GLAST regulates glutamate dynamics and interneuron maturation in the developing cortex

A thesis submitted by

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in partial fulfillment of the requirements for the degree of

PhD

in

Neuroscience

Tufts University

February 2018

Advisor: Chris Dulla, PhD

Abstract

The astrocytic glutamate transporters GLAST and GLT-1 are highly expressed in the neocortex and are critical for regulation of extracellular glutamate concentrations. Glutamate, in turn, regulates many aspects of postnatal cortical development. However, the relative contributions and functional roles of GLAST and GLT-1 in the developing postnatal cortex are not well understood. The main goal of this dissertation is to test the hypothesis that astrocytic glutamate transporters regulate postnatal cortical circuit development. To test this hypothesis, I used heterozygous mouse models where there is 50% reduction of GLAST or GLT-1 protein levels. Using astrocyte glutamate transporter recordings, I show that GLAST is the predominant astrocytic transporter during postnatal cortical development, while GLT-1 becomes dominant in the mature cortex. This functional finding confirms previous studies characterizing GLAST and GLT-1 expression and suggests that GLAST might be critical for the development of cortical circuits. By recording tonic NMDA currents, we show that decreased GLAST function leads to increased ambient glutamate in the developing cortex. Previous work done in the lab had shown that fast-spiking interneurons in the cortex are particularly susceptible to tonic NMDA activation due to their NMDAR subunit composition. Therefore, I tested whether the effects of altered tonic glutamate signaling had cell-type specific effects. Fast-spiking interneurons in the GLAST$^{+/-}$ cortex, but not excitatory pyramidal cells, have altered action potential firing and intrinsic membrane properties during development and altered morphology in the mature somatosensory cortex. Additionally, inhibitory synapse number and interneuron distribution in the cortical layers are altered in the mature GLAST$^{+/-}$ cortex. Finally, I found that the broad deficits in inhibitory circuits

correlate with altered slice hyperexcitability in the mature GLAST$^{+/-}$ cortex, suggesting that loss of GLAST function during development leads to loss of inhibitory control of cortical networks. Since the GLT-1$^{+/-}$ cortex was not hyperexcitable at this mature stage, the results suggest that the aberrant cortical network activity observed results from disruptions during development that persist in the GLAST$^{+/-}$ cortex, rather than from deficits in acute glutamate uptake capacity. Taken together, the results from my thesis project indicate that GLAST, specifically, regulates glutamate in the developing neocortex and that its function is critical for the maturation of inhibitory function in cortical circuits. This main finding is in contrast with earlier work done in the thesis highlighting a role for GLT-1 in the acute response to kainic acid-induced seizures.

Dedication

This thesis is dedicated in memory of Charlene Wang.

Acknowledgements

First and foremost, this entire thesis and completing my degree would not be at all possible without the mentorship of my PhD advisor, Dr. Chris Dulla. Switching labs in the middle of graduate school is something that could have easily derailed my scientific career had I not fortuitously joined the Dulla Lab as a result. In working with Chris, I have learned so much about how to do science right—from the practical aspects (doing e-phys, experimental design, manuscript preparation) to the more theoretical side of things (thinking critically, asking the right questions, the joys of speculation). As a mentor, he has always put my success as a priority, and has gone above and beyond in advocating for me both at Tufts and within the broader scientific community, such as at meetings and symposiums. Additionally, the environment he has created for the lab has been extremely healthy and productive; I at once feel nurtured (ie with food and moral support) as well as highly driven and motivated to get after experiments and data. Working with Chris has made me a better scientist than I thought I could ever be, and I can't thank him enough for putting in the effort to train me.

I would also like to thank all the past and current members of the Dulla Lab that I have had the pleasure of working with: Dr. Lauren Andresen Lau, Dr. Moritz Armbruster, Dr. David Cantu, Danielle Croker, Dr. Liz Hanson, Jenny Koenig, Kyle O'Donnell, and Mary Sommer. I have asked each of these people countless questions on a range of subjects and for countless things over the years and they have always patiently, kindly, and thoroughly answered and helped me out. They are hands-down the best people do science with. In particular, I would like to thank Moritz for being so incredibly intelligent, helping me with so many rig-related issues, and having the answers to all 10 billion of my questions over the years. Additionally, I would like to thank Liz for many

enlightening discussions on glutamate regulation, electrophysiology, American football, internet memes, and a wide range of food-related topics, etc. It was also great to have someone in Liz who I could always count on to take various shenanigans to the next level, such as the incident with the Optogenetics Social sign, the lab flamingo costumes (among other arts and crafts and cooking projects), ice cream cakes, In-N-Out on the UCLA campus… as well as that excursion to the Mexican border. Time flies when you're having fun, and it's really the only way to survive grad school.

There are numerous people at Tufts beyond the Dulla Lab that I would like to recognize. My thesis committee members, Dr. Michele Jacob and Dr. Yongjie Yang, have been instrumental in guiding me through the rough waters that was my PhD circa 2013-2014. They have pushed me to become a better scientist through the years and have been truly invested in my research and my development—for that I am extremely thankful to them. Though they are not on my thesis committee, I would also like to acknowledge Dr. Rob Jackson, who greatly helped me through the lab transition process, as well as with my postdoc search; and Dr. Jamie Maguire for being an excellent and generous role model for women in science (as well as runners in science!), and for spearheading numerous outreach and social activities to keep us all connected. I am also grateful to the Neuroscience admin team for going above and beyond in handling all the thankless behind-the-scenes work of the department. Finally, I would like to thank Dr. Janice Naegele for serving as my external examiner, reading my dissertation, and making the trip out to Tufts from Wesleyan to hear me defend it.

My peers and colleagues have contributed in numerous ways to my well-being over the years; without them I would not have emerged from grad school with my sanity (mostly) intact. These wonderful people include all current and former members of the

Flamingo Lounge, all of the scientists of South Cove 2 from 2014-2017, the renowned distance runner and GABA scientist Dr. Paul Davies, the founding members of Neuroscientology, all of the members of the Glia Family, as well as my California expat friends (none of whom are expats anymore, as it turns out!). I would like to especially acknowledge Dr. Julie Hewitt Coleman, with whom I have shared cupcakes and tales of heartache and happiness. Jaclyn Dunphy could always be relied upon to provide a timely pun or lend a sympathetic ear. I am also grateful to Sam You for all the lunches we spent facing minor midlife/existential crises—thanks for putting up with my endless laments, being a voice of reason, and not making too much fun of my ineptitude/misfortunes with fantasy football.

And of course, without a doubt, my deepest gratitude to my family, who have supported me constantly and endlessly throughout the years, and have always given me the best opportunities to succeed. My mom, my dad, and my sister Evelyn are my most enthusiastic cheerleaders and my greatest sources of inspiration. In particular, whenever things got tough during grad school, I would think of my dad, the OG PhD of the family, who completed his degree while working a full-time job and supporting all of us. Needless to say, it was great motivation for me to get it together. Though my grad school life was somewhat of a black box to them, they somehow always knew (especially my mom!) the exact right thing to say or do to get me through the ups and downs, even though we have been on opposite coasts and in different states this whole time. Being far away didn't even stop Evelyn from showing up to cheer me on at 100% of the Boston Marathons I have run! Knowing that they have my back no matter what is one of the greatest blessings of my life.

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Chapter 2: Shih, J., & Donmez, G. (2013). Mitochondrial sirtuins as therapeutic targets for age-related disorders. *Genes Cancer, 4*(3-4), 91-96. doi:10.1177/1947601912474931
Chapter 3: Shih, J., Liu, L., Mason, A., Higashimori, H., & Donmez, G. (2014). Loss of SIRT4 decreases GLT-1-dependent glutamate uptake and increases sensitivity to kainic acid. *J Neurochem, 131*(5), 573-581. doi:10.1111/jnc.12942

List of abbreviations

ACS2 acetyl-CoA synthetase 2
aCSF artificial cerebrospinal fluid
AHP afterhyperpolarization
ALS amytrophic lateral sclerosis
AMPA α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR AMPA receptor
ANOVA analysis of variance
ANT adenine nucleotide translocator
AP action potential
APV 2-amino-5-phosphonopentanoic acid
ATP adenosine triphosphate
BSA bovine serum albumin
Ca2+ calcium
CNS central nervous system
CPP 3-(2-Carboxypiperazin-4-yl)propyl-1-phosphonic acid
CPS1 carbamoyl phosphate synthetase 1
CTIP2 COUP-TF-interacting protein 2
CUX1 cut-like homeobox 1
Cyt c cytochrome c
DAPI 2-(4-amidinophenyl)-1H -indole-6-carboxamidine
DHK dihydrokainic acid
DIV days in vitro
DNQX 6,7-dinitroquinoxaline-2,3-dione
DPM disintegrations per minute
E embryonic day
EAAC1 excitatory amino acid carrier 1
EAAT excitatory amino acid transporter
EDTA Ethylenediaminetetraacetic acid
EGTA egtazic acid
ENO early network oscillation
EPSC excitatory postsynaptic current
FBS fetal bovine serum
fEPSP field excitatory postsynaptic potential
FS fast-spiking
FOXO3a forkhead box O3
GABA gamma-aminobutyric acid
GABAR GABA receptor
GAD1 glutamate decarboxylase 1
GDH glutamate dehydrogenase
GDP giant depolarizing potential
GFAP glial fibrillary acidic protein
GFP green fluorescent protein
GLAST glutamate aspartate transporter
GLT-1 glutamate transporter 1

GTP guanosine triphosphate
HDAC histone deacetylase
HBSS Hank's balanced salt solution
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF1a hypoxia-inducible factor 1a
HMGCS2 3-hydroxy-3-methylglutaryl-CoA synthase 2
IDE insulin degrading enzyme
IDH2 isocitrate dehydrogenase 2
IPSC inhibitory postsynaptic current
KA kainic acid
KCC2 Potassium-chloride transporter member 5
KCl potassium chloride
KO knockout
L cortical layer
LCAD long chain acyl-CoA dehydrogenase
MAP2 microtubule-associated protein 2
MEF mouse embryonic fibroblast
Mg2+ magnesium
MgCl magnesium chloride
MgSO4 magnesium sulfate
MGE medial ganglionic eminence
mGluR metabotropic glutamate receptor
MK801 dizocilpine
MNI 4-methoxy-7-nitroindolinyl
MnSOD mitochondrial superoxide dismutase
NaCl sodium chloride
NAD+ nicotinamide adenine dinucleotide
NeuN neuronal nuclei
NMDA N-methyl-D-aspartate
NMDAR NMDA receptor
NR1 NMDA receptor subunit 1
NR2 NMDA receptor subunit 2
ns not significant
OTC ornithine transcarbamoylase
P postnatal day
PAGE polyacrylamide gel electrophoresis
PBS phosphate buffered saline
PFA paraformaldehyde
PMSF phenylmethane sulfonyl fluoride
PSD95 postsynaptic density 95
PV parvalbumin
RIPA radioimmunoprecipitation assay
RLU relative luminescent units
ROS reactive oxygen species
SDS sodium dodecyl sulfate
SEM standard error of the mean

SIRT sirtuin
SOD superoxide dismutase
SOM somatostatin
SR-101 sulforhodamine 101
TBOA threo-beta-benzyloxyaspartate
TC thalamocortical
TCA tricarboxylic acid
TEA-Cl Tetraethylammonium chloride
UV ultraviolet
VGAT vesicular GABA transporter
VGLUT1/2 vesicular glutamate transporter 1/2
VIP vasoactive intestinal peptide
WT wild type

Chapter 1: Introduction

1.1 General overview

The goal of this dissertation is to understand how the regulation of glutamate by astrocytic glutamate transporters controls the development of inhibitory cortical circuits. Glutamate is the major excitatory neurotransmitter in the brain; in addition to excitatory neurotransmission, glutamatergic signaling also regulates postnatal neurogenesis (Vicini 2008, Young, Taylor et al. 2011, Jansson and Akerman 2014), synaptogenesis (Kwon and Sabatini 2011), neuronal migration (Komuro and Rakic 1993, Behar, Scott et al. 1999), various types of neural plasticity (Turrigiano 2012, Sur, Nagakura et al. 2013), and excitotoxicity (Choi 1992). These processes contribute to the normal development and function of complex cortical circuits; therefore, the actions of glutamate are critical to the postnatal cortex. However, how glutamate itself is regulated in the early postnatal cortex is still not fully understood. For example, there are still open questions in terms of how extracellular glutamate concentrations are determined in the developing brain, how dynamic they are, and how tightly they are regulated. Additionally, key developmental processes in the cortex can be driven by specific types of neurons. Does glutamatergic signaling in the developing brain have cell-type specific effects? Experiments done to address these two main questions will contribute to our understanding of how glutamate dynamics can be controlled and how they influence cortical circuit maturation. In the context of neurological disease, increased understanding of how altered glutamate signaling could contribute to the mechanisms of neurodevelopmental disorders will lead the way towards more effective therapeutic strategies.

When postnatal development of the cerebral cortex begins, most of the neurons have migrated to their proper laminar localization. However, they are immature and cortical circuits are not fully developed or refined. Rather, the bulk of neuronal maturation and refinement occurs during first two postnatal weeks, when the cortex is highly plastic. Cells finish migrating to their final laminar positions, a process that is still not fully understood (Nadarajah, Alifragis et al. 2003, Miyoshi and Fishell 2011, Kwan, Sestan et al. 2012). Long-range afferents make connections onto relay neurons in Layer IV and local, intracortical connections are formed (Daw, Ashby et al. 2007). Spontaneous activity patterns lay the groundwork for permanent synaptic connections (Shatz 1990, Feller 1999, Yamamoto and Lopez-Bendito 2012). Synapses are formed, both structurally and functionally, and unnecessary connections are pruned away (Blue and Parnavelas 1983, Micheva and Beaulieu 1996, Stevens, Allen et al. 2007, Paolicelli, Bolasco et al. 2011). Neurons and glia begin to express excitatory and inhibitory neurotransmitter receptors, ion channels, and membrane proteins that allow them to receive inputs and process signals (Owens and Kriegstein 2001, Bayraktar, Fuentealba et al. 2014). Together, the cells within a properly formed laminar neocortical circuit work together to receive inputs from subcortical regions and generate outputs to subcortical and subcerebral structures, such as the brainstem and spinal cord. Disruptions in the development of cortical circuitry lead to altered excitation and inhibition balance and has been shown to underlie many pathological conditions in the brain (Manzini and Walsh 2011).

Inhibitory GABAergic interneurons have been shown to be critical for postnatal cortical circuit maturation (Le Magueresse and Monyer 2013). GABAergic interneurons

in the cortex consist of a heterogeneous population (Miyoshi, Hjerling-Leffler et al. 2010), with different interneuron subtypes exhibiting distinct molecular and electrophysiological characteristics. These properties then give rise to specific patterns of GABAergic signaling, which have been shown to shape several aspects of cortical circuit development (Markram, Toledo-Rodriguez et al. 2004). For example, GABAergic neurotransmission guides the morphological maturation of excitatory neurons and affects synaptogenesis (Wang and Kriegstein 2008, Wang and Kriegstein 2011). Additionally, GABA signaling underlies the generation of giant depolarizing potentials in the developing postnatal cortex (Allene, Cattani et al. 2008), an activity pattern thought to be critical for the development of functional cortical circuits. Inhibitory neurotransmission also controls critical windows of plasticity in the cortex, a phenomenon that has been demonstrated extensively in the visual system (Hensch 2005). The development of GABAergic signaling is thought to precede that of glutamatergic signaling, with GABAergic receptors and synapses appearing earlier than glutamatergic ones on both pyramidal cells and interneurons, contributing to the idea GABA signaling guides the development of excitatory neurotransmission and subsequent cortical circuitry (Koller, Siebler et al. 1990, Tyzio, Represa et al. 1999, Hennou, Khalilov et al. 2002, Ben-Ari, Khalilov et al. 2004). However, the production of GABA itself has been shown to be activity- and experience- dependent (Gierdalski, Jablonska et al. 2001, Patz, Wirth et al. 2003, Kinney, Davis et al. 2006), which implies that glutamate reciprocally regulates GABAergic systems. The interplay between these two major neurotransmitter systems in the developing brain contributes to the complexity of cortical circuit development mechanisms.

One of the major ways that glutamate is regulated in the brain is by astrocytic glutamate uptake. Astrocytes are the major glial cell population in the cortex and are important in mediating neural circuit maturation (Chung, Allen et al. 2015, Reemst, Noctor et al. 2016), neuronal function (Auld and Robitaille 2003, Khakh and Sofroniew 2015), and brain metabolism (Belanger, Allaman et al. 2011). One basic function of astrocytes is the active transport of glutamate out of the extracellular space via excitatory amino acid transporters (EAATs), which means that astrocytes have a major role in controlling extracellular glutamate levels in the brain. Astrocytic EAATs are highly abundant in the brain, comprising roughly 1% of total brain protein, and performing 90% of glutamate uptake from the extracellular space in the adult brain (Danbolt 2001). While EAAT function is critical in preventing excitotoxicity (Rothstein, Dykes-Hoberg et al. 1996), its role in regulating extracellular glutamate levels might also have profound effects on cortical circuit formation and maturation. This is the central hypothesis that this dissertation will address.

The body of work described in Chapter 4 of this dissertation aims to first understand how astrocytic glutamate uptake is regulated in the developing postnatal cortex. Further experiments then examine how the unique developmental profile of glutamate uptake function contributes to the maturation of cortical neurons and circuits. In contrast, Chapter 2 is an introduction to earlier work done during the PhD on mitochondrial sirtuins and their role in regulating neurodegeneration. Chapter 3 describes a set of experiments that highlight how sirtuins regulate astrocytic glutamate transporters to mediate the response to excitotoxic insults.

1.2 Postnatal development of cortical circuits

The neocortex is comprised of diverse populations of neurons and glia that are organized into six distinct layers and form the prototypic neocortical circuit (Harris and Shepherd 2015). Cortical neurons can be broadly classified as excitatory projection neurons vs. inhibitory interneurons; further classifications can be based on morphology, firing patterns, types of connections made, and more (Molyneaux, Arlotta et al. 2007). The basic requirements of a cortical circuit are cells that have migrated to the proper location, make the right connections, and are morphologically and functionally mature. The following is a brief overview of these three processes. It should be noted that these processes are not strictly sequential; they can overlap temporally, and in many cases, affect one another.

1.2.1 Neuronal migration

During embryonic development, newly generated excitatory neurons migrate along radial glial processes, moving from the ventricular zone or the subventricular zone into the cortical plate starting on embryonic day (E)10.5 (Rakic 1988, Soriano, Dumesnil et al. 1995). These neurons populate the cortical plate starting with the inner layers first, generating laminar organization in an inside-out manner (Nadarajah, Alifragis et al. 2003). The embryonic migration process continues through E17.5, when projection neurons begin to populate the outermost layer of the cortex. By postnatal day (P)2, radial glial cells differentiate into late-born neurons, astrocytes, and other glial cell types, and disappear from the cortex (Schmechel and Rakic 1979, Voigt 1989, Guo, Eckler et al. 2013).

Inhibitory interneurons, on the other hand, migrate much longer distances from the lateral and medial ganglionic eminences in the telencephalon starting at E12. In this thesis, I will focus on the interneurons that arise from the medial ganglionic eminence (MGE) which make up the majority (65-70%) of interneurons in the neocortex: the parvalbumin-positive (PV+) or somatostatin-positive (SOM+) interneurons (Rudy, Fishell et al. 2011). These interneurons migrate tangentially out of the MGE along axons, reach their cortical column localization, and invade the ventricular zone before migrating radially to a final laminar position (Marin and Rubenstein 2003). Localization of interneurons to their proper laminar position is a complex process that depends on their birthdate, subtype, and origin (Miyoshi, Hjerling-Leffler et al. 2010, Miyoshi and Fishell 2011). It has also been shown that cortical projection neurons and interneurons with the same birthdate will localize to the same layers, a process that is controlled by the projection neurons which have already populated the cortical plate (Lodato, Rouaux et al. 2011). Specific types of projection neurons will guide certain populations of interneurons during radial migration, which explains how pyramidal cells and interneurons that are born at the same time but arrive in the cortex at different times can localize to the same layers. Finally, recent evidence has shown that radial distribution of interneurons during the late stages of migration is activity-dependent and is mediated by glutamatergic signaling (Zechel, Nakagawa et al. 2016). These studies describe some of the cues and mechanisms that coordinate interneuron migration; since interneuron migration is a more elaborate process than pyramidal cell migration, more complex regulation is required.

The migration of excitatory and inhibitory neurons during the embryonic stages is regulated by a variety of cues. These cues can include cell autonomous cues, such as

genes that regulate cytoskeletal architecture (Liu 2011). Neuronal migration is also driven by cell non-autonomous cues, such as the extracellular matrix glycoprotein Reelin secreted from Cajal-Retzius cells (Tissir and Goffinet 2003), anti-cell adhesion signals from radial glial cells (Gongidi, Ring et al. 2004), chemokine signaling (Sanchez-Alcaniz, Haege et al. 2011, Wang, Li et al. 2011), and neurotransmitter levels (Manent, Demarque et al. 2005, Luhmann, Kilb et al. 2015). Since this dissertations focuses on glutamate regulation during development, I will focus this introduction on the role of glutamate in regulating radial and tangential migration of pyramidal cells and interneurons in the postnatal cortex. However, GABA signaling is also critical to migration of both radially migrating projection neurons and tangentially migrating interneurons, which has been shown experimentally (Behar, Li et al. 1996, Behar, Schaffner et al. 1998, Furukawa, Yamada et al. 2014) and reviewed elsewhere (Manent and Represa 2007).

Glutamate acts on ionotropic receptors of migrating neocortical neurons and is generally thought to be a chemoattractant molecule. In both acute cerebellar slice preparations and in vitro embryonic cortical slice cultures, it was shown that blocking NMDA receptors (NMDARs) on migrating neurons using MK801 or APV decreased the migration distance, while blocking glutamate uptake or decreasing Mg2+ increased migration distance (Komuro and Rakic 1993, Behar, Scott et al. 1999). Behar et al. (Behar, Scott et al. 1999) also showed that the pro-migratory effect of glutamate was specific to NMDARs by showing that adding AMPA receptor (AMPAR) antagonists did not decrease neuronal migration. However, there is conflicting evidence on whether NMDARs themselves are absolutely required for neuronal migration. Sustained delivery

of MK801 to the cortical surface of rats led to disruptions in laminar structure in the upper layers of the cortex (Reiprich, Kilb et al. 2005). On the other hand, deletion of the requisite NR1 subunit of NMDARs does not lead to gross disruptions in cortical anatomy (Messersmith, Feller et al. 1997). Deletion of NR1 specifically from excitatory neocortical neurons results in less distinct patterning of Layer IV somatosensory cortex barrels, but does not affect the laminar organization of the barrel cortex or critical period plasticity (Iwasato, Datwani et al. 2000). These findings suggest that other migratory signals and cues, such as GABAergic signaling, may exert more influence on neuronal migration or compensate for the loss of glutamatergic signaling (Manent, Demarque et al. 2005). These studies also do not differentiate between of radial migration of projection neurons or tangential migration of interneurons. However, based on the slice culture systems used in their experiments, it is likely that they describe the effects of glutamate on radial migration of projection neurons. Other studies have specifically examined the role of glutamate in regulating tangential interneuron migration. In contrast to the regulation of radial migration, AMPARs may have an increased role in regulating tangential migration, as both AMPAR and NMDAR antagonists affected interneuron migration (Soria and Valdeolmillos 2002). Of note, it was shown in the hippocampus that AMPARs, but not NMDARs, controlled hippocampal interneuron migration (Manent, Jorquera et al. 2006).

1.2.2 Early network connectivity

The properly formed neocortex is a canonical six-layered structure, with nearly all of the neuronal cell bodies populating Layers II-VI. 70-80% of the neuronal population consists of spiny, excitatory projection neurons, and 20-30% consist of non-spiny,

inhibitory interneurons (DeFelipe and Farinas 1992, Kawaguchi and Kubota 1997). Layer II/III excitatory neurons mainly project within the cortex, sending their axons to local circuits within the same cortical column or to the white matter tracts that make up the corpus callosum. Layer IV is mainly populated with spiny stellate neurons, which serve as relay neurons for sensory information arriving from the thalamus. Layer V/IV neurons are large pyramidal cells that mainly project to subcortical and subcerebral regions, such as striatum, brainstem, and spinal cord. The main input to this stereotypical neocortical circuit comes from thalamocortical (TC) afferents synapsing onto spiny stellate neurons in Layer IV. These neurons then project to neurons in Layer II/III, which then synapse onto Layer V/VI neurons. The neocortical circuit output comes from these deep layer neurons, which send their projections to various other regions of the central nervous system (Harris and Shepherd 2015). Inhibitory neurons are a more diverse population, with a variety of morphological and electrophysiological properties (Markram, Toledo-Rodriguez et al. 2004). They are typically classified named by the molecular markers that they express. The four main interneuron markers are parvalbumin (PV), somatostatin (SOM), vasoactive intestinal peptide (VIP), and reelin (Miyoshi, Young et al. 2015, Wamsley and Fishell 2017). They synapse extensively within local cortical circuits, in some cases with a single interneuron modulating entire neuronal networks (Bonifazi, Goldin et al. 2009). They also inhibit other interneurons to generate specific frequencies of network oscillations (Buzsaki 1997). Different subtypes of inhibitory interneurons preferentially localize to specific cortical layers based on where and when they are born in the ventral telencephalon during embryonic development, which generates distinct patterns of local circuit control in the deep vs. superficial cortical layers (Valcanis and

Tan 2003, Yozu, Tabata et al. 2004, Butt, Fuccillo et al. 2005, Miyoshi, Butt et al. 2007). The laminar organization of interneurons can be further cued by projection neurons in distinct layers of the neocortex (Pla, Borrell et al. 2006, Lodato, Rouaux et al. 2011).

Early functional development of neuronal networks is driven by GABAergic signaling (Ben-Ari, Khalilov et al. 2004). It has been shown extensively in the hippocampus (Tyzio, Represa et al. 1999, Hennou, Khalilov et al. 2002), but also in other brain regions, including the cortex (Koller, Siebler et al. 1990), that GABAergic synapses onto both pyramidal cells and inhibitory neurons are functional before glutamatergic ones. Dendritically targeted inhibitory synapses are formed before perisomatic inhibitory synapses (Tyzio, Represa et al. 1999, Hennou, Khalilov et al. 2002), which correlates well with the recent finding that inputs from dendritically targeted SOM+ INs are required for the maturation of inputs from perisomatically targeted PV+ INs (Tuncdemir, Wamsley et al. 2016).

Postnatally, cortical circuits are formed and refined based on a combination of spontaneous electrical activity and sensory input (Katz and Shatz 1996). Neuronal signaling in the developing neocortex is dominated by spontaneous activity, both in the form of large-scale waves that propagate through entire regions of the cortex (Allene, Cattani et al. 2008) or bursts within local circuits (Minlebaev, Ben-Ari et al. 2007, Minlebaev, Colonnese et al. 2011). These activity patterns contribute to the structure and function of nascent cortical circuits, including the formation of barrels in the somatosensory cortex (Tolner, Sheikh et al. 2012) and coordination of visual circuits prior to visual input (Chiu and Weliky 2002, Ackman, Burbridge et al. 2012). In addition to spontaneously generated activity patterns, the developing cortex relies on sensory input

to guide experience-dependent maturation. The formation of orientation columns and ocular dominance columns in the primary visual system has served as a model for understanding how cortical circuits are formed (Katz and Crowley 2002). During the critical period of plasticity in the visual cortex, cortical circuit refinement is guided by sensory stimuli. Thalamocortical afferents relay sensory input from the eyes to the visual cortex to form a pattern of innervation in Layer IV, which is disrupted by ocular deprivation (Hensch 2005). The critical period is regulated by inhibitory GABAergic signaling, as the disruption of interneuron function and maturation alters critical period timing and ocular dominance plasticity (Hensch, Fagiolini et al. 1998, Di Cristo, Chattopadhyaya et al. 2007), providing further evidence that inhibition is a key driver in regulating circuit maturation.

1.2.3 Postnatal functional maturation of neurons and synapses

Structural and functional synaptogenesis between developing neocortical neurons is a hallmark of neuronal maturation. During neurogenesis, synapses are structurally formed when dendritic filopodia extend and recruit presynaptic contact (Cohen-Cory 2002). In terms of function, neurotransmitter receptors are expressed early in embryonic development and are able to respond to glutamate and GABA, but synaptic transmission does not develop until after birth (Nguyen, Rigo et al. 2001, Demarque, Represa et al. 2002). At birth, glutamatergic synapses in the rodent neocortex are functionally silent as they exclusively contain voltage-dependent NMDARs, and therefore do not respond to glutamatergic signaling at normal resting potentials (Isaac, Nicoll et al. 1995, Wu, Malinow et al. 1996). Over the first postnatal week, spontaneous activity patterns or LTP induction trigger the insertion and functionality of AMPARs, allowing postsynaptic

neurons to respond to glutamatergic signaling (Liao, Hessler et al. 1995, Isaac, Crair et al. 1997, Petralia, Esteban et al. 1999, Zhu, Esteban et al. 2000, Liao, Scannevin et al. 2001). In the rodent somatosensory cortex, silent synapses are present from P2-5 and disappear from the cortex at P8-9 (Isaac, Crair et al. 1997). It has also been demonstrated in the visual cortex that the number of silent synapses and the timeline of when they become functional differs between neurons in the deep vs. superficial layers of the cortex, which may underlie early patterns of connectivity within cortical columns (Rumpel, Kattenstroth et al. 2004). The result of AMPAR insertion into the postsynaptic terminal as a synapse becomes functional is that the ratio of AMPA/NMDA responses increases with maturity at glutamatergic synapses. These events allow glutamatergic signaling to become functionally mature over the first postnatal week.

The functional maturation of GABAergic synapses also follows a similar timeline. Electrical coupling between fast-spiking interneurons in Layer V is not detected before P5 (Pangratz-Fuehrer and Hestrin 2011), and synapses between fast-spiking interneurons and spiny stellate cells in Layer IV are detected at around the same time (Daw, Ashby et al. 2007). In addition to the development of functional GABAergic synapses, the effects of GABA also change through development. In the immature brain, GABA signaling onto pyramidal cells is depolarizing, due to low expression of the chloride cotransporter KCC2 during the first two postnatal weeks (P0-P15) (Rivera, Voipio et al. 1999). KCC2 extrudes intracellular chloride, and therefore the high intracellular chloride concentration in immature neurons leads to net positive inward flow of current and a positive chloride reversal potential relative to the resting potential. Thus, GABA receptors (GABARs) activation leads to depolarization of the cell. Expression of

KCC2 increases after the first two postnatal weeks, and is regulated by a number of factors including neuronal activity, transcriptional control, neurotrophic factors, and pathological conditions (Medina, Friedel et al. 2014). Mature expression of KCC2 then decreases intracellular chloride concentrations and renders GABA signaling inhibitory. Interestingly, interneurons do not show this same developmental shift in intracellular chloride concentration as pyramidal cells do, and as a result GABAergic signaling onto interneurons is inhibitory throughout development (P5-P31) (Banke and McBain 2006). This suggests that there are differential mechanisms for chloride homeostasis in interneurons as compared to pyramidal cells, and evidence suggests that this is indeed the case in adult hippocampal interneurons (Martina, Royer et al. 2001). This result indicates that pyramidal cells and interneurons follow different steps development and may be differentially affected by perturbations during this time.

Since GABAergic neurotransmission by interneurons is thought to drive maturation of cortical circuits postnatally (Ben-Ari, Khalilov et al. 2004, Le Magueresse and Monyer 2013), how interneurons themselves develop and become functional during this time is of great importance. One major mechanism that regulates interneuron maturation is glutamatergic signaling at NMDARs. Blocking NMDARs on interneurons during development alters their firing properties (Jones, Corbin et al. 2014), morphology (Aligny, Roux et al. 2014), and reduces their overall number (de Lima, Opitz et al. 2004). Signaling at NMDARs is also required for the final stages of interneuron radial migration within the cortical plate (Zechel, Nakagawa et al. 2016). NMDARs have a heterotetrameric subunit assembly and consist of two requisite NR1 subunits and some combination of two NR2A-D or NR3A-B subunits (Paoletti, Bellone et al. 2013). The

differential subunit composition of NMDARs determines their unique kinetic properties; for this dissertation, I will focus on the NR2 subunits and how their expression varies with development and cell type (Vicini, Wang et al. 1998). Expression of the NR2 subunits in various brain regions through development has been comprehensively characterized by Monyer et al (Monyer, Burnashev et al. 1994). NR2B and NR2D are expressed in the embryonic brain and during early postnatal development; NR2D expression decreases over the first two postnatal weeks and is expressed at low levels in the adult cortex. NR2B expression is maintained over the first postnatal week but also decreases to low levels in the mature cortex. NR2A expression starts in the early postnatal cortex, and increases over development, inverse to the expression of NR2D. NR2C is expressed later in the more mature brain and is concentrated in the cerebellum and olfactory bulbs. The properties of NR2 subunits give rise to unique NMDAR channel properties, affecting Ca2+ permeability, ion channel open probability, glutamate affinity, and Mg2+ affinity (Vicini, Wang et al. 1998, Traynelis, Wollmuth et al. 2010). In particular, the NR2C and NR2D subunits show decreased affinity for Mg2+ as compared to NR2A and NR2B subunits, which makes NMDARs containing these subunits less voltage-dependent. NMDARs containing NR2C and NR2D also show increased affinity for glutamate, allowing for receptor activation by low glutamate concentrations. Thus, NMDAR function depends on their subunit composition and can be controlled by when and where specific NR2 subunits are expressed.

Specific NMDAR subunits can also mediate interneuron maturation. Early postnatal development of interneurons is mediated by the NR2B subunit, likely due to its enriched expression in the neonatal cortex (Jones, Corbin et al. 2014, Kelsch, Li et al.

2014, Yamasaki, Okada et al. 2014). Loss of this subunit leads to decreased glutamatergic synapses onto interneurons, seizure activity due to a lack of circuit inhibition (Kelsch, Li et al. 2014), and a delay in the onset of the critical period in the somatosensory cortex (Yamasaki, Okada et al. 2014). Furthermore, NR2D, which is also expressed specifically during early postnatal development, was shown to be preferentially expressed in hippocampal interneurons (von Engelhardt, Bocklisch et al. 2015). Loss of NR2D has also been shown to affect circuit maturation in the somatosensory cortex, leading to a premature onset of the critical period (Yamasaki, Okada et al. 2014). These studies suggest that the unique kinetic properties of NMDAR subunits on developing interneurons influence their development.

Maturation of the two main subtypes of cortical interneurons involves changes in gene expression and intrinsic membrane properties. Microarray screens were performed on samples collected from the G42 interneuron reporter mouse line, where 90% of GFP-labeled cells are parvalbumin-positive (PV+) fast spiking interneurons (Chattopadhyaya, Di Cristo et al. 2004) to determine their transcriptional maturation. Principal component analysis showed that there was significant developmental regulation of transcribed genes, and that genes encoding ion channels were upregulated with development (Okaty, Miller et al. 2009). Since changes in ion channel expression largely affects intrinsic membrane properties, electrophysiological properties of fast-spiking interneurons were also characterized through development. There was a significant decrease in the membrane resistance as interneurons matured, corresponding with increased ion channel expression (Okaty, Miller et al. 2009, Pangratz-Fuehrer and Hestrin 2011). In particular, spike frequency adaptation disappears and the maximum action potential firing increases,

correlating with increased expression of the Kv3 potassium channels that enable their sustained fast action potential spiking (Rudy and McBain 2001, Goldberg, Jeong et al. 2011). The electrical coupling of fast-spiking cells observed by Pangratz-Fuehrer and Hestrin (Pangratz-Fuehrer and Hestrin 2011) is also hypothesized to depend on the gap junction protein connexin 36, which increases its expression over development and correlates with decreased membrane resistance. Interestingly, the maturation profile of perisomatically-targeted, fast-spiking interneurons (mainly PV+) differs from that of dendritically targeted SOM+ interneurons. A main difference is in the membrane resistance, which decreases in mature PV+ INs but increases in mature SOM+ INs (Lazarus and Huang 2011). This suggests that the developmental expression of ion channels differs between the two interneuron subtypes, and could contribute to differences in the properties of perisomatic vs. dendritic inhibition. Additionally, action potential properties, such as half-width and after-hyperpolarization, are developmentally regulated in PV+ INs but do not change in SOM+ INs (Lazarus and Huang 2011). Of note, the electrophysiological properties of cortical pyramidal cells also change with development (McCormick and Prince 1987), including a decrease of membrane time constant with neuronal maturation (Kasper, Lubke et al. 1994, Frick, Feldmeyer et al. 2007, Oswald and Reyes 2008, Etherington and Williams 2011).

Neurons in the cortex also mature morphologically. Time-lapse imaging experiments of the optic tectum show that a developing neuron first extends an axonal branch, and then begins to develop the dendritic arbor shortly after (Witte, Stier et al. 1996, Wu and Cline 2003). The dendritic arbor grows rapidly over several days, drastically increasing the total dendritic branch length of the neuron. The growth process

is highly dynamic, with rapid branch additions and retractions, and depends on activity at glutamatergic synapses (Rajan and Cline 1998). As the neuron reaches maturity, its morphology stabilizes and interestingly, glutamatergic signaling now acts to stabilize the dendritic arbor and prevent further growth (Rajan, Witte et al. 1999, Wu, Zou et al. 1999).

1.3 Astrocytes in the developing neocortex

Glia are the most abundant cell type in the central nervous system, making up nearly 50% of the total cell population (Azevedo, Carvalho et al. 2009). Astrocytes are a subtype of glial cell, along with microglia and oligodendrocytes. They have relatively small cell bodies (10-20 μm), but their large domain (66,000 μm^3) is occupied by a dense network of branches, starting with primary branches that gradually ramify into fine processes (Bushong, Martone et al. 2002, Bushong, Martone et al. 2004). The fine processes from a single astrocyte ensheath 300-600 dendrites and roughly 100,000 synapses (Halassa, Fellin et al. 2007). By definition, astrocytes do not fire action potentials (Kuffler 1967, Zhou, Schools et al. 2006). Instead, astrocytes communicate with each other through gap junction signaling, creating a widespread astrocyte syncytium (Houades, Koulakoff et al. 2008, Orellana 2016). More controversial is how astrocytes reciprocally communicate with neurons using calcium signaling (Agulhon, Petravicz et al. 2008, Haydon and Nedergaard 2014, Volterra, Liaudet et al. 2014), although it is widely accepted that astrocytes release gliotransmitters, such as D-serine, glutamate, and ATP, that can modulate behavior and brain states (Araque, Li et al. 2000, Panatier, Theodosis et al. 2006, Jourdain, Bergersen et al. 2007, Zhang, Chen et al. 2007).

In general, astrocytes have low membrane resistance, linear current/voltage relationships, and are known for their high permeability to potassium (Berger, Schnitzer et al. 1991, Kafitz, Meier et al. 2008, Ma, Xu et al. 2014). Accordingly, astrocytes express ion channels and membrane proteins that confer these unique electrical properties (Bordey and Sontheimer 2000, Olsen, Khakh et al. 2015). The main functions originally described of astrocytes in the mature brain include blood-brain-barrier maintenance (Hayashi, Nomura et al. 1997, Abbott, Ronnback et al. 2006), regulation of synaptic transmission (Pfrieger and Barres 1997), buffering of extracellular K+ (Seifert, Huttmann et al. 2009, Sibille, Dao Duc et al. 2015), and regulation of neuron metabolism (Pellerin and Magistretti 2012). However, a number of studies in recent years have highlighted the morphological, molecular, and functional heterogeneity among astrocytes, which caution against excessive generalization of astrocyte function and properties (Zhang and Barres 2010, Bayraktar, Fuentealba et al. 2014, Khakh and Sofroniew 2015, Ben Haim and Rowitch 2017, Morel, Chiang et al. 2017). Astrocytes have been shown to originate from different progenitor pools during gliogenesis (Hochstim, Deneen et al. 2008) and express different genes, even within the same brain region (Doyle, Dougherty et al. 2008, John Lin, Yu et al. 2017, Morel, Chiang et al. 2017), suggesting that astrocytes in the same brain region can have differential functions. Of the many functions that have been discovered, increasing evidence has implicated astrocytes in promoting proper neuronal and circuit function in the cortex. Given the topic of the dissertation, this introduction will first highlight the mechanisms of astrocyte development, and then focus on two of their major functions in the postnatal brain: modulation of synapses and regulation of neurotransmitter release and uptake by astrocytic EAATs.

1.3.1 Astrocyte development

Astrocyte development starts in the late embryonic/early postnatal period. Newborn astrocytes are derived from radial glia in the ventricular zone and neural progenitors in the subventricular zone of the developing cortex (Schmechel and Rakic 1979, Voigt 1989, Cameron and Rakic 1991, Marshall, Suzuki et al. 2003, Kriegstein and Alvarez-Buylla 2009), which generate neurons first, undergo a "gliogenic switch," and then start to generate glial cells, which starts at E16 (Qian, Shen et al. 2000). The two key transcription factors that activate gliogenesis are Sox9 and its target NFIA (Deneen, Ho et al. 2006, Kang, Lee et al. 2012); loss of these transcription factors leads to a delay in gliogenesis and disrupted expression of developing glial markers that regulate migration and metabolism. Newborn glial cells also migrate into cortex along radial glial processes, a process which is thought to rely on extrinsic cues, as astrocyte migration is robust in young, developing tissue and limited in older cortical tissue (Jacobsen and Miller 2003). As astrocytes mature, they acquire mature astrocyte markers, including S100ß, Aldh1L1, Ascgb1, GLT-1, and aquaporin 4 (Molofsky, Krencik et al. 2012).

Following astrocyte differentiation and fate specification, protoplasmic astrocytes (as opposed to fibrous astrocytes of white matter tracts) mature functionally in the gray matter of the postnatal cortex. They develop in size and complexity, increasing their domain size and the ramification of their fine processes, to take on the appearance of a mature protoplasmic astrocyte (Bushong, Martone et al. 2004, Morel, Higashimori et al. 2014). One mechanism that modulates astrocyte maturation is neuronal activity in cortical circuits. Decreased neuronal activity due to loss of the vesicular glutamate transporter VGLUT1 leads to reduced ramification of astrocyte processes, indicating that

fine astrocyte processes rely on glutamatergic signaling for growth (Morel, Higashimori et al. 2014). Additionally, decreased VGLUT1 function is associated with decreased expression of the glutamate transporter GLT-1, a marker of mature astrocytes, further indicating that astrocyte functional maturation is disrupted. Sensory input can also drive astrocyte morphological and molecular maturation, which was shown by increased coverage of dendritic spines and increased glutamate transporter expression in the barrel cortex after whisker stimulation (Genoud, Quairiaux et al. 2006). In the hippocampus, synaptic activity increases the plasticity and the motility of perisynaptic astrocyte processes, prompting increased astrocyte coverage of dendritic spines (Bernardinelli, Randall et al. 2014). Taken together, these studies show that astrocyte maturation is positively correlated with neuronal activity and suggest that there is reciprocal regulation of astrocyte and neuronal circuit maturation. In addition to glutamate transporters, expression of other membrane proteins contributes to the molecular maturation of astrocytes (Rusnakova, Honsa et al. 2013, Olsen, Khakh et al. 2015). These include glutamate receptors (Gallo, Wright et al. 1994, Steinhauser and Gallo 1996, Chew, Fleck et al. 1997, Lalo, Pankratov et al. 2011, Lavialle, Aumann et al. 2011), connexin hemichannels (Dermietzel, Traub et al. 1989, Cheung, Chever et al. 2014), and the inward rectifying K+ channel Kir4.1 (Olsen, Higashimori et al. 2006, Moroni, Inverardi et al. 2015). The full expression of astrocytic ion channels then gives rise to the various functional roles of a mature astrocyte.

1.3.2 Modulation of synaptogenesis

Synaptogenesis in the cortex continues through the first postnatal week, which correlates with the functional maturation of cortical astrocytes (Cahoy, Emery et al. 2008,

Li, Cui et al. 2010). These similar timelines suggest that synaptogenesis and astrocyte development are interrelated and influence one other. As described above, neuronal activity drives the morphological and molecular maturation of astrocytes. Conversely, astrocytes can mediate the structural development of excitatory and inhibitory synapses via contact-mediated mechanisms (Garrett and Weiner 2009) or by releasing secreted factors (Eroglu, Allen et al. 2009). Thrombospondin was one of the first astrocyte secreted factors that was shown to induce excitatory synapse formation by signaling at α2δ1 receptors on neurons (Christopherson, Ullian et al. 2005, Eroglu, Allen et al. 2009). Later studies have identified hevin as another secreted factor that positively regulates the formation of structural synapses, as well as an antagonizing factor SPARC, which negatively regulates synapse formation (Kucukdereli, Allen et al. 2011). Astrocytes can further promote the functional maturation of synapses by secreting factors that induce the insertion of AMPARs into the postsynaptic terminal or increasing the probability of presynaptic neurotransmitter release. These factors include glypicans 4/6, which promote the insertion of AMPA receptors at the postsynaptic density (Allen, Bennett et al. 2012, Farhy-Tselnicker, van Casteren et al. 2017) and cholesterol, which strengthens presynaptic function (Mauch, Nagler et al. 2001). Astrocytes also signal through the BDNF/TrkB pathway to increase postsynaptic GABARs (Elmariah, Oh et al. 2005), indicating that the effect of astrocyte secreted factors on synaptogenesis is not limited to glutamatergic synapses. It should be noted that this is not an exhaustive list of astrocyte factors that influence structural and functional synaptogenesis; furthermore, the same secreted factor can have differential effects on various aspects of structural or functional synapse formation (Chung, Allen et al. 2015). Combined with the finding that astrocytes

also mediate synapse elimination (Chung, Clarke et al. 2013), as well as long-range synaptic contacts (Risher, Patel et al. 2014), the evidence indicates that astrocytes play substantial and complex role in shaping synaptic transmission and cortical networks in the developing brain.

1.4 Excitatory amino acid transporters

1.4.1 Basic overview of the EAAT family

EAATs (excitatory amino acid transporters) are a family of high-affinity glutamate transporters that remove glutamate from the extracellular space (Danbolt 2001). The five members of this group (EAAT1-5) each have unique cellular and regional localization, along with specific properties and functions depending on where and when they are expressed in the CNS (Gegelashvili and Schousboe 1998). Table 1.1 lists a brief overview of the 5 EAAT subtypes, with their general localization in the mature brain and key phenotypes of the null mouse model as observed in the study that originally generated the mouse.

Table 1.1 Overview of the 5 EAAT subtypes.

EAAT subtype	CNS cell type expression	CNS regional expression (Danbolt 2001)	Phenotype of KO mouse model
EAAT1 (GLAST)	Astrocytes (Watase, Hashimoto et al. 1998), Muller glia (Derouiche 1996), oligodendrocytes (Domercq, Sanchez-Gomez et al. 1999)	Cortex, hippocampus, cerebellum, olfactory bulb, retina	Motor deficits, increased cerebellar injury (Watase, Hashimoto et al. 1998)
EAAT2 (GLT-1)	Astrocytes (Tanaka, Watase et al. 1997), neurons (Miyoshi, Young et al. 2015), oligodendrocytes (Martinez-Lozada, Waggener et al. 2014)	Cortex, hippocampus, striatum, thalamus, olfactory bulb, cerebellum, lateral septum, retina	Seizures, postnatal mortality (Tanaka, Watase et al. 1997)
EAAT3 (EAAC-1)	Neurons (Peghini, Janzen et al. 1997), retinal cells except for Muller glia (Danbolt 2001), Purkinje cells (Huang, Dykes-Hoberg et al. 2004), oligodendrocytes (Domercq, Sanchez-Gomez et al. 1999)	Hippocampus, cerebellum, basal ganglia, retina	Dicarboxylic aminoaciduria (Peghini, Janzen et al. 1997), cognitive deficits (Aoyama, Suh et al. 2006)
EAAT4	Purkinje cells (Yamada, Watanabe et al. 1996)	Cerebellum	Regulation of climbing fiber synapses (Huang, Dykes-Hoberg et al. 2004)
EAAT5	Photoreceptor cells, bipolar cells (Arriza, Eliasof et al. 1997, Ochiai, Saito et al. 2010)	Retina	KO mouse does not exist

This dissertation focuses on EAAT1 and EAAT2, which are primarily expressed on astrocytes. Astrocytic EAATs perform the bulk of glutamate uptake in the brain, thus regulating extracellular levels of glutamate released in the normal or pathological brain (Amara and Fontana 2002, Maragakis and Rothstein 2004). The expression of astrocytic EAATs is dependent on a wide variety of regulatory and modulatory mechanisms

(Gegelashvili and Schousboe 1997, Anderson and Swanson 2000). They are subject to transcriptional regulation, which dictates their regional and temporal expression patterns (Martinez-Lozada, Guillem et al. 2016). Posttranslational modifications, such as phosphorylation of GLT-1 (Casado, Bendahan et al. 1993), have also been shown to increase transporter function. EAAT expression is also heavily influenced by neuronal signaling and secreted factors. Co-culturing astrocytes with neurons dramatically increases GLT-1 expression (Gegelashvili, Danbolt et al. 1997, Swanson, Liu et al. 1997), and neurons contribute to the transcriptional control of GLT-1 expression (Yang, Gozen et al. 2009). This suggests that EAATs are also regulated by neurotransmission, an idea which is supported by findings that lesions that disrupt neuronal signaling decrease the expression and function of GLAST and GLT-1 (Ginsberg, Martin et al. 1995, Levy, Lehre et al. 1995). Additionally, both glutamate and AMPAR agonists increase the expression of GLAST (Gegelashvili, Civenni et al. 1996), suggesting that EAATs can respond to increased neuronal activity and dynamically compensate for increased levels of extracellular glutamate.

1.4.2 GLAST vs. GLT-1 in the cortex

This dissertation specifically focuses on how GLAST and GLT-1 function might regulate development of the neocortex. The following is a summary of their expression patterns and known functions in the somatosensory cortex through the first 28 days of age, which covers the bulk of postnatal cortical maturation in that region. Starting at E10 in the ventricular zone, GLAST is expressed on radial glia, which are the major non-neuronal cell type of the embryonic brain (Hartfuss, Galli et al. 2001, Anthony and Heintz 2008). Radial glia serve as both neural progenitor cells, generating a large

proportion of cortical projection neurons, as well as the structural scaffold that guides the migration of newborn neurons into the cortical plate (Noctor, Flint et al. 2001, Campbell and Gotz 2002, Malatesta, Hack et al. 2003). Towards the end of embryonic development, GLAST expression shifts from radial glia and neural precursors in the ventricular zone to gray matter astrocytes, with co-expression with astrocytic GFAP by P7 (Sutherland, Delaney et al. 1996, Shibata, Yamada et al. 1997, Hanson, Armbruster et al. 2015). GLT-1 expression is low in the developing embryonic brain, but has been shown to be expressed in nonsynaptic sites on growing axons (Yamada, Watanabe et al. 1998). Its expression is enriched in the mantle zone, in contrast with the ventricular zone expression of GLAST. Expression of GLT-1 on axons is transient, occurring only during neuronal migration, and disappears from the white matter tracts by P7 (Yamada, Watanabe et al. 1998). In mature cortical astrocytes, GLAST and GLT-1 are expressed throughout astrocytic cell membranes and are enriched in the fine processes that surround synapses; both transporters are found in the same astrocyte but do not multimerize with each other (Chaudhry, Lehre et al. 1995, Haugeto, Ullensvang et al. 1996, Regan, Huang et al. 2007)).

Since glutamate concentrations influence radial neuronal migration (Komuro and Rakic 1993), it was predicted that glutamate transporters regulated neuron migration into the cortical plate during embryonic development. Later studies directly tested the idea that disruption of glutamate transporters perinatally would disrupt cortical development. Using a double knockout of both GLAST and GLT-1, Matsugami et al. found that these mice died in utero and that they had disorganized laminar structure in the cortical plate, decreased neurogenesis, and loss of long-range projections from subcortical regions

(Matsugami, Tanemura et al. 2006). Interestingly, deleting the NR1 subunit of NMDARs in these double knockout mice could rescue the cortical layer defects (Aida, Ito et al. 2012), suggesting that regulation of glutamatergic signaling at NMDARs is critical for brain development.

In the postnatal cortex, GLAST and GLT-1 expression is relatively low at P0, and expression of both transporters increases as the cortex matures (Furuta, Rothstein et al. 1997). However, GLT-1 increases to a much higher level, such that by P14, it is considered the predominant glutamate transporter in the forebrain (Furuta, Rothstein et al. 1997, Ullensvang, Lehre et al. 1997). The dominant function of GLT-1 in the central nervous system is supported by the finding that GLT-1 KO mice die of severe seizures in the third postnatal week (Tanaka, Watase et al. 1997). Deletion of GLAST, on the other hand, is not lethal, does not affect gross brain structure, and has more subtle effects on synaptic function (Watase, Hashimoto et al. 1998). However, the fact that GLAST is expressed more highly during the first two postnatal weeks relative to its adult levels, compared to GLT-1, suggests that it may play a more important role in early postnatal development (Ullensvang, Lehre et al. 1997).

Astrocytic glutamate transporter function has been studied extensively using biochemical and electrophysiological methods. Glutamate uptake by GLAST and GLT-1 is electrogenic, with one net positive charge flowing into the cell with each molecule of glutamate transported (Wadiche, Arriza et al. 1995), and occurs on a submillisecond timescale (Diamond and Jahr 1997). Thus, transporter currents can be recorded in whole cell patch clamp mode from astrocytes, with stimulus-evoked glutamate release, application of exogenous glutamate, or flash-uncaging of glutamate. Using these

methods, it was shown that the rate of uptake increases nearly two-fold in hippocampal astrocytes from the second postnatal week to adulthood (Diamond 2005, Thomas, Tian et al. 2011). This finding was later confirmed in the cortex, where astrocytic uptake was also shown to be significantly slower in the immature brain as compared to the adult (Hanson, Armbruster et al. 2015). Interestingly, maturation of glutamate transport kinetics in the cortex occurs later than in the hippocampus, shown by significantly slower transport kinetics in the cortex during the first two postnatal weeks. The slower glutamate uptake kinetics in the cortex then allows for increased activation of NMDARs in the immature cortex. Given that NMDAR signaling drives critical aspects of cortical circuit development, and that GLAST is expressed at relatively higher levels compared to GLT-1 during at this time, these findings taken together suggest that glutamate uptake by GLAST may shape cortical circuit development.

1.4.3 A special focus on GLAST: cerebellar and cortical phenotypes

GLAST knockout mice were first characterized in 1998 by Watase et al (Watase, Hashimoto et al. 1998). The first published studies on GLAST focused on its function in the cerebellum, where it is highly enriched in Bergmann glia (Rothstein, Martin et al. 1994, Furuta, Rothstein et al. 1997). Given the main function of the cerebellum in promoting motor function, original studies of GLAST function tested motor coordination and circuit function in the cerebellar cortex. Phenotypically, GLAST KO mice showed impaired motor function and increased susceptibility to cold-induced lesions in the cerebellar cortex (Watase, Hashimoto et al. 1998). Interestingly, certain human mutations in the GLAST gene are associated with episodic ataxia, a neurological condition characterized by motor discoordination (Jen, Wan et al. 2005, de Vries, Mamsa et al.

2009, Winter, Kovermann et al. 2012, Cvetanovic 2015). Similar phenotypes observed in GLAST mutant mice and human patients with GLAST mutations indicate that genetic models of GLAST deficiency might be clinically relevant.

Bergmann glia surround parallel fiber synapses or climbing fiber synapses onto GABAergic Purkinje cells (Chan-Palay and Palay 1971). Pharmacological inhibition of glutamate transporters at these synapses prolongs both AMPA- and mGluR-mediated Purkinje cell EPSCs (Barbour, Keller et al. 1994, Takahashi, Kovalchuk et al. 1995, Brasnjo and Otis 2001, Takayasu, Iino et al. 2004). Therefore, it was somewhat surprising that the kinetics of parallel fiber- or climbing fiber-induced EPSCs recorded from Purkinje cells of GLAST KO animals were not different from wild-type Purkinje cell EPSCs (Watase, Hashimoto et al. 1998). Additionally, the rate of clearance of glutamate and the time course of glutamate in the synaptic cleft were also not different, suggesting other glutamate uptake transporters in the cerebellum, such as EAAC1 or EAAT4, play a larger role in regulating synaptically released glutamate. However, the perplexing finding that GLAST does not regulate EPSC kinetics was later resolved in a study where blocking AMPAR desensitization revealed significant differences in the rise time, decay time, and peak amplitude of Purkinje cell EPSCs in GLAST KO mice (Takayasu, Iino et al. 2005), suggesting that the negative findings in earlier studies may have been masked by rapid AMPAR desensitization.

Another key finding in the GLAST KO mice was that they had a higher proportion of multiple innervations from climbing fibers onto Purkinje cells that persisted into adulthood, which could possibly be attributed to the effects of increased glutamate spillover (Watase, Hashimoto et al. 1998, Takayasu, Iino et al. 2006). The original

characterization of GLAST KO mice did not show any gross morphological defects in cerebellar anatomy (Watase, Hashimoto et al. 1998); however, multiple innervations onto one Purkinje cell is a transient phenomenon during development, and in the adult, a one-to-one climbing fiber-Purkinje cell relationship is established (Crepel 1982). The kinetics of these multiply innervated synapses were not different between GLAST KO mice and wild-type, suggesting that other glutamate uptake transporters in the cerebellum can compensate for synaptically released glutamate. Instead, these findings suggest that alterations in GLAST function lead to more subtle changes in connectivity during development that persist in adulthood. A recent study has shown that GLAST expression on Bergmann glia is required for the ensheathment of climbing fiber and parallel fiber synapses onto Purkinje cells; loss of GLAST leads to underdeveloped fine processes in the Bergmann glia that do not wrap the synapses properly (Miyazaki, Yamasaki et al. 2017). The study also showed increased parallel fiber synapses onto Purkinje cells and increased number of Purkinje cells with multiple climbing fiber innervation in GLAST KO mice, consistent with previous findings (Watase, Hashimoto et al. 1998). Multiple innervations at Purkinje cells have also been associated with motor discoordination and ataxia in other genetic deletion mouse models, suggesting it is a common underlying mechanism (Crepel, Delhaye-Bouchaud et al. 1980, Aiba, Kano et al. 1994, Chen, Kano et al. 1995, Kashiwabuchi, Ikeda et al. 1995). Together, these results indicate that glutamate regulation by GLAST during cerebellar development shapes the structure and function of specific neuronal circuits.

The role of GLAST in developing somatosensory circuits has also been previously studied. In GLAST KO mice, gross anatomy of the barrel cortex is normal and

thalamocortical projections to the barrels are not altered (Voutsinos-Porche, Knott et al. 2003, Takasaki, Okada et al. 2008). However, the metabolic response to whisker stimulation, as measured by neuronal 2-deoxyglucose uptake, was transiently decreased in the GLAST KO mice at P9-10. This shows that loss of glutamate uptake alters the functional response to sensory stimulation in neurons during cortical development, and suggests that GLAST can transiently affect neuronal activity (Voutsinos-Porche, Knott et al. 2003). Long-term changes in cortical function were not assessed in this study, but it would be interesting to test whether transient disruptions in the immature cortex could lead to changes in neuronal structure or function that persist to adulthood. Additionally, GLAST KO show decreased lesion-induced plasticity in their barrels as compared to wild-type mice, characterized by smaller changes in barrel size expansion and reduction in response to whisker lesions (Takasaki, Okada et al. 2008). Together, these results are evidence that GLAST function is critical during circuit maturation in the somatosensory cortex and that loss of GLAST can alter both neuronal structure and function during development.

Behavioral characterization of the GLAST KO mouse model shows deficits in schizophrenia-like behaviors. GLAST KO mice exhibit both positive and negative symptoms of schizophrenia, including increased locomotor hyperactivity, decreased sociability, and decreased social novelty preference (Karlsson, Tanaka et al. 2008). They also have cognitive deficits, as shown by impaired pairwise discrimination learning (Karlsson, Tanaka et al. 2009). The social novelty preference appears to be a strong phenotype as it is observed in the GLAST heterozygous mice as well. Some, but not all, of these phenotypes are rescued with the antipsychotic drug haloperidol, as well as an

mGluR2/3 agonist, which restores NMDAR signaling on interneurons and inhibitory control of the prefrontal cortex (Homayoun, Jackson et al. 2005). The results from the pharmacological rescue experiments support the idea that the behavioral deficits in GLAST-deficient mice are related to the pathophysiology of neuropsychiatric disease.

The finding that deficits in GLAST are associated with schizophrenia coincides with the finding from a genome-wide association study by Walsh et al. (Walsh, McClellan et al. 2008) that found a microdeletion spanning a portion of the GLAST gene in a case of childhood onset schizophrenia. The microdeletion structural variant creates a GLAST fusion protein, the expression and function of which is unknown. However, decreased levels of GLAST, but not GLT-1, have been demonstrated in the prefrontal cortex of schizophrenia patients, suggesting that GLAST specifically plays a role in schizophrenia disease mechanisms (Bauer, Gupta et al. 2008). One hypothesis for the pathophysiology of schizophrenia is NMDAR hypofunction in interneurons, which then leads to decreased GABA function and dysregulated network oscillations and neuronal connectivity (Woo, Walsh et al. 2004, Volk and Lewis 2014, Cohen, Tsien et al. 2015). Given that GLAST is specifically decreased in schizophrenia animal models and human patients, the studies suggest that defects in glutamatergic signaling in schizophrenia pathogenesis may be driven in part by defects in GLAST function.

1.4.4 EAATs and neurological conditions

EAATs are critical for controlling levels of extracellular glutamate; therefore, altered EAAT function and/or expression has been implicated in a number of neurological conditions associated with excitotoxic mechanisms. Studies have shown that GLT-1 is downregulated in amyotrophic lateral sclerosis (ALS) (Rothstein, Van Kammen

et al. 1995, Trotti, Rolfs et al. 1999), Alzheimer's disease (Li, Mallory et al. 1997, Masliah, Alford et al. 2000), Parkinson's disease (Ginsberg, Martin et al. 1995), Huntington's disease (Behrens, Franz et al. 2002), ischemia (Fukamachi, Furuta et al. 2001), and glioma (Munch, Penndorf et al. 2001). GLAST is downregulated in Alzheimer's disease (Masliah, Alford et al. 2000, Ikegaya, Matsuura et al. 2002), Parkinson's disease (Ginsberg, Martin et al. 1995), and ischemia (Fukamachi, Furuta et al. 2001, Tao, Lu et al. 2001).

EAATs are also dysregulated as a result of injury (Rao, Baskaya et al. 1998), particularly neonatal injury. Previous work in the lab has shown that astrocyte domains are increased and developmental regulation of EAAT function is disrupted following neonatal freeze lesioning (Armbruster, Hampton et al. 2014), although glutamate transporter current function itself is not altered. Other groups have shown direct changes in glutamate transporter function following freeze lesion injury (Campbell and Hablitz 2008, Campbell, Hablitz et al. 2014), although their experiments were carried out in Layer II/III as opposed to Layer V, which has been shown to have differing network oscillation patterns and glutamate receptor subtypes that underlie neuronal activity (Flint and Connors 1996).

Loss of EAAT function has also been described in animal models of seizures (Ueda, Doi et al. 2001), as increased extracellular glutamate and aberrant glutamate receptor activation are thought to contribute to the pathophysiology of epileptic networks (Meldrum 1994). Notably, GLT-1 KO mice develop severe generalized seizures and die by the third postnatal week (Tanaka, Watase et al. 1997). GLAST KO mice show increased responses to amydala-kindled seizures (Watanabe, Morimoto et al. 1999);

amydaloid kindling has also been shown to decrease GLAST expression (Miller, Levey et al. 1997). However, other studies have shown that GLAST KO mice required more stimulation to reach the same seizure severity as wild-type mice, suggesting that the seizure phenotype is less severe with loss of GLAST (Tsuru, Ueda et al. 2002). However, this result may be explained by the finding that the GABA transporter GAT-1 is decreased in the hippocampus of GLAST KO mice, presumably increasing inhibitory tone to compensate for the loss of glutamate uptake function (Nagatomo, Ueda et al. 2007). Therefore, the direct effects of GLAST on epilepsy are less clear than for GLT-1. Still, taken together, these results suggest that glutamate transporters both contribute to and are regulated by epileptogenesis.

1.4.5 EAATs and ambient glutamate

While EAATs have been widely implicated in a number of neurological conditions, their regulation of glutamate is critical in non-pathological situations as well. The role of EAATs in shaping phasic synaptic transmission have been widely studied and characterized (for review, see (Tzingounis and Wadiche 2007)). However, given that GLAST does not seem to significantly affect synaptically released glutamate at cerebellar synapses, but seems to contribute more to mechanisms resulting from glutamate spillover (Watase, Hashimoto et al. 1998) (but also see discussion in 1.4.3), this introduction will focus on how EAATs control ambient concentrations of glutamate to influence tonic glutamatergic signaling. Tonic glutamatergic signaling activates NMDARs, since they are high affinity and do not rapidly desensitize (Sah, Hestrin et al. 1989, Herman and Jahr 2007, Le Meur, Galante et al. 2007). Though baseline concentrations of glutamate are kept low in the extracellular space by EAATs, ambient glutamate is present in the brain at

nanomolar concentrations, likely as a result of spontaneous release from axon terminals or glial cells, or from spillover from neighboring synapses (Herman and Jahr 2007). Importantly, it has been shown in the hippocampus that these low concentrations of ambient glutamate are sufficient to tonically activate NMDARs (Le Meur, Galante et al. 2007). Tonic signaling at NMDARs has been shown to regulate neuronal excitability, and in particular, interneuron excitability (Povysheva and Johnson 2012, Riebe, Seth et al. 2016), which has then been shown to have effects on network oscillations in the mature hippocampus (Mann and Mody 2010). Additionally, tonic signaling by ambient glutamate, as controlled by EAATs, has been shown to regulate developmental oscillatory patterns in the cortex. In the immature rodent cortex and hippocampus (P0-P5), blocking glutamate uptake with threo-ß-benzyloxyaspartate (TBOA) generates NMDAR-mediated slow oscillatory waves and induces seizures in vivo (Demarque, Villeneuve et al. 2004, Cattani, Bonfardin et al. 2007). This study provides evidence that EAAT activity in the developing brain can influence network-driven oscillations, even before functional synapses have been formed and synaptic transmission is established (Owens and Kriegstein 2001). Specific activity patterns in developing cortical networks have been demonstrated in the developing neocortex and are thought be an integral aspect of functional cortical network maturation (Allene, Cattani et al. 2008). The two patterns described in the neocortex are early network oscillations (ENOs), which depend on glutamate signaling, and giant depolarizing potentials, which depend on GABA signaling. They occur sequentially, with ENOs serving as the dominant network pattern from P0-P5 and GDPs occurring from P6 and on. ENOs respond to tonic glutamate signaling, and together with the finding that EAATs drive network oscillations in the

same time window, the data suggest that glutamate transport could regulate the functional development of cortical networks. Therefore, due to their roles in regulating ambient glutamate concentrations and tonic signaling at NMDARs, glutamate transporters likely influence cortical network activity patterns and cortical maturation processes. These findings prompt further studies into the mechanisms of EAAT regulation of tonic glutamate signaling and subsequent cortical network maturation.

1.5 Hypothesis

Given that (1) GLAST is the predominantly expressed glutamate transporter in the developing neocortex, (2) that glutamate signaling during development may preferentially affect interneurons, and (3) that interneuron function is critical to the development of cortical circuits, I hypothesize that GLAST regulates inhibitory circuits during development to control the maturation of cortical networks. The experiments described in this dissertation test this hypothesis using electrophysiological and immunohistochemistry techniques.

Chapter 2

Mitochondrial sirtuins as therapeutic targets for age-related disorders [1]

[1] Shih J. and G. Donmez (2013). "Mitochondrial sirtuins as therapeutic targets for age-related disorders." Genes Cancer. 4(3-4), 91-96.
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Abstract

Sirtuins are a class of histone deacetylases that have a wide range of regulatory roles in the cell. Three sirtuins, SIRT3-5, localize to and function within the mitochondria. Mitochondrial dysfunction is thought to be the underlying mechanism of several age-related diseases, such as metabolic syndrome, cancer, and neurodegeneration. This review examines current evidence that mitochondrial sirtuins are involved in regulating mitochondrial function and pathogenesis.

Introduction

Over the last decade, many research efforts have been dedicated to understanding the changes in metabolism that contribute to the pathology of cancer, neurodegeneration, and other age-related conditions. An emerging key player in the field is a class of histone deacetylases (HDACs) called sirtuins. Sirtuins, which stands for silent information regulator proteins, are class III HDACs that require nicotinamide adenine dinucleotide (NAD+) for their function (Imai et al., 2000). Sirtuins first became molecules of interest when they were discovered to prolong lifespan in the yeast S. cerevisiae (Kaeberlein et al. 1999). Seven mammalian homologs (SIRT1-7) have since been identified (Frye, 2000), and they were found to have a wide range of roles in mammalian aging processes (reviewed in (Haigis and Guarente, 2006)). In this review, we will focus on the three mitochondrial sirtuins, SIRT3-5, and their recently discovered, emerging metabolic roles associated with cancer, neurodegeneration, and other age-related diseases.

Mitochondrial sirtuins

Mitochondria are the main organelles in the cell that are responsible for energy balance and metabolism. They have been implicated in several human diseases, and an important hypothesis has emerged that mitochondrial dysfunction is associated with the onset of age-related disorders and cancer (Wallace, 2005). Supporting this hypothesis are findings that mitochondrial function is impaired in diseases that occur with age, such as cancer (Xu et al., 1999; Van Remmen et al. 2003), neurodegenerative diseases (Lin and Beal, 2006), and diabetes (Petersen et al., 2004). Therefore, mitochondrial sirtuins, which affect mitochondrial function by affecting metabolic processes, will likely have roles in these diseases and conditions.

There are three mitochondrial sirtuins that localize to and function within the mitochondria. SIRT3 has strong deacetylase activity (Lombard et al., 2007), while SIRT4 has ADP-ribosylation activity only (Ahuja et al., 2007) and SIRT5 has desuccinylase and demalonylase activity, in addition weak deacetylation activity (Du et al., 2011; Schlicker et al., 2008). A number of metabolic substrates have been identified for SIRT3, whereas only a handful of substrates have been identified for SIRT4 and SIRT5 (Table 2.1). These substrates function as part of the basic metabolic machinery of the cell, in tasks such as fatty acid oxidation, electron transport chain function, and urea cycle metabolism (Verdin et al., 2010) (Figure 2.1). The mitochondrial sirtuins therefore have a chance to affect basal and pathological metabolic processes in the cell via their interactions with these metabolic substrates. Below, we will discuss these interactions, and highlight their possible contributions to pathologies such as metabolic syndrome, cancer, and neurodegeneration.

Metabolic syndrome

Metabolic syndrome is generally characterized by obesity, hypertension, insulin resistance, and dyslipidemia. The prevalence of metabolic syndrome today is largely due to a western diet and physical inactivity, but is also associated with aging and other genetic factors (Grundy et al., 2005). The functions of sirtuins in the mitochondria are heavily implicated in metabolic syndrome, since mitochondria contain the key cellular metabolism components that are affected in metabolic syndrome. Importantly, sirtuins mediate the beneficial effects of calorie restriction (Lin et al., 2004), which oppose the effects of metabolic syndrome. Through several studies on sirtuins and calorie restriction, it was shown that sirtuin function is dependent on the energy status of an organism, and sirtuins are able to regulate metabolic pathways accordingly (Imai and Guarente, 2010; Schwer and Verdin, 2008). Additionally, it has also been shown that metabolic enzymes are regulated by acetylation (Wang et al., 2010; Zhao et al., 2010), and therefore the deacetylase capabilities of sirtuins will be crucial for metabolic regulation.

Role of SIRT3

SIRT3 regulates a number of metabolic enzymes in the cell by deacetylation. It is known to respond to the energy level in a cell and in turn, act on various components of the tricarboxylic acid (TCA) cycle, fatty acid oxidation, and oxidative phosphorylation (Verdin et al., 2010). In general, SIRT3 is activated when nutrient levels in the cell are low and NAD+ levels are increased, promoting metabolic processes that are characteristic of a fasting state: amino acid catabolism (Schlicker et al., 2008), basal levels of oxidative phosphorylation (Ahn et al., 2008), fatty acid oxidation (Hirschey et al., 2010), and

ketone body synthesis (Shimazu et al., 2010). SIRT3 also promotes adaptive thermogenesis and promotes activity within brown fat (Shi, Wang, Stieren, and Tong, 2005).

SIRT3 was first discovered to deacetylate and activate acetyl-CoA synthetase 2 (ACS2) (Hallows, Lee, and Denu, 2006). ACS2 converts free acetate to acetyl-CoA, which is the most abundant intermediate metabolism molecule in the cell. Acetyl Co-A can be oxidized in the citric acid cycle (TCA cycle) to produce energy, and impaired acetate metabolism has been demonstrated in diabetic patients (Akanji and Hockaday, 1990). SIRT3 contributes to the fasting state of the cell by promoting fat metabolism and amino acid metabolism. It has been shown to deacetylate and activate long chain acyl-CoA dehydrogenase (LCAD), promoting fatty acid oxidation (Hirschey et al., 2010). Under starvation conditions, when the cell depends on fatty acid catabolism for energy, SIRT3 deacetylates and activates 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2), promoting the formation of ketone body byproducts from acetyl-CoA (Shimazu et al., 2010). During starvation, cells also rely on amino acid catabolism, and SIRT3 has been shown to deacetylate and activate glutamate dehydrogenase, which forms a-ketoglutarate from the amino acid glutamate (Schlicker et al., 2008). SIRT3 also deacetylates and activates ornithine transcarbamoylase, a key component of the urea cycle, which is important to prevent toxic levels of ammonia build-up from amino acid catabolism (Yu et al., 2009; Hallows et al., 2011).

The loss of SIRT3 in vivo leads to hyperacetylation of these enzymes, with implications for altered metabolic function (Lombard et al., 2007). Several studies were then done to assess if and how the loss of SIRT3 and hyperacetylation of metabolic

enzymes contributes to metabolic syndrome. SIRT3 knockout mice, high-fat diet administration, and mouse diabetes models were used in several studies. One study showed that a high fat diet is associated with a decrease in SIRT3 protein expression (Jing et al., 2011). The same study also showed that the loss of SIRT3 led to decreased mitochondrial oxidation and increased reactive oxygen species (ROS) production, which then induces a signaling cascade that ultimately decreases insulin release and contributes to metabolic syndrome characteristics. Another study showed that the SIRT3 knockout mice have hyperacetylated mitochondrial proteins, which ultimately increases their susceptibility to and progression of metabolic diseases (Hirschey et al., 2011). This study identified a polymorphism in the human SIRT3 gene that decreases SIRT3 function and is associated with the development of metabolic syndrome, indicating a genetic basis for the disorder.

Role of SIRT4

SIRT4 was first discovered to ADP-ribosylate and inhibit GDH, an action that inhibits insulin release by the pancreas (Haigis et al., 2006). Insulin is a central metabolic regulator that responds to energy levels in the body, and is released in response to increased levels of glucose in the blood stream or amino acids such as glutamate (Maechler, 2003). Insulin signals the cell to use carbohydrate metabolism for energy. Therefore, by negatively regulating insulin secretion in response to glucose and amino acids, SIRT4 opposes carbohydrate metabolism as seen in calorie restriction (Haigis et al., 2006). This finding indicates that SIRT4 might play a part in metabolic syndrome via GDH by adding another layer of regulation to the metabolic processes that are known to

be involved. Indeed, SIRT4 has also been shown to interact with insulin degrading enzyme (IDE) and the mitochondrial ATP/ADP translocase, ANT2/3, ultimately leading to a decrease in insulin secretion (Ahuja et al., 2007). One function of IDE is to degrade insulin and control insulin levels in the bloodstream; loss of function mutations of IDE can lead to diabetes in experimental mouse models (Farris et al., 2004). Though it is not known what the significance of SIRT4 interaction with IDE is, one can speculate that SIRT4 might also play a role in the onset of metabolic syndrome by affecting IDE function. Similarly, ANT2/3 has roles in regulating the cytosolic levels of ATP. Changing levels of ATP in the cell might trigger responses in many other metabolic pathways, which provides another possible mechanism by which SIRT4 can affect metabolic function in the cell.

Role of SIRT5

SIRT5 was first identified to regulate entry into the urea cycle and promote urea cycle function by deacetylating and activating carbamoyl phosphate synthetase 1 (CPS1) (Nakagawa, Lomb, Haigis, and Guarente, 2009). SIRT5 might also regulate CPS1 via desuccinylation (Du et al., 2011). The urea cycle is important for metabolizing and detoxifying ammonia, which can accumulate during amino acid catabolism. Therefore, it is logical that SIRT5 activates CPS1 and the urea cycle during fasting states or with a high protein diet, when higher levels of amino acid breakdown are required. These situations indicate that there is less fat and carbohydrate available for energy consumption. Since SIRT5 is regulates these metabolic processes during specific energy

states, the loss of SIRT5 function might have role in metabolic diseases, either contributing to or resulting from abnormal energy profiles in these patients.

Cancer

Cancer is a widespread, heterogeneous disease that is the leading cause of death worldwide. It is characterized by oxidative damage, which then leads to metabolic imbalance within the cell, abnormal macromolecule production, and uncontrolled tumor growth. Uncontrolled tumor growth is attributed to a phenomenon called "aerobic glycolysis," where cells have high rates of glycolysis and lactic acid production in the presence of oxidative phosphorylation (Hsu and Sabatini, 2008; Dang and Semenza, 1999). Normally, cells either function anaerobically and produce lactic acid, or consume oxygen and proceed with oxidative phosphorylation; the upregulation of glycolysis in the presence of oxygen does not occur normally and indicates malfunction. Since glycolysis and oxidative phosphorylation are both mitochondrial processes, mitochondrial dysfunction is implicated in cancer progression. It has been shown that oncogenes, such as Ras, Akt kinase, and Myc, promote metabolic processes such as glycolysis and glucose uptake (see (Manning and Cantley, 2007; Gordan, Thompson, and Simon, 2007; Yang et al., 2007) for review). Now, as mitochondrial sirtuins have emerged as metabolic regulators, they are being studied intensely for their possible roles in cancer progression.

Role of SIRT3

Since SIRT3 is a key mitochondrial deacetylase that affects the function of metabolic enzymes and oxidative phosphorylation components, and since abnormal

metabolic profiles in cells is a hallmark of cancer pathology, it seems likely that SIRT3 would play some role in cancer progression. The precise role, however, is unclear, as there is evidence for both tumorigenic and tumor suppressing functions of SIRT3.

On one hand, SIRT3 has been shown to be prosurvival, preventing cell death in response to stress and starvation (Ahn et al., 2008). SIRT3 has also been shown to protect from cell death in response to hypoxia or the apoptotic inducer staurosporine (Pellegrini et al., 2012). These studies support the general role of sirtuins in promoting longevity, but it also might indicate a role in tumor growth. Indeed, SIRT3 has been shown to promote cell proliferation in oral cancer cell lines (Alhazzazi et al., 2011) and to block the growth arrest function of the transcription factor p53 in bladder cancer cells (Li et al., 2010). SIRT3 gene expression levels are also increased in malignant human breast biopsies (Ashraf et al., 2006).

On the other hand, SIRT3 has been shown to be pro-apoptosis under certain pathway conditions (Allison and Milner, 2007). SIRT3 has also been identified as a tumor suppressor in several studies, which directly link SIRT3 to metabolic processes. The loss of SIRT3 in mouse embryonic fibroblasts (MEFs) was first shown induce tumor growth in response to oncogene expression (Kim et al., 2010). SIRT3 expression in MEFs was also shown to destabilize HIF1a, a transcription factor that promotes glycolytic metabolism as dictated by the "Warburg effect" (Warburg, 1956; Bell, Emerling, Ricoult, and Guarente, 2011; Finley et al., 2011). The same study also showed that SIRT3 overexpression in the breast cancer cell lines MCF7, T47D, and CAMA1 reverses the characteristic metabolic profile indicated by the Warburg effect. SIRT3 has also been shown to interact with the FOXO3a transcription factor, which is known to

upregulate apoptotic signaling cascades (Skurk et al., 2004). This is one mechanism by which SIRT3 might act as a tumor suppressor. SIRT3 might also prevent cancer by deacetylating and activating mitochondrial superoxide dismutase (MnSOD), which is thought to control the levels of ROS and prevent oxidative damage that triggers cancer pathology (Qiu, Brown, Hirschey, Verdin, and Chen, 2010; Chen et al., 2011).

These conflicting results show that there are dual hypothetical roles of SIRT3 in cancer and tumor progression. Since SIRT3 is involved in many aspects of cellular metabolism, including fatty acid oxidation, amino acid catabolism, and oxidative phosphorylation, its role in cancer is likely to be complex and multi-dimensional. Different stimuli and environmental effects seem to trigger different responses from SIRT3, and it will be interesting to determine which external stimuli will trigger specific regulatory roles of SIRT3.

Role of SIRT4 and SIRT5

SIRT4 and SIRT5 have not been studied in the context of cancer. However, given their roles in regulating metabolism, it would be very interesting to study whether or not they have tumor suppressor or tumorigenic roles. The metabolic functions of SIRT4 and SIRT5 that have been discovered so far, such as SIRT4 inhibition of insulin release, can possibly mirror or antagonize the established effects of oncogenes on metabolism, and further studies are necessary to place SIRT4 and SIRT5 actions in the context of cancer.

Neurodegeneration

Neurodegeneration is the process by which neurons are lost due to genetic and environmental stressors. There are many types of neurodegenerative diseases, which affect different populations of neurons and cause a range of untreatable symptoms; the most common types are Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS), and Huntington's disease. The central risk factor for neurodegeneration is advanced age. Sirtuins have been implicated in neurodegenerative diseases due to their reputation as aging, stress response genes (Haigis and Guarente, 2006), and due to the fact that they are highly expressed in the brain and central nervous system (CNS) (see (Donmez, 2012) for review). Furthermore, mitochondria are also implicated in aging processes as their function declines (Wallace, 2005; Lin and Beal, 2006). Therefore, mitochondrial sirtuins present promising targets to study in deducing the pathology of neurodegeneration.

Role of SIRT3

SIRT3 is known to deacetylate many substrates in the mitochondria that are important to metabolic processes, but this has mainly been shown in other tissues besides the brain. In general, SIRT3 displays protective effects against reactive oxygen species (ROS) production by upregulating SOD function (Shi, Wang, Stieren, and Tong, 2005; Chen et al., 2011; Jing et al., 2011; Someya, 2010; Kong et al., 2010). Since ROS have been shown to induce neurodegenerative processes (Lin and Beal, 2006), SIRT3 might be protective in neurons against ROS-related damage. SIRT3 was first shown to be protective against excitotoxic injury in primary neuron cultures (Kim, Lu, and Alano,

2011). In this study, SIRT3 was increased following massive ROS production induced by the addition of NMDA, indicating the role of SIRT3 as a downstream effector of ROS injury. Recently, SIRT3 was shown to be neuroprotective in response to Alzheimer's disease-induced cellular stress, in addition to ROS-induced injury (Weir et al., 2012). Possible mechanisms for SIRT3 upregulation in response to neuronal stress include interaction with the FOXO3 transcription factors. SIRT3 has been shown to interact with FOXO3a, but the physiological implications, especially in the context of neurodegeneration, are not clear (Jacobs et al., 2008). FOXO3 transcription factors are thought to offer protection from oxidative stress by activating detoxifying genes such as catalase and MnSOD (Olmos et al., 2009). In addition, while FOXO3 may function as a tumor suppressor in other tissues, its role in apoptosis has implications for sirtuin-mediated neurodegeneration (Liu et al., 2012). More studies are needed to determine whether SIRT3 can be neuroprotective through its interaction with FOXO3a, or if there is another mechanism involved.

Role of SIRT4 and SIRT5

The role of SIRT4 and SIRT5 in neurodegeneration is not clear. While recent discoveries of SIRT4 and SIRT5 substrates in the cell and other metabolic tissues may provide some clues, little is known about SIRT4 and SIRT5 function in brain tissues. The fact that SIRT4 interacts with IDE is one example (Ahuja et al., 2007), since IDE has been shown to degrade pathological beta-amyloid plaques (Kurochkin and Goto, 1994). Another example is SIRT5 interaction with cytochrome c (Schlicker et al., 2008), since cytochrome c is released from the mitochondria during apoptosis (Yang et al., 1997).

Additional studies have to be conducted to understand the functions of mitochondrial sirtuins in the brain.

Concluding remarks

This review highlights the major roles of mitochondrial sirtuins in regulating metabolism, how these roles then affect the progression of metabolic syndrome, cancer, and neurodegeneration. In many cases, the roles of mitochondrial sirtuins seem to have opposing effects in different tissue type and disease settings. For example, the apoptotic functions of mitochondrial sirtuins might be beneficial in preventing tumor growth, but might also facilitate neurodegeneration. This review has speculated on several dual regulatory roles of mitochondrial sirtuins, but much more work is needed to untangle the complex metabolic and regulatory pathways these sirtuins are involved in. Understanding mitochondrial sirtuin function will undoubtedly lead to improved therapeutic strategies for age-related, metabolism-related, and stress-related diseases, benefiting a large proportion of our current population.

Table 2.1: Key metabolic substrates of mitochondrial sirtuins

Disorder	Mitochondrial Sirtuin Involved	Net effect	Key substrates and interactions	Reference
Metabolic Syndrome	SIRT3	Responds to fasting state	ACS2	(Hallows, Lee, and Denu, 2006)
			HMGCS2	(Shimazu et al., 2010)
			LCAD	(Hirschey et al., 2010)
			ETC Complex 1	(Ahn et al., 2008)
			GDH	(Schlicker et al., 2008)
			OTC	(Hallows et al., 2011)
	SIRT4	Opposes calorie restriction	GDH	(Haigis et al., 2006)
			IDE	(Ahuja et al., 2007)
	SIRT5	Responds to fasting state	CPS1	(Nakagawa et al., 2009)
Cancer	SIRT3	Tumorigenic		(Li et al., 2010; Ashraf et al., 2006)
		Tumor suppressor	HIF1a	(Finley et al., 2011; Skurk et al., 2004)
Neurodegeneration	SIRT3	Neuroprotection	FOXO3a	(Olmos et al., 2009)
			IDH2	(Kim, Lu, and Alano, 2011)

Abbreviations: ACS2, acetyl-CoA synthetase 2; HMGCS2, 3-hydroxy-3-methylglutaryl-CoA synthase 2; LCAD, long chain acyl-CoA dehydrogenase; ETC, electron transport chain; GDH, glutamate dehydrogenase; OTC, ornithine transcarbamoylase; IDE, insulin degrading enzyme; CPS1, carbamoyl phosphate synthetase 1; HIF1a, hypoxia-inducible factor 1a; FOXO3a, forkhead box O3; IDH2, isocitrate dehydrogenase 2.

Figure 2.1:

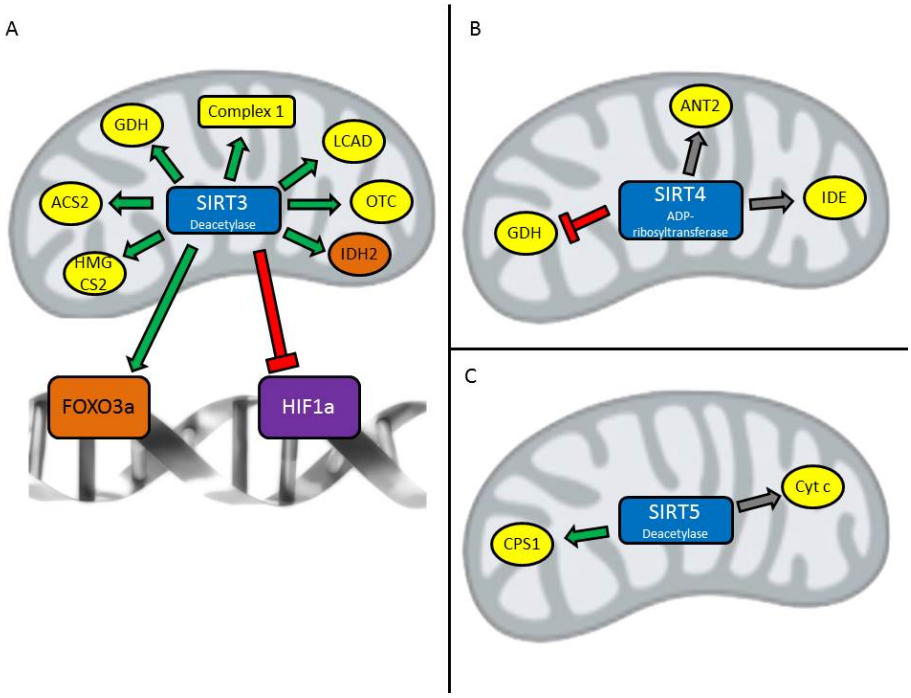

Figure 2.1. Key actions of mitochondrial sirtuins

SIRT3 (A), SIRT4 (B), and SIRT5 (C) act on various substrates in the mitochondria. SIRT3 also interacts with transcription factors. Generally, their enzymatic function activates or promotes the function of a given substrate (green arrows), with the exception of SIRT3 destabilization of the transcription factor HIF1a. Mitochondrial sirtuins also have interactions with substrates where the physiological significance is yet to be confirmed (grey arrows).

Abbreviations: ACS2, acetyl-CoA synthetase 2; HMGCS2, 3-hydroxy-3-methylglutaryl-CoA synthase 2; LCAD, long chain acyl-CoA dehydrogenase; GDH, glutamate dehydrogenase; OTC, ornithine transcarbamoylase; HIF1a, hypoxia-inducible factor 1a; FOXO3a, forkhead box O3; IDH2, isocitrate dehydrogenase 2; IDE, insulin degrading enzyme; ANT2, ATP/ADP translocase 2; CPS1, carbamoyl phosphate synthetase 1; Cyt c, cytochrome c.

Chapter 3

Loss of SIRT4 decreases GLT-1-dependent glutamate uptake and increases sensitivity to kainic acid[2]

[2] Shih, J., L. Liu, A. Mason, H. Higashimori and G. Donmez (2014). "Loss of SIRT4 decreases GLT-1-dependent glutamate uptake and increases sensitivity to kainic acid." J Neurochem 131(5): 573-581.

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Abstract

Glutamate transport is a critical process in the brain that maintains low extracellular levels of glutamate to allow for efficient neurotransmission and prevent excitotoxicity. Loss of glutamate transport function is implicated in epilepsy, traumatic brain injury, and amyotrophic lateral sclerosis (ALS). It is unclear whether or not glutamate transport can be modulated in these disease conditions to improve outcome. Here, we show that SIRT4, a mitochondrial sirtuin, is upregulated in response to treatment with the potent excitotoxin kainic acid. Loss of SIRT4 leads to a more severe reaction to kainic acid and decreased glutamate transporter expression and function in the brain. Together, these results indicate a critical and novel stress response role for SIRT4 in promoting proper glutamate transport capacity and protecting against excitotoxicity.

Introduction

Sirtuins are a class of protein deacetylases, located in the nucleus, cytosol, or mitochondria of a cell, that target various proteins within the cell for post-translational modifications. These post-translational modifications can include ADP-ribosylation, demalonylation, and desuccinylation, in addition to deacetylation. These sirtuin-mediated protein modifications cause a wide range of downstream effects in vivo, including gene silencing or expression and enzyme activation or inhibition (for review, see (Finkel *et al.* 2009; Haigis and Guarente 2006)). Generally, sirtuins are stress-response genes that are activated when an organism is subjected to insults such as hypoxic or oxidative stress (Berdichevsky and Guarente 2006; Wang *et al.* 2007). Physiologically, sirtuins were first discovered to increase lifespan by mediating the effects of calorie restriction (Kennedy *et*

al. 1995; Kaeberlein *et al.* 1999). In general, sirtuins affect a wide range of metabolic pathways, influencing the progression and outcome of metabolic syndrome (Guarente 2006; Newman *et al.* 2012). Eventually, it was discovered that sirtuins also have roles in age-related diseases (Donmez and Guarente 2010; Shih and Donmez 2013), including neurodegeneration (Donmez 2012).

There are seven mammalian sirtuin homologs, SIRT1-7. Three of these homologs, SIRT3-5, are mitochondrial sirtuins (Michishita *et al.* 2005). Less is known about the activity and substrates of mitochondrial sirtuins in the brain, especially SIRT4. Its first known substrate was glutamate dehydrogenase (GDH), and it was shown that SIRT4 ADP-ribosylates and inhibits GDH in pancreatic beta cells (Haigis *et al.* 2006). SIRT4 also interacts with adenine nucleotide translocator 2 (ANT2) and insulin degrading enzyme (IDE) (Ahuja *et al.* 2007). Functionally, SIRT4 has been shown to have roles in insulin response (Haigis *et al.* 2006), fatty acid metabolism (Laurent *et al.* 2013), and tumor suppression (Jeong *et al.* 2013) through regulation of glutamine metabolism. Recent evidence also suggests that SIRT4 positively regulates ATP levels in the muscle and liver, and that this regulation depends on the mitochondrial uncoupling function of ANT2 (Ho *et al.* 2013). In muscle and liver tissues, loss of SIRT4 is associated with decreased levels of ATP. Despite these burgeoning findings regarding the physiological role of SIRT4, its function in the brain has been largely unexplored. Given its role as a regulator of metabolism and energy status, it is likely to have a critical role in the brain, which is the most energy-dependent organ in the body. Many processes in the brain, such as protection from cellular insults and neurotransmission, depend on ATP (for review, see (Pellerin and Magistretti 2004)).

One key neural process that is extremely energy-dependent is sodium (Na)-dependent glutamate transport, which is carried out by a family of excitatory amino acid transporters (EAATs). In the brain, glutamate transport facilitated by the EAATs contributes to normal neuronal function and protects against excitotoxicity. Glutamate is the major excitatory neurotransmitter in the brain, and there exists no catabolic process at the synapse to break it down subsequent to release. Therefore, the brain critically depends on glutamate transport by the EAATs to scavenge excess glutamate at the synapse. Excitotoxicity occurs when glutamate transport is impaired or there is otherwise a build-up of glutamate at the synapse. It can also be induced pharmacologically, such as with kainic acid. The excess extracellular glutamate poisons neurons and causes cell death. Excitotoxicity is associated with acute and chronic CNS diseases, including stroke, traumatic brain injury, and neurodegenerative disease (Choi 1988; Choi 1992). Excitotoxicity caused by defects in glutamate transport is associated with pathology of a number of neurological diseases, such as epilepsy (Tanaka *et al.* 1997), amyotrophic lateral sclerosis (Rothstein *et al.* 1994a; Trotti *et al.* 1999), and Fragile X Syndrome (Higashimori *et al.* 2013).

There are 3 neuronal isoforms and 2 glial isoforms of Na-dependent glutamate transporters; the predominant isoform in the brain is EAAT2, also known as GLT-1 (for review, see (Danbolt 2001)). In many parts of the brain, the glial GLT-1 (EAAT2) is the major glutamate transporter and may perform as much as 95% of glutamate transport (Rothstein *et al.* 1994b; Rothstein *et al.* 1996; Conti and Weinberg 1999). Na-dependent glutamate transporters rely on the sodium gradient created across the cell membrane by the Na/K-ATPase, which, in turn, relies on ATP (Rose *et al.* 2009). Therefore, Na-

dependent glutamate uptake is highly critical and dependent on ATP. This is further supported by the fact that GLT-1 colocalizes with glycolytic enzymes and mitochondria, which allows for energetic support of GLT-1 function (Genda *et al.* 2011). Because of this energy demand, there exists a possible connection between GLT-1 and metabolic regulation by SIRT4.

In this study, we treated wild-type and SIRT4 KO mice with kainic acid, a potent excitotoxin, to induce a stress response and determine whether SIRT4 has a role in excitotoxicity. We then measured glutamate uptake to determine the mechanism of SIRT4 in excitotoxicity. We found that SIRT4 KO mice have enhanced seizure phenotypes, and that in wild-type mice, SIRT4 levels were increased in the hippocampus 24 hours after treatment. In addition, loss of SIRT4 led to decreased glutamate uptake and decreased glutamate transporter expression at the cell surface. This study is the first to report a role of SIRT4 in neural pathophysiology. These results indicate that SIRT4 promotes glutamate uptake, protecting against excitotoxicity and other conditions caused by glutamate transport defects.

Methods

Animals

Wild-type (129S3/SvImJ) and SIRT4 KO (129-Sirt4<tm1Fwa>/J) mice of both genders were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were maintained on a 12-hour light/dark cycle and fed standard chow. All procedures and care were in strict accordance with the NIH "Guide for the Care and Use of Laboratory

Animals" and approved by the Institutional Animal Care and Use Committee at Tufts University.

Kainic acid injections

Adult mice (6-8 weeks of age) were given a single intraperitoneal injection of kainic acid at a dose of 25 mg/kg. Seizure behavior was monitored and observed for 2 hours immediately following the injection. Seizure behavior scores were given based on the Racine scale (Racine 1972). Briefly, a score of 1 indicates a cease in motor activity; a score of 2 indicates twitching and myoclonic jerks of the head and neck; a score of 3 indicates unilateral clonic activity; a score of 4 indicates rearing and forelimb clonus; a score of 5 indicates generalized tonic-clonic seizures and loss of postural stability. Scores were given once every 20 minutes for the duration of observation. To generate a seizure severity index, the scores for each mouse were added up and divided by the minutes of observation (Giménez-Cassina *et al.* 2012). After 2 hours of observation, mice were returned to their home cage and sacrificed 24 hours post-injection.

Western Blots

Mouse brains were power homogenized in RIPA buffer (50mM Tris-HCl pH 8.0, 1mM EDTA, 0.1% SDS, 150mM NaCl, 1% NP-40, 0.1% sodium deoxycholate) with Complete Mini protease inhibitor cocktail (Roche) added. The homogenate was centrifuged for 15 minutes at 14,000 x g and the supernatant was collected for analysis. 75 ug of the supernatant sample was mixed with 1X SDS-PAGE sample buffer (25mM Tris-HCl pH 6.5, 5% glycerol, 1% SDS, 0.05% bromophenol blue) and loaded onto 4-15% Criterion Tris-HCl polyacrylamide gels (Bio-Rad), electrophoresed, and transferred to Optitran nitrocellulose membrane (GE Healthcare). Proteins were immunoblotted with

anti-SIRT4 (generous gift from the lab of Dr. Leonard Guarente; as previously described (Haigis *et al.* 2006)), anti-EAAT2, anti-MAP2, anti-GFAP, and anti-actin antibodies (Millipore).

Cell culture

Cortices and hippocampi from E18 mouse embryos were used to make primary cultures. The appropriate brain tissues were dissected in digesting media (1X sodium pyruvate, 0.1% glucose, 10mM HEPES in HBSS) and dissociated using 0.05% Trypsin-EDTA (Gibco) for 20 minutes at 37C. Following dissociation, the cells were triturated to obtain an even suspension. Cells were counted using a hemacytometer and plated at $1x10^7$ cells per 10 cm dish or $2x10^4$ cells per well in a 96-well plate. The cultures were plated with 10% FBS (Atlanta Biologicals) present for the first 24 hours and then maintained in serum-free Neurobasal media (Invitrogen) with 2% B-27 supplement (Invitrogen), 1% glutamine (Invitrogen), and 1% penicillin-streptomycin. If a mix of astrocytes and neurons were desired in the preparation, cultures were maintained in 2.5%-5% FBS to allow for glial cell growth.

Cell Titer Assay

Hippocampal primary neurons (as described above) were plated at a density of $2x10^4$ cells per well into 96-well plates (Greiner Bio-One) and maintained in standard neuron growth media. Neurons were treated with kainic acid 24 hours before the assay. CellTiter-Glo reagent (Promega) was added to each well 10 minutes prior to reading the plate according the manufacturer instructions. The luminescence was read using a luminometer (Beckman-Coulter) with 400ms of integration time.

Glutamate uptake assay

Crude synaptosome preparations and the glutamate uptake assays were performed as previously described (Higashimori *et al.* 2013). Briefly, crude synaptosomes were prepared from mouse hippocampus using 0.32M sucrose centrifugation. After determination of total protein concentration, 1 uCi L-^{3}H-glutamate and 100uM unlabeled glutamate were added to 25 uL of synaptosome sample in a 96-well MultiScreen filter plate (Millipore). Dihydrokainic acid (DHK, 500uM), a GLT-1 specific antagonist, was added to the wells measuring GLT-1-dependent uptake (Tocris). The reaction incubated for 6 minutes before filtration with the Steriflip vacuum system. Wells were washed at least 6 times with ice-cold PBS to remove any excess L-^{3}H-glutamate. The membrane from each well was removed from the plate and subject to liquid scintillation counting. The disintegrations per minute (DPM) were normalized to the total protein concentration and converted into pmol/ug/minute.

Biotinylation of cell surface proteins

Biotinylation experiments were performed on DIV15 mixed cortical-hippocampal neuron-glia cultures (cultured as described above). 24 hours prior to biotinylation, cultures were treated with 5uM kainic acid or PBS. Cultures were first washed with ice-cold PBS, and then incubated with EZ-Link Sulfo-NHS-SS-Biotin (Thermo Scientific) for 20 minutes at 4C with gentle shaking. The biotin was removed and cultures were washed with ice-cold PBS with 100mM glycine added (PBS-glycine). Cultures were then incubated with PBS-glycine for 45 minutes at 4C with gentle shaking to quench the biotinylation. After washing, cells were lysed with RIPA buffer for 1 hour at 4C with

vigorous shaking. The lysate was collected by centrifugation, total protein concentration was determined, and 450 ug of total protein was used for avidin immunoprecipitation.

Avidin immunoprecipitation

Neutravidin protein (Thermo Scientific) was coupled to AminoLink Coupling Resin (Thermo Scientific) according to the Direct IP kit manufacturer instructions. After the coupling and quenching reactions were complete, 450 ug of protein from the biotinylation was added to the Neutravidin-coupled resin and incubated overnight at 4C with gentle shaking. The supernatant from the incubation was saved as the intracellular fraction, and the cell surface fraction was eluted from the beads by incubation with 2X SDS-PAGE sample buffer (62.5mM Tris-HCl pH 6.5, 12.5% glycerol, 2.5% SDS, 0.125% bromophenol blue) for 10 minutes at 95-100C. SDS-PAGE sample buffer was also added to the intracellular fraction samples and 10% of the total protein samples, which were then boiled at 95-100C and analyzed together with the cell surface fraction by Western blotting.

Statistics

All values obtained were analyzed with GraphPad Prism software (La Jolla, CA, USA). All data are presented as mean$^{+/-}$ SEM, with n indicating the sample size in each experiment. Significance was achieved with $p<0.05$.

Results

SIRT4 KO mice have severe seizure phenotypes

At baseline, SIRT4 KO mice exhibit no obvious behavioral phenotypes. The mice breed normally, do not show cognitive or motor deficits, and have normal gross brain

anatomy (data not shown). They also do not exhibit spontaneous seizures. Therefore, to test their potential vulnerability to excitotoxic insults, we treated wild-type and SIRT4 KO mice with 25 mg/kg kainic acid to induce a stress response. During the two hours immediately following kainic acid injection, SIRT4 KO mice exhibited abnormally severe seizures, as determined by behavioral observation and scoring. The Racine scale was used to score seizures in each mouse, with a higher score indicating a worse seizure phenotype (Racine 1972). At all time points of observation, SIRT4 KO mice showed higher seizure scores than the wild type, and the difference was highest and statistically significant at 40 minutes after injection (Figure 3.1A). To account for any mice that may have died during the two hours of observation, we re-analyzed the seizure data by integrating the scores of an individual mouse over the time it was alive during the experiment. When the seizure data was analyzed this way, SIRT4 KO mice showed a significantly higher seizure severity index, compared to the wild type (Figure 3.1B). There was no difference between wild-type mice and SIRT4 KO mice with regard to mortality rate or percent of animals responding to kainic acid in response to drug treatment (data not shown). Taken together, SIRT4 KO mice exhibit seizure phenotypes that are more severe than those of matched wild-type controls.

SIRT4 levels are upregulated in response to kainic acid

To determine if SIRT4 is involved in the stress response to kainic acid, we examined the protein expression levels of SIRT4 in wild-type mice treated with saline or kainic acid. Western blots were performed on mouse hippocampal brain tissue 24 hours after kainic acid injection. The hippocampus has a high density of kainate receptors and is known to be especially affected by kainic acid treatment (Choi 1992). Western blot

analysis showed a clear increase in SIRT4 protein levels in the hippocampus after kainic acid treatment (Figure 3.2A), suggesting that SIRT4 responds to excitotoxic stress and is upregulated as a result. There was no difference in SIRT4 mRNA levels after kainic acid treatment as compared to control-treated mouse brains (data not shown), suggesting that the observed effect is posttranslational. We then wondered in which cell types SIRT4 might have its effect, since its cell-type specific expression is not known. Since there currently does not exist a suitable antibody for immunohistochemical analysis of SIRT4 localization in the brain, we created predominantly neuronal cultures or predominantly astroglial cultures and tested for SIRT4 expression. Western blot analysis showed that SIRT4 appears to be more highly expressed in the glial cultures, suggesting that it is predominantly a glial protein and that it may have predominantly glial effects (Figure 3.2B).

Glutamate uptake is decreased in SIRT4 KO mouse brains

A key mechanism for maintaining proper glutamate homeostasis in the brain is the Na-dependent glutamate transporters, particularly the glial EAAT2/GLT-1. Proper function of the glutamate transporters is especially crucial following an excitotoxic insult due to increased action potential firing and glutamate release. Therefore, we isolated hippocampal synaptosomes from wild-type and SIRT4 KO mouse brains 24 hours after kainic acid treatment and measured glutamate uptake. Synaptosomal preparations allow for closer study of synaptic functions, since the preparation isolates the protein population near the synaptic density, including neurotransmitter reuptake transporters like GLT-1 (Bai and Witzmann 2007). We examined total glutamate uptake, which accounts for both Na-dependent and Na-independent mechanisms. We also specifically tested

GLT-1-dependent uptake by performing the assay with GLT-1-specific antagonist, dihydrokainic acid (DHK), present in the reaction. At baseline, when mice were control-treated with saline, SIRT4 KO mice had significantly decreased total and GLT-1-dependent glutamate uptake (Figure 3.3A). After kainic acid treatment, SIRT4 KO mice continued to show decreased glutamate uptake as compared to wild-type mice (Figure 3.3B). In both wild type and SIRT4 KO groups, glutamate uptake was increased after kainic acid treatment (Figure 3.3C). However, wild-type mice showed a greater increase in total glutamate uptake than SIRT4 KO mice in response to kainic acid, suggesting that upregulation of SIRT4 following kainic acid treatment (Figure 3.2A) facilitates glutamate uptake. This result also agrees with our finding that SIRT4 appears to be a predominantly glial protein (Figure 3.2B). Taken together, the results so far show that SIRT4 has a neuroprotective role against an excitotoxic insult by facilitating glutamate uptake. At the same time, deficits in glutamate uptake seen in SIRT4 KO mice likely contribute to the more enhanced seizure phenotype observed after kainic acid treatment.

Cell surface expression of GLT-1 is decreased in SIRT4 KO mice

GLT-1 functions at the cell surface, and can be shuttled between the cell surface and intracellular compartments as part of its functional regulation (Robinson 2002). Therefore, to better understand why SIRT4 KO mice have decreased glutamate uptake, the expression of GLT-1 was examined at the cell surface using a mix of primary cortical and hippocampal neurons and glia. Cell surface proteins were biotinylated and isolated with an avidin column. Western blot analysis of the total, intracellular, and cell surface fractions showed a dramatic decrease in GLT-1 cell surface expression in SIRT4 KO cultures, both control-treated and kainic acid-treated (Figure 3.4). The cell surface

expression levels of GLT-1 positively correlate with the levels of GLT-1 dependent glutamate uptake (Figure 3.3). This suggests that decreased glutamate uptake in SIRT4 KO mouse brains may be due to decreased cell surface expression of the major glutamate transporter.

ATP levels are decreased in SIRT4 KO primary hippocampal neurons

Most glial cells, including astrocytes and microglia, appear to be resistant to the direct effects of excitotoxic insults (Matute *et al.* 2002), whereas neurons are extremely sensitive, as they are the direct targets of glutamatergic stimulus. Since we have shown that loss of SIRT4 has deleterious effects on glial cell function, we wondered if there were concurrent deficits in SIRT4 KO neurons. We decided to measure ATP levels, since maintaining cellular energy levels in neurons is essential to protection against excitotoxicity (Novelli *et al.* 1988). In addition, SIRT4 has been shown to regulate ATP levels in the muscle and liver, where loss of SIRT4 is associated with decreased ATP levels (Ho *et al.* 2013). Therefore, we tested whether or not this was the case in neurons as well. A luciferase-based ATP measurement assay in primary hippocampal neurons showed that SIRT4 KO neurons have significantly decreased ATP levels both at baseline and at increasing kainic acid dosages (Figure 3.5). This result suggests that loss of SIRT4 also has adverse effects on neurons, leading to increased susceptibility to excitotoxic insults.

Discussion

The data presented here show that SIRT4 is induced upon and protective against excitotoxic insults. In mice lacking SIRT4, there are more severe seizure phenotypes and

corresponding decreases in glutamate transport function, cell surface expression of glutamate transporters, and ATP levels. In wild-type mice, SIRT4 expression is upregulated following kainic acid treatment, which corresponds with an increase in total glutamate uptake. In SIRT4 KO mice, the decrease in glutamate uptake function is correlated with a decrease in cell surface expression of glutamate transporters. Therefore, SIRT4 may protect against excitotoxicity by promoting glutamate transport in the brain, and loss of SIRT4 increases sensitivity to excitotoxic insults. This is the first study that shows a neuroprotective role of SIRT4 in the adult brain and sheds new light on how glutamate transport can be regulated during excitotoxicity.

Sirtuins are known to be stress response genes, meaning that they are induced and active upon cellular stress or injury. Several previous studies have shown that sirtuins, including SIRT4, are particularly responsive to oxidative stress or cytotoxic injury. In this study, a robust increase in SIRT4 levels are observed following kainic acid treatment (Figure 3.2A), indicating for the first time that SIRT4 has a role in the response to excitotoxins. Excitotoxicity is a major mechanism of neurodegeneration and neuronal injury, and little is known about how sirtuins may be involved in excitotoxicity. Since sirtuins are known metabolic sensors and regulators, and since there is a metabolic aspect to the excitotoxicity mechanism, the connection between sirtuins and excitotoxicity warrants investigation.

Decreased metabolism predicts a decrease in glutamate uptake capacity (Jabaudon *et al.* 2000). And indeed, this is what we observed in our study (Figure 3.3). At baseline, without kainic acid treatment, there is already decreased glutamate uptake in SIRT4 KO mice. After treatment with kainic acid, SIRT4 KO mice continue to show decreased

glutamate uptake as compared to treated wild-type mice. Glutamate uptake increases in both genotypes in response to kainic acid, but total glutamate uptake is increased to a greater extent in wild-type mice. Since glutamate uptake is critical to deal with the increased load of glutamate from the excitotoxic treatment, a greater increase by the wild-type mice in response to kainic acid treatment suggests that SIRT4 is neuroprotective. Furthermore, these data are in agreement with the observed increase in SIRT4 levels exhibited by wild-type mice after kainic acid treatment (Figure 3.2A), suggesting that SIRT4 specifically facilitates this increase in glutamate transport after excitotoxic insult. This result is further corroborated by our finding that SIRT4 is primarily a glial protein (Figure 3.2B). It was previously not known what the cell type expression of SIRT4 was in the brain, although one study had found that it was expressed in the CTX8 radial glial cell line (Komlos *et al.* 2013). That SIRT4 appears to be glial supported our prediction that it would have an effect on glutamate uptake, which is a major role of astrocytes. Interestingly, the glutamate uptake result also correlates with the cell surface expression of GLT-1 result. This provides further evidence that there is dysregulated glutamate uptake in SIRT4 KO mice, both at baseline and in response to excitotoxicity. Our results indicate that the deficits in glutamate uptake and expression are not severe enough to cause any baseline phenotype, but are deleterious in the face of kainic acid, and potentially other excitotoxic conditions.

In the current study, we also show that there are decreased levels of ATP in SIRT4 KO hippocampal neurons (Figure 3.5). Excitotoxicity is considered to be a predominantly neuronal phenomenon, due to their role glutamatergic signaling and high expression of NMDA receptors (Choi 1992). Metabolic impairment can also cause

neurons to become more susceptible to excitotoxic insults, a phenomenon sometimes referred to as "slow" excitotoxicity (Novelli *et al.* 1988). In this model, decreased metabolic function in the cell, characterized by low ATP levels, decreases the function of the Na/K-ATPase, which leads to an elevated membrane potential and decreased glutamate uptake. The cell becomes more sensitive to any excitatory stimulus, and therefore is more susceptible to excitotoxicity and cell death. SIRT4, as a mitochondrial sirtuin, likely has a role in maintaining proper mitochondrial function. Indeed, an interaction between SIRT4 and the adenine nucleotide translocator 2/3 (ANT2/3) has been previously reported (Ahuja *et al.* 2007), and this is one mechanism by which SIRT4 may promote ATP production. Another previous study has also suggested that the role of ANT2 as a mitochondrial uncoupler is regulated by SIRT4, and that loss of SIRT4 leads to decreased ATP levels (Ho *et al.* 2013). Thus, in addition to the dysregulated glutamate transport shown here in SIRT4 KO mice, there may also be slow excitotoxicity mechanisms is at play and contributing to more severe responses to kainic acid.

In summary, we show here for the first time that SIRT4 is induced in the brain by an excitotoxic insult, and acts to protect against deleterious effects by promoting glutamate transporter expression and function. This neuroprotective role of SIRT4 has implications for conditions such as epilepsy, traumatic brain injury, and neurodegeneration. Further study is required to understand how SIRT4 may be used as a therapeutic target against these diseases.

Acknowledgements/Conflicts of interest disclosure

We thank Dr. Yongjie Yang and Dr. Haruki Higashimori for help with the glutamate uptake assay and for providing reagents and antibodies. We thank Dr. Laura

Liscum and members of the Liscum lab for use of radioactivity facilities. We are also thankful to Dr. Lei Liu for assistance with mouse colony maintenance and technical assistance. We would like to thank Dr. Rob Jackson, Dr. Chris Dulla, Dr. Michele Jacob, and Dr. Yongjie Yang for critical reading of manuscript drafts. This study was supported by funds from the Tufts University Department of Neuroscience startup fund (to G.D.), the Tufts Center for Neuroscience Research Pilot Award (to G.D.), and the Charlton Research Award (to G.D.).

The authors disclose that they have no conflicts of interest.

Figures

Figure 3.1:

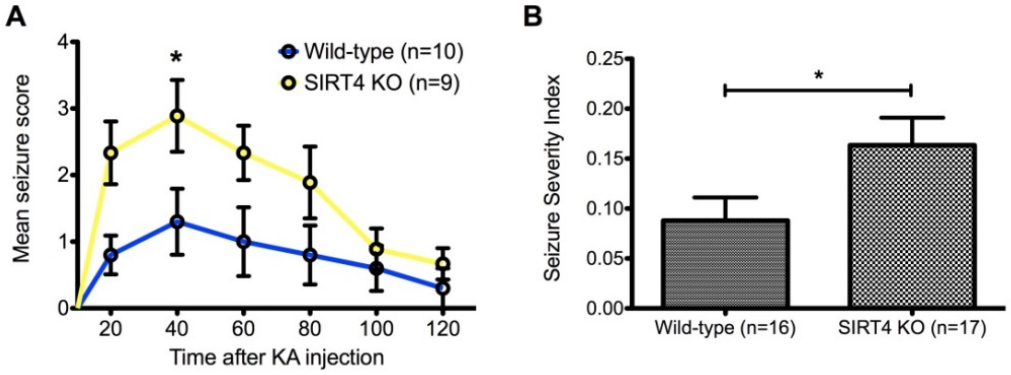

Figure 3.1. SIRT4 KO mice have more severe seizure phenotypes compared to wild-type mice. (A) Mice were intraperitoneally injected with 25 mg/kg of kainic acid and seizures were observed and behaviorally scored for the initial two hours after injection. Seizure scores were given every 20 minutes based on the Racine scale, and each data point indicates the highest observed score during the 20-minute interval. *$p < 0.05$, 2-way ANOVA with Bonferroni correction. (B) Seizure scores were also integrated over the time of the experiment, which allows for the inclusion of any animals that died during the procedure. The seizure severity index for each mouse is the sum of all its seizure scores divided by the duration of observation. *$p < 0.05$, t-test.

WT, wild-type. *sal*, saline. *KA*, kainic acid

Figure 3.2:

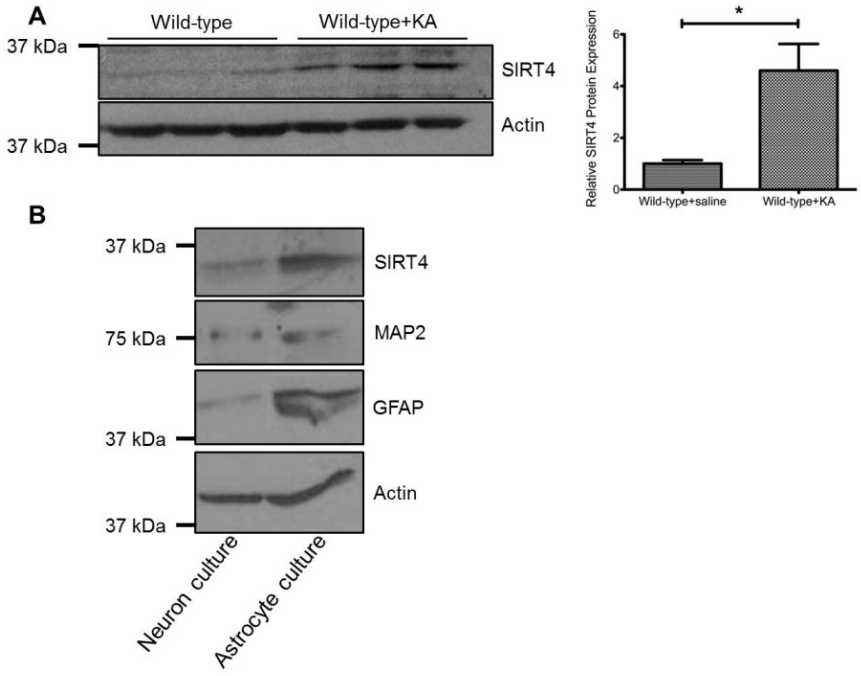

Figure 3.2. SIRT4 levels are increased in the hippocampus 24 hours after kainic acid treatment. (A) A representative Western blot of hippocampal tissue extracts from wild-type mice treated with saline or kainic acid show expression levels of SIRT4, with actin as a loading control. *$p < 0.05$, t-test. (B) Neuron cultures or astrocyte cultures were harvested and probed by Western Blot for the presence of SIRT4, with MAP2 or GFAP indicating purity of cultures and actin as a loading control.

Figure 3.3:

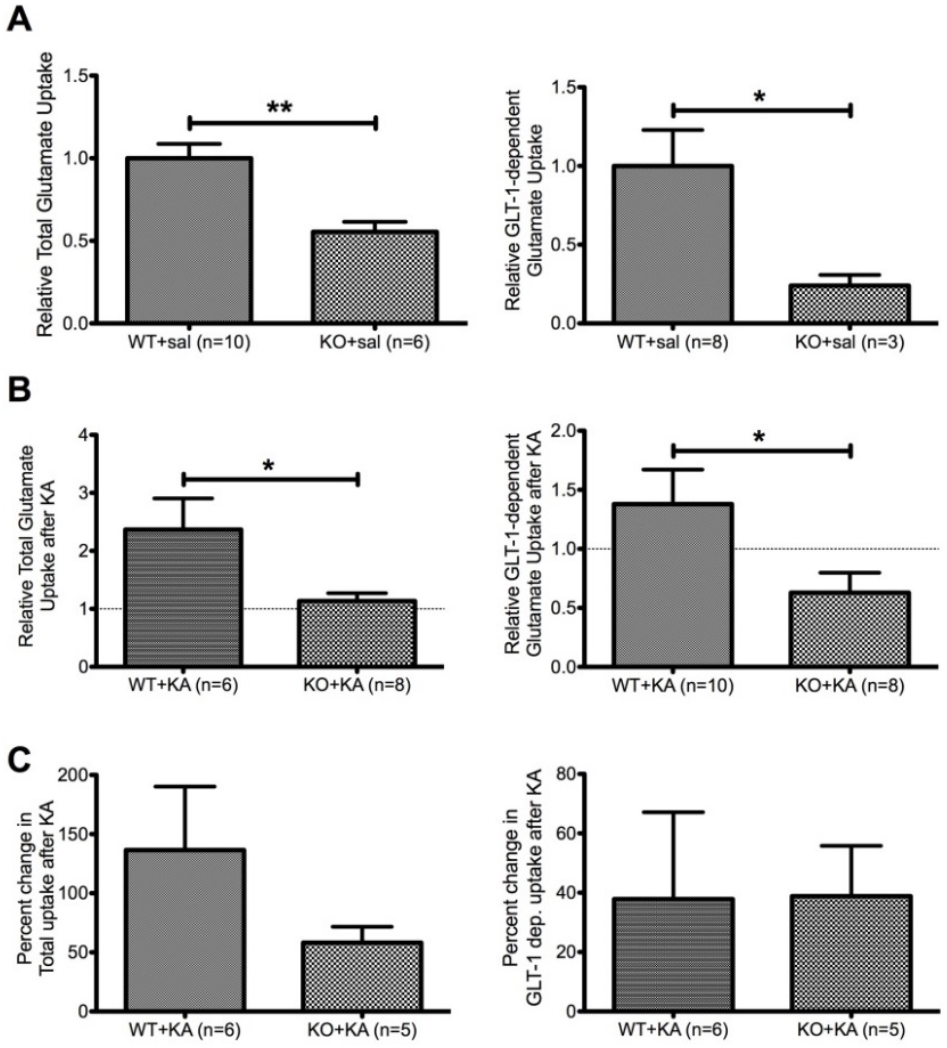

Figure 3.3. Glutamate uptake is decreased in the hippocampus of SIRT4 KO mice. (A) Glutamate uptake was measured in hippocampal synaptosomes from saline-treated wild-type and SIRT4 KO mice. *$p < 0.05$, t-test. (B) Glutamate uptake was measured in hippocampal synaptosomes from kainic acid-treated wild-type and SIRT4 KO mice. The amounts of glutamate uptake was measured 24 hours after treatment and is normalized to the wild-type, saline-treated group. *$p < 0.05$, t-test. (C) The percent increase of glutamate uptake after kainic acid treatment is quantified for wild-type and SIRT4 KO mice.

WT, wild-type. *KO*, SIRT4 KO. *sal*, saline. *KA*, kainic acid

Figure 3.4:

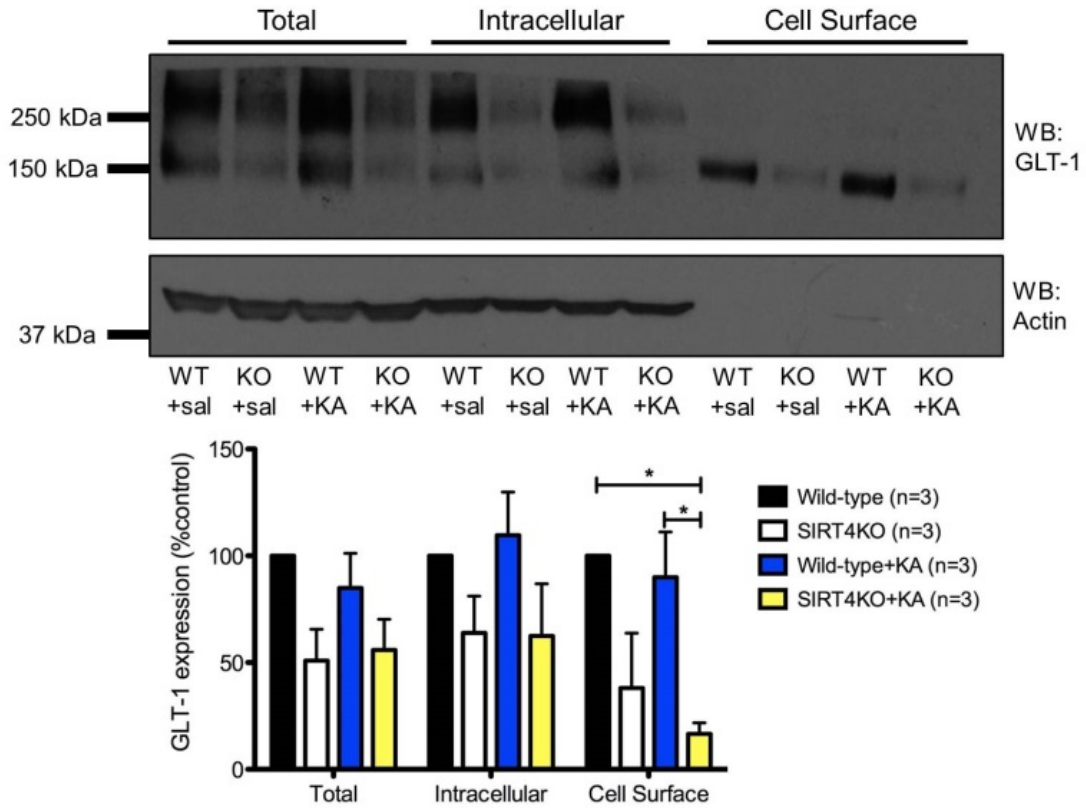

Figure 3.4. Cell surface expression of GLT-1 is decreased in SIRT4 KO primary cultures. (A) Primary cortical/hippocampal tissue cultures were biotinylated to capture and separate cell surface and intracellular protein populations. Western blot shows expression of GLT-1 in total cell culture, intracellular, and cell surface fractions of saline- and kainic acid-treated cultures, with actin serving as a loading control and indicator of fraction purity. (B) Relative protein levels from the representative blot and two other independent experiments are quantified. GLT-1 expression levels are normalized to the wild-type, saline-treated samples.

WT, wild-type. *KO*, SIRT4 KO *sal*, saline. *KA*, kainic acid

Figure 3.5:

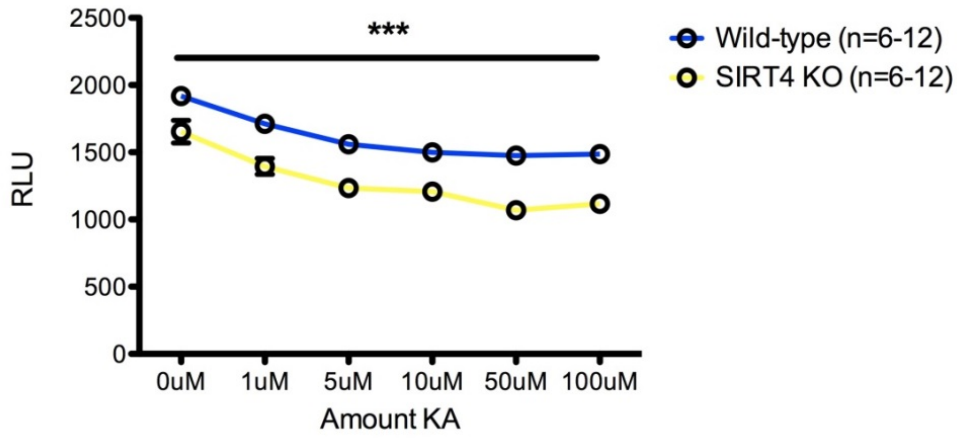

Figure 3.5. ATP levels are further decreased in SIRT4 KO primary hippocampal neurons in response to kainic acid. Wild-type or SIRT4 KO primary hippocampal neurons treated with increasing dosages of kainic acid were mixed with a luciferase reagent that indicates ATP levels. Relative luminescence units (RLU) from each replicate (n=6 or 12 per treatment group) are averaged. ***$p < 0.001$, 2-way ANOVA with Bonferroni correction.

Chapter 4

The astrocytic glutamate transporter GLAST controls neonatal glutamate signaling and the development of fast-spiking cortical interneurons [3]

[3] Shih, J., E. Hanson, M. Armbruster, N.C. Danbolt, Y. Yang, and C. G. Dulla (2017). "The astrocytic glutamate transporter GLAST controls neonatal glutamate signaling and the development of fast-spiking cortical interneurons." To be submitted to *J Neurosci.*

Abstract

Astrocytic excitatory amino acid transporters (EAATs) remove glutamate from the extracellular space and are essential regulators of glutamatergic neurotransmission, synaptic plasticity, and ambient glutamate levels. Two EAAT isoforms, GLAST and GLT-1, predominate in the neocortex and their levels are developmentally regulated. Because GLAST expression and function in the cortex predominates during early postnatal stages, we hypothesized that it may specifically play a role in regulating cortical development. Furthermore, we predicted that GABAergic interneuron maturation, which is glutamate-dependent during early cortical development, may be preferentially affected by disruptions in GLAST function. To test these hypotheses, we genetically manipulated levels of GLAST or GLT-1 using heterozygous mice ($GLAST^{+/-}$ or $GLT\text{-}1^{+/-}$). We found that reducing the expression of GLAST, but not GLT-1, significantly altered glutamate homeostasis during early cortical development (P7-9) and led to cell-type specific effects in fast-spiking (FS) Layer V inhibitory interneurons, but not excitatory pyramidal neurons. FS interneuron intrinsic excitability was transiently increased in neonatal $GLAST^{+/-}$ mice, and was followed by reduced inhibitory synapse number, attenuated FS interneuron dendritic complexity, and altered laminar distribution of parvalbumin-positive interneurons later in development (P14-21). Stimulus-evoked fEPSPs contained abnormal high-frequency (200-500 Hz) activity in $GLAST^{+/-}$, but not $GLT\text{-}1^{+/-}$, mice at P21. Therefore, aberrant network activity observed in the $GLAST^{+/-}$ cortex likely results from developmental changes, rather than acute disruption of glutamate signaling. Together, our results show that GLAST regulates glutamate levels in the neonatal cortex

and significantly shapes interneuron maturation. Disruptions in GLAST lead to abnormal cortical network function consistent with excitatory/inhibitory imbalance.

Significance Statement

The excitatory neurotransmitter glutamate shapes the maturation of GABAergic interneurons. Here, we show that GLAST, an astrocytic glutamate transporter, controls glutamate levels in the developing cortex and contributes to fast-spiking GABAergic interneuron maturation. Long-term disruptions in inhibitory interneuron morphology, inhibitory synapse density, and cortical network function occur when GLAST levels are decreased. Our study identifies a novel interaction between GLAST-mediated control of glutamate and fast-spiking interneuron maturation. These findings may be relevant to the etiology of epilepsy, ataxia, and schizophrenia, in which dysregulation of glutamatergic signaling during development may contribute to excitation/inhibition imbalance and disease pathology.

Introduction

Excitatory amino acid transporters (EAATs) are high-affinity glutamate transporters that remove glutamate from the extracellular space (Danbolt 2001). In the cortex, astrocytic EAATs (EAAT1 (GLAST) and EAAT2 (GLT-1)) perform the bulk of glutamate uptake (Petr, Sun et al. 2015). Robust EAAT function then controls extracellular glutamate levels (Herman and Jahr 2007), neurotransmission (Tzingounis and Wadiche 2007), and synaptic plasticity (Takasaki, Okada et al. 2008), among many other mechanisms that depend on glutamatergic signaling. In particular, many developmental processes in the cortex are glutamate-dependent, but the role of EAATs in their regulation has not been fully characterized.

EAAT levels are especially low in the neonatal cortex, and glutamate receptor activation is largely unconstrained by EAAT function (Hanson, Armbruster et al. 2015). By postnatal day 14 (P14) in mice, however, EAAT expression and function becomes robust (Furuta, Rothstein et al. 1997, Ullensvang, Lehre et al. 1997, Diamond 2005, Thomas, Tian et al. 2011). During this developmental window (birth-P14), glutamatergic signaling contributes to synaptogenesis (Kwon and Sabatini 2011), cellular morphogenesis (Shi, Hayashi et al. 1999, Morel, Higashimori et al. 2014), and spontaneous network activity critical to establishing circuit connectivity (Allene, Cattani et al. 2008, Namiki, Norimoto et al. 2013). At the same time, glutamate signaling helps establish the mature properties of GABAergic interneurons. Glutamatergic activation from thalamocortical inputs is required for radial migration of interneurons within the cortex (Zechel, Nakagawa et al. 2016). Attenuating glutamatergic excitation of interneurons disrupts their morphological development and functional properties (de

Lima, Opitz et al. 2004, Aligny, Roux et al. 2014, Jones, Corbin et al. 2014, Perszyk, DiRaddo et al. 2016). This can lead to circuit-level dysfunction because glutamate-dependent activation of GABAergic interneurons orchestrates early cortical circuit formation (Hensch, Fagiolini et al. 1998, Ben-Ari, Khalilov et al. 2004, Bonifazi, Goldin et al. 2009, Le Magueresse and Monyer 2013).

Since glutamatergic signaling significantly contributes to the developing brain, we aimed to define the specific roles of GLAST and GLT-1 in cortical development. Whether GLAST and GLT-1 have unique roles in the developing cortex is unknown. Biochemical studies show that GLAST is expressed more highly during development relative to its adult levels (Ullensvang, Lehre et al. 1997) and is expressed by radial glia (Shibata, Yamada et al. 1997), both consistent with a developmental role. We also suspected that developing GABAergic interneurons may be especially sensitive to disruptions in glutamate signaling, and therefore EAAT function, because many aspects of their development are glutamate-dependent. How EAATs regulate the excitation of interneurons is largely unknown. In the hippocampus, EAATs keep extracellular glutamate concentrations low, limiting the activation of interneuron metabotropic glutamate receptors (Huang, Sinha et al. 2004, Herman and Jahr 2007). Conversely, minimal EAAT function in the cortex during development may lead to elevated ambient glutamate (Le Meur, Galante et al. 2007), preferentially activating developing interneurons and triggering spontaneous network activity.

To test the hypothesis that GLAST function affects glutamate levels and interneuron maturation, we examined the effects of decreasing either GLAST or GLT-1 expression in the developing cortex. We focused on two developmental ages, P7-9, a

window in which cortical circuits are extremely plastic and EAAT function is low, and P14-21, when cortical networks begin to lose their plasticity and EAAT function is robust. By comparing GLAST heterozygous ($GLAST^{+/-}$) mice with GLT-1 heterozygous ($GLT\text{-}1^{+/-}$) mice, we found that GLAST contributes to the rate of glutamate clearance early in development, while GLT-1 was dominant in the more mature cortex. Decreasing GLAST levels transiently altered interneuron intrinsic properties, decreased the dendritic arborization of fast-spiking interneurons, reduced number of inhibitory synapses, and led to abnormal high-frequency cortical network activity *in vitro* in the mature cortex. Our results identify a unique role for GLAST in regulating glutamate homeostasis and interneuron maturation during neocortical development, which may be relevant to multiple neurodevelopmental disorders associated with EAAT dysfunction (Maragakis and Rothstein 2004).

Materials and Methods

Animals

Animal experiments were performed according to the Guidelines for Care and Use of Animals at Tufts University. Mice deficient in GLAST or GLT-1 were generated as previously described (Tanaka, Watase et al. 1997, Watase, Hashimoto et al. 1998) and mice used in experiments were bred from heterozygous x heterozygous matings and WT x heterozygous matings. When breeding the GLAST mice, both breeding paradigms generated approximate ratios of 50% WT and 50% GLAST$^{+/-}$. Full GLAST knock outs were rarely seen. When breeding the GLT-1$^{+/-}$ mice, heterozygous x heterozygous matings generated approximate ratios of 25% WT, 50% GLT-1$^{+/-}$ and 25% GLT-1 KO; WT x heterozygous matings generated approximate ratios of 50% WT and 50% GLT-1$^{+/-}$. GAD1:eGFP interneuron reporter mice (CB6-Tg(Gad1-EGFP)G42Zjh/J; Jackson Labs Stock # 007677) were obtained from Jackson Labs and crossed with GLAST or GLT-1 heterozygous mice and used in all experiments specifically testing interneurons. Mice were kept on a 12h light/dark cycle with ad libitum access to standard chow and water.

Western blots

Cortical tissue was dissected from brains of wild-type, GLAST$^{+/-}$, and GLT-1$^{+/-}$ mice and solubilized directly in SDS-containing buffer (1% w/v SDS, 1mM PMSF in 20mM sodium phosphate buffer pH 7.4) to avoid formation of insoluble multimeric complexes (Danbolt, Zhou et al. 2016). The soluble fraction of the lysate was collected after centrifugation. 5µg of protein was mixed with sample buffer before they were separated on 10% SDS-PAGE gel (Biorad) and blotted onto nitrocellulose membranes (Biorad). Blots were probed with an anti-GLAST antibody (Anti-A522, RRID:

AB_2314561 (Holmseth, Scott et al. 2009)) or anti-GLT-1 antibody (Anti-B12, RRID: AB_2714081 (Furness, Dehnes et al. 2008)). All protein bands corresponding to EAAT monomers and heteromers were quantified using densitometric analysis (ImageJ) and normalized to actin levels.

Preparation of brain slices for electrophysiology

Cortical brain slices were cut in a coronal orientation and prepared as previously described (Hanson, Armbruster et al. 2015). Briefly, mice were anesthetized with isoflurane and decapitated. Brains were rapidly removed and placed in chilled low-Ca^{2+}, low-Na^{+} sucrose slicing solution consisting of (in mM): 234 sucrose, 11 glucose, 24 $NaHCO_3$, 2.5 KCl, 1.25 NaH_2PO_4, 10 $MgSO_4$, and 0.5 $CaCl_2$. Solutions for electrophysiology were all equilibrated with a mixture of 95%O_2, 5% CO_2. The brain was glued to the stage of a Leica VT1 vibratome and 400 μm-thick slices were cut (300 μm-thick slices for biocytin-filling experiments). The slices were then allowed to recover in oxygenated aCSF consisting of (in mM): 126 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 1 $MgSO_4$, 2 $CaCl_2$, 10 glucose, 26 $NaHCO_2$) for 1 hour. Slices were kept at room temperature after recovery.

Transporter current recording

Acute brain slices were prepared, as described above, and were then bulk loaded with 0.5 μM sulforhodamine 101 (SR-101) for 5 minutes at 32°C immediately after slicing to label astrocytes (Nimmerjahn, Kirchhoff et al. 2004). Slices were then transferred to aCSF without SR-101 and allowed to recover for 1 hour. For recording, brain slices were placed into a submersion recording chamber of an Olympus BX51 microscope and perfused with recirculating aCSF at 32°C. Bath aCSF contained CPP (10

μM) and DNQX (20 μM) to block glutamate receptors, GABAzine (10 μM) to block $GABA_A$ receptors, and 100 μM MNI-glutamate. Astrocytes were identified in Layer V (L5) of the somatosensory cortex based on SR-101 labeling (560 nm excitation, 630 nm emission, filters set = 49008 ET, Chroma) and whole-cell patch clamp recordings were established using borosilicate glass electrodes (6-7 MΩ) containing (in mM): 120 potassium gluconate, 20 HEPES, 10 EGTA, 2 Mg-ATP, 0.2 Na-GTP; pH 7.4, mOsm = 290. Astrocytes were characterized by hyperpolarized membrane potential (-70 to -80 mV) and passive membrane properties. Passive membrane properties were confirmed by a linear input-output relationship in response to current injection steps from -300 to 650 pA, as well as a lack of action potential firing. Intrinsic properties were measured in response to a 5mV, 50 ms depolarizing step. Transporter current recordings were then recorded by flashing a 355 nm UV laser (spot size of 10 μm full width at half maximum) for 1 ms onto the astrocyte cell body while voltage clamping the astrocyte at -80mV. A 5 mV, 50 ms depolarizing pulse was applied 500 ms before the UV-flash in order to normalize evoked transporter currents for changes in access resistance commonly associated with recording from astrocytes (Diamond, Bergles et al. 1998). Additionally, astrocytic recordings with access resistances greater than 25 MΩ were excluded. The resulting glutamate transporter current's decay kinetics were fit using a bi-exponential function, and the time constant of the fast decay was used as a measure of the rate of glutamate uptake.

Tonic NMDA recordings

Neurons were identified in L5 of the somatosensory cortex and whole-cell patch clamp recordings were established using borosilicate glass electrodes with an open-tip

resistance of 2-5 MΩ) containing (in mM): 100 cesium gluconate, 10 HEPES, 5 EGTA, 4 NaCl, 1 $MgCl_2$, 10 TEA-Cl, 10 phosphocreatine, 2 Mg-ATP, 0.2 Na-GTP; pH 7.3, mOsm = 280. Bath aCSF contained DNQX (20 μM), D-serine (200 μM), GABAzine (10 μM), and TTX (1 μM). Cells were voltage-clamped at -60mV and access resistance was allowed to stabilize before increasing the holding potential to +40mV. Holding current was allowed to stabilize and then a 90-second baseline recording was made prior to the addition of 50 μM APV. The change in holding current was allowed to stabilize after the addition of APV before recording 90 seconds of the APV-induced holding current. Access resistance was monitored throughout the recording, and cells with >25% change in access resistance were excluded. The change in holding current after APV wash-on was calculated and normalized to cell capacitance. Capacitance was calculated as $C = \tau\left(\frac{1}{Ra} + \frac{1}{Rm}\right)$ where τ is the decay of the current response to a 5 mV depolarizing step, R_a is access resistance, and R_m is membrane resistance.

Current clamp recordings and biocytin cell filling

GFP-positive cells (inhibitory interneurons) or pyramidal neurons (GFP-negative) were identified in L5 of the somatosensory cortex of GAD1-eGFP reporter mice. Whole-cell patch clamp recordings were established using borosilicate glass electrodes with an open-tip resistance of 2-5 MΩ containing (in mM): 130 potassium gluconate, 10 HEPES, 5 EGTA, 5 KCl, 2 NaCl, 1 $MgCl_2$, 10 TEA-Cl, 10 phosphocreatine, 2 Mg-ATP, 0.2 Na-GTP; pH 7.3, mOsm = 290. Bath aCSF contained DNQX (20 μM) and GABAzine (10 μM). Current steps 250ms long were injected, from -100 pA to 190 pA in 10pA increments (for P7 interneurons), from -25 pA to 600 pA in 25 pA increments (for P21 interneurons), and from -100 pA to 500 pA in 25pA increments (for P7 and P21

pyramidal cells. Current injection was returned to 0 pA for 750 ms between each step. Membrane properties were measured, where capacitance was calculated as $C = \tau\left(\frac{1}{Ra} + \frac{1}{Rm}\right)$, where τ is the decay of the current response to a 5 mV depolarizing step, R_a is access resistance, and R_m is membrane resistance. Action potential properties were measured, and the following parameters were calculated as follows: Membrane potential: the membrane voltage during the 0pA current injection step; Action potential threshold: the membrane voltage at the point where the first action potential peak reached 10% of its peak rise velocity; Action potential after-hyperpolarization: the minimum voltage reached after the first action potential peak; Action potential spike height: the difference between the action potential peak voltage and the after-hyperpolarization voltage; Action potential spike half-width: the width of the action potential waveform at 50% of the action potential spike height. To fill cells with biocytin, 4% biocytin was included in the patch pipette and an outside-out patch was formed after current injection recording, allowing the cell to reseal. Slices were allowed to equilibrate in aCSF for at least 15 minutes before fixing with 4% paraformaldehyde (PFA) overnight.

Cell morphology analysis

Following PFA fixation, slices with biocytin-filled cells were washed 4x in PBS and then incubated overnight at 4ºC in PBS containing streptavidin-conjugated Cy3.5 (1:50), 2% BSA, 10% normal goat serum, and 0.1% Triton X-100. Slices were then washed 3x in PBS followed by a 30 min incubation in 50% glycerol at room temperature. Slices were whole-mounted in Fluoromount-G (Southern Biotech). Mounted sections were then imaged on an A1R confocal microscope (Nikon), with a 20X objective (Olympus). Z stacks were taken, spanning the visible range of the filled cell, at 1 µm

intervals. Two dimensional reconstructions were made by tracing a maximum intensity projection of the entire Z stack with the ImageJ Simple Neurite Tracer plugin. Sholl analysis was performed on the two-dimensional reconstructions with the ImageJ Sholl analysis plugin. Additionally, three dimensional reconstructions were made with Imaris (Bitplane) and branch points of the three-dimensional reconstructions were quantified in Imaris.

Immunohistochemical quantification of inhibitory synapses

Mice were transcardially perfused with phosphate-buffered saline, and the brains were dissected out and immersion-fixed in 4% PFA overnight. After fixation, brains were equilibrated in 30% sucrose in phosphate buffer solution and sectioned into 14 μm thin floating sections using a Thermo Fisher Microm HM 525 cryostat. Brain sections were washed in PBS and placed in blocking buffer (20% normal goat serum in PBS) for 1 h at room temperature. vGAT (1:250, Sigma, AMAb91043) and Gephyrin (1:500, Invitrogen, PA5-19589) antibodies were diluted in antibody solution (PBS containing 0.3% Triton X-100, 10% goat serum). Brain sections were incubated in diluted primary antibody solutions for 48 h at 4 °C then rinsed three times in PBS. Secondary antibodies (goat anti-rabbit Cy3 and goat anti-mouse 647, Jackson Labs; diluted 1:500 in antibody solution) were added to the sections for 2 h at room temperature. Following incubation in the secondary antibodies, sections were washed three times in PBS, with DAPI included in the final wash. Sections were mounted using Fluoromount-G (Southern Biotech). Mounted sections were then used for confocal imaging. Sections were imaged on an A1R confocal microscope (Nikon) with a 60X oil objective (Olympus). Images were taken from L5 of somatosensory cortex. Laser and imaging settings were identical from section

to section and across imaging days. For each section, five sequential miniature Z stacks, each comprised of 3 sections spaced 0.33 μm apart, were taken and maximally projected. Synapses were counted in the maximally projected miniature Z stacks using the ImageJ "puncta analyzer" plugin (Eroglu, Allen et al. 2009).

Immunohistochemical quantification of cortical lamination

P14 WT and GLAST$^{+/-}$ mice were transcardially perfused with phosphate-buffered saline and 4% PFA, and the brains were dissected out and immersion-fixed in 4% PFA overnight. After fixation, brains were equilibrated in 30% sucrose in phosphate buffer solution and sectioned into 20μM (NeuN and Parv staining) or 40μM (CUX1 and CTIP2 staining) free-floating sections using a Thermo Fisher Microm HM 525 cryostat. Brain sections were washed in PBS and placed in blocking buffer (10% normal goat serum, 5% BSA, 0.2% Triton X-100 in PBS) for 1 h at room temperature. NeuN (1:500, Millipore) and PV (1:2500, Swant, PV25), or CUX1 (1:100, Santa Cruz) and CTIP2 (Abcam 1:500) antibodies were diluted in antibody solution (5% normal goat serum, 1% BSA, 0.2% Triton X-100 in PBS). Brain sections were incubated in diluted primary antibody solutions overnight at 4°C then rinsed three times in PBS. Secondary antibodies (goat anti-rabbit Cy3/goat anti-mouse 488 or goat anti-rabbit 488/goat anti-rat 647, Jackson Labs; diluted 1:500 in antibody solution) were added to the sections for 2 h at room temperature. Following incubation in the secondary antibodies, sections were washed 3x in PBS, with DAPI included in the final wash. Sections were mounted using Fluoromount-G (Southern Biotech). Mounted sections were imaged on a Keyence BZ-X700 epifluorescence scope with 20X magnification. Images were taken of the entire somatosensory cortex. Imaging settings were identical from section to section and across

imaging days. Image analysis and quantification were performed in ImageJ (NIH). Background subtraction and autothresholding using the triangle method were applied with the same settings to all images. Identical ROIs spanning the width of the somatosensory cortex and across all cortical layers were applied to each image and cell density was quantified in each ROI using the analyze particles function. For CUX1/CTIP2 analysis, the ROI was divided into deep and superficial layers of the cortex at the Layer IV (L4)-L5 border.

Cortical field EPSP recordings

Slices were placed in an interface chamber maintained at 34°C, superfused with oxygenated aCSF at 2 ml/min and cortical projections were stimulated with a tungsten concentric bipolar electrode at the Layer VI (L6)–white matter boundary. The threshold for stimulation was determined by increasing the stimulator incrementally until a response was detected with a 100 μs pulse but not a 50 μs pulse. Electrical stimulation consisted of 10–50 μA, 150 μs pulses at 30 s intervals delivered by a stimulus isolator (World Precision Instruments). Glass micropipettes (resistance ≅ 1 MΩ) were filled with aCSF and placed in L5 of the cortex directly above the stimulation electrode. Electrophysiological data were recorded with an Axon Multiclamp 700A amplifier and Digidata 1322A digitizer (sampling rate = 20 kHz) with Lab Chart software (AD Instruments). Analysis was performed with pClamp 10 and MATLAB.

Experimental design and statistical analysis

Power analyses were performed to determine the sample size for each experiment and the sample populations used for each experiment are detailed below. Mice of either sex were used for all experiments, with an approximate 1:1 ratio of female to male mice.

Data was not analyzed by gender, but no abnormal distributions of responses were seen. For all immunofluorescence experiments and biocytin cell filling experiments, the experimenter was blind to experimental groups when performing data analysis. For all electrophysiology analysis, data analysis was automated with MATLAB scripts and applied in the same fashion to datasets across all experimental groups. *Western blots*: Data collected from at least 3 animals/group, one cortex sample taken per animal. *Electrophysiology (astrocytic transporter current recordings, tonic NMDA recordings, current clamp recordings)*: Data collected from at least 3 animals/group, 3-5 slices per animal, 1 cell per slice. *Electrophysiology (field EPSP recordings)*: Data collected from at least 3 animals/group, 3-5 slices per animal. *Biocytin cell filling*: Data collected from at least 3 animals/group, 3-5 slices per animal, 1-3 cells per slice. *Immunofluorescence experiments (inhibitory synapse quantification, cortical anatomy quantification)*: Data collected from at least 3 animals/group, 3-5 slices per animal. Detailed information regarding sample sizes and statistical tests are listed in Table 4.1. For all experiments, analysis was done using between-subject comparisons; no within-subject comparisons were made. For comparisons between two experimental groups, a student's t-test was used. For three or more experimental groups, a one-way ANOVA with the Tukey or Bonferroni post-hoc test was used. Values of $P<0.05$ were considered statistically significant. In instances of multiple comparisons, the Holm-Bonferroni Correction was applied (current clamp experiments, input-output analysis) or a mixed effects ANOVA was used (Sholl anlaysis) (Wilson, Sethi et al. 2017). Potential outliers were identified using the Grubbs test, but no outliers were found in any experiments and no data points were removed. Data are reported as mean ± standard error of the mean (SEM) unless

otherwise noted. Bar graphs depicting mean ± SEM values were used to represent the data unless a scatter plot could better depict variability within an experimental group.

Table 4.1: Summary of sample sizes and statistical tests

Experiment (Figure)	Genotype	Age	Total number of animals used	Total N	Statistical test applied
Western blots (Figure 4.1)	WT	P7	4	4 cortical lysates	Two-sample t-test
		P14	3	3 cortical lysates	Two-sample t-test
		P21	4	4 cortical lysates	Two-sample t-test
	GLAST$^{+/-}$	P7	4	4 cortical lysates	Two-sample t-test
		P14	4	4 cortical lysates	Two-sample t-test
		P21	4	4 cortical lysates	Two-sample t-test
	GLT-1$^{+/-}$	P7	4	4 cortical lysates	Two-sample t-test
		P14	3	3 cortical lysates	Two-sample t-test
		P21	4	4 cortical lysates	Two-sample t-test
Astrocytic transporter current recordings (Figure 4.2)	WT	P7	4	18 cells	1-way ANOVA, Tukey posttest
		P21	3	10 cells	1-way ANOVA, Tukey posttest
	GLAST$^{+/-}$	P7	3	13 cells	1-way ANOVA, Tukey posttest
		P21	5	16 cells	1-way ANOVA, Tukey posttest
	GLT-1$^{+/-}$	P7	4	13 cells	1-way ANOVA, Tukey posttest
		P21	3	12 cells	1-way ANOVA, Tukey posttest
Tonic NMDA recordings (Figure 4.3)	WT	P3-6	3	7 cells	Two-sample t-test
		P7-9	4	8 cells	1-way ANOVA, Bonferroni posttest
		P10-19	8	18 cells	Two-sample t-test

	GLAST$^{+/-}$	P3-6	2	5 cells	Two-sample t-test
		P7-9	5	12 cells	1-way ANOVA, Bonferroni posttest
		P10-19	4	9 cells	Two-sample t-test
	GLT-1$^{+/-}$	P7-9	3	7 cells	1-way ANOVA, Bonferroni posttest
IN current clamp recordings (Figure 4.4)	WT	P7	4	18 cells	Two-sample t-test (ad hoc Holm-Bonferroni Correction)
		P21	3	16 cells	Two-sample t-test
	GLAST$^{+/-}$	P7	5	15 cells	Two-sample t-test (ad hoc Holm-Bonferroni Correction)
		P21	3	15 cells	Two-sample t-test
Pyr current clamp recordings (Figure 4.5)	WT	P7	2	11 cells	Two-sample t-test
		P21	3	20 cells	Two-sample t-test
	GLAST$^{+/-}$	P7	2	13 cells	Two-sample t-test
		P21	4	19 cells	Two-sample t-test
IN biocytin cell filling (Figure 4.6)	WT	P21	2	9 cells	Sholl analysis: mixed effects ANOVA
					Branch points: two-sample t-test
	GLAST$^{+/-}$	P21	2	7 cells	Sholl analysis: mixed effects ANOVA
					Branch points: two-sample t-test
Pyr biocytin cell filling (Figure 4.7)	WT	P21	4	14 cells	Mixed effects ANOVA
	GLAST$^{+/-}$	P21	5	25 cells	Mixed effects ANOVA
VGAT-gephyrin	WT	P21	3	12 slices	Two-sample t-test

quantification (Figure 4.8)	GLAST$^{+/-}$	P21	4	16 slices	Two-sample t-test
NeuN-Parv quantification (Figure 4.9A-F)	WT	P21	5	15 slices	Two-sample t-test
	GLAST$^{+/-}$	P21	5	15 slices	Two-sample t-test
CTIP2-CUX1 quantification (Figure 4.9G-I)	WT	P21	5	15 slices	Two-sample t-test
	GLAST$^{+/-}$	P21	5	14 slices	Two-sample t-test
Cortical fEPSPs (Figure 4.10)	WT	P21	8	29 slices	1-way ANOVA, Tukey posttest
	GLAST$^{+/-}$	P21	5	28 slices	1-way ANOVA, Tukey posttest
	GLT-1$^{+/-}$	P21	6	27 slices	1-way ANOVA, Tukey posttest

Results

EAAT expression is reduced in mice heterozygous for GLT-1 and GLAST

To better understand the role of astrocytic glutamate transporters in the development of cortical circuits, we utilized mice heterozygous for either EAAT1 ($GLAST^{+/-}$) or EAAT2 ($GLT\text{-}1^{+/-}$) (Figure 4.1A). The mutant GLAST or GLT-1 alleles each have the neomycin gene inserted into one of their transmembrane domains to disrupt expression (Tanaka, Watase et al. 1997, Watase, Hashimoto et al. 1998). To validate the genetic manipulations, we first tested the effects of heterozygosity on EAAT protein expression in the neocortex. Because EAAT expression is strongly developmentally regulated, we investigated $GLAST^{+/-}$ mice, $GLT^{+/-}$ mice, and WT littermates at 1, 2, and 4 weeks of age. Western blots were performed on total cortical homogenate and, as predicted, the normalized expression of each EAAT was reduced in heterozygous mice by approximately 50% (Figure 4.1B, P7 WT n=4, 1±0.01 vs $GLAST^{+/-}$ n=4, 0.59±0.11, P=0.009, t-test; P14 WT n=3, 1±0.09 vs $GLAST^{+/-}$ n=4, 0.51±0.05, P=0.003, t-test; P28 WT n= 4, 1±0.09 vs $GLAST^{+/-}$- n=4, 0.53±0.04, P=0.002, t-test. Figure 4.1D, P7 WT n=4, 1±0.07 vs $GLT1^{+/-}$ n=4, 0.61±0.02, P=0.002, t-test; P14 WT n=4, 1±0.08 vs $GLT1^{+/-}$ n=3, 0.43±0.03, P=0.002, t-test; P28 WT n= 4, 1±0.04 vs $GLT1^{+/-}$ n=4, 0.44±0.02, P=1.2E-5, t-test). The change in protein expression was specific to the EAAT that was genetically manipulated. GLT-1 levels were not affected in $GLAST^{+/-}$ mice, and vice versa (Figure 4.1C, E). This shows that GLAST and GLT-1 do not compensate for one another in the heterozygous mice, confirming previous findings in the somatosensory cortex (Voutsinos-Porche, Knott et al. 2003).

Reduction of GLAST disrupts astrocytic glutamate uptake in neonatal cortex

How individual EAATs contribute to glutamate astrocytic uptake during cortical maturation is largely unknown. To examine the relative contributions of GLAST and GLT-1 to glutamate homeostasis during cortical development, we utilized an electrophysiological approach in acute brain slices and recorded astrocytic glutamate transporter currents. The uptake of glutamate by EAATs is electrogenic and results in a net positive inwards glutamate transporter current, allowing measurement via whole cell patch clamp electrophysiology (Brew and Attwell 1987, Wadiche, Arriza et al. 1995). Whole cell recordings were made from L5 cortical astrocytes and glutamate transporter currents were evoked by UV-photolysis of MNI-glutamate (Diamond 2005, Hanson, Armbruster et al. 2015). Glutamate uptake rate was quantified by calculating decay constant of the transporter current, which is proportional to the rate of glutamate uptake (Scimemi and Diamond 2013). In the neonatal cortex (P7-10), transporter current decay kinetics were significantly slower in GLAST$^{+/-}$ astrocytes, but normal in GLT-1$^{+/-}$, when compared to decay kinetics measured in WT astrocytes (Figure 4.2A-C, N=18, 13, and 13 cells for WT, GLAST$^{+/-}$, and GLT1$^{+/-}$ respectively, $F(2, 41)=3.528$, 1-way ANOVA. WT vs GLAST$^{+/-}$ $P=0.049$, Tukey Test). However, in the mature somatosensory cortex (P21-24), transporter current decay kinetics were significantly slower in GLT-1$^{+/-}$ astrocytes when compared to WT astrocytes, and unchanged in GLAST$^{+/-}$ (Figure 4.2D-F, N=10, 16, and 12 cells for WT, GLAST$^{+/-}$, and GLT1$^{+/-}$ respectively, $F(2, 35)=4.094$, 1-way ANOVA. WT vs GLT1$^{+/-}$ $P=0.035$, Tukey Test). Astrocyte intrinsic properties were not altered in heterozygous mice, with no differences in resting membrane potential, membrane resistance, IV slope, or capacitance, (Figure 4.2C, 2F, Table 4.2). Together,

our functional data confirms previous expression data (Sutherland, Delaney et al. 1996, Ullensvang, Lehre et al. 1997), indicating that GLAST plays a more significant role in glutamate uptake in the immature, developing neocortex, while GLT-1 function is more critical in the mature cortex.

Ambient glutamate is increased in neonatal GLAST$^{+/-}$ cortex

Since a 50% decrease in GLAST expression resulted in significantly slower glutamate uptake kinetics in the neonatal cortex (Figure 4.2A), we hypothesized that ambient glutamate levels may be altered. To test this, we measured NMDA receptor-mediated tonic currents from L5 cortical pyramidal neurons as a proxy for ambient glutamate levels (Le Meur, Galante et al. 2007). Briefly, whole-cell patch clamp recordings were established in L5Ps, and the holding potential was increased to +40 mV to relieve the voltage-dependent magnesium block of NMDA receptors. This allows unblocked NMDA receptors to act as a sensor for ambient glutamate. We then applied the NMDA receptor selective antagonist APV (50 μM) and measured the change in holding current. Once normalized to capacitance, to account for changes in cell sizes, the effect of APV on the holding current reflects the specific activation of NMDA receptors by ambient glutamate (Figure 4.3A). In P7 WT animals, the change in capacitance-normalized holding current was 0.20±0.04 pA/pF, compared to 0.54±0.10 pA/pF in the P7 GLAST$^{+/-}$ cortex and 0.10±0.06 pA/pF in the P7 GLT$^{+/-}$ cortex (Figure 4.3B-C, N=8, 12, and 7 cells for WT, GLAST$^{+/-}$, and GLT1$^{+/-}$ respectively, $F(2, 24)=8.023$, 1-way ANOVA. WT vs GLAST$^{+/-}$ $P=0.023$, GLAST$^{+/-}$ vs. GLT-1$^{+/-}$ $P=0.004$ Bonferroni Test). The significantly larger difference in the APV-sensitive holding current observed in GLAST$^{+/-}$ L5Ps indicates an increase in tonic NMDA signaling in the GLAST$^{+/-}$ cortex.

Supporting this finding, we observed a 69.9±15.7% decrease in the baseline noise after the addition of APV in GLAST$^{+/-}$ recordings compared to a 17.6±1.92% decrease in WT recordings and 14.5±5.93% decrease in GLT$^{+/-}$ recordings (Figure 4.3D, N=8, 12, and 7 cells for WT, GLAST$^{+/-}$, and GLT1$^{+/-}$ respectively, $F(2, 24)=6.801$, 1-way ANOVA. WT vs GLAST$^{+/-}$ $P=0.015$, GLAST$^{+/-}$ vs. GLT-1$^{+/-}$ $P=0.013$ Tukey Test). The greater reduction in baseline noise measured in the GLAST$^{+/-}$ recording further suggests that tonic NMDA signaling is more prominent in the P7 GLAST$^{+/-}$ cortex. Interestingly, these GLAST$^{+/-}$-specific alterations in tonic NMDA signaling are limited to a small time window in which GLAST is the predominant glutamate transporter in the cortex (Figure 4.2A, Figure 4.3B). Since we did not observe increased ambient glutamate in the developing GLT-1$^{+/-}$ cortex, we focused on the GLAST$^{+/-}$ mice to further investigate the effects of altered glutamate homeostasis in the developing brain.

Intrinsic properties of GAD1-eGFP-postive interneurons are altered in the developing cortex of GLAST$^{+/-}$mice

Because glutamate signaling is known to contribute to the functional maturation of interneurons (Aligny, Roux et al. 2014, Jones, Corbin et al. 2014, Kelsch, Li et al. 2014), we next tested how a reduction in EAAT expression affected neuronal properties of GABAergic. Interneurons were identified using the GAD1-eGFP reporter mouse line (Chattopadhyaya, Di Cristo et al. 2004), which we crossed with the GLAST$^{+/-}$ mice to generate GLAST heterozygous interneuron reporter mice (GAD1-eGFP x GLAST$^{+/-}$). In the GAD1-eGFP reporters, GFP-positive cells in L5 of the mature cortex were reported to be ≈ 90% parvalbumin-positive (PV+) fast-spiking interneurons (Chattopadhyaya, Di Cristo et al. 2004). Whole-cell recordings were established in current clamp mode and

intrinsic properties and action potential firing properties were measured. At P7, GFP-positive interneurons showed electrophysiological properties consistent with immature fast-spiking interneurons (Pangratz-Fuehrer and Hestrin 2011). In GLAST$^{+/-}$ animals, these immature FS interneurons generated more action potentials (APs) in response to current injection, as compared to interneurons in WT littermates (Fig. 4A). Input-output curves show that interneurons in GLAST$^{+/-}$ mice generated on average 3.8 ± 0.2 more APs at current injection steps above 20 pA as compared to interneurons in WT mice (Fig 4B, N=12 and 13 cells, WT and GLAST$^{+/-}$ respectively, t-test adjusted for multiple comparisons with Holm-Bonferroni Correction, α <0.05). The rheobase, defined as the amount of current required to elicit the first AP, was also significantly decreased for interneurons in P7 GLAST$^{+/-}$ mice (Fig. 4C, 38.6±7.23 pA, N=15 cells) as compared to WT (Fig. 4C, 83.4±8.79 pA, N=18 cells, P=5.688E-4, t-test). We further characterized AP properties and found that APs in GLAST$^{+/-}$ mice had an increased spike half-width (Table 4.3, 1.12±0.06 ms vs. 1.51±0.08 ms, WT vs. GLAST$^{+/-}$, N=16 and 15 cells, respectively, P=4.44E-4, t-test), consistent with APs in less mature interneurons (McCormick and Prince 1987). When we quantified intrinsic membrane properties, we found that the membrane resistance of interneurons was increased (Fig. 4D, 698.2±85.7 MΩ, N=15 cells) and the cell capacitance was decreased (Fig. 4E, 65.59±4.39 pF, N=15 cells) in GLAST$^{+/-}$ mice compared to their wild-type littermates at P7 (*Membrane resistance:* 323.0±26.8 MΩ, N=18 cells, P=8.851E-5, t-test; *Cell capacitance:* 86.63±6.92 pF, N=18 cells, P=0.02, t-test). Interestingly, the only difference that was maintained at P21 was the change in interneuron cell capacitance (Figure 4.4E, P21 WT n=16 cells, 114.1±8.84 pF vs P21 GLAST$^{+/-}$ n=15 cells, 78.2±9.55 pF, P=0.01, t-test).

To determine if these changes in action potential firing properties and intrinsic membrane properties were cell-type specific, we performed the same measurements in L5Ps in the GLAST$^{+/-}$ cortex (Figure 4.5A). Here, we did not observe the same major differences in L5P intrinsic properties. There were no differences in action potential firing properties at P7, with pyramidal cells from both genotypes firing similar numbers of action potentials at increasing current injection steps (Fig 5B, N=11 and 13 cells, WT and GLAST$^{+/-}$ respectively). Similarly, rheobase (WT N=11 cells, 43.2±5.93 pA vs. GLAST$^{+/-}$ N=13 cells, 57.7±7.69 pA), membrane resistance (WT N=11 cells, 655.5±70.9 MΩ vs. GLAST$^{+/-}$ N=13 cells, 532.2±74.2 MΩ), and capacitance (WT N=11 cells, 92.3±7.76 pF vs. GLAST$^{+/-}$ N=13 cells, 93.9±11.3 pF) were not different in P7 GLAST$^{+/-}$ L5Ps (Figure 4.5C-E). In P21 L5Ps in the GLAST$^{+/-}$ cortex, we observed a more depolarized action potential threshold (Table 4.3, -45.4±1.70 mV vs. -37.1±1.13 mV, WT vs. GLAST$^{+/-}$, N=16 and 15 cells, respectively, P=4.44E-4, t-test) However, rheobase (WT N=20 cells, 71.2±9.30 pA vs. GLAST$^{+/-}$ N=19 cells, 85.5±11.5 pA), membrane resistance (WT N=15 cells, 182.2±26.4 MΩ vs. GLAST$^{+/-}$ N=17 cells, 196.9±31.7 MΩ), and capacitance (WT N=15 cells, 134.7±13.8 pF vs. GLAST$^{+/-}$ N=17 cells, 161.3±18.8 pF) were not different in P21 GLAST$^{+/-}$ L5Ps (Figure 4.5C-E). This suggests that the effect of GLAST heterozygosity on L5P action potential firing properties is very minor. Overall, our data shows that GLAST heterozygosity alters FS interneuron properties in the cortex, with little effect on the properties of L5Ps.

Layer 5 cortical interneuron morphology is altered in the GLAST$^{+/-}$ mice

Because aberrant activation of neurons during development affects their later morphology (Cancedda, Fiumelli et al. 2007, Aligny, Roux et al. 2014), we next tested

whether interneuron morphology was altered in GLAST$^{+/-}$ mice. Whole-cell recordings were established in L5 GAD1-eGFP-postive interneurons and GFP-negative pyramidal cells in P21 GLAST$^{+/-}$ mice, and cells were filled with biocytin. After filling, cells were reconstructed from confocal Z-stacks. Dendritic morphology was analyzed using Sholl analysis and by quantifying branch points. Sholl analysis revealed decreased dendritic complexity in interneurons from GLAST$^{+/-}$ mice, with a significant difference in the number of intersections with a Sholl radius of 93 μm (Figure 4.6A-B, 10.8 vs 7.6 intersections, N=9 and 7 cells, WT vs GLAST$^{+/-}$ respectively, P=0.027, mixed effects ANOVA). GLAST$^{+/-}$ interneurons also had fewer branch points (12±1.27, N=7 cells) as compared to WT (Figure 4.6C, 16.3±1.05, N=9 cells, P=0.019, t-test). The decreased dendritic complexity observed in GLAST$^{+/-}$ mice is consistent with the decreased cell capacitance measured in GLAST$^{+/-}$ interneurons at P21 (Fig. 4E). We also performed Sholl analysis on P21 L5 pyramidal cells and found no difference in the dendritic arborization (Figure 4.7, N=14 and 25 cells, WT vs GLAST$^{+/-}$ respectively), which correlates with no changes in cell capacitance measured in those cells (Fig. 5E).

Inhibitory synapse density is decreased in GLAST$^{+/-}$ mice

Since we observed decreased dendritic arborization as a result of GLAST heterozygosity, we next analyzed inhibitory synapse number in the P21 GLAST$^{+/-}$ cortex. We utilized an anatomical approach and quantified the co-localization of vGAT, a marker of inhibitory presynaptic terminals, and gephyrin, which marks inhibitory postsynaptic densities. In the cortex of GLAST$^{+/-}$ mice, the number of colocalized puncta (281.2±31.2 per 10,000μm^2) was decreased compared to WT (438.8±77.1 per 10,000μm^2) (Figure 4.8A-C, N=12 vs 16 sections, WT vs GLAST$^{+/-}$, P=0.047, t-test). There was also a

significant decrease in the number of vGAT, but not gephyrin, puncta in GLAST$^{+/-}$ mice (2597.5±136.8 per 10,000μm^2) compared to WT mice (3400.0±323.7 per 10,000μm^2), suggesting that presynaptic, perisomatic inhibitory inputs are decreased in GLAST$^{+/-}$ mice (Figure 4.8D-E, N=12 vs 16 sections, WT vs GLAST$^{+/-}$, P=0.019, t-test). Similar to cell-type specific observations in neuronal morphology, there were no differences in the number of excitatory vGLUT1/PSD95-labeled excitatory synapses (Figure 6.2).

Parvalbumin-positive interneuron density is altered in superficial cortical layers in GLAST$^{+/-}$ mice

The altered interneuron morphology and decreased number of inhibitory synapses in the GLAST$^{+/-}$ cortex suggests that there may be broad deficits in the anatomy of inhibitory circuits. Therefore, we tested whether cortical lamination was disrupted in GLAST$^{+/-}$ mice by quantifying the number of NeuN+ and PV+ cells in the somatosensory cortex. We found no changes in the number of NeuN+ cells across all layers of the GLAST$^{+/-}$ somatosensory region (1523.8±42.1 cells/mm^2, N=15 sections) compared to WT (1413.9±67.7 cells/mm^2, N=15 sections) (Figure 4.9A-B). There were also no differences in the density of PV+ cells, although we did observed non-significant trend towards more PV+ cells in the cortex of GLAST$^{+/-}$ mice (57.2±3.48 cells/mm^2, N=15 sections) compared to WT (48.0±3.51 cells/mm^2, N=15 sections, P=0.07, t-test). We suspected that the difference was layer-specific, and therefore examined PV+ cells in superficial and deep layers of the cortex. We found that PV+ cell density was slightly, but significantly, increased in the superficial layers of the GLAST$^{+/-}$ somatosensory cortex (60.0±4.14 cells/mm^2, N=15 sections) compared to WT (47.8±3.95 48 cells/mm^2, N=15 sections, WT vs GLAST$^{+/-}$ P=0.042, t-test). We also measured the thicknesses of the deep

and superficial layers of the somatosensory cortex, as defined by the borders of CTIP2 and CUX1 (Nieto, Monuki et al. 2004, Arlotta, Molyneaux et al. 2005) expression, respectively, and did not find any differences in the size of the cortical layers in GLAST$^{+/-}$ mice. Therefore, our results suggest that gross anatomy of the cortex in GLAST$^{+/-}$ mice is normal and that there do not appear to be obvious migration defects. However, distribution of PV+ interneurons is slightly altered in GLAST$^{+/-}$ mice.

Cortical network function is disrupted in the mature GLAST$^{+/-}$ cortex

Because we observed deficits in interneuron morphology and inhibitory synapse number in GLAST$^{+/-}$ mice in the mature cortex, we suspected that cortical network function might be altered. Therefore, we measured field excitatory post-synaptic potentials (fEPSPs) in acute cortical brain slices from two- to three-week old WT and GLAST$^{+/-}$ mice. A recording electrode was placed in L5 of the somatosensory region of the cortex and a bi-polar stimulating electrode was placed in the L6-white matter directly below the cortex. A brief (150 μs) constant current stimulation was delivered every 30 seconds and the evoked fEPSP was quantified. The amplitude of the constant current stimulation delivered was not different between genotypes (data not shown). In WT animals, a brief, low amplitude fEPSP was recorded. In GLAST$^{+/-}$ mice, however, we observed an abnormal response, characterized by polyphasic, epileptiform fEPSPs containing high-frequency activity. Power spectrum analysis of fEPSP responses showed that GLAST$^{+/-}$ fEPSPs had increased power in the 200-500Hz range (Figure 4.10A), which correlates with the epileptiform activity observed in GLAST$^{+/-}$ fEPSP traces (Figure 4.10B).The integrated response area of GLAST$^{+/-}$ fEPSPs was compared to WT (N=29, 25, and 27 slices, WT, GLAST$^{+/-}$, and GLT-1$^{+/-}$, respectively. $F(2, 78)=5.0348$,

P=0.00878, 1-way ANOVA. WT vs GLAST$^{+/-}$ P=0.007 Tukey Test). The number of traces recorded per slice that showed epileptiform activity was also increased in GLAST$^{+/-}$ mice (N=29, 25, and 27 slices, WT, GLAST$^{+/-}$, and GLT-1$^{+/-}$, respectively. F(2, 78)=3.218, P=0.045, 1-way ANOVA. WT vs GLAST$^{+/-}$ P=0.035 Tukey Test). Quantification of the power spectrum in the first 750 ms following the stim-evoked response shows a significant increase in 200-500Hz average power in the GLAST$^{+/-}$ fEPSPs (4.71±0.79, N=28 slices) compared to WT fEPSPs (1.99±0.29, N=29 slices) (Figure 4.10C-D, N=29, 28, and 27 slices, WT, GLAST$^{+/-}$, and GLT-1$^{+/-}$, respectively. F(2, 42)=11.94, P=7.862E-5, 1-way ANOVA. WT vs GLAST$^{+/-}$ P=9.54E-4, GLAST$^{+/-}$ vs. GLT-1$^{+/-}$ P=1.57E-4, Tukey Test). Interestingly, fEPSP responses in cortical brain slices from GLT-1$^{+/-}$ mice did not show epileptiform activity or increased integrated fEPSP area, suggesting that the abnormal cortical network phenotype is due to long-term, hardwired changes induced by GLAST heterozygosity, rather than acute glutamate handling effects that would be attributed to GLT-1 function.

Discussion

Excitatory amino acid transporters (EAATs) control glutamatergic excitation (Haugeto, Ullensvang et al. 1996, Tanaka, Watase et al. 1997), but how they contribute to glutamate-dependent inhibitory interneuron development is unknown. We tested the hypothesis that an astrocytic EAAT, GLAST, may be important in early cortical development and thus may have unique effects on inhibitory interneuron maturation. Indeed, we show that decreasing GLAST expression reduced astrocytic glutamate uptake and increased ambient glutamate during early cortical development (P7-9). Decreasing levels of GLAST also increased the intrinsic excitability of developing cortical L5 fast-spiking GABAergic interneurons (as identified by GAD1-eGFP labeling), but not L5 pyramidal neurons. Reduction of GLAST led to alterations in fast-spiking interneuron morphology, inhibitory synapse number, laminar density of PV+ interneurons, and cortical network function in the more mature cortex. Taken together, our findings functionally confirm the importance of GLAST in early cortical development and demonstrate a novel role for GLAST in shaping fast-spiking GABAergic interneuron maturation.

GLAST is highly expressed in the cerebellum, but has also been implicated in cortical development due to its increased expression relative to GLT-1 early in development (Ullensvang, Lehre et al. 1997) and its presence on radial glial and progenitor cells (Shibata, Yamada et al. 1997). Functional demonstration of the developmental role of GLAST, however, is lacking. Our studies show for the first time that reducing the levels of GLAST, but not GLT-1, disrupt glutamate uptake during early neonatal development (Figure 4.2A-C). Conversely, in the more mature cortex, reducing

GLAST levels appears to have little effect of glutamate uptake (Figure 4.2D-F). Complete genetic knockout of GLAST results in abnormal social behavior (Karlsson, Tanaka et al. 2008, Karlsson, Tanaka et al. 2009, Karlsson, Adermark et al. 2012) and decreased electrical seizure kindling (Tsuru, Ueda et al. 2002), in addition to disruptions in cerebellar function (Watase, Hashimoto et al. 1998, Miyazaki, Yamasaki et al. 2017). Our findings suggest that these phenotypes may be mediated by early disruption of cortical development. Because both behavior (Carlen, Meletis et al. 2012) and epilepsy (Cobos, Calcagnotto et al. 2005) are strongly regulated by cortical inhibition, the changes we report in inhibitory interneurons may also be relevant.

Interneurons have complex morphologies, which allow them to constrain neuronal activity with spatiotemporal precision (Markram, Toledo-Rodriguez et al. 2004). Attaining their mature morphology requires precise and controlled glutamatergic activity during development (Cancedda, Fiumelli et al. 2007, Allene, Picardo et al. 2012, Zechel, Nakagawa et al. 2016). Both attenuating neuronal activity via monocular deprivation or reducing glutamatergic activation using pharmacological approaches reduces the morphological complexity of developing interneurons (Rajan and Cline 1998, Cheadle and Biederer 2014, Andreae and Burrone 2015). Conversely, over-activation of NMDARs (George, Baden et al. 2012) and increased synaptic activity leads to compensatory decreases in dendritic arborization of interneurons (Nishimura, Owens et al. 2008, Tripodi, Evers et al. 2008). Interestingly, in GLAST$^{+/-}$ mice specifically, ambient glutamate levels were increased (Figure 4.3) and FS cortical interneurons had smaller, less complex dendritic arbors (Figure 4.6). Our findings show that GLAST

critically controls glutamatergic activity during development and supports the finding that increasing glutamatergic activation can disrupt interneuron morphogenesis.

The density of inhibitory synapses, as labeled by vGAT/gephyrin co-localization, was also decreased in L5 of GLAST$^{+/-}$ somatosensory cortex (Figure 4.8), without a change in the density of vGLUT1/PSD95 labeled excitatory synapses (Figure 6.2). Similar to inhibitory interneuron morphogenesis, inhibitory synapse formation is activity-dependent. Disrupting sensory experience, via whisker trimming, reduces inhibitory synapse number in the somatosensory cortex (Jiao, Zhang et al. 2006). Conversely, direct photo-uncaging of GABA elicits inhibitory synaptogenesis (Oh, Lutzu et al. 2016). Interestingly, changes in sensory experience and GABA uncaging only alters inhibitory synapse number in the cortex during early cortical development. After approximately the first two postnatal weeks, inhibitory synapse number becomes less plastic. How decreased GLAST mechanistically leads to decreased inhibitory synapse number is unknown, but perhaps early activity of developing interneurons inhibits structural plasticity before inhibitory synaptogenesis is complete. Further studies are required to elucidate the link between GLAST function and the molecular mechanisms of inhibitory synaptogenesis.

Glutamatergic activation of cortical interneurons can also shape their migration and radial dispersion into the cortical lamina. Decreasing thalamocortical glutamatergic input into the cortex results in fewer PV+ cells in the superficial layers of the cortex (Zechel, Nakagawa et al. 2016). Conversely, we show that GLAST$^{+/-}$ animals have a small but significant increase in PV+ interneuron density in cortical L2/L3 (Figure 4.9D-F). Because decreasing GLAST function increases ambient glutamate early in

development, the altered L2/L3 density of PV+ interneurons that we observe may be glutamate-dependent. In addition to examining the effects on PV+ interneuron migration, we also investigated whether reduced GLAST levels affected cortical structure more broadly, since glutamate regulates neurogenesis (Jansson and Akerman 2014) and excitatory neuronal migration (Komuro and Rakic 1993). GLAST is also expressed by radial glia during embryonic development (Shibata, Yamada et al. 1997), which provide a physical scaffold for neuronal migration. Surprisingly, we did not find any changes in cortical structure in GLAST$^{+/-}$ mice. Cortical lamination, as measured by the distribution of CTIP2+ or CUX1+ cells, was not altered (Figure 4.9G-I), nor did we find changes in the total number of NeuN+ cells in the cortex (Figure 4.9A-C). This suggests that GLAST heterozygosity does not affect neurogenesis and the subsequent population of the cortical layers. These findings again support that disrupting GLAST function preferentially affects inhibitory interneurons.

We suspect the preferential effects of GLAST heterozygosity on cortical interneurons is mediated by high-affinity NMDARs. GluN2C- and GluN2D-containing NMDA receptors, in addition to their increased affinity for glutamate, have decreased affinity for magnesium (Monyer, Burnashev et al. 1994, Vicini, Wang et al. 1998). The expression of these receptors peaks in early cortical development and in the mature cortex they are expressed by GABAergic interneurons. The properties of these receptors allow them to carry a tonic glutamate current (Le Meur, Galante et al. 2007) which increases interneuron excitability in the adult hippocampus (Sah, Hestrin et al. 1989, Riebe, Seth et al. 2016). Whether immature interneurons express high-affinity NMDA receptors is not known, but changes in the intrinsic properties of developing fast-spiking

interneurons in GLAST$^{+/-}$ mice that we observe are consistent with that possibility (McCormick and Prince 1987, Pangratz-Fuehrer and Hestrin 2011). Regardless of the mechanism, excitability is increased in immature interneurons (Figure 4.4), which likely leads to increased GABA-driven spontaneous network activity that is critical to establishing proper cortical network connectivity in the mature cortex (Allene, Cattani et al. 2008). This early, spontaneous activity is glutamate-dependent and is regulated by glutamate transporters (Demarque, Villeneuve et al. 2004). Therefore, disrupted GLAST function may alter fast-spiking interneuron activity during early cortical development, leading to long-term changes in cortical network function. Consistent with this idea, cortical hyperexcitability was not seen GLT-1$^{+/-}$ mice. This suggests that abnormal cortical network activity is not due to acute glutamate transporter dysfunction, but rather GLAST-mediated in cortical network development.

Our study has a number of important caveats. First, multiple classes of cortical interneurons exist. Here, we have only examined fast-spiking L5 interneurons using GAD1-eGFP labeling. In the adult cortex, this labels predominantly PV+ interneurons (Chattopadhyaya, Di Cristo et al. 2007). Since PV expression becomes robust at around P14 (Doischer, Hosp et al. 2008), we cannot confirm the sub-type categorization of the fast-spiking GFP-positive interneurons we record at P7-9. Next, we cannot confirm that GLAST-mediated effects are due to altered glutamate signaling. Other potential mechanisms include alterations in radial glia cell/cell interactions, changes in chloride driving force cause by decreased GLAST-mediated chloride conductance (Wadiche, Amara et al. 1995), and changes in the morphogenesis of astrocytes, among others. Additionally, we only examine cortical phenotypes up until P21. Future work will

examine which, if any, of these phenotypes persist into the mature cortex. Finally, the developmental time-points we identify here only apply to L5 somatosensory cortex. Other cortical regions may not rely on GLAST, or they may have different developmental windows in which changes occur.

These caveats aside, our findings demonstrate the critical importance of GLAST in shaping the maturation of inhibitory cortical networks. Disruptions in interneuron function is implicated in several neurodevelopmental diseases, including epilepsy (Kobayashi and Buckmaster 2003, Cobos, Calcagnotto et al. 2005) and schizophrenia (Lewis, Hashimoto et al. 2005, Lisman, Coyle et al. 2008). In particular, a leading hypothesis for schizophrenia etiology involves decreased function of PV+ interneurons (Volk and Lewis 2014, Gonzalez-Burgos, Cho et al. 2015). Interestingly, decreased GLAST expression may occur in the prefrontal cortex of schizophrenia patients (Bauer, Gupta et al. 2008), and a genomic microdeletion spanning part of the GLAST gene has been found in a case of childhood-onset schizophrenia (Walsh, McClellan et al. 2008). Schizophrenia-like behaviors have been reported in GLAST KO mice, which show deficits in cognitive and social behavior (Karlsson, Tanaka et al. 2008, Karlsson, Tanaka et al. 2009). Whether GLAST-mediated changes in fast-spiking interneuron properties lead to behavioral changes remains to be seen, but our findings suggest a potential link. Together, our findings show that loss of GLAST function alters the development and function of interneurons and associates decreased GLAST function during development with later deficits in cortical inhibition.

Figures

Figure 4.1:

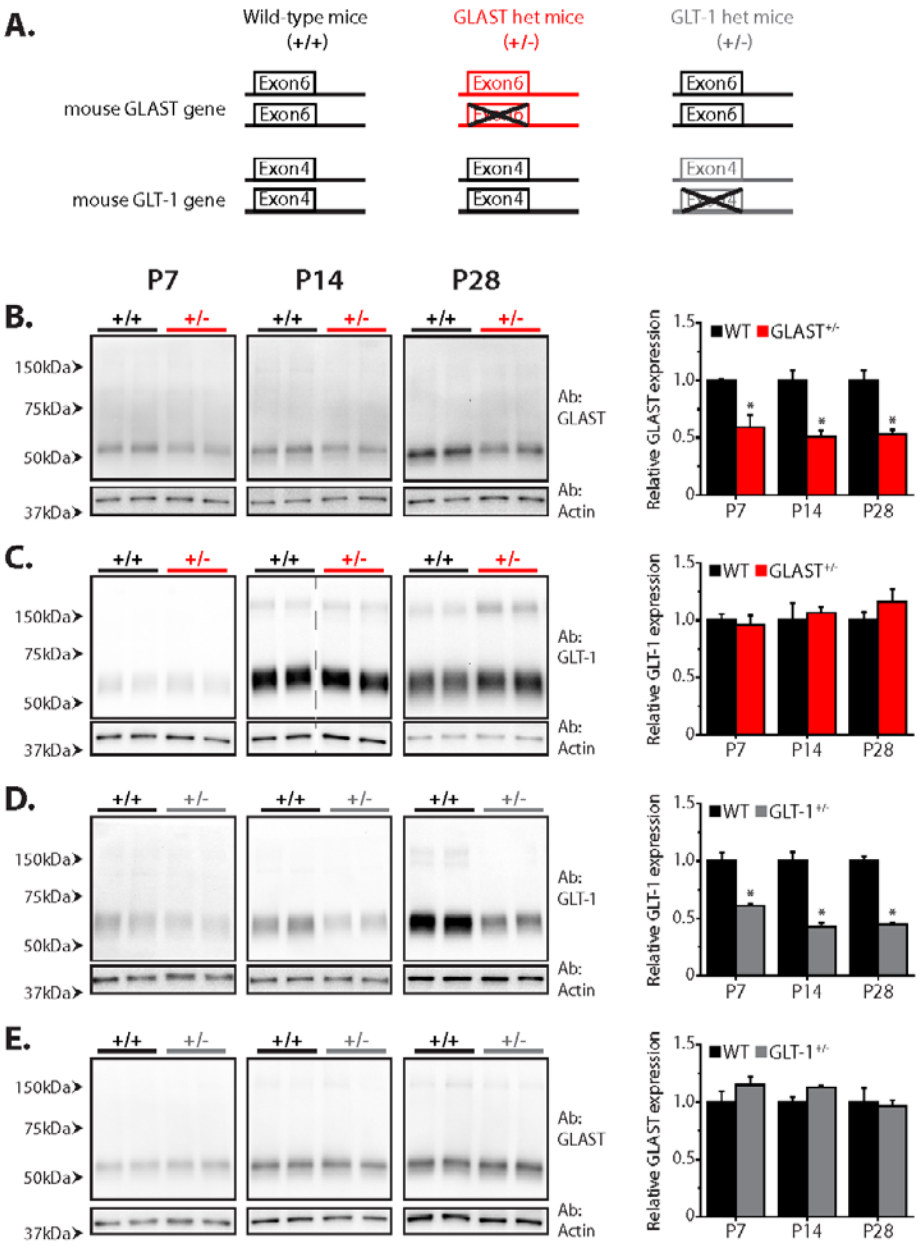

Figure 4.1. Cortical glutamate transporter levels are decreased by roughly 50% in GLAST$^{+/-}$ and GLT-1$^{+/-}$ mice. (A) Schematic diagram of the GLAST and GLT-1 alleles in wild-type, GLAST$^{+/-}$, and GLT-1$^{+/-}$ mice used for experiments. The disrupted exons are indicated. (B) Total cortical lysates from GLAST$^{+/-}$ and wild-type littermate controls were probed for GLAST expression. (C) Total cortical lysates from GLAST$^{+/-}$ and wild-type littermate controls were probed for GLT-1 expression. Dotted line indicates one lane

excluded from the representative blot. (D) Total cortical lysates from GLT-1$^{+/-}$ and wild-type littermate controls were probed for GLT-1 expression. (E) Total cortical lysates from GLT-1$^{+/-}$ and wild-type littermate controls were probed for GLAST expression. In all experiments, GLAST and GLT-1 bands corresponding to the monomer and any multimers were included in the quantification and normalized to actin levels. Relative protein expression from GLAST$^{+/-}$ and GLT-1$^{+/-}$ mice were normalized to wild-type expression levels from littermate controls. *P < 0.05, two-sample t-test.

Figure 4.2:

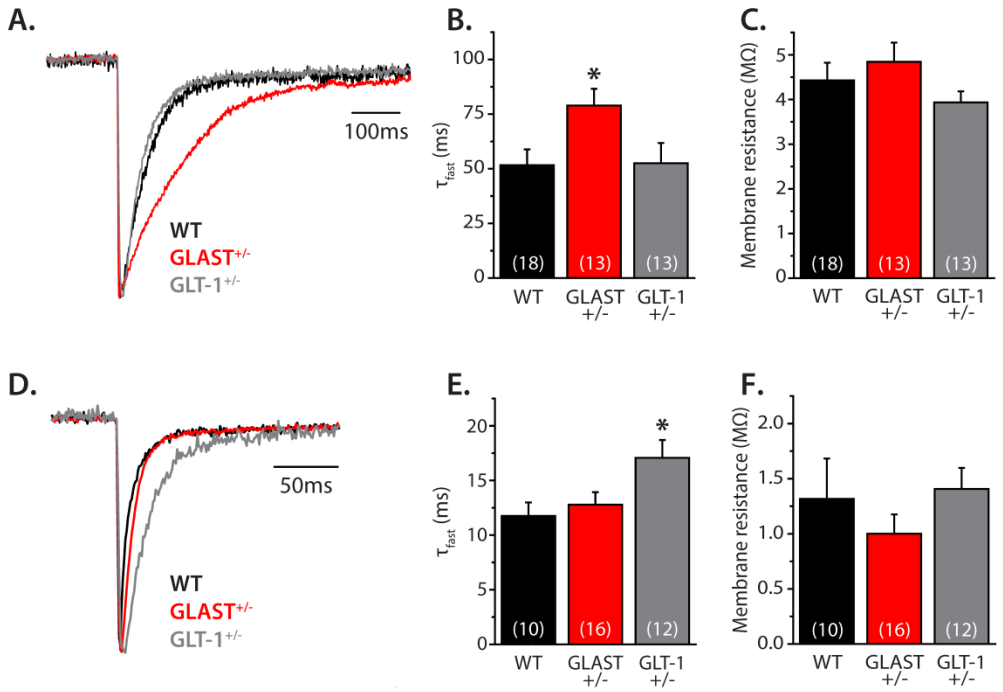

Figure 4.2. Astrocytic glutamate transport relies on GLAST at younger ages and GLT-1 at older ages. (A) Representative peak normalized traces show astrocytic glutamate transporter currents in response to UV flash uncaging of MNI-glutamate in the immature somatosensory cortex of wild-type, GLAST$^{+/-}$, and GLT-1$^{+/-}$ mice at P7-10. (B) The decay of the current was fit with a biexponential function and the fast component was quantified. (C) Astrocyte membrane resistance was measured by the response to a 5 mV depolarizing voltage step. (D) Representative peak normalized traces show astrocytic glutamate transporter currents in response to UV flash uncaging of MNI-glutamate in the mature somatosensory cortex of wild-type, GLAST$^{+/-}$, and GLT-1$^{+/-}$ mice at P21-24. (E) The decay of the current was fit with a biexponential function and the fast component was compared. (F) Astrocyte membrane resistance was measured by the response to a 5mV depolarizing voltage step. *P < 0.05, one-way ANOVA with Tukey posttest.

Figure 4.3:

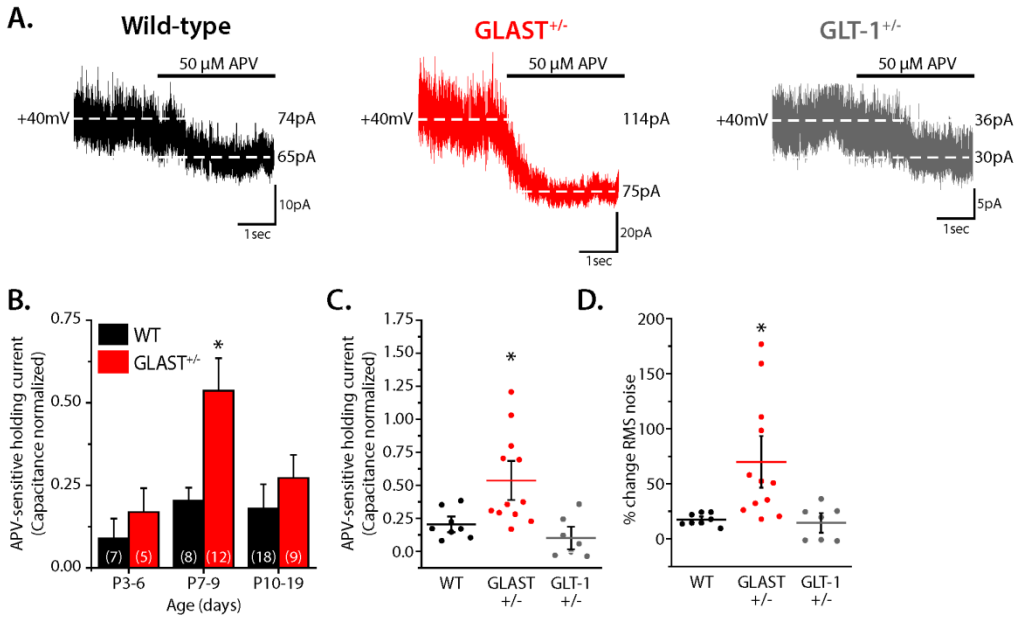

Figure 4.3. Tonic NMDA signaling is increased specifically in the P7 GLAST$^{+/-}$ cortex. (A) Representative traces show holding current required to voltage clamp Layer 5 pyramidal cells at +40 mV. The trace shows the holding current for a two-minute baseline, followed by the decrease in holding current with the addition of 50 µM APV to the bath aCSF. (B) The change in holding current was normalized to cell capacitance for each cell in wild-type and GLAST$^{+/-}$ animals and grouped based on relevant developmental time windows. (C) The APV-sensitive holding current in Layer 5 pyramidal cells was compared between wild-type, GLAST$^{+/-}$, and GLT-1$^{+/-}$ animals at P7. (D) The change in root mean square (RMS) noise was quantified before after APV wash on and the percent change was quantified in recordings from wild-type, GLAST$^{+/-}$, and GLT-1$^{+/-}$ animals at P7. *P < 0.05, one-way ANOVA with Bonferroni posttest.

Figure 4.4:

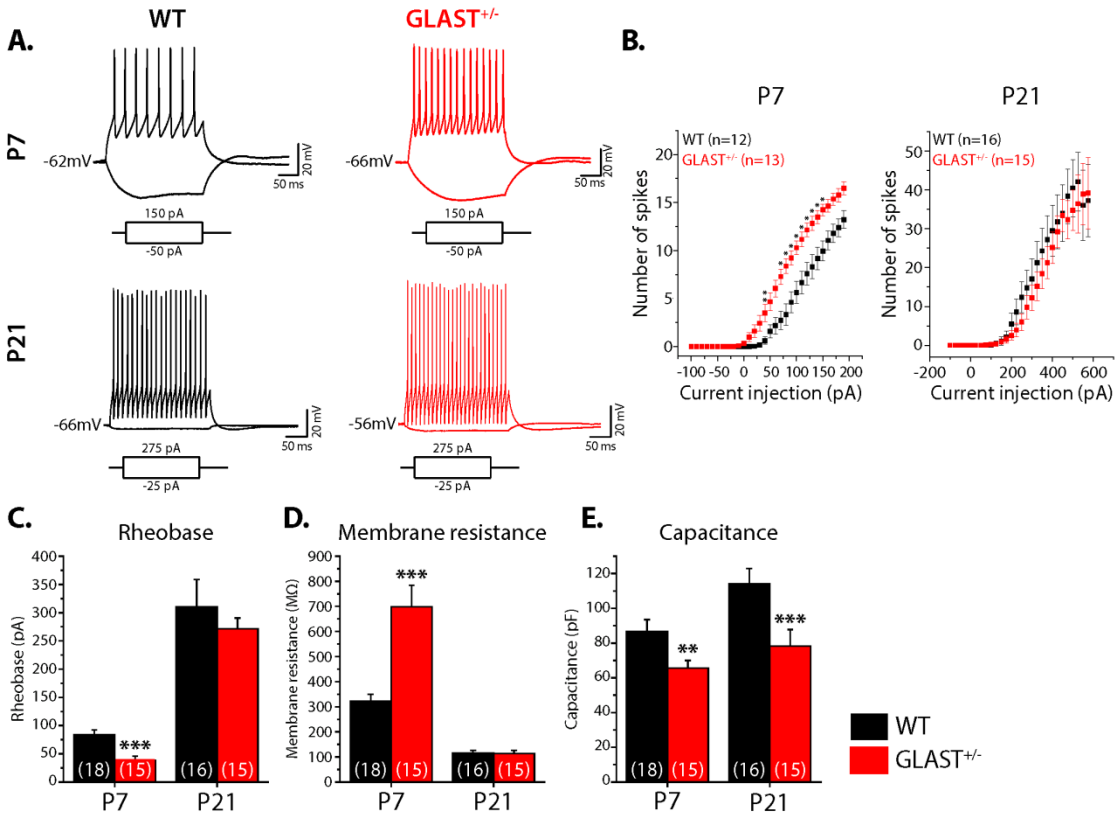

Figure 4.4. Fast-spiking interneurons in Layer 5 of the immature GLAST$^{+/-}$ cortex are more excitable and have altered intrinsic properties. (A) Representative traces show the response of P7 cortical interneurons in Layer 5 in response to -50 pA and +150 pA current injection steps and the response of P21 cortical interneurons in response to -25 pA and +275 pA current injection steps. Interneurons were identified by GFP-positive labeling in GAD1-eGFP reporter mice crossed with the GLAST$^{+/-}$ mice. (B) The input-output relationship of P7 and P21 cortical interneurons is represented by the number of action potential spikes fired at increasing amounts of current injection. P-values were adjusted for multiple comparisons with the Holm-Bonferroni Correction. (C) The threshold current required to elicit the first action potential spike (rheobase) was quantified in P7 and P21 interneurons. (D-E) Membrane properties of P7 and P21 interneurons were also measured by quantifying the response to a 5 mV depolarizing voltage step. *P < 0.05, two-sample t-test. **P < 0.01, two-sample t-test. ***P < 0.001, two-sample t-test.

Figure 4.5:

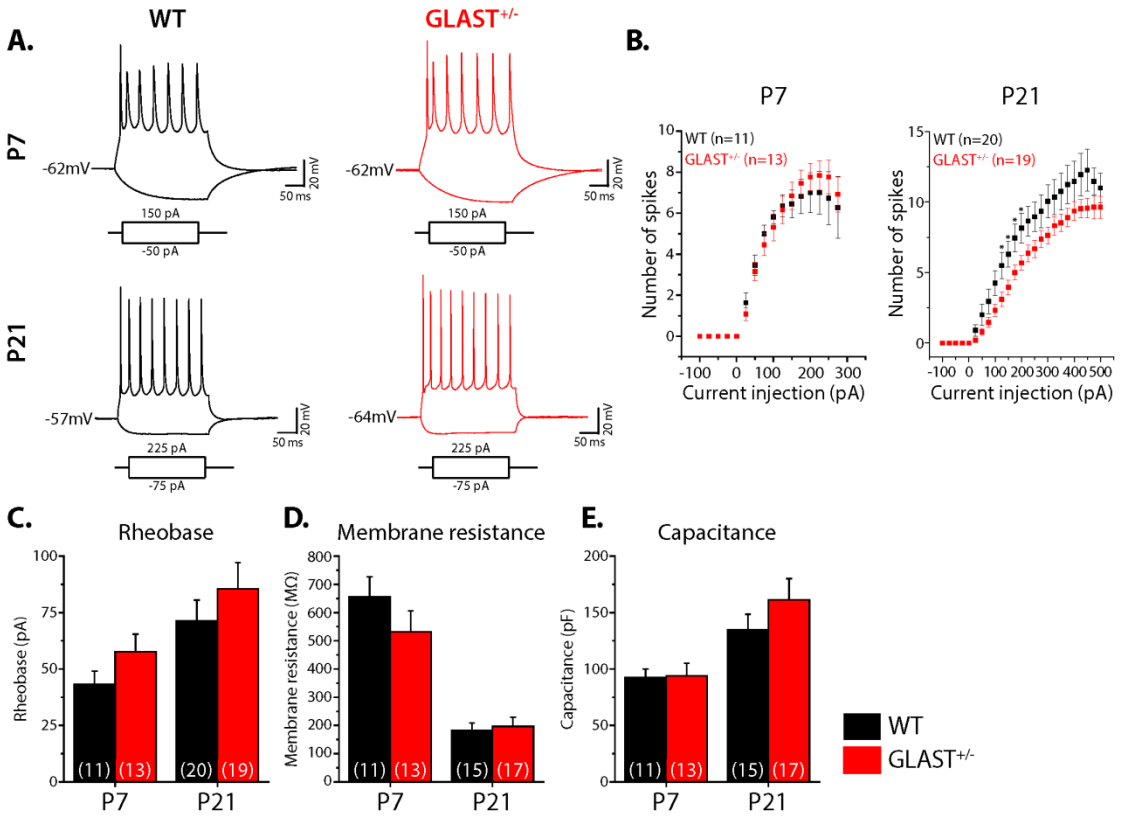

Figure 4.5. Pyramidal cells in Layer 5 of wild-type and GLAST$^{+/-}$ cortex have similar firing and intrinsic properties. (A) Representative traces show the response of P7 Layer 5 pyramidal cells in response to -50 pA and +150 pA current injection steps and the response of P21 cortical interneurons in response to -75 pA and +225 pA current injection steps. Pyramidal cells were identified based on morphology. (B) The input-output relationship of P7 and P21 pyramidal cells is represented by the number of action potential spikes fired at increasing amounts of current injection. (C) The threshold current required to elicit the first action potential spike (rheobase) was quantified in P7 and P21 pyramidal cells. (D-E) Membrane properties of P7 and P21 pyramidal cells were also measured by quantifying the response to a 5 mV depolarizing voltage step.

Figure 4.6:

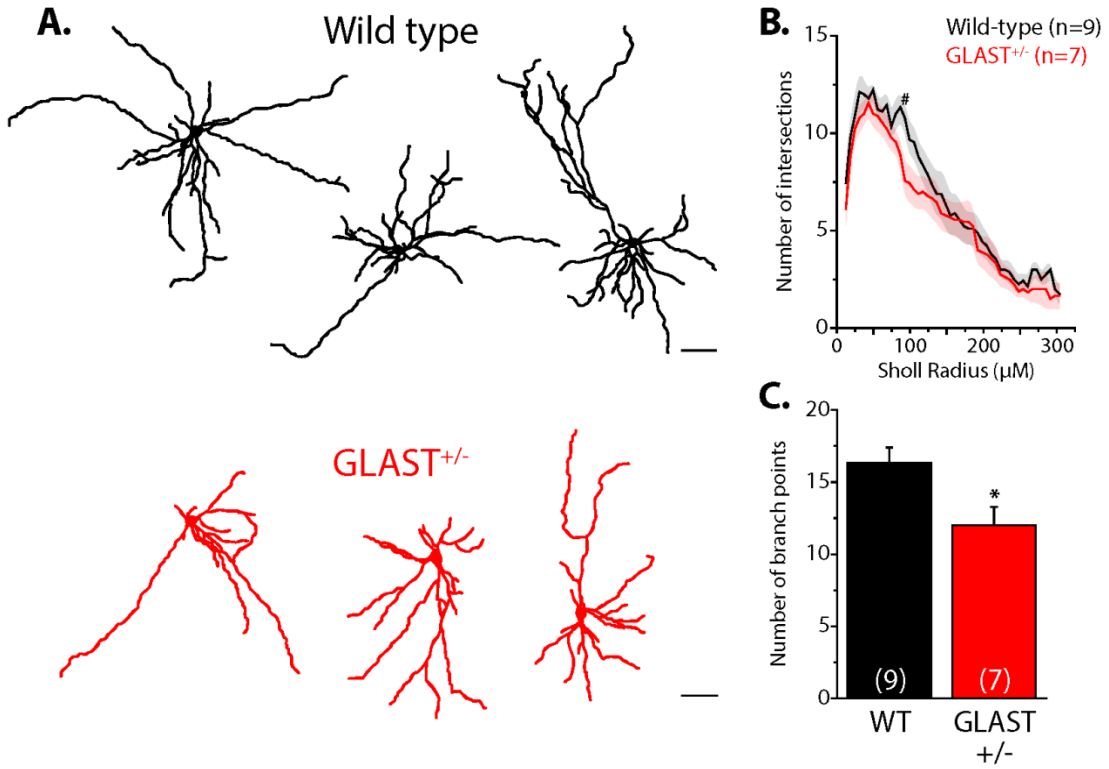

Figure 4.6. GLAST$^{+/-}$ mice have altered Layer 5 interneuron morphology at P21. (A) Layer 5 cortical interneurons were identified by GFP-positive labeling in P21 acute slices from wild-type and GLAST$^{+/-}$ cortex and filled with biocytin. Representative images show line traces from 2D reconstruction of filled interneurons. Scale bar = 50 µm. (B) Sholl analysis was performed on the 2D reconstructed cells using Sholl radii that increased at 6.2 µm steps. (C) The number of branch points in the dendritic arbor was quantified in 3D reconstructed cells. #$P < 0.05$, mixed effects ANOVA. *$P < 0.05$, two-sample t-test.

Figure 4.7:

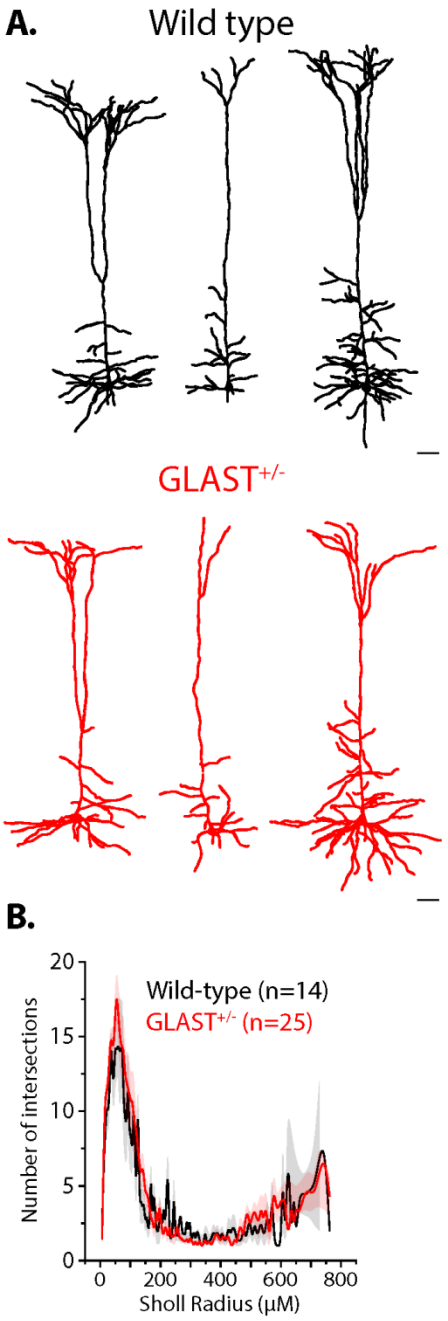

Figure 4.7. Pyramidal cell morphology is not different in Layer 5 of GLAST$^{+/-}$ cortex at P21. (A) Layer 5 pyramidal cells were identified based on morphology in P21 acute slices from wild-type and GLAST$^{+/-}$ cortex and filled with biocytin. Representative images show line traces from 2D reconstruction of filled pyramidal cells. Scale bar = 50 µm. (B) Sholl analysis was performed on the 2D reconstructed cells using Sholl radii that increased at 6.2 µm steps.

Figure 4.8:

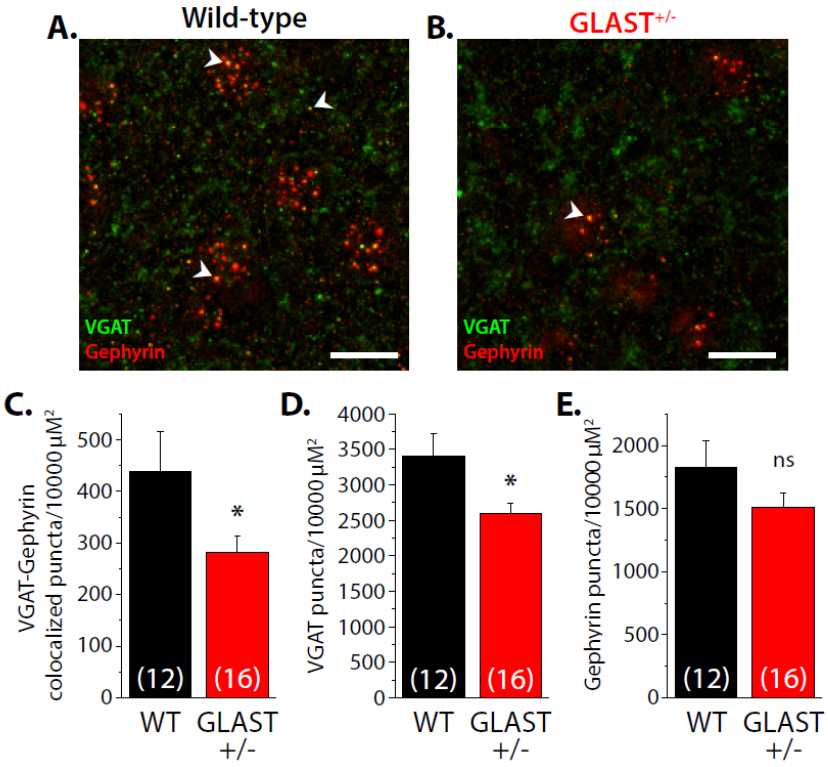

Figure 4.8. Inhibitory synapse number is decreased in Layer 5 of GLAST$^{+/-}$ cortex at P21. (A) Wild-type and GLAST$^{+/-}$ from P21 mice were stained for VGAT and gephyrin to label inhibitory pre- and post- synaptic puncta, respectively. Colocalization of VGAT and gephyrin represent structural inhibitory synapses (white arrowheads). Scale bar = 5 µm. (B-D) The number of colocalized puncta, presynaptic puncta, and postsynaptic puncta were quantified. *P < 0.05, two-sample t-test.

Figure 4.9:

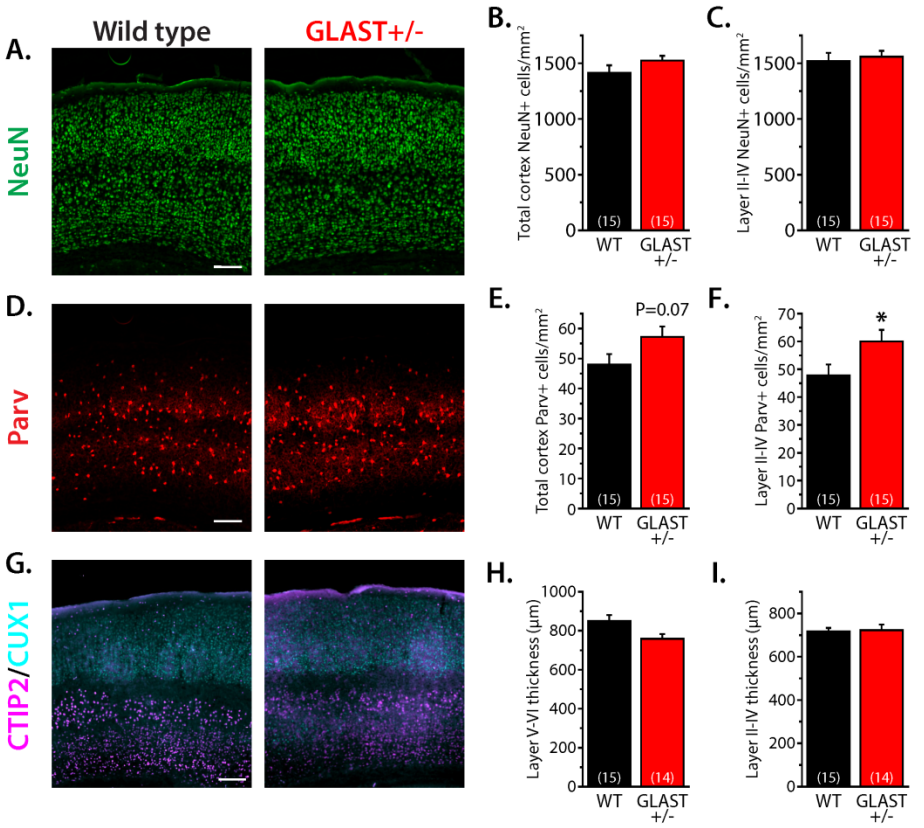

Figure 4.9. Parvalbumin-positive neurons are increased in Layer 2-4 of GLAST$^{+/-}$ cortex at P14. (A-C) Wild-type and GLAST$^{+/-}$ cortical sections from P14 mice were stained for NeuN and total neuron number was quantified across all layers of the somatosensory cortex, as well as in Layer II-IV (L2-L4) specifically. Scale bar = 250 µm. (D-F) Wild-type and GLAST$^{+/-}$ cortical sections from P14 mice were stained for parvalbumin and total interneuron number was quantified across all layers of the somatosensory cortex, as well as in Layer II-IV specifically. Scale bar = 250 µm. (G-I) Wild-type and GLAST$^{+/-}$ cortical sections from P14 mice were stained for CTIP2 and CUX1 to label deep layers and superficial layers of the cortex, respectively. The borders of CTIP2 or CUX1 expression were used to define deep (L5-L6) and superficial (L2-L4) layers, and layer thicknesses were quantified. Scale bar = 250 µm. *P < 0.05, two-sample t-test.

Figure 4.10:

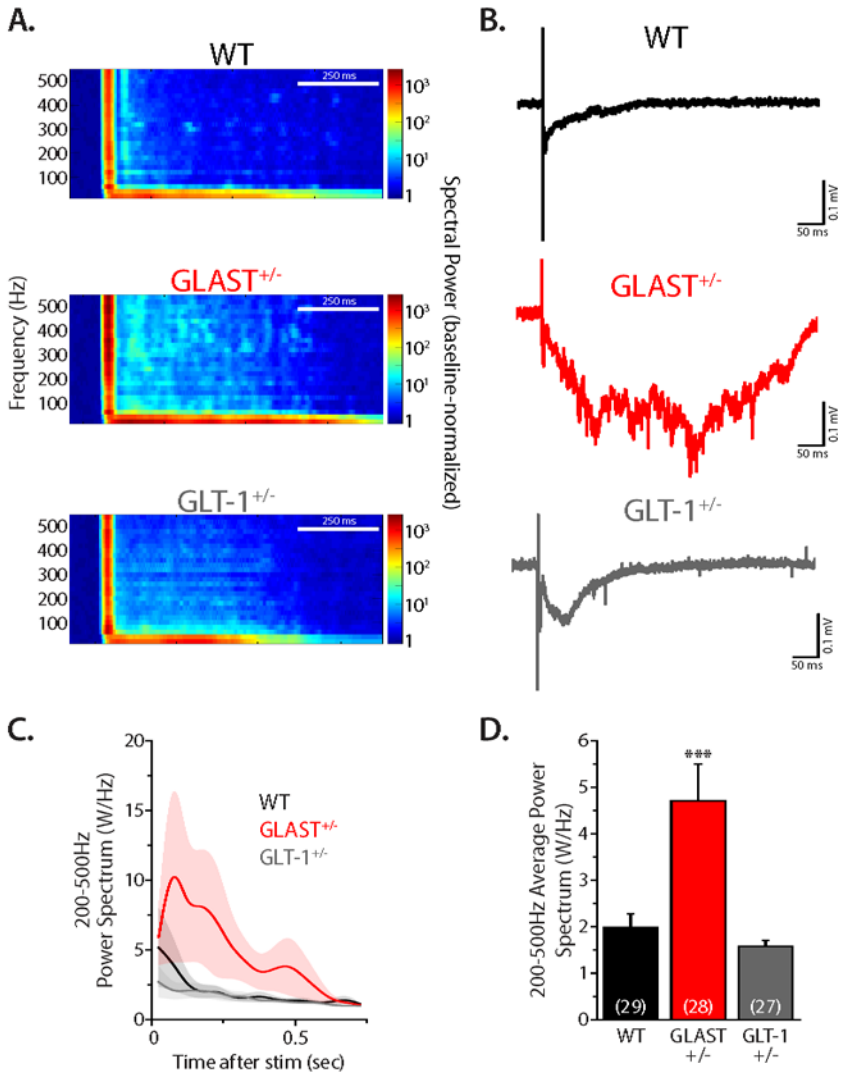

Figure 4.10. Decreased GLAST expression and function leads to hyperexcitability in acute cortical slices. (A) Representative traces show evoked cortical field excitatory postsynaptic potentials (fEPSPs) recorded from wild-type, GLAST$^{+/-}$, and GLT-1$^{+/-}$ mice at P21. Responses were recorded in L5 and evoked by a 150 µs constant current stimulation from the white matter tracts. (B) Spectrograms of averaged traces show power at frequencies ranging from 0-500 Hz. (C) The average power spectrum from 200-500 Hz was quantified over 750 ms after the stim. (D) The average power at 200-500 Hz was integrated over 750 ms after the stim. ***P < 0.001, one-way ANOVA with Tukey posttest.

Table 4.2:

Age	Genotype	Vm (mV)	±SEM	Rm (MΩ)	±SEM	IV slope (mV/pA)	±SEM	Capacitance (pF)	±SEM
P7	WT	-73.4	1.40	4.43	0.39	0.028	0.003	398.4	68.0
P7	GLAST+/-	-78.7	1.75	4.84	0.43	0.020	0.002	422.0	67.2
P7	GLT-1+/-	-78.0	1.68	3.93	0.25	0.030	0.004	575.2	135.1
P21	WT	-73.6	1.71	1.32	0.37	0.021	0.005	6178.7	1474.7
P21	GLAST+/-	-74.2	1.59	1.00	0.18	0.016	0.001	5118.6	2024.7
P21	GLT-1+/-	-71.4	1.45	1.41	0.19	0.022	0.003	3303.3	986.8

Table 4.2. Intrinsic membrane properties of GLAST$^{+/-}$ and GLT-1$^{+/-}$ astrocytes. All values are reported as mean ± SEM. Intrinsic properties were measured in response to a 5 mV, 50 ms depolarizing step.

Table 4.3:

Age	Genotype	Cell type	Vm (mV)	±SEM	AP threshold (mV)	±SEM	AP AHP (mV)	±SEM	AP spike height (mV)	±SEM	AP spike half-width (ms)	±SEM
P7	WT	IN	-61.3	1.30	-40.4	2.25	-53.2	1.62	74.6	2.35	1.12	0.06
P7	GLAST+/-	IN	-58.0	2.51	-38.7	1.57	-52.7	1.42	70.9	2.85	**1.51 (***)**	**0.08**
P21	WT	IN	-64.6	1.31	-27.1	0.94	-53.4	2.40	73.3	5.38	0.55	0.10
P21	GLAST+/-	IN	-65.7	0.79	-31.1	1.80	-54.3	2.38	70.5	4.70	0.48	0.04
P7	WT	Pyr	-55.2	1.65	-38.5	3.00	-30.7	6.00	55.4	9.30	2.27	0.28
P7	GLAST+/-	Pyr	-55.1	1.13	-41.6	2.79	-30.1	5.64	60.8	5.80	2.26	0.27
P21	WT	Pyr	-61.7	1.83	-45.4	1.70	-44.3	1.30	79.0	4.24	1.14	0.05
P21	GLAST+/-	Pyr	-63.1	1.62	**-37.1 (***)**	**1.13**	-42.5	1.14	83.7	2.67	1.18	0.05

Table 4.3. Intrinsic membrane properties and action potential characteristics of GLAST$^{+/-}$ interneurons and pyramidal cells. All values are reported as mean ± SEM. Action potential properties were calculated based on the first action potential fired during the current injection protocol. ***P < 0.001, t-test.

Chapter 5: Discussion

5.1 Summary of experimental results

The experiments described in this dissertation tested the idea that GLAST is the predominant astrocytic glutamate transporter in the developing neocortex and that it regulates the development of inhibitory function to shape cortical network maturation. This role contrasts with the function of GLT-1, which was shown to regulate the acute response to kainic acid treatment, described in Chapter 3 (Shih, Liu et al. 2014). The main findings are that glutamate uptake depends on GLAST in the developing cortex (P7-10) and on GLT-1 in the mature cortex (P21-24). Tonic NMDA signaling is increased specifically in the GLAST$^{+/-}$ cortex at P7, and was not increased at other ages or in the GLT-1$^{+/-}$ cortex. Characterization of action potential firing and intrinsic properties of interneurons showed that fast-spiking interneurons in the GLAST$^{+/-}$ cortex were more excitable and showed phenotypes associated with disrupted maturation. Interestingly, these effects were specific to immature interneurons, as neither interneurons in the P21 GLAST$^{+/-}$ cortex nor pyramidal cells showed significant differences in action potential firing and membrane properties. Disruptions in interneuron properties during development led to altered interneuron morphology and decreased inhibitory synapse number in the mature somatosensory cortex. Again, these findings were specific to inhibitory networks, as differences were not observed in pyramidal cell morphology or excitatory synapses. We did not find any differences in gross cortical anatomy of GLAST$^{+/-}$ mice, but found changes in the distribution of PV+ cells across the cortical layers, further suggesting that inhibitory networks are disrupted as a result of GLAST heterozygosity. Finally, decreased expression of GLAST led to hyperexcitable

fEPSPs, indicating aberrant cortical network function in GLAST$^{+/-}$ mice. Together, these results indicate a novel, critical role for GLAST in regulating glutamate levels and controlling interneuron maturation specifically during development to promote the proper development of cortical circuits.

5.2 Using GLAST$^{+/-}$ and GLT-1$^{+/-}$ mice as a clinically relevant model to understand glutamate regulation in the developing brain

For these experiments, we used heterozygous mice, which have only one functional GLAST or GLT-1 allele, while much of the background literature characterizing GLAST phenotypes used homozygous GLAST KO mice. Using genetic mouse models is a more reliable way to decrease EAAT expression and function, as variable results have been reported with pharmacological methods, particularly with the GLAST-specific inhibitor UCPH-101 (Jensen, Erichsen et al. 2009). In our hands, the compound is ineffective (see Appendix, Figure 6.1), which led us to use genetic models of EAAT knockdown. There were both practical and scientific reasons for choosing heterozygous mouse models for our studies. First, while the original paper that generated these GLAST-deficient mice reported Mendelian ratios of offspring (Watase, Hashimoto et al. 1998), subsequent studies using these mice found a decreased proportion of GLAST KO offspring in litters generated from GLAST$^{+/-}$ x GLAST$^{+/-}$ matings, with 36% WT, 50% GLAST$^{+/-}$, and 14% GLAST KO (Karlsson, Tanaka et al. 2009). In our hands, as well as those of our colleagues (H. Higashimori, personal communication), GLAST KO offspring were extremely rare, with less than one born out of every six litters of pups generated. This is interesting from a developmental perspective, as it suggests that

GLAST KO animals have decreased survival in utero, and further supports the idea that GLAST is more critical earlier in development. From a practical standpoint, we decided to use heterozygous mice, which breed normally, for our experiments. Using mice with only a partial deletion of the GLAST gene and partial reduction in expression is also more relevant from a clinical perspective, as human patients with mutations in the GLAST gene did not have complete loss of protein expression (Jen, Wan et al. 2005). Additionally, while there was decreased expression of GLAST in the prefrontal cortex of schizophrenia patients, GLAST expression was not completely abolished (Bauer, Gupta et al. 2008). These practical and clinical considerations led us to focus on heterozygous mice for our studies.

GLAST and GLT-1 expression in the cortex has been shown to be developmentally regulated, with GLAST expressed at relatively higher levels early in development and GLT-1 expression dominant later in the mature cortex (Ullensvang, Lehre et al. 1997). However, the experiments presented in this dissertation are the first to functionally confirm these developmental expression profiles. Recording astrocytic glutamate transporter currents in the immature (P7-10) versus the mature (P21-24) somatosensory cortex and revealed age-specific contributions of GLAST and GLT-1, and suggest that specific EAAT function in the cortex can be preferentially investigated depending on when during development the experiments are performed. Understanding the relative contributions of GLAST vs GLT-1 at various developmental stages also contributes to our understanding of why there are two major astrocytic glutamate transporters present in the brain. Both have been shown to be expressed in the same astrocyte (Chaudhry, Lehre et al. 1995, Haugeto, Ullensvang et al. 1996), a finding that is

supported by our own data that a glutamate transporter current can be recorded in astrocytes from GLT-1 KO mice (see Appendix, Figure 6.1). Additionally, GLAST and GLT-1 have similar kinetic properties, suggesting that they handle glutamate similarly (Storck, Schulte et al. 1992, Arriza, Fairman et al. 1994, Zerangue, Arriza et al. 1995, Tzingounis and Wadiche 2007), although it has been suggested that GLAST has a tendency to buffer, rather than transport glutamate into the astrocyte (Wadiche and Kavanaugh 1998). Therefore, it is unclear why both are expressed in the cortex and whether they perform similar functions in terms of development, synaptic transmission, and disease pathogenesis. Specific roles of GLAST and GLT-1 within the cortex have not been previously defined, although specific functions of GLAST in the cerebellum, where GLT-1 is also expressed, have been well characterized. The findings described in this dissertation therefore provide the first evidence of differential roles of GLAST and GLT-1 in the cortex, and implicate GLAST as a "developmental EAAT."

5.3 GLAST is the dominant glutamate transporter during neocortical development

The finding that GLAST is the predominant transporter in the neonatal brain is further supported by evidence that there is increased ambient glutamate in the GLAST$^{+/-}$ cortex specifically, and not in the GLT-1$^{+/-}$ cortex. Future studies may address whether ambient glutamate levels are increased in the mature GLT-1$^{+/-}$ cortex, which would support the predominant function of GLT-1 later in development. However, given that there is robust transporter function in the mature brain, as shown by mild effects of the GLT-1 specific antagonist DHK on glutamate transporter kinetics (M. Armbruster, personal communication), it might not be possible to detect a change in tonic NMDA

signaling at adult ages. Increased ambient glutamate in the developing brain has broad implications for neuronal and circuit maturation, as a number of previous studies have shown that early mechanisms of cortical development depend on tonic glutamate signaling, including generation of early network oscillations in the cortex (Allene, Cattani et al. 2008). Our findings suggest that since GLAST controls ambient glutamate during postnatal cortical development, it plays a critical role in how cortical circuits are formed and later function.

GLAST is expressed on radial glia in the embryonic brain (Shibata, Yamada et al. 1997). Radial glia cells are critical for development of the laminar structure of the neocortex, as they serve as both precursors for cortical projection neurons as well as the structural scaffold that guides neuronal migration (Campbell and Gotz 2002). Given that glutamate is known to regulate neurogenesis (Jansson and Akerman 2014) as well as neuronal migration (Komuro and Rakic 1993), it seemed plausible that GLAST could play a role in regulating the laminar structure or composition of the cortical plate. To test whether this was a mechanism by which GLAST regulated cortical circuit development, we examined lamination and the neuronal population in the postnatal GLAST$^{+/-}$ cortex. Since we did not find any differences in the distribution of CTIP2+ or CUX1+ cells at P14, nor did we find differences in the thicknesses of deep and superficial layers of the cortex, our findings suggest that partial loss of GLAST is not sufficient to permanently alter the gross laminar structure of the cortex. We also did not find changes in the total number of PV+ or NeuN+ cells in the cortex, suggesting that GLAST did not affect neurogenesis and the subsequent population of the cortical layers. However, there were subtle changes in interneuron distribution within in the GLAST$^{+/-}$ cortex which warrants

further investigation. Since it was shown that NMDAR signaling onto interneurons affects their radial distribution (Zechel, Nakagawa et al. 2016), the changes in PV+ interneuron distribution in the GLAST$^{+/-}$ cortex that we observed could be driven by altered tonic NMDA signaling. In our experiments, we find that increased ambient glutamate levels during development leads to increased numbers of PV+ cells in the upper layers of the cortex. Interestingly, consistent with our results, it was shown by Zechel et al. (Zechel, Nakagawa et al. 2016) that inhibiting vesicular glutamate release by thalamocortical (TC) axons led to decreased numbers of interneurons in Layers II-IV at P0, and that loss of those axons during development led to decreased numbers of PV+ cells at three weeks of age. One possible future experiment would be to inhibit vesicular release of glutamate by TC axons in the GLAST$^{+/-}$ cortex during development to determine if the distribution of PV+ interneurons is restored. If this is the case, it would suggest that GLAST regulates the development of long-range, thalamocortical inputs to the cortex, which is a major step in the functional maturation of cortical circuits. Additionally, recording inhibitory postsynaptic currents (IPSCs) in deep vs. superficial layers might reveal differences in inhibitory signaling and suggest signaling within the cortical column is disrupted. Finally, while we assessed excitatory synapses by the colocalization of VGLUT1-PSD95 puncta and found no differences (see Appendix, Figure 6.2), VGLUT1-labeled puncta typically label intracortical synapses (Kaneko and Fujiyama 2002). A future experiment to determine the effects of GLAST on thalamocortical inputs to the cortex would be to measure VGLUT2-PSD95 colocalization, as VGLUT2 would identify synapses from thalamic inputs (Kaneko and Fujiyama 2002).

Zechel et al. (Zechel, Nakagawa et al. 2016) also demonstrated that decreased NMDA signaling onto interneurons prematurely increased expression of KCC2, the chloride cotransporter upregulated in mature neurons (Rivera, Voipio et al. 1999). KCC2 is a chloride extrusion pump that hyperpolarizes the chloride reversal potential, which renders GABA signaling inhibitory in the mature brain. However, KCC2 expression is low in immature neurons, and GABAergic signaling is excitatory in these cells. Therefore, the premature expression of KCC2 in interneurons caused by decreased NMDA signaling described by Zechel et al. (Zechel, Nakagawa et al. 2016) suggests that glutamatergic signaling via NMDARs during development regulates functional maturation of interneurons. Conversely, it has also been shown that increased activation of NMDARs decreases KCC2 function (Lee, Deeb et al. 2011), further suggesting that glutamatergic signaling via NMDARs regulates KCC2. Therefore, one possible effect of increased tonic NMDA signaling in the $GLAST^{+/-}$ cortex could be altered expression or function of KCC2. It was previously shown that the excitatory effect of GABA, caused by low KCC2 expression during development, is required for the morphological maturation of interneurons (Cancedda, Fiumelli et al. 2007). Since we found that interneuron morphology was disrupted in $GLAST^{+/-}$ mice, one hypothesis to test in the future is whether or not changes in interneuron morphology are caused by altered KCC2 expression or GABAergic signaling. Future experiments to address this hypothesis include measuring KCC2 expression and the chloride reversal potential in the developing $GLAST^{+/-}$ cortex.

5.4 GLAST regulation of ambient glutamate specifically affects interneuron maturation

Aberrant NMDA signaling alters the function and maturation of interneurons (Aligny, Roux et al. 2014, Jones, Corbin et al. 2014, Kelsch, Li et al. 2014). While these studies demonstrate the effects of decreased signaling at NMDARs, we show here that increased tonic NMDAR activation during development also leads to disruptions in interneuron function, including interneuron excitability. The hypothesis that low concentrations of ambient glutamate tonically activate NMDARs specifically on interneurons is attributed to their unique NMDAR subunit composition. Interneurons preferentially express the NR2D subunit, which has decreased Mg2+ affinity and increased affinity for glutamate, making NMDARs on interneurons less voltage-dependent and more sensitive to low concentrations of glutamate (Standaert, Landwehrmeyer et al. 1996, Paoletti, Bellone et al. 2013, von Engelhardt, Bocklisch et al. 2015). The NR2D subunit is also enriched in the neonatal cortex (Monyer, Burnashev et al. 1994, Yamasaki, Okada et al. 2014, von Engelhardt, Bocklisch et al. 2015). Thus, our findings that increased tonic NMDA signaling specifically and transiently increases the excitability of interneurons, but not pyramidal cells, in the immature (P7-10) neocortex agrees with the expression patterns of NR2D. Our findings that interneurons are preferentially activated are also consistent with previous findings in the lab (E. Hanson, manuscript in review), as well as from other groups (Riebe, Seth et al. 2016). Early studies of tonic NMDA signaling found that it increases neuronal excitability and action potential discharge (Sah, Hestrin et al. 1989). This suggests that our results showing increased action potential firing in GLAST$^{+/-}$ interneurons at P7 is reflective of increased

tonic NMDA signaling demonstrated in the GLAST$^{+/-}$ cortex. By using the GLAST$^{+/-}$ mouse model, where glutamate regulation during development is specifically disrupted, our results demonstrate that the effect of perturbing tonic NMDA signaling and the subsequent alteration of neuronal excitability is dependent on cell type and age in the developing cortex. To date, this is the first study that demonstrates regulation of interneurons via NMDAR signaling by a specific EAAT. Previous findings from Bergles and colleagues had found that both GLAST and GLT-1 mediated interneuron excitability via mGluR signaling in the young adult hippocampus (Huang, Sinha et al. 2004), while Araque and colleages found that astrocytes mediated interneuron signaling onto presynaptic mGluRs to potentiate synaptic transmission (Perea, Gomez et al. 2016). The results from the current study are in line with the idea that astrocytes regulate interneuron function, but advance the theory by identifying novel and specific mechanisms of action.

In our experiments, we tested the properties of fast-spiking interneurons by using reporter mice that expressed GFP under the GAD1 promoter. Initial characterization of these mice suggested that 90% of labeled cells are PV+ basket cells (Chattopadhyaya, Di Cristo et al. 2004), while preliminary data from our lab suggests that roughly 75% of labeled cells co-express PV. Thus, while it is likely that our characterizations of intrinsic properties and morphology characterize this particular class of fast-spiking, PV+ interneuron, it is possible that our sample population is not homogeneous, and that other interneuron subtypes are included in our analysis. A closer look at the electrophysiological characteristics of the current injection traces, such as characterizing spike adaptation or bursting patterns, might be able to reveal whether or not they should be classified as other interneuron subtypes, such as those expressing SOM. Interestingly,

NR2D was specifically shown to be expressed in both of major subtypes in the neocortex, PV+ and SOM+ (Standaert, Landwehrmeyer et al. 1996). Therefore, SOM+ interneurons may also be preferentially activated by ambient glutamate signaling. PV+ and SOM+ interneurons exert perisomatic and dendritic inhibition onto to target neurons, respectively, and were shown to have distinct roles in early cortical circuit development. SOM+ interneurons receive inputs from thalamocortical axons earlier in development, and this early activation was shown to be required for the formation of inputs into PV+ interneurons (Tuncdemir, Wamsley et al. 2016). Therefore, it is possible that alterations in ambient glutamate levels might affect SOM+ innervation, and indirectly contribute to disruption of PV+ interneuron innervation. While the data from the tonic NMDA recording experiments do not show an increase in GLAST$^{+/-}$ at P3-6, which is the time window that SOM+ interneurons were activated (Tuncdemir, Wamsley et al. 2016), preliminary data from glutamate biosensor imaging experiments done in the lab have shown that the increase in ambient glutamate may occur earlier than we can detect using electrophysiological methods (M. Armbruster, unpublished data). Regardless, since GLAST function is reduced during SOM+ interneuron development, future studies may investigate whether or not they are also affected by altered glutamate regulation and if GLAST has any effects prior to P7.

A previous study has also shown that lesion-induced plasticity is diminished in GLAST KO mice and that there is decreased remodeling of the barrel cortex in response to whisker trimming at P2 (Takasaki, Okada et al. 2008). Since inhibitory neurotransmission is known to regulate plasticity during development in both the visual and somatosensory cortex (Hensch 2005), the results presented here showing decreased

GLAST levels causing disruptions in inhibitory function offer a possible explanation for the altered plasticity observed by Takasaki and colleagues. Our results also suggest that GLAST may have a direct role in controlling the onset and closure of critical periods of plasticity in the cortex, the timing of which are controlled largely by the maturation profile of GABAergic interneurons. Eliminating GABAergic signaling in the developing visual cortex results in the loss of critical period plasticity (Hensch, Fagiolini et al. 1998), while accelerating or increasing interneuron function and maturation triggers the premature onset and termination of the criticial period window (Huang, Kirkwood et al. 1999, Fagiolini and Hensch 2000, Di Cristo, Chattopadhyaya et al. 2007). In our experiments, the intrinsic properties of GLAST$^{+/-}$ interneurons at P7 suggest that they are less mature. Therefore, the critical window may be delayed in GLAST$^{+/-}$ somatosensory cortex. Interestingly, Takasaki et al. (Takasaki, Okada et al. 2008) show that critical period termination is unaltered in GLT-1 KO mice, but did not test the window of critical period plasticity in GLAST KO mice. Our findings, which indicate that GLAST, rather than GLT-1, is the predominant glutamate transporter during development and that it regulates interneuron maturation, beg the question of whether critical periods are altered in GLAST$^{+/-}$ mice. Further experiments might also investigate whether rescue of interneuron function or maturation can restore plasticity, which would further confirm the role of GLAST in regulating cortical circuit function by controlling interneuron development.

Since glutamate and glutamate receptor activation is generally thought to promote and accelerate growth and maturation in cortical networks, it is perhaps counterintuitive that increased tonic signaling at NMDA receptors would lead to decreased dendritic

arborization of interneurons and a decrease in inhibitory synapse number. Previous studies found that glutamate accelerated the formation of glutamatergic dendritic spines in Layer II/III of the cortex (Kwon and Sabatini 2011); the fact that we observed decreased inhibitory synapses number suggests that the perhaps the effect of glutamate on spinogenesis is synapse-specific. For example, the pro-synaptogenesis effect of glutamate could be specific to inducing glutamatergic spines or dendritic spines without having the same effect on inhibitory spines or perisomatic synapses. Furthermore, we did not observe persisting changes in excitatory synapse number (see Appendix, Figure 6.2), suggesting that ambient glutamate levels are not sufficient to induce permanent spine formation. Whether there are acute effects remains to be tested, and future experiments can address this by recording excitatory postsynaptic currents in the developing $GLAST^{+/-}$ cortex. Additionally, it has been shown that activation of NMDARs drives dynamic processes underlying dendritic arborization and leads to growth and complexity of the dendritic arbor (Rajan and Cline 1998). However, there is also evidence of bidirectional regulation of dendritic outgrowth mediated by NMDARs (George, Baden et al. 2012). Compensatory decreases in dendritic arborization have also been reported as a result of increased synaptic input or in response to network hyperexcitability (Nishimura, Owens et al. 2008, Tripodi, Evers et al. 2008). While activation of NMDARs promotes neurite growth and synapse formation, overactivation leads to decreases in growth, suggesting that aberrant upregulation of downstream NMDAR effectors can be detrimental to neuron development. Thus, increased tonic signaling observed in $GLAST^{+/-}$ may alter neuronal development by an NMDAR-dependent mechanism, and downstream effectors of NMDARs may be dysregulated by decreased GLAST function.

Together, the results of the present study suggest that GLAST is a major regulator of ambient glutamate concentrations during development, which, in turn, have cell-type and age-specific effects on the complex mechanisms of cortical circuit maturation.

5.5 Transient effects of GLAST during development lead to long-term effects on cortical circuits

Subcortical and subcerebral projections from L5 of the cortex are the major output of a stereotypical cortical circuit and are an indicator of underlying network function, which is a major reason that we chose to study pyramidal cells and interneurons in this particular cortical layer (Silva, Amitai et al. 1991, Solomon and Nerbonne 1993, Harris and Shepherd 2015). Thus, the aberrant fEPSP responses recorded in L5 of GLAST$^{+/-}$ acute cortical slices suggest that there are alterations in the underlying cortical circuit. It has been demonstrated in the hippocampus that the morphology of interneurons affected their control over network oscillations and subsequent maturation of network activity (Allene, Picardo et al. 2012). Since the morphology of interneurons, but not pyramidal cells, was altered, the cortical hyperexcitability that we observed is likely driven by loss of inhibitory function. We also showed decreased inhibitory synaptic puncta in the GLAST$^{+/-}$ cortex at the time hyperexcitability is observed, but no changes in excitatory synapses (see Appendix, Figure 6.2), further suggesting that the aberrant fEPSP is driven by loss of inhibition. An open question remains as to whether or not the hyperexcitability is observed prior to P21, and whether the effect is permanent and extends beyond the ages we tested. To speculate on this, it has been shown in animal models of focal cortical dysplasia, slice hyperexcitability persists for months after injury at P0 (Luhmann 2016).

This provides evidence that disruptions in cortical circuits induced during early postnatal development are long-lasting and can become permanent. Given that we observe numerous structural changes in inhibitory networks in the GLAST$^{+/-}$ cortex, we believe that the fEPSP response would persist.

We did not observe cortical fEPSP hyperexcitability in GLT-1$^{+/-}$ acute slices, which is an intriguing result given that GLT-1 is the dominant transporter in the cortex by P21. Thus, our results suggest that the abnormal fEPSP response is not due to acute glutamate handling defects, but rather alterations during development that persisted in the mature cortex. In contrast to GLAST, GLT-1 function has typically been implicated in the mechanisms of injury response and excitotoxicity, as opposed to development (Rothstein, Dykes-Hoberg et al. 1996, Tanaka, Watase et al. 1997). This is supported by work done earlier in the dissertation, where it was shown that GLT-1 mediates the response to kainic acid-induced seizures, a function that is regulated by the mitochondrial sirtuin SIRT4 (Shih, Liu et al. 2014) (Chapter 3).

Spectral analysis of the fEPSP responses showed increased power at 200-500Hz frequencies, which has been associated with pathological networks (Khosravani, Pinnegar et al. 2005, Zijlmans, Jiruska et al. 2012, Kerber, LeVan et al. 2013), supporting the idea that altered inhibition in the GLAST$^{+/-}$ mice may drive clinically relevant cortical circuit phenotypes. One example is in the pathogenesis of neurodevelopmental disorders. For example, disruptions in interneuron function and loss of inhibitory control of cortical circuits have been implicated in schizophrenia (Lewis, Hashimoto et al. 2005). In particular, one main hypothesis for schizophrenia disease pathogenesis is that decreased function of PV+ interneurons in the prefrontal cortex leads to decreased gamma

oscillations, which are critical for synchronization of neuronal networks and cognitive function (Volk and Lewis 2014, Gonzalez-Burgos, Cho et al. 2015). Interestingly, GLAST has also been associated with the pathophysiology of schizophrenia. Decreased GLAST expression was found in the prefrontal cortex from human patients (Bauer, Gupta et al. 2008), and a genomic microdeletion spanning part of the GLAST gene has been found in a case of childhood-onset schizophrenia (Walsh, McClellan et al. 2008), though it is unclear how the microdeletion affects GLAST expression. Schizophrenia-like behaviors have also been reported in GLAST KO mice, which show deficits in cognitive and social behavior (Karlsson, Tanaka et al. 2008, Karlsson, Tanaka et al. 2009). Together, these findings suggest that decreased GLAST function contributes to schizophrenia pathogenesis, but the mechanism has not been elucidated. Here, we provide evidence that loss of GLAST function specifically alters the development and function of interneurons, thus mechanistically linking decreased GLAST function during development with later inhibitory deficits observed in schizophrenia. One hypothesis of schizophrenia pathophysiology is that NMDA hypofunction on interneurons leads to decreased inhibitory function in the prefrontal cortex (Lisman, Coyle et al. 2008, Cohen, Tsien et al. 2015). The findings in these experiments provide an alternate mechanism for decreasing interneuron function by disrupting their maturation with increased tonic NMDA signaling, ultimately decreasing their function in the mature brain. Thus, our seemingly contradictory findings converge with the NMDA hypofunction hypothesis and potentially lead to the same outcome where cortical circuits are overactive, overstimulate the dopaminergic system, and produce the psychosis associated with schizophrenia. Future studies might explore the function of GLAST in the prefrontal cortex to determine

if it also regulates glutamate during development, which would further implicate GLAST function during development in schizophrenia pathophysiology.

5.6 Conclusion

The work presented in this dissertation identifies a novel interaction between GLAST function and interneuron maturation in the developing cortex. Previously, much of the work in the glutamate transporter field had focused on GLT-1, due to its well-defined and obvious role in excitotoxicity. However, by shifting our attention to GLAST, we have uncovered compelling evidence of its subtle and intricate functions in regulating cortical circuit development. The main conclusion from these experiments is that GLAST, specifically, regulates glutamatergic signaling in the developing neocortex, which has broad implications for the maturation of inhibitory network function. Identifying both an age-specific and a cell type-specific effect of GLAST has allowed us to better define its function in normal brain development. Furthermore, our work has led us to new hypotheses about the pathogenesis of schizophrenia, allowing for future work in this field to build upon these findings and investigate the role of GLAST in neurodevelopmental diseases.

Chapter 6: Appendix

Figure 6.1:

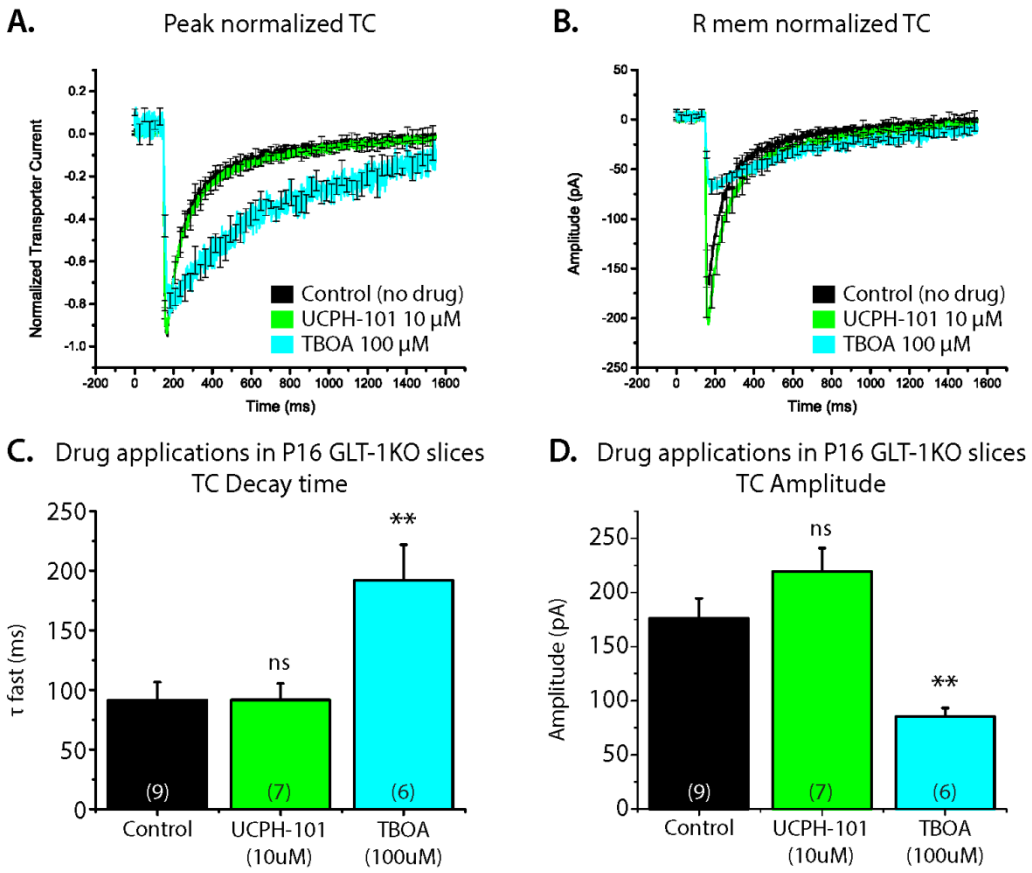

Figure 6.1. The GLAST inhibitor UCPH-101 is ineffective. (A) Averaged astrocyte transporter currents recorded from P16 GLT-1 KO mice were peak normalized and show no effect of 10µM UCPH-101 on transporter current decay kinetics. TBOA (100µM), which blocks all EAATs, was used as a positive control. Astrocyte transporter current recordings were performed as described in Chapter 4. (B) Averaged astrocyte transporter current traces were normalized to membrane resistance and show no effect of 10µM UCPH-101 on transporter current amplitudes. TBOA (100µM), which blocks all EAATs, was used as a positive control. Astrocyte transporter current recordings were performed as described in Chapter 4. (C) The fast component of transporter current decay was quantified. **P<0.01, 1-way ANOVA, Tukey Test. ns, not significant. (D) The transporter current peak amplitude was quantified. **P<0.01, 1-way ANOVA, Tukey Test. ns, not significant.

Figure 6.2:

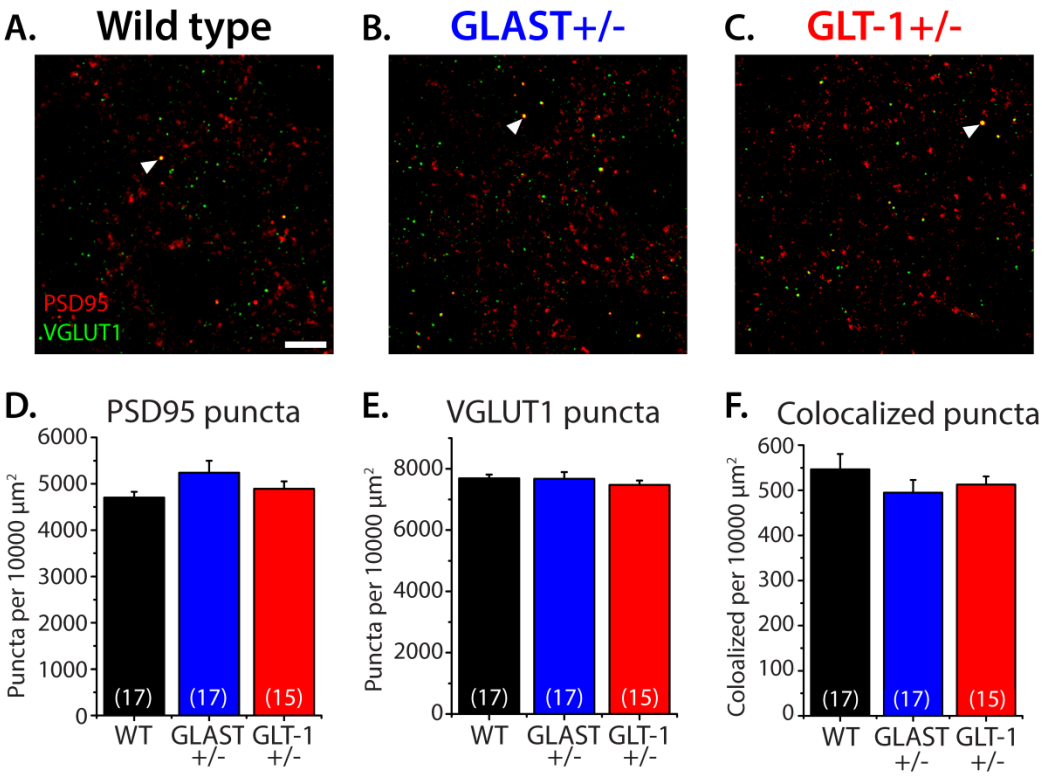

Figure 6.2. Excitatory synapse numbers are not different in Layer 5 of the somatosensory cortex. (A) Wild-type, (B) GLAST$^{+/-}$, and (C) GLT-1$^{+/-}$ sections from P14 mice were stained for VGLUT1 and PSD95 to label pre- and post- synaptic puncta, respectively. Immunohistochemistry was performed as described for inhibitory synapse quantification in Chapter 4. Colocalization of PSD95 and VGLUT1 represent excitatory synapses (white arrowheads). (D-F) The number of postsynaptic, presynaptic, and colocalized puncta are not different between groups. Scale bar is 5 microns.

Chapter 7: Bibliography

Abbott, N. J., L. Ronnback and E. Hansson (2006). "Astrocyte-endothelial interactions at the blood-brain barrier." Nat Rev Neurosci 7(1): 41-53.

Ackman, J. B., T. J. Burbridge and M. C. Crair (2012). "Retinal waves coordinate patterned activity throughout the developing visual system." Nature 490(7419): 219-225.

Agulhon, C., J. Petravicz, A. B. McMullen, E. J. Sweger, S. K. Minton, S. R. Taves, K. B. Casper, T. A. Fiacco and K. D. McCarthy (2008). "What is the role of astrocyte calcium in neurophysiology?" Neuron 59(6): 932-946.

Ahn BH, Kim HS, Song S, et al. A role for the mitochondrial deacetylase Sirt3 in regulating energy homeostasis. Proc Natl Acad Sci 2008;105:14447-52.

Ahuja N, Schwer B, Carobbio S, et al. Regulation of insulin secretion by SIRT4, a mitochondrial ADP-ribosyltransferase. J Biol Chem 2007;282:33583-92.

Ahuja N, Schwer B, Carobbio S, et al. Regulation of insulin secretion by SIRT4, a mitochondrial ADP-ribosyltransferase. J Biol Chem 2007;282:33583-92.

Aiba, A., M. Kano, C. Chen, M. E. Stanton, G. D. Fox, K. Herrup, T. A. Zwingman and S. Tonegawa (1994). "Deficient cerebellar long-term depression and impaired motor learning in mGluR1 mutant mice." Cell 79(2): 377-388.

Aida, T., Y. Ito, Y. K. Takahashi and K. Tanaka (2012). "Overstimulation of NMDA receptors impairs early brain development in vivo." PLoS One 7(5): e36853.

Akanji AO, Hockaday TD. Acetate tolerance and the kinetics of acetate utilization in diabetic and nondiabetic subjects. Am J Clin Nutr 1990;51:112-18.

Alhazzazi TY, Kamarajan P, Joo N, et al. Sirtuin-3 (SIRT3), a novel potential therapeutic target for oral cancer. Cancer 2011;117:1670-78.

Aligny, C., C. Roux, N. Dourmap, Y. Ramdani, J. C. Do-Rego, S. Jegou, P. Leroux, I. Leroux-Nicollet, S. Marret and B. J. Gonzalez (2014). "Ketamine alters cortical integration of GABAergic interneurons and induces long-term sex-dependent impairments in transgenic Gad67-GFP mice." Cell Death Dis 5: e1311.

Allen, N. J., M. L. Bennett, L. C. Foo, G. X. Wang, C. Chakraborty, S. J. Smith and B. A. Barres (2012). "Astrocyte glypicans 4 and 6 promote formation of excitatory synapses via GluA1 AMPA receptors." Nature 486(7403): 410-414.

Allene, C., A. Cattani, J. B. Ackman, P. Bonifazi, L. Aniksztejn, Y. Ben-Ari and R. Cossart (2008). "Sequential generation of two distinct synapse-driven network patterns in developing neocortex." J Neurosci 28(48): 12851-12863.

Allene, C., M. A. Picardo, H. Becq, G. Miyoshi, G. Fishell and R. Cossart (2012). "Dynamic changes in interneuron morphophysiological properties mark the maturation of hippocampal network activity." J Neurosci 32(19): 6688-6698.

Allison SJ, Milner JO. SIRT3 is pro-apoptotic and participates in distinct basal apoptotic pathways. Cell Cycle 2007;6:2669-77.

Amara, S. G. and A. C. Fontana (2002). "Excitatory amino acid transporters: keeping up with glutamate." Neurochem Int 41(5): 313-318.

Anderson, C. M. and R. A. Swanson (2000). "Astrocyte glutamate transport: review of properties, regulation, and physiological functions." Glia 32(1): 1-14.

Andreae, L. C. and J. Burrone (2015). "Spontaneous Neurotransmitter Release Shapes Dendritic Arbors via Long-Range Activation of NMDA Receptors." Cell Rep 10(6): 873-882.

Anthony, T. E. and N. Heintz (2008). "Genetic lineage tracing defines distinct neurogenic and gliogenic stages of ventral telencephalic radial glial development." Neural Dev 3: 30.

Aoyama, K., S. W. Suh, A. M. Hamby, J. Liu, W. Y. Chan, Y. Chen and R. A. Swanson (2006). "Neuronal glutathione deficiency and age-dependent neurodegeneration in the EAAC1 deficient mouse." Nat Neurosci 9(1): 119-126.

Araque, A., N. Li, R. T. Doyle and P. G. Haydon (2000). "SNARE protein-dependent glutamate release from astrocytes." J Neurosci 20(2): 666-673.

Arlotta, P., B. J. Molyneaux, J. Chen, J. Inoue, R. Kominami and J. D. Macklis (2005). "Neuronal subtype-specific genes that control corticospinal motor neuron development in vivo." Neuron 45(2): 207-221.

Armbruster, M., D. Hampton, Y. Yang and C. G. Dulla (2014). "Laser-scanning astrocyte mapping reveals increased glutamate-responsive domain size and disrupted maturation of glutamate uptake following neonatal cortical freeze-lesion." Front Cell Neurosci 8: 277.

Arriza, J. L., S. Eliasof, M. P. Kavanaugh and S. G. Amara (1997). "Excitatory amino acid transporter 5, a retinal glutamate transporter coupled to a chloride conductance." Proc Natl Acad Sci U S A 94(8): 4155-4160.

Arriza, J. L., W. A. Fairman, J. I. Wadiche, G. H. Murdoch, M. P. Kavanaugh and S. G. Amara (1994). "Functional comparisons of three glutamate transporter subtypes cloned from human motor cortex." J Neurosci 14(9): 5559-5569.

Ashraf N, Zino S, MacIntyre A, et al. Altered sirtuin expression is associated with node-positive breast cancer. Br J Cancer 2006;95:1056-061.

Auld, D. S. and R. Robitaille (2003). "Glial cells and neurotransmission: an inclusive view of synaptic function." Neuron 40(2): 389-400.

Azevedo, F. A., L. R. Carvalho, L. T. Grinberg, J. M. Farfel, R. E. Ferretti, R. E. Leite, W. Jacob Filho, R. Lent and S. Herculano-Houzel (2009). "Equal numbers of neuronal and nonneuronal cells make the human brain an isometrically scaled-up primate brain." J Comp Neurol 513(5): 532-541.

Bai F., Witzmann F. A. (2007) Synaptosome proteomics, in Subcellular Proteomics, (Bertrand E. and Faupel M., eds), Vol. 43, pp. 77-98. Springer Netherlands.

Banke, T. G. and C. J. McBain (2006). "GABAergic input onto CA3 hippocampal interneurons remains shunting throughout development." J Neurosci 26(45): 11720-11725.

Barbour, B., B. U. Keller, I. Llano and A. Marty (1994). "Prolonged presence of glutamate during excitatory synaptic transmission to cerebellar Purkinje cells." Neuron 12(6): 1331-1343.

Bauer, D., D. Gupta, V. Harotunian, J. H. Meador-Woodruff and R. E. McCullumsmith (2008). "Abnormal expression of glutamate transporter and transporter interacting molecules in prefrontal cortex in elderly patients with schizophrenia." Schizophr Res 104(1-3): 108-120.

Bayraktar, O. A., L. C. Fuentealba, A. Alvarez-Buylla and D. H. Rowitch (2014). "Astrocyte development and heterogeneity." Cold Spring Harb Perspect Biol 7(1): a020362.

Behar, T. N., A. E. Schaffner, C. A. Scott, C. O'Connell and J. L. Barker (1998). "Differential response of cortical plate and ventricular zone cells to GABA as a migration stimulus." J Neurosci 18(16): 6378-6387.

Behar, T. N., C. A. Scott, C. L. Greene, X. Wen, S. V. Smith, D. Maric, Q. Y. Liu, C. A. Colton and J. L. Barker (1999). "Glutamate acting at NMDA receptors stimulates embryonic cortical neuronal migration." J Neurosci 19(11): 4449-4461.

Behar, T. N., Y. X. Li, H. T. Tran, W. Ma, V. Dunlap, C. Scott and J. L. Barker (1996). "GABA stimulates chemotaxis and chemokinesis of embryonic cortical neurons via calcium-dependent mechanisms." J Neurosci 16(5): 1808-1818.

Behrens, P. F., P. Franz, B. Woodman, K. S. Lindenberg and G. B. Landwehrmeyer (2002). "Impaired glutamate transport and glutamate-glutamine cycling: downstream effects of the Huntington mutation." Brain 125(Pt 8): 1908-1922.

Belanger, M., I. Allaman and P. J. Magistretti (2011). "Brain energy metabolism: focus on astrocyte-neuron metabolic cooperation." Cell Metab 14(6): 724-738.

Bell EL, Emerling BM, Ricoult SJH, Guarente L. SirT3 suppresses hypoxia inducible factor 1α and tumor growth by inhibiting mitochondrial ROS production. Oncogene 2011;30:2986-996.

Ben Haim, L. and D. H. Rowitch (2017). "Functional diversity of astrocytes in neural circuit regulation." Nat Rev Neurosci 18(1): 31-41.

Ben-Ari, Y., I. Khalilov, A. Represa and H. Gozlan (2004). "Interneurons set the tune of developing networks." Trends Neurosci 27(7): 422-427.

Berdichevsky A., Guarente L. (2006) A stress response pathway involving sirtuins, forkhead and 14-3-3 proteins. Cell Cycle. 5, 2588-2591.

Berger, T., J. Schnitzer and H. Kettenmann (1991). "Developmental changes in the membrane current pattern, K+ buffer capacity, and morphology of glial cells in the corpus callosum slice." J Neurosci 11(10): 3008-3024.

Bernardinelli, Y., J. Randall, E. Janett, I. Nikonenko, S. Konig, E. V. Jones, C. E. Flores, K. K. Murai, C. G. Bochet, A. Holtmaat and D. Muller (2014). "Activity-dependent structural plasticity of perisynaptic astrocytic domains promotes excitatory synapse stability." Curr Biol 24(15): 1679-1688.

Blue, M. E. and J. G. Parnavelas (1983). "The formation and maturation of synapses in the visual cortex of the rat. II. Quantitative analysis." J Neurocytol 12(4): 697-712.

Bonifazi, P., M. Goldin, M. A. Picardo, I. Jorquera, A. Cattani, G. Bianconi, A. Represa, Y. Ben-Ari and R. Cossart (2009). "GABAergic hub neurons orchestrate synchrony in developing hippocampal networks." Science 326(5958): 1419-1424.

Bordey, A. and H. Sontheimer (2000). "Ion channel expression by astrocytes in situ: comparison of different CNS regions." Glia 30(1): 27-38.

Brasnjo, G. and T. S. Otis (2001). "Neuronal glutamate transporters control activation of postsynaptic metabotropic glutamate receptors and influence cerebellar long-term depression." Neuron 31(4): 607-616.

Brew, H. and D. Attwell (1987). "Electrogenic glutamate uptake is a major current carrier in the membrane of axolotl retinal glial cells." Nature 327(6124): 707-709.

Bushong, E. A., M. E. Martone and M. H. Ellisman (2004). "Maturation of astrocyte morphology and the establishment of astrocyte domains during postnatal hippocampal development." International Journal of Developmental Neuroscience 22(2): 73-86.

Bushong, E. A., M. E. Martone, Y. Z. Jones and M. H. Ellisman (2002). "Protoplasmic astrocytes in CA1 stratum radiatum occupy separate anatomical domains." J Neurosci 22(1): 183-192.

Butt, S. J., M. Fuccillo, S. Nery, S. Noctor, A. Kriegstein, J. G. Corbin and G. Fishell (2005). "The temporal and spatial origins of cortical interneurons predict their physiological subtype." Neuron 48(4): 591-604.

Buzsaki, G. (1997). "Functions for interneuronal nets in the hippocampus." Can J Physiol Pharmacol 75(5): 508-515.

Cahoy, J. D., B. Emery, A. Kaushal, L. C. Foo, J. L. Zamanian, K. S. Christopherson, Y. Xing, J. L. Lubischer, P. A. Krieg, S. A. Krupenko, W. J. Thompson and B. A. Barres (2008). "A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function." J Neurosci 28(1): 264-278.

Cameron, R. S. and P. Rakic (1991). "Glial cell lineage in the cerebral cortex: a review and synthesis." Glia 4(2): 124-137.

Campbell, K. and M. Gotz (2002). "Radial glia: multi-purpose cells for vertebrate brain development." Trends Neurosci 25(5): 235-238.

Campbell, S. L. and J. J. Hablitz (2008). "Decreased glutamate transport enhances excitability in a rat model of cortical dysplasia." Neurobiol Dis 32(2): 254-261.

Campbell, S. L., J. J. Hablitz and M. L. Olsen (2014). "Functional changes in glutamate transporters and astrocyte biophysical properties in a rodent model of focal cortical dysplasia." Front Cell Neurosci 8: 425.

Cancedda, L., H. Fiumelli, K. Chen and M. M. Poo (2007). "Excitatory GABA action is essential for morphological maturation of cortical neurons in vivo." J Neurosci 27(19): 5224-5235.

Carlen, M., K. Meletis, J. H. Siegle, J. A. Cardin, K. Futai, D. Vierling-Claassen, C. Ruhlmann, S. R. Jones, K. Deisseroth, M. Sheng, C. I. Moore and L. H. Tsai (2012). "A critical role for NMDA receptors in parvalbumin interneurons for gamma rhythm induction and behavior." Mol Psychiatry 17(5): 537-548.

Casado, M., A. Bendahan, F. Zafra, N. C. Danbolt, C. Aragon, C. Gimenez and B. I. Kanner (1993). "Phosphorylation and modulation of brain glutamate transporters by protein kinase C." J Biol Chem 268(36): 27313-27317.

Cattani, A. A., V. D. Bonfardin, A. Represa, Y. Ben-Ari and L. Aniksztejn (2007). "Generation of slow network oscillations in the developing rat hippocampus after blockade of glutamate uptake." J Neurophysiol 98(4): 2324-2336.

Chan-Palay, V. and S. L. Palay (1971). "Tendril and glomerular collaterals of climbing fibers in the granular layer of the rat's cerebellar cortex." Z Anat Entwicklungsgesch 133(3): 247-273.

Chattopadhyaya, B., G. Di Cristo, H. Higashiyama, G. W. Knott, S. J. Kuhlman, E. Welker and Z. J. Huang (2004). "Experience and activity-dependent maturation of perisomatic GABAergic innervation in primary visual cortex during a postnatal critical period." J Neurosci 24(43): 9598-9611.

Chattopadhyaya, B., G. Di Cristo, C. Z. Wu, G. Knott, S. Kuhlman, Y. Fu, R. D. Palmiter and Z. J. Huang (2007). "GAD67-mediated GABA synthesis and signaling regulate inhibitory synaptic innervation in the visual cortex." Neuron 54(6): 889-903.

Chaudhry, F. A., K. P. Lehre, M. van Lookeren Campagne, O. P. Ottersen, N. C. Danbolt and J. Storm-Mathisen (1995). "Glutamate transporters in glial plasma membranes: highly differentiated localizations revealed by quantitative ultrastructural immunocytochemistry." Neuron 15(3): 711-720.

Cheadle, L. and T. Biederer (2014). "Activity-dependent regulation of dendritic complexity by semaphorin 3A through Farp1." J Neurosci 34(23): 7999-8009.

Chen Y, Zhang J, Lin Y, et al. Tumour suppressor SIRT3 deacetylates and activates manganese superoxide dismutase to scavenge ROS. EMBO Reports 2011;12:534-541.

Chen, C., M. Kano, A. Abeliovich, L. Chen, S. Bao, J. J. Kim, K. Hashimoto, R. F. Thompson and S. Tonegawa (1995). "Impaired motor coordination correlates with persistent multiple climbing fiber innervation in PKC gamma mutant mice." Cell 83(7): 1233-1242.

Cheung, G., O. Chever and N. Rouach (2014). "Connexons and pannexons: newcomers in neurophysiology." Front Cell Neurosci 8: 348.

Chew, L. J., M. W. Fleck, P. Wright, S. E. Scherer, M. L. Mayer and V. Gallo (1997). "Growth factor-induced transcription of GluR1 increases functional AMPA receptor density in glial progenitor cells." J Neurosci 17(1): 227-240.

Chiu, C. and M. Weliky (2002). "Relationship of correlated spontaneous activity to functional ocular dominance columns in the developing visual cortex." Neuron 35(6): 1123-1134.

Choi D. W. (1988) Glutamate neurotoxicity and diseases of the nervous system. Neuron. 1, 623-634.

Choi, D. W. (1992). "Excitotoxic cell death." J Neurobiol 23(9): 1261-1276.

Christopherson, K. S., E. M. Ullian, C. C. Stokes, C. E. Mullowney, J. W. Hell, A. Agah, J. Lawler, D. F. Mosher, P. Bornstein and B. A. Barres (2005). "Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis." Cell 120(3): 421-433.

Chung, W. S., L. E. Clarke, G. X. Wang, B. K. Stafford, A. Sher, C. Chakraborty, J. Joung, L. C. Foo, A. Thompson, C. Chen, S. J. Smith and B. A. Barres (2013). "Astrocytes mediate synapse elimination through MEGF10 and MERTK pathways." Nature 504(7480): 394-400.

Chung, W. S., N. J. Allen and C. Eroglu (2015). "Astrocytes Control Synapse Formation, Function, and Elimination." Cold Spring Harb Perspect Biol.

Cline, H. and K. Haas (2008). "The regulation of dendritic arbor development and plasticity by glutamatergic synaptic input: a review of the synaptotrophic hypothesis." J Physiol 586(6): 1509-1517.

Cobos, I., M. E. Calcagnotto, A. J. Vilaythong, M. T. Thwin, J. L. Noebels, S. C. Baraban and J. L. Rubenstein (2005). "Mice lacking Dlx1 show subtype-specific loss of interneurons, reduced inhibition and epilepsy." Nat Neurosci 8(8): 1059-1068.

Cohen, S. M., R. W. Tsien, D. C. Goff and M. M. Halassa (2015). "The impact of NMDA receptor hypofunction on GABAergic neurons in the pathophysiology of schizophrenia." Schizophrenia Res 167(1573-2509 (Electronic)): 98-107.

Cohen-Cory, S. (2002). "The Developing Synapse: Construction and Modulation of Synaptic Structures and Circuits." Science 298(5594): 770-776.

Conti F., Weinberg R. J. (1999) Shaping excitation at glutamatergic synapses. Trends. Neurosci. 22, 451-458.

Crepel, F. (1982). "Regression of functional synapses in the immature mammalian cerebellum." Trends in Neurosciences 5: 266-269.

Crepel, F., N. Delhaye-Bouchaud, J. M. Guastavino and I. Sampaio (1980). "Multiple innervation of cerebellar Purkinje cells by climbing fibres in staggerer mutant mouse." Nature 283(5746): 483-484.

Cvetanovic, M. (2015). "Decreased expression of glutamate transporter GLAST in Bergmann glia is associated with the loss of Purkinje neurons in the spinocerebellar ataxia type 1." Cerebellum 14(1): 8-11.

Danbolt, N. C. (2001). "Glutamate uptake." Progress in Neurobiology 65(0301-0082 (Print)): 1-105.

Danbolt, N. C., Y. Zhou, D. N. Furness and S. Holmseth (2016). "Strategies for immunohistochemical protein localization using antibodies: What did we learn from neurotransmitter transporters in glial cells and neurons." Glia 64(12): 2045-2064.

Dang CV, Semenza GL. Oncogenic alterations of metabolism. Trends Biochem Sci 1999;24:68-72.

Daw, M. I., M. C. Ashby and J. T. Isaac (2007). "Coordinated developmental recruitment of latent fast spiking interneurons in layer IV barrel cortex." Nat Neurosci 10(4): 453-461.

de Lima, A. D., T. Opitz and T. Voigt (2004). "Irreversible loss of a subpopulation of cortical interneurons in the absence of glutamatergic network activity." Eur J Neurosci 19(11): 2931-2943.

De Marco Garcia, N. V., R. Priya, S. N. Tuncdemir, G. Fishell and T. Karayannis (2015). "Sensory inputs control the integration of neurogliaform interneurons into cortical circuits." Nat Neurosci 18(3): 393-401.

de Vries, B., H. Mamsa, A. H. Stam, J. Wan, S. L. Bakker, K. R. Vanmolkot, J. Haan, G. M. Terwindt, E. M. Boon, B. D. Howard, R. R. Frants, R. W. Baloh, M. D. Ferrari, J. C. Jen and A. M. van den Maagdenberg (2009). "Episodic ataxia associated with EAAT1 mutation C186S affecting glutamate reuptake." Arch Neurol 66(1): 97-101.

DeFelipe, J. and I. Farinas (1992). "The pyramidal neuron of the cerebral cortex: morphological and chemical characteristics of the synaptic inputs." Prog Neurobiol 39(6): 563-607.

Demarque, M., A. Represa, H. Becq, I. Khalilov, Y. Ben-Ari and L. Aniksztejn (2002). "Paracrine intercellular communication by a Ca2+- and SNARE-independent release of GABA and glutamate prior to synapse formation." Neuron 36(6): 1051-1061.

Demarque, M., N. Villeneuve, J. B. Manent, H. Becq, A. Represa, Y. Ben-Ari and L. Anikstejn (2004). "Glutamate transporters prevent the generation of seizures in the developing rat neocortex." J Neurosci 24(13): 3289-3294.

Deneen, B., R. Ho, A. Lukaszewicz, C. J. Hochstim, R. M. Gronostajski and D. J. Anderson (2006). "The transcription factor NFIA controls the onset of gliogenesis in the developing spinal cord." Neuron 52(6): 953-968.

Dermietzel, R., O. Traub, T. K. Hwang, E. Beyer, M. V. Bennett, D. C. Spray and K. Willecke (1989). "Differential expression of three gap junction proteins in developing and mature brain tissues." Proc Natl Acad Sci U S A 86(24): 10148-10152.

Derouiche, A. (1996). "Possible role of the Muller cell in uptake and metabolism of glutamate in the mammalian outer retina." Vision Res 36(24): 3875-3878.

Di Cristo, G., B. Chattopadhyaya, S. J. Kuhlman, Y. Fu, M. C. Belanger, C. Z. Wu, U. Rutishauser, L. Maffei and Z. J. Huang (2007). "Activity-dependent PSA expression regulates inhibitory maturation and onset of critical period plasticity." Nat Neurosci 10(12): 1569-1577.

Diamond, J. S. (2005). "Deriving the glutamate clearance time course from transporter currents in CA1 hippocampal astrocytes: transmitter uptake gets faster during development." J Neurosci 25(1529-2401 (Electronic)): 2906-2916.

Diamond, J. S. and C. E. Jahr (1997). "Transporters buffer synaptically released glutamate on a submillisecond time scale." J Neurosci 17(12): 4672-4687.

Diamond, J. S., D. E. Bergles and C. E. Jahr (1998). "Glutamate release monitored with astrocyte transporter currents during LTP." Neuron 21(2): 425-433.

Doischer, D., J. A. Hosp, Y. Yanagawa, K. Obata, P. Jonas, I. Vida and M. Bartos (2008). "Postnatal differentiation of basket cells from slow to fast signaling devices." J Neurosci 28(48): 12956-12968.

Domercq, M., M. V. Sanchez-Gomez, P. Areso and C. Matute (1999). "Expression of glutamate transporters in rat optic nerve oligodendrocytes." Eur J Neurosci 11(7): 2226-2236.

Donmez G. The neurobiology of sirtuins and their role in neurodegeneration. Trends Pharmacol Sci 2012;33:494-501.

Donmez G., Guarente L. (2010) Aging and disease: Connections to sirtuins. Aging Cell. 9, 285-290.

Doyle, J. P., J. D. Dougherty, M. Heiman, E. F. Schmidt, T. R. Stevens, G. Ma, S. Bupp, P. Shrestha, R. D. Shah, M. L. Doughty, S. Gong, P. Greengard and N. Heintz (2008). "Application of a translational profiling approach for the comparative analysis of CNS cell types." Cell 135(4): 749-762.

Du J, Zhou Y, Su X, et al. Sirt5 is a NAD-dependent protein lysine demalonylase and desuccinylase. Science 2011;334:806-9.

Elmariah, S. B., E. J. Oh, E. G. Hughes and R. J. Balice-Gordon (2005). "Astrocytes regulate inhibitory synapse formation via Trk-mediated modulation of postsynaptic GABAA receptors." J Neurosci 25(14): 3638-3650.

Eroglu, C., N. J. Allen, M. W. Susman, N. A. O'Rourke, C. Y. Park, E. Ozkan, C. Chakraborty, S. B. Mulinyawe, D. S. Annis, A. D. Huberman, E. M. Green, J. Lawler, R. Dolmetsch, K. C. Garcia, S. J. Smith, Z. D. Luo, A. Rosenthal, D. F. Mosher and B. A. Barres (2009). "Gabapentin receptor alpha2delta-1 is a

neuronal thrombospondin receptor responsible for excitatory CNS synaptogenesis." Cell 139(2): 380-392.
Etherington, S. J. and S. R. Williams (2011). "Postnatal development of intrinsic and synaptic properties transforms signaling in the layer 5 excitatory neural network of the visual cortex." J Neurosci 31(26): 9526-9537.
Fagiolini, M. and T. K. Hensch (2000). "Inhibitory threshold for critical-period activation in primary visual cortex." Nature 404(6774): 183-186.
Farhy-Tselnicker, I., A. C. M. van Casteren, A. Lee, V. T. Chang, A. R. Aricescu and N. J. Allen (2017). "Astrocyte-Secreted Glypican 4 Regulates Release of Neuronal Pentraxin 1 from Axons to Induce Functional Synapse Formation." Neuron 96(2): 428-445.e413.
Farris W, Mansourian S, Leissring MA, et al. Partial loss-of-function mutations in insulin-degrading enzyme that induce diabetes also impair degradation of amyloid β-protein. Am J Pathol 2004;164:1425-34.
Featherstone, D. E. and S. A. Shippy (2008). "Regulation of synaptic transmission by ambient extracellular glutamate." Neuroscientist 14(2): 171-181.
Feller, M. B. (1999). "Spontaneous correlated activity in developing neural circuits." Neuron 22(4): 653-656.
Finkel T., Deng C. X., Mostoslavsky R. (2009) Recent progress in the biology and physiology of sirtuins. Nature. 460, 587-591.
Finley LWS, Carracedo A, Lee J, et al. SIRT3 opposes reprogramming of cancer cell metabolism through hif1α destabilization. Cancer Cell 2011;19:416-428.
Flint, A. C. and B. W. Connors (1996). "Two types of network oscillations in neocortex mediated by distinct glutamate receptor subtypes and neuronal populations." J Neurophysiol 75(2): 951-957.
Frick, A., D. Feldmeyer and B. Sakmann (2007). "Postnatal development of synaptic transmission in local networks of L5A pyramidal neurons in rat somatosensory cortex." J Physiol 585(Pt 1): 103-116.
Frye RA. Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. Biochem Biophys Res Commun 2000;273:793-98.
Fukamachi, S., A. Furuta, T. Ikeda, T. Ikenoue, T. Kaneoka, J. D. Rothstein and T. Iwaki (2001). "Altered expressions of glutamate transporter subtypes in rat model of neonatal cerebral hypoxia-ischemia." Brain Res Dev Brain Res 132(2): 131-139.
Furness, D. N., Y. Dehnes, A. Q. Akhtar, D. J. Rossi, M. Hamann, N. J. Grutle, V. Gundersen, S. Holmseth, K. P. Lehre, K. Ullensvang, M. Wojewodzic, Y. Zhou, D. Attwell and N. C. Danbolt (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(1): 80-94.
Furukawa, T., J. Yamada, T. Akita, Y. Matsushima, Y. Yanagawa and A. Fukuda (2014). "Roles of taurine-mediated tonic GABAA receptor activation in the radial migration of neurons in the fetal mouse cerebral cortex." Front Cell Neurosci 8: 88.
Furuta, A., J. D. Rothstein and L. J. Martin (1997). "Glutamate Transporter Protein Subtypes Are Expressed Differentially during Rat CNS Development." The Journal of Neuroscience 17(21): 8363-8375.

Gallo, V., P. Wright and R. D. McKinnon (1994). "Expression and regulation of a glutamate receptor subunit by bFGF in oligodendrocyte progenitors." Glia 10(2): 149-153.

Garrett, A. M. and J. A. Weiner (2009). "Control of CNS synapse development by {gamma}-protocadherin-mediated astrocyte-neuron contact." J Neurosci 29(38): 11723-11731.

Gegelashvili, G. and A. Schousboe (1997). "High affinity glutamate transporters: regulation of expression and activity." Mol Pharmacol 52(1): 6-15.

Gegelashvili, G. and A. Schousboe (1998). "Cellular distribution and kinetic properties of high-affinity glutamate transporters." Brain Res Bull 45(3): 233-238.

Gegelashvili, G., G. Civenni, G. Racagni, N. C. Danbolt, I. Schousboe and A. Schousboe (1996). "Glutamate receptor agonists up-regulate glutamate transporter GLAST in astrocytes." Neuroreport 8(1): 261-265.

Gegelashvili, G., N. C. Danbolt and A. Schousboe (1997). "Neuronal soluble factors differentially regulate the expression of the GLT1 and GLAST glutamate transporters in cultured astroglia." J Neurochem 69(6): 2612-2615.

Genda E. N., Jackson J. G., Sheldon A. L., Locke S. F., Greco T. M., O'Donnell J. C., Spruce L. A., Xiao R., Guo W., Putt M. (2011) Co-compartmentalization of the astroglial glutamate transporter, GLT-1, with glycolytic enzymes and mitochondria. J. Neurosci. 31, 18275-18288.

Genoud, C., C. Quairiaux, P. Steiner, H. Hirling, E. Welker and G. W. Knott (2006). "Plasticity of astrocytic coverage and glutamate transporter expression in adult mouse cortex." Plos Biol 4(1545-7885 (Electronic)): 2057-2064.

George, J., D. G. Baden, W. H. Gerwick and T. F. Murray (2012). "Bidirectional influence of sodium channel activation on NMDA receptor-dependent cerebrocortical neuron structural plasticity." Proc Natl Acad Sci U S A 109(48): 19840-19845.

Gierdalski, M., B. Jablonska, E. Siucinska, M. Lech, A. Skibinska and M. Kossut (2001). "Rapid regulation of GAD67 mRNA and protein level in cortical neurons after sensory learning." Cereb Cortex 11(9): 806-815.

Giménez-Cassina A., Martínez-François J. R., Fisher J. K., Szlyk B., Polak K., Wiwczar J., Tanner G. R., Lutas A., Yellen G., Danial N. N. (2012) BAD-Dependent regulation of fuel metabolism and K-ATP channel activity confers resistance to epileptic seizures. Neuron. 74, 719-730.

Ginsberg, S. D., L. J. Martin and J. D. Rothstein (1995). "Regional deafferentation down-regulates subtypes of glutamate transporter proteins." J Neurochem 65(6): 2800-2803.

Goldberg, E. M., H.-Y. Jeong, I. Kruglikov, R. Tremblay, R. M. Lazarenko and B. Rudy (2011). "Rapid Developmental Maturation of Neocortical FS Cell Intrinsic Excitability." Cerebral Cortex 21(3): 666-682.

Gongidi, V., C. Ring, M. Moody, R. Brekken, E. H. Sage, P. Rakic and E. S. Anton (2004). "SPARC-like 1 regulates the terminal phase of radial glia-guided migration in the cerebral cortex." Neuron 41(1): 57-69.

Gonzalez-Burgos, G., R. Y. Cho and D. A. Lewis (2015). "Alterations in cortical network oscillations and parvalbumin neurons in schizophrenia." Biol Psychiatry 77(12): 1031-1040.

Gordan JD, Thompson CB, Simon MC. HIF and c-myc: sibling rivals for control of cancer cell metabolism and proliferation. Cancer Cell 2007;12:108-113.
Grundy SM, Cleeman JI, Daniels SR, et al. Diagnosis and management of the metabolic syndrome. Circulation 2005;112:2735-52.
Guarente L. (2006) Sirtuins as potential targets for metabolic syndrome. Nature. 444, 868-874.
Guo, C., M. J. Eckler, W. L. McKenna, G. L. McKinsey, J. L. Rubenstein and B. Chen (2013). "Fezf2 expression identifies a multipotent progenitor for neocortical projection neurons, astrocytes, and oligodendrocytes." Neuron 80(5): 1167-1174.
Haigis MC, Guarente LP. Mammalian sirtuins--emerging roles in physiology, aging, and calorie restriction. Genes Dev 2006;20:2913-21.
Haigis MC, Mostoslavsky R, Haigis KM, et al. SIRT4 inhibits glutamate dehydrogenase and opposes the effects of calorie restriction in pancreatic β cells. Cell 2006;126:941-54.
Halassa, M. M., T. Fellin, H. Takano, J. H. Dong and P. G. Haydon (2007). "Synaptic islands defined by the territory of a single astrocyte." J Neurosci 27(24): 6473-6477.
Hallows WC, Lee S, Denu JM. Sirtuins deacetylate and activate mammalian acetyl-CoA synthetases. Proc Natl Acad Sci 2006;103:10230-35.
Hallows WC, Yu W, Smith BC, et al. Sirt3 promotes the urea cycle and fatty acid oxidation during dietary restriction. Mol Cell 2011;41:139-49.
Hanson, E., M. Armbruster, D. Cantu, L. Andresen, A. Taylor, N. C. Danbolt and C. G. Dulla (2015). "Astrocytic glutamate uptake is slow and does not limit neuronal NMDA receptor activation in the neonatal neocortex." Glia 63(10): 1784-1796.
Harris, K. D. and G. M. Shepherd (2015). "The neocortical circuit: themes and variations." Nat Neurosci 18(2): 170-181.
Hartfuss, E., R. Galli, N. Heins and M. Gotz (2001). "Characterization of CNS precursor subtypes and radial glia." Dev Biol 229(1): 15-30.
Haugeto, O., K. Ullensvang, L. M. Levy, F. A. Chaudhry, T. Honore, M. Nielsen, K. P. Lehre and N. C. Danbolt (1996). "Brain glutamate transporter proteins form homomultimers." J Biol Chem 271(44): 27715-27722.
Hayashi, Y., M. Nomura, S. Yamagishi, S. Harada, J. Yamashita and H. Yamamoto (1997). "Induction of various blood-brain barrier properties in non-neural endothelial cells by close apposition to co-cultured astrocytes." Glia 19(1): 13-26.
Haydon, P. G. and M. Nedergaard (2014). "How do astrocytes participate in neural plasticity?" Cold Spring Harb Perspect Biol 7(3): a020438.
Hennou, S., I. Khalilov, D. Diabira, Y. Ben-Ari and H. Gozlan (2002). "Early sequential formation of functional GABA(A) and glutamatergic synapses on CA1 interneurons of the rat foetal hippocampus." Eur J Neurosci 16(2): 197-208.
Hensch, T. K. (2005). "Critical period plasticity in local cortical circuits." Nat Rev Neurosci 6(11): 877-888.
Hensch, T. K., M. Fagiolini, N. Mataga, M. P. Stryker, S. Baekkeskov and S. F. Kash (1998). "Local GABA circuit control of experience-dependent plasticity in developing visual cortex." Science 282(5393): 1504-1508.
Herman, M. A. and C. E. Jahr (2007). "Extracellular glutamate concentration in hippocampal slice." J Neurosci 27(36): 9736-9741.

Higashimori H., Morel L., Huth J., Lindemann L., Dulla C., Taylor A., Freeman M., Yang Y. (2013) Astroglial FMRP-dependent translational down-regulation of mGlur5 underlies glutamate transporter GLT1 dysregulation in the fragile X mouse. Hum. Mol. Genet. 22, 2041-2054.

Hirschey M, Shimazu T, Jing E, et al. SIRT3 deficiency and mitochondrial protein hyperacetylation accelerate the development of the metabolic syndrome. Mol Cell 2011;44:177-90.

Hirschey MD, Shimazu T, Goetzman E, et al. SIRT3 regulates mitochondrial fatty-acid oxidation by reversible enzyme deacetylation. Nature 2010;464:121-25.

Ho L., Titus A. S., Banerjee K. K., George S., Lin W., Deota S., Saha A. K., Nakamura K., Gut P., Verdin E. (2013) SIRT4 regulates ATP homeostasis and mediates a retrograde signaling via AMPK. Aging. 5, 835-849.

Hochstim, C., B. Deneen, A. Lukaszewicz, Q. Zhou and D. J. Anderson (2008). "Identification of positionally distinct astrocyte subtypes whose identities are specified by a homeodomain code." Cell 133(3): 510-522.

Holmseth, S., H. A. Scott, K. Real, K. P. Lehre, T. B. Leergaard, J. G. Bjaalie and N. C. Danbolt (2009). "The concentrations and distributions of three C-terminal variants of the GLT1 (EAAT2; slc1a2) glutamate transporter protein in rat brain tissue suggest differential regulation." Neuroscience 162(4): 1055-1071.

Homayoun, H., M. E. Jackson and B. Moghaddam (2005). "Activation of metabotropic glutamate 2/3 receptors reverses the effects of NMDA receptor hypofunction on prefrontal cortex unit activity in awake rats." J Neurophysiol 93(4): 1989-2001.

Houades, V., A. Koulakoff, P. Ezan, I. Seif and C. Giaume (2008). "Gap junction-mediated astrocytic networks in the mouse barrel cortex." J Neurosci 28(20): 5207-5217.

Hsu PP, Sabatini DM. Cancer cell metabolism: Warburg and beyond. Cell 2008;134:703-7.

Huang, Y. H., M. Dykes-Hoberg, K. Tanaka, J. D. Rothstein and D. E. Bergles (2004). "Climbing fiber activation of EAAT4 transporters and kainate receptors in cerebellar Purkinje cells." J Neurosci 24(1): 103-111.

Huang, Y. H., S. R. Sinha, K. Tanaka, J. D. Rothstein and D. E. Bergles (2004). "Astrocyte glutamate transporters regulate metabotropic glutamate receptor-mediated excitation of hippocampal interneurons." J Neurosci 24(19): 4551-4559.

Huang, Z. J., A. Kirkwood, T. Pizzorusso, V. Porciatti, B. Morales, M. F. Bear, L. Maffei and S. Tonegawa (1999). "BDNF regulates the maturation of inhibition and the critical period of plasticity in mouse visual cortex." Cell 98(6): 739-755.

Ikegaya, Y., S. Matsuura, S. Ueno, A. Baba, M. K. Yamada, N. Nishiyama and N. Matsuki (2002). "Beta-amyloid enhances glial glutamate uptake activity and attenuates synaptic efficacy." J Biol Chem 277(35): 32180-32186.

Imai SI, Armstrong CM, Kaeberlein M, Guarente L. Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. Nature 2000;403:795-99.

Imai SI, Guarente L. Ten years of NAD-dependent SIR2 family deacetylases: implications for metabolic diseases. Trends Pharmacol Sci 2010;31:212-20.

Isaac, J. T., M. C. Crair, R. A. Nicoll and R. C. Malenka (1997). "Silent synapses during development of thalamocortical inputs." Neuron 18(2): 269-280.

Isaac, J. T., R. A. Nicoll and R. C. Malenka (1995). "Evidence for silent synapses: implications for the expression of LTP." Neuron 15(2): 427-434.

Iwasato, T., A. Datwani, A. M. Wolf, H. Nishiyama, Y. Taguchi, S. Tonegawa, T. Knopfel, R. S. Erzurumlu and S. Itohara (2000). "Cortex-restricted disruption of NMDAR1 impairs neuronal patterns in the barrel cortex." Nature 406(6797): 726-731.

Jabaudon D., Scanziani M., Gähwiler B. H., Gerber U. (2000) Acute decrease in net glutamate uptake during energy deprivation. Proc. Natl. Sci. Acad. Sci. U. S. A. 97, 5610-5615.

Jacobs KM, Pennington JD, Bisht KS, et al. SIRT3 interacts with the daf-16 homolog Foxo3a in the mitochondria, as well as increases Foxo3a dependent gene expression. Int J Biol Sci 2008;4:291-99.

Jacobsen, C. T. and R. H. Miller (2003). "Control of astrocyte migration in the developing cerebral cortex." Dev Neurosci 25(2-4): 207-216.

Jansson, L. C. and K. E. Akerman (2014). "The role of glutamate and its receptors in the proliferation, migration, differentiation and survival of neural progenitor cells." J Neural Transm (Vienna) 121(8): 819-836.

Jen, J. C., J. Wan, T. P. Palos, B. D. Howard and R. W. Baloh (2005). "Mutation in the glutamate transporter EAAT1 causes episodic ataxia, hemiplegia, and seizures." Neurology 65(4): 529-534.

Jensen, A. A., M. N. Erichsen, C. W. Nielsen, T. B. Stensbol, J. Kehler and L. Bunch (2009). "Discovery of the first selective inhibitor of excitatory amino acid transporter subtype 1." J Med Chem 52(4): 912-915.

Jeong S., Xiao C., Finley L., Lahusen T., Souza A., Pierce K., Li Y. -H., Wang X., Laurent G., German N., Xu X., Li C., Wang R. -H., Lee J., Csibi A., Cerione R., Blenis J., Clish C., Kimmelman A., Deng C. -X., Haigis M. (2013) SIRT4 has tumor-suppressive activity and regulates the cellular metabolic response to DNA damage by inhibiting mitochondrial glutamine metabolism. Cancer Cell. 23, 450-463.

Jiao, Y., C. Zhang, Y. Yanagawa and Q. Q. Sun (2006). "Major effects of sensory experiences on the neocortical inhibitory circuits." J Neurosci 26(34): 8691-8701.

Jing E, Emanuelli B, Hirschey MD, et al. Sirtuin-3 (Sirt3) regulates skeletal muscle metabolism and insulin signaling via altered mitochondrial oxidation and reactive oxygen species production. Proc Natl Acad Sci 2011;108:14608-13.

John Lin, C. C., K. Yu, A. Hatcher, T. W. Huang, H. K. Lee, J. Carlson, M. C. Weston, F. Chen, Y. Zhang, W. Zhu, C. A. Mohila, N. Ahmed, A. J. Patel, B. R. Arenkiel, J. L. Noebels, C. J. Creighton and B. Deneen (2017). "Identification of diverse astrocyte populations and their malignant analogs." Nat Neurosci 20(3): 396-405.

Jones, K. S., J. G. Corbin and M. M. Huntsman (2014). "Neonatal NMDA receptor blockade disrupts spike timing and glutamatergic synapses in fast spiking interneurons in a NMDA receptor hypofunction model of schizophrenia." PLoS One 9(10): e109303.

Jourdain, P., L. H. Bergersen, K. Bhaukaurally, P. Bezzi, M. Santello, M. Domercq, C. Matute, F. Tonello, V. Gundersen and A. Volterra (2007). "Glutamate exocytosis from astrocytes controls synaptic strength." Nat Neurosci 10(3): 331-339.

Kaeberlein M, McVey M, Guarente L. The SIR2/3/4 complex and SIR2 alone promote longevity in saccharomyces cerevisiae by two different mechanisms. Genes Dev 1999;13:2570-80.

Kafitz, K. W., S. D. Meier, J. Stephan and C. R. Rose (2008). "Developmental profile and properties of sulforhodamine 101--Labeled glial cells in acute brain slices of rat hippocampus." J Neurosci Methods 169(1): 84-92.

Kaneko, T. and F. Fujiyama (2002). "Complementary distribution of vesicular glutamate transporters in the central nervous system." Neurosci Res 42(4): 243-250.

Kang, P., H. K. Lee, S. M. Glasgow, M. Finley, T. Donti, Z. B. Gaber, B. H. Graham, A. E. Foster, B. G. Novitch, R. M. Gronostajski and B. Deneen (2012). "Sox9 and NFIA coordinate a transcriptional regulatory cascade during the initiation of gliogenesis." Neuron 74(1): 79-94.

Karlsson, R. M., L. Adermark, A. Molander, S. Perreau-Lenz, E. Singley, M. Solomon, A. Holmes, K. Tanaka, D. M. Lovinger, R. Spanagel and M. Heilig (2012). "Reduced alcohol intake and reward associated with impaired endocannabinoid signaling in mice with a deletion of the glutamate transporter GLAST." Neuropharmacology 63(2): 181-189.

Karlsson, R. M., K. Tanaka, L. M. Saksida, T. J. Bussey, M. Heilig and A. Holmes (2009). "Assessment of glutamate transporter GLAST (EAAT1)-deficient mice for phenotypes relevant to the negative and executive/cognitive symptoms of schizophrenia." Neuropsychopharmacology 34(6): 1578-1589.

Karlsson, R. M., K. Tanaka, M. Heilig and A. Holmes (2008). "Loss of glial glutamate and aspartate transporter (excitatory amino acid transporter 1) causes locomotor hyperactivity and exaggerated responses to psychotomimetics: rescue by haloperidol and metabotropic glutamate 2/3 agonist." Biol Psychiatry 64(9): 810-814.

Kashiwabuchi, N., K. Ikeda, K. Araki, T. Hirano, K. Shibuki, C. Takayama, Y. Inoue, T. Kutsuwada, T. Yagi, Y. Kang and et al. (1995). "Impairment of motor coordination, Purkinje cell synapse formation, and cerebellar long-term depression in GluR delta 2 mutant mice." Cell 81(2): 245-252.

Kasper, E. M., J. Lubke, A. U. Larkman and C. Blakemore (1994). "Pyramidal neurons in layer 5 of the rat visual cortex. III. Differential maturation of axon targeting, dendritic morphology, and electrophysiological properties." J Comp Neurol 339(4): 495-518.

Katz, L. C. and C. J. Shatz (1996). "Synaptic activity and the construction of cortical circuits." Science 274(5290): 1133-1138.

Katz, L. C. and J. C. Crowley (2002). "Development of cortical circuits: lessons from ocular dominance columns." Nat Rev Neurosci 3(1): 34-42.

Kawaguchi, Y. and Y. Kubota (1997). "GABAergic cell subtypes and their synaptic connections in rat frontal cortex." Cereb Cortex 7(6): 476-486.

Kelsch, W., Z. Li, S. Wieland, O. Senkov, A. Herb, C. Gongrich and H. Monyer (2014). "GluN2B-containing NMDA receptors promote glutamate synapse development in hippocampal interneurons." J Neurosci 34(48): 16022-16030.

Kennedy B. K., Austriaco Jr N. R., Zhang J., Guarente L. (1995) Mutation in the silencing gene S/R4 can delay aging in S. cerevisiae. Cell. 80, 485-496.

Kerber, K., P. LeVan, M. Dumpelmann, S. Fauser, R. Korinthenberg, A. Schulze-Bonhage and J. Jacobs (2013). "High frequency oscillations mirror disease activity in patients with focal cortical dysplasia." Epilepsia 54(8): 1428-1436.

Khakh, B. S. and M. V. Sofroniew (2015). "Diversity of astrocyte functions and phenotypes in neural circuits." Nat Neurosci 18(7): 942-952.

Khosravani, H., C. R. Pinnegar, J. R. Mitchell, B. L. Bardakjian, P. Federico and P. L. Carlen (2005). "Increased high-frequency oscillations precede in vitro low-Mg seizures." Epilepsia 46(8): 1188-1197.

Kim H-S, Patel K, Muldoon-Jacobs K, et al.SIRT3 is a mitochondria-localized tumor suppressor required for maintenance of mitochondrial integrity and metabolism during stress. Cancer Cell 2010;17:41 - 52.

Kim SH, Lu HF, Alano CC. Neuronal Sirt3 protects against excitotoxic injury in mouse cortical neuron culture. PLoS One 2011;6:e14731.

Kinney, J. W., C. N. Davis, I. Tabarean, B. Conti, T. Bartfai and M. M. Behrens (2006). "A specific role for NR2A-containing NMDA receptors in the maintenance of parvalbumin and GAD67 immunoreactivity in cultured interneurons." J Neurosci 26(5): 1604-1615.

Kobayashi, M. and P. S. Buckmaster (2003). "Reduced inhibition of dentate granule cells in a model of temporal lobe epilepsy." J Neurosci 23(6): 2440-2452.

Koller, H., M. Siebler, C. Schmalenbach and H. W. Muller (1990). "GABA and glutamate receptor development of cultured neurons from rat hippocampus, septal region, and neocortex." Synapse 5(1): 59-64.

Komlos D., Mann K. D., Zhuo Y., Ricupero C. L., Hart R. P., Liu A. Y. --C., Firestein B. L. (2013) Glutamate dehydrogenase 1 and SIRT4 regulate glial development. Glia. 61, 394-408.

Komuro, H. and P. Rakic (1993). "Modulation of neuronal migration by NMDA receptors." Science 260(5104): 95-97.

Kong X, Wang R, Xue Y, et al.Sirtuin 3, a new target of pgc-1α, plays an important role in the suppression of ROS and mitochondrial biogenesis. PLoS One 2010;5:e11707.

Kriegstein, A. and A. Alvarez-Buylla (2009). "The glial nature of embryonic and adult neural stem cells." Annu Rev Neurosci 32: 149-184.

Kucukdereli, H., N. J. Allen, A. T. Lee, A. Feng, M. I. Ozlu, L. M. Conatser, C. Chakraborty, G. Workman, M. Weaver, E. H. Sage, B. A. Barres and C. Eroglu (2011). "Control of excitatory CNS synaptogenesis by astrocyte-secreted proteins Hevin and SPARC." Proc Natl Acad Sci U S A 108(32): E440-449.

Kuffler, S. W. (1967). "Neuroglial cells: physiological properties and a potassium mediated effect of neuronal activity on the glial membrane potential." Proc R Soc Lond B Biol Sci 168(1010): 1-21.

Kurochkin IV, Goto S. Alzheimer's β-amyloid peptide specifically interacts with and is degraded by insulin degrading enzyme. FEBS Lett 1994;345:33-37.

Kwan, K. Y., N. Sestan and E. S. Anton (2012). "Transcriptional co-regulation of neuronal migration and laminar identity in the neocortex." Development 139(9): 1535-1546.

Kwon, H. B. and B. L. Sabatini (2011). "Glutamate induces de novo growth of functional spines in developing cortex." Nature 474(7349): 100-104.

Lalo, U., Y. Pankratov, V. Parpura and A. Verkhratsky (2011). "Ionotropic receptors in neuronal-astroglial signalling: what is the role of "excitable" molecules in non-excitable cells." Biochim Biophys Acta 1813(5): 992-1002.

Laurent G., German N. J., Saha A. K., de Boer V. C., Davies M., Koves T. R., Dephoure N., Fischer F., Boanca G., Vaitheesvaran B. (2013) SIRT4 coordinates the balance between lipid synthesis and catabolism by repressing malonyl CoA decarboxylase. Mol. Cell. 50, 686-698.

Lavialle, M., G. Aumann, E. Anlauf, F. Prols, M. Arpin and A. Derouiche (2011). "Structural plasticity of perisynaptic astrocyte processes involves ezrin and metabotropic glutamate receptors." Proc Natl Acad Sci U S A 108(31): 12915-12919.

Lazarus, M. S. and Z. J. Huang (2011). "Distinct maturation profiles of perisomatic and dendritic targeting GABAergic interneurons in the mouse primary visual cortex during the critical period of ocular dominance plasticity." J Neurophysiol 106(2): 775-787.

Le Magueresse, C. and H. Monyer (2013). "GABAergic interneurons shape the functional maturation of the cortex." Neuron 77(3): 388-405.

Le Meur, K., M. Galante, M. C. Angulo and E. Audinat (2007). "Tonic activation of NMDA receptors by ambient glutamate of non-synaptic origin in the rat hippocampus." J Physiol 580(Pt. 2): 373-383.

Lee, H. H., T. Z. Deeb, J. A. Walker, P. A. Davies and S. J. Moss (2011). "NMDA receptor activity downregulates KCC2 resulting in depolarizing GABAA receptor-mediated currents." Nat Neurosci 14(6): 736-743.

Lehre, K. P. and N. C. Danbolt (1998). "The number of glutamate transporter subtype molecules at glutamatergic synapses: chemical and stereological quantification in young adult rat brain." J Neurosci 18(21): 8751-8757.

Levy, L. M., K. P. Lehre, S. I. Walaas, J. Storm-Mathisen and N. C. Danbolt (1995). "Down-regulation of glial glutamate transporters after glutamatergic denervation in the rat brain." Eur J Neurosci 7(10): 2036-2041.

Lewis, D. A., T. Hashimoto and D. W. Volk (2005). "Cortical inhibitory neurons and schizophrenia." Nat Rev Neurosci 6(4): 312-324.

Li S, Banck MA, Mujtaba SA, Zhou M-MA, Sugrue MMA, Walsh M. p53-Induced growth arrest is regulated by the mitochondrial SirT3 deacetylase. PLoS One 2010;5:e10486.

Li, M., Z. Cui, Y. Niu, B. Liu, W. Fan, D. Yu and J. Deng (2010). "Synaptogenesis in the developing mouse visual cortex." Brain Res Bull 81(1): 107-113.

Li, S., M. Mallory, M. Alford, S. Tanaka and E. Masliah (1997). "Glutamate transporter alterations in Alzheimer disease are possibly associated with abnormal APP expression." J Neuropathol Exp Neurol 56(8): 901-911.

Liao, D., N. A. Hessler and R. Malinow (1995). "Activation of postsynaptically silent synapses during pairing-induced LTP in CA1 region of hippocampal slice." Nature 375(6530): 400-404.

Liao, D., R. H. Scannevin and R. Huganir (2001). "Activation of silent synapses by rapid activity-dependent synaptic recruitment of AMPA receptors." J Neurosci 21(16): 6008-6017.

Lin MT, Beal MF. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. Nature 2006;443:787-95.
Lin SJ, Ford E, Haigis M, Liszt G, Guarente L. Calorie restriction extends yeast life span by lowering the level of NADH. Genes Dev 2004;18:12-16.
Lisman, J. E., J. T. Coyle, R. W. Green, D. C. Javitt, F. M. Benes, S. Heckers and A. A. Grace (2008). "Circuit-based framework for understanding neurotransmitter and risk gene interactions in schizophrenia." Trends Neurosci 31(5): 234-242.
Liu L, Arun A, Ellis L, Peritore C, Donmez G. Sirtuin 2 (SIRT2) enhances 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP)-induced nigrostriatal damage via deacetylating forkhead box o3a (Foxo3a) and activating Bim protein. J Biol Chem 2012;287:32307-11.
Liu, J. S. (2011). "Molecular genetics of neuronal migration disorders." Curr Neurol Neurosci Rep 11(2): 171-178.
Lodato, S., C. Rouaux, K. B. Quast, C. Jantrachotechatchawan, M. Studer, T. K. Hensch and P. Arlotta (2011). "Excitatory projection neuron subtypes control the distribution of local inhibitory interneurons in the cerebral cortex." Neuron 69(4): 763-779.
Lombard DB, Alt FW, Cheng HL, et al. Mammalian sir2 homolog SIRT3 regulates global mitochondrial lysine acetylation. Mol Cell Biol 2007;27:8807-14.
Luhmann, H. J. (2016). "Models of cortical malformation--Chemical and physical." J Neurosci Methods 260: 62-72.
Luhmann, H. J., W. Kilb and A. Fukuda (2015). "Control of cortical neuronal migration by glutamate and GABA." Frontiers in Cellular Neuroscience 9.
Ma, B., G. Xu, W. Wang, J. J. Enyeart and M. Zhou (2014). "Dual patch voltage clamp study of low membrane resistance astrocytes in situ." Mol Brain 7: 18.
Maechler P. Novel regulation of insulin secretion: The role of mitochondria. Curr Opin Invest Drugs 2003;4:1166-72.
Malatesta, P., M. A. Hack, E. Hartfuss, H. Kettenmann, W. Klinkert, F. Kirchhoff and M. Gotz (2003). "Neuronal or glial progeny: regional differences in radial glia fate." Neuron 37(5): 751-764.
Manent, J. B. and A. Represa (2007). "Neurotransmitters and brain maturation: early paracrine actions of GABA and glutamate modulate neuronal migration." Neuroscientist 13(3): 268-279.
Manent, J. B., I. Jorquera, Y. Ben-Ari, L. Aniksztejn and A. Represa (2006). "Glutamate acting on AMPA but not NMDA receptors modulates the migration of hippocampal interneurons." J Neurosci 26(22): 5901-5909.
Manent, J. B., M. Demarque, I. Jorquera, C. Pellegrino, Y. Ben-Ari, L. Aniksztejn and A. Represa (2005). "A noncanonical release of GABA and glutamate modulates neuronal migration." J Neurosci 25(19): 4755-4765.
Mann, E. O. and I. Mody (2010). "Control of hippocampal gamma oscillation frequency by tonic inhibition and excitation of interneurons." Nat Neurosci 13(2): 205-212.
Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. Cell 2007;129:1261-274.
Manzini, M. C. and C. A. Walsh (2011). "What disorders of cortical development tell us about the cortex: one plus one does not always make two." Curr Opin Genet Dev 21(3): 333-339.

Maragakis, N. J. and J. D. Rothstein (2004). "Glutamate transporters: animal models to neurologic disease." Neurobiol Dis 15(3): 461-473.
Marin, O. and J. L. Rubenstein (2003). "Cell migration in the forebrain." Annu Rev Neurosci 26: 441-483.
Markram, H., M. Toledo-Rodriguez, Y. Wang, A. Gupta, G. Silberberg and C. Wu (2004). "Interneurons of the neocortical inhibitory system." Nat Rev Neurosci 5(10): 793-807.
Marshall, C. A., S. O. Suzuki and J. E. Goldman (2003). "Gliogenic and neurogenic progenitors of the subventricular zone: who are they, where did they come from, and where are they going?" Glia 43(1): 52-61.
Martina, M., S. Royer and D. Pare (2001). "Cell-type-specific GABA responses and chloride homeostasis in the cortex and amygdala." J Neurophysiol 86(6): 2887-2895.
Martinez-Lozada, Z., A. M. Guillem and M. B. Robinson (2016). "Transcriptional Regulation of Glutamate Transporters: From Extracellular Signals to Transcription Factors." Adv Pharmacol 76: 103-145.
Martinez-Lozada, Z., C. T. Waggener, K. Kim, S. Zou, P. E. Knapp, Y. Hayashi, A. Ortega and B. Fuss (2014). "Activation of sodium-dependent glutamate transporters regulates the morphological aspects of oligodendrocyte maturation via signaling through calcium/calmodulin-dependent kinase IIbeta's actin-binding/-stabilizing domain." Glia 62(9): 1543-1558.
Masliah, E., M. Alford, M. Mallory, E. Rockenstein, D. Moechars and F. Van Leuven (2000). "Abnormal glutamate transport function in mutant amyloid precursor protein transgenic mice." Exp Neurol 163(2): 381-387.
Matsugami, T. R., K. Tanemura, M. Mieda, R. Nakatomi, K. Yamada, T. Kondo, M. Ogawa, K. Obata, M. Watanabe, T. Hashikawa and K. Tanaka (2006). "Indispensability of the glutamate transporters GLAST and GLT1 to brain development." Proceedings of the National Academy of Sciences 103(32): 12161-12166.
Matute C., Alberdi E., Ibarretxe G., Sánchez-Gómez M. A. V. (2002) Excitotoxicity in glial cells. Eur. J. Pharmacol. 447, 239-246.
Mauch, D. H., K. Nagler, S. Schumacher, C. Goritz, E. C. Muller, A. Otto and F. W. Pfrieger (2001). "CNS synaptogenesis promoted by glia-derived cholesterol." Science 294(5545): 1354-1357.
McCormick, D. A. and D. A. Prince (1987). "Post-natal development of electrophysiological properties of rat cerebral cortical pyramidal neurones." J Physiol 393: 743-762.
Medina, I., P. Friedel, C. Rivera, K. T. Kahle, N. Kourdougli, P. Uvarov and C. Pellegrino (2014). "Current view on the functional regulation of the neuronal K(+)-Cl(-) cotransporter KCC2." Front Cell Neurosci 8: 27.
Meldrum, B. S. (1994). "The role of glutamate in epilepsy and other CNS disorders." Neurology 44(11 Suppl 8): S14-23.
Messersmith, E. K., M. B. Feller, H. Zhang and C. J. Shatz (1997). "Migration of neocortical neurons in the absence of functional NMDA receptors." Mol Cell Neurosci 9(5-6): 347-357.

Micheva, K. D. and C. Beaulieu (1996). "Quantitative aspects of synaptogenesis in the rat barrel field cortex with special reference to GABA circuitry." J Comp Neurol 373(3): 340-354.

Michishita E., Park J. Y., Burneskis J. M., Barrett J. C., Horikawa I. (2005) Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins. Mol. Biol. Cell. 16, 4623-4635.

Miller, H. P., A. I. Levey, J. D. Rothstein, A. V. Tzingounis and P. J. Conn (1997). "Alterations in glutamate transporter protein levels in kindling-induced epilepsy." J Neurochem 68(4): 1564-1570.

Minlebaev, M., M. Colonnese, T. Tsintsadze, A. Sirota and R. Khazipov (2011). "Early gamma oscillations synchronize developing thalamus and cortex." Science 334(6053): 226-229.

Minlebaev, M., Y. Ben-Ari and R. Khazipov (2007). "Network mechanisms of spindle-burst oscillations in the neonatal rat barrel cortex in vivo." J Neurophysiol 97(1): 692-700.

Miyazaki, T., M. Yamasaki, K. Hashimoto, K. Kohda, M. Yuzaki, K. Shimamoto, K. Tanaka, M. Kano and M. Watanabe (2017). "Glutamate transporter GLAST controls synaptic wrapping by Bergmann glia and ensures proper wiring of Purkinje cells." Proc Natl Acad Sci U S A 114(28): 7438-7443.

Miyoshi, G. and G. Fishell (2011). "GABAergic interneuron lineages selectively sort into specific cortical layers during early postnatal development." Cereb Cortex 21(4): 845-852.

Miyoshi, G., A. Young, T. Petros, T. Karayannis, M. McKenzie Chang, A. Lavado, T. Iwano, M. Nakajima, H. Taniguchi, Z. J. Huang, N. Heintz, G. Oliver, F. Matsuzaki, R. P. Machold and G. Fishell (2015). "Prox1 Regulates the Subtype-Specific Development of Caudal Ganglionic Eminence-Derived GABAergic Cortical Interneurons." J Neurosci 35(37): 12869-12889.

Miyoshi, G., J. Hjerling-Leffler, T. Karayannis, V. H. Sousa, S. J. Butt, J. Battiste, J. E. Johnson, R. P. Machold and G. Fishell (2010). "Genetic fate mapping reveals that the caudal ganglionic eminence produces a large and diverse population of superficial cortical interneurons." J Neurosci 30(5): 1582-1594.

Miyoshi, G., S. J. Butt, H. Takebayashi and G. Fishell (2007). "Physiologically distinct temporal cohorts of cortical interneurons arise from telencephalic Olig2-expressing precursors." J Neurosci 27(29): 7786-7798.

Molofsky, A. V., R. Krencik, E. M. Ullian, H. H. Tsai, B. Deneen, W. D. Richardson, B. A. Barres and D. H. Rowitch (2012). "Astrocytes and disease: a neurodevelopmental perspective." Genes Dev 26(9): 891-907.

Molyneaux, B. J., P. Arlotta, J. R. Menezes and J. D. Macklis (2007). "Neuronal subtype specification in the cerebral cortex." Nat Rev Neurosci 8(6): 427-437.

Monyer, H., N. Burnashev, D. J. Laurie, B. Sakmann and P. H. Seeburg (1994). "Developmental and regional expression in the rat brain and functional properties of four NMDA receptors." Neuron 12(3): 529-540.

Morel, L., H. Higashimori, M. Tolman and Y. Yang (2014). "VGluT1+ neuronal glutamatergic signaling regulates postnatal developmental maturation of cortical protoplasmic astroglia." J Neurosci 34(33): 10950-10962.

Morel, L., M. S. R. Chiang, H. Higashimori, T. Shoneye, L. K. Iyer, J. Yelick, A. Tai and Y. Yang (2017). "Molecular and Functional Properties of Regional Astrocytes in the Adult Brain." J Neurosci 37(36): 8706-8717.

Moroni, R. F., F. Inverardi, M. C. Regondi, P. Pennacchio and C. Frassoni (2015). "Developmental expression of Kir4.1 in astrocytes and oligodendrocytes of rat somatosensory cortex and hippocampus." Int J Dev Neurosci 47(Pt B): 198-205.

Munch, C., A. Penndorf, B. Schwalenstocker, D. Troost, A. C. Ludolph, P. Ince and T. Meyer (2001). "Impaired RNA splicing of 5'-regulatory sequences of the astroglial glutamate transporter EAAT2 in human astrocytoma." J Neurol Neurosurg Psychiatry 71(5): 675-678.

Nadarajah, B., P. Alifragis, R. O. Wong and J. G. Parnavelas (2003). "Neuronal migration in the developing cerebral cortex: observations based on real-time imaging." Cereb Cortex 13(6): 607-611.

Nagatomo, K., Y. Ueda, T. Doi, M. Takaki and N. Tsuru (2007). "Functional role of GABA transporters for kindling development in GLAST KO mice." Neurosci Res 57(2): 319-321.

Nakagawa T, Guarente L. Urea cycle regulation by mitochondrial sirtuin, SIRT5. Aging 2009;1:578-81.

Nakagawa T, Lomb DJ, Haigis MC, Guarente L. SIRT5 deacetylates carbamoyl phosphate synthetase 1 and regulates the urea cycle. Cell 2009;137:560-570.

Namiki, S., H. Norimoto, C. Kobayashi, K. Nakatani, N. Matsuki and Y. Ikegaya (2013). "Layer III neurons control synchronized waves in the immature cerebral cortex." J Neurosci 33(3): 987-1001.

Newman J. C., He W., Verdin E. (2012) Mitochondrial protein acylation and intermediary metabolism: Regulation by sirtuins and implications for metabolic disease. J. Biol. Chem. 287, 42436-42443.

Nguyen, L., J. M. Rigo, V. Rocher, S. Belachew, B. Malgrange, B. Rogister, P. Leprince and G. Moonen (2001). "Neurotransmitters as early signals for central nervous system development." Cell Tissue Res 305(2): 187-202.

Nieto, M., E. S. Monuki, H. Tang, J. Imitola, N. Haubst, S. J. Khoury, J. Cunningham, M. Gotz and C. A. Walsh (2004). "Expression of Cux-1 and Cux-2 in the subventricular zone and upper layers II-IV of the cerebral cortex." J Comp Neurol 479(2): 168-180.

Nimmerjahn, A., F. Kirchhoff, J. N. Kerr and F. Helmchen (2004). "Sulforhodamine 101 as a specific marker of astroglia in the neocortex in vivo." Nat Methods 1(1): 31-37.

Nishimura, M., J. Owens and J. W. Swann (2008). "Effects of chronic network hyperexcitability on the growth of hippocampal dendrites." Neurobiol Dis 29(2): 267-277.

Noctor, S. C., A. C. Flint, T. A. Weissman, R. S. Dammerman and A. R. Kriegstein (2001). "Neurons derived from radial glial cells establish radial units in neocortex." Nature 409(6821): 714-720.

Novelli A., Reilly J. A., Lysko P. G., Henneberry R. C. (1988) Glutamate becomes neurotoxic via the N-methyl-d-aspartate receptor when intracellular energy levels are reduced. Brain. Res. 451, 205-212.

Ochiai, H., M. Saito, T. Maruo and N. Kanemaki (2010). "Molecular cloning of canine excitatory amino acid transporter 5 and its detection in primary lens epithelial cells." Exp Anim 59(4): 449-457.

Oh, W. C., S. Lutzu, P. E. Castillo and H. B. Kwon (2016). "De novo synaptogenesis induced by GABA in the developing mouse cortex." Science 353(6303): 1037-1040.

Okaty, B. W., M. N. Miller, K. Sugino, C. M. Hempel and S. B. Nelson (2009). "Transcriptional and electrophysiological maturation of neocortical fast-spiking GABAergic interneurons." J Neurosci 29(21): 7040-7052.

Olmos Y, Valle I, Borniquel S, et al. Mutual dependence of Foxo3a and PGC-1α in the induction of oxidative stress genes. J Biol Chem 2009;284:14476-84.

Olsen, M. L., B. S. Khakh, S. N. Skatchkov, M. Zhou, C. J. Lee and N. Rouach (2015). "New Insights on Astrocyte Ion Channels: Critical for Homeostasis and Neuron-Glia Signaling." J Neurosci 35(41): 13827-13835.

Olsen, M. L., H. Higashimori, S. L. Campbell, J. J. Hablitz and H. Sontheimer (2006). "Functional expression of Kir4.1 channels in spinal cord astrocytes." Glia 53(5): 516-528.

Orellana, J. A. (2016). "Physiological Functions of Glial Cell Hemichannels." Adv Exp Med Biol 949: 93-108.

Oswald, A. M. and A. D. Reyes (2008). "Maturation of intrinsic and synaptic properties of layer 2/3 pyramidal neurons in mouse auditory cortex." J Neurophysiol 99(6): 2998-3008.

Owens, D. F. and A. R. Kriegstein (2001). "Maturation of channels and receptors: consequences for excitability." Int Rev Neurobiol 45: 43-87.

Panatier, A., D. T. Theodosis, J. P. Mothet, B. Touquet, L. Pollegioni, D. A. Poulain and S. H. Oliet (2006). "Glia-derived D-serine controls NMDA receptor activity and synaptic memory." Cell 125(4): 775-784.

Pangratz-Fuehrer, S. and S. Hestrin (2011). "Synaptogenesis of Electrical and GABAergic Synapses of Fast-Spiking Inhibitory Neurons in the Neocortex." The Journal of neuroscience : the official journal of the Society for Neuroscience 31(30): 10767-10775.

Paoletti, P., C. Bellone and Q. Zhou (2013). "NMDA receptor subunit diversity: impact on receptor properties, synaptic plasticity and disease." Nat Rev Neurosci 14(6): 383-400.

Paolicelli, R. C., G. Bolasco, F. Pagani, L. Maggi, M. Scianni, P. Panzanelli, M. Giustetto, T. A. Ferreira, E. Guiducci, L. Dumas, D. Ragozzino and C. T. Gross (2011). "Synaptic pruning by microglia is necessary for normal brain development." Science 333(6048): 1456-1458.

Patz, S., M. J. Wirth, T. Gorba, O. Klostermann and P. Wahle (2003). "Neuronal activity and neurotrophic factors regulate GAD-65/67 mRNA and protein expression in organotypic cultures of rat visual cortex." Eur J Neurosci 18(1): 1-12.

Peghini, P., J. Janzen and W. Stoffel (1997). "Glutamate transporter EAAC-1-deficient mice develop dicarboxylic aminoaciduria and behavioral abnormalities but no neurodegeneration." Embo j 16(13): 3822-3832.

Pellegrini L, Pucci B, Villanova L, et al. SIRT3 protects from hypoxia and staurosporine-mediated cell death by maintaining mitochondrial membrane potential and intracellular pH. Cell Death Differ 2012;19:1815-825.

Pellerin L., Magistretti P. J. (2004) Neuroenergetics: Calling upon astrocytes to satisfy hungry neurons. The Neuroscientist. 10, 53-62.

Pellerin, L. and P. J. Magistretti (2012). "Sweet sixteen for ANLS." J Cereb Blood Flow Metab 32(7): 1152-1166.

Perea, G., R. Gomez, S. Mederos, A. Covelo, J. J. Ballesteros, L. Schlosser, A. Hernandez-Vivanco, M. Martin-Fernandez, R. Quintana, A. Rayan, A. Diez, M. Fuenzalida, A. Agarwal, D. E. Bergles, B. Bettler, D. Manahan-Vaughan, E. D. Martin, F. Kirchhoff and A. Araque (2016). "Activity-dependent switch of GABAergic inhibition into glutamatergic excitation in astrocyte-neuron networks." Elife 5.

Perszyk, R. E., J. O. DiRaddo, K. L. Strong, C. M. Low, K. K. Ogden, A. Khatri, G. A. Vargish, K. A. Pelkey, L. Tricoire, D. C. Liotta, Y. Smith, C. J. McBain and S. F. Traynelis (2016). "GluN2D-Containing N-methyl-d-Aspartate Receptors Mediate Synaptic Transmission in Hippocampal Interneurons and Regulate Interneuron Activity." Mol Pharmacol 90(6): 689-702.

Petersen KF, Dufour S, Befroy D, Garcia R, Shulman GI. Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes. N Engl J Med 2004;350:664-71.

Petr, G. T., Y. Sun, N. M. Frederick, Y. Zhou, S. C. Dhamne, M. Q. Hameed, C. Miranda, E. A. Bedoya, K. D. Fischer, W. Armsen, J. Wang, N. C. Danbolt, A. Rotenberg, C. J. Aoki and P. A. Rosenberg (2015). "Conditional deletion of the glutamate transporter GLT-1 reveals that astrocytic GLT-1 protects against fatal epilepsy while neuronal GLT-1 contributes significantly to glutamate uptake into synaptosomes." J Neurosci 35(13): 5187-5201.

Petralia, R. S., J. A. Esteban, Y. X. Wang, J. G. Partridge, H. M. Zhao, R. J. Wenthold and R. Malinow (1999). "Selective acquisition of AMPA receptors over postnatal development suggests a molecular basis for silent synapses." Nat Neurosci 2(1): 31-36.

Pfrieger, F. W. and B. A. Barres (1997). "Synaptic efficacy enhanced by glial cells in vitro." Science 277(5332): 1684-1687.

Pla, R., V. Borrell, N. Flames and O. Marin (2006). "Layer acquisition by cortical GABAergic interneurons is independent of Reelin signaling." J Neurosci 26(26): 6924-6934.

Povysheva, N. V. and J. W. Johnson (2012). "Tonic NMDA receptor-mediated current in prefrontal cortical pyramidal cells and fast-spiking interneurons." J Neurophysiol 107(8): 2232-2243.

Qian, X., Q. Shen, S. K. Goderie, W. He, A. Capela, A. A. Davis and S. Temple (2000). "Timing of CNS cell generation: a programmed sequence of neuron and glial cell production from isolated murine cortical stem cells." Neuron 28(1): 69-80.

Qiu X, Brown K, Hirschey MD, Verdin E, Chen D. Calorie restriction reduces oxidative stress by Sirt3-mediated SOD2 activation. Cell Metabolism 2010;12:662-67.

Racine R. J. (1972) Modification of seizure activity by electrical stimulation: II. Motor seizure. Electroencephalogr. Clin. Neurophysiol. 32, 281-294.

Rajan, I. and H. T. Cline (1998). "Glutamate receptor activity is required for normal development of tectal cell dendrites in vivo." J Neurosci 18(19): 7836-7846.
Rajan, I., S. Witte and H. T. Cline (1999). "NMDA receptor activity stabilizes presynaptic retinotectal axons and postsynaptic optic tectal cell dendrites in vivo." J Neurobiol 38(3): 357-368.
Rakic, P. (1988). "Specification of cerebral cortical areas." Science 241(4862): 170-176.
Rao, V. L., M. K. Baskaya, A. Dogan, J. D. Rothstein and R. J. Dempsey (1998). "Traumatic brain injury down-regulates glial glutamate transporter (GLT-1 and GLAST) proteins in rat brain." J Neurochem 70(5): 2020-2027.
Reemst, K., S. C. Noctor, P. J. Lucassen and E. M. Hol (2016). "The Indispensable Roles of Microglia and Astrocytes during Brain Development." Front Hum Neurosci 10: 566.
Regan, M. R., Y. H. Huang, Y. S. Kim, M. I. Dykes-Hoberg, L. Jin, A. M. Watkins, D. E. Bergles and J. D. Rothstein (2007). "Variations in Promoter Activity Reveal a Differential Expression and Physiology of Glutamate Transporters by Glia in the Developing and Mature CNS." The Journal of Neuroscience 27(25): 6607-6619.
Reiprich, P., W. Kilb and H. J. Luhmann (2005). "Neonatal NMDA receptor blockade disturbs neuronal migration in rat somatosensory cortex in vivo." Cereb Cortex 15(3): 349-358.
Riebe, I., H. Seth, G. Culley, Z. Dosa, S. Radi, K. Strand, V. Frojd and E. Hanse (2016). "Tonically active NMDA receptors--a signalling mechanism critical for interneuronal excitability in the CA1 stratum radiatum." Eur J Neurosci 43(2): 169-178.
Risher, W. C., S. Patel, I. H. Kim, A. Uezu, S. Bhagat, D. K. Wilton, L. J. Pilaz, J. Singh Alvarado, O. Y. Calhan, D. L. Silver, B. Stevens, N. Calakos, S. H. Soderling and C. Eroglu (2014). "Astrocytes refine cortical connectivity at dendritic spines." Elife 3.
Rivera, C., J. Voipio, J. A. Payne, E. Ruusuvuori, H. Lahtinen, K. Lamsa, U. Pirvola, M. Saarma and K. Kaila (1999). "The K+/Cl- co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation." Nature 397(6716): 251-255.
Robinson M. B. (2002) Regulated trafficking of neurotransmitter transporters: Common notes but different melodies. J. Neurochem. 80, 1-11.
Rose E. M., Koo J. C., Antflick J. E., Ahmed S. M., Angers S., Hampson D. R. (2009) Glutamate transporter coupling to Na/K-atpase. J. Neurosci. 29, 8143-8155.
Rothstein J. D., Dykes-Hoberg M., Pardo C. A., Bristol L. A., Jin L., Kuncl R. W., Kanai Y., Hediger M. A., Wang Y., Schielke J. P. (1996) Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. Neuron. 16, 675-686.
Rothstein J. D., Martin L., Levey A. I., Dykes-Hoberg M., Jin L., Wu D., Nash N., Kuncl R. W. (1994) Localization of neuronal and glial glutamate transporters. Neuron. 13, 713-725.
Rothstein J.D. (1994a) Excitotoxic mechanisms in the pathogenesis of amyotrophic lateral sclerosis. Adv. Neurol. 68, 7-20.
Rothstein, J. D., L. Martin, A. I. Levey, M. Dykes-Hoberg, L. Jin, D. Wu, N. Nash and R. W. Kuncl (1994b). "Localization of neuronal and glial glutamate transporters." Neuron 13(3): 713-725.

Rothstein, J. D., M. Dykes-Hoberg, C. A. Pardo, L. A. Bristol, L. Jin, R. W. Kuncl, Y. Kanai, M. A. Hediger, Y. Wang, J. P. Schielke and D. F. Welty (1996). "Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate." Neuron 16(3): 675-686.

Rothstein, J. D., M. Van Kammen, A. I. Levey, L. J. Martin and R. W. Kuncl (1995). "Selective loss of glial glutamate transporter GLT-1 in amyotrophic lateral sclerosis." Ann Neurol 38(1): 73-84.

Rudy, B. and C. J. McBain (2001). "Kv3 channels: voltage-gated K+ channels designed for high-frequency repetitive firing." Trends Neurosci 24(9): 517-526.

Rudy, B., G. Fishell, S. Lee and J. Hjerling-Leffler (2011). "Three groups of interneurons account for nearly 100% of neocortical GABAergic neurons." Dev Neurobiol 71(1): 45-61.

Rumpel, S., G. Kattenstroth and K. Gottmann (2004). "Silent synapses in the immature visual cortex: layer-specific developmental regulation." J Neurophysiol 91(2): 1097-1101.

Rusnakova, V., P. Honsa, D. Dzamba, A. Stahlberg, M. Kubista and M. Anderova (2013). "Heterogeneity of astrocytes: from development to injury - single cell gene expression." PLoS One 8(8): e69734.

Sah, P., S. Hestrin and R. A. Nicoll (1989). "Tonic activation of NMDA receptors by ambient glutamate enhances excitability of neurons." Science 246(4931): 815-818.

Sanchez-Alcaniz, J. A., S. Haege, W. Mueller, R. Pla, F. Mackay, S. Schulz, G. Lopez-Bendito, R. Stumm and O. Marin (2011). "Cxcr7 controls neuronal migration by regulating chemokine responsiveness." Neuron 69(1): 77-90.

Schlicker C, Gertz M, Papatheodorou P, Kachholz B, Becker CFW, Steegborn C. Substrates and regulation mechanisms for the human mitochondrial sirtuins Sirt3 and Sirt5. J Mol Biol 2008;382:790-801.

Schmechel, D. E. and P. Rakic (1979). "A Golgi study of radial glial cells in developing monkey telencephalon: morphogenesis and transformation into astrocytes." Anat Embryol (Berl) 156(2): 115-152.

Schwer B, Verdin E. Conserved metabolic regulatory functions of sirtuins. Cell Metab 2008;7:104-12.

Scimemi, A. and J. S. Diamond (2013). "Deriving the time course of glutamate clearance with a deconvolution analysis of astrocytic transporter currents." J Vis Exp 78.

Seifert, G., K. Huttmann, D. K. Binder, C. Hartmann, A. Wyczynski, C. Neusch and C. Steinhauser (2009). "Analysis of astroglial K+ channel expression in the developing hippocampus reveals a predominant role of the Kir4.1 subunit." J Neurosci 29(23): 7474-7488.

Shatz, C. J. (1990). "Impulse activity and the patterning of connections during CNS development." Neuron 5(6): 745-756.

Shi T, Wang F, Stieren E, Tong Q. SIRT3, a mitochondrial sirtuin deacetylase, regulates mitochondrial function and thermogenesis in brown adipocytes. J Biol Chem 2005;280:13560-67.

Shi, S. H., Y. Hayashi, R. S. Petralia, S. H. Zaman, R. J. Wenthold, K. Svoboda and R. Malinow (1999). "Rapid spine delivery and redistribution of AMPA receptors after synaptic NMDA receptor activation." Science 284(5421): 1811-1816.

Shibata, T., K. Yamada, M. Watanabe, K. Ikenaka, K. Wada, K. Tanaka and Y. Inoue (1997). "Glutamate Transporter GLAST Is Expressed in the Radial Glia–Astrocyte Lineage of Developing Mouse Spinal Cord." The Journal of Neuroscience 17(23): 9212-9219.

Shih J., Donmez G. (2013) Mitochondrial sirtuins as therapeutic targets for age-related disorders. Genes. Cancer. 4, 91-96.

Shih, J., L. Liu, A. Mason, H. Higashimori and G. Donmez (2014). "Loss of SIRT4 decreases GLT-1-dependent glutamate uptake and increases sensitivity to kainic acid." J Neurochem 131(5): 573-581.

Shimazu T, Hirschey MD, Hua L, et al. SIRT3 deacetylates mitochondrial 3-hydroxy-3-methylglutaryl coa synthase 2 and regulates ketone body production. Cell Metab 2010;12:654-61.

Sibille, J., K. Dao Duc, D. Holcman and N. Rouach (2015). "The neuroglial potassium cycle during neurotransmission: role of Kir4.1 channels." PLoS Comput Biol 11(3): e1004137.

Silva, L. R., Y. Amitai and B. W. Connors (1991). "Intrinsic oscillations of neocortex generated by layer 5 pyramidal neurons." Science 251(4992): 432-435.

Skurk C, Maatz H, Kim HS, et al. The Akt-regulated forkhead transcription factor Foxo3a controls endothelial cell viability through modulation of the caspase-8 inhibitor FLIP. J Biol Chem 2004;279:1513-25.

Solomon, J. S. and J. M. Nerbonne (1993). "Hyperpolarization-activated currents in isolated superior colliculus-projecting neurons from rat visual cortex." J Physiol 462: 393-420.

Someya S, Yu W, Hallows WC, et al. Sirt3 mediates reduction of oxidative damage and prevention of age-related hearing loss under caloric restriction. Cell 2010;143:802-812.

Soria, J. M. and M. Valdeolmillos (2002). "Receptor-activated calcium signals in tangentially migrating cortical cells." Cereb Cortex 12(8): 831-839.

Soriano, E., N. Dumesnil, C. Auladell, M. Cohen-Tannoudji and C. Sotelo (1995). "Molecular heterogeneity of progenitors and radial migration in the developing cerebral cortex revealed by transgene expression." Proc Natl Acad Sci U S A 92(25): 11676-11680.

Standaert, D. G., G. B. Landwehrmeyer, J. A. Kerner, J. B. Penney, Jr. and A. B. Young (1996). "Expression of NMDAR2D glutamate receptor subunit mRNA in neurochemically identified interneurons in the rat neostriatum, neocortex and hippocampus." Brain Res Mol Brain Res 42(1): 89-102.

Steinhauser, C. and V. Gallo (1996). "News on glutamate receptors in glial cells." Trends Neurosci 19(8): 339-345.

Stevens, B., N. J. Allen, L. E. Vazquez, G. R. Howell, K. S. Christopherson, N. Nouri, K. D. Micheva, A. K. Mehalow, A. D. Huberman, B. Stafford, A. Sher, A. M. Litke, J. D. Lambris, S. J. Smith, S. W. John and B. A. Barres (2007). "The classical complement cascade mediates CNS synapse elimination." Cell 131(6): 1164-1178.

Storck, T., S. Schulte, K. Hofmann and W. Stoffel (1992). "Structure, expression, and functional analysis of a Na(+)-dependent glutamate/aspartate transporter from rat brain." Proc Natl Acad Sci U S A 89(22): 10955-10959.

Sur, M., I. Nagakura, N. Chen and H. Sugihara (2013). Chapter 9 - Mechanisms of Plasticity in the Developing and Adult Visual Cortex. Progress in Brain Research. M. M. Merzenich, M. Nahum and T. M. Van Vleet, Elsevier. 207: 243-254.

Sutherland, M. L., T. A. Delaney and J. L. Noebels (1996). "Glutamate transporter mRNA expression in proliferative zones of the developing and adult murine CNS." J Neurosci 16(7): 2191-2207.

Swanson, R. A., J. Liu, J. W. Miller, J. D. Rothstein, K. Farrell, B. A. Stein and M. C. Longuemare (1997). "Neuronal regulation of glutamate transporter subtype expression in astrocytes." J Neurosci 17(3): 932-940.

Takahashi, M., Y. Kovalchuk and D. Attwell (1995). "Pre- and postsynaptic determinants of EPSC waveform at cerebellar climbing fiber and parallel fiber to Purkinje cell synapses." J Neurosci 15(8): 5693-5702.

Takasaki, C., R. Okada, A. Mitani, M. Fukaya, M. Yamasaki, Y. Fujihara, T. Shirakawa, K. Tanaka and M. Watanabe (2008). "Glutamate transporters regulate lesion-induced plasticity in the developing somatosensory cortex." J Neurosci 28(19): 4995-5006.

Takayasu, Y., M. Iino and S. Ozawa (2004). "Roles of glutamate transporters in shaping excitatory synaptic currents in cerebellar Purkinje cells." Eur J Neurosci 19(5): 1285-1295.

Takayasu, Y., M. Iino, K. Shimamoto, K. Tanaka and S. Ozawa (2006). "Glial glutamate transporters maintain one-to-one relationship at the climbing fiber-Purkinje cell synapse by preventing glutamate spillover." J Neurosci 26(24): 6563-6572.

Takayasu, Y., M. Iino, W. Kakegawa, H. Maeno, K. Watase, K. Wada, D. Yanagihara, T. Miyazaki, O. Komine, M. Watanabe, K. Tanaka and S. Ozawa (2005). "Differential roles of glial and neuronal glutamate transporters in Purkinje cell synapses." J Neurosci 25(38): 8788-8793.

Tanaka, K., K. Watase, T. Manabe, K. Yamada, M. Watanabe, K. Takahashi, H. Iwama, T. Nishikawa, N. Ichihara and T. Kikuchi (1997). "Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1." Science 276(5319): 1699-1702.

Tao, F., S. D. Lu, L. M. Zhang, Y. L. Huang and F. Y. Sun (2001). "Role of excitatory amino acid transporter 1 in neonatal rat neuronal damage induced by hypoxia-ischemia." Neuroscience 102(3): 503-513.

Thomas, C. G., H. Tian and J. S. Diamond (2011). "The relative roles of diffusion and uptake in clearing synaptically released glutamate change during early postnatal development." J Neurosci 31(12): 4743-4754.

Tissir, F. and A. M. Goffinet (2003). "Reelin and brain development." Nat Rev Neurosci 4(6): 496-505.

Tolner, E. A., A. Sheikh, A. Y. Yukin, K. Kaila and P. O. Kanold (2012). "Subplate neurons promote spindle bursts and thalamocortical patterning in the neonatal rat somatosensory cortex." J Neurosci 32(2): 692-702.

Traynelis, S. F., L. P. Wollmuth, C. J. McBain, F. S. Menniti, K. M. Vance, K. K. Ogden, K. B. Hansen, H. Yuan, S. J. Myers and R. Dingledine (2010). "Glutamate receptor ion channels: structure, regulation, and function." Pharmacol Rev 62(3): 405-496.

Tripodi, M., J. F. Evers, A. Mauss, M. Bate and M. Landgraf (2008). "Structural homeostasis: compensatory adjustments of dendritic arbor geometry in response to variations of synaptic input." PLoS Biol 6(10): e260.

Trotti D., Rolfs A., Danbolt N. C., Brown R. H., Hediger M. A. (1999) SOD1 mutants linked to amyotrophic lateral sclerosis selectively inactivate a glial glutamate transporter. Nat. Neurosci. 2, 427-433.

Trotti, D., A. Rolfs, N. C. Danbolt, R. H. Brown, Jr. and M. A. Hediger (1999). "SOD1 mutants linked to amyotrophic lateral sclerosis selectively inactivate a glial glutamate transporter." Nat Neurosci 2(5): 427-433.

Tsuru, N., Y. Ueda and T. Doi (2002). "Amygdaloid kindling in glutamate transporter (GLAST) knockout mice." Epilepsia 43(8): 805-811.

Tuncdemir, S. N., B. Wamsley, F. J. Stam, F. Osakada, M. Goulding, E. M. Callaway, B. Rudy and G. Fishell (2016). "Early Somatostatin Interneuron Connectivity Mediates the Maturation of Deep Layer Cortical Circuits." Neuron 89(3): 521-535.

Turrigiano, G. (2012). "Homeostatic synaptic plasticity: local and global mechanisms for stabilizing neuronal function." Cold Spring Harb Perspect Biol 4(1): a005736.

Tyzio, R., A. Represa, I. Jorquera, Y. Ben-Ari, H. Gozlan and L. Aniksztejn (1999). "The establishment of GABAergic and glutamatergic synapses on CA1 pyramidal neurons is sequential and correlates with the development of the apical dendrite." J Neurosci 19(23): 10372-10382.

Tzingounis, A. V. and J. I. Wadiche (2007). "Glutamate transporters: Confining runaway excitation by shaping synaptic transmission." Nature Reviews Neuroscience 8(12): 935-947.

Ueda, Y., T. Doi, J. Tokumaru, H. Yokoyama, A. Nakajima, Y. Mitsuyama, H. Ohya-Nishiguchi, H. Kamada and L. J. Willmore (2001). "Collapse of extracellular glutamate regulation during epileptogenesis: down-regulation and functional failure of glutamate transporter function in rats with chronic seizures induced by kainic acid." J Neurochem 76(3): 892-900.

Ullensvang, K., K. P. Lehre, J. Storm-Mathisen and N. C. Danbolt (1997). "Differential developmental expression of the two rat brain glutamate transporter proteins GLAST and GLT." Eur J Neurosci 9(8): 1646-1655.

Valcanis, H. and S. S. Tan (2003). "Layer specification of transplanted interneurons in developing mouse neocortex." J Neurosci 23(12): 5113-5122.

Van Remmen H, Ikeno Y, Hamilton M, et al. Life-long reduction in MnSOD activity results in increased DNA damage and higher incidence of cancer but does not accelerate aging. Physiological Genomics 2003;16:29-37.

Verdin E, Hirschey MD, Finley LWS, Haigis MC. Sirtuin regulation of mitochondria: Energy production, apoptosis, and signaling. Trends Biochem Sci 2010;35:669-75.

Vicini, S. (2008). "The role of GABA and glutamate on adult neurogenesis." J Physiol 586(16): 3737-3738.

Vicini, S., J. F. Wang, J. H. Li, W. J. Zhu, Y. H. Wang, J. H. Luo, B. B. Wolfe and D. R. Grayson (1998). "Functional and pharmacological differences between recombinant N-methyl-D-aspartate receptors." J Neurophysiol 79(2): 555-566.

Voigt, T. (1989). "Development of glial cells in the cerebral wall of ferrets: direct tracing of their transformation from radial glia into astrocytes." J Comp Neurol 289(1): 74-88.

Volk, D. W. and D. A. Lewis (2014). "Early developmental disturbances of cortical inhibitory neurons: contribution to cognitive deficits in schizophrenia." Schizophr Bull 40(5): 952-957.

Volterra, A., N. Liaudet and I. Savtchouk (2014). "Astrocyte Ca(2)(+) signalling: an unexpected complexity." Nat Rev Neurosci 15(5): 327-335.

von Engelhardt, J., C. Bocklisch, L. Tonges, A. Herb, M. Mishina and H. Monyer (2015). "GluN2D-containing NMDA receptors-mediate synaptic currents in hippocampal interneurons and pyramidal cells in juvenile mice." Front Cell Neurosci 9: 95.

Voutsinos-Porche, B., G. Knott, K. Tanaka, C. Quairiaux, E. Welker and G. Bonvento (2003). "Glial glutamate transporters and maturation of the mouse somatosensory cortex." Cereb Cortex 13: 1110-1121.

Wadiche, J. I., S. G. Amara and M. P. Kavanaugh (1995). "Ion fluxes associated with excitatory amino acid transport." Neuron 15(3): 721-728.

Wadiche, J. I. and M. P. Kavanaugh (1998). "Macroscopic and microscopic properties of a cloned glutamate transporter/chloride channel." J Neurosci 18(19): 7650-7661.

Wadiche, J. I., J. L. Arriza, S. G. Amara and M. P. Kavanaugh (1995). "Kinetics of a human glutamate transporter." Neuron 14(5): 1019-1027.

Wallace DC. A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: A dawn for evolutionary medicine. Annu Rev Genet 2005;39:359-407.

Walsh, T., J. M. McClellan, S. E. McCarthy, A. M. Addington, S. B. Pierce, G. M. Cooper, A. S. Nord, M. Kusenda, D. Malhotra, A. Bhandari, S. M. Stray, C. F. Rippey, P. Roccanova, V. Makarov, B. Lakshmi, R. L. Findling, L. Sikich, T. Stromberg, B. Merriman, N. Gogtay, P. Butler, K. Eckstrand, L. Noory, P. Gochman, R. Long, Z. Chen, S. Davis, C. Baker, E. E. Eichler, P. S. Meltzer, S. F. Nelson, A. B. Singleton, M. K. Lee, J. L. Rapoport, M. C. King and J. Sebat (2008). "Rare structural variants disrupt multiple genes in neurodevelopmental pathways in schizophrenia." Science 320(5875): 539-543.

Wamsley, B. and G. Fishell (2017). "Genetic and activity-dependent mechanisms underlying interneuron diversity." Nat Rev Neurosci 18(5): 299-309.

Wang F., Nguyen M., Qin F., Tong Q. (2007) SIRT2 deacetylates foxo3a in response to oxidative stress and caloric restriction. Aging Cell. 6, 505-514.

Wang Q, Zhang Y, Yang C, et al. Acetylation of metabolic enzymes coordinates carbon source utilization and metabolic flux. Sci Signaling 2010;327:1004.

Wang, D. D. and A. R. Kriegstein (2008). "GABA regulates excitatory synapse formation in the neocortex via NMDA receptor activation." J Neurosci 28(21): 5547-5558.

Wang, D. D. and A. R. Kriegstein (2011). "Blocking early GABA depolarization with bumetanide results in permanent alterations in cortical circuits and sensorimotor gating deficits." Cereb Cortex 21(3): 574-587.

Wang, Y., G. Li, A. Stanco, J. E. Long, D. Crawford, G. B. Potter, S. J. Pleasure, T. Behrens and J. L. Rubenstein (2011). "CXCR4 and CXCR7 have distinct functions in regulating interneuron migration." Neuron 69(1): 61-76.

Warburg O. On the origin of cancer cells. Science 1956;123:309-314.

Watanabe, T., K. Morimoto, T. Hirao, H. Suwaki, K. Watase and K. Tanaka (1999). "Amygdala-kindled and pentylenetetrazole-induced seizures in glutamate transporter GLAST-deficient mice." Brain Res 845(1): 92-96.

Watase, K., K. Hashimoto, M. Kano, K. Yamada, M. Watanabe, Y. Inoue, S. Okuyama, T. Sakagawa, S. Ogawa, N. Kawashima, S. Hori, M. Takimoto, K. Wada and K. Tanaka (1998). "Motor discoordination and increased susceptibility to cerebellar injury in GLAST mutant mice." Eur J Neurosci 10(0953-816X (Print)): 976-988.

Weir HJM, Murray TK, Kehoe PG, et al. CNS SIRT3 expression is altered by reactive oxygen species and in alzheimer's disease. PLoS One 2012;7:e48225.

Wilson, M. D., S. Sethi, P. J. Lein and K. P. Keil (2017). "Valid statistical approaches for analyzing sholl data: Mixed effects versus simple linear models." J Neurosci Methods 279: 33-43.

Winter, N., P. Kovermann and C. Fahlke (2012). "A point mutation associated with episodic ataxia 6 increases glutamate transporter anion currents." Brain 135(Pt 11): 3416-3425.

Witte, S., H. Stier and H. T. Cline (1996). "In vivo observations of timecourse and distribution of morphological dynamics in Xenopus retinotectal axon arbors." J Neurobiol 31(2): 219-234.

Woo, T. U., J. P. Walsh and F. M. Benes (2004). "Density of glutamic acid decarboxylase 67 messenger RNA-containing neurons that express the N-methyl-D-aspartate receptor subunit NR2A in the anterior cingulate cortex in schizophrenia and bipolar disorder." Arch Gen Psychiatry 61(7): 649-657.

Wu, G. Y. and H. T. Cline (2003). "Time-lapse in vivo imaging of the morphological development of Xenopus optic tectal interneurons." J Comp Neurol 459(4): 392-406.

Wu, G. Y., D. J. Zou, I. Rajan and H. Cline (1999). "Dendritic dynamics in vivo change during neuronal maturation." J Neurosci 19(11): 4472-4483.

Wu, G., R. Malinow and H. T. Cline (1996). "Maturation of a central glutamatergic synapse." Science 274(5289): 972-976.

Xu Y, Krishnan A, Wan XS, et al. Mutations in the promoter reveal a cause for the reduced expression of the human manganese superoxide dismutase gene in cancer cells. Oncogene 1999;18:93-102.

Yamada, K., M. Watanabe, T. Shibata, K. Tanaka, K. Wada and Y. Inoue (1996). "EAAT4 is a post-synaptic glutamate transporter at Purkinje cell synapses." Neuroreport 7(12): 2013-2017.

Yamada, K., M. Watanabe, T. Shibata, M. Nagashima, K. Tanaka and Y. Inoue (1998). "Glutamate Transporter GLT-1 Is Transiently Localized on Growing Axons of the Mouse Spinal Cord before Establishing Astrocytic Expression." The Journal of Neuroscience 18(15): 5706-5713.

Yamamoto, N. and G. Lopez-Bendito (2012). "Shaping brain connections through spontaneous neural activity." Eur J Neurosci 35(10): 1595-1604.

Yamasaki, M., R. Okada, C. Takasaki, S. Toki, M. Fukaya, R. Natsume, K. Sakimura, M. Mishina, T. Shirakawa and M. Watanabe (2014). "Opposing role of NMDA receptor GluN2B and GluN2D in somatosensory development and maturation." J Neurosci 34(35): 11534-11548.

Yang H, Yang T, Baur JA, et al. Nutrient-sensitive mitochondrial NAD+ levels dictate cell survival. Cell 2007;130:1095-1107.

Yang J, Liu X, Bhalla K, et al. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science 1997;5303:1129-32.

Yang, Y., O. Gozen, A. Watkins, I. Lorenzini, A. Lepore, Y. Gao, S. Vidensky, J. Brennan, D. Poulsen, J. Won Park, N. Li Jeon, M. B. Robinson and J. D. Rothstein (2009). "Presynaptic regulation of astroglial excitatory neurotransmitter transporter GLT1." Neuron 61(6): 880-894.

Young, S. Z., M. M. Taylor and A. Bordey (2011). "Neurotransmitters couple brain activity to subventricular zone neurogenesis." Eur J Neurosci 33(6): 1123-1132.

Yozu, M., H. Tabata and K. Nakajima (2004). "Birth-date dependent alignment of GABAergic neurons occurs in a different pattern from that of non-GABAergic neurons in the developing mouse visual cortex." Neurosci Res 49(4): 395-403.

Yu W, Lin Y, Yao J, et al. Lysine 88 acetylation negatively regulates ornithine carbamoyltransferase activity in response to nutrient signals. J Biol Chem 2009;284:13669-75.

Zechel, S., Y. Nakagawa and C. F. Ibáñez (2016). "Thalamo-cortical axons regulate the radial dispersion of neocortical GABAergic interneurons." eLife 5: e20770.

Zerangue, N., J. L. Arriza, S. G. Amara and M. P. Kavanaugh (1995). "Differential modulation of human glutamate transporter subtypes by arachidonic acid." J Biol Chem 270(12): 6433-6435.

Zhang, Y. and B. A. Barres (2010). "Astrocyte heterogeneity: an underappreciated topic in neurobiology." Curr Opin Neurobiol 20(5): 588-594.

Zhang, Z., G. Chen, W. Zhou, A. Song, T. Xu, Q. Luo, W. Wang, X. S. Gu and S. Duan (2007). "Regulated ATP release from astrocytes through lysosome exocytosis." Nat Cell Biol 9(8): 945-953.

Zhao S, Xu W, Jiang W, et al. Regulation of cellular metabolism by protein lysine acetylation. Sci Signaling 2010;327:1000.

Zhou, M., G. P. Schools and H. K. Kimelberg (2006). "Development of GLAST(+) astrocytes and NG2(+) glia in rat hippocampus CA1: mature astrocytes are electrophysiologically passive." J Neurophysiol 95(1): 134-143.

Zhu, J. J., J. A. Esteban, Y. Hayashi and R. Malinow (2000). "Postnatal synaptic potentiation: delivery of GluR4-containing AMPA receptors by spontaneous activity." Nat Neurosci 3(11): 1098-1106.

Zijlmans, M., P. Jiruska, R. Zelmann, F. S. Leijten, J. G. Jefferys and J. Gotman (2012). "High-frequency oscillations as a new biomarker in epilepsy." Ann Neurol 71(2): 169-178.