1 heterozygotes; (2) vHIPP region is

isolated from the brain slice; (3) vHIPP region is cut into microslices of about 150 × 150μm; (4) microslices are plated in minimal medium and allowed to spread overnight; (5) Slice containing NAcc region is obtained from WT brain and NAcc region is isolated from the brain slice; (6) NAcc tissue is digested to obtain dispersed NAcc neurons; (7) dispersed NAcc neurons are plated with vHIPP microslices.

Preliminary results: Presynaptic Nrg1 increases the number of neurites on NAcc neurons

I investigated the effect of presynaptic reduction of Type III-Nrg1 on accumbens neurons in vitro. I incubated N-terminal anti-GluA1 (surface GluA1 staining) and C-terminal anti-GluA1 (total GluA1 staining) in three experimental groups: (i) dispersed accumbens neurons without vHIPP microslices; (ii) dispersed accumbens neurons in co-culture with vHIPP microslices from Type III-Nrg1 heterozygotes and (iii) dispersed accumbens neurons in co-culture with vHIPP microslices from WT animals. I observed that the morphology of NAcc neurons in culture differ, depending on the presynaptic Type III-Nrg1 genotype (Fig 4.2). DIV6 NAcc neurons without vHIPP presynaptic input exhibited a reduced number of primary neurites (neurites originating from the soma). NAcc neurons in co-culture with presynaptic vHIPP microslices from Type III-Nrg1 heterozygotes exhibited an increase in the number of primary neurites compared to dispersed NAcc neurons without presynaptic inputs. NAcc neurons in co-culture with WT presynaptic vHIPP microslices exhibited an increased number of primary neurites (Fig 4.3).

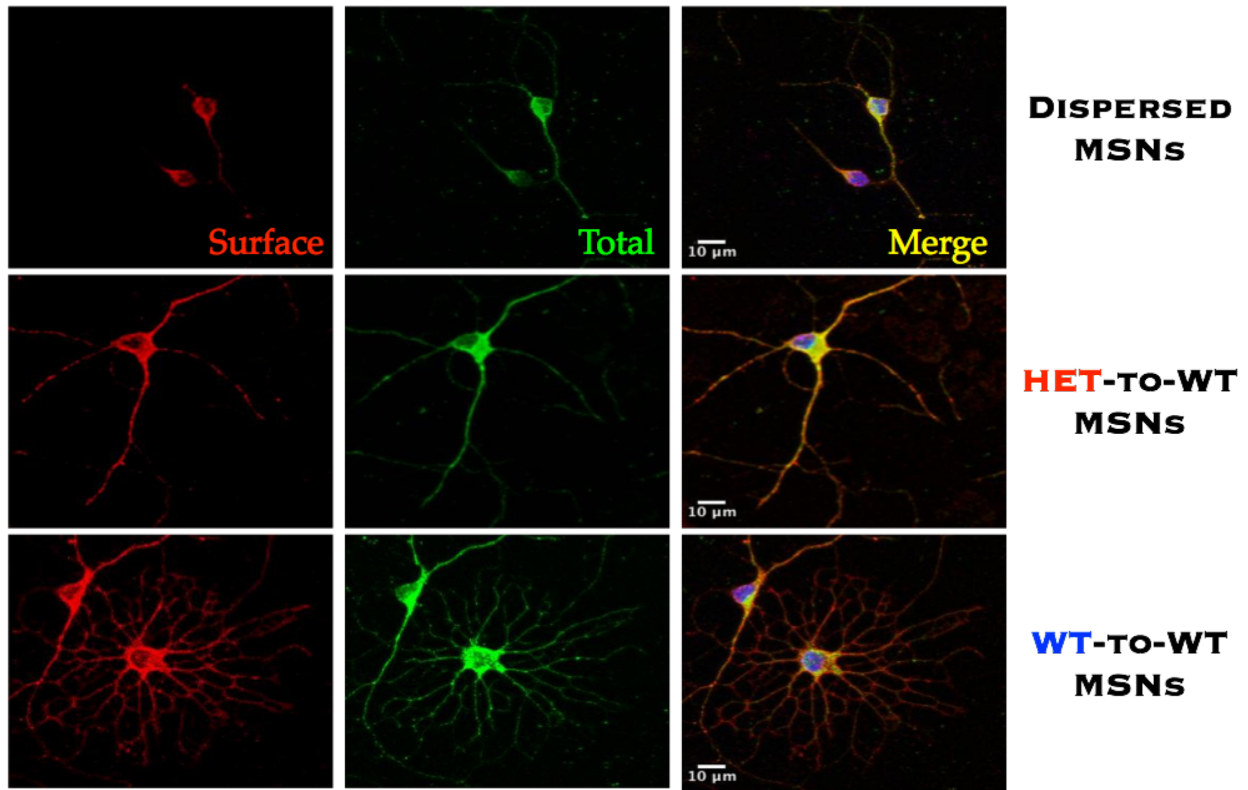

Figure 4.2. Presynaptic Type III-Nrg1 increases the number of neurites in accumbens neurons. Dispersed DIV6 NAcc neurons were processed for immunostaining of surface GluA1 (red) or total GluA1 (green). Endogenous GluA1 was detected both in the cell bodies and in neurites. Blue staining is DAPI. NAcc neurons cultured without vHIPP microslices had fewer primary neurites compared to those cultured with vHIPP from Type III-Nrg1 heterozygotes. NAcc neurons cultured with WT vHIPP microslices displayed an increased number of neurites compared to those cultured with vHIPP microslices from Type III-Nrg1 heterozygotes.

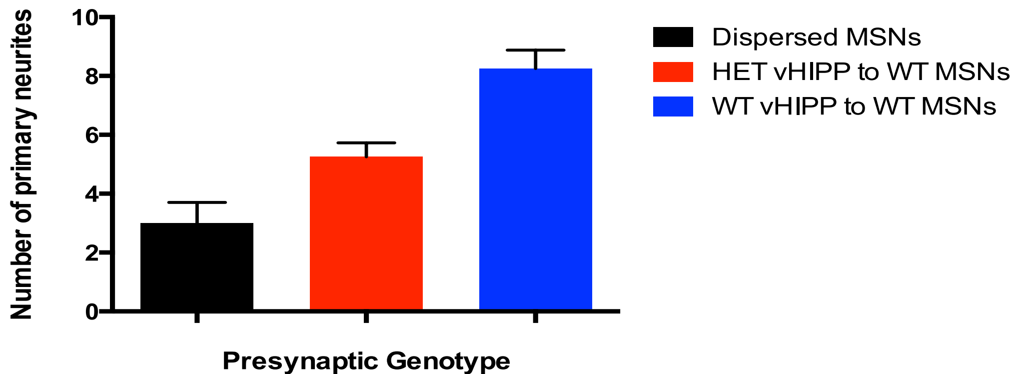

Figure 4.3. Type III-Nrg1 functions in the development of neurites in accumbens neurons. The total number of primary neurites (neurites visibly originating from the soma) was analyzed. NAcc neurons without vHIPP microslices showed fewer numbers of neurites (black bar; 9 neurons, 3 animals). NAcc neurons cultured with vHIPP microslices from Type III-Nrg1 heterozygotes showed increased number of neurites compared to those without vHIPP microslices (red bar; 9 neurons, 3 animals), but fewer numbers of neurites compared to NAcc neurons cultured with vHIPP microslices from WT animals (blue bar; 6 neurons, 2 animals).

My preliminary results suggest that presynaptic Type III-Nrg1 plays a role in the development of neurites in accumbens neurons. I observed that the increase in the number of neurites in NAcc neurons in culture with WT vHIPP microslices occurs in NAcc neurons in close proximity with vHIPP microslice. I speculate that only NAcc neurons in synaptic connections with projections from vHIPP microslices exhibit increased neurite number.

Questions raised by our preliminary results include:

i. What is the role of synaptic activity on NAcc neuron development and GluA1 expression?

ii. What is the effect of presynaptic Type III-Nrg1 on NAcc neuron GluA1 surface/total levels?

iii. What signaling mechanisms of Type III-Nrg1 is utilized in the modulation of NAcc neuron development?

Chapter 5

Materials and Methods

Animals:

Nrg1$^{Lwrtm1+/-}$ (Wolpowitz et al, 2000) and ErbB4$^{-/-}$ Her4^{heart} (Hester et al, 2003) mouse lines were back-crossed onto a C57 BL6 background. Nrg1$^{Lwrtm1-/-}$ mice die at birth and were thus excluded from this study. WT mice were generated from crossings between Type III-Nrg1 heterozygotes. Animals were maintained in a 12hr light/dark cycle and were provided with food and water ad libitum.

Immunohistochemistry:

Isolation and preservation of tissues:

P21 and adult animals were anesthetized with a ketamine:xylazine cocktail, and transcardially perfused with cold PBS followed by 4% PFA. P1 animals were sacrificed by cervical dislocation, and the brains were drop-fixed in 4% PFA. Brains were post-fixed in 4% PFA in PBS overnight and cryoprotected in 30% sucrose for at least 2days, ensuring that they sank to the bottom of the sucrose solution. Brains were embedded immediately in freezing media (TBS, Triangle Biomedical Sciences) and littermates were sectioned in 25µm coronal slices on a vibratome and mounted onto charged, pre-coated slides (Superfrost Plus, Fisher) and processed for immunofluorescent staining. Antigen retrieval of sections was performed in 0.1M citrate buffer, pH 6.0 for 8-10 mins at 94-100°C. Sections were then left in antigen retrieval buffer and allowed to cool to room temperature, then washed by submerging first in water and then twice in PBS for 15 mins each, before blocking and permeabilizing in 10% normal donkey serum/0.5% Triton-X100/0.1M PBS for 1 hr at room temperature. Sections were subsequently incubated in primary

antibodies diluted in blocking solution and incubated overnight at room temperature, followed by three washes in PBS for 15 mins each. Sections were incubated in secondary antibody at room temperature for 45 mins, then washed three times by submerging in PBS for 15min each. Finally, sections were mounted with DAPI/Fluoromount G (Southern Biotech) and allowed to cure at least for 2hours at 4°C

<u>Antibodies:</u>

Species	**Antigen**	**Source**	**Item #**	**Dilution**
Mouse	ErbB4	Neomarker	Ms-270	1:200
Rabbit	ErbB4	Thermo Scientific	Rb-9045	1:200
Chicken	MAP2	Novus	NB300-213	1:10,000
Guinea pig	GFAP	Advanced Immunochemical		1:200
Donkey	Anti-mouse, Anti-rabbit, Anti-chicken, Anti-guinea pig	Invitrogen	Life Technologies	1:500

Table 5.1. Antibodies, source and dilutions

Immunoblot analysis

Nucleus accumbens was isolated from P1, P21 and adult mice and lysed in an NP-40 lysis buffer (50mM Tris pH 7.4, 250mM NaCl, 5mM EDTA, 50mM NaF, 1mM Na_3VO_4, 1% Igepal and 1X

protease inhibitors) and homogenized by passing through a pipette tip several times followed by incubation in an Ultrasonic Cleaner with ice waterbath for at least 20min. Lysate was incubated at 4°C with constant mixing for 30mins, and centrifuged for 10,000rpm at 4°C for 10mins. Supernatant was transferred to a new tube, incubated at 37°C in SDS sample buffer with beta-mercaptoethanol, separated on a 8% SDS-PAGE gel and transferred to a nitrocellulose membrane (Whatman). Membrane was blocked in 5% BSA in Tris-buffered saline with 0.1% Tween-20 (TBS-Tween) overnight at room temperature and incubated in GluA1 (Millipore MAB2263, 1:1000), GluA2 (Santa Cruz sc-7611, 1:500) or β-tubulin (Sigma T8660, 1:1000) primary antibodies in blocking solution, for ≥5hrs at room temperature. Membranes were washed with TBS-Tween three times 15mins each at room temperature, incubated in secondary antibodies conjugated to IRDye-700 or 800 (1:5000, from Rockland) for 45mins-1hr at room temperature. Bands on membranes were visualized using an Odyssey Infrared Imaging System (Li-Cor Biosciences) and analyzed using Odyssey Image Analysis Software. Intensity of GluA1 or GluA2 bands were normalized to that of β-tubulin to control for loading error or differential blot transfer. Ratios of GluA1 or GluA2 to β-tubulin were compared by genotype.

RNA extraction and DNA analysis

<u>RNA extraction</u>

Accumbens tissues were dissected out from an acute slice and homogenized in Trizol Reagent (Invitrogen Cat. # 15596-026) on ice by passing through a narrow 10μl narrow pipette several times. RNA was extracted using the Trizol method. RNA pellets were dissolved in TE buffer with 0.5mM EDTA (pH9.0).

cDNA synthesis

Reverse-transcription of RNA was performed using Superscript III first-strand cDNA synthesis system (Invitrogen Cat # 18080-051). Amplicons were synthesized by PCR using gene-specific primers.

Prime sequences:

	Forward	**Reverse**
Type III-Nrg1	GTACTGTTTGCCGTGATGCTC	TTGGTTCAAGAAGGCAGGGG
ErbB4	CGGCTCCATCTGTGTTGAGT	ACTGTTTGCTCCCTGTAGGC
ErbB3	CGGTTCCGGAGGGGATTATG	TGCCAGTAATCGGGGTTGTC
Dopamine D1 receptor	CATGGGGGTATTCGTGTGCT	CCCACAGCATGAGGGATCAG
Dopamine D2 receptor	CTGGTGGCCACACTGGTTAT	CCATTCTCCGCCTGTTCACT
Parvalbumin	AGACAAAAGTGGCTTCATTG	AGCTTTCAGCCACCAGAGTG
Choline acetyltranseferase	GATCTGGCAACTTCGTCGGA	CCCCAAACCGCTTCACAATG

Table 5.2. Primer sequences

Real-time PCR:

cDNA was synthesized from equal amounts of RNA (100ng) using an equal amount of random hexamers and oligo dT primers. To synthesize control plasmids for generation of calibration curves, amplicons were synthesized from whole brain cDNA using gene-specific primers, cloned into a plasmid, transformed into E. coli, mini-prepped and verified by EcoRI digestion which

releases the amplicon cassette cloned into the plasmid. The following plasmids were generated this way: pTOPO-GluA1, pTOPO-GluA2, pTOPO-GluA3, pTOPO-GluA4, pTOPO-TBP, pTOPO-ErbB4, pTOPO-α7nAChR. Several serial dilutions of each control plasmid was made in 0.1% Tween 20, after which a master mix for each primer set was prepared. Master mixes contained the following: SYBR Green I Premix Ex Taq (Clontech Cat # RR420L), 0.2μM primer pair for each primer set, cDNA and sterile distilled water. Triplicates of each set of dilution points for each primer was pipetted into corresponding wells, followed by samples in duplicates, in a total reaction volume of 15μl. The following conditions were utilized for the PCR run in LightCycler 480 system:

Denature:	95°C, 30sec.	(Ramp rate 4.4°C/sec)	1cycle
PCR (Analysis mode: Quantification):	95°C, 5sec	(Ramp rate 4.4°C/sec)	
	60°C, 30sec	(Ramp rate 2.2°C/sec., acquisition mode: Single)	
	40cycles		
Melting (Analysis mode: Melting Curves):	95°C, 5sec	(Ramp rate 4.4°C/sec)	
	60°C, 1min	(Ramp rate 2.2°C/sec)	
	95°C	(Ramp rate 0.11°C/sec, acquisition mode: Continuous, Acquisitions: 5 per °C	
	1 cycle		
Cooling	50°C, 30sec	(Ramp rate 2.2°C/sec); 1 cycle	

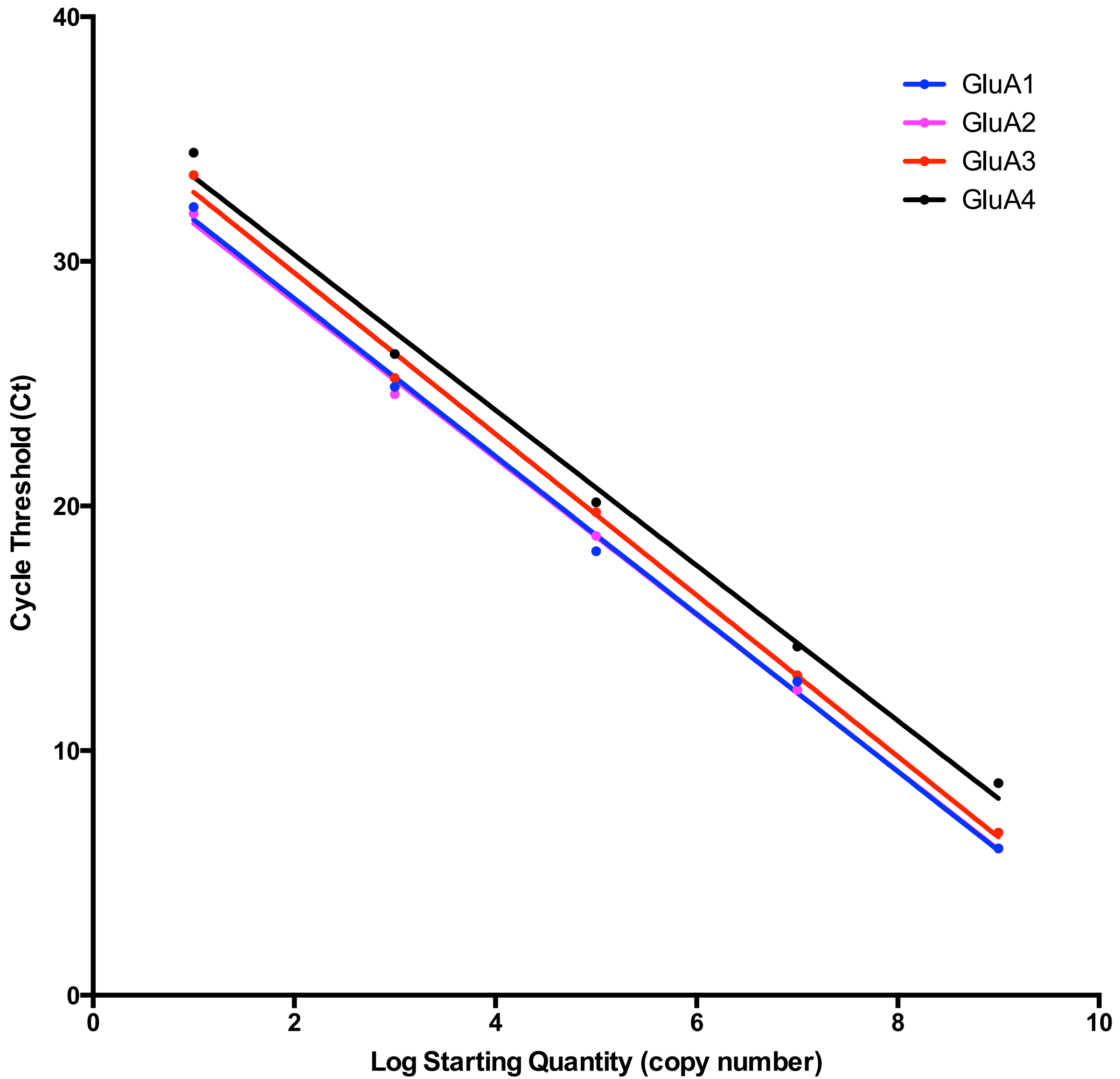

Figure 5.1. Standard curve for GluA1-GluA4 primers used for qPCR. Standard curves were obtained by plotting the Ct values against the log initial quantity (copy number).

<u>Log Fit Values:</u>

GluA1: $R^2 = 0.9974$; $Y = -3.224*X + 34.93$

GluA2: $R^2 = 0.9987$; $Y = -3.199*X + 34.75$

GluA3: $R^2 = 0.9964$; $Y = -3.296*X + 36.13$

GluA4: $R^2 = 0.9937$; $Y = -3.176*X + 36.63$

Primer Sequences for qPCR:

	Forward	**Reverse**
GluA1	AATCAGTTTGTCCTTCAGCTGC	TTCTTCTCGGCGGCTGTATC
GluA2	GCATCGCCACACCTAAAGGA	AGCAACGTTGCTCAGACTGA
GluA3	TGTGGCAACCCCTAAAGGCT	AGGCTTAGAGCACTGGTCTTG
GluA4	TCCAAAGGCTATGGTGTAGCG	AGGCTCAAGGCACTCGTCTT

Table 5.3. Primer sequences for qPCR

Single-cell RT-PCR

Using a recording pipette, cytoplasmic contents of individual cells from the accumbens region in acute coronal slice were aspirated and expelled into a test tube by positive pressure. The pipette tip was broken into the tube and High Capacity RNA-to-cDNA RT buffer was added to the tube. Tubes were placed on a rack and incubated in an Ultrasonic Cleaner with ice waterbath for at least 10min. RT enzyme mix was added and the reaction was incubated at 37°C for at least 1hr followed by 95°C for 5mins. Two rounds of nested PCR was performed using gene-specific primers.

GluA flip/flop analysis

GluA1

1μl cDNA (synthesized from 150ng of accumbens RNA) was used as template for PCR reaction to amplify a 597bp gene fragment spanning exon 13 and 16 (sequence shown below). The region

amplified spans the flip-flop cassette, allowing the primer pair to amplify both flip and flop variants. 4µl of PCR amplicons were cloned into a vector by TOPO-TA cloning (Invitrogen) and transformed into *E. coli*. At least 20 clones were picked from each transformation reaction, each clone was analyzed to determine if it expressed the flip or flop variant either by sequencing, or by a second round of PCR using forward primer from first PCR reaction with a different reverse primer. This amplification yields a 246bp fragment for GluA1flop and a 322bp fragment for GluA1flip that can readily be identified in an agarose gel. Using this method, I confirmed that I could predict the flip/flop ratio by cloning vectors with known amounts of flip or flop variants into *E. coli*, and picking clones to predict the ratio in each sample.

GluA1 flop and flip sequences from first amplification (NM_008165.4). Location of first round PCR primer sequence within GluA1 sequence is shown in bold letters; codons on GluA1 sequence that differ between flip and flop variants are indicated in red; location of reverse primer for second round PCR primer sequence is in bold and underlined.

GluA1Flop sequence (NM_008165.4); 2511-3086)

GAA CCG TCT GTG TTT GTT CGG ACC ACA GAG GAG GGC ATG ATC AGA GTG AGA AAG TCT AAA GGC AAA TAT GCC TAC CTC CTG GAG TCC ACC ATG AAT GAG TAC ATT GAG CAA CGC AAG CCC TGT GAC ACC ATG AAA GTG GGA GGT AAC TTG GAT TCC AAA GGC TAT GGC ATT GCA ACA CCC AAG GGG TCC GCC CTG AGA AAT CCA GTA AAC CTG GCA GTG TTA AAA CT**G AAC GAG CAG GGG CTT TTG** GAC AAA TTG AAA AAC AAA TGG TGG TAC GAC AAG GGC GAG TGC GGC AGC GGG GGA GGT GAC TCC AAG GAC AAG ACC AGT GCT CTG AGC CTG AGC AAT GTG GCA GGC GTG TTC TAC ATC CTG ATT GGA GGG CTG GGA TTG GCC ATG CTG GTT GCC TTA ATC GAG TTC TGC TAC AAA TCC CGT AGC GAG TCG AAG CGG

ATG AAG GGT TTC TGT TTG ATT CCA CAG CAA TCC ATC AAT GAA GCC ATA CGG

ACA TCG ACC CTC CCC AGG AAC AGC GGG GCA GGA GCC AGC GGA GGA AGT

GGC AGT GGA GAG AAT GGC AGA GTG GTC AGC CAG GAC T **TC CCC AAG TCC**

ATG CAA TCC

GluA1 Flip sequence (NM_001113325.2; 2511-3086).

GAA CCG TCT GTG TTT GTT CGG ACC ACA GAG GAG GGC ATG ATC AGA GTG AGA AAG TCT AAA GGC AAA TAT GCC TAC CTC CTG GAG TCC ACC ATG AAT GAG TAC ATT GAG CAA CGC AAG CCC TGT GAC ACC ATG AAA GTG GGA GGT AAC TTG GAT TCC AAA GGC TAT GGC ATT GCA ACA CCC AAG GGG TCC GCC CTG AGA GGT CCC GTA AAC CTA GCG GTT TTG AAA CTC AGT GAG CAA GGC GTC TTA GAC AAG CTG AAA AGC AAA TGG TGG TAC GAT AAA GGG GAA TGT GGA AGC AAG GAC TC**C GGA AGT AAG GAC AAG ACC AGT** GCT CTG AGC CTG AGC AAT GTG GCA GGC GTG TTC TAC ATC CTG ATT GGA GGG CTG GGA TTG GCC ATG CTG GTT GCC TTA ATC GAG TTC TGC TAC AAA TCC CGT AGC GAG TCG AAG CGG ATG AAG GGT TTC TGT TTG ATT CCA CAG CAA TCC ATC AAT GAA GCC ATA CGG ACA TCG ACC CTC CCC AGG AAC AGC GGG GCA GGA GCC AGC GGA GGA AGT GGC AGT GGA GAG AAT GGC AGA GTG GTC AGC CAG GAC T**TC CCC AAG TCC ATG CAA TCC**

GluA1 primer sequences

i. Pan GluA1-For597 (forward): GAACCGTCTGTGTTTGTTCGG

ii.Pan GluA1-Rev597 (reverse): GGATTGCATGGACTTGGGGA

b. Colony PCR using the following primers:

i. Pan GluA1-For597: GAACCGTCTGTGTTTGTTCGG

ii. GluA1-FlipRev322: TGGTCTTGTCCTTACTTCCGG

iii. GluA1-FlopRev246: CAAAAGCCCCTGCTCGTTC

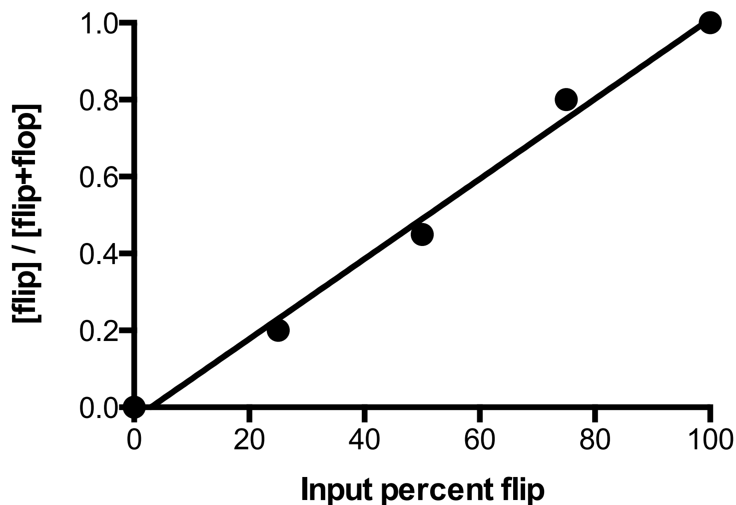

Figure 5.2. Predicting the input flip/flop ratio from samples with known amounts of both flip and flop variants. GluA1flip and flop variants were mixed in different proportions. Using cloning method described above, the proportion of flip/flop variants in each sample could be predicted after picking ≥ 15 colonies for PCR. $R^2 = 0.9912$.

GluA2

GluA2 flip/flop ratio was determined in a similar way to GluA1flip/flop ratio. Second round PCR amplification yields a 469pb fragment for GluA2flop and a 548bp fragment for GluA2flip that can readily be identified in an agarose gel.

First amplification yielding both GluA2flip and GluA2flop. Location of first round PCR primer sequence within GluA2 sequence is shown in bold letters; codons on GluA2 sequence that differ between flip and flop variants are indicated in red; codon for Arginine residue (on the R-G site) is in blue; location of reverse primer for second round PCR primer sequence is in bold and underlined.

GluA2 Flop (NM_013540.2), 2511-3086 (598bp):

A TCT CTC TCT GGG CGC ATT GTT GGA GGT GTG TGG TGG TTC TTT ACC CTC ATC ATC ATC TCC TCC TAC ACG GCT AAC TTA GCT GCC TTC CTG ACT GTA GAG AGG ATG GTG TCG CCC ATC GAA AGT GCT GAG GAT CTG TCT AAG CAA ACA GAA ATT GCT TAT GGA ACA TTA GAC TCT GGC TCC ACT AAA GAG TTT TTC AGG AGA TCT AAA ATT GCA GTG TTT GAT AAA ATG TGG ACT TAT ATG AGG AGT GCA GAG CCC TCT GTG TTT GTG AGG ACT ACG GCA GAA GGA GTA GCC AGA GTC AGG AAA TCC AAA GGG AAG TAT GCC TAC TTG CTG GAG TCC ACA ATG AAT GAG TAC ATC GAG CAG AGG AAG CCT TGC GAC ACC ATG AAA GTG GGC GGC AAC CTG GAT TCC AAA GGC TAC GGC ATC GCC ACA CCT AAA GGA TCC TCA TTA AGA AAT GCG **GTT AAC CTC GCA GTA** CTA AAA CTG AAT GAA CAA GGC CTG TTG GAC AAA TTG AAA AAC AAA TGG TGG TAC GAC AAA GGA GAG TGC GGC AGC GGG GGA GGT GAT TCC AAG GAA AAG ACC AGT GCC C**TC AGT CTG AGC AAC GTT GCT**

GluA2 Flip (NM_001083806.1), 2511-3086 (598bp):

A TCT CTC TCT GGG CGC ATT GTT GGA GGT GTG TGG TGG TTC TTT ACC CTC ATC ATC ATC TCC TCC TAC ACG GCT AAC TTA GCT GCC TTC CTG ACT GTA GAG AGG ATG GTG TCG CCC ATC GAA AGT GCT GAG GAT CTG TCT AAG CAA ACA GAA ATT GCT TAT GGA ACA TTA GAC TCT GGC TCC ACT AAA GAG TTT TTC AGG AGA TCT AAA ATT GCA GTG TTT GAT AAA ATG TGG ACT TAT ATG AGG AGT GCA GAG CCC TCT GTG TTT GTG AGG ACT ACG GCA GAA GGA GTA GCC AGA GTC AGG AAA TCC AAA GGG AAG TAT GCC TAC TTG CTG GAG TCC ACA ATG AAT

GAG TAC ATC GAG CAG AGG AAG CCT TGC GAC ACC ATG AAA GTG GGC GGC AAC CTG GAT TCC AAA GGC TAC GGC ATC GCC ACA CCT AAA GGA TCC TCA TTA **AGA** ACC CCA GTA AAT CTT GCA GTA TTG AAA CTC AGT GAG CAA GGC GTC TTA GAC AAG CTG AAA AAC AAA TGG TGG TAC GAT A**AA GGT GAA TGT GGA GCC AAG G**AC TCG GGA AGT AAG GAA AAG ACC AGT GCC C**TC AGT CTG AGC AAC GTT GCT**

GluA2 primers

i. Pan GluA2-For598: ATCTCTCTCTGGGCGCATTG

ii.Pan GluA2-Rev598: AGCAACGTTGCTCAGACTGA

b. Colony PCR using the following primers:

i. Pan GluA2-For598: ATCTCTCTCTGGGCGCATTG

ii. GluA2-FlipRev548: CCTTGGCTCCACATTCACCTT

iii. GluA2-FlopRev469: TACTGCGAGGTTAACCGCATT

GluA2 A-I (R-G) Editing Analysis:

a. Colony PCR using Pan-GluA2 primers. Amplicons were either sequenced or run on agarose gel following digestion with MseI. Fragment size from digestion of each variant are listed below:

i. GluA2 flip-R: 456 & 142bp fragments

ii.GluA2 flip-G: Uncut (598bp)

iii.GluA2 flop-R: 456 & 142bp fragments

iv.GluA2 flop-G: Uncut (598bp)

GluA3:

Determination of GluA3flip/flop ratio and R-G editing was carried out using a similar cloning method as outlined for GluA1 and GluA2. Amplicons from second round PCR were identified by sequencing using T3 primers located upstream of the cloned cassette in the TOPO-vector.

Pan GluA3 (forward primers): ATACGATGAAAGTTGGTGGAAATC

Pan GluA3 (reverse primers): ACTGGTCTTGTCCTTGGAGTCACC

Data analysis and Statistics

Data analysis was performed using Prism and Statview and are reported as ± SEM. Two-tailed Student's T-test and ANOVAs were used for comparisons. Two-tailed T-test of ANOVA using Tukey's Honest Significance Difference Test was used to correct for multiple comparisons. The asterisk (*) indicates statistical significance at the $p < 0.05$ level in between-group comparisons.

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