

Investigating the Role of Obsessive-Compulsive Disorder Candidate Gene
SLC1A1 in Basal Ganglia and Repetitive Behavior

By

Isaac D. Zike

Dissertation

Submitted to the Faculty of the
Graduate School of Vanderbilt University

In partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

In

Pharmacology

May 31, 2017

Nashville, Tennessee

Approved:

Ariel Y. Deutch, Ph.D.

Gregg Stanwood, Ph.D.

Carrie Jones, Ph.D.

James W. Bodfish, Ph.D.

Christine Konradi, Ph.D.

Jeremy Veenstra-VanderWeele M.D.

I want to dedicate this work to my family,
Mom, Dad,
Gregg, Katharine, Dean, Landon,
Josh, Sarah, Grace, Andrew,
Lauri

There are aspects of all of you in this work.

ACKNOWLEDGEMENTS

There are too many people to thank for helping and supporting me through my Ph.D. training and I hope that if I forget someone, they understand. I'd first like to thank my mentor Jeremy Veenstra-VanderWeele for allowing me to join his lab. The amount of time and effort that Jeremy invested in my training and thesis work can not adequately be represented here so I just want to reiterate that I greatly appreciate everything that he has done for me. While the journey was a bit more winding than most, I would not have the opportunities that I have moving forward without him facilitating my development. Next, I want to thank the other members of my thesis committee, Dr. Ariel Deutch, Dr. Gregg Stanwood, Dr. Carrie Jones, and Dr. Jim Bodfish. This has been a long process and their mentorship during my time both in Nashville and New York City allowed me to become the person that I am now. To the current and previous members of the VVW lab, while I may not have always expressed it, this work wouldn't have been possible without you. I also want to thank Dr. Susanne Ahmari who helped develop the mouse model that my work was founded on.

Next, I would like to thank the faculty and administrative staff in the Pharmacology department at Vanderbilt University. This process wouldn't have been possible without the cumulative teaching and mentorship efforts of all of you. To the other students in the department, thank you. I couldn't have gotten through qualifying exam without everyone helping each other to study and relieve some stress when necessary. I also want to single out Karen Gieg. Her efforts behind the scenes keeping me on track, especially when I was finishing my thesis work in NYC and dealing with all of the less exciting aspects of graduate education are often overlooked but greatly appreciated.

I also need to thank my family, who has provided endless amounts of support during my graduate training. Everyone in my family gave me such strong examples of hard work and perseverance that enabled me to finally reach this point. Mom and Dad, you always had words

of encouragement that especially during the last two years helped navigate me through this process. Gregg and Josh, thank you for always coming through when I needed to vent, often times over a beer. Our trips around the country were always a great stress reliever and they helped me maintain some semblance of sanity.

To the friends that I met outside of graduate school in Nashville, thank you. The relationships that I made with all of you, especially those I played hockey with the past six years, allowed me to take my mind off of research for a few hours a week. The memories that we share will always be remembered fondly and I hope to have the opportunity to make more in the future.

Finally to Kim, I can't state how important you are to me. Moving across the country and restarting research efforts in New York City was the hardest thing that I have ever had to do. I couldn't have made it through without your time, thoughtful input, and unflagging commitment to us.

TABLE OF CONTENTS

	Page
DEDICATION	ii
ACKNOWLEDGEMENTS	iii
LIST OF TABLES.....	vii
LIST OF FIGURES.....	viii
LIST OF SUPPLEMENTARY FIGURES.....	ix
 Chapter	
1. INTRODUCTION.....	1
Introduction to the Glutamate System.....	1
Excitatory Amino Acid Transporter 3.....	6
EAAT3 in Disease	10
Obsessive-Compulsive Disorder.....	15
Convergence of Glutamate, <i>SLC1A1</i> /EAAT3 and Obsessive-Compulsive Disorder.....	22
Mouse Models of Glutamatergic Dysfunction and Repetitive Behavior	25
Specific Aims.....	30
2. RODENT MODELS OF OBSESSIVE-COMPULSIVE DISORDER	31
Introduction	32
Genetic Mouse Models	41
Immunological Models	49
Pharmacological Models	52
Optogenetic Mouse Models	56
Discussion and Future Directions	61
3. BEHAVIORAL ASSESSMENT OF EAAT3 INHIBITORS IN <i>IN VIVO</i> MODELS OF GLUTAMATERGIC AND BASAL GANGLIA DYSFUNCTION	65
Introduction	65
Methods	67
Results	72
Discussion.....	78
4. OCD CANDIDATE GENE <i>SLC1A1</i> /EAAT3 IMPACTS BASAL GANGLIA-MEDIATED ACTIVITY AND STEREOTYPIC BEHAVIOR	85
Introduction	85
Methods	88
Results	102
Discussion.....	110
5. SUMMARY, DISCUSSION, AND FUTURE DIRECTIONS	115
Summary of Results.....	116
Interpretation	122

Dopamine, Glutamate, and OCD Risk	131
Future Directions	131
Potential for EAAT3 Inhibitors in Psychiatric Disorders	136
SUPPLEMENTAL FIGURES	142
APPENDICES	158
1. NEUROCHEMICAL CHARACTERIZATION OF GLUTAMATE, GABA, AND CYSTEINE IN <i>SLC1A1</i> -STOP MICE	159
2. <i>SLC1A1</i> -STOP MICE EXHIBIT NO BASELINE BEHAVIORAL PHENOTYPES IN OCD-RELEVANT SPECIFIC ASSAYS	161
3. CONSEQUENCES OF D1-CRE AND DAT-IRES-CRE BASED RESCUE OF <i>SLC1A1</i> /EAAT3 EXPRESSION ON AMPHETAMINE INDUCED LOCOMOTOR BEHAVIOR.....	164
4. IMPLICATIONS OF A MUSCARINIC ACETYLCHOLINE RECEPTOR M4 POSITIVE ALLOSTERIC MODULATOR IN A PRECLINICAL MODEL OF OCD-RELEVANT BEHAVIOR.....	168
REFERENCES.....	171

LIST OF TABLES

Table	Page
1. Summary of Results.....	123

LIST OF FIGURES

Figure	Page
1. Stoichiometry of glutamate transport via EAAT3 and proposed topology of EAAT3	11
2. Schematics depicting the direct and indirect pathways of the basal ganglia	21
3. Evaluating Mouse Models of OCD-Relevant Behavior and Neurobiology	40
4. Sapap3 null mice express increased levels of striatal EAAT3 however protein function is unaffected.....	73
5. Pretreatment with the EAAT3 inhibitor, NBI-59159, has no affect on grooming behavior in wildtype or <i>Sapap3</i> null mice	75
6. Intracerebroventricular pretreatment with the EAAT3 inhibitor, 2-CFoDA, does not alter amphetamine-induced locomotor behavior	77
7. Direct striatal injection with the EAAT3 inhibitor, 2-CFoDA, affects amphetamine-induced locomotor response at a high, potentially sedating dose	79
8. <i>Slc1a1</i> -STOP mice have reduced <i>Slc1a1</i> /EAAT3 protein expression and function	103
9. <i>Slc1a1</i> -STOP mice have attenuated behavioral response to amphetamine and SKF-38393 challenge.....	105
10. EAAT3 loss affects amphetamine induced <i>cFos</i> expression, dopamine receptor membrane density, and extracellular dopamine concentrations in the dorsal striatum.....	109
11. Viral Cre-mediated rescue of midbrain <i>Slc1a1</i> /EAAT3 expression rescues amphetamine but not SKF-38393 phenotypes in <i>Slc1a1</i> -STOP mice	111
12. Theoretical alterations in midbrain and striatal signaling throughout development in <i>Slc1a1</i> -STOP mice.....	128

LIST OF SUPPLEMENTARY FIGURES

Figure	Page
1. Chemical Structures of EAAT3 Inhibitors.....	142
2. NBI-59159 attenuates amphetamine-Induced locomotor behavior.....	143
3. Injection sites for examining the behavioral consequences of EAAT3 Inhibitors in amphetamine-induced locomotor behavioral assays.....	144
4. No differences in glutamate uptake between wildtype and <i>Slc1a1</i> -STOP mice; high concentration cysteine reveals abolished transporter function in <i>Slc1a1</i> -STOP mice.....	145
5. <i>Slc1a1</i> -STOP mice show no changes in spontaneous behavior.....	146
6. High-dose amphetamine locomotor response is not altered in STOP mice relative to WT, and stereotypic behavior is not observed following saline administration.....	147
7. Representative coronal sections of dorsal striatum, nucleus accumbens, and somatosensory cortex for <i>cFos</i> ⁺ Quantification post-amphetamine challenge.....	148
8. D1-receptor density is not affected in the ventral striatum of <i>Slc1a1</i> -STOP mice, while D2-receptor density is reduced at a trend level in the dorsal striatum.....	150
9. No differences were identified in DAT staining or population estimates in the midbrain of STOP mice.....	151
10. Effects of amphetamine on tissue levels of dopamine and DOPAC in <i>Slc1a1</i> -STOP mice.....	152
11. <i>Slc1a1</i> -STOP mice have reduced baseline dopamine as measured via no-net flux microdialysis, and the main dopamine metabolites are not affected by amphetamine challenge.....	154
12. Injection of an AAV-Cre into the midbrain of <i>Slc1a1</i> -STOP mice results in increased EAAT3 expression.....	155
13. Cre expression is detected in the midbrain and striatum of <i>Slc1a1</i> -STOP mice injected with an AAV-Cre.....	156
14. Viral Cre-mediated rescue of dorsal striatum <i>Slc1a1</i> /EAAT3 expression does not rescue any of the dopaminergic agent behavioral phenotypes observed in <i>Slc1a1</i> -STOP mice ...	157

CHAPTER 1

INTRODUCTION

Introduction to the Glutamate System

Glutamate is an amino acid used as the primary excitatory neurotransmitter in the central nervous system (CNS) and is essential in many brain functions, including behavior, cognition, memory, and learning (Zhou and Danbolt, 2014a). Initial analyses of the molecule indicated that it was important for brain metabolism (Krebs, 1935), and researchers identified its excitatory effect on neurons in the 1950s (Hayashi, 1952, Curtis and Watkins, 1960). Despite this revelation, investigators did not recognize glutamate as the primary excitatory neurotransmitter of the nervous system until over twenty years later (Watkins and Evans, 1981, Fonnum, 1984).

In the CNS, glutamate is packaged into synaptic vesicles within the axon terminal of glutamatergic neurons. Upon the arrival of a propagating action potential at the synaptic terminus, the plasma membrane becomes depolarized and glutamate-containing synaptic vesicles undergo exocytosis. This Ca^{2+} dependent process, termed synaptic vesicular fusion, then releases glutamate into the synaptic space between two neurons, carrying cellular signals from one neuron to another in the form of glutamate (Kandel, 2013). This glutamatergic signal is then interpreted by glutamate receptors that are present on most parts of a neuron (dendrites, nerve terminals, and cell bodies) but usually with the highest density at postsynaptic densities of a synapse. Due to a lack of extracellular enzymes capable to break down glutamate, the only way to control this signal is the removal of glutamate from the extracellular space into both neurons and glial cells surrounding the synapse (Balcar and Johnston, 1972, Logan and Snyder, 1972) via glutamate transporters, also referred to as EAATs, Excitatory Amino Acid Transporters). Spatial and temporal regulation of glutamate concentrations within the CNS by

glutamate transporters is crucial to for two reasons: 1) a constant low concentration of glutamate in the extrasynaptic space allows for a high signal-to-noise ratio resulting in precise cellular communication; 2) prolonged high levels of synaptic glutamate and subsequent glutamate receptor activation is toxic to neurons (Danbolt, 2001, Mehta et al., 2007).

The molecules of glutamate transported from the extracellular space into glial cells are metabolized into glutamine via glutamine synthetase within the cytosol of glial cells or are utilized for cellular metabolism via the tricarboxylic acid cycle. Glial glutamine is then released into the extracellular fluid, taken up by neurons and reconverted back into glutamate by mitochondrial glutaminase (Daikhin and Yudkoff, 2000). Neuronal glutamate is subsequently repackaged into synaptic vesicles via the vesicular glutamate transporter for reuse as a neurotransmitter. This cycle of neuronal glutamate release and recycling through glial cells to be repackaged in neuronal synaptic vesicles is commonly referred to as the glutamine-glutamate cycle (Fonnum, 1993, Westergaard et al., 1995).

Glutamate Receptors

Molecular cloning was used to identify three different families of glutamate receptors (Hollmann et al., 1994, Hollmann and Heinemann, 1994, Borges and Dingledine, 1998, Nakanishi et al., 1998, Ozawa et al., 1998). One class of glutamate receptor was found to be activated by the glutamate analogue, *N*-methyl-d-aspartate (NMDA), thus titled NMDA receptor (Traynelis et al., 2010), and is comprised of varying combinations of structurally distinct subunits (NR1, NR2A, NR2B, NR2C, and NR2D). The second class of glutamate receptor was defined by their activation by α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate. As with NMDA receptors, AMPA receptors are also comprised of structurally distinct subunits (GluR1-4) based on their affinity for AMPA (GluR1-4) or kainate (GluR5-9, KA1-2). These two families are glutamate-gated ion channels that allow for the influx of Na^+ exclusively or both Na^+ and Ca^{2+} ions and are referred to as ionotropic glutamate receptors. Despite both

being activated by glutamate, they have distinct activation profiles and dynamics that result in differential effects on glutamatergic signaling. AMPA receptors are quickly activated by glutamate but also desensitize quickly while NMDA receptors require a depolarized membrane potential in addition to binding of the co-agonist, glycine, along with glutamate activation, for the conductance of cations; however they have a slower desensitization profile (Tang et al., 1989, Ozawa et al., 1998, Traynelis et al., 2010). The third family of glutamate receptors are metabotropic glutamate receptors. These receptors (mGluR1-8) are G-protein coupled receptors and are subdivided into groups I (mGluR1 and mGluR5, G_q -coupled), II (mGluR2 and 3, G_i -coupled), and III (mGluR4-8, G_i -coupled) (Niswender and Conn, 2010). Stimulation of mGluRs result in activation of intracellular signaling pathways resulting in altered intracellular processes, such as receptor trafficking and protein expression (Wan et al., 2011).

Excitatory Amino Acid Transporters

Due to the need to maintain spatiotemporal control of neuronal signaling, and the need to limit excitotoxicity resulting from excessive glutamatergic activation, it is logical that the CNS has highly protective systems in place to remove glutamate from the synaptic cleft. As mentioned previously, glutamatergic signaling is tightly controlled via proteins called excitatory amino acid transporters (EAATs). Following the identification of the glutamine-glutamate cycle, numerous research groups began to study how glutamate is removed from the synapse via EAATs. They found that the transport process was electrogenic and required ion gradients of K^+ and Na^+ (Fonnum, 1984, Kanner and Schuldiner, 1987, Nicholls and Attwell, 1990). The identification and cloning of three types of EAATs showed that the synaptic glutamate removal was more intricate than previously believed (Kanai and Hediger, 1992, Pines et al., 1992, Storck et al., 1992, Kanai et al., 1993, Danbolt, 1994).

There are many different protein families capable of transporting glutamate within the CNS, including high- and low-affinity glutamate transporters, Na^+ -independent carriers, and

glutamate-cystine exchangers. For the focus of this dissertation, emphasis will be placed on the high affinity glutamate transporters (specifically EAAT3); however more information on low affinity glutamate transporters, Na⁺-independent carriers, and glutamate-cystine exchangers can be found elsewhere (Erecinska and Silver, 1990, Warr et al., 1999, Flynn and McBean, 2000). Currently, there are five distinct high-affinity glutamate transporters that have been identified (Saier, 1999, Slotboom et al., 1999). They are titled GLAST (EAAT1), GLT-1 (EAAT2), EAAT3, EAAT4, and EAAT5. All are Na⁺- and K⁺-coupled and transport both L-glutamate and L- and D-aspartate. These five glutamate transporter subtypes share roughly 50-60% sequence homology and all belong to the *SLC1A* gene family (Slotboom et al., 1999). Despite being Na⁺-coupled, these glutamate transporters are a distinct transporter protein family from other neurotransmitter transporters found in the CNS (Borowsky and Hoffman, 1995, Nelson, 1998).

As the focus here is on *SLC1A1/EAAT3*, a brief overview pertaining to the localization and importance of the other high affinity glutamate transporters will be addressed here with a more in depth discussion of EAAT3 to follow. EAAT1 or GLAST (Glutamate ASpartate Transporter) is expressed throughout the CNS by astrocytes (Lehre et al., 1995, Schmitt et al., 1997, Berger and Hediger, 1998), including the retina, where EAAT1 is believed to be the primary form of glutamate uptake (Rauen, 2000, Rauen and Wiessner, 2000). EAAT1 null mice exhibit normal development; however glutamate homeostasis is compromised in brain regions where EAAT1-mediated glutamate uptake predominates (Harada et al., 1998). Behaviorally, phenotypes of EAAT1 null mice include abnormal nesting behavior, sociability, alcohol uptake, and reward (Watase et al., 1998, Stoffel et al., 2004, Karlsson et al., 2009). Mutations of *SLC1A3*, encoding EAAT1, are rare but have been linked to episodic ataxia, hemiplegia, and seizure (Jen et al., 2005, de Vries et al., 2009, Broer and Palacin, 2011).

GLT-1 (Glutamate Transporter 1) is the primary transporter of glutamate at the glutamatergic synapse and was found to be expressed on the membranes of glial cells via immunohistochemistry (Danbolt et al., 1992, Hees et al., 1992, Pines et al., 1992) and use of a

GLT-1 eGFP BAC reporter mouse (de Vivo et al., 2010). Data from transport analysis studies, electrophysiological studies, and *ex vivo* preparations from GLT-1 knockout mice propose that GLT-1 is responsible for ~95% of all glutamate transport in the forebrain (Danbolt et al., 1992, Tanaka et al., 1997, Otis and Kavanaugh, 2000, Holmseth et al., 2012). It is therefore argued that GLT-1 is the only EAAT that is essential for long-term organism viability under in normal conditions (Tanaka et al., 1997, Danbolt, 2001). In agreement with this hypothesis, young GLT-1 null mice display hyperactivity, increased susceptibility to seizures, and reduced body weight compared to littermate controls. By one month of age, the survival rate of GLT-1 deficient mice drops to roughly 50% due to the onset of lethal spontaneous seizures (Tanaka et al., 1997) believed to be linked to increased synaptic glutamate levels (Mitani and Tanaka, 2003, Takasaki et al., 2008).

EAAT4 is localized primarily to dendrites of cerebellar Purkinje cells, particularly the spines (Fairman et al., 1995, Dehnes et al., 1998). It is also reported to be expressed in the forebrain (Dehnes et al., 1998, Massie et al., 2008) and in vestibular hair cells (Dalet et al., 2012). Ablated expression of EAAT4 in mice causes no overt phenotypes; however metabotropic glutamate receptor activation is augmented in cerebellar Purkinje cells (Huang et al., 2004, Nikkuni et al., 2007).

As with EAAT1, EAAT5 function is thought to be preferentially expressed in the retina, while levels elsewhere in the brain are low (Arriza et al., 1997, Eliasof et al., 1998). Confirmation of the exact cellular and subcellular localization of EAAT5 has been difficult due to the lack of a null mouse to be used as a negative control in immunohistochemical studies (Holmseth et al., 2012). As such, more specific information regarding the role and distribution of EAAT5 in the CNS is limited.

Glutamate Exchangers

Another protein involved in the glutamate system is the glutamate-cystine exchanger (system xCT). xCT was identified via molecular cloning (Sato et al., 1999) and characterized as an electroneutral glutamate-cystine exchanger that exchanges extracellular cystine for intracellular glutamate by using the electrochemical gradient of glutamate as a driving force for moving a molecule of glutamate into the extracellular space. Currently, localization of xCT is unresolved; however reports suggest that the exchanger is expressed at relatively low levels, though it may be inducible in response to oxidative stress (Sato et al., 2002, Sato et al., 2004). Interest in xCT has increased due to evidence that xCT expression is increased in glioma and that excessive extracellular glutamate may have toxic effects enabling increased tissue invasion (Sontheimer, 2004, Takeuchi et al., 2013). xCT-deficient mice provide initial evidence that xCT plays a role in extracellular glutamate (De Bundel et al., 2011) and redox-imbalance (Sato et al., 2005) however more work is necessary to resolve xCT's role in these issues.

Excitatory Amino Acid Transporter 3

Structure

Currently, the structure of EAATs is predicted to be comprised of 8-10 transmembrane domains with intracellular N- and C-termini (Figure 1A) (Grunewald and Kanner, 1995, Wahle and Stoffel, 1996, Grunewald et al., 1998, Seal and Amara, 1998, Slotboom et al., 1999, Seal et al., 2000). Recent crystallization of an aspartate transporter from *Pyrococcus horikoshii*, Glt_{Ph}, with ~37% sequence homology to mammalian transporters, identified 8 transmembrane helices (Yernool et al., 2004, Boudker et al., 2007). Glutamate transport into the cell is an electrogenic process and is dependent on electrochemical gradients that exist across the cellular membrane. Na⁺ is necessary for glutamate binding and K⁺ is required for net transport (Kanner and Schuldiner, 1987, Szatkowski et al., 1991). More recent studies utilizing EAAT3 expressed in *Xenopus laevis* oocytes and in mutant CHO cells have shown that the exact stoichiometry of

transport is one glutamate cotransported into the cell with three Na⁺ ions and one H⁺, while one K⁺ is exchanged out of the cell (Figure 1B) (Zerangue and Kavanaugh, 1996, Levy et al., 1998). Stoichiometry of transport of the other high affinity glutamate transporters has not been confirmed, but GLAST has been suggested to exhibit similar transport properties as EAAT3 (Klockner et al., 1993).

Localization

In the CNS, EAAT3 is expressed broadly but shows higher expression in the hippocampus, basal ganglia and cerebellum (Conti et al., 1998b, Kugler and Schmitt, 1999, Holmseth et al., 2012). Outside of the brain, EAAT3 is expressed in the intestine, liver, heart, skeletal muscle, kidneys, placenta, sciatic nerve, dorsal root ganglion and primary afferent fibers terminating in the dorsal spinal horn (Kanai and Hediger, 1992, Velaz-Faircloth et al., 1996, Matthews et al., 1998, Tao et al., 2004). Historically, localization of EAAT3 was more arduous than GLAST and GLT-1 despite its sequence being published in the same *Nature* issue as the GLT-1 sequence (Kanai and Hediger, 1992, Pines et al., 1992) and about 1 month after publication of the GLAST sequence (Storck et al., 1992). This is believed to be a consequence of a few different factors, including a low amount of EAAT3 relative to GLT-1 and GLAST (Haugeto et al., 1996), and a lack of highly specific antibodies necessary for positive identification of an antigen that is expressed at relatively low amounts. Also, contrary to other EAATs, a substantial portion of expressed EAAT3 is located in the cytoplasm, rendering it difficult to be recognized by antibodies (Conti et al., 1998a, Kugler and Schmitt, 1999) and suggesting that EAAT3 can be promptly transported to the plasma membrane (Yang and Kilberg, 2002, Sheldon et al., 2006). Creation of a synthetic peptide corresponding to the C-terminus of EAAT3 enabled localization of the protein (Rothstein et al., 1994) to the neuronal cell bodies and dendrites of several types of neurons including glutamatergic and GABAergic neurons. Recent studies have also identified EAAT3 expression on dopaminergic neurons

(Nafia et al., 2008, Assous et al., 2014) and have shown that EAAT3 rapidly undergoes endocytosis in response to the dopaminergic agent, amphetamine (Underhill et al., 2014). A study using a postembedding immunogold technique reported that EAAT3 is primarily found on the edges of synapses, termed the peri- or extrasynaptic space (He et al., 2000). Despite some *in vitro* reports to the contrary, EAAT3 expression is believed to be completely limited to the neurons as Holmseth et al. detected no identifiable EAAT3 in non-neuronal cells using EAAT3 null mouse verified antibodies in adult rat brain (Holmseth et al., 2012)

Function

The majority of all glutamate uptake in the CNS is mediated via GLT-1, which is not surprising due to it being expressed roughly 100-fold more abundantly than EAAT3 (Holmseth et al., 2012). Some suggest that existing estimates of GLT-1's role in glutamate reuptake may be inflated, since EAAT3 antisense knockdown studies indicate that ~20% of striatal and ~40% of hippocampal glutamate uptake is mediated via EAAT3 (Rothstein et al., 1996). The ongoing hypothesis is the EAAT3 may have a more substantial impact on glutamate exposure in the extrasynaptic regions where it is primarily expressed and where it is expected to modulate local glutamate receptor activation. This has been shown for both AMPA and NMDA receptors on post-synaptic neurons when EAAT3 function has been compromised (Scimemi et al., 2009, Jarzylo and Man, 2012, Underhill et al., 2014, Li et al., 2017). Lack of spatial control over glutamate release can result in synaptic spillover capable of activating NMDA receptors (NMDARs) located on neighboring synapses (Diamond, 2001). EAAT3 was shown to be necessary for the control of spillover glutamate activating NR2B-containing NMDARs specifically (Scimemi et al., 2009). Further, induction of long-term potentiation appeared to decrease EAAT3 activity and further increase local NMDAR activation in hippocampal slice electrophysiology experiments (Scimemi et al., 2009). Activation of hippocampal extrasynaptic NR2B-containing NMDARs via EAAT3 inhibition also led to the reduction of GluR1 and GluR2

via proteasomal degradation in both rat hippocampal and cortical neurons (Jarzylo and Man, 2012).

Another primary function of EAAT3 is the uptake of glutamate for the formation of the neurotransmitter γ -aminobutyric acid (GABA), providing an intracellular pool of glutamate for GABAergic neurons, including the medium spiny neurons (MSNs) of the striatum. (Mathews and Diamond, 2003). Pharmacological inhibition of EAAT3 shows that uptake of spillover glutamate contributes to inhibitory currents in the CA1 region of rat hippocampus (Stafford et al., 2010). Further, pharmacological inhibition of EAAT3 reduces the GABA released by these neurons, as well as the subsequent inhibitory currents resulting from GABA release (Sepkuty et al., 2002, Mathews and Diamond, 2003). Use of an antisense RNA knockdown strategy also revealed a reduction in $^3\text{[H]}$ -GABA synthesis rate and an overall ~50% reduction of hippocampal GABA (Sepkuty et al., 2002).

While all EAATs are able to transport L-glutamate and D-aspartate, recent efforts also show that EAAT3 is able to transport cysteine under physiological conditions (Watts et al., 2014). Transport of cysteine into cells, particularly those under increased levels of oxidative stress, is thought to be a neuroprotective function of EAAT3, providing a neuronal substrate for the synthesis of the anti-oxidant glutathione. The ability of EAAT3 to transport cysteine has been established both in cell culture and animal models (Chen and Swanson, 2003, Aoyama et al., 2006, Nieoullon et al., 2006, Aoyama et al., 2012, Aoyama and Nakaki, 2013, Assous et al., 2014). Investigation in aged EAAT3 null mice revealed increased levels of accumulated oxidative stress, cortical thinning, and enlarged ventricles (Aoyama et al., 2006). As mentioned previously, cell types under high levels of oxidative stress are more susceptible to oxidative stress, and 12-month old EAAT3 null mice exhibit almost ~40% loss of their dopaminergic neurons (Berman et al., 2011). Treatment with the cell permeable cysteine precursor, N-acetylcysteine rescued this loss of dopaminergic neurons in the substantia nigra pars compacta (Berman et al., 2011).

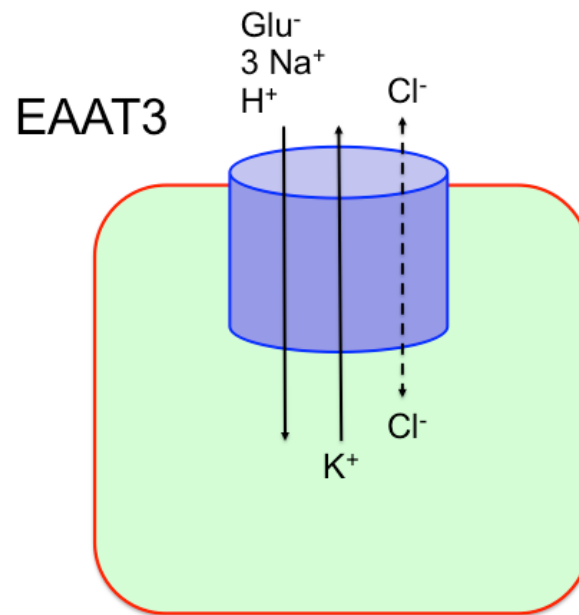
Regulation of glutamate uptake via EAAT3 is thought to occur primarily via intracellular signaling modulation affecting trafficking of the transporter to the cell surface. Early efforts demonstrated that activation of protein kinase C (PKC) augmented glutamate uptake via selective increases in EAAT3 activity while also reducing GLT-1 function (Dowd and Robinson, 1996, Gonzalez and Robinson, 2004). This phenomenon is due to increased levels of EAAT3 at the plasma membrane caused by intracellular activation of the PI3K (phosphoinositide 3-kinase) signaling pathway (Krizman-Genda et al., 2005). Platelet derived growth factor (PDGF) is also able to augment cell surface expression of EAAT3 *in vitro* via activation of the same PI3K pathway (Sims et al., 2000) The C-terminus of EAAT3 is also believed to interact with regulatory cytosolic proteins such as GTRAP3-18, which when physically interacting with EAAT3, inhibits glutamate transport (Lin et al., 2001, Butchbach et al., 2002). GTRAP3-18 knock out mice exhibited reduced body weight, increased levels of EAAT3 and GSH, and enhanced learning and memory in the Morris water maze (Aoyama and Nakaki, 2012, Aoyama et al., 2012). Alternatively from the above intracellular pathways, activation of post-synaptic receptors including the neurotensin 1, endothelin-1, and ionotropic glutamate receptors also modulate EAAT3 cellular localization (Levenson et al., 2002, Najimi et al., 2002, 2005, Waxman et al., 2007) The ability to regulate the cell membrane level of EAAT3 represents the ability of neurons to rapidly induce adaptive changes in the cellular environment that may be useful to control potentially excitotoxic events.

EAAT3 in Disease

Dicarboxylic Aminoaciduria

While *Slc1a1*/EAAT3 is expressed throughout the CNS, it is also the primary transporter of epithelial glutamate and aspartate in the kidneys and intestines. Loss of function mutations in *SLC1A1* have been identified in the human population and lead to dicarboxylic aminoaciduria (Bailey et al., 2011). This condition involves partial reabsorption of negatively charged amino

A



B

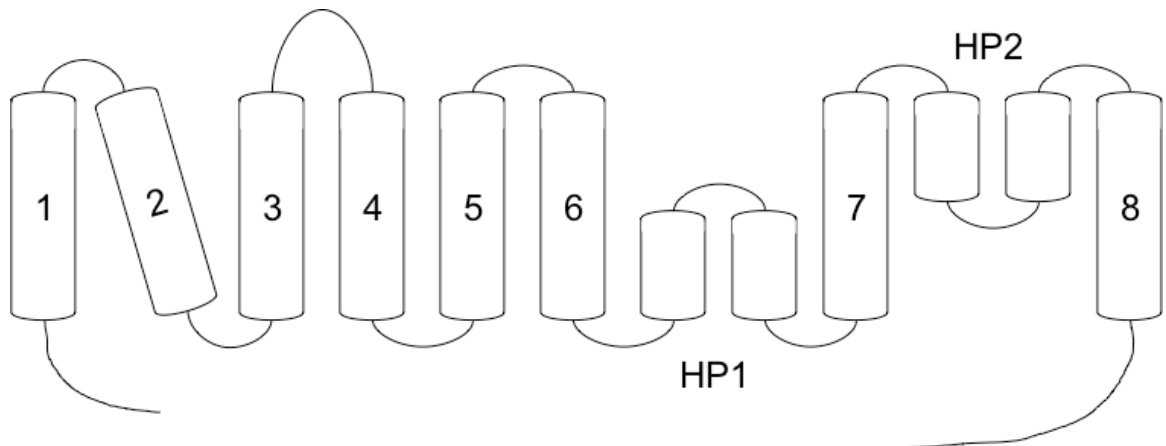


Figure 1. Stoichiometry of glutamate transport via EAAT3 and proposed topology of EAAT3

A. Transport of one glutamate molecule into neurons is coupled to the translocation of three Na^+ ions, one H^+ and counter transport of one K^+ . EAATs also have an anion current that is gated by glutamate but not coupled to transport.

B. Proposed two-dimensional topology diagram of EAAT3 based on the crystal structure of the aspartate transporter, Glt_{Ph} . EAATs are proposed to be composed of eight transmembrane (TM) domains with intracellular N- and C-termini and two reentrant loops between TM 6-7 and TM 7-8.

acids in the kidney which forces affected individuals to expend excessive resources to maintain sufficient cellular levels of anionic amino acids. Lack of functional EAAT3 resulting from these mutations in *SLC1A1* have been suggested to be linked to growth retardation, intellectual disability and metabolic dysfunction (Broer, 2008, Bailey et al., 2011, Broer and Palacin, 2011)

Obsessive-Compulsive Disorder

Family studies suggest a substantial heritable contribution to obsessive compulsive disorder. Genetic association studies have focused on candidate genes in a number of neurotransmitter systems, including glutamate, GABA, dopamine, and serotonin (Haber and Heilbronner, 2013, Bokor and Anderson, 2014, Ivarsson et al., 2015). Links to the serotonin system have primarily been based on the effectiveness of serotonin reuptake inhibitors as the primary form of pharmacotherapy; however little other evidence, genetic or otherwise, has connected the system to the disorder. More evidence for the role of dopamine in the manifestation of OCD symptoms has been reported, primarily via functional imaging and positron emission tomography (PET) studies (Denys et al., 2004, Klanker et al., 2013). Recent studies have focused more on evidence connecting OCD to abnormalities in the glutamatergic system.

Unlike other neurotransmitter systems, evidence implicating the glutamatergic system in OCD has been historically consistent and more robust. Specifics of the involvement of the glutamate system and *Slc1a1*/EAAT3 specifically will be discussed in more depth in the following section. Initial interest in *SLC1A1* in OCD arose from a genomic scan of early-onset OCD patients and immediate family members (Veenstra-VanderWeele et al., 2001). The connection between *SLC1A1* and the disorder was subsequently strengthened via genetic linkage and association studies (Arnold et al., 2006, Dickel et al., 2006). As evidence that alteration of *SLC1A1*/EAAT3 function may affect OCD-relevant behavior and neurobiology grows, the link between the glutamate system and OCD becomes more substantial. Due to the

complexity of neuropsychiatric disorders, it is possible that many different factors contribute to the emergence of OCD; however mounting evidence suggests that pharmacological intervention on EAAT3 function may be a novel therapeutic strategy for the treatment of this debilitating disorder.

Epilepsy

Reduction of EAAT3 mRNA via knockdown strategies increases the susceptibility to seizures in rodents, however it is thought to occur via an alternative mechanism, rather than failure to clear glutamate from the synapse (Rothstein et al., 1996). Acute reductions in EAAT3 have been shown to attenuate tonic inhibition capacity of GABAergic neurons where EAAT3 transport provides a substrate for the synthesis of GABA (Sepkuty et al., 2002). Following kainate and methyazoxymethanol-induced seizures, EAAT3 expression is diminished (Simantov et al., 1999, Harrington et al., 2007). These data mirror one report in human epilepsy patients, where cortical EAAT3 expression was reduced (Rakhade and Loeb, 2008). Interestingly, EAAT3 null animals have not exhibited phenotypes of increased susceptibility to drug-induced seizures (Peghini et al., 1997, Aoyama et al., 2006), perhaps due to a developmental compensatory mechanism often observed in animal models of constitutive protein ablation.

Alternatively, recent studies using EAAT3 null mice argue that loss of EAAT3 is protective in the CA1 region of the hippocampus following pilocarpine-induced status epilepticus (Lane et al., 2014). The authors maintain that under the drug-induced epileptic conditions utilized, reductions in ATP and reversal of the Na⁺ electrochemical gradient across the cellular membrane cause EAAT3 to release intracellular glutamate back into the neuronal cleft which exacerbates glutamatergic signaling dysfunction leading to seizures (Streck et al., 2006, Kovac et al., 2012). In this case, a lack of EAAT3 transporting glutamate back into the synaptic cleft would be protective against glutamate efflux, leading to reductions in neuronal cell death as observed above. The contributions of EAAT3 to epilepsy are currently still under investigation

and, due to reports that EAAT3 is a minor regulator of synaptic glutamate relative to GLT-1, it is possible that dysfunctional EAAT3 would act as more of a risk factor for the exacerbation of epilepsy. Of note, loss-of-function mutations of the EAAT3 are rare in the human population and in a recent report, none of the two probands interviewed reported a history of epilepsy (Bailey et al., 2011).

Schizophrenia

Attempts to understand the underlying pathophysiology of schizophrenia have been ongoing for years. Glutamate has been suggested to be associated with the disorder via evidence from human post-mortem brain tissue studies (Hu et al., 2015), including reductions in EAAT3 transcript in both bipolar and schizophrenia samples (McCullumsmith and Meador-Woodruff, 2002). EAAT3 was decreased in the caudate nucleus of schizophrenic patients in one study (Nudmamud-Thanoi et al., 2007); however other studies report increased EAAT3 in the cortical post-mortem tissue via *in situ* hybridization analyses (Rao et al., 2012) and abnormal expression of the EAAT3 regulating protein JWA, the human ortholog of GTRAP3-18 (Bauer et al., 2008, Aoyama and Nakaki, 2012).

First-line pharmacotherapy of schizophrenia includes treatment with second-generation antipsychotics (SGAs), which have been associated with the induction of obsessive-compulsive (OC) like symptoms in schizophrenia patients (Lykouras et al., 2003, Fonseka et al., 2014). Strengthening the connection to glutamate and to EAAT3 specifically, single nucleotide polymorphisms in *SLC1A1*, the gene encoding EAAT3, have been associated with SGA-induced OC symptoms (Kwon et al., 2009). This finding was independently replicated in a second study, which also reported a significant interaction between *SLC1A1* and the gene coding for the NR2B NMDA subunit, *GRIN2B* (Cai et al., 2013).

Obsessive-Compulsive Disorder

Introduction to Obsessive-Compulsive Disorder

OCD is a chronic, debilitating, and heterogeneous psychiatric disorder affecting between 1-3% of the worldwide population (Koran, 2000, Kessler et al., 2005, Ruscio et al., 2010). Primary symptoms of the disorder include recurrent obsessive and intrusive anxiety-inducing thoughts leading to ritualistic repetitive or compulsive behavior performed to mitigate this anxiety (Calvocoressi et al., 1998, Monzani et al., 2014). Age of onset for OCD ranges from early childhood, with 30-50% of OCD sufferers report onset prior to 10 years of age (Zohar, 1999). Based on higher familial recurrence rates and somewhat different patterns of symptoms, some clinicians suggest that early-onset OCD may in fact be a distinct form of the disorder (Geller et al., 1998, Nakatani et al., 2011).

Treatment

In spite of the societal costs of OCD, reported to be near to \$10 billion in the USA alone (Eaton et al., 2008), available treatments are inadequate for the majority of patients. The initial recommended treatment for most patients is cognitive behavioral therapy (CBT), which appears to be similarly effective in both children and adults, but with the majority of patients showing substantial residual symptoms (Franklin and Foa, 2011). CBT techniques include exposure and response prevention, where a patient is exposed to anxiety-producing stimuli accompanied by the prevention of repetitive behaviors or compulsions that the patient typically performs to decrease their anxiety. Efficacy for CBT is slightly greater relative to treatment with serotonin reuptake inhibitors (SRIs) in some studies (Barrett et al., 2004), and their therapeutic effects are believed to be additive.

First-line pharmacological treatment of OCD is SRIs (Geller et al., 2003); however, only 50-60% of patients exhibit adequate response to available treatments (Koran et al., 2007). For example, a 20-40% decrease in OCD symptoms may result following SRI therapy (Dougherty et

al., 2004), which leaves many with clinically significant residual symptoms. While the effectiveness of SRI's in OCD is empirically validated, evidence for the potential value of therapeutics targeting the dopamine and glutamate system has been mounting in recent years. Dopamine D₂ (and 5-HT_{2A}) receptor blockade in OCD has primarily been studied in the context of augmentation, with a recent meta-analysis of 12 placebo-controlled trials revealing significant improvement in cases of SRI-resistant OCD patients augmented with atypical antipsychotic medications compared to placebo (Dold et al., 2015). These results should be taken cautiously due to the heterogeneous nature of the methodology, dosage, inclusion criteria and other factors incorporated into the meta-analysis; however it does provide preliminary evidence for the use of D₂ blockade in OCD as an augmentation therapy, particularly for risperidone (Bloch et al., 2006, Skapinakis et al., 2007, Dold et al., 2013). A few drugs that target the glutamate system have shown promise as an augmentation therapy in OCD. The glutamate-modulating drug riluzole, FDA-approved for the treatment of amyotrophic lateral sclerosis, has emerging, suggestive evidence for reducing OCD symptoms in pediatric and adult patients (Coric et al., 2005, Grant et al., 2007, Pittenger et al., 2008). Other drugs targeting the glutamatergic system, including agents targeting the NMDA receptor such memantine (Poyurovsky et al., 2005, Pasquini and Biondi, 2006, Haghghi et al., 2013) and D-cycloserine (Wilhelm et al., 2008, Storch et al., 2016) showed suggestive results when used as augmentation; however more thorough investigation is needed to fully understand the therapeutic potential of these compounds in OCD. Other augmentation therapies, such as NMDA receptor antagonists and the glutathione precursor, N-acetylcysteine, are also being evaluated, but evidence for their utility is limited (Arumugham and Reddy, 2013).

In some refractory OCD cases, deep brain stimulation (DBS) has also been used (Sturm et al., 2003, Goodman et al., 2010). The most common targets for DBS are the anterior limb of the internal capsule (VC/VS) (Greenberg et al., 2010), the nucleus accumbens (van den Munckhof et al., 2013, Ooms et al., 2014), and the subthalamic nucleus (Mallet et al., 2008). As

this field evolves, novel targets and stimulation techniques may be identified, ideally allowing for improved therapeutic efficacy while maintaining minimal off-target effects.

Heritability

As with many neuropsychiatric conditions, the underlying causes of OCD are unknown and are likely to involve both genetic and environmental factors. Abundant evidence for a heritable component of OCD stems from twin and family studies (Grados and Wilcox, 2007, Pauls, 2008). As reviewed elsewhere (Pauls, 2010), OCD symptoms are estimated to be 40-65% heritable in children and 27-47% heritable in adults (van Grootheest et al., 2005). Family studies indicate that OCD is twice as common in first-degree relatives of affected adults and ten times as likely in relatives of affected children (Pauls, 2008). These studies support the premise that OCD risk is derived from a complex combination of genetic and non-genetic factors.

Genetic analysis of OCD

Genetic linkage studies have yet to generate genome-wide significant findings for the core diagnosis of OCD, likely because of lack of statistical power in limited sample sizes (Hanna et al., 2002a, Mathews et al., 2012). The most promising linkage signal is on chromosome 9p24, based upon a suggestive linkage peak in the first genome-wide linkage study that was subsequently directly replicated in a study targeting only this region (Hanna et al., 2002a, Willour et al., 2004a). Another suggestive linkage peak on chromosome 15q14 was identified in two genome-wide linkage studies (Shugart et al., 2006). To date, genome-wide association studies (GWAS) have been similarly underpowered in OCD (Stewart et al., 2013b, Mattheisen et al., 2015). A single polymorphism near *BTBD3* reached genome-wide significance in a family-based subset of one GWAS analysis, but it was only suggestive in the overall sample (Stewart et al., 2013b).

Candidate genes for OCD have been identified based upon proximity to linkage peaks, biomarker findings, and relationship to pharmacological targets. The strongest candidate gene association findings in OCD focus on *SLC1A1*, which encodes the neuronal glutamate transporter, EAAT3 (Arnold et al., 2006, Dickel et al., 2006, Stewart et al., 2007, Kwon et al., 2009, Shugart et al., 2009, Wendland et al., 2009a, Samuels et al., 2011, Stewart et al., 2013b, Wu et al., 2013). Interest in *SLC1A1* stemmed from its location under the chromosome 9p24 linkage peak, as well as biomarker studies implicating the glutamate system in OCD. Additional candidate genes in the glutamate system have also been studied, including *GRIN2B*, which shows evidence of association in some but not all studies (Arnold et al., 2004, Alonso et al., 2012, Cai et al., 2013). The other leading candidate gene is the serotonin transporter, *SLC6A4*, with both common and rare variants exhibiting association in some studies (Ozaki et al., 2003, Dickel et al., 2007, Grados et al., 2007, Saiz et al., 2008, Wendland et al., 2008), but not all (Taylor, 2013). The dopaminergic system (*COMT and DRD4*) has been implicated via gene association studies; however these findings have yet to be replicated (Billett et al., 1998, Pooley et al., 2007). A lack of clear susceptibility genes contributes to the difficulty of modeling OCD in animals, though this issue is shared by most complex heterogeneous neuropsychiatric disorders.

Neurobiology of OCD

As imaging methods have improved over the last thirty years, our ability to non-invasively examine neural substrates and brain regions in OCD sufferers has led to significant advancement of our understanding of the underlying pathophysiology of the disorder. As will be discussed more thoroughly below, neuroimaging analyses led researchers to focus on a cortico-striatal-thalamo-cortical (CSTC) model (also known as a frontostriatal or corticostriatal model) of OCD (Saxena and Rauch, 2000, Maia et al., 2008, Rotge et al., 2008, Ting and Feng, 2011). This model has become the predominant model proposed to reflect the important neural regions

involved in the pathophysiology of OCD; however recent adaptations have been suggested (Milad and Rauch, 2012, Wood and Ahmari, 2015).

Within the basal ganglia circuitry, the indirect pathway has the capacity to exert inhibitory control on excitatory output from the thalamus to the cortex; whereas the direct pathway exerts excitatory control (Figure 2). Based on human imaging studies, hyperactivity through the direct pathway due to a lower activation threshold may result in subsequent excessive activity of the CSTC and manifest itself in the form of repetitive thoughts and behavior (Saxena and Rauch, 2000).

Structural Neuroimaging

The most consistent brain regions implicated via structural analyses have been the orbitofrontal cortex (OFC), the anterior cingulate cortex (ACC), and the striatum (Pittenger et al., 2011, Rodman et al., 2012, de Wit et al., 2014). A large MRI study reported reductions in medial OFC gray matter (Di Martino et al., 2008) and increased grey matter of the ventral striatum (Pujol et al., 2004) in OCD. Meta-analyses have indicated consistent findings of reduced volumes of the left ACC and bilateral OFC, and increased thalamic volume relative to control samples (Rotge et al., 2009). A separate meta-analysis identified increased bilateral gray matter in the striatum as well as reductions in bilateral ACC volume (Radua and Mataix-Cols, 2009). Also of note is a study that combined structural imaging and behavioral testing of OCD patients and non-affected first degree relatives, demonstrating that impairment on a response-inhibition task correlated with reductions in OFC gray matter and increased great matter in the ACC and striatum (Menziés et al., 2008). While structural studies have been somewhat inconsistent, alterations in the OFC, ACC, and striatum are most frequently reported.

Functional Imaging

In agreement with the structural imaging reports, the most consistently implicated brain regions in functional neuroimaging via positron emission tomography (PET) and functional magnetic resonance imaging (fMRI), are the OFC, ACC, and the caudate (specifically the head) (Rauch and Savage, 1997, Rotge et al., 2008). Hyperactivity is reported in all of these regions at baseline and following symptom provocation (Rauch et al., 1997), supporting the model of excessive signaling in the CSTC circuitry. In contrast, studies of SRI or CBT treatment demonstrate activity reductions in the OFC or caudate following successful treatment (Rauch et al., 2002, Nakao et al., 2005). Recent resting-state connectivity analyses using fMRI further support the hypothesis of dysfunctional CSTC circuitry, specifically the OFC, ACC, ventral striatum, dorsal striatum, putamen, and anterior thalamus (Beucke et al., 2013, Harrison et al., 2013, Anticevic et al., 2014, Posner et al., 2014). While three of the resting state connectivity studies have reported increased connectivity between the OFC and the striatum (Harrison et al., 2009, Beucke et al., 2013, Harrison et al., 2013), one has reported the opposite (Posner et al., 2014). Justification for the discrepancy may be that the majority of the subjects in the Posner study were clear of medication (either treatment naïve or ~2 years on average without medication), along with differing analytical methodologies.

Elevated glutamate in cerebrospinal fluid

The most direct evidence for glutamatergic dysfunction in OCD comes from studies using overlapping samples, where the authors analyzed the glutamate concentrations of cerebrospinal fluid (CSF) of drug-naïve adult OCD patients and normal controls. In the first study, glutamate concentrations of the OCD patients was significantly elevated relative to

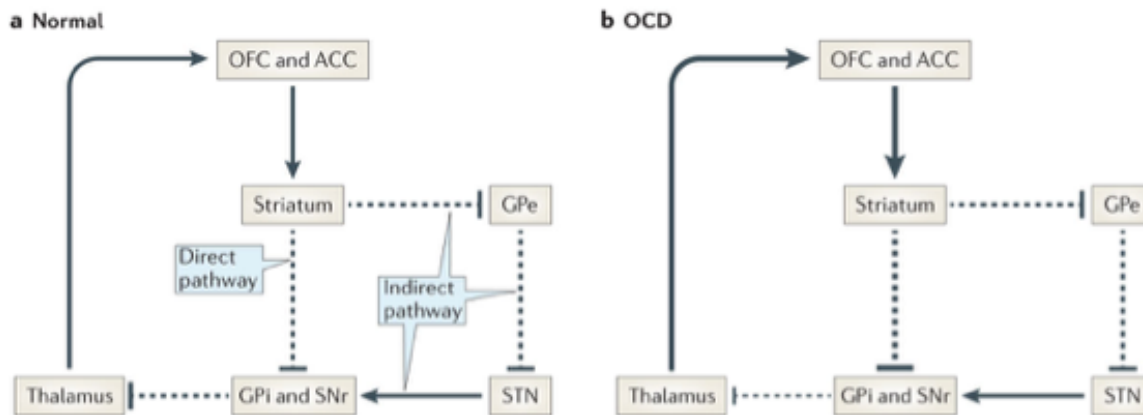


Figure 2. Schematics depicting the direct and indirect pathways of the basal ganglia

Solid arrows depict glutamate (excitatory) pathways and dashed lines depict GABAergic (inhibitory) pathways.

A. In the normally functioning cortico–striato–thalamo–cortical circuit, glutamatergic signals from the frontal cortex (specifically, the orbitofrontal cortex (OFC) and anterior cingulate cortex (ACC)) lead to excitation in the striatum. Through “direct pathway”, striatal activation increases inhibitory GABA signals to the globus pallidus interna (GPi) and the substantia nigra (SNr). This decreases the inhibitory GABA output from the GPi and SNr to the thalamus, resulting in excitatory glutamatergic output from the thalamus to the frontal cortex. This direct pathway is a positive-feedback loop. In the indirect pathway, the striatum inhibits the globus pallidus externa (GPe), which decreases its inhibition of the subthalamic nucleus (STN). The STN is then free to excite the GPi and SNr and thereby inhibit the thalamus.

B. In patients with obsessive–compulsive disorder (OCD), a hypothetical imbalance between the direct and indirect pathways results in excess tone in the former over the latter.

Adapted from *Obsessive-compulsive disorder: An integrative genetic and neurobiological perspective* (Pauls, 2014)

controls, without any effects of age, sex, or duration of illness (Chakrabarty et al., 2005a). The second study, performed on the same samples, used a more in-depth analysis on subjects over the age of 15 by also analyzing additional amino acids, as well as autoantibodies against the basal ganglia and thalamus. The authors reproduced their initial findings of elevated glutamate, and also reported elevated levels of the NMDAR co-agonist, glycine, in OCD patients relative to controls (Bhattacharyya et al., 2009). The authors made note that elevations in glycine were driven by OCD patients with autoantibodies to the basal ganglia or the thalamus, indicating that immune system alteration may be connected to elevations in glutamate and glycine. Despite a low number of studies, these results do suggest that alterations in glutamate homeostasis may be linked to OCD.

Convergence of Glutamate, *SLC1A1*/EAAT3, and Obsessive-compulsive disorder

Based on the efficacy observed in the treatment of OCD and SRIs, the serotonin system was the first neurotransmitter system suspected to be involved in OCD. However, a significant portion of the OCD patient population display minimal clinical benefit resulting from serotonin-enhancing drugs (Soomro et al., 2008), while some but not all of refractory cases are improved by augmentation with dopamine modulating drugs (Arumugham and Reddy, 2013). Novel evidence from genetic analysis, neuroimaging and neurochemical studies, observations from animal models, and off-label trials of FDA-approved glutamate-modulating drugs suggest that dysregulation of glutamate may be involved in the pathophysiology of OCD.

First, the prevailing glutamate hypothesis of OCD argues that genes related to the glutamatergic system would be feasible candidate genes for the disorder. *SLC1A1*, the gene coding for the neuronal excitatory amino acid transporter, EAAT3, is the most consistently implicated OCD candidate gene. *SLC1A1* was first identified via genome-wide linkage study of the disorder and was subsequently replicated (Hanna et al., 2002a, Willour et al., 2004b). Soon after, a significant genetic association was found with a SNP in the 3' end of the gene (Arnold et

al., 2006, Dickel et al., 2006). While the specific SNPs and haplotypes associated with OCD have not been reproduced consistently, the majority of the SNPs have been located in the 3' region of the gene (Arnold et al., 2006, Dickel et al., 2006, Stewart et al., 2007, Kwon et al., 2009, Shugart et al., 2009, Wendland et al., 2009a, Samuels et al., 2011, Stewart et al., 2013a). Interestingly, recent genetic investigations of the gene propose that polymorphisms of *SLC1A1* are associated with *de novo* obsessive-compulsive symptoms induced via atypical antipsychotic treatment in schizophrenia patients (Kwon et al., 2009, Ryu et al., 2011, Schirmbeck et al., 2012, Cai et al., 2013), suggesting an interaction between the dopamine system and glutamate neurotransmission.

The consistent implication of *SLC1A1* in OCD via genetic studies does strengthen the hypothesis that the gene is in fact a risk factor for OCD; however limited data provide insight into how abnormal expression or function of EAAT3 may be responsible for OCD symptoms. An EAAT3 null mouse exhibited cortical neurodegenerative effects and behavioral phenotypes, but only at an advanced age and without OCD-relevant behavioral characterization (Peghini et al., 1997, Aoyama et al., 2006). One of the two studies examining the role of EAAT3 in the CSTC circuitry found that exposure to amphetamine triggers endocytosis of EAAT3 from the cell membrane, leading to augmented glutamatergic signaling via AMPA and NMDA glutamate receptors (Underhill et al., 2014, Li et al., 2017). Additional studies of EAAT3's role in modulating OCD-relevant behavior and circuitry are needed to elucidate its contribution to the underlying pathophysiology of OCD-relevant behavior.

Abnormal Regulation of Glutamate in the Central Nervous System

The primary neurotransmitters in the CSTC circuit are glutamate and GABA (Figure 2). The medium spiny neurons (MSNs) of the striatum C receive glutamatergic input via projections from the cerebral cortex, thalamus, and hippocampus. MSNs of the striatum, however, are GABAergic and act to inhibit the neuronal activity of their targets. These targets include the

globus pallidus pars interna (GPI), the globus pallidus pars externa (GPe), and the substantia nigra pars reticulata (SNr). Neurons in these MSN target regions are themselves GABAergic and act to inhibit their targets, including the subthalamic nucleus (indirect pathway) and thalamus (direct pathway). In turn, glutamatergic thalamic neurons innervate the neocortex, including the OFC and ACC. As such, the majority of neurons, aside from a subset of interneurons, in the CSTC circuit use glutamate or GABA as their primary neurotransmitters. Potential excessive activity throughout this circuit could be reflected by increased levels of glutamate in brain regions innervated by glutamatergic neurons. In order to test this, they began using MRS in order to quantify glutamate in specific brain regions implicated in OCD.

Use of magnetic resonance spectroscopy allows quantification of tissue levels of a number of common brain chemicals, but its ability to analyze glutamatergic concentrations has limitations. Currently, all MRS analysis studies of glutamatergic levels have been performed at low magnetic strengths. Thus, the subtle molecular differences between glutamate, glutamine, and GABA are unable to be resolved. Another caveat is that glutamate is an important biosynthetic building block for cellular metabolism, not exclusively for neurotransmission, and Glx in different cellular compartments are unable to be differentiated. Most Glx signal in MRS studies therefore likely corresponds to intracellular glutamate, which may or may not indicate a change in glutamatergic signaling. Despite these technical limitations, alterations in Glx in the CSTC circuit have consistently been reported in OCD. Early MRS studies described elevations in the caudate Glx that normalized following SRI treatment in pediatric patients (Moore et al., 1998, Rosenberg et al., 2000, Rosenberg et al., 2001). Decreased ACC Glx was separately reported in pediatric patients (Rosenberg et al., 2004). An MRS study in adult women with OCD showed that symptom severity was linked to reductions in ACC Glx (Yucel et al., 2008). One study in treatment-naïve adults described increased Glx signal in orbitofrontal white matter of OCD patients relative to controls that was associated with symptom severity (Whiteside et al.,

2006); however a similar study reported no Glx differences in the OFC (Bedard and Chantal, 2011).

These MRS studies, together with CSF measurement of glutamate, suggests glutamatergic dysfunction in the CSTC circuit of OCD patients. These data must be judiciously interpreted, however, because many studies had small sample sizes and were unable to differentiate glutamate alone from the Glx signal. Due to the limitations of the imaging techniques, it is also unclear whether increases in glutamate are due to excessive glutamate projection, changes in glutamate release, altered glutamate transport, or changes in glial glutamate exchange. Regardless, taken together with the structural neuroimaging findings, there is strong evidence that abnormal regulation of the glutamate may be involved in the pathophysiology of the disorder.

Mouse Models of Glutamatergic Dysfunction and Repetitive Behavior

A broader array of mouse models of compulsive-like behavior will be discussed in Chapter 2; therefore only models with alteration of the glutamate system will be addressed here. No animal models accurately reproduce the heterogeneous characteristics of OCD symptomology. This is an inherent limitation on animal models of most psychiatric disease, but this constraint is even more pronounced in OCD, since obsessions are purely subjective, thus leaving researchers to examine repetitive behaviors and deficits in neurotransmitter systems with strong prior evidence, such as the glutamate system.

D1CT-7 Mice

One of the first transgenic mouse models relevant to OCD was the D1CT-7 model (Campbell et al., 1999) that expresses an intracellular form of cholera toxin under control of the dopamine receptor 1 gene (*DRD1*) promoter. By constricting expression of the toxin to *DRD1*-expressing neurons, researchers were able to limit chronic activation of stimulatory G-protein

(G_s) to the direct pathway of the basal ganglia in an effort to mimic the hypothesis that hyperactivity of the direct pathway underlies the pathophysiology of OCD. This model exhibits a myriad of compulsive-like behaviors, including perseverance of normal behaviors, repetitive nonaggressive biting, and leaping (Campbell et al., 1999). Onset of motor tic-like behavior was described in juveniles, with increased prevalence in males (Nordstrom and Burton, 2002), reminiscent of the psychiatric disorder Tourette's Syndrome (TS). While direct evidence of glutamatergic dysfunction at baseline was absent, addition of a non-competitive NMDA receptor antagonist, resulting in stimulation of cortical-limbic glutamate output, exacerbated compulsive-like behavior (McGrath et al., 2000).

***Sapap3* Null Mice**

One of the most prominent genetic mouse models of repetitive behavior is the *Sapap3* null model. SAPAP3 is a post-synaptic density scaffolding protein that colocalizes to glutamatergic synapses within the CSTC circuit. Not only do these mice exhibit increased anxiety-like behavior and repetitive behavior, their elevated grooming phenotype responds to subchronic levels of SRI administration. Evidence from electrophysiological studies identified cortico-striatal glutamatergic signaling dysfunction in *Sapap3* nulls, including increased NMDA receptor activity and altered NMDA receptor subunit composition (Welch et al., 2007, Wan et al., 2014). Genetic evidence supporting *SAPAP3* in OCD is lacking; however an association was identified in a small subset of OCD patients exhibiting grooming symptoms, such as trichotillomania (Bienvenu et al., 2009). Despite a weak case for construct validity, *Sapap3* nulls are a valuable model of repetitive behavior exhibiting face and predictive validity, alongside dysfunctional cortical-striatal glutamatergic transmission.

***Slitrk5* Null Mice**

Interestingly, ablation of another post-synaptic protein, SLITRK5, also led to a genetic model of OCD-relevant behavior. Another *SLITRK* family member, *SLITRK1*, was previously reported to be associated with TS (Abelson et al., 2005). The function of SLITRK5 is still being explored, but it is believed to be important for neuronal survival and synapse formation (Proenca et al., 2011). *Slitrk5* null mice display excessive grooming and anxiety similar to SAPAP3 null mice, and as well as improvement with chronic SRI treatment (Shmelkov et al., 2010b). Notably with regards to the glutamate hypothesis, *Slitrk5* nulls also showed reduced cortico-striatal transmission and increased indirect markers of neuronal activity in the OFC (Shmelkov et al., 2010b).

Repeated stimulation of OFC-Ventral striatum projections

Optogenetic and chemogenetic approaches allow manipulations of the activity of specific cell populations with varying degrees of temporal control. Optogenetic techniques take advantage of exogenous, light-activated ion channels to activate or silence neurons with temporal and cell-type specificity. To test the glutamatergic hyperactivity hypothesis in a specific component of the CSTC circuit, Ahmari and colleagues expressed channelrhodopsin-2 (ChR2) in the glutamatergic neurons projecting from the OFC to the ventral striatum (VS). Acute stimulation had no OCD-relevant behavior consequences; however repeated stimulation of OFC-VS glutamatergic neurons over multiple days led to a perseverative grooming behavior that persisted even after the cessation of the stimulation protocol (Ahmari et al., 2013). High-dose chronic (but not acute) SRI administration rescued the observed grooming phenotype and normalized the evoked firing rate elicited by optogenetic stimulation. These findings provide yet another piece of evidence for the role of CSTC glutamatergic signaling in OCD.

Summary of Data Linking OCD and Glutamate

The hypothesis that aberrant glutamatergic regulation is connected to the pathophysiology of OCD was established near the beginning of the new millennium (Moore et al., 1998, Rosenberg and Keshavan, 1998). This foundational hypothesis was structured around glutamate being the primary neurotransmitter in the CSTC circuit, which was recognized as connecting the brain regions consistently implicated in structural and functional neuroimaging analyses of the OCD patient population. The hypothesis further proposes that hyperactivity of the direct pathway or hypoactivity of the indirect pathway may lead to an overactive CSTC circuit that could result in repetitive thoughts and behaviors (Saxena et al., 2001).

The first animal model aimed at recapitulating this hardwired hyperactive state of the CSTC circuit was the D1CT-7 model, resulting in OCD- and tic-like behavior that was exacerbated by NMDA receptor antagonists (Campbell et al., 1999, McGrath et al., 2000). More convergent evidence followed, identifying abnormal glutamatergic concentrations in MRS studies of specific brain regions in the CSTC circuit (Rosenberg et al., 2000, Rosenberg et al., 2004) and elevated glutamate concentrations in the CSF of OCD patients (Chakrabarty et al., 2005a, Bhattacharyya et al., 2009).

Due to the accumulating support for glutamatergic dysregulation in OCD, genetic analyses focused on potential candidate genes relevant to the glutamate system. The gene with the most consistent genetic association to OCD is *SLC1A1*, coding for the neuronal glutamate transporter (EAAT3). EAAT3 is localized on the post-synaptic and extrasynaptic membrane of neurons receiving glutamate input, with high levels of expression in cortical and striatal brain regions. Another candidate gene with preliminary evidence for association is *GRIN2B* (Arnold et al., 2004), which encodes the NR2B subunit of NMDARs. NR2B-containing NMDARs are also predominantly expressed on the extrasynaptic membrane and have been shown to interact with EAAT3 (Scimemi et al., 2009, Jarzylo and Man, 2012). Secondly, the increase in evidence linking glutamatergic dysfunction with OCD has also coincided with the proliferation of

transgenic animal models. Two mouse models relevant to OCD, the *Sapap3* and *Slitrk5* null mouse models not only result in SRI-responsive repetitive behaviors but also exhibit cortico-striatal glutamatergic signal dysfunction and altered glutamate receptor expression (Welch et al., 2007, Shmelkov et al., 2010b, Wan et al., 2011, Wan et al., 2014). These models elicited considerable excitement in the field, especially with regard to the glutamate hypothesis; however evidence of specific genetic alterations in their corresponding genes in the human OCD population are less strong. The first optogenetic mouse model strengthened both the hyperactive CSTC circuit and glutamate dysfunction hypotheses by showing that long term repeated stimulation of glutamatergic OFC projections innervating the ventral striatum led to the emergence of SRI-responsive repetitive grooming and evoked firing (Ahmari et al., 2013).

There are many difficulties with accurately modeling neuropsychiatric disorders in mouse models. Direct translation of genetic abnormalities identified in the OCD patient population into animal models is limited due to a lack of known rare genetic variants that elicit a large effect, as well as the inability of genome-wide association studies to identify common variants that reach genome-wide significance. However, findings in transgenic and optogenetic animal models have furthered our understanding of potential mechanisms linked to the CSTC circuit and repetitive- or compulsive-like behavior. With these caveats in mind, my thesis research focused on taking advantage of the most replicated genetic evidence in the field, suggesting that altered expression or function of *Slc1a1/EAAT3*, a neuronal glutamate transporter prominently expressed in the CSTC circuit, is linked to the underlying pathophysiology of OCD. The goal of this research was to investigate the consequences of altered *Slc1a1/EAAT3* expression in an OCD-relevant behavioral context and to provide novel insights into the generation of rationally designed therapeutics aimed at improving the unmet therapeutic needs of the OCD patient population.

Specific Aims

The research presented herein was undertaken in an effort to understand the OCD-relevant consequences of reduced *Slc1a1*/EAAT3 expression and to further our knowledge of the functional role that EAAT3 plays in OCD-implicated neurocircuitry.

- 1) Characterize the biochemical effects of reduced *Slc1a1*/EAAT3 expression in the *Slc1a1*-STOP mouse model.
- 2) Identify baseline behavioral deficits resulting from reduced *Slc1a1*/EAAT3 expression in the *Slc1a1*-STOP mouse model.
- 3) Pharmacologically probe OCD-implicated brain circuitry in the *Slc1a1*-STOP mouse model in order to evaluate the effect of reduced *Slc1a1*/EAAT3 expression on striatum-dependent repetitive behavior.
- 4) Examine and localize the effects of *Slc1a1*/EAAT3 loss on cortico-striatal circuits.

CHAPTER 2

RODENT MODELS OF OBSESSIVE-COMPULSIVE BEHAVIOR

*As published in: Rodent Models of Obsessive Compulsive Disorder: Evaluating Validity to Interpret Emerging Neurobiology. Neuroscience (2016)
Isaac Zike, Tim Xu, Natalie Hong, Jeremy Veenstra-VanderWeele*

Abstract

Obsessive Compulsive Disorder (OCD) is a heterogeneous neuropsychiatric disorder with unknown molecular underpinnings. Progress in neuroimaging has linked the cortico-striatal-thalamo-cortical (CSTC) circuit within the brain to the symptomatic obsessions and compulsions of OCD patients. Genetic and non-genetic risk factors have also been identified resulting in animal models created using transgenic, immunological, pharmacological, and optogenetic tools. These models enable researchers to manipulate known OCD risk factors such as genes, or for the ability to perturb specific neurocircuitry implicated in the disorder and examine behavioral, biochemical, and electrophysiological responses. These model systems are essential to hypothesis testing however they have inherent limitations and are unable to fully reproduce the human condition. With the end goal of further understanding the molecular mechanisms underlying OCD-relevant behavior in rodents potentially leading to novel intervention strategies, relatively rigorous validation criteria (predictive, face, and construct) may be used to evaluate their utility with respect to how well they mirror the human disorder. Currently, assessment of available models has shown that no single model is likely to substantially encompass all validation criteria. As new molecular and genetic findings as well as novel technologies emerge, intricate dissection of implicated genes and circuits may be utilized to create models more representative of the human condition in preference of manipulations that elicit OCD-relevant behavior in rodents.

Introduction

Obsessive compulsive disorder (OCD) is a common, chronic condition characterized by persistent, intrusive obsessions, repetitive behavior, and anxiety (Calvocoressi et al., 1998). The disorder affects 1-3% of the population and is among the top ten causes of disability worldwide (Kessler et al., 2005, Koran et al., 2007, Michael S. Ritsner, 2007). First line forms of therapy include serotonin reuptake inhibitors (SRIs) and cognitive behavioral therapy; however, only 50-60% of patients show adequate response to available treatments (Koran et al., 2007). For example, a 20-40% decrease in OCD symptoms may result following SRI therapy (Dougherty et al., 2004), which leaves many with clinically significant residual symptoms. In some refractory OCD cases, deep brain stimulation (DBS) has also been used as a treatment alternative (Sturm et al., 2003, Goodman et al., 2010). Other augmentation therapies, such as antipsychotics and glutamatergic agents, are also being evaluated but have limited evidence for their utility (Arumugham and Reddy, 2013).

Ideally, more effective therapeutics would emerge from an understanding of the etiology of OCD. As with many neuropsychiatric conditions, the underlying causes of OCD are unknown and likely to involve both genetic and environmental factors. Hypotheses based on genetic and neuroimaging data have led researchers to create animal models that recapitulate the hallmarks of the disorder, with the aim of probing the underlying neurobiology. Here, we will discuss the growing number of proposed rodent models of OCD, including pharmacologically-induced, genetic, and optogenetic animal models, with a focus on assessment of the validity of the model in relation to knowledge of the human condition. Building upon the initial data on validity, we will describe the emerging understanding of neurobiological mechanisms in each model. Finally, we will discuss the approaches to take growing knowledge from these rodent models and translate it into novel treatments that can be applied in patients with OCD.

Genetic and non-genetic factors likely contribute to OCD risk

To understand the validity of rodent models, we must first understand the risk factors that may contribute to OCD. Abundant evidence for a heritable component of OCD stems from twin and family studies (Grados and Wilcox, 2007, Pauls, 2008). As reviewed elsewhere (Pauls, 2010), OCD symptoms are estimated to be 40-65% heritable in children and 27-47% heritable in adults (van Grootheest et al., 2005). Family studies indicate that OCD is twice as common in first-degree relatives of affected adults and ten times as likely in relatives of affected children (Pauls, 2008). These studies support the premise that OCD risk is derived from a complex combination of genetic and non-genetic factors.

Genetic linkage studies have yet to generate genome-wide significant findings for the core diagnosis of OCD, likely because of lack of statistical power in limited sample sizes (Hanna et al., 2002a, Mathews et al., 2012). The most promising linkage signal is on chromosome 9p24, based upon a suggestive linkage peak in the first genome-wide linkage study that was subsequently directly replicated in a study targeting only this region (Hanna et al., 2002a, Willour et al., 2004a). Another suggestive linkage peak on chromosome 15q14 was identified in two genome-wide linkage studies (Shugart et al., 2006). Genome-wide association studies (GWAS) have been similarly underpowered, to date, in OCD (Stewart et al., 2013b, Mattheisen et al., 2015). A single polymorphism near *BTBD3* reached genome-wide significance in a family-based subset of one GWAS analysis, but was only suggestive in the overall sample (Stewart et al., 2013b).

Candidate genes for OCD have been identified based upon proximity to linkage peaks, biomarker findings, and relationship to pharmacological targets. The strongest candidate gene association findings in OCD focus on the *SLC1A1*, which encodes the neuronal glutamate transporter, EAAT3 (Arnold et al., 2006, Dickel et al., 2006, Stewart et al., 2007, Kwon et al., 2009, Shugart et al., 2009, Wendland et al., 2009a, Samuels et al., 2011, Stewart et al., 2013b, Wu et al., 2013). Interest in *SLC1A1* stemmed from its location under the chromosome 9p24

linkage peak, as well as biomarker studies implicating the glutamate system in OCD. Elevated cerebrospinal fluid glutamate levels in two studies (Chakrabarty et al., 2005b, Bhattacharyya et al., 2009) are matched by increased glutamatergic signal in magnetic resonance spectroscopy studies in OCD (Moore et al., 1998, Rosenberg et al., 2000). Additional candidate genes in the glutamate system have also been studied, including *GRIN2B*, which shows evidence of association in some but not all studies (Arnold et al., 2004, Alonso et al., 2012, Cai et al., 2013). The other leading candidate gene is the serotonin transporter, *SLC6A4*, with both common and rare variants exhibiting association in some studies (Ozaki et al., 2003, Dickel et al., 2007, Grados et al., 2007, Saiz et al., 2008, Wendland et al., 2008), but not all (Taylor, 2013). The dopaminergic system (*COMT and DRD4*) has also been implicated via gene association studies; however these findings have yet to be replicated (Billett et al., 1998, Pooley et al., 2007). A lack of clear susceptibility genes contributes to the difficulty of modeling OCD in animals, though this issue is shared by most complex heterogeneous neuropsychiatric disorders.

Apart from genetics, autoimmunity has also received considerable attention as a potential risk factor for OCD. Observation of neuropsychiatric manifestations co-occurring with rheumatic fever and Sydenham's chorea lead to the description of Pediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal infections (PANDAS). As reviewed elsewhere (Williams and Swedo, 2015), children with this presentation may develop compulsions, tics, or other psychiatric symptoms very rapidly following an infection. Emerging data suggest that non-streptococcal infections may also serve as triggers for symptoms, leading to the more recent description of Pediatric Acute-onset Neuropsychiatric Syndrome (Chang et al., 2015). Anti-brain antibodies, including antibodies to the basal ganglia, have been identified in some cases of PANDAS, but with little evidence that antibodies consistently separate cases from controls or increase with symptom exacerbations (Pavone et al., 2004, Singer et al., 2004, Singer et al., 2005, Morris-Berry et al., 2013). In contrast, studies in the larger population of

patients with idiopathic OCD have more consistently reported elevated anti-basal ganglia antibodies in serum, as supported by a recent meta-analysis (Pearlman et al., 2014), as well as one study in cerebrospinal fluid (Bhattacharyya et al., 2009). It is not clear, however, whether and how these antibodies might be causally connected to the pathophysiology of OCD.

Basal ganglia circuitry is implicated in OCD

Evidence from neuroimaging studies has consistently implicated the basal ganglia as being involved in the development of OCD, specifically the cortico-striatal-thalamo-cortical (CSTC) loop (Saxena and Rauch, 2000, Ting and Feng, 2011). The most consistently implicated basal ganglia subregions in OCD patients are the caudate nucleus and the putamen, which make up the human striatum (Rauch et al., 2001, Rosenberg et al., 2001). Structural neuroimaging studies have consistently demonstrated abnormalities in caudate volume in OCD (Radua and Mataix-Cols, 2009, Radua et al., 2010). Functional imaging studies have also identified hyperactivity in cortico-striatal circuits, both at baseline and after symptom provocation (Menzies et al., 2008). The caudate is extrinsically innervated primarily by excitatory inputs originating from the cerebral cortex, the thalamus, and the substantia nigra pars compacta (Parent and Hazrati, 1995). Many studies have found that increased activity within the cortex, specifically the anterior cingulate (ACC) and orbitofrontal cortex (OFC) are seen in OCD patients (Chamberlain et al., 2008, MacMaster et al., 2008, Menzies et al., 2008). These studies, along with further meta-analysis of functional neuroimaging data in OCD patients (Whiteside et al., 2004, Whiteside et al., 2006), suggest that there is a functional abnormality resulting in hyperactivity of the basal-ganglia loop. A common model of this increased CSTC signaling suggests that it could arise from hyperactivity of the direct-pathway or by hypoactivity of the indirect pathway resulting in repetitive and compulsive behaviors (Ting and Feng, 2011).

Validation of mouse models relevant to OCD

Rodent models allow hypothesis testing via manipulation of OCD risk factors, as well as stimulation or inhibition of the circuitry implicated in OCD. The ability to perform such experimental manipulations is also accompanied by immediate access to the brain that allows for detailed probing of molecular and circuitry changes that may underlie the pathophysiology of OCD. Unfortunately, behavioral assessment in rodents is limited to compulsive-like behaviors, since obsessions are an internal experience that can only be measured via self-report. Animal studies aimed at eliciting OCD-relevant behaviors through the use of pharmacological, transgenic, immunologic, and optogenetic tools have resulted in a wide range of behavioral phenotypes with varying degrees of similarity to compulsive behaviors in the human population. Biochemical and electrophysiological analyses of the underlying cells and circuits believed to be involved in OCD are beginning to provide data that may enable researchers to develop novel therapeutics closer to the pathophysiology of the disorder.

Model systems are necessary to test hypotheses and probe the underlying neurobiology, but models are inherently limited and cannot fully recapitulate the human disorder. To assess the potential utility of a given model, three criteria are typically used, as first described by Willner in 1991: 1) construct validity, 2) face validity, and 3) predictive validity (Willner, 1991). By using these criteria to assess how well an animal model mirrors the human condition, we can better contextualize the significance of resulting findings, potentially allowing us to target our attempts at translation to subgroups of the human population that may more clearly map onto the risk factor, behavioral profile, or circuitry implicated in the rodent.

Construct Validity

The first and likely most important criterion for assessing animal model validity is construct validity. This is best understood in the context of genetic models, where a putative OCD risk gene is manipulated in mice to observe its behavioral and neurobiological

consequences. Similarly, an immunological trigger, such as the antibodies that have been detected in some cases of Pediatric Autoimmune Neuropsychiatric Disorders Associated with *Streptococcal* infections (PANDAS), could allow direct transfer into an animal model (Swedo, 2002, Snider and Swedo, 2004). Drug-induced worsening of OCD symptoms, such as has been reported with the anti-migraine 5-HT_{1B/D} agonists (Koran et al., 2001a) and psychostimulants (Varley et al., 2001, Bloch et al., 2009), may also provide construct validity. Since these drugs seem to exacerbate but not cause OCD, this approach to validity may not work independently from another risk factor, as discussed in the predictive validity section below.

Since circuitry is better defined than risk factors, could circuits be a source of construct validity? Or does this represent face or predictive validity? Often, validation criterion may overlap, as is the case when thinking about brain circuitry. The differentiation between the validity criteria when it comes to brain circuits may come down to experimental design. If alteration of circuit activity is the foundation of the animal model, as is the case in some recent optogenetic work, then construct validity may be supported. Conversely, for genetic models that result in electrophysiological changes in brain regions implicated in OCD, the circuitry findings may be better classified as face validity, as described below.

Face Validity

The second criterion, face validity, is usually based on the emergence of observable behaviors reminiscent of compulsive behavior, since OCD is a behaviorally defined disorder. The most commonly observed rodent behavior that supports face validity is excessive grooming, which corresponds to an increase in a behavior that follows a stereotyped pattern even in wildtype mice (Welch et al., 2007, Shmelkov et al., 2010a, Ahmari et al., 2013); although the stereotyped pattern itself may also be distorted in some models. In contrast, other models lead to a novel stereotyped, repetitive pattern of behavior, as is seen with dopaminergic (quinpirole) and serotonergic (8-OHDPAT) agonists that induce perseverative locomotor

behavior in the Y-maze or open field chambers (Yadin et al., 1991, Szechtman et al., 1998). Other measures, such as reversal learning or prepulse inhibition deficits, parallel findings that are not explicitly diagnostic but are instead thought to underlie the symptoms of OCD, such as cognitive rigidity or motor inhibition deficits (Chamberlain et al., 2006, Remijnse et al., 2006, Gu et al., 2008, Valerius et al., 2008, Andersen et al., 2010, Ahmari et al., 2012, Bissonette and Powell, 2012, Brigman et al., 2012, Remijnse et al., 2013, Hatalova et al., 2014, Zhang et al., 2015).

Face validity may also be established in animal models by identifying alterations in putative biomarkers that mirror those previously identified in the human OCD population. As reviewed above, several such biomarkers could be used to assess a rodent model of OCD, such as abnormal caudate volume (Radua and Mataix-Cols, 2009, Radua et al., 2010), hyperactivity in the cortico-striatal circuit (Menziés et al., 2008), increased glutamate concentrations in the caudate (Moore et al., 1998, Whiteside et al., 2006, Starck et al., 2008), and elevated cerebrospinal fluid glutamate levels (Chakrabarty et al., 2005a). Identification of other abnormalities in previously hypothesized neurotransmitter system, such as serotonin, dopamine, or glutamate (Dickel et al., 2007, Taylor et al., 2010, Pittenger et al., 2011), could also be asserted to represent face validity, but we must be careful not to stretch too far in assessing what “looks like” our conception of OCD.

Predictive Validity

Predictive validity of animal models of OCD may be determined in a few ways. Unfortunately, there is no straightforward way to model the cognitive behavioral therapy that should be the first-line treatment in OCD; however compulsive-like behaviors may be rescued or protected against through the administration of SRIs, as these are the first line medications. Additionally, response to SRIs in OCD requires several weeks of administration, which could be used to add specificity, as has been suggested in a few models that show response to chronic

or sub-chronic but not acute dosing. Adjunctive medications, such as atypical antipsychotics, which have some evidence of benefit in OCD, may also be expected to improve compulsive-like behavior, though this may be less specific since they also benefit tics in the human population. Invasive treatments, such as capsulotomy or high frequency stimulation (HFS) protocols similar to deep brain stimulation (DBS), have shown benefit in SRI-resistant OCD and could be used to assess predictive validity with a clearer relationship to any observed changes in circuitry (Greenberg et al., 2006). From the opposite perspective, common anxiolytics or antidepressants that are ineffective in people suffering from OCD, including benzodiazepines and noradrenergic reuptake inhibitors (NRIs), should be expected to be ineffective in rescuing the behavioral phenotypes observed in a valid mouse model of OCD. One challenge for the field is that many people with OCD show only modest or even minimal response to available treatments, and lack of predictive validity could indicate that a model corresponds to this subset – the precise group that we would most like to help.

One question is how to conceptualize drugs that appear to worsen OCD symptoms. When administered to wildtype animals, these drugs may provide support for construct validity; although there is little evidence for drugs inducing persistent OCD symptoms once the drug has been stopped. In contrast, within an existing model of an OCD risk factor, a drug targeting the serotonin or dopamine system may be a way to reveal the worsened symptoms that have been reported with certain drugs in OCD patients, including clozapine, stimulants, and sumatriptan and its analogs (Broocks et al., 1998, Koran et al., 2001b, Gross-Isseroff et al., 2004, Bloch et al., 2009, Fonseka et al., 2014). Ultimately, assessing a given model does not necessarily require checking off each box in the three types of validity but instead assessing the overall strength of the data and carefully drawing boundaries around the human population that it models. For example, if clozapine were used to induce repetitive behavior and CSTC circuit abnormalities in a particular inbred strain of mouse, then the initial step in translation would

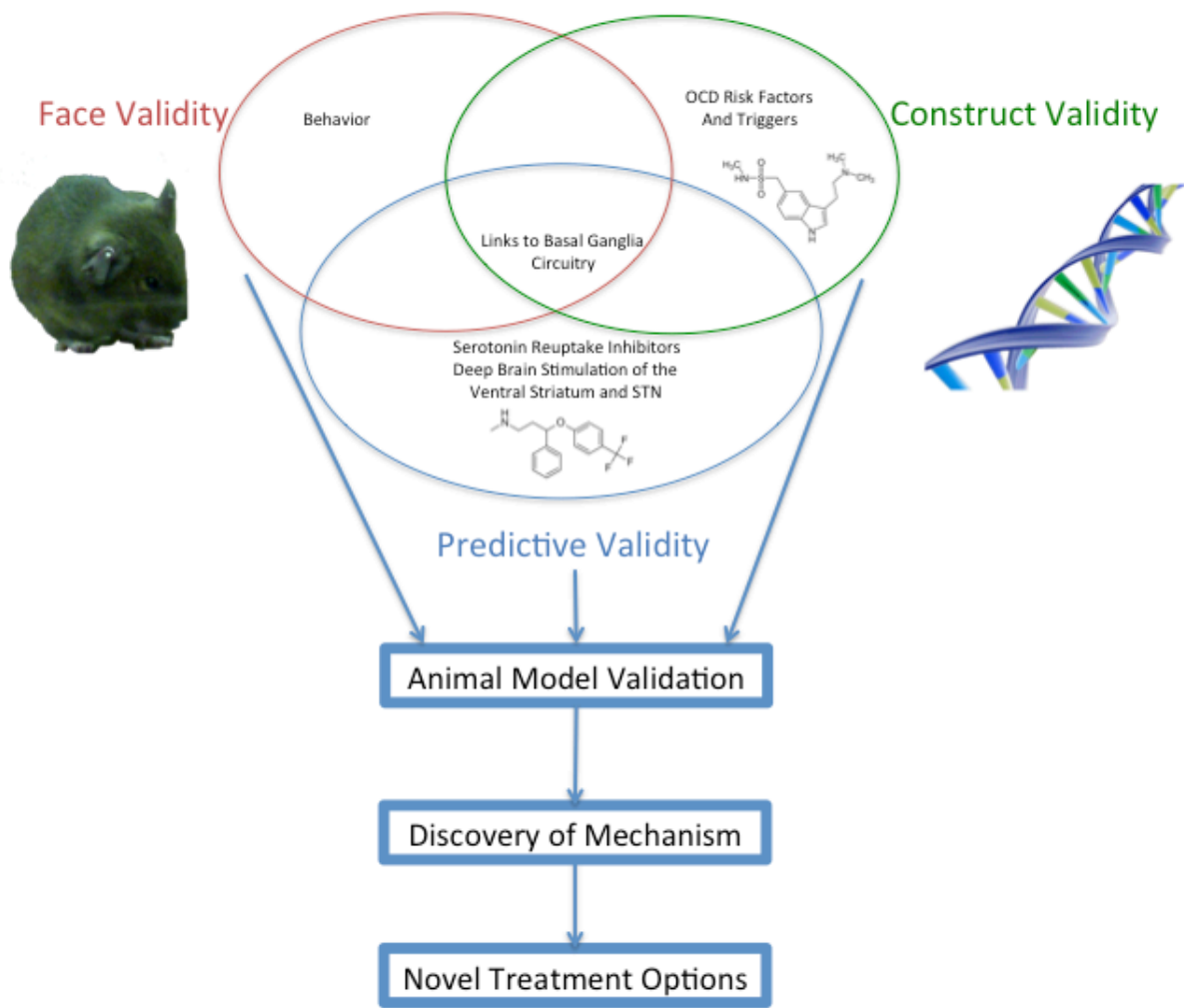


Figure 3. Evaluating Mouse Models of OCD-Relevant Behavior and Neurobiology

The most commonly used criteria to evaluate rodent models of Obsessive Compulsive Disorder are: 1) construct validity, 2) face validity, and 3) predictive validity. Each criterion contains exclusive components (grooming behavior for face validity or behavioral response to serotonin reuptake inhibitors for predictive validity). Some findings, such as the implication of cortico-striatal circuitry, may contribute to validation in any one of the three domains, depending upon context. For example, an optogenetic mouse model was constructed via chronic stimulation of cortical inputs to the striatum (Ahmari et al., 2013). Abnormal corticostriatal signaling is seen in a couple of genetic mouse models (Welch et al., 2007, Shmelkov et al., 2010), a form of face validity paralleling human neuroimaging findings. Finally, a different optogenetic paradigm seeks predictive validity by rescuing compulsive-like behavior by stimulating a cortico-striatal circuit (Burguiere et al., 2013).

target the human population that experiences treatment-emergent compulsions while taking clozapine.

Genetic Mouse Models

***Sapap3* Null Mice**

At the time of the initial description of OCD-related phenotypes in mice lacking *Sapap3*, (Sap90/PSD-95 associated protein 3) (Welch et al., 2007, Burguiere et al., 2013), no genetic data were available to examine its potential involvement in OCD. A subsequent study identified rare amino acid variants in the human *Sapap3* ortholog, *DLGAP3* (Discs, Large (Drosophila) Homolog-Associated Protein 3), in OCD and trichotillomania populations; although the degree to which these variants contribute to OCD risk remains somewhat unclear (Zuchner et al., 2009). Another association study found nominally significant association with a number of common polymorphisms and haplotypes in individuals with a grooming disorder (trichotillomania, excoriation, or nail-biting) in addition to OCD, but not in OCD alone (Bienvenu et al., 2009). Additionally, the largest genome-wide association study in OCD found some suggestive but not significant evidence of association with another DLGAP family member, *DLGAP1* (Stewart et al., 2013b). Collectively, these individual pieces of data provide plausible hints of construct validity without replicated evidence of involvement of *DLGAP3* in human OCD.

The face validity and predictive validity of the *Sapap3* null mouse is quite appealing and provided an example for subsequent studies of putatively compulsive-like behavior. Between 4-6 months, *Sapap3*-null mice exhibit a perseverative grooming phenotype that results in open skin wounds, as well as increased anxiety-like behavior, which together parallel the common co-occurrence of maladaptive repetitive behavior and anxiety in human OCD. It is also possible, however, to argue that increased, self-injurious grooming in the mouse maps less well onto the cleaning symptoms commonly seen in OCD, such as compulsive hand-washing, and more neatly onto the OC-spectrum conditions of trichotillomania or excoriation/skin-picking. Without access to “obsessions” that may drive the grooming behavior, it is impossible to clearly separate

these possibilities. It may be most useful, then, to conceptualize this mouse as a model of OC-spectrum behavior. Importantly, the excessive grooming is alleviated by sub-chronic (6 days), but not acute administration of fluoxetine, an SRI. It is important to note that chronic administration of SRIs for a month or more is necessary to achieve efficacy in the human OCD population; however differences in brain circuitry in mice compared to humans could underlie this difference.

SAPAP3 (SAP90/PSD95-associated protein 3 or DLGAP3) is a post-synaptic scaffolding protein that interacts with Post Synaptic Density Protein 95 and Shank protein families (Takeuchi et al., 1997), and is highly expressed within glutamatergic synapses of the striatum, providing plausibility for its involvement in OCD risk (Saxena and Rauch, 2000, Welch et al., 2007, Menzies et al., 2008, Ting and Feng, 2011). The initial characterization of *Sapap3* null mice demonstrated cortico-striatal glutamatergic transmission abnormalities, including defective synaptic transmission and reduction in N-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) glutamate receptor function (Welch et al., 2007). Further electrophysiological work with this model has revealed that loss of SAPAP3 enhances AMPA receptor endocytosis via an mGlu5-dependent mechanism (Wan et al., 2011). Interestingly, it was also found that constitutive and induced deletion of *Mc4r*, the gene coding for the melanocortin 4 receptor (MC4R) was able to rescue the elevated grooming phenotype and normalize the striatal signaling abnormalities of the *Sapap3*-null mouse (Xu et al., 2013). MC4R impacts feeding behavior and polymorphisms within the gene, *MC4R*, are the most common cause of hyperphagia and morbid obesity (Farooqi, 2008). Prior work with MC4R showed that stimulation of the receptor resulted in a compulsive-like grooming phenotype (Alvaro et al., 2003) and led the researchers to hypothesize that deletion of *Mc4r* within *Sapap3*-null mice may rescue the previously described grooming phenotype. At this point it is not understood how links between obesity or metabolism and compulsive behavior may be impactful, this double knockout of *Sapap3* and *Mc4R* is useful to show that intervention resulting

in the amelioration of striatal signaling abnormalities may be capable of rescuing OCD-relevant behavioral phenotypes as observed in the *Sapap3*-null mouse. Additional defects in inhibitory neurotransmission of *Sapap3*-null mice have also been identified, including reductions of parvalbumin-positive fast spiking interneurons, resulting in abnormalities in a learning related behavioral task that will be discussed in future sections (Burguiere et al., 2013), further supporting the hypothesis of dysfunctional neurotransmission within the cortico-striatal circuit in this model. As the first genetic mouse model to be described with SRI-responsive, striatally-dependent repetitive behavior, the *Sapap3* null mouse has already begun to yield insights that could potentially translate into human OC-spectrum disorders. In the absence of clear genetic data, however, it remains difficult to assess whether findings in this mouse offer a direct parallel to human OCD.

***Slitrk5* Null Mice**

Interest in the *SLIT* (*slit guidance ligand*)- and *TRK* (*trunk*)-like (*Slitrk*) gene family initially emerged from a chromosomal translocation and follow-up rare variant association study linking *Slitrk1* and Tourette's syndrome (Abelson et al., 2005), with support in some but not all subsequent studies (Keen-Kim et al., 2006, Scharf et al., 2008, Miranda et al., 2009, O'Roak et al., 2010, Karagiannidis et al., 2012, Ozomaro et al., 2013). SLITRKs are single-transmembrane proteins containing two extracellular leucine-rich repeat domains, similar to SLIT proteins, and a carboxy-terminal domain similar to tyrosine kinase receptors (Proenca et al., 2011). Like SLIT proteins, SLITRK proteins are widely expressed throughout the brain and are believed to direct neurite outgrowth. Tic-like or compulsive-like behavior has not been described in *Slitrk1*-null mice, but these animals do exhibit increased anxiety-like behaviors and elevated norepinephrine levels in the prefrontal cortex and nucleus accumbens (Katayama et al., 2010). On the other hand, knockout of another *SLITRK* family member, *Slitrk5*, resulted in a phenotype similar to that previously described in the *Sapap3* null mice. It is unclear whether genetic association data

at *SLITRK1* should be seen as contributing to construct validity of *Slitrk5* null mice as a model of OC-spectrum behavior. Similarly, a recent genome-wide study in OCD found suggestive association near *PTPRD* (*protein tyrosine phosphatase, receptor type D*), which encodes a member of the receptor protein tyrosine phosphatase family that interacts with SLITRK3, providing more circumstantial evidence for involvement of this protein family, if not *SLITRK5* itself.

Shmelkov and colleagues (2010) developed and characterized a *Slitrk5*-null mouse that displayed face-validity in the form of elevated grooming resulting in facial hair loss and skin lesion by three months of age (Shmelkov et al., 2010b). It is also of note that hemizygous *Slitrk5* mice developed similar lesions but with a delayed onset at seven to nine months of age, which has not been reported in the *Sapap3* heterozygous animals. *Slitrk5*-null mice also displayed increased anxiety-like behaviors in the elevated plus maze and open-field test. With regards to predictive-validity, chronic treatment with the SRI, fluoxetine, reversed the elevated grooming phenotype in *Slitrk5*-nulls; although the consequences of acute treatment were not shown.

The *Slitrk5* null animals also showed biochemical and structural findings in the CSTC circuit implicated in OCD. Increased FosB immunoreactivity in the OFC in these animals may parallel functional imaging finding in OCD (Chamberlain et al., 2008). Similarly, they show decreased striatal volume that may parallel reports of reduced striatal volume relative to whole-brain measurements in OCD patients (Rosenberg et al., 1997). As with the *Sapap3* null, defective neurotransmission was measured in striatal slice preparations from *Slitrk5* null mice, linking abnormalities in brain regions previously implicated in OCD to this model. As an almost direct parallel to the established pattern of validation in the *Sapap3* null mouse, the *Slitrk5* null mouse model shows SRI-sensitive, excessive grooming behavior in the context of altered corticostriatal structure and function. Again, more genetic data will be needed to evaluate whether this provides a direct model of OCD or OC-spectrum risk; however it is possible that

insights from this model will translate to SRI-dependent repetitive behavior across species, regardless of whether this particular genetic mechanism underlies OCD itself.

***HoxB8* Null Mice**

HOXB8 is a component of the mammalian Hox (Homeobox-containing) complex, a family of 39 transcription factors important in determining the anteroposterior axis during development (Capecchi, 1997). In contrast to the above models, where some genetic data, albeit limited, point to potential genetic involvement in OCD or OC-spectrum disorders, no data implicate HOXB8 or its gene family in the human disorder. On the other hand, Greer and Capecchi (2002) noted that *HoxB8*-null mice show excessive grooming resulting in extensive hair loss and skin lesions (Greer and Capecchi, 2002). *HoxB8*-null animals self-groom excessively and also have increased social grooming of control animals. A subsequent report from another group using a different *Hoxb8* null mouse line suggested that deficits in spinal cord function led to changes in sensitivity to pain, potentially underlying the excessive, self-injurious grooming behavior (Holstege et al., 2008). A follow-up study conducted by the Capecchi group in the original *Hoxb8* null mice, however, clearly demonstrated that central nervous system function was responsible for excessive grooming in these animals (Holstege et al., 2008, Chen et al., 2010). Remarkably, targeted knockout of *Hoxb8* in the hematopoietic system alone was found to induce excessive grooming in these mice, and bone marrow transplant resulted in rescue of the grooming phenotype in *Hoxb8* null animals. Taken together, these findings suggest that dysfunction resulting from peripherally-derived microglia accounts for compulsive-like grooming in these animals.

While the latest study on targeted *Hoxb8* knockout ruled out peripheral nervous system (PNS) defects as the cause of the grooming phenotype, the broad expression of *Hoxb8* in microglia of the brain prevents straightforward localization of the implicated brain region(s). As noted above, this model lacks clear construct validity, and predictive validity has not been

assessed with an SRI rescue experiment. Despite this, the novelty of microglial involvement in excessive grooming provides an interesting new direction for potential treatment of compulsive-like behavior. As noted above, immune or autoimmune mechanisms have been explored in OCD; although without conclusive data tying a particular mechanism to emerging symptoms (Swedo, 2002, Martino et al., 2009). Intravenous immunoglobulin or plasmapheresis have been explored for treatment of Streptococcal infection-associated compulsive behaviors (Snider and Swedo, 2004); however, no current treatments have targeted the monocyte lineages leading to microglia. The *Hoxb8* null model, then, may be an example of a genetic mouse model limited to face validity that nevertheless generates a very novel hypothesis about OCD risk or treatment. Without construct or predictive validity at the outset, however, translational studies targeting peripherally-derived microglia seem quite risky and should likely be reserved for truly refractory OCD unless more data emerge implicating this mechanism in a subgroup of patients with OCD.

***Slc1a1*/EAAT3 null mice**

As a counter example to these three mouse models that show compulsive-like behavior but without strong construct validity, the neuronal glutamate transporter gene *SLC1A1* (*Solute Carrier Family 1 Member 1*) has the most genetic evidence to date, but behavioral assessment of mice lacking *Slc1a1* has not suggested altered compulsive-like behavior, to date. As noted above, both linkage and association data point to *SLC1A1* in OCD, particularly in males with early-onset symptoms (Veenstra-VanderWeele et al., 2001, Hanna et al., 2002b, Willour et al., 2004b, Arnold et al., 2006, Dickel et al., 2006, Stewart et al., 2007, Shugart et al., 2009, Wendland et al., 2009b, Samuels et al., 2011). Adding circumstantial evidence, a recent study of 3 patients with dicarboxylic aminoaciduria identified rare loss-of-function mutations in *SLC1A1*, with one of the probands reporting long-standing checking and hand-washing behaviors that could be OCD-related but were not formally evaluated (Bailey et al., 2011). *Slc1a1* mRNA and its corresponding protein, EAAT3 (Excitatory Amino Acid Transporter 3), are

strongly expressed in the cortico-striatal regions implicated in OCD (Nieoullon et al., 2006). EAAT3 is also involved in mediating neuronal cysteine transport, an essential rate-limiting step in the synthesis of the antioxidant glutathione (Aoyama et al., 2006, Aoyama and Nakaki, 2013).

Initial publication of an *Slc1a1*/EAAT3 null mouse suggested little behavioral relevance to OCD (Peghini et al., 1997), with the only significant change being decreased spontaneous open-field locomotor activity. Subsequent studies with the EAAT3 null mouse focused on the potential neurodegenerative effects associated with the loss of neuronal cysteine transport. Aoyama et al. (2006) reported reduced neuronal glutathione levels and age-dependent neurodegeneration, shown by cortical thinning and enlarged ventricles. As they aged, EAAT3 null mice were observed to show increased aggression and to appear disheveled. At eleven months of age, but not at two months of age, EAAT3 null mice showed hippocampal atrophy and declines in learning on the Morris water maze (Aoyama et al., 2006). The available data, then, certainly do not suggest face validity for the EAAT3 null animals in relation to OCD. A lack of face validity in these mice is not surprising, as the majority of genetic evidence suggests that increased *SLC1A1*/EAAT3 expression, rather than decreased expression, as in the null animals, would be predicted from the OCD-associated polymorphisms (Wendland et al., 2009b). By not creating a transgenic model accurately reproducing the genetic changes in *Slc1a1* predicted to result from common OCD-associated SNPs, construct validity of the EAAT3 null mice is limited at best. Further work to evaluate the impact of *Slc1a1*/EAAT3 should focus on modeling increased expression. Alternatively, face validity could potentially be demonstrated by attenuation of pharmacologically- or genetically-mediated compulsive-like behaviors in mice with diminished EAAT3 expression.

Other genetic manipulations also lead to increased grooming

Excessive grooming is a common behavioral phenotype described in these mouse models of compulsive-like behavior, but it is not exclusive to OCD-like mouse models. One early

mouse model created to mimic the hypothesized hyperactivity of the direct basal ganglia pathways postulated to underlie obsessive and compulsive behavior in humans, is the *DICT-7* transgenic mouse (Campbell et al., 1999). These mice express cholera toxin (CT) in dopamine receptor 1 expressing neurons, resulting in potentiation of CSTC circuitry, providing limited construct validity to the model. While face validity of this model is supported via increased anxiety-like and repetitive behaviors, any measures exploring predictive validity with SRIs are absent. Notably, this model is a precursor to the use of optogenetics to affect the activity of neuronal activity in implicated circuitry as in the Ahmari model evaluated below. Many other genetic manipulations similarly generate increased levels of grooming, especially those related to autism spectrum disorders (ASD), where one hallmark of the disorder is excessive repetitive behavior that may or may not be OCD-like (Jacob et al., 2009). ASD-related models, including the *Cntnap2* null, *Neurexin1* null, *Shank3* null, and *Integrin beta 3* null, are primarily based on genes that have been associated with ASD and other neurodevelopmental disorders (Etherton et al., 2009, Carter et al., 2011, Peca et al., 2011, Penagarikano et al., 2011). The *Cntnap2* null and the *Shank3* null mice also show altered striatal structure or function, supporting relevance to OCD despite construct validity primarily related to ASD. Although the construct validity of these models specifically to OCD may be tenuous, the above genetic models thought to be most relevant to OCD are also lacking in either construct or face validity. Importantly, none of the ASD genetic models have been evaluated for response to SRIs, which limits evaluation of predictive validity. On the other hand, the *Cntnap2* null mouse does show decreased grooming with a non-sedating dose of risperidone, an atypical antipsychotic medication frequently used in ASD but also used as an adjunctive medication in OCD (Bloch et al., 2006). Overall, the emerging pattern of increased grooming in genetic mouse models suggests that this species-typical repetitive behavior may be a useful readout for altered corticostriatal signaling, regardless of whether the model is most relevant to OCD or ASD (Kalueff et al., 2016).

Immunological Models

Alternative models aimed at understanding the potential role of the immune system and the induction abnormal neuropsychiatric behavior may also be a fruitful path in relation to OCD. One model with potential construct validity is based on the observation that streptococcal infections may induce obsessive-compulsive behavior and tics in children, as noted above (Swedo et al., 1998). Mouse models intended to mimic PANDAS have shown mixed results. The primary hypothesis of these investigations is that immune response to streptococcal infection would alter neuronal function and lead to the induction of repetitive behavior reminiscent of those reported in PANDAS. Since mice are not naturally infected with group A beta-hemolytic *streptococcus pyogenes* (GABHS) it is challenging to develop a direct model of the human pathophysiology. An alternative would be to transfer antibodies or sera from human patients into mice; although this also has challenges due to the differences between protein antigens across species.

The first attempt at a PANDAS model used repeated injection of a GABHS homogenate together with Freund's adjuvant to generate an immune response (Hoffman et al., 2004). In comparison to animals injected with adjuvant alone, sera from these GABHS-exposed mice showed greater immunoreactivity to the globus pallidus and thalamus with the CSTC loop implicated in OCD; however the strongest finding was greater immunoreactivity to the deep cerebellar nuclei (DCN) (Hoffman et al., 2004). Animals with enhanced immunoreactivity to the DCN exhibited increased rearing behavior in the open field arena and in hole board tests compared to controls and to GABHS-immunized mice without increased DCN immunoreactivity. A follow-up study from the same group tested if passive transfer of antibodies from GABHS-immunized animals to non-immunized animals was capable of inducing similar behavioral disturbances (Yaddanapudi et al., 2010). Increased rearing behavior was again observed in the open-field, and a more extensive behavioral evaluation also demonstrated reduced aggression

and social interaction in resident-intruder behavioral tasks, as well as motor coordination deficits. In theory, the rearing behavior observed in this model could represent a repetitive, compulsive-like behavior, but rearing is also an exploratory behavior that may indicate decreased anxiety-like behavior or increased exploratory drive. Motor coordination deficits and broad changes in emotional reactivity are sometimes reported in PANDAS, but this seems less relevant to OCD symptoms in particular (Swedo et al., 1998). Evaluating predictive validity in a PANDAS model is a little challenging, since serotonin reuptake inhibitors have not been explicitly tested in this subpopulation of OCD.

Rats have also been utilized in the creation of a rodent PANDAS model. The goal of the researchers was to create an animal model that duplicated the behavioral, pharmacological, and immunological phenotypes of streptococcal-related neuropsychiatric disorders. Male rats exposed to GAS (Group A *streptococcus*) antigen had impaired motor symptoms (food manipulation and beam walking) while also exhibiting elevated induced-grooming, which was rescued by dopamine D2 antagonist and SRI administration respectively (Brimberg et al., 2012). As previously described in the mouse PANDAS model, the GAS-exposed rats had increased levels of immunoreactivity in the previously implicated OCD-relevant basal ganglia circuit. This study was also able to show the sera isolated from the GAS exposed rats reacts with both D1 and D2 dopamine receptors, providing face validity based on the implication of the dopamine system in both PANDAS and OCD. The model exhibits relatively strong construct validity specifically for PANDAS while it's face validity may be more reminiscent of disorders such as Sydenham Chorea, in which patients display both motor and compulsive or tic-like symptoms. The predictive validity of the model is more tenuous when looked at specifically with regards to OCD. Administration of dopamine D2 receptor antagonists rescued the motor phenotype however dopamine D2 receptor antagonists in OCD have been utilized primarily as augmentation therapy to SRIs in OCD (Li et al., 2005), in contrast to the standalone manner they were used in this model. As noted previously, SRI's are the preeminent form of

pharmacological therapy used in the treatment of OCD however they typically require weeks of administration prior to observable efficacy and in this rat PANDAS model, three days of SRI treatment attenuated the compulsive-like phenotypes. It is debatable how much predictive validity that this provides due to the differing timelines for onset of efficacy when framed specifically for OCD and the lack of studies evaluating the effectiveness of SRIs in the PANDAS subpopulation. A follow up study from the same group expanded on the previous rat PANDAS findings by asking if intra-striatal passive transfer of isolated antibodies of GAS-exposed rats was able to induce similar behavioral abnormalities observed above. In this study, rats striatally infused with IgG purified from Group A *streptococcus*-exposed rats (GAS-I), took longer to traverse a narrow beam however no induced-grooming phenotypes emerged relative to naïve rats or those infused with total IgG from adjuvant-exposed rats (Lotan et al., 2014). Interestingly, the GAS-I rats did exhibit increased marble burying behavior compared to Control-I and naïve rats, which had been previously observed in GAS-exposed rats but not published. Face validity of elevated marble burying is uncertain due to benzodiazepines being able to attenuate the behavior in wild-type mice (Nicolas et al., 2006) despite their ineffectiveness in the human OCD population (Crockett et al., 2004). Taken together, this expanded PANDAS model was able to reproduce some of the behavioral abnormalities previously published and further identify behavioral phenotypes that may be relevant to OCD while isolating a specific brain region where autoantibodies produced following Group A *streptococcus* exposure may be exerting their pathogenicity.

Injecting sera from human patients with PANDAS into rodents may be a more valid approach, given that mice are not typically infected with GABHS; although species differences could also distort patterns of immunoreactivity. One study infused sera isolated from patients with PANDAS or Tourette syndrome into either the ventral or ventrolateral striatum of rats. They found no behavioral abnormalities and concluded that antibody reactivity in the striatum was unlikely to mediate the behaviors observed in PANDAS (Loiselle et al., 2004). The body of

literature on immunological models of OCD is quite small and difficult to evaluate at this point because of the limitation of studies using GABHS in rodents. On the other hand, interest in immune-mediated mechanisms continues to grow in light of the growing literature on PANDAS and PANS as well as the rescue of the excessive grooming behavior in the *Hoxb8* null mouse by bone marrow transplantation.

Pharmacological Models

Pharmacological models of OCD-relevant behavior are based on drug-induced behavioral phenotypes reminiscent of OCD behaviors observed in human patients (e.g., perseveration, compulsive checking). In the models evaluated, drugs that affect previously implicated neurotransmitter systems drive the OCD-relevant behavior. Due to numerous reports of alterations of both the dopaminergic and serotonergic system in people with OCD (Denys et al., 2004, Fineberg et al., 2012), it is reasonable to believe that manipulation of these neurotransmitter systems could lead to OCD-relevant behaviors in rodents. It is unclear, however, exactly how dopamine and serotonin are involved in the underlying pathogenesis of the disorder, thus the validity of each model must be carefully evaluated when making conclusions from these data, with predictive validity becoming even more important. In contrast, two drug classes have been reported to exacerbate or trigger OCD in the human population. One of these, the 5-HT_{1B} agonist drugs in the triptan class, has led to a pharmacological model, as described below. The other, atypical antipsychotic drugs, with clozapine as the primary offender (Fonseka et al., 2014), has not yet been examined as a potential trigger of compulsive-like behavior in rodents.

OH-DPAT induced decrease in spontaneous alternation

One of the initial drugs to be studied in relation to compulsive-like behavior was 8-hydroxy-2-(di-n-propylamino)-tetraline (8-OH-DPAT), a serotonin 5-HT_{1A} receptor agonist that also has some activity at 5-HT₇ (Yadin et al., 1991). Of note, no compulsive-like response has been described in humans in response to 5-HT_{1A} agonists, such as buspirone, so construct validity is limited to the general implication of the serotonin system in OCD. Acute administration of 8-OH-DPAT to rats results in a decrease in spontaneous alternation behavior in the T-maze. Instead of spontaneously switching the arm that is entered, rats who receive the drug tend to return repeatedly to the same arm, which may parallel the perseverative behavior often observed in OCD but could also indicate defects in working memory (Dek et al., 2015). Chronic fluoxetine prevents this drug-induced behavior, potentially providing predictive validity; whereas desipramine does not, providing evidence of specificity (Fernandez-Guasti et al., 2003). Importantly, however, diminution of serotonin receptor-mediated behavior with chronic administration of a serotonin reuptake inhibitor is expected due to receptor down-regulation in the context of tonic increases in extracellular serotonin levels. It is difficult, therefore, to know whether this truly indicates predictive validity or whether it simply represents an expected homeostatic mechanism. An alternative approach to predictive validity would be to parallel neuromodulatory treatments in OCD. Both bilateral lesions and bilateral low-frequency stimulation of the thalamic reticular nucleus diminished perseverative response to 8-OH-DPAT; although high-frequency stimulation, which more clearly parallels the response to deep brain stimulation in OCD, had no effect (Andrade et al., 2009, Andrade et al., 2010). More recent work suggests that 8-OH-DPAT may also induce perseverative behavior in other contexts, including returning repeatedly to the same location in an open field arena, which has been described as “compulsive checking” behavior (Alkhatib et al., 2013). Overall, despite interesting data, this model is challenged by difficulties in establishing clear validation in any of the three domains.

Quinpirole induced “compulsive checking”

Chronic administration of the D2/3 receptor agonist quinpirole to rats results in a perseverative exploration phenotype also described as “compulsive checking” behavior (Szechtman et al., 1998). This model has some degree of construct validity with regard to the dopamine system generally, since stimulant drugs and dopamine agonists can lead to repetitive behaviors, or frank compulsive behavior, in some contexts, such as with treatment of Parkinson’s disease or attention deficit hyperactivity disorder (Borcherding et al., 1990, Djamshidian et al., 2011, Weintraub et al., 2015). On the other hand, stimulants do not appear to be a common cause of worsening in OCD patients themselves and may even lead to improvements in some patients (Insel et al., 1983). The quinpirole model uses an open field chamber containing four small cubes, and rats receiving 5 or more weeks of drug treatment show more rapid and excessive returns to preferred objects, as well as ritual-like motor behaviors, in comparison to rats receiving vehicle injections (Szechtman et al., 1998). Chronic treatment with clomipramine, an SRI in the tricyclic class, delayed but did not eliminate this behavior. High frequency stimulation and temporary inactivation of the subthalamic nucleus (STN) was able to attenuate this compulsive-like behavior (Winter et al., 2008), paralleling one of the most common neuromodulatory targets in OCD (Mallet et al., 2002, Fontaine et al., 2004). Similarly, high-frequency stimulation of the OCD-linked nucleus accumbens, another common target in OCD (Sturm et al., 2003, Greenberg et al., 2006, Rauch et al., 2006), also reduced quinpirole-induced checking (Mundt et al., 2009). In sum, the predictive validity of this model is impressive, with interesting behavioral parallels to OCD as well, but the construct validity is more difficult to evaluate.

RU24969 induced repetitive circling and prepulse inhibition deficits

Serotonin 5-HT_{1B} receptor agonist drugs, such as sumatriptan, are used to treat migraines and have been reported to worsen OCD in some patients (Koran et al., 2001a, Gross-

Isseroff et al., 2004). Based upon this observation, treatment with the 5-HT_{1B} agonist RU24969, which also has some activity at 5-HT_{1A}, has been examined as a possible construct valid pharmacological model of OCD. Acute treatment with RU24969 in mice results in a dramatic increase in activity, and this activity is dominated by perseverative circling around the perimeter of an open field chamber (Shanahan et al., 2009). Direct injection of a 5-HT_{1B} antagonist demonstrated that 5-HT_{1B} in the OFC is necessary for this perseverative circling response (Shanahan et al., 2009, Shanahan et al., 2011), paralleling neuroimaging data implicating the OFC in OCD (Menzies et al., 2008). Furthermore, RU24969 treatment results in prepulse inhibition (PPI) deficits that may parallel data in OCD, as well as in a number of other neuropsychiatric conditions' (Swerdlow et al., 1993, Shanahan et al., 2009, Ahmari et al., 2012). Chronic treatment but not acute treatment with fluoxetine, but not desipramine, rescued both the repetitive circling behavior and the PPI deficits (Shanahan et al., 2009). This provides some degree of predictive validity but with the same challenge of circularity when examining 5-HT receptor response after chronically increasing extracellular 5-HT with an SRI. Considering all of the evidence, RU24969 treatment is one of the most convincing pharmacologically-induced models due to the most impressive construct validity, coupled with some evidence for both face and predictive validity.

Neonatal clomipramine

Neonatal exposure to fluoxetine has previously been described to lead to long-term increases in anxiety-like behavior in mice (Ansorge et al., 2004), supporting the possibility that such exposure could also lead to risk of compulsive-like behavior. No epidemiological studies have yet examined this hypothesis in human studies, but the Andersen lab administered clomipramine, an SRI in the tricyclic class, to rat pups from postnatal day 9-16, followed by assessment of relevant behaviors in adulthood (Andersen et al., 2010). Exposed rats showed increased anxiety-like behavior on the elevated plus maze, increased marble burying, and

increased “hoarding” of food pellets at the bottom of the cage, as well as deficits in OCD-relevant cognitive tasks (spontaneous alternation, reversal learning, and working memory-related tasks). All of the above behaviors are evocative of symptoms or cognitive deficits observed in OCD patients (Krebs and Heyman, 2015). They also reported increased 5-HT_{2C} receptor mRNA in the OFC and dopamine D₂ receptor mRNA in the striatum of experimental rats compared to controls, providing some parallel to neuroimaging studies in OCD (Remijnse et al., 2006, Menzies et al., 2008). This developmental model shows the broadest range of OCD-relevant phenotypes with perhaps greater face validity than any other model described to date, but it lacks construct and predictive validity at this point. These intriguing findings, however, raise the possibility that early life programming, either via pharmacological exposure or via genetics, may result in adaptive changes in OCD-relevant brain regions that lead to later emergence of compulsive-like behavior.

Optogenetic Mouse Models

As both human neuroimaging data and electrophysiology data from genetic mouse models continue to refine our understanding of the underlying circuitry, optogenetic approaches can be used to test these hypotheses by directly stimulating or inhibiting components of the cortico-striatal-thalamo-cortical network. By taking an alternative approach from targeting genes identified from OCD genetic studies, the optogenetic approach may point to circuitry-based treatment options such as repetitive transcranial stimulation or deep brain stimulation, rather than focusing on a molecular understanding or potential pharmacological treatments. These techniques permit alteration of neural activity with brain region, genetic, and temporal precision. Optogenetic technology has recently been used to in two separate studies in an attempt to 1) rescue OC-relevant behaviors in a previously studied genetic mouse model via stimulation of neurons projecting from the lateral OFC to striatum, and 2) induce compulsive-like behaviors

using optogenetic stimulation of neurons projecting from the OFC to the ventral medial striatum (VMS). Whether used in conjunction with previously validated transgenic models or on their own, optogenetic tools may revolutionize the study of disease-relevant circuits in animal models of OCD.

Optogenetic induction of excessive grooming behavior

Ahmari and colleagues used optogenetics to test circuitry-based hypotheses about compulsive behavior (Ahmari et al., 2013). Rather than focusing on a genetic or immune hypothesis of OCD risk, they focused on the intermediate phenotype of hyperactivation of the OFC and VMS in OCD (Insel and Winslow, 1992, Rosenberg and Hanna, 2000, Pauls et al., 2014), matched by the observation that deep brain stimulation of the ventral striatum is effective in reducing OCD symptoms in some patients (Rodriguez-Romaguera et al., 2012). Based upon these lines of evidence in humans with OCD, they hypothesized that increasing signaling from the OFC to the VMS would result in increased compulsive-like behavior in mice. The optogenetic approach is very different from genetic manipulations that attempt to establish construct validity by linking to the most proximal cause of OCD risk. Instead, this type of experiment may be thought to primarily examine the plausibility of the cortico-striatal-thalamo-cortical circuit hypothesis of OCD. Remarkably, however, whereas the results may not directly inform our understanding of the proximal risk factors contributing to OCD risk, the results suggest that manipulation of this circuit could be used to understand the dysfunction that generates compulsive-like behavior, and potentially to develop new treatments based upon this understanding.

Ahmari and colleagues introduced the light-sensitive cation channel, Channelrhodopsin via viral injection to the OFC. They then applied light stimulation in the VMS to establish specificity for the OFC-VMS circuit. Contrary to the initial hypothesis, acute OFC-VMS

hyperstimulation did not increase repetitive behavior. In contrast, repeated hyperstimulation of the OFC-VMS projection generated a progressive increase in grooming, which persisted for two weeks after the stimulation was stopped. No self-injury was described in these animals, but the increase in grooming suggests that the OFC projection to the VMS can alter this species-typical stereotyped behavior that is also increased in genetic models with face and predictive validity for OCD. Importantly, electrophysiologic measurements were also taken and a progressive increase in light-evoked firing was observed with repeated stimulation, paralleling the increase in repetitive grooming behavior.

Moving beyond testing the plausibility of the CSTC hypothesis, they then examined whether an SRI could rescue the induced grooming. Remarkably, treatment with two weeks of fluoxetine following a seven-day optogenetic stimulation protocol was able to normalize light-evoked neuronal activity and attenuate grooming behavior, providing evidence of predictive validity. Taken as a whole, these data move beyond establishing plausibility for altered corticostriatal signaling as an intermediate phenotype in compulsive-like behavior. The increase in VMS response to light-induced activity with repeated hyperstimulation suggests that plasticity at OFC-VMS synapses induces long-lasting alterations that prime the OFC-VMS synapses, resulting in a reduced activation threshold during subsequent bouts of stimulation. Increased OFC-VMS activity may transmit information through the CSTC circuit, leading to multiple downstream events that generate a repetitive pattern of behavior. The combination of construct, face, and predictive validity make this model very appealing for further dissecting the impact on local microcircuits in the VMS, downstream effects on CSTC signaling, as well as the molecular events underlying the observed synaptic plasticity, with the possibility that other manipulations, beyond SRIs, could also rescue the altered corticostriatal signaling and compulsive-like behavior.

Optogenetic rescue in *Sapap3* null mice

Burguire and colleagues (2013) used optogenetics to take the opposite approach. Rather than using inducing compulsive-like behavior, they evaluated the ability of circuit manipulation to rescue compulsive-like behavior in the *Sapap3* null mouse model. They first designed a novel delayed conditioning task to achieve temporal and conditioned control over the previously described excessive grooming phenotype. They presented a tone followed by an unconditioned stimulus of a water drop delivered to the nose, which induced grooming behavior in both the wildtype and the *Sapap3* null mice. Probe trials, with the tone presented alone without the water drop, were interspersed with conditioning trials. In mid-training, both wildtype and *Sapap3* null mice showed grooming response to the tone alone, but later in training this was suppressed in the wildtype but not the mutant mice. This suggests that *Sapap3* null mice are unable to inhibit their conditioned grooming response to the tone, even after observing only intermittent presentation of the unconditioned water drop stimulus. In parallel, they found that medium spiny neuron activity was higher in the *Sapap3* null animals late in the training period when the response pattern diverged. Interestingly, they noted a decrease in striatal parvalbumin positive (PV⁺) interneurons in the *Sapap3* null animals, suggesting that defective inhibition could account for the electrophysiological and behavioral defects.

To assess whether cortical input could be used to rescue inhibition deficits in the striatum, Burguire and colleagues used optogenetics to stimulate axon terminals of the lateral orbitofrontal cortex (l-OFC) within the striatum, based on previous findings implicating these regions in OCD (Chamberlain et al., 2005, Remijnse et al., 2006, Chamberlain et al., 2008). Remarkably, activation of l-OFC input to the striatum in the *Sapap3* null mice was able to attenuate their elevated MSN activity late in training, presumably by enhanced feed-forward inhibition driven by cortical activation of fast-spiking striatal interneurons. Beyond the electrophysiological rescue, they also found that optogenetic stimulation rescued the tone-

response inhibition deficits observed in the mutants. Even outside the context of the delayed conditioning task, they found that optogenetic activation of I-OFC input to the striatum decreased compulsive-like grooming in the mutant mice. Importantly, this optogenetic rescue makes use of I-OFC input to striatum, despite no clear evidence that I-OFC activity is defective in the *Sapap3* null mice (Burguiere et al., 2013). Such an approach could be paralleled by deep brain stimulation in the human population, where the manipulation depends upon an understanding of the circuits implicated in OCD, without knowing specifically what node in the circuit may be dysfunctional in an individual patient. This idea could be further tested by extending this optogenetic paradigm to other mouse models with compulsive-like grooming behavior due to other genetic manipulations, such as the *Slitrk5* or *Hoxb8* null mice.

At first glance, these two optogenetic studies seem to have conflicting results. One is able to rescue elicited compulsive-like behavior, whereas the other rescues it, despite activating similar brain regions. It is important to note that there are some fundamental differences. Burguiere and colleagues (2013) took advantage of a genetic knockout mouse model with established repetitive behavioral phenotypes that result from abnormal neural activity resulting from the genetic manipulation. In contrast, Ahmari and colleagues (2013) used wild-type mice that had undergone normal neural development prior to introduction of the channelrhodopsin and stimulation of the OFC-VMS circuit. Initial work characterizing the *Sapap3*-null mouse showed abnormal cortico-striatal function that may be a result of abnormal neural development (Welch et al., 2007), suggesting that the circuits in the two studies may be fundamentally different at the onset. Secondly, the stimulation protocols are actually quite different, with different stimulation durations in either the lateral OFC and primarily dorsal striatum (Burguiere) or the medial OFC and ventral striatum (Ahmari). These are distinctly separate cell populations that were previously posited to have alternative roles in the pathophysiology of the disorder

(Milad and Rauch, 2012). Clearly, more work will be needed to better understand the stimulus pattern, subregion, and cell type specificity in relation to compulsive-like behavior.

Discussion and Future Directions

The primary limitation across rodent models of OCD is the lack of clear risk factors that would support construct validity. At least one model in each of the four major categories reviewed here shows both face and predictive validity, but construct validity is considerably more challenging. The genetic category is likely the easiest domain to establish construct validity, but the field is limited by the lack of power in OCD linkage and genome-wide association studies. The published genetic models with increased grooming that responds to SRI treatment are limited to circumstantial genetic evidence supporting their potential involvement in OCD in the human population. Conversely, those genes with the strongest support in the OCD literature have not yet been adequately evaluated in rodent models, either for compulsive-like behavior or even in relation to their function in the circuits implicated in OCD. Beyond genetic linkage and association studies, no postmortem studies have been published to date in OCD, leaving us without the RNA, protein, or cellular profiles that have guided development of animal models in schizophrenia or autism spectrum disorder (Berg et al., 2015, Brown et al., 2015).

Construct validity is harder to establish for immunological, pharmacological, or optogenetic studies. PANDAS and PANS are exciting clinical syndromes that are poorly understood and offer technical challenges for rodent models. Our knowledge of pharmacological triggers is primarily limited to triptans and atypical antipsychotics, but these are not thought to cause OCD itself but typically to worsen symptoms, and even then only in a subset of patients. The circuitry-based models are perhaps the most exciting and can offer us an opportunity to test hypotheses based upon intermediate phenotypes in OCD, rather than requiring a clear understanding of the original factors that contributed to risk in an individual. It may be difficult to

assess construct validity in these models, but they seem to offer substantial promise for understanding how the circuitry implicated in OCD works, even if they do not directly model the disorder itself. We must be careful, however, to not over-interpret models based upon expressing a foreign, light-sensitive protein in an ectopic pattern and then stimulating neurons in a temporal pattern that would not typically occur in the brain.

The difficulty in establishing construct validity also complicates our understanding of the most valid behavioral or cognitive tests that are relevant to OCD. If we had several genes clearly implicated in OCD with mouse models that mimic the human risk variant, we would be able to survey the phenotypes in those animals to understand the true range of “OCD-like” behavior in the rodent. Just as OCD is often difficult to diagnose in the human population, some of those animals would likely be engaging in subtle rituals or compulsive-like behaviors that we may not anticipate or detect without extensive characterization. In the absence of construct valid models to serve as examples, we are restricted to behaviors that fit our expectations for how compulsivity might present in a rodent. In this context, a broad assessment that includes both spontaneous behaviors, such as grooming, as well as cognitive measures reported to be abnormal in OCD (Dittrich and Johansen, 2013), is important to better understand the strengths and limitations of a given model. None of the genetic models have had a significant assessment of cognitive function reported to date; although the conditioned-grooming assay in the *Sapap3* model begins to move toward an understanding of cognitive function in these animals, albeit only in the context of the already identified grooming phenotype. Some intermediate phenotypes identified in OCD, such as reversal learning deficits or impaired prepulse inhibition, can be assessed across species; although no single cognitive or sensory gating measure is specific for OCD to the exclusion of other neuropsychiatric disorders.

In contrast to the challenges in understanding the risk factors and molecular etiology of OCD, neuroimaging has made significant progress in identifying affected brain regions through the use of continually advancing techniques. Likewise, the use of deep brain stimulation points

to brain regions or tracts that are likely to at least reflect downstream consequences of the pathophysiology of OCD. This understanding of brain regions and circuits has become one of the key arguments for the validity of particular genetic or pharmacological models, rather than the construct being modeled. Abnormalities in the OFC or the striatum, or corticostriatal signaling specifically, are reassuring findings across models, but we should be particularly excited to see more specific findings arise, such as the decrease in fast-spiking striatal interneurons in the *Sapap3* null mice. Even in the absence of construct or predictive validity, the sheer novelty of the *Hoxb8* microglia rescue raises the possibility that an animal model will transform our understanding of OCD pathophysiology, even if just in a small subset of the overall population.

When assessing the available models, a few conclusions can be drawn. First, no single model is likely to be all-encompassing, with each model likely corresponding to a subset of the disorder and potentially to a particular symptom type or cognitive deficit. Second, excessive grooming is a species-appropriate, stereotyped behavior that seems particularly sensitive to perturbations that affect the CSTC circuit implicated in OCD and may be a very useful readout across models. Importantly, rodent grooming also increases in novel or stressful environments (Hammamieh et al., 2012, Xu et al., 2015), and anxiolytic response to SRIs may therefore complicate assessment of predictive validity specific to compulsive-like behavior. Third, emerging techniques to manipulate specific circuits offer the possibility of working back and forth between genetic, immunological, and pharmacological models that perturb specific circuits, and optogenetic models that can evaluate whether an observed change in circuit function is necessary and specific to generate the observed compulsive-like behavior, with optogenetic rescue of the *Sapap3* null mice as the pioneering example. The rapid advance in technologies to develop and study rodent models is moving forward at an astonishing pace, and we may expect more genetic models to emerge that may force us to test construct validity after observing a rodent phenotype, as in the *Sapap3* and *Slitrk5* null mice. We can also expect that

optogenetic findings will direct the attention of human neuroimaging studies to specific circuits or nodes that may otherwise have been ignored. Ideally, however, we will also continue to pursue models with explicit construct validity in order to maintain a focus on factors that impact upon OCD risk in humans, rather than the myriad of manipulations that may generate compulsive-like behavior in rodents.

CHAPTER 3

BEHAVIORAL ASSESSMENT OF EAAT3 INHIBITORS IN *IN VIVO* MODELS OF GLUTAMATERGIC AND BASAL GANGLIA DYSFUNCTION

Introduction

In the past two decades, investigation into the etiology of obsessive-compulsive disorder (OCD) has converged upon the cortical-striatal-thalamo-cortical (CSTC) circuit and the glutamatergic system. OCD is a debilitating neuropsychiatric condition characterized by persistent, intrusive obsessions, repetitive behavior, and anxiety (Calvocoressi et al., 1998). OCD has a worldwide prevalence rate of 2% and is tenth in the World Health Organization's ranking of the leading causes of disability (Michael S. Ritsner, 2007). Serotonin reuptake inhibitors (SRIs) are the primary form of pharmacotherapy; however only 50-60% of patients show adequate response (Koran et al., 2007). A mean 20-40% decrease in OCD symptoms are observed following SRI therapy (Dougherty et al., 2004), which leaves many with clinically significant residual symptoms. Recent evidence in the field suggests that further efforts focused on the glutamatergic system could yield rationally designed therapeutics needed to address the underlying pathophysiology of the disorder.

The ability to non-invasively investigate neural structures, activity, and chemical substrates within the brain with neuroimaging techniques has revolutionized our understanding of neuropsychiatric disease. Collective interpretation of the studies in OCD suggests that dysfunction of the CSTC circuit, including hyperactivity of the direct pathway or hypoactivity of the indirect pathway, may underlie the manifestation of OCD (Saxena and Rauch, 2000, Maia et al., 2008, Rotge et al., 2008, Ting and Feng, 2011). Structural analyses via magnetic resonance imaging (MRI) identified abnormalities within the CSTC circuit, including reductions in grey matter of the orbitofrontal cortex (OFC) (Di Martino et al., 2008), and increased grey matter in

the ventral striatum (Pujol et al., 2004). Structural alterations in gray matter volume of structures within the CSTC also correlated with cognitive impairment in OCD patients relative to non-affected first-degree relatives (Menziés et al., 2008). Functional imaging analysis identified hyperactive states of the OFC, ACC, and the caudate at baseline and following symptom provocation in OCD patients (Rauch et al., 1997), as well as reductions in the activity of the OFC and caudate following treatment (Rauch et al., 2002, Nakao et al., 2005). The CSTC circuit dysfunction hypothesis is also supported by resting-state connectivity analyses with functional MRI indicating increased connectivity between the OFC, ACC, striatum, and thalamus, (Harrison et al., 2009, Beucke et al., 2013, Anticevic et al., 2014).

With glutamate being the major excitatory neurotransmitter in the CSTC circuit, studies aimed at identifying alterations in the glutamatergic system have also been performed in the OCD patient population. Via proton magnetic resonance spectroscopy ($^1\text{H-MRS}$), abnormalities in a cumulative glutamate signal, Glx (summation of glutamate, glutamine, and GABA), are found in the caudate and ACC of pediatric and adult patients irrespective of their treatment history (Rosenberg et al., 2000, Rosenberg et al., 2004, Starck et al., 2008, Yucel et al., 2008). The elevation in Glx levels from $^1\text{H-MRS}$ analysis corresponds to data from two studies describing increased glutamate in the cerebrospinal fluid of OCD patients (Chakrabarty et al., 2005a, Bhattacharyya et al., 2009).

Many insights into the underlying causes of psychiatric disorders originate from genetic investigation of the patient population. Linkage analysis supported a preliminary link between OCD and the chromosome 9p24, which included *SLC1A1* (Hanna et al., 2002b, Willour et al., 2004b). This piqued researcher's interest in *SLC1A1* as it codes for the neuronal glutamate transporter, excitatory amino acid transporter 3 (EAAT3). Ensuing genetic analysis identified significant genetic association of *SLC1A1* and OCD (Arnold et al., 2006, Dickel et al., 2006), which has been consistently replicated (Stewart et al., 2007, Shugart et al., 2009, Wendland et al., 2009a, Samuels et al., 2011, Stewart et al., 2013b). The most common OCD-associated

polymorphism, rs301430C, affects *SLC1A1* expression by leading to increases in the encoded protein, EAAT3. The C-allele is also linked to increased *SLC1A1* expression in lymphoblastoid cells, human post-mortem brain, and in a luciferase reporter assay (Wendland et al., 2009a). When taken together, this suggests that OCD-susceptibility may result from increased *SLC1A1* expression and that inhibition of EAAT3 function may be therapeutic.

To examine the hypothesis that increased *SLC1A1*/EAAT3 expression may contribute to glutamatergic dysfunction within the OCD-linked CSTC circuit, we utilized two EAAT3 inhibitors (NBI-59159 and 2-CFoDA) in two distinct *in vivo* models of OCD-relevant repetitive behavior and basal ganglia dysfunction. The first model, *Sapap3* null mice, exhibits excessive self-injurious grooming behavior and glutamatergic signaling dysfunction at the cortical-striatal synapse (Welch et al., 2007). As an alternative to the *Sapap3* null transgenic model, amphetamine was used to behaviorally probe the CSTC circuit. Amphetamine challenge leads excessive dopamine release in the striatum and robust hyperlocomotor behavior. Utilization of these two *in vivo* EAAT3 inhibitors, NBI-59159 and 2-CFoDA, allows us to translate findings from human genetic studies into *in vivo* assessment of how reductions in the activity of EAAT3, the protein coded for by the most consistently replicated OCD-associated gene, *SLC1A1*, affects repetitive behavior in OCD-relevant mouse models.

Methods

Mice

SAPAP3 null mice were donated from the Feng Lab (Massachusetts Institute of Technology, Cambridge, MA). The colony was maintained via heterozygous breeding to ensure littermate controls for all behavioral experiments. SAPAP3 null mice used for experiments were maintained on a C57BL/6J background and aged 4-6 months to ensure emergence of repetitive grooming phenotype. All mice used for intracerebellar ventricular (ICV) and direct striatal 2-

CFoDA injection studies were wildtype C57BL/6J mice obtained from The Jackson Laboratory (Stock #000664) aged 12-16 weeks.

Immunoblotting Studies

Brains were harvested from mice following rapid decapitation. Brains were immediately placed on an ice-cold metal platform, and the striatum was dissected. Dissected tissue was placed into 3 mL of homogenization buffer [130 mM NaCl, 1.3 mM KCl, 2.2 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 10 mM Hepes (pH 7.4), 10 mM glucose, ascorbic acid, and 0.32 M sucrose] and homogenized using a Potter Elvehjem homogenizer (Wheaton, Millville, NJ, USA). Protein concentrations of all samples were determined by a bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific). Equal amounts of protein were incubated with Laemmli sample buffer for 10 min at room temperature and analyzed by using SDS/PAGE and Western blotting. EAAT3 protein was visualized in samples blotted to Immobilon-P PVDF membrane (EMD Millipore, Billerica, MA, USA) by using a rabbit anti-EAAC1/EAAT3 antibody (EAAC11-A, 1:1,000 dilution; Alpha Diagnostics, San Antonio, TX, USA). β -actin was visualized by using a mouse anti- β -actin antibody (A5316, 1:10,000 dilution; Sigma-Aldrich, St. Louis, MO, USA) as a loading control. Appropriate HRP-conjugated, secondary antibodies were obtained from GE Healthcare Life Sciences (Pittsburgh, PA, USA). Secondary antibody labeling was detected by using Amersham ECL Prime Western Blotting Detection Reagents and visualized via chemiluminescence using FluorChem M System (Protein Simple, San Jose, CA, USA). Multiple exposures were obtained to ensure linearity of band detection. Western blots were quantified by using NIH ImageJ software.

Synaptosomal Cysteine Transport Assays

Brains were harvested from mice following rapid decapitation and immediately placed on an ice-cold metal platform so that striatum could be dissected. Striatal tissue samples were

placed into 3 mL of homogenization buffer [130 mM NaCl, 1.3 mM KCl, 2.2 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 10 mM Hepes (pH 7.4), 10 mM glucose, ascorbic acid, and 0.32 M sucrose] and homogenized by using a Potter-Elvehjem homogenizer (Wheaton). Homogenates were centrifuged at 500 × g at 4 °C for 10 min. Supernatants were removed and centrifuged at 12,000 × g at 4 °C for 10 min. The resulting synaptosome-enriched pellets were then resuspended in 2.5 mL of assay buffer [130 mM NaCl, 1.3 mM KCl, 2.2 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 10 mM Hepes (pH 7.4), 10 mM glucose and ascorbic acid], and protein concentrations were determined by using the bicinchoninic acid (BCA) Protein Assay (Thermo Fisher Scientific). This material (hereafter called synaptosomes) was then diluted to 30 µg of total protein per 100 µL. Synaptosomes were incubated with 200 µM [³⁵S]-cysteine (Perkin-Elmer, Waltham, MA, USA) (1% labeled and 99% unlabeled). Assays were terminated by rapid filtration over 0.3% polyethyleneimine-soaked GF/B glass microfiber filters (Whatman, GE Life Science) and washed three times with ice-cold Krebs-Ringers-Hepes buffer [130 mM NaCl, 1.3 mM KCl, 2.2 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, and 10 mM Hepes (pH 7.4)]. Filters placed into scintillation vials with 7 mL of Ecoscint H (National Diagnostics, Atlanta, GA, USA) scintillation fluid, shaken overnight at room temperature, and then radioactivity was quantified by using a TriCarb 2900TR scintillation counter (Perkin-Elmer). Specific [³⁵S]-cysteine transport was assayed in triplicate for all samples.

Spontaneous Grooming

Mice were placed alone in an empty novel cage and allowed to habituate for 30 minutes. Mouse behavior was then recorded for 10 minutes. Total time spent grooming was manually decoded by an observer blind to genotype. For NBI-159 grooming studies, mice were pretreated with NBI-59159 (5 mg/kg, IP), 30 minutes prior to testing.

Guide Cannula Implantation

Mice were anesthetized with isoflurane (5% for induction, 2% for maintenance during surgery) and placed in a stereotaxic frame using mouse-specific ear bars (Kopf Instruments, Tujunga, CA). A guide cannula (CMA7) was lower through a skull burr hole to rest above the injection site (ICV AP 0.0 mm, M/L \pm 0.75 mm, DV -1.5 mm; Striatal injection AP +0.5 mm, ML \pm 1.5 mm, DV -2.5 mm; all measurements relative to bregma) and secured to the skull using glass ionomer cement (Instech Solomon, Plymouth Meeting, PA). Mice received post-op care daily (5 mg/kg Ketoprofen, Sigma, St. Louis, MO) and were allowed to recover for one week prior to behavioral testing.

2-CFoDA Injection

Mice were anesthetized with isoflurane (5% for induction, 2% for maintenance during injection) and placed in a stereotaxic frame using mouse-specific ear bars (Kopf Instruments, Tujunga, CA). Injection cannula was connected to a 5 μ L Hamilton syringe (Hamilton, Reno, NV) placed into a nano-syringe pump (KDS310, KD Scientific, Holliston, MA). The injection cannula was front loaded with the 1 μ L of the desired concentration of 2-CFoDA then inserted to into the guide cannula. The injection cannula extended ventrally from the guide cannula (ICV 0.5 mm; Striatal 1.0 mm) to reach the desired injection coordinates in lateral ventricles for ICV injection (AP 0.0 mm, M/L \pm 0.75 mm, DV -2.0 mm) and dorsal medial striatum (AP +0.5 mm, ML \pm 1.5 mm, DV -3.5 mm). A total of 1 μ L of 2-CFoDA was infused at a rate of 0.5 μ L/min. The injection cannula was left in place for 10 minutes after completion of infusion to eliminate backflow then removed from the guide cannula. This process was then repeated on the contralateral side. Following bilateral 2-CFoDA infusion, mice were immediately placed into locomotor chambers for amphetamine induced locomotion assays.

Amphetamine Induced Locomotion

Locomotor activity in the open field was measured by using 27 x 27 x 20.5 cm chambers (Med Associates) placed within light- and air-controlled sound-attenuating boxes (64 x 45 x 42 cm). Locomotion was detected by interruption of infrared beams by the body of the mouse (16 photocells in each horizontal axis located 1 cm above the activity chamber floor, as well as 16 photocells elevated 4 cm above the chamber floor to detect rearing and jumping behaviors). Data were collected and analyzed by Med Associates Activity Monitor software. Mice were acclimated to the activity chambers during a 30-min session 2–3 d before data recording began. On day 1 of the experimental sequence, mice were weighed, infused with 2-CFoDA, then placed into activity chambers, and activity was monitored for 60 min. The mice were then removed from the activity chambers and received an intraperitoneal injection of 0.9% saline or amphetamine (Sigma-Aldrich) (3 mg/kg). Mice were returned to the activity chambers and locomotor activity was recorded for 75 min. Mice were returned to their home cage for a week to allow for drug wash out then given alternative treatment. Time course data were analyzed by using non-linear curve line fit analysis and cumulative measures of behavior (i.e., total distance traveled) during the entire 75-minute period following amphetamine administration.

Statistical Analysis

Data were analyzed using Prism (GraphPad, La Jolla, CA, USA). Two-tailed, unpaired Student *t*-test or two-way ANOVA with Sidak's post-tests were used to analyze the primary data, except for locomotor data, which was analyzed using non-linear curve fit analysis. Specific statistical analyses for each data set are described in results and in the figure legends. In the text and figures, all data are reported as the mean \pm standard error of the mean. Bar graphs depict the mean \pm standard error of the mean.

Results

Loss of SAPAP3 Expression Affects Expression But Not Function of EAAT3

To investigate the hypothesis that abnormal expression and or function of *Slc1a1*/EAAT3 may contribute to OCD-relevant repetitive behaviors in mouse models of glutamatergic dysfunction, the SAPAP3 null mouse model was utilized (Welch et al., 2007). Immunoblots of striatal synaptosome preparations revealed an increase of EAAT3 in SAPAP3 nulls relative to wildtype (WT) controls (Figure 4a and b, $P=0.011$, $n=4$ per genotype). We next examined EAAT3 function via striatal synaptosome transport assays (Nieoullon et al., 2006) to assess if EAAT3 function mirrored the observed elevation in EAAT3 expression. Because EAAT3 is the primary source for neuronal cysteine (Aoyama et al., 2012, Watts et al., 2014), [^{35}S]-cysteine (200 μM) was used as the substrate for EAAT3 synaptosome uptake assays. A significant effect of the EAAT inhibitor TBOA (100 μM) and a trend level genotype effect were observed in synaptosomes prepared from SAPAP3 null mouse striatum relative to WT synaptosomes (Figure 4c, Two-way ANOVA; TBOA $F(1,20)=14.55$, $P=0.001$; genotype $F(1,20)=3.8$ $P=0.06$, $n=2$ per genotype). These data indicate that loss of SAPAP3 affects EAAT3 expression; however this change may not be matched by a change in the functional capabilities of the transporter in SAPAP3 null mice.

Pharmacological Inhibition of EAAT3 Does Not Rescue OCD-relevant Grooming Behavior in SAPAP3 Null Mice

With the evidence from immunoblots that EAAT3 showed increased expression in the striatum of SAPAP3 null mice (Figure 4a), we next wanted to determine if pretreatment with an EAAT3 inhibitor, NBI-59159, would attenuate the repetitive grooming behavior observed in SAPAP3 null mice (Welch et al., 2007). (S)-4-(9Hfluoren-2-ylamino)-2-amino-4-oxobutanoic acid (NBI-59159) (Supplementary Figure 1) was reported to show an *in vitro* preference for EAAT3 over EAAT2 in HEK cells, yielding IC_{50} values of 0.09 μM for EAAT3 and 1.4 μM for

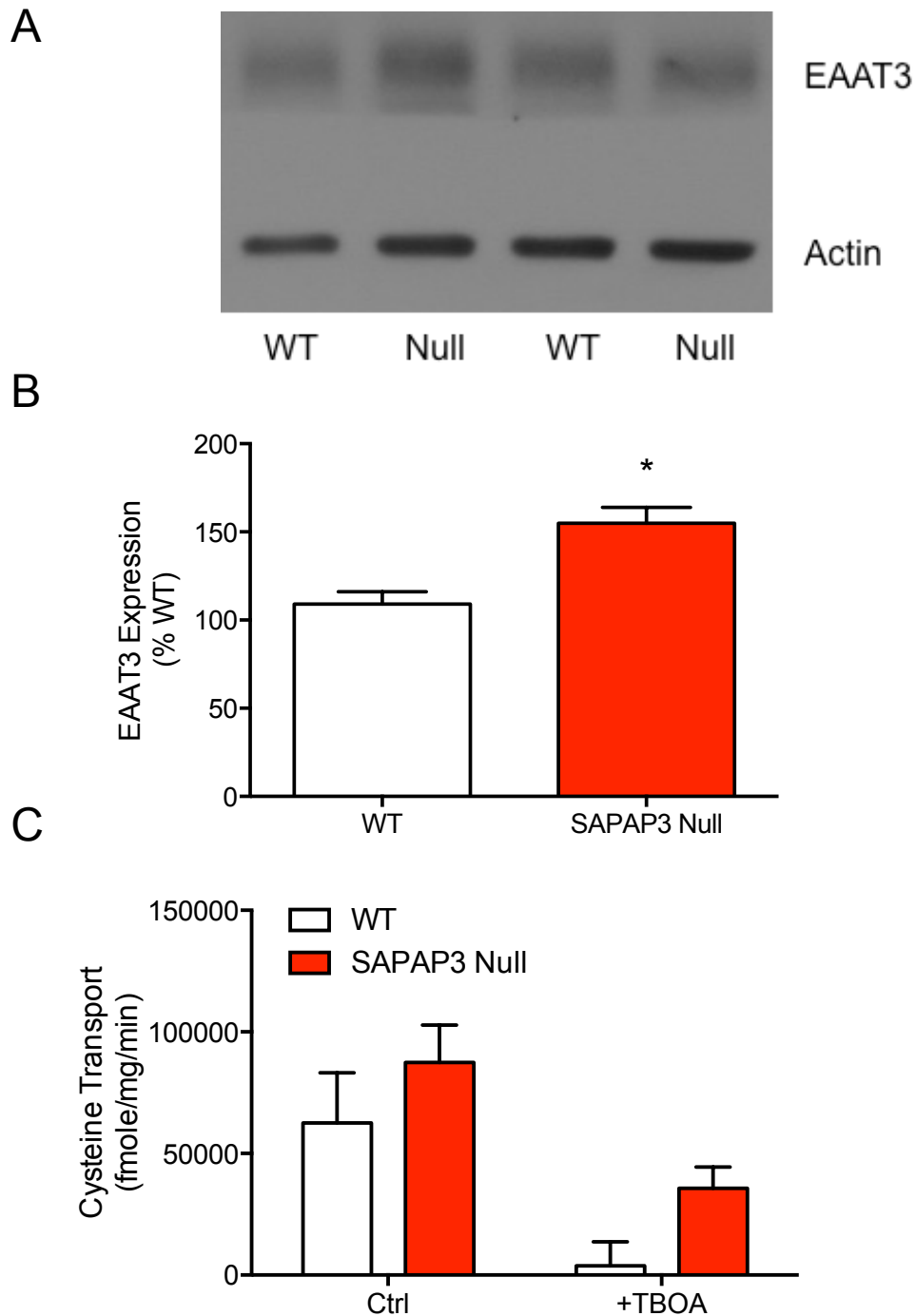


Figure 4. Sapap3 null mice express increased levels of striatal EAAT3 however protein function is unaffected.

(A) Representative immunoblot from striatal synaptosome preparations from SAPAP3 null (Lane 2 and 4) and wildtype (WT) (Lane 1 and 3) mice. EAAT3 (top band) was the protein of interest and actin (bottom band) was the loading control.

(B) EAAT3 protein expression is elevated in striatal synaptosome preparations from SAPAP3 null mice relative to WT controls. (unpaired t-test, $t = 3.26$, $*P = 0.011$, $n = 5$ per genotype. Figure is representative of 3 separate experiments). Average protein expression is demonstrated in bar graph.

(C) EAAT3 function assayed via cysteine uptake is unchanged in Sapap3 null mice relative to WT controls (Two-way ANOVA; genotype $F(1,20) = 3.83$, $^{\wedge}P = 0.64$; TBOA $F(1,20) = 14.55$, $***P = 0.001$; $n = 2$ per genotype).

GLT-1 (Greenfield et al., 2005). Prior behavioral assessment of NBI-59159 suggested that pretreatment of mice with the EAAT3 inhibitor prior to challenge with amphetamine attenuated the locomotor response to the psychostimulant amphetamine; however the behavioral effect was modest and published without experimental detail (Supplementary Figure 2) (Dunlop and Marquis, 2006). For this experiment in SAPAP3 null mice, we pretreated SAPAP3 null and WT littermate controls with 5 mg/kg NBI-59159 (intraperitoneal, IP), 60 minutes prior to manual scoring of time spent grooming over a 10-minute observation period. Following pretreatment with either NBI-59159 or saline, no effect of NBI-59159 on overall time spent grooming was observed in SAPAP3 null or WT mice (Figure 5a, Two-way ANOVA; genotype $F(1,46)=35.41$, **** $P<0.0001$; NBI-59159 $F(1,46)=0.66$, $P=0.42$). We did observe a main effect of genotype and a stark increase in time spent grooming by SAPAP3 null mice relative to WT controls regardless of their pretreatment condition (Figure 5a, Sidak's multiple comparison test; ** $P<0.01$).

Basal Ganglia Dependent Amphetamine-Induced Locomotion is Unaffected by Intracerebellar Ventricular Delivery of an EAAT3 Inhibitor

One of the most consistently identified biomarkers in studies examining the OCD patient population is glutamatergic dysfunction in the CSTC circuit. In order to probe one component of this circuit pharmacologically and induce elevated levels of striatal dopamine release, we challenged WT mice with the psychostimulant, amphetamine. As mentioned previously, systemic challenge of low to moderate dose amphetamine causes a hyperlocomotor behavioral response due to increased dopamine and glutamate release in the striatum (Yates et al., 2007). Prior work with the EAAT3 inhibitor NBI-59159 showed that pretreatment with the EAAT3 inhibitor attenuated the psychostimulant induced locomotor response (Supplementary Figure 2). Due to the unavailability of larger amounts of NBI-59159, we transitioned to using a novel EAAT3 inhibitor, 2-CFoDA (Supplementary Figure 1). As with NBI-59159, 2-CFoDA inhibits all

A

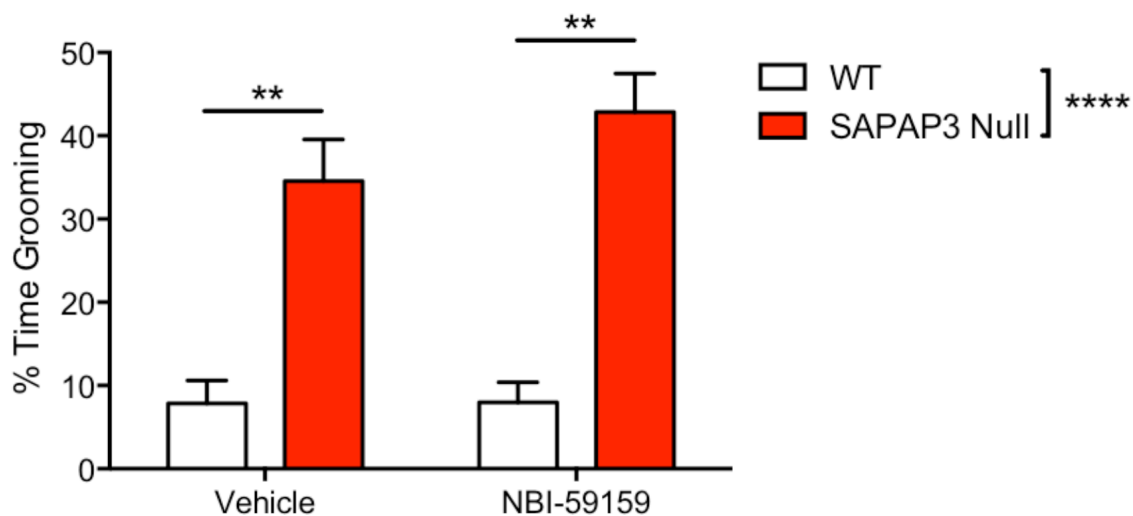


Figure 5. Pretreatment with the EAAT3 inhibitor, NBI-59159, has no effect on grooming behavior in wildtype or SAPAP3 null mice

(A) 60 minute pretreatment with the EAAT3 inhibitor, NBI-59159, did not affect grooming behavior in wildtype (WT) or SAPAP3 null mice (Two-way ANOVA, genotype $F(1,46)=35.41$, **** $P<0.0001$; NBI-59159 $F(1,46)=0.66$, $P=0.42$; Sidak's multiple comparison test; ** $P<0.01$)

EAATs, but is reported to be the most potent at EAAT3 (*in vitro* IC₅₀=0.09 uM vs 20 uM and 1.44 uM for GLT-1 and GLAST respectively) (Ye, 2009). Effects of 2-CFoDA were never before characterized *in vivo* so in order to maximize our experimental capabilities with the inhibitor, we first implanted guide cannula into the lateral ventricles of WT mice so that the EAAT3 inhibitor could be delivered via intracerebroventricular (ICV) injection (Supplementary Figure 3). Following two weeks of recovery, the mice were pretreated with one of four pretreatment conditions (saline, 0.1 mg/ml 2-CFoDA, 1 mg/ml 2-CFoDA, or 5 mg/ml 2-CFoDA, all 1 uL volume). Sixty minutes following pretreatment, mice were challenged with amphetamine (3 mg/kg, IP) and their locomotor behavior was assessed. Following acute amphetamine challenge, no changes in amphetamine induced locomotor behavior were observed at any of the doses of 2-CFoDA relative to saline (Figure 6a-d). Analysis via non-linear curve fit of the data resulted in overlapping curve fits for all comparisons except 0.1 mg/ml 2-CFoDA (Figure 6a-c), which revealed higher activity in comparison to the saline control. Further analysis performed by calculating the area under the locomotor curves following amphetamine challenge from the two groups indicated that their overall activity was not different from one another, or the other 2-CFoDA doses (Figure 6d, One-way ANOVA; $F(3,28)=0.92$, $P=0.45$). Due to the previously uncharacterized nature of 2-CFoDA *in vivo*, it is difficult to assess the effectiveness of the inhibitor in this behavioral context, as many of the variables important for effective drug action of 2-CFoDA in the CNS are unknown.

Basal Ganglia Dependent Amphetamine-Induced Locomotion is Attenuated by Direct Striatal Delivery of High Dose 2-CFoDA

We were unable to ascertain if inhibition of EAAT3 was ineffective in altering the locomotor response to amphetamine via ICV delivery, or if 2-CFoDA was unable to reach its site of action. We therefore transitioned to an alternative experimental strategy. As before, the mice

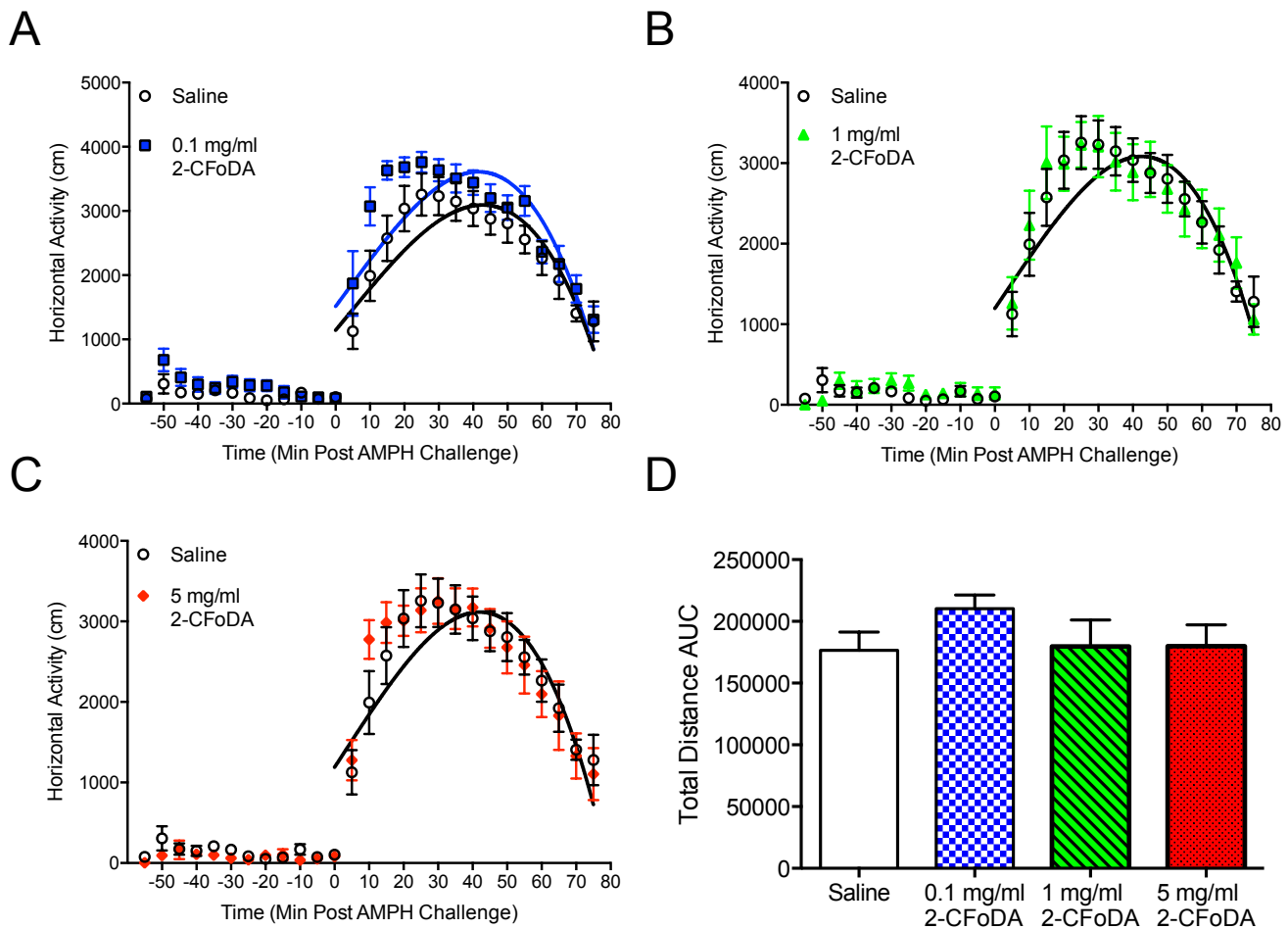


Figure 6. Pretreatment with the EAAT3 inhibitor, 2-CFoDA, via intracerebroventricular (ICV) injection does not alter amphetamine-induced locomotor behavior

(A-D) Following acute D-amphetamine challenge (3.0 mg/kg, IP), WT mice treated with the EAAT3 inhibitor 2-CFoDA (ICV) do not exhibit an altered locomotor response to the psychostimulant. While curve fit analysis of 0.1 mg/ml 2-CFoDA vs. saline resulted in different curve fits (A), Area under the curve (AUC) analysis of amphetamine locomotor response was unchanged.

A) 0.1 mg/ml 2-CFoDA pretreatment (Non-linear curve fit analysis $T=0-60$; $F(4,424)=6.151$, $****P<0.0001$, $n=8$ per treatment)

B) 1.0 mg/ml 2-CFoDA pretreatment (Non-linear curve fit analysis $T=0-60$; $F(4,424)=0.26$, $P=0.90$, $n=8$ per treatment)

C) 5.0 mg/ml 2-CFoDA pretreatment (Non-linear curve fit analysis $T=0-60$; $F(4,424)=0.48$, $P=0.75$, $n=8$ per treatment)

D) Area under the curve analysis of amphetamine induced locomotor response of each 2-CFoDA treatment condition relative to saline (One-way ANOVA; $F(3,28)=0.92$, $P=0.45$, $n=8$ per treatment).

were pretreated with 2-CFoDA (5 or 10 mg/ml, 1 μ L volume), then challenged with amphetamine (3 mg/kg, IP); however they were now administered the drug directly into the striatum (AP +0.52 mm, ML \pm 1.5 mm, DV -3.5 mm, all relative to bregma) (Supplementary Figure 3) via guide and injection cannula prior to amphetamine challenge. At the pretreatment dose of 5 mg/ml 2-CFoDA, subtle differences in the locomotor response curve were identified as above (Figure 7a, 5 mg/ml, Non-linear curve fit analysis T=0-60; $F(4,235)=2.95$, $P=0.021$, $n=4$ saline, $n=5$ 2-CFoDA); however further AUC analysis identified no differences in the cumulative locomotor response between pretreatment with saline or 5 mg/ml 2-CFoDA (Figure 7b, t-test, $t=0.54$, $P=0.61$, $n=4$ saline, $n=5$ 2-CFoDA). At the highest 2-CFoDA pretreatment dose tested (10 mg/ml), substantial reductions in locomotor response curves were observed in response to direct striatal injection of the EAAT3 inhibitor (Figure 7c, 10 mg/ml, Non-linear curve fit analysis T=0-60; $F(4,397)=49.12$, $P<0.0001$, $n=8$ saline, $n=7$ 2-CFoDA). Subsequent area under the curve analysis agreed with the non-linear curve fit analysis providing strong evidence of reductions in amphetamine-induced locomotor response when pretreated with 2-CFoDA directly into the striatum (Figure 7d, t-test, $t=3.07$, $P=0.0089$, $n=8$ saline, $n=7$ 2-CFoDA).

Discussion

One of the main difficulties in understanding the underpinnings of heterogeneous neuropsychiatric disorders such as obsessive-compulsive disorder is the identification of candidate risk genes and putative biomarkers in the patient population that allow for rational examination in a pre-clinical context. In the studies described in this chapter, we aimed to incorporate two of the most consistent findings in OCD, genetic evidence implicating *SLC1A1* in OCD (Hanna et al., 2002b, Willour et al., 2004b, Arnold et al., 2006, Dickel et al., 2006, Stewart et al., 2007, Kwon et al., 2009, Shugart et al., 2009, Samuels et al., 2011) and glutamatergic dysfunction in the CSTC circuit (Rosenberg et al., 2000, Rosenberg et al., 2004, Chakrabarty et al., 2005a, Starck et al., 2008, Yucel et al., 2008, Bhattacharyya et al., 2009). Using *in vivo*

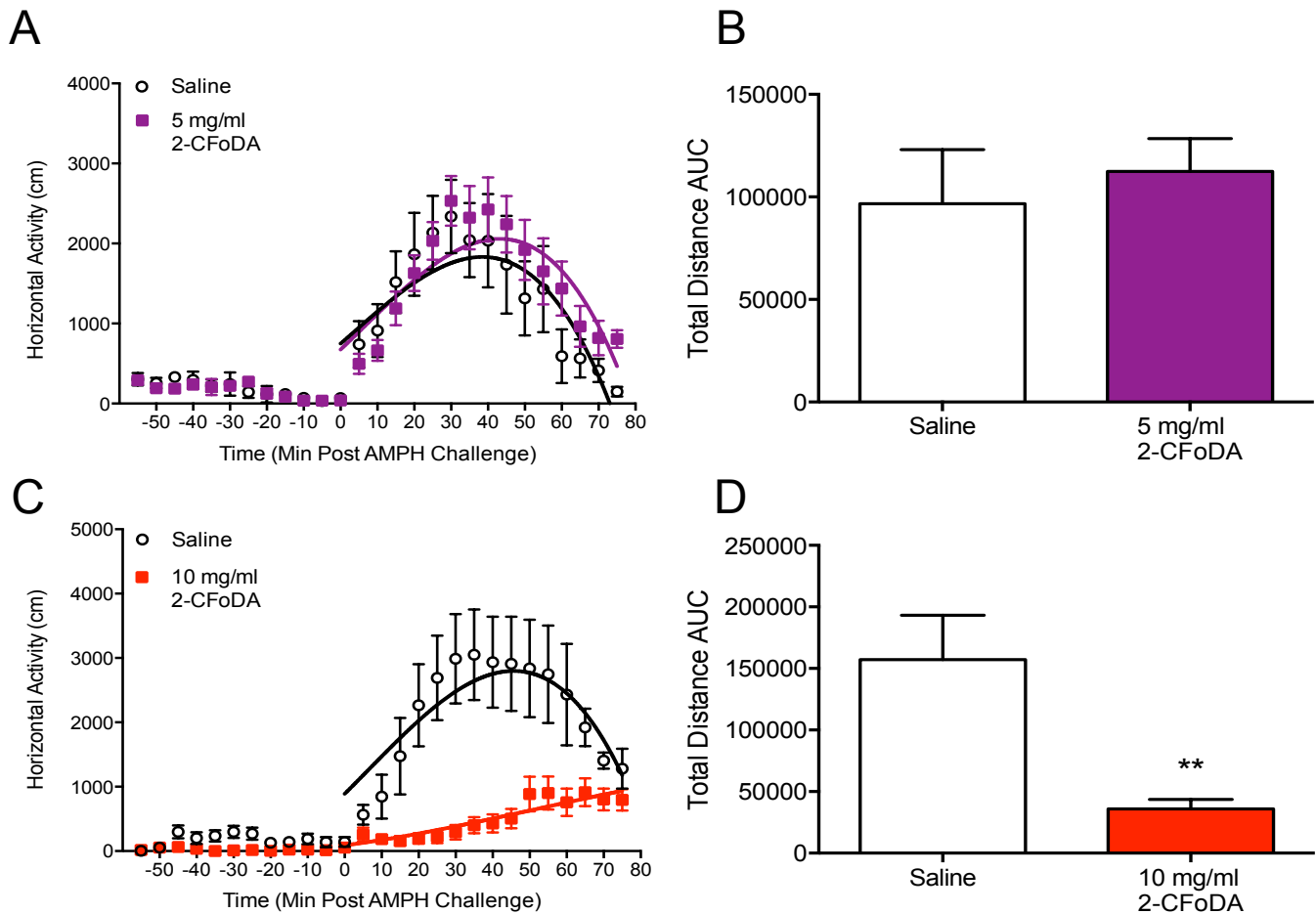


Figure 7. Pretreatment with the EAAT3 inhibitor, 2-CFoDA, via direct striatal injection only affects amphetamine-induced locomotor response at a high, potentially sedating, dose

Amphetamine locomotor response curves in mice pretreated with 5 mg/ml 2-CFoDA relative to saline pretreated mice are different however, AUC analysis of locomotor curves do not segregate, suggesting a lack of an overall effect on amphetamine-induced locomotor behavior at this dose. Locomotor response to amphetamine at a high dose of 2-CFoDA (10 mg/ml) does result in separate curve fit relative to saline pretreatment and lower AUC for amphetamine locomotor response curves, potentially due to off-target sedating effects.

A) 5 mg/ml 2-CFoDA pretreatment via direct site injection (Non-linear curve fit analysis T=0-60; $F(4,235)=2.95$, $*P=0.021$, $n=4$ saline, $n=5$ 2-CFoDA).

B) Area under the curve analysis of amphetamine induced locomotor response of striatal direct site injection of 5 mg/ml 2-CFoDA treatment relative to saline is unchanged (T-test, $t=0.54$, $P=0.61$, $n=4$ saline, $n=5$ 2-CFoDA).

C) 10 mg/ml 2-CFoDA pretreatment via direct site injection inhibits the amphetamine induced locomotor response (Non-linear curve fit analysis T=0-60; $F(4,397)=49.12$, $****P<0.0001$, $n=8$ saline, $n=7$ 2-CFoDA).

D) Area under the curve analysis of amphetamine induced locomotor response of striatal direct site injection of 10 mg/ml 2-CFoDA treatment relative to saline is reduced (T-test, $t=3.07$, $**P=0.0089$, $n=8$ saline, $n=7$ 2-CFoDA).

EAAT3 inhibitors in mouse models of repetitive behavior and glutamatergic dysfunction enabled us to examine whether these convergent findings in the human population may yield potential benefit in preclinical models.

The SAPAP3 null mouse is a well characterized model of repetitive behavior and glutamatergic cortico-striatal dysfunction however no prior studies have investigated the impact of SAPAP3 loss on EAAT3. We identified an increase in the striatal expression of EAAT3 in SAPAP3 null mice relative to WT controls (Figure 4a-b). This is consistent with prior identification of an OCD-associated *SLC1A1* polymorphism that results in increased *SLC1A1* expression (Wendland et al., 2009a). In order to determine if this increase in EAAT3 expression in SAPAP3 null mice corresponded to increased EAAT3 function, [³⁵S]-cysteine uptake experiments were performed. Uptake experiments using striatal synaptosome preparations from nulls and WT controls identified only a trend-level genotype effect (Figure 4c); albeit in a small sample size. One explanation is that SAPAP3 null mice may express increased levels of EAAT3; however this increased expression does not result in elevated increased protein function at the synaptic membrane as a significant portion of the transporter is proposed to be localized in the cytoplasm of neurons (Conti et al., 1998a, Kugler and Schmitt, 1999). Importantly, the sample size was relatively small, and further functional assessment of EAAT3 in Sapap3 nulls may identify a main genotype effect once the sample size is expanded.

Examination of grooming behavior in potential animal models of OCD is common since the behavior has direct correlates to many symptoms observed in the human patient population (O'Sullivan et al., 2000). We next took advantage of this to assess if pretreatment of SAPAP3 null mice with the EAAT3 inhibitor NBI-59159, would lessen their elevated spontaneous grooming phenotype. Pretreatment of SAPAP3 null mice and WTs with NBI-59159 (5 mg/kg) had no effect on grooming in either genotype regardless of pretreatment condition (Figure 5). There are many factors that may help interpret this lack of effectiveness of NBI-59159 to lessen the grooming behavior in the null mice. The first is that prior *in vivo* assessment of the

compound in animal models used a shorter pretreatment period (15 min vs 60 min, Supplementary Figure 2) (Dunlop and Marquis, 2006) so by the time we performed our grooming analysis, the inhibitor may have been metabolized to lower concentrations in the CNS. Another potential explanation is that recent investigation of SAPAP3 null mice identified increased striatal signaling via mGluR5 (Ade et al., 2016). Due to the proximal localization of the mGluR5 to EAAT3 and EAAT3's ability to limit glutamate availability in a manner that affects mGluR5 signaling (Otis et al., 2004), inhibition of EAAT3 function via NBI-59159 administration may not lead to reductions in mGluR5 signaling, and therefore we may not observe alterations in repetitive behaviors that are linked to increased striatal mGluR5 signaling in SAPAP3 nulls.

Ensuing investigation focused on the ability of EAAT3 inhibitors to impact the CSTC circuit used a separate compound, 2-CFoDA. 2-CFoDA was synthesized as part of broader effort to improve GLT-1 inhibitors and was reported to exhibit an improved inhibitory profile at EAAT3 relative to NBI-59159, though the details regarding *in vitro* inhibition assays used for the two compounds were different. Due to a lack of information on the drug metabolism or pharmacokinetic properties of 2-CFoDA, we designed our amphetamine-induced locomotion experiments so that the mice were initially pretreated with 2-CFoDA via ICV injection in order to bypass the blood brain barrier. Our ICV pretreatment of mice with 2-CFoDA did not decrease amphetamine-induced locomotor behavior at any of the three doses used (0.1, 1, and 5 mg/ml) (Figure 6). Curve fit analysis identified alterations in post amphetamine locomotion in the 0.1 mg/ml condition relative to saline (Figure 6a). These data suggest that pretreatment with a potent EAAT3 inhibitor sixty minutes prior to psychostimulant challenge has no affect on basal ganglia dependent behavior; however there are some limitations of the experiment to consider. The first caveat is that the CSF of a mouse poorly penetrates the brain tissue, primarily due to the limitations of diffusion. The second caveat is that turnover of CSF is rapid in the mouse, approximately two hours. Since the pretreatment time of the mice in this experiment was 60 minutes, half of the total CSF volume will have been removed in that pretreatment period.

Thirdly, our knowledge of the mechanism in which 2-CFoDA is metabolized is unknown. All previous work with the compound was performed *in vitro* without any further investigation of its *in vivo* drug metabolism and pharmacokinetic properties. Taken together, whatever portion of 2-CFoDA that does reach the striatum (without being metabolized or removed from brain CSF completely) is still limited by diffusion into the brain tissue. It is more likely than not that the results from these experiments do not provide a conclusive resolution to the ability of EAAT3 inhibitors to impact behavior relevant to glutamatergic dysfunction in the CSTC or OCD.

In order to minimize the caveats discussed above, we transitioned to an alternative experimental strategy by delivering 2-CFoDA directly into the striatum of WT mice prior to amphetamine challenge. At 5 mg/ml, subtle alterations in the locomotor curve following amphetamine administration were observed relative to saline (Figure 7a); however calculations of total distance traveled via area under the curve analysis identified no differences between 2-CFoDA and saline pretreatment condition (Figure 7b). By increasing the pretreatment dose of 2-CFoDA to 10 mg/ml, we observed significantly different amphetamine locomotor response curves (Figure 7c), and total distance traveled via area under the locomotor curve analysis was substantially reduced in mice pretreated with 10 mg/ml 2-CFoDA relative to saline (Figure 7d). As with prior data in this chapter, this finding of reduced amphetamine-induced locomotion following pretreatment with high dose 2-CFoDA must be interpreted judiciously. One confounding aspect of the data is that the locomotor curves from the mice pretreated with 10 mg/ml 2-CFoDA do not resemble control conditions, as their eventual increase in locomotion is relatively linear while mice in the saline condition exhibit abrupt and almost exponential increases in activity following amphetamine administration. Additionally, the 10 mg/ml curve does not resemble amphetamine-induced locomotor curves from subsequent chapters where a significant reduction in amphetamine-induced locomotor behavior is observed. Due to the relatively high dose and lack of knowledge of the pharmacodynamic profile of 2-CFoDA, the inhibitor may have off-target sedative liability. Another consideration is that GLT-1 is reported to

be expressed ~100 fold more than EAAT3 (Holmseth et al., 2012). Despite 2-CFoDA's preference for EAAT3 versus GLT-1, we may be observing unforeseen behavioral consequences resulting from the inequalities of EAAT3 and GLT-1 expression in the striatum. It is also possible inhibition of EAAT3 at an alternative site in the brain would be more impactful in inhibiting amphetamine-induced locomotion. Of note, recent studies investigating the molecular consequences of amphetamine administration have shown that EAAT3 undergoes endocytosis from the neuronal membrane in dopaminergic neurons of the midbrain, which are responsible for the release of dopamine into the striatum following amphetamine challenge. Investigation of 2-CFoDA's *in vivo* pharmacodynamic profile and further effective dose studies would be an important step towards understanding the potential effectiveness of 2-CFoDA *in vivo*.

The search for efficacious pharmacological agents acting on the glutamatergic system has been ongoing ever since dysfunction of the neurotransmitter was identified as a putative biomarker for obsessive-compulsive disorder. Many different types of glutamatergic modulators have been investigated as novel therapeutic options for the disorder, primarily as augmenting agents in refractory OCD cases. These include riluzole (Coric et al., 2005, Grant et al., 2007, Grant et al., 2014), a glutamate modulator that inhibits release of the neurotransmitter via inhibition of voltage gated Na⁺-channels, and NMDA blockers including memantine (Pasquini and Biondi, 2006, Haghghi et al., 2013), D-cycloserine (Kushner et al., 2007, Storch et al., 2016), and ketamine (Rodriguez et al., 2011, Bloch et al., 2012, Rodriguez et al., 2013). As is the issue with most forms of pharmacotherapy for neuropsychiatric disorders, the treatments may be efficacious in certain subsets of the patient population but rarely, if ever, are they effective in all. More studies aimed at understanding the underlying cause of the disorder must be done in order to isolate specific proteins that are implicated in the disorder. At this time, the strongest evidence in OCD is for *SLC1A1/EAAT3*; however little effort has been put forth to develop compounds with the capacity to exert physiological effects *in vivo*. It is irrational to argue that a panacea drug will be found to cure all of the ills of patients suffering from OCD, as

the patient population undergoes a diverse set of symptoms. However, until rationally designed compounds aimed at modulating strongly implicated protein targets are developed, this patient population will continue to be treated with therapeutic strategies aimed at treating only their symptoms, not the underlying pathophysiology of the disorder.

CHAPTER 4

OCD CANDIDATE GENE *SLC1A1/EAAT3* IMPACTS BASAL GANGLIA-MEDIATED ACTIVITY AND STEREOTYPIC BEHAVIOR

As published in: OCD candidate gene SLC1A1/EAAT3 impacts basal ganglia-mediated activity and stereotypic behavior. Proc Natl Acad Sci USA (2017).

Isaac D. Zike, Muhammad O. Chohan, Jared M. Kopelman, Emily Krasnow, Daniel Flicker, Katherine M. Nautiyal, Michael Bubser, Christoph Kellendonk¹, Carrie K. Jones, Gregg D. Stanwood, Kenji F. Tanaka, Holly Moore, Susanne E. Ahmari, Jeremy Veenstra-VanderWeele

Introduction

Obsessive-compulsive disorder (OCD) is a common neuropsychiatric condition that ranks among the top ten causes of disability worldwide (Koran et al., 2007, Michael S. Ritsner, 2007). Primary forms of therapy include serotonin reuptake inhibitors (SRIs) and cognitive behavioral therapy; however, only 50-60% of patients exhibit adequate response to current treatment approaches, with clinically significant residual symptoms in most patients (Dougherty et al., 2004, Koran et al., 2007). Other agents, including antipsychotic medications and glutamatergic agents, have been investigated to augment SRIs, but have shown limited evidence of efficacy to date (Simpson et al., 2013, Grant et al., 2014, Sarris et al., 2015). Surgical intervention with deep-brain stimulation (DBS) in the ventral capsule/ventral striatum or subthalamic nucleus shows promise, but is reserved for the most severely ill patients (Aouizerate et al., 2004, Fontaine et al., 2004). New treatments based on greater understanding of pathophysiology are therefore needed.

Multiple lines of evidence indicate that the basal ganglia are critically affected in OCD. Structural neuroimaging studies demonstrate altered caudate volume in OCD (Rosenberg et al., 1997, Radua and Mataix-Cols, 2009), and functional imaging has identified hyperactivity in cortico-striatal circuits, both at baseline and with symptom provocation (Menzies et al., 2008).

Interestingly, some reports using magnetic resonance spectroscopy (MRS) describe elevated striatal glutamatergic signal in the caudate (Whiteside et al., 2006, Starck et al., 2008), suggesting increased intracellular glutamate and/or gamma-aminobutyric acid (GABA). Recent work in genetic and optogenetic mouse models of SRI-sensitive compulsive-like grooming behavior have also implicated cortico-striatal signaling, suggesting relevance of dysfunctional basal ganglia signaling to repetitive behavior across species (Welch et al., 2007, Shmelkov et al., 2010b, Ahmari et al., 2013, Burguiere et al., 2013).

Family studies support a significant role for genetics in OCD, with increased heritability in early-onset OCD (Grados and Wilcox, 2007, Pauls, 2008). Suggestive linkage to the chromosome region 9p24, which contains *SLC1A1*, in addition to other genes, was initially established in a genome-wide linkage scan of OCD and then independently replicated (Hanna et al., 2002b, Willour et al., 2004b). Analysis of *SLC1A1*, which codes for the neuronal glutamate transporter EAAT3 (excitatory amino acid transporter 3), identified significant association in the 3' region, with stronger evidence in males (Veenstra-VanderWeele et al., 2001, Arnold et al., 2006, Dickel et al., 2006). Some, but not all, subsequent studies also identified association with polymorphisms in the 3' gene region, with the most evidence for association with the rs301430C allele (Arnold et al., 2006, Dickel et al., 2006, Stewart et al., 2007, Shugart et al., 2009, Wendland et al., 2009a, Stewart et al., 2013a), which is linked to elevated *SLC1A1* expression in lymphoblastoid cells, human postmortem brain, and a luciferase reporter assay (Wendland et al., 2009a). Taken together, these data suggest that OCD susceptibility may result from elevated *SLC1A1* expression, and that decreasing EAAT3 activity could therefore be a therapeutic target. Association findings, gene expression differences, and deletions of *SLC1A1* have also been reported in schizophrenia and bipolar disorder (Horiuchi et al., 2012, Myles-Worsley et al., 2013, Afshari et al., 2015), indicating a potential role for EAAT3 in a broader array of neuropsychiatric disorders.

SLC1A1 mRNA and EAAT3 protein are strongly expressed in the cortex and the striatum, and in mesolimbic and nigrostriatal dopaminergic neurons (Nieoullon et al., 2006, Nafia et al., 2008, Holmseth et al., 2012, Underhill et al., 2014, Bjorn-Yoshimoto and Underhill, 2016). EAAT3 localizes to peri- and postsynaptic regions (Conti et al., 1998a), where it serves three apparent functions: 1) buffering glutamate concentrations around peri/extrasynaptic N-methyl-D-aspartate (NMDA) and metabotropic glutamate receptors (Otis et al., 2004); 2) taking up glutamate as an intracellular precursor for GABA synthesis (Sepkuty et al., 2002, Mathews and Diamond, 2003); and 3) taking up cysteine for glutathione synthesis and protection from oxidative stress (Aoyama et al., 2006, Watts et al., 2014).

One logical place for *SLC1A1*/EAAT3 to impact cortico-striatal signaling is in the GABAergic medium spiny neurons (MSNs) of the striatum, which receive glutamatergic inputs from the cortex and provide output to the thalamus via the direct and indirect pathways of the basal ganglia. EAAT3 activity limits NR2B-containing NMDA receptor-dependent signaling at hippocampal glutamatergic synapses (Scimemi et al., 2009), and could similarly impact postsynaptic signaling in striatal MSNs (Cahill et al., 2014). In addition, recent reports suggest that EAAT3 is expressed in dopaminergic neurons projecting from the ventral tegmental area (VTA) and the substantia nigra (SN) to the ventral and dorsal striatum respectively (Nafia et al., 2008, Underhill et al., 2014). It is therefore possible that the subpopulation of EAAT3 in midbrain dopaminergic neurons could impact OCD-implicated basal ganglia circuitry via neuromodulation. This is especially relevant in light of recent evidence that amphetamine elicits endocytosis of EAAT3 and causes elevated signaling at glutamate receptors in dopaminergic neurons (Underhill et al., 2014).

Despite its genetic association with OCD, no studies have addressed the functional impact of *Slc1a1*/EAAT3 on OCD-relevant brain circuits or on OCD-like behaviors. To explore these questions, we used a flexible knock-in approach (Tanaka et al., 2010) to generate mice with constitutively reduced *Slc1a1*/EAAT3 expression (*Slc1a1*-STOP mice). We hypothesized

that these animals would show decreased liability to repetitive behaviors, based on the implication of increased *SLC1A1* expression in OCD risk. Beyond assessing spontaneous repetitive behaviors, which occur at low baseline frequency, we examined sensitivity to pharmacologically induced compulsive-like behaviors using amphetamine (which causes dopamine efflux and increased synaptic dopamine levels) and the dopamine D₁ receptor agonist SKF-38393. Our flexible knock-in approach also permitted targeted excision of the STOP cassette, allowing us to localize the impact of EAAT3 loss on repetitive behaviors.

Methods

Slc1a1-STOP Mouse Line Generation

Generation of targeting vector: We constructed a pNeoSTOPtetO plasmid using BAC recombineering (Tanaka et al., 2010). Our targeting vector has a 10kb 5'-homology arm, NeoSTOPtetO cassette, 1.7kb 3'-homology arm, and diphtheria toxin A subunit. We inserted the STOP-tetO cassette just upstream of the *Slc1a1* translation initiation site (Figure 8a). Multicloning (MCS) site1 (PacI/NotI/ BamHI), loxP, FRT, PGK-EM7-Neo-HSV thymidine kinase poly A minigene, STOP sequence, FRT, tetO sequence, loxP, and MCS2 (EcoRV/EcoRI/PacI/Sall) were connected in tandem. 400 bp DNA fragments upstream and downstream of the translation initiation site were amplified with PCR primers containing appropriate restriction enzyme sites, and respectively inserted into each MCS of pNeoSTOP-tetO plasmid. To perform BAC recombination, the linearized NeoSTOP-tetO cassette with 400 bp homology arms was transferred into bacteria carrying the pBADTcTypeG plasmid and the BAC encompassing the *Slc1a1* coding frame; BAC genomic clones containing *Slc1a1* promoter and regulatory regions were obtained from BacPac (RP23-475B5). The targeting vector was isolated from the recombined, kanamycin resistant clone using a retrieving technique into pMCS-DTA plasmid.

Construct insertion. 8 colonies were found to be kanamycin-resistant, and 2/8 were found to contain the NeoSTOP-tetO cassette via colony direct PCR. Expected band sizes based on primer positions and location of NeoSTOP-tetO sequence were seen: 685 bp (5' arm) and 644 bp (3' arm).

Generation of ES Cells: The targeting vector was electroporated into a 129/SvEvTac mouse ES cell line in the Duke Embryonic Stem Cell Core. Homologous recombinants were detected using PCR for the 3' arm of the targeting vector using a primer complementary to the 3' homology arm, and an external primer complementary to the genomic sequence located 3' of the 3' homology arm of the targeting vector. A subset of positive clones were tested by PCR for homologous targeting of the 5' arm. Transgene incorporation was verified using Southern blot with probe position from -11,256 to -10,717 bases upstream of the translation initiation site, outside the homology arm, which generated the predicted band sizes of 15kb (WT) and 18kb (*Slc1a1*-STOP-tetO). Positive ES clones were injected into C57BL/6J blastocysts to obtain chimeric mice, which were crossed to 129S6/SvEvTac females to obtain germline transmission. Germline transmitted offspring were then established as *Slc1a1* STOP-tetO heterozygous knock-ins on a pure 129S6/SvEvTac background.

Quantitative-Real Time Polymerase Chain Reaction

RNA Isolation

RNA from fresh striatal tissue was isolated using Qiagen RNeasy Mini Kit with DNase I (Qiagen, Valencia, CA, USA) treatment per manufacturer's instructions. RNA quality and yield were assessed by NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Quantitative PCR

For quantitative PCR (qPCR) experiments, cDNA was generated using High Capacity cDNA

Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA) per manufacturer's instructions. qPCR was performed using *Slc1a1* (Mm00436590_m1) and *Gapdh* (Mm99999915_g1) TaqMan gene expression assays run on a 7900HT system (Life Technologies). All samples were run in triplicate with non-template and reverse transcriptase negative controls. Differential gene expression was calculated using the $\Delta\Delta$ CT method with *Gapdh* as the endogenous control.

Immunoblotting Studies

Brains were harvested from mice following rapid decapitation. Brains were immediately placed on an ice-cold metal platform, and the striatum was dissected. Dissected tissue was placed into 3 mL of homogenization buffer [130 mM NaCl, 1.3 mM KCl, 2.2 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 10 mM Hepes (pH 7.4), 10 mM glucose, ascorbic acid, and 0.32 M sucrose] and homogenized using a Potter Elvehjem homogenizer (Wheaton, Millville, NJ, USA). Protein concentrations of all samples were determined by a bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific). Equal amounts of protein were incubated with Laemmli sample buffer for 10 min at room temperature and analyzed by using SDS/PAGE and Western blotting. EAAT3 protein was visualized in samples blotted to Immobilon-P PVDF membrane (EMD Millipore, Billerica, MA, USA) by using a rabbit anti-EAAC1/EAAT3 antibody (EAAC11-A, 1:1,000 dilution; Alpha Diagnostics, San Antonio, TX, USA). β -actin was visualized by using a mouse anti- β -actin antibody (A5316, 1:10,000 dilution; Sigma-Aldrich, St. Louis, MO, USA) as a loading control. Appropriate HRP-conjugated, secondary antibodies were obtained from GE Healthcare Life Sciences (Pittsburgh, PA, USA). Secondary antibody labeling was detected by using Amersham ECL Prime Western Blotting Detection Reagents and visualized via chemiluminescence using FluorChem M System (Protein Simple, San Jose, CA, USA). Multiple exposures were obtained to ensure linearity of band detection. Western blots were quantified by using NIH ImageJ software.

Synaptosomal Glutamate and Cysteine Transport Assays.

Brains were harvested from mice following rapid decapitation and immediately placed on an ice-cold metal platform so that striatum could be dissected. Striatal tissue samples were placed into 3 mL of homogenization buffer [130 mM NaCl, 1.3 mM KCl, 2.2 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 10 mM Hepes (pH 7.4), 10 mM glucose, ascorbic acid, and 0.32 M sucrose] and homogenized by using a Potter-Elvehjem homogenizer (Wheaton). Homogenates were centrifuged at 500 × g at 4 °C for 10 min. Supernatants were removed and centrifuged at 12,000 × g at 4 °C for 10 min. The resulting synaptosome-enriched pellets were then resuspended in 2.5 mL of assay buffer [130 mM NaCl, 1.3 mM KCl, 2.2 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 10 mM Hepes (pH 7.4), 10 mM glucose and ascorbic acid], and protein concentrations were determined by using the bicinchoninic acid (BCA) Protein Assay (Thermo Fisher Scientific). This material (hereafter called synaptosomes) was then diluted to 30 µg of total protein per 100 µL. Synaptosomes were incubated with 20 µM of [³H]-glutamate (10% labeled and 90% unlabeled), 50 µM and 200 µM [³⁵S]-cysteine (Perkin-Elmer, Waltham, MA, USA) (1% labeled and 99% unlabeled). Assays were terminated by rapid filtration over 0.3% polyethyleneimine-soaked GF/B glass microfiber filters (Whatman, GE Life Science) and washed three times with ice-cold Krebs-Ringers-Hepes buffer [130 mM NaCl, 1.3 mM KCl, 2.2 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, and 10 mM Hepes (pH 7.4)]. Filters placed into scintillation vials with 7 mL of Ecoscint H (National Diagnostics, Atlanta, GA, USA) scintillation fluid, shaken overnight at room temperature, and then radioactivity was quantified by using a TriCarb 2900TR scintillation counter (Perkin-Elmer). Specific [³H]-glutamate and [³⁵S]-cysteine transport was assayed in triplicate for all samples.

Behavioral Assays.

All behavioral experiments were performed in the Laboratory for Neurobehavior Core Facility at Vanderbilt, operated by the Vanderbilt Brain Institute, or the Rodent Neuroanalytical Core, operated by the New York State Psychiatric Institute. Behavioral testing was performed between 10:00 and 16:00 using mice 8-16 wk old. Animals were habituated to testing rooms for 15–20 min before the start of each experiment.

Spontaneous Locomotor Activity

Spontaneous locomotor activity in the open field was measured by using 27 x 27 x 20.5 chambers (Med-Associates, St. Albans, VT, USA) placed within light- and air-controlled sound-attenuating boxes (64 x 45 x 42 cm). Locomotion was detected by interruption of infrared beams by the body of the mouse (16 photocells in each horizontal axis located 1 cm above the activity chamber floor, as well as 16 photocells elevated 4 cm above the chamber floor). Data were collected and analyzed by Med Associates Activity Monitor software. Mice were acclimated to the activity chambers during a 30-min session 2–3 d before data recording began.

Elevated Zero Maze

Anxiety behaviors were examined by using an elevated zero maze (62.5 cm outer diameter, 5 cm path width, 15 cm wall height in closed segments; Stoelting, Wood Dale IL, USA) with recordings lasting for 5 min. At the start of each trial, mice were placed onto an open portion of the maze, adjacent to and facing one of the closed segments. Each session was recorded by a ceiling-mounted video camera connected to a computer for digital video acquisition and analysis with ANY-maze software (Stoelting). Data analyzed include the percent of time spent in the open zone, number of open and closed zones entries, and distance traveled within the maze.

Light-Dark Emergence

Light-dark emergence was measured by using 27 x 27 x 20.5 chambers (Med Associates) placed within light- and air-controlled sound-attenuating boxes (64 x 45 x 42 cm). An opaque black insert is placed into the open- field chamber that creates equal sized light and dark compartments with an open door. Mice were placed into the dark chamber through a trap door located on top of the insert. Mouse location was detected by interruption of infrared beams by the body of the mouse (16 photocells in each horizontal axis located 1 cm above the activity chamber floor. The distance traveled in each chamber, the total number of transitions, time spent in the each chamber, and latency to enter the light chamber are recorded by the Med Associates software.

Acoustic Startle and Prepulse Inhibition

Acoustic startle response and prepulse inhibition of acoustic startle were measured by using the Acoustic Startle Reflex Test Compartments (Med Associates). Mice were acclimated to background white noise of 65 dB for 5 min in a Plexiglas holding cylinder. Mice were then presented with seven trial types in six discrete, randomized blocks of trials for a total of 42 trials with an intertrial interval of 10 to 20 s. One trial measured baseline movement and one trial measured response to the 120-dB, 50-ms startle stimulus alone. The other five trials used an acoustic prepulse of 74, 78, 82, 86, or 90 dB preceding the acoustic startle stimulus by 100 ms. Startle amplitude was measured every millisecond over a 65 ms period beginning at the onset of the startle stimulus. The dependent variable was the maximum startle over the sampling period. Prepulse inhibition was calculated by dividing the difference between baseline startle and startle following prepulse by baseline startle.

Home Cage Scan

To evaluate possible repetitive behavior, individual mice of each genotype were video-recorded alone in their home cage for 24 h while maintaining their 12 h/12 h light/ dark schedule.

Automated video analysis was conducted by using HomeCageScan (Clever Sys, Reston, VA, USA) to index time spent performing individual behaviors.

Spontaneous Grooming

Mice were placed in an empty novel cage and allowed to habituate for 30 minutes. Mouse behavior was then recorded for 10 minutes. Time spent grooming was then manually determined with a stopwatch while observing the video, with the observer blind to genotype.

Amphetamine-Induced Locomotion

Locomotor activity in the open field was measured by using 27 x 27 x 20.5 cm chambers (Med Associates) placed within light- and air-controlled sound-attenuating boxes (64 x 45 x 42 cm). Locomotion was detected by interruption of infrared beams by the body of the mouse (16 photocells in each horizontal axis located 1 cm above the activity chamber floor, as well as 16 photocells elevated 4 cm above the chamber floor to detect rearing and jumping behaviors). Data were collected and analyzed by Med Associates Activity Monitor software. Mice were acclimated to the activity chambers during a 30-min session 2–3 d before data recording began. On day 1 of the experimental sequence, mice were weighed then placed into activity chambers, and activity was monitored for 30 min. The mice were then removed from the activity chambers and received an intraperitoneal (I.P.) injection of 0.9% saline or amphetamine (Sigma-Aldrich) (1.8, 3, or 8 mg/kg) Mice were returned to the activity chambers and locomotor activity was recorded for 60 min. Mice were returned to their home cage for a week to allow for drug wash out then given the alternative treatment. Time course data were analyzed by using non-linear curve line fit analysis and cumulative measures of behavior (i.e., total distance traveled, time in stereotypy, number of stereotype behaviors, time rearing, and number of rearing behaviors) during the entire 60-min recording was analyzed.

Amphetamine-Induced Stereotypy

Mice were weighed one hour prior to the beginning of behavioral assay then administered with 8 mg/kg amphetamine (Sigma Aldrich). Mice were then promptly placed into novel clear empty cages and behavior was recorded for 90 minutes with a video camera. Mouse behavior was analyzed for a stationary shuffling and sniffing-like stereotypy previously observed in pilot experiments. Observers blind to genotype performed analysis by post hoc scoring of video recordings. The observer recorded time spent performing this stationary shuffling and sniffing-like stereotypy manually with a stopwatch. Time spent undergoing stereotypy was analyzed for two minutes 50 and 80 min post amphetamine administration.

SKF-38393-Induced Grooming

Mice were weighed then placed in an open field apparatus measuring by using 27 x 27 x 20.5 cm chambers (Med Associates) placed within light- and air-controlled sound-attenuating boxes (64 x 45 x 42 cm). Mice were allowed to habituate for 30 min then administered saline or SKF-38393 (10 mg/kg, I.P. Tocris Biosciences, Pittsburgh, PA, USA) and placed back into the open field for 60 additional minutes. Behavior was recorded via cameras located above the open field for the entirety of the experiment. Following a one-week wash-out period, mice received the alternative treatment from week 1. Trained observers, blind to genotype and drug treatment, manually scored grooming duration during sample periods (first two minutes of every ten-minute bin) for one hour following SKF-38393 administration.

cFos⁺ Immunohistochemistry

To measure an indirect marker of neuronal activation, we immunostained coronal slices of mouse striatum and nucleus accumbens challenged with amphetamine (3.0 mg/kg, I.P.) or saline. Mice were then perfused with 4% paraformaldehyde and post-fixed overnight at 4 °C, transferred to 30% sucrose in 0.1 M phosphate buffer until sunk, and sectioned on a microtome

to a thickness of 40µm. Striatum containing sections were peroxidase quenched in .3% H₂O₂ in methanol for 30 min, blocked in PBS with 5% normal donkey serum and 0.3% Triton X-100 for 2 hr at room temperature, and incubated in rabbit anti-cFos primary antibody (1:5000; Santa Cruz, Dallas, TX, USA) for 72 hr at 4 °C. After washing in PBS with 0.3% Triton X-100, sections were incubated in biotinylated donkey anti-rabbit (1:500; Jackson ImmunoResearch, West Grove, PA, USA) secondary antibody for 2 hr at room temperature. Washed sections were incubated with ABC reagent and DAB substrate (Vector Labs, Burlingame, CA, USA) per manufacturer's instruction for visualization of stained cells.

cFos⁺ Cell Quantification

cFos immunostaining was identified using StereoInvestigator software (MBF Biosciences, Williston, VT, USA) and an Axio Imager M2 microscope (Zeiss) with a 10X objective coupled to a Retiga 2000R camera (Qimaging). 3-4 coronal brain sections corresponding to dorsal medial striatum (A/P +0.9 mm, M/L ±1.5 mm, D/V -3 mm relative to bregma), nucleus accumbens core (A/P +1.2 mm, M/L ±0.75 mm, D/V -4.5 mm relative to bregma) and nucleus accumbens shell (A/P +1.2 mm, M/L ±0.5 mm, D/V -4.5 mm relative to bregma), and somatosensory cortex (AP +0.9 mm, M/L ±3.0 mm, DV -3.0 mm relative to bregma) were identified for each treatment condition and matched between genotypes using the Allen Brain Atlas.(Franklin and Paxinos, 2013) For each animal (n=6 DS WT and ST, n=4 NAc WT and ST), the total number of *cFos⁺* cells within a unilateral region of interest was estimated using a sampling frame (dorsal striatum 90 x 67.5 µm; NAcc, and somatosensory cortex 35 x 35 µm) and the cell counter plugin for ImageJ (NIH). For each mouse, total number of *cFos⁺* cells within the counting frame were counted, averaged within subject, and then normalized within genotype to the saline condition. Analysis was performed using two-way ANOVA's to identify interactions between drug and genotype as well as main effects. All quantification was done blind to genotype and treatment. Raw counts are included in Table 1.

Binding

Fresh striatal tissue was dissected and frozen as noted above then placed in 2 mL of binding buffer [50 mM Tris (pH 7.4), 5 mM MgCl₂, 5 mM KCl, 1 mM EDTA, and 120 mM NaCl] and homogenized by using an Omni-Tip handheld homogenizer (Omni International) at 10,000–12,000 rpm for 10–15 s. Homogenates were then centrifuged at 20,000 × g at 4 °C for 20 min. Membrane pellets were resuspended in 3 mL of binding buffer and homogenized again as described above. Homogenates were preincubated at 37 °C for 15 min and then centrifuged at 20,000 × g at 4 °C for 20 min. Membrane pellets were again resuspended in 3 mL of binding buffer and homogenized as described above. Protein concentration was determined by BCA protein assay (Thermo Fisher Scientific). Samples were stored at –80 °C until used for binding assays. Membrane samples were then thawed and (100–150 µg for striatum) incubated in a final reaction volume of 1 mL at room temperature for 75 min in the presence of 3 nM [3 H]-SCH 23390 (D1R assays; 84.3 Ci/mmol; Perkin-Elmer) or 90 min in the presence of 3 nM [3 H]-methylspiperone (D2R assays; 84.3 Ci/mmol; Perkin-Elmer). Binding reactions were terminated by the addition of 8 mL of ice-cold wash buffer [50 mM Tris (pH 7.4)] followed by rapid filtration over water-moistened S&S (#5) (Schleicher and Schuell Bioscience) or Whatman GF/B (Whatman) glass fiber filter by using a Millipore vacuum manifold. Each filter was then washed two times with 8 mL of ice-cold wash buffer. Filters were placed in scintillation vials, and 10 mL of Biosafe II scintillation fluid (Research Products International, Mt. Prospect, IL, USA) was added to each vial. Vials were shaken overnight at room temperature, and then radioactivity was counted by using a TriCarb 2900TR scintillation counter (Perkin-Elmer). Nonspecific binding was determined by using parallel incubations as above with the addition of 2 µM butaclamol to samples with binding values from these samples subtracted from radioligand-only samples to determine specific binding. All samples (total and nonspecific binding) were assayed in triplicate.

Microdialysis

Anesthetized (isoflurane) 6-8 week old mice were implanted unilaterally with Microdialysis Guide Cannula MBR-5 (BASi, MBR-2255) using standard stereotactic techniques (AP +0.9 mm, ML 1.7 mm, DV -2.0 mm; relative to bregma). Microdialysis was performed following a recovery period of 3 days. On the day of microdialysis a Microdialysis Probe MBR-2-5 (BASi, MB-2212) with a confirmed in vitro recovery of > 7% was lowered into the guide cannula. The probe was then perfused with an artificial cerebral spinal fluid (aCSF; 145 mM NaCl, 2.7 mM KCl, 1.0 mM MgCl₂, 1.2 mM CaCl₂, pH 7.4) at a rate of 1 µL/min for a period of 120 mins prior to collecting the first sample. Following this equilibration period, dialysis samples were collected into vials containing 5 µl of HeGA preservative every 40 min. Once stable basal dopamine levels were established four different concentrations of dopamine (0, 1, 10, 20 nM) in aCSF were perfused in a random order and loss or gain of dopamine was measured. Following completion of this no net flux protocol, aCSF was again perfused until steady levels of dopamine were established; at which point 3 mg/kg amphetamine was administered (i.p). Four 40 mins samples were then collected. Dialysis samples were immediately analyzed using HPLC using a 100mm long column (Pursuit XRs C18 100 x 3.0mm, Agilent Technologies) and a mobile phase consisting of 1.5% acetonitrile, 7% methanol, 75 mM sodium phosphate monobasic monohydrate, 25 µM EGTA, 2.28 mM 1-octanesulfonic acid soap with a pH of 3.9 prepared no more than 24hrs before the run. DA and metabolite peaks were detected using an electrochemical microdialysis cell (ESA, model 5014B) coupled to a CoulArray detector (ESA, model 5600A) with CoulArray software. After the experiments, mice were deeply anesthetized and transcardially perfused, brains sliced and probe positions confirmed.

DAT and Cre Immunohistochemistry

Mice (12 weeks for DAT and 16-24 weeks for Cre) were perfused with 4% paraformaldehyde and post-fixed overnight at 4°C, transferred to 30% sucrose in 0.1 M phosphate buffer until sunk, and sectioned on a cryostat to a thickness of 40µm. Midbrain containing sections were washed with PBS and 0.3% Triton X-100 3 times for ten minutes, peroxidase quenched in 0.3% H₂O₂ in methanol for 30 min, and then blocked in PBS with 5% normal donkey serum and 0.3% Triton X-100 for 1 hr at room temperature. Sections were then incubated overnight at 4°C with either rat anti-DAT (1:1000, Millipore, Billerica, MA, USA) or mouse anti-Cre (1:1000, Millipore) antibody. After washing in PBS with 0.3% Triton X-100, sections were incubated in biotinylated donkey anti-Mouse (Cre, 1:500; Jackson Immuno Research, West Grove, PA, USA) or biotinylated donkey anti-Rat (DAT, 1:500; Jackson Immuno Research) secondary antibody for 2 hr at room temperature. Washed sections were incubated with ABC reagent and DAB substrate (Vector Labs, Burlingame, CA, USA) per manufacturer's instruction for visualization of stained cells. DAT and Cre immunostaining was identified using StereoInvestigator software (MBF Biosciences, Williston, VT, USA) and an Axio Imager M2 microscope (Zeiss) coupled to a Retiga 2000R camera (Qimaging). Areas corresponding to VTA (AP -2.9 -3.4 mm; ML ± 0.4 mm; DV -4.5 mm relative to bregma) were identified (Allen Brain Atlas) (Franklin and Paxinos, 2013).

DAT Stereological Quantification

Unbiased stereology was used to estimate mean total number of DAT⁺ neurons in the midbrain via the optical fractionator method (Pakkenberg and Gundersen, 1989, Furness et al., 2008, Gondre-Lewis et al., 2016) This design-based method allows an estimation of cell number that is independent of volume estimates. Data collection was performed using the StereoInvestigator program (MBF Biosciences). The system used an X-Y-Z motorized stage. The program was integrated with an Axio Imager M2 microscope (Zeiss) with coupled to a Retiga 2000R camera

(Qimaging). Region of interest was outlined at low power using a 2.5X objective. ROIs were determined based on stereotactic coordinates provided by Paxinos and Franklin atlas (Franklin and Paxinos, 2013) at -2.9 - -3.5 mm from bregma for the midbrain (VTA and SNc). Cells were counted using a 20X objective (N.A.= 0.5). Sampling scheme, sampling grid sizes (170 μ M x 170 μ M) and counting frames (45 μ M x 45 μ M), were optimized to obtain individual estimates of DAT⁺ neuron number with a mean Gundersen coefficient of error (CE) \leq 0.1 (Pakkenberg and Gundersen, 1989). Four mice per genotype (WT and *Slc1a1*-STOP), obtained via heterozygous breeding, were analyzed using StereoInvestigator. Sampling parameters are listed below. 15 sections per animal were used for estimates with the first section being selected randomly within the first three sections. Section thickness was measured at every fifth sampling location, and the mean thickness was used for computation of DAT⁺ neuron estimates. At each sampling location, microscope was focused down through the dissector sample to count any cell according to dissector counting rules. Since the fractionator method does not require a measurement of tissue volume or any other dimensional quality, the cell number estimate is valid, even if the tissue volume changes during processing. (West et al., 1991, West et al., 1996)

Sampling Parameters

	Number of Mouse Sections	Section Evaluation Interval	Distance Between Sections (μ M)	Sampling Grid Area (μ M)	Counting Frame (μ M)	Dissector Height (μ M)	Guard Zones (μ M)	Average Section Thickness (μ M; range)	CE (m=1)
Wildtype	15	3	40	170 x 170	45 x 45	14	2	18 (16-19.6)	0.10
<i>Slc1a1</i> - STOP	15	3	40	170 x 170	45 x 45	14	2	17.8 (15-19.5)	0.09

HPLC Analysis of Tissue Biogenic Amines

Neurochemical measures were obtained in the Neurochemistry Core Facility at Vanderbilt University Medical Center operated by the Vanderbilt Brain Institute. Brain regions, harvested

and dissected as noted above, were flash-frozen in liquid nitrogen and stored at $-80\text{ }^{\circ}\text{C}$. Frozen brain tissue was homogenized by using a tissue dismembrator (Misonix XL-2000; Qsonica, Newtown, CT, USA) in 100–750 μL of a solution containing 100 mM TCA, 10 mM $\text{NaC}_2\text{H}_3\text{O}_2$, 100 μM EDTA, 5 ng/mL isoproterenol (an internal standard), and 10.5% (vol/vol) methanol (pH 3.8). Samples were spun in a microcentrifuge at $10,000 \times g$ for 20 min, and the supernatants were stored at $-80\text{ }^{\circ}\text{C}$ until assayed. Before assay, thawed supernatants were centrifuged at $10,000 \times g$ for 20 min before being analyzed by HPLC. Twenty microliters of each sample were injected by using a Waters 2707 autosampler onto a Phenomenex Kintex (2.6 μm , 100 A) C18 HPLC column (100 \times 4.6 mm). Biogenic amines were eluted with a mobile phase [89.5% 100 mM TCA, 10 mM $\text{NaC}_2\text{H}_3\text{O}_2$, 100 μM EDTA, and 10.5% methanol (pH 3.8)] delivered at 0.6 mL/min by using a Waters 515 HPLC pump. Analytes were detected by using an Antec Decade II (oxidation: 0.4) (3 mm GC WE, HYREF) electrochemical detector operated at $33\text{ }^{\circ}\text{C}$. HPLC instrument control and data acquisition was managed by Empower software.

In Vivo Viral Infusion

Stereotaxic surgery was performed followed standard procedures, under sterile conditions. Adult male (8-12 week old) *Slc1a1*-STOP homozygous and wildtype controls were anesthetized with an i.p. injection of xylazine (10 mg/kg; Sigma Aldrich) and ketamine (100 mg/kg; Hospira, Lake Forest, IL, USA) and place into a mouse stereotaxic apparatus. A 5- μL Hamilton syringe (Hamilton, Reno NV, USA) attached to a nano syringe pump (KDS310; KD Scientific, Holliston, MA, USA) was lowered through a skull burr hole into the ventral tagmental area or dorsal striatum at the coordinates: VTA (AP -3.3 mm; ML \pm 0.4 mm; DV -4.5 mm relative to bregma; dorsal striatum (AP +0.9 mm; ML \pm 1.7 mm; -2.5 mm relative to bregma) with reference to the mouse brain atlas of Paxinos (Franklin and Paxinos, 2013). A total of 0.3 μL of virus was infused bilaterally at a flow rate of 0.1 $\mu\text{L}/\text{min}$. The syringe was left in place for 10 min after completion of infusion to eliminate backflow and then slowly withdrawn. This process was repeated on the

other side to produce bilateral viral injections. Cranial incision was closed with Vetbond (3M, Maplewood, MN, USA) and mice were returned to their home cage for a minimum of 2 weeks postsurgery. Mice received either AAVrh10.CMV.PI.Cre.rBG (University of Pennsylvania Vector Core, Philadelphia, PA, USA) or AAVrh10.CMV.PI.eGFP.WPRE.bGH (University of Pennsylvania Vector Core) at a titer of 1×10^{13} genomic copies per milliliter.

Statistical Analysis

Data were analyzed using Prism (GraphPad, La Jolla, CA, USA). Two-tailed, unpaired Student *t*-test or two-way ANOVA with Sidak's post-tests were used to analyze the primary data, except for locomotor data, which was analyzed using non-linear curve fit analysis. Specific statistical analyses for each data set are described in results and in the figure legends. In the text and figures, all data are reported as the mean \pm standard error of the mean. Bar graphs depict the mean \pm standard error of the mean.

Results

***Slc1a1*/EAAT3 Expression and Transporter Function is Reduced in *Slc1a1*-STOP Mice**

To investigate the functional impact of *Slc1a1*/EAAT3 expression in OCD-relevant behavioral assays, we used the Flexible Accelerated STOP Tetracycline operator knock-in (FAST) system (Tanaka et al., 2010) to create a knock-in mouse with globally reduced *Slc1a1*/EAAT3 expression (Figure 8a). As expected, *Slc1a1* mRNA was reduced in *Slc1a1*-STOP (ST) mice relative to wildtype (WT) littermate controls (Figure 8b) via qRT-PCR of the dorsal striatum (unpaired *t*-test; $P < 0.0001$, $n = 5$ per genotype). Immunoblots of whole striatum synaptosomes also demonstrated ablated EAAT3 protein expression in *Slc1a1*-STOP mice compared to WT littermate controls ($P = 0.0001$, $n = 6$ per genotype) (Figure 8c).

We next probed the functional consequences of reduced EAAT3 expression using striatal synaptosome transport assays (Nieoullon et al., 2006). Because EAAT3 is the primary

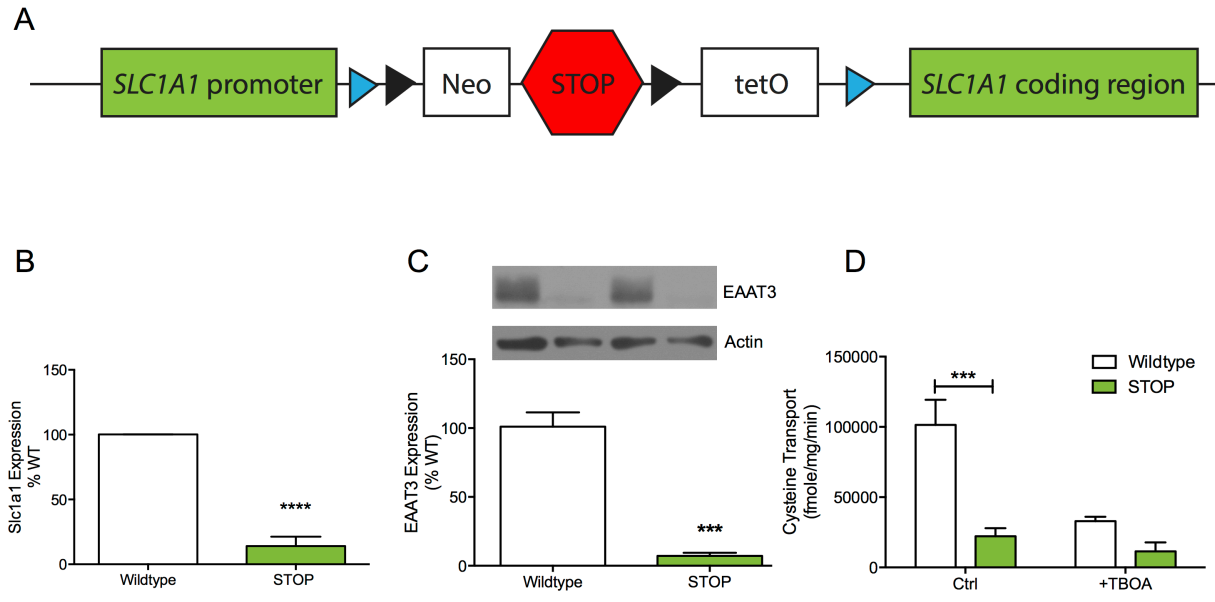


Figure 8. *Slc1a1*-STOP mice have reduced *Slc1a1*/EAAT3 protein expression and function

(A) *Slc1a1*-STOP construct schematic. Closed triangles represent flippase recognition target sequences and open triangles represent LoxP sites. STOP: Stop signal. Neo: PGK-EM7-NEO minigene. tetO: tetracycline operon.

(B) *Slc1a1*-STOP mice have reduced *Slc1a1* mRNA expression as measured by qRT-PCR of dorsal striatum. (unpaired t-test; $t=11.81$, **** $P<0.0001$, $n=5$ per genotype)

(C) EAAT3 protein expression is reduced in striatal synaptosome preparations from *Slc1a1*-STOP (ST, Lane 2 and 4) mice, relative to wildtype (WT, Lane 1 and 3) mice. (unpaired t-test; $t=8.84$, *** $P=0.0001$, $n=6$ per genotype. Figure is representative of three separate experiments). Average protein expression is demonstrated in bar graph.

(D) Na^+ -dependent uptake of L-cysteine (50 μM) is abolished in striatal synaptosome preparations from *Slc1a1*-STOP (ST) mice. Non-selective EAAT inhibitor threo- β -benzyloxyaspartate (TBOA) (100 μM) reduced wildtype (WT) synaptosome uptake but did not affect uptake from ST synaptosomes. (two-way ANOVA; inhibitor x genotype $F(1,20)=8.361$, ** $P=0.009$; inhibitor $F(1,20)=15.7$, *** $P=0.0008$; genotype $F(1,20)=25.34$, **** $P<0.0001$, $n=6$ per genotype; post hoc Sidak's multiple comparison test *** $P<0.05$). Figure is representative of three separate experiments.

source for neuronal cysteine (Watts et al., 2014), [³⁵S]-cysteine was used as the substrate for EAAT3 synaptosome uptake. Na⁺-dependent uptake of cysteine in synaptosomes prepared from *Slc1a1*-STOP mice was ablated relative to WT synaptosomes (Figure 8d; two-way ANOVA; inhibitor x genotype F(1,20)=8.361, *P*=0.009; inhibitor F(1,20)=15.7, *P*=0.0008; genotype F(1,20)=25.34, *P*<0.0001, n=6 per genotype; post hoc Sidak's multiple comparison test *P*<0.05). As expected from previous reports (Holmseth et al., 2012), we were unable to detect a difference in Na⁺-dependent glutamate uptake in striatal synaptosomes from *Slc1a1*-STOP mice relative to WT littermate controls in either the presence or absence of the EAAT inhibitor dihydrokainic acid (DHK) (Supplementary Figure 4a).

***Slc1a1*-STOP Mice Show No Changes in Spontaneous Behavior**

Slc1a1-STOP mice and littermate controls were subjected to a battery of behavioral tasks to determine if baseline behavioral differences were present. No anxiety-like phenotypes, compulsive-like phenotypes, or deficits in sensorimotor gating (Ahmari et al., 2012) were observed in *Slc1a1*-STOP mice as measured by changes in open field activity, time spent in the open arms of the elevated zero maze, light-dark emergence, prepulse inhibition, or spontaneous grooming relative to wildtype littermate controls (Supplementary Figure 5).

Pharmacological Probing of Basal Ganglia Circuitry Reveals Reductions in Basal Ganglia-Dependent Repetitive Behavior in *Slc1a1*-STOP Mice

To induce basal ganglia-mediated locomotor and repetitive behaviors, D-amphetamine was administered acutely in *Slc1a1*-STOP and WT littermate controls. At a moderate dose (1.8 mg/kg), amphetamine-induced locomotion was significantly attenuated in *Slc1a1*-STOP mice relative to controls (Figure 9a; Curve fit analysis; F(4,496)=6.89, *P*<0.0001). A higher dose (3.0 mg/kg) further accentuated this difference (Figure 9b; Curve fit analysis; F(4,496)=13.32, *P*<0.0001). At the highest amphetamine dose tested (8.0 mg/kg), at which stereotypic behavior

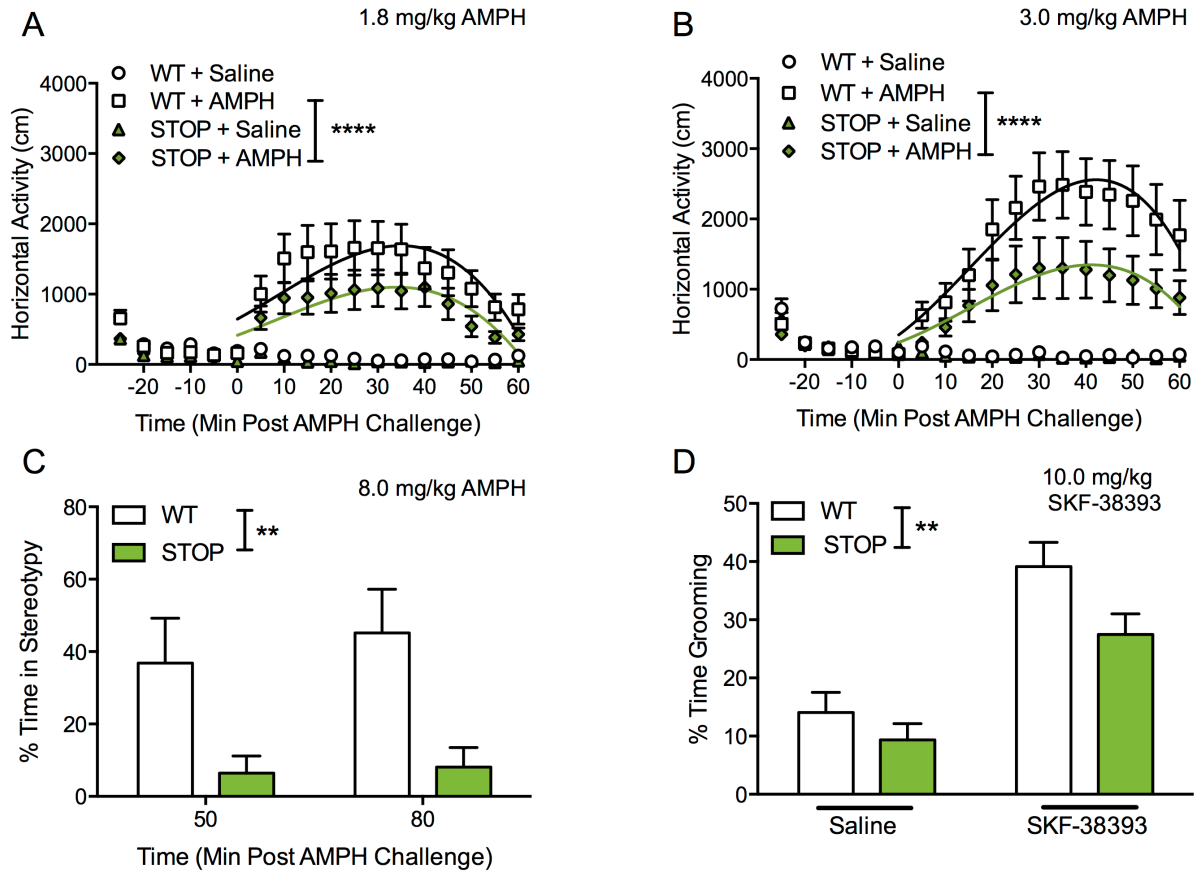


Figure 9. *Slc1a1*-STOP mice have attenuated behavioral response to amphetamine and SKF-38393 challenge

(A-B) Following acute D-amphetamine challenge (1.8 and 3.0 mg/kg), WT and *Slc1a1*-STOP mice demonstrate locomotor hyperactivity, which was attenuated in *Slc1a1*-STOP mice at both 1.8 mg/kg (A: curve fit analysis T=30-90; $F(4,496)=6.891$, **** $P<0.0001$, n=14 per genotype) and 3.0 mg/kg (B: curve fit analysis T=30-90; $F(4,496)=13.32$, **** $P<0.0001$, n=14 per genotype).

(C) A main effect of genotype is observed on stereotypy following high dose amphetamine challenge (8.0 mg/kg) in *Slc1a1*-STOP mice and wildtype littermate controls. Stereotypic behavior was scored by trained blind independent observers at 50 and 80 minutes post challenge (two-way ANOVA; genotype $F(1,42)=12.09$, ** $P=0.001$, n=12 per genotype).

(D) SKF-38393 challenge reveals a main effect of drug and genotype for grooming behavior following agonist challenge in *Slc1a1*-STOP and wildtype controls. Grooming behavior was scored by a trained blind independent observers (two-way repeated measures ANOVA; drug x genotype, $F(1,26)=0.74$, $P=0.40$; drug, $F(1,26)=28.5$, *** $P<0.0001$; genotype, $F(1,26)=7.95$, ** $P=0.0091$; n=14 per genotype)

dominates over locomotion (Castellan Baldan et al., 2014), there were no differences in overall locomotor behavior (Supplementary Figure 6a). However, when a separate cohort of animals was tested at this 8.0 mg/kg dose, blinded video scoring of stereotypic behavior at 50 and 80 minutes post amphetamine revealed a main effect of genotype in *Slc1a1*-STOP and WT littermate controls (Figure 9c; two-way ANOVA; genotype $F(1,42)=12.09$, $P=0.0012$, $n=12$ per genotype). Mice did not undergo stereotypic behavior following saline challenge (Supplementary Figure 6b).

To examine the impact of ablated EAAT3 expression independent of pre-synaptic dopamine release triggered by amphetamine administration, we acutely challenged *Slc1a1*-STOP mice with the dopamine D₁ receptor (D₁) agonist SKF-38393 (SKF) (10 mg/kg, IP) to induce perseverative grooming (Starr and Starr, 1986, Taylor et al., 2010). Via two-way ANOVA, a main effect of genotype was identified following SKF challenge in *Slc1a1*-STOP mice and controls (Figure 9d; two-way Repeated Measures ANOVA; Drug, $F(1,26)=28.5$, $P<0.0001$; Genotype, $F(1,26)=7.95$, $P=0.0091$, $n=14$ per genotype).

Amphetamine Dependent *cFos*⁺ Induction is Decreased in the Dorsal Striatum of *Slc1a1*-STOP mice

Amphetamine-induced locomotion and stereotypy are dependent on discrete subregions of the striatum (Rebec et al., 1997, Ikemoto, 2002). We therefore quantified *cFos* immunoreactivity in the dorsal striatum and the nucleus accumbens core and shell in response to amphetamine (3.0 mg/kg). In the dorsal striatum, a main effect of genotype was observed in *cFos*⁺ cells, with an amphetamine-induced increase in *cFos*⁺ cells in WT littermate controls that was absent in *Slc1a1*-STOP mice (Figure 10a, Supplementary Figure 7a; two-way ANOVA; drug x genotype; $F(1,17)=9.91$, $P=0.006$; genotype; $F(1,17)=9.91$, $P=0.006$; $n=5-6$ per genotype; post hoc Sidak's multiple comparison test, $P<0.05$). A similar but less robust main effect of amphetamine was observed in the nucleus accumbens (NAc) core (Figure 10b,

Supplementary Figure 7b; two-way ANOVA; drug x genotype; $F(1,12)=1.55$, $P=0.24$; drug; $F(1,12)=17.52$, $P=0.001$; $n=4$ per genotype) and shell (Figure 10c, Supplementary Figure 7c; two-way ANOVA; drug x genotype $F(1,12)=4.74$, $P=0.05$; drug $F(1,12)=13.59$, $P<0.003$; $n=4$ per genotype) in WT and *Slc1a1*-STOP mice.

Dopamine Receptor Density is Reduced in Striatal Membranes of *Slc1a1*-STOP Mice

To assess whether the blunted dopamine agonist response in *Slc1a1*-STOP mice could be explained by changes in striatal dopamine receptor density, we performed binding experiments in striatal membrane preparations from both dorsal and ventral striatum using a dopamine D₁ receptor (D₁) antagonist, [³H]-SCH-23390. D₁ binding was decreased in *Slc1a1*-STOP membranes isolated from dorsal striatum relative to WT littermate controls (Figure 10d; unpaired t-test; $P=0.008$, $n=6$ WT, $n=8$ ST) however, binding estimates of D₁ density in the ventral striatum were not affected (Supplementary Figure 8a). We also measured dorsal striatal membrane binding of the D₂ receptor using a D₂ antagonist, [³H]-methylspiperone (NMSP) and observed a trend towards decreased binding (Supplementary Figure 8b; unpaired t-test; $P=0.058$, $n=6$ WT, $n=8$ ST).

Decreased striatal dopaminergic transmission in *Slc1a1*-STOP mice

To corroborate a previous report of no change in dopamine neuron numbers or morphology in *Slc1a1* null mice at 3 months of age (Berman et al., 2011), we performed dopamine transporter immunohistochemistry in the midbrain of 10-12 week old *Slc1a1*-STOP mice and littermate controls and found no differences in stereological DAT⁺ cell number estimates (Supplementary Figure 9a-c). In order to explore pre-synaptic mechanisms that could account for altered response to dopamine agonists in the *Slc1a1*-STOP mice, we measured tissue levels of dopamine and its major metabolite, 3,4-Dihydroxyphenylacetic acid (DOPAC), 30 minutes after amphetamine (3.0 mg/kg IP) or saline injection. Via two-way ANOVA,

significant drug effects were observed on substantia nigra (SN) dopamine and DOPAC, VTA DOPAC, and dorsal striatum dopamine (Supplementary Figure 10). A main effect of genotype was only observed in substantia nigra DOPAC (Supplementary Figure 10).

We next investigated if extracellular striatal dopamine levels were altered in *Slc1a1*-STOP mice relative to wildtype controls. Basal, steady-state extracellular dopamine levels were first measured using the quantitative technique of “no net flux” microdialysis (Chefer et al., 2006). In freely moving mice, dorsal striatal extracellular dopamine levels were found to be significantly lower in *Slc1a1*-STOP mice than in WT littermate controls (Figure 10e; unpaired t-test; $P=0.0006$, $n=6$ per genotype). No differences were observed in dopamine clearance as measured by the slope of no net flux regression line (Supplementary Figure 11a). Conventional microdialysis revealed a significant elevation in dopamine levels following amphetamine (3 mg/kg, IP) in both WT and *Slc1a1*-STOP mice compared to baseline levels. However, absolute levels of dopamine after amphetamine were significantly reduced in *Slc1a1*-STOP mice compared to wildtype controls (Figure 10f; repeated measures two-way ANOVA; time x genotype, $F(7,70)=2.52$, $P=0.0226$; genotype $F(1,10)=9.34$, $P=0.01$; Sidak’s multiple comparison; $P<0.001$; $*P<0.05$; $n=6$ per genotype). No genotypic differences were seen in dopamine metabolite levels at baseline or in response to amphetamine challenge (Supplementary Figure 11b-c).

Viral-Mediated Rescue of *Slc1a1*/EAAT3 in the Midbrain Attenuates Amphetamine-Induced Behavioral Deficits in *Slc1a1*-STOP Mice

To test the hypothesis that our findings could be explained by the impact of EAAT3 ablation on midbrain dopaminergic neurons, we took advantage of the FAST construct to restore *Slc1a1*/EAAT3 expression in the midbrain of *Slc1a1*-STOP mice via Cre-Lox recombination. *Slc1A1*-STOP mice were bilaterally infused with either AAVrh10-CMV.Cre (ST:Cre) or AAVrh10-CMV.eGFP (ST:GFP) in the central midbrain (-3.3 mm AP, ± 0.4 mm ML, -

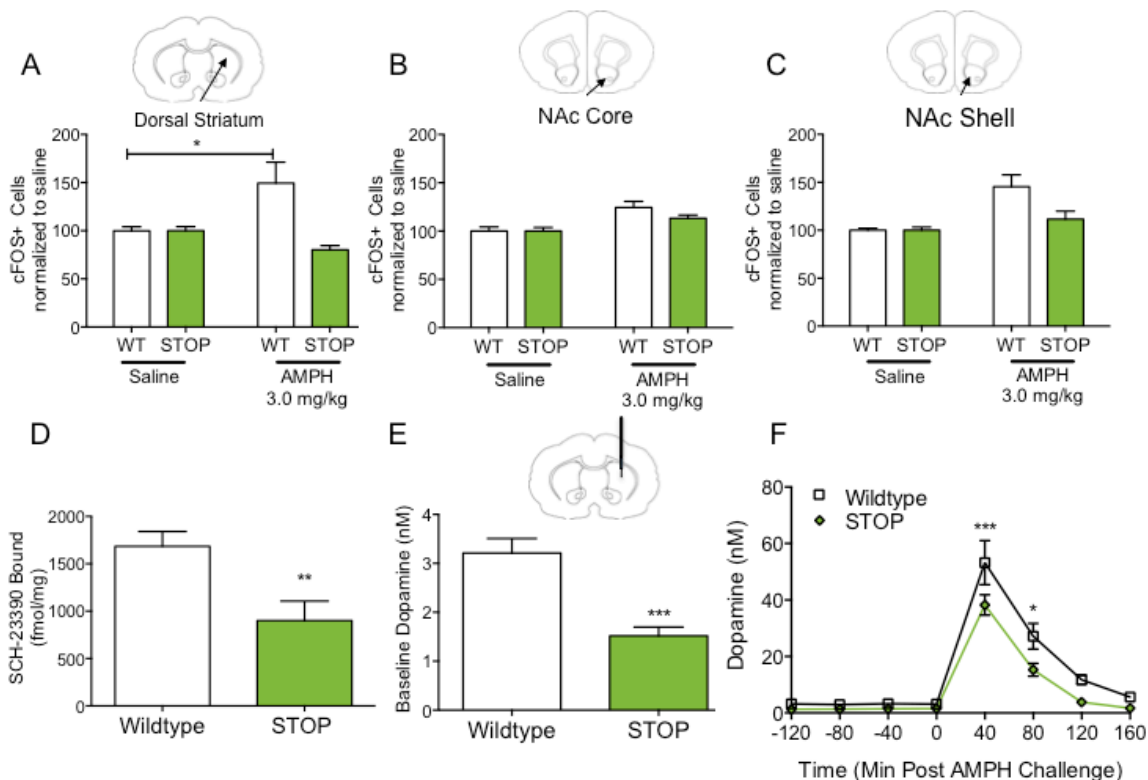


Figure 10. EAAT3 loss affects amphetamine induced *cFos* expression, dopamine receptor membrane density, and extracellular dopamine concentrations in the dorsal striatum

Quantification of *cFos*⁺ cells was performed in the dorsal striatum (left), nucleus accumbens (NAc) core, (middle), and shell (right).

(A) Staining for *cFos*⁺ cells reveals an amphetamine (3.0 mg/kg, IP) dependent increase in the dorsal striatum (DS) of WT mice that is absent in *Slc1a1*-STOP mice. (Two-way ANOVA; drug x genotype; $F(1,17)=9.91$, $**P=0.006$; genotype; $F(1,17)=9.91$, $**P=0.006$; $n=5-6$ per genotype; post hoc Sidak's multiple comparison test, $*P<0.05$).

(B) Staining for *cFos*⁺ cells reveals a main effect of amphetamine on *cFos*⁺ cells in the nucleus accumbens (NAc) core of WT and STOP mice. (Two-way ANOVA; drug; $F(1,12)=17.52$, $**P<0.01$; $n=4$ per genotype)

(C) Staining for *cFos*⁺ cells reveals a main effect of amphetamine on *cFos*⁺ cells in the NAc shell of WT and STOP mice and a trend level interaction and genotype effect. (Two-way ANOVA; drug x genotype and genotype $F(1,12)=4.74$, $^{\wedge}P=0.05$; drug $F(1,12)=13.59$, $**P<0.01$; $n=4$ per genotype)

(D) Dopamine D₁ receptor density estimated with [³H]-SCH-23390 binding in dorsal striatum membrane preparations (unpaired t-test; $t=3.1$, $**P=0.008$, $n=8$ wildtype, $n=6$ STOP).

(E) *Slc1a1*-STOP mice have significantly lowered dopamine levels at baseline as measured by the extrapolation of linear regression using no-net flux microdialysis (unpaired-test, $t=4.89$, $***P=0.0006$, $n=6$ per genotype).

(F) Dorsal striatal dopamine levels are significantly reduced in *Slc1a1*-STOP mice relative to wildtype controls following systemic administration of amphetamine (3 mg/kg, IP) (Repeated Measures Two-way ANOVA; time x genotype, $F(7,70)=2.52$, $*P=0.0226$; genotype $F(1,10)=9.34$, $*P=0.01$; Sidak's Multiple Comparison; $***P<0.001$, $*P<0.05$; $n=6$ per genotype).

4.5 mm DV), and wildtype littermate controls were bilaterally infused with AAVrh10-CMV.Cre (WT-Cre) (Figure 11a). After a two week incubation period, we found that ST:Cre animals showed a greater locomotor response to amphetamine (3.0 mg/kg) compared to ST:GFP control animals (Figure 11b; curve fit analysis; $F(4,478)=6.84$, $P<0.0001$, $n=12$ ST:GFP, $n=15$ ST:Cre); however, their locomotor response remained less than wildtype animals. We also observed a significant main effect of Cre virus in ST:Cre and ST:GFP controls following high dose amphetamine-induced stereotypy (8.0 mg/kg) (Figure 11c; two-way ANOVA; Cre virus $F(1,46)=9.45$, $P=0.0035$). No difference in perseverative grooming was observed between ST:GFP and ST:Cre mice after injection of the D_1 agonist, SKF (Figure 11d; two-way ANOVA; drug, $F(1,50)=19.2$, $P<0.0001$; virus, $F(1,50)=0.13$, $P=0.72$; $n=12$ ST:GFP, $n=15$ ST:Cre). Rescue of EAAT3 expression and viral spread in the midbrain of ST:Cre mice was confirmed via western blot (Supplementary Figure 12a-b) and immunohistochemistry (Supplementary Figure 13). To verify the specificity of midbrain rescue, we assessed the impact of viral Cre-mediated restoration of EAAT3 in the dorsal striatum, and found no differences in amphetamine- or SKF-mediated repetitive behavior in comparison to GFP controls (Supplementary Figure 14).

Discussion

Multiple studies have identified linkage and association of *SLC1A1*/EAAT3 with obsessive-compulsive disorder (Veenstra-VanderWeele et al., 2001, Arnold et al., 2006, Dickel et al., 2006, Stewart et al., 2007, Shugart et al., 2009, Wendland et al., 2009a, Samuels et al., 2011, Veenstra-VanderWeele et al., 2012); however, this is the first *in vivo* study to assess whether changes in EAAT3 expression affect repetitive behavior. By using amphetamine as well as a dopamine D_1 receptor agonist, we were able to probe the effects of EAAT3 ablation on basal ganglia-mediated behavior. As hypothesized, we detected a decrease in hyperlocomotion, stereotypic behavior, and striatal dopamine concentrations in response to these drugs in *Slc1a1*-STOP mice (Figure 9-10). Coupled with our findings of partial Cre-mediated rescue in

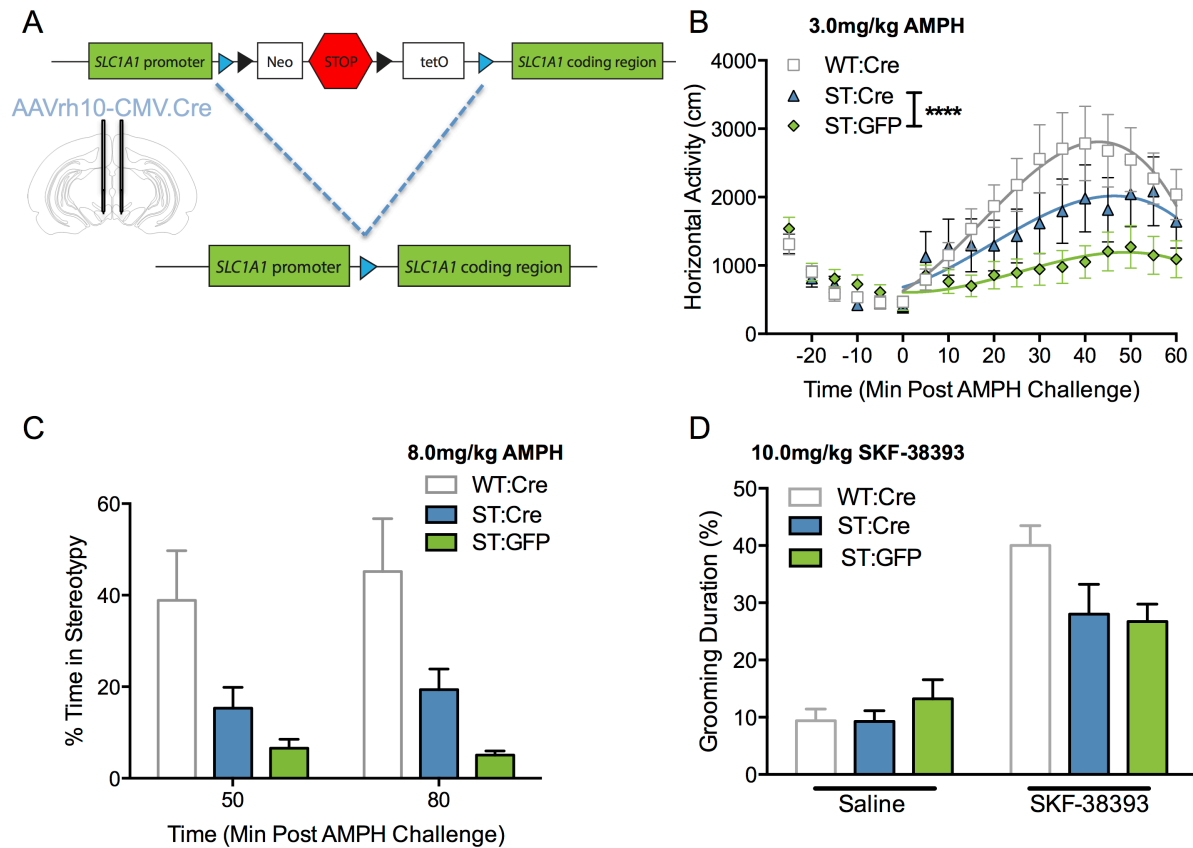


Figure 11. Viral Cre-mediated rescue of midbrain *Slc1a1*/EAAT3 expression rescues amphetamine but not SKF-38393 phenotypes in *Slc1a1*-STOP mice

(A) Schematic of Cre-mediated excision of the neo-STOP-tetO cassette in *Slc1a1*-STOP mice, leading to endogenous *Slc1a1*/EAAT3 expression. Blue triangles represent LoxP sites. Reference section indicates injection position in the central midbrain of *Slc1a1*-STOP and wildtype controls (-3.3 mm AP, ±0.4 mm ML, -4.5 mm DV).

(B) *Slc1a1*-STOP mice injected with AAV-Cre (ST:Cre) exhibit increased hyperlocomotor response to 3.0 mg/kg amphetamine in comparison to *Slc1a1*-STOP littermate controls injected with AAV-GFP (ST:GFP) (curve fit analysis T=30-90; F(4,478)=6.84, ****P<0.0001; n=12 ST:GFP, n=15 ST:Cre).

(C) A main effect of Cre virus is observed on stereotypy following high dose amphetamine challenge (8.0 mg/kg) in ST:Cre and ST:GFP controls. Stereotypic behavior was scored by trained blind independent observers at 50 and 80 minutes post challenge (two-way ANOVA; Cre virus, F(1,46)=9.453, **P=0.0035; n=12 ST:GFP, n=14 ST:Cre).

(D) ST:Cre mice showed no difference in stereotyped grooming behavior in response to 10 mg/kg SKF-38393 in comparison to ST:GFP littermate controls (two-way ANOVA; drug, F(1,50)=19.2, P<0.0001; virus, F(1,50)=0.13, P=0.72; n=12 ST:GFP, n=15 ST:Cre).

the midbrain, this is consistent with multiple previous lines of evidence supporting a role for dopaminergic pathology in compulsive-like behavior. Most immediately relevant to our findings, the *Hdc* null mouse model, based upon a family demonstrating a complex neuropsychiatric phenotype including complete penetrance for Tourette syndrome and partial penetrance for OCD, displayed elevated stereotypy in response to amphetamine challenge (Castellan Baldan et al., 2014). In humans, dopamine agonists, including amphetamine and the dopamine precursor L-DOPA, are well-known triggers of repetitive behavior, from simple motor movements to frankly compulsive behavior in disorders linked to altered dopamine homeostasis (Varley et al., 2001, Parraga et al., 2007, Voon et al., 2009, Madruga-Garrido and Mir, 2013). Indirect evidence also suggests an interaction between the dopamine system and *SLC1A1* in humans. Atypical antipsychotic medications, which act in part as dopamine receptor antagonists, trigger OC symptoms in some patients, and recent evidence suggests that polymorphisms in *SLC1A1* moderate susceptibility to this uncommon drug-induced compulsivity (Kwon et al., 2009, Cai et al., 2013).

Our data show that EAAT3 ablation leads to decreased immediate early gene activation in dorsal striatal neurons in response to amphetamine (Figure 10a), in addition to reductions in extracellular dopamine concentrations in the striatum at baseline and following amphetamine challenge (Figure 10e-f). These data are consistent with a change in pre-synaptic dopaminergic neuron function as a result of EAAT3 loss, and align with a recent study demonstrating that EAAT3 impacts glutamatergic input onto midbrain dopaminergic neurons (Underhill et al., 2014). Specifically, Underhill and colleagues found that EAAT3 is internalized in response to amphetamine, resulting in increased glutamate exposure and potentiation of AMPA and NMDA glutamate receptor-mediated synaptic transmission in midbrain dopamine neurons (Underhill et al., 2014). Based on these convergent data, we hypothesized that chronic increases in peri-synaptic glutamate levels at dopaminergic neurons may elicit a homeostatic mechanism in *Slc1a1*-STOP mice that underlies the attenuated response to amphetamine. In support of this

idea, we found that viral-mediated rescue of midbrain *Slc1a1*/EAAT3 resulted in increased amphetamine-induced locomotor and stereotypy behavior compared to *Slc1a1*-STOP mice infused with a control virus (Figure 11b-c). This contrasted with rescue of *Slc1a1*/EAAT3 in the striatum of *Slc1a1*-STOP mice, which had no impact on amphetamine-induced behavior (Supplementary Figure 14). Importantly, the lack of complete rescue of amphetamine response, as well as lack of change in D₁ agonist response, suggests that EAAT3 ablation in dopaminergic neurons (at least in adult animals) may not be the only mechanism impacting response to dopamine agonists in *Slc1a1*-STOP mice. Further study of EAAT3 ablation, restoration, and overexpression will be needed to dissect its importance in specific brain regions, neuronal subtypes, and importantly, within particular developmental windows.

Of note, the initial published evaluation of *Slc1a1*/EAAT3 null mice reported decreased activity in the open field, although this was not consistent with a later report, which described no baseline differences but found impaired Morris Water Maze performance in aged animals due oxidative stress-mediated neuronal loss (Peghini et al., 1997, Aoyama et al., 2006). In our baseline assessment of activity, anxiety-like behavior, and compulsive-like behavior, we found no significant changes in the *Slc1a1*-STOP animals. This is consistent with some data suggesting that the OCD-associated *SLC1A1* alleles lead to increased, not decreased, expression; however, postmortem studies are needed to clarify the direction of *SLC1A1* expression change in OCD (Wendland et al., 2009a, Porton et al., 2013). Examining the impact of *SLC1A1* overexpression on OCD-relevant behaviors in mice would also more directly assess this hypothesis. These data may also indicate the difficulty of detecting a potential decrease in low levels of spontaneous compulsive-like behavior (Wendland et al., 2009a). In addition, one subsequent report in *Slc1a1*/EAAT3 null mice described oxidative stress-mediated loss of dopamine neurons in animals at 12 months of age but no differences at 3 months (Berman et al., 2011). Even though our model does retain some degree of preserved EAAT3 function (Figure 8), we therefore restricted our work to younger animals and ruled out decreases in

dopamine transporter immunohistochemistry (Supplementary Figure 9) or diminished dopamine or DOPAC levels in the midbrain (Supplementary Figure 10). Furthermore, the results of our midbrain viral rescue (Figure 11) are not consistent with dopamine neuron loss as a mechanism of altered response to amphetamine or SKF-38393.

As with many studies aimed at unraveling pathophysiology in a preclinical context, it is important not to over-interpret these data in relation to the human condition. We therefore believe that the *Slc1a1*-STOP mouse should be considered as a putative model of reduced liability to dopamine-induced and basal ganglia-mediated repetitive behaviors (Castellan Baldan et al., 2014). Although heterozygous *SLC1A1* deletions have been reported in schizophrenia and schizoaffective disorder (Myles-Worsley et al., 2013, Afshari et al., 2015), our findings do not clearly indicate a psychosis-like phenotype, due to no observed changes in baseline behavior or prepulse inhibition. In the context of psychotic disorders, our observation of decreased sensitivity to amphetamine could be considered opposite to what might be expected, since amphetamine can induce psychosis in humans (Murray et al., 2013). Because of this apparent contradiction, further work using the *Slc1a1*-STOP mice, including heterozygous animals, is warranted to better understand the potential contribution of *SLC1A1* deletions to risk of psychosis.

In summary, we report the first evaluation of the OCD candidate gene *Slc1a1*/EAAT3 in relation to OCD-relevant circuitry and behavior in an animal model. Using dopaminergic agonism as a probe, we demonstrate the relevance of EAAT3 to striatal dopaminergic neurotransmission and to repetitive behavior. The partial rescue of dopamine agonist response by restoration of EAAT3 expression in the midbrain demonstrates an *in vivo* functional impact that matches previous cell model and *ex vivo* reports of EAAT3 effects in dopaminergic neurons. More work is needed to examine the effects of manipulating EAAT3 expression in other proposed models of striatally-mediated repetitive behavior (Welch et al., 2007, Shmelkov et al., 2010b) and in cognitive tasks relevant to OCD (Dittrich and Johansen, 2013, Krebs and

Heyman, 2015). Our results also suggest that EAAT3 antagonists should be evaluated in relation to dopamine agonist response and, perhaps, more broadly in relation to basal ganglia-mediated repetitive behavior across species.

CHAPTER 5

SUMMARY, DISCUSSION, AND FUTURE DIRECTIONS

Glutamate is the primary excitatory neurotransmitter in the CNS and is critical for behavior, cognition, memory, and learning (Zhou and Danbolt, 2014b). Glutamatergic dysfunction has been identified in many neurological disorders including epilepsy (Tanaka et al., 1997), schizophrenia (McCullumsmith and Meador-Woodruff, 2002, Nudmamud-Thanoi et al., 2007, Hu et al., 2015), amyotrophic lateral sclerosis (Blasco et al., 2014), Parkinson's disease (Gardoni and Di Luca, 2015), addiction (Tzschentke and Schmidt, 2003) and obsessive-compulsive disorder (Pittenger et al., 2011, Pittenger, 2015). In the Veenstra-VanderWeele lab, our primary focus has been on investigating the impact of glutamatergic dysfunction in obsessive-compulsive disorder. Glutamatergic deficits in OCD have been identified in the CSTC circuit (Rosenberg et al., 2001, Rosenberg et al., 2004, MacMaster et al., 2008, Starck et al., 2008), the same brain region previously implicated in OCD via structural imaging analysis (Saxena et al., 1998, Saxena et al., 2001, Maia et al., 2008, Rotge et al., 2008). The combination of these findings and evidence from other studies of the CSTC circuit (Rauch et al., 1997, Rauch et al., 2001, Harrison et al., 2009, Beucke et al., 2013) led to a preliminary model where hyperactivity of the direct pathway or hypoactivity of the indirect pathway may lead to overall elevated activity of the CSTC circuit and subsequent emergence of OCD symptoms.

Following the identification of glutamatergic dysfunction in the CSTC circuit as a putative biomarker of OCD, evidence from subsequent genetic analysis relevant to the glutamate system became of particular interest. Fitting with the glutamatergic hypothesis, the most consistently implicated gene in OCD is *SLC1A1*, which codes for the neuronal glutamate transporter excitatory amino acid transporter 3 (EAAT3, also known as EAAC1). *SLC1A1* was initially suspected in OCD based on a genome-wide linkage study, which uncovered a peak on

chromosome 9p24, where the gene is located (Hanna et al., 2002b, Willour et al., 2004b). A number of studies subsequently identified association between polymorphisms in and around *SLC1A1* in OCD (Arnold et al., 2006, Dickel et al., 2006, Kwon et al., 2009, Shugart et al., 2009, Samuels et al., 2011, Veenstra-VanderWeele et al., 2012, Stewart et al., 2013a). Of note, recent investigation of *SLC1A1* suggests that polymorphisms within the gene are also associated with *de novo* obsessive-compulsive symptoms induced by antipsychotic medication in schizophrenia (Kwon et al., 2009, Ryu et al., 2011, Schirmbeck et al., 2012, Cai et al., 2013), implying that there is an interaction between the dopamine and glutamate system relevant to repetitive behavior.

Accurate preclinical modeling of neuropsychiatric disorders is always difficult, especially in the case of OCD where rare genetic variants eliciting a large effect are unavailable and genome-wide association studies have yet to identify common variants that reach genome-wide significance. Consistent identification of *SLC1A1* in association studies provides support for *SLC1A1* as a putative risk gene for OCD; however little evidence is available to identify how alterations in its expression or function may lead to dysfunction of the CSTC circuit or to compulsive behavior. My work in the Veenstra-VanderWeele lab used genetic and pharmacological manipulations in mice to understand whether altered *SLC1A1*/EAAT3 expression or function could explain the convergence of neurochemical and genetic data implicating the glutamate system generally and this gene specifically in compulsive behavior and its underlying neural circuitry.

Summary of Results

Initial efforts to identify abnormal expression and/or function of EAAT3 in models of repetitive behavior and dysfunctional glutamatergic neurotransmission focused on *Sapap3* null mice. These mice exhibit excessive SRI-responsive grooming and exhibit deficits in cortical-striatal glutamatergic neurotransmission. We identified increases in EAAT3 expression in striatal

synaptosomes of *Sapap3* null mice relative to WT controls; however this increase was not mirrored in *ex vivo* assays of striatal EAAT3 function, albeit with low statistical power (Supplementary Figure 4). We next transitioned to examining the capabilities of a novel EAAT3 inhibitor (NBI-59159), previously shown to attenuate amphetamine-induced hyperlocomotion, a basal ganglia dependent behavior (Dunlop and Marquis, 2006), to alleviate the grooming phenotype observed in *Sapap3* null mice. Despite the prior reports of the inhibitors *in vivo* efficacy, acute pretreatment of *Sapap3* null and wildtype littermate controls with NBI-59159 prior to assessment of spontaneous grooming had no effect on grooming behavior, regardless of genotype. It is possible that more thorough investigation of the behavioral consequences of NBI-59159 dosing on *Sapap3* null phenotypic behavior would have resulted in data supporting the compounds efficacy; however we were only able to acquire a limited amount of the compound, allowing for a small pilot study at the dose reported to impact behavior in a previous study.

Recent reports using *Sapap3* null mice suggest that increased local glutamate concentration around EAAT3 due to pharmacological inhibition might not be expected to alleviate repetitive grooming behavior. Using pharmacological techniques, the Calakos lab found that *Sapap3* null mice show persistent activation of the metabotropic glutamate receptor mGlu5, leading to repetitive behavior (Ade et al., 2016). Since mGluR5 colocalizes to the extrasynaptic space surrounding synapses, along with EAAT3, increasing the local glutamate concentration via inhibition of EAAT3 seems unlikely to rescue the repetitive behavior grooming phenotype observed in *Sapap3* null mice.

To date, the striatum has been repeatedly implicated in OCD via numerous experimental approaches ranging from neuroimaging of patients (Rosenberg et al., 2000, Whiteside et al., 2006, Starck et al., 2008) to optogenetic rodent models (Ahmari et al., 2013, Burguiere et al., 2013). Taking note of this, we sought to directly probe the striatum pharmacologically to assess the *in vivo* efficacy of a novel EAAT3 inhibitor, 2-CFoDA, as had been reported previously with NBI-59159 (Dunlop and Marquis, 2006). Acute administration of systemic amphetamine causes

a robust increase in striatal synaptic dopamine concentrations, resulting in increased activation of dopamine receptors and overall disinhibition of the basal ganglia. Using amphetamine to induce basal ganglia dependent locomotor behavior enabled us to determine how blockade of EAAT3 function may affect behavior dependent on a node of the CSTC circuit. The initial characterization of 2-CFoDA in the Bridges lab was primarily focused on assessing its efficacy *in vitro*. Thus, the compound's *in vivo* profile was not well understood. We opted to deliver the compound into the CSF of WT mice via ICV injection in an attempt to bypass the blood brain barrier to deliver the drug throughout the brain. Despite prior reports of NBI-59159's capability to attenuate amphetamine induce locomotor behavior; we did not observe any differences in locomotor behavior following amphetamine administration at any of the doses of 2-CFoDA delivered ICV (Figure 6). It is possible that delivery of 2-CFoDA into the CSF did not allow for the inhibitor to reach its intended site of action in the striatum. Thus, we then transitioned to direct striatal administration of 2-CFoDA. At a moderate dose of 2-CFoDA, no *in vivo* efficacy was observed (Figure 7); however we found that a high dose of the inhibitor did reduce amphetamine-induced locomotor behavior (Figure 7). However, these data suggested sedating effects at this high dose because minimal overall activity was observed in 2-CFoDA pretreated mice following amphetamine challenge.

Taken together, the data from our behavioral pharmacology studies with EAAT3 inhibitors were unable to shed light on the potential utility of EAAT3 targeting compounds for impacting behavior dependent on the basal ganglia. In the time since *SLC1A1/EAAT3* has been identified as a potential candidate risk gene for OCD, development of compounds efficacious *in vivo* has not been a priority for drug development groups for a few reasons. First, establishing specificity for EAAT3 versus other glutamate transporters has proven challenging, and even modest off-target effects at GLT-1 could generate substantial adverse effects. Second, the observed association in OCD has been with common variants that have uncertain impact on *SLC1A1* expression or function, and rare variants have not yet been clearly implicated in OCD.

Third, previous studies have not clarified how EAAT3 function may impact glutamatergic signaling in the specific brain regions relevant to the OCD. Until more is known about EAAT3's role in local glutamatergic signaling and how modulation of transporter function may impact OCD-relevant brain regions, there is little motivation to invest substantial resources into the development of compounds effectively targeting EAAT3 *in vivo*.

Despite the convergence of evidence from putative glutamatergic biomarker analysis and consistent genetic association studies implicating of the gene, no studies had focused on assessing the functional impact of *Slc1a1*/EAAT3 on OCD-relevant circuits or behavior. In an effort to understand whether alteration of *Slc1a1*/EAAT3 expression may affect OCD-relevant behavior or neural circuitry, the Veenstra-VanderWeele lab, along with our collaborators from the Ahmari lab at the University of Pittsburgh, developed an *Slc1a1*/EAAT3 transgenic mouse line, termed *Slc1a1*-STOP mice. *Slc1a1*-STOP mice were made via homologous recombination to introduce a FAST construct (Tanaka et al., 2010), including a floxed-Neo-STOP cassette, upstream of the native *Slc1a1* locus, resulting in a constitutive model of reduced *Slc1a1*/EAAT3 expression (Figure 8a). *Slc1a1*-STOP mice were biochemically characterized by assessing *Slc1a1*/EAAT3 expression and function. Both *Slc1a1* and EAAT3 expression was reduced to negligible levels in striatal tissue of *Slc1a1*-STOP mice relative to WT mice (Figure 8b-c). EAAT3 function, examined via [³⁵S]-cysteine synaptosome uptake assays, was completely absent in synaptosomes isolated from *Slc1a1*-STOP mice compared to WT synaptosomes (Figure 8d).

Next, the spontaneous behavior of *Slc1a1*-STOP mice was assessed via a battery of assays aimed at identifying any baseline behavioral phenotypes. No anxiety-like phenotypes, compulsive-like phenotypes, or deficits in sensorimotor gating were observed in *Slc1a1*-STOP mice (Supplementary Figure 5). This is consistent with preliminary evidence suggesting that OCD-associated *SLC1A1* alleles cause increased, not decreased, expression (Wendland et al., 2009a). We could also have observed a floor effect, since reductions in OCD-relevant behavior

are unlikely to be seen in *Slc1a1*-STOP mice in tasks where WT mice exhibit low levels of spontaneous OCD-relevant behavior.

Based on these initial data, our hypothesis was that *Slc1a1*-STOP mice could represent an animal model of reduced liability to the induction of basal ganglia mediated repetitive or stereotypic behavior. To test this hypothesis, amphetamine was administered to induce basal ganglia mediated locomotor and repetitive behavior. *Slc1a1*-STOP mice exhibited reductions in amphetamine-induced locomotion at low and intermediate doses relative to WT mice, supporting our hypothesis (Figure 9a-b). We also measured locomotor behavior at high dose amphetamine; however no genotype differences were observed, perhaps because at this dose mice transition to a non-locomotor stereotypic behavior (Supplementary Figure 6). Conversely, when high dose amphetamine-induced stereotypy was measured by video scoring, reductions in repetitive stereotypic behavior were identified in *Slc1a1*-STOP mice (Figure 9c). We also explored the impact of ablated *Slc1a1*/EAAT3 expression on dopaminergic signaling in the striatum independent of pre-synaptic dopamine release by challenging mice with the dopamine D₁ receptor agonist SKF-38393, which causes perseverative grooming/scratching behavior. This face valid OCD-relevant behavior was blunted in *Slc1a1*-STOP mice relative to WT littermate controls, mirroring the results from the amphetamine-induced behavior (Figure 9d). These were the first OCD-relevant behavioral findings suggesting that alteration of *Slc1a1*/EAAT3 has a functional impact on face valid behavior dependent on the neural circuitry implicated in OCD.

Since the striatum has distinct subregions that are linked to amphetamine-induced locomotion and stereotypy specifically (Rebec et al., 1997, Ikemoto, 2002), we next quantified the activation of the immediate early gene *cFos* by amphetamine in the dorsal striatum and the nucleus accumbens core and shell. As expected, our data showed that ablation of EAAT3 expression in *Slc1a1*-STOP mice results in reduced *cFos*⁺ immunoreactivity broadly in all striatal subregions measured following amphetamine challenge, as well as reductions in dorsal striatal dopamine receptor binding in adult mice (Figure 11a). These data support the hypothesis that

EAAT3 loss primarily affects striatal MSNs; however they are also consistent with potential changes in pre-synaptic dopaminergic neuron function.

In order to address the possibility of changes in pre-synaptic dopaminergic neuronal function and to corroborate a previous report of no change in dopamine neuron numbers or morphology in *Slc1a1* null mice at 3 months of age (Berman et al., 2011), we performed dopamine transporter immunohistochemistry. No differences in stereological DAT⁺ cell number estimates were identified in the midbrain of young adult *Slc1a1*-STOP mice relative to wildtype littermate controls (Supplementary Figure 9). To further explore pre-synaptic mechanisms that could account for altered response to dopamine agonists in the *Slc1a1*-STOP mice, we measured tissue levels of dopamine and its major metabolite, 3,4-Dihydroxyphenylacetic acid (DOPAC), 30 minutes after amphetamine (3.0 mg/kg IP) or saline injection. Via two-way ANOVA, significant drug effects were observed on substantia nigra (SN) dopamine and DOPAC, VTA DOPAC, and dorsal striatum dopamine. A main effect of genotype was only observed in substantia nigra DOPAC.

We next investigated if extracellular striatal dopamine levels were altered in *Slc1a1*-STOP mice relative to wildtype controls. Basal, steady-state extracellular dopamine levels were first measured using the quantitative technique of “no net flux” microdialysis (Chefer et al., 2006). In freely moving mice, dorsal striatal extracellular dopamine levels were found to be significantly lower in *Slc1a1*-STOP mice than in WT littermate controls (Figure 10e). Absolute levels of dopamine after amphetamine challenge were also significantly reduced in *Slc1a1*-STOP mice compared to wildtype controls (Figure 10f).

Based on these findings indicating that EAAT3 loss influences striatal dopamine levels, as well as recent reports of amphetamine-induced endocytosis of EAAT3 impacting glutamate receptor mediated neurotransmission in the midbrain (Underhill et al., 2014), we hypothesized that EAAT3 loss in midbrain dopaminergic neurons accounted for the change in amphetamine sensitivity. We therefore utilized the loxP sites surrounding the STOP-tetO cassette in the

Slc1a1-STOP mice to restore *Slc1a1*/EAAT3 expression in the midbrain using a viral based expression rescue strategy. After injection of an AAV-Cre virus into the midbrain, *Slc1a1*-STOP mice underwent Cre-Lox recombination, and expression of *Slc1a1*/EAAT3 was restored (Supplementary Figure 12-13). Restoration of midbrain *Slc1a1*/EAAT3 expression in *Slc1a1*-STOP mice led to increases in locomotion and stereotypy at moderate and high doses of amphetamine respectively when compared to *Slc1a1*-STOP mice injected with a control GFP virus (Figure 11b-c). It is of note however that their response remained less than that of WT mice treated with the same AAV-Cre virus. Furthermore, induction of perseverative grooming behavior via SKF-38393 was not affected by restoration of midbrain EAAT3 expression. The inability to rescue the D₁-agonist induced grooming phenotype by restoring midbrain expression of *Slc1a1*/EAAT3 implies that there are alternative mechanisms involving EAAT3 and the behavioral response to dopaminergic agents that are responsible for the reduced SKF induced grooming observed in *Slc1a1*-STOP mice.

Interpretation

Our work is the first known attempt to examine how changes in *Slc1a1*/EAAT3 expression affect the dopaminergic system and repetitive behavior dependent on the striatum. Experimental evidence supports our hypothesis that reduced *Slc1a1*/EAAT3 expression attenuates induced striatal-dependent repetitive and stereotypic behavior, which our data suggest is driven, at least in part, by reductions in dopamine concentrations in the striatum of *Slc1a1*-STOP mice. Identifying a link between midbrain EAAT3 expression and amphetamine-induced repetitive behavior was an unexpected finding; although previous evidence did support a role of dopamine in compulsive-like behavior (Szechtman et al., 1998, Denys et al., 2004, Voon et al., 2009, Denys et al., 2013, Schmidt et al., 2013, Castellán Baldan et al., 2014). Since all of the observed OCD-relevant behavioral deficits observed in *Slc1a1*-STOP mice were

	Wildtype	<i>Slc1a1</i>-STOP	<i>Slc1a1</i>-STOP Cre (Mibdrain)	<i>Slc1a1</i>-STOP Cre (Striatum)
EAAT3 Expression and/or Function	Normal	Absent	Intermediate	Intermediate
AMPH Locomotor Response	Normal	Reduced	Intermediate	Reduced
AMPH Stereotypy Response	Normal	Reduced	Intermediate	Reduced
SKF-38393 Grooming Response	Normal	Reduced	Reduced	Reduced
Dorsal Striatum DA Receptor Density	Normal	Reduced (D1)	-	-
Striatal <i>cFos</i>+ AMPH Response	Normal	Reduced	-	-
Baseline Striatal DA Concentrations	Normal	Reduced	-	-
AMPH Induced Striatal DA Concentrations	Normal	Reduced	-	-

Table 1. Summary of Results

Included above are results from biochemical and behavioral assays for wildtype, *Slc1a1*-STOP, *Slc1a1*-STOP Cre (midbrain), and *Slc1a1*-STOP Cre (striatum) mice.

dependent on dopaminergic agents, it would be imprudent to not consider the interdependence of *Slc1a1*/EAAT3 and dopamine in relation to OCD.

Another mouse model was recently reported to have altered response to dopaminergic agents as a potential model of OC-spectrum disorders. The histidine decarboxylase (*Hdc*) null mouse was studied based upon a family with an apparent dominant negative mutation in *HDC* exhibiting complex neuropsychiatric phenotypes including full penetrance for Tourette syndrome (TS) and partial penetrance for OCD. *Hdc* null mice exhibited augmented striatal *cFos*⁺ immunoreactivity, baseline striatal dopamine concentrations, and amphetamine-induced stereotypic behavior (Castellan Baldan et al., 2014). Although these data are in the opposite direction from the amphetamine-induced behavioral deficits observed in *Slc1a1*-STOP mice, they complement our findings because the *Hdc* null mouse was designed to recapitulate the molecular consequences of rare mutations found in TS, while *Slc1a1*-STOP mice were hypothesized to be a model of reduced liability to basal ganglia dependent repetitive behavior.

There are considerable similarities between TS and OCD, including clinical symptomology, genetics, implicated brain structures, and treatment strategies. Despite these shared characteristics, it is unknown to what extent the underlying pathogenic mechanisms of the disorders overlap. Dopamine receptor antagonists are an effective monotherapy for the treatment of TS and have been used as adjunct strategy to SRIs in OCD (Vulink et al., 2005, Denys, 2006). Furthermore, dopamine agonists, including amphetamine and the dopamine precursor L-DOPA, have been reported to elicit tics and induce obsessive-compulsive symptoms (Varley et al., 2001, Parraga et al., 2007, Voon et al., 2009, Madruga-Garrido and Mir, 2013, Shakeri et al., 2016). Of particular interest to our efforts, certain second generation antipsychotic medications, such as clozapine, which acts at dopamine and serotonin receptors, can also induce obsessive-compulsive symptoms, and recent evidence indicates that polymorphisms in *SLC1A1* may moderate susceptibility to this drug-induced compulsivity (Kwon et al., 2009, Ryu et al., 2011, Cai et al., 2013).

Though the data in Chapter 4 are the first to link functional alterations of *Slc1a1*/EAAT3 to OCD-relevant behavior and the dopaminergic system *in vivo*, prior evidence has linked EAAT3 and dopaminergic neurons. EAAT3 has been of particular interest to researchers investigating disorders involving degeneration of dopaminergic neurons, specifically Parkinson's disease. Dopaminergic neurons, which are inherently more susceptible to oxidative stress, are preferentially affected when cysteine transport via EAAT3 is inhibited *in vitro* (Nafia et al., 2008). Further, *in vivo* investigation showed that acute intranigral injection of a pan-EAAT inhibitor selectively caused degeneration of substantia nigra pars compacta dopaminergic neurons and that coinjection of NMDA receptor antagonists was protective for dopaminergic neurons, suggesting that inhibiting mechanisms of glutamate uptake results in increased, toxic NMDA receptor activity in the substantia nigra (Assous et al., 2014). Even more pertinent to our efforts, a report using EAAT3 null mice identified a ~40% loss in dopaminergic neurons in the substantia nigra pars compacta in aged mice (but not in younger mice that parallel our study subjects) that was not observed in a separate group of EAAT3 null mice chronically dosed with the glutathione precursor N-acetylcysteine (Berman et al., 2011), which bypasses EAAT3. When considered in relation to our data, specifically the amphetamine-induced behavioral data, a potential explanation could have been that *Slc1a1*-STOP mice were losing dopaminergic neurons in the midbrain leading to reductions in amphetamine dependent behavior. For this reason, we investigated DAT staining in the midbrain of young adult *Slc1a1*-STOP and WT mice using stereology, which did not identify any significant differences in estimates of the dopaminergic neuron population (Supplementary Figure 9).

A recent publication from the Amara lab identified reductions in cell membrane levels of EAAT3 in dopaminergic neurons of the VTA following acute administration of amphetamine (Underhill et al., 2014). Interestingly, they report augmented glutamatergic neurotransmission at glutamate receptors in dopaminergic neurons of the midbrain following amphetamine-driven endocytosis of EAAT3, primarily due to NR2B-containing NMDA receptors (Li et al., 2017).

While these and prior studies investigating EAAT3's impact on glutamatergic signaling in midbrain dopaminergic neurons show that acute alteration of EAAT3 function affects glutamatergic neurotransmission (Assous et al., 2014, Underhill et al., 2014, Li et al., 2017), it was previously unclear how chronic loss of glutamate uptake via EAAT3 would affect dopaminergic neuron function over the longer term. Based on our data, it is most likely that the deficits observed in amphetamine response in *Slc1a1*-STOP mice are driven by our observation of reduced striatal dopamine levels both at baseline and in response to the psychostimulant.

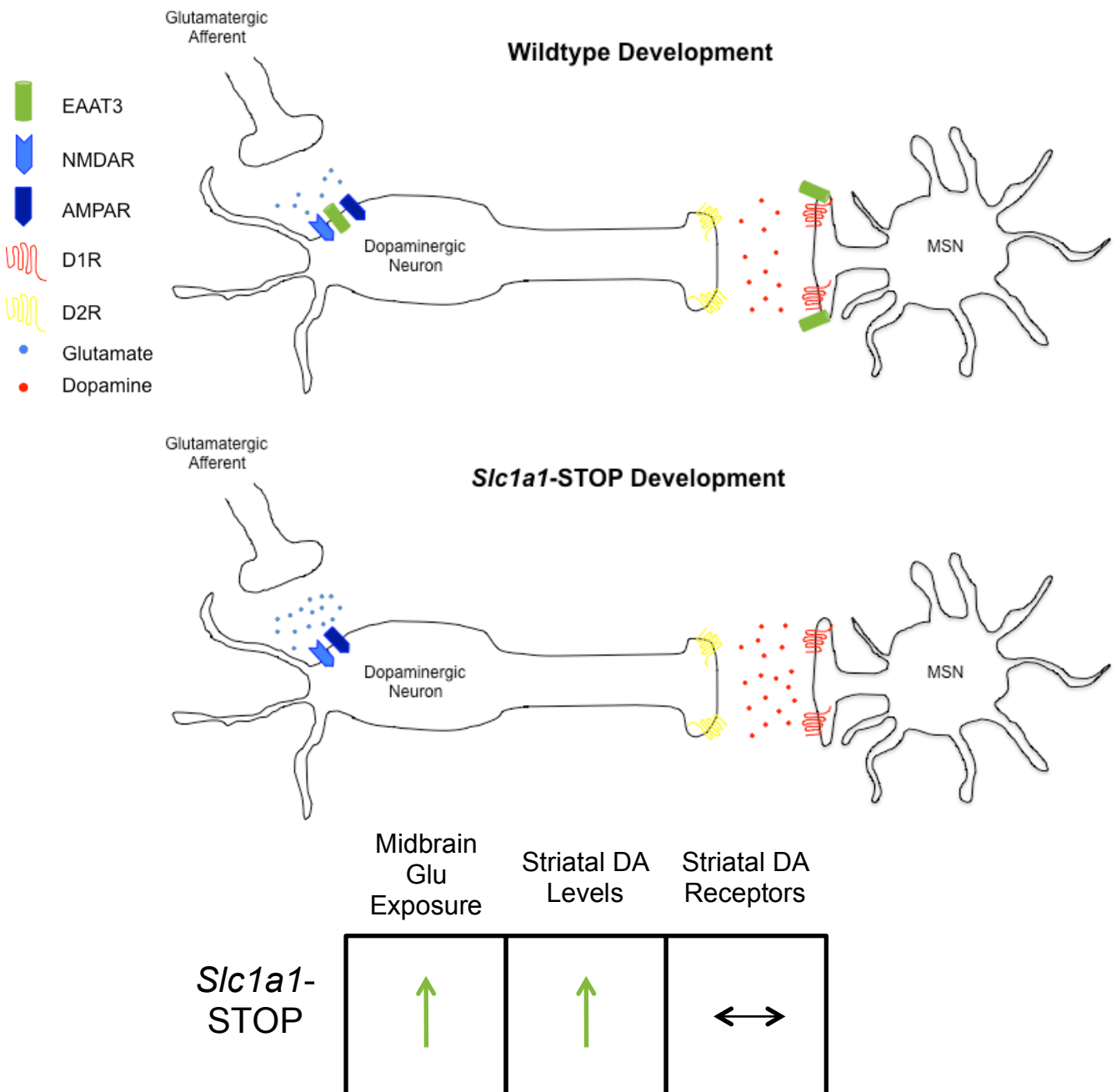
One distinct difficulty in constructing a mechanistic explanation of our behavioral results in *Slc1a1*-STOP mice is that prior published data involved acute inhibition of EAAT3 function in wildtype mice; whereas EAAT3 function in the dopaminergic neurons of *Slc1a1*-STOP mice is chronically absent. Inherently, it is unclear how many of the insights gleaned from these distinct experiments may overlap to help us understand our results. In particular, it is challenging to explain how hypothetical chronic increases in local glutamate receptor exposure in the dopaminergic neurons of the midbrain may result in reduced dopamine concentrations in the striatum, both at baseline and after amphetamine administration.

The findings from the Amara and Assous groups showing the consequences of acute EAAT3 loss in dopaminergic neurons offer two logical hypotheses for extrapolation into *Slc1a1*-STOP mice. The first would be that chronically increased local glutamate concentrations in the midbrain of *Slc1a1*-STOP mice lead to an activity-dependent downregulation of glutamate receptor expression or signaling in the midbrain. Previous data show that increased glutamate signaling and resultant elevations in intracellular Ca^{2+} can lead to NMDA receptors trafficking away from the membrane, presumably as a homeostatic mechanism to prevent excitotoxicity (Hardingham and Bading, 2010, Wild et al., 2014). Within the context of the midbrain of *Slc1a1*-STOP mice, increased signaling at NMDA receptors over time could hypothetically lead to compensatory reductions in the cell membrane population of glutamate receptors. This chronic reduction of cell membrane NMDA receptors available to respond to glutamate may help explain

the dopaminergic deficits in the striatum and the observed attenuated amphetamine-induced behavioral responses observed in *Slc1a1*-STOP mice. A potential issue with this explanation is that Wild and colleagues (2014), used high frequency stimulation of substantia nigra pars compacta neurons to induce this activity-dependent trafficking of NMDA receptors off of the cellular membrane. In contrast, EAAT3 is thought to be largely responsible for the uptake of extra-synaptic and peri-synaptic glutamate, with relatively minor contributions to the removal of glutamate from the synapse (Holmseth et al., 2012). These high frequency stimulation data therefore may not offer an ideal parallel for changes in chronic glutamate concentration in *Slc1a1*-STOP mice. Although preliminary, no alterations in cell membrane levels of glutamate receptors in the midbrain of *Slc1a1*-STOP mice have been identified (data not shown). This explanation also does not account for the observed reductions in dopamine receptor density detected in the striatum of *Slc1a1*-STOP mice.

The second hypothesis that could explain the discrepancy in acute versus chronic effects of EAAT3 loss is that consistently increased ambient glutamate acting upon midbrain DA neurons leads to alterations in development of the dopaminergic system. A chronic increase in glutamate receptor activation may lead to increased extracellular dopamine in early developmental stages that leads to compensatory changes in the dopaminergic system over time. Potential compensatory mechanisms include increased autoreceptor activity, which could drive reductions in basal striatal extracellular dopamine levels (Figure 10e-f). Developmental increase in extracellular dopamine levels could also potentially result in a homeostatic decrease in membrane density of dopamine receptors in the striatum, as we observe in *Slc1a1*-STOP mice (Figure 10d). Due to the relatively small portion of glutamate that EAAT3 is responsible for removing from the glutamatergic synapse relative to GLT-1, synaptic DA levels would hypothetically be moderately affected and alterations in relevant baseline behaviors would not be expected, as is the case for *Slc1a1*-STOP mice. This explanation of increased tonic DA

A



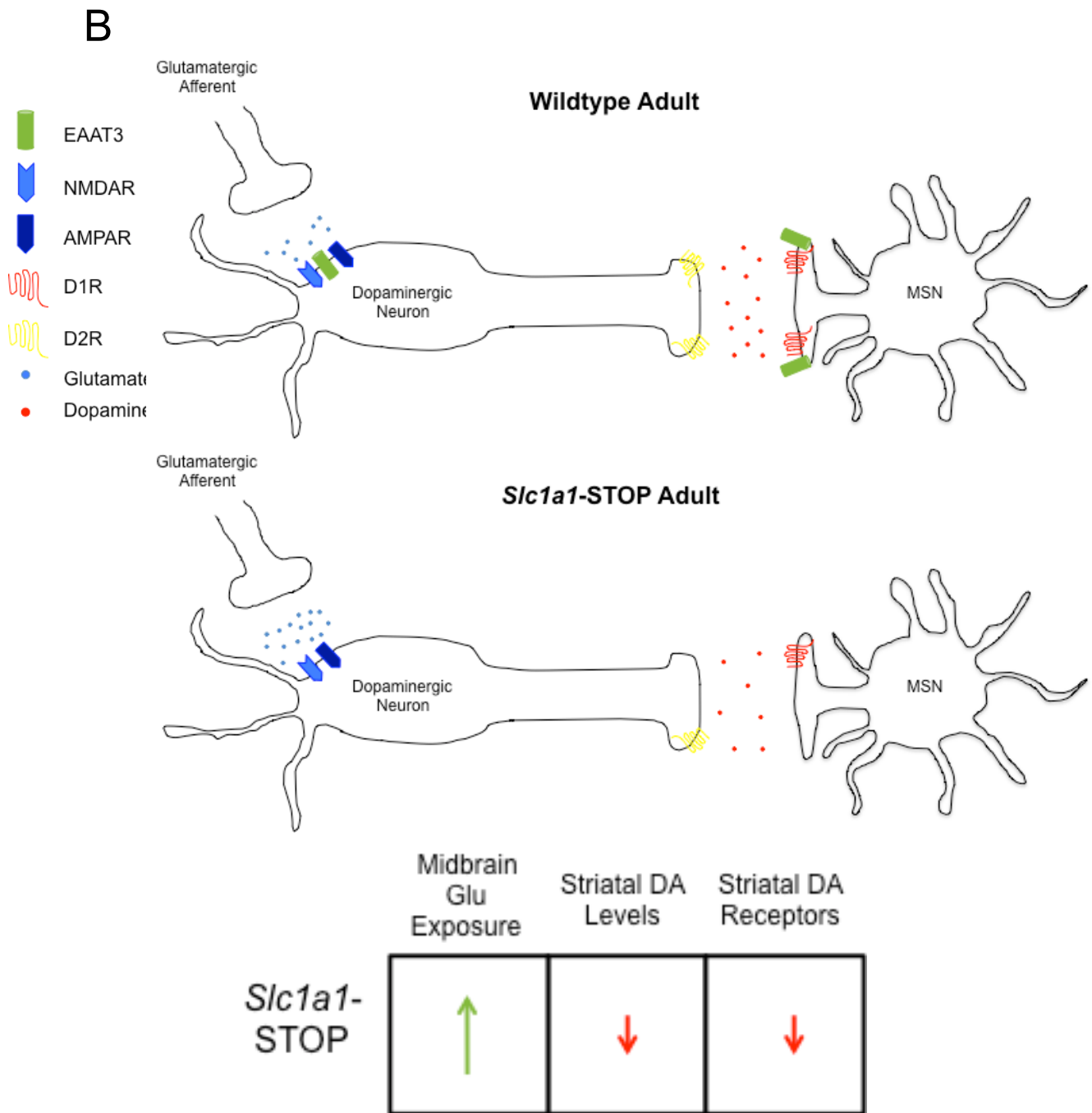


Figure 12. Theoretical alterations in midbrain and striatal signaling throughout development in *Slc1a1*-STOP mice.

(A) Schematic displaying increased glutamate exposure in the midbrain of *Slc1a1*-STOP mice during development due to the lack of EAAT3 expression. Hypothetically, chronic elevations in glutamate exposure of dopaminergic neurons may lead to increased dopamine release in the striatum during development compared to wildtype mice.

(B) Schematic displaying observed reductions in striatal cell membrane levels of dopamine receptors and extracellular dopamine of adult *Slc1a1*-STOP mice. These reductions may represent a compensatory mechanism driven by excessive midbrain glutamate receptor exposure throughout development due to a lack of functional EAAT3 in *Slc1a1*-STOP mice.

release resulting in compensatory changes of the dopaminergic system across development also matches our findings of attenuated amphetamine-induced behavior and striatal *cFos*⁺ immunoreactivity in adult *Slc1a1*-STOP mice relative to WT controls.

In addition to the deficits observed in amphetamine induced repetitive behavior, a blunted behavioral response to SKF-38393 was found in *Slc1a1*-STOP mice. Our finding of reduced membrane density of the D₁ dopamine receptor in the dorsal striatum of these mice relative to controls likely explains this. These data are also consistent with our hypothesis that loss of EAAT3 in dopaminergic neurons results in compensatory changes in the dopaminergic system, which in turn leads to adaptive changes in receptor expression and reduced response to dopamine receptor agonists in the striatum. With regard to our data showing that our *Slc1a1*-STOP:Cre midbrain viral injection does not rescue of SKF-38393 induced grooming, we hypothesize that compensatory changes yielding reduced striatal cell membrane density of dopamine receptors occur at an early developmental stage and that partial rescue of midbrain *Slc1a1*/EAAT3 expression in adult mice therefore does not rescue SKF-38393 induced repetitive grooming phenotypes in *Slc1a1*-STOP:Cre (midbrain) mice. It is also possible that EAAT3 expression is necessary in both the dopaminergic cells of the midbrain and the striatal MSNs to generate wildtype-like behavior in response to the D₁ agonist, as we have shown that rescued expression of *Slc1a1*/EAAT3 in either site alone does not rescue this particular behavior.

At this point, it is unclear how these fascinating and thought-provoking data fit into the glutamate hypothesis of OCD and how they may complement other animal models of repetitive behavior. As we have described, *Slc1a1*-STOP mice do not seem to recapitulate any behaviors commonly associated with animal models of spontaneous OCD-like behavior. Instead, *Slc1a1*-STOP mice may be thought of as an “anti-model” of basal ganglia induced repetitive behavior. We would hypothesize that EAAT3 ablation or blockade could alleviate repetitive behavior in preclinical models relevant to OCD such as *SAPAP3*, *SLITRK5*, or *Hdc* null mice.

Dopamine, Glutamate, and OCD Risk

Our data on altered dopamine system function in mice with reduced expression of a neuronal glutamate transporter add further to accumulating evidence pointing to an interaction between the glutamatergic and dopamine systems, including in relation to OCD risk and OCD treatment (Fonseka et al., 2014, Dold et al., 2015). Despite the evidence for glutamatergic dysfunction of the CSTC circuit in OCD, it is likely that the disorder involves dysfunction in modulatory neurotransmitters as well, based on the degree to which the dopamine and serotonin systems impact this circuit. For example, serotonin reuptake inhibitors, the primary pharmacotherapy for OCD at this point, normalize glutamatergic signal in the striatum and the cingulate cortex in neuroimaging studies (Moore et al., 1998, Rosenberg et al., 2000, Hansen et al., 2002). Further, dopamine receptor antagonists are an effective augmentation strategy in OCD (Li et al., 2005, Bloch et al., 2006, Skapinakis et al., 2007, Veale et al., 2014, Dold et al., 2015). It is likely that alterations in protein function derived from abnormalities in multiple serotonergic, dopaminergic, or glutamatergic risk genes may result in abnormal signaling within the CSTC circuit, thereby leading to compulsive behavior.

Future Directions

Based on our initial findings using a mixed genetic and behavioral pharmacology approach in the *Slc1a1*-STOP mouse, many avenues are available to better understand how loss of *Slc1a1*/EAAT3 function affects signaling in the midbrain and striatum, the two brain regions that we hypothesize are most impacted in the *Slc1a1*-STOP mouse. Future work focused on delineating the glutamatergic and dopaminergic changes that lead to the amphetamine-dependent behavioral phenotypes described in *Slc1a1*-STOP mice are imperative. Currently, the most plausible explanation of how loss of EAAT3 function affects amphetamine-dependent repetitive behavior based on our data is that increased ambient

glutamate in the midbrain during development leads to the observed reductions of striatal dopamine levels in adult mice, both at baseline and after amphetamine, which we believe underlie the observed behavioral phenotypes. Overall reductions in extracellular dopamine levels in the striatum may then explain the attenuation of repetitive amphetamine-induced behaviors identified in *Slc1a1*-STOP mice as well as reductions in *cFos*⁺ immunoreactivity in the striatum following amphetamine challenge.

Based on preliminary data obtained using a genetic strategy to rescue *Slc1a1*/EAAT3 expression in either D1 dopamine receptor or DAT expressing neurons (Appendix 3), the hypothesized developmental mechanism underlying the observed behavioral deficits in *Slc1a1*-STOP mice most likely includes both dopaminergic neurons and other neurons in the CSTC circuit, most likely MSNs. Exclusive expression of *Slc1a1*/EAAT3 in either neuronal population referenced above is unable to rescue amphetamine induced locomotor phenotypes of *Slc1a1*-STOP mice, suggesting that expression of EAAT3 is necessary in both neuronal populations throughout development for wildtype-like behavioral response. Use of a D1-Cre and DAT-Cre double rescue strategy may be a viable alternative to alleviate the observed phenotypes in *Slc1a1*-STOP mice and would provide more insight into how amphetamine induced behavior is affected by EAAT3 function in these neuronal populations during development.

In order to more fully elucidate EAAT3's role in the striatum, our collaborators in the Ahmari lab at the University of Pittsburgh have strategically designed experiments to understand how global loss of *Slc1a1*/EAAT3 impacts striatal neuron function. They have stereotactically injected Ca²⁺ indicators into the dorsal striatum of *Slc1a1*-STOP mice and WT controls in order to visualize changes in neuronal activity with microendoscopy in response to amphetamine and SKF-38393, while concurrently monitoring locomotor and repetitive behavior. The findings of these experiments will reveal any alterations in independent or synchronized firing of *Slc1a1*-STOP MSNs that may explain our observed reductions in dopaminergic agent induced repetitive behavior.

To further isolate where loss of EAAT3 has an impact on glutamatergic signaling abnormalities in *Slc1a1*-STOP mice leading to the observed phenotypes, identification of potential alterations of AMPA and NMDA glutamate receptor activity in the midbrain of *Slc1a1*-STOP mice would be helpful. Electrophysiological experiments aimed at isolating glutamate receptor specific components of the post-synaptic response in dopaminergic neurons of the midbrain in *Slc1a1*-STOP mice and controls revealed through the use of AMPAR and NR2B-selective antagonists would allow us to determine how loss of EAAT3 affects their activation profiles. Such a change could potentially help to explain the decrease in extracellular dopamine in the striatum at baseline. Alternatively, this same experiment could also be performed in the MSNs of the striatum in order to understand whether the absence of EAAT3 has a similar effect at other sites in the CSTC circuitry.

It would also be useful to investigate if there are electrophysiological changes following amphetamine challenge that could explain the behavioral phenotypes observed in *Slc1a1*-STOP mice. This could be tested in two different ways. First, bath application could identify the acute effects of amphetamine on dopamine neuron firing in the absence of EAAT3. Second, administering amphetamine to the animal prior to harvesting neural tissue could identify whether EAAT3 loss alters adaptive changes in dopaminergic neurons, allowing an assessment of Underhill and colleagues' findings that EAAT3 internalization mediates an increase in glutamate sensitivity following amphetamine administration. Alternatively, if more efficacious and selective EAAT3 inhibitors were available, it would also be useful to understand whether acute or chronic pharmacological blockade of the transporter impacts glutamatergic signaling, either in the striatum and midbrain. In particular, it would be interesting to compare the effects of chronic absence of EAAT3 with the effects of acute EAAT3 blockade, which we might expect to differ based upon our failure to restore D₁ agonist response with Cre-mediated viral rescue of EAAT3 expression in the midbrain. Currently, the impact of reduced EAAT3 function in electrophysiological studies has only been assessed via amphetamine-induced endocytosis of

the transporter. Acute application of selective EAAT3 inhibitors would allow for a clearer understanding of how short-term loss of EAAT3 function may impact glutamatergic signaling in the absence of other adaptive changes that might be expected following amphetamine administration.

While no identifiable abnormalities of baseline dopaminergic activity in the midbrain of anesthetized *Slc1a1*-STOP mice were identified via single unit recordings (Chohan, et al, in preparation), further investigation could focus on dopaminergic activity of these mice. For example, application of NMDA or ifenprodil, an NR2B-specific inhibitor, during these recordings may detect inherent differences of dopaminergic neuronal firing that are dependent on NR2B-containing NMDA receptors. Further, systemically administering amphetamine at both high and low doses while assessing activity of dopaminergic neurons would help to determine whether challenge with the psychostimulant impacts neuronal firing, including whether this impact is evenly distributed across midbrain dopaminergic nuclei or is biased toward the substantia nigra or ventral tegmental area.

As noted above, an OCD-linked polymorphism, rs301430C, is associated with increased *SLC1A1* expression in lymphoblastoid cells, human postmortem tissue, and a luciferase reporter assay (Wendland et al., 2009a). These data indicate that overexpression of *SLC1A1*/EAAT3, particularly in males with early-onset OCD (Dickel et al., 2006), may contribute to OCD susceptibility. By taking advantage of the FAST construct in *Slc1a1*-STOP mice, we could drive overexpression of *Slc1a1*/EAAT3 and investigate how augmenting expression affects baseline and drug-induced OCD-relevant behavior. Via cross-breeding strategies, we removed the NeoSTOP cassette in *Slc1a1*-STOP mice, yielding *Slc1a1*-tetO mice that we subsequently crossed with a CaMKIIa-tTA (tet-OFF) line. These *Slc1a1*-tetO:CaMKIIa-tTA mice drastically overexpress EAAT3 in the striatum, confirming that our experimental system results in effective tTA-mediated overexpression of EAAT3. Our priority would be to focus initially on animals with overexpression in dopaminergic neurons using a TH-tTA driver, since our viral

rescue data localize to the midbrain. The *Slc1a1*-tetO:CamKIIA-tTA animals may allow us to pursue a parallel overexpression experiment emphasizing the striatum, based upon previous work from the Kellendonk lab. Both lines of animals could be evaluated for baseline OCD-relevant behavior, such as baseline and spray-induced grooming, marble burying, nestlet shredding, prepulse inhibition, and reversal learning. Based upon the human genetic data and our mouse studies, we would hypothesize that overexpression of *Slc1a1*/EAAT3 will result in increased spontaneous OCD-relevant behavior. It is also possible that no overt behavioral phenotypes emerge in either line of *Slc1a1* overexpressing mice, since overexpression is associated with OCD risk but is unlikely to act in a deterministic way but likely requires the presence of other genetic or environmental risk factors to result in compulsive behavior. In this case, we would evaluate response to dopaminergic agonists, paralleling our work in the *Slc1a1*-STOP mice.

Based on our current hypothesis that constitutive loss of *Slc1a1*/EAAT3 expression results in compensatory changes in the dopaminergic system that underlie the observed behavioral phenotypes in *Slc1a1*-STOP mice, it would also be useful to understand how loss of *Slc1a1*/EAAT3 expression or function exclusively in adulthood impacts OCD-relevant behavior. Using currently available resources, alternative strategies utilizing tetracycline trans-suppressor (tTS)-mediated reduction of *Slc1a1*/EAAT3 expression would also be beneficial, as little work has been done investigating the developmental impact of *Slc1a1*/EAAT3 expression on OCD-relevant behavior. Due to the scarcity of cell type specific tTS lines, examining the impact of *Slc1a1*/EAAT3 loss in exclusive developmental stages could also be achieved by using an inducible knockout mouse line containing a LoxP bound *Slc1a1* crossed with an inducible Cre recombinase. Due to the complexity of neural development and the described impact of EAAT3 function on the dopamine system, it is possible that temporally controlled global or cell-type specific tTS- or Cre-mediated reductions in *Slc1a1*/EAAT3 expression could have a different

effect on the glutamatergic and dopaminergic systems and provide further understanding of the EAAT3's role in OCD-relevant behavior.

Alternatively, if a selective, potent, and bioavailable EAAT3 inhibitor was readily available, temporally controlled chronic loss of EAAT3 function could also be achieved pharmacologically. By using such a pharmacological tool in a chronic dosing strategy, we would be able to investigate similar questions as above; however fewer resources would need to be invested if the compound was already developed and its properties were well understood. Importantly, it is not uncommon for psychiatric pharmacotherapies to reach efficacy only upon chronic administration, as is the case for SRIs in both preclinical models of OCD-like behavior and in treatment of OCD patients. If chronic loss of EAAT3 function via pharmacological inhibition normalized OCD-relevant behavior in induced or spontaneous preclinical models, strong support would be provided for the potential utility of EAAT3 inhibitors in OCD treatment.

Potential for EAAT3 Inhibitors in Psychiatric Disorders

The majority of the OCD patient population is left with significant impairment following the primary forms of pharmacotherapy and behavioral therapy. Alternative rationally designed therapeutics that impact the underlying mechanism of the disorder are needed to better serve the patient population. As discussed previously, an ancillary aim of this project was to preliminarily ascertain whether EAAT3 could be an appropriate target for novel therapeutics in OCD. Our data are the first to evaluate whether ablating this leading OCD candidate gene reverses behavior relevant to the disorder. The two novel EAAT3 inhibitors utilized in Chapter 3, NBI-59159 and 2-CFoDA, were previously uncharacterized *in vivo*, and it was uncertain how effective they would be in rodent models of repetitive behavior and glutamatergic dysfunction. Based on our data indicating a lack of efficacy or significant off-target side effects *in vivo* for these compounds, at least at the doses we were able to test, it is clear that alternative

compounds are needed to investigate the value of EAAT3 inhibition in a preclinical OCD-relevant context.

Currently, the lack of effective preclinical *in vivo* EAAT3 inhibitors for use in induced models of repetitive behavior is understandable. Preliminary data implicating *Slc1a1*/EAAT3 in OCD had been confined predominantly to human genetic analysis, while functional investigation of altered EAAT3 function in animal models has focused on its role in the supply of cysteine to neurons for the production of glutathione and downstream effects on oxidative stress. Outside of our findings describing the striatal dopaminergic deficits and amphetamine-induced behavioral deficits incurred by reduced *Slc1a1*/EAAT3 expression, no other group has adequately investigated how altered EAAT3 function may impact repetitive behavior or the neural circuitry relevant to OCD. Due to this previous lack of significant preclinical data, it is understandable that development efforts are at their earlier stages, however more potent, selective, and bioavailable compounds to inhibit EAAT3 function are being developed.

We hope that our data using a genetic model of EAAT3 loss will help motivate further development of EAAT3 inhibitors. If a compound with a desirable pharmacodynamic and metabolic profile were available, it would be of substantial benefit to the field. First, a potent inhibitor of EAAT3 would be useful in studies aimed at understanding how EAAT3 contributes to glutamatergic signaling via electrophysiology. Current studies attempting to understand this are primarily constrained to using pan-EAAT inhibitors, thus they are unable to delineate the signaling consequences of individual EAAT blockade in brain regions with concurrent expression of multiple EAATs. Previous work has suggested that alteration in EAAT3 function affects AMPA, NMDA, and mGluR5 receptor activity but have not isolated the EAAT3 specific contributions to baseline glutamatergic signaling in normal conditions (Otis et al., 2004, Underhill et al., 2014, Li et al., 2017). Further study using specific EAAT3 inhibitors would help the field understand how changes in transporter function may contribute to alterations in glutamatergic signaling and thereby confer OCD risk. Furthermore, EAAT3 inhibitors could also

be utilized *in vivo* in preclinical animal models of genetic, spontaneous, or drug-induced models OCD-like behavior (See Chapter 2). Despite the majority of these models having limited construct validity, they have been behaviorally characterized and in some cases, have demonstrated abnormalities in CSTC signaling. Examining the ability of EAAT3 blockade to rescue behavior or signaling abnormalities in these animal models would provide strong evidence for clinical EAAT3 inhibitor development aimed at testing alternative treatment strategies for OCD patients.

As is the case for any theoretical experimental strategy, there are important points of concern to address as well. Achieving *in vivo* specificity for EAAT3 versus other EAAT proteins is a significant hurdle. While the structure of EAATs has been extrapolated from an analogous aspartate transporter found in *Pyrococcus horikoshii* (Boudker et al., 2007), the exact structure of mammalian EAATs has yet to be resolved. It is unlikely that there are significant structural differences across the protein family with regard to their orthosteric site, therefore making specificity difficult. It is likely that strategies aimed at allosteric modulation of EAAT3, as has been done in the mGluR protein family (Conn et al., 2009), may be more practical. The drastic expression difference in EAAT3 relative to other EAATs also makes specificity of inhibitors paramount. In addition to an effective pharmacodynamic profile, a potential EAAT3 inhibitor also needs to possess a suitable pharmacokinetic and metabolic profile. If the compound is unable to reach its intended site of action, then it becomes less useful outside of *in vitro* or *ex vivo* experimental strategies. Potential off-target effects would also need to be addressed, specifically with regard to increased oxidative stress due to loss of cysteine transport. As noted above, treatment with N-acetylcysteine could be used to bypass EAAT3 and serve as a substrate for glutathione synthesis, potentially preventing any impact on oxidative stress susceptibility due to an EAAT3 inhibitor. Loss of function mutations of *SLC1A1* lead to dicarboxylic aminoaciduria (Bailey et al., 2011), which would be expected to also arise from a chronic dosing regimen of an EAAT3 inhibitor. Neither glutamate nor aspartate is an

essential amino acid, and individuals with dicarboxylic aminoaciduria did not appear to have other significant medical problems, so this effect of EAAT3 inhibition likely does not interfere with drug development. Of note, if a compound capable of effectively inhibiting EAAT3 function while maintaining a desirable pharmacokinetic profile was developed, it is possible that it could be of use in other psychiatric disorders as well.

Outside of the data supporting glutamatergic dysfunction in OCD, evidence has also implicated glutamatergic dysfunction in schizophrenia, substance abuse, mood disorders, Alzheimer's disease, and autism spectrum disorder (Moghaddam and Javitt, 2012). Previously reported links between polymorphisms in *SLC1A1* and increased susceptibility to second generation antipsychotic-induced obsessive-compulsive symptoms, as well as our reports of dopaminergic alterations in *Slc1a1*-STOP mice, are of particular interest when thinking about additional disorders in which EAAT3 inhibition may be advantageous. It is also of note that association of *GRIN2B* polymorphisms to schizophrenia has been reported (Qin et al., 2005, Martucci et al., 2006, Allen et al., 2008), in addition to data identifying overall NMDA receptor hypofunction in the disorder (Coyle, 2012). *GRIN2B* codes for the NR2B subunit of NMDA receptors, and its activity is impacted by EAAT3 function specifically (Scimemi et al., 2009, Jarzylo and Man, 2012). It is possible that EAAT3 inhibition may have beneficial effects by increasing ambient glutamate concentrations around NR2B-containing NMDA receptors and facilitating NMDA receptor mediated neurotransmission.

Our data linking altered *Slc1a1*/EAAT3 expression and function to the dopaminergic system also suggests that pharmacological blockade of the transporter may be useful for studying disorders connected to dopamine signaling, including Parkinson's disease, schizophrenia, mood disorders, and Tourette syndrome (Goto and Grace, 2007, Taylor et al., 2010, Denys et al., 2013, Castellán Baldan et al., 2014, Gardoni and Di Luca, 2015, Goto et al., 2016). The phenotypic and genetic overlap between OCD and TS has previously been well described (Grados, 2010). Of particular interest, a recent investigation of the casual relationship

between a rare histidine decarboxylase (*Hdc*) mutation and TS-relevant behavior stemmed from the identification of a hypomorphic *Hdc* mutation in a family that included nine members with Tourette syndrome, four of whom had comorbid OCD symptoms (Castellan Baldan et al., 2014). The authors identified abnormalities in dopaminergic modulation of the basal ganglia, including elevations in basal striatal dopamine levels, amphetamine-induced striatal *cFos*⁺ immunoreactivity and repetitive stereotypy in mice expressing a nonsense *Hdc* mutation. These findings seem to be the mirror image of those identified in *Slc1a1*-STOP mice, further supporting the potential utility of EAAT3 inhibitors in preclinical models of psychiatric disorders characterized by aberrant dopaminergic function. Use of novel EAAT3 inhibitors in animal models of schizophrenia, Tourette syndrome or any alternative psychiatric condition purportedly exhibiting glutamatergic and/or dopaminergic dysfunction is an exciting opportunity that may improve our understanding of the mechanisms underlying these debilitating disorders, leading to improved therapeutic options. Of note, a genetic approach could also be taken by crossing *Slc1a1*-STOP mice with *Hdc* null mice or other models of dopamine-dependent behavior.

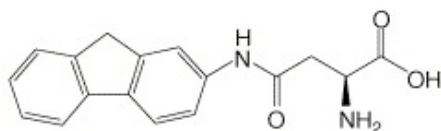
In conclusion, the data described and discussed herein are the first attempts to understand how alterations in EAAT3 function affect repetitive behavior and the neural circuitry relevant to OCD. Up to this point, the field of OCD research has largely been confined to human genetic and neuroimaging strategies that have yet to be strategically integrated to provide mechanistic understanding of pathophysiology. Despite substantial evidence separately implicating *SLC1A1*, glutamatergic and dopaminergic signaling, and hyperactivity of the basal ganglia in OCD, there has been a distinct lack of functional investigation linking all three of these pieces of human data using *in vivo* models. Our data in the *Slc1a1*-STOP mouse allows us to connect *Slc1a1*/EAAT3 to basal ganglia-dependent repetitive behavior and the dopaminergic system. These findings now frame further investigation of *SLC1A1*/EAAT3's role in the pathophysiology of OCD. Specifically, we hypothesize that EAAT3 function impacts striatal-dependent repetitive behavior by modulating neurotransmission at extrasynaptic

receptors in dopaminergic neurons, thereby affecting both baseline striatal dopamine levels and response to dopamine agonists. Continued investigation of *Slc1a1*-STOP-tetO mice offers a unique opportunity to understand the role of EAAT3 function in OCD-implicated neural circuits, potentially motivating the development of a new, rationally-designed treatment for the OCD patient population.

SUPPLEMENTAL FIGURES

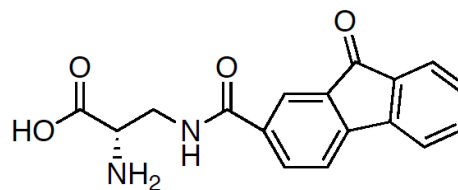
A

NBI-59159



B

2-CFoDA

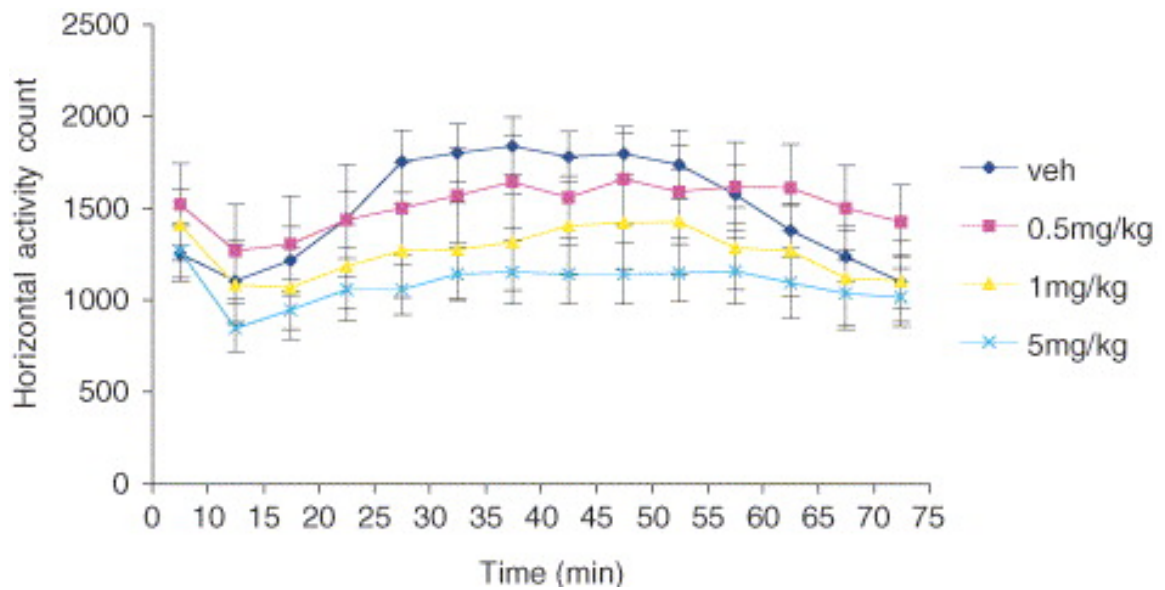


Supplementary Figure 1. Chemical Structures of EAAT3 Inhibitors

(A) Chemical structure of the EAAT3 inhibitor, NBI-59159, used in SAPAP3 null mouse repetitive grooming experiments.

(B) Chemical structure of the EAAT3 inhibitor, 2-CFoDA, used in ICV and direct striatal injection amphetamine challenge experiments.

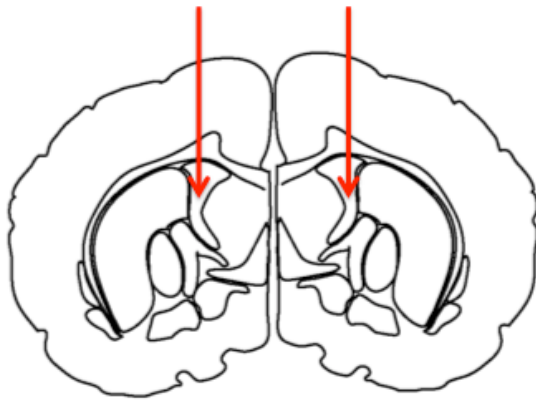
A



Supplementary Figure 2. NBI-59159 attenuates amphetamine-induced locomotor behavior

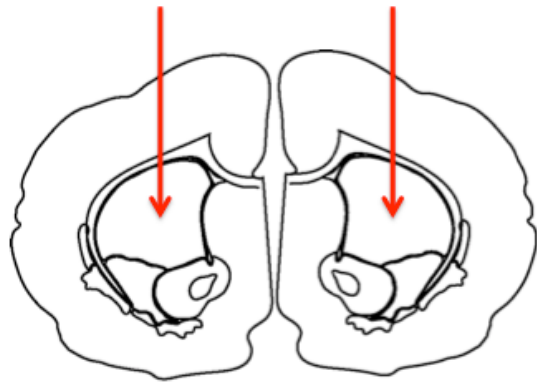
A) NBI-59159 was administered to mice 15 minutes prior to amphetamine (3 mg/kg, subcutaneous) challenge. Overall time versus treatment effect was observed ($P=0.018$). Post-hoc analysis revealed a significant effect for the 5 mg/kg dose at 25-50 minutes. Figure was adapted from *Glutamate Transport Inhibitors as Targets for Treating Psychosis* (Dunlop, 2006).

A



Intracerebroventricular Injection Site

B

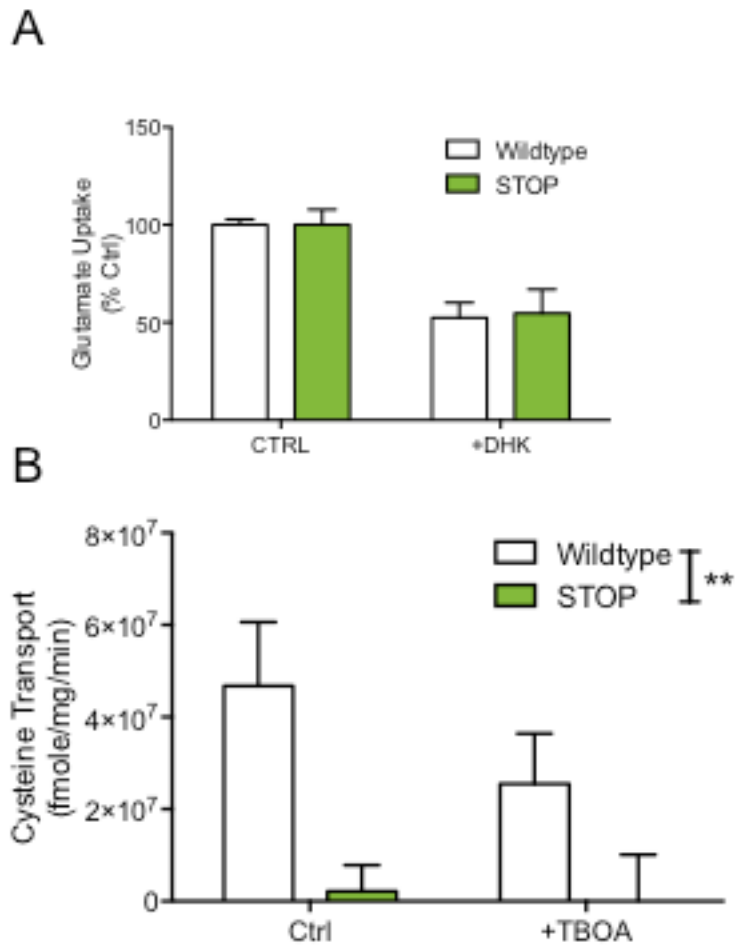


Direct Striatal Injection Site

Supplementary Figure 3. Injection sites for examining the behavioral consequences of EAAT3 Inhibitors in amphetamine-induced locomotor behavioral assays.

A) Site of intracerebroventricular (ICV) injection cannula (AP 0.0 mm, ML \pm 0.75 mm, DV -2.0 mm, all relative to bregma).

B) Site of direct striatal injection site (AP +0.5 mm, ML \pm 1.5 mm, DV -3.5 mm, all relative to bregma).

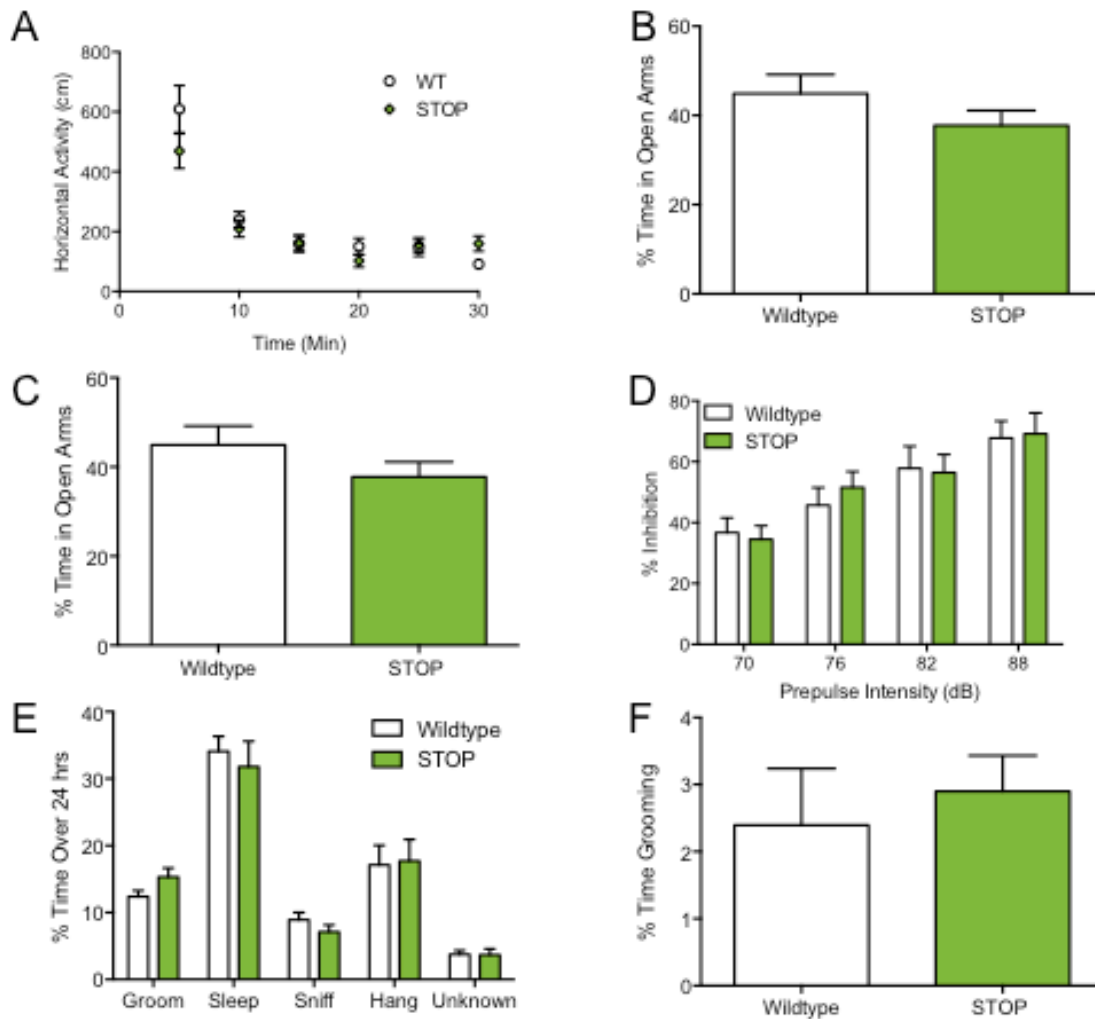


Supplementary Figure 4.

No differences in glutamate uptake between wildtype and *Slc1a1*-STOP mice; high concentration cysteine reveals abolished transporter function in *Slc1a1*-STOP mice.

(A) Na⁺-dependent uptake of L-glutamate (100 μ M) is not altered in synaptosome preparations from *Slc1a1*-STOP mice. EAAT inhibitor: dihydrokainic acid (DHK) (100 μ M) (two-way ANOVA; genotype $F(1,8)=0.02$, $P=0.9$, $n=3$; Figure is representative of three separate experiments).

(B) Na⁺-dependent uptake of L-cysteine (200 μ M) is absent in striatal synaptosome preparations from *Slc1a1*-STOP (ST) mice (two-way ANOVA; genotype $F(1,20)=10.89$, $P=0.0036$, $n=6$ per genotype). Figure is representative of three separate experiments.



Supplementary Figure 5.

***Slc1a1*-STOP mice show no changes in spontaneous behavior.**

Slc1a1-STOP (STOP) mice display no behavioral abnormalities in assays relevant to anxiety or OCD-like behavior.

(A) Spontaneous locomotor behavior (two-way ANOVA; genotype $F(1,53)=0.4378$; $P=0.44$. $n=14$ per genotype).

(B) Elevated zero maze (unpaired t-test; $t=1.3$, $P=0.20$. $n=14$ per genotype).

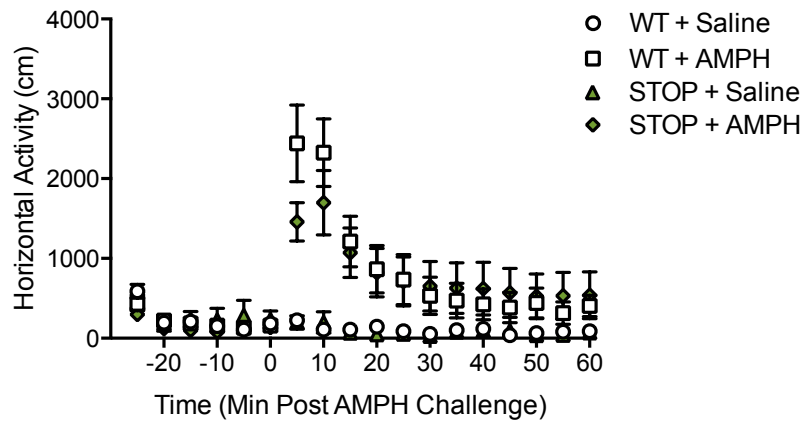
(C) Light-dark emergence (unpaired t-test; $t=0.5$, $P=0.63$. $n=14$ per genotype).

(D) Prepulse Inhibition (two-way ANOVA; genotype $F(1,91)=0.05$; $P=0.83$, $n=14$ per genotype).

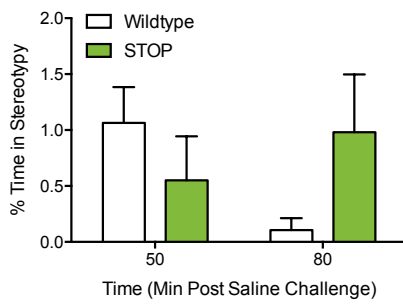
(E) Home Cage Scan (unpaired t-test; grooming $P=0.08$, $p>0.05$ for all other behaviors analyzed separately, $n=14$ per genotype).

(F) Spontaneous Grooming (unpaired t-test; $t=0.5$, $p=0.62$, $n=14$ per genotype).

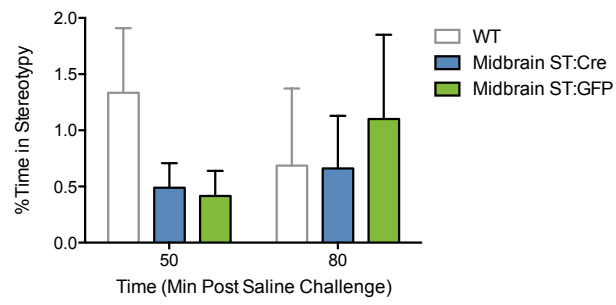
A



B



C



Supplementary Figure 6.

High-dose amphetamine locomotor response is not altered in STOP mice relative to WT, and stereotypic behavior is not observed following saline administration

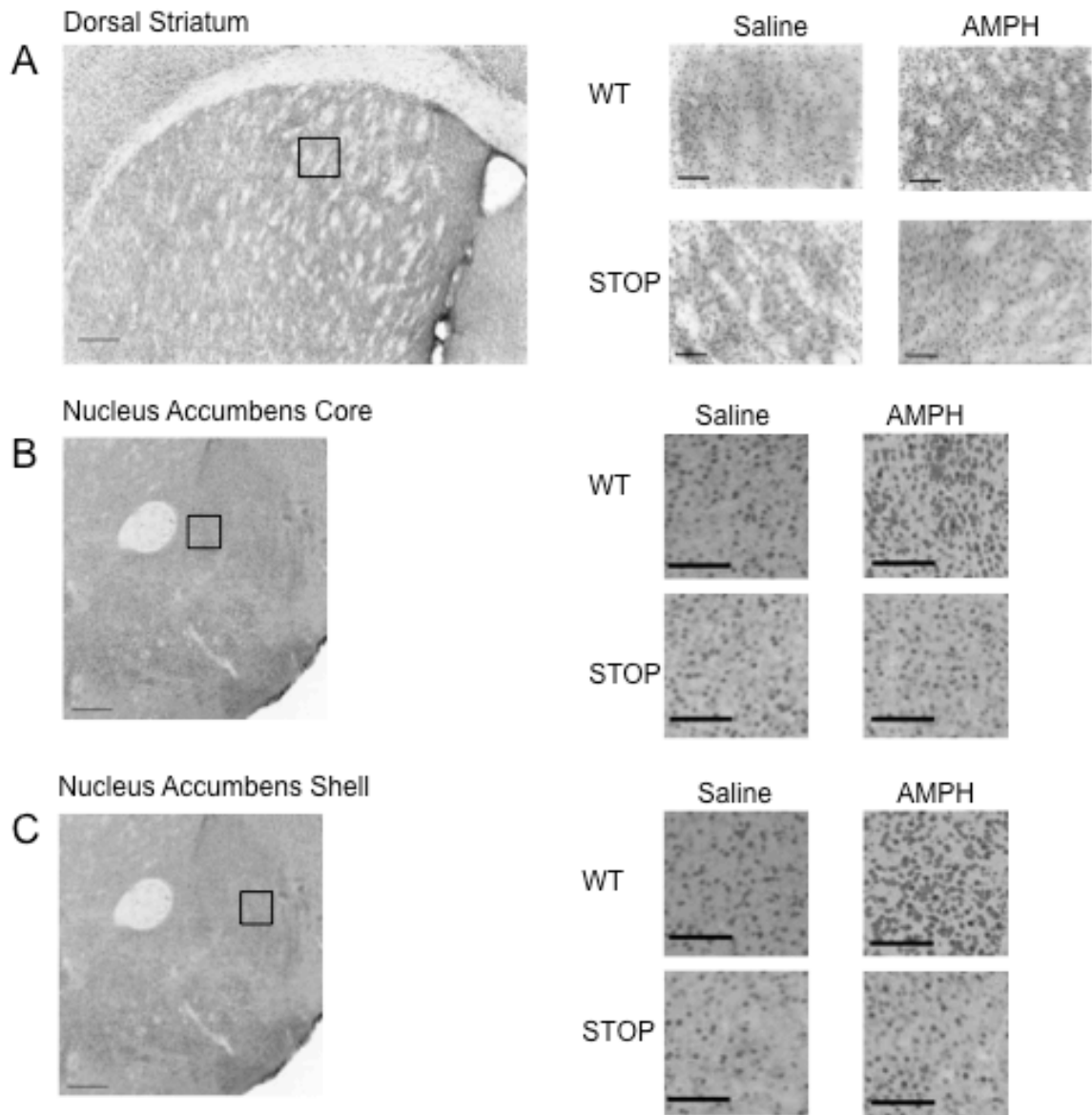
Following acute D-amphetamine challenge (8.0 mg/kg), WT and STOP mice initially underwent locomotor activation and then transitioned to stationary stereotypy.

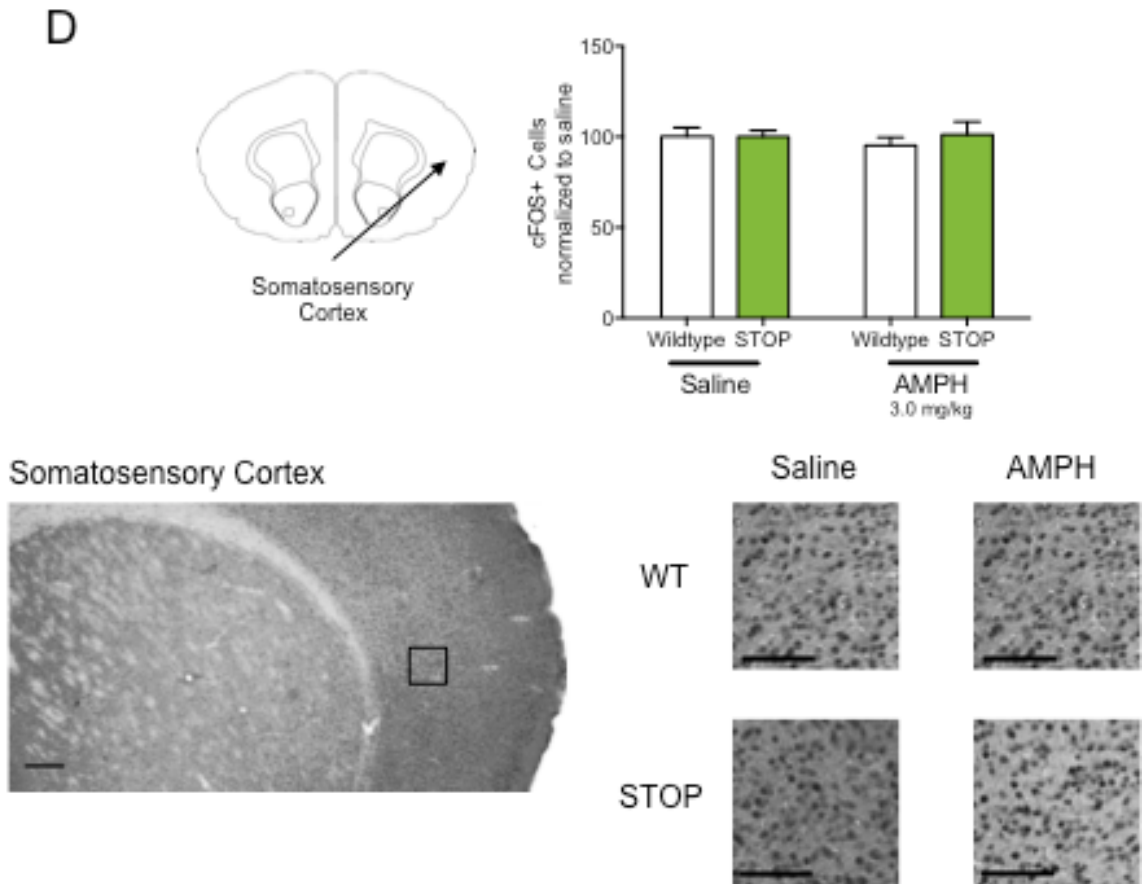
(A) 8.0 mg/kg D-amphetamine (IP) (curve-Fit Analysis; $F(4,496)=1.17$, $P=0.32$, $n=14$ per genotype).

Saline treated mice do not undergo phenotypic shuffling or sniffing stereotypy behavior. All mice in panels B and C are vehicle treated (i.e. receive only saline).

(B) Saline induced stereotypy (Two way ANOVA: time x genotype $F(1,44)=0.75$, $P=0.39$; time $F(1,44)=0.41$, $P=0.52$, genotype $F(1,44)=0.42$, $P=0.42$; $n=12$ per genotype)

(C) Saline induced stereotypy (Two way ANOVA; time x virus $F(1,50)=0.32$, $P=0.58$; time $F(1,50)=0.89$, $P=0.35$; virus $F(1,50)=0.69$, $n=12$ ST:GFP, 15 ST:Cre).





Supplementary Figure 7.

Representative coronal sections of dorsal striatum, nucleus accumbens, and somatosensory cortex for *cFos*⁺ Quantification post-amphetamine challenge

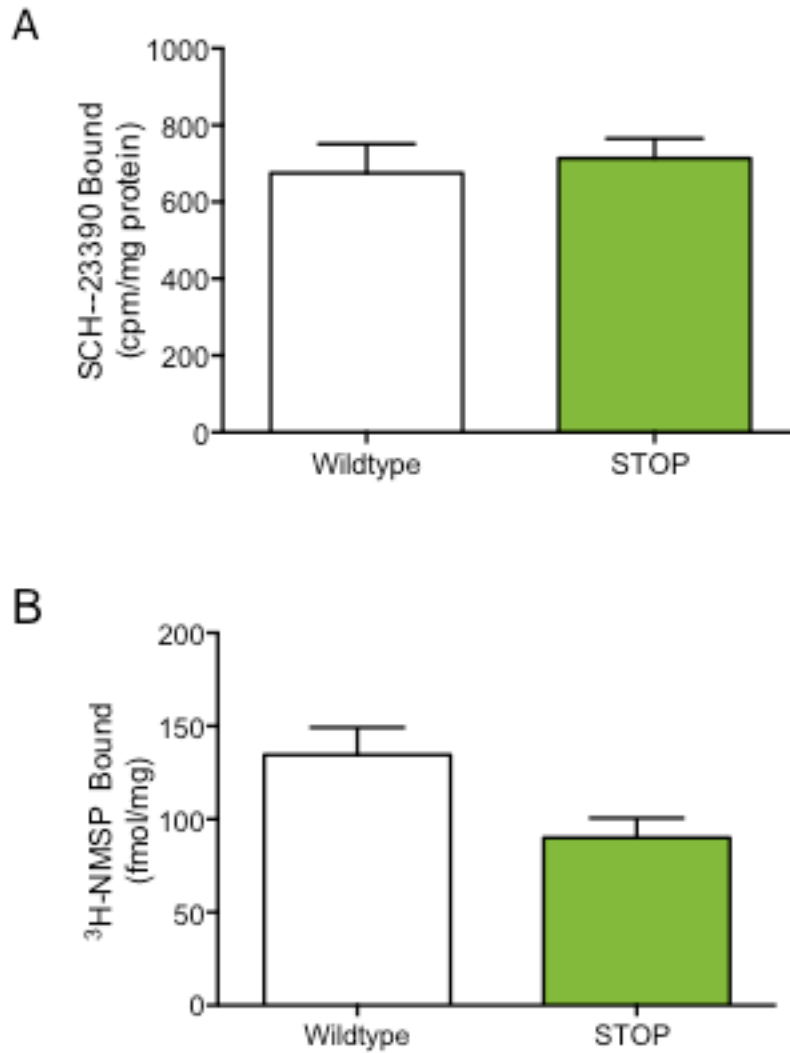
Low and high magnification images are in the left and right columns, respectively. Wildtype (top) and *Slc1a1*-STOP (bottom) mice were challenged with saline (left) and amphetamine (right), and then subjected to *cFos*⁺ immunohistochemistry. Region of quantification is indicated with black box in low magnification images. Scale bars are 250 μ m and 15 μ m on low and high magnification images respectively.

(A) Dorsal striatum (A/P +0.9 mm, M/L \pm 1.5 mm, D/V -3 mm)

(B) Nucleus Accumbens Core (A/P +1.2 mm, M/L \pm 0.75 mm, D/V -4.5 mm)

(C) Nucleus Accumbens Shell (A/P +1.2 mm, M/L \pm 0.5 mm, D/V -4.5 mm)

(D) Analysis of *cFos*⁺ staining in the somatosensory cortex (A/P +0.9 mm, M/L \pm 3.0 mm, D/V -3.0 mm) was performed as a negative control for both amphetamine and genotype. Analysis reveals no main effects on *cFos*⁺ cells in the somatosensory cortex of WT and STOP mice. (Two-way ANOVA; drug x genotype $F(1,12)=0.36$, $P=0.56$; drug $F(1,12)=0.13$, $P=0.72$; genotype $F(1,12)=0.36$, $P=0.56$; $n=4$ per genotype)



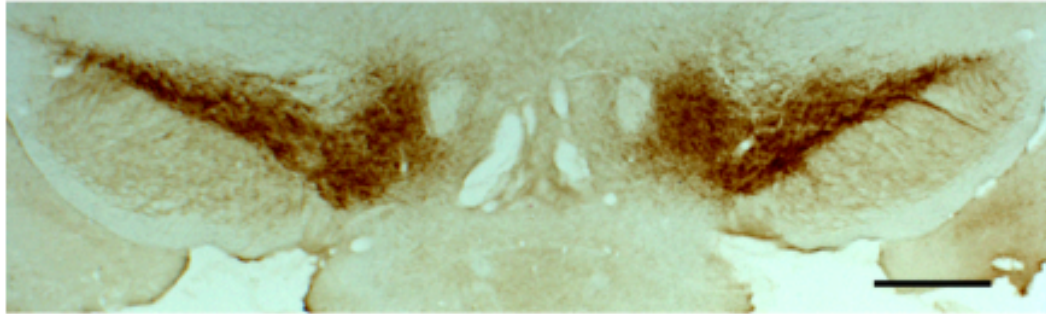
Supplementary Figure 8.

D1-receptor density is not affected in the ventral striatum of *Slc1a1*-STOP mice, while D2-receptor density is reduced at a trend level in the dorsal striatum

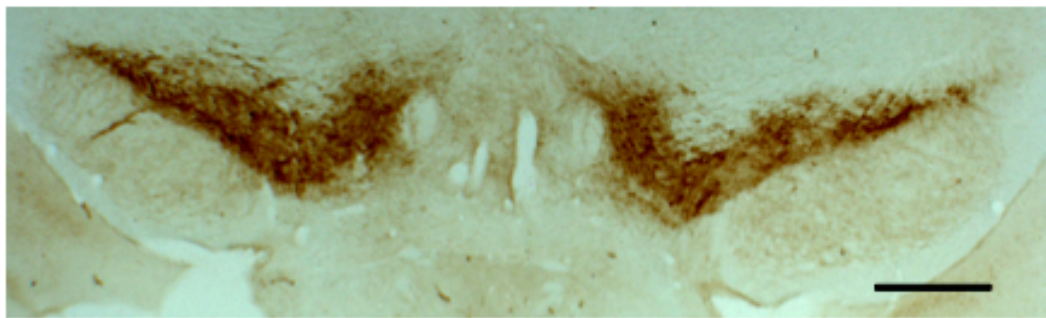
(A) Dopamine D₁ receptor density estimated with [³H]-SCH-23390 binding in ventral striatum membrane preparations (unpaired T-test; $t=0.39$, $P=0.7$, $n=9$ WT, $n=7$ STOP).

(B) Dopamine D₂ receptor density estimated with [³H]-methylspiperone (NMSP) binding in dorsal striatum membrane preparations (unpaired t-test; $t=2.1$, $^{\wedge}P=0.06$, $n=9$ wildtype, $n=6$ STOP).

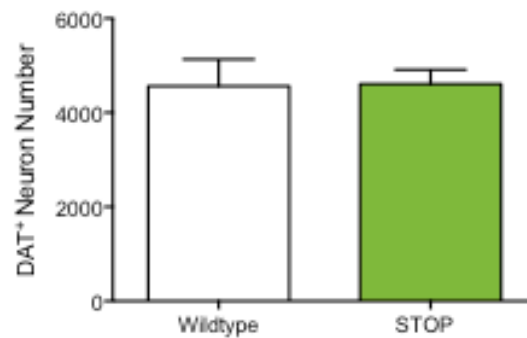
A WT DAT



B STOP DAT



C

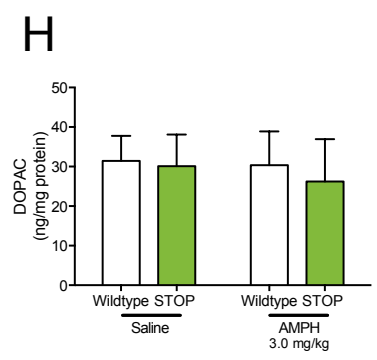
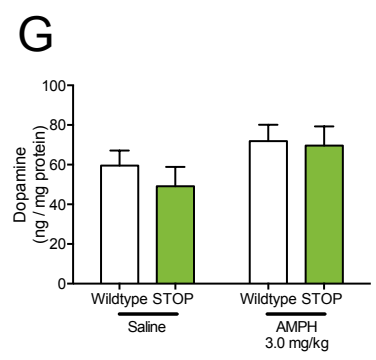
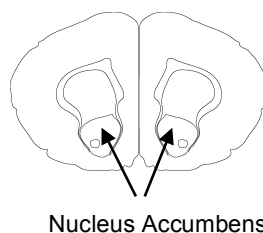
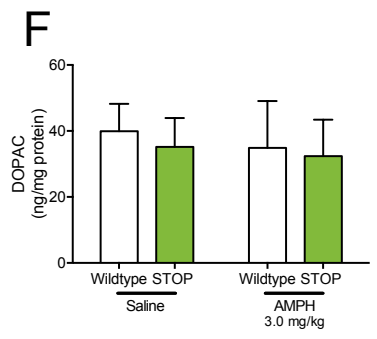
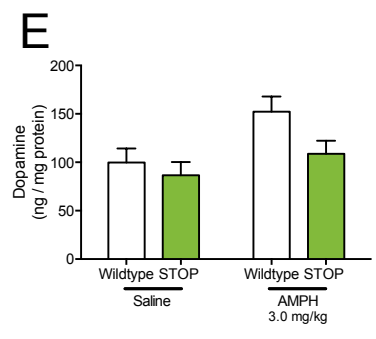
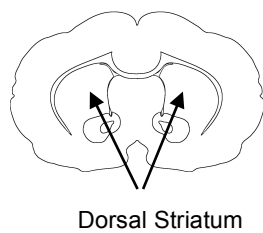
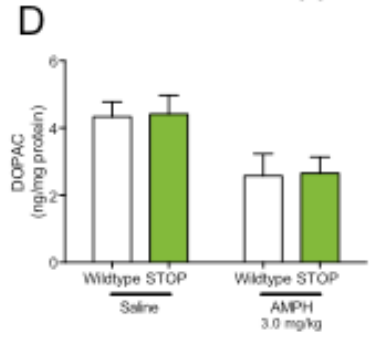
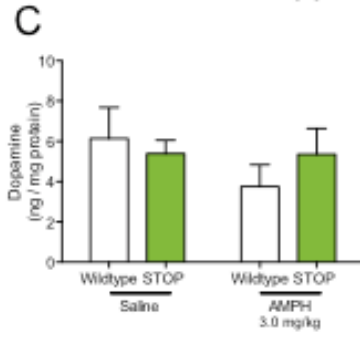
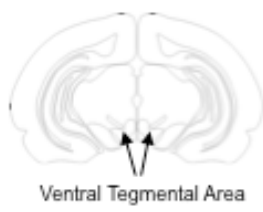
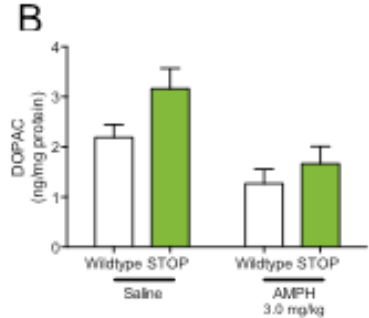
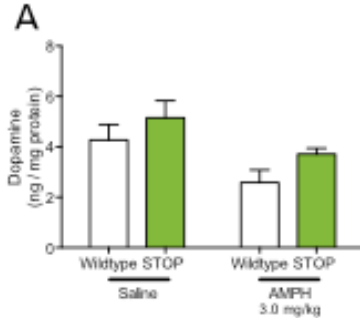
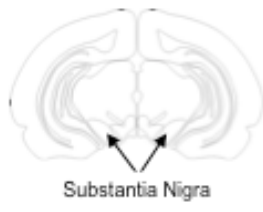


Supplementary Figure 9.

No differences were identified in DAT staining or population estimates in the midbrain of STOP mice

(A-B) Representative midbrain coronal sections of DAT staining in WT (A) and *Slc1a1*-STOP (B) mice. AP -3.1 mm. Scale bar: 500 μ M.

(C) Stereological analysis of DAT⁺ revealed no difference in the midbrain of WT and *Slc1a1*-STOP mice using the optical fractionator method. Coronal midbrain sections used for analysis of DAT staining were from -2.9 – -3.4 mm relative to bregma, (N=4 WT and *Slc1a1*-STOP).



Supplementary Figure 10.

Effects of amphetamine on tissue levels of dopamine and DOPAC in *Slc1a1*-STOP mice

(A) A main effect of amphetamine is observed in a two-way ANOVA of amphetamine's effect on DA levels in the substantia nigra (SN) of WT and *Slc1a1*-STOP mice. (two-way ANOVA; drug x genotype $F(1,23)=0.05$, $P=0.83$; drug $F(1,23)=7.95$, $P<0.01$; genotype $F(1,23)=3.29$, $P=0.08$. $n=7$ per genotype per treatment).

(B) A main effect of amphetamine and genotype is observed in a two-way ANOVA of amphetamine's effect on DOPAC levels in the substantia nigra (SN) of WT and *Slc1a1*-STOP mice (two-way ANOVA; drug x genotype, $F(1,23)=0.78$, $P=0.39$; drug $F(1,23)=13.54$, $P<0.01$, genotype $F(1,23)=4.35$, $P<0.05$. $n=7$ per genotype per treatment).

(C) Neither amphetamine nor genotype had a significant effect on tissue levels of dopamine in the ventral tegmental area (VTA) (two-way ANOVA; drug x genotype $F(1,23)=0.99$, $P=0.33$; genotype $F(1,23)=0.1294$, $P=0.72$, $n=7$ per genotype per treatment)

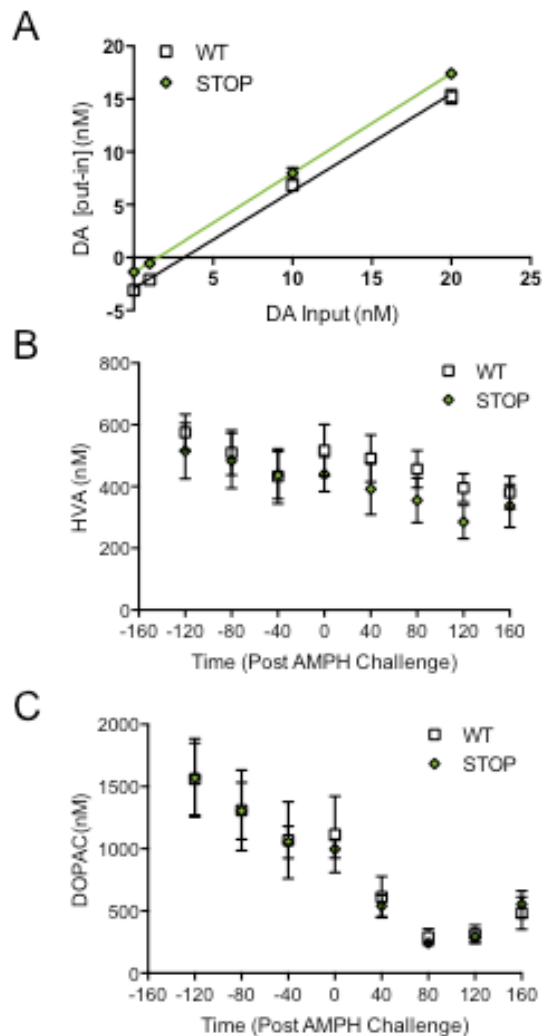
(D) A main effect of amphetamine is observed in a two-way ANOVA of amphetamine's effect on DOPAC levels in the VTA of WT and *Slc1a1*-STOP mice. (two-way ANOVA; drug x genotype $F(1,23)<0.0001$, $P=0.99$; drug $F(1,23)=10.3$, $P<0.01$, $n=7$ per genotype per treatment)

(E) A main effect of amphetamine is observed in a two-way ANOVA of amphetamine's effect on DA levels in the dorsal striatum (DS) of WT and *Slc1a1*-STOP mice. (two-way ANOVA; drug x genotype $F(1,24)=1.13$, $P=0.30$; drug $F(1,24)=6.8$, $P=0.01$; genotype $F(1,24)=3.92$, $P=0.06$; $n=7$ per genotype per treatment).

(F) Neither amphetamine nor genotype had a significant effect on DS DOPAC levels (two-way ANOVA; drug x genotype $F(1,24)=0.01$, $P=0.92$; genotype $F(1,24)=0.11$, $P=0.74$, $n=7$ per genotype per treatment).

(G) Neither amphetamine nor genotype had a significant effect on nucleus accumbens (NAcc) DA tissue levels (two-way ANOVA; drug x genotype $F(1,24)=0.2$, $P=0.65$; drug $F(1,24)=3.43$, $P=0.08$; genotype $F(1,23)=0.51$, $P=0.48$, $n=7$ per genotype per treatment).

(H) Neither amphetamine nor genotype had a significant effect on NAcc DOPAC tissue levels (two-way ANOVA; drug x genotype $F(1,24)=0.03$, $P=0.87$; genotype $F(1,24)=0.11$, $P=0.75$, $n=7$ per genotype per treatment).

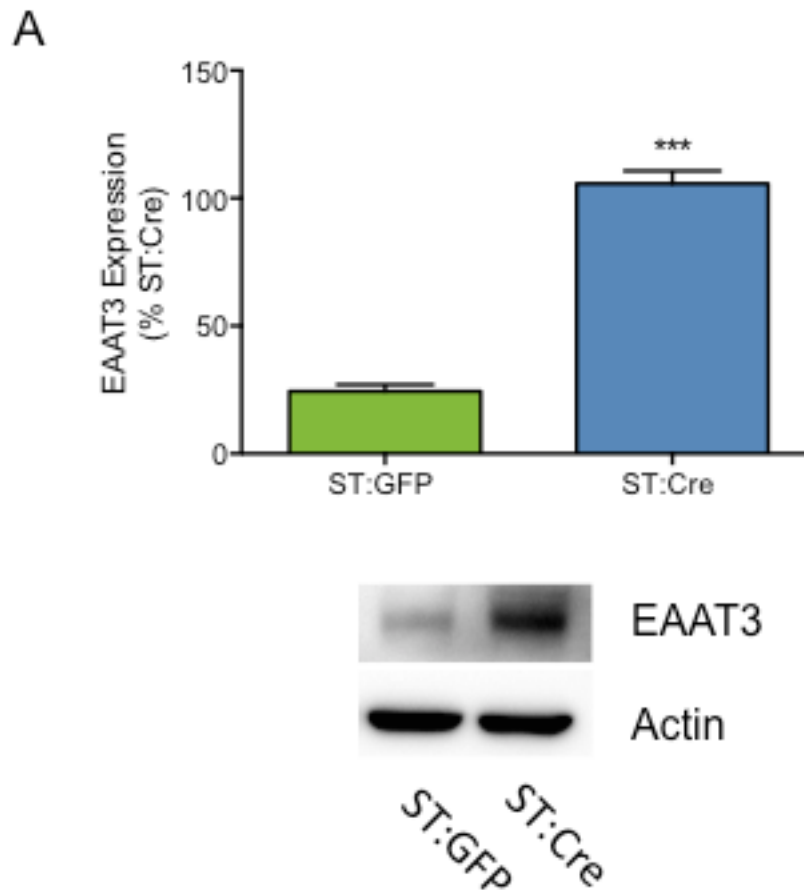


Supplementary Figure 11.

***Slc1a1*-STOP mice have reduced baseline dopamine as measured via no-net flux microdialysis, and the main dopamine metabolites are not affected by amphetamine challenge.**

(A) *Slc1a1*-STOP mice have significantly lowered dopamine levels at baseline as measured by the extrapolation of linear regression using no-net flux microdialysis. (WT slope=0.92±0.03, x-intercept=3.17; STOP slope=0.94±0.19, x-intercept=1.52; Slope comparison F(1,44)=0.44, *P*=0.51; n=6 per genotype)

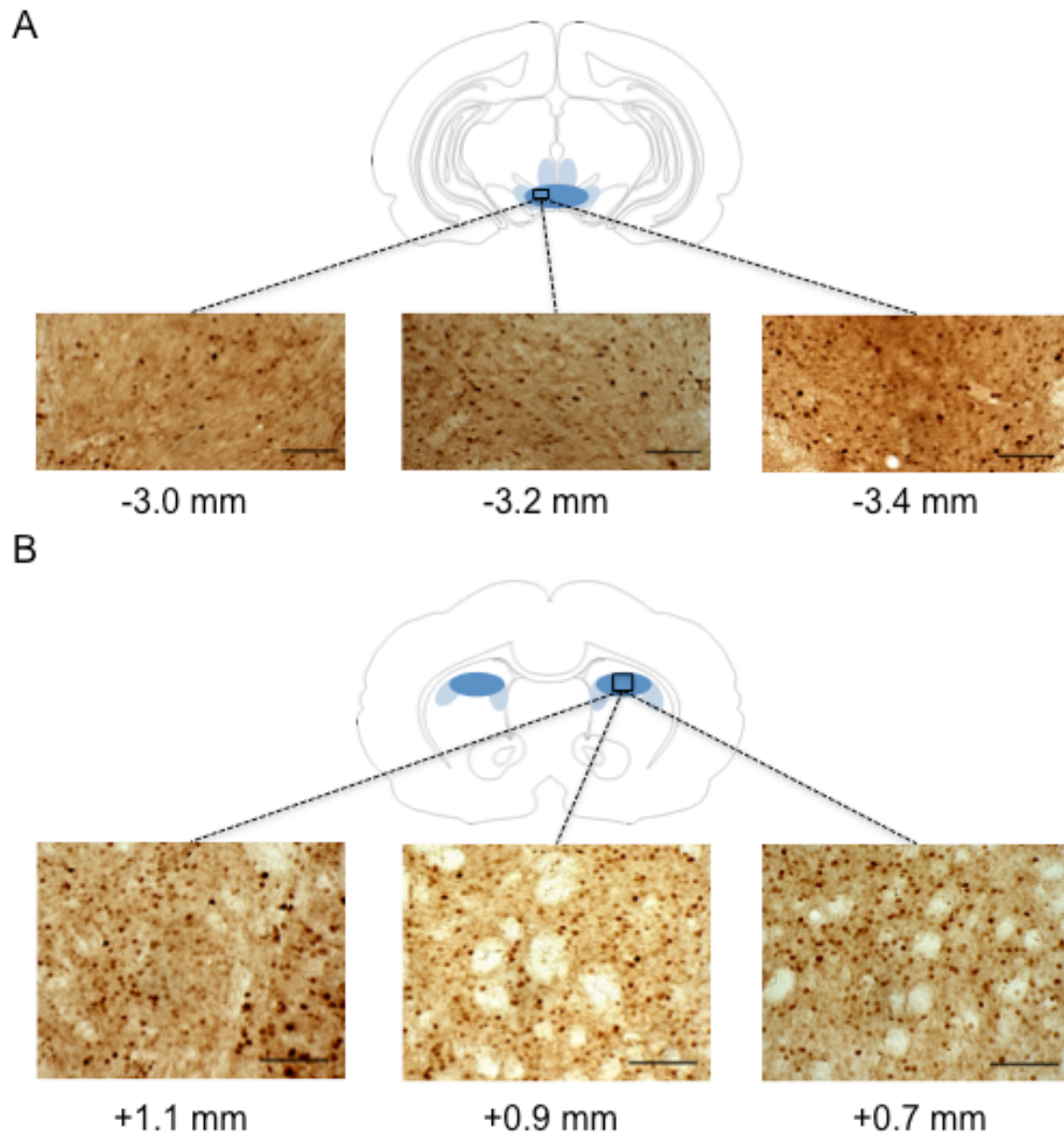
(B-C) Dorsal striatal levels of the dopamine metabolites HVA (B) and DOPAC (C) are not different in *Slc1a1*-STOP mice relative to wildtype controls following systemic administration of amphetamine (3 mg/kg, IP) (B) Repeated measures two-way ANOVA; time x genotype, F(7,70)=0.96, *P*=0.47; (C) Repeated measures two-way ANOVA; time x genotype F(7,70)=0.12; n=6 per genotype).



Supplementary Figure 12.

Injection of an AAV-Cre into the midbrain of *Slc1a1*-STOP mice results in increased EAAT3 expression

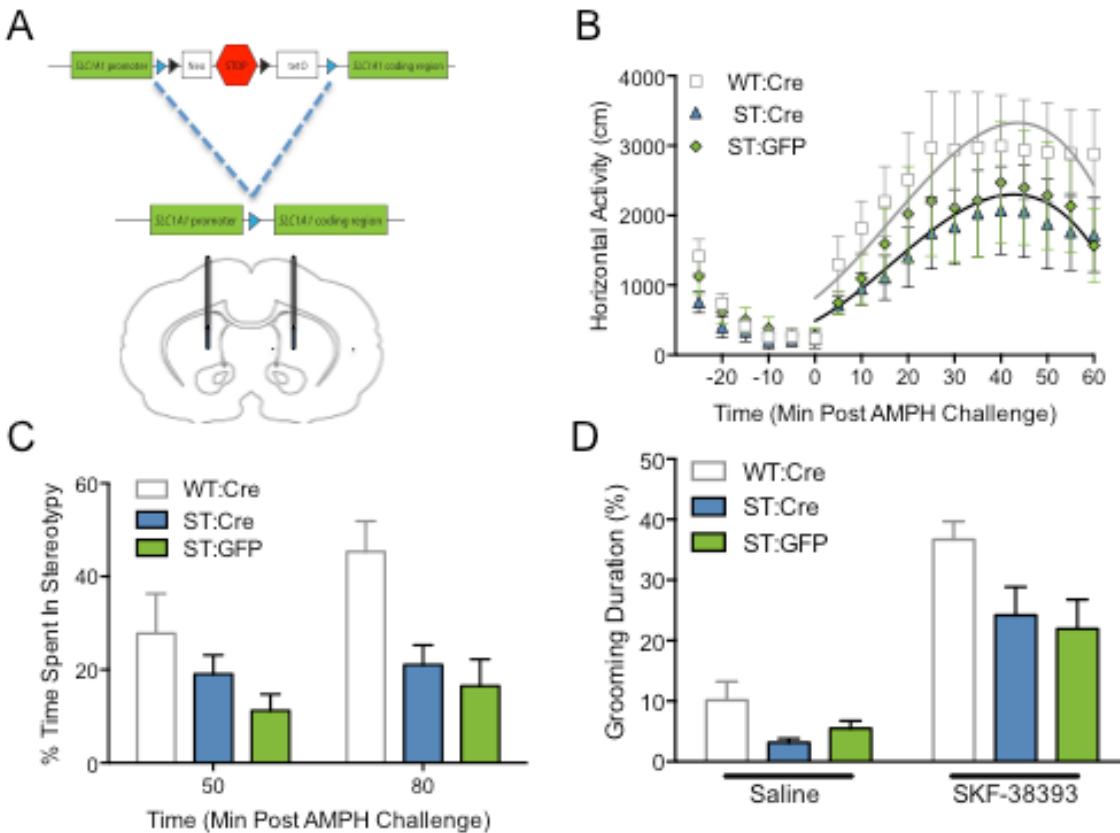
(A) EAAT3 protein expression is increased in midbrain synaptosome preparations from *Slc1a1*-STOP mice injected with Cre virus (ST:Cre, Lane 2), relative to *Slc1a1*-STOP mice injected with GFP (ST:GFP, Lane 1). (Unpaired t-test; $t=14.6$, $***P=0.0001$, $n=3$ per virus. Figure is representative of three separate experiments). Average protein expression \pm SEM is demonstrated in bar graph.



Supplementary Figure 13.

Cre expression is detected in the midbrain and striatum of *Slc1a1*-STOP mice injected with an AAV-Cre.

(A-B) Cre expression is detected in the midbrain and striatum of *Slc1a1*-STOP mice injected with an AAV-Cre. Representative coronal sections are shown for both midbrain (-3 - -3.4 mm AP relative to bregma) and striatum (1.1 – 0.7 mm AP relative to bregma). High magnification images were taken at 20X, scale bar is 100 μ M.



Supplementary Figure 14.

Viral Cre-mediated rescue of dorsal striatum *Slc1a1*/EAAT3 expression does not rescue any of the dopaminergic agent behavioral phenotypes observed in *Slc1a1*-STOP mice

(A) Cre-mediated excision of the Flexible Accelerated STOP-tetO knock-in in *Slc1a1*-STOP mice, leading to endogenous *Slc1a1*/EAAT3 expression. Blue triangles represent *LoxP* sites. Reference section indicates injection position in the dorsal striatum of *Slc1a1*-STOP and wildtype controls (A/P +0.9 mm, M/L \pm 1.5 mm, D/V -3 mm).

(B) *Slc1a1*-STOP mice injected with AAV-Cre (ST:Cre) in the dorsal striatum exhibit a similar hyperlocomotor response to 3.0 mg/kg amphetamine in comparison to *Slc1a1*-STOP littermate controls injected with AAV-GFP (ST:GFP) (Curve fit analysis T=0-60; $F(4,370)=0.83$, $P=0.50$, $n=9$ ST:GFP, $n=12$ ST:Cre).

(C) Stereotyped behavior in ST:Cre mice in response to 8.0 mg/kg amphetamine is not different in comparison to ST:GFP littermate controls (Two-way ANOVA; Time x Virus, $F(1,32)=0.14$, $P=0.71$; Time, $F(1,32)=0.68$, $P=0.42$; Virus, $F(1,32)=1.95$, $P=0.17$; $n=9$ ST:GFP, $n=10$ ST:Cre).

(D) ST:Cre mice showed no difference in stereotyped grooming behavior in response to 10 mg/kg SKF-38393 in comparison to ST:GFP littermate controls (Two-way ANOVA; Drug x Virus $F(1,32)=0.45$, $P=0.51$; Drug, $F(1,32)=29.74$, $P<0.0001$; Virus, $F(1,32)<0.0001$, $P=0.99$; $n=9$ ST:GFP, $n=10$ ST:Cre).

APPENDICES

In addition to the data presented in the previous chapters, which has been submitted for publication, I have pursued numerous other studies. Although these findings are not published, they have informed previous and ongoing efforts in the Veenstra-VanderWeele lab.

Appendix 1: Neurochemical Characterization of Glutamate, GABA, and Cysteine in *Slc1a1*-STOP Mice

Appendix 2: *Slc1a1*-STOP mice exhibit no baseline behavioral phenotypes in OCD-relevant specific assays

Appendix 3: Consequences of D1-Cre and DAT-IRES-Cre Based Rescue of *Slc1a1*/EAAT3 Expression on Amphetamine Induced Locomotor Behavior

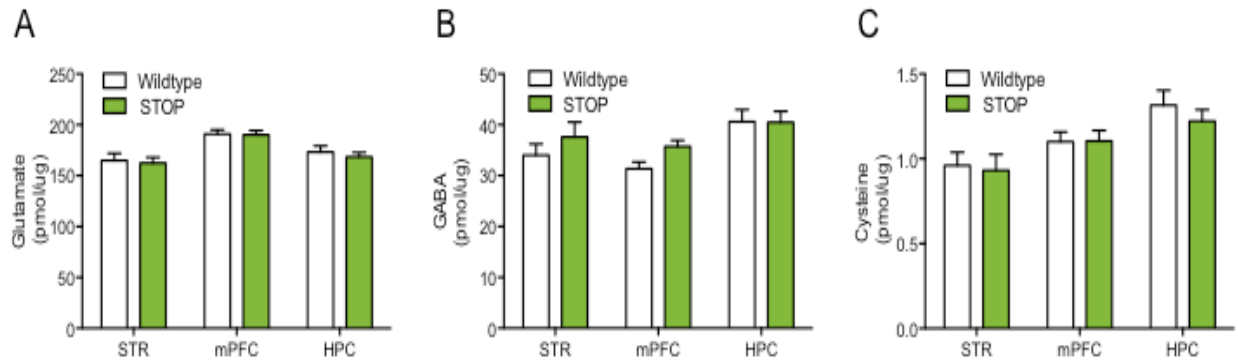
Appendix 4: Implications of a Muscarinic Acetylcholine Receptor M4 Positive Allosteric Modulator in a Preclinical Model of OCD-Relevant Behavior

APPENDIX 1

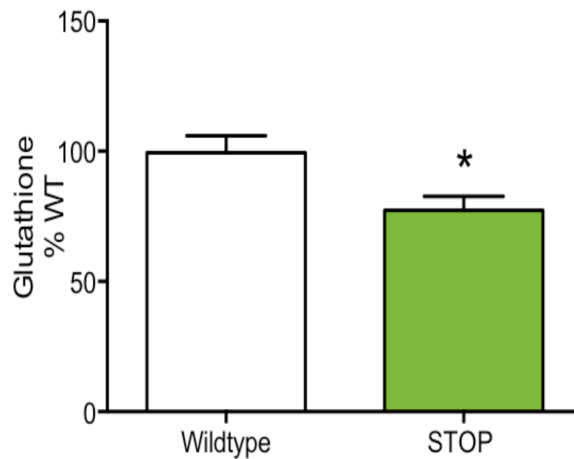
Neurochemical Characterization of Glutamate, GABA, and Cysteine in *Slc1a1*-STOP Mice

Alterations in the concentration of glutamate, GABA, and cysteine have been observed in the human OCD population and in studies investigating the downstream impact of altered EAAT3 function (Moore et al., 1998, Sepkuty et al., 2002, Mathews and Diamond, 2003, Rosenberg et al., 2004, Chakrabarty et al., 2005a, Aoyama et al., 2006, Whiteside et al., 2006, Yucel et al., 2008, Bailey et al., 2011, Aoyama and Nakaki, 2013, Watts et al., 2014). EAAT3 is strongly expressed in the cortico-striatal regions implicated in these studies and is proposed to have three functions: 1) regulate extrasynaptic glutamate levels; 2) uptake of GABA to provide a substrate for GABA synthesis in GABAergic neurons; and 3) uptake of cysteine for glutathione synthesis (Nieoullon et al., 2006). In order to determine how disrupted function of EAAT3 affects these functions in striatum, medial prefrontal cortex, and hippocampus of *Slc1a1*-STOP mice, fresh tissue was isolated from each region and submitted for analysis of the aforementioned amino acids via high performance liquid chromatography. No abnormal tissue concentrations were identified in for the amino acids analyzed in any of brain regions (Appendix Figure 1). Due to our previous data showing that cysteine uptake is absent in synaptosomes of *Slc1a1*-STOP mice relative to wildtype mice (Chapter 4) and previous reports of EAAT3 null mice having increased levels of oxidative-stress and lower levels of the anti-oxidant glutathione (Aoyama et al., 2006, Aoyama and Nakaki, 2013), we also performed a colorimetric based assay to quantify glutathione levels in striatal tissue of *Slc1a1*-STOP mice. A reduction of ~23% of total striatal glutathione was identified in *Slc1a1*-STOP mice relative to wildtype controls (Appendix Figure 2). These data support our previous functional data showing that baseline glutamate uptake is not affected in *ex vivo* preparations as well as agreeing with reports from other groups showing that downstream products of EAAT3 function are affected as a result of reduced EAAT3

expression in the striatum. As glutathione deficiencies resulting from a lack of EAAT3 function are exacerbated in aged EAAT3 null mice (Aoyama et al., 2006), future work related to these efforts in *Slc1a1*-STOP mice may look at more specific and prolonged time points to garner further insight into the consequences of disrupted EAAT3 function on downstream biochemical processes.



Appendix Figure 1. No differences were observed in (A) glutamate, (B) GABA, or (C) cysteine tissue concentrations in the striatum (STR), medial prefrontal cortex (mPFC), or in the hippocampus (HPC) of *Slc1a1*-STOP mice relative to controls (t-test, $P > 0.05$; $n=5$ per genotype per region).



Appendix Figure 2. Total striatal glutathione levels are reduced in *Slc1a1*-STOP mice normalized to wildtype controls (t-test; $t=2.66$, $*P=0.023$, $n=6$ per genotype, each sample was analyzed in triplicate).

Appendix 2

***Slc1a1*-STOP mice exhibit no baseline behavioral phenotypes in OCD-relevant specific assays**

In order to assess any baseline behavior deficits relevant to OCD, *Slc1a1*-STOP mice were assayed in behavioral assays believed to be more pertinent of the disorder. As with many preclinical animal behavioral models of psychiatric disorders, symptoms observed in the human population are unable to be tested in rodent models and individual assay's validity (Chapter 2) may be subject to discussion with regards to the relevance to the disorder they are sought to be modeling.

Marble Burying

Initial analysis of marble burying by rodents was performed as a manner to test novel compounds for anxiolytic activity as rodents commonly use bedding to bury novel objects (Andersen et al., 2010). However, upon the finding that SRIs reduced this behavior, and SRIs known therapeutic effect in OCD patients, marble burying has thus been used as a screen to test novel preclinical models of the disorder. Briefly, twenty marbles in a four by five grid were laid out in a novel cage with clean bedding. *Slc1a1*-STOP mice and wildtype controls were placed into the novel cage alone for thirty minutes. At the end of the assay, mice were removed and pictures were taken to score the number of buried marbles by two scorers blind to genotype. No differences were observed in the total number of marbles buried by *Slc1a1*-STOP mice relative to wildtype controls, suggesting a lack of OCD-relevant behavioral phenotype in this assay (Appendix Figure 3a).

Nestlet Shredding

Nestlet shredding is another behavioral assay that takes advantage of rodent's natural behavior to shred materials in order to construct a nest. As with marble burying, nestlet shredding is believed to be a relatively high throughput behavioral assay relevant to OCD based

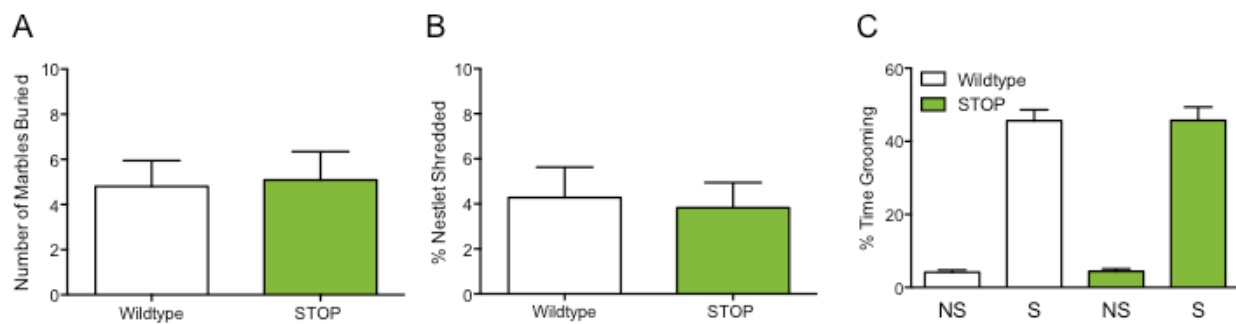
on the observed effectiveness of SRIs to limit the behavior in mouse models (Li et al., 2006, Angoa-Perez et al., 2013). One mouse was placed into a cage with clean bedding along with a single preweighed nestlet and left undisturbed for thirty minutes. Following the completion of the thirty minutes, mice were removed and the unshredded nestlet was allowed to dry overnight then weighed. As with the marble burying, no observed differences were identified in *Slc1a1*-STOP mice relative to wildtype controls (Appendix Figure 3b).

It is important to point out that while marble burying and nestlet shredding are commonly used due to their purported relevance to OCD, many drugs, which are ineffective in the treatment of OCD, have shown efficacy in these assays, lessening these assay's predictive validity (Angoa-Perez et al., 2013).

Water Spray Induced Grooming

Increased spontaneous grooming is a commonly observed behavioral phenotype observed in mouse models reported to be relevant to OCD (Welch et al., 2007, Shmelkov et al., 2010b, Ahmari et al., 2013). However, as previously described (Chapter 4), *Slc1a1*-STOP mice exhibit no clear spontaneous grooming phenotypes at baseline. As the majority of the behavioral deficits in *Slc1a1*-STOP mice were induced, I wanted to examine if *Slc1a1*-STOP mice also displayed reduced induced behavior, which is suggested to be relevant to OCD. Mice were placed into an empty novel cage and allowed to habituate for five minutes. Mice were then videotaped for five minutes then removed from the cage and lightly misted with water from a hand spray bottle at a distance of twelve inches three times to coat the mouse's dorsal surface. Mice were then placed back into the cage and videotaped for another five minutes. Following the completion of the assay, two observers blind to genotype scored grooming behavior. As with baseline grooming, no differences in spontaneous or water spray induced grooming in *Slc1a1*-STOP mice relative to wildtype littermate controls (Appendix Figure 3c). Initially, it may be

surprising that no behavioral abnormalities were identified in *Slc1a1*-STOP mice following the induction of a behavior reported to be relevant to OCD, as many of the behavioral deficits previously described were induced behaviors. However, it is important to point out that all of the previous behavioral phenotypes were induced pharmacologically by probing the dopamine system while the induction of grooming in the manner described has not been reported to affect any specific neurotransmitter system.



Appendix Figure 3. *Slc1a1*-STOP mice do not exhibit OCD-relevant behavioral phenotypes at baseline or when induced via water spray.

(A) No differences were observed in number of marbles buried by *Slc1a1*-STOP mice relative to wildtype controls (t-test, $t=0.16$, $P=0.87$, $n=10$ WT, 12 STOP).

(B) No differences were observed in nestlet shredding behavior by *Slc1a1*-STOP mice relative to wildtype controls (t-test, $t=0.25$, $P=0.81$, $n=10$ WT, 12 STOP).

(C) Grooming behavior was unchanged in *Slc1a1*-STOP mice exclusively in no spray (NS) or water-spray induced (S) behavioral paradigms relative to wildtype controls (Two-way ANOVA, Spray; $F(1,40)=99.3$; $P<0.0001$; Genotype $F(1,40)=0.008$, $P=0.92$; $n=10$ WT and 12 STOP).

Appendix 3

Consequences of D1-Cre and DAT-IRES-Cre Based Rescue of *Slc1a1*/EAAT3 Expression on Amphetamine Induced Locomotor Behavior

D1-Cre

Prior to the AAV-Cre (midbrain) dependent findings of partial rescue of amphetamine induced behaviors in *Slc1a1*-STOP mice (Chapter 4), an alternative experimental strategy was used to attempt to rescue the behavioral phenotypes observed in *Slc1a1*-STOP mice. Having identified initial reductions in both dopamine D1R agonist sensitivity via SKF-38393 challenge and reduced density estimates of the receptor in the striatal cell membrane preparations in *Slc1a1*-STOP mice (Chapter 4), I wanted to ascertain if cell-type specific rescue of *Slc1a1*/EAAT3 in D1R expressing neurons throughout development would rescue the amphetamine induced locomotor phenotypes of *Slc1a1*-STOP mice. Mice carrying a dopamine D1R specific cre-recombinase were crossbred with *Slc1a1*-STOP mice to yield mice heterozygous for D1R-Cre and homozygous for *Slc1a1*-STOP mice (*Slc1a1*-STOP:D1-Cre). Female *Slc1a1*-STOP:D1-Cre mice were then bred with male *Slc1a1*-STOP mice to yield littermate pairs to be used for behavioral characterization. Rescued expression of *Slc1a1* was confirmed via qRT-PCR in striatal tissue samples (Appendix Figure 4a, One-way ANOVA; $F(2,5)=18.4$, $P=0.0049$, $n=3$ per genotype; Tukey's multiple comparison test *Slc1a1*-STOP vs. *Slc1a1*-STOP:D1-Cre, $P<0.05$). As in previous amphetamine-induced locomotor assays, mice were placed into locomotor chambers, allowed to habituate for thirty minutes then challenged with amphetamine (3.0 mg/kg, IP). I found that rescue of *Slc1a1*/EAAT3 expression in D1R expressing cells did affect amphetamine-induced locomotion, unexpectedly however, it led to an exaggerated reduced behavioral locomotor phenotype relative to *Slc1a1*-STOP mice (Appendix Figure 4b, Non-linear curve fit analysis, $F(4,280)=6.64$, $P<0.0001$; $n=9$ per genotype).

I also tested how rescued expression of *Slc1a1*/EAAT3 in D1R-expressing neurons affects D1R agonist behavioral sensitivity as I hypothesized that rescued expression of EAAT3

in D1R-MSNs of the striatum may effect SKF-38393 induced grooming behavior despite the results observed in response to amphetamine. A main effect of SKF on grooming behavior was identified however no genotypes differences were observed when *Slc1a1*-STOP:D1-Cre mice were challenged with the D1R agonist relative to *Slc1a1*-STOP controls (Appendix Figure 4c; Two-way ANOVA; SKF-38393 $F(1,30)=27.56$, $P<0.0001$; $n=9$ per genotype).

These data were certainly intriguing however they were unexpected. They suggest that the observed behavioral deficits observed in *Slc1a1*-STOP mice were not dependent exclusively on EAAT3 function in D1R-expressing neurons and that EAAT3 expression has an impact on dopamine dependent behaviors outside of the striatum. Second, these data also show that expression of EAAT3 in the D1R-expressing cells alone does not underlie the reductions in D1R cell membrane estimates or behavioral sensitivity to SKF-38393. Due to the amplified reduction of amphetamine induced locomotion observed in *Slc1a1*-STOP:D1-Cre mice relative to *Slc1a1*-STOP mice, it can also be hypothesized that EAAT3 plays an important development role on the neurocircuitry involved in amphetamine dependent behaviors.

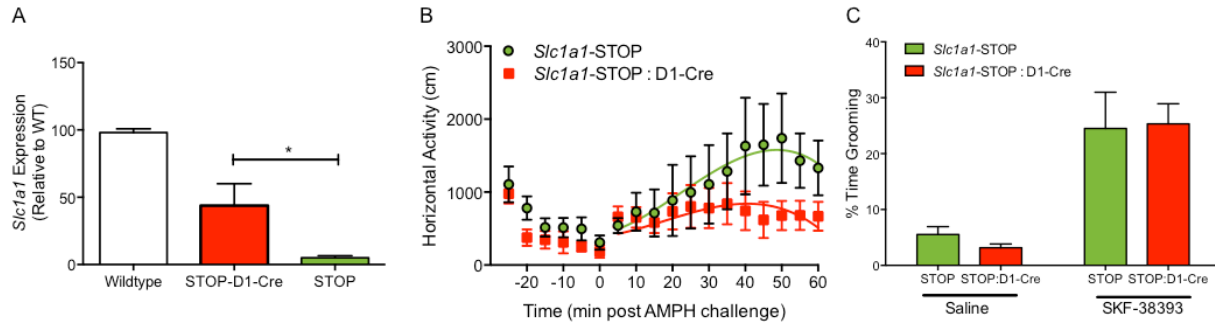
DAT-IRES-Cre

Following the behavioral results identified in Chapter 4 showing that AAV-Cre mediated rescue of *Slc1a1*/EAAT3 in the midbrain led to partial rescue of amphetamine dependent behavior in *Slc1a1*-STOP mice, I followed up on that study by using another transgenic line, DAT-IRES-Cre, to rescue *Slc1a1*/EAAT3 expression in DAT expressing neurons throughout development. *Slc1a1*-STOP:DAT-Cre mice were generated as above. It was my hypothesis that rescuing *Slc1a1*/EAAT3 expression in dopaminergic neurons throughout development would completely rescue the amphetamine dependent behavioral phenotypes previously described in *Slc1a1*-STOP mice. Amphetamine induced locomotor behavioral assays were performed as previously described (3.0 mg/kg, IP) in *Slc1a1*-STOP:DAT-Cre mice and *Slc1a1*-STOP controls. Interestingly, as previously observed in *Slc1a1*-STOP:D1-Cre mice, amphetamine challenge led

to reduced locomotor behavior in *Slc1a1*-STOP:DAT-Cre mice relative to *Slc1a1*-STOP controls (Appendix Figure 5; Non-linear curve fit analysis; $F(4,406)=11.49$, $P<0.0001$, $n=12$ per genotype).

More comprehensive discussion of the implications of this work is included in Chapter 5 however brief discussion and interpretation is included here. The results of these studies are quite fascinating however their implications on the underlying mechanisms that elicit reduced amphetamine and basal ganglia dependent behavioral phenotypes in *Slc1a1*-STOP mice are relatively unclear. First, when the results from our viral and transgenic rescue studies are interpreted comprehensively, it can be deduced that there is a significant developmental aspect of *Slc1a1*-STOP mice's behavioral phenotypes. Hypothetically, it may be that the underlying mechanisms leading to reduced dopamine driven behaviors in *Slc1a1*-STOP mice occur early during development and are dependent on EAAT3 function in both presynaptic DAergic and postsynaptic MSNs.

The comparison between the midbrain viral Cre and DAT-Cre rescue studies also bring about other questions. How can rescued expression of *Slc1a1*/EAAT3 in the midbrain exclusively in adulthood partially rescue amphetamine induced locomotion however D1-Cre or DAT-Cre driven rescued expression of *Slc1a1*/EAAT3 cause an opposite behavioral effect? It is possible that rescue of midbrain EAAT3 expression and function in adult mice affects DAergic neurotransmission in an alternative manner that is not intrinsically related to the cause of the observed phenotypes in *Slc1a1*-STOP mice regardless of the intervention's ability to moderately mitigate the amphetamine-induced behavioral phenotypes. Further examination of loss of EAAT3 function on dopaminergic and glutamatergic neurotransmission in each of the implicated nodes of dopamine dependent behavior are necessary in order to more fully comprehend potential underlying signaling deficits related to the behavioral phenotypes observed in *Slc1a1*-STOP mice.

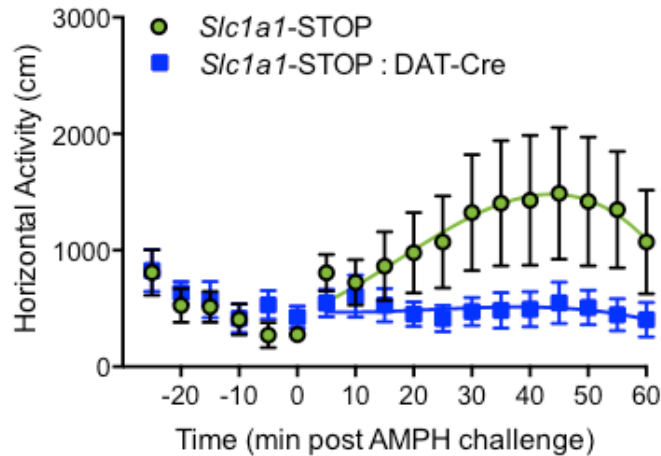


Appendix Figure 4. D1-Cre driven rescue of *Slc1a1*/EAAT3 Expression leads to reductions in amphetamine induced locomotion but does not affect SKF-38393 induced grooming.

(A) *Slc1a1* expression is partially rescued in tissue samples harvested from the striatum of *Slc1a1*-STOP:D1-Cre mice relative to *Slc1a1*-STOP littermate controls (One-way ANOVA; $F(2,5)=18.4$, $**P=0.0049$, $n=3$ per genotype; Tukey's multiple comparison test, $*P<0.05$).

(B) Amphetamine (3.0 mg/kg, IP) causes minimal locomotor activation in *Slc1a1*-STOP:D1-Cre mice relative to *Slc1a1*-STOP littermate controls (Non-linear curve fit analysis, $F(4,280)=6.64$, $P<0.0001$; $n=9$ per genotype).

(C) No genotype effect is observed following SKF-38393 challenge in *Slc1a1*-STOP:D1-Cre mice relative to *Slc1a1*-STOP littermate controls (Two-way ANOVA; SKF-38393 $F(1,30)=27.56$, $P<0.0001$; Genotype $F(1,30)=0.04$, $P=0.85$; $n=9$ per genotype).



Appendix Figure 5. DAT-Cre driven rescue of *Slc1a1*/EAAT3 Expression leads to reductions in amphetamine induced locomotion.

Amphetamine (3.0 mg/kg, IP) causes minimal locomotor activation in *Slc1a1*-STOP:DAT-Cre mice relative to *Slc1a1*-STOP littermate controls (Non-linear curve fit analysis, $F(4,406)=11.49$, $***P<0.0001$; $n=12$ per genotype).

Appendix 4

Implications of a Muscarinic Acetylcholine Receptor M4 Positive Allosteric Modulator in a Preclinical Model of OCD-Relevant Behavior

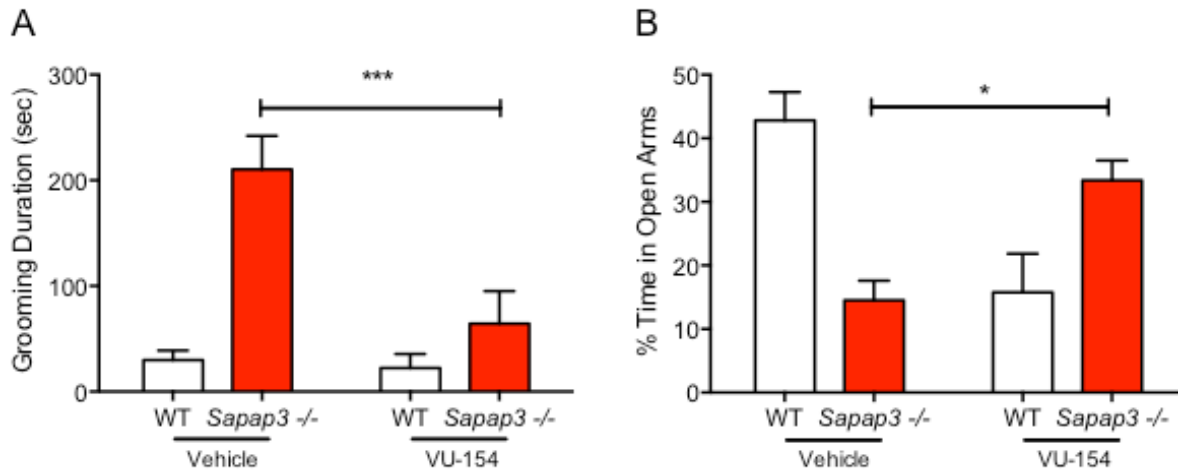
In collaboration with the Vanderbilt Center for Neuroscience Drug Discovery (VCNDD) and as an alternative to my work in *Slc1a1*-STOP mice, I also performed behavioral pharmacology in *Sapap3* null mice, a previously described preclinical model of repetitive and anxiety like behavior (Chapter 2 and 3). Briefly, *Sapap3* null mice exhibit cortico-striatal signaling deficits, increased anxiety-like behavior, and SRI responsive perseverative grooming behavior (Welch et al., 2007, Zuchner et al., 2009). While the overall human genetic data implicating the human ortholog of *Sapap3*, *DLGAP3*, in OCD is largely speculative; the *Sapap3* nulls are a useful model for preliminary screening of compounds thought to impact repetitive-like behavior. In collaboration with the VCNDD, I sought to determine if the robust behavioral phenotypes observed in *Sapap3* null mice would respond to a novel positive allosteric modulator (PAM) of the M4 muscarinic acetylcholine receptor (M4R), VU-154. Previous work from the VCNDD showed the VU-154 was able to reverse behavioral and cognitive deficits induced by the noncompetitive NMDAR antagonist, MK-801 (Bubser et al., 2014). Physiologically, it was also shown that that potentiation of M4R via VU-154 administration impacts glutamate release from cortico-striatal terminals (Pancani et al., 2014), suggesting that VU-154 may also be effective in ameliorating the behavioral phenotypes observed in *Sapap3* null mice.

To test this hypothesis, VU-154 was used in behavioral assays to examine its ability to attenuate both repetitive grooming and anxiety-like phenotypes described in *Sapap3* null mice. Spontaneous grooming was assessed by placing single mice into novel clean cages where their behavior was recorded via videotape then subsequently analyzed for total time spent grooming by independent observers blind to genotype. Thirty minute pretreatment of *Sapap3* null mice with VU-154 (3.0 mg/kg, IP) led a reduction in perseverative grooming behavior that was not

observed in wildtype littermate controls (Appendix Figure 6a; Two-way ANOVA; VU-154 x Genotype $F(1,24)=7.28$, $P=0.01$; VU-154 $F(1,24)=8.92$, $P=0.005$; Genotype $F(1,24)=18.67$, $P=0.0001$; Multiple Comparisons $***P<0.001$, $n=7$ per genotype).

As in the grooming assays, thirty minute pretreatment of *Sapap3* nulls and wildtype littermate controls with VU-154 was utilized to determine the M4R PAMs ability to reduce anxiety-like behavior in *Sapap3* null mice in the elevated zero maze. Via two-way ANOVA, a significant VU-154 by genotype interaction was observed with *Sapap3* nulls exhibiting a VU-154 responsive anxiety-like behavioral phenotype relative to wildtype controls (Appendix Figure 6b; Two-way ANOVA; VU-154 x Genotype $F(1,18)=32.67$, $P<0.0001$; Multiple Comparisons $***P<0.001$, $*P<0.05$; $n=5$ WT, 7 *Sapap3* null).

While these behavioral assays were relatively low-powered, they do indicate the potential utility of cortico-striatal normalizing compounds in the preclinical models of repetitive and anxiety-like behavior. Future efforts investigating VU-154's effect on alternative models of OCD-relevant behavior with improved construct validity relative to *Sapap3* nulls are important to garner further insight into the potential efficacy of the M4R PAMs as a novel therapeutic option in the treatment of OCD and related disorders.



Appendix Figure 6. M4R PAM VU-154 attenuates repetitive and anxiety-like behavioral phenotypes in *Sapap3* null mice.

(A) Perseverative grooming behavior is attenuated by VU-154 treatment in *Sapap3* null mice (Two-way ANOVA; VU-154 x Genotype $F(1,24)=7.28$, $**P=0.01$; VU-154 $F(1,24)=8.92$, $**P=0.005$; Genotype $F(1,24)=18.67$, $***P=0.0001$; Multiple Comparisons $***P<0.001$, $n=7$ per genotype).

(B) Percent time spent in open arms of elevated zero maze is increased by VU-154 treatment in *Sapap3* null mice (Two-way ANOVA; VU-154 x Genotype $F(1,18)=32.67$, $***P<0.0001$; Multiple Comparisons $***P<0.001$, $*P<0.05$; $n=5$ WT, 7 *Sapap3* null).

REFERENCES

- Abelson JF, Kwan KY, O'Roak BJ, Baek DY, Stillman AA, Morgan TM, Mathews CA, Pauls DL, Rasin MR, Gunel M, Davis NR, Ercan-Sencicek AG, Guez DH, Spertus JA, Leckman JF, Dure LS, Kurlan R, Singer HS, Gilbert DL, Farhi A, Louvi A, Lifton RP, Sestan N, State MW (2005) Sequence variants in SLITRK1 are associated with Tourette's syndrome. *Science* 310:317-320.
- Ade KK, Wan Y, Hamann HC, O'Hare JK, Guo W, Quian A, Kumar S, Bhagat S, Rodriguiz RM, Wetsel WC, Conn PJ, Dzirasa K, Huber KM, Calakos N (2016) Increased Metabotropic Glutamate Receptor 5 Signaling Underlies Obsessive-Compulsive Disorder-like Behavioral and Striatal Circuit Abnormalities in Mice. *Biological psychiatry* 80:522-533.
- Afshari P, Myles-Worsley M, Cohen OS, Tiobech J, Faraone SV, Byerley W, Middleton FA (2015) Characterization of a Novel Mutation in SLC1A1 Associated with Schizophrenia. *Mol Neuropsychiatry* 1:125-144.
- Ahmari SE, Risbrough VB, Geyer MA, Simpson HB (2012) Impaired sensorimotor gating in unmedicated adults with obsessive-compulsive disorder. *Neuropsychopharmacology* : official publication of the American College of Neuropsychopharmacology 37:1216-1223.
- Ahmari SE, Spellman T, Douglass NL, Kheirbek MA, Simpson HB, Deisseroth K, Gordon JA, Hen R (2013) Repeated cortico-striatal stimulation generates persistent OCD-like behavior. *Science* 340:1234-1239.
- Alkhatib AH, Dvorkin-Gheva A, Szechtman H (2013) Quinpirole and 8-OH-DPAT induce compulsive checking behavior in male rats by acting on different functional parts of an OCD neurocircuit. *Behav Pharmacol* 24:65-73.
- Allen NC, Bagade S, McQueen MB, Ioannidis JP, Kavvoura FK, Khoury MJ, Tanzi RE, Bertram L (2008) Systematic meta-analyses and field synopsis of genetic association studies in schizophrenia: the SzGene database. *Nature genetics* 40:827-834.
- Alonso P, Gratacos M, Segalas C, Escaramis G, Real E, Bayes M, Labad J, Lopez-Sola C, Estivill X, Menchon JM (2012) Association between the NMDA glutamate receptor GRIN2B gene and obsessive-compulsive disorder. *J Psychiatry Neurosci* 37:273-281.
- Alvaro JD, Taylor JR, Duman RS (2003) Molecular and behavioral interactions between central melanocortins and cocaine. *The Journal of pharmacology and experimental therapeutics* 304:391-399.
- Andersen SL, Greene-Colozzi EA, Sonntag KC (2010) A novel, multiple symptom model of obsessive-compulsive-like behaviors in animals. *Biological psychiatry* 68:741-747.
- Andrade P, Carrillo-Ruiz JD, Ramirez Y, Jimenez F (2010) Effects of Thalamic Reticular Nucleus Electrical Stimulation in Rats in a T-maze Perseverative Behavior Model Induced by 8-OH-DPAT. *Neuromodulation* 13:2-9.
- Andrade P, Fernandez-Guasti A, Carrillo-Ruiz JD, Ulloa RE, Ramirez Y, Reyes R, Jimenez F (2009) Effects of bilateral lesions in thalamic reticular nucleus and orbitofrontal cortex in

- a T-maze perseverative model produced by 8-OH-DPAT in rats. *Behav Brain Res* 203:108-112.
- Angoa-Perez M, Kane MJ, Briggs DI, Francescutti DM, Kuhn DM (2013) Marble burying and nestlet shredding as tests of repetitive, compulsive-like behaviors in mice. *J Vis Exp* 50978.
- Ansorge MS, Zhou M, Lira A, Hen R, Gingrich JA (2004) Early-life blockade of the 5-HT transporter alters emotional behavior in adult mice. *Science* 306:879-881.
- Anticevic A, Hu S, Zhang S, Savic A, Billingslea E, Wasylink S, Repovs G, Cole MW, Bednarski S, Krystal JH, Bloch MH, Li CS, Pittenger C (2014) Global resting-state functional magnetic resonance imaging analysis identifies frontal cortex, striatal, and cerebellar dysconnectivity in obsessive-compulsive disorder. *Biological psychiatry* 75:595-605.
- Aouizerate B, Cuny E, Martin-Guehl C, Guehl D, Amieva H, Benazzouz A, Fabrigoule C, Allard M, Rougier A, Bioulac B, Tignol J, Burbaud P (2004) Deep brain stimulation of the ventral caudate nucleus in the treatment of obsessive-compulsive disorder and major depression. Case report. *Journal of neurosurgery* 101:682-686.
- Aoyama K, Nakaki T (2012) Inhibition of GTRAP3-18 may increase neuroprotective glutathione (GSH) synthesis. *Int J Mol Sci* 13:12017-12035.
- Aoyama K, Nakaki T (2013) Neuroprotective properties of the excitatory amino acid carrier 1 (EAAC1). *Amino Acids* 45:133-142.
- Aoyama K, Suh SW, Hamby AM, Liu J, Chan WY, Chen Y, Swanson RA (2006) Neuronal glutathione deficiency and age-dependent neurodegeneration in the EAAC1 deficient mouse. *Nature neuroscience* 9:119-126.
- Aoyama K, Watabe M, Nakaki T (2012) Modulation of neuronal glutathione synthesis by EAAC1 and its interacting protein GTRAP3-18. *Amino Acids* 42:163-169.
- Arnold PD, Rosenberg DR, Mundo E, Tharmalingam S, Kennedy JL, Richter MA (2004) Association of a glutamate (NMDA) subunit receptor gene (GRIN2B) with obsessive-compulsive disorder: a preliminary study. *Psychopharmacology* 174:530-538.
- Arnold PD, Sicard T, Burroughs E, Richter MA, Kennedy JL (2006) Glutamate transporter gene SLC1A1 associated with obsessive-compulsive disorder. *Archives of general psychiatry* 63:769-776.
- Arriza JL, Eliasof S, Kavanaugh MP, Amara SG (1997) Excitatory amino acid transporter 5, a retinal glutamate transporter coupled to a chloride conductance. *Proceedings of the National Academy of Sciences of the United States of America* 94:4155-4160.
- Arumugham SS, Reddy JY (2013) Augmentation strategies in obsessive-compulsive disorder. *Expert Rev Neurother* 13:187-202; quiz 203.
- Assous M, Had-Aissouni L, Gubellini P, Melon C, Nafia I, Salin P, Kerkerian-Le-Goff L, Kachidian P (2014) Progressive Parkinsonism by acute dysfunction of excitatory amino acid transporters in the rat substantia nigra. *Neurobiol Dis* 65:69-81.

- Bailey CG, Ryan RM, Thoeng AD, Ng C, King K, Vanslambrouck JM, Auray-Blais C, Vandenberg RJ, Broer S, Rasko JE (2011) Loss-of-function mutations in the glutamate transporter SLC1A1 cause human dicarboxylic aminoaciduria. *The Journal of clinical investigation* 121:446-453.
- Balcar VJ, Johnston GA (1972) The structural specificity of the high affinity uptake of L-glutamate and L-aspartate by rat brain slices. *Journal of neurochemistry* 19:2657-2666.
- Barrett P, Healy-Farrell L, March JS (2004) Cognitive-behavioral family treatment of childhood obsessive-compulsive disorder: a controlled trial. *J Am Acad Child Adolesc Psychiatry* 43:46-62.
- Bauer D, Gupta D, Harotunian V, Meador-Woodruff JH, McCullumsmith RE (2008) Abnormal expression of glutamate transporter and transporter interacting molecules in prefrontal cortex in elderly patients with schizophrenia. *Schizophr Res* 104:108-120.
- Bedard MJ, Chantal S (2011) Brain magnetic resonance spectroscopy in obsessive-compulsive disorder: the importance of considering subclinical symptoms of anxiety and depression. *Psychiatry Res* 192:45-54.
- Berg JM, Lee C, Chen L, Galvan L, Cepeda C, Chen JY, Penagarikano O, Stein JL, Li A, Oguro-Ando A, Miller JA, Vashisht AA, Starks ME, Kite EP, Tam E, Gdalyahu A, Al-Sharif NB, Burkett ZD, White SA, Fears SC, Levine MS, Wohlschlegel JA, Geschwind DH (2015) JAKMIP1, a Novel Regulator of Neuronal Translation, Modulates Synaptic Function and Autistic-like Behaviors in Mouse. *Neuron* 88:1173-1191.
- Berger UV, Hediger MA (1998) Comparative analysis of glutamate transporter expression in rat brain using differential double in situ hybridization. *Anat Embryol (Berl)* 198:13-30.
- Berman AE, Chan WY, Brennan AM, Reyes RC, Adler BL, Suh SW, Kauppinen TM, Edling Y, Swanson RA (2011) N-acetylcysteine prevents loss of dopaminergic neurons in the EAAC1^{-/-} mouse. *Ann Neurol* 69:509-520.
- Beucke JC, Sepulcre J, Talukdar T, Linnman C, Zschenderlein K, Endrass T, Kaufmann C, Kathmann N (2013) Abnormally high degree connectivity of the orbitofrontal cortex in obsessive-compulsive disorder. *JAMA Psychiatry* 70:619-629.
- Bhattacharyya S, Khanna S, Chakrabarty K, Mahadevan A, Christopher R, Shankar SK (2009) Anti-brain autoantibodies and altered excitatory neurotransmitters in obsessive-compulsive disorder. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* 34:2489-2496.
- Bienvenu OJ, Wang Y, Shugart YY, Welch JM, Grados MA, Fyer AJ, Rauch SL, McCracken JT, Rasmussen SA, Murphy DL, Cullen B, Valle D, Hoehn-Saric R, Greenberg BD, Pinto A, Knowles JA, Piacentini J, Pauls DL, Liang KY, Willour VL, Riddle M, Samuels JF, Feng G, Nestadt G (2009) Sapap3 and pathological grooming in humans: Results from the OCD collaborative genetics study. *Am J Med Genet B Neuropsychiatr Genet* 150B:710-720.

- Billett EA, Richter MA, Sam F, Swinson RP, Dai XY, King N, Badri F, Sasaki T, Buchanan JA, Kennedy JL (1998) Investigation of dopamine system genes in obsessive-compulsive disorder. *Psychiatr Genet* 8:163-169.
- Bissonette GB, Powell EM (2012) Reversal learning and attentional set-shifting in mice. *Neuropharmacology* 62:1168-1174.
- Bjorn-Yoshimoto W, Underhill SM (2016) The importance of the excitatory amino acid transporter 3 (EAAT3). *Neurochem Int*.
- Blasco H, Mavel S, Corcia P, Gordon PH (2014) The glutamate hypothesis in ALS: pathophysiology and drug development. *Curr Med Chem* 21:3551-3575.
- Bloch MH, Landeros-Weisenberger A, Kelmendi B, Coric V, Bracken MB, Leckman JF (2006) A systematic review: antipsychotic augmentation with treatment refractory obsessive-compulsive disorder. *Molecular psychiatry* 11:622-632.
- Bloch MH, Panza KE, Landeros-Weisenberger A, Leckman JF (2009) Meta-analysis: treatment of attention-deficit/hyperactivity disorder in children with comorbid tic disorders. *J Am Acad Child Adolesc Psychiatry* 48:884-893.
- Bloch MH, Wasylink S, Landeros-Weisenberger A, Panza KE, Billingslea E, Leckman JF, Krystal JH, Bhagwagar Z, Sanacora G, Pittenger C (2012) Effects of ketamine in treatment-refractory obsessive-compulsive disorder. *Biological psychiatry* 72:964-970.
- Bokor G, Anderson PD (2014) Obsessive-compulsive disorder. *J Pharm Pract* 27:116-130.
- Borcherding BG, Keysor CS, Rapoport JL, Elia J, Amass J (1990) Motor/vocal tics and compulsive behaviors on stimulant drugs: is there a common vulnerability? *Psychiatry Res* 33:83-94.
- Borges K, Dingledine R (1998) AMPA receptors: molecular and functional diversity. *Progress in brain research* 116:153-170.
- Borowsky B, Hoffman BJ (1995) Neurotransmitter transporters: molecular biology, function, and regulation. *Int Rev Neurobiol* 38:139-199.
- Boudker O, Ryan RM, Yernool D, Shimamoto K, Gouaux E (2007) Coupling substrate and ion binding to extracellular gate of a sodium-dependent aspartate transporter. *Nature* 445:387-393.
- Brigman JL, Powell EM, Mittleman G, Young JW (2012) Examining the genetic and neural components of cognitive flexibility using mice. *Physiol Behav* 107:666-669.
- Brimberg L, Benhar I, Mascaro-Blanco A, Alvarez K, Lotan D, Winter C, Klein J, Moses AE, Somnier FE, Leckman JF, Swedo SE, Cunningham MW, Joel D (2012) Behavioral, pharmacological, and immunological abnormalities after streptococcal exposure: a novel rat model of Sydenham chorea and related neuropsychiatric disorders. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* 37:2076-2087.

- Broer S (2008) Amino acid transport across mammalian intestinal and renal epithelia. *Physiol Rev* 88:249-286.
- Broer S, Palacin M (2011) The role of amino acid transporters in inherited and acquired diseases. *The Biochemical journal* 436:193-211.
- Broocks A, Pigott TA, Hill JL, Canter S, Grady TA, L'Heureux F, Murphy DL (1998) Acute intravenous administration of ondansetron and m-CPP, alone and in combination, in patients with obsessive-compulsive disorder (OCD): behavioral and biological results. *Psychiatry Res* 79:11-20.
- Brown JA, Ramikie TS, Schmidt MJ, Baldi R, Garbett K, Everheart MG, Warren LE, Gellert L, Horvath S, Patel S, Mirnics K (2015) Inhibition of parvalbumin-expressing interneurons results in complex behavioral changes. *Mol Psychiatry* 20:1499-1507.
- Bubser M, Bridges TM, Dencker D, Gould RW, Grannan M, Noetzel MJ, Lamsal A, Niswender CM, Daniels JS, Poslusney MS, Melancon BJ, Tarr JC, Byers FW, Wess J, Duggan ME, Dunlop J, Wood MW, Brandon NJ, Wood MR, Lindsley CW, Conn PJ, Jones CK (2014) Selective activation of M4 muscarinic acetylcholine receptors reverses MK-801-induced behavioral impairments and enhances associative learning in rodents. *ACS Chem Neurosci* 5:920-942.
- Burguiere E, Monteiro P, Feng G, Graybiel AM (2013) Optogenetic stimulation of lateral orbitofronto-striatal pathway suppresses compulsive behaviors. *Science* 340:1243-1246.
- Butchbach ME, Lai L, Lin CL (2002) Molecular cloning, gene structure, expression profile and functional characterization of the mouse glutamate transporter (EAAT3) interacting protein GTRAP3-18. *Gene* 292:81-90.
- Cahill E, Salery M, Vanhoutte P, Caboche J (2014) Convergence of dopamine and glutamate signaling onto striatal ERK activation in response to drugs of abuse. *Front Pharmacol* 4:172.
- Cai J, Zhang W, Yi Z, Lu W, Wu Z, Chen J, Yu S, Fang Y, Zhang C (2013) Influence of polymorphisms in genes SLC1A1, GRIN2B, and GRIK2 on clozapine-induced obsessive-compulsive symptoms. *Psychopharmacology* 230:49-55.
- Calvocoressi L, Libman D, Vegso SJ, McDougle CJ, Price LH (1998) Global functioning of inpatients with obsessive-compulsive disorder, schizophrenia, and major depression. *Psychiatric services* 49:379-381.
- Campbell KM, de Lecea L, Severynse DM, Caron MG, McGrath MJ, Sparber SB, Sun LY, Burton FH (1999) OCD-Like behaviors caused by a neuropotentiating transgene targeted to cortical and limbic D1+ neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 19:5044-5053.
- Capecchi MR (1997) Hox genes and mammalian development. *Cold Spring Harb Symp Quant Biol* 62:273-281.

- Carter MD, Shah CR, Muller CL, Crawley JN, Carneiro AM, Veenstra-VanderWeele J (2011) Absence of preference for social novelty and increased grooming in integrin beta3 knockout mice: initial studies and future directions. *Autism Res* 4:57-67.
- Castellan Baldan L, Williams KA, Gallezot JD, Pogorelov V, Rapanelli M, Crowley M, Anderson GM, Loring E, Gorczyca R, Billingslea E, Wasylink S, Panza KE, Ercan-Sencicek AG, Krusong K, Leventhal BL, Ohtsu H, Bloch MH, Hughes ZA, Krystal JH, Mayes L, de Araujo I, Ding YS, State MW, Pittenger C (2014) Histidine decarboxylase deficiency causes tourette syndrome: parallel findings in humans and mice. *Neuron* 81:77-90.
- Chakrabarty K, Bhattacharyya S, Christopher R, Khanna S (2005a) Glutamatergic dysfunction in OCD. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* 30:1735-1740.
- Chakrabarty K, Bhattacharyya S, Christopher R, Khanna S (2005b) Glutamatergic dysfunction in OCD. *Neuropsychopharmacology* 30:1735-1740.
- Chamberlain SR, Blackwell AD, Fineberg NA, Robbins TW, Sahakian BJ (2005) The neuropsychology of obsessive compulsive disorder: the importance of failures in cognitive and behavioural inhibition as candidate endophenotypic markers. *Neuroscience and biobehavioral reviews* 29:399-419.
- Chamberlain SR, Fineberg NA, Blackwell AD, Robbins TW, Sahakian BJ (2006) Motor inhibition and cognitive flexibility in obsessive-compulsive disorder and trichotillomania. *The American journal of psychiatry* 163:1282-1284.
- Chamberlain SR, Menzies L, Hampshire A, Suckling J, Fineberg NA, del Campo N, Aitken M, Craig K, Owen AM, Bullmore ET, Robbins TW, Sahakian BJ (2008) Orbitofrontal dysfunction in patients with obsessive-compulsive disorder and their unaffected relatives. *Science (New York, NY)* 321:421-422.
- Chang K, Frankovich J, Cooperstock M, Cunningham MW, Latimer ME, Murphy TK, Pasternack M, Thienemann M, Williams K, Walter J, Swedo SE, Consortium PC (2015) Clinical evaluation of youth with pediatric acute-onset neuropsychiatric syndrome (PANS): recommendations from the 2013 PANS Consensus Conference. *J Child Adolesc Psychopharmacol* 25:3-13.
- Chefer VI, Zapata A, Shippenberg TS, Bungay PM (2006) Quantitative no-net-flux microdialysis permits detection of increases and decreases in dopamine uptake in mouse nucleus accumbens. *J Neurosci Methods* 155:187-193.
- Chen SK, Tvrdik P, Peden E, Cho S, Wu S, Spangrude G, Capecchi MR (2010) Hematopoietic origin of pathological grooming in Hoxb8 mutant mice. *Cell* 141:775-785.
- Chen Y, Swanson RA (2003) The glutamate transporters EAAT2 and EAAT3 mediate cysteine uptake in cortical neuron cultures. *Journal of neurochemistry* 84:1332-1339.
- Conn PJ, Christopoulos A, Lindsley CW (2009) Allosteric modulators of GPCRs: a novel approach for the treatment of CNS disorders. *Nat Rev Drug Discov* 8:41-54.

- Conti F, DeBiasi S, Minelli A, Rothstein JD, Melone M (1998a) EAAC1, a high-affinity glutamate transporter, is localized to astrocytes and gabaergic neurons besides pyramidal cells in the rat cerebral cortex. *Cerebral cortex* (New York, NY : 1991) 8:108-116.
- Conti F, DeBiasi S, Minelli A, Rothstein JD, Melone M (1998b) EAAC1, a high-affinity glutamate transporter, is localized to astrocytes and gabaergic neurons besides pyramidal cells in the rat cerebral cortex. *Cerebral cortex* 8:108-116.
- Coric V, Taskiran S, Pittenger C, Wasylink S, Mathalon DH, Valentine G, Saksa J, Wu YT, Gueorguieva R, Sanacora G, Malison RT, Krystal JH (2005) Riluzole augmentation in treatment-resistant obsessive-compulsive disorder: an open-label trial. *Biological psychiatry* 58:424-428.
- Coyle JT (2012) NMDA receptor and schizophrenia: a brief history. *Schizophr Bull* 38:920-926.
- Crockett BA, Churchill E, Davidson JR (2004) A double-blind combination study of clonazepam with sertraline in obsessive-compulsive disorder. *Ann Clin Psychiatry* 16:127-132.
- Curtis DR, Watkins JC (1960) THE EXCITATION AND DEPRESSION OF SPINAL NEURONES BY STRUCTURALLY RELATED AMINO ACIDS. *Journal of neurochemistry* 6:117-141.
- Daikhin Y, Yudkoff M (2000) Compartmentation of brain glutamate metabolism in neurons and glia. *The Journal of nutrition* 130:1026S-1031S.
- Dalet A, Bonsacquet J, Gaboyard-Niay S, Calin-Jageman I, Chidavaenzi RL, Venteo S, Desmadryl G, Goldberg JM, Lysakowski A, Chabbert C (2012) Glutamate transporters EAAT4 and EAAT5 are expressed in vestibular hair cells and calyx endings. *PloS one* 7:e46261.
- Danbolt NC (1994) The high affinity uptake system for excitatory amino acids in the brain. *Progress in neurobiology* 44:377-396.
- Danbolt NC (2001) Glutamate uptake. *Progress in neurobiology* 65:1-105.
- Danbolt NC, Storm-Mathisen J, Kanner BI (1992) An [Na⁺ + K⁺]coupled L-glutamate transporter purified from rat brain is located in glial cell processes. *Neuroscience* 51:295-310.
- De Bundel D, Schallier A, Loyens E, Fernando R, Miyashita H, Van Liefferinge J, Vermoesen K, Bannai S, Sato H, Michotte Y, Smolders I, Massie A (2011) Loss of system x(c)- does not induce oxidative stress but decreases extracellular glutamate in hippocampus and influences spatial working memory and limbic seizure susceptibility. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 31:5792-5803.
- de Vivo L, Melone M, Bucci G, Rothstein JD, Conti F (2010) Quantitative analysis of EAAT4 promoter activity in neurons and astrocytes of mouse somatic sensory cortex. *Neuroscience letters* 474:42-45.
- de Vries B, Mamsa H, Stam AH, Wan J, Bakker SL, Vanmolkot KR, Haan J, Terwindt GM, Boon EM, Howard BD, Frants RR, Baloh RW, Ferrari MD, Jen JC, van den Maagdenberg AM (2009) Episodic ataxia associated with EAAT1 mutation C186S affecting glutamate reuptake. *Arch Neurol* 66:97-101.

- de Wit SJ, Alonso P, Schweren L, Mataix-Cols D, Lochner C, Menchon JM, Stein DJ, Fouche JP, Soriano-Mas C, Sato JR, Hoexter MQ, Denys D, Nakamae T, Nishida S, Kwon JS, Jang JH, Busatto GF, Cardoner N, Cath DC, Fukui K, Jung WH, Kim SN, Miguel EC, Narumoto J, Phillips ML, Pujol J, Remijnse PL, Sakai Y, Shin NY, Yamada K, Veltman DJ, van den Heuvel OA (2014) Multicenter voxel-based morphometry mega-analysis of structural brain scans in obsessive-compulsive disorder. *The American journal of psychiatry* 171:340-349.
- Dehnes Y, Chaudhry FA, Ullensvang K, Lehre KP, Storm-Mathisen J, Danbolt NC (1998) The glutamate transporter EAAT4 in rat cerebellar Purkinje cells: a glutamate-gated chloride channel concentrated near the synapse in parts of the dendritic membrane facing astroglia. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 18:3606-3619.
- Dek EC, van den Hout MA, Engelhard IM, Giele CL, Cath DC (2015) Perseveration causes automatization of checking behavior in obsessive-compulsive disorder. *Behav Res Ther* 71:1-9.
- Denys D (2006) Pharmacotherapy of obsessive-compulsive disorder and obsessive-compulsive spectrum disorders. *The Psychiatric clinics of North America* 29:553-584, xi.
- Denys D, de Vries F, Cath D, Figeo M, Vulink N, Veltman DJ, van der Doef TF, Boellaard R, Westenberg H, van Balkom A, Lammertsma AA, van Berckel BN (2013) Dopaminergic activity in Tourette syndrome and obsessive-compulsive disorder. *Eur Neuropsychopharmacol* 23:1423-1431.
- Denys D, Zohar J, Westenberg HG (2004) The role of dopamine in obsessive-compulsive disorder: preclinical and clinical evidence. *The Journal of clinical psychiatry* 65 Suppl 14:11-17.
- Di Martino A, Scheres A, Margulies DS, Kelly AM, Uddin LQ, Shehzad Z, Biswal B, Walters JR, Castellanos FX, Milham MP (2008) Functional connectivity of human striatum: a resting state FMRI study. *Cerebral cortex* 18:2735-2747.
- Diamond JS (2001) Neuronal glutamate transporters limit activation of NMDA receptors by neurotransmitter spillover on CA1 pyramidal cells. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 21:8328-8338.
- Dickel DE, Veenstra-VanderWeele J, Bivens NC, Wu X, Fischer DJ, Van Etten-Lee M, Himle JA, Leventhal BL, Cook EH, Jr., Hanna GL (2007) Association studies of serotonin system candidate genes in early-onset obsessive-compulsive disorder. *Biological psychiatry* 61:322-329.
- Dickel DE, Veenstra-VanderWeele J, Cox NJ, Wu X, Fischer DJ, Van Etten-Lee M, Himle JA, Leventhal BL, Cook EH, Jr., Hanna GL (2006) Association testing of the positional and functional candidate gene SLC1A1/EAAC1 in early-onset obsessive-compulsive disorder. *Archives of general psychiatry* 63:778-785.
- Dittrich WH, Johansen T (2013) Cognitive deficits of executive functions and decision-making in obsessive-compulsive disorder. *Scand J Psychol* 54:393-400.

- Djamshidian A, Auerbeck BB, Lees AJ, O'Sullivan SS (2011) Clinical aspects of impulsive compulsive behaviours in Parkinson's disease. *J Neurol Sci* 310:183-188.
- Dold M, Aigner M, Lanzenberger R, Kasper S (2013) Antipsychotic augmentation of serotonin reuptake inhibitors in treatment-resistant obsessive-compulsive disorder: a meta-analysis of double-blind, randomized, placebo-controlled trials. *The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum* 16:557-574.
- Dold M, Aigner M, Lanzenberger R, Kasper S (2015) Antipsychotic Augmentation of Serotonin Reuptake Inhibitors in Treatment-Resistant Obsessive-Compulsive Disorder: An Update Meta-Analysis of Double-Blind, Randomized, Placebo-Controlled Trials. *The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum* 18.
- Dougherty DD, Rauch SL, Jenike MA (2004) Pharmacotherapy for obsessive-compulsive disorder. *Journal of clinical psychology* 60:1195-1202.
- Dowd LA, Robinson MB (1996) Rapid stimulation of EAAC1-mediated Na⁺-dependent L-glutamate transport activity in C6 glioma cells by phorbol ester. *Journal of neurochemistry* 67:508-516.
- Dunlop J, Marquis K (2006) Glutamate transport inhibitors as targets for treating psychosis. *Drug Discovery Today: Therapeutic Strategies* 3:533-537.
- Eaton WW, Martins SS, Nestadt G, Bienvenu OJ, Clarke D, Alexandre P (2008) The burden of mental disorders. *Epidemiol Rev* 30:1-14.
- Eliasof S, Arriza JL, Leighton BH, Amara SG, Kavanaugh MP (1998) Localization and function of five glutamate transporters cloned from the salamander retina. *Vision Res* 38:1443-1454.
- Erecinska M, Silver IA (1990) Metabolism and role of glutamate in mammalian brain. *Progress in neurobiology* 35:245-296.
- Etherton MR, Blaiss CA, Powell CM, Sudhof TC (2009) Mouse neurexin-1alpha deletion causes correlated electrophysiological and behavioral changes consistent with cognitive impairments. *Proceedings of the National Academy of Sciences of the United States of America* 106:17998-18003.
- Fairman WA, Vandenberg RJ, Arriza JL, Kavanaugh MP, Amara SG (1995) An excitatory amino-acid transporter with properties of a ligand-gated chloride channel. *Nature* 375:599-603.
- Farooqi IS (2008) Monogenic human obesity. *Front Horm Res* 36:1-11.
- Fernandez-Guasti A, Ulloa RE, Nicolini H (2003) Age differences in the sensitivity to clomipramine in an animal model of obsessive-compulsive disorder. *Psychopharmacology* 166:195-201.

- Fineberg NA, Reghunandan S, Brown A, Pampaloni I (2012) Pharmacotherapy of obsessive-compulsive disorder: Evidence-based treatment and beyond. *The Australian and New Zealand journal of psychiatry*.
- Flynn J, McBean GJ (2000) Kinetic and pharmacological analysis of L-[35S]cystine transport into rat brain synaptosomes. *Neurochem Int* 36:513-521.
- Fonnum F (1984) Glutamate: a neurotransmitter in mammalian brain. *Journal of neurochemistry* 42:1-11.
- Fonnum F (1993) Regulation of the synthesis of the transmitter glutamate pool. *Prog Biophys Mol Biol* 60:47-57.
- Fonseka TM, Richter MA, Muller DJ (2014) Second generation antipsychotic-induced obsessive-compulsive symptoms in schizophrenia: a review of the experimental literature. *Curr Psychiatry Rep* 16:510.
- Fontaine D, Mattei V, Borg M, von Langsdorff D, Magnie MN, Chanalet S, Robert P, Paquis P (2004) Effect of subthalamic nucleus stimulation on obsessive-compulsive disorder in a patient with Parkinson disease. Case report. *Journal of neurosurgery* 100:1084-1086.
- Franklin KBJ, Paxinos G (2013) Paxinos and Franklin's The mouse brain in stereotaxic coordinates. Amsterdam: Academic Press, an imprint of Elsevier.
- Franklin ME, Foa EB (2011) Treatment of obsessive compulsive disorder. *Annu Rev Clin Psychol* 7:229-243.
- Furness DN, Dehnes Y, Akhtar AQ, Rossi DJ, Hamann M, Grutle NJ, Gundersen V, Holmseth S, Lehre KP, Ullensvang K, Wojewodzic M, Zhou Y, Attwell D, Danbolt NC (2008) A quantitative assessment of glutamate uptake into hippocampal synaptic terminals and astrocytes: new insights into a neuronal role for excitatory amino acid transporter 2 (EAAT2). *Neuroscience* 157:80-94.
- Gardoni F, Di Luca M (2015) Targeting glutamatergic synapses in Parkinson's disease. *Curr Opin Pharmacol* 20:24-28.
- Geller D, Biederman J, Jones J, Park K, Schwartz S, Shapiro S, Coffey B (1998) Is juvenile obsessive-compulsive disorder a developmental subtype of the disorder? A review of the pediatric literature. *J Am Acad Child Adolesc Psychiatry* 37:420-427.
- Geller DA, Biederman J, Stewart SE, Mullin B, Martin A, Spencer T, Faraone SV (2003) Which SSRI? A meta-analysis of pharmacotherapy trials in pediatric obsessive-compulsive disorder. *The American journal of psychiatry* 160:1919-1928.
- Gondre-Lewis MC, Darius PJ, Wang H, Allard JS (2016) Stereological analyses of reward system nuclei in maternally deprived/separated alcohol drinking rats. *J Chem Neuroanat* 76:122-132.
- Gonzalez MI, Robinson MB (2004) Protein kinase C-dependent remodeling of glutamate transporter function. *Mol Interv* 4:48-58.

- Goodman WK, Foote KD, Greenberg BD, Ricciuti N, Bauer R, Ward H, Shapira NA, Wu SS, Hill CL, Rasmussen SA, Okun MS (2010) Deep brain stimulation for intractable obsessive compulsive disorder: pilot study using a blinded, staggered-onset design. *Biological psychiatry* 67:535-542.
- Goto Y, Grace AA (2007) The dopamine system and the pathophysiology of schizophrenia: a basic science perspective. *Int Rev Neurobiol* 78:41-68.
- Goto Y, Lee YA, Yamaguchi Y, Jas E (2016) Biological mechanisms underlying evolutionary origins of psychotic and mood disorders. *Neuroscience research*.
- Grados M, Wilcox HC (2007) Genetics of obsessive-compulsive disorder: a research update. *Expert review of neurotherapeutics* 7:967-980.
- Grados MA (2010) The genetics of obsessive-compulsive disorder and Tourette syndrome: an epidemiological and pathway-based approach for gene discovery. *J Am Acad Child Adolesc Psychiatry* 49:810-819, 819 e811-812.
- Grados MA, Samuels J, Shugart YY, Willour VL, Wang Y, Cullen B, Bienvenu OJ, Hoehn-Saric R, Valle D, Liang KY, Riddle MA, Wendland JR, Murphy DL, Nestadt G, Detera-Wadleigh S (2007) Rare plus common SERT variants in obsessive-compulsive disorder. *Molecular psychiatry* 12:422-423.
- Grant P, Lougee L, Hirschtritt M, Swedo SE (2007) An open-label trial of riluzole, a glutamate antagonist, in children with treatment-resistant obsessive-compulsive disorder. *Journal of child and adolescent psychopharmacology* 17:761-767.
- Grant PJ, Joseph LA, Farmer CA, Luckenbaugh DA, Lougee LC, Zarate CA, Jr., Swedo SE (2014) 12-week, placebo-controlled trial of add-on riluzole in the treatment of childhood-onset obsessive-compulsive disorder. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* 39:1453-1459.
- Greenberg BD, Gabriels LA, Malone DA, Jr., Rezai AR, Friehs GM, Okun MS, Shapira NA, Foote KD, Cosyns PR, Kubu CS, Malloy PF, Salloway SP, Giftakis JE, Rise MT, Machado AG, Baker KB, Stypulkowski PH, Goodman WK, Rasmussen SA, Nuttin BJ (2010) Deep brain stimulation of the ventral internal capsule/ventral striatum for obsessive-compulsive disorder: worldwide experience. *Molecular psychiatry* 15:64-79.
- Greenberg BD, Malone DA, Friehs GM, Rezai AR, Kubu CS, Malloy PF, Salloway SP, Okun MS, Goodman WK, Rasmussen SA (2006) Three-year outcomes in deep brain stimulation for highly resistant obsessive-compulsive disorder. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* 31:2384-2393.
- Greenfield A, Grosanu C, Dunlop J, McIlvain B, Carrick T, Jow B, Lu Q, Kowal D, Williams J, Butera J (2005) Synthesis and biological activities of aryl-ether-, biaryl-, and fluorene-aspartic acid and diaminopropionic acid analogs as potent inhibitors of the high-affinity glutamate transporter EAAT-2. *Bioorganic & medicinal chemistry letters* 15:4985-4988.
- Greer JM, Capecchi MR (2002) Hoxb8 is required for normal grooming behavior in mice. *Neuron* 33:23-34.

- Gross-Isseroff R, Cohen R, Sasson Y, Voet H, Zohar J (2004) Serotonergic dissection of obsessive compulsive symptoms: a challenge study with m-chlorophenylpiperazine and sumatriptan. *Neuropsychobiology* 50:200-205.
- Grunewald M, Bendahan A, Kanner BI (1998) Biotinylation of single cysteine mutants of the glutamate transporter GLT-1 from rat brain reveals its unusual topology. *Neuron* 21:623-632.
- Grunewald M, Kanner B (1995) Conformational changes monitored on the glutamate transporter GLT-1 indicate the existence of two neurotransmitter-bound states. *The Journal of biological chemistry* 270:17017-17024.
- Gu BM, Park JY, Kang DH, Lee SJ, Yoo SY, Jo HJ, Choi CH, Lee JM, Kwon JS (2008) Neural correlates of cognitive inflexibility during task-switching in obsessive-compulsive disorder. *Brain* 131:155-164.
- Haber SN, Heilbronner SR (2013) Translational research in OCD: circuitry and mechanisms. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* 38:252-253.
- Haghighi M, Jahangard L, Mohammad-Beigi H, Bajoghli H, Hafezian H, Rahimi A, Afshar H, Holsboer-Trachsler E, Brand S (2013) In a double-blind, randomized and placebo-controlled trial, adjuvant memantine improved symptoms in inpatients suffering from refractory obsessive-compulsive disorders (OCD). *Psychopharmacology* 228:633-640.
- Hammamieh R, Chakraborty N, De Lima TC, Meyerhoff J, Gautam A, Muhie S, D'Arpa P, Lumley L, Carroll E, Jett M (2012) Murine model of repeated exposures to conspecific trained aggressors simulates features of post-traumatic stress disorder. *Behav Brain Res* 235:55-66.
- Hanna G, Veenstra-Vander Weele J, Cox N, Boehnke M, Himle J, Curtis G, Leventhal B, Cook E (2002a) Genome-wide linkage analysis of families with obsessive-compulsive disorder ascertained through pediatric probands. *American Journal of Medical Genetics (Neuropsychiatric Genetics)* 114:541-552.
- Hanna GL, Veenstra-VanderWeele J, Cox NJ, Boehnke M, Himle JA, Curtis GC, Leventhal BL, Cook EH (2002b) Genome-wide linkage analysis of families with obsessive-compulsive disorder ascertained through pediatric probands. *American journal of medical genetics* 114:541-552.
- Hansen ES, Hasselbalch S, Law I, Bolwig TG (2002) The caudate nucleus in obsessive-compulsive disorder. Reduced metabolism following treatment with paroxetine: a PET study. *The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum* 5:1-10.
- Harada T, Harada C, Watanabe M, Inoue Y, Sakagawa T, Nakayama N, Sasaki S, Okuyama S, Watase K, Wada K, Tanaka K (1998) Functions of the two glutamate transporters GLAST and GLT-1 in the retina. *Proceedings of the National Academy of Sciences of the United States of America* 95:4663-4666.

- Hardingham GE, Bading H (2010) Synaptic versus extrasynaptic NMDA receptor signalling: implications for neurodegenerative disorders. *Nat Rev Neurosci* 11:682-696.
- Harrington EP, Moddel G, Najm IM, Baraban SC (2007) Altered glutamate receptor - transporter expression and spontaneous seizures in rats exposed to methylazoxymethanol in utero. *Epilepsia* 48:158-168.
- Harrison BJ, Pujol J, Cardoner N, Deus J, Alonso P, Lopez-Sola M, Contreras-Rodriguez O, Real E, Segalas C, Blanco-Hinojo L, Menchon JM, Soriano-Mas C (2013) Brain corticostriatal systems and the major clinical symptom dimensions of obsessive-compulsive disorder. *Biological psychiatry* 73:321-328.
- Harrison BJ, Soriano-Mas C, Pujol J, Ortiz H, Lopez-Sola M, Hernandez-Ribas R, Deus J, Alonso P, Yucel M, Pantelis C, Menchon JM, Cardoner N (2009) Altered corticostriatal functional connectivity in obsessive-compulsive disorder. *Archives of general psychiatry* 66:1189-1200.
- Hatalova H, Radostova D, Pistikova A, Vales K, Stuchlik A (2014) Spatial reversal learning in chronically sensitized rats and in undrugged sensitized rats with dopamine d2-like receptor agonist quinpirole. *Front Behav Neurosci* 8:122.
- Haugeto O, Ullensvang K, Levy LM, Chaudhry FA, Honore T, Nielsen M, Lehre KP, Danbolt NC (1996) Brain glutamate transporter proteins form homomultimers. *The Journal of biological chemistry* 271:27715-27722.
- Hayashi T (1952) A PHYSIOLOGICAL STUDY OF EPILEPTIC SEIZURES FOLLOWING CORTICAL STIMULATION IN ANIMALS AND ITS APPLICATION TO HUMAN CLINICS. *The Japanese Journal of Physiology* 3:46-64.
- He Y, Janssen WG, Rothstein JD, Morrison JH (2000) Differential synaptic localization of the glutamate transporter EAAC1 and glutamate receptor subunit GluR2 in the rat hippocampus. *The Journal of comparative neurology* 418:255-269.
- Hees B, Danbolt NC, Kanner BI, Haase W, Heitmann K, Koepsell H (1992) A monoclonal antibody against a Na(+)-L-glutamate cotransporter from rat brain. *The Journal of biological chemistry* 267:23275-23281.
- Hoffman KL, Hornig M, Yaddanapudi K, Jabado O, Lipkin WI (2004) A murine model for neuropsychiatric disorders associated with group A beta-hemolytic streptococcal infection. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 24:1780-1791.
- Hollmann M, Boulter J, Maron C, Heinemann S (1994) Molecular biology of glutamate receptors. Potentiation of N-methyl-D-aspartate receptor splice variants by zinc. *Ren Physiol Biochem* 17:182-183.
- Hollmann M, Heinemann S (1994) Cloned glutamate receptors. *Annu Rev Neurosci* 17:31-108.
- Holmseth S, Dehnes Y, Huang YH, Follin-Arbelet VV, Grutle NJ, Mylonakou MN, Plachez C, Zhou Y, Furness DN, Bergles DE, Lehre KP, Danbolt NC (2012) The density of EAAC1 (EAAT3) glutamate transporters expressed by neurons in the mammalian CNS. *The*

Journal of neuroscience : the official journal of the Society for Neuroscience 32:6000-6013.

- Holstege JC, de Graaff W, Hossaini M, Cardona Cano S, Jaarsma D, van den Akker E, Deschamps J (2008) Loss of Hoxb8 alters spinal dorsal laminae and sensory responses in mice. *Proceedings of the National Academy of Sciences of the United States of America* 105:6338-6343.
- Horiuchi Y, Iida S, Koga M, Ishiguro H, Iijima Y, Inada T, Watanabe Y, Someya T, Ujike H, Iwata N, Ozaki N, Kunugi H, Tochigi M, Itokawa M, Arai M, Niizato K, Iritani S, Kakita A, Takahashi H, Nawa H, Arinami T (2012) Association of SNPs linked to increased expression of SLC1A1 with schizophrenia. *Am J Med Genet B Neuropsychiatr Genet* 159B:30-37.
- Hu W, MacDonald ML, Elswick DE, Sweet RA (2015) The glutamate hypothesis of schizophrenia: evidence from human brain tissue studies. *Annals of the New York Academy of Sciences* 1338:38-57.
- Huang YH, Dykes-Hoberg M, Tanaka K, Rothstein JD, Bergles DE (2004) Climbing fiber activation of EAAT4 transporters and kainate receptors in cerebellar Purkinje cells. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 24:103-111.
- Ikemoto S (2002) Ventral striatal anatomy of locomotor activity induced by cocaine, D-amphetamine, dopamine and D1/D2 agonists. *Neuroscience* 113:939-955.
- Insel TR, Hamilton JA, Guttmacher LB, Murphy DL (1983) D-amphetamine in obsessive-compulsive disorder. *Psychopharmacology (Berl)* 80:231-235.
- Insel TR, Winslow JT (1992) Neurobiology of obsessive compulsive disorder. *The Psychiatric clinics of North America* 15:813-824.
- Ivarsson T, Skarphedinsson G, Kornor H, Axelsdottir B, Biedilae S, Heyman I, Asbahr F, Thomsen PH, Fineberg N, March J, Accreditation Task Force of The Canadian Institute for Obsessive Compulsive D (2015) The place of and evidence for serotonin reuptake inhibitors (SRIs) for obsessive compulsive disorder (OCD) in children and adolescents: Views based on a systematic review and meta-analysis. *Psychiatry Res* 227:93-103.
- Jacob S, Landeros-Weisenberger A, Leckman JF (2009) Autism spectrum and obsessive-compulsive disorders: OC behaviors, phenotypes and genetics. *Autism Res* 2:293-311.
- Jarzylo LA, Man HY (2012) Parasynaptic NMDA receptor signaling couples neuronal glutamate transporter function to AMPA receptor synaptic distribution and stability. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 32:2552-2563.
- Jen JC, Wan J, Palos TP, Howard BD, Baloh RW (2005) Mutation in the glutamate transporter EAAT1 causes episodic ataxia, hemiplegia, and seizures. *Neurology* 65:529-534.
- Kalueff AV, Stewart AM, Song C, Berridge KC, Graybiel AM, Fentress JC (2016) Neurobiology of rodent self-grooming and its value for translational neuroscience. *Nat Rev Neurosci* 17:45-59.

- Kanai Y, Hediger MA (1992) Primary structure and functional characterization of a high-affinity glutamate transporter. *Nature* 360:467-471.
- Kanai Y, Smith CP, Hediger MA (1993) A new family of neurotransmitter transporters: the high-affinity glutamate transporters. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 7:1450-1459.
- Kandel ER (2013) *Principles of neural science*. New York: McGraw-Hill.
- Kanner BI, Schuldiner S (1987) Mechanism of transport and storage of neurotransmitters. *CRC Crit Rev Biochem* 22:1-38.
- Karagiannidis I, Rizzo R, Tarnok Z, Wolanczyk T, Hebebrand J, Nothen MM, Lehmkuhl G, Farkas L, Nagy P, Barta C, Szymanska U, Panteloglou G, Miranda DM, Feng Y, Sandor P, Barr C, TsgeneSee, Paschou P (2012) Replication of association between a SLITRK1 haplotype and Tourette Syndrome in a large sample of families. *Mol Psychiatry* 17:665-668.
- Karlsson RM, Tanaka K, Saksida LM, Bussey TJ, Heilig M, Holmes A (2009) Assessment of glutamate transporter GLAST (EAAT1)-deficient mice for phenotypes relevant to the negative and executive/cognitive symptoms of schizophrenia. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* 34:1578-1589.
- Katayama K, Yamada K, Ornthanalai VG, Inoue T, Ota M, Murphy NP, Aruga J (2010) Slitrk1-deficient mice display elevated anxiety-like behavior and noradrenergic abnormalities. *Molecular psychiatry* 15:177-184.
- Keen-Kim D, Mathews CA, Reus VI, Lowe TL, Herrera LD, Budman CL, Gross-Tsur V, Pulver AE, Bruun RD, Erenberg G, Naarden A, Sabatti C, Freimer NB (2006) Overrepresentation of rare variants in a specific ethnic group may confuse interpretation of association analyses. *Hum Mol Genet* 15:3324-3328.
- Kessler RC, Chiu WT, Demler O, Merikangas KR, Walters EE (2005) Prevalence, severity, and comorbidity of 12-month DSM-IV disorders in the National Comorbidity Survey Replication. *Archives of general psychiatry* 62:617-627.
- Klanker M, Feenstra M, Denys D (2013) Dopaminergic control of cognitive flexibility in humans and animals. *Front Neurosci* 7:201.
- Klockner U, Storck T, Conradt M, Stoffel W (1993) Electrogenic L-glutamate uptake in *Xenopus laevis* oocytes expressing a cloned rat brain L-glutamate/L-aspartate transporter (GLAST-1). *The Journal of biological chemistry* 268:14594-14596.
- Koran LM (2000) Quality of life in obsessive-compulsive disorder. *The Psychiatric clinics of North America* 23:509-517.
- Koran LM, Hanna GL, Hollander E, Nestadt G, Simpson HB (2007) Practice guideline for the treatment of patients with obsessive-compulsive disorder. *The American journal of psychiatry* 164:5-53.

- Koran LM, Pallanti S, Quercioli L (2001a) Sumatriptan, 5-HT(1D) receptors and obsessive-compulsive disorder. *European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology* 11:169-172.
- Koran LM, Pallanti S, Quercioli L (2001b) Sumatriptan, 5-HT(1D) receptors and obsessive-compulsive disorder. *Eur Neuropsychopharmacol* 11:169-172.
- Kovac S, Domijan AM, Walker MC, Abramov AY (2012) Prolonged seizure activity impairs mitochondrial bioenergetics and induces cell death. *Journal of cell science* 125:1796-1806.
- Krebs G, Heyman I (2015) Obsessive-compulsive disorder in children and adolescents. *Arch Dis Child* 100:495-499.
- Krebs HA (1935) Metabolism of amino-acids. IV The synthesis of glutamine from glutamic acid and ammonia, and the enzymic hydrolysis of glutamine in animal tissues 29:1951-1969.
- Krizman-Genda E, Gonzalez MI, Zelenai O, Robinson MB (2005) Evidence that Akt mediates platelet-derived growth factor-dependent increases in activity and surface expression of the neuronal glutamate transporter, EAAC1. *Neuropharmacology* 49:872-882.
- Kugler P, Schmitt A (1999) Glutamate transporter EAAC1 is expressed in neurons and glial cells in the rat nervous system. *Glia* 27:129-142.
- Kushner MG, Kim SW, Donahue C, Thuras P, Adson D, Kotlyar M, McCabe J, Peterson J, Foa EB (2007) D-cycloserine augmented exposure therapy for obsessive-compulsive disorder. *Biological psychiatry* 62:835-838.
- Kwon JS, Joo YH, Nam HJ, Lim M, Cho E-Y, Jung MH, Choi J-S, Kim B, Kang D-H, Oh S, Park T, Hong KS (2009) Association of the glutamate transporter gene SLC1A1 with atypical antipsychotics-induced obsessive-compulsive symptoms. *Archives of general psychiatry* 66:1233-1241.
- Lane MC, Jackson JG, Krizman EN, Rothstein JD, Porter BE, Robinson MB (2014) Genetic deletion of the neuronal glutamate transporter, EAAC1, results in decreased neuronal death after pilocarpine-induced status epilepticus. *Neurochem Int* 73:152-158.
- Lehre KP, Levy LM, Ottersen OP, Storm-Mathisen J, Danbolt NC (1995) Differential expression of two glial glutamate transporters in the rat brain: quantitative and immunocytochemical observations. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 15:1835-1853.
- Levenson J, Weeber E, Selcher JC, Kategaya LS, Sweatt JD, Eskin A (2002) Long-term potentiation and contextual fear conditioning increase neuronal glutamate uptake. *Nature neuroscience* 5:155-161.
- Levy LM, Attwell D, Hoover F, Ash JF, Bjoras M, Danbolt NC (1998) Inducible expression of the GLT-1 glutamate transporter in a CHO cell line selected for low endogenous glutamate uptake. *FEBS letters* 422:339-342.

- Li MH, Underhill SM, Reed C, Phillips TJ, Amara SG, Ingram SL (2017) Amphetamine and Methamphetamine Increase NMDAR-GluN2B Synaptic Currents in Midbrain Dopamine Neurons. *Neuropsychopharmacology* : official publication of the American College of Neuropsychopharmacology.
- Li X, May RS, Tolbert LC, Jackson WT, Flournoy JM, Baxter LR (2005) Risperidone and haloperidol augmentation of serotonin reuptake inhibitors in refractory obsessive-compulsive disorder: a crossover study. *The Journal of clinical psychiatry* 66:736-743.
- Li X, Morrow D, Witkin JM (2006) Decreases in nestlet shredding of mice by serotonin uptake inhibitors: comparison with marble burying. *Life sciences* 78:1933-1939.
- Lin CI, Orlov I, Ruggiero AM, Dykes-Hoberg M, Lee A, Jackson M, Rothstein JD (2001) Modulation of the neuronal glutamate transporter EAAC1 by the interacting protein GTRAP3-18. *Nature* 410:84-88.
- Logan WJ, Snyder SH (1972) High affinity uptake systems for glycine, glutamic and aspartic acids in synaptosomes of rat central nervous tissues. *Brain research* 42:413-431.
- Loiselle CR, Lee O, Moran TH, Singer HS (2004) Striatal microinfusion of Tourette syndrome and PANDAS sera: failure to induce behavioral changes. *Mov Disord* 19:390-396.
- Lotan D, Benhar I, Alvarez K, Mascaro-Blanco A, Brimberg L, Frenkel D, Cunningham MW, Joel D (2014) Behavioral and neural effects of intra-striatal infusion of anti-streptococcal antibodies in rats. *Brain Behav Immun* 38:249-262.
- Lykouras L, Alevizos B, Michalopoulou P, Rabavilas A (2003) Obsessive-compulsive symptoms induced by atypical antipsychotics. A review of the reported cases. *Progress in neuro-psychopharmacology & biological psychiatry* 27:333-346.
- MacMaster FP, O'Neill J, Rosenberg DR (2008) Brain imaging in pediatric obsessive-compulsive disorder. *J Am Acad Child Adolesc Psychiatry* 47:1262-1272.
- Madruga-Garrido M, Mir P (2013) Tics and other stereotyped movements as side effects of pharmacological treatment. *Int Rev Neurobiol* 112:481-494.
- Maia TV, Cooney RE, Peterson BS (2008) The neural bases of obsessive-compulsive disorder in children and adults. *Development and psychopathology* 20:1251-1283.
- Mallet L, Mesnage V, Houeto JL, Pelissolo A, Yelnik J, Behar C, Gargiulo M, Welter ML, Bonnet AM, Pillon B, Cornu P, Dormont D, Pidoux B, Allilaire JF, Agid Y (2002) Compulsions, Parkinson's disease, and stimulation. *Lancet* 360:1302-1304.
- Mallet L, Polosan M, Jaafari N, Baup N, Welter ML, Fontaine D, du Montcel ST, Yelnik J, Chereau I, Arbus C, Raoul S, Aouizerate B, Damier P, Chabardes S, Czernecki V, Ardouin C, Krebs MO, Bardinet E, Chaynes P, Burbaud P, Cornu P, Derost P, Bougerol T, Bataille B, Mattei V, Dormont D, Devaux B, Verin M, Houeto JL, Pollak P, Benabid AL, Agid Y, Krack P, Millet B, Pelissolo A, Group SS (2008) Subthalamic nucleus stimulation in severe obsessive-compulsive disorder. *The New England journal of medicine* 359:2121-2134.

- Martino D, Defazio G, Giovannoni G (2009) The PANDAS subgroup of tic disorders and childhood-onset obsessive-compulsive disorder. *J Psychosom Res* 67:547-557.
- Martucci L, Wong AH, De Luca V, Likhodi O, Wong GW, King N, Kennedy JL (2006) N-methyl-D-aspartate receptor NR2B subunit gene GRIN2B in schizophrenia and bipolar disorder: Polymorphisms and mRNA levels. *Schizophr Res* 84:214-221.
- Massie A, Cnops L, Smolders I, McCullumsmith R, Kooijman R, Kwak S, Arckens L, Michotte Y (2008) High-affinity Na⁺/K⁺-dependent glutamate transporter EAAT4 is expressed throughout the rat fore- and midbrain. *The Journal of comparative neurology* 511:155-172.
- Mathews CA, Badner JA, Andresen JM, Sheppard B, Himle JA, Grant JE, Williams KA, Chavira DA, Azzam A, Schwartz M, Reus VI, Kim SW, Cook EH, Hanna GL (2012) Genome-wide linkage analysis of obsessive-compulsive disorder implicates chromosome 1p36. *Biol Psychiatry* 72:629-636.
- Mathews GC, Diamond JS (2003) Neuronal glutamate uptake contributes to GABA synthesis and inhibitory synaptic strength. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 23:2040-2048.
- Mattheisen M, Samuels JF, Wang Y, Greenberg BD, Fyer AJ, McCracken JT, Geller DA, Murphy DL, Knowles JA, Grados MA, Riddle MA, Rasmussen SA, McLaughlin NC, Nurmi EL, Askland KD, Qin HD, Cullen BA, Piacentini J, Pauls DL, Bienvenu OJ, Stewart SE, Liang KY, Goes FS, Maher B, Pulver AE, Shugart YY, Valle D, Lange C, Nestadt G (2015) Genome-wide association study in obsessive-compulsive disorder: results from the OCGAS. *Molecular psychiatry* 20:337-344.
- Matthews JC, Beveridge MJ, Malandro MS, Rothstein JD, Campbell-Thompson M, Verlander JW, Kilberg MS, Novak DA (1998) Activity and protein localization of multiple glutamate transporters in gestation day 14 vs. day 20 rat placenta. *Am J Physiol* 274:C603-614.
- McCullumsmith RE, Meador-Woodruff JH (2002) Striatal excitatory amino acid transporter transcript expression in schizophrenia, bipolar disorder, and major depressive disorder. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* 26:368-375.
- McGrath MJ, Campbell KM, Parks CR, Burton FH (2000) Glutamatergic drugs exacerbate symptomatic behavior in a transgenic model of comorbid Tourette's syndrome and obsessive-compulsive disorder. *Brain research* 877:23-30.
- Mehta SL, Manhas N, Raghubir R (2007) Molecular targets in cerebral ischemia for developing novel therapeutics. *Brain Res Rev* 54:34-66.
- Menzies L, Chamberlain SR, Laird AR, Thelen SM, Sahakian BJ, Bullmore ET (2008) Integrating evidence from neuroimaging and neuropsychological studies of obsessive-compulsive disorder: the orbitofronto-striatal model revisited. *Neuroscience and biobehavioral reviews* 32:525-549.
- Michael S. Ritsner AGA (2007) Quality of Life Impairment in Schizophrenia, Mood and Anxiety Disorders. *New Perspectives on Research and Treatment*.

- Milad MR, Rauch SL (2012) Obsessive-compulsive disorder: beyond segregated cortico-striatal pathways. *Trends in cognitive sciences* 16:43-51.
- Miranda DM, Wigg K, Kabia EM, Feng Y, Sandor P, Barr CL (2009) Association of SLITRK1 to Gilles de la Tourette Syndrome. *Am J Med Genet B Neuropsychiatr Genet* 150B:483-486.
- Mitani A, Tanaka K (2003) Functional changes of glial glutamate transporter GLT-1 during ischemia: an in vivo study in the hippocampal CA1 of normal mice and mutant mice lacking GLT-1. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 23:7176-7182.
- Moghaddam B, Javitt D (2012) From revolution to evolution: the glutamate hypothesis of schizophrenia and its implication for treatment. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* 37:4-15.
- Monzani B, Rijdsdijk F, Harris J, Mataix-Cols D (2014) The structure of genetic and environmental risk factors for dimensional representations of DSM-5 obsessive-compulsive spectrum disorders. *JAMA Psychiatry* 71:182-189.
- Moore GJ, MacMaster FP, Stewart C, Rosenberg DR (1998) Case study: caudate glutamatergic changes with paroxetine therapy for pediatric obsessive-compulsive disorder. *Journal of the American Academy of Child and Adolescent Psychiatry* 37:663-667.
- Morris-Berry CM, Pollard M, Gao S, Thompson C, Tourette Syndrome Study G, Singer HS (2013) Anti-streptococcal, tubulin, and dopamine receptor 2 antibodies in children with PANDAS and Tourette syndrome: single-point and longitudinal assessments. *J Neuroimmunol* 264:106-113.
- Mundt A, Klein J, Joel D, Heinz A, Djodari-Irani A, Harnack D, Kupsch A, Orawa H, Juckel G, Morgenstern R, Winter C (2009) High-frequency stimulation of the nucleus accumbens core and shell reduces quinpirole-induced compulsive checking in rats. *The European journal of neuroscience* 29:2401-2412.
- Murray RM, Paparelli A, Morrison PD, Marconi A, Di Forti M (2013) What can we learn about schizophrenia from studying the human model, drug-induced psychosis? *Am J Med Genet B Neuropsychiatr Genet* 162B:661-670.
- Myles-Worsley M, Tiobech J, Browning SR, Korn J, Goodman S, Gentile K, Melhem N, Byerley W, Faraone SV, Middleton FA (2013) Deletion at the SLC1A1 glutamate transporter gene co-segregates with schizophrenia and bipolar schizoaffective disorder in a 5-generation family. *Am J Med Genet B Neuropsychiatr Genet* 162B:87-95.
- Nafia I, Re DB, Masméjean F, Melon C, Kachidian P, Kerkerian-Le Goff L, Nieouillon A, Had-Aissouni L (2008) Preferential vulnerability of mesencephalic dopamine neurons to glutamate transporter dysfunction. *Journal of neurochemistry* 105:484-496.
- Najimi M, Maloteaux JM, Hermans E (2002) Cytoskeleton-related trafficking of the EAAC1 glutamate transporter after activation of the G(q/11)-coupled neurotensin receptor NTS1. *FEBS letters* 523:224-228.

- Najimi M, Maloteaux JM, Hermans E (2005) Pertussis toxin-sensitive modulation of glutamate transport by endothelin-1 type A receptors in glioma cells. *Biochimica et biophysica acta* 1668:195-202.
- Nakanishi S, Nakajima Y, Masu M, Ueda Y, Nakahara K, Watanabe D, Yamaguchi S, Kawabata S, Okada M (1998) Glutamate receptors: brain function and signal transduction. *Brain Res Brain Res Rev* 26:230-235.
- Nakao T, Nakagawa A, Yoshiura T, Nakatani E, Nabeyama M, Yoshizato C, Kudoh A, Tada K, Yoshioka K, Kawamoto M, Togao O, Kanba S (2005) Brain activation of patients with obsessive-compulsive disorder during neuropsychological and symptom provocation tasks before and after symptom improvement: a functional magnetic resonance imaging study. *Biological psychiatry* 57:901-910.
- Nakatani E, Krebs G, Micali N, Turner C, Heyman I, Mataix-Cols D (2011) Children with very early onset obsessive-compulsive disorder: clinical features and treatment outcome. *J Child Psychol Psychiatry* 52:1261-1268.
- Nelson N (1998) The family of Na⁺/Cl⁻ neurotransmitter transporters. *Journal of neurochemistry* 71:1785-1803.
- Nicholls D, Attwell D (1990) The release and uptake of excitatory amino acids. *Trends Pharmacol Sci* 11:462-468.
- Nicolas LB, Kolb Y, Prinssen EP (2006) A combined marble burying-locomotor activity test in mice: a practical screening test with sensitivity to different classes of anxiolytics and antidepressants. *Eur J Pharmacol* 547:106-115.
- Nieouillon A, Canolle B, Masméjean F, Guillet B, Pisano P, Lortet S (2006) The neuronal excitatory amino acid transporter EAAC1/EAAT3: does it represent a major actor at the brain excitatory synapse? *Journal of neurochemistry* 98:1007-1018.
- Nikkuni O, Takayasu Y, Iino M, Tanaka K, Ozawa S (2007) Facilitated activation of metabotropic glutamate receptors in cerebellar Purkinje cells in glutamate transporter EAAT4-deficient mice. *Neuroscience research* 59:296-303.
- Niswender CM, Conn PJ (2010) Metabotropic glutamate receptors: physiology, pharmacology, and disease. *Annu Rev Pharmacol Toxicol* 50:295-322.
- Nordstrom EJ, Burton FH (2002) A transgenic model of comorbid Tourette's syndrome and obsessive-compulsive disorder circuitry. *Molecular psychiatry* 7:617-625, 524.
- Nudmamud-Thanoi S, Piyabhan P, Harte MK, Cahir M, Reynolds GP (2007) Deficits of neuronal glutamatergic markers in the caudate nucleus in schizophrenia. *J Neural Transm Suppl* 281-285.
- O'Roak BJ, Morgan TM, Fishman DO, Saus E, Alonso P, Gratacos M, Estivill X, Teltsh O, Kohn Y, Kidd KK, Cho J, Lifton RP, State MW (2010) Additional support for the association of SLITRK1 var321 and Tourette syndrome. *Mol Psychiatry* 15:447-450.

- O'Sullivan RL, Mansueto CS, Lerner EA, Miguel EC (2000) Characterization of trichotillomania. A phenomenological model with clinical relevance to obsessive-compulsive spectrum disorders. *The Psychiatric clinics of North America* 23:587-604.
- Ooms P, Mantione M, Figeo M, Schuurman PR, van den Munckhof P, Denys D (2014) Deep brain stimulation for obsessive-compulsive disorders: long-term analysis of quality of life. *J Neurol Neurosurg Psychiatry* 85:153-158.
- Otis TS, Brasnjo G, Dzubay JA, Pratap M (2004) Interactions between glutamate transporters and metabotropic glutamate receptors at excitatory synapses in the cerebellar cortex. *Neurochemistry international* 45:537-544.
- Otis TS, Kavanaugh MP (2000) Isolation of current components and partial reaction cycles in the glial glutamate transporter EAAT2. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 20:2749-2757.
- Ozaki N, Goldman D, Kaye WH, Plotnicov K, Greenberg BD, Lappalainen J, Rudnick G, Murphy DL (2003) Serotonin transporter missense mutation associated with a complex neuropsychiatric phenotype. *Molecular psychiatry* 8:933-936.
- Ozawa S, Kamiya H, Tsuzuki K (1998) Glutamate receptors in the mammalian central nervous system. *Progress in neurobiology* 54:581-618.
- Ozomaro U, Cai G, Kajiwara Y, Yoon S, Makarov V, Delorme R, Betancur C, Ruhrmann S, Falkai P, Grabe HJ, Maier W, Wagner M, Lennertz L, Moessner R, Murphy DL, Buxbaum JD, Zuchner S, Grice DE (2013) Characterization of SLITRK1 variation in obsessive-compulsive disorder. *PLoS One* 8:e70376.
- Pakkenberg B, Gundersen HJ (1989) New stereological method for obtaining unbiased and efficient estimates of total nerve cell number in human brain areas. Exemplified by the mediodorsal thalamic nucleus in schizophrenics. *APMIS : acta pathologica, microbiologica, et immunologica Scandinavica* 97:677-681.
- Pancani T, Bolarinwa C, Smith Y, Lindsley CW, Conn PJ, Xiang Z (2014) M4 mAChR-mediated modulation of glutamatergic transmission at corticostriatal synapses. *ACS Chem Neurosci* 5:318-324.
- Parent A, Hazrati LN (1995) Functional anatomy of the basal ganglia. I. The cortico-basal ganglia-thalamo-cortical loop. *Brain Res Brain Res Rev* 20:91-127.
- Parraga HC, Parraga MI, Harris DK (2007) Tic exacerbation and precipitation during atomoxetine treatment in two children with attention-deficit hyperactivity disorder. *Int J Psychiatry Med* 37:415-424.
- Pasquini M, Biondi M (2006) Memantine augmentation for refractory obsessive-compulsive disorder. *Progress in neuro-psychopharmacology & biological psychiatry* 30:1173-1175.
- Pauls DL (2008) The genetics of obsessive compulsive disorder: a review of the evidence. *American journal of medical genetics Part C, Seminars in medical genetics* 148C:133-139.

- Pauls DL (2010) The genetics of obsessive-compulsive disorder: a review. *Dialogues Clin Neurosci* 12:149-163.
- Pauls DL, Abramovitch A, Rauch SL, Geller DA (2014) Obsessive-compulsive disorder: an integrative genetic and neurobiological perspective. *Nat Rev Neurosci* 15:410-424.
- Pavone P, Bianchini R, Parano E, Incorpora G, Rizzo R, Mazzone L, Trifiletti RR (2004) Anti-brain antibodies in PANDAS versus uncomplicated streptococcal infection. *Pediatr Neurol* 30:107-110.
- Pearlman DM, Vora HS, Marquis BG, Najjar S, Dudley LA (2014) Anti-basal ganglia antibodies in primary obsessive-compulsive disorder: systematic review and meta-analysis. *Br J Psychiatry* 205:8-16.
- Peca J, Feliciano C, Ting JT, Wang W, Wells MF, Venkatraman TN, Lascola CD, Fu Z, Feng G (2011) Shank3 mutant mice display autistic-like behaviours and striatal dysfunction. *Nature* 472:437-442.
- Peghini P, Janzen J, Stoffel W (1997) Glutamate transporter EAAC-1-deficient mice develop dicarboxylic aminoaciduria and behavioral abnormalities but no neurodegeneration. *The EMBO journal* 16:3822-3832.
- Penagarikano O, Abrahams BS, Herman EI, Winden KD, Gdalyahu A, Dong H, Sonnenblick LI, Gruver R, Almajano J, Bragin A, Golshani P, Trachtenberg JT, Peles E, Geschwind DH (2011) Absence of CNTNAP2 leads to epilepsy, neuronal migration abnormalities, and core autism-related deficits. *Cell* 147:235-246.
- Pines G, Danbolt NC, Bjoras M, Zhang Y, Bendahan A, Eide L, Koepsell H, Storm-Mathisen J, Seeberg E, Kanner BI (1992) Cloning and expression of a rat brain L-glutamate transporter. *Nature* 360:464-467.
- Pittenger C (2015) Glutamatergic agents for OCD and related disorders. *Curr Treat Options Psychiatry* 2:271-283.
- Pittenger C, Bloch MH, Williams K (2011) Glutamate abnormalities in obsessive compulsive disorder: neurobiology, pathophysiology, and treatment. *Pharmacol Ther* 132:314-332.
- Pittenger C, Kelmendi B, Wasyluk S, Bloch MH, Coric V (2008) Riluzole augmentation in treatment-refractory obsessive-compulsive disorder: a series of 13 cases, with long-term follow-up. *J Clin Psychopharmacol* 28:363-367.
- Pooley EC, Fineberg N, Harrison PJ (2007) The met(158) allele of catechol-O-methyltransferase (COMT) is associated with obsessive-compulsive disorder in men: case-control study and meta-analysis. *Molecular psychiatry* 12:556-561.
- Porton B, Greenberg BD, Askland K, Serra LM, Gesmonde J, Rudnick G, Rasmussen SA, Kao HT (2013) Isoforms of the neuronal glutamate transporter gene, SLC1A1/EAAC1, negatively modulate glutamate uptake: relevance to obsessive-compulsive disorder. *Transl Psychiatry* 3:e259.

- Posner J, Marsh R, Maia TV, Peterson BS, Gruber A, Simpson HB (2014) Reduced functional connectivity within the limbic cortico-striato-thalamo-cortical loop in unmedicated adults with obsessive-compulsive disorder. *Hum Brain Mapp* 35:2852-2860.
- Poyurovsky M, Weizman R, Weizman A, Koran L (2005) Memantine for treatment-resistant OCD. *The American journal of psychiatry* 162:2191-2192.
- Proenca CC, Gao KP, Shmelkov SV, Rafii S, Lee FS (2011) Slitrks as emerging candidate genes involved in neuropsychiatric disorders. *Trends in neurosciences* 34:143-153.
- Pujol J, Soriano-Mas C, Alonso P, Cardoner N, Menchon JM, Deus J, Vallejo J (2004) Mapping structural brain alterations in obsessive-compulsive disorder. *Archives of general psychiatry* 61:720-730.
- Qin S, Zhao X, Pan Y, Liu J, Feng G, Fu J, Bao J, Zhang Z, He L (2005) An association study of the N-methyl-D-aspartate receptor NR1 subunit gene (GRIN1) and NR2B subunit gene (GRIN2B) in schizophrenia with universal DNA microarray. *Eur J Hum Genet* 13:807-814.
- Radua J, Mataix-Cols D (2009) Voxel-wise meta-analysis of grey matter changes in obsessive-compulsive disorder. *The British journal of psychiatry : the journal of mental science* 195:393-402.
- Radua J, van den Heuvel OA, Surguladze S, Mataix-Cols D (2010) Meta-analytical comparison of voxel-based morphometry studies in obsessive-compulsive disorder vs other anxiety disorders. *Archives of general psychiatry* 67:701-711.
- Rakhade SN, Loeb JA (2008) Focal reduction of neuronal glutamate transporters in human neocortical epilepsy. *Epilepsia* 49:226-236.
- Rao JS, Kellom M, Reese EA, Rapoport SI, Kim HW (2012) Dysregulated glutamate and dopamine transporters in postmortem frontal cortex from bipolar and schizophrenic patients. *J Affect Disord* 136:63-71.
- Rauch SL, Dougherty DD, Malone D, Rezai A, Friehs G, Fischman AJ, Alpert NM, Haber SN, Stypulkowski PH, Rise MT, Rasmussen SA, Greenberg BD (2006) A functional neuroimaging investigation of deep brain stimulation in patients with obsessive-compulsive disorder. *Journal of neurosurgery* 104:558-565.
- Rauch SL, Savage CR (1997) Neuroimaging and neuropsychology of the striatum. Bridging basic science and clinical practice. *The Psychiatric clinics of North America* 20:741-768.
- Rauch SL, Savage CR, Alpert NM, Fischman AJ, Jenike MA (1997) The functional neuroanatomy of anxiety: a study of three disorders using positron emission tomography and symptom provocation. *Biological psychiatry* 42:446-452.
- Rauch SL, Shin LM, Dougherty DD, Alpert NM, Fischman AJ, Jenike MA (2002) Predictors of fluvoxamine response in contamination-related obsessive compulsive disorder: a PET symptom provocation study. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* 27:782-791.

- Rauch SL, Whalen PJ, Curran T, Shin LM, Coffey BJ, Savage CR, McInerney SC, Baer L, Jenike MA (2001) Probing striato-thalamic function in obsessive-compulsive disorder and Tourette syndrome using neuroimaging methods. *Adv Neurol* 85:207-224.
- Rauen T (2000) Diversity of glutamate transporter expression and function in the mammalian retina. *Amino Acids* 19:53-62.
- Rauen T, Wiessner M (2000) Fine tuning of glutamate uptake and degradation in glial cells: common transcriptional regulation of GLAST1 and GS. *Neurochem Int* 37:179-189.
- Rebec GV, White IM, Puotz JK (1997) Responses of neurons in dorsal striatum during amphetamine-induced focused stereotypy. *Psychopharmacology* 130:343-351.
- Remijnse PL, Nielen MM, van Balkom AJ, Cath DC, van Oppen P, Uylings HB, Veltman DJ (2006) Reduced orbitofrontal-striatal activity on a reversal learning task in obsessive-compulsive disorder. *Archives of general psychiatry* 63:1225-1236.
- Remijnse PL, van den Heuvel OA, Nielen MM, Vriend C, Hendriks GJ, Hoogendijk WJ, Uylings HB, Veltman DJ (2013) Cognitive inflexibility in obsessive-compulsive disorder and major depression is associated with distinct neural correlates. *PLoS one* 8:e59600.
- Rodman AM, Milad MR, Deckersbach T, Im J, Chou T, Dougherty DD (2012) Neuroimaging contributions to novel surgical treatments for intractable obsessive-compulsive disorder. *Expert Rev Neurother* 12:219-227.
- Rodriguez CI, Kegeles LS, Flood P, Simpson HB (2011) Rapid resolution of obsessions after an infusion of intravenous ketamine in a patient with treatment-resistant obsessive-compulsive disorder. *The Journal of clinical psychiatry* 72:567-569.
- Rodriguez CI, Kegeles LS, Levinson A, Feng T, Marcus SM, Vermes D, Flood P, Simpson HB (2013) Randomized controlled crossover trial of ketamine in obsessive-compulsive disorder: proof-of-concept. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* 38:2475-2483.
- Rodriguez-Romaguera J, Do Monte FH, Quirk GJ (2012) Deep brain stimulation of the ventral striatum enhances extinction of conditioned fear. *Proceedings of the National Academy of Sciences of the United States of America* 109:8764-8769.
- Rosenberg DR, Hanna GL (2000) Genetic and imaging strategies in obsessive-compulsive disorder: potential implications for treatment development. *Biological psychiatry* 48:1210-1222.
- Rosenberg DR, Keshavan MS (1998) A.E. Bennett Research Award. Toward a neurodevelopmental model of obsessive-compulsive disorder. *Biological psychiatry* 43:623-640.
- Rosenberg DR, Keshavan MS, O'Hearn KM, Dick EL, Bagwell WW, Seymour AB, Montrose DM, Pierri JN, Birmaher B (1997) Frontostriatal measurement in treatment-naive children with obsessive-compulsive disorder. *Archives of general psychiatry* 54:824-830.

- Rosenberg DR, MacMaster FP, Keshavan MS, Fitzgerald KD, Stewart CM, Moore GJ (2000) Decrease in caudate glutamatergic concentrations in pediatric obsessive-compulsive disorder patients taking paroxetine. *Journal of the American Academy of Child and Adolescent Psychiatry* 39:1096-1103.
- Rosenberg DR, MacMillan SN, Moore GJ (2001) Brain anatomy and chemistry may predict treatment response in paediatric obsessive-compulsive disorder. *The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum* 4:179-190.
- Rosenberg DR, Mirza Y, Russell A, Tang J, Smith JM, Banerjee SP, Bhandari R, Rose M, Ivey J, Boyd C, Moore GJ (2004) Reduced anterior cingulate glutamatergic concentrations in childhood OCD and major depression versus healthy controls. *J Am Acad Child Adolesc Psychiatry* 43:1146-1153.
- Rotge J-Y, Guehl D, Dilharreguy B, Cuny E, Tignol J, Bioulac B, Allard M, Burbaud P, Aouizerate B (2008) Provocation of obsessive-compulsive symptoms: a quantitative voxel-based meta-analysis of functional neuroimaging studies. *Journal of psychiatry & neuroscience : JPN* 33:405-412.
- Rotge JY, Guehl D, Dilharreguy B, Tignol J, Bioulac B, Allard M, Burbaud P, Aouizerate B (2009) Meta-analysis of brain volume changes in obsessive-compulsive disorder. *Biological psychiatry* 65:75-83.
- Rothstein JD, Dykes-Hoberg M, Pardo CA, Bristol LA, Jin L, Kuncl RW, Kanai Y, Hediger MA, Wang Y, Schielke JP, Welty DF (1996) Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. *Neuron* 16:675-686.
- Rothstein JD, Martin L, Levey AI, Dykes-Hoberg M, Jin L, Wu D, Nash N, Kuncl RW (1994) Localization of neuronal and glial glutamate transporters. *Neuron* 13:713-725.
- Ruscio AM, Stein DJ, Chiu WT, Kessler RC (2010) The epidemiology of obsessive-compulsive disorder in the National Comorbidity Survey Replication. *Molecular psychiatry* 15:53-63.
- Ryu S, Oh S, Cho E-Y, Nam HJ, Yoo JH, Park T, Joo YH, Kwon JS, Hong KS (2011) Interaction between genetic variants of DLGAP3 and SLC1A1 affecting the risk of atypical antipsychotics-induced obsessive-compulsive symptoms. *American journal of medical genetics Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics* 156B:949-959.
- Saier MH, Jr. (1999) Eukaryotic transmembrane solute transport systems. *Int Rev Cytol* 190:61-136.
- Saiz PA, Garcia-Portilla MP, Arango C, Morales B, Bascaran MT, Martinez-Barrondo S, Florez G, Sotomayor E, Paredes B, Alvarez C, San Narciso G, Carreno E, Bombin I, Alvarez V, Coto E, Fernandez JM, Bousoño M, Bobes J (2008) Association study between obsessive-compulsive disorder and serotonergic candidate genes. *Progress in neuro-psychopharmacology & biological psychiatry* 32:765-770.

- Samuels J, Wang Y, Riddle MA, Greenberg BD, Fyer AJ, McCracken JT, Rauch SL, Murphy DL, Grados MA, Knowles JA, Piacentini J, Cullen B, Bienvenu OJ, Rasmussen SA, Geller D, Pauls DL, Liang K-Y, Shugart YY, Nestadt G (2011) Comprehensive family-based association study of the glutamate transporter gene SLC1A1 in obsessive-compulsive disorder. *American journal of medical genetics Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics* 156B:472-477.
- Sarris J, Oliver G, Camfield DA, Dean OM, Dowling N, Smith DJ, Murphy J, Menon R, Berk M, Blair-West S, Ng CH (2015) N-Acetyl Cysteine (NAC) in the Treatment of Obsessive-Compulsive Disorder: A 16-Week, Double-Blind, Randomised, Placebo-Controlled Study. *CNS Drugs* 29:801-809.
- Sato H, Nomura S, Maebara K, Sato K, Tamba M, Bannai S (2004) Transcriptional control of cystine/glutamate transporter gene by amino acid deprivation. *Biochemical and biophysical research communications* 325:109-116.
- Sato H, Shiiya A, Kimata M, Maebara K, Tamba M, Sakakura Y, Makino N, Sugiyama F, Yagami K, Moriguchi T, Takahashi S, Bannai S (2005) Redox imbalance in cystine/glutamate transporter-deficient mice. *The Journal of biological chemistry* 280:37423-37429.
- Sato H, Tamba M, Ishii T, Bannai S (1999) Cloning and expression of a plasma membrane cystine/glutamate exchange transporter composed of two distinct proteins. *The Journal of biological chemistry* 274:11455-11458.
- Sato H, Tamba M, Okuno S, Sato K, Keino-Masu K, Masu M, Bannai S (2002) Distribution of cystine/glutamate exchange transporter, system x(c)-, in the mouse brain. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 22:8028-8033.
- Saxena S, Bota RG, Brody AL (2001) Brain-behavior relationships in obsessive-compulsive disorder. *Seminars in clinical neuropsychiatry* 6:82-101.
- Saxena S, Brody AL, Schwartz JM, Baxter LR (1998) Neuroimaging and frontal-subcortical circuitry in obsessive-compulsive disorder. *Br J Psychiatry Suppl* 26-37.
- Saxena S, Rauch SL (2000) Functional neuroimaging and the neuroanatomy of obsessive-compulsive disorder. *The Psychiatric clinics of North America* 23:563-586.
- Scharf JM, Moorjani P, Fagerness J, Platko JV, Illmann C, Galloway B, Jenike E, Stewart SE, Pauls DL, Tourette Syndrome International Consortium for G (2008) Lack of association between SLITRK1var321 and Tourette syndrome in a large family-based sample. *Neurology* 70:1495-1496.
- Schirmbeck F, Nieratschker V, Frank J, Englisch S, Rausch F, Meyer-Lindenberg A, Rietschel M, Zink M (2012) Polymorphisms in the glutamate transporter gene SLC1A1 and obsessive-compulsive symptoms induced by second-generation antipsychotic agents. *Psychiatric genetics* 22:245-252.
- Schmidt TT, Rea E, Shababi-Klein J, Panagis G, Winter C (2013) Enhanced reward-facilitating effects of d-amphetamine in rats in the quinpirole model of obsessive-compulsive

- disorder. *The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum* 16:1083-1091.
- Schmitt A, Asan E, Puschel B, Kugler P (1997) Cellular and regional distribution of the glutamate transporter GLAST in the CNS of rats: nonradioactive in situ hybridization and comparative immunocytochemistry. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 17:1-10.
- Scimemi A, Tian H, Diamond JS (2009) Neuronal transporters regulate glutamate clearance, NMDA receptor activation, and synaptic plasticity in the hippocampus. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 29:14581-14595.
- Seal RP, Amara SG (1998) A reentrant loop domain in the glutamate carrier EAAT1 participates in substrate binding and translocation. *Neuron* 21:1487-1498.
- Seal RP, Leighton BH, Amara SG (2000) A model for the topology of excitatory amino acid transporters determined by the extracellular accessibility of substituted cysteines. *Neuron* 25:695-706.
- Sepkuty JP, Cohen AS, Eccles C, Rafiq A, Behar K, Ganel R, Coulter DA, Rothstein JD (2002) A neuronal glutamate transporter contributes to neurotransmitter GABA synthesis and epilepsy. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 22:6372-6379.
- Shakeri J, Farnia V, Karimi AR, Tatari F, Juibari TA, Alikhani M, Bajoghli H, Holsboer-Trachsler E, Brand S (2016) The prevalence and clinical features of amphetamine-induced obsessive compulsive disorder. *Drug Alcohol Depend* 160:157-162.
- Shanahan NA, Holick Pierz KA, Masten VL, Waeber C, Ansorge M, Gingrich JA, Geyer MA, Hen R, Dulawa SC (2009) Chronic reductions in serotonin transporter function prevent 5-HT1B-induced behavioral effects in mice. *Biol Psychiatry* 65:401-408.
- Shanahan NA, Velez LP, Masten VL, Dulawa SC (2011) Essential role for orbitofrontal serotonin 1B receptors in obsessive-compulsive disorder-like behavior and serotonin reuptake inhibitor response in mice. *Biological psychiatry* 70:1039-1048.
- Sheldon AL, Gonzalez MI, Robinson MB (2006) A carboxyl-terminal determinant of the neuronal glutamate transporter, EAAC1, is required for platelet-derived growth factor-dependent trafficking. *The Journal of biological chemistry* 281:4876-4886.
- Shmelkov SV, Hormigo A, Jing D, Proenca CC, Bath KG, Milde T, Shmelkov E, Kushner JS, Baljevic M, Dincheva I, Murphy AJ, Valenzuela DM, Gale NW, Yancopoulos GD, Ninan I, Lee FS, Rafii S (2010a) Slitrk5 deficiency impairs corticostriatal circuitry and leads to obsessive-compulsive-like behaviors in mice. *Nat Med* 16:598-602, 591p following 602.
- Shmelkov SV, Hormigo A, Jing D, Proenca CC, Bath KG, Milde T, Shmelkov E, Kushner JS, Baljevic M, Dincheva I, Murphy AJ, Valenzuela DM, Gale NW, Yancopoulos GD, Ninan I, Lee FS, Rafii S (2010b) Slitrk5 deficiency impairs corticostriatal circuitry and leads to obsessive-compulsive-like behaviors in mice. *Nature medicine* 16:598-602, 591p following 602.

- Shugart YY, Samuels J, Willour VL, Grados MA, Greenberg BD, Knowles JA, McCracken JT, Rauch SL, Murphy DL, Wang Y, Pinto A, Fyer AJ, Piacentini J, Pauls DL, Cullen B, Page J, Rasmussen SA, Bienvenu OJ, Hoehn-Saric R, Valle D, Liang KY, Riddle MA, Nestadt G (2006) Genomewide linkage scan for obsessive-compulsive disorder: evidence for susceptibility loci on chromosomes 3q, 7p, 1q, 15q, and 6q. *Mol Psychiatry* 11:763-770.
- Shugart YY, Wang Y, Samuels JF, Grados MA, Greenberg BD, Knowles JA, McCracken JT, Rauch SL, Murphy DL, Rasmussen SA, Cullen B, Hoehn-Saric R, Pinto A, Fyer AJ, Piacentini J, Pauls DL, Bienvenu OJ, Riddle MA, Liang KY, Nestadt G (2009) A family-based association study of the glutamate transporter gene SLC1A1 in obsessive-compulsive disorder in 378 families. *American journal of medical genetics Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics* 150B:886-892.
- Simantov R, Crispino M, Hoe W, Broutman G, Tocco G, Rothstein JD, Baudry M (1999) Changes in expression of neuronal and glial glutamate transporters in rat hippocampus following kainate-induced seizure activity. *Brain Res Mol Brain Res* 65:112-123.
- Simpson HB, Foa EB, Liebowitz MR, Huppert JD, Cahill S, Maher MJ, McLean CP, Bender J, Jr., Marcus SM, Williams MT, Weaver J, Vermes D, Van Meter PE, Rodriguez CI, Powers M, Pinto A, Imms P, Hahn CG, Campeas R (2013) Cognitive-behavioral therapy vs risperidone for augmenting serotonin reuptake inhibitors in obsessive-compulsive disorder: a randomized clinical trial. *JAMA Psychiatry* 70:1190-1199.
- Sims KD, Straff DJ, Robinson MB (2000) Platelet-derived growth factor rapidly increases activity and cell surface expression of the EAAC1 subtype of glutamate transporter through activation of phosphatidylinositol 3-kinase. *The Journal of biological chemistry* 275:5228-5237.
- Singer HS, Hong JJ, Yoon DY, Williams PN (2005) Serum autoantibodies do not differentiate PANDAS and Tourette syndrome from controls. *Neurology* 65:1701-1707.
- Singer HS, Loisel CR, Lee O, Minzer K, Swedo S, Grus FH (2004) Anti-basal ganglia antibodies in PANDAS. *Mov Disord* 19:406-415.
- Skapinakis P, Papatheodorou T, Mavreas V (2007) Antipsychotic augmentation of serotonergic antidepressants in treatment-resistant obsessive-compulsive disorder: a meta-analysis of the randomized controlled trials. *Eur Neuropsychopharmacol* 17:79-93.
- Slotboom DJ, Konings WN, Lolkema JS (1999) Structural features of the glutamate transporter family. *Microbiol Mol Biol Rev* 63:293-307.
- Snider LA, Swedo SE (2004) PANDAS: current status and directions for research. *Molecular psychiatry* 9:900-907.
- Sontheimer H (2004) Ion channels and amino acid transporters support the growth and invasion of primary brain tumors. *Mol Neurobiol* 29:61-71.
- Soomro GM, Altman D, Rajagopal S, Oakley-Browne M (2008) Selective serotonin re-uptake inhibitors (SSRIs) versus placebo for obsessive compulsive disorder (OCD). *Cochrane Database Syst Rev* CD001765.

- Stafford MM, Brown MN, Mishra P, Stanwood GD, Mathews GC (2010) Glutamate spillover augments GABA synthesis and release from axodendritic synapses in rat hippocampus. *Hippocampus* 20:134-144.
- Starck G, Ljungberg M, Nilsson M, Jönsson L, Lundberg S, Ivarsson T, Ribbelin S, Ekholm S, Carlsson A, Forssell-Aronsson E, Carlsson ML (2008) A 1H magnetic resonance spectroscopy study in adults with obsessive compulsive disorder: relationship between metabolite concentrations and symptom severity. *Journal of neural transmission* (Vienna, Austria : 1996) 115:1051-1062.
- Starr BS, Starr MS (1986) Grooming in the mouse is stimulated by the dopamine D1 agonist SKF 38393 and by low doses of the D1 antagonist SCH 23390, but is inhibited by dopamine D2 agonists, D2 antagonists and high doses of SCH 23390. *Pharmacology, biochemistry, and behavior* 24:837-839.
- Stewart SE, Fagerness JA, Platko J, Smoller JW, Scharf JM, Illmann C, Jenike E, Chabane N, Leboyer M, Delorme R, Jenike MA, Pauls DL (2007) Association of the SLC1A1 glutamate transporter gene and obsessive-compulsive disorder. *American journal of medical genetics Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics* 144B:1027-1033.
- Stewart SE, Mayerfeld C, Arnold PD, Crane JR, O'Dushlaine C, Fagerness JA, Yu D, Scharf JM, Chan E, Kassam F, Moya PR, Wendland JR, Delorme R, Richter MA, Kennedy JL, Veenstra-VanderWeele J, Samuels J, Greenberg BD, McCracken JT, Knowles JA, Fyer AJ, Rauch SL, Riddle MA, Grados MA, Bienvenu OJ, Cullen B, Wang Y, Shugart YY, Piacentini J, Rasmussen S, Nestadt G, Murphy DL, Jenike MA, Cook EH, Pauls DL, Hanna GL, Mathews CA (2013a) Meta-analysis of association between obsessive-compulsive disorder and the 3' region of neuronal glutamate transporter gene SLC1A1. *Am J Med Genet B Neuropsychiatr Genet* 162B:367-379.
- Stewart SE, Yu D, Scharf JM, Neale BM, Fagerness JA, Mathews CA, Arnold PD, Evans PD, Gamazon ER, Davis LK, Osiecki L, McGrath L, Haddad S, Crane J, Hezel D, Illman C, Mayerfeld C, Konkashbaev A, Liu C, Pluzhnikov A, Tikhomirov A, Edlund CK, Rauch SL, Moessner R, Falkai P, Maier W, Ruhrmann S, Grabe HJ, Lennertz L, Wagner M, Bellodi L, Cavallini MC, Richter MA, Cook EH, Jr., Kennedy JL, Rosenberg D, Stein DJ, Hemmings SM, Lochner C, Azzam A, Chavira DA, Fournier E, Garrido H, Sheppard B, Umana P, Murphy DL, Wendland JR, Veenstra-VanderWeele J, Denys D, Blom R, Deforce D, Van Nieuwerburgh F, Westenberg HG, Walitza S, Egberts K, Renner T, Miguel EC, Cappi C, Hounie AG, Conceicao do Rosario M, Sampaio AS, Vallada H, Nicolini H, Lanzagorta N, Camarena B, Delorme R, Leboyer M, Pato CN, Pato MT, Voyiaziakis E, Heutink P, Cath DC, Posthuma D, Smit JH, Samuels J, Bienvenu OJ, Cullen B, Fyer AJ, Grados MA, Greenberg BD, McCracken JT, Riddle MA, Wang Y, Coric V, Leckman JF, Bloch M, Pittenger C, Eapen V, Black DW, Ophoff RA, Strengman E, Cusi D, Turiel M, Frau F, Macciardi F, Gibbs JR, Cookson MR, Singleton A, North American Brain Expression C, Hardy J, Database UKBE, Crenshaw AT, Parkin MA, Mirel DB, Conti DV, Purcell S, Nestadt G, Hanna GL, Jenike MA, Knowles JA, Cox N, Pauls DL (2013b) Genome-wide association study of obsessive-compulsive disorder. *Molecular psychiatry* 18:788-798.

- Stoffel W, Korner R, Wachtmann D, Keller BU (2004) Functional analysis of glutamate transporters in excitatory synaptic transmission of GLAST1 and GLAST1/EAAC1 deficient mice. *Brain Res Mol Brain Res* 128:170-181.
- Storch EA, Wilhelm S, Sprich S, Henin A, Micco J, Small BJ, McGuire J, Mutch PJ, Lewin AB, Murphy TK, Geller DA (2016) Efficacy of Augmentation of Cognitive Behavior Therapy With Weight-Adjusted d-Cycloserine vs Placebo in Pediatric Obsessive-Compulsive Disorder: A Randomized Clinical Trial. *JAMA Psychiatry* 73:779-788.
- Storck T, Schulte S, Hofmann K, Stoffel W (1992) Structure, expression, and functional analysis of a Na(+)-dependent glutamate/aspartate transporter from rat brain. *Proceedings of the National Academy of Sciences of the United States of America* 89:10955-10959.
- Streck EL, Feier G, Burigo M, Franzon R, Dal-Pizzol F, Quevedo J, Wyse AT (2006) Effects of electroconvulsive seizures on Na(+),K(+)-ATPase activity in the rat hippocampus. *Neuroscience letters* 404:254-257.
- Sturm V, Lenartz D, Koulousakis A, Treuer H, Herholz K, Klein JC, Klosterkötter J (2003) The nucleus accumbens: a target for deep brain stimulation in obsessive-compulsive- and anxiety-disorders. *J Chem Neuroanat* 26:293-299.
- Swedo SE (2002) Pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections (PANDAS). *Molecular psychiatry* 7 Suppl 2:S24-25.
- Swedo SE, Leonard HL, Garvey M, Mittleman B, Allen AJ, Perlmutter S, Lougee L, Dow S, Zamkoff J, Dubbert BK (1998) Pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections: clinical description of the first 50 cases. *The American journal of psychiatry* 155:264-271.
- Swerdlow NR, Benbow CH, Zisook S, Geyer MA, Braff DL (1993) A preliminary assessment of sensorimotor gating in patients with obsessive compulsive disorder. *Biological psychiatry* 33:298-301.
- Szatkowski M, Barbour B, Attwell D (1991) The potassium-dependence of excitatory amino acid transport: resolution of a paradox. *Brain research* 555:343-345.
- Szechtman H, Sulis W, Eilam D (1998) Quinpirole induces compulsive checking behavior in rats: a potential animal model of obsessive-compulsive disorder (OCD). *Behavioral neuroscience* 112:1475-1485.
- Takasaki C, Okada R, Mitani A, Fukaya M, Yamasaki M, Fujihara Y, Shirakawa T, Tanaka K, Watanabe M (2008) Glutamate transporters regulate lesion-induced plasticity in the developing somatosensory cortex. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 28:4995-5006.
- Takeuchi M, Hata Y, Hirao K, Toyoda A, Irie M, Takai Y (1997) SAPAPs. A family of PSD-95/SAP90-associated proteins localized at postsynaptic density. *The Journal of biological chemistry* 272:11943-11951.
- Takeuchi S, Wada K, Toyooka T, Shinomiya N, Shimazaki H, Nakanishi K, Nagatani K, Otani N, Osada H, Uozumi Y, Matsuo H, Nawashiro H (2013) Increased xCT expression

- correlates with tumor invasion and outcome in patients with glioblastomas. *Neurosurgery* 72:33-41; discussion 41.
- Tanaka K, Watase K, Manabe T, Yamada K, Watanabe M, Takahashi K, Iwama H, Nishikawa T, Ichihara N, Kikuchi T, Okuyama S, Kawashima N, Hori S, Takimoto M, Wada K (1997) Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1. *Science* 276:1699-1702.
- Tanaka KF, Ahmari SE, Leonardo ED, Richardson-Jones JW, Budreck EC, Scheiffele P, Sugio S, Inamura N, Ikenaka K, Hen R (2010) Flexible Accelerated STOP Tetracycline Operator-knockin (FAST): a versatile and efficient new gene modulating system. *Biological psychiatry* 67:770-773.
- Tang CM, Dichter M, Morad M (1989) Quisqualate activates a rapidly inactivating high conductance ionic channel in hippocampal neurons. *Science* 243:1474-1477.
- Tao F, Liaw WJ, Zhang B, Yaster M, Rothstein JD, Johns RA, Tao YX (2004) Evidence of neuronal excitatory amino acid carrier 1 expression in rat dorsal root ganglion neurons and their central terminals. *Neuroscience* 123:1045-1051.
- Taylor JL, Rajbhandari AK, Berridge KC, Aldridge JW (2010) Dopamine receptor modulation of repetitive grooming actions in the rat: potential relevance for Tourette syndrome. *Brain research* 1322:92-101.
- Taylor S (2013) Molecular genetics of obsessive-compulsive disorder: a comprehensive meta-analysis of genetic association studies. *Molecular psychiatry* 18:799-805.
- Ting JT, Feng G (2011) Neurobiology of obsessive-compulsive disorder: insights into neural circuitry dysfunction through mouse genetics. *Current opinion in neurobiology* 21:842-848.
- Traynelis SF, Wollmuth LP, McBain CJ, Menniti FS, Vance KM, Ogden KK, Hansen KB, Yuan H, Myers SJ, Dingledine R (2010) Glutamate receptor ion channels: structure, regulation, and function. *Pharmacol Rev* 62:405-496.
- Tzschentke TM, Schmidt WJ (2003) Glutamatergic mechanisms in addiction. *Molecular psychiatry* 8:373-382.
- Underhill SM, Wheeler DS, Li M, Watts SD, Ingram SL, Amara SG (2014) Amphetamine Modulates Excitatory Neurotransmission through Endocytosis of the Glutamate Transporter EAAT3 in Dopamine Neurons. *Neuron* 83:404-416.
- Valerius G, Lumpp A, Kuelz AK, Freyer T, Voderholzer U (2008) Reversal learning as a neuropsychological indicator for the neuropathology of obsessive compulsive disorder? A behavioral study. *J Neuropsychiatry Clin Neurosci* 20:210-218.
- van den Munckhof P, Bosch DA, Mantione MH, Figeo M, Denys DA, Schuurman PR (2013) Active stimulation site of nucleus accumbens deep brain stimulation in obsessive-compulsive disorder is localized in the ventral internal capsule. *Acta neurochirurgica Supplement* 117:53-59.

- van Grootheest DS, Cath DC, Beekman AT, Boomsma DI (2005) Twin studies on obsessive-compulsive disorder: a review. *Twin Res Hum Genet* 8:450-458.
- Varley CK, Vincent J, Varley P, Calderon R (2001) Emergence of tics in children with attention deficit hyperactivity disorder treated with stimulant medications. *Compr Psychiatry* 42:228-233.
- Veale D, Miles S, Smallcombe N, Ghezai H, Goldacre B, Hodsoll J (2014) Atypical antipsychotic augmentation in SSRI treatment refractory obsessive-compulsive disorder: a systematic review and meta-analysis. *BMC Psychiatry* 14:317.
- Veenstra-VanderWeele J, Kim SJ, Gonen D, Hanna GL, Leventhal BL, Cook EH (2001) Genomic organization of the SLC1A1/EAAC1 gene and mutation screening in early-onset obsessive-compulsive disorder. *Molecular psychiatry* 6:160-167.
- Veenstra-VanderWeele J, Xu T, Ruggiero AM, Anderson LR, Jones ST, Himle JA, Kennedy JL, Richter MA, Hanna GL, Arnold PD (2012) Functional studies and rare variant screening of SLC1A1/EAAC1 in males with obsessive-compulsive disorder. *Psychiatr Genet* 22:256-260.
- Velaz-Faircloth M, McGraw TS, alandro MS, Fremeau RT, Jr., Kilberg MS, Anderson KJ (1996) Characterization and distribution of the neuronal glutamate transporter EAAC1 in rat brain. *Am J Physiol* 270:C67-75.
- Voon V, Fernagut PO, Wickens J, Baunez C, Rodriguez M, Pavon N, Juncos JL, Obeso JA, Bezard E (2009) Chronic dopaminergic stimulation in Parkinson's disease: from dyskinesias to impulse control disorders. *Lancet Neurol* 8:1140-1149.
- Vulink NCC, Denys D, Westenberg HGM (2005) Bupropion for patients with obsessive-compulsive disorder: an open-label, fixed-dose study. *The Journal of clinical psychiatry* 66:228-230.
- Wahle S, Stoffel W (1996) Membrane topology of the high-affinity L-glutamate transporter (GLAST-1) of the central nervous system. *The Journal of cell biology* 135:1867-1877.
- Wan Y, Ade KK, Caffall Z, Ilcim Ozlu M, Eroglu C, Feng G, Calakos N (2014) Circuit-selective striatal synaptic dysfunction in the Sapap3 knockout mouse model of obsessive-compulsive disorder. *Biological psychiatry* 75:623-630.
- Wan Y, Feng G, Calakos N (2011) Sapap3 deletion causes mGluR5-dependent silencing of AMPAR synapses. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 31:16685-16691.
- Warr O, Takahashi M, Attwell D (1999) Modulation of extracellular glutamate concentration in rat brain slices by cystine-glutamate exchange. *J Physiol* 514 (Pt 3):783-793.
- Watase K, Hashimoto K, Kano M, Yamada K, Watanabe M, Inoue Y, Okuyama S, Sakagawa T, Ogawa S, Kawashima N, Hori S, Takimoto M, Wada K, Tanaka K (1998) Motor discoordination and increased susceptibility to cerebellar injury in GLAST mutant mice. *The European journal of neuroscience* 10:976-988.

- Watkins JC, Evans RH (1981) Excitatory amino acid transmitters. *Annu Rev Pharmacol Toxicol* 21:165-204.
- Watts SD, Torres-Salazar D, Divito CB, Amara SG (2014) Cysteine transport through excitatory amino acid transporter 3 (EAAT3). *PLoS one* 9:e109245.
- Waxman EA, Bacongus I, Lynch DR, Robinson MB (2007) N-methyl-D-aspartate receptor-dependent regulation of the glutamate transporter excitatory amino acid carrier 1. *The Journal of biological chemistry* 282:17594-17607.
- Weintraub D, David AS, Evans AH, Grant JE, Stacy M (2015) Clinical spectrum of impulse control disorders in Parkinson's disease. *Mov Disord* 30:121-127.
- Welch JM, Lu J, Rodriguiz RM, Trotta NC, Peca J, Ding J-D, Feliciano C, Chen M, Adams JP, Luo J, Dudek SM, Weinberg RJ, Calakos N, Wetsel WC, Feng G (2007) Cortico-striatal synaptic defects and OCD-like behaviours in Sapap3-mutant mice. *Nature* 448:894-900.
- Wendland JR, Moya PR, Kruse MR, Ren-Patterson RF, Jensen CL, Timpano KR, Murphy DL (2008) A novel, putative gain-of-function haplotype at SLC6A4 associates with obsessive-compulsive disorder. *Human molecular genetics* 17:717-723.
- Wendland JR, Moya PR, Timpano KR, Anavitarte AP, Kruse MR, Wheaton MG, Ren-Patterson RF, Murphy DL (2009a) A haplotype containing quantitative trait loci for SLC1A1 gene expression and its association with obsessive-compulsive disorder. *Archives of general psychiatry* 66:408-416.
- Wendland JR, Moya PR, Timpano KR, Anavitarte AP, Kruse MR, Wheaton MG, Ren-Patterson RF, Murphy DL (2009b) A haplotype containing quantitative trait loci for SLC1A1 gene expression and its association with obsessive-compulsive disorder. *Arch Gen Psychiatry* 66:408-416.
- West MJ, Ostergaard K, Andreassen OA, Finsen B (1996) Estimation of the number of somatostatin neurons in the striatum: an in situ hybridization study using the optical fractionator method. *The Journal of comparative neurology* 370:11-22.
- West MJ, Slomianka L, Gundersen HJ (1991) Unbiased stereological estimation of the total number of neurons in the subdivisions of the rat hippocampus using the optical fractionator. *The Anatomical record* 231:482-497.
- Westergaard N, Sonnewald U, Schousboe A (1995) Metabolic trafficking between neurons and astrocytes: the glutamate/glutamine cycle revisited. *Developmental neuroscience* 17:203-211.
- Whiteside SP, Port JD, Abramowitz JS (2004) A meta-analysis of functional neuroimaging in obsessive-compulsive disorder. *Psychiatry Res* 132:69-79.
- Whiteside SP, Port JD, Deacon BJ, Abramowitz JS (2006) A magnetic resonance spectroscopy investigation of obsessive-compulsive disorder and anxiety. *Psychiatry research* 146:137-147.

- Wild AR, Jones S, Gibb AJ (2014) Activity-dependent regulation of NMDA receptors in substantia nigra dopaminergic neurones. *J Physiol* 592:653-668.
- Wilhelm S, Buhlmann U, Tolin DF, Meunier SA, Pearlson GD, Reese HE, Cannistraro P, Jenike MA, Rauch SL (2008) Augmentation of behavior therapy with D-cycloserine for obsessive-compulsive disorder. *The American journal of psychiatry* 165:335-341; quiz 409.
- Williams KA, Swedo SE (2015) Post-infectious autoimmune disorders: Sydenham's chorea, PANDAS and beyond. *Brain Res* 1617:144-154.
- Willner P (1991) Methods for Assessing the Validity of Animal Models of Human Psychopathology. In: *Animal Models in Psychiatry*, I, vol. 18 (Boulton, A. et al., eds), pp 1-23: Humana Press.
- Willour VL, Yao Shugart Y, Samuels J, Grados M, Cullen B, Bienvenu OJ, 3rd, Wang Y, Liang KY, Valle D, Hoehn-Saric R, Riddle M, Nestadt G (2004a) Replication study supports evidence for linkage to 9p24 in obsessive-compulsive disorder. *Am J Hum Genet* 75:508-513.
- Willour VL, Yao Shugart Y, Samuels J, Grados M, Cullen B, Bienvenu OJ, Wang Y, Liang K-Y, Valle D, Hoehn-Saric R, Riddle M, Nestadt G (2004b) Replication study supports evidence for linkage to 9p24 in obsessive-compulsive disorder. *American journal of human genetics* 75:508-513.
- Winter C, Mundt A, Jalali R, Joel D, Harnack D, Morgenstern R, Juckel G, Kupsch A (2008) High frequency stimulation and temporary inactivation of the subthalamic nucleus reduce quinpirole-induced compulsive checking behavior in rats. *Experimental neurology* 210:217-228.
- Wood J, Ahmari SE (2015) A Framework for Understanding the Emerging Role of Corticolimbic-Ventral Striatal Networks in OCD-Associated Repetitive Behaviors. *Front Syst Neurosci* 9:171.
- Wu H, Wang X, Xiao Z, Yu S, Zhu L, Wang D, Jiang K, Wang Z, Zhang T, Fralick D (2013) Association between SLC1A1 gene and early-onset OCD in the Han Chinese population: a case-control study. *J Mol Neurosci* 50:353-359.
- Xu M, Li L, Ohtsu H, Pittenger C (2015) Histidine decarboxylase knockout mice, a genetic model of Tourette syndrome, show repetitive grooming after induced fear. *Neuroscience letters* 595:50-53.
- Xu P, Grueter BA, Britt JK, McDaniel L, Huntington PJ, Hodge R, Tran S, Mason BL, Lee C, Vong L, Lowell BB, Malenka RC, Lutter M, Pieper AA (2013) Double deletion of melanocortin 4 receptors and SAPAP3 corrects compulsive behavior and obesity in mice. *Proceedings of the National Academy of Sciences of the United States of America* 110:10759-10764.
- Yaddanapudi K, Hornig M, Serge R, De Miranda J, Baghban A, Villar G, Lipkin WI (2010) Passive transfer of streptococcus-induced antibodies reproduces behavioral

- disturbances in a mouse model of pediatric autoimmune neuropsychiatric disorders associated with streptococcal infection. *Molecular psychiatry* 15:712-726.
- Yadin E, Friedman E, Bridger WH (1991) Spontaneous alternation behavior: an animal model for obsessive-compulsive disorder? *Pharmacology, biochemistry, and behavior* 40:311-315.
- Yang W, Kilberg MS (2002) Biosynthesis, intracellular targeting, and degradation of the EAAC1 glutamate/aspartate transporter in C6 glioma cells. *The Journal of biological chemistry* 277:38350-38357.
- Yates JW, Meij JT, Sullivan JR, Richtand NM, Yu L (2007) Bimodal effect of amphetamine on motor behaviors in C57BL/6 mice. *Neuroscience letters* 427:66-70.
- Ye R (2009) MASS SPECTROMETRIC CHARACTERIZATION AND FLUOROPHOREASSISTED LIGHT INACTIVATION OF HUMAN EXCITATORY AMINO ACID TRANSPORTER 2. In: Department of Chemistry, vol. Doctor of Philosophy, p 134 Missoula, Montana: University of Montana.
- Yernool D, Boudker O, Jin Y, Gouaux E (2004) Structure of a glutamate transporter homologue from *Pyrococcus horikoshii*. *Nature* 431:811-818.
- Yucel M, Wood SJ, Wellard RM, Harrison BJ, Fornito A, Pujol J, Velakoulis D, Pantelis C (2008) Anterior cingulate glutamate-glutamine levels predict symptom severity in women with obsessive-compulsive disorder. *The Australian and New Zealand journal of psychiatry* 42:467-477.
- Zerangue N, Kavanaugh MP (1996) Flux coupling in a neuronal glutamate transporter. *Nature* 383:634-637.
- Zhang J, Yang X, Yang Q (2015) Neuropsychological dysfunction in adults with early-onset obsessive-compulsive disorder: the search for a cognitive endophenotype. *Rev Bras Psiquiatr* 37:126-132.
- Zhou Y, Danbolt NC (2014a) Glutamate as a neurotransmitter in the healthy brain. *J Neural Transm (Vienna)* 121:799-817.
- Zhou Y, Danbolt NC (2014b) Glutamate as a neurotransmitter in the healthy brain. *Journal of Neural Transmission* 121:799-817.
- Zohar AH (1999) The epidemiology of obsessive-compulsive disorder in children and adolescents. *Child Adolesc Psychiatr Clin N Am* 8:445-460.
- Zuchner S, Wendland JR, Ashley-Koch AE, Collins AL, Tran-Viet KN, Quinn K, Timpano KC, Cuccaro ML, Pericak-Vance MA, Steffens DC, Krishnan KR, Feng G, Murphy DL (2009) Multiple rare SAPAP3 missense variants in trichotillomania and OCD. *Molecular psychiatry* 14:6-9.