Control of striatal activity by neuronal glutamate transporters

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CONTROL OF STRIATAL ACTIVITY
BY NEURONAL GLUTAMATE TRANSPORTERS

by

Modhurika De

A Thesis
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Abstract

The basal ganglia are subcortical nuclei that control the execution of learnt motor behaviors and emotions. The striatum, the main output nucleus of the basal ganglia, receives glutamatergic inputs from the cortex and thalamus and sends GABAergic inputs to the sub-thalamic nucleus via the axonal projections of D1- and D2-receptor (D1R and D2R) expressing medium spiny neurons (D1- and D2-MSNs, respectively). The dorsolateral striatum (DLS) is mainly involved in movement execution, whereas the ventromedial striatum (VMS) regulates emotions. Excitatory inputs onto D1- and D2-MSNs are mediated by post-synaptic ionotropic (GluA, GluN) and metabotropic glutamate receptors (mGluR1). The diffusion distance of glutamate from its release sites, is important to regulate the time course of excitatory transmission and depends on the local expression of glutamate transporters. Although the vast majority of glutamate transporters are localized in astrocytes, neuronal glutamate transporters are the closest to active release sites. In the hippocampus, the neuronal glutamate transporter EAAC1, limits glutamate spillover and inter-synaptic cross-talk. The Excitatory Amino Acid Carrier 1 (EAAC1) is localized in the peri-synaptic environment of excitatory synapses in striatal MSNs. In humans, non-functional polymorphisms in the gene encoding EAAC1 are associated with Obsessive Compulsive Disorder (OCD). What remains unknown are the molecular mechanisms by which EAAC1 regulates excitatory synaptic transmission onto striatal MSNs.

Here we address this question using behavioral and molecular approaches. Previous data shows that, in mice, EAAC1 expression limits grooming and the expression of anxiety-like behaviors, both of which are controlled by the DLS. Previous work shows that at the cellular level, EAAC1 limits mGluR1 activation and, by doing so, it promotes D1R expression with no effect on D2R expression. These effects are important because they ensure expression of long-term plasticity in the DLS. The results of this thesis show that cell-
specific activation of signaling cascades coupled to mGluRI in the DLS recapitulates the molecular and behavioral phenotype of mice devoid of EAAC1, even in the presence of this transporter.

The results of previous experiments identify EAAC1 as a key regulator of fast synaptic transmission and long-term plasticity in the DLS. Like glial glutamate transporters, EAAC1 prevents extra-synaptic receptor activation. However, the identity of the extra-synaptic receptors protected by EAAC1 varies across brain regions. In the hippocampus, these are primarily GluN receptors, abundantly expressed extra-synaptically. In the DLS, the expression of GluN receptors is significantly lower than in the hippocampus, but mGluRIs are abundant. These are the receptors protected by EAAC1. Preventing mGluRI activation promotes D1R expression. Although we identify one of the intracellular signaling molecules mediating this regulatory effect, others might also be involved. The expression of D2Rs, in contrast, is not affected by EAAC1. This suggests the existence of different intracellular mechanisms controlling dopaminergic signaling in D1- and D2-MSNs. Overall, our data identify EAAC1 as a powerful molecular switch capable of regulating both glutamatergic and dopaminergic transmission in the DLS and this, in turn may have profound implications for the coordinated execution of learnt motor behaviors.
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Modhurika De
1. Introduction

1.1 Glutamate transporters and synaptic transmission: an overview

The function of the brain depends upon synaptic function and synaptic transmission. Fast synaptic transmission mainly comes in two flavors: excitatory transmission and inhibitory transmission. These two forms of transmission differ for the neurotransmitters mediating them. Excitatory synaptic transmission is mediated, among others, by the neurotransmitter glutamate. Inhibitory synaptic transmission is mediated by the neurotransmitter GABA. Glutamate is a major excitatory neurotransmitter in the central nervous system (CNS). Upon its release, glutamate interacts with post-synaptic glutamate receptors (GluRs) and in part, diffuses out into the extracellular space. Due to its potency as an excitatory neurotransmitter, in very high concentrations, glutamate is toxic to cells. In other words, over-excitation due to a build-up of glutamate in the extracellular space is capable of causing cell death. In order to regulate the extracellular concentration of glutamate, glutamate transporters have to continuously remove it from the extra-synaptic space. Glutamate-aspartate transporter (GLAST) and glutamate transporter 1 (GLT-1), which are expressed on the surface of astrocytes, have the highest abundance among the population of glutamate transporters (Zhou & Danbolt, 2014). Most of the glutamate that diffuses into the extracellular space is taken up by GLAST and GLT-1. Apart from astrocytes, neurons also express glutamate transporters. The neuronal glutamate transporter Excitatory Amino Acid Carrier 1 (EAAC1), which is the main focus of this thesis, makes up a small fraction of all the glutamate transporters in the brain. Compared to GLAST and GLT-1, EAAC1 has limited distribution over the different brain regions. According to the study by (Holmseth et al., 2012) the highest levels of EAAC1 are expressed in the young adult rat hippocampus (roughly 130 molecules per μm$^3$), but these levels are still 100 times lower than GLT-1. However, the brain region where EAAC1 is most abundantly expressed is, in fact, the striatum
EAAC1 is also abundantly expressed in the kidney, placenta and heart, but its role in these organs is unclear (Danbolt, 2001).

Quantitatively, GLAST and GLT1 have a greater contribution to removing glutamate from the extracellular space, compared to EAAC1. Among glutamate transporters, EAAC1 makes up the smallest population and is expressed on the surface of neurons. This low expression is the reason why EAAC1’s role in glutamate transport has long been debated. (Holmseth et al., 2012) have shown that there is no appreciable impairment in glutamate uptake (measured as the total concentration of extracellular glutamate) in the absence of EAAC1. In agreement with these findings, knockout studies in mice have shown that the loss of GLAST and GLT-1 impact the levels of extracellular glutamate more than the absence of EAAC1 (Rothstein et al., 1996). (Peghini et al., 1997) have shown EAAC1-deficient mice only have a very mild phenotype. Altogether, these results suggest that EAAC1 has a negligible contribution to glutamate uptake compared to its astrocytic counterparts.

Alternative roles for EAAC1 have been suggested, such as regulating the distribution of glutamate in the extracellular space. This is done either by modulating the ratio of synaptic and extra-synaptic glutamate, or by altering the activation of NMDA receptors (NMDARs) leading to functional abnormalities (Pittenger et al., 2011). At CA1 synapses onto hippocampal pyramidal cells, EAAC1 buffers synaptically-released glutamate and prolongs its lifetime in the extracellular space (Scimemi et al, 2009). EAAC1 also limits NMDA receptor (NMDAR) activation at extracellular localizations by binding glutamate and preventing its spillover (Diamond, 2001; Scimemi et al, 2009). By regulating the activation of NMDARs, EAAC1 controls the expression of NMDAR-activation-dependent forms of long-term plasticity (Scimemi et al, 2009). The following sections will describe the structure and function of these glutamate receptors (NMDARs, AMPARs and KARs) and transporters (GLAST, GLT-1 and EAAC1) in more detail.
The activity of EAAC1 is influenced by its location. Immunogold electron microscopy shows that EAAC1 localizes peri-synaptically (i.e. at the periphery of the post-synaptic neuron) at excitatory synapses (He et al., 2000). This puts EAAC1 in an advantageous position when glutamate is released from the pre-synaptic terminal. This means EAAC1 can bind glutamate diffusing out of and away from the synaptic cleft, before it reaches astrocytic transporters or other extra-synaptic receptors. In this way, EAAC1 prevents glutamate spillover (Diamond, 2001).

Despite its low abundance of expression, EAAC1 has been associated with the onset of the symptoms of OCD (Porton et al., 2013). OCD is a neuropsychiatric disorder characterized by the repeated execution of stereotyped movements. This disorder involves persistent, intrusive thoughts (obsessions), and the need to alleviate anxiety caused by such thoughts through a ritualistic repetition of these stereotyped movements (compulsions; Ting & Feng, 2008; Jenike, 2004). The anxiety from these obsessions is due to a patient’s awareness that these actions are irrational and/or excessive. OCD affects approximately 1-3% of the world’s population (Ting & Feng, 2008). The cortico-striatal-thalamo-cortical pathway (CSTC pathway; the circuit of information exchange between the cortex, striatum and thalamus) receives serotonergic innervation from the brainstem raphe nuclei, making serotonin a likely therapeutic target. Therefore, current treatments for OCD target mainly the serotonin system. Selective Serotonin Reuptake Inhibitors (SSRIs) block the reuptake of serotonin by blocking the pre-synaptic serotonin transporter (5-HTT), therefore increasing the concentration of serotonin in the brain (Ting, & Feng, 2008). However, SSRRI therapy is only effective in approximately 50% of patients, suggesting that neurotransmitters other than serotonin might be involved in the onset of OCD. In addition to SSRIs, OCD patients are often treated with cognitive-behavioral therapy called Exposure and Response Prevention. Here, patients keep exposing themselves to anxiety-inducing stimuli that induce obsessions (for example, exposure to germs), followed by prevention of the compulsions that would otherwise be acted upon in response (for example, hand washing). This repeated exposure is intended to build up resistance towards performing compulsive
actions that alleviate the anxiety caused by a patient’s obsessions (Abramowitz, 2006). However, almost 30% of all patients treated using this method do not respond well, or respond minimally, to treatment (Chakrabarty et al., 2005). Due this low rate of effectiveness among existing therapies for OCD, it is necessary to look into alternative molecules, cellular pathways and mechanisms that are implicated in the onset of the symptoms of this disorder.

The general etiology of OCD is largely unknown. Family-based linkage analysis has implicated loss of function polymorphisms in the gene \textit{Slc1a1}, which encodes the expression of EAAC1, in the onset of OCD symptoms (Porton et al., 2013). Functional Magnetic Resonance Imaging (fMRI) studies on patients with OCD have shown hyperactivity in the CSTC pathway of the brain. Specifically, glutamatergic synaptic dysfunction within the CSTC circuit has been implicated in the onset of OCD (Pittenger et al., 2011; Chakrabarty et al., 2005; Ting & Feng, 2008). This suggests that the glutamate transporter EAAC1 could be involved in the onset of OCD. Indirect evidence of \textit{Slc1a1} being involved in OCD comes from studies in transgenic mice, but there are genes other than \textit{Slc1a1} that have been implicated in the onset of OCD and its symptoms. These include \textit{Sapap3}, \textit{Grin2B} and \textit{Grik2}.

There is no conclusive information about how EAAC1 modulates synaptic transmission in the striatum. Here, we determine the molecular mechanisms employed by EAAC1 to control excitatory synaptic transmission in the striatum, with a combination of molecular and viral approaches using EAAC1\textsuperscript{−/−} and transgenic mice. In the following sections, I will provide a description of the cell composition, input and output connectivity of the striatum. I will additionally describe glutamatergic and dopaminergic synaptic transmission in the brain, along with the molecular and chemogenetic techniques we have used to manipulate them.
1.2 The Basal Ganglia

The basal ganglia are a group of subcortical nuclei involved in processing sensory information and in coordinating it with movement execution, cognition and emotions (Groenewegen, 2003). They include the globus pallidus (GP; Fig. 1A, right), nucleus accumbens (NAc; Fig. 1B, left), olfactory tubercle (OT; Fig. 1C, left) and the caudate putamen (CPu; Fig. 1A, middle).

The GP is responsible for regulating voluntary movements. In rodents, the GP external segment (GPe) is located caudomedially with respect to the striatum and it is separated from it by the internal capsule. The GP is composed of inhibitory GABAergic neurons which project to the thalamus and brainstem (Fig. 2; (Parent, Lévesque, & Parent, 1999)). The medial medullary lamina separates the GP internal segment (GPi) from the GPe (Kaphingst, Persky, & Lachance, 2010). GP neurons are GABAergic (Saunders, Huang, & Sabatini, 2016). Of these, neurons in the GPe are immuno-positive for enkephalin. Neurons in the GPi are immuno-positive for substance P. The GPe receives GABAergic inputs from the striatum and sends GABAergic outputs to the subthalamic nucleus (STN). The GPi receives excitatory glutamatergic afferents from the STN and inhibitory GABAergic afferents from the striatum. The GPi sends GABAergic projections onto the thalamus (Fig. 2).

The NAc (Fig. 1B, left) is responsible for processing reward information. This structure consists of a core (the inner region) and a shell (the outer region). About 95% of all neurons in the NAc are GABAergic MSNs which express either D1R or D2R. Cholinergic aspiny neurons make up roughly 1-2% of the cell population in the NAc (Robison & Nestler, 2011). The NAc receives glutamatergic afferents from the hippocampus, prefrontal cortex and amygdala. The NAc sends GABAergic efferents to the hypothalamus, substantia nigra (SN) and pallidum (Fig. 2; (Salgado & Kaplitt, 2015)).

The OT (Fig. 1D) is responsible for processing sensory information and regulating responses to odor information and locomotor behavior (Gadizola et al., 2015; Wesson & Wilson, 2011). It is located in the
basal forebrain, rostrally with respect to the optic chiasm (Xiong & Wesson, 2016). The OT is composed of cholinergic neurons resembling striatal MSNs, granule cells and a population of cells known as crescent cells (Millhouse & Heimer, 1984). The OT receives direct inputs from the olfactory bulb and the cortex. The OT also receives inputs from brain regions such as the thalamus, hypothalamus, hippocampus and amygdala, making it a highly interconnected brain region. The OT densely innervates the NAc and the striatum with cholinergic projections (Fig. 2).

The striatum (Fig. 1A, left), known as the CPu in rodents, controls motor behaviors and cognitive functions (Grahn, Parkinson, & Owen, 2009). It is a heterogeneous structure that will be discussed in more detail in Chapter 1.3.

The SN controls reward-seeking behaviors, motor planning and eye movement (Nicola, Surmeier, & Malenka, 2000). The SN lies ventral to the thalamus and is composed of two regions: the substantia nigra pars compacta (SNc; Fig. 1C, right) and the substantia nigra pars reticulata (SNr; Fig. 1C, middle). Excitatory inputs to the SNr arrive from the STN (Gerfen & Wilson, 1996). The SNc contains dopaminergic neurons that project to the striatum. The SNr receives GABAergic inputs from the striatum and GPe. The neurons in the SNr are GABAergic and send inhibitory outputs to the thalamus and brainstem (Fig. 2).

The STN (Fig. 1B, left) controls action selection. It is located rostral to the SN. Neurons in the STN are glutamatergic and project to the GPe (Rafols & Clement, 1976). The STN receives glutamatergic inputs from the cerebral cortex and thalamus, GABAergic inputs from the GPe and dopaminergic inputs from the SNc (Lanciego, Luquin, & Obeso, 2012). The STN provides glutamatergic inputs to the GPi (Fig. 2).
1.3 The striatum

The striatum is the largest nucleus of the basal ganglia, located deep within each cerebral hemisphere. In rodents, the striatum consists of two major components: the CPu and the GP. The CPu comprises the neostriatum, a phylogenetically new component of the brain. The GP comprises the paleostriatum, a more ancient component of the brain. In rodents, the CPu occupies the DLS, whereas the NAc and the OT occupy the VMS. The striatum is implicated in the control and expression of a variety of behaviors. The DLS controls the expression of habitual actions (Hilario, Holloway, Jin & Costa, 2012; Gremel & Costa, 2014). The VMS mediates reward cognition, motivational salience and reinforcement.

The striatum is composed of two distinct compartments: striosomes (also known as patches) and matrix (Fig. 3; Graybiel & Ragsdale, 1978)). Striosomes make up 10-20% of the entire striatal volume, the rest being formed by the matrix. Striosomes and matrix differ from one another in their expression of specific protein markers, embryonic development and connectivity (Graybiel & Ragsdale, 1978; Lopez-Huerta et al., 2016; Pert, Kuhar, & Snyder, 1976). Striosomes express µ-opioid receptors (MORs; a class of opioid receptors with a high affinity for enkephalin) and acetylcholinesterase (AChE; the degrading enzyme for acetylcholine). The matrix expresses enkephalin, the ligand of MORs present in striosomes (Koshimizu et al., 2008; Lopez-Huerta et al., 2016) and the calcium binding protein, calbindin (Fig. 3).

Striosomes receive excitatory inputs from pyramidal neurons in layers Vb and VI of the limbic cortex, and the matrix receives excitatory inputs from layer V pyramidal neurons in the sensorimotor cortex (Donoghue & Herkenham, 1986). GABAergic MSNs in striosomes project to dopaminergic neurons in the SNr. In contrast, GABAergic MSNs in the matrix project to the SNr, GPe and GPi (Fig. 4; Banghart et al., 2015)). The mesostrial system is composed of dopamine-sensitive neurons that project from the midbrain to the striatum. These dopaminergic neurons project to the striosomes and matrix in a biochemically distinct manner. For instance, dopaminergic neurons projecting to the matrix express
calbindin. This calbindin expression is not detected in the dopaminergic neurons projecting to the striosome (Gerfen, Baimbridge, & Thibault, 1987). In addition to these differences, electrophysiological recordings in mice have shown that there is no striatal connectivity between the striosomes and the matrix (Lopez-Huerta et al., 2016). This is because the dendrites and axon collaterals of MSNs remain restricted within the striatal compartment where they are located despite having very dense arborization throughout the striatum (Penny, Wilson, & Kitai, 1988; Lanciego et al., 2012).

### 1.3.1 Cell composition of the striatum.

The striatum is composed of GABAergic MSNs, cholinergic interneurons (ACh INs) and GABAergic interneurons (GABA INs). GABAergic MSNs represent more than 95% of all neurons in the striatum (Tepper, Koos, & Wilson, 2004). There are two classes of MSNs in the striatum, which can be distinguished based on their expression of receptors, neuropeptides and their output connectivity. The first class of MSNs expresses D1Rs, adenosine A1 receptors, dynorphin and substance P and are commonly referred to as D1-MSNs. D1-MSNs project to the SNc, SNr and to the entopeduncular nucleus of the GPi. The projections to the GPi and SNr form the direct pathway (Fig. 5). The second class of MSNs expresses D2Rs, adenosine A1 and A2A receptors and enkephalin. D2-MSNs project to the GPe which, in turn, relays information to the SNr (Ferré et al., 1997; Gerfen, 2004; Quiroz et al., 2009). This pathway is commonly referred to as the indirect pathway (Fig. 5; Paxinos & Franklin, 2001).

GABA INs that are distinct from MSNs compose 3-4% of all striatal neurons. There are three main types of GABA INs that can be distinguished histochemically. One type co-expresses the peptides somatostatin, neuropeptide Y and the enzymes NADPH diaphorase and nitric oxide synthase (SOM INs). The other two types of GABA INs express either the calcium binding protein parvalbumin (PV INs) or the calcium binding protein calretinin (CR INs). SOM INs comprise 0.6% of the population of GABA INs. SOM INs provide dendritic GABAergic inhibition to MSNs. PV INs are the best characterized group of GABA INs and make
up 0.7% of the striatal GABA IN population. They are present in both striosomes and matrix (Cowan et al., 1990). PV INs receive cholinergic and dopaminergic inputs from the cortex (Kubota et al., 1988) and GABAergic inputs from MSNs and other PV INs in the GPe (Bevan, Booth, Eaton, & Bolam, 1998; Ramanathan et al., 2002). PV INs receive excitatory inputs from the primary somatosensory cortex (SS1), primary motor cortex (M1) and the thalamus (Kita, 1993). PV INs provide axo-somatic GABAergic inhibition to MSNs (Tepper & Bolam, 2004; Tepper et al., 2010). CR INs make up approximately 0.5% of all GABA INs (Rymar et al., 2004) but are less well characterized from a functional standpoint, compared to the other types of GABA INs (Tepper & Bolam, 2004). CR INs were first discovered in the rat striatum (Jacobowitz & Winsky, 1991; Resibois and Rogers, 1992), and later studied in mice to characterize their morphological features, topographical distribution and relative density in the striatum (Petryszyn, Beaulieu, Parent, & Parent, 2014).

ACh INs represent 1-2% of all striatal neurons. These INs are characterized by a larger soma (>15 μm) compared the other INs. They are aspiny and have unique electrophysiological properties. For instance, they are tonically active, unlike phasically active MSNs (Zhou, Wilson & Dani, 2002). This means they fire tonic action potentials with frequencies of around 3 Hz to 10 Hz (Wilson et al., 1990; O.Lim, Kang & McGehee, 2014). They possess depolarized resting membrane potentials of -60 mV and very high input resistances of around 200 MQ (Calabresi et al., 1998). Despite their low abundance, ACh INs possess extremely dense arborizations, especially in the striatum (Zhou, Wilson & Dani, 2002). This is why the striatum has the most abundant expression of ACh in the brain. ACh INs project onto dendritic spine necks in the striatum (Bolam et al., 1984).

1.3.2 Long-range inputs to the striatum.

Long-range inputs to the striatum originate in the cortex, thalamus and the brain stem and terminate either on MSNs or INs (Fig. 2). Cortical inputs to the striatum express the vesicular glutamate transporter
isoform 1 (VGLUT1). These inputs mainly arise from layer V and layer III pyramidal neurons. Cortico-striatal inputs control goal-directed behaviors, emotions and motivation. The area of the cortex most closely involved with these functions is the frontal cortex. The frontal cortex can further be divided into: (i) medial prefrontal cortex (mPFC), (ii) dorsal PFC (dPFC), (iii) premotor areas and (iv) M1. The mPFC is involved in controlling emotions and motivation, the dPFC controls higher cognitive functions, the premotor areas are involved in motor panning and the M1 controls the execution of these plans. Cortico-striatal glutamatergic inputs target both D1- and D2-MSNs, but these inputs originate from different populations of pyramidal cells in the cortex. D1-MSNs are preferentially targeted by ipsi- and contralateral intratelencephalic (IT) pyramidal neurons. D2-MSNs are targeted by ipsilateral pyramidal tract (PT) neurons (Lei, 2004).

Cortico-striatal projections target dendritic spine heads of MSNs. The DLS receives glutamatergic projections from these cortical areas along with projections from the parietal cortex associated with somatosensory function. Labelling studies from the 1980’s suggested that cortico-striatal inputs are almost exclusively glutamatergic. These projections also inhibit the activity of striatal neurons. One of the possible mechanisms would be cortical stimulus activating inhibitory cells within the striatum. Recently, a separate class of inhibitory inputs from cortical SOM-INs to the DLS was identified (Rock, Zurita, Wilson, & Apicella, 2016). These inhibitory inputs are part of feed-forward or feed-back circuits (Tepper, Wilson, & Koos, 2008). The feed-forward circuit is formed by inhibitory inputs from the cortex onto GABA-INs, while the feed-back circuit is formed by axon collaterals from MSNs onto neighboring MSNs (Calabresi et al., 1991; Roma & Raimondo, 1991). Given that the striatum receives both excitatory and inhibitory inputs from the cortex and INs respectively, the sequence and/or timing of these inputs relative to one another determines the net effect on striatal neurons and activity. There is a transient increase in the activity of both direct and indirect pathway neurons prior to action initiation, but not during phases of inactivity (Cui et al., 2013). Experiments using retrograde tracers showed that the majority of long-range inputs onto
both direct and indirect pathway neurons (61.1% of total inputs to direct pathway neurons and 69.6% of total inputs onto indirect pathway neurons) originate from the SS1, MC and limbic cortex (Wall, DeLaParra, Callaway, & Kreitzer, 2013). Despite corticostriatal inputs reaching both the direct and indirect pathway neurons, a higher proportion of somatosensory and limbic innervation from the cortex preferentially targets the direct pathway neurons (Wall, DeLaParra, Callaway, & Kreitzer, 2013; Reiner, & Deng, 2017).

Thalamic inputs to the striatum express the vesicular glutamate transporter isoform 2 (VGLUT2; (Lanciego, Luquin, & Obeso, 2012)). The thalamostriatal system mainly originates in the midline and intralaminar nuclei (Dube, Smith, & Bolam, 1988). Two other thalamic nuclei involved in providing glutamatergic inputs to the striatum include the central lateral and parafascicular nuclei. Thalamostriatal projections target both MSNs and INs within the striatum. These projections preferentially target the dendritic shafts of these neurons (Lanciego et al., 2012; Smith, Raju, Pare, & Sidibe, 2004), although later findings suggest a more complex organization where thalamostriatal afferents from the parafascicular nuclei target dendritic shafts but projections from the central lateral nuclei prefer spine heads (Raju et al., 2006). The thalamus provides 22% of total inputs onto direct pathway neurons and 25.5% of total inputs onto indirect pathway neurons in the striatum (Wall et al., 2013). There is a bias in the innervation of thalamo-striatal inputs, with a higher proportion of these inputs targeting indirect pathway neurons compared to direct pathway neurons. This differential innervation is a potential mechanism to suppress competing actions in the overall sequence of action selection.

Inputs from the SNc and ventral tegmental area (VTA) to the striatum are GABAergic and dopaminergic, terminating onto both dendritic spines and shafts of MSNs. Brainstem inputs control behaviors linked to learning and motor control. The dopaminergic system, made up of inputs from the SNc and VTA, has been implicated in reward-based learning, addiction and plasticity (Nestler & Malenka, 2004). D1R signaling enhances excitability in striatonigral MSNs (i.e. MSNs projecting from the striatum onto the SN) and D2R
signaling exerts an inhibitory effect on striatopallidal MSNs (i.e. MSNs projecting from the striatum onto the GP; Nicola, Surmeier, & Malenka, 2000; Surmeier, Ding, Day, Wang, & Shen, 2007). These findings stem from the classical model of dopaminergic modulation of striatal activity which suggests that D1Rs excite direct striatonigral pathway MSNs and D2Rs inhibit indirect striatopallidal pathway MSNs (Fig. 5; Albin et al., 1989).

In addition to glutamatergic, GABAergic and dopaminergic inputs, the striatum also receives cholinergic inputs from the OT (Fig. 2), PPNc and DLT (Dautan et al., 2014; Silberberg & Bolam, 2015). The DLS is targeted by the PPNc whereas the VMS is targeted by the DLT. MOR staining has shown that, interestingly, cholinergic afferents from the PPNc seem to avoid projecting onto striosomes (Silberberg & Bolam, 2015).

### 1.3.3 Long-range outputs from the striatum.

The striatum sends long-range projections to two basal ganglia nuclei: the GP (both GPe and GPI) and the SNr (Fig. 5). D1-MSNs innervate the GPI and SNr. D2-MSNs innervate the GPe. Striatonigral MSNs abundantly express D1Rs. D1Rs are positively coupled to adenylyl cyclase through G proteins like Golf. Alterations in the activation of adenylyl cyclase leads to increases in cytosolic cAMP levels and the subsequent activation of protein kinase A (PKA). This is also accompanied by the phosphorylation of many intracellular targets such as the Dopamine- and cAMP-regulated phosphoprotein, MW 32 KDa (DARPP-32; Svenningsson et al., 2004). The D1-PKA cell-signaling cascade affects the function as well the trafficking of glutamate receptors on the cell membrane, such as AMPARs and NMDARs, which will be further discussed in the next chapter. Striato-pallidal MSNs abundantly express D2Rs. These D2Rs are coupled to G proteins such as G

Cell-signaling cascades coupled to these proteins leads to the inhibition of adenylyl cyclase through Ga subunits. There is a subsequent reduction in the opening of voltage-gated Ca\(^{2+}\) channels on the dendritic membrane at glutamatergic synapses (Surmeier, Ding, Day, Wang, & Shen, 2007). This generates the compound diacylglycerol (DAG) and activates protein kinase C (PKC). As a result,
inositol (1,4,5)-triphosphate (IP$_3$) is released and intracellular Ca$^{2+}$ stores are mobilized. Much like D1Rs, D2Rs also affect glutamate receptors in the DLS in that, activation of D2Rs leads to a reduction in the amplitude of AMPAR currents (Cepeda et al., 1993) and phosphorylates specific sites on AMPAR subunits. These effects will be elaborated on, in the following chapter. The activation of D2Rs reduces the amount of pre-synaptic glutamate that is released, and therefore also reduce the excitability of striato-pallidal neurons (Surmeier, Ding, Day, Wang, & Shen, 2007).

### 1.3.4 Local synaptic connections of the striatum.

In addition to projecting out from the striatum, MSNs also possess local axon collaterals. Given that striatal MSNs are GABAergic, these collaterals mediate inhibitory synaptic transmission. Such synaptic transmission is additionally aided by GABA-INs, which are known to make synaptic connections with MSNs, especially in the DLS (Czubayko & Plenz, 2002). The most prominent intra-striatal connections are the GABAergic synapses formed between PV-INs and MSNs (Koos & Tepper, 1999; Silberberg & Bolam, 2015). Each PV-IN contacts MSNs within a 100 µm radius of itself (Planert, Szydlowski, Hjorth, Grillner, & Silberberg, 2010). A single PV-IN is capable of targeting both D1- and D2-MSNs, but with a propensity for targeting D1-MSNs. PV-INs provide unidirectional feed-forward inhibition to MSNs. Some studies have shown that PV-INs provide inhibition onto their neighboring MSNs but selectively avoid certain other cell types such as ACh-INs (Szydlowski et al., 2013). ACh-INs also inhibit MSNs via di-synaptic pathways; one, by activating GABAergic inhibition in MSNs, and two, by directly activating neuropeptide Y-expressing INs that elicit GABAergic inhibition in MSNs (English et al., 2012).

Inhibitory synaptic transmission between striatal MSNs is also modulated by dopamine (i.e. individual connections between MSNs exhibit either D1R-mediated facilitation or D2R-mediated facilitation). Electrophysiology recordings show that synaptic transmission between MSNs are either facilitated by D1R activation or inhibited by the activation of D2Rs. At times, both D1Rs and D2Rs can regulate synaptic
transmission (Tecuapetla, Koos, Tepper, Kabbani, & Yeckel, 2009). This difference arises from a differential expression of D1- and D2Rs between MSNs, as discussed earlier. Therefore, dopamine’s ability to facilitate or inhibit synaptic transmission relies heavily on the whether the presynaptic neuron in question is a D1- or D2-MSN. The contribution of DRs to synaptic transmission will be discussed in detail in chapter 1.5.
1.4 Glutamate receptors

Glutamate is the main excitatory neurotransmitter in the mammalian central nervous system (CNS) and is involved in a range of functions such as learning, memory and synaptic plasticity (Willard & Koochekpour, 2013). Excitatory synaptic transmission depends on the release of glutamate from a presynaptic neuron and its detection by specific glutamate receptors on the postsynaptic neuron. There are two main classes of glutamate receptors: ionotopic (iGluRs) and metabotropic glutamate receptors (mGluRs) (Hollman & Heinemann, 1994). These two classes differ mainly for their mechanism of action. A detailed description of these classes is provided below.

1.4.1 Ionotropic glutamate receptors.

iGluRs are ligand-gated ion channels composed of four conserved subunits (Fig. 6), each with an extracellular amino terminal domain (NTD), the ligand binding domain (LBD), a transmembrane domain (TMD) and an intracellular carboxy terminal domain (CTD; Willard & Koochekpour, 2013). The NTD is implicated in helping the receptor subunits assemble, receptor trafficking and its localization (Sobolevsky, Rosconi, & Gouaux, 2009). The ligand binding site is formed by the NTD and the extracellular loop between the third and fourth transmembrane domains (Fig. 6, 7). An AMPAR has four glutamate binding sites (Mayer, 2005). When two glutamate molecules are bound, the extracellular loops shift and the pore opens. Further binding of more molecules of glutamate opens the channel further and increases the channel conductance and subsequently the current flowing through it (Armstrong, Jasti, Beich-Frandsen, & Gouaux, 2006). The LBD, as its name suggests, binds glutamate or other agonists. This leads to conformational changes in the TMD which opens the pore in the channel, allowing the influx of Na⁺, K⁺ and Ca²⁺ ions. This in turn, causes specific downstream effects within the cell. iGluRs can be further classified as NMDARs, AMPARs and KARs, based on the agonists they bind, their subunit composition and kinetics.
AMPARs get their name from their specific agonist α-amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid. In hippocampal excitatory synapses, AMPARs localize at the post-synaptic density (PSD) opposite the pre-synaptic active zone in order to ensure fast and efficient synaptic transmission (Scheefhals & MacGilliavry, 2018). Studies in other brain regions such as the cortex have shown that AMPARs may also localize in the periphery of PSDs. This suggests that AMPAR localization varies (Bernard, Somogyi, & Bolam, 1997).

AMPARs are composed of four subunits: GluA1, GluA2, GluA3 and GluA4 (Fig. 6A), which are arranged as tetramers in the cell membrane (Shi et al., 1999; Song & Huganir, 2002). AMPARs can form heterotetramers, consisting of a symmetric dimer of dimers of GluA2 with either one of the three remaining subunits. The GluA2 subunit governs the permeability of an AMPAR to Ca²⁺, Na⁺ and K⁺ ions, through a pore enclosed by the NTD, LBD and TBD (Scheefhals & MacGilliavry, 2018).

AMPARs are responsible for mediating fast excitatory synaptic transmission (Dingledine et al., 1999; Platt, 2007). AMPARs are susceptible to rapid desensitization once open. Desensitization also depends on receptor occupancy, such that, even if a single subunit is occupied, an AMPAR can be desensitized. AMPARs are not all equally susceptible to desensitization, nor do they recover from it at the same rate (Robert & Howe, 2003).

There are two main forms of long-term plasticity: long-term potentiation (LTP) and long-term depression (LTD). In brain regions such as the hippocampus, LTP is expressed due to an increased recruitment of AMPARs to the membrane of the post-synaptic neuron. When glutamate binds to AMPARs, the channels are opened and the post-synaptic neuron is depolarized. This depolarization relieves the Mg²⁺ block on NMDARs and renders them conductive. NMDA receptors are permeable to Na⁺, K⁺ and Ca²⁺, so influx of Ca²⁺ via these conductive NMDA receptors upregulates the AMPA receptors being trafficked to the cell membrane and results in LTP (Maren, Tocco, Stanley, Baudry, & Thompson, 1993). In the
striatum in particular, LTP depends on the activation of NMDARs and D1- or D2Rs at glutamatergic synapses, resulting in an increase in post-synaptic responsiveness. LTD expression at striatal glutamatergic synapses, on the other hand, involves the activation of mGluRs and D2Rs (Lovinger, 2010).

Studies in perinatal rodents have shown that compared to mature neurons, immature neurons have a lower sensitivity to the AMPAR agonist kainate, a compound known to cause cell-death in mature neurons. A developmental increase in sensitivity to kainate can be explained by a corresponding increase in the expression of AMPARs (Manzini et al., 2007). This suggests that AMPAR activation is implicated in neurodegeneration and cell death. Antagonists such as 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo[f]quinoxaline (NBQX) have been extensively used to block AMPARs and counter glutamate toxicity in rodent models (Pitt, Werner, & Raine, 2000).

NMDARs are so called since they selectively bind the molecule N-methyl-D-aspartate. They preferentially localize at the center of the PSD although they may also be found in extra-synaptic locations (Hardingham & Bading, 2003; Scheefals & MacGillavry, 2018). These receptors are co-activated by the binding of two molecules- glutamate and a co-agonist (either D-serine or glycine; Kleckner & Dingledine, 1988). The NMDAR is a tetramer and is composed of three subunits: NR1, NR2 and NR3 (Fig. 6, 7). Of these, the NR1 subunit has eight subtypes; NR2 has four (NR2A-NR2D) and NR3 has two (NR3A and NR3B). All the subunits share a common membrane topology with an extracellular NTD, a TMD of three segments, an extracellular loop between the TMD and an intracellular CTD (Fig. 6; Cull-Candy, Brickley, & Farrant, 2001; Dingledine, Borges, Bowie, & Traynelis, 1999).

Each of the domains comprising the NMDAR acts as a functional unit in itself and are arranged in three layers such that the NTD is at the ‘top’, the LBD in the ‘middle’ and the TMD at the ‘bottom’ (Lee & Gouaux, 2011; Lee at l., 2014). The extracellular NTD is composed of two globular structures- one is modulatory and the other is an LBD (also known as the S1S2 domain). NR1 subunits can bind the co-agonist glycine
while NR2 subunits can bind glutamate (Fig. 7C, 8). The agonist-binding module is linked to the TMD. The cytoplasmic CTD is composed of residues that can be modified by protein kinases and phosphatases along with structural and scaffolding proteins. These are responsible for conformational changes in the receptor, affecting its capacity to bind its ligands. The NR2B subunit imparts basic structure and function to the NMDAR in that it is the site of glutamate binding and the Mg$^{2+}$ block.

A feature which distinguishes NMDARs from AMPARs is that in order to be activated, the receptor has to bind glycine and glutamate, and overcome a Mg$^{2+}$ block. Despite being primarily ligand-gated, the flow of current through NMDARs is voltage dependent. When agonists first bind to an NMDAR, extracellular Mg$^{2+}$ ions bind to specific sites within the receptor (Fig. 8) and block the passage of other ions through the pore. When the cell is depolarized, the Mg$^{2+}$ block is released, following which Na$^+$ ions and Ca$^{2+}$ ions flow into the cell and K$^+$ ions flow out of the cell. At this stage, the NMDAR is active and conducting.

The (hyper or hypo) activation of NMDARs can affect neuronal activity. This is because NMDARs are involved in the regulation of activity-dependent changes in synaptic strength underlying learning and memory formation (Bliss & Collingridge, 1993). NMDAR-dependent Ca$^{2+}$ influx is required for the synthesis of genes controlled by transcription factors such as cAMP-response-element-binding-protein (CREB; Hardingham & Bading, 2003). This indicates that NMDARs are required for cell survival and these receptors have a neuroprotective role in the CNS. Conversely, NMDARs are also implicated in neurodegeneration and cell-death if chronically activated (Lipton & Rosenberg, 1994). In such cases, blocking NMDARs using antagonists such as 2-amino-5-phosphonopentanoic acid (APV), can help prevent neurodegeneration and the corresponding symptoms. Therefore, neuronal health and proper functioning relies on the well-balanced activity of NMDARs.

Kainate receptors (KARs) are named after their selective agonist kainate, which also activates AMPARs. These receptors localize both pre- and post-synaptically in brain regions such as the hippocampus,
amygdala, cerebral cortex and striatum. Their role in synaptic transmission differs based on their localization. There are studies that explore the location-dependent activity of KARs in more detail (Chittajallu et al., 1999). However, these roles are not relevant for the purpose of my research. Therefore, my descriptions will be limited to a broad overview of the receptor’s basic functions.

Structurally, KARs have five subunits (Fig. 7): GluK1, GluK2, GluK3, GluK4 and GluK5 (previously known as GluK5, GluK6, GluK7, KA1 and KA2 respectively; Lerma & Marques, 2013). These subunits, like those of AMPARs and NMDARs, form tetramers (Collingridge et al., 2010; Dingledine et al., 1999). While GluK1, GluK2 and GluK3 can exist either as homomers or heteromers, GluK4 and GluK5 can only form functional units in combination with one of the GluK1-3 subunits.

Each of the KAR subunits is composed of a 400-residue extracellular NTD. This is followed by the first half of the neurotransmitter-binding cleft, S1. The next segment is a TMD, also known as M1. A part of the following segment, M2, is within the membrane. The rest of it juts out into the cytoplasm. This segment is therefore known as the ‘p loop’. The segment M3, is another TMD, which emerges on the extracellular face of the cell membrane and forms the remainder of the neurotransmitter binding site, S2. The last segment, M4, begins on the extracellular face of the membrane and crosses through to the cytoplasmic face to form the CTD (Fig. 6).

KARs form ion channels which are mainly permeable to Na\(^+\) and K\(^+\) ions, with very slight permeability to Ca\(^{2+}\) ions (Huettner, 2003). KARs also have a second mode of signaling mediated by G proteins, especially at GABAergic hippocampal CA1 synapses, therefore giving them characteristics of mGluRs as well as iGluRs (Bhangoo & Swanson, 2013). These receptors have three main functions in the brain: (i) they mediate post-synaptic depolarization and conduct synaptic current, (ii) they can modulate the release of glutamate and GABA, and (iii) they are involved in the maturation of neural circuits during development (Lerma &
KARs are desensitized by high concentrations of glutamate. These receptors can be blocked pharmacologically by NBQX, a drug that is also used as an AMPAR antagonist.

1.4.2. Metabotropic glutamate receptors.

mGluRs are distinguished from their ionotropic counterparts by their area of localization on the postsynaptic terminal and their mode and mechanism of activation. Unlike iGluRs, which are expressed in the core of the PSD, mGluRs may be found in the peri-synaptic domain (defined as an annular ring of 100-200 nm surrounding the PSD; Scheefhals & Macgillavry, 2018). Briefly, G-proteins are composed of a heterotrimeric complex with three subunits: α, β and γ (Fig. 8). When the α-subunit binds the compound guanosine 5'-diphosphate (GDP), the G-protein is inactive. Exchanging GDP with its phosphorylated form guanosine 5'-triphosphate (GTP) breaks the heterotrimer of the three subunits and activates the G-protein. Conversely, when GTP is hydrolyzed back to GDP, the heterotrimer reforms and G-proteins are inactivated (Fig. 8). Therefore, mGluRs are not channels, but rather activate specific cell-signaling cascades when bound to their ligands. This in turn modifies other proteins and leads to changes in pre- and postsynaptic transmission (Bonsi et al., 2005; Platt, 2007).

In terms of structure, mGluRs consist of an extracellular, bi-lobed N-terminal domain or NTD. Due to the unique crystal structure of this domain, the NTD is also referred to as the Venus Flytrap domain (VFD) and it acts as the glutamate binding site (Niswender & Conn, 2010). There is a cysteine-rich domain (CRD) which plays an important role in dimerization and activation of the receptors. The CRD is composed of nine cysteine residues, of which eight are linked by disulfide bridges. The signals generated by ligands binding to the VFD are transmitted via the CRD. This domain is followed by the classic seven pass α-helical TMD and finally there is an intracellular CTD (Fig. 6; Platt, 2007). The CTD is subject to phosphorylation
and alternative splicing, allowing it to control G-protein coupling (Niswender & Conn, 2010; Rondard & Pin, 2015).

There are eight types of mGluRs and three main groups: Group I (mGluR1 and 5), Group II (mGluR2 and 3) and Group III (mGluR4, 6, 7 and 8) based on agonist-binding pharmacology and the specific second messenger system activated upon glutamate binding. The amino acid sequence homology within each group is >70% but between groups only about 45% (Willard & Koochekpour, 2013). For the purpose of my work, I have described Group I mGluRs in detail and provided a brief overview of the other two groups below.

Group I mGluRs (mGluRIs) are G\text{q}\text{-coupled receptors that localize post-synaptically, on the extra-synaptic portions of the dendritic membrane of hippocampal pyramidal cells and principal cells (Lujan et al., 1996). Both mGluR1 and mGluR5 bind the agonist (S)-3,5-dihydroxyphenylglycine ((S)-3,5-DHPG). When stimulated, mGluRIs cause the enzyme phospholipase C (PLC) to hydrolyze phosphoenositide phospholipids in the plasma membrane of the cell (Bonsi et al., 2005; Endoh, 2004). As a result, IP\text{3} and DAG are formed. IP\text{3} then travels to the endoplasmic reticulum and induces the opening of intracellular Ca\textsuperscript{2+} channels, increasing the cytosolic Ca\textsuperscript{2+} concentrations. DAG remains localized to the cell membrane and is a co-factor for PKC activation. Depending upon whether they are located pre-synaptically or post-synaptically, mGluRs can be either excitatory (enabling more glutamate to be released from the pre-synaptic terminal) or inhibitory (preventing glutamate release and modulating voltage-dependent Ca\textsuperscript{2+} channels; Endoh, 2004).

mRNA levels of mGluR1 are either stable or increase with age (Casabona et al., 1997) but mGluR5 mRNA levels in the cerebellum and hypothalamus decreases during postnatal development. mGluR1 has a higher
expression in the neonatal hippocampus, hypothalamus and olfactory bulb and mGluR5 has a higher expression in all brain regions in P9 rats, compared to adult mice (Casabona et al., 1997). In the striatum, the region of the brain we studied, mGluRIs are expressed post-synaptically, on the extra-synaptic regions of MSNs and INs (Paquet & Smith, 2003).

mGluRIs have a limited probability of activation due to their extra-synaptic localization (Paquet & Smith, 2003). Glutamate released from a single synaptic vesicle binds to post-synaptic receptors when it is released from a pre-synaptic neuron. Some residual glutamate also diffuses out of the synaptic cleft where both mGluRIs and peri-synaptic transporters such as EAAC1, compete to bind it. Here, glutamate transporters have a positional advantage over mGluRIs, being the first to encounter the residual glutamate such that mGluRIs rarely activate during single-release events (Scheefhals & Macgillavry, 2018a). In the striatum, mGluRIs are involved in the expression of long-term plasticity and have neuroprotective roles. When mGluRIs are pharmacologically blocked in hippocampal CA1 neurons, LTD expression is abolished (Volk, Daly, & Huber, 2006). mGluRI-dependent LTD induction is linked to the activation of the intracellular second messenger PKC and Ca\(^{2+}\) being released from intracellular stores via the activation of IP\(_3\). However, mGluRIs alone are not responsible for the expression of LTD. Post-synaptic interactions between mGluRIs and DRs are also necessary for LTD induction (Paquet & Smith, 2003). Common signaling pathways and direct protein-protein interactions may be involved in the joint control of LTD expression by mGluRIs and DRs. The neuroprotective actions of mGluRIs have been evidenced by studies that report DHPG (a mGluRI agonist) as an effective inhibitor of glutamate-induced neuronal death (Nagasawa et al., 2004).

Group II mGluRs (mGluRIIs) are found on both pre- and post-synaptic membranes. Their role is to inhibit neurotransmitter release at glutamatergic, GABAergic or cholinergic synapses. The first group of selective
mGluRII agonists to be identified included (2S,2/R,3/R)-2-(2/R,3/R)-Dicarboxycyclopropyl) glycine (DCG-IV) and (2R,4R)-4-Aminopyrrolidine-2,4-dicarboxylate (2R,4R)-ADPC. Group III mGluRs (mGluRIIIs) have a predominantly pre-synaptic localization. Of its subtypes, mGluR4 is abundantly expressed in the cerebellum, with lower levels of expression in the basal ganglia, OT and hippocampus. mGluR6 has the least abundant expression of all the mGluRIIIs and is mainly found on retinal bipolar cells (Nakajima et al., 1993). mGluR7 is widely distributed throughout the brain. This receptor is localized in the synaptic active zone and exhibits a very low affinity for glutamate (Shigemoto et al., 1997). This subtype only activates when extracellular glutamate levels are extremely high. mGluR8 is also widely distributed throughout the brain although its expression levels are lower than that of mGluR4 and mGluR7. Both mGluRII (mGluR2 and 3) and III (mGluR4, 6, 7 and 8) are coupled to G_{i/o} proteins that inhibit the formation of adenyl cyclase, which forms cAMP from ATP (Bonsi et al., 2005). This leads to the subsequent activation of K^+ channels and the inhibition of Ca^{2+} channels.
1.5 Dopamine receptors

Dopamine (DA) or 3,4-dihydroxyphenethylamine is a neurotransmitter in the mammalian CNS. A DA molecule has a catechol structure with one benzene ring and two hydroxyl side groups. It has an amine group attached via an ethyl chain. DA has the simplest structure among the members of the catecholamine family such as epinephrine and norepinephrine. Its direct precursor L-DOPA is derived from the amino acids phenyl alanine and tyrosine. Tyrosine is modified by the enzyme tyrosine hydroxylase, producing DOPA. The enzyme DOPA decarboxylase then removes carbon dioxide from DOPA to form DA. In the striatum, DA is involved in the direct and indirect pathways, as mentioned in chapter 1.3 (Gerfen & Surmeier, 2011; Gerfen & Wilson, 1996). DA is also implicated in the expression of reward related behavior, motor control, motivation and reinforcement (Mink, 1996).

DA in itself is neither excitatory nor inhibitory, and its action depends upon the type of receptor present on the target cells. These DA receptors (DRs) function as metabotropic G protein-coupled receptors (GPCRs), so a second messenger complex is involved in signaling. They use two main signal transduction pathways for cell signaling: the phosphatidylinositol (PI) pathway and the cyclic AMP (cAMP) pathway. Two main subtypes of DA receptors have been identified in mammals: D1 and D2. D1-like receptors include D1 (D1R) and D5 (D5R) receptors and D2-like receptors include D2 (D2R), D3 (D3R) and D4 (D4R) receptors (Gerfen & Surmeier, 2011). Both families of subtypes share sequence homology (Fig. 9; Jaber et al., 1996) with an extracellular NTD, three intracellular loops (IL) and an intracellular CTD.

The D1-like family of receptors consists of D1Rs and D5Rs, which are Gs-coupled receptors and share a high sequence homology of 78% (Jaber et al., 1996). The activation of these receptors leads to an increase in intracellular cAMP levels via the activation of adenylate cyclase (Araki et al., 2007; Tiberi & Caron, 1994). D5Rs constitutively activate cAMP in response to agonist (Jaber et al., 1996). D1R activation has also been coupled to changes in intracellular Ca²⁺ concentrations. Activation of D1-like receptors can cause a net
excitation or inhibition via mechanisms such as the opening of Na\(^+\) and K\(^+\) channels respectively. The D2-like family of receptors is composed of D2Rs, D3Rs and D4Rs. These are coupled to the inhibitory G proteins G\(_i\) and G\(_o\) and block the activity of the compound adenylyl cyclase. The first DR cDNA to successfully be cloned was that of the D2R. Activation of D2-like receptors can cause a net inhibition of the target neurons.

In a study conducted by (Thibault et al., 2013) in BAC transgenic mice, it was shown that in vivo, at embryonic day 18 (E18), at birth (P0) and at postnatal day 14 (P14), D1R and D2R reporter genes had only a 10% colocalization. There was also a gradual decrease in the percentage of colocalization to under 5% from E18 to P0 and P14. This segregation was seen to be maintained in vitro, independent of the identity of neuronal populations interacting with MSNs (mesencephalic and/or cortical neurons). Of the different receptor subtypes, D1R and D2R mRNA was most abundant in all the regions of the mature brain (Araki et al., 2007). D1Rs were more densely expressed than the D2R in the frontal and cingulate cortices. The reverse was true in the striatum and GP. In the embryonic forebrain, D3-, D4- and D5- receptors were predominant. Only D3R and D4R showed developmental regulation in the striatum and cortex. All five receptor-mRNA are expressed in the striatum (from E12 to P60). Although D1R and D2R mRNAs were seen to be most abundantly expressed at all ages examined, D2R expression was overall more abundant than D1R expression.
1.6 Glutamate uptake

Glutamate uptake from the extracellular environment is mediated by Na⁺-dependent transport systems, expressed in the tissue surrounding release sites. At excitatory synapses, transporters regulate the reuptake of glutamate. After uptake, glutamate can re-accumulate at the synaptic cleft either via release from pre-synaptic terminals or via recycling once it is taken up by transporters (Fig. 11). The activity of glutamate transporters therefore influences the dynamics of excitatory synaptic transmission, as mediated by glutamate. Glutamate is not only an important neurotransmitter in the CNS, but is also excitotoxic at high concentrations. The build-up of glutamate in the extracellular space is known to cause cell death due to overstimulation (excitotoxicity). Glutamate transporters are responsible for the uptake and transport of glutamate across the cell membrane, reducing the extracellular concentrations of the neurotransmitter and preventing excitotoxicity. Glutamate transporters are also involved in the glutamate-glutamine cycle (Fig. 12) which restores released glutamate into synaptic vesicles (Rauen, Tanui, & Grewer, 2014). To elaborate briefly, after glutamate is released into the synaptic cleft, it either binds to receptors on the post-synaptic membrane or taken up by glutamate transporters on the surface of astrocytes. Astrocytes convert glutamate to glutamine using the enzyme glutamine synthetase. Once glutamine is released from the astrocyte, it is taken up by the neuron and converted back into glutamate for release, and the cycle starts over.

Yernool et al. (2004) crystallized a glutamate transporter homologue from *Pyrococcus horikoshii* (GltPh) sharing 37% sequence similarity with human Excitatory Amino Acid Transporter 2 (EAAT2; Fig. 13; Yernool et al., 2004). GltPh was crystallized in an outward facing configuration as well as an inward facing configuration. This led to the ‘alternating access hypothesis’ according to which glutamate transporters possess an intermembrane glutamate binding site with a gate on either side. As a result of this conformation, glutamate can ‘alternatively access’ either the intracellular or extracellular solution
Glutamate transport is associated with the co-transport of glutamate with three Na$^+$ ions and one proton, along with the counter-transport of one K$^+$ ion (Zerangue & Kavanaugh, 1996). Based on the location of glutamate transporters, glutamate uptake can occur either at the glial level or the neuronal level via EAATs. I will be limiting my descriptions to EAATs since the activity of vesicular glutamate transporters (VGLUTs) is not relevant to this work.

In the striatum, EAATs are responsible for removing glutamate from the synaptic cleft and its reuptake into glial cells and neurons. Following uptake by EAATs, glutamate is converted into glutamine and carried back into the presynaptic neuron. Here, it is once again converted into glutamate via a glutamate-glutamine cycle and taken up into synaptic vesicles by VGLUTs (Shigeri et al., 1994). Apart from glutamate, acidic molecules such as aspartate and serine are also substrates for EAATs. Neutral amino acids such as serine and alanine, however, have a very low binding affinity ($K_m$, a parameter which determines the extent to which the transporters bind glutamate during the process of uptake) of around 1 mM, compared to a $K_m$ of 45 µM for glutamate (Koch et al., 1999; Watts et al., 2014). The specificity and high affinity for binding acidic substrates comes from the presence of a positively charged arginine residue (R447) which is conserved across all EAATs, and the lack of neutral threonine residues in these transporters.

There are five subtypes of EAATs: EAAT1-5 (encoded by the genes Slc1a3, Slc1a2, Slc1a1, Slc1a6 and Slc1a7 respectively). Within the EAAT subtypes, glial glutamate transport is mediated by EAAT1 and EAAT2. Neuronal glutamate transport is mediated mainly by EAAT3, and also by EAAT4 and EAAT5. In this chapter, I will be focusing mainly on the glial glutamate uptake by EAAT1 and EAAT2 and neuronal glutamate uptake by EAAT3, since the activity of EAAT4 and EAAT5 are beyond the scope of my research.

EAAT1 is also known as GLAST and is encoded by the Slc1a3 gene. It is mainly expressed in the plasma membrane (Lehre et al., 1995). In vivo, GLAST is a homotrimer and mediates the transport of glutamate and aspartic acid along with the co-transport of three Na$^+$ and one H$^+$. One K$^+$ ion is counter-transported.
EAAT2 is also known as GLT-1 and is encoded by the \textit{Slc1a2} gene. It is responsible for 90% of the glutamate uptake in the CNS (Holmseth et al., 2009; Zhou & Danbolt, 2014) in the adult brain. GLT-1 is a member of the solute carrier protein family and is the principal transporter that clears glutamate from the extracellular space in the CNS. GLT-1 is also expressed in neurons at early stages of development (Rauen et al., 2014). Approximately 10% of GLT-1 molecules are expressed in axon terminals, despite it being a predominantly astrocytic transporter (Zhou & Danbolt, 2014). Mutations in GLT-1 expression have been linked to the onset of amyotrophic lateral sclerosis (ALS), and upregulation of GLT-1 has been implicated in schizophrenia (Bellesi & Conti, 2010). Impairment of glutamate transport by GLT-1 has also been implicated in the onset of traumatic brain injury, Alzheimer’s disease and stroke. GLT-1 is distinguishable from the other members of its family on the basis of its high sensitivity to Kainic acid (KA) and Dihydrokainic acid (DHK). In voltage clamp, most inhibitors act as substrates for GLT-1, except KA and DHK, which specifically block GLT-1-mediated glutamate transport competitively.

This work primarily deals with EAAT3 (known as EAAC1 in rodents) which is encoded by the \textit{Slc1a1} gene. EAAC1 is expressed in both glutamatergic and GABAergic neurons, making it a uniquely neuronal transporter. Compared to GLAST and GLT-1, EAAC1 has a very low abundance of expression (Holmseth et al., 2012). Due to its low abundance the role of EAAC1 in transporting glutamate has been heavily debated. The loss of EAAC1 has not been found to have a major effect on the extracellular concentrations of glutamate (Holmseth et al., 2012). This suggests that in the absence of EAAC1; GLAST, and GLT-1 in particular (shown to be approximately 100 times more abundant than EAAC1 in the brain; Holmseth et al., 2012) are able to regulate extracellular glutamate concentrations. On the other hand, owing to its peri-
synaptic localization on the cell membrane (He et al., 2000) EAAC1 is able to bind glutamate readily upon its release.

EAAC1 is also a known transporter of L-Cysteine and D-Aspartate. The ability of EAAC1 to transport cysteine allows neurons to obtain cysteine for glutathione synthesis. Glutathione is a molecule required to counteract neurotoxicity and oxidative stress. Overall, EAAC1 has a 10-fold higher affinity for cysteine compared to all other EAATs. Cysteine transport is required to maintain intracellular redox potential (Aoyama & Nakaki, 2013), making this role of EAAC1 especially significant.

The role of EAAC1 varies between brain regions. In the hippocampus, EAAC1 regulates synaptic transmission by altering the activation of NMDARs (Scimemi et al., 2009). However, electrophysiology experiments in the DLS show that EAAC1 operates via a different mechanism in this brain region (Bellini et al., 2018). In other words, in the absence of EAAC1, blocking NMDARs with their agonist APV does not affect the activation of this group of receptors (Bellini et al., 2018). Instead, EAAC1 limits mGluRI activation in the striatum (Bellini et al., 2018).

Despite its low abundance in the brain, EAAC1 has been implicated in the onset of the symptoms of OCD. Three alternate isoforms of Slc1a1 have been identified that negatively modulate the function of EAAC1, inhibiting it from transporting glutamate (Porton et al., 2013). There is not much information regarding the molecular mechanism via which EAAC1 alters excitatory synaptic transmission in the striatum. Not only does the striatum express EAAC1 abundantly, but it is also hyperactive in patients of OCD (Burguiere et al., 2015). Therefore, there is likely a connection between the role of EAAC1 specifically in the striatum and the onset of the symptoms of OCD. I will elaborate on this connection further in a later section, when I introduce my research question for this thesis.
1.7 The Cre-Lox recombination system

Cre-Lox recombination is a technology used to carry out deletions, insertions, translocations and inversions in a site-specific manner within DNA (Sauer, 1987; Sauer & Henderson, 1988) and is particularly useful in studying the neural circuitry of the brain. The system consists of an enzyme Cre-recombinase (a site-specific DNA recombinase), which recombines a pair of target sequences known as LoxP sequences. Both the enzyme and the Lox site are derived from the bacteriophage PI. Depending on where the LoxP sites are placed, genes can be modulated (activated, repressed or exchanged with other genes). It is possible to activate the Cre-recombinase enzyme in a specific cell type via certain external stimuli such as chemicals or heat shock.

The protein Cre-recombinase consists of four subunits and two domains: a smaller NTD and a larger CTD (the catalytic site of the enzyme). The entire protein has 343 amino acids. The LoxP (locus of X over P1) site is made up of 34 base pairs including an asymmetric eight base pair sequence between two sets of symmetric and palindromic 13 base pair sequences. It is this 13-base-pair sequence which gives the LoxP sites their directionality. When both LoxP sites are in the same direction, the floxed site (sequence flanked by two LoxP sites) is excised, but if both LoxP sites are in an opposite orientation, the floxed site is inverted. When LoxP sites exist on two separate strands of DNA, the action of Cre on these sites leads to translocation of the DNA fragments (Fig. 14).

When cells that have LoxP sites in their genome also express Cre-recombinase, a recombination event may occur between the LoxP sites. Cre-recombinase targets the first and last 13 base pairs of one of the Lox sites to form a dimer, which then binds to the dimer on another lox site and forms a tetramer. Since lox sites are directional, the two sites joined by the tetramer have a parallel orientation. Cre-recombinase excises the DNA at both loxP sites and DNA ligase re-joins them. The directionality of LoxP sites determines the outcome of the recombination. For two LoxP sites situated on the same chromosome, inverted LoxP
sites will cause an inversion, direct repeats of the LoxP site will cause deletions. If the LoxP sites are on different chromosomes, Cre recombination may cause translocation.

For our experiments, we used mice that expressed Cre-recombinase under the control of either a D1R or an adenosine receptor 2 promoter, the latter being specific to D2Rs (Bellini et al., 2018). These mice will be referred to as D1\textsuperscript{Cre/+} and A2A\textsuperscript{Cre/+}, respectively. These transgenic mice were used to selectively target and activate Gq signaling cascades similar to the ones coupled to mGluRIs, in either D1-MSNs (in D1\textsuperscript{Cre/+} mice) or D1-MSNs (in A2A\textsuperscript{Cre/+} mice). Both groups of mice were crossed with a mouse line expressing a red fluorescent protein (RFP) reporter gene Ai9, such that when mice from the subsequent litters expressed Cre-recombinase under the control of D1R or A2A promoter (D1\textsuperscript{Cre/+};Ai9\textsuperscript{Tg/Tg} and A2A\textsuperscript{Cre/+};Ai9\textsuperscript{Tg/Tg} respectively) specifically D1- or D2-MSNs would express the RFP. Such mice were generated on both a WT and EAAC1\textsuperscript{−/−} background and used as per the experimental requirements.
**1.8 Chemogenetic approaches for cell-specific control of signaling pathways**

GPCRs are a highly versatile group of receptors which regulate an assortment of cellular responses including homeostasis and endocrine signaling. They recognize a large variety of ligands such as neurotransmitters, lipids, hormones, odorants and even photons (Zhu & Roth, 2014), making them important therapeutic targets. Our ability to assess their function was for long limited to the use of transgenic mice and/or *in vivo* pharmacological studies. GPCRs are expressed in various types of tissue (Regard et al., 2008) and the drugs used in pharmacological studies are non-specific. This non-specificity allows drugs to act on GPCRs in all tissue types in which they are expressed. As a result, the interpretation of studies using these drugs, is confounded. In other words, using the above approaches, there is no way to activate GPCR-coupled signaling cascades in a cell-specific manner.

GPCRs have been modified to favor binding a synthetic substrate over a natural one, allowing us to limit the activation of these receptors to only when the synthetic substrate is supplied. They used a molecular evolution approach to create a family of muscarinic receptors which are activated by a pharmacologically inert compound Clozapine-N-Oxide (CNO) but not by their native ligand ACh (Armbruster et al., 2007; Gomez et al., 2017). These engineered receptors were called Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) and were shown to have little to no constitutive activity *in vivo* (Zhu & Roth, 2014). Since then, DREADDs have been used in a wide variety of neuroscientific applications such as the manipulation of cellular activity and the investigation of disease mechanisms. For example, the receptor hM3Dq, used in our experiments, is coupled to $G_q$ signaling cascades and induces neuron firing. On the other hand, hM4Di receptors are coupled to $G_i$ signaling cascades and mediate synaptic silencing.

Despite DREADDs being widely used in the above-mentioned biological applications, the mechanism underlying their activation via CNO was only described very recently in 2017 (Gomez et al., 2017). CNO itself is an inert compound as it cannot cross the blood brain barrier. Therefore, when it is administered
via systemic drug injections, it has a low affinity for DREADDs. However, after injection, CNO converts into Clozapine in vivo, a form in which it is able to enter the brain and which shows a high affinity for DREADDs. Clozapine is a potent activator of DREADDs and has been shown to induce DREADD-related behavioral responses (Gomez et al., 2017). Converted Clozapine has been shown to reach its highest concentrations in the CSF around 2 to 3 hours after injection (Raper et al., 2017). For this reason, in our experiments we sacrificed mice 2 hours after they had received I/P CNO, in order to collect striatal tissue for Western Blots.

Overall, the use of DREADDs involves three main steps: (i) transgenic or virus-mediated expression of DREADDs in a specific cell-type, (ii) a gap of 2-3 weeks for transfection of viral constructs in case of viral transduction and (iii) administration of the designer drug CNO to activate the GPCR-coupled signaling cascades in a cell-specific manner (Fig. 15). For our experiments, we took advantage of the above timeline to activate Gq signaling pathways specifically in D1- or D2-MSNs. The rationale behind using this approach as well as the steps we followed are explained in more detail the following sections.
2. The question

EAAC1’s role in the brain has long been ambiguous owing to its low abundance compared to GLAST and GLT-1 (Holmseth et al., 2012). Despite this, as mentioned in chapter 1.6, loss-of-function polymorphisms in \textit{Slc1a1}, have been implicated in the onset of the symptoms of OCD. Experiments in EAAC1\(^{-/-}\) mice have shown that in the absence of EAAC1, grooming behavior is altered. Not only do EAAC1\(^{-/-}\) mice have a significantly higher frequency of grooming, but these mice also tend to spend a larger proportion of their total grooming time, grooming their paws compulsively (Bellini et al., 2018). This behavior is reminiscent of ritualistic hand-washing exhibited by human OCD patients. Since the striatum is responsible for controlling the expression of grooming behavior in mice (Kalueff et al., 2016), this compulsive behavior of EAAC1\(^{-/-}\) mice is an indication that EAAC1 alters striatal function.

Extracellular field recordings in the DLS show that EAAC1 limits the activation of mGluRIs in this brain region (Bellini et al., 2018). As mentioned in chapter 1.4.2, mGluRIs are responsible for controlling the expression of long-term plasticity in the striatum. In fact, in the absence of EAAC1, the loss of LTD in the striatum can be explained by EAAC1’s ability to alter mGluRI activation (Bellini et al., 2018). However, LTP is also abolished in the striatum in the absence of EAAC1 (Bellini et al., 2018). This outcome is puzzling since LTP expression depends on the activation of NMDARs, and EAAC1 does not alter the activation of these receptors in the striatum (Bellini et al., 2018). Apart from NMDAR activation, the expression of striatal LTP also depends on the expression of D1Rs (Calabresi et al., 1992; Partridge, Tang, & Lovenberg, 2000; Luscher & Malenka, 2012). In the absence of EAAC1, D1R expression is significantly reduced (Bellini et al., 2018). But what is the relationship between EAAC1’s control of mGluRI activation and D1R expression? DARPP-32 is a known integrator of glutamatergic and dopaminergic neurotransmission (Svenningson et al., 2004). This molecule is involved in EAAC1 limiting mGluRI activation and facilitating D1R expression. Although the total expression level of DARPP-32 does not vary significantly in the absence
of EAAC1, the activity of DARPP-32 depends not only on its total expression levels, but also on the phosphorylation of four specific sites on the molecule. These sites are: Threonine 34 (T34), Threonine 75 (T75), Serine 97 (S97) and Serine 130 (S130). There is a significant increase in phosphorylation at pDARPP-32S130 (Bellini et al., 2018), a site which happens to be activated by mGluRI-coupled Gq signaling cascades in the striatum (Liu et al., 2001). These findings suggest that mGluRI-coupled Gq-signaling cascades control the expression of long-term plasticity, D1R expression and pDARPP-32S130 phosphorylation in the striatum. Therefore, EAAC1 shapes excitatory synaptic transmission in this brain region via its control of mGluRI activation. These results are further corroborated by the fact that when mGluRIs are pharmacologically blocked using specific antagonists, long-term plasticity, D1R expression and phosphorylation levels at pDARPP-32S130 in the striatum are rescued (Bellini et al., 2018). However, the caveat at this point is an unforeseen alteration of phosphorylation levels at pDARPP-32T75, an outcome not evident in earlier experiments (Bellini et al., 2018). When brain slices are treated with mGluRI antagonists, there is no specificity as to which region of the brain the receptor is blocked in. In other words, mGluRIs could be blocked not just in the striatum, but also in a region such as the cortex. This could be causing alterations in the phosphorylation level of pDARPP-32T75.

To address this issue, it is necessary to be able to manipulate Gq-signaling cascades only in specific cells in the striatum. How can this cell-specific activation of Gq-signaling cascades be achieved? For my research, I have answered this very question using chemogenetics, particularly DREADDs. In the following sections, I will explain how we used a combination of DREADDs and transgenic mice to activate Gq-signaling cascades specifically in D1- and D2-MSNs, along with the molecular and behavioral implications of this approach.
3. Materials and methods

3.1 Ethics statement

The protocols followed for all the experiments conducted in this thesis have been approved by the Institutional Animal Care and Use Committee (IACUC), at the University at Albany, SUNY. The protocols are in accordance with the guidelines specified in the ‘Guide for the Care and Use of Laboratory Animals’ from the National Institute of Health (NIH).

3.2 Mice

The mice used for our experiments were housed in a 12-hour light/12-hour dark cycle. Food and water were provided to them ad libitum. C57BL/6 (WT) mice and EAAC1$^{-/-}$ mice were used for our research. The EAAC1$^{-/-}$ mice used in these experiments were obtained using the method detailed by Peghini et al., 1997. A pgk Neomycin cassette was inserted into exon1 of the Slc1a1 gene to disrupt it. From there, EAAC1$^{-/-}$ breeder mice were obtained according to the methods of Scimemi et al., 2009. Transgenic mice were also used for our experiments. D1$^{Cre/+}$ mice (MMRRC Cat# 030778-UCD; STOCK Tg(Drd1-cre)EY217Gsat/Mmucd) and A2A$^{Cre/+}$ mice (MMRRC Cat# 036158 B6.FVB(Cg)-Tg(Adora2a-cre)KG139Gsat/Mmucd) were kindly provided by Dr A.V. Kravitz and Dr C.F. Gerfen from the NIH/NIDDK. Both these strains of mice express the protein Cre-recombinase under the control of either the D1R or adenosine receptor 2 (which colocalizes with the D2R) respectively. In addition to the Cre mice, a line of Ai9$^{Tg/T8}$ conditional reporter mice (The Jackson Laboratory Cat# 007909; B6.Cg-Gt(Rosa)26Sortm9(CAG-tdTomato)Hze)) were used, provided by Dr. P. E. Forni at SUNY Albany. The strain of D1$^{tdTomato}$ mice used for our research were purchased from the Jackson Laboratory (Cat# 016204; Tg(Drd1a-tdTomato)6Calak)).
3.3 Behavioral tests

Grooming in mice is a behavior controlled by the DLS (Barnes et al., 2005). Therefore, analysis of grooming behavior in EAAC1\(^{-/-}\) and WT mice, gives us an idea of how EAAC1 affects the function of the DLS. For instance, obsessive grooming in mice is reminiscent of the ritualistic hand-washing behavior exhibited by human patients of OCD. Grooming videos were acquired in a behavioral arena sectioned into four chambers that had a clear bottom and side walls (L 15 cm x W 25 cm x H 15 cm). A DSLR camera (Canon EOS Rebel T3i with EF-S 18-55 mm f/3.5-5.6 IS lens, 60 fps) was positioned 6 to 12” under the chamber to record 10 min long videos monitoring the grooming behavior of each mouse. Further analysis of the videos was carried out by watching each 10 min acquisition and taking note of two main factors for each mouse: (i) the start and end times of each grooming episode (as described by Kalueff et al., 2007), from which the inter-event interval and grooming frequency was calculated, and (ii) the total duration of grooming.

3.4 Genotyping

Routine genotyping was performed on all the mice before they were used in any experiments. The mice were genotyped between the ages of P7 and P10, with tissue taken from their toes. The toes from young mice were digested overnight in a digestion buffer (Bellini et al., 2018) at 55°C overnight with constant shaking. A NanoDrop Microvolume Spectrophotometer (ThermoFisher) was used to determine the DNA concentration (in ng/μl) from the digested tissue samples. Samples were only processed for further PCR analysis if their DNA concentration was ≥ 500 ng/μl. Samples with DNA concentrations higher than 500 ng/μl were diluted down to 500 ng/μl with nuclease-free water. The DNA extracts from our mice were then processed for PCR analysis by adding specific primers (Table 1; Bellini et al., 2018). All reactions except that for D1\(^{tdTomato}\) were performed using a standard Taq Polymerase (Cat# R2523, Millipore Sigma, St.Louis, MO). For D1\(^{tdTomato}\), a HotStart Taq polymerase was used (Cat# KK5621; KAPA Biosystems, Wilmington, MA). For each reaction, the pertinent PCR protocols were followed (Tables 2-5; Bellini et al.,
After PCR, samples were run on a 2% agarose gel (Cat# A9539, Millipore Sigma, St.Louis, MO) and gel was imaged in a UV transilluminator (c300, Azure Biosystems, Dublin, CA). Genotypes were determined by comparing the band size (in bps) with the expected band size for each gene (EAAC1\textsuperscript{+/+}: 358 bps, EAAC1\textsuperscript{−/−}: 680 bps, D1\textsuperscript{Cre/+}: 340 bps, A2A\textsuperscript{Cre/+}: 350 bps, Ai9\textsuperscript{Tg/Tg}: 196 bps, Ai9\textsuperscript{0/0}: 297 bps, D1\textsuperscript{tdTomato}: 750 bps).

### 3.5 Western Blot

Western Blot experiments were carried out on tissue samples collected from the striatum of male and female WT and EAAC1\textsuperscript{−/−} mice aged P21 to P36. The tissue samples were then processed to extract two fractions of proteins: the cytoplasmic fraction and the membrane fraction, using the MemPer Plus Membrane Protein Extraction Kit (Cat# 89842; Thermo Fisher Scientific, Waltham, MA), according to the manufacturer’s instructions. In order to prevent protein degradation due to the action of proteases, a Halt Protease Inhibitor Cocktail (10 μl/ml, Cat# 78441; Thermo Fisher Scientific, Waltham, MA) was added to the tissue samples during extraction. The cytoplasmic protein extracts were used to probe for mCherry and pDARPP-32 (T34, T75, S97 and S130) protein levels. The membrane protein extracts were used to probe for D1R and D2R protein levels. For each of the above mentioned proteins, their specific primary antibodies were used as follows: mCherry (Cat# 600-401-P16 Rockland Antibodies & Assays, Limerick, PA); rabbit antibodies against the different isoforms of pDARPP-32 (pDARPP-32T34, 1:500, Cat# 12438; Cell Signaling Technology, Danvers, MA), (pDARPP-32T75 and pDARPP-32S97, 1:1,000, Cat# 2301 and Cat# 3401 respectively; Cell Signaling Technology, Danvers, MA), (pDARPP-32S130, 1:500, Cat# p1025-137; PhosphoSolutions, Aurora, CO); rabbit anti DARPP-32 (1:1,000, Cat# 2306; Cell Signaling Technology, Danvers, MA); rabbit anti D1R and D2R (1:200, Cat# ADR-001 and ADR-002 respectively; Alomone Labs, Jerusalem, Israel). All protein levels were normalized against β-actin (1:1,000, Cat# 4970, Cell Signaling Technology, Danvers, MA). Protein concentrations were measured before probing, using the NanoDrop Microvolume Spectrophotometer (Thermo Fisher). Cytoplasmic proteins were run on a 12% acrylamide gel.
gel and membrane proteins were run on a 10% acrylamide gel. After the gels were run, they were transferred onto a PVDF membrane (Cat# P2563; Millipore Sigma, St. Louis, MO) using a semi-dry transfer apparatus (Cat# 1703940; Bio-Rad, Hercules, CA). The membrane was then incubated in blocking buffer (5% non-fat dry milk dissolved in TBS buffer with 0.1% Tween-20, pH-7.6) at RT for 2 hours. The blocked membrane was then washed in Tris-Buffered Saline (TBS, with 0.1% Tween-20) three times for five minutes each and incubated in the relevant primary and secondary antibodies as follows: membranes probed with anti-mCherry, anti-D1R, anti-D2R, anti-T34, anti-T75 and anti-S97 primary antibodies were incubated at 4°C overnight. Membranes probed with anti-S130 antibody were incubated at RT overnight (Bellini et al., 2018). After 3 washes with TBS-0.1% Tween 20 (5 min each), the membranes were incubated in secondary antibody (biotinylated horse anti-rabbit IgG, Cat# BA-1100; Vector Laboratories, Burlingame, CA) at RT for 2 hours. Once the membrane had been incubated in secondary antibody, it was washed 3 times with TBS-0.1% Tween 20 (5 min each) and then incubated in the Vectastain ABC kit solution (Cat# PK-6100; Vector Laboratories, Burlingame, CA; 1:1000 for T34 and 1:2000 for all other antibodies as per Bellini et al., 2018). The membranes were then developed using the Clarity Western ECL Kit (Cat# 170-5060; Bio-Rad, Hercules, CA). The membrane was then imaged using an UV transilluminator (ChemiDoc, Bio-Rad, Hercules, CA or c300, Azure Biosystems, Dublin, CA), to obtain 16-bit images for analysis. The images were converted to 8-bit images on Fiji (https://fiji.sc/). Three images were selected where there was no pixel saturation, and these were analyzed for the mean gray values (MGV) within a specified region of interest. The MGVs of the protein bands of interest were all normalized to their respective bands of β-actin.

3.6 Stereotaxic surgeries

Stereotaxic surgeries were performed on the DLS of WT, D1<sup>Cre/+</sup> and A2A<sup>Cre/+</sup> mice of either sex aged between P14 and P16. Before beginning the surgery, mice were placed under anesthesia, at the rate of 5% in 100% O<sub>2</sub> at 1-2 l/min for induction and 3% in 100% O<sub>2</sub> at 1-2 l/min for maintenance. To prepare the
stereotaxic frame (Neurostar, Tubingen, Germany) used for these surgeries it was wiped down with 70% ethanol and a heating pad (Hand Warmer) was fastened onto the stage where the mouse was to be placed. The heating pad was used regulate the animal’s body temperature during surgery. The anesthetized mouse’s head was shaved using Nair hair removal cream, and the mouse was then head-fixed onto the stereotaxic frame. The mouse’s snout was placed into the nose cone and ear bars were inserted into its ear canals. The shaved skin on its head was then sterilized using betadine and ethanol. After sterilization, Lidocaine was applied to the skin and allowed to rest for 2 minutes. An incision was made onto the skin and 100 nl of the virus (AAV-hSyn-DIO-hM3D(Gq)-mCherry (University of North Carolina, Chapel Hill, NC) was injected using a Hamilton syringe at the rate of 100 nl/min. The injections were unilateral, and aimed at the DLS (coordinates from bregma were: AP: -2.5 mm, ML: ±2.0 mm, DV: +5.0 mm; Bellini et al., 2018). Mice were allowed to recover from the surgery in their home cages for 7 days. After this period of rest, mice received daily I/P injections of 100 nl saline for 7 days, to accustom them to the sensation of receiving injections and ensuring that the final injection of CNO did not elicit a stress response in them. A total of 2 weeks after the surgery, the mice received an I/P injection of 5 mg/Kg CNO (Enzo Life Sciences, Farmingdale, NY) in NaCl 0.9%. One hour after the injection, the mice were taken to a behavioral arena. 10-minute videos of these mice were acquired for behavioral analysis. The mice were then sacrificed and the injected striatum was collected for Western Blots. The non-injected striatum and the cortex were also collected as control tissue samples to perform Western Blot experiments on.

3.7 Data Analysis

All the data shown in this thesis is represented as mean ± S.E.M, unless otherwise stated. Student’s paired or unpaired t-test was used to determine the statistical significance of the data on Microsoft Office Excel. p<0.05 (*p<0.05; **p<0.01; ***p<0.001) was considered significant.
4. Result

4.1 Cell-specific activation of signaling pathways coupled to mGluR1 in D1-MSNs of mice expressing EAAC1 recapitulates the molecular and behavioral phenotype of EAAC1−/− mice

EAAC1 is known to limit the activation of mGluR1s in the striatum (Bellini et al., 2018). In this brain region, mGluR1s are expressed in both D1- and D2-MSNs (Tallakasen-Greene et al., 1998). In EAAC1−/− mice, there is a significant reduction in the expression of D1R, but no significant difference in D2R is observed (Bellini et al., 2018). This suggests that D1-MSNs could be more sensitive to mGluR1 activation when compared to D2-MSNs. If this were true, then it is conceivable that cell-specifically activating Gq-signaling pathways similar to those coupled to mGluR1s, only in D1-MSNs, would mimic the molecular and behavioral phenotype of EAAC1−/− mice. To test this, we used a chemogenetic approach based on the use of BAC transgenic mouse lines and DREADDs (Armbruster et al., 2007). Conditionally expressing DREADDs in D1Cre/+ mice allowed us to specifically activate Gq-signaling cascades like those coupled to mGluR1s, in D1-MSNs.

We performed unilateral stereotaxic injections of AAV-hSyn-DIO-Hm3d(Gq)-mCherry in the DLS of D1Cre/+ mice between the ages P14-16 (Fig. 16A). To confirm the success of our DREADDs transfection, we measured the expression of the protein mCherry in the striatum and cortex of the injected as well as non-injected brain. For internal controls, we used the non-injected striatum and the adjacent cortices from the injected and non-injected brain, which would not be expected to express mCherry. One week after the surgery, the mice were given daily I/P saline injections for seven days to habituate them to receiving injections. This was done to avoid biasing our results by acute stress responses to the injections of the drug CNO two weeks after the surgery. One hour after CNO was administered, we took behavioral videos.
of the mice and one hour after that we sacrificed them to extract the striatum and cortex for Western blot. To confirm the accuracy of the injections, we measured the levels of mCherry expressed in the injected and non-injected striatum and cortex. The level of Cherry was significantly higher in the injected striatum, compared to the non-injected striatum and cortex sample (Cortex: 1.22±0.20 (n=10), p=0.28; hM3D(Gq) Striatum: 2.89±0.80 (n=10), *p=0.040; Fig. 16B, left). In the samples from the injected and non-injected striatum and cortex, we also probe for the levels of D1R, D2R and pDARPP-32. Our analysis showed a decrease in the expression of D1R (hM3D(Gq): 0.91±0.10 (n=8), p=0.35; Fig. 16C) and an increase in phosphorylation specifically at pDARPP-32\textsuperscript{S130} (hM3D(Gq): 2.48±0.49 (n=11), **p=5.6e-3; Fig. 16D). These results show that a cell-specific activation of G\textsubscript{q} signaling cascades in D1-MSNs is able to mimic the molecular phenotype of EAAC1\textsuperscript{-/-} mice. We also repeated these injections in the DLS of A2A\textsuperscript{Cre/+} mice, targeting the activation of G\textsubscript{q} signaling cascades specifically in D2-MSNs. Again, we confirmed the efficacy of our injections through Western blots to check the protein levels for mCherry, which showed a significant increase only in the injected striatum (hM3D(Gq) Cortex: 1.18±0.31 (n=8), p=0.59; hM3D(Gq) Striatum: 2.06±0.52 (n=23), *p=0.035; Fig. 17A). The activation of G\textsubscript{q} signaling cascades specifically in D2-MSNs did not alter the expression of either D1R (hM3D(Gq): 1.09±0.12 (n=10), p=0.63) or D2R (hM3D(Gq): 0.85±0.19 (n=6), p=0.47; Fig. 17B) in the DLS of A2A\textsuperscript{Cre/+} mice. It also did not affect the phosphorylation levels of pDARPP-32\textsuperscript{S130} (hM3D(Gq): 1.22±0.26 (n=8), p=0.41; Fig 17C). These results are consistent with previous findings that the expression of D2R is not affected in EAAC1\textsuperscript{-/-} mice (Bellini et al., 2018), showing that these receptors are less sensitive to the modulation of mGluRs than D1R.

We analyzed the behavioral videos that were obtained one hour after injecting the mice with CNO. The behavior of WT mice (referred to here as ‘Sham’, same age as mice that received CNO) who had been injected only with saline instead of CNO, was analyzed as a control. This was done to ensure that the injection itself was not responsible for any behavioral changes. Upon analysis, D1\textsuperscript{Cre/+} mice that had
received the injection of CNO showed an increased grooming frequency compared to Sham mice (D1^{Cre/+}: 8.3±1.1e-3 Hz (n=28); Sham: 5.4±0.5e-3 Hz (n=18); D1^{Cre/+} vs Sham: **p=6.0e-3; Fig. 18A). No change in the mean duration of grooming episodes was observed in these mice (D1^{Cre/+}: 45.2±7.3 s (n=36), p=0.39; Sham: 37.4±5.5 s (n=23); Fig. 18B). When the same analysis was repeated on A2A^{Cre/+} mice that had received CNO injections, there was no significant change in either the grooming duration or frequency compared to the Sham mice (A2A^{Cre/+}: 6.0±0.4e-3 Hz (n=50), p=0.38; 42.3±3.9 s (n=58), p=0.08; Fig. 17A, B). This shows that the selective activation of Gq signaling cascades in D2-MSNs did not alter the grooming frequency or duration in A2A^{Cre/+} mice. Therefore, cell-specific activation of Gq signaling pathways is able to recapitulate the molecular and behavioral phenotype of EAAC1^{-/-} mice only in D1-MSNs (in D1^{Cre/+} mice) and not D2-MSNs (in A2A^{Cre/+} mice). These results identify EAAC1 as a key player in the regulation of glutamatergic and dopaminergic signaling in the striatum, and implicate it in controlling the execution of stereotyped motor behaviors.
5. Discussion

5.1 EAAC1 controls glutamatergic and dopaminergic transmission

There has long been some ambiguity regarding the role of EAAC1 in glutamate uptake and excitatory synaptic transmission. Owing to its low abundance, EAAC1 does not significantly contribute to glutamate uptake (Holmseth et al., 2012). However, EAAC1 limits the activation of mGluRIs in the striatum, therefore playing a role in the modulation of glutamatergic transmission (Bellini et al., 2018). mGluRIs in turn, are implicated in the control and expression of long-term plasticity in the striatum, particularly LTD. The loss of LTD observed in EAAC1\(^{-/-}\) mice is therefore consistent with its observed effect on mGluRI activation. Previous findings also show that both LTP and LTD are lost in the absence of EAAC1 (Bellini et al., 2018). This is significant because the expression of LTP does not depend on the activation of mGluRIs, but rather the activation of NMDA receptors and the expression of D1Rs. EAAC1 does not alter the activation of NMDA receptors in the striatum. Data from Western Blot experiments to probe for protein levels of D1R and D2R in the striatum show that loss of EAAC1 reduces the expression of D1Rs without significantly affecting the expression of D2Rs. This not only provides an explanation for the observed loss of LTP in the absence of EAAC1, but it also demonstrates the role of EAAC1 as a modulator of both glutamatergic and dopaminergic transmission in the striatum (Bellini et al., 2018).

Data from previous experiments (Bellini et al., 2018) as well as the results of this thesis confirm that EAAC1 is able to modulate both glutamatergic and dopaminergic signaling in the striatum, by altering the phosphorylation levels of DARPP-32\(^{5130}\), a known integrator of synaptic transmission. Striatal MSNs express high levels of DARPP-32 (approximately 50 µM; (Svenningsson et al., 2004)).
DARPP-32 is involved in signaling cascades which are integral to cell differentiation, gene expression, neuronal plasticity and metabolism. Studies in DARPP-32 deficient mice have shown that this protein is involved in regulating the interactions between dopamine and other neurotransmitters (Reis et al., 2007). Works such as that of (Svenningson et al., 2004) implicate this protein in the integration of cellular function, suggesting the possibility of using it as a therapeutic target. Our result that selective activation of mGluRI-coupled Gq signaling cascades in D1-MSNs leads to increased phosphorylation at DARPP-32S130, agrees with the role of DARPP-32 as a known integrator of glutamatergic and dopaminergic signaling in the striatum. This makes it a potentially useful tool to manipulate the activity of EAAC1, allowing us to move one step closer to finding targeted therapies for neuropsychiatric diseases like OCD. In a broader sense, DARPP-32 is also a potential therapeutic target for a few other neurological disorders. DARPP-32 has also been implicated in the onset of Parkinson’s Disease, Huntington’s Disease and Schizophrenia, all of which are caused by dopaminergic dysfunction. Patients of Parkinson’s disease have significantly reduced levels of DARPP-32 in their putamen, SNc and SNr (Cash, Reisman, Ploska, & Agid, 1987). Patients of Schizophrenia have been shown to have reduced levels of DARPP-32 in their dorsolateral pre-frontal cortex (Albert et al., 2002). Given these findings, it is safe to say that a deeper understanding of DARPP-32 and its associated signaling cascades will ultimately help us treat the above neurological disorders more effectively than is currently possible.
5.2 The role of neuronal glutamate transporters differs across the brain

As discussed in chapter 1.6, when glutamate is released from a pre-synaptic terminal, it can either be taken up by astrocytic glutamate transporters, or, very rarely, be bound by the neuronal glutamate transporter EAAC1. Due to its limited expression in the brain, the role of EAAC1 in glutamate uptake has always been debatable. The two regions of the brain where EAAC1 is most abundantly expressed are the hippocampus and the striatum (Danbolt, 2001; Holmseth, 2012).

In the hippocampus, EAAC1 alters the activation of NMDA receptors, allowing it to shape hippocampal activity (Scimemi et al., 2009). Repeating the same electrophysiology experiments in the striatum have not provided any evidence towards EAAC1 having an effect on the activation of NMDA receptors (Bellini et al., 2018). Immunogold labelling studies have shown the presence of another group of glutamate receptors on the extra-synaptic regions of post synaptic neurons. These receptors are mGluRs (Paquet & Smith, 2003). In particular, the activation of mGluRIs is affected by the perisynaptic location of EAAC1 (Bellini et al., 2018). When glutamate is released from the presynaptic terminal, it is taken up by glutamate transporters on neighboring astrocytes. Part of this glutamate also diffuses into the extra-synaptic space. Here, the presence of EAAC1 at the periphery of postsynaptic neurons allows it to be the first to bind glutamate, preventing it from spilling over into the extracellular regions. By binding glutamate in this manner, EAAC1 prevents glutamate from interacting with or binding to extra-synaptically expressed glutamate receptors like mGluRIs. This is why, the presence of EAAC1 limits the activation of mGluRIs (Bellini et al., 2018).

Together, these findings show that the activity of EAAC1 differs between the hippocampus and the striatum, suggesting that EAAC1 has different roles in different brain regions. These findings provide important insight into the mechanisms used by EAAC1 to modulate synaptic transmission in the striatum.
5.3 How do neuronal and glial glutamate transporters shape synaptic transmission?

Glutamate is the main excitatory neurotransmitter in the CNS. The extent of its effects is determined, among others, by its lifetime in the synaptic cleft. Once released, glutamate binds to its receptors and mediates post-synaptic effects. Owing to its capability to cause excitotoxic cell-death in high concentrations, glutamate must be removed from the synaptic space constantly, a task carried out by glutamate transporters. The effectiveness of neurotransmission at glutamatergic synapses depends not only on the release of glutamate into the synaptic cleft, but also the phenomena that follow. In other words, proper recycling of the excess glutamate and even the used synaptic vesicles is important to ensure smooth synaptic transmission. This is carried out by both glial as well as neuronal transporters.

The activity of glial glutamate transporters such as GLAST and GLT-1 regulates the lifetime of glutamate in the synaptic cleft. The work of (Beurrier et al., 2009) shows that when a large amount of glutamate is released into the synaptic cleft at corticostriatal synapses, glial glutamate transporters (GLAST, GLT-1) are responsible for regulating synaptic transmission (Beurrier et al., 2009). GLAST and GLT-1 are also involved in the glutamate-glutamine cycle (as described in chapter 1.6; Fig. 12), which cycles glutamate released at the synapse back into the pre-synaptic terminal. This cycle has been implicated in the regulation of synaptic transmission (especially at inhibitory synapses) and therefore also inhibitory currents at hippocampal synapses (Bergles, & Edwards, 2008).

At corticostriatal synapses, neuronal glutamate transporters like EAAC1 regulate glutamate transmission and the kinetics of excitatory post-synaptic currents. Owing to its peri-synaptic localization, EAAC1 prevents glutamate from spilling over into the extra-synaptic regions. This prevents extra-synaptically expressed receptors like mGluRIs from being activated by glutamate (Bellini et al., 2018). Glutamate uptake is an essential component of long-term plasticity. The rate of glutamate uptake in the hippocampus has been shown to increase in the early stages of LTP. In the case of EAAC1 in particular,
LTP causes an increase in the translocation of this transporter to the neuronal membrane, specifically in the CA1 region of the hippocampus. This shows that the rate of glutamate uptake determines synaptic strength in LTP (Nieoullon et al., 2006).

The types of glutamate transporters in the CNS are greatly diverse. Therefore, they are capable of modulating synaptic transmission in their regions of expression in the above stated, and other varied was. Synapses in the CNS are not static, but rather undergo changes depending on their environmental cues. It is safe to say that the role of neuronal and glutamate transporters at these synapses is also similarly dynamic, changing in response to their surroundings and the requirements of the synapse (Bergles, & Edwards, 2008). It is this versatility that makes glutamate transporters integral to the regulation of synaptic transmission.
5.4 Limitations and open questions for future work

Taken together with previous findings, the results from this work provide a better understanding of how EAAC1 operates in the striatum. EAAC1 is able to alter the activation of mGluRIs and affect the expression of D1Rs in the striatum, indicating that it works differently in this region of the brain compared to others such as the hippocampus. By regulating the level of phosphorylation of DARPP-32S\textsuperscript{130}, EAAC1 influences the G\textsubscript{q} signaling cascades coupled to mGluRIs and ultimately D1R expression. In addition, these mGluRI-coupled G\textsubscript{q} signaling cascades can be activated in a cell-specific manner using chemogenetics in the form of DREADDs. By doing so, cell-specificity can be achieved. We find that activating these mGluRI-coupled G\textsubscript{q} signaling cascades in D1-MSNs recapitulates the molecular and behavioral phenotype of EAAC1\textsuperscript{-/-} mice but doing so in D2-MSNs does not have the same effect. This suggests that D1-MSNs might be more sensitive to the control of mGluRI-coupled signaling cascades. This also indicates that EAAC1 regulates dopaminergic signaling differently in these two populations of neurons.

These findings are significant because they identify EAAC1 as a powerful molecular switch that modulates both glutamatergic and dopaminergic signaling in the striatum. However, our results alone are not sufficient to determine if these mechanisms alone mediate EAAC1’s control on striatal excitatory transmission. Other pathways may also be involved. EAAC1 is not exclusively expressed at excitatory synapses. It is also expressed pre-synaptically on inhibitory synapses where it has a different mechanism of action (Rothstein et al., 1994; He et al., 2000). This, among others, is a question that could be addressed in the future.
Figure 2
Figure 3

(Modified from (Graybiel & Ragsdale, 1978))
Figure 4
Figure 5

(Adapted from Macpherson et al., 2014)
Figure 6
Figure 7

(Sobolevsky et al., 2009; Furukawa et al., 2005)
Figure 8
Figure 9
Figure 12
(Yernool et al., 2004)

Figure 13
Figure 14
(Dawn of the DREADDs-https://neuwritesd.org/2016/01/28/dawn-of-the-dreadd/)
Figure 16

A

B

C

D

Figure 16
Figure 17
Figure 18
### 7. Tables

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence for Forward Primer</th>
<th>Sequence for Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAAC1&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>5’ AGAAGCTCCTCGGTGGGACAC 3’</td>
<td>5’ GAGAGCAGCGCCAGTCATGATTC 3’</td>
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<tr>
<td>EAAC1&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>5’ CTGTGCTCGACGTTGACTCAGT 3’</td>
<td>5’ GAGAGCAGCGCCAGTCATGATTC 3’</td>
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<tr>
<td>D1&lt;sup&gt;Cre/+&lt;/sup&gt;</td>
<td>5’ GCTATGGAGATGCTCCTGATGGGAA 3’</td>
<td>5’ CGGCAAACGGACAGAAGGCATT 3’</td>
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<tr>
<td>A2A&lt;sup&gt;Cre/+&lt;/sup&gt;</td>
<td>5’ CGTGAAGAAGCCTTTGGGAAGCT 3’</td>
<td>5’ CGGCAAACGGACAGAAGGCATT 3’</td>
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<td>Ai9&lt;sup&gt;0/0&lt;/sup&gt;</td>
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<td>D1td&lt;sup&gt;Tomato/+&lt;/sup&gt;</td>
<td>5’ CTTCTGAGGCAGAAAGAACC 3’</td>
<td>5’ TTTCTGATTGAGAGCATGC 3’</td>
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**Table 1.** Primer sequences used for PCR (modified from Bellini et al., 2018)

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
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</thead>
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<td>Initiation</td>
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<tr>
<td>Denaturation</td>
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</tr>
<tr>
<td>Annealing</td>
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</tr>
<tr>
<td>Elongation</td>
<td>72°C</td>
<td>1.5 min</td>
</tr>
<tr>
<td>Amplification</td>
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<td>10 min</td>
</tr>
<tr>
<td>Hold</td>
<td>4°C</td>
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**Table 2.** EAAC1 PCR protocol (modified from Bellini et al., 2018)
<table>
<thead>
<tr>
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<th>Annealing</th>
<th>Elongation</th>
<th>Amplification</th>
<th>Hold</th>
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</thead>
<tbody>
<tr>
<td>94°C</td>
<td>94°C</td>
<td>65-55°C</td>
<td>72°C</td>
<td>72°C</td>
<td>4°C</td>
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<tr>
<td>5 min</td>
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<td>0.5 min</td>
<td>0.17 min</td>
<td>5 min</td>
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</tr>
<tr>
<td>1 cycle</td>
<td></td>
<td></td>
<td></td>
<td>40 cycles</td>
<td>1 cycle</td>
</tr>
</tbody>
</table>

(1-10: T\text{Anneal} -1°C/cycle)

### Table 3. D1\textsuperscript{Cre/+} and A2A\textsuperscript{Cre/+} PCR protocol (modified from Bellini et al., 2018).

<table>
<thead>
<tr>
<th>Initiation</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Elongation</th>
<th>Amplification</th>
<th>Hold</th>
</tr>
</thead>
<tbody>
<tr>
<td>94°C</td>
<td>94°C</td>
<td>65-60°C</td>
<td>72°C</td>
<td>72°C</td>
<td>10°C</td>
</tr>
<tr>
<td>2 min</td>
<td>0.33 min</td>
<td>0.25 min</td>
<td>0.17 min</td>
<td>2 min</td>
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</tr>
<tr>
<td>1 cycle</td>
<td></td>
<td></td>
<td></td>
<td>38 cycles</td>
<td>1 cycle</td>
</tr>
</tbody>
</table>

(1-10: T\text{Anneal} -0.5°C/cycle)

### Table 4. Ai9 PCR protocol (modified from Bellini et al., 2018)

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<th>Annealing</th>
<th>Elongation</th>
<th>Amplification</th>
<th>Hold</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C</td>
<td>95°C</td>
<td>60°C</td>
<td>72°C</td>
<td>72°C</td>
<td>4°C</td>
</tr>
<tr>
<td>3 min</td>
<td>0.25 min</td>
<td>0.25 min</td>
<td>0.25 min</td>
<td>1 min</td>
<td>∞</td>
</tr>
<tr>
<td>1 cycle</td>
<td></td>
<td></td>
<td></td>
<td>35 cycles</td>
<td>1 cycle</td>
</tr>
</tbody>
</table>

### Table 5. D1\textsuperscript{tdTomato} PCR protocol (modified from Bellini et al., 2018)
8. Figure legends

Figure 1. Structures comprising the basal ganglia in mice. (adapted from the Allen Mouse Brain Atlas)

(A) The position of the striatum (left), CPu (middle) and GP (right) in the transverse plane. (B) The position of the NAc (left), SNr (middle) and SNC (right) in the transverse plane. (C) The position of the STN (left) and VTA (right) in the transverse plane. (D) The position of the OT in the transverse plane.

Figure 2. Basal ganglia inputs and outputs. GPe and GPI receive glutamatergic inputs (green arrows) from the STN. The GPe sends GABAergic outputs (red arrows) to the STN. The GPI sends GABAergic outputs to the thalamus. The NAc receives glutamatergic inputs from the cortex and hippocampus. The NAc receives glutamatergic inputs from the cortex and hippocampus. The NAc sends GABAergic output to the SN (dotted line enclosing the SNr and SNC). The OT sends cholinergic outputs (purple arrows) to the striatum and NAc. The OT receives glutamatergic inputs from the cortex and thalamus. The SNr sends GABAergic outputs to the thalamus. The SNr receives glutamatergic inputs from the STN and GABAergic inputs from the NAc and striatum. The SNC sends dopaminergic outputs to the striatum (blue arrow) and receives GABAergic inputs from the NAc. The STN receives GABAergic inputs from the GPe. The STN sends glutamatergic inputs to the GP and SNr.

Figure 3. The striosome (patch) and matrix compartments of the striatum (Adapted from Graybiel & Ragsdale, 1978). A schematic representation of the striosome and matrix compartments of the striatum (left) and a histological staining of the human striatum showing the same compartments (right; (Graybiel & Ragsdale, 1978)). The striosome is immunopositive for MORs and AChE. The matrix is immunopositive for enkephalin and calbindin.

Figure 4. Connections of the striosome and matrix. Striosomes receive excitatory inputs (thick green arrows) from the associative cortex and layer Vb and VI of the limbic cortex. Striosomes send GABAergic
projections (thin red arrows) to the SNC. The matrix receives excitatory inputs from layer V of the sensorimotor cortex. The matrix sends GABAergic projections to the SNr, GPi and GPe.

**Figure 5. The direct and indirect pathways of the striatum (Adapted from Macpherson et al., 2014).** The striatum receives glutamatergic input from the cortex. It receives dopaminergic innervation from the SNC. D1-MSNs in the striatum send GABAergic projections onto the SNr and VTA, which then send GABAergic projections to the thalamus. This makes up the direct pathway. D2-MSNs in the striatum send GABAergic projections onto the GPe, which sends GABAergic projections to the STN. The STN sends dopaminergic projections to the SNr and VTA, which ultimately send GABAergic projections to the thalamus. These connections make up the indirect pathway of the striatum.

**Figure 6. Glutamate receptor domains.** (A) AMPARs and NMDARs have the same domain structure composed of an extracellular NTD, 4 TMDs and an intracellular CTD. The LBD is formed between the extracellular portions of the NTD and the loop connecting TMD3 and TMD4. (B) KARs are composed of an extracellular NTD, 4 TMDs and an intracellular CTD. Their ligand binding sites are between two segments S1 and S2, formed between the extracellular portions of the NTD and the loop connecting TMD3 and TMD4. (C) mGluRs are composed of an extracellular NTD, a group of 7 TMDs and an intracellular CTD. Their LBD is present on a segment of the extracellular loop ending in the NTD.

**Figure 7. AMPAR and NMDAR crystal structure.** (A) Crystal structure of rat AMPAR homomer (Sobolevsky et al., 2009) showing the NTD, LBD and TMD. (B) Axis of symmetry (black dotted line) for AMPAR homomer. (C) Crystal structure of an NMDAR heterodimer showing the NR1 (green) and NR2A (blue) subunits, complexed with glycine and glutamate respectively. The grey arrow indicates the axis of symmetry (Furukawa et al., 2005).

**Figure 8. Subunit composition of iGluRs.** (A) AMPARs are composed of four subunits: GluA1, GluA2, GluA3 and GluA4. Between these subunits is the cation channel (blue dotted arrow), which conducts Na⁺
ions. The glutamate binding sites are on the GluA1 subunit. (B) NMDARs are composed of three subunits: NR1, NR2 and NR3. The cation channel is enclosed by these subunits (blue dotted arrow) and conducts Na\(^+\) and Ca\(^{2+}\) ions. There is a specific binding site where Mg\(^{2+}\) ions bind and block the receptor. The glutamate binding site is located on the NR2 subunit and the glycine binding site is located on the NR1 subunit. (C) KARs are composed of five subunits: GluK\(_5\), GluK\(_6\), GluK\(_7\), KA1 and KA2. These subunits enclose the cation channel (blue dotted arrow) which conducts Na\(^+\) and K\(^+\) ions.

**Figure 9. Mechanism of activation of second messengers via mGluRs.** (Adapted from motifolio.com) mGluRs are GPCRs. This means that when ligands are bound to them, they activate certain G protein coupled signaling cascades. In the inactive state, the three subunits of G proteins (α, β and γ) exist as a complex and bind GDP. When GDP is phosphorylated to GTP, the β and γ subunits detach from the complex. Specific enzymes can then bind to the α subunit and regulate the activation of second messenger systems such as PLC, IP\(_3\) and DAG in the cell.

**Figure 10. Dopamine receptor domains.** (A) Domain composition of D1-like family of DRs. These are composed of seven TMDs, three intracellular loops (IL-1-IL-3), an extracellular NTD and an intracellular CTD. (B) Domain composition of D2-like family of DRs. They are composed of seven TMDs like the D1-like family DRs. There are two small intracellular loops (IL-1,2) and a third large one (IL-3). They possess an extracellular NTD and an intracellular CTD.

**Figure 11. A schematic representation of the glutamatergic synapse.** When glutamate is released from the pre-synaptic terminal it binds NMDARs, AMPARs and KARs (shown here on the PSD). Glutamate also diffuses out of the synaptic cleft and is taken up by astrocytic glutamate transporters GLAST and GLT-1. In the event that glutamate reaches the peri-synaptic region of the post-synaptic neuron, it is bound by EAAC1, and very rarely binds to mGluRs.
Figure 12. The glutamate-glutamine cycle. Glutamine is converted into glutamate in the presence of polyamineglycol (PAG). This glutamate is then taken up by EAATs expressed on the surface of astrocytes, from where it is converted back into glutamine. Glutamine is then released back into the extracellular space to re-produce glutamate and continue the cycle.

Figure 13. The crystal structure of GltPh. (Yernool et al., 2004) described an 80 Angstrom structure of a glutamate transporter homologue from Pyrococcus horikoshii. The transporter is described as a bowl-shaped trimer, containing a solvent-filled extracellular region that spans the membrane bilayer.

Figure 14. The three outcomes of Cre-Lox recombination. (A) When a strand of DNA is present between two LoxP sites that are facing the same direction, Cre recombinase excises the DNA fragment between the two LoxP sites. (B) When a strand of DNA is present between two LoxP sites oriented in the opposite directions, Cre recombinase transposes the fragment of DNA. (C) When two LoxP sites exist between two DNA fragments on separate strands, Cre recombinase action causes translocation of the DNA strands.

Figure 15. Steps involved in the use of DREADDs. (Dawn of the DREADDs-https://neuwritesd.org/2016/01/28/dawn-of-the-dreadd/). DNA that encodes for the expression of a designer receptor is tagged with a fluorescent protein and inserted into a viral vector (maybe AAVs). The viral construct that encodes this designer receptor is injected into a desired brain region, where upon being incorporated, cells express the fluorescent-tagged DREADD. After a period of rest, allowing the virus to diffuse into the brain, the designer drug CNO is applied (orally or intravenously) in order to selectively activate or inhibit the brain region.

Figure 16. Cell specific activation of mGluR1-coupled Gq signaling cascades in D1-MSNs results in reduced expression of D1R protein and increased phosphorylation at pDARPP-32S130 in the DLS. (A) Experimental timeline followed for stereotaxic injection of DREADDs into the mouse DLS (B) Expression levels of mCherry, in the injected and non-injected striatum of D1 Cre/+ mice. There is a significant increase
in mCherry expression only in the injected striatum, and not the non-injected striatum or cortex of D1\textsuperscript{Cre/+} mice (n=10, \*p=0.040). (C) D1R expression is significantly reduced in the injected striatum of D1\textsuperscript{Cre/+} mice (n=9, \*p=0.013), with no change in the expression of D2R (n=8, p=0.35). (D) Phosphorylation at pDARPP-32\textsuperscript{S130} is significantly increased in D1\textsuperscript{Cre/+} mice (pDARPP-32\textsuperscript{T34} n=10, p=0.92; pDARPP-32\textsuperscript{T75} n=11, p=0.18; pDARPP-32\textsuperscript{S97} n=11, p=0.13, pDARPP-32\textsuperscript{S130} n=11, **p=5.6e-3) upon selective activation of Gq signaling cascades in D1-MSNs.

Figure 17. Cell specific activation of mGluR1-coupled Gq signaling cascades in D2-MSNs does not affect the expression of D1R protein or phosphorylation at pDARPP-32\textsuperscript{S130} in the DLS. (A) Expression levels of mCherry, in the injected and non-injected striatum of A2A\textsuperscript{Cre/+} mice. There is a significant increase in mCherry expression only in the injected striatum, and not in the control striatum, or the cortex of A2A\textsuperscript{Cre/+} mice (n=23, \*p=0.035), similar to that of D1\textsuperscript{Cre/+} mice. (B) There is no significant change in the expression of either D1R (n=10, p=0.54) or D2R (n=6, p=0.47) in A2A\textsuperscript{Cre/+} mice. (C) The expression of pDARPP-32\textsuperscript{S130} is not significantly different as a result of the injections in A2A\textsuperscript{Cre/+} mice (pDARPP-32\textsuperscript{T34} n=11, p=0.85; pDARPP-32\textsuperscript{T75} n=10, p=0.57; pDARPP-32\textsuperscript{S97} n=10, p=0.36, pDARPP-32\textsuperscript{S130} n=8, p=0.47).

Figure 18. Cell-specific activation of mGluR1-coupled Gq signaling cascades in D1\textsuperscript{Cre/+} mice recapitulates the behavioral phenotype of EAAC1\textsuperscript{-/-} mice. (A) Comparison between the grooming frequencies of Sham and injected D1\textsuperscript{Cre/+} and A2A\textsuperscript{Cre/+} mice (Sham (n=18), D1\textsuperscript{Cre/+} (n=28), Sham vs D1\textsuperscript{Cre/+} \*p=0.019, A2A\textsuperscript{Cre/+} (n=50), Sham vs A2A\textsuperscript{Cre/+} p=0.38). (B) Comparison between the mean bout duration of Sham and injected D1\textsuperscript{Cre/+} and A2A\textsuperscript{Cre/+} mice (Sham (n=23), D1\textsuperscript{Cre/+} (n=36), p=0.39, Sham vs D1\textsuperscript{Cre/+} p=0.12, A2A\textsuperscript{Cre/+} (n=58), Sham vs A2A\textsuperscript{Cre/+} p=0.08).
9. References


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