Effects of neuronal glutamate transporters on repetitive and time sensitive reward-based learning and behavior

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Effects of Neuronal Glutamate Transporters on Repetitive and Time Sensitive Reward-Based Learning and Behavior

by

Benjamin A. Bennink

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College of Arts & Sciences
Department of Biological Sciences

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1 Acknowledgements

“No man is an island, entire of itself; every man is a piece of the continent, a part of
the main.”

— John Donne, 1624

"If I have seen further it is by standing on the shoulders of Giants."

— Isaac Newton, 1675

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2 Abstract

The basal ganglia are a collection of subcortical nuclei responsible for action selection. The striatum is the primary input of the basal ganglia and forms two distinct feedback loops with the motor cortex. Within the striatum, projection neurons receive cortical inputs via glutamatergic synapses, and modulation via cholinergic synapses and dopaminergic projections. These projection neurons create two separate pathways to the principal output of the basal ganglia, the substantia nigra pars reticulata. The striatum uses these two pathways to filter cortical motor inputs and integrate those inputs with dopaminergic motor and reward inputs to determine action selection output. The GABAergic striatal projection neurons are distinguished based on whether they express D1-receptors or D2-receptors. Previous work has been conducted indicating that the loss of the neuronal glutamate transporter, EAAC1, expressed within the striatum by the projection neurons, results in a variety of behavioral changes including compulsion-like stereotyped grooming behaviors. What remains unknown is how EAAC1 impacts differences in reward-motivated voluntary motor actions.

Herein, we seek to answer this question using behavioral approaches, and discover a novel phenotype previously undescribed in EAAC1\(^{-/-}\) mice. These mice show an impulsive behavioral phenotype in which they are faster than WT mice to initiate a reward-motivated behavior action sequence. This lays the groundwork for future research on how EAAC1 affects the overall function of the striatum and basal ganglia in determining action selection on the molecular level. This also suggests evidence, for the first time, of a tangible effect of spillover due to the supposed role of EAAC1 at glutamatergic synapses.
3 Introduction

Motor action and reward-based responses are multifaceted paradigms involving several brain regions and a variety of neurotransmitters. Our research focuses on the basal ganglia integrating glutamatergic signals from the motor cortex, dopaminergic signals from the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc), cholinergic signals from the latero-dorsal tegmental area (LDT) and pedunculopontine tegmental area (PPT), as well as local cholinergic modulation by cholinergic interneurons (CINs) to coordinate GABAergic output from striatal projection neurons. Each of these elements plays a unique role in response to reward stimulus or initiation, cessation, or continuation of motor action. We investigate the specific contributions of neuronal glutamate transporters in this coordination with the intent to describe behavioral differences and explore the underlying mechanisms that cause these changes.

3.1 Glutamate Transporters

Glutamate is the main excitatory neurotransmitter in the vertebrate central nervous system (CNS). Glutamate is abundant in the brain, but mostly intracellular, with physiological extracellular concentrations of 25 nM in the hippocampus and the nucleus accumbens (NAc) (Chiu & Jahr, 2017; Herman et al., 2011; Herman & Jahr, 2007). In addition to mediating fast excitatory transmission, glutamate can also trigger excitotoxicity (Zhou & Danbolt, 2014). For this reason, its concentration in the brain neuropil must be accurately controlled in both space and time. Glutamate is released into the extracellular space through different mechanisms, including synaptic release, reversed glutamate uptake, and through a poorly-understood mechanism known as swelling-activated excitatory amino acid release (Danbolt, 2001). The majority of this release is non-vesicular, and is continuous: blocking glutamate uptake pharmacologically leads to rapid
extracellular buildup of glutamate (Jabaudon et al., 1999). There are no extracellular enzymes which breakdown glutamate in the CNS, and so glutamate transporters are necessary to maintain the extracellular concentrations of glutamate at low nanomolar levels (Chiu & Jahr, 2017; Herman et al., 2011; Herman & Jahr, 2007).

There are five known glutamate transporters. In humans, they are called excitatory amino acid transporters 1-5 (EAAT1-5). In rodents, these correspond to GLAST, GLT-1, EAAC1, EAAT4, and EAAT5. These are secondary active transporters, meaning that the energy necessary to support the translocation of glutamate across the membrane comes from the dissipation of the ionic gradients. Accordingly, glutamate transporters translocate glutamate across the membrane by coupling it to the co-transport of three Na\(^+\) and one H\(^+\), and the counter-transport of one K\(^+\) ion (Wadiche, Amara, et al., 1995; Wadiche, Arriza, et al., 1995; N. Zerangue & Kavanaugh, 1996). As a result, there is a net influx of two positive charges per transport cycle, leading to the generation of a stoichiometrically coupled transporter current. In addition to this stoichiometrically coupled transporter current, glutamate transporters are also capable of generating a non-stoichiometrically coupled anion current. This non-stoichiometrically coupled current is carried by Cl\(^-\), and this ion moves across the membrane according to the direction set by the driving force. In astrocytes, the intracellular Cl\(^-\) concentration is much lower than that present in the extracellular space \((E_{\text{Cl}} = -60 \text{ mV})\), and the resting membrane potential of these cells is close to -90 mV. As a result, the non-stoichiometrically-coupled Cl\(^-\) current is inwardly directed and leads to membrane depolarization. In neurons, the membrane potential varies across cells and in time, meaning that here the non-stoichiometrically coupled current can be hyperpolarizing, shunting (i.e. change the
membrane resistance without generating a change in membrane voltage) or depolarizing (Scimemi, 2014).

Different glutamate transporters shuttle glutamate at different rates. For example, heterologous expression of GLAST, GLT-1, and EAAC1 in *Xenopus laevis* oocytes shows that the $K_m$ values for these transporters are 11, 17, and 12 µM respectively. By contrast, $K_m$ values for EAAT4 and EAAT5 are 3.3 µM and 1.3 µM respectively (Danbolt, 2001). GLAST, GLT-1, and EAAC1 share similar cycling/turnover rates and capture efficiency, and have a glutamate steady state affinity of ~12-20 µM. However, the pre-steady state glutamate affinity, a closer measure of the glutamate-binding and unbinding constants, is substantially different among glutamate transporter subtypes. For example, GLT-1 has a $K_m$ of 12 µM and a $K_d$ of 140 µM (Tzingounis & Wadiche, 2007). EAAT4 and 5 have the highest Cl⁻ conductance of any of the EAATs. Due to their ability to bind glutamate, limited glutamate transport efficiency and high Cl⁻ permeability, EAAT4 and EAAT5 act more like inhibitory glutamate receptors than as glutamate transporters (Zhou & Danbolt, 2014). EAAT4 is predominantly expressed in the dendritic spines of cerebellar Purkinje cells where its expression is developmentally regulated, peaking at P26 in rats. Although EAAT4 is also detected in a subset of forebrain neurons and vestibular hair cells, the expression in these cells reaches a maximum level around P10-P16 and further decreases with age (Furuta et al., 1997). EAAT5 is mostly expressed in the retina and its expression peaks at P21 in rats (Pow & Barnett, 2000). EAAT5 is also expressed in vestibular hair cells along with EAAT4 (Zhou & Danbolt, 2014).
GLT-1 was the first glutamate transporter to be purified and identified using chromatographic fractionation (Zhou & Danbolt, 2014). GLT-1 is expressed throughout the CNS during embryonic development, reaching adult levels of expression by post-natal day 26 (P26). There is transient prenatal expression of GLT-1 along axonal pathways interconnecting the neocortex, basal ganglia, and thalamus, but it is localized exclusively to astrocytes in the adult brain (Furuta et al., 1997). In the adult brain, GLT-1 is responsible for up to 95% of the total glutamate removal from the extracellular space (Holmseth et al., 2012). GLAST was the second glutamate transporter isolated through partial proteolysis of the then-unknown protein which co-purified with galactosyltransferase. The GLAST amino acid sequence was then identified using degenerate oligonucleotide probes and screening of a rat brain cDNA library (Zhou & Danbolt, 2014). GLAST is expressed in astrocytes throughout the CNS. The loss of glial glutamate transporters GLAST or GLT-1 produced elevated extracellular glutamate levels, neurodegeneration characteristic of excitotoxicity, and a progressive paralysis (Rothstein et al., 1996).

GLAST−/− mice show reduced glutamate uptake capacity in brain regions where GLAST is the most abundant glutamate transporter, and exhibit poor nesting, abnormal sociability, and reduced alcohol intake and reward. Human EAAT1 mutations are associated with episodic ataxia (Zhou & Danbolt, 2014). The glutamate transporter EAAT3 (excitatory amino acid carrier 1, EAAC1 in mice) is the transporter upon which this thesis will focus its analysis.

3.2 The Excitatory Amino Acid Carrier 1 (EAAC1)

EAAC1 is an exclusively neuronal glutamate transporter expressed in a variety of brain tissues. It is expressed in the striatum, neocortex (which includes the hippocampus), olfactory bulb, and
the thalamus. Using immunoblotting techniques, it is estimated that in the hypothalamus, EAAC1 is expressed at approximately half the levels measured in the hippocampus, while in the cerebellum, mesencephalon, pons, and medulla, EAAC1 is expressed at levels ranging from one third to one fourth of those measured in the hippocampus (Holmseth et al., 2012). Immunogold electron microscopy studies show that in the hippocampus, EAAC1 is expressed peri-synaptically and post-synaptically at excitatory synapses and pre-synaptically at inhibitory synapses (He et al., 2000).

Like other glutamate transporters, EAAC1 has a rapid binding rate for glutamate but a low (53%) transport efficiency. Rapid binding without transport serves to delay the diffusion of glutamate to astrocytic membranes where it is cleared by GLT-1. This means that EAAC1 acts mainly by rapidly buffering glutamate, not by transporting it across the membrane (Scimemi et al., 2009; Tzingounis & Wadiche, 2007). EAAC1 is the only glutamate transporter that can transport the uncharged amino acid L-cysteine (Watts et al., 2014), with a steady state affinity of 190 μM (Noa Zerangue & Kavanaugh, 1996). Transport via EAAC1 is proposed by Watts et al. as an important mechanism by which neurons obtain cysteine to synthesize glutathione, as EAAC1 deficient mice show decreased levels of neuronal glutathione. Other routes for cysteine uptake include the XₐG- and, to a lesser extent, the ASC Na⁺-dependent systems (Shanker et al., 2001). Glutathione synthesis from cysteine is accomplished by deprotonating cysteine at the EAAC1 binding site to thiolate and then rapidly re-protonating the thiolate after transport (Watts et al., 2014). Glutathione is a powerful antioxidant and is vital to prevent oxidative stress and neurodegeneration (Schulz et al., 2000). In addition to transporting cysteine, EAAC1 has been implicated as being upstream of protein kinase A (PKA) in a pathway to facilitate membrane trafficking of GluR1, one of the main
subunits of AMPA glutamate receptors, though its exact location in this pathway remains unknown (Cao et al., 2014).

While total tissue content of EAAC1 is about 100 times lower than that of GLT-1 (Holmseth et al., 2012), loss of EAAC1 has notable behavioral effects. For example, constitutive EAAC1−/− mice exhibit less freezing behavior in tone-related fear conditioning, and an increased time to identify the target hole in the Barnes maze test (Lee et al., 2012; Wang et al., 2014). EAAC1−/− mice also exhibit anxiety-like behaviors including increased fidgeting, higher proportion of time in the closed arms of an elevated plus maze, and burying more marbles in a marble-burying test (Bellini et al., 2018). At one year of age, EAAC1−/− mice show loss of dopaminergic neurons in the SNc (Berman et al., 2011). Since this effect can be rescued by feeding mice with cysteine water, this effect is thought to be due to the oxidative stress from insufficient synthesis of glutathione (Aoyama et al., 2006; Berman et al., 2011). Finally, EAAC1 promotes D1 dopamine receptor (D1R) expression in striatal neurons (Scimemi et al., 2009). The implication of EAAC1 expression in dopaminergic and striatal circuits will be a major focus of this thesis.

3.3 Acetylcholine

Acetylcholine (ACh) is the main excitatory neurotransmitter at peripheral neuromuscular junctions (NMJs), but is also used as a neurotransmitter in the CNS. Here, ACh functions as a powerful neuromodulator. There are two predominant types of cholinergic neurons in the brain: (1) phasic-firing projection neurons which extend from the PPT, LDT, medial habenula (MHb), and basal forebrain (BF) to areas throughout the CNS; (2) tonically-active interneurons located in the striatum and NAc. The neuromodulatory effects of ACh, which refer to its ability to change
how neurons respond to excitatory or inhibitory inputs, are accomplished through two types of receptors, each with subtypes: metabotropic muscarinic receptors (mAChR) and ionotropic nicotinic receptors (nAChR). mAChRs are coupled to $G_q$, $G_i$, or $G_o$ proteins depending on subtype and are expressed both pre- and post-synaptically throughout the brain, leading to a large diversity of signaling cascades including those tied to activation of phospholipase C or inhibition of adenylate cyclase. nAChRs are non-selective excitatory cation channels expressed in presynaptic terminals, cell bodies, and axons. Depending on their subunit composition, these receptors can undergo rapid desensitization. For example, calcium permeable $\alpha_7$-subunit containing nAChRs desensitize rapidly, while $\alpha_7$-subunits (which are present in dopaminergic axons in the striatum) are less desensitized and recover faster than other subtypes (Grady et al., 2012). Differently from the NMJ, nAChRs in the CNS are not clustered post-synaptically. Different subtypes of nAChRs are responsible for increasing the synaptic release of glutamate, GABA, DA, ACh, norepinephrine, and 5-HT depending on brain region (Picciotto et al., 2012).

Cholinergic projection neurons from the LDT and PPT to the striatum preferentially synapse onto CINs (76%) compared to medium spiny neurons (MSNs, 24%). While these projection neurons decrease the firing rate of MSNs, they cause an excitatory effect on CINs. In contrast to cholinergic projection neurons, CINs have an inhibitory effect on other CINs. These differences in neuronal effect contribute to differences in behavior. Specifically, inhibition of LDT axons in rats prevent them from developing goal-directed behavior, while blocking ACh transmission in the striatum from the PPT or from striatal CINs results in impaired habitual action control (Dautan et al., 2020). While CINs facilitate integration of new learning into old strategies, projection neurons
update behavioral states triggered by changing contingencies. The inhibition of either CINs or projection neurons, however, impairs the encoding of action strategies (Dautan et al., 2020).

3.4 Dopamine

The monoamine dopamine (DA) is a hormone and neuromodulator, which in the CNS is produced by neurons in the SNc and the VTA. The primary biosynthetic pathway for dopamine is: L-Phenylalanine → L-Tyrosine → L-DOPA → DA. The minor biosynthetic pathway is: L-Phenylalanine → m-Tyrosine → m-Tyramine → DA. The direct precursor of dopamine, L-DOPA, can be synthesized indirectly from the essential amino acid phenylalanine or directly from the non-essential amino acid tyrosine. Dopamine is broken down into inactive metabolites by the sequential activation of the enzymes monoamine oxidase (MAO), catechol-O-methyl transferase (COMT), and aldehyde dehydrogenase (ALDH).

As a paracrine messenger, dopamine acts by inhibiting norepinephrine release, vasodilation, and reducing insulin production. As a neuromodulator, increase or reduction of dopamine release in various pathways mediate movement execution, reward-motivated behaviors, arousal, and other functions including lactation and nausea.

There are five dopamine receptors, D1-D5, which belong to two major groups. D1-type dopamine receptors include D1 and D5 receptors. D2 type dopamine receptors include D2-4 receptors. D1-type receptors are Gs coupled receptors which lead to transcription changes within the nucleus through activation of the cAMP/PKA pathway. D2-type receptors are Gi coupled receptors which inhibit adenyl cyclase and activate K+ channels by inhibiting the cAMP/PKA pathway (Bhatia &
Abnormal dopaminergic transmission is associated with neurodegenerative disease like Parkinson’s Disease, neuropsychiatric disorders like schizophrenia and addiction.

### 3.4.1 Role of dopamine in motor function

Dopaminergic neurons in the SNc are autonomous pacemakers, meaning that they show rhythmic spontaneous firing. This activity is responsible for the sustained release of dopamine necessary for the proper functioning of target structures, such as the striatum (Guzman et al., 2009). The mechanisms underlying autonomous pacemaking have been the subject of investigation for more than a decade. At present, the dominant view is that, in adult SNc dopaminergic neurons, pacemaking is dependent on Ca\(^{2+}\) channels that open at relatively hyperpolarized membrane potentials (Amini et al., 1999; Nicola B. Mercuri et al., 1994; Nedergaard et al., 1993; Puopolo et al., 2007; Wilson & Callaway, 2000). It was initially shown that blocking the hyperpolarization-activated cation current I\(_h\) has no effect on pacemaking in most midbrain dopamine neurons (N. B. Mercuri et al., 1995). However, later works suggest that the reliance of pacemaking on I\(_h\) appears to vary depending on the age and species of animals used, as well as on the location and neurochemical properties of dopamine neurons recorded (Chan et al., 2007; Guzman et al., 2009; Khaliq & Bean, 2010; Neuhoff et al., 2002; Okamoto et al., 2006; Puopolo et al., 2007). In contrast to most other pacemaking neurons in the CNS where blocking calcium entry has little effect on pacemaking or even speeds it (Beurrier et al., 2000; Bevan & Wilson, 1999; Do & Bean, 2003; Llinas & Alonso, 1992; Raman & Bean, 1999; Takakusaki & Kitai, 1997), midbrain dopamine neurons are unusual in that blocking calcium current by cadmium or cobalt completely silences pacemaking (Fujimura & Matsuda, 1989; Grace & Onn, 1989; Harris et al., 1989). The pacemaking firing of dopaminergic neurons builds upon a subthreshold oscillatory mechanism.
established by the interplay of persistent Na+ and Ca$^{2+}$ and Ca$^{2+}$-activated K$^+$ conductances. The persistent Ca$^{2+}$ and Na$^+$ channels activate at relatively hyperpolarized potentials (-60 mV), and gradually depolarize the membrane potential of SNc neurons until the concentration of Ca$^{2+}$ is sufficiently high to activate a Ca$^{2+}$-dependent K$^+$ channels. These, in turn, hyperpolarize the resting membrane potential until Ca$^{2+}$ concentrations are low enough to begin this subthreshold cycle again (Morikawa & Paladini, 2011).

Since the SNc projects to the striatum, the pace making activity of SNc neurons leads to tonic release of dopamine into the striatum. Here, the two main types of long-range projection neurons (the MSNs) give rise to the direct and indirect pathways. Traditionally, the direct and indirect pathways are associated with a GO and NO-GO signal, respectively. Activation of the direct GO pathway ultimately leads to disinhibition of the thalamus, which promotes cortical activity. Activation of the indirect NO-GO pathway ultimately leads to inhibition of the thalamus, which prevents cortical activity (Cohen & Frank, 2009). This traditional explanation is overly simplified and is discussed in greater detail in section 3.5 on the striatum.

### 3.4.2 Role of dopamine in reward-motivated behavior

Dopaminergic neurons in the VTA also exhibit pacemaker activity, but through a different mechanism than those found in the SNc. Here, two types of Na$^+$ currents underlie the pacemaker activity: one mediated by fast-activating and fast-inactivating voltage-gated Na$^+$ channels, and a second one mediated by a voltage-independent persistent Na$^+$ current (Khaliq & Bean, 2010; Raman et al., 2000). Traditional models assumed that the VTA projections to the ventral striatum dominated reward-motivated behaviors, while SNc projections to the dorsal striatum were solely responsible for motor-functions. Recent evidence has shifted the accepted paradigm to reflect a
spectrum, where both dopamine projections shape motor and reward functions, resulting in a more subtly complex function of basal ganglia regions than previously described.

Early models of dopamine’s role in reward-motivated behavior predicted that dopamine release correlated with reward value. This gave rise to the Reward Prediction Error (RPE) model, in which dopamine actually encodes the temporal difference between the expected and actual reward time (Kakade & Dayan, 2002). Dopamine levels increase in a ramp-like pattern during learning of spatial, temporal, or sequence tasks (Collins et al., 2016). These ramps reach peak amplitude at different times during learning. Early in training, the peak amplitude coincides with reward delivery, as predicted by the temporal difference model. During training, the peak amplitude shifts to earlier times during the tasks until the point when asymptotic performance is reached. Once asymptotic performance is reached, the peak dopamine occurs before initiation of the action sequence. The amplitude of the dopamine peak positively correlates with learning speed, as measured by the number of sessions needed to reach asymptotic performance, during training, and with motivation, measured by the speed of performance of the multi-action task, after training. In a sequence lever press task, in which pressing one lever made another lever available and pressing of that lever resulted in a sugar-water reward, rats showing higher amplitude dopamine peaks during training learned the sequence tasks faster and rats showing higher amplitude dopamine peaks after training accomplished these full task more quickly (Collins et al., 2016).

Differently from what is predicted by the RPE model, dopamine release also occurs during non-rewarding novel or aversive stimulus presentation (Kakade & Dayan, 2002). While dopamine release from the VTA to the ventral striatum is consistent with the traditional RPE model,
dopamine release from the SNc to the caudal portion (tail) of the striatum (TS) encodes avoidance (Menegas et al., 2018). Normally when mice are exposed to novel objects, they will behave in a characteristic approach and retreat pattern (Menegas et al., 2018). During this pattern, the mice will approach the novel object and then retreat, and this behavior pattern will repeat multiple times. Ablation of dopamine projections to the TS does not impair the initial avoidance of an aversive stimulus or the periodic retreat during novel object investigation, but results in decreased subsequent avoidance response in later trials within the same session. This implies that dopamine does not cause retreat from aversive stimuli or novel objects, but reinforces that aversion increasing the future probability of retreat behaviors (Menegas et al., 2018). There is also a correlation between dopamine release levels and passive (freezing) avoidance during inescapable foot shock, indicating that dopamine also plays a role in maintaining passive avoidance in addition to active avoidance.

3.5 The striatum

The striatum is the primary input nucleus of the basal ganglia. In humans, it is composed of the caudate nucleus and putamen, but these domains cannot be distinguished in rodents. The striatum can be functionally distinguished in different domains: the dorsolateral (motor) and the ventromedial striatum (reward). The NAc is located within the ventral striatum. Excitatory glutamatergic cortical inputs to the striatum are divided depending on which region the afferents are from.

In humans, the striatum is divided into distinct regions including the caudate and putamen. The caudate head receives inputs from the frontal lobe, including the premotor cortex and
supplementary motor areas. The putamen also receives some input from the frontal lobes, including the primary motor and somatosensory cortices. The caudate body receives projections from the occipital lobe, and the caudate tail receives input from the temporal lobe. These divisions are even further segregated in motor regions giving rise to a motor homunculus in the striatum that correlates to that found in the motor cortex. This topographical projection from the cortex to the striatum is one reason why the caudate nucleus is traditionally associated with cognition while the putamen has been associated with motor control. There is, however, complex interactions between cortical connections and connections within and between the caudate and putamen that challenge this traditional view, suggesting integration between cognition, motor function, and reward (Haber, 2016).

In rodents, the dorsal striatum is one of the largest undivided structures and does not lend itself to subdivision by traditional means. Here, the dorsolateral striatum is sometimes referred to as the caudo-putamen because its role is similar to that detected in humans, although separate caudate and putamen regions cannot be identified anatomically. Despite this lack of formal division, a similar topography of the descending cortico-striatal projections along the rostral/caudal, dorsal/ventral, and medial/lateral axes has been mapped in the mouse brain (Hintiryan et al., 2016; Mathis et al., 2017).

Within the striatum there are two major neuronal cell types: MSNs and interneurons. MSNs are GABAergic projection neurons and are the primary recipients of striatal afferents from the cortex and thalamus. These MSNs are grouped into D₁ and D₂-MSNs depending on which dopamine receptor type they express. Interneurons form connections entirely within the striatum between
MSNs. These interneurons are further divided into CINs and GABAergic interneurons. Lastly, the striatum receives dopaminergic inputs from both the VTA and the SNc. Classically, the SNc was considered to project dopaminergic axons into only the NAc affecting reward, while the VTA projected only into the dorsal striatum affecting movement. More recent research has suggested that these dopaminergic afferents are better represented as a spectrum of SNc/VTA to NAc/striatum connections affecting both reward and motor functions (Morikawa & Paladini, 2011). The two outputs from the striatum via MSNs are known as the direct and indirect pathways.

3.5.1 D₁-MSNs and the direct pathway

The direct pathway is formed by the axonal long-range projections of D₁-MSNs. D₁-MSNs form a feedback loop circuit in which they receive excitatory inputs from the sensorimotor cortex and thalamus, and project directly to the internal capsule of the globus pallidus (GPi). The GPi inhibits the thalamus, which sends excitatory projections to the primary motor cortex, meaning that the net effect of exciting the D₁-MSNs is to excite the cortex as positive feedback. In addition, D₁-MSNs directly inhibit the substantia nigra pars reticulata (SNr), the primary output of the basal ganglia to the motor thalamus. This has led to the traditional role of the direct pathway being hypothesized as necessary for action initiation. Experiments involving optogenetic activation of striatal circuits during action sequences have shown that the direct pathway increases activity in the motor cortex while no modulation was seen during optogenetic activation of the indirect pathway. Similar experiments presented independent of movement resulted in modulation in the motor cortex when both pathways were activated, but a fraction of cortical neurons remained unaffected by each pathway, suggesting distinct regions of the motor cortex affected by each pathway (Ian Oldenburg et al., 2015).
3.5.2 D2-MSNs and the indirect pathway

The indirect pathway is formed by the axonal long-range projections of D2-MSNs. Like D1-MSNs, D2-MSNs also receive excitatory inputs from the sensorimotor cortex and thalamus to form a feedback loop. They project onto the external segment of the globus pallidus (GPe). The GPe inhibits the sub-thalamic nucleus (STN), which in turn excites the GPi. The GPi connection to the thalamus and ultimately the primary motor cortex is shared by both pathways. Recently, the role of the STN in this chain of events has been disputed, with evidence that the GPe neurons directly inhibit the GPi allowing inhibiting a specific response as opposed to a broad, more global modulatory function (Cohen & Frank, 2009). Through this chain of inhibition and excitation, the net effect of excitation of D2-MSNs is to inhibit the cortex as a form of negative feedback. The indirect pathway is formed where D2-MSNs inhibit the STN which in turn excites the SNr, and has been suggested to suppress movement. Prior to action initiation, 5 s x 5 Hz and 14 Hz optogenetic excitation and inhibition of the direct pathway both slow or pause action initiation, while the same excitation and inhibition of the indirect pathway both resulted in aborted action and action selection switching. However, when that optogenetic inhibition of either pathway occurred after initiation impaired ongoing execution. After action initiation, excitation of the indirect pathway sustained performance of learned behaviors while the activation of the direct pathway caused aborted performance. This suggests that both pathways functioning in a coordinated manner are necessary for proper action initiation (Tecuapetla et al., 2016). Other optogenetic experiments showed transient excitation preceding decreased activity in the motor cortex caused by a subset of cells when activating the indirect pathway, challenging the accepted role as purely suppressive of motor activity; rather than performing a NO-GO function, the indirect
pathway coordinates with the direct pathway and inhibits competing motor signals (Ian Oldenburg et al., 2015).

### 3.5.3 Other local pathways

In addition to the excitation input from the cortex, the striatum receives other input and also forms local pathways and networks within itself. D$_1$ and D$_2$-MSNs synapse onto each other; receive cholinergic input from CINs and neurons projecting from the LDT and PPT; and dopaminergic input from the VTA and SNc. The dopaminergic inputs have direct and indirect effects on the MSNs of the striatum. Dopamine bursts pause the tonic CIN firing, activate D$_1$-MSNs, and inhibit D$_1$-MSNs due to different results from different dopamine receptor subtypes (Chuhma et al., 2014). MSNs form GABAergic synapses onto dopaminergic afferent axons within the striatum, providing a local negative feedback loop on dopaminergic modulation (Kramer et al., 2020). CINs also synapse onto these dopaminergic afferents to help control dopaminergic modulation (Rice & Cragg, 2004; Zhang & Sulzer, 2004).

Ongoing experiments including immuno-fluorescent imaging and RNA-scope in our lab suggest that EAAC1 is expressed in D$_1$-MSNs but not D$_2$-MSNs. It is the intent of this thesis to explore the impact of EAAC1 on reward-motivated behaviors through the combination of previously mentioned factors: (1) loss of dopamine projection to the striatum; (2) the effects of dopamine on CINs and MSNs; and (3) the reciprocal effects of CINs and MSNs on dopamine release. We analyzed variations of lever-press experiments to measure effects on ability to learn a task, time to complete longer behavior sequences, and speed to initiate performance of those sequences when cued.
4 Materials and methods

4.1 Ethics statement

All experimental procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at the State University of New York at Albany and guidelines described in the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals.

4.2 Mice

Unless otherwise stated, all C57BL/6NCrl mice of either sex, aged 8 weeks were group housed and kept under 12H:12H L:D conditions (lights on at 7 AM; lights off at 7 PM). Food and water were available ad libitum to all mice throughout each 24 hour period. Both WT and EAAC1−/− mice were bred in house, and sampled from multiple litters.

4.3 Genotyping

Routine genotyping was performed on all the mice before they were used in any experiments. Experimental mice were genotyped between the ages of P6 and P12 with tissue taken from their toes, while breeder mice were retyped using tail tissue post-P12. Tissue was digested overnight in DNA extraction buffer (100 mM Tris pH 8, 5 mM EDTA, 200 mM NaCl, 0.2% SDS) containing Proteinase K diluted 1:200 at 55°C overnight with constant shaking. Samples were then held for 10 min at 97°C to deactivate the Proteinase K and centrifuged for 5 min at 13,000 RPM at 4°C. From these centrifuged samples, 20 µl of supernatant was added to 20 µl of nuclease-free water. The diluted DNA extracts were then processed for PCR analysis by adding commercially obtained primers and HotStart Taq polymerase was used (Cat# KK5621; KAPA Biosystems, Wilmington,
MA). 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<sup>+/+</sup>: 358 bps, EAAC1<sup>−/−</sup>: 680 bps).

4.4 Behavioral tests

We used 7 µl water as a reward for all reward-based behavior tests. Three days prior to the first test, water in the home-cage dispenser was replaced with 2% citric acid (w/v), so that water alone could then be perceived as a reward (Reinagel, 2018). We measured changes in body weight induced by this procedure. Typically, a 20% body loss occurred immediately after citric acid dispensation, which recovered in 7 days. Behavioral tests were conducted during the light-cycle hours for ten minutes per session at most one training session per day based on availability of data collectors. Lighting in the experimental conditions matched those in home-cage conditions using ambient lighting in the animal facility. All behavior tests were conducted in a two-sided custom 3D-printed operant conditioning chamber (Length: 19 cm, Width: 14.5 cm, Height: 13 cm). Each chamber contained one lever and one water valve controlled by custom code in MATLAB 2019b (MathWorks, Natick, MA) for the Bpod state machine r2 (Sanworks, Stony Brook). During each behavior test, the mouse had free access to the lever and water valve. The consequences of pressing the lever varied by experiment. The first session of training was used for experimental data collection and for acclimation to the chamber. No confounding effects from novelty were expected as novelty increases the impact of dopamine response encoding RPE (Schultz, 2016).
4.4.1 Lever press experiments

To train the mice to press the lever, and to analyze differences in learning and performance of this trained task, we used a simple lever press experiment. Seventeen WT mice (eight male and nine female from seven total litters) and eighteen EAAC1\(^{-/-}\) mice (eight male and ten female from five total litters) were tested. During the lever press experiment, each press resulted in delivery of water reward. In this task, mice received reinforcement (water) each time they pressed the lever on a continuous reinforcement schedule, also known as fixed ratio 1 (FR1) schedule (Fig. 1A). Each mouse was tested for a total of nine sessions. For each trial, the total number of lever presses and the time of each lever press (measured from the time of chamber entry) was measured. Number of presses per trial were analyzed (Fig. 1B-D, left) as well as the temporal profile of the presses within the first (Fig. 1B-D, middle) and last (Fig. 1B-D, right) session to determine the differences between WT and EAAC1\(^{-/-}\) mice in both the naïve and trained state.

4.4.2 Sequence lever press experiments

Because other research in our lab indicates a difference in expression of EAAC1 in D1- and D2-MSNs, we expected EAAC1\(^{-/-}\) mice to exhibit behavior differences reflecting similarities to those in which the direct pathway was manipulated. We designed an experiment modeled after the experiments in Tecuapetla et al., 2018, in which optogenetic manipulations of both circuits were performed on mice during FR8 lever press tasks. Fourteen WT (ten male, four female, from seven different litters), and twelve EAAC1\(^{-/-}\) mice (ten male, two female, from four different litters) were used. Mice were first trained in the FR1 experiment, then placed on an FR8 lever press schedule in which the water reward was delivered only after the lever had been pressed eight times (Fig. 2A). We analyzed the total number of presses and rewards per session (Fig. 2C), the temporal
profile of the presses and rewards within the first (Fig. 2D) and last (Fig. 2E) session, the average
time taken to perform each 8-press sequence (Fig. 2F, left), and the time between sequences (Fig. 2F, right).

4.4.3 Timed sequence lever press experiments

To characterize further the time-course of behaviors, and to determine whether increased presses were based on compulsion or reward-motivated, we designed a time-sensitive sequence experiment. Mice that had not been tested in the sequence press experiment, but that had been trained previously on the FR1 schedule, were tested in a timed sequence lever press task. Fifteen WT (five male and ten female from six different litters) and fifteen EAAC1−/− mice (five male and ten female from three different litters) were analyzed. Rewards in this experiment were delivered only if all eight presses occurred within 6 s after the first lever press. If the timer ran out before 8 lever presses were completed, the counter was reset (Fig. 3A). We analyzed number of total presses (Fig. 3D, left), number of presses that were part of a rewarded sequence (Fig. 3D, middle), and number of presses that occurred outside of a rewarded sequence (Fig. 3D, right). We also analyzed the temporal profile of total presses, total rewards, and out-of-sequence presses in session 1 and session 10 (Fig. 3C).

4.4.4 Cued sequence lever press experiments

Based on the results of the previous experiments, we hypothesized that EAAC1−/− mice started new sequences based on faster response to reward-motivated behavior cues. We used the behavioral experiments in Ian Oldenburg et al., 2015 to create an experiment with reward, neutral, and punishment periods of similar design criteria. We used mice which were previously trained
using the FR1 schedule but not in other experiments. Twelve WT (eight male and four female from seven different litters) and ten EAAC1\textsuperscript{-/-} mice (seven male and three female from three different litters) were analyzed. In this experiment, an LED housed within the water valve unit was turned on for 6 s, to indicating the reward period. During the reward period, a single lever press would result in delivery of a water reward and transition to the neutral period. The neutral period consisted of a 2 s time interval at the end of the reward period, either due to time expiring or the lever being pressed. At the conclusion of the neutral period, a punishment period began. During the 2 s punishment period, pressing the lever resulted in resetting the timer for the punishment period. When the punishment period finished, the LED would turn on signifying another reward period (Fig. 6A). Mice were assessed on latency to press after the LED cue turned, as well as the reduction in response during the punishment period over time (Fig. 6 B-E).

4.5 Data acquisition and analysis

Experimental data from behavioral tests were automatically exported from MATLAB as .xlsx files and copied into Igor Pro 6.37 for analysis. All data shown in this thesis is presented as mean±S.E.M., unless otherwise stated. Within and between group analysis of genotype and sex was performed in Microsoft Excel for Mac version 16.53.1. Student’s paired and unpaired t-tests were conducted using Igor Pro 6.37 to determine statistical significance; p<0.05 (*p<0.05; **p<0.01; ***p<0.001) was considered statistically significant.
5 Results

5.1 Loss of EAAC1 affects the timing of reward-based behaviors

Anatomical data shows that EAAC1 is expressed in the striatum, but its implication for striatal-based behaviors remains unknown (Holmseth et al., 2012). Since the striatum is involved in the execution of reward-based motor tasks, we first tested 8-12 week old male and female WT and EAAC1−/− mice with a simple fixed ratio 1:1 (FR1) reward task. Here, mice were tested over a 10 min time window and received a 7 µl water reward every time they pressed a lever (Fig. 1A).

From session 1 (S1) to session 9 (S9) of the FR1 lever press experiments, WT and EAAC1−/− mice had a similar number of lever presses and improved over the course of training by similar amounts (WT: S1 3.9±1.0, n=18, S9 100.3±5.7; EAAC1−/−: S1 6.3±1.7, S9 100.7±5.5, n=17; WT versus EAAC1−/− p=0.27; Fig. 1B, left). However, when we analyzed the timing of the lever presses, we noticed that EAAC1−/− mice pressed the lever sooner over the course of the 10 min duration of this test. This can be detected in Fig. 1B (middle: S1; right: S9), where we overlaid a frequency count of the lever presses in a binned time interval of 20 s (left axis) with a cumulative plot of the lever presses at different times (right axis). The differences in the timing of the lever presses in S1 and S9 were significantly different (S1: ***p=5.1e-6; S9: ***p=1.4e-5, cumulative presses). Two-factor repeated measure ANOVA showed no significant differences in performance between sexes during FR1 testing.

These results are important, because they suggest that EAAC1 may control the timing with which mice engage in reward-motivated behaviors, with no effects on the overall performance or learning. The results also indicate that there is not difference in satiety between WT and
EAAC1<sup>−/−</sup> mice, based on the fact that the number of lever presses remains comparable between these two groups.

### 5.2 Loss of EAAC1 results in shorter time between lever-press sequences

Because EAAC1<sup>−/−</sup> mice exhibit repetitive stereotyped behaviors (Bellini et al., 2018), we hypothesized they would press more than WT mice in a lever press task that required repetitive pressing. To test this, we designed the FR8 experiment in which mice previously trained in the FR1 experiment needed to perform 8 lever presses to obtain a single reward. While we did find that EAAC1<sup>−/−</sup> mice received more rewards total, we were surprised to see that this difference was due to shorter inter-sequence intervals as opposed to faster performance of a sequence. Over the course of training from S<sub>1</sub> to S<sub>10</sub> in the FR8 lever-press task, WT and EAAC1<sup>−/−</sup> mice both increased the number of their lever presses, but EAAC1<sup>−/−</sup> mice consistently pressed significantly more throughout training (WT: S<sub>1</sub> 269.3±41.5, S<sub>10</sub> 433.1±28.4, n=14; EAAC1<sup>−/−</sup>: S<sub>1</sub> 361.3±50.6, S<sub>10</sub> 503.3±33.3, n=12; ***p=4.5e-6; Fig. 2C, left). Two factor ANOVA showed a significant effect of sex on press and reward F(1, 36)=13.05, p=9.18e-4, a significant effect of genotype on press and reward F(1, 36)=74.06, p=2.92e-10, and a significant combined effect F(1, 36)=57.57, p=5.65e-9. However, power analysis shows that the analysis of sex is grossly underpowered, and this statistical significance cannot be used to imply a real effect without further experiments with larger samples. The difference in the number of presses/rewards was due to the fact that EAAC1<sup>−/−</sup> mice began a new sequence of lever presses sooner than WT mice (WT: S<sub>1</sub> 5.9±1.6 s, S<sub>10</sub> 5.3±0.7 s, n=14; EAAC1<sup>−/−</sup>: S<sub>1</sub> 3.3±0.7 s, S<sub>10</sub> 3.4±0.6 s, n=12; ***p=1.7e-4; Fig. 2F, right). By contrast, the speed with which the sequences were performed was similar between mice strains (WT: S<sub>1</sub> 24.4±6.3 s, S<sub>10</sub> 6.3±0.6 s, n=14; EAAC1<sup>−/−</sup>: S<sub>1</sub> 14.4±3.5 s, S<sub>10</sub> 6.6±1.0 s, n=12; p=0.05;
This showed us that the timing difference observed in the FR1 experiment was due to a faster initiation of a new sequence after receiving a reward.

5.3 Loss of EAAC1 results in more rewards during time-sensitive repetitive reward task

To further explore the differences in time-courses observed in previous behavior tasks, we designed the timed sequence experiments to require all presses within the FR8 paradigm to be performed within a 6 s window which began when the first press of the sequence was performed (Fig. 3-5A). Due to the possibility of a complete lack of reward if the mice did not perform within the allotted 6 s, we were not surprised that some mice exhibited extinction of the lever press behavior (WT: 60% extinction rate, n=15; EAAC1^{-}/: 54% extinction rate, n=15; Fig. 3-5B, left). Of the mice that were able to successfully perform the timed sequence task, while both groups increased the number of presses and rewards over the course the training from S_1 to S_{10}, EAAC1^{-}/ mice consistently performed more total lever presses (WT: S_1 150.5±32.7 presses, S_{10} 384.0±50.5 presses, n=6; EAAC1^{-}/: S_1 284.9±67.0 presses, S_{10} 471.7±61.6 presses, n=7; ***p=7.4e-6; Fig. 4C-D, left) and received more rewards than WT mice (WT: S_1 1.5±0.8 rewards, S_{10} 27.0±10.4 rewards, n=6; EAAC1^{-}/: S_1 17.4±7.1 rewards, S_{10} 44.1±9.5 rewards, n=7; ***p=4.5e-8; Fig. 4C-D, middle) as in the FR8 experiment with no time dependence. Of particular note, by S_{10}, EAAC1^{-}/ mice were performing significantly fewer lever presses that were not part of a rewarded sequence (*p=0.02; Fig. 4C, bottom right). Two-factor repeated measure ANOVA showed no significant differences in performance between sexes in timed sequence testing. We hypothesize that a compulsive increase in lever pressing would have increased the number of presses both within and outside the reward period, and that this provided further evidence that the larger number of lever presses performed by EAAC1^{-}/ mice was not due to compulsion-like
behaviors. Had the reduced inter-sequence interval been due to compulsion-like repetition of the lever press behavior, we would have expected to see a larger number of presses performed by EAAC1<sup>−/−</sup> outside of rewarded sequences. In fact, both strains showed extremely similar numbers and time-course for presses outside of rewarded sequences during session 1 (p= 0.83; Fig 4C, top right).

5.4 Loss of EAAC1 results in a faster response to visually cued behavior

Based on the results of the previous trials, we hypothesized that the reduced time for EAAC1<sup>−/−</sup> mice to begin a new sequence of presses was due to a faster reaction to implicit cues that reward was available again (in previous trials this cue would be the water reward, indicating they may start again). To test this, we designed a cued experiment in which the mice would have six seconds to press the lever in response to a visual cue (illuminated LED) (Fig. 6A). A punishment period, beginning two seconds prior to the beginning of the next cued reward period was included to reduce the likelihood of the mice simply learning the normal time between cued trials, as well as to measure any difference between WT and EAAC1<sup>−/−</sup> mice in response to a subtle punishment stimulus. Over the course of training from S<sub>1</sub> to S<sub>10</sub> both groups increased the number of rewards received (WT: S<sub>1</sub> 58.4±5.9 rewards, S<sub>10</sub> 91.1±4.5 rewards, n=11, ***p=3.8e-4; EAAC1<sup>−/−</sup>: S<sub>1</sub> 62.9±5.7 rewards, S<sub>10</sub> 82.1±4.5 rewards, n=10; *p=0.04; no significance between groups, p=0.92; Fig. 6D). It was unsurprising that both groups increased the total number of rewards received over the course of training, but of interest: EAAC1<sup>−/−</sup> mice responded significantly faster to the cue over the course of training while WT mice did not (WT peak response: S<sub>1</sub> 0.4±0.1 s, S<sub>10</sub> 0.3±0.1 s, n=11, p=0.78; EAAC1<sup>−/−</sup> peak response: S<sub>1</sub> 0.8±0.2 s, S<sub>10</sub> 0.3±0.1 s, n=10, *p=0.02; Fig. 5E). This supports the hypothesis that EAAC1<sup>−/−</sup> mice respond faster
than WT to external stimuli which signal the opportunity to initiate a behavior. Because no significant differences were seen in punished presses between any groups (Fig 6D, right), and because the addition of punishment to this experiment added a great deal of complexity, we plan to repeat this experiment in the future with a simpler design which lacks the punishment aspect. Sex differences were not analyzed in the current design but will be analyzed in the upcoming simplified experimental design.
6 Discussion

There is ongoing contention over the functional role of the neuronal glutamate transporter EAAC1 in the brain. Immunolabeling and western blot approaches show they constitute only 1% of all glutamate transport in the brain (Holmseth et al., 2012), leading Holmseth and Danbolt to doubt the relative importance of the neuronal glutamate transporter. Additionally, electrophysiological recordings show that there is no detectable current mediated by these transporters in CA1 hippocampal pyramidal cells (Bergles & Jahr, 1998; Holmseth et al., 2012). Despite this, Scimemi et al. show that EAAC1 can limit spillover onto extrasynaptic glutamate receptors in the hippocampus and dorsolateral striatum (Bellini et al., 2018; Scimemi et al., 2009). Holmseth et al. show that while EAAC1 is not highly expressed in general, it is most abundantly expressed in the striatum, cortex, thalamus, and hippocampus. In 2014, Wang et al. showed significant expression of EAAC1 in the amygdala using commercially available rabbit polyclonal antibody (Holmseth et al., 2012; Wang et al., 2014). The hippocampus is known for its important roles in spatial learning and memory consolidation, the striatum is important for reward-mediated learning and motor function, and the amygdala is the primary input for emotional valence processing. These functions give context to and support previous behavioral findings showing EAAC1−/− mice exhibit impaired fear memory consolidation, increased anxiety-like behavior, and repetitive habitual behaviors (Bellini et al., 2018; Lee et al., 2012; Wang et al., 2014).

There are two lines of evidence that can be used to reconcile the seemingly paradoxical findings about EAAC1. First, EAAC1 is located in dendrites and transporter currents are heavily filtered as they propagate from the dendrites to the cell soma during whole-cell patch clamp recordings. This means that the glutamate transporter mediated currents may be large locally (i.e. at the
synapse), but attenuated as they propagate towards the soma. If EAAC1 is, in fact, responsible for more significant glutamate transport than indicated by Bergles & Jahr and Holmseth et al.’s data, then this transport may play an important role in the behavioral functions described due more to localized expression as opposed to overall abundance. Second, like all other glutamate transporters the glutamate translocation efficiency of EAAC1 is only 50%. This means that only 50% of all bound glutamate molecules are eventually translocated across the plasma membrane. The remaining 50% are released back in the extracellular space. Consequently, EAAC1 can prolong the lifetime of glutamate in the extracellular space, which is eventually taken up by abundantly expressed glial glutamate transporters. The ability of EAAC1 to prevent spillover depend critically on the ability of this transporter to bind glutamate, not necessarily on its ability to translocate it across the membrane. Experimental evidence that EAAC1 limits glutamate spillover away from the synaptic cleft was first provided by Diamond, 2001. His experiments relied on the use of the broad-spectrum glutamate transporter antagonist TBOA, because to date there are no specific antagonists that target EAAC1 without affecting glial transporters. However, his findings were later validated by Scimemi et al, 2009 using constitutive EAAC1−/− mice.

The alternative hypothesis in the field for the behavioral effects of loss of EAAC1 is that the metabolic role is more significant than its ability to transport or buffer glutamate. EAAC1 is one of three pathways for cysteine uptake in neurons, along with XAG- and ASC Na+-dependent systems (Shanker et al., 2001; Watts et al., 2014). This argument states that EAAC1’s contribution to cysteine uptake is of sufficient magnitude that the loss of EAAC1 results in insufficient cysteine uptake, especially by dopaminergic neurons in the VTA and SNc. This insufficient uptake of cysteine in turn leads to insufficient production of glutathione, a powerful antioxidant. The lack of glutathione results in oxidative stress causing death or loss of function of these dopaminergic
neurons, and this loss of dopamine functionality supposedly describes the behavioral effects observed (Aoyama et al., 2006; Berman et al., 2011). EAAC1 is not expressed at elevated levels in these dopaminergic regions, and ongoing experiments in our lab have been unable to reproduce Berman et al.’s results. For these reasons, we are cautiously skeptical of the metabolic impact and choose to more aggressively explore the loss of buffering action and increased synaptic spillover as more likely mechanisms of the behavioral phenotypes observed.

6.1 Loss of EAAC1 results in a previously undescribed behavioral phenotype

Previous descriptions of behavioral phenotypes expressed by EAAC1−/− mice have focused on hippocampus-dependent or amygdala-regulated behaviors (Lee et al., 2012; Wang et al., 2014) or the behaviors dependent on the dorsolateral striatum, responsible largely for habitual motor function (Bellini et al., 2018). Our behavioral experiments focus on functions controlled by both the dorsolateral and the ventromedial striatum, responsible for goal-motivated behavior (Redgrave et al., 2010). Our findings show that EAAC1−/− mice have an impulsive behavioral phenotype in addition to previously described increases in anxiety-like behaviors and stereotyped grooming behaviors (Bellini et al., 2018; Lee et al., 2012; Wang et al., 2014). This results in a faster initiation of goal-motivated motor action after the cue for that action is presented. In the simple lever press experiment, this manifested in an overall shorter latency to lever press. The fact that both WT and EAAC1−/− mice reached a similar level of lever presses in this experiment suggests this effect is not related to differences in satiety but is indeed a difference in motor-movement initiation speeds, which suggests a difference in motivation salience; i.e., these differences are regulated by dopaminergic effects. In sequence lever press experiments, this new behavioral phenotype presented as a reduced time to begin a new sequence (pressing the lever after receiving reward
from the previous sequence) with the reward from one sequence serving as a cue to begin the next. That EAAC1<sup>−/−</sup> mice received a greater number of rewards in these experiments is likely due to the 10 minute experimental duration being insufficient to reach satiety, which is consistent with analysis of no apparent slowdown in representative raster plots of presses during these experiments (Fig. 2B and 4B). The experiment which most apparently quantified this behavioral phenotype is the cued lever press experiment in which EAAC1<sup>−/−</sup> mice directly showed a faster response to a visual cue once the cue was learned.

The discovery of a significant behavioral phenotype suggests that the relative abundance of EAAC1 transporters in different brain regions, and the specific location of EAAC1 within the synapse, plays a larger role than its total membrane concentration. This explanation is supported by the deficiencies observed in the fear conditioning in EAAC1<sup>−/−</sup> mice (Lee et al., 2012; Wang et al., 2014) in which hippocampal memory consolidation is affected. Goal-driven and habitual behaviors are mediated by separate circuits within the striatum (Redgrave et al., 2010). Over the course of learning, which circuit controls a given behavior changes as the subject progresses from a naïve to experienced learner and the task shifts from goal-oriented to habitual (Lehéricy et al., 2005). While Bellini et al. explored habitual behavioral effects due to loss of EAAC1 in the dorsolateral striatum, our experiments are the first to explore goal-driven ventromedial striatal learning effects. It is interesting to compare the differences in the effect of the loss of EAAC1 on behaviors controlled by different brain regions; specifically that while hippocampal-dependent behavioral effects are diminished, striatal-dependent behavioral effects are enhanced. We see the increase of habitual behaviors controlled by the dorsolateral striatum evident in Bellini et al.’s data and a reduced time to act controlled by the ventromedial striatum in our results. The different
results between hippocampal and striatal effects might be due to the difference in the types of neurons mediating different types of motor behaviors, and location of EAAC1 within these cells. The hippocampus contains both excitatory and inhibitory synapses which express EAAC1 peri-synaptically and post-synaptically at excitatory synapses and pre-synaptically at inhibitory synapses (He et al., 2000). In the striatum, EAAC1 would only be capable of acting on the excitatory synapses from the cortex and thalamus onto MSNs. While EAAC1’s localization relative to synapses in the striatum is still unverified, it is reasonable to assume the peri-synaptic expression on the post-synaptic membrane seen at excitatory synapses in other brain regions holds true. If so, the lack of pre-synaptic expression in the striatum may explain why striatal learning is affected in an opposite manner of hippocampal learning upon loss of EAAC1.

6.2 Evidence for behavioral significance of synaptic spillover

While it is no longer questioned whether synaptic spillover from glutamatergic synapses occurs (Diamond, 2001) the question of whether spillover is of physiological and behavioral? significance has remained controversial (Okubo & Iino, 2011). Further, it has recently been established that dopaminergic afferents in the striatum are sensitive to GABA via GABA\textsubscript{A} receptors (Kramer et al., 2020) in addition to cholinergic modulation via nicotinic receptors (Rice & Cragg, 2004; Zhang & Sulzer, 2004). The buffering mechanism by which EAAC1 may reduce synaptic glutamate spillover and increase glial uptake proposed by Scimemi et al. in 2009 should in turn increase the glial recycling of glutamate into glutamine. In the striatum, this glutamine is used by MSNs to synthesize GABA. EAAC1, therefore, would play an important role in the adequate production of GABA. EAAC1\textsuperscript{−/−} mice may produce reduced levels of GABA in striatal MSNs, which in turn reduces GABAergic inhibition of dopaminergic afferents in the striatum. Our experimental results
are consistent with this hypothesis. Reduced dopaminergic inhibition of dopaminergic afferents in the striatum would increase the size of dopamine spikes during lever press tasks. This would explain increased motivation of the new behavioral phenotype we have observed in EAAC1\(^{-}\) mice based on the correlation between dopamine spike size and motivation (Collins et al., 2016). If this hypothesis is supported by future experiments, it would be the first time that a large-scale physiological effect of synaptic spillover has been so demonstrated.

### 6.3 Future directions

Our future goals are to determine the molecular basis for the observed impulsive initiation of reward-motivated action behavioral phenotype. We hypothesize that this behavioral phenotype arises from a difference in dopaminergic modulation within the striatum. There are several methods of dopaminergic modulation previously mentioned which we plan to explore. First, to verify a difference in dopaminergic activity in the striatum between WT and EAAC1\(^{-}\) mice, we will be conducting imaging experiments using an optical dopamine sensor, dLight (Patriarchi et al., 2018). This will confirm or refute the hypothesis that the behavioral differences noted by our experiments result from increased dopamine release. This is an important demonstration, as the only previously explored link between EAAC1 and dopamine is a reduction of D_{1}R expression shown in EAAC1\(^{-}\) mice (Bellini et al., 2018).

Next, we will be performing electrophysiological experiments with pharmaceutical controls to determine the contribution of both cholinergic and GABAergic modulation of dopaminergic afferent axons in the striatum in both EAAC1\(^{-}\) mice and WT mice. Specifically, we will measure dopamine release in the striatum when stimulating either CINs or MSNs in the presence of
pharmaceutical blockers of cholinergic or GABAergic receptors. This will allow us to support or reject the hypothesis that synaptic spillover in EAAC1−/− mice results in reduced production of GABA, as well as elucidating the relative roles of GABAergic and cholinergic modulation of dopamine release in the striatum and how they differ between wild type and EAAC1−/− mice.

Finally, we will explore the relevant differences in dopaminergic, cholinergic, and GABAergic striatal activity in vivo between EAAC1−/− and WT mice while they perform lever press tasks using fiber photometry. This will provide direct evidence for the increased dopamine spike size and earlier time course of dopamine spiking in EAAC1−/− mice compared to WT. At the conclusion of all planned experiments, we should have a much more complete understanding of the role of EAAC1 on molecular, cellular, and behavioral levels.
7 Figures and figure legends

**Figure 1. Loss of EAAC1 affects the timing of reward-based behaviors.**

**A.** Experiment conditions: mouse is in operant conditioning chamber with lever and valve, each press gives one reward.

**B.** WT and EAAC1−/− mice reach similar numbers of presses over training, EAAC1−/− mice press sooner.

**C.** Results among only males is similar but less pronounced.

**D.** Results among only females is similar but more pronounced.
Figure 2. Loss of EAAC1 results in shorter time between lever-press sequences. A. Experimental conditions: mouse is in operant conditioning chamber with lever and valve, any sequence of 8 lever presses results in water reward. B. Representative raster plots of each session for WT (Willow) and EAAC1⁻/⁻ (Kobe). C. EAAC1⁻/⁻ mice perform more total lever presses and receive more total rewards. D. EAAC1⁻/⁻ mice consistently press more and receive more rewards throughout S1. E. EAAC1⁻/⁻ mice consistently press more throughout S10. F. WT and EAAC1⁻/⁻ mice take similar times to perform each sequence, EAAC1⁻/⁻ mice start new sequences sooner.
Figure 3. Loss of EAAC1 increases rewards received during time-sensitive repetitive reward task. A. Experimental conditions: mouse is in operant conditioning chamber with lever and valve. A sequence of 8 lever presses results in a water reward if it is completed within 6 s of sequence initiation. B. Percentage of mice by genotype that experienced extinction (left). Representative Raster of EAAC1<sup>−/−</sup> (Klara) and WT (Whiteclaw) performance in sessions 1 and 10 (right). C. EAAC1<sup>−/−</sup> mice performed more presses and got more rewards compared to WT in both S1 and S10. D. EAAC1<sup>−/−</sup> mice consistently performed more lever presses and received more rewards throughout all sessions compared to WT. No significant differences between the number of presses occurring outside of timed intervals.
Figure 4. Loss of EAAC1 increases rewards received during time-sensitive repetitive reward task in task-performing mice. A. Experimental conditions: mouse is in operant conditioning chamber with lever and valve. A sequence of 8 lever presses results in a water reward if it is completed within 6 s of sequence initiation. B. Percentage of mice by genotype that experienced extinction (left). Representative Raster of EAAC1−/− (Karma) and WT (Wharton) performance in sessions 1 and 10 (right). C. EAAC1−/− mice performed more presses and got more rewards compared to WT in both S1 and S10. D. EAAC1−/− mice consistently performed more lever presses and received more rewards throughout all sessions compared to WT. No significant differences between the number of presses occurring outside of timed intervals.
Figure 5. Loss of EAAC1 does not affect rewards received during time-sensitive repetitive reward task in task in non-performing mice. A. Experimental conditions: mouse is in operant conditioning chamber with lever and valve. A sequence of 8 lever presses results in a water reward if it is completed within 6 s of sequence initiation. B. Percentage of mice by genotype that experienced extinction (left). Representative Raster of EAAC1−/− (Kayden) and WT (Willa) performance in sessions 1 and 10 (right). C. Both groups performed similar number of lever presses in S1, WT performed nearly twice as many presses in S10, neither group received many rewards in either session D. Both groups showed typical extinction curves due to lack of reinforcement on average, with individuals experiencing typical extinction bursts.
Figure 6. Loss of EAAC1 results in a faster response to visually cued behavior. A. Experimental conditions: mouse is in operant conditioning chamber with lever and valve. LED illuminates for 6 s, during which a lever press results in reward. Immediately after the 6 s interval or if a lever press occurs, a 2 s neutral period begins during which a lever press has no effect. Last, a 2 s punishment period begins, which is reset by any lever press. At the conclusion of the punishment period, the LED illuminates again. B. Normalized proportion of presses. Both EAAC1<sup>−/−</sup> and WT mice showed a reduction in punishment and an increase in reward. C. Over the course of training, both groups reduced punishment and increased reward. EAAC1<sup>−/−</sup> mice pressed the lever more quickly after the cue appeared. D. Both groups increased number of total presses between sessions 1 and 10. E. EAAC1<sup>−/−</sup> mice reduced the time to press after cue appeared while WT mice did not.
8 References


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