# Morphological characterization of medium spiny neuron development *in vitro*

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Rachel Dobrof Penrod-Martin

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Dr. Lorene Lanier, Co-Advisor
Dr. Mark Thomas, Co-Advisor

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## Dedication

This dissertation is dedicated to Pallas Athena. As goddess of wisdom, skill, craft, courage, and inspiration I have called on her everyday during the course of my dissertation research. I hope my efforts have earned me a spot in her role as patron of heroes.

**Abstract**

Medium Spiny Neurons (MSNs) are the primary cell type of the striatum, a structure critically involved in motivation, memory, and movement. MSN structure and function are altered in Parkinson's and Huntington's disease as well as following repeated exposure to drugs of abuse. One site on the MSN that changes in all of these diseases is the dendritic spine, a sub-micron protrusion responsible for receiving excitatory (glutamatergic) signals. Dendritic spines undergo structural and functional changes during development, normal aging, and disease. To date, no studies have specifically characterized the developmental changes undergone by MSN dendritic spines and investigation into the molecules that regulate MSN dendritic spines has been limited to *in vivo* manipulations in adults or characterization of global knockout of specific molecules. Research into MSN dendritic spine development and plasticity mechanisms were likely limited by the absence of an *in vitro* primary neuronal culture system that produced MSNs with mature *in vivo*-like characteristics. The experiments detailed in this thesis describe the development and characterization of such a system as well as a morphological analysis of MSN development in culture. MSNs cultured using this technique develop gross morphological characteristics similar to *in vivo* cells. They also develop high densities of dendritic spines with a high variety of mature morphologies and have synaptic and intrinsic physiological characteristics like MSNs *in vivo*. This work represents a significant contribution to the field, supporting future research into the molecules that regulate MSN development and plasticity in both normal and disease models.

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## Chapter 1: Introduction

*In vivo*, MSN dendritic spines undergo density and morphology changes during development (DiFiglia et al., 1980) and in association with numerous disease and experience-dependent states (Deutch et al., 2007; Russo et al., 2010). Although MSN dendritic spines are known to be a locus for structural and functional plasticity, research into the molecular mechanisms of their development and plasticity has been limited in part by the lack of an *in vitro* system. In contrast, previous research in the well established cortical or hippocampal neuron culture system has provided significant information regarding the mechanisms of spinogenesis and plasticity in pyramidal neurons (Ethell and Pasquale, 2005; Tada and Sheng, 2006; Schubert and Dotti, 2007). These studies have been aided by the reproducible nature of spine development in pyramidal culture systems and the detailed protocols available. In contrast, commonly used protocols for the culturing of MSNs often rely on striatal mono-culture (Ventimiglia et al., 1998) a system that produces high numbers of MSNS that retain a relatively simple morphology with low densities of dendritic spines when compared to MSNs co-cultured with glutamatergic neurons (Segal et al., 2003; Tian et al., 2010; Penrod et al., 2011). This dissertation details a streamlined protocol that produces MSNs developing numerous *in vivo*-like characteristics. The development of MSNs *in vitro* is examined, including the first detailed characterization of dendritic spine morphological development.

Striatal anatomy and MSN connectivity

The striatum is a brain region critically involved in functions like sensory processing, movement, motivation, and cognition (Pennartz et al., 2009; Devan et al., 2011; van der Meer and Redish, 2011). The striatum is a highly heterogeneous structure, consisting of multiple neuron types and receiving a number of afferents (Gerfen, 1988; Bolam et al., 2000; Tepper et al., 2008). Medium Spiny Neurons (MSNs) are the major neuron type of the striatum (Kemp, 1968; Kemp and Powell, 1971b; Graveland and DiFiglia, 1985; Rafols et al., 1989; Matamales et al., 2009) and are characterized by medium-sized cell bodies, complex dendritic arbors, a high density of dendritic spines, and a high level of expression of dopamine and cyclic AMP regulated phosphoprotein of 32kDa (DARPP-32, (Anderson and Reiner, 1991))

The striatum can be divided into two gross compartments (dorsal and ventral) and further divisions within those compartments (patch/matrix, core/shell). MSNs are the primary output neurons of all of these regions and receive two primary types of afferents,

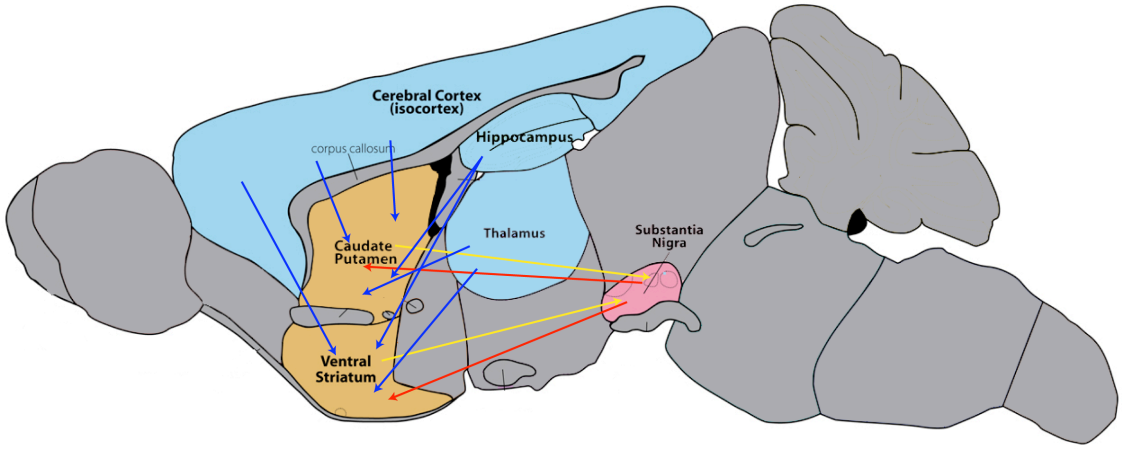


**Figure 1. Simplified schematic of striatal connectivity**
The adult mouse striatum receives glutamatergic (blue arrows) input from regions of the cortex, the hippocampus, the thalamus, and other regions. The striatum also receives dopaminergic input (red arrows) from the substantia nigra/ventral tegmental area. MSNs of the striatum send inhibitory inputs back to the dopaminergic structures as well as other regions.

glutamatergic and dopaminergic, regardless of their location (Figure 1). Many of these afferents terminate on the dendritic spines of MSNs (Groves, 1980; Kaiya and Namba, 1981; Bouyer et al., 1984; Hattori et al., 1991; Groves et al., 1994). The major sources of glutamatergic input into the dorsal striatum come from multiple areas of the cortex (Kemp and Powell, 1970; McGeorge and Faull, 1989), thalamic nuclei (Kemp and Powell, 1971a; Groenewegen and Berendse, 1994; McFarland and Haber, 2000), and the hippocampus (Groenewegen et al., 1987). The corticostriatal connections are thought to play a critical role in decision-making (Pennartz et al., 2009) and are a part of the cortico-striatal-thalamic loop that is critical in movement (Obeso et al., 2008). The primary source of glutamatergic input into the ventral striatum is from "limbic structures" that include the amygdala (Kita and Kitai, 1990) and hippocampus (Groenewegen et al., 1987), as well as intralaminar and parafascicular nuclei of the thalamus (Berendse et al., 1992; Groenewegen and Berendse, 1994), and pre-frontal cortex (Berendse et al., 1992). These projections are a critical component in the mesolimbic dopamine system, which controls motivated behavior (Koob, 1996; Berridge and Robinson, 1998; Wightman and Robinson, 2002). The thalamostriatal connection appears to enhance dopamine transmission in the striatum (Kilpatrick and Phillipson, 1986), regulate MSN neurotransmitter expression (Nieoullon et al., 1985; Samuel et al., 1988), and contribute to a large amount of the glutamate tone in the striatum (Touchon et al., 2004). Many of the regions of the cortex that innervate the striatum also innervate regions of the thalamus that project to the striatum, generating functional "loops within loops" that coordinate motor and sensory integration (Kimura et al., 2004; Haber and Calzavara, 2009).

Projections from the intralaminar nuclei of the thalamus are critically involved in certain learning tasks (Porter et al., 2001) and mediate alterations in striatal glutamate following cocaine exposure (McKee et al., 2010). In all regions of the striatum, glutamatergic afferents from both the thalamus and cortex synapse, in large part, onto dendritic spine heads (Doig et al., 2010).

Both the dorsal and ventral striatum receive substantial innervation from midbrain dopaminergic afferents (Groves, 1980). The primary dopaminergic input to the dorsal striatum comes from the substantia nigra pars compacta (Fallon and Moore, 1978; Veening et al., 1980) and the primary source of dopaminergic input into the ventral striatum is the ventral tegmental area (Fallon and Moore, 1978; Lindvall and Björklund, 1978; Moore and Bloom, 1978; Veening et al., 1980; Kalivas and Miller, 1984). In both compartments, dopaminergic afferents synapse on the dendritic shaft but also onto the necks of MSN dendritic spines (Bouyer et al., 1984; Freund et al., 1984; Smith and Bolam, 1990).

MSNs are the output neuron of the striatum and send GABAergic projections (Kita and Kitai, 1988) to a number of brain regions as well as recurrent collaterals within the striatum (Preston et al., 1980; Bishop et al., 1982; Chang et al., 1982; Penny et al., 1988)). MSNs can be divided into two broad subcategories based on their dopamine receptor expression and projections. MSNs in dorsal striatum are commonly referred to as “direct” and “indirect”, referring to the fact that D1-receptor containing MSNs project directly to the medial globus pallidus (the output nucleus of the basal ganglia) and substantia nigra pars reticulata (also referred to as striatonigral) while the D2-receptor

containing MSNs project "indirectly" to the internal globus pallidus and subthalamic nuclei (also referred to as striatopallidal). The MSNs of these two types also differ in their expression of other proteins, including muscarinic acetylcholinergic and adenosine receptors (nigral and pallidal respectively) and certain neuropeptides such as substance P and ekephalin (nigral and pallidal respectively; Bertran-Gonzalez et al., 2010). Although the projections from MSNs of the ventral striatum are differ, going to the ventral pallidum, lateral hypothalamus, and the substantia nigra pars compacta (Phillipson, 1979; Heimer et al., 1991), this segregation of MSN subtypes is maintained at the level of receptor and protein expression differences. Ultimately, the MSNs of the striatum, regardless of compartment or subtype, are at the core of numerous critical circuits, upon which their proper function is required.

Striatal development

The striatum develops from a transient embryonic structure of the telencephalic primordium, the ganglionic (or ventricular) eminence (GE). MSNs are born and differentiate within the lateral ganglionic eminence (LGE, (Deacon et al., 1994; Nakao et al., 1994; Olsson et al., 1995; Olsson et al., 1998)), while the medial ganglionic eminence (MGE) generates cholinergic interneurons of the striatum and other brain regions (Olsson et al., 1998) as well as GABAergic interneurons of the striatum and cortex (Marin et al., 2000; Wichterle et al., 2001). Cells in the GE are already postmitotic, having migrated from the ventricular and subventricular zones near the lateral ventricle. By embryonic day (E16) in the mouse, neurons in the GE have already reached a final differentiation

step and are determined to form GABAergic projection neurons (MSNs), cholinergic interneurons, or GABAergic interneurons (Deacon et al., 1994).

As previously noted, the two primary afferents into the striatum are dopaminergic and glutamatergic. These connections are formed initially during embryonic development and refined up to early postnatal stages. Dopaminergic midbrain neurons are born and differntiated around E10-14 in mouse (Bayer et al., 1995) and continue to mature between E15-16 in mouse (Perrone-Capano and Di Porzio, 2000). Dopaminergic neurites start to extend around E14 in rat (E12.5 in mouse) and by E15 (E13.5 mouse) these fibers have reached much of the ganglionic eminence (Voorn et al., 1988). In the rat, these fibers have formed synapses by birth, mainly on the dendrites of MSNs, and upon arrival of cortical afferents and dendritic spine formation these contacts are reorganized to synapse on dendritic spine necks (Antonopoulos et al., 2002). Dopamine projections into the striatum are at first unorganized, with projections from both the substantia nigra and ventral tegmental area intermingled, and have a more mature appearance around post natal day 0 (P0) through pruning steps (Hu et al., 2004). Corticostriatal fibers are believed to be collaterals of corticofugal axons that first pass through the striatum around E12. These axons form collaterals into the striatum as early as E18 with synapses formed by P2 and continuing to mature over the first postnatal week (Nisenbaum et al., 1998; Sheth et al., 1998). *In vivo* studies in rat demonstrate that the majority of the cortical inputs are elaborating and forming asymmetric synapses with MSN dendritic spines during the first through third weeks of postnatal development (Hattori and McGeer, 1973; Sharpe and Tepper, 1998).

MSN dendritic spines

MSNs are named, in part, due to the high density of dendritic spines that stud their dendritic arbors (Figure 2). Dendritic spines are small protrusions from the dendrite shaft that are the primary site of excitatory neurotransmission. Dendritic spines consist of two

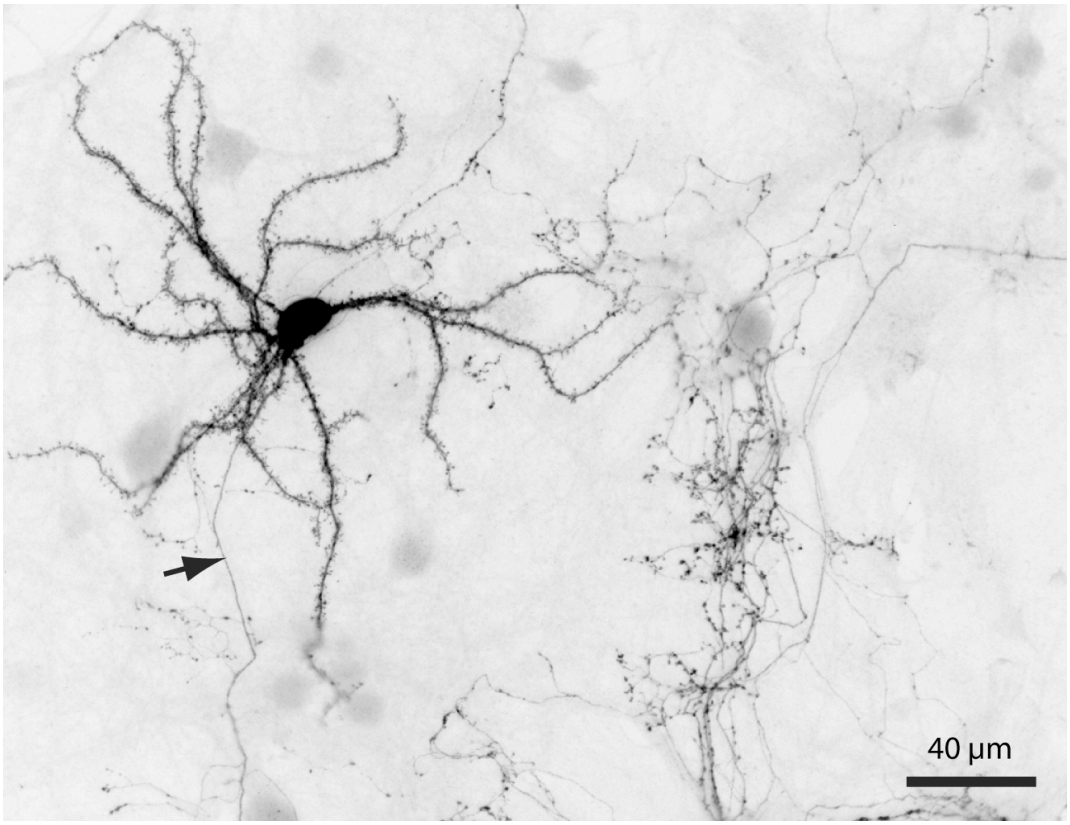


**Figure 2. DARPP-32 positive mature MSN *in vitro***
DARPP-32 positive MSN, note radial, highly branched dendrite arbor studded with high densities of dendritic spines.

general domains, the head and neck (Sorra and Harris, 2000). The head is the site of the glutamatergic synapse. It is an f-actin rich structure (Dunaevsky et al., 1999; Matus; Capani et al., 2001) that contains the post-synaptic density, associated receptors and channels, as well as numerous downstream signaling cascades (Sheng and Hoogenraad,

2007). The neck connects the spine head to the dendrite shaft and is the site for many of the MSNs dopaminergic synapses (Bouyer et al., 1984; Freund et al., 1984; Smith and Bolam, 1990). While the head is thought to be a biochemical compartment where local rises in $Ca^{2+}$ are able to influence local signaling (Müller and Connor, 1991; Koch and Zador, 1993; Connor et al., 1994; Jaffe et al., 1994; Segal; Yuste and Denk, 1995; Yuste et al., 2000; Sabatini et al.), the neck is often viewed as a biochemical barrier that isolates the head from the dendrite shaft (Svoboda et al., 1996; Sabatini et al., 2001; Noguchi et al., 2005; Grunditz et al., 2008; Bloodgood et al., 2009). Although the spine neck provides a diffusional barrier to local $Ca^{2+}$ and signaling cascades in the head, the presence of additional channels and dopaminergic synapses on spines of MSNs may enable the spine neck to serve as a modulator of signals (Figure 3).

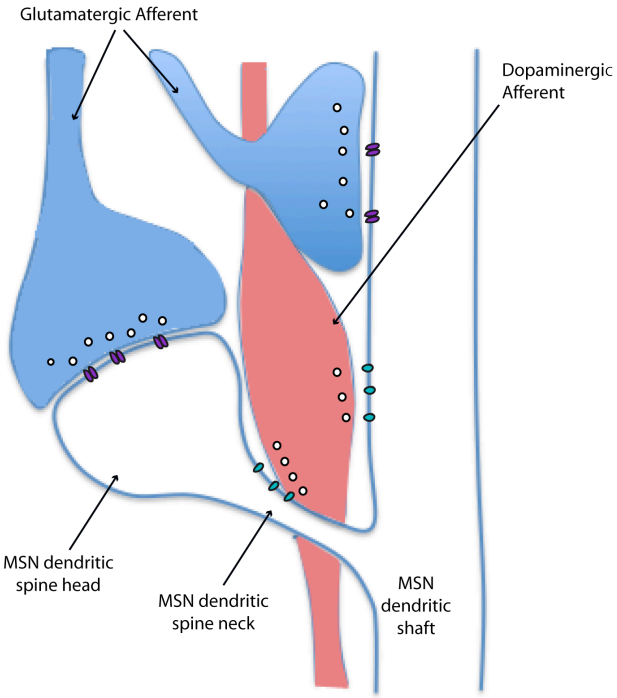


**Figure 3. Simplified schematic of MSN dendritic spine synapses**
MSN dendrites receive glutamatergic (blue) and dopaminergic (red) synapses. Most glutamatergic synapses are formed onto dendritic spine heads. Dopaminergic synapses are formed with the spine neck and dendritic spine shaft. Adapted from Kotter et al., Prog Neurobio, 1994

The shape of the dendritic spine is believed to affect its function (Kasai et al., 2003). Previous research, primarily in pyramidal

(hippocampal and cortical) neurons has supported the idea that larger spine heads and shorter spine necks are associated with stronger synapses. This is due to the positive correlation between spine head diameter, post-synaptic density size (Kasai et al 2003, Bourne and Harris, 2008) and AMPA receptor complement (Nusser et al., 1998) and thus, synaptic strength (Matsuzaki et al., 2001). In addition, the reduction in neck length may reduce the diffusional barrier (Majewska et al., 2000b; Majewska et al., 2000a; Holthoff et al., 2002), increasing $Ca^{2+}$ transients and signaling molecule diffusion from the spine to the dendrite shaft. Many predictions about MSN dendritic spine structure/function relationships and morphological maturation have been extrapolated from this data. Although the morphological maturation of pyramidal neuron dendritic spines has been previously described (Figure 4; Papa et al., 1995; Boyer et al., 1998; Bourne and Harris, 2008), similar experiments have not been conducted with MSNs. Mature MSNs have high densities of dendritic spines with highly variable morphologies (Wilson et al., 1983) but correlated structure/function experiments have yet to establish whether structure/function relationships found in pyramidal neuron dendritic spines are the same for MSN spines.

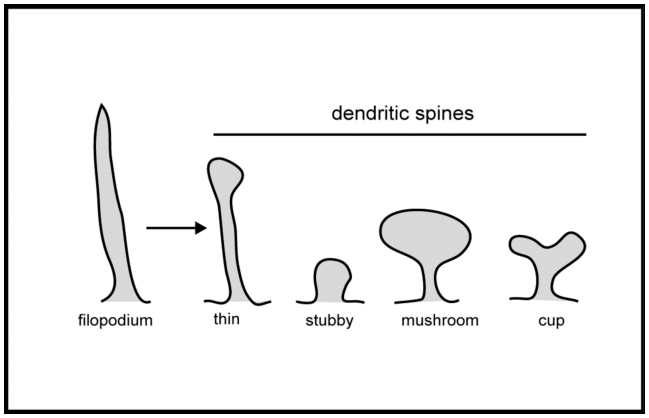


**Figure 4. Dendritic spine morphologies**
Examples of developing and mature dendritic spine morphologies. Adapted from Ethell and Pasquale, Prog Neurobio, 2005

Significant work in the pyramidal culture system has defined a number of molecules that mediate dendritic spine development and plasticity (Frost et al., 2010; Shen and Cowan, 2010; Fortin et al., 2011; Tolias et al., 2011). Far less work has been done investigating the molecules that mediate MSN dendritic spine development and plasticity. Investigations of *in vivo* postnatal development of MSNs in a variety of mammals have described the gross morphological changes and increases in spine density with development (Chronister et al., 1976; DiFiglia et al., 1980; Tanaka, 1980; Hull et al., 1981), but no specific investigations of MSN dendritic spine morphological development have ever been conducted. Only a few proteins have been directly implicated in MSN dendritic spine development; for example, knockout of spinophilin (Feng et al., 2000), Cerebellin-1 (Kusnoor et al., 2010), PSD-95 (Vickers et al., 2006), or Brain Derived Neurotrophic Factor (BDNF, (Baquet et al., 2004; Saylor et al., 2006; Rauskolb et al., 2010)) alters MSN dendritic spine density *in vivo*.

Despite interest in MSN dendritic spine formation and maturation, investigations into the molecular mechanisms for these findings has been limited. Most of the examinations of molecules that regulate MSN spine morphology and density have been focused on delineating pathways mediating experience and disease dependent changes with little focus on developmental mechanisms. Although there may be a number of shared mechanisms for dendritic spine morphogenesis and plasticity between spiny neuron types, the expression of different densities of neurotransmitter receptors and signaling molecules indicate the mechanisms for spine formation, maturation, and plasticity in MSNs may diverge from pyramidal neurons. A primary neuron culture

system in which MSN dendritic spine development can be monitored and manipulated would support future research into the molecular mechanisms that regulate MSN spine morphogenesis.

MSNs in disease

The structure and function of MSNs are altered in a number of disease states of importance to human health. Alterations in MSN structure have been reported in human brain tissue from subjects with schizophrenia (Roberts et al., 1996), Parkinson's disease (Zaja-Milatovic et al., 2005), and Huntington's disease (Graveland et al., 1985). Animal models of repeated drug exposure ("addiction"), Parkinson's, and Huntington's (Robinson and Kolb, 2004; Deutch et al., 2007; van Spronsen and Hoogenraad, 2010) also report alterations in MSN structure and function. These alterations occur at the level of the dendrite but also at the level of the dendritic spine. In Huntington's disease, MSNs are progressively lost from the striatum, producing alterations in movement, mood, and motivation that ultimately prove lethal. In Parkinson's disease the degeneration of dopaminergic afferents cause structural and functional changes in MSNs that produce motoric and behavioral deficits. And finally, in drug addiction, alterations in the mesolimbic dopamine system lead to functional and structural changes in MSN that contribute to addiction-related behaviors. In each case, the dendritic spines of MSNs are altered, in number, morphology, or both.

Huntington's disease (HD) is produced by an autosomal dominant mutation in the huntintin gene (Group, 1993). Accumulation of the mutant protein, typified by a large

CAG (poly-glutamine) expansion (Persichetti et al., 1995), ultimately leads to death of the MSN, primarily MSNs of the indirect pathway that express D2 receptors (Albin et al., 1989). This leads to an imbalance of activity through the indirect pathway, which usually inhibits movement, producing the characteristic chorea movements (Haddad and Cummings, 1997). Although huntingtin is expressed throughout the organism, this mutation appears to primarily affect MSN health through an unknown mechanism (Thomas et al., 2011). In animal models of HD where mutated human huntingtin is expressed in MSNs, dendritic spine morphology (Spires et al.) and density is altered (Klapstein et al., 2001; Lerner et al., 2012), an observation also found in human HD brains (Graveland et al., 1985; Ferrante et al., 1991). Much of the pathological mechanism of the mutation is unknown, including how it exerts its effect on dendritic spines. Transcriptional profiling of HD-model MSNs show disruption in numerous proteins related to both dopaminergic and glutamatergic signaling (Thomas et al., 2011), indicating that these post-synaptic changes are likely cell autonomous and linked directly to the gain of function mutation in huntingtin. Multiple investigations using different models of HD have repeatedly found dysfunction at MSN corticostriatal synapses including changes in efficacy (Cepeda et al., 2003; Cepeda et al., 2007; Milnerwood and Raymond, 2007) and increased *N*-methyl-D-aspartate (NMDA) glutamate receptor induced excitotoxicity (Estrada Sánchez et al., 2008). Clearly, dendritic spine signaling and function are related to MSN dysfunction and death and further research into these mechanisms could improve treatments for HD. Although numerous mouse models of mutant huntingtin have been generated (Menalled and Chesselet, 2002) there is a lack of

knowledge about the basic mechanisms of HD. Research into the cell biological mechanisms of HD have used *in vitro* systems, investigating MSN related proteins in pyramidal neurons (Fan et al., 2012), non-neuronal cells (Chen et al., 1999; Zeron et al., 2001) and mono-cultured MSNs (Zeron et al., 2002; Tang et al., 2003; Tang et al., 2004; Tang et al., 2007). Although these studies improve our understanding of HD pathology, the use of a primary cell culture system optimized for MSN development will support future investigations into differences in development, function, and plasticity of MSNs in a system where molecular manipulations are more tractable.

Parkinson's disease (PD) is produced by the progressive death of dopaminergic cell bodies in the substantia nigra, causing a loss of dopaminergic input to MSNs and altering the activity of the cortico-striatal-thalamic system involved in motor production. Although the primary neurons affected are the midbrain dopaminergic neurons, the loss of this input into the striatum has profound impacts on the structure and function of MSNs. The loss of dopamine inputs to MSNs of both direct and indirect pathway disrupts activity in both pathways, leading to increased inhibition of the thalamus, decreasing excitation to the cortex and leading to the inhibition of movement. MSN dendrites and dendritic spines are altered in the brains of human PD patients (McNeill et al., 1988; Meredith et al., 2000) and the loss or disruption of dopaminergic input alters the activity of MSNs (Cepeda et al., 1998; Day et al., 2006). Models of PD that deplete dopamine or lesion dopaminergic inputs reduce spine density and alter spine morphology in the striatum (Ingham et al., 1989 2000; Zaja-Milatovic et al., 2005). Treatments for PD are often focused on replenishing dopamine tone, either via agonist treatment,

dopamine precursor treatment, or grafting of embryonic dopaminergic cells into the striatum. In the case of agonist or precursor treatments, there can often be side effects due to plastic changes in other portions of the cortico-striatal-thalamic loop (Calabresi et al., 2007) and post-mortem studies demonstrate that drug treatments do not restore normal morphology to MSNs (Stephens et al., 2005), likely contributing to ongoing dysfunction in MSN activity. Although grafts will synapse on MSNs, these synapses are unlike those in normal striatum, in part due to the loss of MSN dendritic spines (Soderstrom et al., 2008). This loss of spinous sites for dopaminergic terminals produces abnormal terminations that impact the efficacy of the graft to alter motor behaviors (Soderstrom et al., 2010).

Previous research has shown a $Ca^{2+}$ dependent mechanism for dopamine deinnervation induced MSN spine loss (Day et al., 2006) and that treatment with $Ca^{2+}$ channel blockers can prevent dopamine deinnervation induced spine loss (Soderstrom et al., 2010); however, it is unclear that these treatments could restore normal spine morphology in PD patients already expressing MSN structural dysfunction. These findings may also have implications for mechanisms of MSN dendritic spine development and maturation. A primary cell culture system could be used to examine other mechanisms of spine retention and reformation in a system that allows for investigation into shared developmental and mature pathways regulating MSN dendritic spines. Better understanding of the molecules and mechanisms regulating MSN dendritic spine development and synaptic plasticity will enable the design of better treatments for PD.

Repeated exposure to drugs of abuse produce behavioral, cellular, and structural changes. Abused substances are thought to exert their addictive action through activation of the mesolimbic dopamine system, including the ventral striatum (Koob and Bloom, 1988; Bayer et al.; Self and Nestler) as well as the "habit system" that includes the dorsal striatum (Paulson and Robinson, 1995; Porrino et al., 2004; Vanderschuren et al., 2005). Although the mechanisms vary, as do the resultant changes in MSNs, these drugs produce changes in MSN spine density and morphology (Russo et al.). Repeated psychostimulant exposure is an often-studied paradigm for drug addiction as it produces a number of addiction-like behaviors (Shippenberg and Koob, 2002). Psychostimulant exposure and the acquisition or expression of these behaviors is correlated with changes in MSN activity (Alcantara et al., 2011; Dobi et al., 2011; Saddoris et al., 2011) and structural plasticity of MSN spines (Li et al., 2003; Ferrario et al., 2005; Lee et al., 2006; Ball et al., 2009; Dobi et al., 2011; Martin et al., 2011). Research has shown that the structural (Robinson and Kolb, 1997; Robinson and Kolb, 1999; Brown and Kolb, 2001; Robinson et al., 2001; Kolb et al., 2003; Li et al., 2003; Lee et al., 2006; Jedynak et al., 2007), functional (Thomas et al., 2001; Kourrich et al., 2007; Dobi et al., 2011; Wu et al., 2012), and behavioral effects (Brown and Kolb, 2001) of repeated drug administration can persist beyond the last drug exposure, potentially contributing to the ongoing risk of relapse in drug users. The correlated changes in structure and function of MSNs has led to the belief that dendritic spine changes are coordinated with physiological changes that regulate behavioral responses to drug. Investigations into the relationship between structural plasticity and behavioral responses to psychostimulants have yielded

contrasting results that are often difficult to reconcile (Pulipparacharuvil et al., 2008; Russo et al., 2009). For example, in one case preventing cocaine induced increases in spine density by expressing a constitutively active form of MEF2 (myocyte enhancing factor 2, a transcription factor that negatively regulates synapse number) in the nucleus accumbens, increased sensitivity to cocaine (increased acquisition rate of sensitization, Pulipparacharvil et al 2007), indicating that MSN spine density increases may be a compensatory mechanism reducing sensitivity to the rewarding effects of cocaine. In an independent set of experiments, blocking cocaine-induced increases in dendritic spine density, by expression of a dominant negative form of NFκB (nuclear factor κ B, a transcription factor that regulates synaptic plasticity) *decreased* sensitivity to cocaine (reduction in cocaine place conditioning, Russo et al 2009), indicating that spine density increases may enhance cocaine reward. The conflicting findings of these experiments may be due to specific differences in cocaine exposures, behavioral tests, or targets used to alter spine density (MEF2c v NFκB respectively), but it highlights the still unknown relationship between spine structure, MSN function, and animal behavior. A primary dissociated MSN culture system would allow for more proximal determinants of spine structure and function to be identified. This system would also allow for molecules previously associated with psychostimulant exposure to be tested for their role in MSN spine morphology or function and would provide future avenues for characterizing structure/function relationships caused by experience dependent plasticity at MSN dendritic spines.

Concluding comments and rationale

MSNs are a locus for structural and functional changes during development and experience-induced plasticity. Although multiple groups have identified alterations in MSN spines and investigated possible molecular mediators, a number of basic developmental and plasticity mechanisms remain completely unexplored. Current research utilizing a number of *in vivo* techniques have furthered our understanding of MSN function and structure in the adult animal and identified molecules involved in experience dependent plasticity. Other research using a striatal mono-culture *in vitro* system has greatly expanded our understanding of signaling cascades within MSNs. Despite these advances, research into the molecules and extracellular environments involved in MSN development and plasticity has been limited by the lack of a robust primary cell culture system that produces MSNs with mature *in vivo*-like characteristics.

Primary cell culture provides a number of benefits for the examination of developmental and plasticity mechanisms. The cell culture system allows for neurons to be tracked across their development by examinations at fixed time points or live-cell imaging. This allows for manipulations that affect structure and function to be studied as they are happening, on a much finer timescale than allowed by *in vivo* examinations. In addition, in primary cell cultures the external environment (media, substrate, supplements) is relatively well defined, allowing for the addition or removal of specific cues or factors for discrete time periods. Primary cell culture also supports manipulations of specific cells using various transduction techniques. The ability to alter protein expression during discrete time points will allow for careful dissection of their

contribution to specific development or plastic events and enable structure function correlations. Cells *in vitro* can be examined using simultaneous imaging and electrophysiological techniques, allowing for close temporal correlation during plasticity events. These and other benefits of primary cell culture have specific applications towards understanding MSN dendritic spine morphogenesis and plasticity.

Previous research into structure/function relationships in spiny neurons has relied heavily on the use of the highly reproducible pyramidal (hippocampal or cortical) neuron *in vitro* system. The widely available, fairly standardized protocols for pyramidal cultures employed by many researchers provides a high reliability in system for studying developmental timing and final morphology. This enables researchers to characterize specific molecules and pathways within a system where numerous other molecules and pathways have already been defined, with each successive experiment adding to the fields greater understanding of the context. This experimental paradigm has produced a wealth of information regarding the molecules that regulate pyramidal neuron development and plasticity, especially of dendritic spines. These findings have been the basis for the fields somewhat dogmatic understanding of structure/function relationships at spines and the molecules that regulate spine development and morphological plasticity.

In contrast to pyramidal neurons, MSNs dendritic spines receive a high density of coordinated glutamate and dopamine signaling. The signaling cascades and subsequent downstream effect of activity at MSN spines are likely to be different than those activated at pyramidal spines. A large body of literature demonstrates the importance of dopamine in MSN spine morphology and plasticity in the adult, but the mechanisms that underlie

these effects have not been completely defined. Additionally, although the time course for MSN gross morphological and electrophysiological development have been characterized, the molecular underpinnings of and contribution of morphological changes of MSN dendritic spines to this development have not been delineated. These specific cascades are likely be critical for the function and plasticity of MSNs and can not be explored in the current widely used MSN *in vitro* system, due to the lack of spine development. The experiments described in dissertation project were carried out to fill a knowledge gap into MSN-specific dendritic spine development and plasticity research.

The goal of this thesis work was to develop and characterize an in vitro system for MSNs that supported the development *in vivo*-like characteristics. In order to produce this system, numerous variables known to affect *in vitro* survival and development were examined. MSN survival and development were compared to the more commonly used mono-culture protocol. MSN gross morphology was quantified using Sholl analysis and dendritic spine density and morphology were assessed. MSN synaptic and intrinsic excitability was examined, as was synaptic marker localization. Using the optimized *in vitro* system, MSN morphological development was characterized. DARPP-32+ MSNs were examined at time periods spanning one week to one month of *in vitro* development. Gross morphology, dendritic spine density, and morphology were examined across the time course. This research has produced reliable, reproducible streamlined protocol for maintaining MSNs with numerous *in vivo*-like characteristics that can be manipulated by expressing exogenous proteins of interest (constitutively or temporally restricted) in cells that can be maintained in culture for at least one month.

## Chapter 2: Development and characterization of an *in vitro* system for examining MSN development

Introduction

Although MSN dendritic spine morphology changes during development and in association with numerous disease and experience-dependent states (Robinson and Kolb, 2004; Deutch et al., 2007; van Spronsen and Hoogenraad, 2010), limited progress has been made in defining the molecules that regulate these changes. Previous research in the well-established cortical and hippocampal neuron culture system has provided significant information regarding the mechanisms of spinogenesis and plasticity in pyramidal neurons (Ethell and Pasquale, 2005; Tada and Sheng, 2006; Schubert and Dotti, 2007). These studies have been aided by the reproducible nature of spine development in these culture systems and the detailed protocols available. In contrast, commonly used protocols for the culturing of MSNs often rely on striatal mono-culture (Ventimiglia et al., 1998), a system that generally produces morphologically immature MSNs with low densities of dendritic spines when compared to MSNs co-cultured with glutamatergic neurons (Segal et al., 2003; Tian et al., 2010). Importantly, it appears that excitatory afferent activity, produced by the inclusion of cortical or hippocampal pyramidal neurons, is required for the development of MSN dendritic spines in culture (Segal et al., 2003).

In order to provide a reliable, reproducible system for the study of MSN dendritic spines, a detailed method for the preparation of an *in vitro* system that supports MSN development was developed. This method can be used to generate cortical-striatal co-

cultures that support the survival and development of MSNs with *in vivo*-like characteristics. This method takes advantage of the large number and relative robustness of striatal cells in the embryonic striatum (a.k.a. the ganglionic eminence), making it possible to produce multiple dishes of primary neurons from a single embryo. This straightforward protocol provides an *in vitro* system that can be applied to a wide variety of investigations into the molecular mechanisms that regulate MSN dendritic spine morphology and plasticity.

## Methods

### *Animals*

Animal procedures were performed at the University of Minnesota in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and in accordance with protocols approved by the University of Minnesota IACUC, as well as the principles outlined in the National Institute of Health *Guide for the Care and Use of Laboratory animals*.

### *Coverslip Preparation*

German glass 12mm coverslips (Bellco; 1943-10012) were acid washed in a 1M HCl solution overnight at 55˚C, washed twice for 30 minutes with distilled water and then rinsed 30 minutes each in 50%, 75%, and 90% solutions of ethanol. Following washing, coverslips were dried and maintained in at 225˚C oven until immediately prior to coating.

One to two days before cultures were prepared, acid-washed coverslips were transferred to 35 mm petri dishes (5 coverslips per 35 mm dish) and overlaid with 50µl of a mixture of 100 µg/ml Poly-D-Lysine (PDL) and 4 µg/ml laminin. PDL was prepared by dissolving 100 mg of PDL in 0.1 M borate buffer (pH 8.5). PDL/borate solution was then sterilized through a 0.2 µm filter, aliquoted and stored at -80˚C. Laminin was thawed on ice, aliquoted and stored at -80˚C. Immediately prior to coverslip coating, PDL was thawed at 32˚C and laminin was thawed on ice. When working with laminin, care must be taken to avoid warming the solution too quickly, which can cause laminin to aggregate, reducing it's ability to serve as a substrate for cells. After overlying 50 µl of PDL/laminin on each coverslip, the dishes were sealed with parafilm to prevent evaporation and coverslips were incubated overnight at room temperature. Coverslips were then rinsed three times with sterile water. Each 35mm dish was filled with 2ml of neuronal plating media and stored in the tissue culture incubator until needed.

*Dissection of striatal and cortical tissues*

Figure 5 presents a flow chart of the culturing protocol. Embryonic day 16 (E16) pregnant mice were euthanized by $CO_2$ asphyxiation and the uterus was removed. Within a sterile dissecting hood, embryos were removed from the uterus and decapitated. Heads were stored in room temperature $Ca^{2+}$/$Mg^{2+}$-free Hank's Balance Salt Solution with 10mM HEPES (CMF-HBSS, the same solution in which all dissections took place). All dissecting equipment was sterilized in 70% ethanol and was routinely dipped in 70% ethanol during the dissection, especially during transitions from exterior structures to

interior structures (i.e. between removing brain from skull and working with isolated brain). These steps were undertaken to maintain the sterile environment and avoid contamination. Routine use of this procedure eliminates the need for antibiotic administration at any point during the preparation or maintenance of the cultures. Brains were removed from the skull using forceps and transferred to a wax bottom 35 mm petri dish (5 ml of paraffin wax in the bottom of dissecting dishes helps protect the tips of delicate tools). The hemispheres were removed from the cerebral peduncles with a sharp forceps. The meninges were removed and hemispheres were placed medial surface up. A region of cortex, roughly corresponding to the pre-frontal cortex, was removed from the medial side of the anterior aspect of the cortex. The remaining cortex overlying the lateral ventricle was folded away to expose the ventricle and ganglionic eminences. The ganglionic eminences (medial and lateral; lateral being the presumptive striatum (Wichterle et al., 2001)) were removed using angled forceps to "scoop out" the entire eminence from the lateral ventricle and separated from the overlying cortex. The cortex and ganglionic eminence regions were transferred to separate CMF-HBSS containing petri dishes. After dissection of all pups, tissue was minced using a clean, sterile razor blade.

*Tissue dissociation*

Minced cortical and striatal tissue was transferred to separate sterile 15 ml conical tubes and allowed to settle to the bottom of the tube. CMF-HBSS was removed and replaced by either papain or trypsin digestion solutions. For papain treatment, minced

tissue pieces were resuspended in 5 ml of 20-25 units/ml papain (Worthington, LS003126) solution including 1.1 mM EDTA and 5.5 mM cysteine in CMF-HBSS and incubated for 30 min at 37˚C. Cells were allowed to settle to the bottom of the tube, digestion solution was removed and 5ml of inhibition solution (750 µg/ml DNAse1, 10 mg/ml BSA, and 10 mg/ml Type II-O Trypsin inhibitor) was then added and the incubation continued for 15 min. Tissue was then centrifuged at 1000 x g (~3000 rpm) for five minutes, resuspended in neuronal plating media (10 mM HEPES, 10 mM sodium pyruvate, 0.5 mM glutamine, 12.5 µM glutamate, 10% Newborn Calf Serum, 0.6% Glucose in EMEM (Minimal Essential Media, plus Earl's salt)) and triturated with a fire polished Pasteur pipette. For trypsin digestion, 10X Trypsin-EDTA (Sigma-Aldrich, T4174) was added to the cell suspension at a final concentration of 0.25%Trypsin and incubated for 15-30 min at 37˚C with occasional gentle agitation. Tissue was monitored during this period and 3 mg/ml DNAse1 (Sigma-Aldrich, DN25) added if tissue pieces appear to be connected by a stringy mass of DNA. After trypsinization, cells were centrifuged at 1000 x g for five minutes, solution was removed and replaced with neuronal plating media and triturated using sterile flame-polished glass pipettes. For all experiments investigating MSN development, cells were digested using Trypsin only. For either protease treatment, properly digested tissue should be fully dissociated after 10 triturations. Excessive trituration will reduce cell viability, as will introduction of air/bubbles during the trituration. Following dissociation, cells were counted using trypan blue (to determine cell viability) and a hemocytometer. Cell viability should be ≥ 90%.

*Plating and maintenance of neuronal cultures*

Cells were plated at a total density of 2 x $10^5$ cells/35 mm dish. For co-cultures, a ratio of 2 parts striatal to 3 parts cortical cells was used. One to three hours after plating the neuronal plating media was replaced with neuronal growth media (50x B27, 0.5 mM glutamine, Neurobasal) that had been conditioned 24-48 hr on confluent glia cultures (see Glial Cultures section). Every seven days one half of the media was replaced with new glial conditioned neuronal growth media.

*Plasmid DNAs*

Three different types of plasmids were used to test DNA transduction methods and subsequently label neurons: (1) pEGFP-C1 (Clonetech) expresses enhanced green fluorescent protein (EGFP) under control of the CMV promoter, (2) pCAG-EGFP (constructed in the Lanier lab) expresses EGFP under the control of the CMV immediate early enhancer and the chicken beta actin promoter (pCAG, (Alexopoulou et al., 2008)), (3) EGFP was cloned into pTRE-tight (Clontech) and co-transfected with pCAG-rtTA-advanced (produced in the Lanier lab by subcloning the rtTA-advanced tet transactivator from pTet-On-Advanced (Clonetech) into pCAG).

*Electroporations*

For some experiments, striatal cells were electroporated using the Lonza Nucleofector system and the mouse neuron transfection reagent (cat. VAPG-1001). We

have found that best results are obtained if the two parts of the transfection solution are mixed on the day of transfection (rather than mixed in advance as per the manufacturer's instructions) and if the transfection kit is used within 3 months of purchase. For this reason, we buy the smaller kit (10 reactions) unless we plan to do more than 10 transfections in 2 months time. For transfection, $1x10^6$ dissociated striatal cells were transferred to a microfuge tube and centrifuged at 1000 x g for 5 min, then plating media was removed and cells were gently resuspended in 100 µl of complete transfection reagent containing 10-12 µg of pCAG-EGFP of pEGFP-C1 or a mix of 10 µg of pTRE-tight-EGFP and 17 µg of pCAG-rtTA plasmid DNA per 1 million cells (always keeping the total volume of DNA <10 µl). Following electroporation, cells were quickly transferred to 2 ml of pre-warmed plating media and samples were taken for hemocytometer counting using trypan blue. Cell viability should be ≥ 90%. We have found that the inclusion of unelectroporated neurons at plating helps the survival and development of electroporated neurons. Therefore, in the co-culture condition, cortical neurons were unelectroporated and all the striatal neurons were electroporated. In the mono-culture condition electroporated striatal neurons are mixed 1:1 with unelectroporated striatal neurons at plating.

*Glia Cultures*

Glia cultures were prepared from cortices of postnatal (P1-2) mice with the striatal tissue removed. Minced tissue was incubated in CMF-HBSS plus 0.25% trypsin-EDTA and DNAse (either 3 mg/ml DNAseI or 1 µl/ml Benzonase (Novagen, 70664-3))

at 37ºC for 30 min. After trypsinization, an equal volume of glia plating media (EMEM, 10 mM HEPES, 1 mM Sodium Pyruvate, 2 mM glutamine, 10% NCS, 0.6% Glucose, 1x Penicillin-Streptomycin) was added to inhibit the trypsin and the tissue was collected by centrifugation (1000 x g for 2 min). Tissue was resuspended in glia plating media, mechanically dissociated using a flame-polished glass pipette and filtered through a 0.7 µm cell strainer (BD Biosciences, 352350). Cells from one litter of pups were plated one pup per dish onto 10 cm tissue culture dishes (untreated, because coating with PDL does not increase the yield). Glia plating media was replaced one day after culturing and once per week each subsequent week. The first day after plating dishes tend to have a large amount of debris and significant cell division does not occur until approximately 3 *days in vitro* (DIV). After 7-10 DIV plates have reached 70-100% confluence and are suitable for use in the preparation of conditioned media. These cultures will be >90% astroglia and appear as large, flat cells that can be somewhat difficult to view under phase contrast.

Alternatively, confluent dishes of glial cells can be frozen and stored in liquid nitrogen following standard protocols. Briefly, glia cultures are trypsinized, resuspended in an equal volume of glia plating media, centrifuged and resuspended in cold freezing media (EMEM/20% NCS/10% DMSO). Glia should be aliquoted one 10 cm dish of cultured cells per freezing vial. Vials are transferred to a styrofoam container to slow freeze overnight at -80˚C, and then transferred to liquid nitrogen. To thaw glia, agitate one vial of cells in a 37˚C water bath until the contents are just thawed (do not let vial warm up). Immediately transfer the cell suspension to a tube containing 5 volumes of warm glia plating media, centrifuge and resuspend cells in warm glia plating media.

Plate one vial of cells per 10 cm dish. If freezing and storage was done properly, >90% of the cells should survive. It should take about 2 weeks for glia to reach confluence and be suitable for conditioning.

Glial-conditioned growth media (GCM) was prepared by removal of glia plating media and replacing with 7-10 ml of neuronal growth media for 24-48 hrs. Once glial conditioning of neuronal growth media was complete, the GCM was removed and replaced with glia plating media. GCM is used within 24 hrs of reclamation from glial plates and stored in the 37˚C/5% $CO_2$ incubator when not in use. Glia plates are typically used only once per week for conditioning media and, if used to condition multiple times in one week, given at least 48 hrs in glia plating media between conditioning sessions. Confluent glia plates can be maintained in this fashion for three or more months and are discarded if significant microglial proliferation is observed (microglia appear as bright, refractile cells, usually growing on top of the astroglia).

*Immunofluorescence*

At time points designated for the experiments cultures were processed for immunofluorescent imaging. Coverslips were fixed in 4% Paraformaldehyde/ PHEM (60 mM PIPES pH 7.0, 25 mM K-HEPES pH 7.0, 10 mM EGTA, 2 mM MgCl2)/ 0.12 M sucrose buffered fixative for 15-20 min at 4˚C. Following fixation, cells were rinsed with Phosphate Buffered Saline (PBS) and blocked in 3% fatty acid free bovine serum albumin (BSA; Roche 03117057001) in PBS for 30 min at room temperature or overnight at 4˚C. The use of a BSA blocking step before and after permeabilization

enhances preservation of fine cell structure, especially cytoskeletal components. In addition, use of BSA, rather than serum, makes the blocking compatible with phospho-epitope specific antibody staining. Cells were permeabilized for 10 min at room temperature in 0.2% Triton X-100 in PBS after which coverslips were rinsed for 5 min in room temperature PBS. After permeabilization, cells were again blocked in 3% BSA in PBS for a minimum of 15 min at room temperature. Coverslips were incubated overnight at 4˚C with 50 µl of primary antibody mixture per slip in 1% BSA in PBS. The following antibodies were used: polyclonal Rabbit anti-DARPP-32 1:250 (Cell Signaling, 2302), monoclonal mouse anti-EGFP 1:1000 (Invitrogen, A11120), monoclonal mouse anti-βIII Tubulin 1:2000 (Promega, G7121), polyclonal mouse anti-SV2 1:100 (DSHB, SV2), and monoclonal mouse anti-PSD-95 1:100 (Chemicon, MAB1598). The protocol described above was used for most combinations of antibodies. The only exception was in staining using the PSD-95 antibody. For best exposure of this epitope, cultures were fixed as described above and then treated to 5 min with -20˚C methanol. Following overnight incubation at 4˚C with primary antibody, coverslips were washed in room temperature PBS and incubated for 1 hour at room temperature with secondary antibody and phalloidin combinations in 1% BSA in PBS. The following secondary antibodies were used: donkey anti-rabbit and anti-mouse conjugated to AMCA, Texas Red, FITC, or Cy5 (Jackson Immunoresearch), all used at 1:100 dilutions. Alexa-488 or -594 phalloidins (Molecular Probes) were diluted 1:200. Following the secondary incubation period, coverslips were washed for 5 min in room temperature PBS. For nuclear staining, coverslips were submerged in 1 µg/ml DAPI in PBS for 30 sec and rinsed in room

temperature PBS for 5 min before mounting. Coverslips were mounted on glass slides with 2.5% 1,4-Diazabicyclo-[2.2.2]Octane, 150mM Tris pH 8.0, and 80% glycerol mountant to reduce photobleaching.

*pCREB stimulation*

At 19-21 DIV co-cultures were tested for dopamine receptor activation-induced CREB phosphorylation. All experiments were conducted in Tyrode (129 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 30 mM glucose, 25 mM HEPES, 0.1 mM glycine) media at 37˚ in the tissue culture incubator. Cultures were treated for three hours with Tetrodotoxin (TTX, 1 μm) and D(-)-2-amino-5-phosphono-pentanoic acid (AP-5; 25 μm) in order to silence basal activity induced CREB phosphorylation. After the silencing period, cultures were stimulated for 15 min with either 6-chloro-pb (500 nM; D1-agonist) or potassium chloride (20K; 20 mM). Some coverslips were fixed prior to silencing to serve as baseline, another set of coverslips were fixed after the silencing period and the remaining coverslips were fixed after stimulation. Coverslips were processed for immunofluorescence imaging as described above; cells were stained with DARPP-32 antibody, DAPI, and monoclonal mouse anti-pCREB 1:1000 (Santa Cruz Biotechnology, sc-81486). Exposure times for DARPP-32 and DAPI channel were set based on an unanalyzed sample cell. The exposure time for pCREB was set using a high intensity signal from the 20K group, such that the image was not saturated. DARPP-32+ MSNs were identified and the DAPI signal was used to determine the mid-nuclear plane. pCREB signal was acquired last in the sequence of imaging and was not examined prior

to collection. A minimum of 20 DARPP-32+ cells per coverslip were imaged and cells from coverslips receiving the same treatment were pooled. Images were collected using a Zeiss Axiovert 200M microscope and Openlab software (Improvision/Perkin Elmer). pCREB fluorescence intensity was determined within Openlab software. The outline of the cell nucleus was drawn using the DAPI channel and that ROI was transferred to sample the corresponding pCREB image. The average fluorescence intensity within the ROI was calculated and the measurements for all analyzed cells were exported. Experiments were performed multiple times and a representative data set is displayed. All statistical analyses were conducted as described in the next section and displayed as MEAN + SEM.

*Imaging, Quantification, and Statistical Analysis*

Images for Sholl analysis, percent cell calculations, and antibody localization were collected using a Zeiss Axiovert 200M microscope and Openlab software (Improvision/Perkin Elmer). Image adjustments were made using Photoshop CS3 (Adobe) with brightness and intensity changes standardized for each experiment (all conditions). All image analysis was conducted using one or more plugin for ImageJ (NIH, http://rsbweb.nih.gov/ij/; detailed below). All statistical analysis was conducted using GraphPad Prism v4.0 (GraphPad Software).

For experiments calculating the percentage of DARPP-32 positive neurons, a minimum of 20 fields per coverslip was imaged at 10x magnification. Exposure times for each channel were standard across conditions. Multi-channel images of the same

field were merged into a single "stacked" image in ImageJ and the cell-counter plug-in (http://rsbweb.nih.gov/ij/plugins/cell-counter.html) was used to count and label DAPI stained nuclei, βIII-Tubulin-positive neurons, and DARPP-32 staining. To be counted as DARPP-32 positive, complete soma staining and a visible staining in the dendrites was required. This inclusion criterion was used for all experiments identifying MSNs and is relatively strict when compared to other reports that require detection of DARPP-32 only in the soma (e.g. Ivkovic and Ehrlich, 1999). The percentage of DARPP-32 positive cells per field was calculated and each field was treated as an independent observation. Replications from 2 independent cultures were pooled. Percentages were compared between time points using Mann-Whitney U to compare two groups of non-Gaussian distribution, $p<0.05$ was considered significant.

For experiments investigating the morphological complexity of DARPP-32 positive neurons, Sholl analysis was performed (Sholl, 1953). Isolated DARPP-32 positive MSNs were imaged at 20x magnification using standard exposure time across conditions. At earlier time points when DARPP-32 expression does not fill the entire neuron, EGFP signal was used for Sholl analysis. In mature MSNs EGFP and DARPP-32 over-lap to fill the entire MSN. The ImageJ concentric rings plugin (http://rsbweb.nih.gov/ij/plugins/concentric-circles.html) was used to place concentric rings every 10 μm out to 150 μm from the cell's center. The cell-counter plugin was used to count and label processes crossing each ring starting 20 μm or 30 μm from the center of the cell. Replications from 2 or 3 independent cultures were pooled. Crossings were compared across the distance measured and between conditions using a two-way

ANOVA with Bonferonni's post-test to determine distances of significant difference, $p<0.05$ was considered significant. Measurements are reported and displayed as mean ± SEM.

For experiments determining the spine density and morphology in striatal mono-culture and striatal-cortical co-culture, EGFP positive, DARPP-32 positive neurons were imaged at 100x magnification using a standard exposure time across conditions. Images were collected on an Olympus ix71 microscope outfitted with a Personal Deltavision module (Applied Precision). Terminal tips of isolated MSN dendrites were identified and z-stack images were collected at 0.15-0.2 μm intervals through the dendrite and the stacks were deconvolved using softWoRx software (Applied Precision). The deconvolved tiff files were imported into Neuronstudio (Mount Sinai School of Medicine, Rodriguez et al, 2008) and were subjected to semi-automated spine analysis. Densities calculated from individual segments of dendrite were treated as independent observations and densities were calculated from 2-3 independent culture preps were pooled. For dendritic spine morphology comparisons a trained analyst confirmed that spines identified by Neuronstudio met criteria for inclusion (eg. observable neck connected to the dendrite shaft). For Neuronstudio collects three parameters used to automatically qualify dendritic spines into morphological categories of mushroom, stubby, and thin (see Rodriguez et al, 2008, figure 8). Occasionally Neuronstudio identifies spines but was unable to collect the neck diameter parameter. In these cases spines were manually categorized based on the neck length to head diameter ratio and the absolute diameter of the head. Spines were categorized as mushroom if the ratio was less

than one (indicating the head diameter is greater than the length of the spine neck) and the absolute head diameter was greater than 0.35 μm. Spines with a neck length to head diameter of greater than 2.5 (indicating the neck is much longer than the head is wide) were classified as thin and all other spines were classified as stubby. Densities and morphological measurements were compared between conditions using the appropriate non-parametric analysis (due to non-Gaussian distributions) depending on the number of conditions, $p<0.05$ was considered significant. Measurements are reported and displayed as mean ± SEM.

*Electrophysiology*

Coverslips were transferred to a recording chamber superfused with artificial cerebral spinofluid (ACSF) at 22–23°C saturated with 95% $O_2$/5% $CO_2$ and containing 119 mM NaCl, 2.5 mM KCl, 1.0 mM $NaH_2PO_4$, 1.3 mM $MgSO_4$, 2.5 mM $CaCl_2$, 26.2 mM $NaHCO_3$, and 11 mM glucose. Picrotoxin (100 mM) was added to block GABA-A receptor-mediated IPSCs (inhibitory post-synaptic currents). Cells were visualized using infrared-differential interference contrast (DIC) optics. MSNs were identified by their morphology (i.e. soma size, dendrite organization, dendritic spines) and hyperpolarized resting membrane potential. In some cultures, DIC observations were confirmed by EGFP expression (Figure 10, left panel)

To assess excitatory synaptic transmission, neurons were voltage clamped at -70 mV using a Multiclamp 700A amplifier (Molecular Devices, Foster City, CA) and miniature EPSCs (excitatory post-synaptic currents) were collected in the presence of

lidocaine hydrochloride (1 mM). Electrodes (3–5 MΩ) contained 117 mM cesium gluconate, 2.8 mM NaCl, 20 mM HEPES, 0.4 mM EGTA, 5 mM tetraethylammonium-Cl, 2 mM MgATP, and 0.3 mM MgGTP, pH 7.2–7.4 (285–295 mOsm). Series resistance (10–30 MΩ) and input resistance were monitored online with a 4 mV depolarizing step (100 ms) at 0.1 Hz. Data were low-pass filtered at 2 kHz, digitized at 10 kHz, and collected and analyzed using custom software (Igor Pro; Wavemetrics, Lake Oswego, OR). Quantal events were analyzed using Minianalysis software (Synaptosoft, Decatur, GA) and verified by eye.

To assess firing properties, kynurenic acid (2 mM) was used to block glutamatergic transmission during recording. Whole-cell current-clamp recordings were performed with electrodes (3–5 MΩ) containing 120 mM K-gluconate, 20 mM KCl, 10 mM HEPES, 0.2 mM EGTA, 2 mM $MgCl_2$, 4 mM $Na_2ATP$, and 0.3 mM Tris–GTP. Data were low-pass filtered at 5 kHz, digitized at 10 kHz, and collected and analyzed using custom software (Igor Pro; Wavemetrics). Membrane potentials were held at approximately -70 mV. Series resistances ranged from 10 to 18 MΩ and input resistances ($R_i$) were monitored on-line with a +40 pA, 150 ms current injection given before every 800 ms current injection stimulus. Firing was obtained through a series of hyperpolarizing and depolarizing current injections (800 ms duration at 0.1 Hz, -20 to +260 pA range with a 20 pA step increment). Resting membrane potentials were corrected for liquid junction potential (~ 14mV).

The mEPSC amplitudes and frequencies are presented as mean ± SEM in the results section and shown in a box-and-whisker plot in Figure 10A. The box-and-whisker

plot shows maximum, upper quartile, median, lower quartile and minimum. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software; La Jolla, CA). Statistical significance was assessed using two-tailed Student's t tests, $p<0.05$ was considered significant.

## Results

### *Qualitative evaluation of reagents*

A flow-chart detailing the optimized final dissection protocol is presented in Figure 5. Previous reports on the co-culture of striatal and cortical neurons have used different ages and species of animals, as well as different matrices, medias, and cell densities (Segal et al., 2003; Shen et al., 2007; Day et al., 2008; Gertler et al., 2008; Sun et al., 2008; Sun and Wolf, 2009; Tian et al., 2010). We sought to establish a streamlined procedure in which all tissue was collected from a single animal or litter at an embryonic stage compatible with other commonly used hippocampal and cortical culture techniques. In the process, many of the published parameters were tested and their affect on the survival and development of DARPP-32+ MSNs monitored over a month in culture (summarized in Table 1).

### Source of tissue

The source of tissue is an important consideration because it can affect the yield of cells, the relative enrichment of particular types of cells, and the resiliency of neurons. Published striatal mono-culture methods have used a variety of pre- or postnatal stages, while the published co-culture methods have combined pre- and postnatal tissues, sometimes even using a combination of mouse and rat tissues. In an attempt to develop a streamlined method, several developmental stages were tested to determine if both cortical and striatal tissue could be harvested from the same animal. When early postnatal (P1 or P2 mouse) tissues were used, live cell yields were high at plating, but

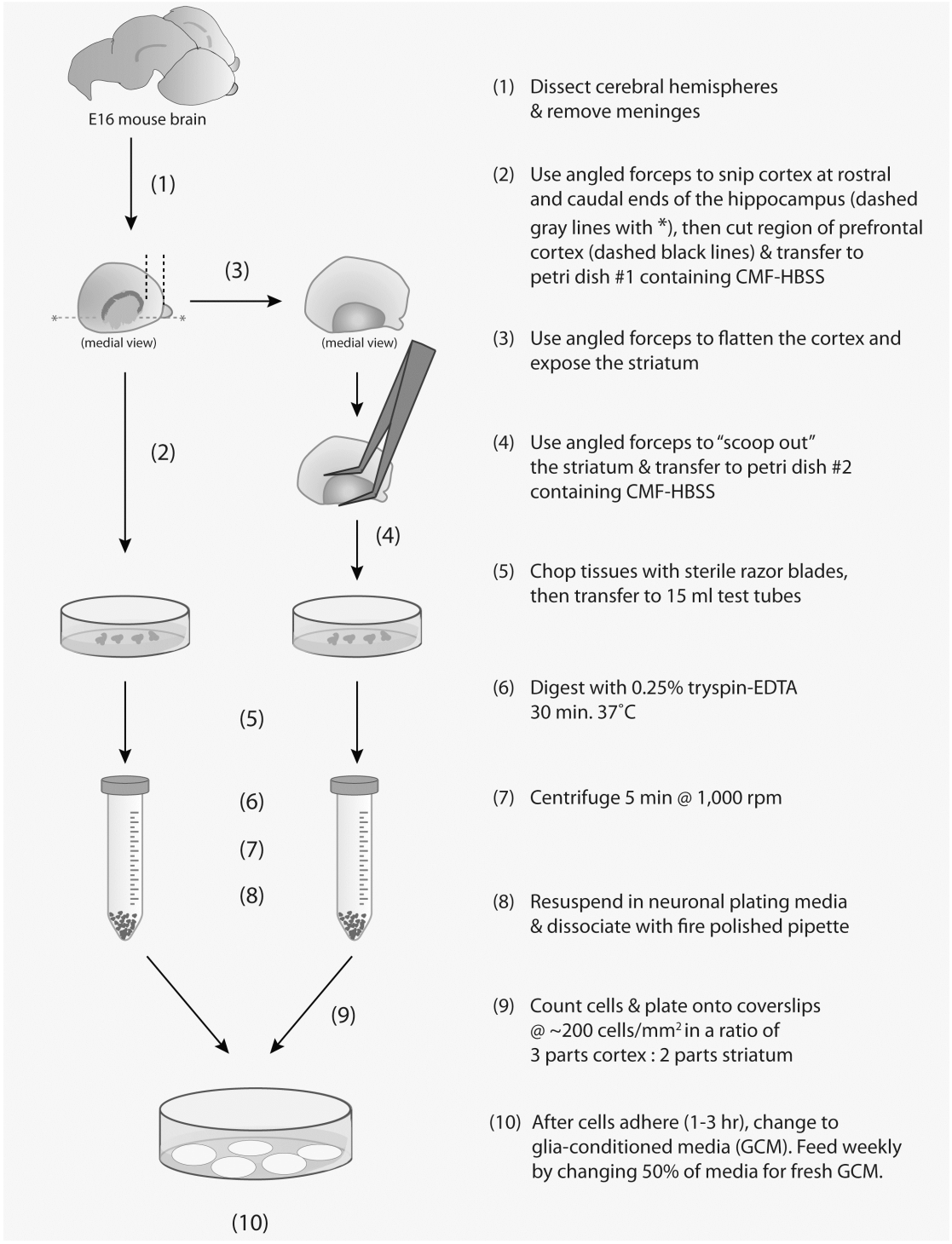


**Figure 5. Simplified schematic of dissection protocol**

these postnatal cultures consistently showed poor adhesion and experienced survival issues, with the majority of neurons dying before a week in culture. When working with postnatal tissues, increases in plating density (up to five times higher than that used with embryonic tissue) appeared to improve survival, but cultures never appeared to be as robust as those derived from prenatal tissue. These problems were eliminated when using embryonic tissues. The use of embryonic tissues made it possible to harvest cortical and striatal tissues from the same animal and improved cell yields at plating, allowing for more coverslips to be generated from a single litter and for single pups to generate sufficient tissue to be plated in isolation. Finally, the use of embryonic day 16 (E16) tissue made the protocol compatible with currently used hippocampal culture techniques, allowing for multiple types of cultures to be generated from single litters.

Matrix

The type of matrix used to support neurons in culture can have significant effects on adherence, survival, and rate of development. Therefore, several matrices were tested for their effect on embryonic tissues. Matrigel (1:50 in plating media, BD Biosciences 354234) allowed for adherence and survival of striatal and cortical neurons, but morphological development was no better than on PDL/laminin and the thick nature of Matrigel made imaging studies more difficult. In addition, the undefined nature of Matrigel would confound future studies into the role of matrix on MSN development. Laminin or poly-d-lysine alone gave poor adherence and growth. In contrast, high levels of adherence, survival, and development were achieved with a mixture of 4 μg/ml

laminin and 100 μg/ml poly-D-lysine, with the additional improvement that cells developed in a near mono-layer, allowing for easier imaging of dendritic arbors. Increasing laminin concentrations (from 4 μg/ml to 20 μg/ml) did not improve adherence, survival, or development above the lower concentration.

Digestion solution

In the absence of protease treatment, mechanical dissociation of brain tissue causes significant cell death. Therefore, the efficacies of papain and trypsin digestion were compared. For postnatal tissue, papain digestion was more effective and yielded higher viability than trypsin. For embryonic tissues, papain and trypsin gave identical results, both in terms of cell viability immediately after dissociation and subsequent long-term survival and differentiation (data not shown). Because of its significantly lower cost, ease of preparation and routine usage in other types of cell culture, trypsin dissociation became the method of choice.

Glial conditioning and growth factors

The embryonic cortex and striatum have a high density of glial cells that can proliferate in culture. These glia cells secrete factors that enhance neuronal survival and development, including Brain Derived Neurotrophic Factor (BDNF), which has been shown to promote survival and development of striatal neurons in mono-culture (Mizuno et al., 1994; Widmer and Hefti, 1994; Nakao et al., 1995; Ventimiglia et al., 1995; Ivkovic and Ehrlich, 1999). Despite the presence of some glia in both the mono and co-

culture conditions, routine feeding of glia-conditioned media (GCM) improved the survival and development of MSNs when compared to unconditioned neuronal growth media. Inhibition of glial proliferation with anti-mitotic agents had a negative impact on MSN survival and BDNF application did not improve the survival or development of MSNs above the level produced by GCM.

DNA transduction

The ability to transduce MSNs with exogenous DNA will be critical for future experiments investigating the development and plasticity of MSN dendritic spines. Several transduction methods were tested in the co-culture condition, with the goal of finding a method that efficiently transduced MSNs. Electroporation of plasmid DNA using the Lonza/Amaxa system prior to plating transduced approximately 10–20% of MSNs in culture with no detectable effect on survival or differentiation. Furthermore, using plasmids with a hybrid CMV early enhancer/chicken beta-actin promoter (pCAG-EGFP), expression was maintained for more than 21 days. In contrast, expression from pEGFP-C1 (CMV promoter) decreased markedly during the first week in culture and was barely detectable by 21DIV (data not shown). In addition to successfully transducing a constitutively active plasmid, we have had success using a two plasmid, tet-ON system to allow temporally restricted expression of EGFP. When using the tet-ON system, the transactivator plasmid (driven by the constitutively active CAG promoter) and a plasmid containing the tet-response element (expressing soluble EGFP) were simultaneously electroporated. Expression of EGFP was induced by maintaining cultures in GCM

containing 1 µg/ml doxycycline and reached maximal levels after about 4–7 days (ex. Figure 6). Expression could be maintained following induction for more than 21 days.

In contrast to electroporation, calcium phosphate transfection, although effective at transducing cortical pyramidal neurons on the coverslip, proved inappropriate for use in the MSN co-cultures. Calcium phosphate simply failed to transfect MSNs at any time point (<<1% transfection or 1–2 MSNs per coverslip). In addition, both adenovirus and adeno-associated viruses 5 (AAV5), yielded high levels of glia infection that obscured the visualization of infected MSNs. Initial tests with Adeno-associated virus 2 (AAV2) was efficacious at transducing the pyramidal neurons but failed to transduce significant numbers of MSNs. Tests are currently underway to determine if another AAV serotype might be more efficacious for MSN transduction.

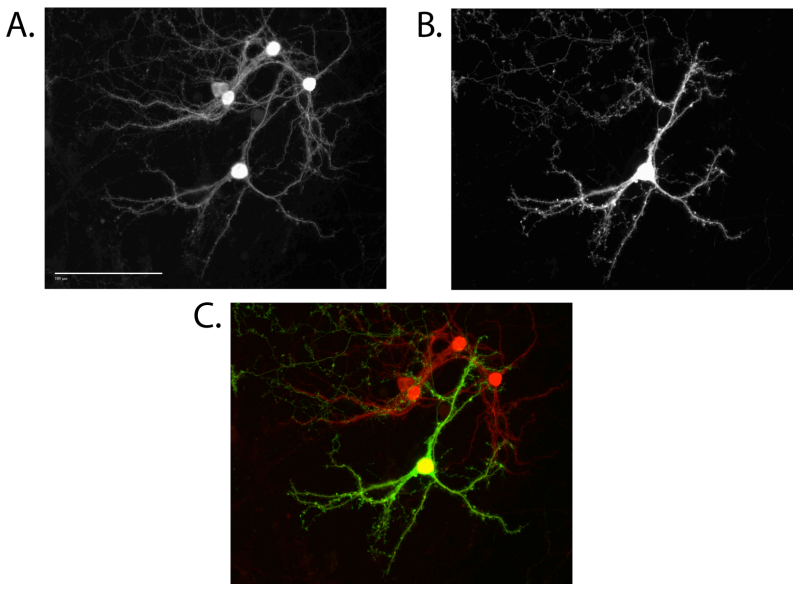


**Figure 6. Tet-inducible EGFP expression in mature MSNs**
25DIV MSN (A, DARPP antibody) electroporated at plating with a two plasmid tet-inducible EGFP (B, EGFP expression) induced at 21DIV with 1µg/ml Doxycycline. (C, merge) Expression is limited to a subset of MSNs allowing for better reconstruction dendrites.

## Table 2. Observations from reagent testing

| | Variables Tested | Observations | Final Selected |
|---|---|---|---|
| Matrix | Matrigel (1:50) | Cells from embryonically and postnatally sourced tissue adhere well, but thick substrate makes imaging and assessing the development of neurons difficult | Not used |
| | Poly-d-lysine (100µg/ml) | Cells from both sources adhere well but develop slowly | Not used |
| | Poly-d-lysine (100µg/ml) and Laminin (4µg/ml) | Cells adhere as well as in higher laminin concentration and develop faster than PDL alone. Develop in near mono-layer, able to image neurons easily | Selected as final matrix due to good adherence, survival, development, and imaging |
| Cell Source | P1-3 striatum dissected from coronal slice | Initial cell numbers are high, but cultures fail to survive to three weeks *in vitro* | Not used |
| | E16 "striatum" dissected as whole ganglionic eminence | This technique provides a large number of cells that survive well in culture, but also contributes more non-MSNs than the postnatal source | Despite the increase in non-MSN cells from this source, it consistently produces MSNs that can survive past three weeks in culture |
| Cell Density | $1*10^6$ cells/35mm dish | This density was used in attempt to overcome the failure of postnatal tissue to survive *in vitro*. In embryonically sourced tissue, this density was far too high for imaging. | Not used |
| | $2*10^5$ cells/35mm dish | In cultures from postnatal tissue this density was initially promising, but cultures failed to survive to three weeks in culture. In embryonically sourced tissue this density gave sufficient for good MSN survival and ease of imaging. | This density, in a 2:3 striatum to cortex ratio, produces embryonic cultures that survive past three weeks in culture with high numbers of mature MSNs |
| Media | Glial Conditioned Neurobasal Growth Media | Despite the presence of glia in the cultures, the conditioning of growth media on confluent glial plates improved the survival and development of cultures above unconditioned media. | All cultures are maintained in glial conditioned growth media that is supplemented weekly |
| Supplements | BDNF | The addition of BDNF to glial conditioned growth media did not improve survival or development of MSNs | Not used |
| | Anti-mitotic agents (67.5µm 5-Fluoro-2'-deoxyuridine and 137µm uridine at 3, 7, and 14div) | Although anti-mitiotic agents reduced the number of proliferating glia in the cultures they produced a pronounced loss of neurons that were associated with the glial cells. The addition of glial conditioned media did not augment this effect, indicating that the presence of glia may be helping MSNs to survive in culture | Not used |
| | Antibiotics | Antibiotics are not needed in cultures sourced from embryonic tissue but required to reduce contamination seen in postnatally sourced tissue. | Not used |
| Digestion Solution | Papain Digestion | Digested cells well, produced cells that were easily dissociated manually | Not used |
| | Trypsin Digestion | Digested cells well, produced cells that were easily dissociated manually | Although both digestion techniques worked well, trypsin digestion was already in routine use and was selected for future experiments |
| Transduction | Calcium Phosphate | Effective at transducing the cortical pyramidal neurons but failed to transduce MSNs | Not used |
| | Adeno-virus | Infected the high levels of glia in the culture with greater efficiency than neurons and non-MSNs with greater effciency than neurons | Not used |
| | Electroporation (AMAXA) | Effective in transducing ~10-20% of MSNs in mature culture when introduced at plating. Electroporation of striatal cells in culture precludes transduction of pyramidal neurons. | Only effective method at consistently transducing an appreciable number of MSNs in culture. |
| Coverslip Preparation | German Glass (ex. Bellco) | Improves adherence and survival after 5div | Used |
| | Acid and Ethanol washing | Reduces fasiculation of neurites during development. Nitric acid can also be used, but is more difficult to dispose of | Used |
| | Maintained in 200˚C oven | Eliminates seasonal air quality differences that impact cell adherence | Used |

*Effect of culture condition on the development of DARPP-32+ expressing MSNs*

The survival and development of MSNs were compared between the co-culture and mono-culture conditions in order to determine when the number of DARPP-32 expressing MSNs was maximal and dendritic arborization and spine density had reached mature levels. In both culture conditions, DARPP-32 expression was first detectable at 7DIV, but cells were still morphologically immature (data not shown). By 14DIV, DARPP-32 expression had increased and was easily detectable in morphologically complex neurons. Using βIII Tubulin to identify neurons and DAPI to identify all cellular nuclei, the percentage of neurons in the culture was analyzed (Table 2). At 14DIV there was no significant difference in the percentage of neurons between culture conditions. However, when DARPP-32 antibody staining was used to specifically identify MSNs, it was revealed that there was a significantly higher percentage of neurons that were MSNs in the co-culture condition (6.08% co vs. 3.08% mono, $n = 100$, $p < .001$ two-tailed). After 21DIV, the percentage of total cells that were neurons was significantly lower in the co-culture compared to the mono-culture (39.04% co vs. 56.05% mono, $n = 100$, $p < .001$ two-tailed), likely due to the proliferation of glia in the co-culture condition. In contrast, at 21DIV the percentage of total cells that were MSNs was not significantly different between culture conditions (1.94% co vs. 2.39% mono, $n = 100$, $p = 0.1590$ two-tailed). In the co-cultures, the proliferation of the glia, rather than the dying of MSNs, likely accounts for the apparent decrease from 14 to 21DIV in the percentage of total cells that were MSNs. This percent of MSNs in the co-culture is

consistent with published estimates of the number of MSNs in the developing striatum (see discussion).

**Table 3. Effect of culture condition on MSN yield**

| | | % neuron of total cells | % MSN of total neuron | % MSN of total cells | Cells/mm$^2$ [a] | Neurons/mm$^2$ | MSN/mm$^2$ |
|---|---|---|---|---|---|---|---|
| MONO | 14div | 51.33 | 3.08 | 1.67 | 228.60 | 186.91 | 4.03 |
| | 21div | 56.05 | 3.58 | 2.39 | 229.97 | 115.59 | 3.36 |
| CO | 14div | 53.93 | 6.08 | 3.13 | 143.41 | 113.18 | 5.52 |
| | 21div | 39.04 | 3.88 | 1.94 | 255.67 | 82.56 | 3.47[d] |
| Published Reports | Adult Striatum | 20[b] | ~99[b] | ~20 | - | - | - |
| | Embryonic LGE | ~55[c] | ~23[c] | ~12.7 | - | - | - |
| a: density at plating = ~200cells/mm$^2$ | | | | | | | |
| b: Schröder et al. J Hirnforsch (1975) vol. 16 (4) pp. 333-50 | | | | | | | |
| c: Skogh et al. Neuroscience (2003) vol. 120 (2) pp. 379-85 | | | | | | | |

*Morphological development of MSNs in different culture conditions*

Culture condition can affect the rate and complexity of morphological development. To assess the complexity of MSN processes, a Sholl analysis (Figure 7) was performed on DARPP-32+ neurons from both culture conditions. At 14DIV, morphological complexity was greater in the co-culture condition, with significantly more crossings at 40–70 μm from the cell body (Fig. 6; bonferroni post hocs allowed paired comparisons, $p < 0.05$). The increased complexity in the co-culture condition was maintained at 21DIV, with significantly more crossings at 30–100 μm from the cell body (Figure 7). Although the co-cultures showed increased complexity of the more distal dendrites from 14 to 21DIV, there were no significant differences in complexity between time-points within a given condition. The apparent decrease in complexity in the mono-culture from 14 to 21DIV was due, at least in part, to increased fasciculation in mono-

cultured MSN dendrites (for example, see Figure 7, boxed region). As mono-cultured MSNs developed, their dendrites tended to bundle together, making it difficult to resolve single dendrites and their associated spines. In the co-culture condition MSN dendrites remained spatially separated from one another.

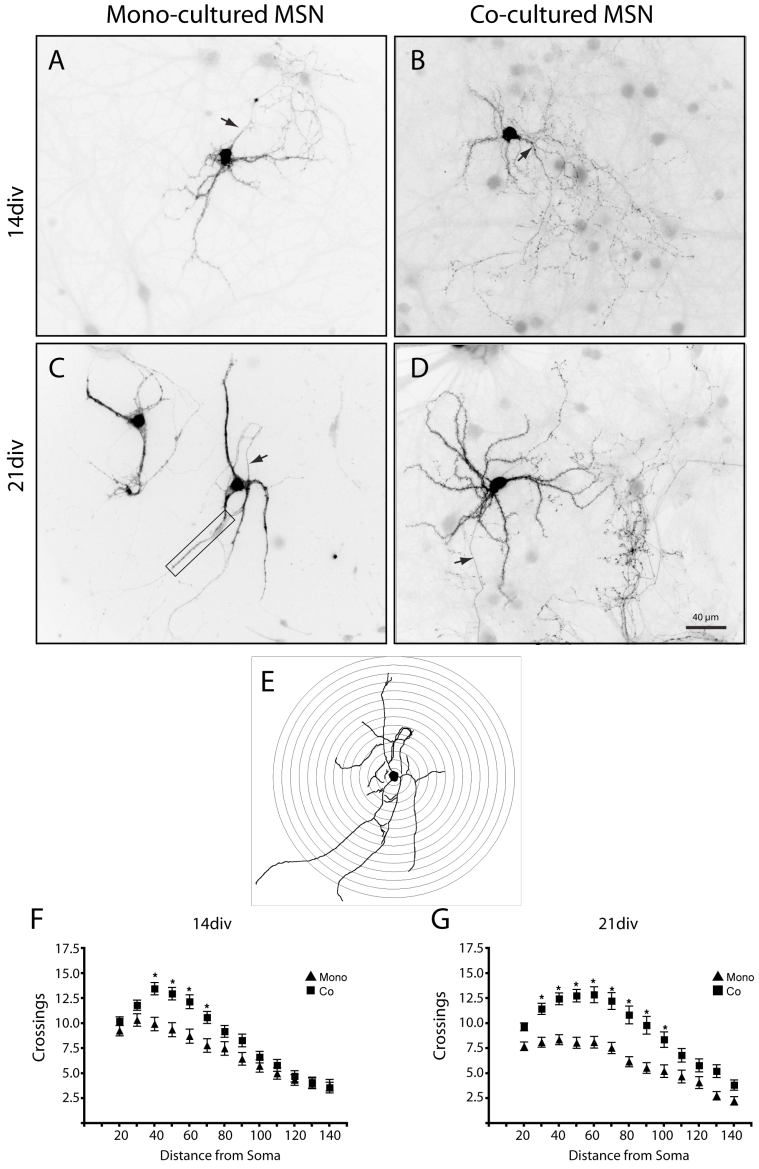


**Figure 7. Sholl analysis of morphological complexity between culture conditions** DARPP-32+ MSNs (A-D) were analyzed for morphological complexity using Sholl analysis (E). Co-cultured MSNs were found to be more complex than mono-cultured MSNs at 14 and 21DIV (F, G). Boxed area highlights region of fasciculation, arrowheads indicate axon, scale bar 40µm.

*Quantification of dendritic spines in different culture conditions*

Medium spiny neurons are so named due to the high density of spines that stud their dendritic arbors. To assess the density of dendritic spines, EGFP filled, DARPP-32+ MSNs were examined at 21DIV, a time point where morphological maturation was maximal and DARPP-32 expression levels were high. At this time point, there was a significantly higher density of dendritic spines in the co-culture condition (10.1 ± 0.7 spines/10 μm co vs. 4.4 ± 0.5 spines/10 μm mono, $n_{co}$ = 42 segments, $n_{mono}$ = 33 segments, $p < .0001$, two-tailed). This significant difference in spine densities can be seen as an increase in the relative frequency of spine densities greater than 10 spines/10 μm in the co-culture conditions (Figure 8).

In addition to an increased density of dendritic spines produced by co-culturing, the morphology of spines was significantly different between conditions. Morphometric analyses showed that spine head diameter was significantly larger in co-cultured MSNs (Figure 8, 0.44 ± 0.004 μm co vs. 0.38 ± 0.008 μm mono, $n_{co}$ = 42 segments, $n_{mono}$ = 33 segments, $p < .0001$, two-tailed). In addition to larger heads, co-cultured MSN dendritic spines had significantly longer spine necks than mono-cultured MSN spines (Fig. 4, 1.143 ± 0.01 μm co vs. 0.79 ± 0.02 μm mono, $n_{co}$ = 42 segments, $n_{mono}$ = 33 segments, $p < .0001$, two-tailed). Spines can be categorized based on their morphologies into varied classifications including mushroom, thin, and stubby. In the mono-culture condition approximately 25% of spines had mushroom-like morphologies (neck length to head diameter less than one and head diameter greater than 0.35 μm), nearly 35% of spines had thin-like morphologies (neck length to head diameter greater than 2.5), and the

remaining 40% of spines had a stubby-like morphology. The co-culture condition had a greater percentage of spines with mushroom-like morphologies (52%) and smaller proportions of thin (28%) and stubby (20%) morphologies (Figure 8).

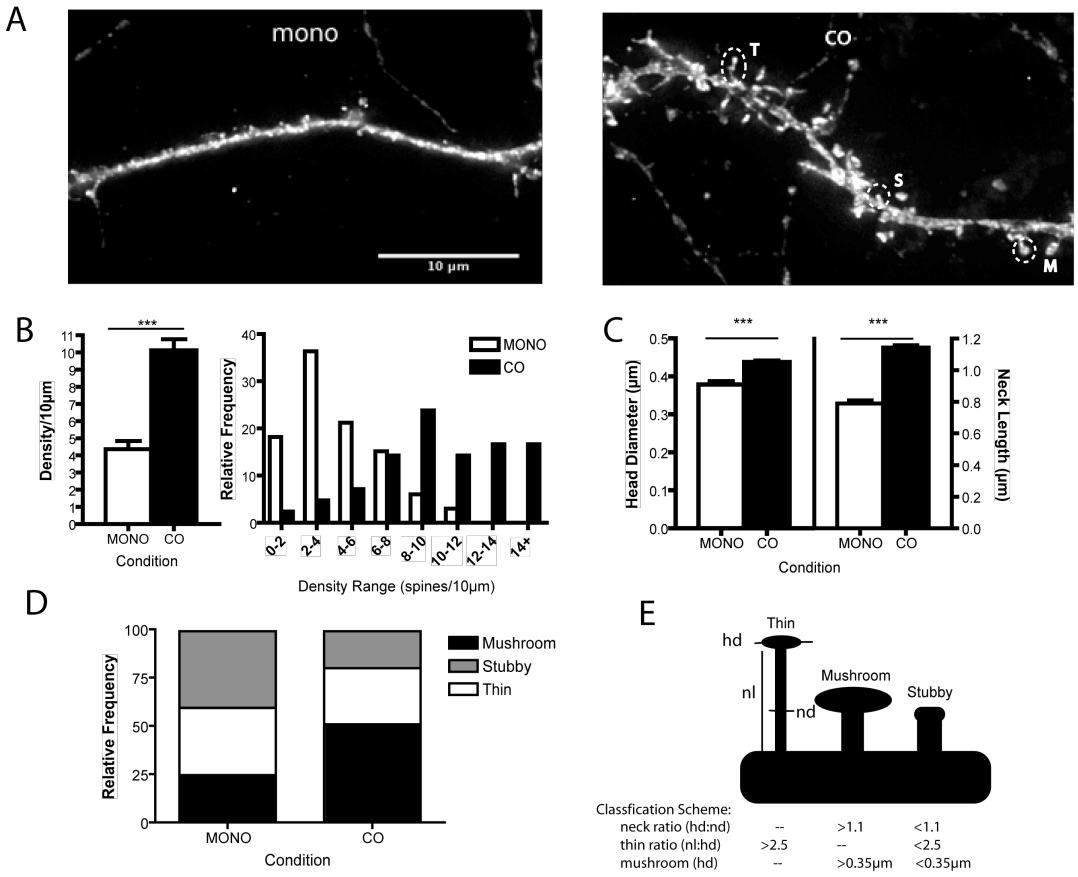


**Figure 8. Effect of culture condition on MSN dendritic spines**
(A) 21DIV mono (left) and co-cultured (right) MSN dendrites and spines expressing EGFP. Note the presence of a variety of spine morphologies in co-cultured MSNs. Co-cultured MSNs have thin (t), stubby (s), and mushroom (m) spiens found on the same dendrite. (B) Co-cultured MSNs have significantly greater density of dendritic spines. (C) Co-cultured MSN spines have larger heads and longer necks than mono-cultured MSNs. (D) Co-cultured MSN dendritic spines are predominately mushroom and stubby type, whereas mono-cultured MSN dendritic spines are predominately stubby and thin type. (E) Explanatory figure of Neuronstudio three-type classification system.

*Immunochemical analysis of dendritic spines*

In order for dendritic spines to receive and respond to stimulation they must have intact pre- and post-synaptic machinery. Because co-cultured MSNs were significantly more developed than those in mono-cultures, immunocytochemical analysis of dendritic spines was restricted to 21DIV co-cultures. In the co-cultures, DARPP-32 was detected in all of the dendritic spines, where it appeared to partially co-localize with EGFP (Figure 9). Spine heads were filled with filamentous actin (f-actin; Figure 9 B'), were apposed to SV2 containing pre-synaptic compartment (Figure 9 C') and contained a PSD-95 rich post-synaptic density (Figure 9 D'), indicative of the presence of functional excitatory synapses.

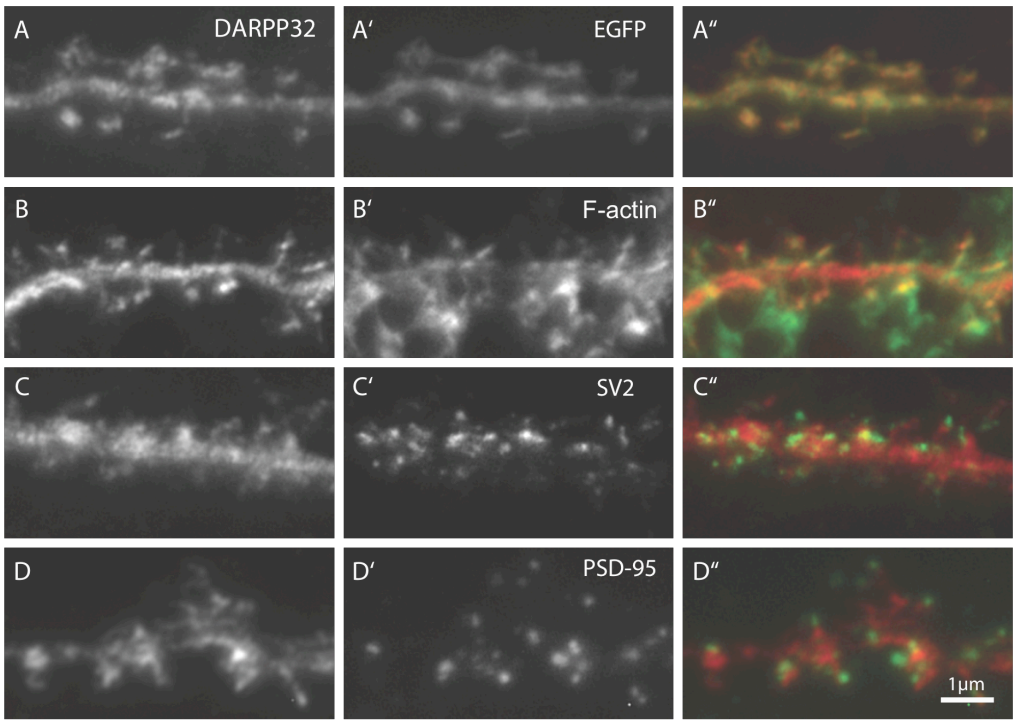


**Figure 8. MSN synaptic marker localization.**
21DIV co-cultured DARPP-32+ (left column) MSNs have mature synaptic marker localization. (A) EGFP fills MSN dendritic spines. (B) MSN dendritic spine heads are f-actin rich, (C) apposed to SV2, (D) and contain PSD-95

*Electrophysiological characteristics of MSNs in co- and mono-culture conditions*

The presence of PSD-95 and SV2 puncta indicate that dendritic spines of co-cultured MSNs could be functional. In order to compare the synaptic function of mono – and co-cultured MSNs, whole-cell patch clamp recordings were conducted on 19-24DIV cultures (Figure 10). MSNs were identified by morphology (i.e. soma size, dendrite organization) as well as by their hyperpolarized resting membrane potential. Specifically, compared to interneurons (GABA and ACh interneurons), which exhibit a depolarized resting potential (Vrest, around −50/−60 mV), MSNs exhibit a hyperpolarized Vrest (around −80 mV). However, another type of striatal neurons, fast spiking interneurons (FSNs), exhibit a relatively hyperpolarized Vrest (around −74 mV), which might lead to potential confusion with MSNs. Nonetheless, FSNs represent less than 5% of total number of striatal neurons in the adult striatum and possess a morphology (number of dendritic branches and dendritic architecture) distinguishable from MSNs (Kreitzer, 2009; Govindaiah et al., 2010), which makes the level of possible contamination extremely low. Co-cultured MSNs had significantly higher mEPSC frequency (4.28 ± 0.48 Hz co vs. 1.56 ± 0.48 Hz mono, $t_{13} = 3.05$, $p < 0.01$), consistent with the higher numbers of functional dendritic spines in co-cultures. Unexpectedly, mono-cultured MSNs exhibited higher mEPSC amplitude compared to co-cultured MSNs (9.73 ± 0.92 pA co vs. 13.95 ± 1.11 pA mono, $t_{13} = 2.87$, $p < 0.05$). Due to different experimental conditions between brain slices and cultured neurons, it is difficult to compare electrophysiological results obtained in these two preparations. However, it is noteworthy to mention that the level of mEPSC amplitude and frequency in co-cultured

conditions are comparable to those observed in *in vitro* sagittal striatal slice recordings (in which glutamatergic fibers from the PFC are left intact) made in the presence of lidocaine to isolate responses that reflect only activity-independent glutamate release (e.g. (Kourrich et al., 2007).

Overall neuronal excitability, and thus neuronal function, depends on both synaptic and intrinsic factors (i.e. voltage-gated ion channel conductances). To test if characteristics of intrinsic excitability have properly developed in our co-culture model, we assessed firing properties of co-cultured MSNs. Recordings showed firing characteristics typical of MSNs (Figure 10) and comparable to those recorded *in vivo* (O'Donnell and Grace, 1995) and in mature brain slices (Nisenbaum et al., 1994; Kasanetz and Manzoni, 2009; Kourrich and Thomas, 2009; Kreitzer, 2009). Specifically, co-cultured MSNs displayed a hyperpolarized resting membrane potential, a slowly depolarizing ramp when depolarized close to or slightly above firing threshold, regular spiking and a delayed firing latency during incremental depolarizing steps (Kita et al., 1985; Bargas et al., 1989; Nisenbaum et al., 1994; Kawaguchi, 1997; Belleau and Warren, 2000).

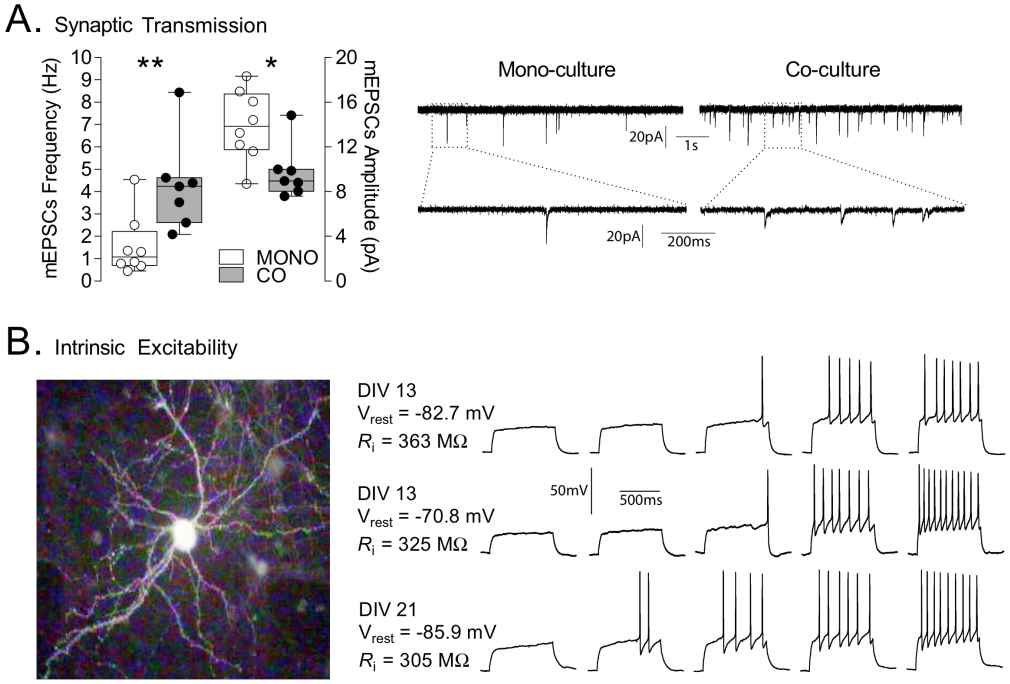


**Figure 9. Electrophysiological characterization of mono- and co-cultures.**
(A) Analysis of synaptic transmission. Left, co-cultured MSNs (CO, n=7cells) exhibit significantly greater mEPSC frequency and lower amplitude compared to mono-cultured MSNs (MONO, n=8cells). Right, sample traces of mEPSCs from mono- and co-cultured MSNs. Calibration: 1s, 20pA and 200ms, 20pA; the lower traces on each side show an expanded sweep of a portion of the upper traces. * $p<0.05$, ** $p<0.01$ (B) Analysis of intrinsic excitability. Left, image of a recorded EGFP-expressing MSN taken directly from the recording rig. Right, sample traces from co-cultured MSNs at 13DIV (top, at 100, 120, 140, 180, and 200pA; middle, at 80, 100, 120, 160, and 200pA) and 21DIV (bottom, at 80, 100, 120, 140, and 160 pA). Calibration: 500ms, 50mV

*Dopamine receptor stimulation induced CREB phosphorylation*

In striatal neurons D1-type dopamine receptor activation leads to an increase in CREB (Cyclic-AMP Response Element-Binding protein) phosphorylation (Konradi et al., 1994) through a $G_s$/adenylyl cyclase pathway (Das et al., 1997) as well as a cAMP/Akt pathway (Brami-Cherrier et al., 2002). In order to assess co-cultured MSNs sensitivity to dopamine receptor activation, CREB phosphorylation was assessed following stimulation with a D1-specific agonist (500 nM 6-chloro-PB) or potassium depolarization (20mM KCl). Mature (20DIV) co-cultured MSNs had a high baseline level of CREB phosphorylation (Figure 11A, E), likely produced by excitatory stimulation from cortical neurons. CREB phosphorylation was significantly reduced following 3 hours incubation with 25 μm APV (to block NMDA-induced $Ca^{2+}$ influx) and 1 μm TTX (to block action potentials; Figure 11 B, E; Dunn's multiple comparison, $p<0.001$). Incubation in 20 mM KCl for 15mins induced a significant increase in MSN CREB phosphorylation compared to silenced MSNs (Figure 11 C, E; Dunn's multiple comparison, $p<0.001$), but which was not significantly different from pre-silenced baseline. Similarly, incubation with 500 nM 6-chloro-PB induced a significant increase in CREB phosphorylation (Figure 11 D, E; Dunn's multiple comparison, $p<0.001$) that was not significantly different from baseline.

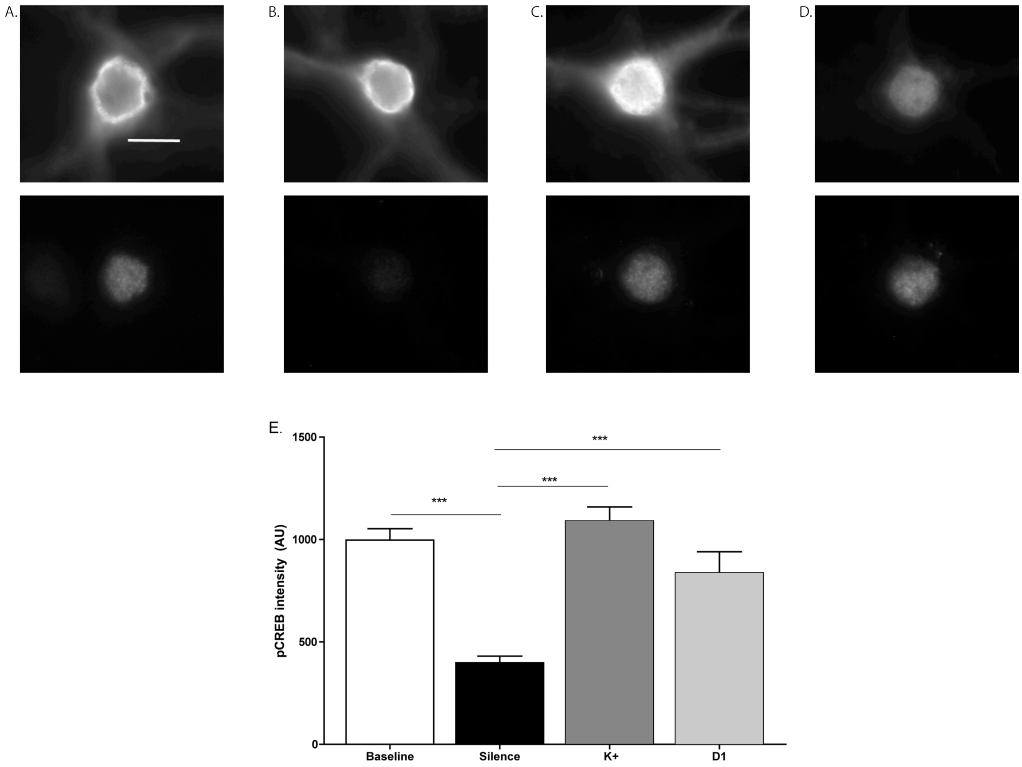


**Figure 10. Stimulation induced CREB phosphorylation in co-cultured MSNs**
A-D) 20DIV co-cultured MSNs showing DARPP-32 (top) and pCREB (bottom) staining. A) pCREB signal is high in unsilenced, unstimulated cultures. B) Incubation for 3hrs with 25µm APV/1µm TTX significantly decreases pCREB levels. C) Stimulation for 15mins with 20mM KCl significantly increases pCREB to a level not significantly different from baseline. D) Stimulation for 15mins with 500nM 6-chloro-PB (D1-agonist) significantly increases pCREB to a level not significantly different from baseline. E) Quantification of pCREB intensity in DARPP-32+ MSNs. A Kruskal-Wallis test revealed a significant effect of treatment on pCREB intensity ($\chi^2 = 68.56$, $p<0.0001$) and Dunn's multiple comparisons test identified treatments of significant difference (***$p<0.001$).

Discussion

After testing myriad conditions, it was determined that optimal MSN development and survival were obtained when MSNs from the embryonic striatum were co-cultured with embryonic cortical neurons, plated on a mixture of poly-d-lysine and laminin and grown in glial-conditioned Neurobasal/B27 media. This culture system takes advantage of the high viability of neurons in the embryonic striatum, enabling plating of large numbers of coverslips from a single litter. Additionally, the technique described in this paper can be adapted for use with transgenic animals, with the cells from single pups contributing to several dishes of coverslips.

MSNs in co-culture appeared to be quite refractory to most methods of DNA transduction, such that only electroporation of the striatal cell suspension immediately prior to plating reliably transduced a significant number of MSNs. Fortunately, using a strong CAG promoter it was possible to maintain high levels of gene expression for >21 days, with no effect on cell health. By comparison, expression from the CMV promoter gradually decreased and was generally lost by 14div (data not shown). Using electroporation, it was also possible to co-transfect a tet-on expression plasmid with a tet-transactivator expressed from a CAG promoter and induce the tet promoter expression of EGFP at any time from 3 to 21DIV (e.g. Figure 6). Robust levels of expression were achieved within 72 hr of induction with 1 μg/ml doxycyclin and could be maintained for at least 21 days with weekly feeding of fresh doxycyclin-free glial conditioned media. Thus, both constitutive and inducible gene expression can be obtained in the co-culture system.

Interestingly, development of the MSNs in co-culture appeared to require the physical presence of glia in the dish; addition of anti-mitotic agents to limit glial expansion affected both the development and survival of MSNs and the requirement for glia could not be substituted for with glial-conditioned media. Despite the presence of glia in the in culture dish, glial-conditioned media improved survival and development of MSNs beyond what is seen in dishes maintained in unconditioned media. It would appear that both physical contact with the glia in the dish and secreted factors provided by the glial-conditioned media are supporting enhanced survival and development. The most likely explanation for the need for GCM is that during early development *in vitro* the number of glial cells in the culture dish is not sufficient to provide the secreted factors necessary to support the MSNs. This conclusion is in agreement with previous reports indicating that the presence of a small number of proliferating astrocytes enhances the survival and differentiation of striatal neurons in culture (Sebben et al., 1990; Skogh and Campbell, 2003).

*In vivo*, the number of DARPP-32+ cells increases significantly during the first few weeks of postnatal development (Foster et al., 1987). Our finding that the number of DARP32+ MSNs did not increase from 14 to 21DIV (roughly corresponding to postnatal days 10–17) suggests that dissociation of the tissue disrupted signaling events that are critical to MSN differentiation and/or DARPP-32 expression (Jain et al., 2001). Although is has been reported that inclusion of BDNF in E17 striatal mono-culture increases the number of DARPP32+ cells (Ivkovic and Ehrlich, 1999), we found no such effect in the co-culture system (data not shown).

At first glance, the fact that only ~2% of the cells in the co-culture were MSNs might seem unexpectedly low given the often quoted fact that in the adult striatum, ≥95% of the neurons are MSNs (Table 2); however, it is important to remember that due to the abundance of glia cells, only 20–55% of the total cells in the adult striatum are in fact MSNs (the value depends on whether the analysis is limited to the caudate (Kataoka et al., 2010) or includes the entire striatum (Schröder et al., 1975) and likely varies between species). In the co-culture, the entire ganglionic eminence (GE) was dissected as a source of MSNs, but the majority of DARPP32+ cells are found in the lateral ganglionic eminence (LGE; (Deacon et al., 1994)). It has been estimated that the LGE comprises about 50% of the total GE and that ~12.7% of LGE cells are DARPP32+ (Skogh et al., 2003). Since only 40% of the cells in the co-culture came from the ganglionic eminence, at plating only ~2.5% of cells should be MSNs (2.5% = 50% of GE is LGE × 12.7% of LGE cells are MSNs × 40% of plated cells are from GE). This yield of MSNs is similar to that reported for other embryonic striatal culture systems (Watts et al., 1997). Thus, the current yield of MSNs suggests that maximal survival of MSNs has been achieved in the co-culture.

Although mono-culture is capable of supporting the survival of MSNs, our data indicate that MSNs in the mono-culture condition were less morphologically complex than those in the co-culture condition. The fasciculation of dendrites in the mono-culture condition could complicate further experiments designed to investigate the development and plasticity of dendritic spines by limiting the sampling area to only the terminal tips of dendrites. MSNs grown in co-culture had 10.12 ± 0.7 spines/10 μm, a value remarkably

similar to that reported for *in vivo* analysis of mature MSNs in adult mouse and hamster striatum (Jedynak et al., 2007; Neely et al., 2007; Staffend et al., 2011). In addition to having a significantly greater density of dendritic spines, spines in the co-culture condition were morphologically more like MSNs *in vivo*. Spines on co-cultured MSNs had larger heads and longer necks than those on mono-cultured MSNs. This difference in spine morphologies was reflected as a change in the relative proportion of different spine types. In mono-cultured MSNs, only about a quarter of spines had mushroom-like morphologies, contrasted with more than half of all spines being this type in co-culture. Importantly, using similar morphometric classification, MSNs in adult mouse striatum were shown to have similar ratios of mushroom, thin and stubby spines (Jedynak et al., 2007).

The presence of any spines or excitatory post-synaptic currents in the mono-cultured MSNs is likely still dependent on cortical innervation. Given the method of collection of the striatal neurons (removal of the eminence from over-lying cortex) it is likely that there is contamination by cortical pyramidal neurons in the mono-culture and that the variable presence of this glutamatergic input contributes to the limited development of spines in mono-culture. Whether the morphology of dendritic spines in the mono-culture represent an immature morphology or a compensatory change due to decreased afferent activity is beyond the scope of the current study.

Electrophysiological characterization of co-cultured MSNs demonstrated that the basic synaptic and intrinsic properties are comparable to those observed in mature MSNs recorded in slice and in *in vivo* preparations (O'Donnell and Grace, 1995; Belleau and

Warren, 2000; Kasanetz and Manzoni, 2009). These results suggest that the present co-culture method provides appropriate conditions for supporting the development of mature, *in vivo*-like MSNs. Previous research indicated that mono-cultured MSNs grown in Neurobasal/B27 media fail to develop mature firing properties, but that MSNs grown in a serum containing DMEM media are able to develop mature firing properties (Rubini et al., 2006). In the current co-culture system, MSNs grown in Neurobasal/B27 media do develop mature firing properties, suggesting that in previous experiments factors other than the media were allowing for mature intrinsic property development. Since the inclusion of serum in the media will enhance glial survival and proliferation (Goslin, 1998), it is possible that the reported effect of media may have been due to the influence of glia on MSN development.

Despite obvious differences in the two preparations, it is striking that mEPSC frequency and amplitude measured in co-cultured MSNs were similar to those observed in slices in which the glutamatergic fibers are left intact (Kourrich et al., 2007). Unexpectedly, mono-cultured MSNs exhibited higher mEPSCs amplitude when compared to co-cultured MSNs. This increased mEPSC amplitude in mono-cultures may reflect a developmental homeostatic adaptation, such as synaptic scaling (Turrigiano et al., 1998; Turrigiano, 2008), intended to compensate for the low level of excitatory afferents produced by low levels of cortical contamination in the mono-culture condition. Reductions in network activity, produced by changes in excitatory input density (Ivenshitz and Segal, 2010) or chronic blockade with TTX (Segal et al., 2003; Fishbein and Segal, 2011), produce significant increases in mEPSC amplitude and that there is a

correlation between increased AMPAR insertion and increased mEPSC amplitude (Matsuzaki et al., 2001); however, given that MSNs in mono-culture have smaller spine head diameters than those in co-culture, it seems unlikely that the increased mEPSC amplitude seen mono-cultured MSN is due entirely to increased AMPR insertion. This may suggest there is a mechanism other than AMPA receptor insertion for increasing mEPSC amplitude (such as alterations in subunit composition favoring GluR2-lacking, $Ca^{2+}$ in mono-cultured MSNs or that the correlation between AMPA receptor complement and spine head size is not as tightly coupled in MSNs *in vitro*.

Alternatively, it is possible that, rather than a change in postsynaptic strength, the enhanced mEPSC amplitude seen in the mono-cultured MSNs is related to the significant reduction in spine neck length. The spine neck serves as a barrier to the diffusion of calcium produced by depolarization of the spine head (Schmidt and Eilers, 2009) and there is an inverse correlation between neck length and depolarization at the soma (Araya et al., 2006). Given that the dendritic spines of monocultured MSNs have shorter necks and smaller heads, $Ca^{2+}$ transients in the head would be more likely to diffuse into the dendritic shaft, contributing to greater depolarization at the soma. Thus, it is quite possible that a combination of synaptic scaling and changes in calcium diffusion could contribute to increasing the amplitude of mEPCSs in mono-cultured MSNs.

MSNs in co-culture have a high level of basal CREB phosphorylation, indicating that the cortico-striatal synapses are producing sufficient $Ca^{2+}$ influx to activate CREB phosphorylation pathways. Since MSNs in co-culture developed in the absence of dopamine afferents, it was possible that they might not express surface targeted,

functional D1-type dopamine receptors; however, co-cultured MSNs showed a significant increase in CREB phosphorylation when stimulated with a D1-agonist following silencing with APV/TTX. This increase was similar to one induced by potassium depolarization. This finding demonstrates that, despite the lack of dopaminergic input, the D1-receptor appears to be functionally coupled to appropriate downstream cascades to induce CREB phosphorylation. Interestingly, D1-receptor activation appeared to change the localization pattern of DARPP-32 to favor somatic and perhaps nuclear enrichment (Figure 11D). Although not quantified in this experiment, the observation is consistent with a previous report of D1-receptor induced DARPP-32 nuclear translocation (Stipanovich et al., 2008). This observation indicates that in addition to intact CREB signaling cascades, D1-receptor activation is also properly coupled to the cAMP/PKA/PP2/DARPP-32 cascade.

In summary, we describe and characterize a simple, streamlined culture system capable of supporting the development and maturation of *in vivo*-like MSNs, complete with high densities of dendritic spines. These MSNs survive for more than a month *in vitro* and display electrophysiological and morphological characteristics consistent with mature MSNs. Future research in this system can be used to address mechanisms of MSN dendritic spine development and plasticity.

## Chapter 3: Morphological analysis of MSN development *in vitro*

Introduction

Alterations in MSN spine density are known to occur during postnatal development (Chronister et al., 1976; DiFiglia et al., 1980; Tanaka), in diseases such as Parkinson's (Stephens et al., 2005; Zaja-Milatovic et al., 2005) and Huntington's (Ferrante et al., 1991), and after repeated psychostimulant exposure (Robinson and Kolb, 2004). Although there is significant interest in changes in mature MSN spine density and morphology, there has yet to be a detailed characterization of the morphological development of MSN dendritic spines. Given that MSN dendritic spines are known to be a locus for structural and functional plasticity, understanding their morphological development will provide insights into the relationship of structure and function in mature MSNs and the molecules regulating their development and plasticity. Here we characterize the development of MSNs *in vitro*, including the first morphological analysis of dendritic spine development. We show that MSN dendrite elaboration and spine density increase during development *in vitro* until they stabilize to *in vivo*-like levels. MSN dendritic spine morphology is found to be highly variable across development, with small but significant increases in head diameter and neck length with time. This study identifies time points important for *in vitro* MSN dendrite development, periods of spine density increases and stabilization, and trends in spine morphological development that expand our knowledge of *in vitro* MSN development and highlights the morphological diversity of MSN spines.

Methods

*Cell Culture and transfection*

Primary striatal-cortical co cultures were prepared as previously described (Penrod et al., 2011). Briefly, the ganglionic eminence (presumptive striatum) and pre-frontal cortex were removed from E16 mouse embryos. Tissues were separately digested at 37˚ for 15-30 min in a final concentration of 0.25% trypsin-EDTA (Sigma-Aldrich, T4174), rinsed briefly in CMF/HBSS, and resuspended in neuronal plating media (10 mM HEPES, 10 mM sodium pyruvate, 0.5 mM glutamine, 12.5 µm glutamate, 10% Newborn Calf Serum, 0.6% glucose in minimal essential media plus Earl's salt (EMEM)). Tissues were separately dissociated by trituration with a fire-polished pipette. Following dissociation, cells were counted using trypan blue and a hemocytometer. Cells were plated in 35 mm dishes containing 5 12 mm glass coverslips coated with 100 µg/ml PDL/ 4 µg/ml Laminin, filled with neuron plating media (10 mM HEPES, 10 mM sodium pyruvate, 0.5 mM glutamine, 12.5 µM glutamate, 10% Newborn Calf Serum, 0.6% Glucose in EMEM (Minimal Essential Media, plus Earl's salt)). Cells were plated in a 3:2 cortex to striatum ratio at a final cell density of 200,000 cells/dish. 1-3 hours after plating, plating media was removed and replaced with glial-conditioned growth media (Neurobasal, 1x B27, 0.5 mM glutamine). Dishes were maintained in a 37˚C/5% $CO_2$ incubator and half of the media was replaced with fresh glial conditioned media once per week. Experiments were conducted on cultures ranging in age from 7 to 28DIV.

Prior to plating, the striatal cell population was electroporated using the Lonza Nucleofector system and the mouse neuron transfection reagent. For transfection $1X10^6$

dissociated striatal cells were centrifuged at 1000 x g for 5mins, plating media was removed, and cells were resuspended in 100 µl of complete transfection reagent containing 10 µg of pCAG-EGFP plasmid. Immediately following electroporation, cells were quickly moved into 2 ml of pre-warmed plating media. After a short (less than 2mins) equilibration period, cells were counted using a hemocytometer and trypan blue.

Glia cultures were prepared as previously described (Penrod et al., 2011). Briefly, the cortices of postnatal (P1-2) mice were removed, incubated with 0.25% trypsin-EDTA and 1 µl/ml Benzonase (Novagen, 70664-3) or 3 mg/ml DNAse1 for 30 min at 37˚. After incubation, an equal volume of glia plating media (10 mM HEPES, 1 mM Sodium Pyruvate, 2 mM glutamine, 10% Newborn Calf Serum, 0.6% Glucose, 1x Penicillin-Streptomycin) was added and cells were collected by centrifugation (1000 x g for 2min). Tissue was resuspended in glia plating media, triturated using a flame-polished glass pipette and filtered through a 0.7 µm cell strainer. Cells were plated one pup per dish on uncoated 10 cm tissue culture dishes. Glia media was replaced one day after culture and once per week each subsequent week. Glial-conditioned media was prepared by incubating 10 mls of neuron growth media on confluent glial plates (usually 10DIV, or older) for 48 hrs. Once condition period is complete, conditioned media is removed and replaced with glia plating media.

*Immunofluorescence*

At designated time points, coverslips were fixed at 4˚ with 4% Paraformaldehyde/PHEM (60 mM PIPES pH 7.0, 25 mM K-HEPES pH 7.0, 10 mM

EGTA, 2 mM $MgCl_2$)/0.12 M sucrose buffered fixative for 15-20 min at 4˚C. Following fixation, cells were rinsed in 1XPBS and blocked in 3% bovine serum albumin (BSA) for 30 min at room temperature or 4˚ overnight prior to permeabilization. Cells were permeabalized using 0.2% TritonX-100 in PBS for 10 min at room temperature, rinsed in 1XPBS, and blocked for at least 15 min in 3% BSA prior to staining. All antibody mixtures were prepared in 1% BSA and coverslips were incubated overnight at 4˚C with 50 µl of primary antibody mixture per coverslip. For dendritic spine morphology, coverslips were stained with DARPP-32 (Cell signaling, 2302, 1:250) and mouse monoclonal EGFP (Invitrogen, A11120, 1:1000). Following overnight primary incubation coverslips are rinsed in 1X PBS and incubated in secondary antibody mixture for 1hr at room temperature. The following secondary antibodies were used: donkey anti-rabbit conjugated to Texas Red or TRITC, donkey anti-mouse conjugated to AMCA or FITC (Jackson Immunoresearch). All secondaries were used at 1:100. Following secondary incubation, coverslips are rinsed in 1XPBS and mounted on glass slides with 2.5% 1,4-Diazabicyclo-[2.2.2]Octane, 150 mM Tris pH 8.0, and 80% glycerol mountant to reduce photobleaching.

*Imaging, Quantification, and Analysis*

For Sholl analysis (Sholl, 1953), images were collected using Openlab software (Improvision/Perkin Elmer) on a Zeiss Axiovert 200M microscope using a 20x objective and image analysis was conducted using ImageJ (NIH). Isolated MSNs were identified using DARPP-32 staining. At 7DIV, DARPP-32 expression is visible in the soma but

does not fill the entire cell. When DARPP-32 expression did not fill the entire neuron, EGFP signal was used for Sholl analysis. In mature MSNs EGFP and DARPP-32 overlaps to fill the entire MSN (data not shown). Neurites were counted as individual processes at the soma edge. The ImageJ concentric rings plugin (http://rsbweb.nih.gov/ij/plugins/concentric-circles.html) was used to place concentric rings every 10μm out to 150μm from the cell's center. The cell-counter plugin (http://rsbweb.nih.gov/ij/plugins/cell-counter.html) was used to count processes crossing each ring starting 30μm from the center of the cell. Replications from two independent cultures were pooled. Statistical analysis was conducted using GraphPad Prism v4.0 (GraphPad Software). Crossings were compared across the distance measured and between time points using a two-way ANOVA with Bonferonni's post-test to determine distances of significant difference, $p<0.05$ was considered significant. Measurements are reported and displayed as mean ± SEM.

For dendritic spine development, images were collected on an Olympus ix71 microscope outfitted with a personal Deltavision module. MSNs were identified using DARPP-32 staining and only stretches of dendrites that included the terminal tip were imaged using a 100x objective. Z-stack images were taken at 0.15-0.2 μm intervals through entire focal range of dendrite and stacks were deconvolved using softWoRx software (Applied Precision). Neuronstudio (Mount Sinai School of Medicine, (Rodriguez et al., 2008)) was used for semi-automated spine analysis of deconvolved stacks. For dendritic spine morphological comparisons, a trained analyst confirmed that spines identified by Neuronstudio met criteria for inclusion (ex. observable neck

connected to dendritic shaft, neck correctly attached to dendrite shaft, head diameter placed in center of head). Densities were calculated from individual segments of dendrite greater than 10μm in length. Densities calculated from individual segments were treated as independent observations and densities calculated from 3-4 independent cultures were pooled. Neuronstudio calculated the head diameter and neck length for all identified spines. These measurements were treated as independent observations and parameters from 3-4 independent cultures were pooled. Neuronstudio will automatically classify measured spines into three morphological categories (mushroom, thin, stubby; see (Rodriguez et al., 2008) for classification scheme) based on previous studies of mature pyramidal neurons (Jones and Powell, 1969; Harris et al., 1992). Occasionally Neuronstudio identified spines but was unable to collect the spine neck diameter. In these cases spines were manually categorized based on the neck length to head diameter ratio and absolute head diameter. Spines were categorized as mushroom if they had a neck length to head diameter ratio of less than 1 and an absolute head diameter greater than 0.35 μm. Spines with a neck length to head diameter ratio of greater than 2.5 were classified as thin and all other spines were classified as stubby.

In order to define morphological categories that are found across the entire developmental spectrum, all spine head and neck measurements from 7-28DIV were pooled and the median values determined. Using a median split, spines were categorized as follows; **1**: long neck, small head (≥ median neck length (NL), ≤ median head diameter (HD)), **2**: long neck, big head (≥ median NL, ≥ median HD), **3:** short neck, small head (≤ median NL, ≤ median HD), **4:** short neck, big head (≤ median NL, ≥ median HD). Type

1 spines were further categorized in order to identify filopodial-like spines (type **5**: extreme long neck and extreme small head, ≥75% NL and ≤25% HD). Following assignment into the categories, the proportion of each type of spine per segment length was determined by calculating the relative proportion of each spine per dendritic segment and pooling all segments from a time point to determine the mean proportion. Densities and morphological measurements were compared across time points using Kruskal-Wallis one-way ANOVA followed by Dunn's multiple comparison test to determine time points of significant difference, $p<0.05$ was considered significant. Measurements are reported as mean ± SEM.

## Results

### *Dendritic arborization*

The organization of dendrites affects the availability of sites for synapses to be formed and impacts the transmission of signals back to the soma. In diseases that affect MSNs, dendrite structure is often altered (Klapstein et al., 2001), potentially contributing to MSN dysfunction. Future investigations into the mechanisms of MSN dendrite development will benefit from characterization of periods of rapid elaboration and subsequent stabilization. In order to determine how MSN dendritic branch complexity changed during development *in vitro*, Sholl analysis was performed (SHOLL, 1953).

DARPP-32 positive MSNs were imaged at 20x magnification and a series of concentric circles were overlaid on the cell, with rings every 10 μm from the soma center covering 150 μm, starting 30 μm from the soma center. DARPP-32 is first detectable in MSN soma at 7DIV, therefore this was the first time point examined; however, primary neurites had formed prior to this and the culture as a whole had stable neurite/dendrite number up to this point (data not shown). By 7DIV MSNs had an average of 4.7 primary dendrites and the highest level of dendrite complexity was found close to the soma with very little dendrite elaboration past 120 μm from the soma.

Dendrite elaboration occurred primarily in two waves, with a distal increase occurring between 10 and 13DIV and a proximal increase occurring between 13 and 16DIV. There was a significant increase in dendritic branching over time (Figure 11, Table 3, two-way ANOVA, significant interaction DIV x distance, $F(84, 3536)=3.692$, $p<0.0001$; significant main effects of distance and DIV, $p<0.0001$). Significant increases

in dendrite elaboration were found between 10 and 13DIV and 13 and 16DIV. Between 10 and 13DIV there were significant increases in crossings at 60-80µm from the soma. Between 13 and 16DIV there was a significant increase in crossings at 30-50µm from the soma. There was an additional increase in complexity at 50µm between 16 and 19DIV. Dendritic complexity was stable by 19DIV, with no further changes for the remaining developmental period examined. During development the number of primary neurites on DARPP-32+ MSNs was between 4 and 6.

**Table 3. MSN dendritic complexity increases during *in vitro* development**

| | 30µm | | | 40µm | | | 50µm | | | 60µm | | | 70µm | | | 80µm | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| DIV | MEAN | SEM | N | MEAN | SEM | N | MEAN | SEM | N | MEAN | SEM | N | MEAN | SEM | N | MEAN | SEM | N |
| 7 | 6.08 | 1.24 | 24 | 5.71 | 1.17 | 24 | 5.04 | 1.03 | 24 | 3.58 | 0.73 | 24 | 3.33 | 0.68 | 24 | 2.79 | 0.57 | 24 |
| 10 | 6.43 | 1.34 | 23 | 6.48 | 1.35 | 23 | 5.43 | 1.13 | 23 | 4.65 | 0.97 | 23 | 3.61 | 0.75 | 23 | 2.87 | 0.60 | 23 |
| 13 | 6.20 | 1.13 | 30 | 6.83 | 1.25 | 30 | 7.13 | 1.30 | 30 | 7.20* | 1.31 | 30 | 6.60** | 1.20 | 30 | 5.57** | 1.02 | 30 |
| 16 | 8.29* | 1.29 | 41 | 9.54*** | 1.49 | 41 | 9.44** | 1.47 | 41 | 9 | 1.41 | 41 | 7.46 | 1.17 | 41 | 6.34 | 0.99 | 41 |
| 19 | 9.39 | 1.34 | 49 | 10.78 | 1.54 | 49 | 11.16* | 1.59 | 49 | 9.88 | 1.41 | 49 | 8.63 | 1.23 | 49 | 7.55 | 1.08 | 49 |
| 22 | 8.31 | 1.41 | 35 | 9.63 | 1.63 | 35 | 10.11 | 1.71 | 35 | 9.69 | 1.64 | 35 | 8.54 | 1.44 | 35 | 6.83 | 1.15 | 35 |
| 25 | 9.52 | 1.44 | 44 | 11.34 | 1.71 | 44 | 11.73 | 1.77 | 44 | 10.73 | 1.62 | 44 | 9.45 | 1.43 | 44 | 8.59 | 1.30 | 44 |
| 28 | 8.06 | 1.38 | 34 | 10.21 | 1.75 | 34 | 10.56 | 1.81 | 34 | 10.03 | 1.72 | 34 | 9.03 | 1.55 | 34 | 7.62 | 1.31 | 34 |

Mean, SEM, and N (cell) for MSNs at all time points and distances of significant difference. MSN dendrite arborization increases with time *in vitro*. Between 10DIV and 13DIV there is a significant increase in crossings 60-80µm from the soma. Elaboration continues from 13DIV to 16DIV, with significant increases 30-59µm from the soma. After a final increase at 50µm from the soma between 16DIV and 19DIV there are no further changes in dendrite complexity. Dunn's multiple comparisons; *$p<0.05$; **$p<0.01$; ***$p<0.001$. Asterices represent significant difference with the preceding time point at the same distance.

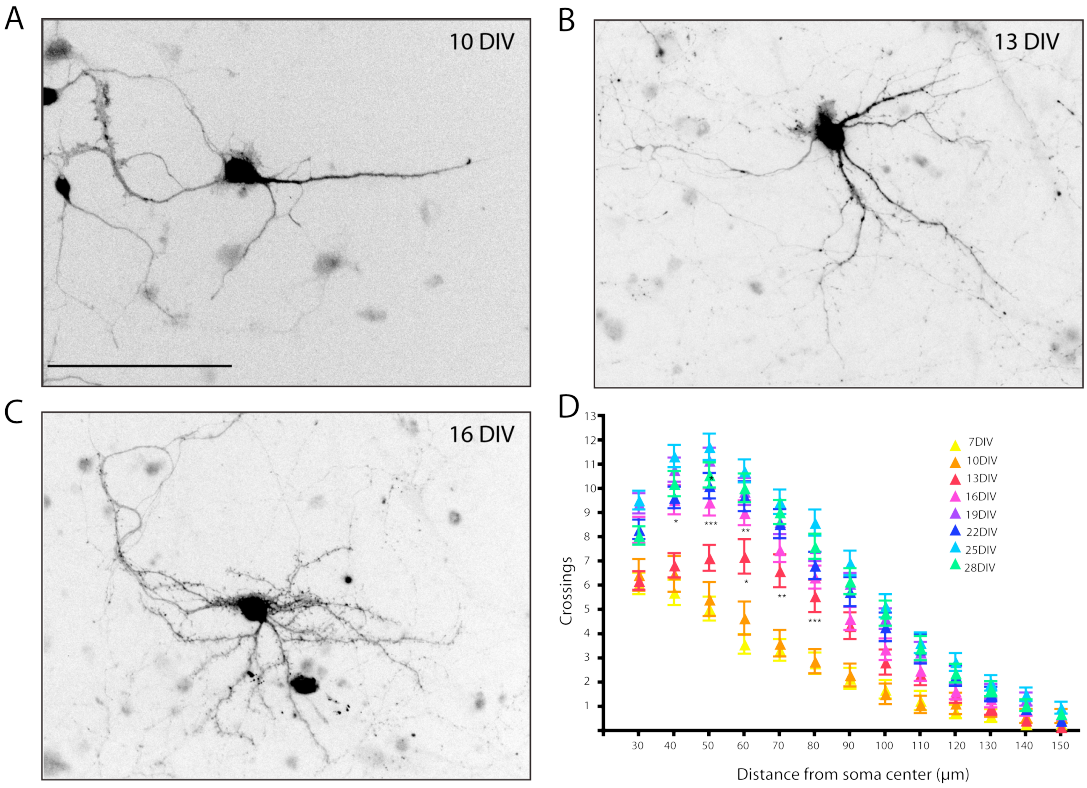


**Figure 11. Developmental analysis of MSN morphological complexity**
EGFP fill of dARPP-32+ MSNs at 10DIV (A), 13DIV (B), and 16DIV (C). DARPP-32+ MSNs undergo a time dependent increase in dendrite branching (D) significant interaction, main effect of time and distance (p's<0.001). Astericks represent significant difference from preceeding time point (*P<0.05, **p<0.01, ***p<0.001). Data displayed as Mean ± SEM, scale bar 100µm

*Spine Density*

We found that *in vitro* dendritic spine density increased steadily over time and plateaued to a final level similar to reports from *in vivo* (DiFiglia et al., 1980). Spine density increased progressively from 7 to 19DIV ($H(7) = 198$, $p<0.0001$) and reached final density of approximately 13 spines/10 µm by 28DIV (Figure 13). At 7DIV spine density was less than one spine per 10µm and significantly lower than density at 13DIV (0.74 ± 0.08 spines/10µm v 3.41 ± 0.33 spines/10 µm; Dunn's multiple comparison, $p<0.01$). Density continued to increase over time; spine density at 19DIV was significantly greater than density at 13DIV (3.41 ± 0.33 spines/10µm v 9.03 ± 0.56 spines/10 µm; Dunn's multiple comparison, $p<0.001$). By 19DIV spine density plateaued as evidenced by no further significant differences with time points 22-28DIV (Dunn's multiple comparison, $p>0.05$).

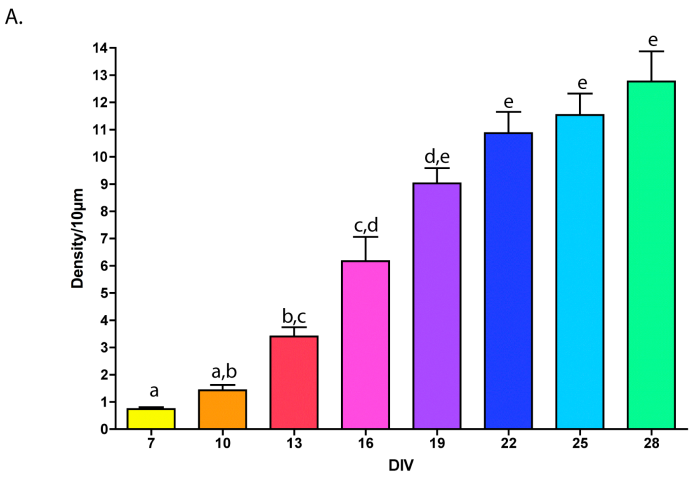


**Figure 12. MSN spine density increases during *in vitro* development**
Dendritic spine density of developing MSNs increased over time (A). Letters represent time points that are not significantly different from one another. Spine density increased from 7DIV to 13DIV (Dunn's multiple comparisons, $p<0.01$), from 10DIV to 16DIV ($p<0.001$), from 13DIV to 19DIV ($p<0.001$), from 16DIV to 22DIV ($p<0.05$), and was stable from 19DIV onward. B. Raw values of densities across development, N is segments analyzed

*Spine Morphology*

The relationship between spine head diameter and neck length, as well as their absolute values, determines the "type" of spine and leads to predictions regarding the strength and plasticity of a given spine. In mature MSNs, the head diameter has been positively correlated with the synaptic membrane area (Wilson et al., 1983), indicating that head diameter is related to synaptic function. Mature MSNs *in vivo* are known to have highly heterogeneous dendritic spine morphologies (Wilson et al., 1983), but the developmental time course of spine morphology has not been examined. We find that *in*

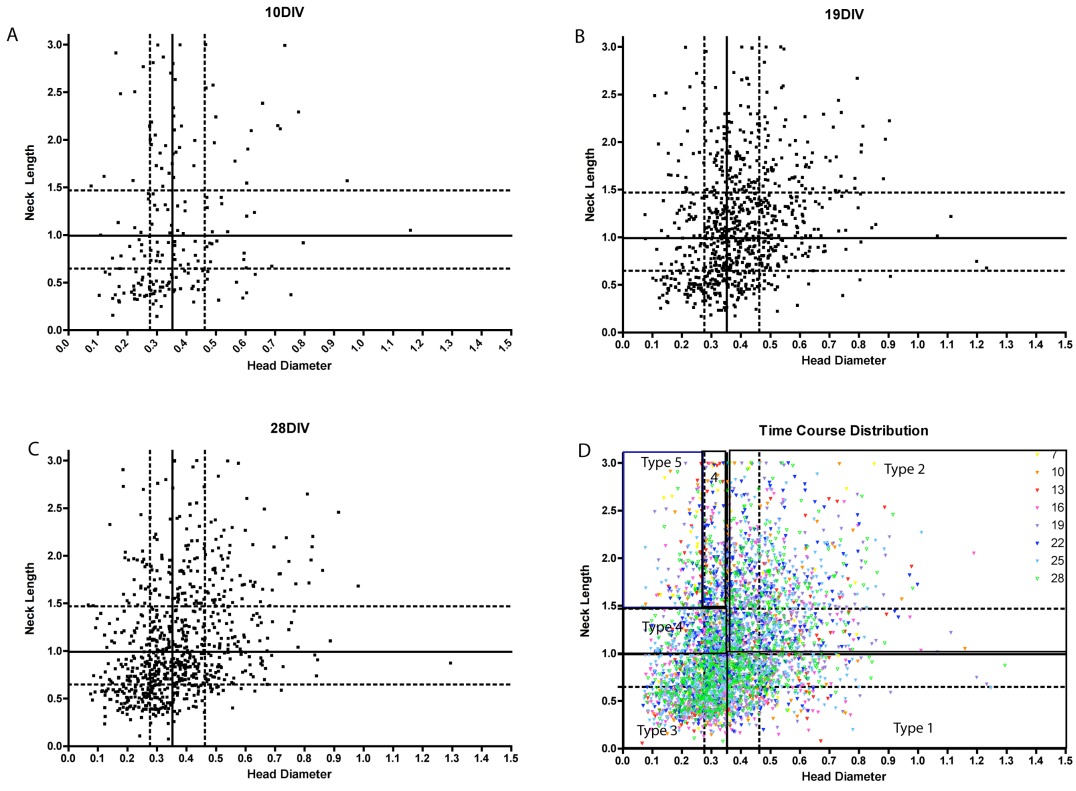


**Figure 13. MSN spine morphology is highly variable across development.**
Scatter plots of paired neck length (Y-axis) and head diameter (X-axis) measurements for spines at 10DIV (A), 19DIV (B), 28DIV (C), and across the entire developmental range examined (D). Medians are determined from the entire population and displayed as solid lines with 75$^{th}$ and 25$^{th}$ percentile as dotted lines. Portions of the graph the graph that reflect different spine types are marked and labeled by type (D).

*vitro* MSN dendritic spine morphology is highly variable across development (Figure 14). There is a small but significant increase in mean spine head diameter and decrease in neck length with development (Table 4), indicating that spines are increasing in size as they mature; however MSNs appear to maintain a wide variety of head diameters and neck lengths even at mature time points.

**Table 4. MSN dendritic spines have a small but significant increase in diameter and decrease in length with development.**

| | **10DIV** | **19DIV** | **28DIV** |
|---|---|---|---|
| Mean Head Diameter (μm) | 0.365 ± 0.010 | 0.397± 0.007 | 0.369 ± 0.005 |
| Mean Neck Length (μm) | 1.097 ± 0.071 | 1.171 ± 0.020 | 1.084 ± 0.019 |

Mean MSN dendritic spine head diameter increases with development after having a transient spike (Mean ± SEM, Dunn's multiple comparison, $p<0.001$ for all groups). MSN dendritic spine neck length decreases with development after having a transient spike (Mean ± SEM, Dunn's multiple comparison, $p<0.001$ for all groups

In order to highlight changes between developmentally significant time points, spine head and neck lengths were compared at early (10DIV), intermediate (19DIV), and mature (28DIV) time points. 10DIV was selected to represent an early developmental time point because dendrite organization is very simple and spine density is low. 19DIV represents an intermediate time point because dendrite elaboration and spine density has just stabilized. By 28DIV MSN morphology is stable with no further changes in spine density or dendrite arborization. There was a small but highly significant, increase in

mean dendritic spine head diameter with development (Table 4, H (2) =28.44, $p<0.0001$; Dunn's multiple comparison, 10DIV v. 28DIV, $p<0.001$). Interestingly, at 19DIV there appears to be a transient increase in dendritic spine head diameter greater than the final head diameter reached at 28DIV (Dunn's multiple comparison, 19DIV v. 28DIV, $p<0.001$). Neck length is also found to increase with development (Table 4, H (2)=10.88, $p<0.01$; Dunn's multiple comparison, 10DIV v. 28DIV, $p<0.001$). As with head diameter, there appears to be a period of transient spine neck length increase greater than the final length reached at 28DIV (Dunn's multiple comparison, 19DIV v. 28DIV $p<0.001$).

Previous research has used a three-group classification system (thin, mushroom, and stubby) for categorizing types of spines (Jones and Powell, 1969; Harris et al., 1992). These categories are derived from types of mature morphologies observed in pyramidal neurons. When dendritic spines from developing MSNs were classified into these three groups, there are significant differences between types during development (Figure 15A, two-way ANOVA interaction DIV x type, $F(4,324)= 7.38$, $p=0.0001$; significant main effect of type, $p<0.0001$). At all time points, thin-type spines were the predominant spine type. Post-hoc comparisons showed there was a significant increase in the proportion of mushroom-type spines during development (10DIV v 19DIV, $p<0.001$; 10DIV v 28DIV, $p<0.01$), but no significant differences between any spine types at mature time points (19DIV v 28DIV, $p>0.05$ for all types). The increase in the proportion of mushroom-type spines coincided with a decrease in both thin and stubby-type spines, but this was not statistically significant. This coding scheme shows an early developmental increase

in a single type of spine (mushroom), but stable proportions of all spine types at intermediate and mature time points.

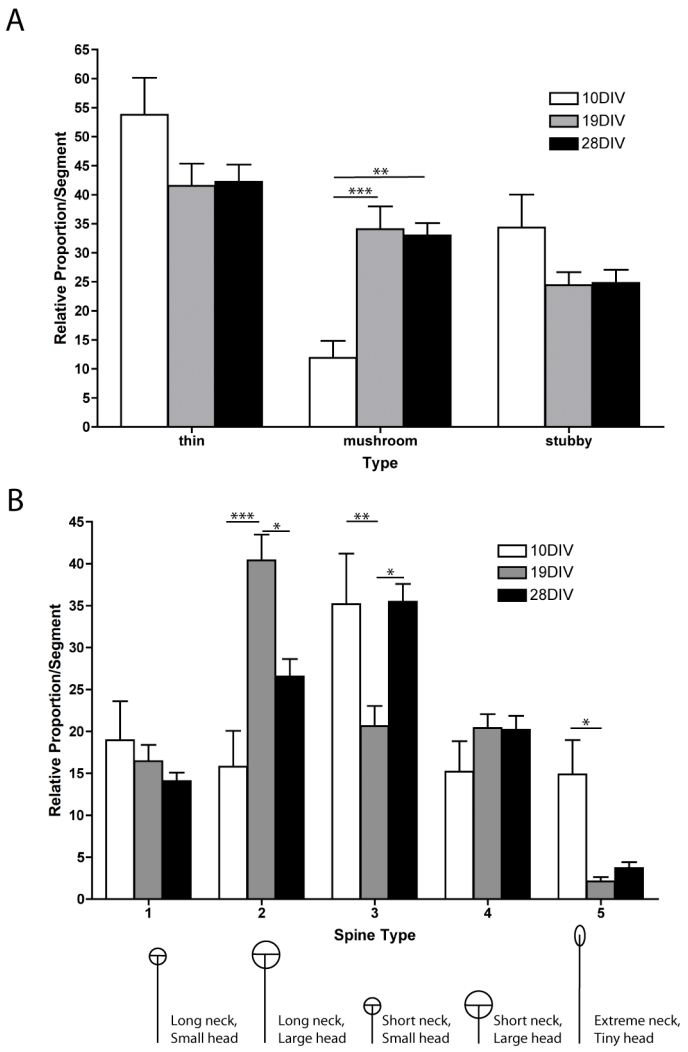


**Figure 14. Spine type coding schemes applied to developing MSN spines**
A. Using the classical three spine type coding scheme, there is an increase in the proportion of mushroom spines from 10DIV to 19DIV ($p<0.01$) and the proportion of different spine types is stable from 19DIV to 28DIV. B. Using the median split spine type coding scheme, based on observed MSN morphologies across development, there is a transient increase in type 2 spines (10DIV v 19DIV, $p<0.01$) and the proportion of spine types is otherwise stable across development. Representative morphologies and descriptions for the median split are displayed below panel B.

Our study is the first to date that examines MSN spine morphology at multiple developmental time points. To account for the increased heterogeneity associated with development, we developed a categorization scheme that could be used to classify the entire population of spines examined at all time points across development. These categories represent a wider range of morphologies and increase the sensitivity of detecting developmental or experience-dependent changes in a specific population of spines. Using a median split procedure, we generating 5 types of MSN spines based on all observed head diameters and neck lengths across development (Figure 3D). This procedure identified spines by the relationship between their absolute head diameter and neck length values compared to the median and interquartile range observed in the whole data set (Figure 3D, Figure 4B). This led to the generation of four primary spine types, 1-4, featuring spines with necks greater than and heads smaller than the median (type 1), spines with necks greater than and heads greater than the median (type 2), spines with necks shorter than and heads smaller than the median (type 3), and spines with necks shorter than and heads greater than the median (type 4). The type 1 spine category was further divided into an extreme type 5, with spines having neck lengths greater than the 75% percentile and heads smaller than the 25% percentile. Spines with this morphology likely represent the immature spine precursor, a filopodial-like protrusion (Papa et al., 1995; Ziv and Smith, 1996; Fiala et al., 1998; Marrs et al 2001).

At intermediate and mature time points, type 2 and 3 spines were the predominant spine type, representing two extremes of head diameter and neck length relationships (Figure 15B). As expected from the transient increase in diameter and length at 19DIV,

there was a significant increase in type 2 spines from 10 to 19DIV (Two-way ANOVA interaction DIV x type, $F(8,595)=6.87$, $p<0.0001$; significant main effect of type, $p<0.0001$; Bonferroni post-hoc, 10DIV v 19DIV, $p<0.001$; 19DIV v 28DIV, $p<0.05$). This was partnered with a transient decrease in type 3 spines (10DIV v 19DIV, $p<0.01$; 19DIV v 28DIV, $p<0.05$). Additionally, there is a significant decrease in type-5, filopodial-like, spines between 10DIV and 19DIV ($p<0.05$).

These two schemes of spine coding highlight the importance of representing spine types based on actual observed morphologies. When developing MSN dendritic spines are coded using types based on mature pyramidal neuron spines, thin-type spines are the dominant type at all time points, there is a significant increase in mushroom type-spines with development, and stable proportions of the three types at intermediate and mature time points. When developing MSN dendritic spines are coded using types based on the observed morphologies across development type 2 and 3 (lollipop and stubby-like) spines are the predominant types at all time points. The observed transient increase in mean head diameter and neck length is represented by a transient increase in the proportion of type-2 (lollipop-like) spines. This transient increase appears to be due to a significant decrease in type 3 spines. Type 5 (filopodial-like) spines decrease with development, while the relative proportion of the remaining spine types was stable with development.

Discussion

Although significant effort has been made to characterize the role of MSN dendritic spines in disease and behavior, a detailed morphological examination of their development has yet to be undertaken. Here we characterize the developmental changes in dendrite elaboration, spine density, and spine morphology of MSNs *in vitro*. In concordance with previous examinations of *in vivo* MSN development, MSNs *in vitro* undergo significant increases in dendrite complexity and dendritic spine density that is maintained in mature MSNs. Previous examinations of this system have shown that by 21DIV MSNs have numerous *in vivo*-like characteristics, including electrophysiological characteristics and spine densities very similar to those reported in previous literature (Penrod et al., 2011).

It has been reported that alterations in MSN dendrite complexity occur in animal models and in human brains with neurodegenerative diseases that involve the striatum (McNeill et al.; Ferrante et al., 1991; Stephens et al.; Zaja-Milatovic et al., 2005). Some of these studies indicate that proper dendrite elaboration and organization impacts the function of MSNs (Klapstein et al., 2001). In order to understand the functional impact of dendrite complexity and examine the molecules that may regulate MSN dendrite elaboration, a time course for developmental changes in morphological complexity must be defined. We find that MSNs *in vitro* undergo two distal-proximal waves of dendrite elaboration. An initial distal elaboration occurs from 10 to 13DIV, producing a significant increase in dendrite crossings 60-80μm from the cell body. A second wave of dendrite elaboration occurs more proximally, 30-50μm from the cell body, from 13-

16DIV. By 19DIV, dendrite complexity is maximal and maintained with no further differences up to 28DIV (the last time point examined). Similarly, it has been reported that during early postnatal stages *in vivo* the greatest peaks in branching complexity are proximal to the soma (Rauskolb et al., 2010; Berlanga et al., 2011; Lee and Sawatari, 2011). The mechanism for these two waves of branching is unknown. One possible explanation is that morphological complexity is regulated by interactions with the glutamatergic afferents. This is supported by previous findings that MSNs cultured in the presence of very low levels of glutamatergic input fail to develop complex dendritic arborization even after three weeks *in vitro* (Penrod et al., 2011). Perhaps the distal to proximal increase is related to signaling events initiated by interaction with glutamatergic afferents that form first at the elaborating distal dendrites. It is unlikely that this branching pattern is regulated solely by transport of materials from the soma, which would be predicted to produce accumulation of these branch-inducing molecules proximally first, further supporting a role for signaling from afferents. One possible molecular cascade mediating this elaboration could be BDNF release from excitatory afferents (Altar et al., 1997), as previous research has found decreased dendrite complexity in BDNF knockout animals (Rauskolb et al., 2010).

MSN dendritic spines receive coordinated glutamate and dopamine inputs, both known to regulate MSN spine density in mature MSN (Ingham et al., 1989; Chen and Hillman, 1990; Ingham et al., 1993; Meredith et al., 1995; Cheng et al., 1997). Studies *in vivo* have described increases in dendritic spine density during postnatal development (Chronister et al., 1976; DiFiglia et al., 1980; Tanaka; Hull et al., 1981), finding that the

most prominent increases in spine density are found during the period when excitatory afferents (primarily from the cortex) are synapsing in the striatum (Hattori and McGeer, 1973; Tepper and Trent, 1993). Interestingly, in our system, where these excitatory afferents are available from plating, MSNs still undergo a progressive increase in spine density. This is in contrast to a rapid increase early in development that might be expected if afferent availability was the only factor regulating MSN spine development. Whether the relatively slow time-dependent increase in spine density, despite the availability of excitatory afferents, represents an intrinsic limitation on spine formation could be addressed in this system.

This progressive increase in spine density occurs from 7-19DIV, after which spine density stabilizes and plateaus to a final density of approximately 9-13 spines/10 μm. This density is in a range previously reported for *in vivo* MSNs (Jedynak et al., 2007; Neely et al., 2007; Christoffel et al., 2011; Ding et al., 2011). Much like the case for dendrite complexity, this stably maintained spine density reflects the overall health of the MSNs *in vitro*, lending further support for the use of this system in studying factors mediating development and maintenance of mature MSN characteristics.

We found that as a population, MSN dendritic spines had highly variable morphologies across development, even at the most mature time points, as has been noted in previous descriptions of mature MSN spines *in vivo* (Wilson et al., 1983). There was a small but significant increase in MSN dendritic spine head diameter and neck length with development and a transient spike in both parameters at 19DIV. The finding that MSN dendritic spines exhibit wide variability across development has interesting functional

implications. Although MSN spines tended to increase in size over development, they still maintained variety in absolute neck lengths and head diameters. During development of the commonly studied pyramidal neurons, decreased neck length and increased head width is associated with maturation (Harris et al., 1992; Papa et al., 1995). These findings in pyramidal neurons lead to the development of specific structure/function hypotheses about mature dendritic spines (Ethell and Pasquale, 2005); with shorter neck, larger head spines being viewed as the most mature morphology. In contrast, MSN dendritic spines did not undergo a population change in morphology associated with development. Instead, MSN spines seem to retain relatively long necks and have large heads throughout development and into the mature state. Future work in this system will be able to examine structure/function relationships during development and determine the functional implications of maintained spine heterogeneity during development.

In addition to measuring the absolute values for head diameter and neck length, previous work has relied upon the use of spine morphological sub-types to better describe changes occurring in populations of spines. Our definition of spine types has relied heavily on data from mature pyramidal (hippocampal or cortical) neurons. Although we are able to classify MSN spines using these categories (mushroom, thin, and stubby) and our mature MSNs have proportions of the three types similar to those reported previously (Figure 15A, (DiFiglia et al., 1980; Christoffel et al., 2011)), we believe these categories may not accurately capture the variety of morphological types seen across development. When MSN spines are categorized using these groups, there is an increase in mushroom-

like spines from 10DIV to 19DIV, but otherwise the relative proportion of different spine types is maintained across development. Although these categories do show the maintained heterogeneity of spine morphology observed in the absolute measurements (Figure 13D), it fails to capture the significant, transient increase in both head diameter and neck length at 19DIV. Because a detailed morphological characterization of MSN dendritic spine development *in vivo* was not previously available, we utilized the large dataset we have generated from our in vitro studies to devise a classification based on the median split procedure; this generates five sub-types of MSN spines based on the absolute size of spine heads and necks. These sub-types introduce an important spine type for coding purposes, the type-2 or "lollipop" like spine. Spines of this type feature long spine necks partnered with large spine heads and are the dominant spine type of *in vitro* MSNs. This spine type also reflects the transient increase at 19DIV, a period immediately following the last wave of dendrite elaboration. Whether this transient increase is related to changes in MSN function or sensitivity to exogenous cues can be addressed in future research. It is interesting that at all time points examined the dominant spine types 2 and 3 represented two extremes of spine morphology. Although not explicitly compared, it does appear that the proportion of these two spine types may be correlated. At the 19DIV when there is a significant increase in the type 2 spines, there is also a significant decrease in type 3 and type 5 spines, with no changes in other spine types. Whether these two spines represent an interconvertable population or two functional states can be addressed in future examinations.

Although this system recapitulates a number of morphological characteristics similar to *in vivo* MSNs, this system does not include the large number of dopaminergic afferents that MSNs receive *in vivo*. By embryonic day 16, the date of dissection, dopaminergic afferents have entered the ganglionic eminence, but mature connections have likely not been made (Voorn et al., 1988; Hu et al., 2004). Although we cannot eliminate the possibility that MSNs in culture have received dopamine stimulation prior to dissection, one can conclude that the described developmental events do not require ongoing dopaminergic input. It is interesting that MSN development of numerous *in vivo*-like characteristics does not require dopaminergic signaling given the known importance in regulating the morphology and function of mature MSNs. Clearly, the interaction of dopamine and glutamate signaling within MSN spines contributes to their structure and function (Neely et al., 2007; Garcia et al., 2010), and future research using exogenously applied dopamine or the inclusion of dopaminergic neurons can address how that interaction affects MSN development and plasticity.

Here we report the time course of dendrite complexity and spine density changes during *in vitro* MSN development. Our findings support the use of this system for examining the factors and molecules regulating MSN development and identify time points *in vitro* for studying these developmental events. In this study we also characterize the morphological development of MSN dendritic spines for the first time. The similar results obtained in the present analysis of MSN development *in vitro* and the qualitative descriptions of postnatal development of MSN spines *in vivo* (DiFiglia et al., 1980) and the quantitative analysis of mature *in vivo* MSNs (Wilson et al., 1983), indicate

that the development of MSNs in striatal-cortical culture likely recapitulates many key aspects of MSN development *in vivo*. Future studies in this system can address intrinsic and extrinsic factors affecting MSN spine development and determine how these factors affect plasticity.

## Chapter 4: Conclusions and Discussion

The structure and function of MSNs are of interest due to their critical role in regulating numerous behaviors as well as their involvement in diseases that affect humans. Dendritic spines of MSNs are of specific interest due to their association with these states. Despite this interest in the basic function of MSNs, there is still a great deal unknown about them. The molecules that regulate gross morphological development, spine morphogenesis and maturation, and numerous pathological mechanisms are largely undefined. One reason that MSN-specific pathways are not well-described is the lack of a reliable, reproducible primary cell culture system that supports the development of MSNs with *in vivo* like characteristics. The ability to examine MSNs as they develop and respond to molecular or environmental manipulations will allow for delineation of the specific molecules and factors that regulate MSN morphological and synaptic plasticity. These finding will inform our understanding of structure/function relationships, disease pathologies, and provide insights into treatments.

### Strengths of *in vitro* systems

Primary cell culture has a number of advantages when studying the molecular mechanisms of cell biology. Primary neuronal cultures can be examined at numerous times during development, repeatedly or for continuous periods. This sort of accessibility is particular helpful when asking questions of development. Neurons can be identified and examined across development or before, during, and after a specific manipulation. Cultured neurons can be examined using a variety of techniques, many of them

simultaneously. The function of cultured neurons can be assessed using electrophysiological techniques, voltage-sensitive dyes, and neurotransmitter receptor trafficking. These techniques can be applied at the single cell and network level, providing a better sense of how a cells function contributes to the wider network. Cell culture also allows for better access to molecular manipulations. Cultured neurons can be transduced to express proteins of interest, knockdown constructs, and fluorescent tags. Expression of these constructs can be temporally controlled, which paired with access to the cell during development, allows for a detailed investigation of the role of certain proteins at discrete time points. Expression can also be limited to a specific cell population depending on the promoters used or the technique employed for transduction. The expression and localization patterns of endogenous proteins can be tracked in cultured neurons. Using immunofluorescent microscopy, tagged constructs can be visualized in live or fixed tissues, proteins can be localized to specific regions of the cell and the changes in localization during development or manipulation can be assessed. The variety of techniques and the ability to use them in combination in a reduced system will support future research into the basic biology of MSNs and their dendritic spines.

MSN survival is influenced by physical interactions with glia

The system described in this dissertation represents a novel contribution to the field in that we provide a detailed procedure for producing *in vitro* MSNs with mature, *in vivo*-like characteristics. During the optimization of this procedure numerous observations were made regarding the *in vitro* environment that best supported MSN

survival and development. Cultures prepared from embryonic tissue were found to be more robust, requiring a lower starting cell density at plating. Interestingly, the presence of glia in the culture dish seemed necessary for the long-term survival of the MSNs, but not the cortical neurons. When cultures were treated with an antimitotic agent, which stops proliferation of glia with no effect on the post-mitotic neurons, MSN survival declined. Observations in dying cultures showed that although the cortical neurons continued to adhere and develop, the only MSNs in the culture dish appeared to be those still associated with the remaining glia. If antimitotic agents were delivered repeatedly, as is commonly done in pyramidal cultures with no ill effect, MSNs were essentially lost from the culture dish. This relationship of MSN survival and glial association appeared to require a physical contact because cultures are maintained in glial conditioned media that should contain secreted factors necessary for survival. The mechanism for this effect is unknown, but future research could determine what interactions mediate this effect and if they could be exploited to enhance MSN survival in disease states.

DARPP-32 expression *in vitro*

DARPP-32 is expressed in post-mitotic MSNs that have completed migration from the SVZ into the presumptive striatum (Foster et al., 1988; Gustafson et al., 1992) making it a useful marker for determined MSNs even at embryonic stages. Although the yield of DARPP-32+ MSNs was reasonable given the source tissue, embryonic stage, and plating density, it may not have represented the maximal possible yield of MSNs. Research in mono-cultured MSNs from a similar stage/source has reported a greater yield

of MSNs (Ivkovic and Ehrlich, 1999). One possible explanation for this could be a difference in inclusion criteria (with our criteria requiring greater DARPP-32 expression intensity), but it may also be that DARPP-32 is not being expressed at a significant level in cells that might otherwise be MSNs (i.e. cells that are most, but not all of the way down the differentiation path to becoming MSNs). Although research has shown a role for BDNF signaling in inducing DARPP-32 expression in MSN mono-culture (Ivkovic and Ehrlich, 1999), we found that in our co-culture system exogenously applied BDNF failed to increase endogenous DARPP-32 expression level or cell number, and it did not induce expression of EGFP driven by a DARPP-32 promoter (unpublished observations, DARPP32-EGFP mice provided by M. Ehrlich). It is possible that some factor in the co-culture is limiting expression of DARPP-32 through an unknown mechanism. It seems unlikely that this "brake" on DARPP-32 expression is due to enhanced excitatory drive from the glutamatergic afferents because pilot experiments limiting activity (through either TTX or APV/CNQX application) during the first week *in vitro* failed to increase DARPP-32 levels. One possible source for this "brake" signal may be a secreted or contact derived signal from the high levels of glia in the culture dish. Given the previously described relationship between MSN survival and glial contact, it would appear that some aspects of *in vitro* MSN survival and development are sensitive to glial signaling. Given the presence of cortical neurons, which are known to release BDNF (Altar et al., 1997), BDNF levels in the co-culture may already be saturated, explaining why exogenous application has no additional effect. Future research could expand our understanding of the regulation of DARPP-32 and other MSN-associated proteins,

contributing to our understanding of how their regulation may be disrupted in pathological states.

MSN dendrite development is dependent on extrinsic factor

The effect of excitatory afferents in MSN spine development has been previously established (Segal et al., 2003; Tian et al., 2010), but the effect on dendritic arborization has not been examined. Co-culturing was found to increase the dendritic complexity of MSNs, producing a more *in vivo*-like dendritic arbor, but the mechanisms mediating MSN dendritic arborization are unknown. Developmental studies showed that MSN dendrite complexity occurred in two waves from 10-13 and 13-16DIV. MSN dendrites first elaborated distally then proximally, until stabilizing for the remainder of the time examined. When compared with developing co-cultured MSNs, it appears that mono-cultured MSN dendritic arborization resembles immature co-cultured MSN organization, indicating that mono-cultured MSNs may be in a developmentally delayed or halted state. Given that the major difference in the conditions is the presence of glutamatergic afferents, this suggests that a factor released by and/or a cascade activated by association with glutamatergic afferents may be helping regulate dendritic development. There is further support for a cell-extrinsic factor regulating MSN dendrite development based on the branching pattern observed. Given that complexity increases distally to proximally, this branching is unlikely to be determined by the accumulation of some transported factor from the soma. If cell-intrinsic expression and transport of factors were the determining step in arborization, one would expect proximal branching to occur first as

materials are transported out to distal branches. Further research into the developmental mechanisms of MSN dendrite elaboration could provide targets for restoring normal MSN gross morphology in disease states and elucidate the importance of dendrite organization in MSN function.

Mono-cultured MSNs may be developmentally immature

Mono-cultured MSNs were less morphologically complex, but did have low densities of dendritic spines; this likely reflects a small amount of underlying cortex that is removed with the GE dissection. When examined electrophysiologically, co-cultured MSNs had a greater frequency, but lower amplitude of mEPSCs compared to mono-cultured MSNs. Although the greater frequency of mEPSCs in the co-culture is consistent with a higher density of dendritic spines, the lower amplitude was surprising. One possible reason for this increase in amplitude of mono-cultured MSN minis is a homeostatic synaptic scaling explanation (Turrigiano, 2008), where neurons deprived of their normal level of excitatory (or inhibitory) drive alter the expression, function, and/or localization of neurotransmitters in order to adjust the strength of individual synapses to restore normal levels of activity. Given the similarity of mono-cultured MSNs to immature MSNs (in terms of gross morphology and spine density), it is also possible that this level of activity is associated with a particular stage of development. Future investigations of the functional development of co-cultured MSNs will provide insight into this finding. Determining the relationship between synaptic and morphological

maturation of MSN dendritic spines will expand our understanding of the physiological and structural plasticity seen in development, maturation, and pathological states.

Intrinsic limitations on MSN spine morphogenesis

Despite the interest in plasticity of mature MSN dendritic spines, previous studies have not detailed the morphological development of MSN spines *in vivo* or *in vitro*. The density of MSN dendritic spines increased during the first two weeks *in vitro* and stabilized to a maintained density shortly before the third week. Research has described a similar time course for *in vivo* postnatal MSN dendritic spine formation, finding that there is a large increase in spine density as glutamatergic afferents into the striatum are being refined (Hattori and McGeer, 1973; Tepper and Trent, 1993). If MSN dendritic spine formation was purely dependent on the presence of afferents, we might expect rapid increases in spine density much earlier *in vitro* given that cortical neurons are co-plated with MSNs. Instead, we find a steady increase in density that plateaus, perhaps representing an intrinsic brake on spine formation or a gradual development of the molecules required for spine formation. Future research into the mechanisms mediating MSN spine formation may find that spines formation is dependent not only on the presence of excitatory afferents, but also some cell intrinsic factor that may be developmentally regulated. If this is the case, such a molecule or cascade may be a valuable target for inducing spine formation or maturation in mature MSNs.

MSN dendritic spine morphology is heterogeneous

Our research on developing co-cultured MSNs highlights the maintained diversity of MSN dendritic spine morphology. At all developmental time points examined the relative proportion of different spine types were fairly steady and dominated by two morphological extremes; stubby-like (type 3) spines with short necks and small heads and lollipop-like (type 2) spines with long necks and large heads. Previous electrophysiological examinations in this system demonstrated functional MSN excitatory synapses by 19DIV and immunofluorescent localization demonstrated mature patterns of pre- and post-synaptic markers by that same time point (data not shown). The finding of a transient, but highly significant spike in head diameter and neck length at 19DIV, which increases the proportion of type 2 spines, is of unknown importance. The functional consequences and the possible mechanisms for this transient shift in spine morphology will be a focus for future work. This spike corresponds to a time point at which other morphological characteristics (spine density and dendrite arborization) have become stable. It is possible that this transient increase represents an MSN-specific, cell intrinsic morphological preparation for dopaminergic inputs, which *in vivo* would synapse on MSN spine necks. Alternatively, this transient increase may occur in all developing spines and because of the large increase in spine density during the previous time point, a large number of recently developed spines are undergoing the transient stage simultaneously, producing the mean and proportional increases. Future experiments could determine the time course of synaptic and morphological development and determine what, if any, relationship there is between MSN dendritic spine structure and function. Future studies that take advantage of live cell imaging of SEP (Super-Ecliptic

Phlourin, a pH sensitive GFP construct that tracks membrane insertion) tagged neurotransmitter receptors would correlate the relationship between neurotransmitter receptor localization and spine morphology during development. Extensions of these experiments that take advantage of labeled MSNs for morphological and/or electrophysiological examination would further elucidate the relationship between structure and function of developming MSN dendritic spines. These studies could also elucidate the importance of the transient morphological peak found as MSNs are transitioning to their final, stable morphologies. Understanding these relationships will help future researchers to understand the possible functional consequences of dendritic spine morphological plasticity and inform hypotheses related to disruptions of MSN spine morphology.

Dopaminergic afferents are not required for the development of mature MSN characteristics

Of particular interest for future experiments is characterizing the role of dopamine in regulating MSN development and plasticity. Although we cannot exclude the early embryonic role of dopamine in establishing MSN determination or protein expression, our studies show that ongoing dopamine inputs are not required for MSNs to develop a number of *in vivo*-like characteristics, including dendritic spine density and morphology. This is in contrast to the clear and well-reported effect that changes in dopamine inputs have on the morphology and function of mature MSNs. Research in striatal mono-culture has demonstrated a role for dopamine receptor activity in regulating GABAergic

synaptogenesis (Goffin et al., 2010), but further research into the role of dopamine in MSN development has been limited. Studies in our system have demonstrated that D1-receptor activation can induce CREB phosphorylation and we have seen a similar response to application of dopamine. This finding led to a series of pilot experiments, not detailed in this dissertation, that established the concentration, duration, and tolerance of co-cultures for treatment with exogenously applied dopamine and other compounds. In the future, the role of dopamine in MSN development and plasticity will be examined using both the physical presence of dopaminergic afferents (by co-plating with ventral midbrain dopaminergic neurons) and the acute, repeated, or sustained administration of exogenously applied dopamine (and related compounds). Given the now known time course of MSN development, we can manipulate dopaminergic tone at discrete time periods to assess the effect on normal MSN *in vitro* development. These findings would add substantially to the fields' understanding of the role dopamine plays in MSN development and have the potential to identify developmental pathways that may be altered in disease states.

## Concluding Remarks

In summary, this dissertation describes the development and characterization of an *in vitro* primary neuronal culture system designed to support the development and maturation of MSNs. This system produces MSNs with *in vivo*-like dendritic arbors, spine densities, and electrophysiological characteristics. The system is designed for mouse striatal neurons, allowing future research to take advantage of genetic models for

labeling specific cells, disrupting proteins of interest, and/or modeling diseases. MSNs grown under these conditions survive for up to or exceeding a month *in vitro*, during this time they undergo time-dependent increases in dendritic arborization and dendritic spine density that plateau to maintained, *in vivo*-like levels by the third week in culture. This system has provided the first morphological characterization of MSN spine development, revealing a small but significant increase in the size of MSN spines and highlighting their high morphological variability. With the establishment of this system, a number of questions regarding MSN development and plasticity can be addressed, including the molecules/pathways that regulate MSN survival, dendrite arborization, and dendritic spine development and the role of dopamine in regulating MSN development and plasticity.

There is reason to believe that the mechanisms for MSN dendritic spine development, signaling, and plasticity will diverge from the commonly studied pyramidal neuron system. MSNs and cortical neurons are differentially sensitive to NMDA-induced excitotoxicity, in part due to the different localization of NMDA receptors (Kaufman et al., 2012). Given the known role of NMDA receptor expression and localization in pyramidal spine development (Tian et al., 2007; Ultanir et al., 2007), the establishment of this differential localization may be related to different mechanisms for MSN spine development. The substantial dopamine innervation and the clear role of dopamine in maintaining mature MSN structure and function, indicates that pathways regulating MSN spine structure, function, and plasticity would be more dependent on dopamine than those in pyramidal neurons. DARPP-32 is used as a marker of MSNs for the purpose of this

dissertation, but it also has a critical role in activity-dependent signaling, serving as a inhibitor of protein phosphatase 1 and a substrate for numerous kinases and phosphatases that alter it's activity and localization (Walaas et al., 2011). The enrichment of DARPP-32 in MSNs and position as a mediator of glutamatergic and dopaminergic signaling (Svenningsson et al., 2004) could have important implications for it's role in MSN dendritic spine development and plasticity, roles it is unlikely to have in pyramidal neurons. Future work in this system will be able to provide new insights into the cell biology of MSNs, structure/function relationships in dendritic spines, and the pathology and treatment of MSN-related diseases.

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