FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTOCTORALES

uOttawa

L'Université canadienne
Canada's university

FACULTY OF GRADUATE AND POSDOCTORAL STUDIES

Kheïra Jolin-Dahel
AUTEUR DE LA THÈSE / AUTHOR OF THESIS

M.Sc. (Neuroscience)
GRADE / DEGREE

Department of Cellular and Molecular and Medicine
FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

The role of Cholinergic Interneurons in Regulating Corticostriatal Culture Response to Dopaminergic Stimulation
TITRE DE LA THÈSE / TITLE OF THESIS

William Staines
DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

Mario Tiberi

Jean-Claude Béique

Gary W. Slater
Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

The role of cholinergic interneurons in regulating corticostriatal culture response to dopaminergic stimulation

Prepared by:
Kheira Jolin-Dahel

MSc Thesis submitted to the
Department of Neurosciences

University of Ottawa

Library and Archives Canada

Published Heritage Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et Archives Canada

Direction du Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence
ISBN: 978-0-494-79670-2
Our file Notre référence
ISBN: 978-0-494-79670-2

NOTICE:

The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

AVIS:

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'Internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

Canada

Authorization

SPRINGER LICENSE
TERMS AND CONDITIONS

Nov 03, 2010

This is a License Agreement between Kheira Jolin-Dahel ("You") and Springer ("Springer") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Springer, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	2467621459754
License date	Jul 14, 2010
Licensed content publisher	Springer
Licensed content publication	Histochemistry and Cell Biology
Licensed content title	Striatal interneurons in dissociated cell culture
Licensed content author	S. C. Schock
Licensed content date	Jan 1, 2010
Volume number	134
Issue number	1
Type of Use	Thesis/Dissertation
Portion	Figures
Author of this Springer article	Yes and you are a contributor of the new work
Order reference number	
Title of your thesis / dissertation	Role of striatal cholinergic interneurons in basal ganglia function
Expected completion date	Jul 2010
Estimated size(pages)	150
Total	0.00 USD

Abstract

Introduction: We investigated the cholinergic regulation of striatal response to dopaminergic stimulation.

Methods: Rat neurons for dissociated striatal cultures were harvested from E18.5. E14.5 neurons were supplemented to the cultures to provide cholinergic interneurons. Cultures were stimulated with dopamine D1R or D2R agonist and antagonist and changes in c-FOS and EGR-1 expression and DARPP-32 phosphorylation were assessed by Western blots. Electrophysiological recordings were performed using multielectrode array.

Results: Molecular studies: Although no significant changes were observed, trends showed that cholinergic innervation produced a differential regulation of c-FOS and EGR-1 expression associated with D1R and D2R signaling. **Electrophysiological analysis:** Significantly more activity was detected in cultures without cholinergic interneurons. Moreover, an increase in firing was seen in cultures with cholinergic interneurons following the addition of a muscarinic 1 receptor antagonist.

Conclusion: Our results suggest a differential impact of cholinergic regulation on D1R and D2R signaling pathways.

Table of Content

List of Tables

List of Figures

List of Abbreviations

6-OH DOPA	6-hydroxydopamine
Ach	Achetylcholine
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
apo	Apomorphine (non specific DA agonist)
BDNF	Brain derived neurothophic factor
cAMP	Cyclic adenosine monophosphate
cdk5	Cycline dependent kinase 5
D1R	Dopamine D1 receptor
D2R	Dopamine D2 receptor
DA	Dopamine
DARPP-32	Dopamine and cAMP regulated phosphoprotein 32 kDa
DIV	Days *in vitro*
EdU	5 ethynyl-2-deoxyuridine
EGR-1	Early growth response response-1
EnK	Enkephaline
FACS	Fluorescent activated cell sorting
GABA	Gamma-amino butyric acid
GFP	green fluorescent protein
GPe	External segment of the globus pallidus
GPi	Internal segment of the globus pallidus
Kir 2.1	Potassium inward rectifying channel
KCNQ	Voltage gated potassium channel 7
LTP	Long term potentiation
M1R	Muscarinic M1 receptor
M4R	Muscarinic M4 receptor
MEA	Multi electrode array
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MSN	Mediun size spiny neurons
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
PD	Parkinson's disease
PKA	Protein kinase A
PKC	protein kinase C
PP-1	Protien phosphatase 1
Rac	Raclopride tartrate (D2R antagonist)
RIPA	Radioimmunoprecipitation assay buffer
SNc	Subtantia nigra pars compacta
SNr	Subtantia nigra pars reticulata
SNT	Subthalamic nucleus
Sub P	Subtance P

Thal	Thalamus
Thr	Threonine
vGAT	Vesicular GABAergic transportor
vGLUT	Vesicualr glutamatergic transporter

Acknowledgments

I would like to express my gratitude to Dr William Staines who was an exceptional mentor and remarkable professor. I would also like to thanks my fellow lab members, Dr Sarah Schock and Paul Schock, for their technical support and completion of some of the work presented here. Finally, I would like to thank Drs Arbuthnott and Garcia-Munoz for travel, training, friendship and funding through the Okinawa Institute of Science and Technology Promotion Corporation.

Introduction

1.1 Basal Ganglia Circuits

The basal ganglia are a group of interconnected nuclei that are important in regulating motor function. The striatum, comprised of the caudate nucleus and the putamen, is the main input nucleus of the basal ganglia. It receives excitatory glutamatergic input from practically all regions of the cerebral cortex and sends outputs to the external segment of the globus pallidus (GPe), internal segment of the globus pallidus (GPi) and substantia nigra pars reticulata (SNr). The striatum also receives dopaminergic input from the substantia nigra par compacta (SNc) and glutamatergic input from the thalamus (Bolam et al, 2000).

Neurons in the striatum are either projection neurons, i.e. medium spiny neurons (MSNs), or interneurons. The projection neurons carry the output information of the striatum and are divided into two anatomical sets, the direct and indirect pathways. Neurons (MSNs) in these two pathways differ in their connections with other basal ganglia nuclei, in the type of neuropeptide neurotransmitter they use and in the type of dopamine receptor they express. MSNs of the direct pathway project to the GPi/SNr, synthesize gamma-amino butyric acid (GABA) and the neuropeptide substance P as neurotransmitters and express the D1 subtype of dopamine receptor. MSNs of the indirect pathway project first to the external segment of the globus pallidus which in turn projects to the subthalamic nucleus (STN) before reaching the GPi/SNc. They also synthesize the neurotransmitter GABA in addition to the neuropeptide enkephalin and express the D2 dopamine receptor subtype (Figure 1) (Smith et al, 1998).

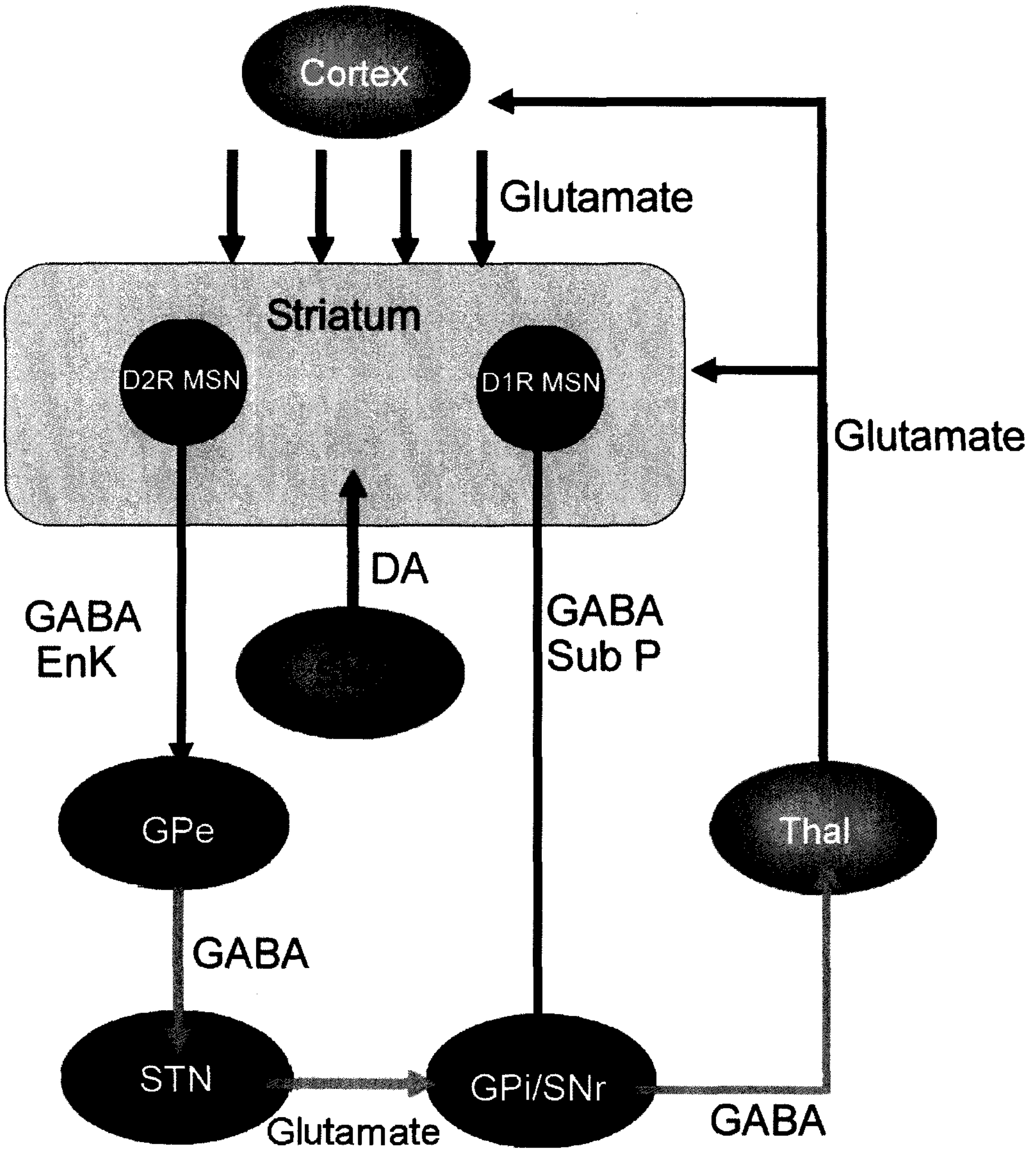

Figure 1: Connections between the basal ganglia nuclei

1.2 Striatal Interneurons

Projection neurons (MSNs) are the most abundant type of neurons in the striatum. The remaining neurons, representing the other 5% of the striatal volume in rat, are composed of four subtypes of typical interneurons including three types of inhibitory GABAergic interneurons co-expressing either parvalbumin, calretinin or somatostatin/neuropeptide Y /nitric oxide synthase. There is also a non-GABAergic, giant aspiny cholinergic interneuron (Tepper et al, 2004). It has been recently recognized that there is a small population of atypical GABAergic interneurons noted for their ability to synthesize and release DA under unique circumstances (Ibanez-Sandoval et al 2010).

Despite their small numbers, striatal interneurons are very important in the regulation of striatal function. Electrical activation of the GABAergic interneurons is strong enough to delay and even completely inhibit the cortically driven spiking activity of the projection neurons (Tepper et al, 2004). The electrophysiological differences between the three subtypes of classical striatal inhibitory interneurons suggest that circumstances under which they are activated are particular to each subtype.

The role of the cholinergic interneurons in basal ganglia function is somewhat better understood than that of the other interneurons. These neurons, also known as the tonically active striatal neurons, display a typical slow and steady firing in the absence of input stimulation but can express a variety of firing patterns with stimulation (Apicella, 2007). They have long been though to participle in regulation of motor function but it is only recently that the details of this mechanism are being understood. A salient stimulus will cause ongoing motor activity to stop and attention to be focused on the salient stimuli. This happens via activation of the thalamostriatal neurons that will induce a burst followed by a short pause in striatal cholinergic firing (Ding et al, 2010). This cholinergic burst will lead to a presynaptic inhibition of corticostriatal innervation of both MSNs population as well as an enhanced sensitivity of the indirect pathway MSNs. The pause in cholinergic firing ceases inhibition of corticostriatal glutamate release, leading to an increased firing of the indirect pathway MSNs. Neurons from the indirect pathways are known to mediate inhibition of ''none selected'' movement so that the attention can be focused of the ''movement to execute'' by the direct pathway MSNs. Thus, activation of the indirect pathway MSNs will lead to inhibition of ongoing movement. Similarly, a burst-pause pattern in cholinergic firing is seen in reward learning mechanism (Aosaki, et al, 2010).

The cholinergic interneuron also targets the other subtypes of interneurons, enhancing their role in striatum regulation (Contant et al, 1996).

1.3 Cholinergic Receptor distribution and Function in the striatum:

The importance of cholinergic regulation in the striatum is paralleled by the variety and abundance of cholinergic receptors expressed there.

Both populations of MSNs express the muscarinic M1 receptor (ref) and 39% of direct pathway MSNs also express the M4R (Bernard et al, 1992). Activation of the M1R modulates MSN in discussion up and down states (see section number 4.5). M1R signalling will inhibit the activation of various potassium channels, such as Kir2 and

KCNQ, which are responsible in part for maintaining neuronal polarization. Thus, their inactivation is excitatory and favours the up state. MSNs from the indirect pathway express particularly high levels of the Kir channel, suggesting that cholinergic modulation of the up and down state is more potent on these MSNs.

The effect of M4R activation of the subpopulation of direct MSNs is not fully understood. However, M4 activation decreases PKC activity which is required for inhibition of the KCNQ channel (ref). I postulate that M4R acts as an additional factor to dampen the cholinergic effect on direct pathway MSN excitability, enhancing the relatively greater increase in excitability of the indirect MSN .The specific response of the various GABAergic interneurons to acetylcholine stimulation are not as well studied as are the MSN responses. Reports so far describe an inhibition of GABA release following cholinergic stimulation. (Sugita, *et al,* .1991)

Cholinergic interneurons also exert autoregulation in various manners. First, they express the M2R which is known to act as an auto receptor to decrease cholinergic activity (Lester et al, 2010). Second, cholinergic activation of the GABAergic interneurons inhibit cholinergic interneurons suggesting that the latter are indirectly interconnected via GABAergic interneurons (Sullivan et al, 2008).

This somewhat contradicts the report stating that acetylcholine leads to a decrease in GABA release. However, the variety of cholinergic receptors expresses by the GABAergic interneurons may elicit various responses. In addition, the complex interactions taking place between this network of interneurons may indirectly affect cholinergic response of a specific sub population of neurons.

Acetylcholine has been found to have a dual effect on dopamine release:

Striatal acetylcholine acting directly on dopaminergic nerve terminals will increase dopamine release via both muscarinic (M5R) (Vilaro et al, 1990) and nicotinic ($\alpha4\beta2$ or $\alpha6\beta2$) receptors (Zhou et al, 2002). Expression of nicotinic and muscarinic receptor gives an additional control over the rapidity of response to cholinergic stimulation. Nicotinic receptors will lead to a rapid depolarization whereas the increase in dopamine release seen with M5R will happen in a much slower fashion

It is when acting indirectly that striatal acetylcholine with have a dual effect on dopamine release.

Signaling via the M4R inhibit GABAergic neurons which lead to an increase in DA release (Zhang et al, 2002, Marchi et al, 1990 and Kemel et al 1989). However, activation of the M3R on corticostriatal synapses inhibits glutamate release, which normally facilitates dopamine release (Sugita et al 1991).

1.4 The Dopamine/Acetylcholine Balance Hypothesis

There has long been an interest in the striatal cholinergic interneurons as they are known to be involved in a balanced interaction with the dopaminergic inputs from the SNc in the maintenance of striatal function. Cholinergic function has a direct impact on motor control and reward learning mechanisms. Disruption of DA/ACh balance leads to pathological states such as Parkinson diseases (PD), Huntington's disease and schizophrenia (Calabresi et al, 2000). The classical hypothesis of ACh and DA balance in the regulation of striatal function was originally based on the improvement of symptoms seen in patients with PD when given a cholinergic antagonist (Vale, 2008). More recently, studies on the effect of chronic deprivation of dopaminergic input to the striatum have reported important changes in gene expression and neurotransmitter activity as well as significant reorganization of the cholinergic innervation of the MSNs (Salin et al, 2009 and Meurers et al, 2009). Taken together these findings suggest that the regulation of ACh by DA in the striatum is more complex than originality thought. Finally, DA and ACh closely regulate each other and striatal function either via direct neurotransmitter effects or indirectly by impacting on interneurons, networks of inter-MSNs axon collaterals and glutamatergic input from both the cortex and the thalamus (Kreitzer et al, 2009). Electrophysiology in behaving animals shows that the tonically active neuron - cholinergic interneuron - exhibits a pause in firing in response to novel behaviorally relevant stimuli (Morris et al, 2004). This is proposed to facilitate increased DA release and behavioral plasticity or learning. Thus we see that the ACh DA balance deduced from clinical observations in the middle of the last century has maintained relevance on into the most current models of striatal function. Despite indications that cholinergic function is of equal importance, most of the literature focuses of the DA side of the balance. Most studies on cholinergic interneurons deal with their electrophysiological properties rather than their contribution to striatal function.

1.5 Dissociated Culture Model

Much of the data on the function of striatal cholinergic interneurons has been collected from organotypic slice cultures. My interest, on the other hand, is in dissociated cultures as they present a different set of advantages to study function, such as better patch clamp analysis, calcium imaging for networks analysis and transgenic labeling of dispersed cells. One of the major advantages specific to our interests is that dissociated cultures offer us the possibility to create an in vitro preparation in which the cholinergic interneurons are absent. When using the conventional embryonic time point for rat neuronal dissociated cultures (E18.5), no cholinergic interneurons are present. This is because of their earlier birthdates and migration to the striatum, compared to other striatal neurons, which causes the cholinergic interneurons to die during the dissociation protocol. This can be corrected by harvesting striatal neurons from an earlier time point, at which the cholinergic interneurons survive the dissociation protocol. By mixing the two gestational culture time points together, we can create striatal cultures containing cholinergic interneurons (Figure 2). This gives us the opportunity to compare function and pharmacology of striatal dissociated cultures containing or lacking cholinergic interneurons. Moreover, dissociated cultures give us the opportunity to add or remove other components of the basal ganglia circuits such as cortical, nigral or thalamic inputs.

1.6 Research Hypothesis

Our hypothesis is that the presence and absence of cholinergic interneurons will have major effects on DA signaling in striatal circuits. To reveal the differences, we will study a striatal dissociated culture model with and without cholinergic interneurons which receives excitatory cortical input. The differences observed will provide a better understanding of key aspects of cholinergic signaling function and will serve as an excellent starting point for future experimental design.

My research focuses on the electrophysiological and neurochemical differences between corticostriatal cultures with and without striatal ACh interneurons. I investigated changes seen in the regulation of signaling pathways that are known to be regulated by DA in the striatum, and determined how they were affected by the absence of cholinergic regulation. Specifically, I studied changes in the regulation of DARPP-32, c-FOS and EGR-1. As the striatum is comprised of 95% GABAergic inhibitory neurons, we decided to study these changes in the presence of cortical inputs which provide a constant baseline of neural excitation in the striatal neurons.

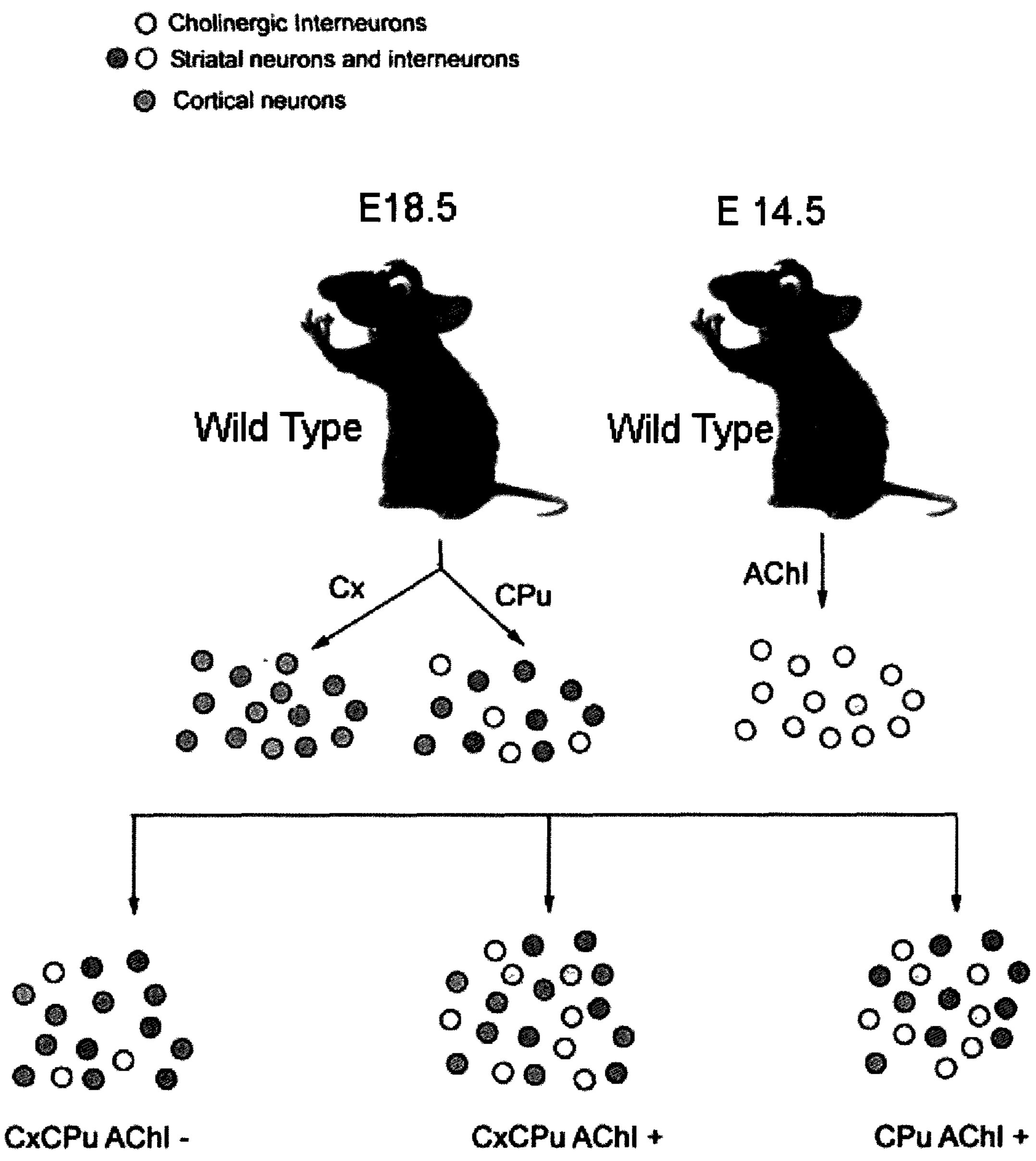

Figure 2: Rat Corticostriatal dissociated cultures models. Rat striatal neurons harvested at E18.5 lack the cholinergic interneurons. Cholinergic interneurons can be successfully harvested from E14.5. Neurons collected from both time point can then be added together to create cultures containing and lacking cholinergic interneurons. A similar process can be followed with cortical neurons harvested at E18.5.

1.6 Molecular Studies

1.7.1 DARPP-32

Several transduction pathways exist to regulate MSN excitability. The dopamine and cAMP regulated phosphoprotein-32kDa (DARPP-32) signaling pathway is known to be differentially activated by the D1 and D2 receptors (Borgkvist et al, 2007; Ouimet et al, 1988). The downstream targets of DARPP-32 are varied and can lead to biochemical, electrophysiological and behavioral responses (Nairn et al, 2004). DARPP-32's actions depend on its phosphorylation state. Its two main phosphorylation sites are threonine (Thr) 34 by protein kinase A (PKA) and threonine 75 by cyclin dependant kinase 5 (cdk5) (Walaas et al, 1983 and Bibb et al, 1999). Both dopamine and glutamate come into play in regulating DARPP-32's phosphorylation state. Glutamate increases the Thr 34 phosphorylation by inhibition of protein phosphatase 1 (PP-1) which is responsible for dephosphorylation at this site (Qi et al, 2010). Moreover, DA can regulate DARPP-32's phosphorylation differently depending on which receptor it activates. Dopamine D1 receptor (D1R) stimulation increases intracellular cAMP levels and leads to increased phosphorylation at the Thr 34 site (Walaas et al, 1983). On the other hand, dopamine D2 receptor (D2R) stimulation decreases intracellular cAMP levels and results in an increase in phosphorylation at the Thr 75 site (Figure 3) (Bateup et al, 2008).

In addition to this dual phosphorylation, DARPP-32's function is regulated by positive feedback on itself. Phosphorylation on the Thr 34 site will inhibit PP-1 whereas phosphorylation of Thr 75 site will inhibit PKA (Hemmings et al, 1984 and Bibb et al, 1999). The inhibition of PP-1 potentiates the D1-cAMP-PKA signaling pathway by reducing dephosphorylation of its downstream targets (Narin et al, 2004). Finally, activation of the D1R will decrease phosphorylation of the Thr75 site (Usui et al, 1998, Bibb et al, 1999, Nish et al, 2000).

The regulation of DARRP-32 is essential in eliciting the behavioral responses seen with psychostimulants (Zachariou et al, 2006) such as amphetamine and cocaine. For instance, the hyperlocomotor response observed after treatment with cocaine is severely decreased in DARPP-32 knock outs (Fienberg et al, 1998). Moreover, cocaine can also result in differential phosphorylation of DARPP-32 with an acute or chronic exposure (Borgkvist et al, 2007).

Considering DARRP-32's role in the integration of the cAMP-signaling pathways, its impact on MSNs and its regulation by dopamine, we decided to investigate its regulation in the presence and absence of cholinergic interneurons.

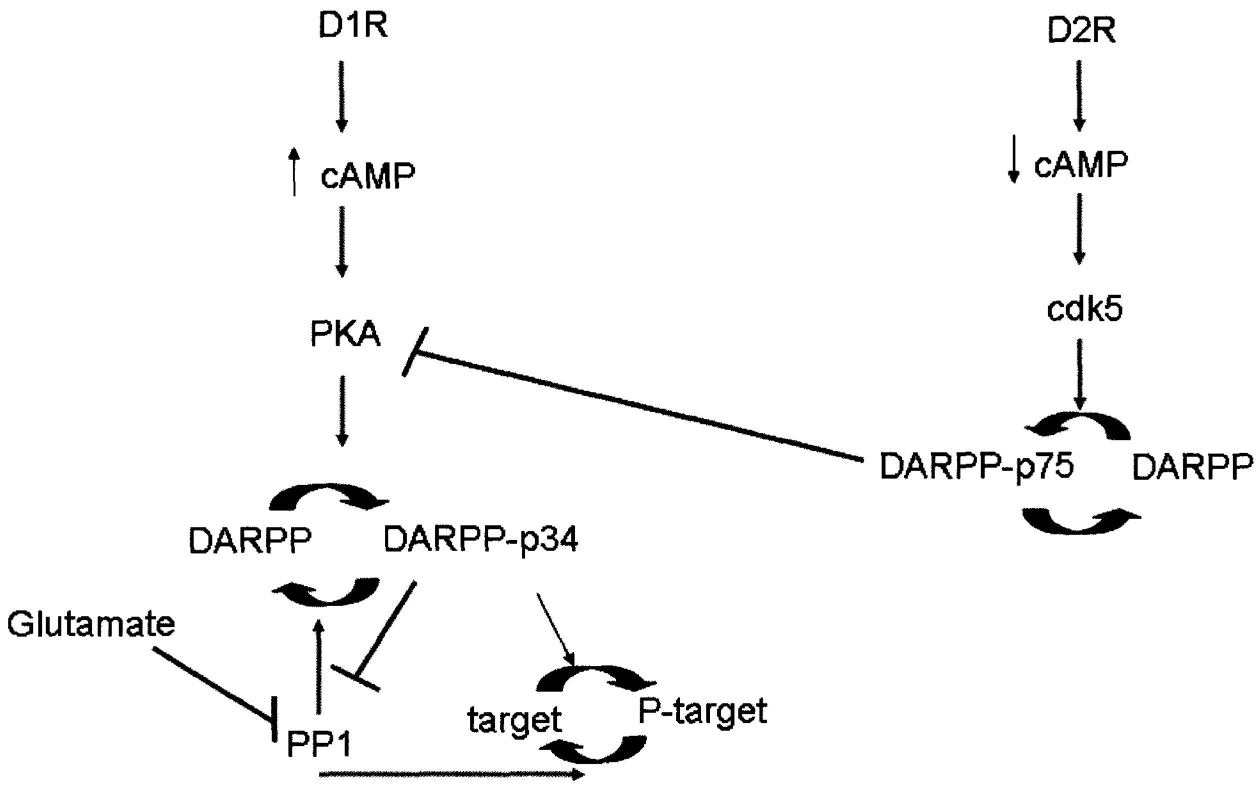

Figure 3: Effect of D1R or D2R stimulation on the DARPP-32 Signalling Pathway. Stimulation via the D1 receptor increases phosphorylation of DARPP-32 on Thr 34, promoting neuronal excitability. Signalling via the D2 receptor increases phosphorylation of DARPP-32 on Thr 75 inhibiting neuronal excitability.

1.7.2 c-FOS

c-FOS is a transcription factor belonging to the immediate early gene family. It combines with c-jun to form the AP-1 transcription factor complex. Genes from the Fos family function to translate short term extracellular stimuli into long term intracellular changes. Their downstream targets include genes involved in cell proliferation, differentiation, apoptosis, and transformation (Durchdewald et al, 2009). Several regulatory responses influence c-FOS expression. Complex interactions between these elements rather than independent regulation are responsible for the differential regulation of c-FOS under a wide range of stimuli (Robertson et al, 1995).

Under normal conditions, c-FOS expression in the striatum is undetectable but can be rapidly induced by behavioral or drug stimuli (Graybiel et al, 1990). In rats, RNA levels of c-FOS are known to peak 30 to 45 min after systemic injection of antipsychotic drug, whereas proteins can be detected 2 hrs after injection. A differential regulation of c-FOS by typical and atypical antipsychotics has led to the discovery of both D1 and D2 dependent regulation (Svenningsson et al, 2000 and Nguyen et al, 1992).

Increases in c-FOS expression are seen after administration of a D1 agonist or a D2 antagonist (Roberson et al, 1992). Decreases in c-FOS expression in the striatum have been described in both 6-OH-DOPA and MPTP lesion mice and coincide with the appearance of motor symptoms (Gross et al, 2009 and Kano et al, 1995). However, in these conditions, the increase in c-FOS associated with stimulation of the D1R is lost but the effect seen with a D2R antagonist persists (Roberson et al, 1992 and Dragunow et al, 1990).

Based on this information, we can infer that c-FOS regulation reflects certain acute drug effects relevant to the context of striatal function.

1.7.3 EGR-1

Early growth response -1 (EGR-1) is another transcription factor part of the immediate early gene family. Its expression can be stimulated by many extracellular signaling molecules including hormones, neurotransmitters, growth, differentiation factors and nitric oxide (NO) (Contestabile, 2008). It is induced within minutes following the onset of a stimulus and decays within hours thereafter (Nguyen et al, 1992).

In the striatum, EGR-1 expression is induced by dopamine and glutamate via an intracellular Ca2+ dependent mechanism. (Knapska et al, 2004). More specifically, the efficacy of induction was dependent on the type of receptor activated, the more potent being the NMDA receptor (Cordorelli et al, 1994 and Keefe et al, 1996).

Similarly to c-FOS, EGR-1 is differentially regulated by the D1 and D2 receptors and under certain conditions, its induction is specific to the direct or indirect pathway (Keefe et al, 1996).

Since the expression and/or phosphorylation of these three proteins (DARPP-32, c-FOS and EGR-1) are tightly regulated in the striatum and are widely used as indicators of striatal function, we decided to investigate how they would be affected by the absence of cholinergic interneurons when stimulated by different dopaminergic agonists and antagonists.

1.8 Electrophysiological analysis

Neuronal network activity can be very easily analyzed in tissue culture preparation by multielectrode array (MEA). MEAs are culture dishes that contain electrodes, on which neurons are grown. Upon connection of the culture dish to an amplifier and recording device, activity at each electrode can be recorded. The signal detected at each electrode can be spatially and temporally integrated to represent network activity.

Despite their morphological similarities, membrane properties of striatal MSNs appear to differ (Day et al, 2008). Under normal conditions, corticostriatal stimulation will increase excitability of direct pathway MSNs but will decrease firing rate of indirect pathway MSNs (Flores-Barrera et al, 2010). Acetylcholine signaling can modulate the activity of membrane ion channels and thereby impact MSN excitability (Shen et al, 2005). In addition, cholinergic signaling impacts differentially the two populations of striatal MSNs, and this regulation is altered by the absence of dopaminergic signaling (Shen et al, 2007).

In addition to the impact of cholinergic interneurons on signal transduction, we studied changes in network firing activity of corticostriatal cultures (AChI+/-), using multielectrode array analysis.

1.9 Research Goals

1. Develop an *in vitro* striatal dissociated neuronal model that includes the AChI. Standard CPu cultures lack the cholinergic interneuron and as such we had to work out the appropriate conditions to reconstitute it from earlier embryonic sources.

2. Develop an *in vitro* corticostriatal model. The largely inhibitory striatal culture requires cortical input to maintain a baseline firing rate. We combined cortex and striatum in culture and then sought evidence of functional innervation.

3. Investigate cortical cell cultures to determine neuronal developmental maturity. Using a simplified culture model that only contains cortical neurons, assess, by means of molecular and electrophysiological studies, their development milestone *in vitro,* to insure that pharmacological studies, performed in 4. below, are not carried during a period of rapidly changing synaptic organization.

4. Assess the impact of the presence and absence of AChI on DA signaling in striatal circuits. Provide further understating of AChI functional relevance to striatal regulation.

Methods

2.1 Dissociated cell cultures

Striatal neurons were prepared from Sprague-Dawley rats at either gestational day 14.5 or 18.5. Cortical neurons were prepared from Sprague-Dawley rats at gestational day 18.5. Dissection procedures were approved by the University of Ottawa Animal Care Committee. From each embryo, the CPu primordia MGE/LGE (medial/lateral ganglionic eminence) was removed and placed into ice cold 1X Hank's buffered salt solution (HBSS; Sigma Aldrich). Neurons were centrifuged at 1000 RPM for 5 min prior to mechanical dissociation in the presence of DNAse (200 units/ml, Sigma Aldrich). Exclusion of Trypan blue (0.4%, MP Biomedicals) was used to assess live cell number. Neurons were cryopreserved (QBM cell science, Ottawa, Canada) and stored in liquid nitrogen until used.

Neurons were thawed and resuspended in Neurobasal/B27 (2% final volume, Invitrogen), 100 units/ml of penicillin, 100 μg/ml of streptomycin (pen/strep), L-glutamine (L-glut: 2 mM) and nerve growth factor (NGF; 100 ng/ml). Neurons were plated at a density of $1x10^5$ cells per well on 96 well culture dishes which were pre-coated with poly-D-lysine. Cultures contained E18.5 striatal neurons (no AChI) with E18.5 cortical neurons and/or E14.5 striatal neurons (AChI) (Figure 2). Cells were incubated (5% CO2/95% air) at 37 °C, at least 35 DIV with media change every 3-4 days, replacing water lost (Potter and DeMarse, 2001) by adding 160 μl sterile water per 1ml media to be added.

2.2 Immunocytochemistry

Cultures were fixed in 4% paraformaldehyde containing 7% (v/v) saturated picric acid for 45 min at room temperature. Cells were rinsed three times with 10 mM phosphate buffered saline (PBS) and stored at 4°C until assessed. Cultures were incubated with primary antibodies (Table 1) diluted in 10 mM PBS containing 0.3% triton X overnight at 4°C, then rinsed with 10 mM PBS for 5 min. Appropriate secondary antibodies were used at a 1:400 dilution in 10 mM PBS containing 0.3% triton X and were incubated for 35 min at 37°C, then rinsed again for 5 min prior to analysis using a Zeiss Axiovert 200M fluorescence microscope or a Zeiss Axioplan fluorescence microscope.

2.3 Counting and Statistics

Cell counting was performed with the public domain ImageJ software (NIH Image). Successive steps were (i) linear adjustment of threshold so that only the NeuN positive cells were highlighted, (ii) analyzing the particles with a pixel size of 20-infinity. Results obtained were reliable as tested with several independent counts by hand of the same culture. Counts were made on 2 wells from 3 independent experiments. Significance was determined by a one-way ANOVA. The range indicated standard deviation.

Primary Antibody	Species	Concentration	Supplier
Calretinin	Goat	IH: 1:400	Swant, Bellinzona
Somatostatin	Mouse	IH: 1:200	Dr. Brown, University of British Columbia
NOS	Rabbit	IH: 1:200	Santa Cruz
ParV	Mouse	IH: 1:4000	Sigma
vAChT	Rabbit	IH: 1:1000	Synaptic-Systems
TH	Mouse	IH: 1:50	Boehringer Mannheim Biochemica
NeuN	Mouse	IH: 1:10	Dr Mullen, University of Utah
DARPP-32	Rbt	IH: 1:100 WB: 1:2000	Cell Signalling
DARRP-32 phosphoThr^{34}	Rabbit	WB: 1:1000	Chemicon
DARRP-32 phosphoThr^{75}	Rabbit	WB 1:1000	Millipore
EGR-1	Rabbit	WB 1:1000	Santa Cruz
c-FOS	Rabbit	WB 1:1000	Abcam
vGAT	Rabbit	IH: 1:500 WB: 1:2000	Alpha Diagnostics
vGLUT	Guinea Pig	IH: 1:500 WB: 1:1000	Millipore
Synapsin	Rabbit	IH: 1:500 WB 1:1000	Phosphosolutions
Actin	Mouse	WB 1:1000	Santa Cruz

Table 1: Antibodies used for immunocytochemistry (IC) and Western Blot analysis (WB).

2.4 Pharmacological studies

Cultures were grown up to 35 DIV before receiving drug treatments. Cultures received either: 20 uM of SKF 38393 (Sigma Aldrich), a dopamine D1 receptor specific agonist for 45 min, overnight treatment with 20 uM of apomorphine (Sigma Aldrich), a non specific dopamine agonist or over night treatment with 20 uM of apomorphine, a non specific dopamine agonist followed by 45 min with 20 uM of raclopride tartrate (Sigma Aldrich), a Dopamine D2 specific antagonist.

2.5 Western Blot

Protein levels were determined by Western blotting using the antibodies listed in Table1. To harvest cells, plates were washed 2X with PBS, and lysed in radioimmunoprecipitation assay buffer (RIPA: Tris 50mM; NaCl 150 mM; SDS 0.1 %; Na.Deoxycholate 0.5 %; Triton X 100 or NP40 1%) buffer with protein inhibitors (Sigma Aldrich) for 30 min on ice, vortexing every 5 min. Lysates were centrifuged at 10,000 × *g* at 4°C for 2 min. Protein concentration was determined by Bradford method (BioRad). Protein (15 μg) were added to 4X reducing sample buffer (100 mM Tris–Cl pH 6.8, 200 mM dithiothretiol, 4% SDS, 20% glycerol and 0.2% Bromophenol blue) and heated at 100°C for 5 min. Samples were electrophoresed on 12% polyacrylamide gel under constant current (100 mV). Separated proteins were transferred onto a PDVF membrane (Perkin-Elmer). Blots were blocked with 1% milk solution in TBST (20 mM Tris–HCl pH 7.6, 137 mM NaCl, 0.05% Tween-20) for 1 h at room temperature and incubated with primary antibody two hours at room temperature, washed 3X with TBST and incubated for two hours with horseradish peroxidase (HRP)-conjugated second antibody

(Santa Cruz Biotechnology). Membranes were washed three times with TBST and the immunoblots were visualized on X-ray films (Interscience) after exposure to enhanced chemiluminescence reagent (ECL) (Amersham). Actin bands were monitored on the same blot to verify consistency of protein loading. Immunoblots were stripped with Reblot Plus solution (Chemicon) for 30 min at room temperature. Blots were probed with anti-actin primary antibody and second antibody (anti-mouse, Santa Cruz) as described above. The molecular size of the protein was determined by running prestained protein markers in an adjacent lane (Sigma).

2.6 Quantification of Western Blots

Image J densitometry software (National Institutes of Health, USA) was used for quantitative densitometric analysis of Western blots. Selected bands were quantified based on their relative intensities. Results were expressed in fold increase or decrease compare to control condition.

2.7 Electrophysiological analysis

Mutielectrode arrays (MEAs) recordings were performed on cultures. Dissociated cells were plated at a density of $0.5x10^6$ cells on poly-D-lysine coated multielectrode arrays with a square grid 8x8 of 60 planar Ti/TiN-microelectrodes (MultiChannel Systems, Reutlingen, Germany). Extracellular potentials from all 60 electrodes were simultaneously recorded at a sampling frequency of 25 kHz, visualized and stored using the standard software MC_Rack provided by MultiChannel Systems (Reutlingen, Germany). Spike detection was performed offline using the software SpAnNer (Medizinische Analyseverfahren, Tönisvorst, Germany; for details see Otto *et al.* 2003).

At the beginning of the experiments cells were transferred to the recording apparatus and placed into a 37°C incubator with 5% CO_2 and left to settle for 15 min. Recordings were then performed for 10 min per MEA once every 7 days for 42 DIV and then again at 46 DIV. Pharmacological studies were performed at 46 DIV. Drugs used were 200 nM of muscarinic toxin 7 (MT 7) (Peptide Institute Inc, Osaka Japan), 50 uM SKF 38393 (Sigma Aldrich), and 50 uM raclopride tartrate (Sigma Aldrich). Addition of water was used as a control and recording lasted 5 min per drug.

Results

3.1 Striatal cultures

Standard dissociated striatal cultures (harvested from E18.5) were cultured for 35 DIV and were examined using immunohistochemistry, for the presence of cholinergic interneurons, as well as other types of striatal neurons. As seen in preliminary experiments, all interneuronal types were represented, except for the cholinergic interneuron. The presence of the TH interneurons is explained by the lack of dopaminergic innervation to our cultures which creates an ideal environment for TH expression in the striatum (Ibanez-Sandoval et al, 2010). When mixing the two gestational dissections together (E18.5 with E14.5) all types of striatal neurons, including the cholinergic interneurons and the MSNs were detected (Figure 4). Morphologically, the interneurons in both the E18.5 and the mixed gestational cultures showed typical morphological characteristics. The parvalbumin interneurons displayed aspiny dendrites with modest arborisation as well as some heterogeneity in the size of the cell soma. The cholinergic interneuron was easily identifiable as a giant aspiny neuron. The NOS and CalR neurons also displayed their typical morphological characteristics (Tepper et al, 2004).

3.1.1 Proportion of interneurons in culture models

The proportion of interneurons harvested from each time point was established and compare to values from *in vivo* reports (Table 2) (Tepper J et al. 2004). As expected, we found very sparse cholinergic interneurons in the typical dissection date for striatal cultures (0.01 ±0.01%). The cultures collected from the earlier time point (E14.5) and the mixed gestational cultures both contained significantly more cholinergic interneurons (0.22±0.04% and 0.08±0.03%, respectively). This represents an almost 10 fold increase in the number of cholinergic neurons in mixed culture and approaches the values seen *in vivo*.

3.1.2 Proportion of neuronal progenitors in our culture model

Since the AChI are harvested from a much earlier time point then typical striatal cultures, we worried about contaminating our mixed gestational model with neuronal progenitors from the E14.5 dissection. Because of this, other members of my lab further characterized the mixed gestational cultures in order to assess the proportion of AChI to other neuronal progenitors in the E14.5 cultures, using EdU assays (a recent improvement on the BrdU technique). The proportion of progenitor neurons was significantly higher in the E14.5 culture than in the E18.5 culture. However, no significant differences in the proportion of progenitors were found between the E18.5 culture and the mix gestational culture (E18.5 with E14.5), suggesting that the proportion of neuronal progenitors added from the E14.5 is negligible in our mixed gestational culture model.

3.1.3 Neuronal death over time

Since our cultures are incubated for 35 DIV before being used for experiments, another potential limitation to our culture model is neuronal death over time. This was assessed by counting the number of NeuN positive neurons in cultures at different points during their development. We found no significant changes in neuron numbers between 7, 21 and 35 DIV (data not shown).

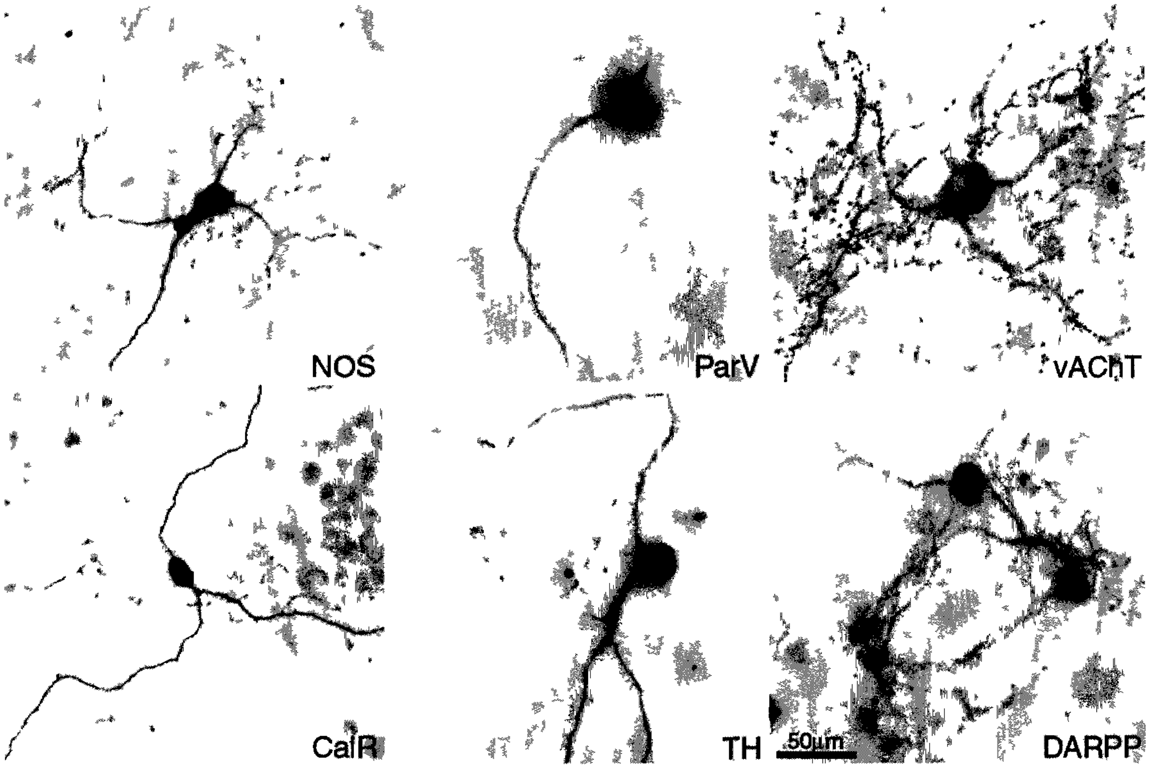

Figure 4: Striatal neurons in dissociated cultures. Inverted immunohistochemical figure showing striatal interneurons and MSNs in dissociated cultures. All 4 types of typical interneurons are present in cultures in addition to the atypical tyrosine hydroxylase (TH) interneuron. DARPP staining represents striatal MSNs.

%	**E18.5**	**E14.5**	**E18.5/E14.5**	**CPu *in vivo***
ParV	0.27 ±0.04	0.18±0.04	0.37±0.07	0.7
NOS	1.11±0.19	0.24±0.01*	0.93±0.18	0.8
vAChT	0.01±0.01	0.22±0.04*	0.08±0.03*	0.3
CalR	0.96±0.28	1.01±0.05	0.68±0.23	0.8

Table 2: Proportion of striatal interneurons in dissociated cultures. Numbers represent percentage of interneuron detected by immunocytochemistry ± standard deviation. * $p < 0.01$ for dissection dates in comparison to the typical striatal dissection date (E18.5). (*In vivo* data from Tepper et al, 2004)

3.2 Cortical innervation of striatal interneurons

Mixed gestational striatal cultures (E18.5 with E14.5) were cultured together with E18.5 cortical neurons. When comparing the morphology of interneurons in corticostriatal cultures to striatal cultures, we consistently found more intense immunostaining together with more pronounced dendritic and axonal outgrowth of interneurons in cultures with cortical innervation (Figure 5). This effect was particularly prominent for the parvalbumin and cholinergic interneurons. Failed attempts at quantifying these changes based on immunostaining methods directed us toward WB analysis. Changes in protein expression of each interneuronal marker were assessed by other members of my lab, and confirmed my results that cortical innervation resulted in an increase in vAChT and parvalbumin expression, with no significant changes in NOS or DARPP-32. Furthermore, these increases were mimicked by glutamate agonist and the effect of cortical innervation was reversible by the addition of glutamate antagonist (unpublished data for our lab). These observations indicate functional innervation of striatal interneurons by cortical input in our cultures. They are further supported by patch clamp analysis performed by our collaborators in Japan, which confirmed functional cortical innervation of striatal neurons in our culture model (Randall et al, 2009).

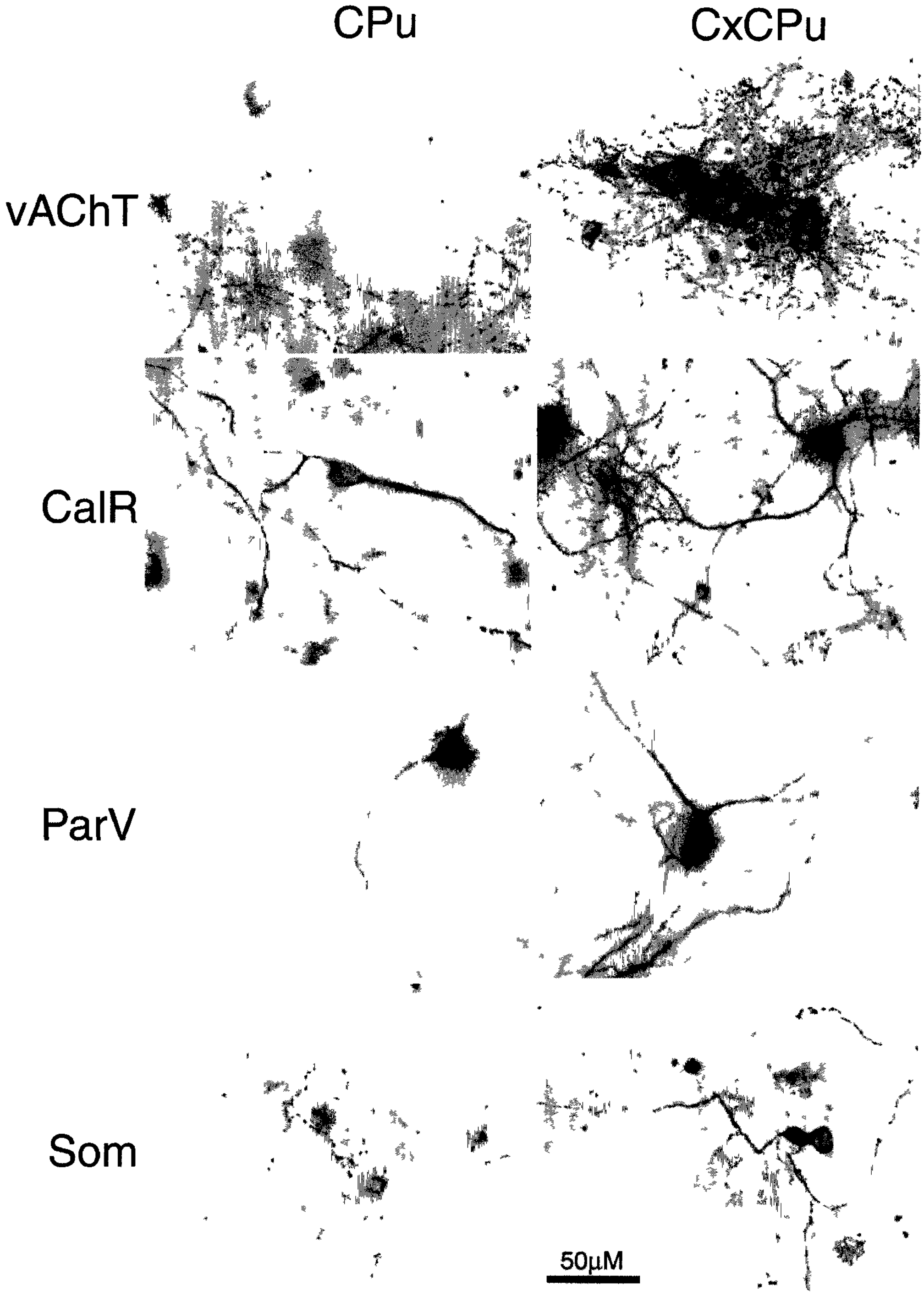

Figure 5: Effect of cortical innervation on striatal interneurons. Inverted immunohistochemical figure showing the effect of cortical innervation on dentritic and axonal outgrowth of striatal interneurons.

3.3 Development of dissociated cortical cultures

Rat cortical neurons (E18.5) *in vitro* development was assessed at weekly time points. The expression of synaptic markers such as synapsin, vGAT and vGLUT were assessed by immunocytochemistry, and Western blotting to follow synaptogenesis. These results were performed in collaboration with coauthors in my lab.

3.3.1 Synapsin expression in cortical cultures

Immunocytochemistry and WB analysis showed that synapsin expression was negligible during the two first week of development, but significantly increased after 21 DIV ($p= 0.037$ for IM, $p= 0.0009$ for WB) (Figure 6). Immunohistochemical results indicate that synapsin levels stabilize after 21 DIV, while WB analysis showed a 2-fold increase between 21 and 42 DIV ($p= 0.001$).

3.3.2 vGAT and vGLUT expression in cortical cultures

Immunocytochemistry showed a modest increase in vGAT expression throughout the time course followed (Figure 7). WB results show a similar pattern of expression with an 11 fold increase after 21 DIV ($p=0.002$). vGLUT showed a pattern of expression similar to vGAT with a 3.1-fold after 21 DIV, showed by WB ($p<0.02$) (Figure 8).

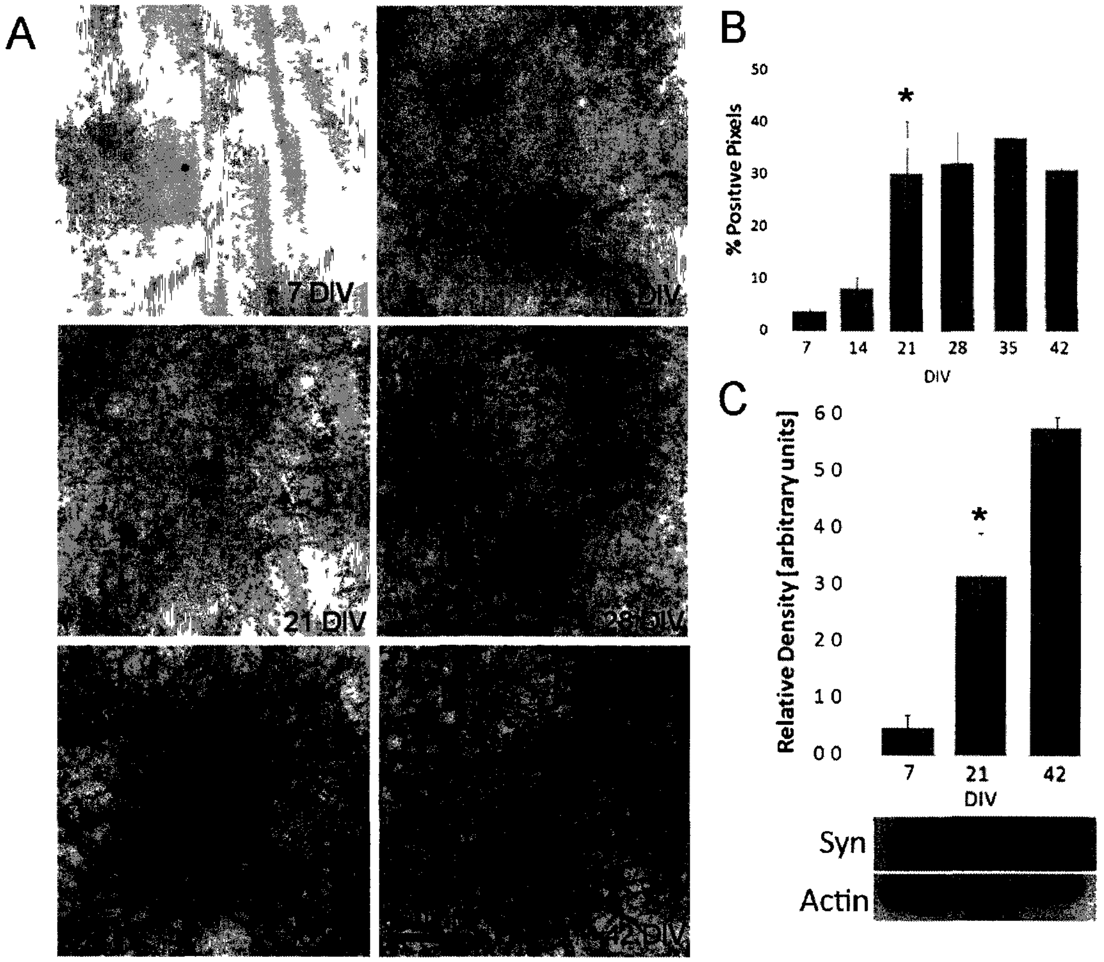

Figure 6 : Synapsin expression in developing cortical cultures. Expression was assessed using immunocytochemistry and WB. A: Inverted histochemical figures showing synapsin expression at every week. B: Graph showing quantification of immunohistochemical data. C: Graph and gel showing WB results. * shows significance compared to the previous week at $p < 0.001$ using one way ANOVA. Note that actin controls are the same for figure 6, 7 and 8 since they come from the same sample.

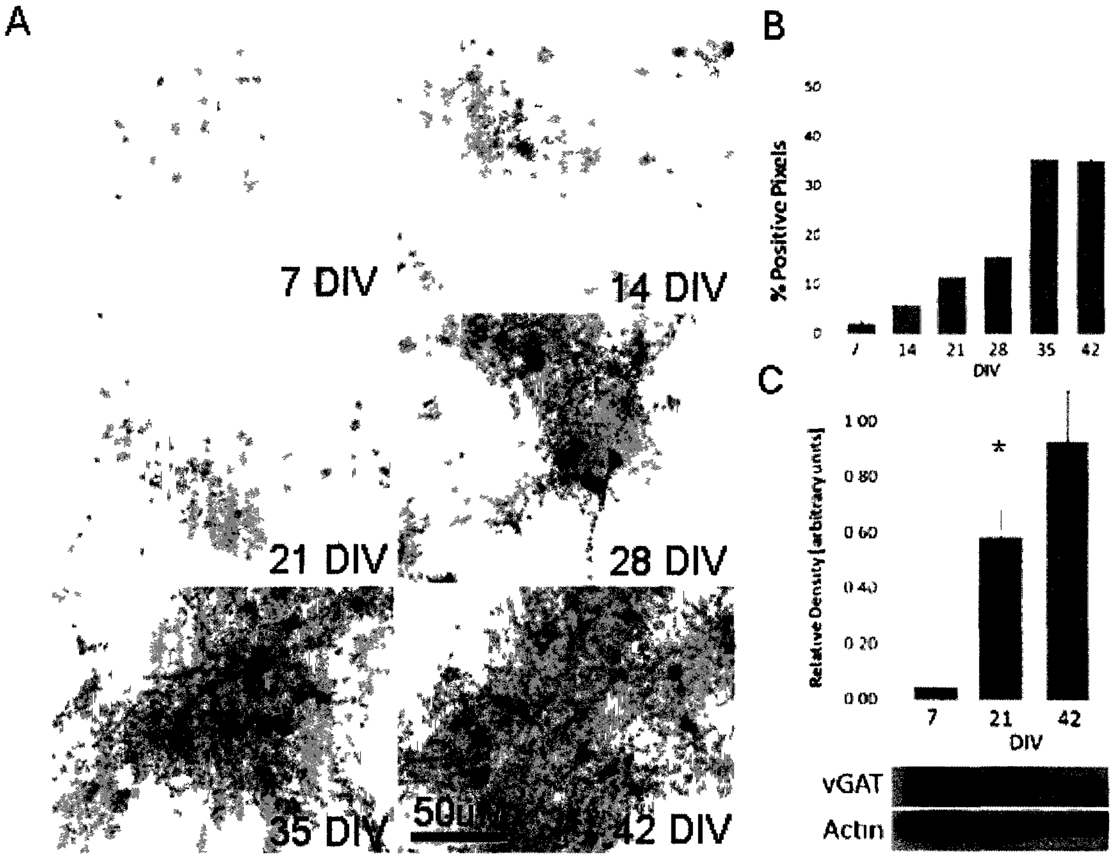

Figure 7: vGAT expression in developing cortical cultures. Expression was assessed using immunocytochemistry and WB. A: Inverted histochemical figures showing vGAT expression at every week. B: Graph showing quantification of immunohistochemical data. C: Graph and gel showing WB results. * shows significance compared to the previous week at p <0 001 using one way ANOVA.

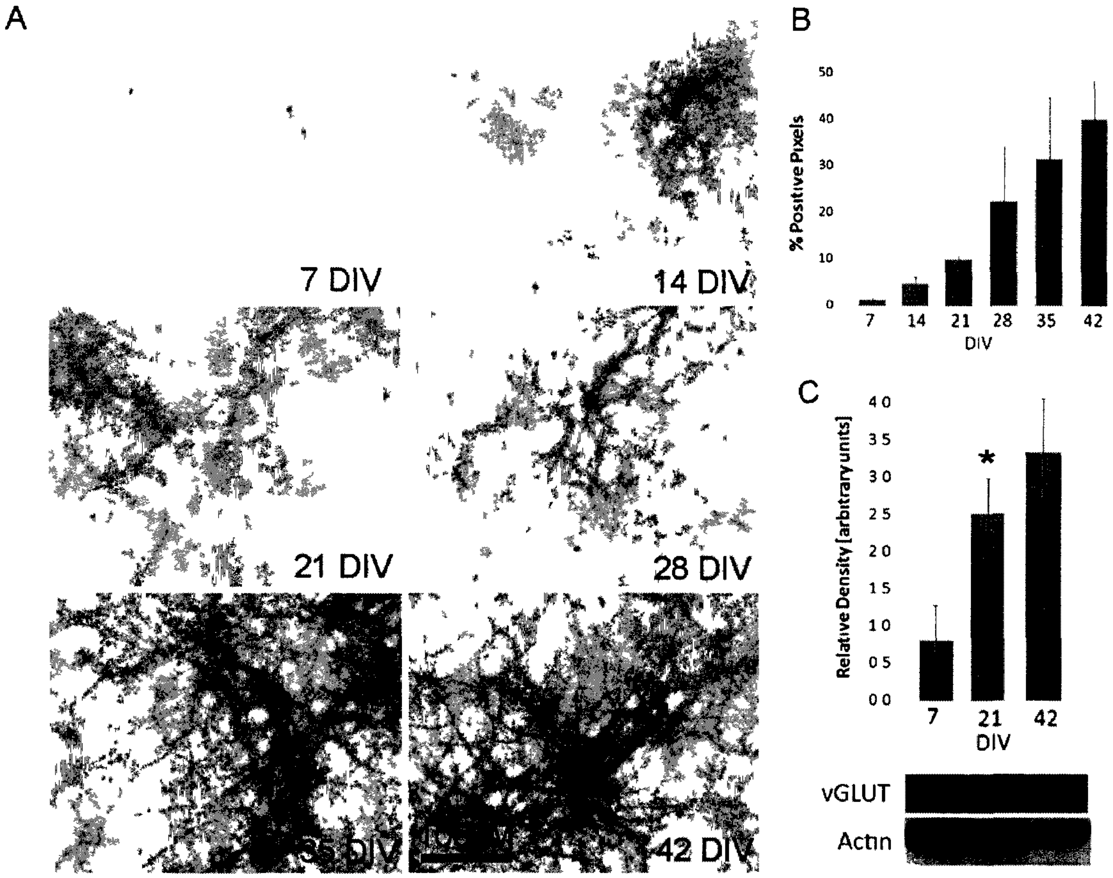

Figure 8 : vGLUT expression in developing cortical cultures. Expression was assessed using immunocytochemistry and WB. A: Inverted histochemical figures showing vGLUT expression at every week. B: Graph showing quantification of immunohistochemical data. C: Graph and gel showing WB results. * shows significance compared to the previous week at $p < 0\ 001$ using one way ANOVA

3.3.3 Electrophysiological analysis of developing cortical cultures

In addition to the molecular characterisation, we assessed culture maturity based on electrophysiological activity. Spikes detection showed minimal activity at 7, 14 and 21 DIV, with a 10-fold increase detected at 28 DIV. A significant increase in spike detection was observed at each of the time points following (Figure 9 A).

Network activity does not always represent network maturity. In addition to spike detection, we studied network synchronicity as a way to evaluated maturity of our cultures. Network synchronicity analysis was based on population burst detection. These values represent the number of electrodes from which an action potential is detected at one point in time. When the number of electrodes firing exceeded a set threshold, it was considered a population burst.

Due to the insufficient firing, this analysis could not be performed prior to 35 DIV. From 35 to 42 DIV, there was a 2.2-fold increase in the number of population bursts detected ($p<0.0005$) (Figure 9B). There were no significant changes observed between 42 and 46 DIV. These data suggest that even though overall network activity kept increasing during the entire time period from which we recorded, network maturity, i.e. synchronicity, was reached at 42 DIV.

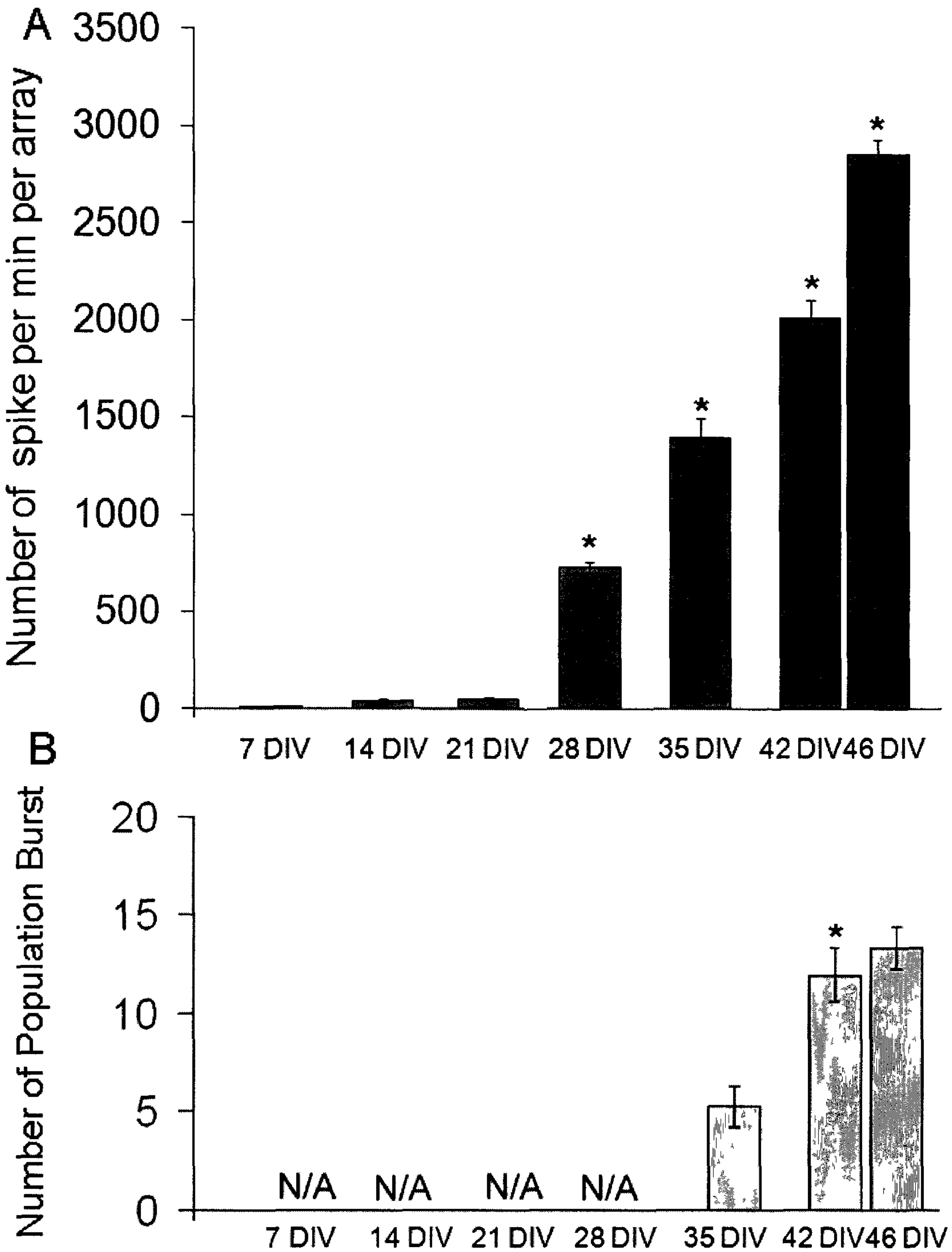

Figure 9: Electrophysiological analysis of cortical cultures maturity in vitro. A: Number of spike detected per minutes per array, for recordings at each weeks. B: Population burst analysis: represent network synchronicity. * shows $p < 0.001$ with one way ANOVA, when compared to the previous week.

3.4 Effect of cholinergic interneurons on striatal response

To study if cholinergic interneurons had an impact on MSNs' regulation by dopaminergic signaling, we assessed changes in protein levels of genes that are known to be affected by dopamine stimulation. Due to a small number of replicates, none of these results have reached statistical significance yet. Nevertheless, the trends seen in the different types of cultures with the addition of dopaminergic drugs are interesting and will be the basis of my discussion.

3.4.1 c-FOS

c-FOS protein expression was evaluated using Western blot analysis. No changes were observed in the CxCPu AChI+ cultures after stimulation with SFK38393 (D1R agonist) or apomorphine (DA agonist) (Figure 10). However, when cultures treated with apomorphine were given raclopride tartrate, (D2R antagonist), an increase in c-FOS expression was observed. Indicating that in these cultures a disinhibition of the indirect pathway results in an increase in c-FOS expression.

On the other hand, treatment with both SKF 38393 (D1R agonist) and apomorphine (DA agonist) elicited an increase in c-FOS expression in CxCPu AChI- cultures (Figure 10). This increase was not reversible with the addition of raclopride tartrate (D2R antagonist), indicating that the apomorphine response was due to D1R stimulation.

In the CPu AChI+ cultures, addition of apomorphine (DA agonist) resulted in a decrease in c-FOS expression. Addition of raclopride tartrate (D2R antagonist) not only reversed the effect of apomorphine but also resulted in an increased c-FOS expression (Figure 10). This over-compensation may be due to a supersensitivity of the D2R to an antagonist. No differences were observed with the addition of SKF 38393 (D1R agonist).

3.4.2 EGR-1

EGR-1 levels were also assessed by Western blot analysis (Figure 11). Addition of both SKF 38393 (D1R agonist) and apomorphine (DA agonist) to CxCPu AChI+ cultures elicited an increase in EGR-1 (Figure 11). This increase was not reversible with the addition of raclopride tartrate (D2R antagonist), indicating that the effect seen with apomorphine treatment was most likely due to stimulation of the D1R.

In the CxCPu AChI- cultures, addition of SKF 38393 (D1R agonist) or apomorphine (DA agonist) resulted in an increase in EGR-1 expression. The higher increase seen with addition of apomorphine suggests that the increase in EGR-1 expression is mediated by both activation of the D1R and D2R. This increase was reversible with the addition of raclopride tartrate.

Finally, no changes in EGR-1 expression were observed in the CPu AChI+ cultures (Figure 11), indicating a role for corticostriatal stimulation in regulation of EGR-1.

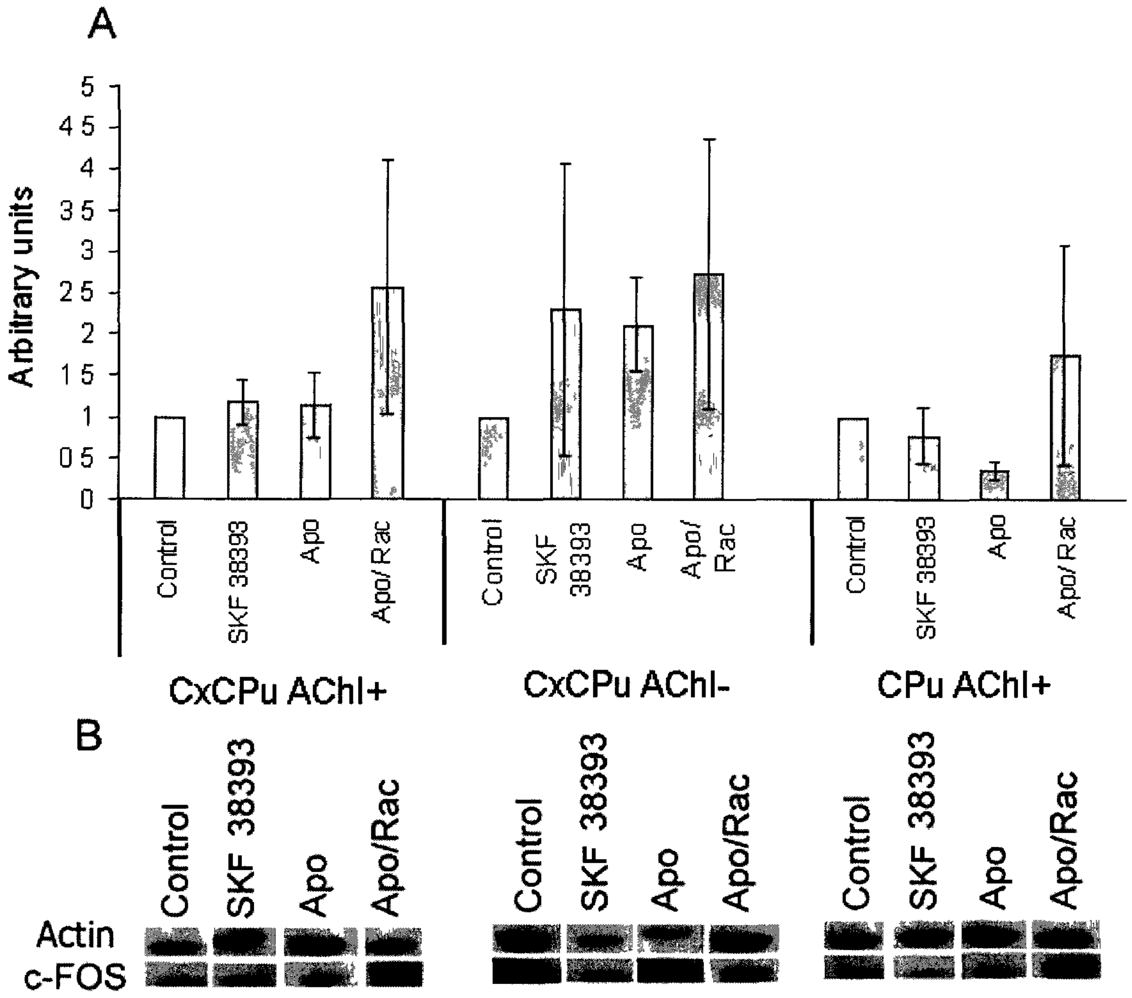

Figure 10: Western blot quantification of c-FOS. All results are expressed as a fold increase compared to control drug condition (n=3). CxCPu AChI+: corticostriatal cultures with cholinergic interneurons, CxCPu AChI-: corticostriatal cultures without cholinergic interneurons and CPu AChI+: striatal cultures with cholinergic interneurons.

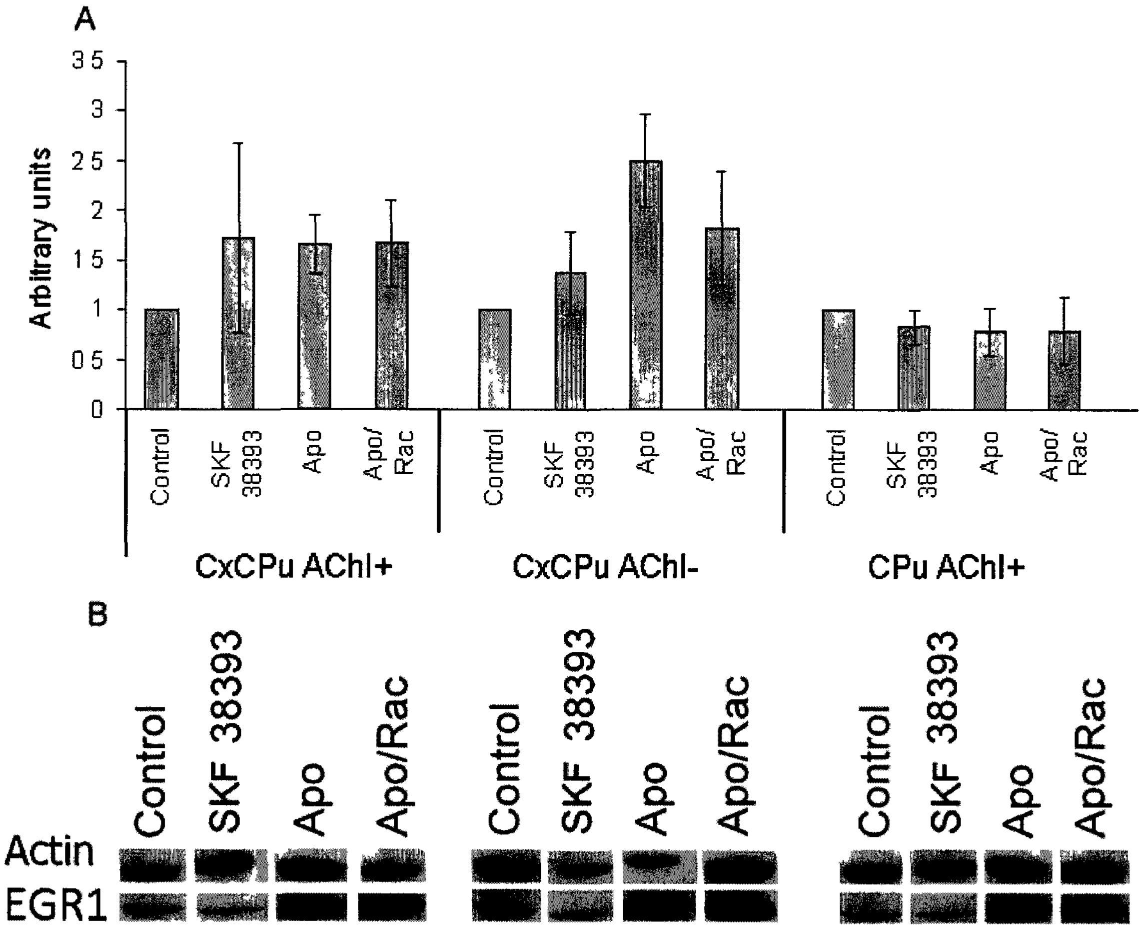

Figure 11: Western blot quantification of EGR-1. All results are expressed as a fold increase compared to control drug condition (n=3). CxCPu AChI+: corticostriatal cultures with cholinergic interneurons, CxCPu AChI-: corticostriatal cultures without cholinergic interneurons and CPu AChI+: striatal cultures with cholinergic interneurons.

3.4.3 DARPP-32

Using WB analysis, we studied the effect of cholinergic interneurons on DARPP-32 basal expression level as well as its phosphorylation state on two major regulatory sites Thr 34 and Thr 75.

Despite apparent variation in DARPP-32 expression levels seen after quantitative methods, the pictures of the WB seem to indicate little variation in DARPP-32 basal expression levels with the addition of the different drugs. However, CPu AChI+ cultures seem to have overall lower levels of expression than cultures containing cortical innervation (Figure 12)

3.4.4 DARPP-32 Phosphorylation

An important variation amongst replicates is observed making these results difficult to interpret at this point (Figure 13).

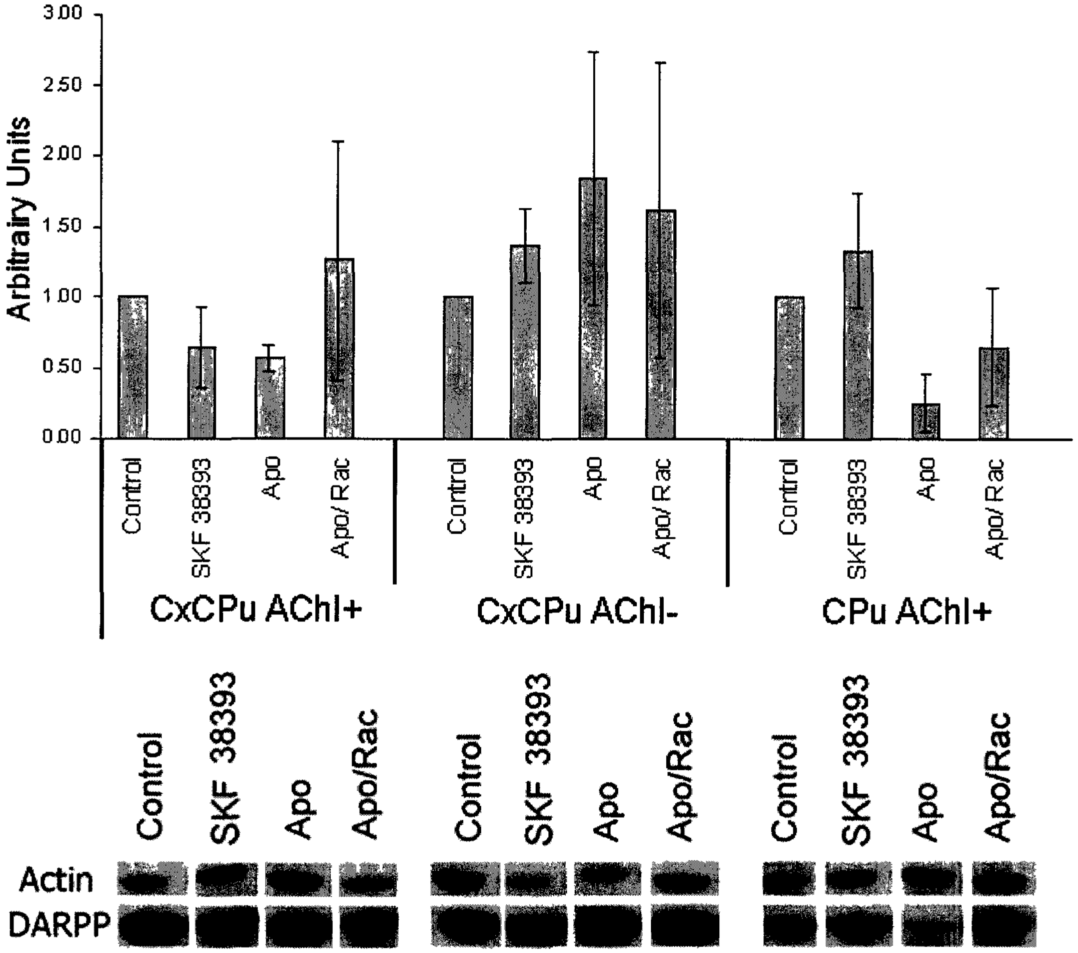

Figure 12: Western blot quantification of DARPP-32 expression in striatal cultures. All results are expressed as a fold increase compared to control drug condition (n=3). CxCPu AChI+: corticostriatal cultures with cholinergic interneurons, CxCPu AChI-: corticostriatal cultures without cholinergic interneurons and CPu AChI+: striatal cultures with cholinergic interneurons

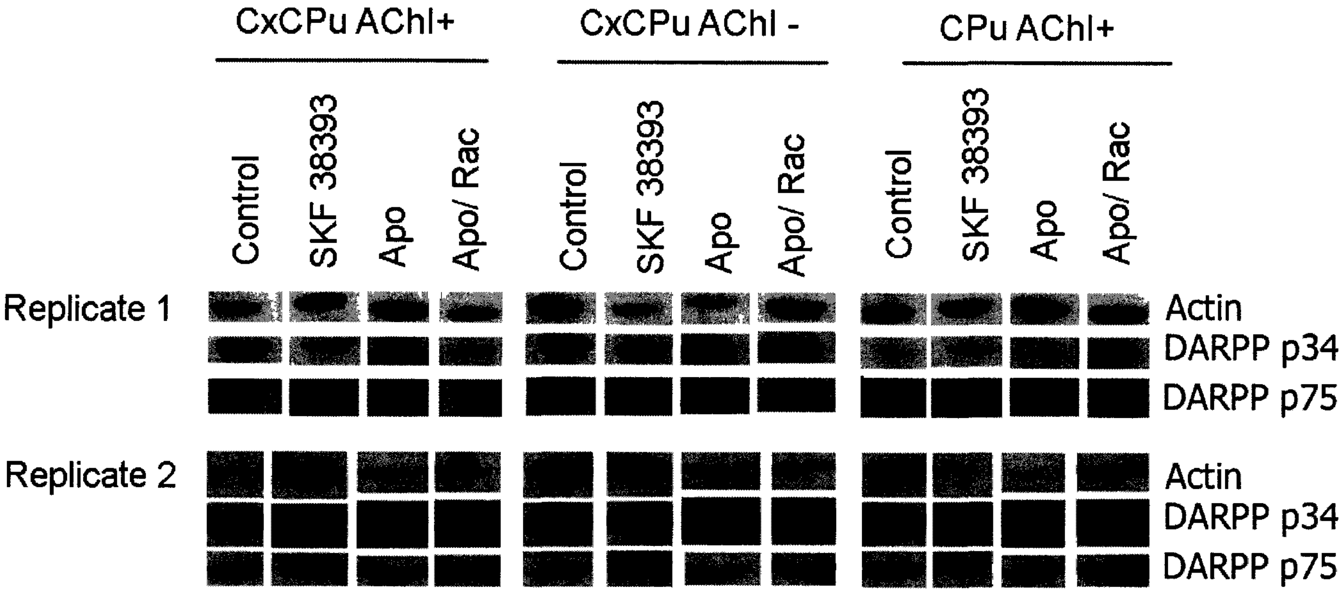

Figure 13: Western blot of DARPP-32 phosphorylation. Showing blot results for phosphorylation on the two main regulatory sites Thr 34 and Thr 75. CxCPu AChI+: corticostriatal cultures with cholinergic interneurons, CxCPu AChI-: corticostriatal cultures without cholinergic interneurons and CPu AChI+: striatal cultures with cholinergic interneurons

3.5 Effect of AChI on striatal network activity

Multi electrode array (MEA) recordings of our cultures were performed to study their electrophysiological properties. The parameter used to quantify activity was the number of spikes per minute (Figure 14). Firing activity of the cultures started to appear after 14 DIV and considerably increased thereafter. No firing was detected in the CPu AChI+ cultures. Throughout their development, there was significantly more activity detected in the CxCPu AChI- cultures than in the CxCPu AChI+ cultures, suggesting an inhibition of the MSNs by cholinergic interneurons

In addition we stimulated the cultures with various cholinergic and dopaminergic drugs to see how they would affect the cultures (Figure 15). Under control conditions, we found that the CxCPu AChI- culture had significantly more spikes per minute than the CxCPu AChI+ cultures. Addition of muscarinic toxin 7 (MT 7), an M1R inhibitor, resulted in a 2.5 fold increase ($p< 0.0001$) in the number of spikes per minute in the CxCPu AChI+ cultures with no effect on the CxCPu AChI- cultures. Similarly, we observed a 1.3 fold decrease ($p< 0.0001$) in firing in the CxCPu AChI+ cultures after the addition of raclopride tartrate. No effect was observed in the CxCPu AChI- cultures.

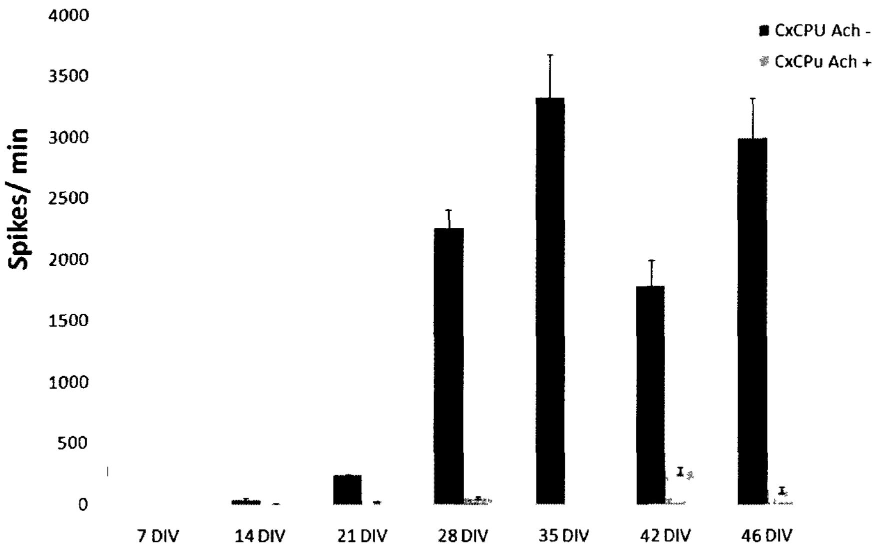

Figure 14: Electrophysiological activities of cultures on multielectrode arrays. The graph shows the average number of spikes per minute measured at every week of the culture development. Error bars represent standard error of the mean. Note, no activity was detected in the striatal cultures lacking cortical input. CxCPu AChI+: corticostriatal cultures with cholinergic interneurons, CxCPu AChI+: corticostriatal cultures without cholinergic interneurons, DIV: days *in vitro*.

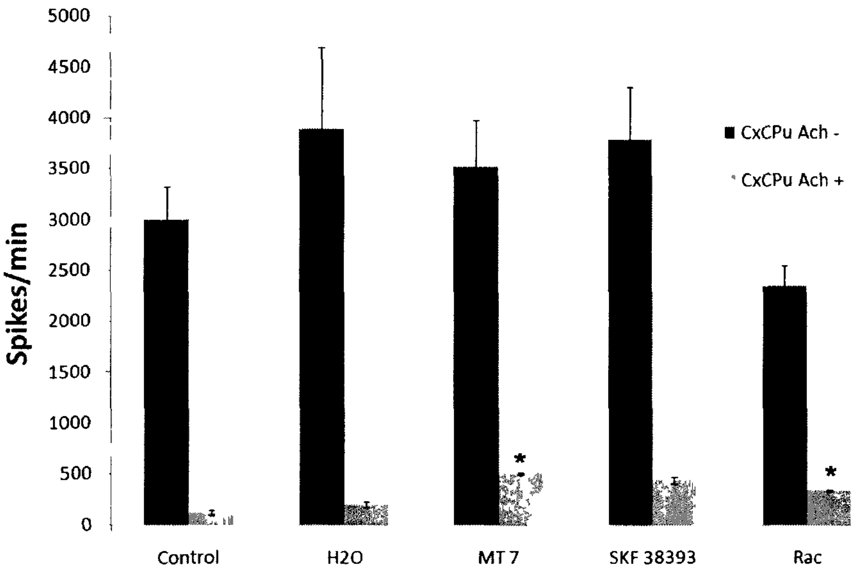

Figure 15: Electrophysiological activities of cultures on multielectrode arrays following drug treatment. The graph shows the average number of spikes per minute following drug treatment, measured at 46 DIV. CxCPu AChI+: corticostriatal cultures with cholinergic interneurons, CxCPu AChI+: corticostriatal cultures without cholinergic interneurons, MT 7: Muscarinic toxin 7 (M1R antagonist). * $p<0.01$ when compared to H2O measurements.

Discussion

4.1 Striatal cultures

The striatum originates from the lateral ganglionic eminence (LGE) (Marin et al, 2001). The striatal cholinergic interneurons however are born in the medial ganglionic eminence (MGE) and migrate to the LGE very early during development (Van Vulpen et al, 1998). They start their neurite outgrowth before the other striatal neurons and are already sending out processes while other striatal neurons have only a soma. Because of this, cholinergic interneurons die during the dissociation protocol for typical striatal cultures and are not present in the final dissociated cultures. However, cholinergic interneurons can be harvested from an earlier dissection time point and supplemented to the cultures in order to create a complete striatal cultures.

Our mixed gestational culture successfully allows us to obtain dissociated striatal cultures containing or lacking cholinergic interneurons. In addition, it creates the possibility of adding back other components of the basal ganglia circuit, such as cortical, nigral or thalamic innervations, and studying their impact on striatal neurons.

In parallel to these rat striatal cultures, we have developed a similar protocol using mouse striatal neurons (Schock et al, 2010). This opens the door to the use of transgenic mice in which specific subpopulations of neurons can be labeled. For example, we recently acquired a transgenic mouse in which the AChI express the green fluorescent protein (GFP). Therefore, using fluorescence activated cell sorting (FACS), we can isolate these neurons from our dissection and specifically supplement them in a controlled proportion to our standard striatal cultures.

Similarly, we could remove a GFP-tagged interneuron subtype from a population of striatal neurons, allowing us to create a culture model in which that particular interneuron subtype would be lacking. This is equivalent to the creation of a knock out mouse but much superior, not only in its simplicity, but in the certainty that the interneuron subtype is lacking from the population rather than simply not expressing a specific marker, making it invisible to immunochemical staining protocols.

In addition, transgenic mice also create the opportunity to study changes in gene expression of specific neuronal populations eg: MSNs of the direct versus indirect pathway, giving further insight on striatal regulation (Valjent et al, 2009).

Finally, GFP-tagged neurons can also be used to differentiate between neuronal populations from different nuclei. For example, cortical neurons could be expressing GFP together in culture with wildtype striatal neurons, allowing us to easily differentiate between these two populations.

4.2 Cortical innervation of striatal neurons

We have demonstrated a functional impact of cortical innervation on striatal interneuron morphology. The fact that the changes seen were reversible with the addition of glutamate antagonist suggests they were dependent on changes in neuronal excitability rather than secretion of growth factors by the cortical neurons. They also confirm that there was functional innervation of the striatal neurons by the cortical neurons in our cultures. These results are part of a manuscript in preparation.

We and others (Segal et al, 2003) have shown that cortical stimulation has both functional and morphological impact on striatal neurons. Adding back the cortical input is our first step in recreating a basal ganglia circuit *in vitro*. We are now ready to supplement the cultures with dopaminergic inputs from the SNc, and are working on dissection protocols for thalamic neurons. Ultimately, we will study functional differences in striatal regulation in the presence and absence of cholinergic interneurons, when all major inputs to the striatum are present.

4.3 Development of cortical cultures

Striatal studies using live animal models are based on a mature neuronal network. Since our neurons for culture are harvested at an embryonic time point, we have to insure they have reached maturity prior to beginning our experiments. If striatal functions are assessed to early during development, the synaptogenesis process that occurs can create an unstable environment for scientific studies and are not representative of *in vivo* conditions.

To assess the development of dissociated neurons *in vitro*, we studied a simplified model based on cortical cultures. We followed synaptogenesis during their development based on expression of synaptic markers such as synapsin, vGAT and vGLUT, as well as network maturity based on electrophysiological recordings.

4.3.1 Synaptogenesis in cortical cultures

Synapsin's expression could be considered in two ways; the number of varicosities and the amount of protein in those varicosities. Our immunostaining results, representing the number of varicosities showed that these have reached a maximum after 21 DIV. In contrast, the WB results, representing the quantity of protein, shows a persistent increase even after 21 DIV.

In contrast to the synapsin data, vGAT and vGLUT expression showed a constant increase, throughout the time course followed, with both immunostaining and WB analysis. These data suggest that synaptogenesis is not completed until at least 35 DIV, and that cultures should not be assessed for pharmacological experiments prior to this. Moreover, the earlier rise in synapsin compare to the markers of vesicular storage suggests a role for synapsin earlier in development.

4.3.2 Electrophysiological maturation of cortical cultures

Using multielectrode array analysis, we recorded electrophysiological activities during development of our cultures. Detection of spike activity was very sparse prior to 28 DIV, which is in accordance with our molecular studies of synaptogenesis. Spike activity kept increasing past the 46 DIV time point followed for our study, suggesting that perhaps maturity of network had not been reached at 46 DIV. However, studies of network synchronicity showed that at 42 DIV cultures had reached a plateau in term of synchronicity. These suggest that firing activity may not be the best measure to estimate network maturity and that network synchronicity should be considered instead.

Finally, our molecular and electrophysiological data indicate that cultures should be maintained for 42 DIV prior to use for analysis.

4.4 Effect of cholinergic interneurons on striatal response

We studied functional responses to dopaminergic stimulation of corticostriatal cultures in the presence and absence of cholinergic interneurons. Our dopaminergic stimulation consisted of stimulation with a D1R specific agonist (SKF 38393), a non specific dopamine agonist (apomorphine) or a specific D2R antagonist (raclopride tartrate). Because of the differential expression of dopamine receptors by the MSNs the D1R agonist specifically stimulates MSNs from the direct pathway, whereas a D2R antagonist specifically acts on MSNs from the indirect pathway (Figure 16). Finally, apomorphine can stimulate both the D1R and D2R and therefore act on both populations of MSNs.

Due to some technical challenges with the culturing, these results are based on a small number of replicates (n=2 or 3). Because of this, none of the results have significance at a statistical level. Nonetheless, the trends observed are of great interest from a physiological point of view and point to differences between cultures with and without the cholinergic interneurons.

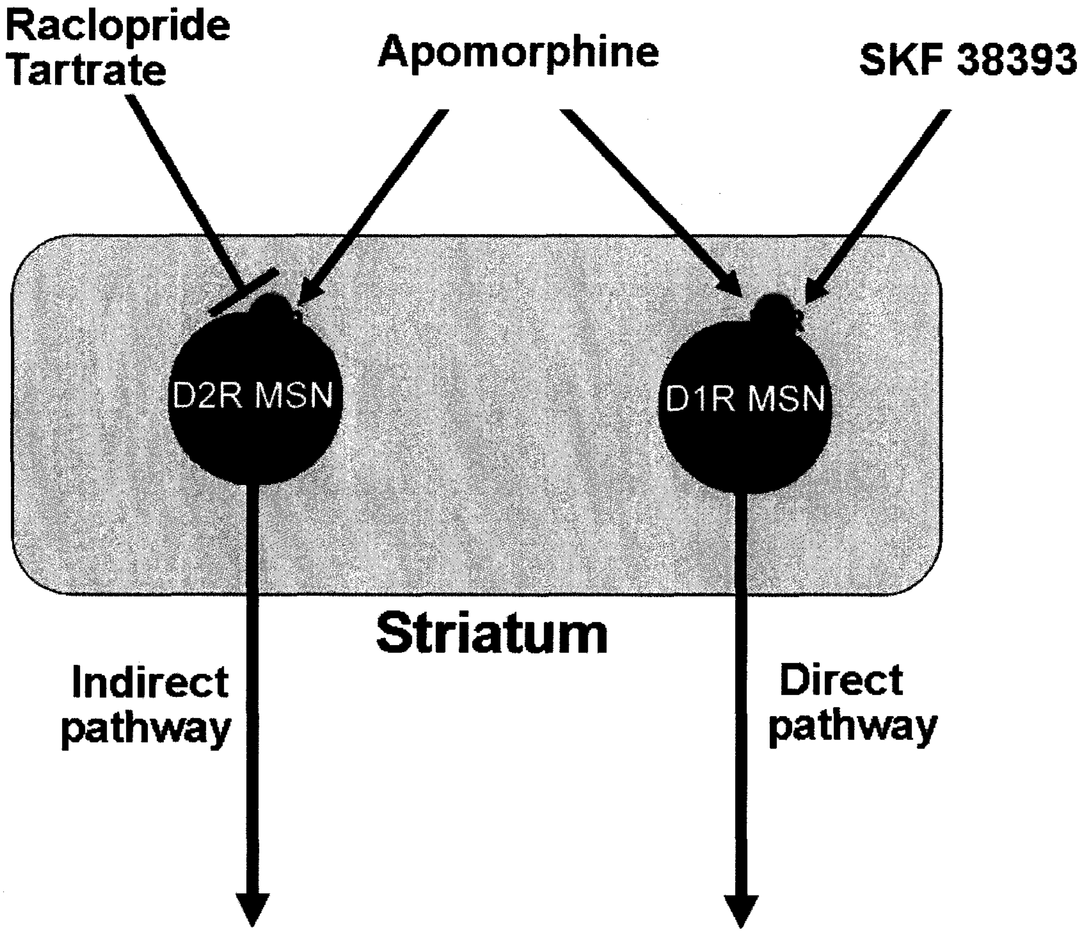

Figure 16: Pharmacological studies: Impact of dopaminergic drugs on MSN population.

4.4.1 c-FOS

In the dopamine deprived striatum, c-FOS expression can be induced via the ERK/MAPK pathway (Gerfen et al, 2002). This induction requires combined interaction between D1R and NMDA/mGLUR (Gross et al, 2009). Stimulation with a D2R antagonist also produces an increase in c-FOS expression but this is achieved via an ERK/MAPK independent mechanism (Bertran-Gonzalez et al, 2008).

We expect to see an increase in c-FOS expression after stimulation of the D1R (SKF 38393 or apomorphine), or after inhibition of the D2R (raclopride tartrate) (Pollack et al, 2010). However, only the withdrawal response after stimulation with a D2R antagonist is observed in our CxCPu AchI + cultures. On the other hand, we do see the expected increase in c-FOS expression after stimulation with of D1R in the CxCPu AChI- cultures but the increase seen with inhibition of the D2R is lost. Taken together, these results suggest that the cholinergic interneurons inhibit activation of the D1R/ NMDA-mGLUT stimulation of c-FOS expression in MSNs expressing the D1R but are required for increased c-FOS expression in MSNs expressing the D2R, indicating a differential regulation of the two neuronal pathways by cholinergic interneurons. Finally, our results suggest that the regulation c-FOS expression in MNS from the indirect pathway function via a corticostriatal innervation independent mechanism, since the increase in c-FOS seen after Apo/Rac stimulation persists in CPu AChI+ cultures. This is consistent with a previous report that c-FOS expression after stimulation of the D2R with an antagonist does not require interaction with glutamate receptors (Brami-Cherrier et al, 2009).

I suggest that a potential mechanism for the regulation c-FOS expression by cholinergic interneurons most likely involves intracellular signaling pathways regulating c-FOS expression. Moreover, differential regulation of the two populations of MSNs by acetylcholine may be explained by the pattern of muscarinic receptor they express. Both populations of MSNs express the M1 and M4 mucarinic receptor, however, the M4 muscarinic receptor is more widely expressed on neurons from the direct pathway (Bernard et al, 1992). Activation of the M1R receptor results in activation of PKC, which can activate both ERK and PKA and also results in mobilization of the intracellular calcium (Felder, 1995). Activation of the M4R is negatively coupled to adenylyl cyclase which will result in inhibition of PKA. Therefore, the inhibition of the induction of c-FOS expression following SKF 38393 when the AChI are present may be regulated via activation of the M4R. On the other hand, the increase in c-FOS expression seen with disinhibition of the D2R may be associated with activation of the M1R. Previous studies had reported a positive effect of cholinergic agonist on c-FOS expression in MSNs from the indirect and of a positive effect of cholinergic antagonist on c-FOS expression in MSNs from the direct pathways, which are in agreement with the results presented here (Bernard et al, 1993). Finally, AChI may also indirectly impact regulation of MSNs by affecting other interneuron subtypes (Sullivan et al, 2008).

4.4.2 EGR-1

EGR-1 is a member of the immediate early gene (IEG) transcription factors. Similarly to c-FOS, EGR-1 expression is regulated by the ERK/ MAPK pathway, however, its regulation depends on different downstream intermediaries (Brami-Cherrier et al, 2009).

Activation of the ERK/MAPK pathway with glutamate, dopamine or acetylcholine has been associated with an increase in EGR-1 expression (Grimes et al, 1999 and Vaccarino et al, 1992).

Previous reports associated an increase in EGR-1 expression with stimulation of the D1R, and little effect was seen with inhibition of the D2R (Cole et al, 1992). From this, we should expect to see an increase in EGR-1 expression with SKF38393 (D1R agonist) and apo (non specific DA agonist) stimulation and no effect with addition of rac (D2R antagonist), which is what we observed in the CxCPu AChI+ cultures. However, when we removed the AChI from the cultures, an important increase in EGR-1 was seen following stimulation with apo. This increase was reversible with the addition of rac. These results suggest that in the absence of cholinergic stimulation, expression of EGR-1 can be induced via D2R signaling. Cultures lacking the AChI also demonstrate a lesser increase in EGR-1 expression following SKF 38393 stimulation suggesting that the AChI may potentiate this increase. Finally, the lack of changes seen in the CPu AChI+ cultures agrees with a previous report suggesting that corticostriatal innervation is important in regulation of EGR-1 expression (Knapska et al, 2004).

Interestingly, it seems like the effect of AChI on the two populations of MNS is opposite in terms of c-FOS and EGR-1 expression (Figure 17). More specifically, in MSNs from the indirect pathways, AchI seems to be required for the expression of c-FOS but has an inhibitory effect on the expression of EGR-1. Since both of these genes are under the control of the ERK/MAPK pathway, this implies that AChI have a differential regulation of both arms of this downstream pathway.

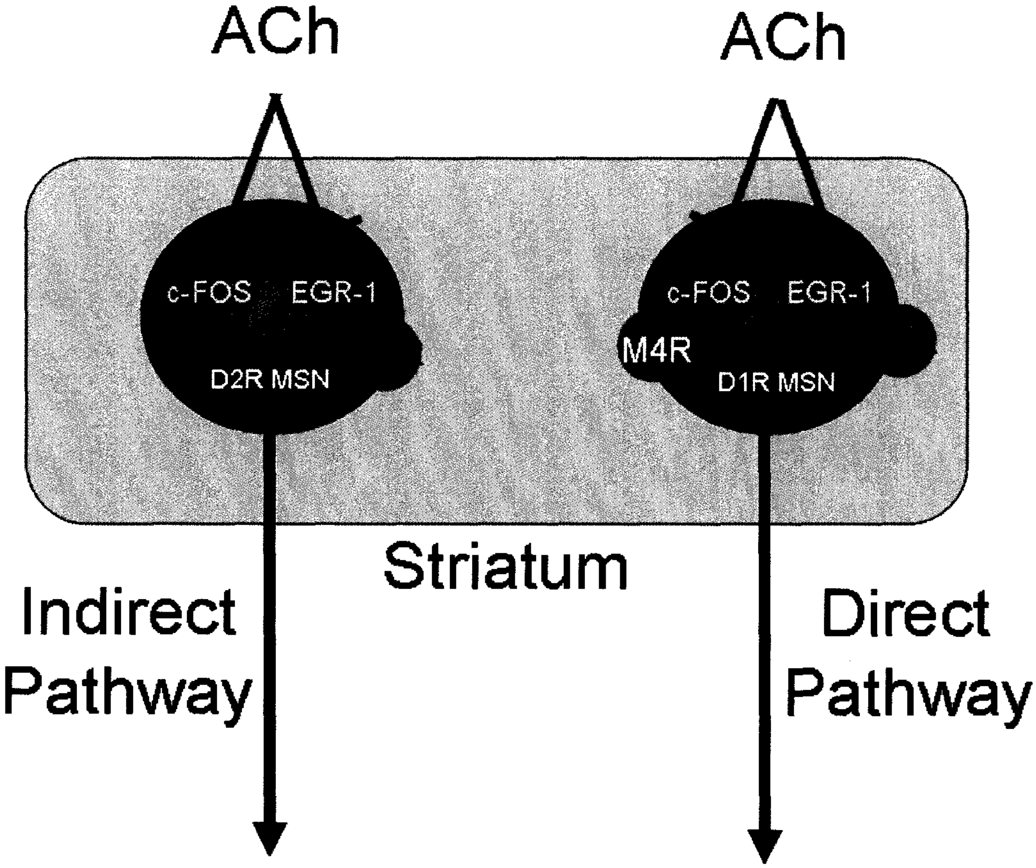

Figure 17: Differential regulation of striatal MSNs by AChI. A proposed mechanism for the differential regulation of striatal MSNs by AChI is the pattern of expression of the muscarinic receptors.

4.4.3 DARPP

Downstream targets of DARPP-32 include voltage dependent calcium channels as well as AMPA, NMDA, and GABA receptors (Borgkvist et al, 2007). DARPP-32 phosphorylation on Thr 34 activates these proteins, resulting in increasing MSN excitability.

DARPP-32 is an important intracellular mediator of dopamine signaling (Qi et al, 2010). Its phosphorylation state, rather than its expression levels are under the control of dopamine receptors (Nishi et al, 2000). In culture models, basal levels of DARPP-32 expression are severely diminished compared to in vivo models, but can be induced by addition of BDNF (Ivkovic et al, 1999).

We believe that changes observed in DARPP-32 basal expression levels are likely resulting from loading differences between replicates rather than expression differences associated with dopaminergic or cholinergic signalisation.

4.4.4 DARPP-32 phosphorylation

DARPP-32 phosphorylation state is heavily regulated by dopamine. We expected stimulation with SKF 38393 to cause an increase in Thr34 phosphorylation and a decrease in Thr 75 phosphorylation. We also expected an increase in phosphorylation of Thr 34 after addition of Rac to the cultures. Because of great variation between the replicates, interpretation of DARPP-32 phosphorylation state is difficult. A higher number of repeats is necessary.

4.5 Effect of AChI on striatal network activity

From a developmental point of view, significant firing was detected in cultures after 28 DIV. This is similar to data that we obtained in cortical cultures from which we characterized the development more extensively (manuscripts in preparation) and is what guided us toward using cultures that had reached at least 35 DIV before using them for analysis.

The lack of activity detected in the CPu AChI+ cultures was to be expected since striatal cultures are comprised of 98% inhibitory GABAergic neurons. The interesting results are the differences in firing activity between the CxCPu AChI+ and CxCPu AChI- cultures.

Striatal MSN membrane properties differ in their responses to neurotransmitter stimulation. MSNs can be in two physiological excitability states known as ''up'' or ''down'' state. MSNs can only fire when they are in their "up state" (Galiñanes et al, 2009).

Cholinergic interneurons increase MSN excitability by decreasing the opening of membrane ion channels such as KCNQ and Kir2 channels (Shen et al 2005 and Shen et al, 2007). Therefore, we would expect the presence of cholinergic interneurons to increase overall MSN excitability in our cultures. However, cultures without AChI displayed more spontaneous activity. Moreover, addition of a M1R antagonist (MT 7) resulted in increased firing in cultures with AChI. These data suggest that in our culture model, cholinergic interneurons exert an inhibitory effect on MSN excitability. This result is opposite to the impact of AChI on MSN excitability described in the literature, including DA depleted striatum. The differences observed could be explained by several factors.

First, our results describe overall network activity and not single neuron excitability. Therefore even if at the single neuron level, AChI have an excitatory effect, it does not mean it will be translated to the overall network activity. Second, our culture model represents an incomplete basal ganglia circuit. Not only is it lacking the thalamic inputs, which is the main excitatory inputs to the cholinergic interneurons (Lapper et al, 1992) but it also differs in its dopaminergic depletion. In animal studies, neurons reach maturity in the presence of dopaminergic nigral input, which is then lesioned for PD models. In our cultures, the nigral inputs have been absent from the beginning. It is possible that the lack of thalamic and nigral inputs to AchI has affected their ability to regulate MSN excitability. Finally, in our cultures, the architectural organization of the striatum is destroyed which could impact on MSN regulation (Svenningsson et al, 2000).

It is interesting to notice however, that our MEA results indicate a decrease in network firing with addition of D2R antagonist raclopride tartrate, but no changes with addition of D1R agonist SKF 38393. Several studies have indicated a more prominent regulation of indirect pathway MSNs (expressing the D2R) by AChI in DA depleted striatum (Shen et al, 2007 and Salin et al, 2009). Therefore, even if in our culture model AChI the effect of striatal MSNs seems to be opposite to what we expected, it still maintains a preferential regulation of D2R MSNs.

Conclusion and Future Experiments

We have successfully created a dissociated culture model in which the presence of cholinergic interneurons can be manipulated. Our preliminary results indicate that AChI differentially regulate the two different MSN populations, and that this regulation is sometimes dependent on cortical innervation.

Future work will first include , confirmation of these results by increasing the number of replicates; second, continuation of reconstructing basal ganglia circuitry *in vitro* and third specifically studying changes in both MSN populations using transgenic mouse culture models.

5.1 Confirmation of preliminary results

By increasing the number of replicates, we can reach statistical significance for the results presented here. If the trends observed so far persist, deeper understanding of differential cholinergic regulation could be obtained by repeating molecular experiments in the presence of muscarinic receptor specific antagonists, particularly M1R and M4R antagonists. Ultimately, we could also gain deeper understanding of interactions between intracellular signalling pathways by looking at changes in ERK/MAPK, PKA or PKC phosphorylation states.

5.2 Reconstruction of Basal Ganglia Circuits

The next step in reconstruction of basal ganglia circuits *in vitro* is the addition of dopaminergic input from SNc. We have successfully harvested and grown nigral dopaminergic neurons with striatal cultures and are in the process of repeating the molecular analysis done on corticostriatal cultures. Adding back the dopaminergic side of the balance will provide insight into cholinergic regulation of dopamine secretion at the presynaptic levels.

Thalamic innervation is another important input to the striatum and dissection and culturing protocols are now being optimized.

5.3 Differential regulation of MSN populations

We have translated our culture models to mouse neurons which offer the possibility of transgenic labeling. Being able to separate MSNs for the direct and indirect pathway prior to molecular analysis will enable us to more specifically study changes in their gene expression.

References

Aosaki, T, Graybiel, A.M, and Kimura, M. 1994, Effect of the nigrostriatal dopamine system on acquired neural responses in the striatum of behaving monkeys. *Science 265; 412*

Apicella P. 2007 Leading tonically active neurons of the striatum from reward detection to context recognition , *Trends in Neuroscience 30: 299*

Bateup HS, Svenningsson P, Kuroiwa M, Gong S, Nishi A, Heintz N, Greengard P. 2008 Cell type–specific regulation of DARPP-32 phosphorylation by psychostimulant and antipsychotic drugs, *Nature Neuroscience 11 : 932*

Bernard V, Normand E, Bloch B. 1992 Phenotypical characterization of the rat striatal neurons expressing muscarinic receptor genes, *Journal of Neuroscience 12: 3591*

Bernard V, Dumartin B, Lamy E, Bloch D. 1993 Fos Immunoreactivity after Stimulation or Inhibition of Muscarinic Receptors Indicates Anatomical Specificity for Cholinergic Control of Striatal Efferent Neurons and Cortical Neurons in the Rat, *Europeen Journal of Neuroscience 5: 1218*

Bertran-Gonzalez J, Bosch C, Maroteaux M, Matamales M, Herve D, Valjent E, Girault J A.. 2008 Opposing patterns of signalingactivation in dopamine D1 and D2 receptor-expressing striatal neurons in response to cocaine and haloperidol *Journal of Neuroscience*. 28: 5671

Bibb JA, Snyder GL, Nishi A, Yan Z, Meijer L, Fienberg AA, Tsai LH, Kwon YT, Girault JA, Czernik AJ, Huganir RL, Hemmings HC Jr, Nairn AC, Greengard P. 1999 Phosphorylation of DARPP by cdk5 modulates dopamine signaling in neurons, *Nature 402: 588*

Bolam JP, Hanley JJ, Booth PA, Bevan MD. 2000 Synaptic organisation of the Basal ganglia, *Journal of Anatomy 196:527*

Borgkvist A, Fisone G. 2007 Psychoactive drugs and regulation of cAMP/PKA/DARPP-32 cascade in striatal medium spiny neurons, *Neuroscience and Biobehavioral Reviews 31:79*

Brami-Cherrier K, Roze E, Girault JA, Betuing S, Caboche J. 2009 Role of the ERK/MSK1 signaling pathway in chromatin remodelling and brain responses to drugs of abuse *Journal of Neurochemistry 108: 1323*

Calabresi P, Centonze D, Gubellini P, Pisani A, Bernardi G. 2000 Acetylcholine-mediated modulation of striatal function , *Trends in Neuroscience 23: 120*

Cole AJ, Bhat RV, Patt C, Worley PF, Baraban JM. 1992, D1 dopamine recpetor activation of multiple transcription factors in genes in rat striatum, *Journal of Neurochemistry 58: 1420*

Contant C, Umbriaco D, Garcia S, Watkins KC, Descarries L. 1996, Ultrastructural characterization of the acetylcholine innervation in adult rat neostriatum , *Neuroscience 51: 533*

Contestabile A 2008, Regulation of transcription factors by nitric oxide in neurons and in neural-derived tumor cells, *Progress in Neurobiology 84: 317*

Condorelli DF, Dell'Albani P, Amico C, Lukasiuk K, Kaczmarek L, Giuffrida-Stella AM. 1994, Glutamate receptor-driven activation of transcription factors in primary neuronal cultures , *Neurochemical Research 19: 489*

Day M, Wokosin D, Plotkin JL, Tian X, Surmeier DJ 2008, Differential excitability and modulation of striatal medium spiny neurons dendrites , *J. Neurosci 25: 11603*

Ding JB, Guzman JN, Peterson JD, Goldberg JA, Surmeier DJ 2010, Thalamic gating of corticostriatal signaling by cholinergic interneurons, Neuron 67; 175

Dragunow M, Robertson GS, Faull RL, Robertson HA, Jansen K 1990, D2 Dopamine receptor antagonists induce c-Fos and related proteins in rat striatal neurons, *Neurosciences 37: 287*

Durchdewald M, Angel P, Hess J 2009, The transcription factor Fos: a Janus-type regulator in health and disease, *Histology and Histopathology 24: 1451*

Felder CC, 1995 Muscarinic acetylcholine receptors: signal transduction through multiple effectors, *The FASEB Journal 9: 619*

Fienberg AA, Hiroi N, Mermelstein PG, Song W, Snyder GL, Nishi A, Cheramy A, O'Callaghan JP, Miller DB, Cole DG, Corbett R, Haile CN, Cooper DC, Onn SP, Grace AA, Ouimet CC, White FJ, Hyman SE, Surmeier DJ, Girault J, Nestler EJ, Greengard P, 1998 DARPP-32: regulator of the efficacy of dopaminergic neurotransmission , *Science 281: 838*

Flores-Barrera E, Vizcarra-Chac0n BJ, Tapia D, Bargas J, Galarraga E, 2010, Different corticostriatal integration in spiny projection neurons from direct and indirect pathway, *Frontiers in systems Neuroscience 4: 15*

Galinanes GL et al, 2009 Dopamine-dependent periadolescent maturation of corticostriatal functional connectivity in mouse, *J Neurosci 29: 2496*

Gerfen CR, Miyachi S, Paletzki R, Brown P, 2002 D1 dopamine receptor super sensitivity in the dopamine-depleted striatum results from a switch in the regulation of ERK1/2/MAP kinase, *Journal of Neuroscience 22: 5042*

Graybiel AM, Moratalla R, Robertson HA, 1990 Amphetamine and cocaine induce drug-specific activation of the c-fos gene in striosome-matrix compartments and limbic subdivisions of the striatum , *Pro Natl Acad Sci USA 87: 6912*

Grimes CA, Jope R.S 1999 Cholinergic Stimulation of Early Growth Response-1 DNA Binding Activity Requires Protein Kinase C and Mitogen- Activated Protein Kinase Kinase Activation and Is Inhibited by Sodium Valproate in SH-SY5Y Cells, *Journal of Neurochemistry 73: 1384*

Gross NB, Marshall JF, 2009 Striatal dopamine and glutamate receptors modulate methamphetamine-induced cortical Fos expression, *Neuroscience 161: 1114*

Hemmings HC Jr, Greengard P, Tung HY, Cohen P, 1984 DARPP-32, a dopamine-regulated phosphoprotein, is a potent inhibitor of protein phosphatase-1, *Nature 310: 503*

Ibáñez-Sandoval O, Tecuapetla F, Unal B, Shah F, Koós T, Tepper JM 2010 Electrophysiological and morphological characteristics and synaptic connectivity of tyrosine hydroxylase-expressing neurons in adult mouse striatum., *The Journal of Neuroscience, 30: 6999*

Ivkovic S, Ehrlich ME, 1999 Expression of the Striatal DARPP-32/ARPP-21 Phenotype in GABAergic Neurons Requires Neurotrophins in Vivo and In Vitro, *The Journal of Neuroscience, 19: 5409*

Kano T, Suzuki Y, Shibuya M, Kiuchi K, Hagiwara M, 1995 Cocaine-induced CREB phosphorylation and c-FOS expression are supressed in Parkinsonim model mice, *Neuroreport 6: 2197*

Keefe KA, Gerfen CR, 1996 D1 dopamine receptor-mediated induction of zif268 and c-fos in the dopamine-depleted striatum: differential regulation and independence from NMDA receptors, The *Journal of Comparative Neurology 367: 165*

Kemel M-L, Desban M, Glowinski J, Gauchyh C. 1989 Distinct presynaptic control of dopamine release in striosomal and matrix areas of the cat caudate nucleus. *Proceedings of the Naional Academy of Sciences 86;900*

Knapska E, Kaczmarek L, 2004 A gene for neuronal plasticity in the mammalian brain: Zif268/Egr-1/NGFI-A/Krox-24/TIS8/ZENK? , *Progress in Neurobiology 74: 183*

Kreitzer AC 2009 Physiology and Pharmacology of striatal neurons, *Annual reviews in neuroscience, 32:127*

Lapper SR, Bolam JP1992 Input from the frontal cortex and parafascicular nucleus to cholinergic interneurons in the dorsal striatum of the rat, *Neuroscience 51: 533*

Lester D, Rogers T, Blaha C, 2010 Acetylcholine–Dopamine Interactions in the Pathophysiology and Treatment of CNS Disorders, *CNS Neuroscience & Therapeutics 16; 137*

Marchi M, Sanguineti P, Raiteri M 1990. Muscarinic receptors mediate direct inhibition of GABA release from rat striatal nerve terminals. ***Neuroscience Letter 116****; 347*

Marín O, Rubenstein JL, 2001 A long remarkable journey: tangential migration in the telencephalon, *Nature Review in Neuroscience 11: 780*

Meurers BH, Dziewczapolski G, Shi T, Bittner A, Kamme F, Shults CW, 2009
Dopamine depletion induces distinct compensatory gene expression changes in DARPP-32 signal transduction cascades of striatonigral and striatopallidal neurons, *The Journal of Neuroscience 29: 6828*

Morris G, Arkadir D, Nevet A, Vaadia E, Bergman H, 2004 Coincident but distinct messages of midbrain dopamine and striatal tonically active neurons, *Neurons 43: 133*

Nairn AC, Svenningsson P, Nishi A, Fisone G, Girault JA, Greengard P, 2004 The role of DARPP in the action of drug abuse *Neuropharmacology 47: 14*

Nguyen TV, Kosofsky BE, Birnbaum R, Cohen BM, Hyman SE, 1992 Differential expression of c-fos and zif268 in rat striatum after haloperidol, clozapine, and amphetamine, *Proceedings of the National Academy of Sciences 89: 4270*

Nishi A, Bibb JA, Snyder GL, Higashi H, Nairn AC, Greengard P 2000 Amplification of dopaminergic signaling by positive feedback loop, *Proceedings of the National Academy of Sciences 97: 12840*

Otto F, Görtz P, Fleischer W, Siebler M, 2003 Cryopreserved rat cortical cells develop functional neuronal networks on microelectrode arrays, *The Journal of Neuroscience Methods 128:173*

Ouimet CC, Langley-Gullion KC, Greengard P, 1998, Quantitative immunocytochemistry of DARPP-32-expressing neurons in the rat caudatoputamen , *Brain Research 808: 8*

Pollack AE, Thomas LI , 2010 D1 priming enhances both D1-and D2-mediated rotational behavior and striatal FOS epxression in 6-hydroxydopamine lesioned rats, *Pharmacology, Biochemistry and Behaviour 94: 346*

Potter SM, DeMarse TB, 2001 A new approach to neural cell culture for long-term studies, *The Journal of Neuroscience Methods 110: 17*

Qi Z, Miller GW, Voit EO, 2010 The internal state of medium spiny neurons varies in response to different inpu signals, *BMC systeme biology 17: 26*

Randall F, Garcia-Munoz M, Staines W and arbuthnott G 2009 The corticostriatal system in dissociated cell culture, *Jounal of physiology 15 C12*

Robertson LM, Kerppola TK, Vendrell M, Luk D, Smeyne RJ, Bocchiaro C, Morgan JI, Curran T, 1995 Regulation of c-fos expression in transgenic mice requires multiple interdependent transcription control elements, *Neurons 14: 241*

Salin P, López IP, Kachidian P, Barroso-Chinea P, Rico AJ, Gómez-Bautista V, Coulon P, Kerkerian-Le Goff L, Lanciego JL, 2009 Changes to interneuron-driven striatal microcircuits in a rat model of Parkinson's disease, *Neurobiology of Disease 34: 545*

Schock SC, Jolin-Dahel KS, Schock PC, Staines WA, Garcia-Munoz M, Arbuthnott GW, 2010 Striatal interneurons in dissociated cell cultures, *Histochemistry and Cell Biology, 134: 1*

Segal M and Greenberger V, 2003 Formation of dendritic spines in cultured striatal neurons depends on excitatory afferent activity, *European Jounal of Neuroscience 17; 2573*

Shen W, Tian X, Day M, Ulrich S, Tkatch T, Nathanson NM, Surmeier DJ, 2007 Cholinergic modulation of Kir2 channels selectively elevates dendritic excitalility in striatopallidal neurons, *Nature Neuroscience 10, 1458*

Shen W, Hamilton SE, Nathanson NM, Surmeier DJ, 2005 Cholinergic suppression of KCNQ channel currents enhances excitability of striatal medium spiny neurons, *The Journal of Neuroscience 25: 7449*

Smith Y, Bevan MD, Shink E, Bolam JP, 1998 Microcircuitry of the direct and indirect pathway of the basal ganglia, *Neuroscience 86: 353*

Sugita S, Uchimura N, Jiang ZG, North *RA*, 1991, Distinct muscarinic receptors inhibit release of GABA and excitatory amino acids in mammalian brain, *Proc. Natl. Acad. Sci. U. S. A.* 88; 2608

Sullivan MA, Chen H, Morikawa H, 2008 Recurrent inhibitory network among striatal cholinergic interneurons, *The Journal of Neuroscience 28: 8682*

Svenningsson P, Fredholm BB, Bloch B, Le Moine C, 2000 Co-stimulation of D(1)/D(5) and D(2) dopamine receptors leads to an increase in c-fos messenger RNA in cholinergic interneurons and a redistribution of c-fos messenger RNA in striatal projection neurons, *Neurocience 98: 749*

Tepper JM, Bolam JP, 2004 Functional diversity and specificity of neostriatal interneurons, *Current Opinion in Neurobiology 14: 685*

Usui H, Inoue R, Tanabe O, Nishito Y, Shimizu M, Hayashi H, Kagamiyama H, Takeda M, 1998 Activation of protein phosphatase A2 by cAMP-dependent protein kinase catalysed of the 74kDa B regulatory subunit in vitro and identification of phosphorylation sites, *FEBS letters 430, 312*

Vale S 2008, Current management of cognitive dysfunction in Parkinson's disease: how far have we come? *Experimental Biology and Medecine 233: 941*

Valjent E, Bertran-Gonzalez J, Hervé D, Fisone G, Girault JA, 2009 Looking BAC at striatal signaling: cell-specific analysis in new transgenic mice, *Trends in Neuroscience 32: 538*

van Vulpen E, van der Kooy D , 1998 Striatal cholinergic interneurons: birthdates predict compartmental localization, *Developmental Brain Research 109: 51*

Vilaro´MT, Palacios JM, Mengod G, 1990 Localization of m5 muscarinic receptor mRNA in rat brain examined by in situ hybridization histochemistry. *Neuroscience Letters 114;154*

Walaas SI, Aswad DW, Greengard P, 1983 A dopamine and cyclic-AMP-regulated phosphoprotein enriched in dopamine innervated brain regions *Nature 301: 69*

Zachariou V, Sgambato-Faure V, Sasaki T, Svenningsson P, Berton O, Fienberg AA, Nairn AC, Greengard P, Nestler EJ, 2006 Phosphorylation of DARPP-32 at Threonine-34 is required for cocaine action., *Neuropsychopharmacology 31: 555*

Zhang W, Yamanda M, Gomeza J, Basile AS, Wess J. 2002 Multiple muscarinic acetylcholine receptor subtypes modulate striatal dopamine release, as studied with M1–M5 muscarinic receptor knock-out mice. *Journal of Neuroscience 22;634*

Zhou F, Wilson C, Dani JA, 2002 Cholinergic interneuron characteristics and nicotinic properties in the striatum. *Journal of Neurobiology 53;590*