Characterization of diverse excitatory currents in Melanin-Concentrating hormone neurons

By

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Abstract

Positive energy balance and sleep are coordinated by a lateral hypothalamic neuronal subpopulation known as Melanin concentrating hormone (MCH) neurons. The excitatory transmitter glutamate plays an important role in shaping the activity of MCH neurons, which is regulated by glutamate transporters present on astrocytes and neurons. Here, we aimed to investigate the role of controlling excitatory signaling via glutamatergic receptors in MCH neurons. We performed *in vitro* electrophysiological (Whole-cell patch clamp) recordings from acute brain slices to investigate the effect of ambient glutamate by using a glutamate transporter blocker. We identified and characterized three kinetically diverse excitatory currents in addition to excitatory postsynaptic currents (EPSCs): tonic inward current (TIC), step currents and slow inward currents (SICs). TIC had the longest time course and amplitude followed by the step currents and SICs. These currents are mediated by glutamatergic (non-NMDA and NMDA) receptors and dependent on the action potential-dependent exocytotic release. 12 h fasting reduced the amplitude and frequency of TIC and SICs respectively, suggesting that ambient glutamate regulation by glutamate transporter is altered under homeostatic challenge, therefore the excitability of MCH neurons. Taken together, MCH neurons are under the influence of ambient glutamate modulated by glutamate transporters. This could possibly have physiological implications in energy homeostasis.

General Summary

Glutamate, an excitatory neurotransmitter is known to elicit food intake behavior within the lateral hypothalamus. One possible mediator of this glutamate action is melanin concentrating hormone (MCH) neurons that promote food intake and decrease energy expenditure. To understand how glutamate regulates MCH neurons, we investigated the role of glutamate transporters that control the extracellular glutamate levels. We used patch-clamp electrophysiology technology to study ionic currents in the MCH neurons. We found that non-specific glutamate transporter inhibitor induced three types of excitatory currents in MCH neurons with distinct time courses. We also found that fasting mice for 12 hours also affects the excitatory current in MCH neurons. Our results suggest that glutamate transporters control the excitability of MCH neurons by regulating the ambient glutamate. Hence, this may play a vital role in the control of appetite and energy homeostasis.

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Co-Authorship Statement

I, Mohammed Sohel Chowdhury designed the experiments along with my supervisor Dr. Michiru Hirasawa. I performed all the experiments except the rat study which was performed by Sherri Bowes and the data were used for comparison with mice. A portion of SIC and step current kinetics data analysis were done by undergraduate students Bana Sakkar, Monawar Shahwan, Jacob power and graduate student Shona Campbell and validated by myself. And I concur that, I am the sole author of this manuscript.

Table of Contents

Abstract ii

General Summary iii

Acknowledgements iv

Co-Authorship Statement v

Table of Contents vi

List of Figures x

List of Abbreviations xi

1.0 Introduction 1

- 1.1 General overview 1
- 1.2 Glutamate: A major excitatory chemical messenger in the brain 1
  - 1.2.1 Glutamate synthesis 2
  - 1.2.2 Neuronal Release of glutamate 3
- 1.3 Glutamatergic receptors 4
- 1.4 Ambient glutamate 6
- 1.5 Non-neuronal sources of glutamate 7
- 1.6 Glutamate transporters: Regulating homeostasis of glutamate in the brain 7
  - 1.6.1 Astroglial and neuronal glutamate transporters 8
- 1.7 Lateral hypothalamus 11

1.8 Melanin concentrating hormone (MCH) neurons 12

1.9 Rationale and Hypotheses 14

2.0 Methods 16

2.1 Animals 16

2.2 In vitro whole cell patch clamp 16

2.2.1 Preparing brain slices 16

2.2.2 Electrophysiological recording 17

2.3 Immunohistochemistry 18

2.4 Drugs 19

2.5 Statistical analysis 19

3.0 Results 22

3.1 Discrete excitatory currents are induced by Glu transporter blockade in MCH neuron 22

3.1.1 Non-specific glutamate transport blockade induces large tonic inward current (TIC) in MCH neurons 23

3.1.2. Non-specific glutamate transport blockade reveals heterogenous kinetics of discrete excitatory currents. 23

3.2 Role of ionotropic glutamate receptors in TBOA-induced excitatory currents 33

3.3 TBOA-induced excitatory currents are mediated by postsynaptic ionotropic NMDAR 34

i) NMDAR dependent metabotropic signaling 35

ii) Involvement of postsynaptic NMDARs 36

3.4 Ambient Glutamate is dependent on action potential exocytosis 41
3.5 Acute food deprivation modulates the TBOA-induced excitatory currents in MCH neuron 43
4.0 Discussion 45
4.1 Non-specific glutamate transporter blockade reveals heterogenous excitatory currents in MCH neuron 45
4.2 Excitatory tonic inward current (TIC) 45
4.3 Cellular mechanism underlying TIC 47
4.3.1 AMPAR and KAR activation is controlled by glutamate transporters 47
4.3.2 Glutamate transporters control the activation of postsynaptic ionotropic NMDARs 48
4.4 Ambient glutamate is dependent on neuronal activity in LH 50
4.5 Slow inward currents (SICs) and step currents 50
4.6 Role of TIC and SICs in MCH neurons 54
4.7 Limitation of the study and future directions 58
4.7.1 Source of ambient glutamate 58
4.7.2 NMDAR on astrocytes? 58
4.7.3 Are heterogenous currents mediated by synaptic or extrasynaptic receptors? 59
4.7.4 Space clamp issue 59
4.7.5 Heterogenous GABAergic signaling? 59

4.8 Conclusion/Perspectives 60

5.0 References 62

APPENDIX A: Animal Ethics Approval 80

List of Figures

Figure 1 : Identification of MCH neurons 21

Figure 2 : Glu transporter blockade induces distinct excitatory currents in MCH neurons. 27

Figure 3 : Glutamate transport blockade induces large tonic inward current in MCH neurons. 28

Figure 4 :Heterogenous kinetics of excitatory currents in MCH neurons. 29

Figure 5 : Slow inward currents (SICs) can be statistically distinguished from Step currents. 30

Figure 6 : Step currents and Slow inward currents (SICs) have discrete features. 31

Figure 7 : Step and SICs arises at specific time point after TBOA application 32

Figure 8 : Glutamate transporter controls the activation of non-NMDA ionotropic glutamate receptors 37

Figure 9 : TBOA-induced excitatory currents are mediated by NMDA receptors. 38

Figure 10 : Metabotropic signaling may not be involved in mediating discrete excitatory currents. 39

Figure 11 : Postsynaptic NMDAR mediates TBOA-TIC in MCH neurons. 40

Figure 12 : TBOA-induced currents are dependent on neuronal activity. 42

Figure 13 : Food deprivation attenuates TBOA induced excitatory currents on MCH neurons in mice. 44

Figure 14 : Possible localization of glutamate receptors mediating distinct currents in MCH neuron. 57

List of Abbreviations

ACSF: Artificial Cerebrospinal Fluid

AgRP: Agouti-related Peptide

AMPAR: a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic Receptor

ANOVA: Analysis of variance

ATP: Adenosine Triphosphate

CNS: Central Nervous System

D-AP5: D-(-)-2-Amino-5-phosphonopentanoic Acid

DIC: Differential Interference Contrast

DNQX: 6,7-dinitroquinoxaline-2,3-dione

EAAC1: Excitatory Amino Acid Carrier 1

EAAT: Excitatory Amino Acid Transporter

EGTA: ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid

EPSCs: Excitatory Postsynaptic Currents

EPSP: Excitatory Postsynaptic Potential

GABA: γ-Aminobutyric Acid

GAD: Glutamic Acid Decarboxylase

GFAP: Glial Fibrillary Acidic Protein

GLAST: Glutamate Aspartate Transporter

GLT1: Glutamate Transporter 1

GPCR: G-protein-coupled Receptor

GTP: Guanosine Triphosphate

GYKI52466: 4-(8-Methyl-9H-1,3-dioxolo[4,5-h][2,3]benzodiazepin-5-yl)- benzenamine dihydrochloride

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

iGluRs:	Ionotropic Glutamate Receptors
KAR:	Kainate Receptor
LH:	Lateral Hypothalamus
LTP:	Long Term Potentiation
MCH:	Melanin-concentrating Hormone
MCHR1:	Melanin-concentrating Hormone Receptor 1
MCHR2:	Melanin-concentrating Hormone Receptor 2
mGluR:	Metabotropic Glutamate Receptor
MK-801:	(5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate
mM:	Millimolar
ms:	Millisecond
mV:	Millivolt
NMDAR:	N-Methyl-D Aspartate Receptor
NMDG:	N-Methyl-D-glucamine
NPY:	Neuropeptide Y
ORX:	Orexin
P2X7:	P2X purinoceptor 7
pA:	Picoampere
PAR:	Protease-activated Receptor
PSD:	Post-Synaptic Density
PVN:	Paraventricular Hypothalamic Nucleus
REM:	Rapid Eye Movement
RT-PCR:	Reverse Transcriptase Polymerase Chain Reaction
SEM:	Standard Error of the Mean

SIC: Slow Inward Current

SLC1: Solute Carrier 1

SN1: System N Transporter 1

SON: Supraoptic Nucleus

TFB-TBOA: (3S)-3-[[3-[[4-(Trifluoromethyl)benzoyl]amino]phenyl]methoxy]-L-aspartic acid

TIC: Tonic Inward Current

TTX: Tetrodotoxin

UBP310: S-1-(2-Amino-2-carboxyethyl)-3-(2-carboxy-thiophene-3-yl-methyl)-5-methylpyrimidine-2,4-dione

VGLUT: Vesicular Glutamate Transporter

μm: Micrometer

μM: Micromolar

1.0 Introduction

1.1 General overview

Glutamate, a primary excitatory neurotransmitter in the central nervous system, plays various roles in maintaining optimal physiological function. Glutamate transporters act as a gatekeeper that regulates the level of extracellular glutamate and prevents overactivation of neurons. Therefore, glutamate transporters are critical for physiological functions and dysfunctional glutamate transporters may result in abnormal glutamatergic transmission and result in neurotoxicity. In this thesis, we will investigate the role of glutamate transporters in heterogenous distinct glutamatergic signaling in melanin concentrating hormone (MCH) neurons, a neuronal subtype in the lateral hypothalamus implicated in sleep and energy homeostasis. Further, we will also investigate the effect of acute homeostatic disruption on the glutamatergic signaling in the MCH neuron. Understandings of different glutamatergic signaling in MCH neuron may provide us fundamental insights into possible implications of ambient glutamate in homeostatic regulation.

1.2 Glutamate: A major excitatory chemical messenger in the brain

Glutamate, an anion of amino acid (glutamic acid) is a vital chemical necessary for communication between neurons. Since the 1980s, glutamate has been widely known to be the principal excitatory neurotransmitter in the nervous system of vertebrates (Fonnum, 1984). Glutamate acts as an immediate point-to-point information transmitter between neurons. Glutamatergic synapses between two neurons can

undergo synaptic plasticity such as long term potentiation (LTP), which is the foundation for adaptation, learning and memory in the brain (McEntee & Crook, 1993). Importantly, glutamate is involved in neuronal development (differentiation, migration and survival) in the brain mainly through facilitation of Ca^{2+} entry into the cell (Hack & Balázs, 1994; Llorente-Folch et al., 2016; Yano et al., 1998).

Glutamate is also released from non-excitable cells such as astrocytes, which can modulate excitatory neurotransmission (Kristian Enkvist & McCarthy, 1994). Also, presence of glutamate receptors in peripheral tissues outside of the CNS, e.g., pancreatic β-cells (Weaver et al., 1996), osteoblasts and osteoclasts (Patton et al., 1998) and others (Gill & Pulido, 2001) is known. Glutamate also acts as a key metabolic intermediary in several metabolic pathways (Nedergaard et al., 2002). Therefore, glutamate plays a pivotal role not only in the functioning of the central nervous system (CNS) but also at peripheral sites.

1.2.1 Glutamate synthesis

Glutamate is found at higher concentrations in the plasma than cerebrospinal fluid. Under normal physiological conditions, glutamate is impermeable to the blood brain barrier, thus CNS is the main source of glutamate (Hawkins, 2009). Glutamine is a primary precursor in the synthesis of glutamate. De novo synthesis of glutamine from glutamate takes place in the astroglial cells (Nedergaard et al., 2002). Glutamate is taken up by the astrocytic excitatory amino acid transporters (EAATs) and then converted to glutamine in the presence of glutamine synthetase enzyme, followed by release of this substrate back into the extracellular space. Glutamine is then taken up by the neurons at the presynaptic terminal and converted back to glutamate by

mitochondrial enzyme glutaminase. Synthesized glutamate is packaged into synaptic vesicles for later release (Hackett & Ueda, 2015; Purves et al., 2001). Hence, this glutamate-glutamine cycle plays a significant role in supplying glutamate for synaptic transmission.

Furthermore, the glutathione cycle can also act as a physiological reservoir for glutamate if the glutamate-glutamine cycle is inhibited (Sedlak et al., 2019). Astrocytes release glutamine to the extracellular space by sodium-dependent transporter, SN1 (Mahmoud et al., 2019). These glutamine transporters are also responsible for glutamine influx into neurons.

Glutamine present in neurons is a precursor to neurotransmitters such as glutamate and GABA which are packed inside synaptic vesicles waiting to be released again during synaptic transmission (Bak et al., 2006; Bröer & Brookes, 2001; Mahmoud et al., 2019). If the concentration of extracellular glutamate is less than 0.2 mM, glutamate is metabolized via the glutamate-glutamine cycle. However, at higher concentrations, glutamate is oxidatively metabolized to a tricarboxylic acid intermediate, α-ketoglutarate, serving as a substrate for production of ATP (McKenna et al., 2016). Oxidative metabolism of glutamate can also cause astrocytes to produce ATP exceeding the ATP required for glutamate uptake (Mahmoud et al., 2019).

1.2.2 Neuronal Release of glutamate

Glutamate packaged and stored in the vesicles at the presynaptic terminal is a major source of extracellular glutamate. Generally, when an action potential propagates to the nerve terminal, it allows opening of the N- and P/Q-type voltage-dependent Ca^{2+}

channels (Birnbaumer et al., 1994). The transiently increased concentration of Ca^{2+} at the presynaptic terminal triggers synaptic vesicle fusion with the plasma membrane, i.e., exocytosis, releasing the content of the vesicles into the synaptic cleft and initiating synaptic transmission (Pang & Südhof, 2010). Based on immunoisolated synaptic vesicles study, it is assumed that vesicular glutamate concentration is approximately ~100 mmol/L (Burger et al., 1989) and release of such concentrated glutamate from a single vesicle generates an excitatory postsynaptic potential (EPSP) as glutamate binds to the postsynaptic receptors (Meldrum, 2000). Interestingly, a wide range of presynaptic modulators can regulate the vesicular release of glutamate, including cholinergic, adenosine (A1), κ-opioid, γ-aminobutyric acid ($GABA_B$), cholecystokinin and neuropeptide Y (Y2) receptors (Meldrum, 1998, 2000). Glutamate can also act on autoreceptors expressed on the presynaptic terminals to inhibit its own release (Kwon & Castillo, 2008).

Non-vesicular release mechanism such as i) the reversed operation of Na^+-dependent glutamate transporters (D. J. Rossi et al., 2000) and ii) cystine-glutamate exchangers present on both astrocytes and neurons can also contribute to the release of glutamate (Warr et al., 1999).

1.3 Glutamatergic receptors

Glutamate effects are mediated via a diverse family of glutamatergic receptors (GluRs) (Reiner & Levitz, 2018). They can be primarily categorized into ionotropic GluRs (iGluRs) and metabotropic GluRs (mGluRs).

The iGluRs are ligand-gated ion channels that allow the influx of cations (Na^+, K^+, and Ca^{2+}) when the ligand binds to the receptor mediating excitatory transmission. iGluRs are assembled as mainly heteromeric (tetrameric or pentameric) protein subunits (Dingledine et al., 1999) specific to the three subtypes (AMPA, Kainate and NMDA receptors). The structural composition of the receptor subtype dictates the type of cation entry into the cell, affecting overall receptor function (Platt, 2007).

Postsynaptic AMPA receptors (AMPARs) mediate fast synaptic transmission responsible for an early element of EPSP (Meldrum, 2000). This is due to the channel opening and closing quickly (~1 ms) (Kim et al., 2001). Kainate receptors (KARs) are also fast gated but with slower kinetics than AMPARs in mediating postsynaptic potentials (Huettner, 2003). NMDA receptors (NMDARs) are comprised of endogenous agonist glutamate and co-agonists glycine and D-serine binding sites. They respond slowly (activation time ~ 10 ms) and have higher Ca^{2+} permeability in comparison to AMPARs and KARs, inducing downstream signaling cascades (Reiner & Levitz, 2018). One prominent feature of NMDARs is its voltage sensitivity due to the channel blockade by Mg^{2+} (Cull-Candy et al., 2001; Paoletti et al., 2013). Its activation requires depolarization of postsynaptic membrane which dislodges and relieves Mg^{2+} from the pore, allowing cation entry into the cell. Unlike AMPAR and KARs, NMDARs do not show prominent ligand induced receptor desensitization (Traynelis et al., 2010). While all three iGluRs can mediate the excitatory effect of endogenous glutamate, AMPARs and KARs exhibit lower affinity to glutamate than NMDARs (Dingledine et al., 1999).

mGluRs are slower acting G-protein-coupled receptors (GPCRs) comprised of seven trans-membrane domains, an extracellular N-terminus and intracellular C-

terminus (Kunishima et al., 2000). mGluRs primarily work indirectly by activating GTP-binding proteins and different downstream intracellular second messengers (Rousseaux, 2008). mGluR are coupled to different downstream signaling cascades based on their subtypes and hence regulating different functions (Niswender & Conn, 2010). There are 7 subtypes of mGluRs that are divided into three subfamilies; Group I (mGluR1 and 5) are primarily Gq coupled excitatory receptors largely expressed on postsynaptic membranes (Doumazane et al., 2011), whereas Group II and III are Gi/o coupled inhibitory receptors located predominantly at the presynaptic sites and their activation inhibits neurotransmitter release (Endoh, 2004).

1.4 Ambient glutamate

Extracellular glutamate is not restricted to synaptic clefts but also present at the peri and extrasynaptic regions. This is known as ambient glutamate. Ambient glutamate could originate from the spillover of glutamate from the synaptic cleft following neuronal vesicular release. Ambient glutamate can be also from non-neuronal sources through different mechanisms (Pál, 2018) including astrocytic release, which can induce tonic activation of extrasynaptic NMDARs and mGluRs, therefore playing a role in regulating neural excitability (Balmer et al., 2021; Herman & Jahr, 2007; le Meur et al., 2007).

It is generally accepted that extracellular concentration of glutamate is maintained at a low level (nanomolar to micromolar range) outside the synaptic cleft to prevent continuous activation or desensitization of receptors (le Meur et al., 2007). This is due to rapid removal of glutamate by the excitatory amino acid transporters (EAATs) present on astrocytes and neurons thereby maintaining short and reliable synaptic

transmission (Balmer et al., 2021). The level of ambient glutamate has been estimated to range from 25-89 nM in the brain (hippocampal) slices (Cavelier & Attwell, 2005; Herman & Jahr, 2007; le Meur et al., 2007). In vivo studies using intracerebral microdialysis reported concentrations of more than 2 μM, however, this may be due to probe artefact (Moldavski et al., 2020; Nyitrai et al., 2006).

1.5 Non-neuronal sources of glutamate

Astrocytes can release glutamate via Ca^{2+} dependent vesicular release (Panatier & Robitaille, 2016; Skowrońska et al., 2019) and through other mechanisms, most notably via i) ionotropic purinergic receptors (P2X7), ii) hemichannels (connexin/pannexin), iii) reversal transport by glutamate transporters, iv) cystine-glutamate antiport (Harada et al., 2016). These mechanisms allow a bidirectional communication between astrocytes and neurons hence modulating neurotransmission and maintenance of glutamate homeostasis (Hubbard & Binder, 2016).

Microglia and oligodendroglia can also be another non-neuronal source of extrasynaptic glutamate (Fairhall & Caramazza, 2013; Frühbeis et al., 2013). The process of releasing neuroactive chemicals from glial cells is known as gliotransmission and the substrates released are referred as gliotransmitters.

1.6 Glutamate transporters: Regulating homeostasis of glutamate in the brain

Glutamate transporters are the primary transporter protein involved in moving glutamate across the cell membrane. Those expressed on the plasma membrane are essential in maintaining low extracellular glutamate concentrations. When an action potential triggers release of glutamate, it is quickly removed from the extracellular space

by these transporters. This results in termination of synaptic transmission, preventing accumulation of excessive extracellular glutamate and therefore maintaining homeostatic balance within the CNS (Blutstein & Haydon, 2014).

1.6.1 Astroglial and neuronal glutamate transporters

Glutamate transporters can be classified based on their cell specific expression pattern and dependency on electrochemical ion gradient. Na^+-dependent glutamate transporters (EAATs) are expressed on the plasma membrane of both astroglia and neurons (Shigeri et al., 2004). Na^+-independent, Cl^--dependent cystine-glutamate antiporters are localized on the plasma membrane of mainly astrocytes but also found in microglia and neurons (Bridges et al., 2012). Vesicular glutamate transporters (VGLUTs) are primarily found in the synaptic vesicles but also in the astrocytes (Danbolt, 2001).

In mammals, there are 5 different isoforms of EAATs (1-5) identified and are responsible for the majority of glutamate reuptake from extracellular space. These transmembrane amino acid transporters belong to the solute carrier 1 (SLC1) family. Nomenclature of these proteins in rodents referred to as GLAST (glutamate-aspartate transporter, corresponds to human EAAT1), GLT-1 (glutamate transporter 1, EAAT2) and EAAC1 (excitatory amino acid carrier 1, rabbit version of EAAT3) (Malik & Willnow, 2019; Vandenberg & Ryan, 2013). Generally, EAATs have high affinity for glutamate (responsible for ~90% of glutamate uptake) with varying degree in transport rates depending on the subtypes. The stoichiometry of these transporters involves the establishment of Na^+/K^+ gradient primarily by the Na^+/K^+ ATPase to transport glutamate against its concentration gradient. Each cycle of the transporter carries one molecule of

glutamate while bringing in 3 Na^+ ions along with 1 H^+ and extruding 2 K^+ which results in a net flow of positive charge into the cell (Levy et al., 1998; Rothstein et al., 1995; Wadiche et al., 1995). Hence, glutamate transporters depend on transmembrane Na^+, K^+ and H^+ concentration gradient.

During adulthood, EAAT1/GLAST is expressed throughout the CNS. Mature astrocytes express a large proportion of GLAST including Bergmann glia in the cerebellum (Rothstein et al., 1994), Müller glia in the retina (Rauen et al., 1996), and cochlear glial cells in the inner ear (Glowatzki et al., 2006). In addition, in adults, GLAST is expressed to a lesser extent in microglia and oligodendrocytes (Kondo et al., 1995). EAAT2/GLT-1 are the membrane bound proteins having similar structure to GLAST and are also majorly expressed in mature astrocytes (Rothstein et al., 1994). Functionally, GLAST and GLT-1 have a similar glutamate turnover rate of 16 s-1 and 14.6 s-1 respectively (Vandenberg & Ryan, 2013; Wadiche et al., 1995) , however, GLT-1 is responsible for more than ~90% of glutamate uptake predominantly in the forebrain (Tanaka et al., 1997). Both of these transporters are distributed in perisynaptic astrocytic processes in contact with glutamatergic synapses. The upregulation of GLT-1 transporters is positively correlated to glutamate release from adjacent glutamatergic neurons (Mahmoud et al., 2019; Poitry-Yamate et al., 2002).

EAAT3/EAAC1, EAAT4 and EAAT5 are localized on the dendrites and axon terminals of neurons (Rothstein et al., 1994; Shashidharan et al., 1997) . EAAT3/EAAC1 protein concentration is highest in the hippocampus, cerebellum, and basal ganglia (Holmseth et al., 2012) . EAAC1 is expressed by both glutamatergic and GABAergic neurons. Expression of EAAC1 on GABAergic terminals helps maintain GABA levels by

supplying glutamate necessary for GABA synthesis (Murphy-Royal et al., 2017; Sepkuty et al., 2002). Immunolabeling studies showed that EAAC1 is also present in dendritic shafts and spines encompassing active zones as well as in axon terminals but not in astrocytes (Holmseth et al., 2012; Scimemi et al., 2009). In addition to transporting glutamate, the buffering action of EAAC1 through glutamate binding and sequestering limits the activation of perisynaptic NMDARs and increase the LTP induction threshold (Holmseth et al., 2012; Scimemi et al., 2009). In the adult CNS, EAAT4 is expressed in the plasma membrane of Purkinje cells soma and dendrites including the spines (Dehnes et al., 1998). Double-labeled immunofluorescent staining, confocal image analysis, and reverse transcriptase-polymerase chain reaction (RT-PCR) demonstrated that EAAT4 is also expressed in astrocytes (Hu et al., 2003). Finally, EAAT5 is only found in the retina where it is primarily localized to photoreceptors and bipolar neurons (A. Lee et al., 2012).

Na^+ independent cystine-glutamate transporters mediate the exchange of anionic cystine and glutamate in both directions. Since, there is a lower concentration of cystine intracellularly and higher glutamate concentration than the extracellular space, this antiporter can uptake cystine and release glutamate at 1:1 ratio (Bridges et al., 2012). Extracellular glutamate can potentially inhibit the antiporter system; therefore, these transporters can play a role in excitatory signaling independently of vesicular glutamate release (Lewerenz et al., 2013).

In contrast to transporters that transport glutamate across the plasma membrane, vesicular glutamate transporters (VGLUTs) store glutamate into synaptic vesicles for release through exocytosis. VGLUTs are dependent on proton gradient that is created

by H^+-ATPase that transports H^+ into the synaptic vesicle. This creates a pH gradient across the vesicle membrane that drives transport of glutamate anion into synaptic vesicles (Özkan & Ueda, 1998). There are 3 major vesicular glutamate transporters (VGLUT 1-3). VGLUT1 is localized mainly in the neocortex (I-III), piriform cortex, hippocampus, amygdala and subiculumn whereas VGLUT2 is highly expressed in olfactory bulb, layer IV of cerebral cortex, thalamus, hypothalamus and in the brain stem (Li et al., 2003). In the CNS, VGLUT1 and VGLUT2 are complementarily expressed in all known glutamatergic neurons essential in glutamatergic transmission. VGLUT3 is localized in a number of glutamatergic neurons of different brain regions such as neocortex, hippocampus, olfactory bulb, hypothalamus, and substantial nigra (Schäfer et al., 2002; Shigeri et al., 2004).

1.7 Lateral hypothalamus

Lateral hypothalamus (LH) is a neuroanatomical structure comprised of heterogeneous cell population responsible for regulating feeding behavior, sleep/wake cycle, and energy balance (Arrigoni et al., 2019). Electrical stimulation of the LH showed increased food intake behavior, whereas its ablation reduced food consumption (J. Lee et al., 2021). In addition, LH is also involved in coordination of certain motivated behaviors like copulation, drinking, nest building etc. (J. Lee et al., 2021; Stuber & Wise, 2016).

LH receives a diverse array of afferent connections from midbrain, brainstem cortical and subcortical regions. Excitatory fibers from the hippocampus innervates LH via fornix (Berthoud & Münzberg, 2011; Stuber & Wise, 2016). Neurons projecting to the

LH from other intrahypothalamic structures, notably arcuate nucleus and periventricular hypothalamus, are reported to be involved in feeding behavior (Betley et al., 2013; Wu et al., 2015). VGlutT2 containing glutamatergic neurons are also abundantly found in the LH. In addition to innervating locally, LH also sends glutamatergic output to ventral tegmental area, parabrachial nucleus, intrahypothalamic arcuate and paraventricular nucleus (PVN) and other regions (Godfrey & Borgland, 2019; Stuber & Wise, 2016). To conclude, LH contains extensively interconnected circuitry acting as an integration unit for afferent and efferent information, which play a role in regulating homeostatic, appetitive and motivated behavior functions.

1.8 Melanin concentrating hormone (MCH) neurons

LH predominantly expresses two intermingled populations of neurons that synthesize distinct neuropeptides (Orexin and MCH). These neurons control energy balance, sleep, and motivated behavior (Arrigoni et al., 2019). Orexin neuropeptide producing neurons are known to promote wakefulness and energy expenditure, whereas MCH neurons promote REM sleep and food intake, and decrease energy expenditure (González et al., 2016). For the theme of this thesis, we will primarily focus on MCH neurons.

MCH, a cyclic peptide was initially isolated from the pituitary gland of teleost fish necessary for skin pigmentation, and later identified in the mammalian brain (Bittencourt, 2011). MCH expression is more abundant in the LH and to a lesser extent rostromedially in the Zona incerta (Bittencourt et al., 1992). This peptide binds to its G protein coupled receptors MCHR1 and MCHR2 (the latter is not present or inactive in

rodents) (Hill et al., 2001). MCHR1 activation can be either excitatory through intracellular Ca^{2+} increase, or inhibitory by activating G-protein inward rectifier channel and subsequently hyperpolarizing the target neurons (J. Lee et al., 2021; Saito et al., 1999). MCH neurons are reported to co-express VGluTs and GABA synthesizing enzyme GAD 65/67 and can release both excitatory glutamate and inhibitory GABA as co-neurotransmitters (Chee et al., 2015; Harthoorn et al., 2005).

MCH neurons play a significant role in maintaining energy homeostasis. Ablation of MCH neurons resulted in hypophagia, hyperactivity and leanness, whereas intracerebroventricular injection of MCH or its overexpression stimulated food intake, increased body weight, and resulted in insulin resistance (Arrigoni et al., 2019; J. Lee et al., 2021; Qu et al., 1996; M. Rossi et al., 1997). Furthermore, increased MCH mRNA levels were also observed following fasting (Qu et al., 1996). MCH activation also promotes REM sleep duration and resting, thereby conserving energy consumption (Konadhode et al., 2013; Yamashita & Yamanaka, 2017). MCH neuron activity during REM sleep is also implicated in forgetting hippocampus dependent memories (Izawa et al., 2019). These reports indicate that MCH neurons play physiological roles in homeostatic and cognitive functions. Hence, their activities are required to be optimally regulated to maintain these functions.

MCH neuron receive several inputs from different intrahypothalamic structures relevant to energy homeostasis. In the energy balance circuitry, the first order Agouti-related peptide/ neuropeptide Y (AgRP/NPY) neurons in the arcuate nucleus are the primary sensor of peripheral energy balance. These neurons project to MCH neurons and coordinate food intake (Bäckberg et al., 2004; Bittencourt, 2011). Neuromodulators

like oxytocin and vasopressin from the PVN excite MCH neuron through either direct or indirect mechanism (Yao et al., 2012). Within the LH, MCH neurons receive glutamatergic input from orexin neuron (van den Pol et al., 2004). MCH neurons also have a vast projection to the CNS evident from their receptor profile. Particularly, their projection to PVN and parabrachial nucleus may be involved in food intake (J. Lee et al., 2021). They also send glutamatergic terminals innervating lateral septum (Chee et al., 2015). Moreover, MCH neurons also receive inputs and send their fibres densely into the brainstem suggesting involvement in the feeding and autonomic response (Bittencourt, 2011). To conclude, the intricate circuitry that feeds into and out of MCH neuron are vital in maintaining autonomic and homeostatic function. Thus, understanding underlying glutamatergic signaling mechanism in MCH neuron and their implication in homeostatic dysregulation is necessary.

1.9 Rationale and Hypotheses

MCH neurons are important in energy expenditure, appetite, and sleep, and likely mediate the known role of glutamate in the LH in regulating feeding and body weight (Khan et al., 1999). Thus, it is imperative to understand fundamental mechanisms of glutamatergic transmission in MCH neurons and how they change under different physiological conditions. Previously, our lab showed that excitatory synapses to MCH neurons display plasticity during different dietary conditions (Linehan et al., 2020; Linehan & Hirasawa, 2022). Moreover, sleep deprivation resulted in plasticity of excitatory transmission via increased GLT-1 apposition to MCH neuron (Briggs et al., 2018). These results suggest that glutamate transporters have a functional role in regulating excitatory transmission to MCH neurons in normal state and under

homeostatic challenges. Consequently, this influences the equilibrium of ambient glutamate in the extracellular space and spatial and temporal kinetics of glutamatergic transmission. Modulation of ambient glutamate can likely shape the excitability of MCH neurons.

Hence, this thesis work aimed to address the following hypotheses:

1) Glutamate transporters regulate excitatory currents induced by ambient glutamate, which are mediated by glutamate receptors in MCH neurons.
2) Acute disruption in energy homeostasis alters how glutamate transporters regulate ambient glutamate.

To test the First hypothesis, we took advantage of non-specific glutamate transporter blocker as a tool to reveal the effect of ambient glutamate regulated by glutamate transporters. This uncovered multiple types of excitatory currents in MCH neurons. Therefore, these currents were characterized in detail in this thesis. Next, we addressed the second hypothesis by depriving mice for 12 h which differentially affected these currents. This thesis work demonstrates that glutamate transporters are essential for tuning the excitability of MCH neuron by regulating the ambient glutamate levels. This may indicate a novel intercellular signaling mechanism that regulates MCH neurons associated with metabolic control.

2.0 Methods

All procedures involving animals were conducted following the guidelines by Canadian Council of Animal Care and were approved by the Memorial University Institutional Animal Care Committee.

2.1 Animals

Experiments were performed using mice of both sexes aged 4-13 weeks. Wild-type C57BL/6 (strain code: 027, Charles River Laboratories, MA, USA), MCH-cre transgenic mice and MCH-tdTomato mice were used. MCH-tdtomato mice were bred at Memorial University, generated by crossing MCH-cre mouse (originally generated by Dr. Brad Lowell, Beth Israel Deaconess Medical Center, USA and provided by Dr. Melissa Chee, Carleton University, Canada) with tdTomato reporter mouse (stock number 007909, Jackson Laboratory). These transgenic mice were useful in visualizing MCH neuron in the hypothalamic region and Zona incerta. All animals were kept under 12h light/12h dark cycle and fed with standard chow diet (Prolab RMH 3000) *ad libitum* at the animal care facility in the Health Sciences Centre.

2.2 In vitro whole cell patch clamp

2.2.1 Preparing brain slices

Mice were deeply anesthetized using 4% isoflurane and decapitated; their brains were carefully removed. Coronal slices of 250 µm thickness containing the hypothalamus were cut in cold artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 18 $NaHCO_3$, 2.5 glucose, 2 $CaCl_2$ using a

vibratome (Leica Microsystems, VT 1000S and Campden instruments, 7000 smz2). Following sectioning, brain slices were first incubated in a NMDG based recovery solution containing (in mM): 92 NMDG, 2.5 KCl, 1.25 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 2 thiourea, 5 ascorbic acid, 3 sodium pyruvate, 0.5 $CaCl_2 \cdot 2H_2O$, and 10 $MgSO_4 \cdot 7H_2O$ for 15 mins at 32°C and then transferred to ACSF for another 15 mins at 32°C. Recovery solution was added to the procedure to obtain healthier brain slices for patch-clamp recordings (Ting et al., 2018). Slices were then left at room temperature until recording. Each solution was continuously bubbled with carbogen (95% O_2 & 5% CO_2). The pH of all solution was adjusted to 7.29-7.31 and osmolarity to 290- 300 mOsm.

2.2.2 Electrophysiological recording

The slices were visualized using a differential interference contrast microscope (DIC) (DM LFSA, Leica Microsystems). The recordings were performed on hemisected slices continuously perfused with ACSF at 27-29°C at a flow rate of 1.8-2.1 ml/min. Cluster of neurons were located in the lateral hypothalamic area and Zona incerta. In wild-type cells with apparent large diameter of 10-20 µm were selected and in MCH-tdTomato mice, MCH neurons were identified by red fluorescence for whole cell patch clamp recording. A standard internal solution (containing in mM: 123 K-gluconate, 2 $MgCl_2$, 8 KCl, 0.2 EGTA, 10 HEPES, 5 Na_2-ATP, 0.3 Na-GTP, and 2.7 biocytin) was filled into glass microelectrodes (tip resistance: 2.5-5 MΩ) to record and label the patched cells unless otherwise noted. Neurons with whole cell access resistance with 5-20 MΩ only were included for analysis. Recordings were obtained using Multiclamp

700B amplifier and Clampex software version 9, 10 and 11 (Molecular Devices, Sunnyvale, CA, USA) were used for recording visualizations and analysis.

After whole cell access was obtained, MCH neurons were identified by their unique electrophysiological properties previously established in our lab (Linehan & Hirasawa, 2018). Specifically, under current clamp mode, a series of 600 ms hyperpolarizing and depolarizing currents (ranges from -200pA to +200pA) were applied (Figure 1A). Typically, their electrophysiological phenotype included a lack of spontaneous firing at rest and H-current during hyperpolarized current. However, they display action potentials with spike adaptations during positive current injection.

For all other recordings, membrane currents were measured under voltage clamp mode while holding the membrane potential at -70 mV. Membrane currents were filtered at 1kHz and digitized at 10kHz.

2.3 Immunohistochemistry

We also confirmed the neurochemical phenotype for both wild-type and transgenic mice. Following experiments, slices were fixed in 10% formalin solution for a minimum of 24 h at 4°C. Then, slices were incubated in rabbit anti-MCH IgG (1:1000; H-070-47, Phoenix Pharmaceuticals, CA, USA) for 3 days at 4°C. Then, slices were incubated with Alexa-488-conjugated anti-rabbit antibody and Alexa-350 conjugated streptavidin overnight at 4°C. Finally, with an epifluorescence microscope MCH neuropeptide colocalization with biocytin was confirmed (Figure 1B).

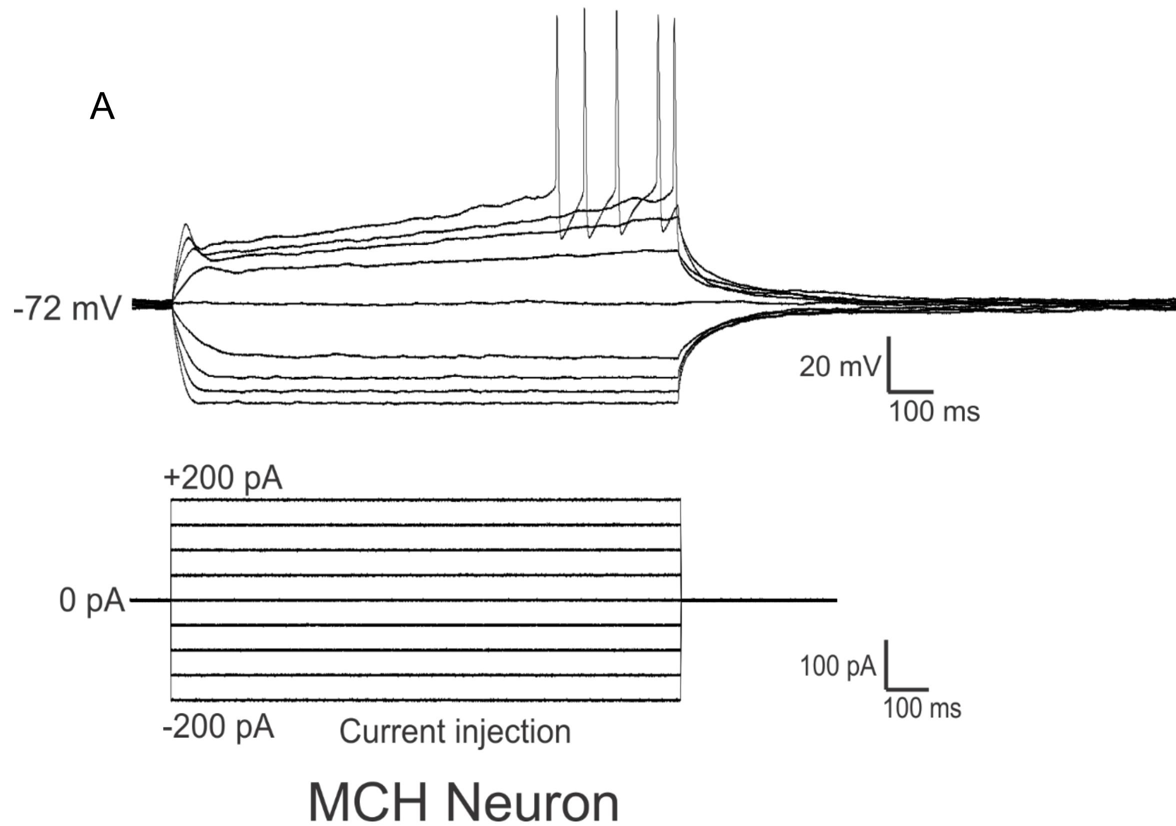
2.4 Drugs

Different drugs were prepared as a stock solution and stored at -20°C in aliquots, which was diluted in ACSF to their final concentration immediately before perfusing into the recording chamber. Picrotoxin (50 μM, Sigma-Aldrich, Oakville, ON, Canada) was always present in the ACSF during recordings to block $GABA_A$ receptor currents and record only excitatory currents. NMDAR antagonists (DAP-5, MK-801), KAR antagonist (UBP310), AMPAR antagonist (GYKI52466) were purchased from Abcam (Toronto, ON, Canada). AMPAR and KAR antagonist (DNQX), glutamate transporter blocker (TFB-TBOA) were purchased from Tocris Bioscience (Minneapolis, MN, USA). Na^+ channel blocker, Tetrodotoxin (TTX) was purchased from Alomone labs (Jerusalem, Israel). Intracellular MK-801 solution was prepared from 20 mM stock solution and added to the internal solution to make a final 1 mM concentration (pH ~ 7.31, Osmolarity ~ 292 mOsm).

2.5 Statistical analysis

Results are reported as the mean ± SEM. n and N values represent the number of cells and number of animals used, respectively. The amplitude of tonic inward current (TIC) was measured as the difference between the baseline and the peak. To measure amplitude and kinetics of step current and slow inward current (SIC), experimental time and current (pA) values were gathered at the start, peak and end of individual events by using Clampfit 10 (Molecular Devices, Sunnyvale, CA, USA). To compare the effects of different drug treatments between groups, one-way ANOVA or two-way ANOVA was performed; post hoc analysis was done using Dunnett's or sidak's multiple comparison test as appropriate. Two tailed Students t-test (paired and unpaired) were performed

where necessary. Statistical significance was determined as $p<0.05$. All statistical analyses were made using Prism 8 and 9 (GraphPad Software, La Jolla, CA).



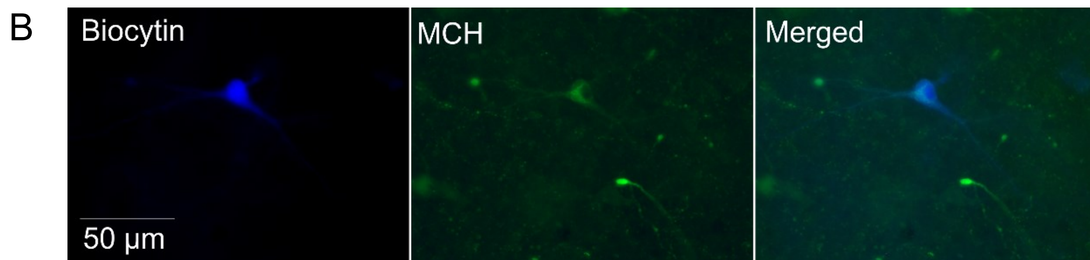


Figure 1 : Identification of MCH neurons

A) Example of electrophysiological profile of a MCH neuron during a series of both positive (depolarizing) and negative (hyperpolarizing) current injection of -200 to 200 pA with the increment of 50 pA.
B) Cells were labelled with biocytin during whole cell recording via patch pipette. Brain slices were later stained for biocytin (Blue) and proMCH (Green). Merged image shows co-labeling of the markers confirming MCH neurochemical phenotype.

3.0 Results

3.1 Discrete excitatory currents are induced by Glu transporter blockade in MCH neurons

To determine the role of glutamate transporters in the excitability of MCH neurons, the non-specific glutamate transporter blocker TFB-TBOA was tested (referred as TBOA onward). We have identified four different types of excitatory currents in MCH neuron based on their unique kinetics when Glu transporters are blocked by 5 μM TBOA (Figure 2- See inset Figures). Firstly, an unambiguous robust tonic inward current (TIC) seen as a downward shift in holding current was observed upon application of TBOA, which has the largest amplitude and longest duration lasting for 10-15 minutes. Secondly, this TIC was overlapped with several step currents (comprises step-wise feature) with rapid downward shifts, each lasting for tens of seconds following the application of TBOA. Thirdly, slow inward currents (SICs) that last for a few seconds were also seen. These currents were more prominent when TBOA was washing away and TIC was decaying. Finally, these excitatory currents can be explicitly separated from fast excitatory currents lasting less than 10 ms, which is consistent with the time course of excitatory postsynaptic currents (EPSCs).

To further understand the kinetics of these distinct excitatory events, we have separately characterized these currents in the following subsection.

3.1.1 Non-specific glutamate transport blockade induces large tonic inward current (TIC) in MCH neurons

A 5-minute application of TBOA to mice MCH neurons resulted in a robust TIC that appeared several minutes into the application period, peaked after the drug washout and slowly decayed back to the baseline (Peak mean amplitude of TBOA = -495.1 ±50.8 pA, paired t test, $t(25) = 9.740$; $p<0.0001$, baseline vs. TBOA; n/N=26/19; Figure 3, B&C). This TBOA-induced tonic inward current (TBOA-TIC) was concentration dependent, as the TIC amplitude was significantly larger at 5 μM compared to 1 μM (unpaired t test with Welch's correction, $t(25.4) = 9.030$; $p<0.0001$; 1 μM, n/N = 3/2 vs. 5 μM, n/N = 26/19; Figure 3D). TBOA-TIC was also observed in previous lab study in rat MCH neurons which was similar in magnitude as those in mice (unpaired t test $t(36) = 0.4367$; $p = 0.6649$; rats n/N = 12/8 vs. mice n/N = 26/19; Figure 3E). Similarly, step currents, SICs and EPSCs were also observed upon visual inspection in rats. As no significant difference was seen in the TBOA-TIC between different rodent models, the remaining experiments and analyses were performed using mice.

3.1.2. Non-specific glutamate transport blockade reveals heterogenous kinetics of discrete excitatory currents.

Next, we characterized transient excitatory currents observed in MCH neurons. Obvious EPSCs with fast rise and decay as previously reported were excluded from this analysis (Linehan & Hirasawa, 2022). Initially, different criteria (amplitude, rise time, decay time & total duration) of 307 excitatory events (n/N = 7/6) were analyzed, which showed multimodal frequency distribution with multiple peaks (Figure 4A1-A4). This indicates that heterogeneous transient excitatory currents exist in MCH neurons that

can be differentiated into subgroups (i.e., SICs and step currents). Hence, to further characterize their heterogeneity, step currents and SICs were identified by eye and relative frequency distribution histograms were separately plotted for each of the criterion above. This revealed that SICs have overall smaller amplitude (Figure 4B1) and faster kinetics (Figure 4B2-B4), whereas step currents had a higher proportion of events with larger amplitude (Figure 4C1) and considerably slower kinetics (Figure 4C2-C4).

Therefore, a direct comparison of these different parameters between step currents and SICs were performed (Figure 5A-D, Table 1). The mean amplitude of step current was significantly different from SICs' amplitude (unpaired t test, $t(17.02) = 4.870$, $p = 0.0001$), although some overlap in amplitudes was seen between the two types of currents. Similarly, rise time of step current was different from that of SIC with some overlap (unpaired t test, $t(17.06) = 6.358$, $p < 0.0001$). Decay time was more distinguishable with little overlap between the two populations of excitatory currents (unpaired t test, $t(17.01) = 5.945$, $p < 0.0001$). Notably, no overlap was seen in the total duration between these two currents unpaired t test, $t(17.01) = 6.639$, $p < 0.0001$). This clearly indicates that, despite overlap of some parameters (See Table 1), step currents and SICs are generally distinguishable and categorized based on their amplitude and kinetics.

Table 1: Summary of amplitude and kinetics of step currents and SICs in MCH neurons. Mean ± SEM and range of each measure are shown.

	Amplitude (pA)	Rise time (s)	Decay time (s)	Total duration (s)
Step				
(Mean)	120.70 ± 20.24	7.625±1.101	47.774± 7.687	55.399 ±7.939
(Range)	16.9 to 391.2	2.62 to 19.23	10.14 to 117.16	19.19 to 25.55
SIC				
(Mean)	22.08 ± 0.45	0.616±0.047	2.066±0.095	2.682±0.119
(Range)	4.93 to 53.1	0.02 to 6.67	0.08 to 10.3	0.10 to 13.7

To determine whether a combination of parameters can be used to reliably distinguish SICs and step currents, the relationship between amplitude and rise time (Figure 6A1-A2), decay time (Figure 6B1-B2) and total duration (Figure 6C1-C2) were examined. The resultant scatter plot diagrams for step currents illustrate a significant positive linear correlation between the amplitude and decay or total duration, whereas SICs showed no correlation between amplitude and all the aforementioned criteria (Figure 6A2-C2). Importantly, the x-y scatter plot between the amplitude and decay time or total duration showed a clear distinction between step currents and SICs (Figure 6B1, C1). Thus, these parameter combinations can be used to define the two types of excitatory currents.

Furthermore, occurrences of step and SICs were time-dependent relative to TBOA application. The frequency of step currents were greatest after 5 to 10 minutes of post-TBOA application, and significantly different from the rest of the experimental time

points (One-way ANOVA, $F(4, 30) = 31.56$, $p<0.0001$; Figure 7A). Similarly, the frequency of SICs increased following TBOA application (One-way ANOVA, $F(4, 30) = 6.085$, $p = 0.0010$; Figure 7B). Note that a trend of frequent SICs existed even after 15 minutes of washout of TBOA.

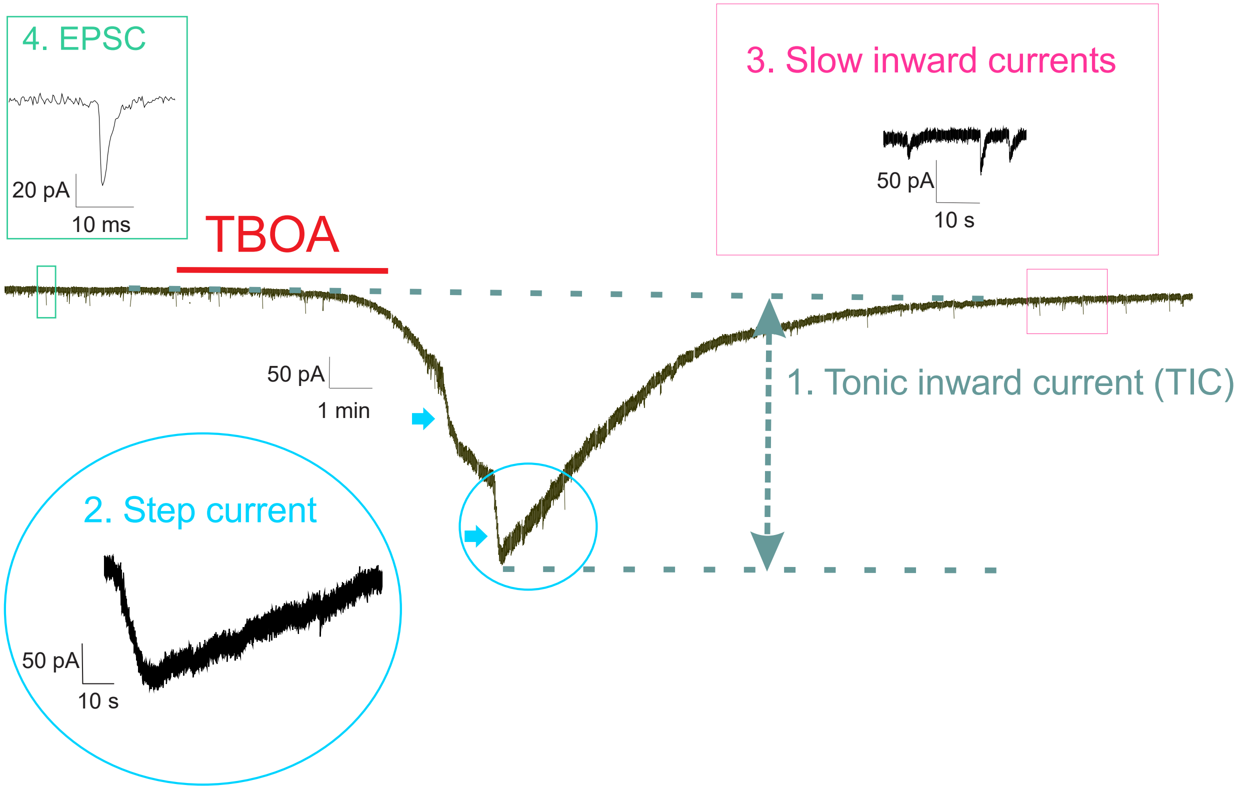


Figure 2 : Glu transporter blockade induces distinct excitatory currents in MCH neurons.

Representative voltage clamp trace recorded from a MCH neuron, displaying 4 types of excitatory currents with distinct time courses in response to the non-specific Glu transporter blocker TFB-TBOA (5 µM). From long to short currents: 1) tonic inward current (TIC), 2) step current, 3) slow inward current (SIC) and 4) EPSC. Inset figures show expanded view of each of the discrete events.

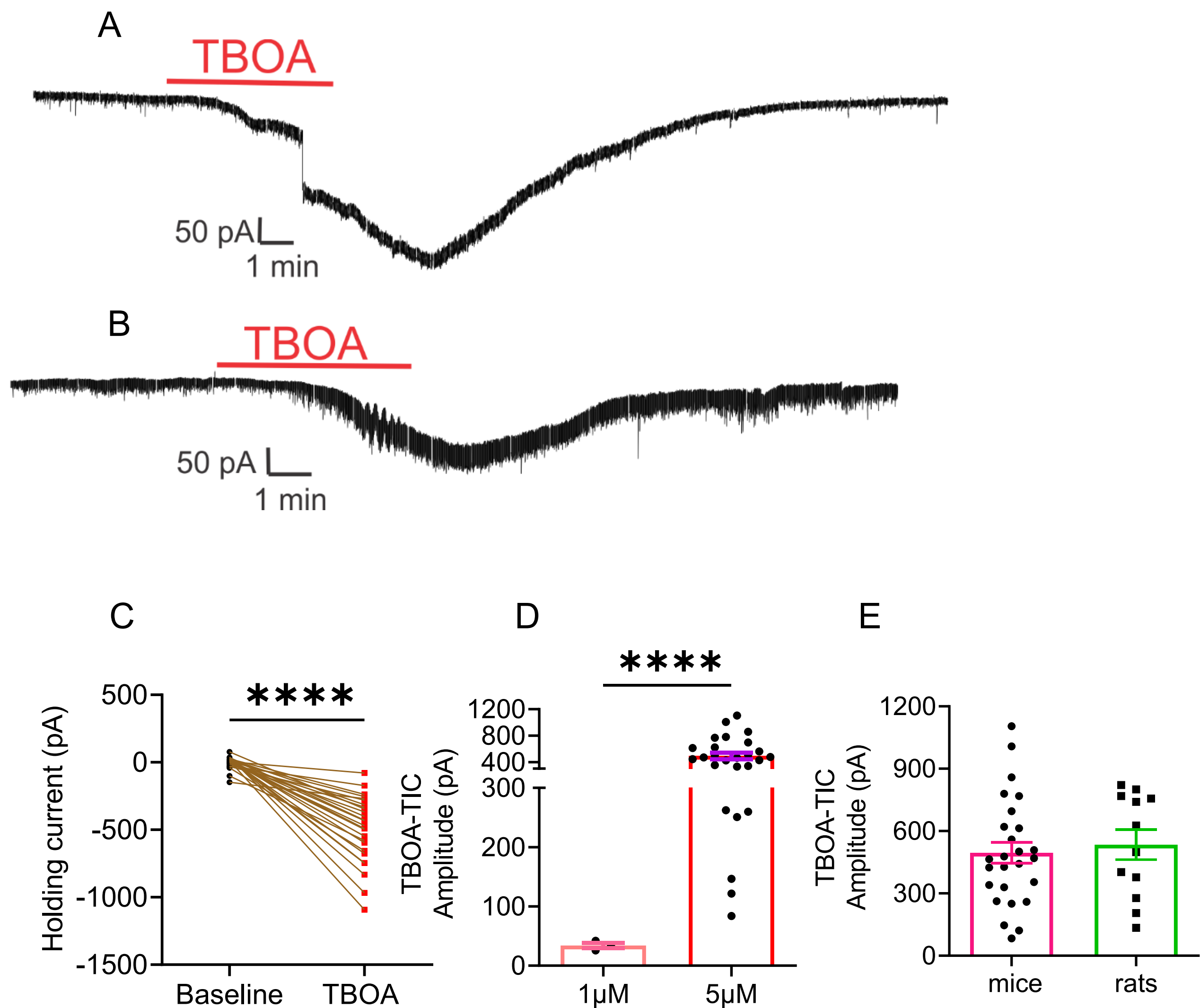


Figure 3 : Glutamate transport blockade induces large tonic inward current in MCH neurons.

Representative voltage clamp trace recorded from a mouse MCH neuron (A) and Orexin neuron (B) illustrating that the non-specific Glu transporter blocker TBOA (5 μM) induces a large reversible tonic inward current (TBOA-TIC). C) Summary plot depicting the peak of TBOA effect on the holding current compared to baseline. D) TBOA effect is concentration dependent. E) TBOA (5 μM) induces TIC in different rodent models.
Paired t-test in Figure 3C and unpaired t-test in Figure 3D&E as indicated by ****p<0.0001

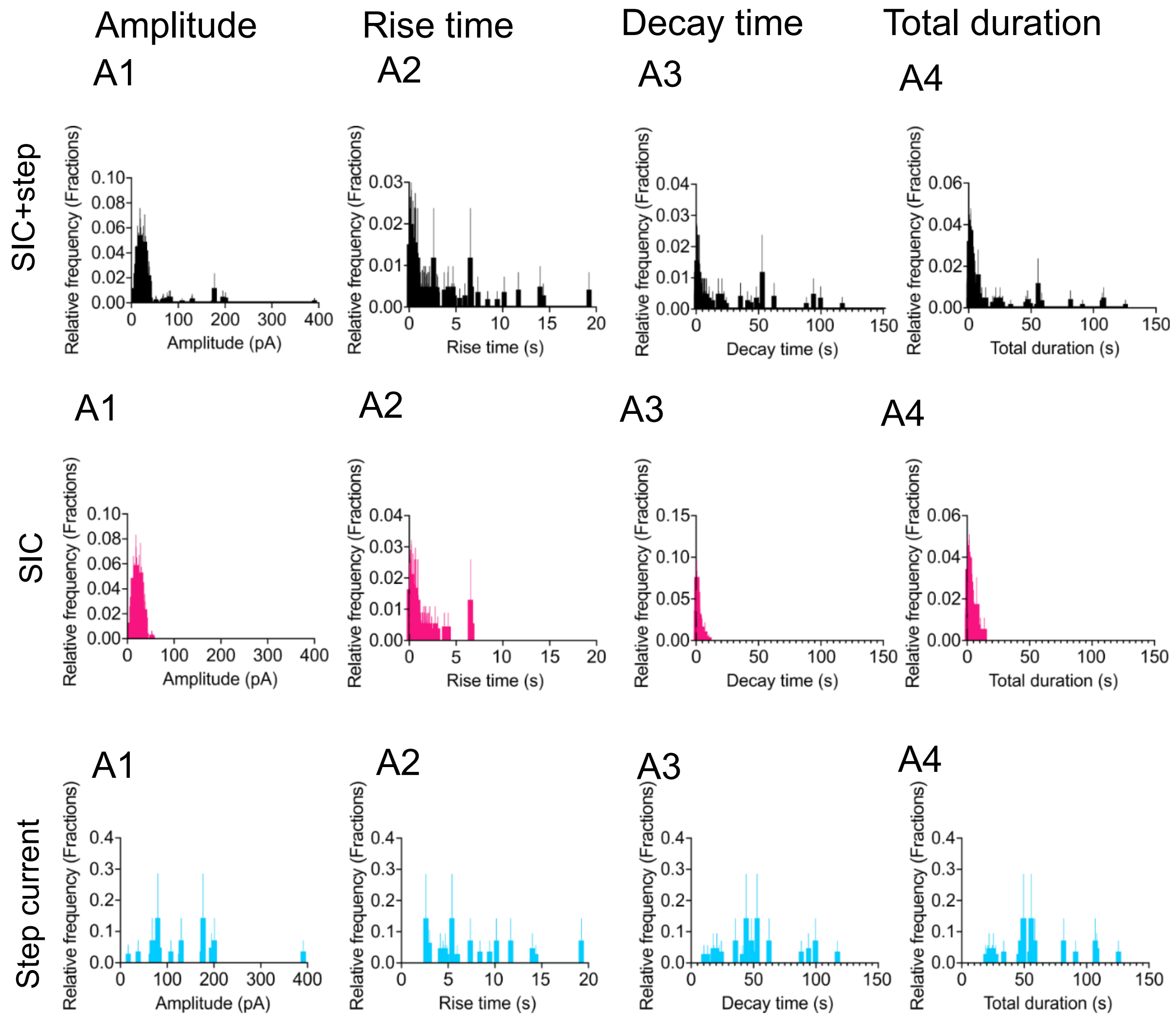


Figure 4 :Heterogenous kinetics of excitatory currents in MCH neurons.

Relative frequency distribution histograms of the amplitude, rise time, decay time and total duration of SICs and step currents. The two types of currents were distinguished by eye.
(A1-A4) both SICs and step current
(B1-B4) SICs only
(C1-C4) step currents only
Each histogram represents mean +/-S.E.M of n = 7 cells/N = 6 mice.

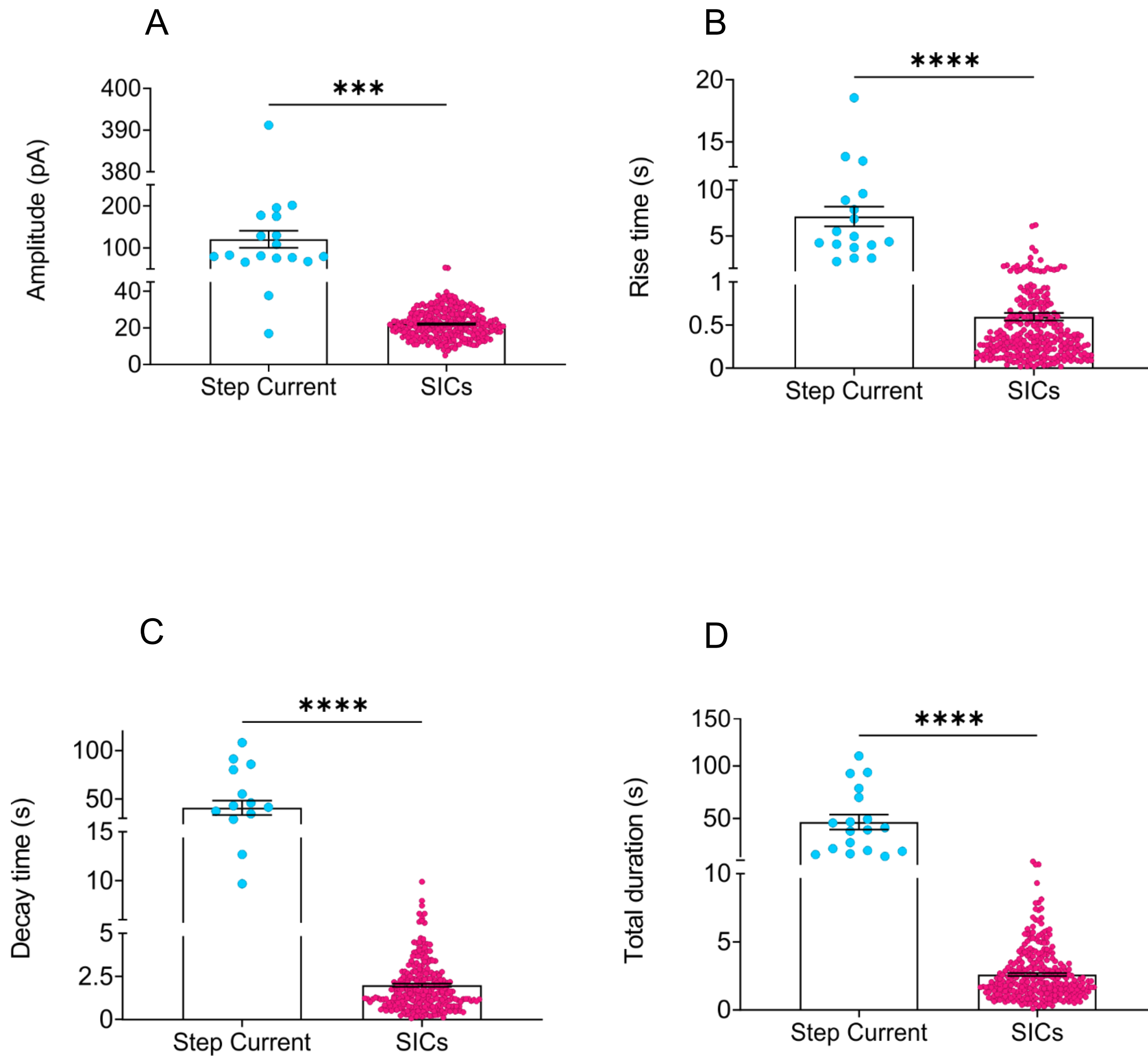


Figure 5 : Slow inward currents (SICs) can be statistically distinguished from Step currents.

(A-D) Summary of different parameters of SICs and step currents as indicated. Each dot indicates an individual current, error bars indicate SEM. Unpaired t test indicated by ***p<0.001 and ****p<0.0001. Gaps in the Y-axis are added to show an overall data distribution between both groups.

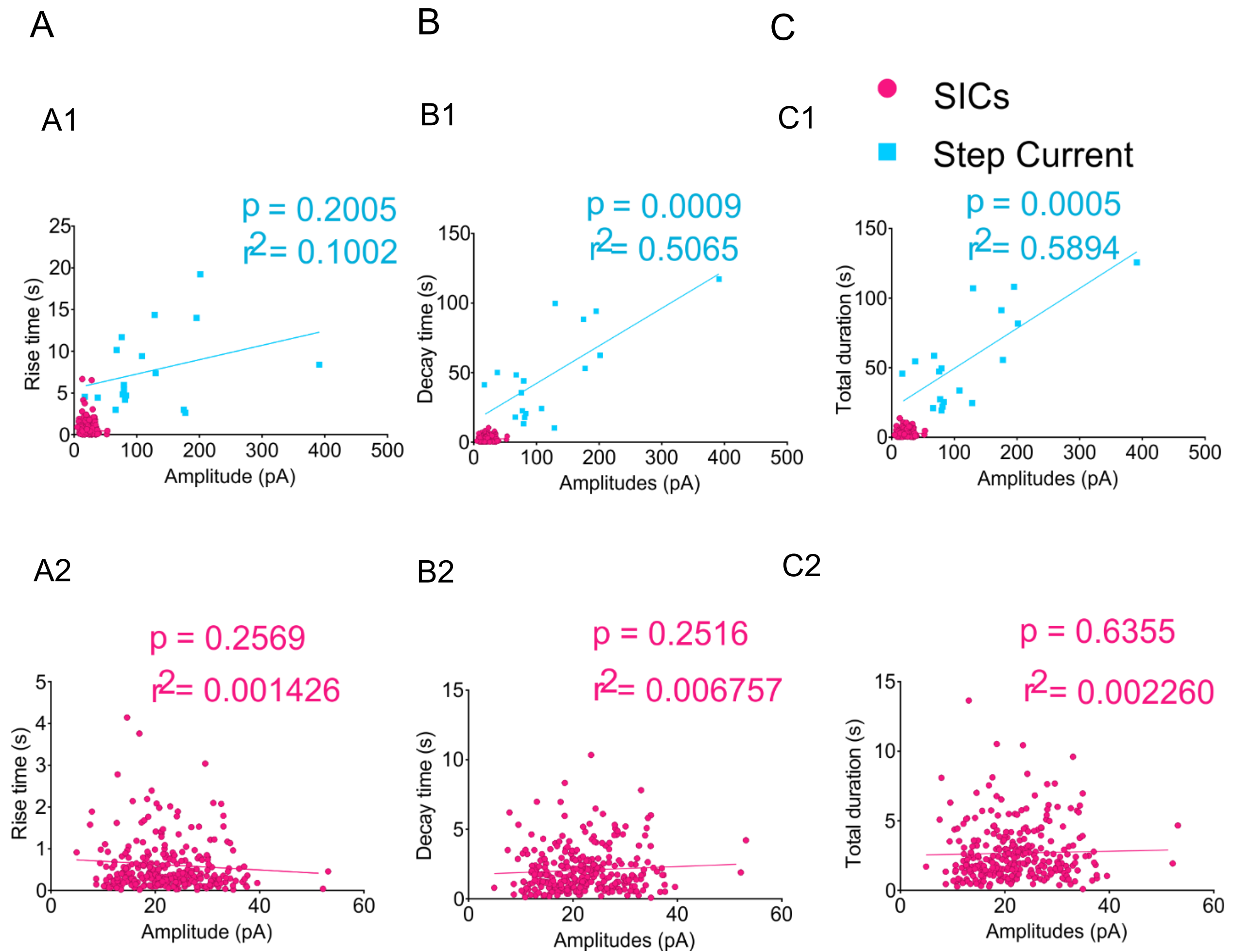


Figure 6 : Step currents and Slow inward currents (SICs) have discrete features.

A1-C1) Scatterplot diagram representing the relationship between the amplitude and rise time (A1), decay time (B1), and total duration (C1) of both SICs (magenta) and step currents (Orange).
A2-C2) Same data as shown in A1-C1; zoomed in portion of the SIC cohort only. The slope is significantly non-zero for B1 & C1 only.
Each dot represents one event pooled from n = 7 cells, N = 6 mice. Solid line indicates the linear regression.

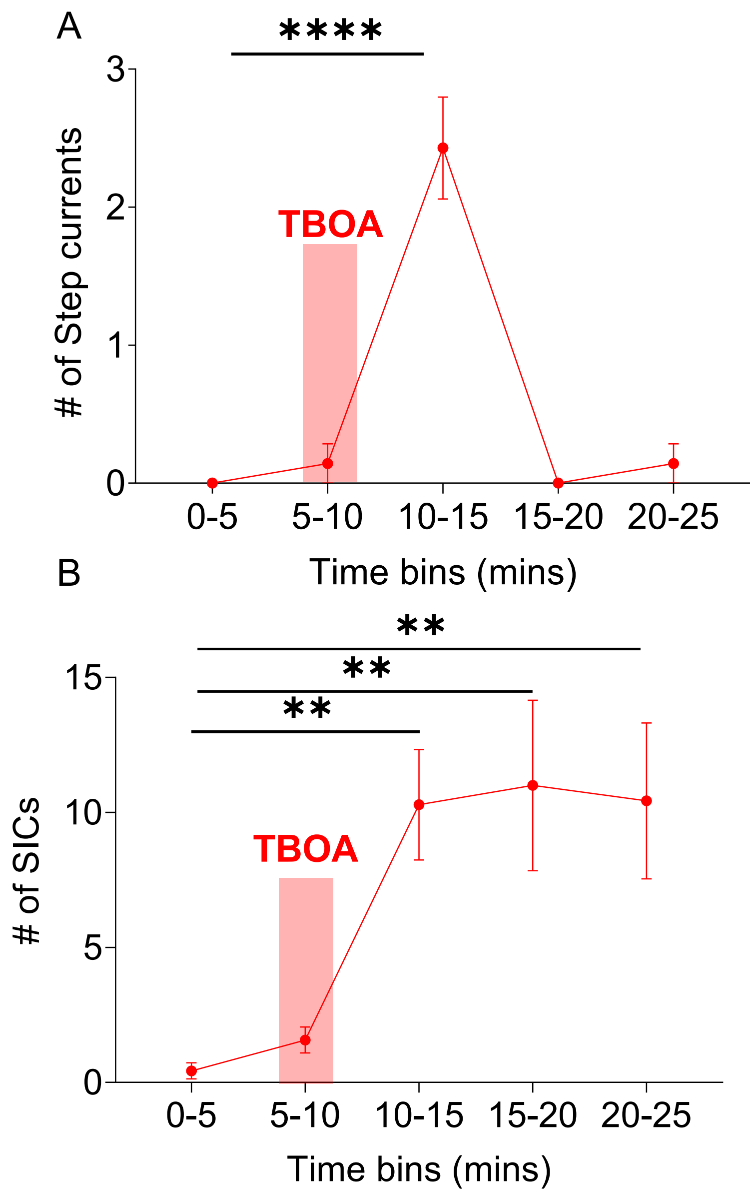


Figure 7 : Step and SICs arises at a specific time point after TBOA application

A and B represents the change in frequency of step currents and SICs following TBOA application respectively. Rose colored bar indicates the time of TBOA application (5-10 mins time bins). One-way ANOVA, Dunnett's test, **p<0.01, ****p<0.0001 vs baseline.

3.2 Role of ionotropic glutamate receptors in TBOA-induced excitatory currents

A blockade of glutamate transporters can be expected to increase the extracellular levels of glutamate, which would activate glutamate receptors. To determine which glutamate receptors mediate TBOA-TIC, step current and SICs, antagonists for different glutamate receptors were tested. Thus, we first assessed the role of non-NMDA glutamatergic receptors such as AMPA receptor (AMPAR) and kainate receptor (KAR). We found that bath application of DNQX (10 μM (Moldavski et al., 2020), AMPAR/ KAR antagonist, n/N = 4/2) blocked TBOA-TIC significantly compared to TBOA alone (One-way ANOVA, $F(3, 33) = 4.399$, $p = 0.0104$; Figure 8 A, B). However, AMPAR antagonist GYKI52466 (100μM (Kopach et al., 2011), n/N = 4/2) or KAR antagonist UBP310 (10 μM (Pinheiro et al. 2013), n/N = 5/3) alone were not as effective on TBOA-TIC (Figure 8B). These results suggest co-activation of AMPAR and KAR by ambient glutamate induces a robust tonic current in MCH neurons, which is regulated by glutamate transporters. Furthermore, DNQX completely abolished the occurrence of step currents (TBOA alone vs. DNQX + TBOA two-way ANOVA, $F(4, 39) = 12.19$, $p<0.0001$; Figure 8C). The frequency of SICs was also significantly reduced by DNQX (TBOA alone vs. DNQX + TBOA two-way ANOVA, $F(4, 39) = 2.948$, $p =0.0320$; Figure 8D). Moreover, DNQX significantly reduced the SICs amplitude (25 SICs for DNQX+TBOA vs. 284 SICs for TBOA only unpaired t-test $t(46.62) = 15.33$, $p<0.0001$; Figure 8E). Note that the amplitude of step currents could not be assessed, as there were none detected in the presence of DNQX. The above results show a co-involvement of AMPAR and KAR in mediating these excitatory currents.

Next, to determine the role of NMDAR in TBOA-induced currents, D-AP5 (50 μM), an NMDAR antagonist was bath applied 10 minutes prior to TBOA application and continued throughout the experiment. D-AP5 significantly reduced the amplitude of TBOA-TIC (unpaired t-test $t(18) = 7.202$; $p<0.0001$, TBOA alone (n/N = 26/19) vs TBOA + D-AP5 (n/N = 3/2); Figure 9A, B). Furthermore, upon application of TBOA in the presence of D-AP5, step currents were nullified completely (TBOA vs. D-AP5 + TBOA two-way ANOVA, $F(4, 40) = 12.62$, $p<0.0001$; Figure 9C). Similarly, D-AP5 application reduced the SIC frequency (TBOA vs. D-AP5 + TBOA two-way ANOVA, $F(1, 40) = 12.71$, $p =0.0010$; Figure 9D). Furthermore, SIC amplitude was significantly reduced by D-AP5 application (unpaired t-test $t(20.66) = 3.701$; $p = 0.0014$, TBOA alone (284 SICs) vs. TBOA+D-AP5 (19 SICs); Figure 9E). Overall, these results suggest that TBOA-TIC, step currents and SICs are dependent on NMDA receptor activation.

3.3 TBOA-induced excitatory currents are mediated by postsynaptic ionotropic NMDAR

Our result showing the involvement of NMDARs in these TBOA-induced excitatory currents is somewhat perplexing as our recording condition (holding potential = -70 mV) should have prevented NMDAR activation on recorded MCH neurons due to Mg^{2+} blockade. However, it is possible that NMDARs on the distal dendrites could escape voltage clamp (Povysheva & Johnson, 2012) and be relieved from the Mg^{2+} blockade. This could be due to AMPAR and KAR activation leading to sufficient membrane depolarization to permit NMDAR activation (Blanke & VanDongen, 2009). Alternatively,

it may involve mechanisms that are insensitive to Mg^{2+} block on NMDAR. To elucidate this mechanism, we performed experiments to test possible signaling of NMDARs.

i) NMDAR dependent metabotropic signaling

It is known that NMDAR can mediate ionotropic and metabotropic signaling (Nabavi et al., 2013; Salter & Kalia, 2004). While Mg^{2+} blocks the the ionotropic signaling, it does not block the metabotropic function of NMDAR. Thus, metabotropic NMDA signaling can explain the lack of apparent Mg^{2+} sensitivity of TBOA-TIC. To distinguish ionotropic NMDAR signaling from metabotropic ones, MK-801 (NMDAR open channel blocker) can be used, which is known to selectively block ionotropic function of NMDAR (Song et al., 2018). We found that MK-801 (20 µM; n/N = 4/4) blocked TBOA-TIC as effectively as D-AP5 that blocks both ionotropic and metabotropic NMDAR signaling (One-way ANOVA, $F(2, 28) = 9.389$, $p = 0.0008$; Figure 10A, B).

In addition, MK-801 significantly reduced the frequency of step currents induced by TBOA (TBOA alone vs. MK-801 + TBOA, two-way ANOVA, $F(4, 40) = 8.553$, $p<0.0001$; Figure 10C). Likewise, the frequency of SICs was significantly reduced by MK-801 (TBOA alone vs. MK-801 + TBOA, two-way ANOVA, $F(2, 49) = 8.639$, $p =0.0006$; Figure 10D). The amplitude of SICs in TBOA+MK-801 was also smaller in contrast to those induced by TBOA alone (33 SICs, unpaired t-test, $t (36.03) = 2.307$; $p = 0.0269$ for TBOA+MK-801 vs TBOA alone Figure 10E). This suggests that NMDAR metabotropic signaling may not be involved; instead, ionotropic NMDAR are responsible for mediating TBOA-TIC, step current and SICs.

ii) **Involvement of postsynaptic NMDARs**

Ambient glutamate accumulated by blocking glutamate transporters would likely activate glutamate receptors on not only the recorded cell but also those on surrounding neurons and astrocytes, which could in turn release neuroactive substances that affect the recorded cells (S. Y. Lee & Haydon, 2007) (Rusnakova et al., 2013). These possibilities cannot be distinguished by bath application of receptor antagonists. Thus, to distinguish NMDAR on MCH neurons from that on other cells, we applied MK-801 (1 mM, n/N =6/4) intracellularly through the patch pipette to selectively block NMDARs on the recorded cell. Intracellular MK-801 application significantly blocked TBOA-TIC to a similar degree as bath application of D-AP5 (One-way ANOVA, $F (2, 30) = 11.42$, $p = 0.0002$; Figure 11 A, B). This indicates that postsynaptic NMDAR are likely mediating the TIC. To further test for a role of NMDAR on MCH neurons, we performed the experiment in Mg^{2+} free ACSF to relieve NMDAR of Mg^{2+} blockade while holding the cell at -70 mV. There was a significant difference between TBOA-TIC amplitude in the presence or absence of extracellular Mg^{2+}, where TBOA-TIC recorded in Mg^{2+} free ACSF was larger than that in standard ACSF (mean amplitude = 1082± 152.3 pA, unpaired t-test, $t (9.783) = 3.559$; $p = 0.0054$, 1.2mM vs 0mM Mg^{2+} (n/N =9/5); Figure 11C). These results together support a critical role of postsynaptic NMDARs in mediating TBOA-TIC.

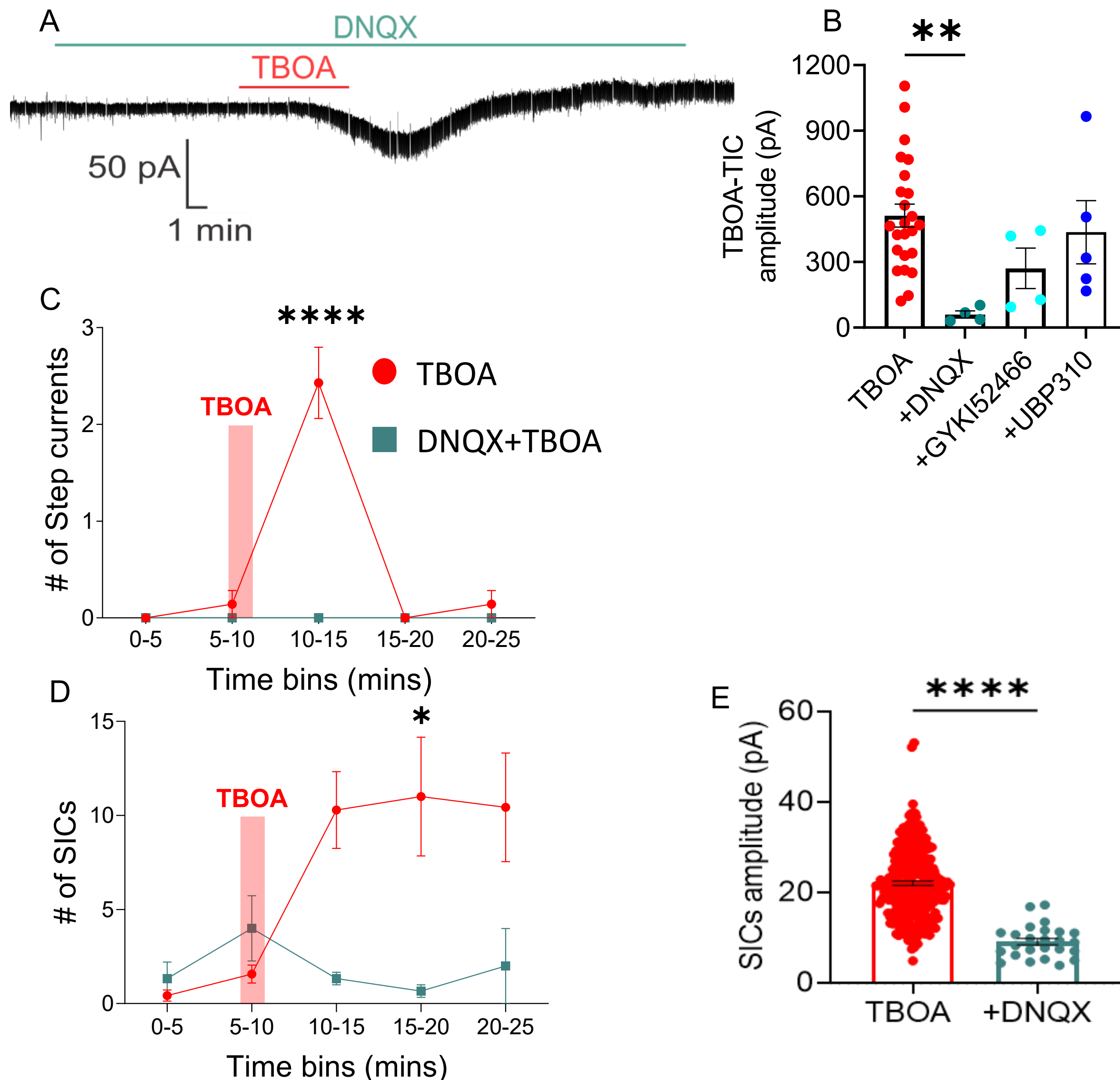


Figure 8 : Glutamate transporter controls the activation of non-NMDA ionotropic glutamate receptors.

A) Representative voltage clamp traces illustrating that the TBOA-TIC is significantly reduced by 10 μM DNQX.
B) Grouped data summarizing the effect of antagonists for AMPAR and KAR (DNQX, 10 μM), AMPAR only (GYKI52466, 100 μM), KAR only (UBP310, 10 μM) on TBOA-TIC. **p<0.01 compared to TBOA alone; One-way ANOVA, Dunnett's test.
C, D) Time course of the appearance of step currents (C) and SICs (D) induced by TFB-TBOA in the absence or presence of DNQX. *p<0.05, ****p<0.0001; DNQX+TBOA vs TBOA alone (two-way ANOVA and Sidak's test).
E) Effect of non-NMDAR antagonist on SIC amplitude.
****p<0.0001 (Unpaired t test); DNQX+TBOA vs TBOA alone.

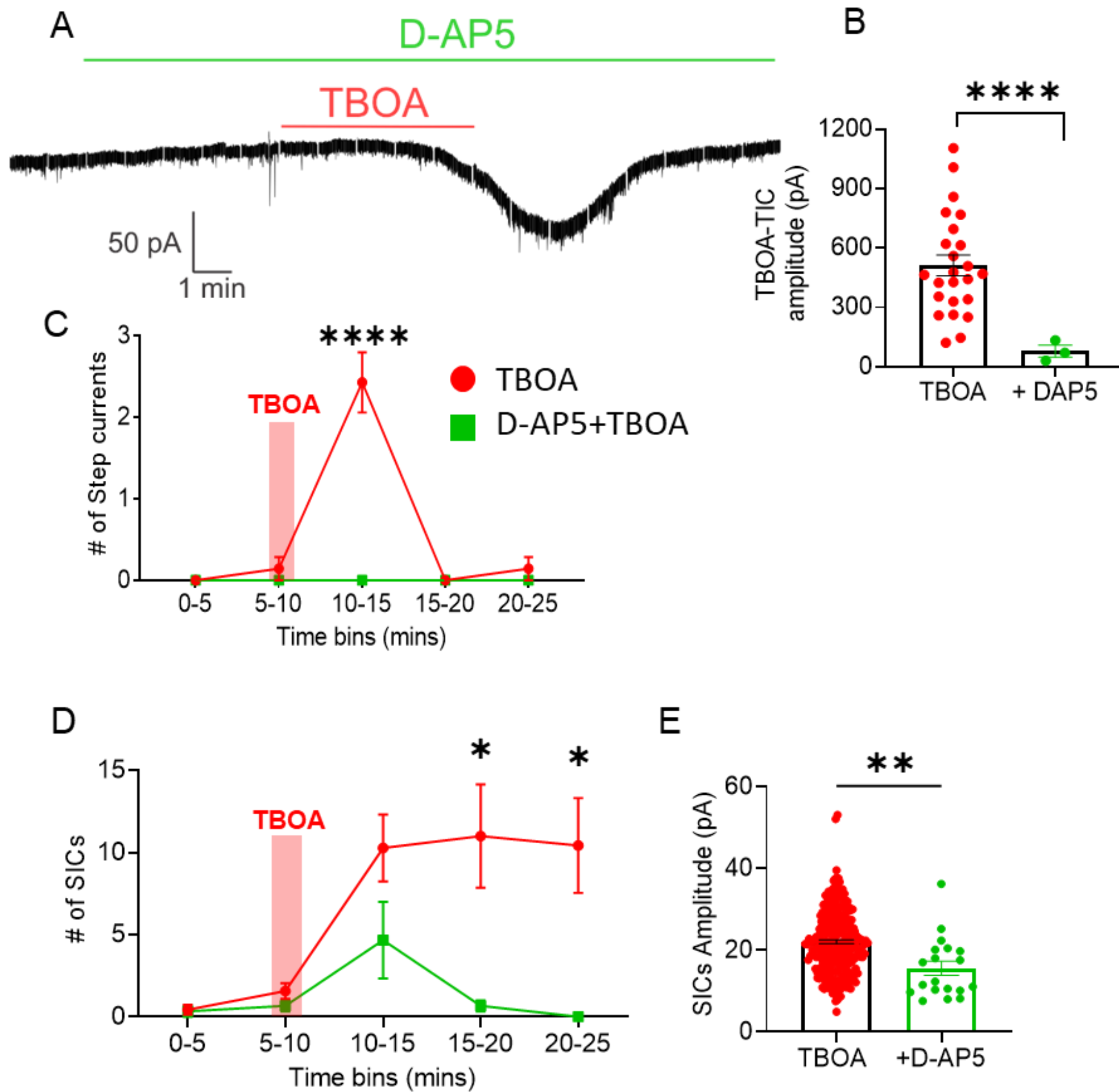


Figure 9 : TBOA-induced excitatory currents are mediated by NMDA receptors.

A) Representative voltage clamp traces illustrating that TBOA induces a modest TIC in the presence of 50 µM D-AP5.

B) Summary graph depicting the effect of TBOA in the absence or presence of D-AP5.

C, D) Time course of the appearance of step currents (C) and SICs (D) induced by TBOA in the absence or presence of NMDAR antagonist D-AP5 (50 µM). TBOA application started at 5-minute stamp. *p<0.05, ****p<0.0001; D-AP5+TBOA vs TBOA alone (two-way ANOVA and Sidak's test)

E) Effect of NMDA receptor antagonists on SICs amplitude.

****p<0.0001, **p<0.01 (Unpaired t test for B & E); D-AP5+TBOA vs TBOA alone.

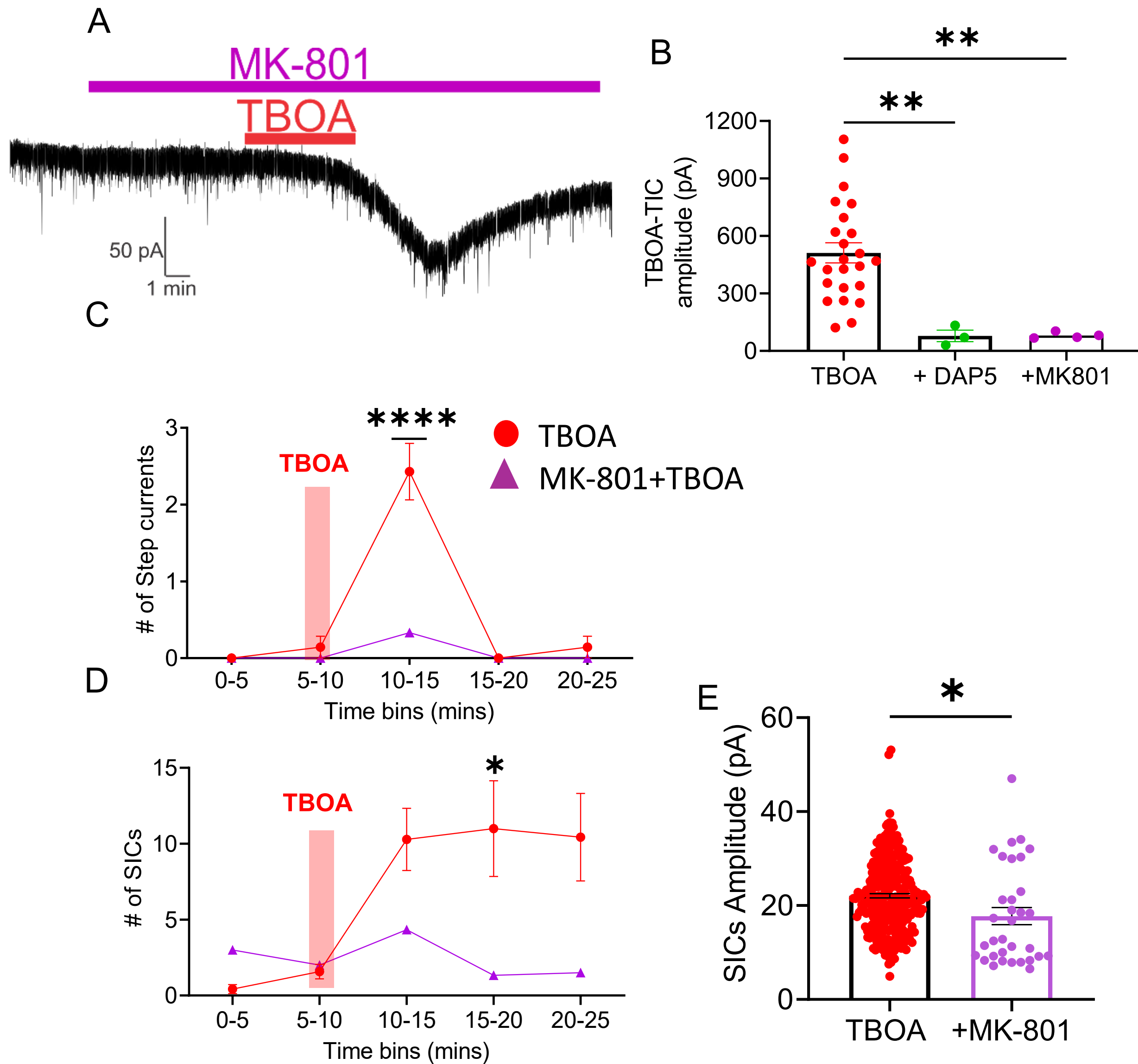


Figure 10 : Metabotropic signaling may not be involved in mediating discrete excitatory currents.

A) Representative voltage clamp trace illustrating that the TIC is significantly reduced by the open NMDAR channel blocker MK-801 (20 μM). B) Grouped data summarizing the effect of NMDAR antagonists on TBOA-TIC in MCH neurons. MK801 blocks TBOA-TIC similar to D-AP5. **p<0.01, one-way ANOVA and Dunnett's test. C, D) Time course of the appearance of Step currents (C) and SICs (D) induced by TBOA in the absence or presence of MK-801, *p<0.05, ****p<0.0001 MK-801+TBOA vs TBOA alone (two-way ANOVA and Sidak's test. E) Effect of NMDAR open channel blocker on SICs amplitude. *p<0.05 (Unpaired t test); MK-801+TBOA vs TBOA alone.

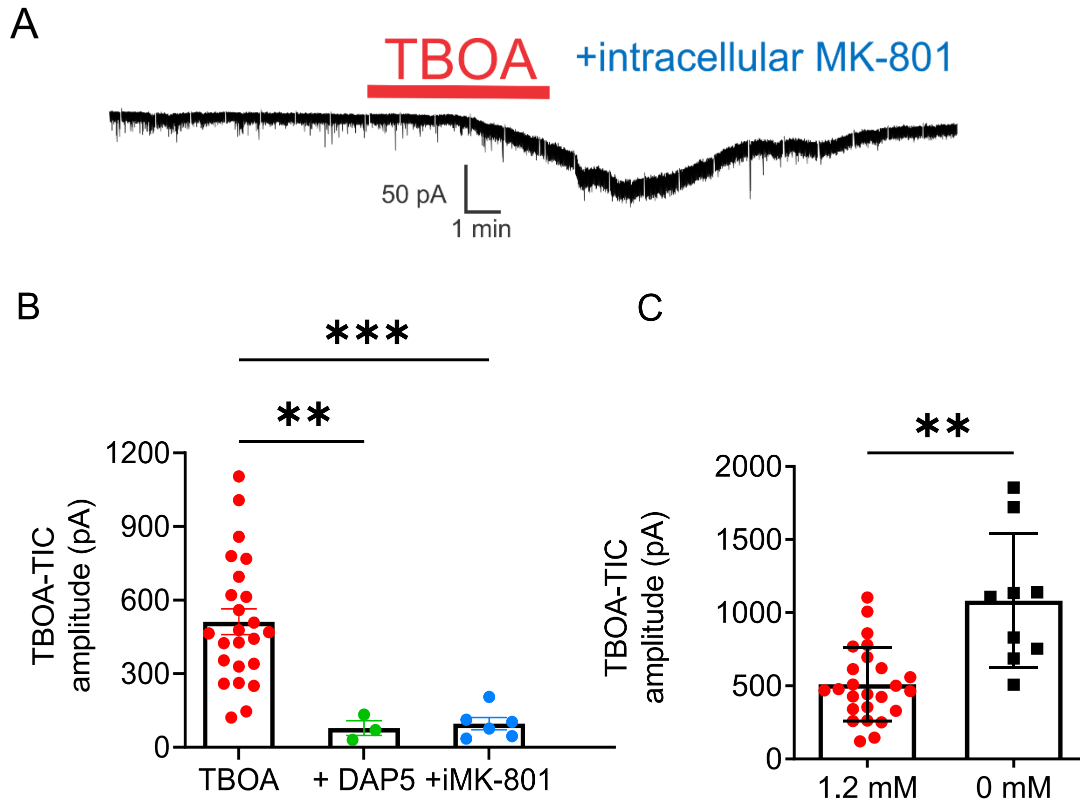


Figure 11 : Postsynaptic NMDAR mediates TBOA-TIC in MCH neurons.

A) Representative voltage clamp trace showing that TBOA-TIC is largely blocked by intracellular application of MK-801 (iMK-801) (1 mM).

B) Grouped data showing that the effect of postsynaptic NMDAR blockade by iMK-801 on TBOA-TIC is comparable to bath application of NMDAR antagonist DAP5. **$p<0.01$, ***$p<0.001$ TBOA alone vs D-AP5 and iMK-801 respectively (one-way ANOVA and Dunnett's test).

C) Summary graph of the amplitude of TBOA-TIC recorded in standard 1.2 mM Mg^{2+} or Mg^{2+}-free ACSF. **$p<0.01$ (Unpaired t test).

3.4 Ambient Glutamate is dependent on action potential evoked exocytosis

One potential source of extracellular glutamate is glutamatergic neurons located in the vicinity, which could fire action potentials and release neurotransmitters to send signals to the recorded MCH neuron. This signal is likely to be sensitive to tetrodotoxin (TTX) which blocks action potentials by inhibiting voltage gated sodium channels. To test this, TBOA was applied in the presence of TTX (1 μM, n/N = 4/2). This significantly reduced the amplitude of TBOA-TIC by 81.6% (unpaired t-test, $t(21) = 7.493$; $p<0.0001$, for TBOA+TTX vs TBOA alone; Figure 12, A&B). TTX also completely abolished step currents (TBOA only vs. TTX+TBOA, two-way ANOVA, $F(4, 45) = 17.21$, $p<0.0001$; Figure 12C) and significantly diminished the rate of SICs (TBOA only vs. TTX+TBOA, two-way ANOVA, $F(1, 45) = 19.05$, $p<0.0001$; Figure 12D). Furthermore, SIC amplitude was smaller with TTX than with TBOA alone (unpaired t-test, $t(28.98) = 8.131$; $p<0.0001$ for TBOA+TTX (22 SICs) vs TBOA alone; Figure 12E). This result suggests that the main source of ambient glutamate is from spontaneously active nearby neurons.

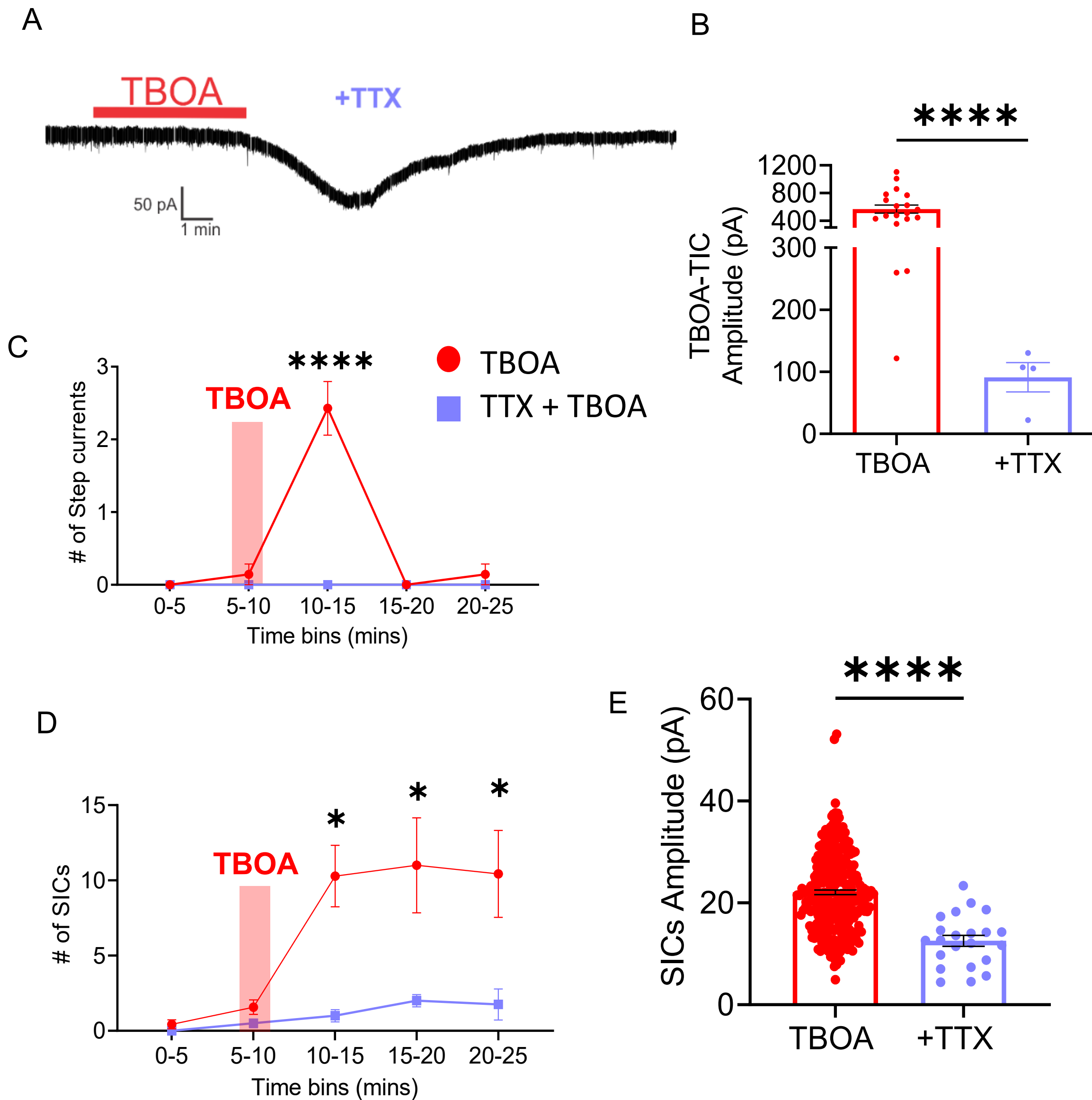


Figure 12 : TBOA-induced currents are dependent on neuronal activity.

A) Representative voltage clamp trace illustrating that the TIC is significantly reduced in the presence of TTX. B) Summary graph depicting the effect of TBOA in the presence of TTX. C & D) Time-effect plot of the effect of TTX (1 μM) on the frequency of Step currents (C) and SICs (D). ****p<0.0001 *p<0.05 compared to TBOA alone. E) TTX also attenuates the SICs amplitude; ****p<0.0001 vs TBOA; Unpaired Welch's t test performed for Figure B and E.

3.5 Acute food deprivation modulates the TBOA-induced excitatory currents in MCH neuron

Lateral hypothalamic (LH) injection of glutamate is known to elicit food intake behavior in satiated rats (Glenn Stanley et al., 1993) suggesting glutamatergic receptors involvement in the control of feeding behavior. MCH neurons in the LH promote feeding behavior and decrease energy expenditure (Bittencourt et al., 1992), and acute (12h) fasting has been shown to depolarize these neurons in rats (Linehan & Hirasawa, 2022). It is possible that acute fasting affects the excitability of MCH neurons by modulating glutamate transport. Thus, to understand how acute fasted condition may affect the TBOA effect on MCH neuron, mice were food deprived for 12h. Fasted mice had significantly attenuated TBOA-TIC in comparison to satiated mice (n/N = 8/3, $p = 0.0335$ for fasted vs fed group, unpaired t-test; Figure 13A). The frequency and amplitude of Step currents in fasted mice did not differ compared to Fed mice (2.6 ± 0.872/5 min at 10-15 time point, n/N = 5/3, $p = 0.117$ for fasted group; fed vs fasted, two-way ANOVA, $F(4, 50) = 0.9375$, $p = 0.450$; Figure 13 B). In contrast, the frequency of SICs was significantly lower post-TBOA application compared to the fed mice (fed vs fasted, two-way ANOVA, $F(1, 50) = 14.57$, $p = 0.0004$; Figure 13 D). However, SIC amplitude was comparable between the groups (unpaired t-test, $t(67.87) = 0.3509$; $p = 0.7268$ for fed vs fasted; Figure 13E). Overall, these results indicate that glutamatergic receptor signaling in MCH neurons may be sensitive to feeding status of the animal.

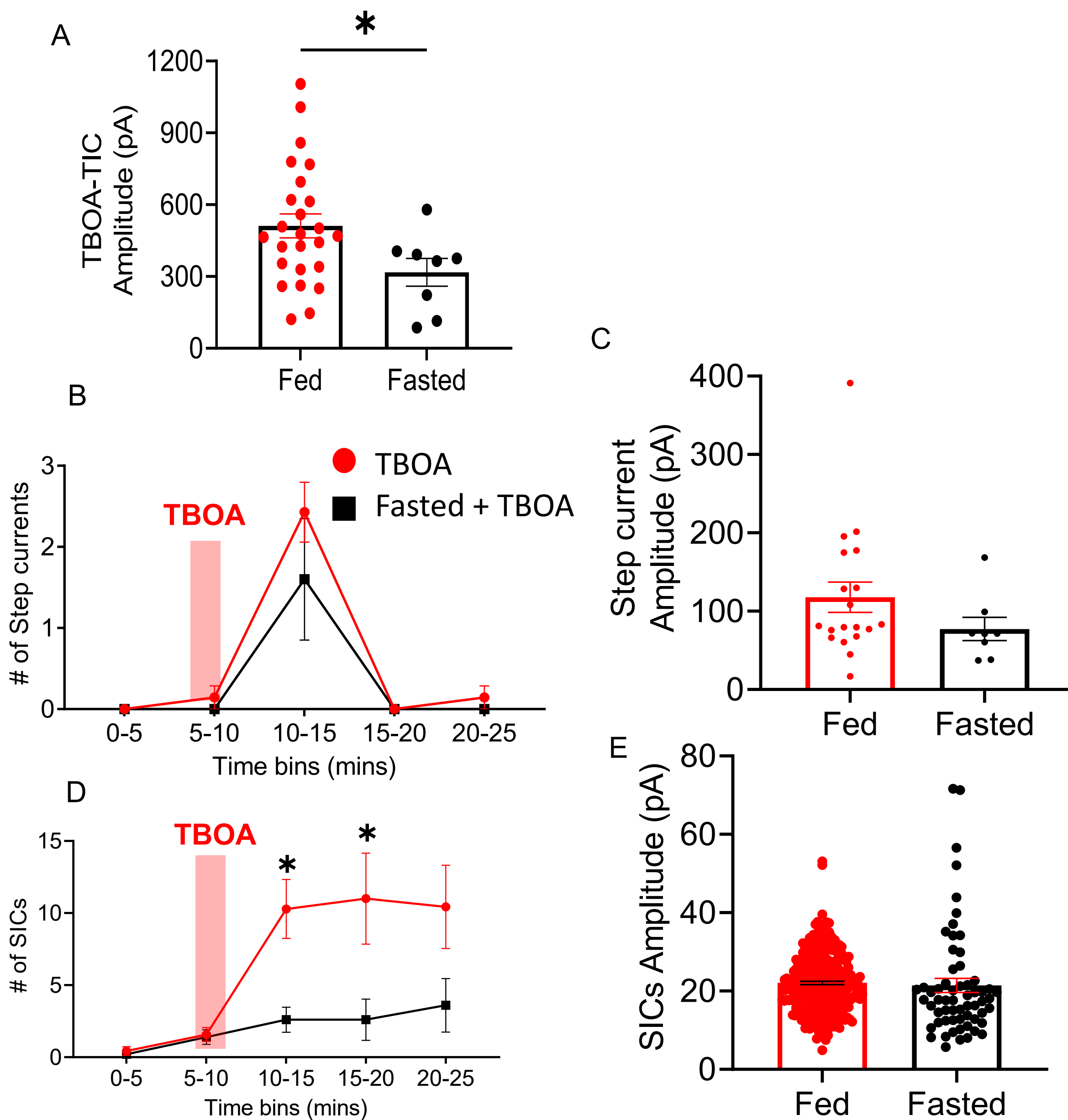


Figure 13 : Food deprivation attenuates TBOA induced excitatory currents on MCH neurons in mice.

A) The amplitude of TBOA-TIC is significantly reduced by 12h fasting; *p<0.05 vs Fed (Unpaired t test). B, C) data illustrating the frequency (B) and amplitude (C) of step currents respectively in satiated and fasted mice. Fasting significantly reduced the frequency of SICs (D) but not Step currents, Two-way ANOVA, Sidak's multiple comparison test; *p<0.05 vs Fed group. No significant difference was observed in the amplitude of step currents (C) and SICs (E) vs Fed.

4.0 Discussion

4.1 Non-specific glutamate transporter blockade reveals heterogenous excitatory currents in MCH neuron

TFB-TBOA (TBOA) was first synthesized by (Shimamoto et al., 2004) as a highly potent glutamate transporter inhibitor of EAAT1 (GLAST), EAAT2 (GLT-1) and EAAT3 (EAAC1) with IC50 values of 17, 22 and 300 nM respectively. Moreover, TBOA shows no affinity for NMDA receptors in comparison to its more widely used counterpart DL-TBOA, making it a powerful tool to investigate glutamate transporters (Shimamoto et al., 2004; Tsukada et al., 2005). Glutamate transporters expressed on the cell surface essentially regulates the level of extracellular glutamate spatially and temporally, keeping its concentration at low levels (in nanomolar to micromolar range) (Yang et al., 2015). Specifically, glutamate transporters tightly regulate ambient glutamate, which can be distinguished from ephemeral glutamate present at the synapse(Pál, 2018) . Thus, TBOA can prompt accumulation of such ambient glutamate, resulting in elevated excitatory responses in neurons. Using TBOA as a tool, we found that blocking Na^+ dependent glutamate transporters induce diverse excitatory currents with three distinguishable kinetics in MCH neurons, suggesting the physiological role of glutamate transport. Hence, this thesis work aimed to identify and characterize these excitatory currents.

4.2 Excitatory tonic inward current (TIC)

Ambient neurotransmitter can induce tonic currents also referred to as background currents. Tonic currents can be inhibitory or excitatory, which depend on

the type of neurotransmitter and receptor involved (Balmer et al., 2021). For instance, ambient GABA can produce tonic inhibitory current via activation of extrasynaptic $GABA_A$ receptors (Kaneda et al., 1995). Similarly, ambient glutamate has been shown to induce tonic activation of extrasynaptic NMDARs (present study, (Sah et al., 1989) and metabotropic glutamate receptors (Bandrowski et al., 2003), both of which can result in TIC.

TICs have been reported in many parts of the brain such as hippocampus (Angulo et al., 2004; le Meur et al., 2007), Purkinje cells of cerebellum (Sasaki et al., 2012), and spinal dorsal horn (Nie et al., 2010). In the hypothalamus, parvocellular neuron (Herrera Moro Chao et al., 2022), and magnocellular neurosecretory cells (Pai et al., 2016; M. Zhang et al., 2017) paraventricular nucleus and supraoptic nucleus (SON) (Fleming et al., 2011) also exhibit tonic currents. This response can be elicited or potentiated by manipulating the recording condition using glutamate transporter blockers (Pál, 2015). To the best of my knowledge, this thesis is the first to report that hypothalamic MCH neurons also display TIC.

A long term application of this potent TBOA (100 nM-2 μM) exhibits an inward current in different brain regions including hypothalamic parvocellular (M. Zhang et al., 2017) and magnocellular neurons (Gunn et al., 2013), nucleus tractus solitarius (Martinez et al., 2020), hippocampus (Bao et al., 2021; Tsukada et al., 2005), cerebellar unipolar brush cells and T-stellate cell, and retinal ganglion cells (Balmer et al., 2021; Yang et al., 2015). We found that TBOA induced TIC in mice MCH neurons in a concentration dependent manner. At lower concentrations (e.g., 1 μM), TBOA is known to block EAAT1 and EAAT2, whereas higher concentrations (≥5 μM) also inhibit EAAT3

(Balmer et al., 2021; Shigeri et al., 2004). In the present study, TBOA induced a modest TIC at 1 μM, whereas 5 μM resulted in a robust TIC. Hence, it is likely that neuronal EAAT3 is involved in glutamate clearance encompassing MCH neurons. We also found that orexin neurons, another cell population intermingled with MCH neurons in the lateral hypothalamus, showed a much smaller TIC at a high TBOA concentration (Figure 3B). This suggests that glutamate transporters differentially regulate the effect of ambient glutamate dependent on the cell type in the lateral hypothalamus. Alternatively, heterogeneity in receptor expression between MCH and orexin neurons may also explain the differences in TIC (Mickelsen et al., 2017). Taken together, in addition to EAAT1 and EAAT2, EAAT3 in MCH neurons may also regulate extracellular glutamate levels, and the resulting tonic current is dependent on diverse receptors present on phenotypically distinct neurons.

4.3 Cellular mechanism underlying TIC

This thesis work aimed to elucidate the cellular mechanism of the persistent tonic excitatory current influenced by increased ambient glutamate in MCH neurons. To assess the glutamatergic receptors involved in mediating tonic changes in excitability by extracellular glutamate, we used different glutamate receptor antagonists in the presence of TBOA.

4.3.1 AMPAR and KAR activation is controlled by glutamate transporters

As described earlier, tonic effect of extracellular glutamate can be mediated by AMPAR and KAR in different brain regions such as hippocampus (le Meur et al., 2007) , unipolar brush cells (Balmer et al., 2021) and supraoptic magnocellular neurosecretory

cells (Pai et al., 2016). This is consistent with our results in MCH neurons as TBOA-TIC was significantly reduced by DNQX (AMPAR/KAR antagonist). However, individual application of AMPAR and KAR specific antagonists failed to significantly reduce the TBOA-TIC. These results found in mice was further consolidated by previous work in our lab showing insignificant blockade of TIC by these specific antagonists in rat MCH neurons. AMPARs and KARs are generally active at the resting membrane potential. Hence, we assume that AMPAR and KAR additively contribute to TIC. This idea can be tested by co-application of both AMPAR and KAR antagonists (GYKI52466 and UBP310), which would inhibit TBOA-TIC to a similar to that of DNQX.

In general, AMPAR and KAR are fast kinetic receptors opening (~1 ms) and desensitize quickly and are localized at the synapse producing fast EPSCs (Traynelis et al., 2010). In contrast, extrasynaptic glutamatergic receptors are known to mediate slow tonic currents (Pai et al., 2016; Ramos et al., 2022). It is likely that TBOA-TIC is mediated by AMPARs and KARs present at the extrasynaptic site that are activated by ambient and spillover of glutamate facilitated by EAATs blockade.

4.3.2 Glutamate transporters control the activation of postsynaptic ionotropic NMDARs

In consensus with our previous study in rats, bath application of competitive NMDAR antagonist (D-AP5) resulted in significant blockade of TBOA-TIC in MCH neuron in mice. This implicates a tonic activation of NMDARs by elevated ambient glutamate. However, NMDARs are likely to remain closed due to voltage dependent Mg^{2+} blockade during voltage clamp recording (Mayer et al., 1984). Hence, we hypothesized that NMDAR metabotropic signaling may mediate this tonic current.

Interestingly, preventing ion influx through NMDAR channel using MK-801, an NMDAR open channel blocker, produced a comparable result to that of D-AP5 which blocks ionotropic and metabotropic NMDARs. This suggests that ionotropic NMDAR signaling, rather than metabotropic signaling, mediates TBOA-TIC. Another possible site of D-AP5 action is NMDARs on neighboring astrocytes (Skowrońska et al., 2019) and neurons (Lituma et al., 2021). These cells may consequentially release excitatory transmitter that could modulate the excitability of MCH neurons. However, selectively blocking NMDARs in recorded cell by intracellular MK-801 application inhibited TBOA-TIC just like bath-applied MK-801. Altogether, these results suggest that ambient glutamate directly activates ionotropic NMDARs to induce TBOA-TIC in MCH neurons.

Immunohistochemical, qRT-PCR and in-situ hybridization evidence indicates that GluN1, GluN2A, and GluN2B NMDAR subunits are most abundant in the lateral hypothalamic area, where MCH neurons are localized (Eyigor et al., 2001; Hernandez et al., 2020; Khan et al., 1999). Ambient glutamate arising from synaptic spillover and other sources can potentially activate extrasynaptic NMDARs. Reports in several neuronal types suggest that GluN2B-NMDAR subtype with higher glutamate affinity and low desensitization rates are located preferentially at the extrasynaptic site and mediate tonic NMDAR current (Fleming et al., 2011; le Meur et al., 2007; Papouin et al., 2012). Moreover, (Kuner & Schoepfer, 1996) reported that NMDAR subunits assembled in GluN1-GluN2A or GluN1-Glun2B conformation are strongly blocked by Mg^{2+}, while the present study showed that TBOA-TIC in MCH neurons was further potentiated in the absence of extracellular Mg^{2+}. Taken together with the abundance of GluN2B in the lateral hypothalamus, it is possible that TBOA-TIC is mediated by GluN2B-NMDARs in

MCH neurons. Further experiments are needed to identify and conclude which NMDAR subunits mediate this tonic current.

4.4 Ambient glutamate is dependent on neuronal activity in LH

Glutamate transporters play a significant role by preventing or tightly controlling diffusion of glutamate from the synaptic cleft. Under strong synaptic activity, glutamate can spillover and activate extrasynaptic receptors (Asztely et al., 1997; Lozovaya et al., 2004). We found that blocking the propagation of action potential in neurons using TTX reduced TBOA-TIC in MCH neurons. This indicates that ambient glutamate arises from neuronal activity driving synaptic release of glutamate, which can spillover from the synaptic cleft. It is also possible that a persistent Na^+ current contributes to TBOA-TIC and its blockade may contributes to the effect of TTX. Evidence of such persistent Na^+ current have been reported elsewhere (Balmer et al., 2021), including the hypothalamus (Llinas & Alonso, 1992; Uteshev et al., 1995).

While our experiment using TTX clearly supports the neuronal source of ambient glutamate, we cannot rule out the possibility of other ambient glutamate sources from non-synaptic origin. Ample research evidently suggests that tonic currents induced by ambient glutamate are independent of action potential-driven neurotransmitter release (Angulo et al., 2004; Fleming et al., 2011; le Meur et al., 2007).

4.5 Slow inward currents (SICs) and step currents

In contrast to TIC, neuronal SICs are defined as phasic depolarizing currents with kinetics that are significantly slower than spontaneous and miniature EPSCs (Pál, 2015). SICs have been reported in several CNS areas such as hippocampus (Angulo et

al., 2004; Fellin et al., 2004), nucleus accumbens (D'Ascenzo et al., 2007), thalamus (Pirttimaki & Parri, 2012), brainstem (Kovács & Pál, 2017) and prefrontal cortex (Y. Zhang et al., 2019). In accordance with the literature, the observed SICs in MCH neurons are unambiguously different from EPSCs due to their slow rise and decay times (Bardoni et al., 2010; Kovács & Pál, 2017; Shigetomi et al., 2008). To the best of our knowledge, our current work is the first to report the existence of SICs in MCH neurons.

SICs are often elicited by using different mechanistic and physiological manipulations, for example electrical and optogenetic stimulation, extracellular Mg^{2+} free condition, mGluR agonists, glutamate transporter blockers etc (Pál, 2015). In MCH neurons, we found that SICs become more apparent during the washout period of glutamate transporter blocker TBOA, after the TIC returns to the baseline. This may be due to the increased ambient glutamate levels favoring the likelihood of SICs with slower kinetics (Angulo et al., 2004). However, that doesn't explain the return of TIC back to the baseline, therefore mechanism underlying remains to be explored further.

The frequency and amplitude of TBOA-induced SICs were significantly inhibited by blockade of AMPARs + KARs. Thus, these receptors may mediate SICs or promote their occurrence. Considering the slower kinetics, it is likely that extrasynaptic AMPARs and KARs, rather than synaptic receptors, are involved in SICs. Evidently, extrasynaptic AMPARs with GluA1/A2 heteromers and KARs are observed in different regions of the brain (Kopach et al., 2011; Lu et al., 2009; Ramos et al., 2022). It is also possible that NMDARs located extrasynaptically are also involved in SICs. Indeed, SICs can result from GluN2B NMDAR activation typically located at the extrasynaptic location (Angulo

et al., 2004; D’Ascenzo et al., 2007; Fellin et al., 2004; Kovács & Pál, 2017). We recorded SICs in the presence of extracellular Mg^{2+} which would block NMDAR, however, AMPAR and KAR activation may induce sufficient local membrane depolarization required for NMDAR activation due to space clamp limitation. Indeed, the frequency and amplitude of SICs were reduced in the presence of NMDAR antagonists.

SIC frequency and amplitude were also reduced by the Na^{+} channel blocker TTX, suggesting that they are dependent on action potential propagation in the neuronal network. It is possible that synaptically released glutamate diffuses away from the synaptic cleft leading to activation of glutamatergic receptors located distally to generate slow SICs. (Huang & van den Pol, 2007) observed that slow EPSCs were completely blocked by TTX in MCH neuron. This indicates a presence of intricate neuronal network effects and/or persistent sodium current modifying the kinetics of SICs (Huang & van den Pol, 2007; Kovács & Pál, 2017).

There has been substantial amount of research showing the astrocytic involvement in generating SICs. Astrocytic glutamate release can occur via Ca^{2+} dependent vesicular or non-vesicular release. Triggering calcium transients in astrocytes via various mechanisms, including optogenetic activation, mGluR agonists, prostaglandin E2, Ca^{2+} uncaging, PAR-1 receptor stimulation, increased the occurrence of SICs (Angulo et al., 2004; D’Ascenzo et al., 2007; Fellin et al., 2004; Shigetomi et al., 2008). Non-vesicular release via volume-regulated anion channel, cystine-glutamate antiporters, purinergic receptors, hemichannels or reverse operation of glutamate transporters may also generate SICs (Bardoni et al., 2010; Danbolt, 2001). This thesis did not investigate the involvement of glutamate release from astrocytes. However,

increased frequency of SICs during TBOA application contradicts the involvement of glutamate transporter in reverse mode (Angulo et al., 2004). It is also established that Mg^{2+} insensitive NMDAR and AMPAR expressed on astrocytes are functionally active at the resting state (Dzamba et al., 2015; Matthias et al., 2003). Thus, it is possible that ambient glutamate surplus activates these receptors and downstream signaling, leading to gliotransmitter release to generate SICs. Overall, astrocytic processes in close proximity to MCH neuron may be a source of transient glutamate mediating SICs. Additional studies are essential to elucidate the contribution of astrocytes to extracellular glutamate that regulates the excitability of MCH neuron.

Upon TBOA application, we also observed a very peculiar step current riding along with the TIC. This was observed in MCH neurons but not Orexin neurons (Figure 3B). Their occurrence rate was the highest at 5 minutes post TBOA application and more apparent at a higher concentration (i.e., 5 μM). These events can be unequivocally distinguished from SICs based on their much slower rise and decay times, total duration and larger amplitude. It is possible that accumulation of excessive extracellular glutamate causes subsequent prolonged activation of glutamatergic receptors contributing to sustained depolarization (R. wei Zhang et al., 2019). Surely, step currents were completely blocked in the presence of ionotropic glutamatergic receptor antagonists (DNQX, D-AP5 and MK-801). Furthermore, by observing the recorded traces, intracellular MK-801 application was also found to inhibit this current, suggesting that postsynaptic NMDARs contributed to the step current. Step currents were also abolished by TTX, indicating that these currents are dependent on neuronal activity.

While SIC and step currents are distinguishable by their individual kinetics (rise and decay time), the time course if their occurrence is also distinct. It is evident that step currents contribute to the peak of the TIC, while SICs occur more frequently during and following the decay of TIC. Nevertheless, these distinct currents involve similar receptors and have similar sensitivity to TTX, suggesting that there may be common regulatory mechanisms for these currents. It remains unclear which glutamate transporter subtype is important in the regulation of these excitatory currents in MCH neurons, however, neuronal EAAC1 may play a role. Upon inspection of recordings of rat MCH neurons from a previous study in our lab, step currents were not observed with selective GLAST or GLT-1 blockers while they appeared in the presence of saturating concentrations of TBOA. Additionally, in the present study, we did not observe step currents at 1 μM TBOA, which supports the involvement of EAAC1 that has low sensitivity to TBOA blockade. Further direct approaches targeting EAAC1 are required to conclude its role in modulating these events.

4.6 Role of TIC and SICs in MCH neurons

The physiological function of MCH neuropeptide is known in feeding and energy metabolism; however, the role of glutamatergic action on MCH neuron in these functions is understudied. It has been reported that microinjections of glutamatergic receptor agonists in the lateral hypothalamic area increase feeding behaviors in satiated rats (Glenn Stanley et al., 1993), and endogenous glutamate is also known to regulate feeding and body weight via NMDARs (Stanley et al., 1996). These glutamate effects on energy balance may be mediated by MCH neurons. If so, it is possible that metabolic challenges causing energy imbalance may affect the excitability of MCH neurons.

Indeed, we found that 12 h food deprivation significantly reduced TBOA-TIC amplitude. This blunted effect of TBOA during short term nutrient shortage may involve altered expression and/or function of EAATs, which would affect astrocytic control of ambient glutamate. (Fuente-Martiń et al., 2012) showed that 24 h fasting upregulated astrocytic marker GFAP and glutamate transporters in the hypothalamus, possibly due to decreased circulating leptin levels. Moreover, EAAT1 and EAAT2 expression decreased in the arcuate nucleus and hippocampus after high fat diet (Liu & Zheng, 2019; Tsai et al., 2018). My study found that fasting also decreased SIC frequency but not the amplitude. It is possible that nutritional changes may affect the astrocytic Ca^{2+} and EAAT activity and therefore altering release and clearance of glutamate (Herrera Moro Chao et al., 2022). Furthermore, occurrence of SICs were inversely proportional to the distance between astrocytic and neuronal somata (Kovács & Pál, 2017). Taken together, fasting condition affects ambient glutamate regulation by glutamate transporters, and this may contribute to altered astroglial and neuronal communication and excitability of MCH neurons (Figure 14). Hence, changes in TBOA-TIC and SICs may represent physiological mechanisms involved in energy homeostasis.

It is important to note that TIC and SICs in MCH neuron were observed in the presence of an EAAT blocker. This indicates that activation of glutamate receptors on MCH neurons by ambient glutamate may be limited in basal conditions, and it remains unknown under which conditions they appear. Our laboratory has previously found that astrocytic GLT1 apposition is increased in MCH neurons while decreased in orexin neurons following sleep deprivation, resulting in distinct plasticity of excitatory neurotransmission (Briggs et al., 2018). Such changes in glutamate transporter

localization could alter ambient glutamate dynamics. In other brain areas, involvement of extrasynaptic NMDAR-mediated TICs were reported in seizures, depression, cocaine addiction and Alzheimer's disease (Bao et al., 2021; Miller et al., 2014; Montiel et al., 2005; Ortinski et al., 2013). Hypothalamic neurosecretory cells showed diminished TIC in heart failure and dehydrated rats, which was associated with altered glutamate transport activity (Fleming et al., 2011; Potapenko et al., 2012). Such homeostatic challenges can also bring about functional and morphological changes in astrocytes, which could alter ambient glutamate level and consequently modulating neuronal excitability (Pál, 2018; Wang & Hatton, 2009). On the other hand, SICs can be involved in neuronal synchronization (Angulo et al., 2004). Our finding in MCH neurons showing decreased SICs after food deprivation suggests that neuronal synchronization is possibly attenuated under fasting condition.

In summary, we identified distinct excitatory currents in MCH neurons. Although functional roles of these excitatory modalities are yet to be determined, their detailed characterization presented in this thesis could be a foundation for future studies on regulatory mechanisms of MCH neurons and can potentially be used as an experimental model to study astrocyte-neuron communication.

Figure 14 : Possible localization of glutamate receptors mediating distinct currents in MCH neuron.

Ambient glutamate arises mainly from the presynaptic vesicular exocytosis that acts on postsynaptic receptors to generate EPSCs. Glutamate spillover occurs following glutamate transporter blocker TBOA that likely activates extrasynaptic AMPA, Kainate and NMDA receptors; sustained activation of these receptors give rise to tonic inward current (TIC), step current, and SIC (slow inward current). Acute fasting can also alter control of ambient glutamtate by affecting glutamate transporter expression. Figure was created using Motifolio Toolkit (Motifolio Inc, Ellicott City, Md).

4.7 Limitation of the study and future directions

4.7.1 Source of ambient glutamate

In our study, we observed that heterogenous excitatory currents in MCH neurons were attenuated by blocking voltage gated sodium channel, suggesting spontaneous firing of neurons elevate ambient glutamate. However, action potential independent glutamate release can still occur. Therefore, to confirm the neuronal source of glutamate release, a future study can use bafilomycin A1 to inhibit vacuolar H^{+}-ATPase to block neuronal vesicular release. Additionally, there has been a plethora of studies in different regions of the brain suggesting astrocytic glutamate release (both vesicular and non-vesicular) (Lalo et al., 2021). The role of astrocytes as a source of ambient glutamate in the LH can be tested by using specific gliotoxins like fluorocitrate or optogenetic tools to inhibit astrocytic function (Nie et al., 2010).

4.7.2 NMDAR on astrocytes?

Astrocytic NMDARs with low to Mg^{2+} sensitivity and Ca^{2+} permeability is reported in several brain regions (Lalo et al., 2021). Their activation can consequently lead to release of gliotransmitters and modulation of the excitability of MCH neurons. To test the role of astrocytic NMDAR, the NMDAR antagonist MK-801 can be infused intracellularly into astrocytes via a patch pipette to selectively block astrocytic NMDARs (Palygin et al., 2011). This will allow us to identify whether astrocytic NMDAR are involved in an upstream intercellular signaling component mediating TIC and SICs such as triggering gliotransmitter release.

4.7.3 Are heterogenous currents mediated by synaptic or extrasynaptic receptors?

Glutamate receptor antagonists used in our experiments do not distinguish between synaptic and extrasynaptic receptors. In the future, characterization of the receptors' spatial location mediating TIC and SICs can be investigated in MCH neurons. For example, we can take advantage of the use-dependent open channel NMDAR blocker (MK-801) and low frequency stimulation protocol to selectively block synaptic NMDARs and isolate extrasynaptic NMDARs response (Harris & Pettit, 2007). Furthermore, putative extrasynaptic GluN2B NMDARs antagonists like memantine and ifenprodil can be used to make such distinction (Bao et al., 2021; le Meur et al., 2007).

4.7.4 Space clamp issue

Distal dendritic sites of MCH neurons may suffer from poor space clamp during voltage clamp recording. This can be not only be permissive to NMDAR activation but also lead to incorrect current estimation as voltage dependent NMDAR current may be underestimated (Povysheva & Johnson, 2012). This space clamp error can be alleviated by adding Cs^+ to the patch pipette to block potassium channels. Likewise, recordings at different holding potential can be done to validate the consistency of our findings and evaluate NMDAR voltage dependency that may occur due to space clamp errors.

4.7.5 Heterogenous GABAergic signaling?

Although understudied, tonic inhibitory current and slow outward currents mediated by GABA receptors are also reported in the central nervous system (Pál,

2015). In MCH neurons, GABA receptors are known to undergo dynamic changes following sleep deficit (Toossi et al., 2016). Nevertheless, the role of GABA transporters and GABAergic currents in regulating MCH neurons and in homeostatic function remains to be investigated.

4.8 Conclusion/Perspectives

Ambient glutamate control via glutamate transporters is fundamental to maintaining healthy neuronal/non-neuronal communication. This thesis demonstrated that glutamate transporters tightly regulate the extracellular glutamate concentration and act as a key modulator of MCH neuronal activity preventing overactivation. Three kinetically distinct excitatory currents were revealed in MCH neurons by blocking glutamate transporters, indicating that multiple forms of glutamate-mediated intercellular communication are actively ongoing in the LH. Furthermore, a change in nutrient status modulates these excitatory modalities, suggesting that the balance between glutamate release and uptake in this brain region may be sensitive to homeostatic challenges and may be involved in counterregulatory responses. Further studies are necessary to understand how ambient glutamate control contributes to energy homeostasis and metabolic disorders.

Despite the ubiquitous distribution of glutamate and its importance as a chemical messenger in the brain, toxicity by this amino acid is prevalent. Elevated concentrations of glutamate in the extracellular space can lead to overstimulation, damage and/or death of neurons, a phenomenon known as “excitotoxicity” (Velasco et al., 2017).

Glutamate transporters dynamically maintain physiological homeostasis of glutamate concentration and prevent neurotoxic effects (Kruyer et al., 2022).

Thus, glutamate and its transporters are critically implicated in physiology and pathology of the nervous system. This thesis contributes to advancing our understanding of the glutamatergic system in the brain by revealing novel mechanisms of glutamate signaling in MCH neurons and by highlighting the role of glutamate transporters. This may help in the advent of potential therapy in metabolic or sleep disturbance conditions given the role of MCH in sleep and energy homeostasis.

5.0 References

Angulo, M. C., Kozlov, A. S., Charpak, S., & Audinat, E. (2004). Glutamate Released from Glial Cells Synchronizes Neuronal Activity in the Hippocampus. *Journal of Neuroscience*, *24*(31), 6920–6927. https://doi.org/10.1523/JNEUROSCI.0473-04.2004

Arrigoni, E., Chee, M. J. S., & Fuller, P. M. (2019). To eat or to sleep: That is a lateral hypothalamic question. *Neuropharmacology*, *154*, 34–49. https://doi.org/10.1016/J.NEUROPHARM.2018.11.017

Asztely, F., Erdemli, G., & Kullmann, D. M. (1997). Extrasynaptic Glutamate Spillover in the Hippocampus: Dependence on Temperature and the Role of Active Glutamate Uptake. *Neuron*, *18*(2), 281–293. https://doi.org/10.1016/S0896-6273(00)80268-8

Bäckberg, M., Ultenius, C., Fritschy, J. M., & Meister, B. (2004). Cellular localization of GABAA receptor α subunit immunoreactivity in the rat hypothalamus: Relationship with neurones containing orexigenic or anorexigenic peptides. *Journal of Neuroendocrinology*, *16*(7), 589–604. https://doi.org/10.1111/j.1365-2826.2004.01207.x

Bak, L. K., Schousboe, A., & Waagepetersen, H. S. (2006). The glutamate/GABA-glutamine cycle: aspects of transport, neurotransmitter homeostasis and ammonia transfer. *Journal of Neurochemistry*, *98*(3), 641–653. https://doi.org/10.1111/J.1471-4159.2006.03913.X

Balmer, T. S., Borges-Merjane, C., & Trussell, L. O. (2021). Incomplete removal of extracellular glutamate controls synaptic transmission and integration at a cerebellar synapse. *Elife*, *10*, e63819. https://doi.org/10.7554/ELIFE.63819

Bandrowski, A. E., Huguenard, J. R., & Prince, D. A. (2003). Baseline glutamate levels affect group I and II mGluRs in layer V pyramidal neurons of rat sensorimotor cortex. *Journal of Neurophysiology*, *89*(3), 1308–1316. https://doi.org/https://doi.org/10.1111/j.1471-4159.2006.03913.x

Bao, Y., Yang, X., Fu, Y., Li, Z., Gong, R., & Lu, W. (2021). NMDAR-dependent somatic potentiation of synaptic inputs is correlated with β amyloid-mediated neuronal hyperactivity. *Translational Neurodegeneration*, *10*(1), 1–15. https://doi.org/10.1186/S40035-021-00260-3/FIGURES/6

Bardoni, R., Ghirri, A., Zonta, M., Betelli, C., Vitale, G., Ruggieri, V., Sandrini, M., & Carmignoto, G. (2010). Glutamate-mediated astrocyte-to-neuron signalling in the rat dorsal horn. *The Journal of Physiology*, *588*(5), 831–846. https://doi.org/10.1113/JPHYSIOL.2009.180570

Berthoud, H. R., & Münzberg, H. (2011). The lateral hypothalamus as integrator of metabolic and environmental needs: From electrical self-stimulation to opto-

genetics. *Physiology & Behavior*, *104*(1), 29–39. https://doi.org/10.1016/J.PHYSBEH.2011.04.051

Betley, J. N., Cao, Z. F. H., Ritola, K. D., & Sternson, S. M. (2013). Parallel, Redundant Circuit Organization for Homeostatic Control of Feeding Behavior. *Cell*, *155*(6), 1337–1350. https://doi.org/10.1016/J.CELL.2013.11.002

Birnbaumer, L., Campbell, K. P., Catterall, W. A., Harpold, M. M., Hofmann, F., Horne, W. A., Mori, Y., Schwartz, A., Snutch, T. P., Tanabe, T., & Tsien, R. W. (1994). The naming of voltage-gated calcium channels. *Neuron*, *13*(3), 505–506. https://doi.org/10.1016/0896-6273(94)90021-3

Bittencourt, J. C. (2011). Anatomical organization of the melanin-concentrating hormone peptide family in the mammalian brain. *General and Comparative Endocrinology*, *172*(2), 185–197. https://doi.org/10.1016/J.YGCEN.2011.03.028

Bittencourt, J. C., Presse, F., Arias, C., Peto, C., Vaughan, J., Nahon, J. -L, Vale, W., & Sawchenko, P. E. (1992). The melanin-concentrating hormone system of the rat brain: an immuno- and hybridization histochemical characterization. *The Journal of Comparative Neurology*, *319*(2), 218–245. https://doi.org/10.1002/CNE.903190204

Blanke, M. L., & VanDongen, A. M. J. (2009). Activation Mechanisms of the NMDA Receptor. *Biology of the NMDA Receptor*, 283–312. https://doi.org/10.1201/9781420044157.ch13

Blutstein, T., & Haydon, P. G. (2014). The Tripartite Synapse: A Role for Glial Cells in Modulating Synaptic Transmission. *The Synapse: Structure and Function*, 155–172. https://doi.org/10.1016/B978-0-12-418675-0.00005-5

Bridges, R. J., Natale, N. R., & Patel, S. A. (2012). System xc- cystine/glutamate antiporter: an update on molecular pharmacology and roles within the CNS. *British Journal of Pharmacology*, *165*(1), 20–34. https://doi.org/10.1111/J.1476-5381.2011.01480.X

Briggs, C., Hirasawa, M., & Semba, K. (2018). Sleep Deprivation Distinctly Alters Glutamate Transporter 1 Apposition and Excitatory Transmission to Orexin and MCH Neurons. *Journal of Neuroscience*, *38*(10), 2505–2518. https://doi.org/10.1523/JNEUROSCI.2179-17.2018

Bröer, S., & Brookes, N. (2001). Transfer of glutamine between astrocytes and neurons. *Journal of Neurochemistry*, *77*(3), 705–719. https://doi.org/10.1046/J.1471-4159.2001.00322.X

Burger, P. M., Mehl, E., Cameron, P. L., Maycox, P. R., Baumert, M., Lottspeich, F., de Camilli, P., & Jahn, R. (1989). Synaptic vesicles immunoisolated from rat cerebral cortex contain high levels of glutamate. *Neuron*, *3*(6), 715–720. https://doi.org/10.1016/0896-6273(89)90240-7

Cavelier, P., & Attwell, D. (2005). Tonic release of glutamate by a DIDS-sensitive mechanism in rat hippocampal slices. *The Journal of Physiology*, *564*(2), 397–410. https://doi.org/10.1113/JPHYSIOL.2004.082131

Chee, M. J. S., Arrigoni, E., & Maratos-Flier, E. (2015). Melanin-Concentrating Hormone Neurons Release Glutamate for Feedforward Inhibition of the Lateral Septum. *Journal of Neuroscience*, *35*(8), 3644–3651. https://doi.org/10.1523/JNEUROSCI.4187-14.2015

Cull-Candy, S., Brickley, S., & Farrant, M. (2001). NMDA receptor subunits: diversity, development and disease. *Current Opinion in Neurobiology*, *11*(3), 327–335. https://doi.org/10.1016/S0959-4388(00)00215-4

Danbolt, N. C. (2001). Glutamate uptake. *Progress in Neurobiology*, *65*(1), 1–105. https://doi.org/10.1016/S0301-0082(00)00067-8

D'Ascenzo, M., Fellin, T., Terunuma, M., Revilla-Sanchez, R., Meaney, D. F., Auberson, Y. P., Moss, S. J., & Haydon, P. G. (2007). mGluR5 stimulates gliotransmission in the nucleus accumbens. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(6), 1995–2000. https://doi.org/10.1073/PNAS.0609408104

Dehnes, Y., Chaudhry, F. A., Ullensvang, K., Lehre, K. P., Storm-Mathisen, J., & Danbolt, N. C. (1998). The Glutamate Transporter EAAT4 in Rat Cerebellar Purkinje Cells: A Glutamate-Gated Chloride Channel Concentrated near the Synapse in Parts of the Dendritic Membrane Facing Astroglia. *Journal of Neuroscience*, *18*(10), 3606–3619. https://doi.org/10.1523/JNEUROSCI.18-10-03606.1998

Dingledine, R., Borges, K., Bowie, D., & Traynelis, S. F. (1999). The glutamate receptor ion channels. *Pharmacological Reviews*, *51*(1), 7–62.

Doumazane, E., Scholler, P., Zwier, J. M., Trinquet, E., Rondard, P., & Pin, J.-P. (2011). A new approach to analyze cell surface protein complexes reveals specific heterodimeric metabotropic glutamate receptors. *The FASEB Journal*, *25*(1), 66–77. https://doi.org/10.1096/FJ.10-163147

Dzamba, D., Honsa, P., Valny, M., Kriska, J., Valihrach, L., Novosadova, V., Kubista, M., & Anderova, M. (2015). Quantitative Analysis of Glutamate Receptors in Glial Cells from the Cortex of GFAP/EGFP Mice Following Ischemic Injury: Focus on NMDA Receptors. *Cellular and Molecular Neurobiology*, *35*(8), 1187–1202. https://doi.org/10.1007/s10571-015-0212-8

Endoh, T. (2004). Characterization of modulatory effects of postsynaptic metabotropic glutamate receptors on calcium currents in rat nucleus tractus solitarius. *Brain Research*, *1024*(1–2), 212–224. https://doi.org/10.1016/J.BRAINRES.2004.07.074

Eyigor, O., Centers, A., & Jennes, L. (2001). Distribution of ionotropic glutamate receptor subunit mRNAs in the rat hypothalamus. *Journal of Comparative Neurology*, *434*(1), 101–124. https://doi.org/10.1002/CNE.1167

Fairhall, S. L., & Caramazza, A. (2013). Brain Regions That Represent Amodal Conceptual Knowledge. *Journal of Neuroscience*, *33*(25), 10552–10558. https://doi.org/10.1523/JNEUROSCI.0051-13.2013

Fellin, T., Pascual, O., Gobbo, S., Pozzan, T., Haydon, P. G., & Carmignoto, G. (2004). Neuronal synchrony mediated by astrocytic glutamate through activation of extrasynaptic NMDA receptors. *Neuron*, *43*(5), 729–743. https://doi.org/10.1016/j.neuron.2004.08.011

Fleming, T. M., Scott, V., Naskar, K., Joe, N., Brown, C. H., & Stern, J. E. (2011). State-dependent changes in astrocyte regulation of extrasynaptic NMDA receptor signalling in neurosecretory neurons. *The Journal of Physiology*, *589*(16), 3929–3941. https://doi.org/10.1113/JPHYSIOL.2011.207340

Fonnum, F. (1984). Glutamate: A Neurotransmitter in Mammalian Brain. *Journal of Neurochemistry*, *42*(1), 1–11. https://doi.org/10.1111/J.1471-4159.1984.TB09689.X

Frühbeis, C., Fröhlich, D., Kuo, W. P., Amphornrat, J., Thilemann, S., Saab, A. S., Kirchhoff, F., Möbius, W., Goebbels, S., Nave, K. A., Schneider, A., Simons, M., Klugmann, M., Trotter, J., & Krämer-Albers, E. M. (2013). Neurotransmitter-Triggered Transfer of Exosomes Mediates Oligodendrocyte–Neuron Communication. *PLOS Biology*, *11*(7), e1001604. https://doi.org/10.1371/JOURNAL.PBIO.1001604

Fuente-Martiń, E., García-Cáceres, C., Granado, M., de Ceballos, M. L., Sánchez-Garrido, M. Á., Sarman, B., Liu, Z. W., Dietrich, M. O., Tena-Sempere, M., Argente-Arizón, P., Díaz, F., Argente, J., Horvath, T. L., & Chowen, J. A. (2012). Leptin regulates glutamate and glucose transporters in hypothalamic astrocytes. *The Journal of Clinical Investigation*, *122*(11), 3900–3913. https://doi.org/10.1172/JCI64102

Gill, S. S., & Pulido, O. M. (2001). Glutamate receptors in peripheral tissues: current knowledge, future research, and implications for toxicology. *Toxicologic Pathology*, *29*(2), 208–223. https://doi.org/10.1080/019262301317052486

Glenn Stanley, B., Ha, L. H., Spears, L. C., & Dee, M. G. (1993). Lateral hypothalamic injections of glutamate, kainic acid,d,l-α-amino-3-hydroxy-5-methyl-isoxazole propionic acid or N-methyl-d-aspartic acid rapidly elicit intense transient eating in rats. *Brain Research*, *613*(1), 88–95. https://doi.org/10.1016/0006-8993(93)90458-Y

Glowatzki, E., Cheng, N., Hiel, H., Yi, E., Tanaka, K., Ellis-Davies, G. C. R., Rothstein, J. D., & Bergles, D. E. (2006). The Glutamate–Aspartate Transporter GLAST

Mediates Glutamate Uptake at Inner Hair Cell Afferent Synapses in the Mammalian Cochlea. *Journal of Neuroscience*, *26*(29), 7659–7664. https://doi.org/10.1523/JNEUROSCI.1545-06.2006

Godfrey, N., & Borgland, S. L. (2019). Diversity in the lateral hypothalamic input to the ventral tegmental area. *Neuropharmacology*, *154*, 4–12. https://doi.org/10.1016/J.NEUROPHARM.2019.05.014

González, J. A., Iordanidou, P., Strom, M., Adamantidis, A., & Burdakov, D. (2016). Awake dynamics and brain-wide direct inputs of hypothalamic MCH and orexin networks. *Nature Communications 2016 7:1*, *7*(1), 1–9. https://doi.org/10.1038/ncomms11395

Gunn, B. G., Cunningham, L., Cooper, M. A., Corteen, N. L., Seifi, M., Swinny, J. D., Lambert, J. J., & Belelli, D. (2013). Dysfunctional Astrocytic and Synaptic Regulation of Hypothalamic Glutamatergic Transmission in a Mouse Model of Early-Life Adversity: Relevance to Neurosteroids and Programming of the Stress Response. *The Journal of Neuroscience*, *33*(50), 19534. https://doi.org/10.1523/JNEUROSCI.1337-13.2013

Hack, N., & Balázs, R. (1994). Selective stimulation of excitatory amino acid receptor subtypes and the survival of granule cells in culture: effect of quisqualate and AMPA. *Neurochemistry International*, *25*(3), 235–241. https://doi.org/10.1016/0197-0186(94)90067-1

Hackett, J. T., & Ueda, T. (2015). Glutamate Release. *Neurochemical Research 2015 40:12*, *40*(12), 2443–2460. https://doi.org/10.1007/S11064-015-1622-1

Harada, K., Kamiya, T., & Tsuboi, T. (2016). Gliotransmitter release from astrocytes: Functional, developmental, and pathological implications in the brain. *Frontiers in Neuroscience*, *9*(JAN), 499. https://doi.org/10.3389/FNINS.2015.00499/BIBTEX

Harris, A. Z., & Pettit, D. L. (2007). Extrasynaptic and synaptic NMDA receptors form stable and uniform pools in rat hippocampal slices. *The Journal of Physiology*, *584*(Pt 2), 509. https://doi.org/10.1113/JPHYSIOL.2007.137679

Harthoorn, L. F., Sañé, A., Nethe, M., & van Heerikhuize, J. J. (2005). Multi-Transcriptional Profiling of Melanin-Concentrating Hormone and Orexin-Containing Neurons. *Cellular and Molecular Neurobiology 2005 25:8*, *25*(8), 1209–1223. https://doi.org/10.1007/S10571-005-8184-8

Hawkins, R. A. (2009). The blood-brain barrier and glutamate. *The American Journal of Clinical Nutrition*, *90*(3), 867S-874S. https://doi.org/10.3945/AJCN.2009.27462BB

Herman, M. A., & Jahr, C. E. (2007). Extracellular Glutamate Concentration in Hippocampal Slice. *Journal of Neuroscience*, *27*(36), 9736–9741. https://doi.org/10.1523/JNEUROSCI.3009-07.2007

Hernandez, G., Poirier, E., Lebied, K., Kouwenhoven, W. M., Lévesque, D., & Rompré, P.-P. (2020). Glutamate NMDA receptors containing GluN2C subunit relay the reward signal of the ventral tegmental area upon dorsal raphe stimulation. *BioRxiv*, 2020.07.26.222125. https://doi.org/10.1101/2020.07.26.222125

Herrera Moro Chao, D., Kirchner, M. K., Pham, C., Foppen, E., Denis, R. G. P., Castel, J., Morel, C., Montalban, E., Hassouna, R., Bui, L.-C. C., Renault, J., Mouffle, C., García-Cáceres, C., Tschöp, M. H., Li, D., Martin, C., Stern, J. E., & Luquet, S. H. (2022). Hypothalamic astrocytes control systemic glucose metabolism and energy balance. *Cell Metabolism*, *34*(10), 1532-1547.e6. https://doi.org/10.1016/J.CMET.2022.09.002

Hill, J., Duckworth, M., Murdock, P., Rennie, G., Sabido-David, C., Ames, R. S., Szekeres, P., Wilson, S., Bergsma, D. J., Gloger, I. S., Levy, D. S., Chambers, J. K., & Muir, A. I. (2001). Molecular Cloning and Functional Characterization of MCH2, a Novel Human MCH Receptor. *Journal of Biological Chemistry*, *276*(23), 20125–20129. https://doi.org/10.1074/jbc.M102068200

Holmseth, S., Dehnes, Y., Huang, Y. H., Follin-Arbelet, V. v., Grutle, N. J., Mylonakou, M. N., Plachez, C., Zhou, Y., Furness, D. N., Bergles, D. E., Lehre, K. P., & Danbolt, N. C. (2012). The Density of EAAC1 (EAAT3) Glutamate Transporters Expressed by Neurons in the Mammalian CNS. *Journal of Neuroscience*, *32*(17), 6000–6013. https://doi.org/10.1523/JNEUROSCI.5347-11.2012

Hu, W. H., Walters, W. M., Xia, X. M., Karmally, S. A., & Bethea, J. R. (2003). Neuronal glutamate transporter EAAT4 is expressed in astrocytes. *Glia*, *44*(1), 13–25. https://doi.org/10.1002/GLIA.10268

Huang, H., & van den Pol, A. N. (2007). Rapid Direct Excitation and Long-Lasting Enhancement of NMDA Response by Group I Metabotropic Glutamate Receptor Activation of Hypothalamic Melanin-Concentrating Hormone Neurons. *Journal of Neuroscience*, *27*(43), 11560–11572. https://doi.org/10.1523/JNEUROSCI.2147-07.2007

Hubbard, J. A., & Binder, D. K. (2016). Gliotransmitters. *Astrocytes and Epilepsy; Academic Press: Cambridge, MA, USA*, 53–73.

Huettner, J. E. (2003). Kainate receptors and synaptic transmission. *Progress in Neurobiology*, *70*(5), 387–407. https://doi.org/10.1016/S0301-0082(03)00122-9

Izawa, S., Chowdhury, S., Miyazaki, T., Mukai, Y., Ono, D., Inoue, R., Ohmura, Y., Mizoguchi, H., Kimura, K., Yoshioka, M., Terao, A., Kilduff, T. S., & Yamanaka, A. (2019). REM sleep-active MCH neurons are involved in forgetting hippocampus-dependent memories. *Science (New York, N.Y.)*, *365*(6459), 1308. https://doi.org/10.1126/SCIENCE.AAX9238

Kaneda, M., Farrant, M., & Cull-Candy, S. G. (1995). Whole-cell and single-channel currents activated by GABA and glycine in granule cells of the rat cerebellum. *The Journal of Physiology*, *485*(2), 419–435. https://doi.org/10.1113/JPHYSIOL.1995.SP020739

Khan, A. M., Currás, M. C., Dao, J., Jamal, F. A., Turkowski, C. A., Goel, R. K., Gillard, E. R., Wolfsohn, S. D., & Stanley, B. G. (1999). Lateral hypothalamic NMDA receptor subunits NR2A and/or NR2B mediate eating: immunochemical/behavioral evidence. *The American Journal of Physiology*, *276*(3). https://doi.org/10.1152/AJPREGU.1999.276.3.R880

Kim, D. Y., Kim, S. H., Choi, H. B., Min, C. K., & Gwag, B. J. (2001). High Abundance of GluR1 mRNA and Reduced Q/R Editing of GluR2 mRNA in Individual NADPH-Diaphorase Neurons. *Molecular and Cellular Neuroscience*, *17*(6), 1025–1033. https://doi.org/10.1006/MCNE.2001.0988

Konadhode, R. R., Pelluru, D., Blanco-Centurion, C., Zayachkivsky, A., Liu, M., Uhde, T., Glen, W. B., van den Pol, A. N., Mulholland, P. J., & Shiromani, P. J. (2013). Optogenetic Stimulation of MCH Neurons Increases Sleep. *Journal of Neuroscience*, *33*(25), 10257–10263. https://doi.org/10.1523/JNEUROSCI.1225-13.2013

Kondo, K., Hashimoto, H., Kitanaka, J. ichi, Sawada, M., Suzumura, A., Marunouchi, T., & Baba, A. (1995). Expression of glutamate transporters in cultured glial cells. *Neuroscience Letters*, *188*(2), 140–142. https://doi.org/10.1016/0304-3940(95)11408-O

Kopach, O., Kao, S. C., Petralia, R. S., Belan, P., Tao, Y. X., & Voitenko, N. (2011). Inflammation alters trafficking of extrasynaptic AMPA receptors in tonically firing lamina II neurons of the rat spinal dorsal horn. *PAIN®*, *152*(4), 912–923. https://doi.org/10.1016/J.PAIN.2011.01.016

Kovács, A., & Pál, B. (2017). Astrocyte-dependent slow inward currents (SICs) participate in neuromodulatory mechanisms in the pedunculopontine nucleus (PPN). *Frontiers in Cellular Neuroscience*, *11*, 16. https://doi.org/10.3389/FNCEL.2017.00016/BIBTEX

Kristian Enkvist, M. O., & McCarthy, K. D. (1994). Astroglial Gap Junction Communication Is Increased by Treatment with Either Glutamate or High K+ Concentration. *Journal of Neurochemistry*, *62*(2), 489–495. https://doi.org/10.1046/j.1471-4159.1994.62020489.x

Kruyer, A., Kalivas, P. W., & Scofield, M. D. (2022). Astrocyte regulation of synaptic signaling in psychiatric disorders. *Neuropsychopharmacology 2022*, 1–16. https://doi.org/10.1038/s41386-022-01338-w

Kuner, T., & Schoepfer, R. (1996). Multiple structural elements determine subunit specificity of Mg2+ block in NMDA receptor channels. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *16*(11), 3549–3558. https://doi.org/10.1523/JNEUROSCI.16-11-03549.1996

Kunishima, N., Shimada, Y., Tsuji, Y., Sato, T., Yamamoto, M., Kumasaka, T., Nakanishi, S., Jingami, H., & Morikawa, K. (2000). Structural basis of glutamate recognition by a dimeric metabotropic glutamate receptor. *Nature 2000 407:6807*, *407*(6807), 971–977. https://doi.org/10.1038/35039564

Kwon, H. B., & Castillo, P. E. (2008). Role of Glutamate Autoreceptors at Hippocampal Mossy Fiber Synapses. *Neuron*, *60*(6), 1082. https://doi.org/10.1016/J.NEURON.2008.10.045

Lalo, U., Koh, W., Lee, C. J., & Pankratov, Y. (2021). The tripartite glutamatergic synapse. *Neuropharmacology*, *199*, 108758. https://doi.org/10.1016/J.NEUROPHARM.2021.108758

le Meur, K., Galante, M., Angulo, M. C., Audinat, E., Meur, K. le, Galante, M., Angulo, M. C., & Audinat, E. (2007). Tonic activation of NMDA receptors by ambient glutamate of non-synaptic origin in the rat hippocampus. *The Journal of Physiology*, *580*(2), 373–383. https://doi.org/10.1113/JPHYSIOL.2006.123570

Lee, A., Anderson, A. R., Barnett, N. L., Stevens, M. G., & Pow, D. v. (2012). Alternate splicing and expression of the glutamate transporter EAAT5 in the rat retina. *Gene*, *506*(2), 283–288. https://doi.org/10.1016/J.GENE.2012.07.010

Lee, J., Raycraft, L., & Johnson, A. W. (2021). The dynamic regulation of appetitive behavior through lateral hypothalamic orexin and melanin concentrating hormone expressing cells. *Physiology & Behavior*, *229*, 113234. https://doi.org/10.1016/J.PHYSBEH.2020.113234

Lee, S. Y., & Haydon, P. G. (2007). Astrocytic glutamate targets NMDA receptors. *The Journal of Physiology*, *581*(3), 887–888. https://doi.org/10.1113/JPHYSIOL.2007.134676

Levy, L. M., Warr, O., & Attwell, D. (1998). Stoichiometry of the Glial Glutamate Transporter GLT-1 Expressed Inducibly in a Chinese Hamster Ovary Cell Line Selected for Low Endogenous Na+-Dependent Glutamate Uptake. *Journal of Neuroscience*, *18*(23), 9620–9628. https://doi.org/10.1523/JNEUROSCI.18-23-09620.1998

Lewerenz, J., Hewett, S. J., Huang, Y., Lambros, M., Gout, P. W., Kalivas, P. W., Massie, A., Smolders, I., Methner, A., & Pergande, M. (2013). The cystine/glutamate antiporter system xc− in health and disease: from molecular mechanisms to novel therapeutic opportunities. *Antioxidants & Redox Signaling*, *18*(5), 522–555. https://doi.org/10.1089/ARS.2011.4391

Li, J. L., Fujiyama, F., Kaneko, T., & Mizuno, N. (2003). Expression of vesicular glutamate transporters, VGluT1 and VGluT2, in axon terminals of nociceptive primary afferent fibers in the superficial layers of the medullary and spinal dorsal horns of the rat. *Journal of Comparative Neurology*, *457*(3), 236–249. https://doi.org/10.1002/CNE.10556

Linehan, V., Fang, L. Z., Parsons, M. P., & Hirasawa, M. (2020). High-fat diet induces time-dependent synaptic plasticity of the lateral hypothalamus. *Molecular Metabolism*, *36*. https://doi.org/10.1016/J.MOLMET.2020.100977

Linehan, V., & Hirasawa, M. (2018). Electrophysiological properties of melanin-concentrating hormone and orexin neurons in adolescent rats. *Frontiers in Cellular Neuroscience*, *12*. https://doi.org/10.3389/fncel.2018.00070

Linehan, V., & Hirasawa, M. (2022). Short-term Fasting Induces Alternate Activation of Orexin and Melanin-concentrating Hormone Neurons in Rats. *Neuroscience*. https://doi.org/https://doi.org/10.1016/j.neuroscience.2022.04.006

Lituma, P. J., Kwon, H. B., Alviña, K., Luján, R., & Castillo, P. E. (2021). Presynaptic nmda receptors facilitate short-term plasticity and bdnf release at hippocampal mossy fiber synapses. *ELife*, *10*. https://doi.org/10.7554/ELIFE.66612

Liu, X., & Zheng, H. (2019). Leptin-Mediated Sympathoexcitation in Obese Rats: Role for Neuron–Astrocyte Crosstalk in the Arcuate Nucleus. *Frontiers in Neuroscience*, *13*, 1217. https://doi.org/10.3389/FNINS.2019.01217/BIBTEX

Llinas, R. R., & Alonso, A. (1992). Electrophysiology of the mammillary complex in vitro. I. Tuberomammillary and lateral mammillary neurons. *Journal of Neurophysiology*, *68*(4), 1307–1320. https://doi.org/10.1152/JN.1992.68.4.1307

Llorente-Folch, I., Rueda, C. B., Pérez-Liébana, I., Satrústegui, J., & Pardo, B. (2016). l-Lactate-Mediated Neuroprotection against Glutamate-Induced Excitotoxicity Requires ARALAR/AGC1. *Journal of Neuroscience*, *36*(16), 4443–4456. https://doi.org/10.1523/JNEUROSCI.3691-15.2016

Lozovaya, N. A., Grebenyuk, S. E., Tsintsadze, T. S., Feng, B., Monaghan, D. T., & Krishtal, O. A. (2004). Extrasynaptic NR2B and NR2D subunits of NMDA receptors shape 'superslow' afterburst EPSC in rat hippocampus. *The Journal of Physiology*, *558*(2), 451–463. https://doi.org/10.1113/JPHYSIOL.2004.063792

Lu, W., Shi, Y., Jackson, A. C., Bjorgan, K., During, M. J., Sprengel, R., Seeburg, P. H., & Nicoll, R. A. (2009). Subunit Composition of Synaptic AMPA Receptors Revealed by a Single-Cell Genetic Approach. *Neuron*, *62*(2), 254–268. https://doi.org/10.1016/J.NEURON.2009.02.027

Mahmoud, S., Gharagozloo, M., Simard, C., & Gris, D. (2019). Astrocytes maintain glutamate homeostasis in the CNS by controlling the balance between glutamate uptake and release. *Cells*, *8*(2), 184. https://doi.org/10.3390/CELLS8020184

Malik, A. R., & Willnow, T. E. (2019). Excitatory Amino Acid Transporters in Physiology and Disorders of the Central Nervous System. *International Journal of Molecular Sciences*, *20*(22), 5671. https://doi.org/10.3390/IJMS20225671

Martinez, D., Rogers, R. C., Hermann, G. E., Hasser, E. M., & Kline, D. D. (2020). Neural Control: Astrocytic glutamate transporters reduce the neuronal and physiological influence of metabotropic glutamate receptors in nucleus tractus solitarii. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*, *318*(3), R545. https://doi.org/10.1152/AJPREGU.00319.2019

Matthias, K., Kirchhoff, F., Seifert, G., Hüttmann, K., Matyash, M., Kettenmann, H., & Steinhäuser, C. (2003). Segregated Expression of AMPA-Type Glutamate Receptors and Glutamate Transporters Defines Distinct Astrocyte Populations in the Mouse Hippocampus. *Journal of Neuroscience*, *23*(5), 1750–1758. https://doi.org/10.1523/JNEUROSCI.23-05-01750.2003

Mayer, M. L., Westbrook, G. L., & Guthrie, P. B. (1984). Voltage-dependent block by Mg2+ of NMDA responses in spinal cord neurones. *Nature*, *309*(5965), 261–263. https://doi.org/10.1038/309261A0

McEntee, W. J., & Crook, T. H. (1993). Glutamate: its role in learning, memory, and the aging brain. *Psychopharmacology*, *111*(4), 391–401. https://doi.org/10.1007/BF02253527

McKenna, M. C., Stridh, M. H., McNair, L. F., Sonnewald, U., Waagepetersen, H. S., & Schousboe, A. (2016). Glutamate oxidation in astrocytes: Roles of glutamate dehydrogenase and aminotransferases. *Journal of Neuroscience Research*, *94*(12), 1561–1571. https://doi.org/10.1002/JNR.23908

Meldrum, B. S. (1998). The glutamate synapse as a therapeutical target: perspectives for the future. *Progress in Brain Research*, *116*, 441–458. https://doi.org/10.1016/S0079-6123(08)60454-9

Meldrum, B. S. (2000). Glutamate as a Neurotransmitter in the Brain: Review of Physiology and Pathology. *The Journal of Nutrition*, *130*(4), 1007S-1015S. https://doi.org/10.1093/JN/130.4.1007S

Mickelsen, L. E., Kolling, F. W., Chimileski, B. R., Fujita, A., Norris, C., Chen, K., Nelson, C. E., & Jackson, A. C. (2017). Neurochemical Heterogeneity Among Lateral Hypothalamic Hypocretin/Orexin and Melanin-Concentrating Hormone Neurons Identified Through Single-Cell Gene Expression Analysis. *ENeuro*, *4*(5), 13–17. https://doi.org/10.1523/ENEURO.0013-17.2017

Miller, O. H., Yang, L., Wang, C. C., Hargroder, E. A., Zhang, Y., Delpire, E., & Hall, B. J. (2014). GluN2B-containing NMDA receptors regulate depression-like behavior and are critical for the rapid antidepressant actions of ketamine. *ELife*, *2014*(3). https://doi.org/10.7554/ELIFE.03581

Moldavski, A., Behr, J., Bading, H., & Bengtson, C. P. (2020). A novel method using ambient glutamate for the electrophysiological quantification of extrasynaptic NMDA receptor function in acute brain slices. *The Journal of Physiology*, *598*(4), 633–650. https://doi.org/10.1113/JP278362

Montiel, T., Camacho, A., Estrada-Sánchez, A. M., & Massieu, L. (2005). Differential effects of the substrate inhibitor l-trans-pyrrolidine-2,4-dicarboxylate (PDC) and the non-substrate inhibitor DL-threo-beta-benzyloxyaspartate (DL-TBOA) of glutamate transporters on neuronal damage and extracellular amino acid levels in rat . *Neuroscience*, *133*(3), 667–678. https://doi.org/10.1016/J.NEUROSCIENCE.2004.11.020

Murphy-Royal, C., Dupuis, J., Groc, L., Oliet, S. H. R. R., Murphy-Royal, C., Dupuis, J., Groc, L., & Oliet, S. H. R. R. (2017). Astroglial glutamate transporters in the brain: regulating neurotransmitter homeostasis and synaptic transmission. *Journal of Neuroscience Research*, *95*(11), 2140–2151. https://doi.org/10.1002/JNR.24029

Nabavi, S., Kessels, H. W., Alfonso, S., Aow, J., Fox, R., & Malinow, R. (2013). Metabotropic NMDA receptor function is required for NMDA receptor-dependent long-term depression. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(10), 4027–4032. https://doi.org/10.1073/pnas.1219454110

Nedergaard, M., Takano, T., & Hansen, A. J. (2002). Beyond the role of glutamate as a neurotransmitter. *Nature Reviews Neuroscience*, *3*(9), 748–755. https://doi.org/10.1038/nrn916

Nie, H., Zhang, H., & Weng, H. R. (2010). Bidirectional neuron-glia interactions triggered by deficiency of glutamate uptake at spinal sensory synapses. *Journal of Neurophysiology*, *104*(2), 713–725. https://doi.org/10.1152/JN.00282.2010/ASSET/IMAGES/LARGE/Z9K0081002700008.JPEG

Niswender, C. M., & Conn, P. J. (2010). Metabotropic Glutamate Receptors: Physiology, Pharmacology, and Disease. *Annual Review of Pharmacology and Toxicology*, *50*, 295. https://doi.org/10.1146/ANNUREV.PHARMTOX.011008.145533

Nyitrai, G., Kékesi, K. A., & Juhász, G. (2006). Extracellular level of GABA and Glu: in vivo microdialysis-HPLC measurements. *Current Topics in Medicinal Chemistry*, *6*(10), 935–940. https://doi.org/10.2174/156802606777323674

Ortinski, P. I., Turner, J. R., & Christopher Pierce, R. (2013). Extrasynaptic targeting of NMDA receptors following D1 dopamine receptor activation and cocaine self-administration. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *33*(22), 9451–9461. https://doi.org/10.1523/JNEUROSCI.5730-12.2013

Özkan, E. D., & Ueda, T. (1998). Glutamate transport and storage in synaptic vesicles. *The Japanese Journal of Pharmacology*, *77*(1), 1–10. https://doi.org/10.1254/JJP.77.1

Pai, Y. H., Lim, C. S., Park, K.-A. A., Cho, H. S., Lee, G.-S. S., Shin, Y. S., Kim, H.-W. W., Jeon, B. H., Yoon, S. H., & Park, J. B. (2016). Facilitation of AMPA receptor-mediated steady-state current by extrasynaptic NMDA receptors in supraoptic magnocellular neurosecretory cells. *Korean Journal of Physiology and Pharmacology*, *20*(4), 425. https://doi.org/10.4196/KJPP.2016.20.4.425

Pál, B. (2015). Astrocytic actions on extrasynaptic neuronal currents. *Frontiers in Cellular Neuroscience*, *9*(DEC), 474. https://doi.org/10.3389/FNCEL.2015.00474/BIBTEX

Pál, B. (2018). Involvement of extrasynaptic glutamate in physiological and pathophysiological changes of neuronal excitability. *Cellular and Molecular Life Sciences*, *75*(16), 2917–2949. https://doi.org/10.1007/S00018-018-2837-5

Palygin, O., Lalo, U., & Pankratov, Y. (2011). Distinct pharmacological and functional properties of NMDA receptors in mouse cortical astrocytes. *British Journal of Pharmacology*, *163*(8), 1755. https://doi.org/10.1111/J.1476-5381.2011.01374.X

Panatier, A., & Robitaille, R. (2016). Astrocytic mGluR5 and the tripartite synapse. *Neuroscience*, *323*, 29–34. https://doi.org/10.1016/J.NEUROSCIENCE.2015.03.063

Pang, Z. P., & Südhof, T. C. (2010). Cell biology of Ca2+-triggered exocytosis. *Current Opinion in Cell Biology*, *22*(4), 496–505. https://doi.org/10.1016/J.CEB.2010.05.001

Paoletti, P., Bellone, C., & Zhou, Q. (2013). NMDA receptor subunit diversity: impact on receptor properties, synaptic plasticity and disease. *Nature Reviews Neuroscience*, *14*(6), 383–400. https://doi.org/10.1038/nrn3504

Papouin, T., Ladépêche, L., Ruel, J., Sacchi, S., Labasque, M., Hanini, M., Groc, L., Pollegioni, L., Mothet, J.-P. P., Oliet, S. H. R., Ladé Pê Che, L., Rô Me Ruel, J., Sacchi, S., Labasque, M., Hanini, M., Groc, L., Pollegioni, L., Mothet, J.-P. P., Phane, S., & Oliet, H. R. (2012). Synaptic and Extrasynaptic NMDA Receptors Are Gated by Different Endogenous Coagonists. *Cell*, *150*(3), 633–646. http://dx.doi.org/10.1016/j.cell.2012.06.029

Patton, A. J., Genever, P. G., Birch, M. A., Suva, Lj. J., & Skerry, T. M. (1998). Expression of an N-methyl-D-aspartate-type receptor by human and rat osteoblasts and osteoclasts suggests a novel glutamate signaling pathway in bone. *Bone*, *22*(6), 645–649. https://doi.org/10.1016/S8756-3282(98)00061-1

Pinheiro, P. S., Lanore, F., Veran, J., Artinian, J., Blanchet, C., Crepel, V., ... & Mulle, C. (2013). Selective block of postsynaptic kainate receptors reveals their function at

hippocampal mossy fiber synapses. Cerebral cortex, 23(2), 323-331. https://doi.org/10.1093/cercor/bhs022

Pirttimaki, T. M., & Parri, H. R. (2012). Glutamatergic input–output properties of thalamic astrocytes. *Neuroscience*, *205*, 18–28. https://doi.org/10.1016/J.NEUROSCIENCE.2011.12.049

Platt, S. R. (2007). The role of glutamate in central nervous system health and disease–a review. *The Veterinary Journal*, *173*(2), 278–286. https://doi.org/10.1016/J.TVJL.2005.11.007

Poitry-Yamate, C. L., Vutskits, L., Rauen, T., Poitry-Yamate, C. L., Vutskits, L., & Rauen, T. (2002). Neuronal-induced and glutamate-dependent activation of glial glutamate transporter function. *Journal of Neurochemistry*, *82*(4), 987–997. https://doi.org/10.1046/J.1471-4159.2002.01075.X

Potapenko, E. S., Biancardi, V. C., Zhou, Y., & Stern, J. E. (2012). Altered astrocyte glutamate transporter regulation of hypothalamic neurosecretory neurons in heart failure rats. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology*, *303*(3), 291–300. https://doi.org/https://doi.org/10.1152/ajpregu.00056.2012

Povysheva, N. v., & Johnson, J. W. (2012). Tonic NMDA receptor-mediated current in prefrontal cortical pyramidal cells and fast-spiking interneurons. *Journal of Neurophysiology*, *107*(8), 2232–2243. https://doi.org/10.1152/JN.01017.2011

Purves, D., Augustine, G. J., Fitzpatrick, D., Katz, L. C., LaMantia, A.-S., McNamara, J. O., & Williams, S. M. (2001). Glutamate receptors. *Neuroscience*, 137–147.

Qu, D., Ludwig, D. S., Gammeltoft, S., Piper, M., Pelleymounter, M. A., Cullen, M. J., Mathes, W. F., Przypek, J., Kanarek, R., & Maratos-Flier, E. (1996). A role for melanin-concentrating hormone in the central regulation of feeding behaviour. *Nature 1996 380:6571*, *380*(6571), 243–247. https://doi.org/10.1038/380243a0

Ramos, C., Lutzu, S., Yamasaki, M., Yanagawa, Y., Sakimura, K., Tomita, S., Watanabe, M., & Castillo, P. E. (2022). Activation of Extrasynaptic Kainate Receptors Drives Hilar Mossy Cell Activity. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *42*(14), 2872–2884. https://doi.org/10.1523/JNEUROSCI.0922-21.2022

Rauen, T., Rothstein, J. D., & Wässle, H. (1996). Differential expression of three glutamate transporter subtypes in the rat retina. *Cell and Tissue Research*, *286*(3), 325–336. https://doi.org/10.1007/S004410050702

Reiner, A., & Levitz, J. (2018). Glutamatergic signaling in the central nervous system: ionotropic and metabotropic receptors in concert. *Neuron*, *98*(6), 1080–1098. https://doi.org/10.1016/J.NEURON.2018.05.018

Rossi, D. J., Oshima, T., & Attwell, D. (2000). Glutamate release in severe brain ischaemia is mainly by reversed uptake. *Nature*, *403*(6767), 316–321. https://doi.org/10.1038/35002090

Rossi, M., Choi, S. J., O’Shea, D., Miyoshi, T., Ghatei, M. A., & Bloom, S. R. (1997). Melanin-Concentrating Hormone Acutely Stimulates Feeding, But Chronic Administration Has No Effect on Body Weight. *Endocrinology*, *138*(1), 351–355. https://doi.org/10.1210/ENDO.138.1.4887

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*(3), 713–725. https://doi.org/10.1016/0896-6273(94)90038-8

Rothstein, J. D., van Kammen, M., Levey, A. I., Martin, L. J., & Kuncl, R. W. (1995). Selective loss of glial glutamate transporter GLT-1 in amyotrophic lateral sclerosis. *Annals of Neurology*, *38*(1), 73–84. https://doi.org/10.1002/ANA.410380114

Rousseaux, C. G. (2008). A review of glutamate receptors I: current understanding of their biology. *Journal of Toxicologic Pathology*, *21*(1), 25–51. https://doi.org/10.1293/TOX.21.25

Rusnakova, V., Honsa, P., Dzamba, D., Ståhlberg, A., Kubista, M., & Anderova, M. (2013). Heterogeneity of astrocytes: from development to injury–single cell gene expression. *PLoS One*, *8*(8), e69734. https://doi.org/10.1371/JOURNAL.PONE.0069734

Sah, P., Hestrin, S., & Nicoll, R. A. (1989). Tonic activation of NMDA receptors by ambient glutamate enhances excitability of neurons. *Science*, *246*(4931), 815–818. https://doi.org/10.1126/SCIENCE.2573153

Saito, Y., Nothacker, H. P., Wang, Z., Lin, S. H. S., Leslie, F., & Civelli, O. (1999). Molecular characterization of the melanin-concentrating-hormone receptor. *Nature 1999 400:6741*, *400*(6741), 265–269. https://doi.org/10.1038/22321

Salter, M. W., & Kalia, L. v. (2004). Src kinases: a hub for NMDA receptor regulation. *Nature Reviews Neuroscience*, *5*(4), 317–328. https://doi.org/10.1038/nrn1368

Sasaki, T., Beppu, K., Tanaka, K. F., Fukazawa, Y., Shigemoto, R., & Matsui, K. (2012). Application of an optogenetic byway for perturbing neuronal activity via glial photostimulation. *Proceedings of the National Academy of Sciences*, *109*(50), 20720–20725. https://doi.org/10.1073/PNAS.1213458109/SUPPL_FILE/SM01.MOV

Schäfer, M. K. H., Varoqui, H., Defamie, N., Weihe, E., Erickson, J. D., Schäfer, M. K.-H., Varoqui, H., Defamie, N., Weihe, E., & Erickson, J. D. (2002). Molecular cloning and functional identification of mouse vesicular glutamate transporter 3 and its expression in subsets of novel excitatory neurons. *Journal of Biological Chemistry*, *277*(52), 50734–50748. https://doi.org/10.1074/jbc.M206738200

Scimemi, A., Tian, H., & Diamond, J. S. (2009). Neuronal transporters regulate glutamate clearance, NMDA receptor activation, and synaptic plasticity in the hippocampus. *Journal of Neuroscience*, *29*(46), 14581–14595. https://doi.org/10.1523/JNEUROSCI.4845-09.2009

Sedlak, T. W., Paul, B. D., Parker, G. M., Hester, L. D., Snowman, A. M., Taniguchi, Y., Kamiya, A., Snyder, S. H., & Sawa, A. (2019). The glutathione cycle shapes synaptic glutamate activity. *Proceedings of the National Academy of Sciences*, *116*(7), 2701–2706. https://doi.org/10.1073/PNAS.1817885116/SUPPL_FILE/PNAS.1817885116.SAPP.PDF

Sepkuty, J. P., Cohen, A. S., Eccles, C., Rafiq, A., Behar, K., Ganel, R., Coulter, D. A., & Rothstein, J. D. (2002). A neuronal glutamate transporter contributes to neurotransmitter GABA synthesis and epilepsy. *Journal of Neuroscience*, *22*(15), 6372–6379. https://doi.org/10.1523/JNEUROSCI.22-15-06372.2002

Shashidharan, P., Huntley, G. W., Murray, J. M., Buku, A., Moran, T., Walsh, M. J., Morrison, J. H., & Plaitakis, A. (1997). Immunohistochemical localization of the neuron-specific glutamate transporter EAAC1 (EAAT3) in rat brain and spinal cord revealed by a novel monoclonal antibody. *Brain Research*, *773*(1–2), 139–148. https://doi.org/10.1016/S0006-8993(97)00921-9

Shigeri, Y., Seal, R. P., & Shimamoto, K. (2004). Molecular pharmacology of glutamate transporters, EAATs and VGLUTs. *Brain Research Reviews*, *45*(3), 250–265. https://doi.org/10.1016/J.BRAINRESREV.2004.04.004

Shigetomi, E., Bowser, D. N., Sofroniew, M. v., & Khakh, B. S. (2008). Two Forms of Astrocyte Calcium Excitability Have Distinct Effects on NMDA Receptor-Mediated Slow Inward Currents in Pyramidal Neurons. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *28*(26), 6659–6663. https://doi.org/10.1523/JNEUROSCI.1717-08.2008

Shimamoto, K., Sakai, R., Takaoka, K., Yumoto, N., Nakajima, T., Amara, S. G., & Shigeri, Y. (2004). Characterization of novel L-threo-β-benzyloxyaspartate derivatives, potent blockers of the glutamate transporters. *Molecular Pharmacology*, *65*(4), 1008–1015. https://doi.org/10.1124/MOL.65.4.1008

Skowrońska, K., Obara-Michlewska, M., Zielińska, M., & Albrecht, J. (2019). NMDA Receptors in Astrocytes: In Search for Roles in Neurotransmission and Astrocytic Homeostasis. *International Journal of Molecular Sciences*, *20*(2), 309. https://doi.org/10.3390/IJMS20020309

Song, X., Jensen, M. O. Ø., Jogini, V., Stein, R. A., Lee, C.-H. H., McHaourab, H. S., Shaw, D. E., & Gouaux, E. (2018). Mechanism of NMDA receptor channel block by MK-801 and memantine. *Nature*, *556*(7702), 515–519. https://doi.org/10.1038/s41586-018-0039-9

Stanley, B. G., Willett, V. L., Donias, H. W., Dee, M. G., & Duva, M. A. (1996). Lateral hypothalamic NMDA receptors and glutamate as physiological mediators of eating and weight control. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology*, *270*(2 39-2), 443–449. https://doi.org/10.1152/AJPREGU.1996.270.2.R443

Stuber, G. D., & Wise, R. A. (2016). Lateral hypothalamic circuits for feeding and reward. *Nature Neuroscience 2016 19:2*, *19*(2), 198–205. https://doi.org/10.1038/nn.4220

Tanaka, K., Watase, K., Manabe, T., Yamada, K., Watanabe, M., Takahashi, K., Iwama, H., Nishikawa, T., Ichihara, N., & Kikuchi, T. (1997). Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1. *Science*, *276*(5319), 1699–1702. https://doi.org/10.1126/science.276.5319.1699

Ting, J. T., Lee, B. R., Chong, P., Soler-Llavina, G., Cobbs, C., Koch, C., Zeng, H., & Lein, E. (2018). Preparation of Acute Brain Slices Using an Optimized N-Methyl-D-glucamine Protective Recovery Method. *Journal of Visualized Experiments : JoVE*, *132*, 53825. https://doi.org/10.3791/53825

Toossi, H., del Cid-Pellitero, E., & Jones, B. E. (2016). GABA Receptors on Orexin and Melanin-Concentrating Hormone Neurons Are Differentially Homeostatically Regulated Following Sleep Deprivation. *ENeuro*, *3*(3), 420–424. https://doi.org/10.1523/ENEURO.0077-16.2016

Traynelis, S. F., Wollmuth, L. P., McBain, C. J., Menniti, F. S., Vance, K. M., Ogden, K. K., Hansen, K. B., Yuan, H., Myers, S. J., & Dingledine, R. (2010). Glutamate Receptor Ion Channels: Structure, Regulation, and Function. *Pharmacological Reviews*, *62*(3), 405–496. https://doi.org/10.1124/PR.109.002451

Tsai, S. F., Wu, H. T., Chen, P. C., Chen, Y. W., Yu, M., Wang, T. F., Wu, S. Y., Tzeng, S. F., & Kuo, Y. M. (2018). High-fat diet suppresses the astrocytic process arborization and downregulates the glial glutamate transporters in the hippocampus of mice. *Brain Research*, *1700*, 66–77. https://doi.org/10.1016/J.BRAINRES.2018.07.017

Tsukada, S., Iino, M., Takayasu, Y., Shimamoto, K., & Ozawa, S. (2005). Effects of a novel glutamate transporter blocker, (2S, 3S)-3-{3-[4-(trifluoromethyl)benzoylamino]benzyloxy}aspartate (TFB-TBOA), on activities of hippocampal neurons. *Neuropharmacology*, *48*(4), 479–491. https://doi.org/10.1016/J.NEUROPHARM.2004.11.006

Uteshev, V., Stevens, D. R., & Haas, H. L. (1995). A persistent sodium current in acutely isolated histaminergic neurons from rat hypothalamus. *Neuroscience*, *66*(1), 143–149. https://doi.org/10.1016/0306-4522(94)00593-T

van den Pol, A. N., Acuna-Goycolea, C., Clark, K. R., & Ghosh, P. K. (2004). Physiological Properties of Hypothalamic MCH Neurons Identified with Selective Expression of Reporter Gene after Recombinant Virus Infection. *Neuron*, *42*(4), 635–652. https://doi.org/10.1016/S0896-6273(04)00251-X

Vandenberg, R. J., & Ryan, R. M. (2013). Mechanisms of glutamate transport. *Physiological Reviews*, *93*(4), 1621–1657. https://doi.org/10.1152/physrev.00007.2013

Velasco, M., Rojas-Quintero, J., Chávez-Castillo, M., Rojas, M., Bautista, J., Martínez, M. S., Salazar, J., Mendoza, R., & Bermúdez, V. (2017). Excitotoxicity: an organized crime at the cellular level. *Journal of Neurology and Neuroscience*, *8*(3), 0. https://doi.org/10.21767/2171-6625.1000193

Wadiche, J. I., Arriza, J. L., Amara, S. G., & Kavanaugh, M. P. (1995). Kinetics of a human glutamate transporter. *Neuron*, *14*(5), 1019–1027. https://doi.org/10.1016/0896-6273(95)90340-2

Wang, Y. F., & Hatton, G. I. (2009). Astrocytic Plasticity and Patterned Oxytocin Neuronal Activity: Dynamic Interactions. *Journal of Neuroscience*, *29*(6), 1743–1754. https://doi.org/10.1523/JNEUROSCI.4669-08.2009

Warr, O., Takahashi, M., & Attwell, D. (1999). Modulation of extracellular glutamate concentration in rat brain slices by cystine-glutamate exchange. *The Journal of Physiology*, *514*(3), 783–793. https://doi.org/10.1111/j.1469-7793.1999.783ad.x

Weaver, C. D., Yao, T. L., Powers, A. C., & Verdoorn, T. A. (1996). Differential expression of glutamate receptor subtypes in rat pancreatic islets. *Journal of Biological Chemistry*, *271*(22), 12977–12984. https://doi.org/10.1074/jbc.271.22.12977

Wu, Z., Kim, E. R., Hao Sun, X., Xu, Y., Mangieri, L. R., Li, D.-P., Pan, H.-L., Xu, Y., Arenkiel, B. R., & Tong, X. Q. (2015). GABAergic projections from lateral hypothalamus to paraventricular hypothalamic nucleus promote feeding. *Soc Neuroscience*. https://doi.org/10.1523/JNEUROSCI.3720-14.2015

Yamashita, T., & Yamanaka, A. (2017). Lateral hypothalamic circuits for sleep–wake control. *Current Opinion in Neurobiology*, *44*, 94–100. https://doi.org/10.1016/J.CONB.2017.03.020

Yang, Y., Xu-Fried, M. A., & Xu-Friedman, M. A. (2015). Different pools of glutamate receptors mediate sensitivity to ambient glutamate in the cochlear nucleus. *Journal of Neurophysiology*, *113*(10), 3634–3645. https://doi.org/10.1152/JN.00693.2014

Yano, S., Tokumitsu, H., & Soderling, T. R. (1998). Calcium promotes cell survival through CaM-K kinase activation of the protein-kinase-B pathway. *Nature*, *396*(6711), 584–587. https://doi.org/10.1038/25147

Yao, Y., Fu, L. Y., Zhang, X., & van den Pol, A. N. (2012). Vasopressin and oxytocin excite MCH neurons, but not other lateral hypothalamic GABA neurons. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology*, *302*(7), 815–824. https://doi.org/10.1152/AJPREGU.00452.2011/ASSET/IMAGES/LARGE/ZH60061278800007.JPEG

Zhang, M., Biancardi, V. C., & Stern, J. E. (2017). An increased extrasynaptic NMDA tone inhibits A-type K+ current and increases excitability of hypothalamic neurosecretory neurons in hypertensive rats. *The Journal of Physiology*, *595*(14), 4647–4661. https://doi.org/10.1113/JP274327

Zhang, R. wei, Du, W. jie, Prober, D. A., & Du, J. lin. (2019). Müller Glial Cells Participate in Retinal Waves via Glutamate Transporters and AMPA Receptors. *Cell Reports*, *27*(10), 2871-2880.e2. https://doi.org/10.1016/J.CELREP.2019.05.011

Zhang, Y., Wu, S., Xie, L., Yu, S., Zhang, L., Liu, C., Zhou, W., & Yu, T. (2019). Ketamine within clinically effective range inhibits glutamate transmission from astrocytes to neurons and disrupts synchronization of astrocytic SICs. *Frontiers in Cellular Neuroscience*, *13*, 240. https://doi.org/10.3389/FNCEL.2019.00240/BIBTEX

APPENDIX A: Animal Ethics Approval

Animal Care Committee (ACC)

St. John's, NL, Canada A1C 5S7 Tel: 709 777-6620, acs@mun.ca

https://www.mun.ca/research/about/acs/acc/

Researcher Portal File No.: 20220767
Animal Care File: 21-01-MH

Entitled: (21-01-MH) Stereotaxic AAV vector injections for studying feeding-related neurons in the brain Status: Active
Related Awards:

Awards File No	Title	Status	
20171018	Mechanism for high fat diet-induced activation of MCH neurons and its role in obesity	Active	1. Research Grant and Contract Services (RGCS)–St. John's and Grenfell Campuses
20210181	Neuronal and non-neuronal excitatory transmission in the hypothalamus	Active	1. Research Grant and Contract Services (RGCS)–St.

			John's and Grenfell Campuses

Ethics Clearance Terminated: November 2, 2024

Your Animal Use Protocol has been renewed for a three-year term. This file replaces previous 18-03-MH and 19-01- MH that you have combined into the current file. As such, 18-03-MH and 19-01-MH will be closed. Please note the new file ID and Animal Care ID when referring to this protocol.

This ethics clearance includes the following Team Members: Dr. Michiru Hirasawa (Principal Investigator) Dr. Matthew Parsons (Co-Investigator)

This ethics clearance includes the following related awards:

Awards File No	Title	Status	
20171018	Mechanism for high fat diet-induced activation of MCH neurons and its role in obesity	Active	1. Research Grant and Contract Services (RGCS)–St. John's and Grenfell Campuses
20210181	Neuronal and non-neuronal excitatory transmission in the hypothalamus	Active	1. Research Grant and Contract Services (RGCS)–St. John's and Grenfell Campuses

An Event [Annual Report] will be required following each year of protocol activity.

Should you encounter an unexpected incident that negatively affects animal welfare or the research project relating to animal use, please submit an Event [Incident Report].

Any alterations to the protocol requires prior submission and approval of an Event [Amendment].