Synaptic Vesicle Glycoprotein 2A alterations in focal epilepsy

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Abstract

Positron emission tomography (PET) with [^{11}C]UCB-J, which targets the synaptic vesicle glycoprotein 2A (SV2A), can demonstrate synaptic loss within the epileptogenic hippocampus of mesial temporal lobe epilepsy (mTLE) patients *in vivo*. To investigate mTLE-associated synaptic changes *in vitro*, we conducted a saturation autoradiography study with [^{3}H]UCB-J, a radioligand that specifically binds to this protein. We aimed to quantify SV2A density (B_{max}) and [^{3}H]UCB-J dissociation constants (K_D) in surgical hippocampal specimens from mTLE patients, compared to non-epilepsy post-mortem control hippocampi from necropsy. We observed a 45.5% decrease in B_{max} in mTLE compared to controls, which was independent of age at brain tissue collection and of post-mortem delay (PMD) in the control group. This decrease suggests synapse loss, downregulation of SV2A, or loss of synaptic vesicles. mTLE specimens also showed a 50.6% decrease in K_D, which was also independent of age and PMD. This change in K_D may represent an alteration to SV2A conformational state associated with the pathophysiology of mTLE or to the occurrence of seizures. We observed significant correlations between B_{max} and age at epilepsy onset/duration of epilepsy. Duration of epilepsy was also correlated with K_D. These findings are consistent with further progression of synaptic changes as duration of epilepsy increases. This is not surprising as epilepsy is a network disease and synaptic abnormalities are an underlying feature of epileptogenicity.

Furthermore, dysregulation of neurotransmitter systems and their constituents has been associated with the etiology of epilepsy. Abnormalities in the expression and availability of the metabotropic glutamate receptor type 5 (mGluR5) have been observed within the epileptogenic hippocampus of mTLE patients. In particular, using the radioligand [^{3}H]ABP688, which binds to mGluR5's allosteric site, we have previously demonstrated reduced mGluR5 availability within

surgical hippocampal specimens from intractable mTLE patients. To determine if there exists a relationship between synaptic changes and observed mGluR5 tissue abnormalities within the epileptogenic hippocampus of mTLE patients, we correlated the B_{max} values from our previous [^{3}H]ABP688 study with the [^{3}H]UCB-J B_{max} values from the current study. We observed a significant positive correlation between [^{3}H]ABP688 B_{max} and [^{3}H]UCB-J B_{max}, suggesting that a relationship exists between SV2A and mGluR5 receptor density in the epileptogenic hippocampus of mTLE patients.

Focal cortical dysplasia (FCD) is another intrinsically epileptogenic lesion resulting from a developmental malformation of cortex. SV2A abnormalities have also been observed within the borders of FCD lesions, however, to the best of our knowledge, SV2A receptor density has not yet been quantified within these lesions. We therefore characterized for the first time in FCD lesions, SV2A density and [^{3}H]UCB-J-SV2A binding affinity within the lesion's borders.

Résumé

La tomographie par émission de positrons (TEP) avec [^{11}C]UCB-J, qui cible la glycoprotéine 2A de la vésicule synaptique (SV2A), peut démontrer une perte synaptique dans l'hippocampe épileptogène des patients atteints d'épilepsie du lobe temporal mésial (ELTm) *in vivo*. Pour étudier les changements synaptiques associés à ELTm *in vitro*, nous avons mené une étude d'autoradiographie à saturation avec [^{3}H]UCB-J, un radioligand qui se lie spécifiquement à cette protéine. Nous avons cherché à quantifier la densité de SV2A (B_{max}) et les constantes de dissociation [^{3}H]UCB-J (K_D) dans des spécimens chirurgicaux d'hippocampe de patients ELTm par rapport à des hippocampes de contrôle post-mortem non épileptiques provenant d'une autopsie. Nous avons observé une diminution de 45,5 % de B_{max} dans le ELTm par rapport aux contrôles, ce qui était indépendant de l'âge au moment de la collecte des tissus cérébraux et du délai post-mortem (DPM) dans le groupe contrôle. Cette diminution suggère une perte de synapse, une régulation négative de SV2A, ou une perte de vésicules synaptiques. Les échantillons ELTm ont également montré une diminution de 50,6 % du K_D, qui était également indépendante de l'âge et de la DPM. Ce changement de K_D peut représenter une altération de l'état conformationnel du SV2A associée à la physiopathologie de ELTm ou à la survenue de crises. Nous avons observé des corrélations significatives entre B_{max} et l'âge au début de l'épilepsie/la durée de l'épilepsie. La durée de l'épilepsie était également corrélée avec la K_D. Ces résultats sont cohérents avec une progression supplémentaire des modifications synaptiques à mesure que la durée de l'épilepsie augmente. Cela n'est pas surprenant car l'épilepsie est une maladie de réseau et les anomalies synaptiques sont une caractéristique sous-jacente de l'épileptogénicité.

De plus, la dérégulation des systèmes de neurotransmetteurs et de leurs constituants a été associée à l'étiologie de l'épilepsie. Des anomalies dans l'expression et la disponibilité du récepteur

métabotropique du glutamate de type 5 (mGluR5) ont été observées dans l'hippocampe épileptogène de patients atteints de ELTm. En particulier, en utilisant le radioligand [^{3}H]ABP688, qui se lie au site allostérique de mGluR5, nous avons précédemment démontré une disponibilité réduite de mGluR5 dans des échantillons chirurgicaux d'hippocampe de patients ELTm intraitables. Pour déterminer s'il existe une relation entre les changements synaptiques et les anomalies tissulaires mGluR5 observées dans l'hippocampe épileptogène des patients atteints de ELTm, nous avons corrélé les valeurs B_{max} de notre précédente étude [^{3}H]ABP688 avec les valeurs [^{3}H]UCB-J B_{max} de l'étude actuelle. Nous avons observé une corrélation positive significative entre [^{3}H]ABP688 B_{max} et [^{3}H]UCB-J B_{max}, suggérant qu'il existe une relation entre la densité des récepteurs SV2A et mGluR5 dans l'hippocampe épileptogène des patients atteints de ELTm.

La dysplasie corticale focale (DCF) est une autre lésion intrinsèquement épileptogène résultant d'une malformation du développement du cortex. Des anomalies SV2A ont également été observées dans les limites des lésions DCF, cependant, à notre connaissance, la densité des récepteurs SV2A n'a pas encore été quantifiée dans ces lésions. Nous avons donc caractérisé pour la première fois la densité de SV2A et l'affinité de liaison [^{3}H]UCB-J-SV2A dans les limites de la lésion.

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Contribution of Authors

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Common Abbreviations

B_{max}	Maximum Specific Binding
CA	*Cornu Ammonis*
FCD	Focal Cortical Dysplasia
GABA	Gamma-Aminobutyric Acid
HS	Hippocampal Sclerosis
IHC	Immunohistochemistry
K_D	Dissociation Constant
LEV	Levetiracetam
LTP	Long-Term Potentiation
LTD	Long-Term Depression
mGluR5	Metabotropic Glutamate Receptor Type 5
(m)TLE	(Mesial) Temporal Lobe Epilepsy
MTS	Mesial Temporal Sclerosis
PET	Positron Emission Tomography
PMD	Post-Mortem Delay
ROI	Region of Interest
SV2(A/B/C)	Synaptic Vesicle Glycoprotein 2(A/B/C)
VFD	Venus Flytrap Domain

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Introduction

Epilepsy is a chronic neurological disorder marked by an imbalance of excitatory and inhibitory neural activity leading to recurrent and unprovoked seizures (Staley, 2015). It affects upwards of 50 million people worldwide, accounting for a significant proportion of the world's disease burden (Behr et al., 2016; Ngugi et al., 2010).

In temporal lobe epilepsy (TLE), the most common type of focal epilepsy (Blair, 2012), seizures often originate within the mesial temporal structures, particularly within the hippocampus (Tatum, 2012). Mesial temporal lobe seizures are often resistant to pharmacological treatment (Téllez-Zenteno & Hernández-Ronquillo, 2012), and patients with medically intractable seizures may be offered surgical treatment to remove the affected brain tissue. The mechanisms underlying onset of temporal lobe seizures and mesial TLE (mTLE) are not well understood, limiting the development of effective disease-modifying interventions. The most common underlying neuropathology in mTLE is mesial temporal sclerosis (MTS).

Focal cortical dysplasia (FCD), which is a form of developmental malformation of the cortex, is the most common cause of refractory epilepsy in children, and the second most common cause of intractable epilepsy in adults, in particular in extra-temporal epilepsies (Kabat & Król, 2012). FCD is characterized by abnormal cytoarchitecture within the cerebral cortex and is related to faulty neuronal migration and/or differentiation (Bast et al., 2006; Crino, 2015; Tahta & Turgut, 2020; Thom et al., 2005). The mechanism through which these abnormal neurons cause seizures is still poorly understood.

Epilepsy is associated with various changes at the molecular, cellular, and structural levels of brain organization and function (Badawy et al., 2009). Epileptogenicity (i.e., the capacity to develop spontaneous seizures) is currently understood to be a consequence of the establishment of

an abnormal neuronal network. Reorganization of cells and networks within the brain are thought to enable the neuronal hyperexcitability characteristic of the disorder. These modifications are extremely diverse, some of which may be specific to certain epilepsies and anatomical locations, while others could play a common role in a broader epileptogenic mechanism shared by different types of neuropathology abnormalities.

Dysregulation of several neurotransmitter systems has been associated with the etiology of epilepsies. Decreased inhibition, resulting from impairment of the gamma-aminobutyric acid (GABA) neurotransmission system, and increased excitation, resulting from hyperactivation of the glutamatergic neurotransmission system, are equally thought to contribute to increased excitation. Observed abnormalities in catecholamine signalling could also play a role in the loss of balance between neural excitation and inhibition (Engelborghs et al., 2000). Although these neurotransmitter systems have been shown to be altered in epilepsy, the molecular underpinnings leading to such an imbalance, and their association with observed neurophysiological and structural changes, remain unclear.

The synaptic vesicle glycoprotein 2A (SV2A), a transmembrane protein expressed on synaptic vesicles throughout the brain, plays an important role in the regulation of neurotransmission (Stout et al., 2019). Although the exact function of SV2A remains elusive, it has been proposed to regulate the activity of synaptotagmin, a calcium-sensitive modulator of transmitter release, and serve as a galactose transporter (Madeo et al., 2014; Rossi et al., 2022). As SV2A is expressed ubiquitously at synapses throughout the brain, it has gained interest not just for its role in neurotransmission, but also for its potential as a marker of synaptic density. In addition, changes to SV2A expression have been associated with epilepsy in humans and in mouse models of mTLE, which may or may not be linked to changes to synaptic density (Crèvecœur et al., 2014;

Crowder et al., 1999; Janz et al., 1999). In humans, SV2A immunostaining demonstrated decreased SV2A expression in the hippocampus of temporal lobe epilepsy patients with hippocampal sclerosis (HS) within areas of synaptic loss (Crèvecœur et al., 2014). SV2A knockout mouse models were shown to exhibit severe seizures and die postnatally (Crowder et al., 1999; Janz et al., 1999).

Neuronal and synaptic loss as well as reactive gliosis have been demonstrated in the sclerotic hippocampi resected from drug-resistant mTLE patients (Crespel et al., 2002; Kandratavicius et al., 2013; Looney et al., 1999). *In vivo* positron emission tomography (PET) with [^{11}C]UCB-J, a radiopharmaceutical that binds to SV2A, enables whole brain *in vivo* imaging of reduced synaptic density in mTLE patients (Finnema et al., 2016; Finnema et al., 2020).

The objective of this project is to quantify SV2A in surgically-resected hippocampi of mTLE patients, using *in vitro* [^{3}H]UCB-J saturation autoradiography, to shed light on synaptic changes associated with mTLE. We will also investigate the binding affinity of [^{3}H]UCB-J for SV2A to determine if the protein undergoes any conformational changes associated with pathophysiology of mTLE. A control group will consist of post-mortem hippocampal specimens from non-epilepsy controls. Furthermore, we aim to characterize for the first time SV2A density and binding affinity in the dysplastic cortex of FCD patients.

Background

Mesial Temporal Lobe Epilepsy (mTLE)

mTLE is the most common form of epilepsy observed in adults (Blair, 2012). In mTLE, seizures originate from mesial temporal regions such as the hippocampus and amygdala, with resistance to antiseizure medications (ASM) in approximately one-third of cases (Blümcke et al., 1999; Fernandes et al., 2015). Seizures in mTLE patients will generally manifest as focal onset seizures with or without impaired awareness (Nayak & Bandyopadhyay, 2022). Focal seizures can further be classified as motor (e.g., automatisms such as lip-smacking or wandering) or non-motor (e.g., cognitive seizures, which could involve impairments to language or spatial perception). Less often, a seizure may start focally and spread bilaterally, known as a focal to bilateral tonic-clonic seizure (Fisher, 2017). Many patients with intractable mTLE have a clinical history of febrile seizures during early childhood (French et al., 1993).

Patients with intractable mTLE may be offered surgical treatment, which considerably diminishes or abolishes seizures in most patients (Mathon et al., 2015; Téllez-Zenteno et al., 2005). The most common neuropathological finding in the surgical specimens from these patients is HS, which shows a characteristic pattern of severe neuronal loss and gliosis (Blümcke et al., 2012). HS can be divided into subtypes based on the pattern of severe neuronal loss and gliosis within hippocampal subregions. The subtypes of HS include severe neuronal loss and gliosis predominantly in *cornu ammonis* 1 (CA1) and CA4 (HS type 1), CA1 predominant severe neuronal loss and gliosis (HS type 2) and CA4 predominant severe neuronal loss and gliosis (HS type 3) (Blümcke et al., 2013).

Focal Cortical Dysplasia (FCD)

FCD is another common cause of intractable epilepsy, in particular extratemporal lobe epilepsies (Kabat & Król, 2012). Seizures usually begin in childhood, although seizure onset may occur in later life as well (Fauser et al., 2006). Disruption of cellular migration and/or differentiation during development can lead to abnormal cortical lamination, neuronal maturation and/or differentiation, which can result in neurological disorders such as epilepsy and cognitive impairment (Gaitanis & Donahue, 2013). Seizure semiology in FCD is determined by the anatomical location of the lesion. For example, patients with occipital lobe seizures may report visual symptoms, whereas patients with a frontal lobe lesion may present with sleep-related seizures (Crino, 2015).

Surgical resection of the epileptogenic cortex is also an option for FCD patients, in whom seizures are typically drug-resistant, with 43-75% of patients achieving seizure freedom or freedom from disabling seizures after surgery (Edwards et al., 2000; Tassi et al., 2002; Widdess-Walsh et al., 2005).

FCD can be subdivided based on histopathological findings. FCD type I presents as cortical dyslamination, with either abnormal radial cortical lamination (Ia), abnormal tangential cortical lamination (Ib), or both (Ic). FCD type II consists of cortical dyslamination and the presence of dysmorphic neurons, without (IIa) or with (IIb) balloon cells. Dysmorphic neurons are defined by a significantly enlarged cell body and nucleus, abnormal intracellular distribution of Nissl substance, and the accumulation of neurofilaments in the cytoplasm . Balloon cells also present with an enlarged cell body, generally with multiple nuclei, and may be derived from the abnormal development of neural stem cells (Blümcke et al., 2011; Oh et al., 2008). FCD type III consists of FCD associated with another lesion/pathology, including hippocampal sclerosis (IIIa), tumours

(IIIb), vascular malformations (IIIc), or early-life lesions (IIId) (Blümcke et al., 2011; Gaitanis & Donahue, 2013).

Synapses and Synaptic Density

Synapses are crucial components of the nervous system that enable signalling throughout the brain. Proper synaptic communication is essential for normal brain function. Perturbations of synaptic structure and machinery have the potential to disrupt the brain's homeostasis, leading to faulty neuronal signalling and the development of pathology (Lepeta et al., 2016). Evidence accumulated thus far points to a role for aberrant synaptic structure and function in various neurological and neurodevelopmental disorders, including Alzheimer's and Parkinson's diseases, autism spectrum disorder, and epilepsy (Lepeta et al., 2016; van Spronsen & Hoogenraad, 2010).

Synaptic density alterations underlie several neuropathological features within epileptogenic foci, contributing to the establishment of an aberrant network that enables seizure onset and propagation. Reduced dendritic branching, dendritic spine loss, and neuronal and synaptic loss have been observed *ex vivo* in epilepsy patients (Alonso-Nanclares et al., 2011; von Campe et al., 1997). Immunohistochemistry with synaptophysin, a marker of synaptic density, has also demonstrated synaptic loss in the hippocampus of mTLE patients (Looney et al., 1999; Proper et al., 2000). Moreover, synaptic density changes correlate with abnormalities in neuroreceptor expression and function within the seizure zone.

Synaptic Vesicle Glycoprotein 2A (SV2A)

SV2A is an integral transmembrane protein expressed ubiquitously on synaptic vesicles throughout the brain (Löscher et al., 2016; Madeo et al., 2014). It is a member of the major

facilitator superfamily of transporters, a group of proteins involved in the transport of various substrates across biological membranes (Quistgaard et al., 2016). SV2A has 12 transmembrane domains, an amino domain, a loop region, and an extracellular domain, and has been shown to possess two major conformational states, however, little else is known about its structure (Bajjalieh et al., 1992; Löscher et al., 2016; Lynch et al., 2008). SV2A is one of three members of the SV2 family (SV2A, SV2B, and SV2C) all encoded by separate genes with approximately 60% sequence homology (Bartholome et al., 2017). Each SV2 protein is believed to play a crucial role in synaptic function. In neurons, SV2A has been proposed to function as a modulator of calcium-mediated neurotransmitter release, a regulator of calcium sensor protein synaptotagmin expression and trafficking and may also serve as a galactose transporter (Madeo et al., 2014; Rossi et al., 2022).

SV2A emerged as a protein of interest in the study of epilepsy after the discovery that it was the binding site of the anti-seizure medication levetiracetam (LEV) (Lynch et al., 2004). In addition, SV2A knockout mouse models were shown to express seizures at early postnatal age, which has been linked to loss of hippocampal CA3 inhibitory GABA transmission (Crowder et al., 1999; Janz et al., 1999; Kaminski et al., 2009). In mTLE patients, reduced hippocampal SV2A expression has been shown by immunocytochemistry and Western blot analyses (Crèvecœur et al., 2014; van Vliet et al., 2009). Reductions in SV2A immunoreactivity closely matched the pattern of synaptophysin staining (Crèvecœur et al., 2014). Additionally, a homozygous mutation in the SV2A gene has been identified in an individual with intractable epilepsy, suggesting SV2A abnormalities as underlying pathophysiological mechanisms (Serajee & Huq, 2015). Furthermore, when mouse neuronal cultures were genetically-engineered to express this SV2A mutant identified in human disease, the mutant SV2A was found to be mislocalized from synaptic vesicles to the

plasma membrane. In addition, the mutant SV2A demonstrated reduced binding to synaptotagmin-1 (Harper et al., 2020).

Recently, the PET radioligand [^{11}C]UCB-J, which specifically binds to SV2A, has been validated as a tool for *in vivo* imaging of synaptic density in the human brain, correlating highly with the synaptic marker synaptophysin (Finnema et al., 2016). The advantages of *in vivo* imaging of SV2A are many. It allows evaluation of the whole brain as compared to tissue resected from surgery and therefore provides means to determine whether changes are specific to the brain regions generating seizures or diffusely found in the epileptic brain. Moreover, it can evaluate patients that are not surgical candidates and healthy subjects, allowing assessment of abnormalities as a factor of presence of disease and disease burden.

Reduced *in vivo* PET [^{11}C]UCB-J binding, compared to non-epilepsy control hippocampi, has been correlated to synaptic loss in the epileptogenic hippocampus of mTLE patients (Finnema et al., 2016; Finnema et al., 2020). The observed alterations to SV2A expression and availability suggest that either synaptic density changes, or specific SV2A changes, play an important role in the pathophysiology of mTLE.

Changes to SV2A expression and availability are not specific to mTLE but can be found in other forms of epilepsy as well. In FCD type IIB, an intrinsically epileptogenic lesion, reduced SV2A immunoreactivity along with reduction in protein expression confirmed via Western blot analysis have been described (Toering et al., 2009). Furthermore, synaptic loss within the FCD lesion has been demonstrated *in vivo* using PET with another radioligand specific for SV2A named 18F-SynVesT-1 (Y. Tang et al., 2022).

Glutamatergic Abnormalities in Epilepsy

Glutamate is the primary excitatory neurotransmitter in the human brain; thus, the glutamatergic system is of high interest in the study of epilepsy. Glutamate mediates neuronal excitation by binding to several classes of receptors to initiate fast excitatory neurotransmission and slower long-term plastic changes (Barker-Haliski & White, 2015). Heightened levels of glutamate can cause excitotoxicity – wherein excessive activation of ionotropic glutamate receptors leads to cell death and loss of synaptic structures (Lau & Tymianski, 2010). Therefore, glutamate is typically maintained at low levels extracellularly, and its expression is tightly controlled (Mahmoud et al., 2019).

Levels of glutamate have been shown to be chronically elevated in patients with various medically refractory epilepsies (Çavuş et al., 2016). In epileptogenic hippocampi, glutamate levels were found to be increased even further – to potentially neurotoxic concentrations – during a seizure (During & Spencer, 1993). In rodent epilepsy models, agonists of the inotropic glutamate receptors have been used to elicit seizures, whereas antagonists have demonstrated potent antiepileptic effects (Faught, 2014; Hanada et al., 2011; Kaminski et al., 2004; Kienzler-Norwood et al., 2017; Yen et al., 2004). In addition, in mTLE patients with HS, increased expression of ionotropic glutamate receptor mRNA has been observed (Mathern et al., 1997).

Metabotropic Glutamate Receptor Type 5

The metabotropic glutamate receptor type 5 (mGluR5) is a G-protein coupled transmembrane protein expressed mainly in the periphery of the postsynaptic terminal of neurons, and within glial cells (Niswender & Conn, 2010; Spampinato et al., 2018). The receptor is normally expressed as dimer bound by disulfide bonds, with each protomer containing a large N-terminal Venus flytrap domain (VFD), seven transmembrane domains, and an intracellular C-terminus

(Niswender & Conn, 2010; Romano et al., 1996). When extracellular glutamate binds to the orthosteric site within the VFD, the ensuing conformational change to the receptor activates the G protein. Once activated, G-proteins will initiate signalling cascades that can affect the function of various enzymes, ion channels, and transcription factors (Niswender & Conn, 2010), alter gene transcription at transcriptional and translational levels, and cause the release of Ca2+ from intracellular stores (Wang & Zhuo, 2012).

mGluR5 functions as a regulator of neuronal excitability and a promoter of synaptic plasticity under normal conditions (Anwyl, 1999). Depending on the level of intracellular calcium, mGluR5 may potentiate or inhibit NMDA (N-methyl-D-aspartate) receptor responses (Gerber et al., 2007) affecting long-term potentiation (LTP) or long-term depression (LTD) (Harney et al., 2006). Furthermore, modulation of hippocampal mGluR5 activity by both agonists and antagonists has been associated with altered LTP and LTD (Bikbaev et al., 2008; Watabe et al., 2002).

Activation of mGluR5 has been associated with persistent ictogenic effects and neuronal hyperexcitability (Bianchi et al., 2012; Merlin, 2002; Qian & Tang, 2016). Furthermore, abnormal mGluR5 expression has been observed in surgical specimens resected from drug-resistant mTLE patients and in rat models of mTLE. Immunohistochemistry (IHC) studies have shown increased mGluR5 immunoreactivity within the epileptogenic hippocampus of drug-resistant mTLE patients, independent of a neuropathological diagnosis of hippocampal sclerosis (Kandratavicius et al., 2013; Notenboom et al., 2006; F.-R. Tang et al., 2002). Abnormalities in mGluR5 expression have also been demonstrated *in vivo*. PET studies with [^{11}C]ABP688 (a highly-selective negative allosteric modulator of mGluR5), have shown reduced mGluR5 availability in the involved hippocampus of mTLE patients (Lam et al., 2019) and within the epileptogenic lesion of patients with FCD (DuBois et al., 2016).

Reduced [^{3}H]ABP688 Binding in mTLE Hippocampi

We have previously shown reduced [^{3}H]ABP688 binding in the surgically-resected hippocampi of drug-resistant mTLE patients that is not attributable to changes to the binding affinity of the radioligand-receptor pair (Zimmermann et al., 2022). We have proposed that these reductions may signify mGluR5 internalization or conformational changes that reduce [^{3}H]ABP688 binding. As a receptor expressed post-synaptically, alterations to mGluR5 expression or availability may be linked to changes to synaptic density. However, it is not currently known if reduced [^{3}H]ABP688 binding relates to the synaptic losses observed in the epileptogenic hippocampus of drug-resistant mTLE patients.

Saturation Autoradiography

Autoradiography is a gold standard technique for investigating the tissue distribution of radiolabelled molecules *in vitro* by exposing biological material (such as brain tissue) labelled with a radioisotope to a detection screen, such as an X-ray film or phosphor imaging plate. Saturation autoradiographic techniques can be used to generate saturation binding curves, from which the maximum specific binding (B_{max}) and dissociation constants (K_D) can be extrapolated. In quantitative saturation autoradiography, the maximum specific binding typically corresponds to the density of the target protein/receptor. Therefore, B_{max} is often used interchangeably with 'receptor density'. Dissociations constants represent the tendency of the radioligand to separate from its protein target, therefore, the inverse of K_D represents the affinity of the radioligand-receptor or radioligand-protein pair. Many of the radioligands used in autoradiography are common PET tracers as well, making saturation autoradiography an advantageous technique for

validating results from *in vivo* PET studies, compared to other *ex vivo* techniques, such as immunohistochemistry.

Rationale and Aims

1) Assessing [^{3}H]UCB-J Binding (B_{max}) and Affinity (K_D) for SV2A in mTLE Hippocampi

Although reductions in [^{11}C]UCB-J binding potentials in the mesial structures of mTLE patients have been demonstrated *in vivo*, these findings remain to be quantitatively validated *in vitro*. Therefore, the *first aim* of this study is to assess *in-vitro* changes to synaptic density in surgically-extracted hippocampi of drug-resistant mTLE patients compared to non-epileptic control hippocampi from necropsy, using [^{3}H]UCB-J autoradiography.

We therefore *hypothesize that* (1) reductions in [^{3}H]UCB-J binding will be observed in the hippocampus of mTLE patients compared to non-epileptic control specimens, and (2) [^{3}H]UCB-J binding affinity for SV2A will not significantly differ between groups.

2) Correlating [^{3}H]UCB-J and [^{3}H]ABP688 Binding (B_{max}) in mTLE Hippocampi

The exact mechanism underlying mGluR5 abnormalities in mTLE, as well as their role in epileptogenicity have not yet been elucidated. Neuronal loss and synaptic changes being hallmarks of the epileptogenic hippocampus, evaluating the interplay between synaptic density alterations and changes in mGluR5 expression *in vitro* could elucidate a possible cause or consequence role for tissue mGluR5 abnormalities.

Hence, the *second aim* of this study is to assess the relationship between *in vitro* synaptic density levels and mGluR5 expression within mTLE patient hippocampi.

We hypothesize that observed reductions in synaptic density will positively correlate with the decreases in mGluR5 seen via previous [^{3}H]ABP688 autoradiography (Zimmermann et al., 2022).

3) Characterizing [^{3}H]UCB-J Binding (B_{max}) and Affinity (K_D) in FCD

Although qualitative SV2A reductions within FCD lesions of patients have been described previously (Y. Tang et al., 2022; Toering et al., 2009), to the best of our knowledge, no study has yet been undertaken to quantify the expression of this protein within the borders of the lesion. Additionally, the binding affinity of UCB-J to SV2A within this type of lesion is unknown.

Therefore, we aim to characterize for the first time the *in vitro* SV2A density, and the binding affinity between UCB-J and SV2A, within the FCD lesion surgically resected from drug-resistant epilepsy patients.

Methods

Ethics

This study was approved by the Montréal Neurological Institute Research Ethics Board. All patients provided written informed consent for research on their resected brain tissue.

Brain Tissue Sampling and Preparation

Flash-frozen specimens from mTLE hippocampi (N=26, age at surgery 18-67) and FCD lesions (N=6, age at surgery 22-55) were obtained from drug-resistant patients who underwent surgery. All patients underwent surgical resection of their epileptogenic brain tissue following standard of care pre-surgical evaluation at the Epilepsy Monitoring Unit of the Montréal Neurological Institute. Samples for this study were obtained at the Neuropathology Department, consisting of sections deemed no further contributory after the clinical neuropathological diagnosis was made.

Non-epilepsy post-mortem control samples (Table 1) from necropsy (14, aged 18-91) were procured from the Douglas Bell Canada Brain Bank (Montréal, Canada).

Using a cryostat at -20°C, each brain tissue block was cut into serial 20μm-thick sections and thaw-mounted on microscope slides. The slides were stored at -80°C prior to the start of the experiment. Hippocampal specimens were cut in a coronal orientation, and FCD specimens were cut perpendicularly to the pial surface, to display gray matter and white matter whenever present.

[^{3}H]UCB-J and [^{3}H]ABP688 Autoradiography

[^{3}H]UCB-J was provided by UCB through an Investigator Initiated Agreement for use in research. On the day of the binding assay, the prepared frozen tissue sections were thawed to room

temperature (approximately 20 minutes) and pre-incubated in a 50mM Tris-HCl buffer solution (50mM, 0.9% NaCl), with a pH of 7.4 (adjusted by HCl) for 20 minutes. The sections were then air-dried and incubated in the same buffer solution containing one of six different concentrations (0.625-20nM) of [^{3}H]UCB-J for 1 hour. After the incubation with [^{3}H]UCB-J, the sections were washed 3 times in ice-cold incubation buffer (5 minutes/wash) and dipped once in ice-cold distilled water for 30 seconds. The sections were then air-dried at room temperature and placed in a desiccator with paraformaldehyde powder for mild fixation (1 day). Sections were then exposed on tritium-sensitive phosphor imaging plates for 1 week (Fujifilm). Autoradiographic calibration was performed using industrial tritium standards (American Radiolabelled Chemicals) exposed on the same imaging plates. The plates were scanned on an Amersham Typhoon biomolecular imager (spatial resolution 20μm).

The method for the previous [^{3}H]ABP688 autoradiography is described in Zimmermann et al. (2022). In brief, the protocol was the same as for the above [^{3}H]UCB-J autoradiography, with the exception of an Na HEPES solution (30mM Na HEPES, 110mM NaCl, 5mM KCl, 2.5mM $CaCl_2$, 1.2mM $MgCl_2$, with a pH of 7.4 adjusted by NaOH) used as the buffer, and 10μM MPEP was used as a blocking agent to assess non-specific binding in 3 sections per specimen. [^{3}H]ABP688 concentrations ranged from 0.25-8nM.

Image Analysis

The average grey value per square pixel was measured for each region of interest (ROI) using ImageJ software (Fiji, https://imagej.net/software/fiji/, RRID:SCR_002285).

Because not all mTLE patients underwent an *en bloc* anterior temporal resection involving complete removal of the hippocampus, not all specimens displayed a complete section of all

hippocampal subfields in a coronal tissue cut. Hippocampal ROIs included the dentate gyrus, *cornu ammonis* (CA) areas, and subiculum, when visible. ROIs for FCD specimen consisted of all grey matter visible within the slice.

Non-specific binding in all groups was determined via sampling of white matter at 3 non-overlapping regions adjacent to the hippocampus/cortical grey matter. Radioactivity concentrations in the tissue (nCi/mg) were determined using a calibration curve constructed from the tritium standards, with values corrected for tissue equivalency. SV2A concentrations were expressed in fmol/mg using the specific activity of the [^{3}H]UCB-J sample (24 Ci/mmol). Using GraphPad Prisms' (RRID:SCR_002798) one-site saturation binding model, specific binding was calculated as the difference between total and non-specific binding. Saturation binding parameters B_{max} (maximum specific binding) and K_D (dissociation constant) were extrapolated from the saturation binding curve. B_{max} values correspond to receptor density. Dissociation constants (K_D) were assessed to determine the binding affinity of [^{3}H]UCB-J to SV2A in each group.

Statistical Analysis of [^{3}H]UCB-J Data

Differences in B_{max} values between non-epilepsy controls and mTLE hippocampi were assessed with an unpaired t-test, while differences in K_D were assessed with a Mann Whitney U test. Group differences in age and sex distribution were assessed with a Mann Whitney U test and Chi square test, respectively. Correlations between B_{max} and age (at death, controls; at surgery, mTLE) were assessed to demonstrate the independence of B_{max} from age in both groups. Similarly, the correlation between age and K_D was assessed in both groups. Spearman correlations were used to demonstrate the independence of B_{max} and K_D from post-mortem delay (PMD) in the control group. Where an exact PMD was not available (2 controls), PMD was estimated as the center of

the post-mortem interval. Differences in B_{max} and K_D between males and females were assessed with Mann Whitney U tests.

Correlations between saturation binding parameters (B_{max} and K_D) and age at epilepsy onset, duration of epilepsy (years until surgery), age at surgery, and frequency of seizures (focal impaired awareness seizures per month in the year prior to surgery) for the mTLE group were assessed with the appropriate Pearson or Spearman correlation. Information on age of epilepsy onset, duration of epilepsy, and frequency of seizures was not available for all 25 patients (2, 2, and 3, respectively), therefore, the analyses were limited to patients for whom the information was available. The correlation between age at epilepsy onset and epilepsy duration in the mTLE group was also assessed with a Spearman correlation.

Statistical differences in B_{max} and K_D between mTLE patients who were treated with LEV prior to surgery (n = 5) and those who were not treated with LEV (n = 20) were assessed with the appropriate two-sample test. Differences in B_{max} and K_D were also assessed between mTLE patients who achieved seizure freedom following surgery (n = 18) and patients who did not achieve seizure freedom (n = 7), patients whose resected tissue was obtained from the left (n = 12) vs. right (n = 13) hemisphere, and patients whose pathologic diagnosis of the resected specimen displayed no definite abnormality (n = 5) vs. those who demonstrated either gliosis or mesial temporal sclerosis (MTS) (n = 20). Seizure freedom status was determined by Engel classification (Ia = seizure free; Ib–IV = non-seizure-free).

As we did not obtain cortical specimens from necropsy controls that corresponded to the exact anatomical location for each FCD specimen evaluated in this study, no comparison with non-epilepsy tissue was made for this group.

Correlating [^{3}H]UCB-J and [^{3}H]ABP688 Autoradiography

Hippocampal tissue sections from 14 mTLE patients were included in both the present [^{3}H]UCB-J autoradiography and the previous [^{3}H]ABP688 autoradiography (Zimmermann et al., 2022). Slides for [^{3}H]UCB-J contained cryostat slices sequential to those used for [^{3}H]ABP688 autoradiography.

Images from the prior [^{3}H]ABP688 autoradiography were re-analyzed to match as closely as possible to the hippocampal ROIs selected for the [^{3}H]UCB-J analysis. Updated B_{max} values from the [^{3}H]ABP688 autoradiography were correlated with B_{max} values from the present [^{3}H]UCB-J autoradiography to investigate the relationship between synaptic density and mGluR5 receptor density in the hippocampus of mTLE patients.

Characterizing SV2A Density and Binding Affinity in FCD Specimens

B_{max} values obtained from Prisms' one-site saturation binding model were used to determine mean receptor density in the FCD specimens. K_D values were used to characterize median binding affinity of [^{3}H]UCB-J to SV2A within the dysplastic cortex.

Results

Demographics

One mTLE specimen (P31) was excluded from all analyses due to poor fitting of the one-site saturation binding model (resulting in unreliable parameter estimation), constituting an outlier.

There was no significant difference in sex distribution between mTLE patients (Table 2) and controls: $\chi 2(1) = 0.3003$; $p = 0.5837$. Further analysis revealed that there was also no significant difference in B_{max} ($U(20,19) = 183$; $p = 0.8567$) or K_D ($U(20,19) = 180$; $p = 0.7919$) between sexes.

There was a significant difference in median age between the mTLE and control groups: $U(14,25) = 54$; $p = 0.0002$. However, correlation analysis of B_{max} and age (at death/at surgery for controls and mTLE patients, respectively) revealed that age was not correlated with B_{max} in either group: Spearman $r = -0.06381$; $p = 0.8286$ (controls); Pearson $r = -0.1175$; $p = 0.5758$ (mTLE). Similarly, age was uncorrelated with K_D in both groups: Spearman $r = 0.3300$; $p = 0.2476$ (controls); Spearman $r = -0.3638$; $p = 0.0738$ (mTLE). There was a significant correlation between age at epilepsy onset and epilepsy duration (in years until surgery) for the mTLE group (Spearman $r = -0.7072$, $p = 0.0002$***).

There were no significant correlations between PMD and B_{max} (Spearman $r = -0.2659$; $p = 0.3573$) or K_D (Spearman $r = -0.1033$; $p = 0.7270$) in the control group, supporting their use as adequate controls for the mTLE surgical specimens.

Table 1: Non-Epilepsy Controls from Necropsy

ID	Gender	Age at Death (years)	Post-Mortem Delay (hours)	Cause of Death
1	M	18	2	Natural death cardiovascular *
2	F	66	57.6	Traumatic injury from motor vehicle accident *
3	M	31	9.2	Natural death, cause unknown *
4	M	59	17-41 [29] **	Pancreatic carcinoma
5	M	65	16.5-40.5 [28.5] **	Unknown complications following hemicolectomy
6	F	74	11	N/A
7	M	71	76.07	Cardiac arrest *
8	F	78	19.75	Renal failure
9	F	76	10.42	Old myocardial infarction with severe and generalized coronary arteriosclerosis *
10	M	79	21.92	Pulmonary edema *
11	M	68	18.5	Lung abscess and pulmonary emphysema *
12	M	80	13	N/A
13	F	91	26.42	Cachexia in the context of neoplasia of the pharynx and esophagus *
14	F	79	17.3	Pancreatic neoplasia *

Legends: M: male, F: female, N/A = not available; *As recorded by the coroner. **Where only post-mortem interval was specified, PMD was estimated as the center value of the range.

Synaptic Density Reductions in mTLE

A two-tailed unpaired t-test revealed a significant decrease (45.5%) in B_{max} in mTLE hippocampi compared to controls: $t(37) = 7.032$; $p < 0.0001$; $n_{mTLE} = 25$, $n_{control} = 14$ (Figure 1). A saturation binding curve is presented in Figure 2A. The mean SV2A density in the mTLE group was 522.7 ± 160.0 fmol/mg tissue equivalent (mean ± SD), while the mean SV2A density in the control group was 959.7 ± 226.6 fmol/mg tissue equivalent (Figure 2B).

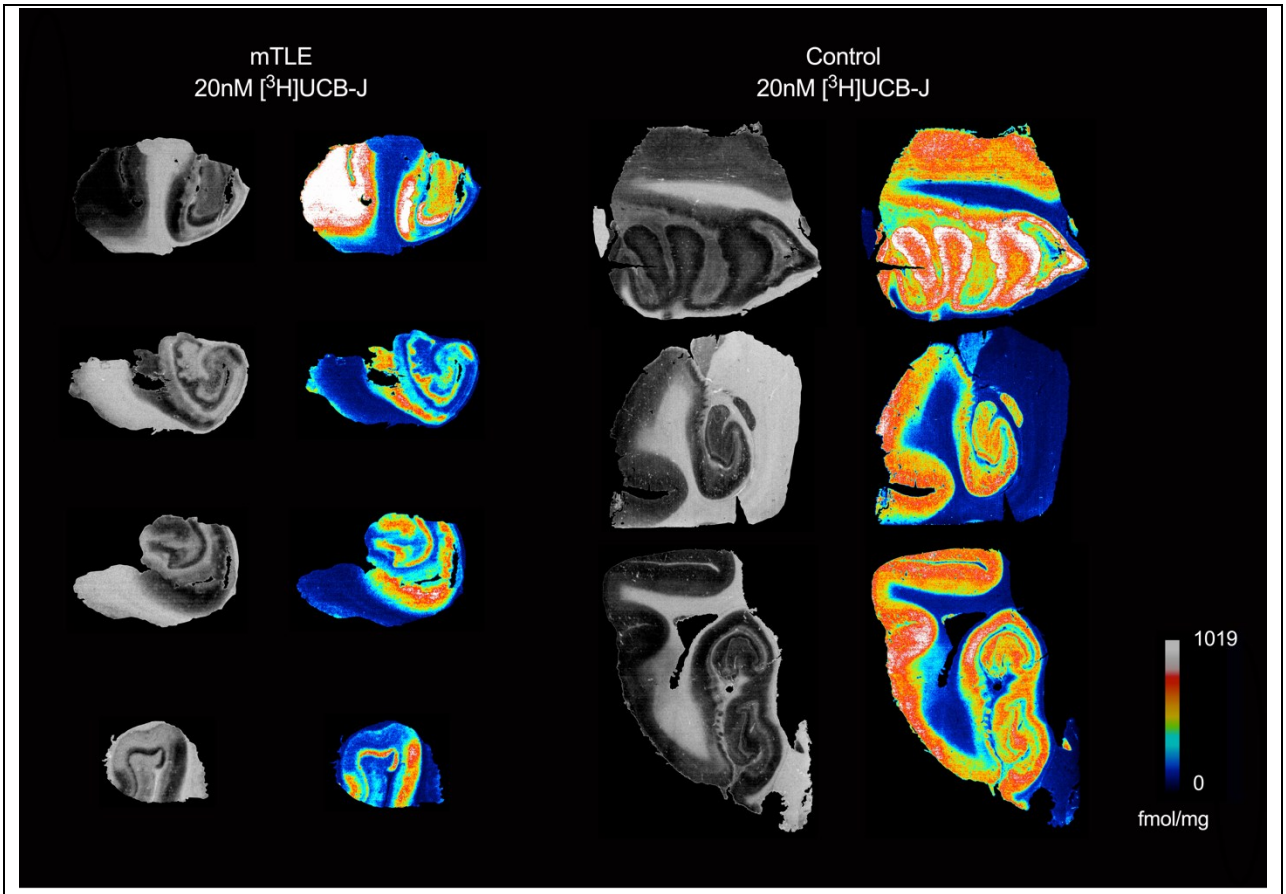

Figure 1: Hippocampal [^{3}H]UCB-J Binding in mTLE patients and Non-epilepsy Controls

Autoradiography with 20nM [^{3}H]UCB-J in the hippocampus of 3 non-epilepsy controls from necropsy and 4 drug-resistant mesial temporal lobe epilepsy (mTLE) patients. Greyscale images demonstrate hippocampal structure.

Increased SV2A Binding Affinity in mTLE

A two-tailed Mann Whitney U Test demonstrated a significant decrease (50.6%) in K_D in mTLE hippocampi compared to controls: $U(14,25) = 10$; $p < 0.0001$. The median K_D in the mTLE group was 8.173 (IQR = 10.180-7.458), while the median K_D in the control group was 16.56 (IQR = 23.198-14.168) (Figure 2C).

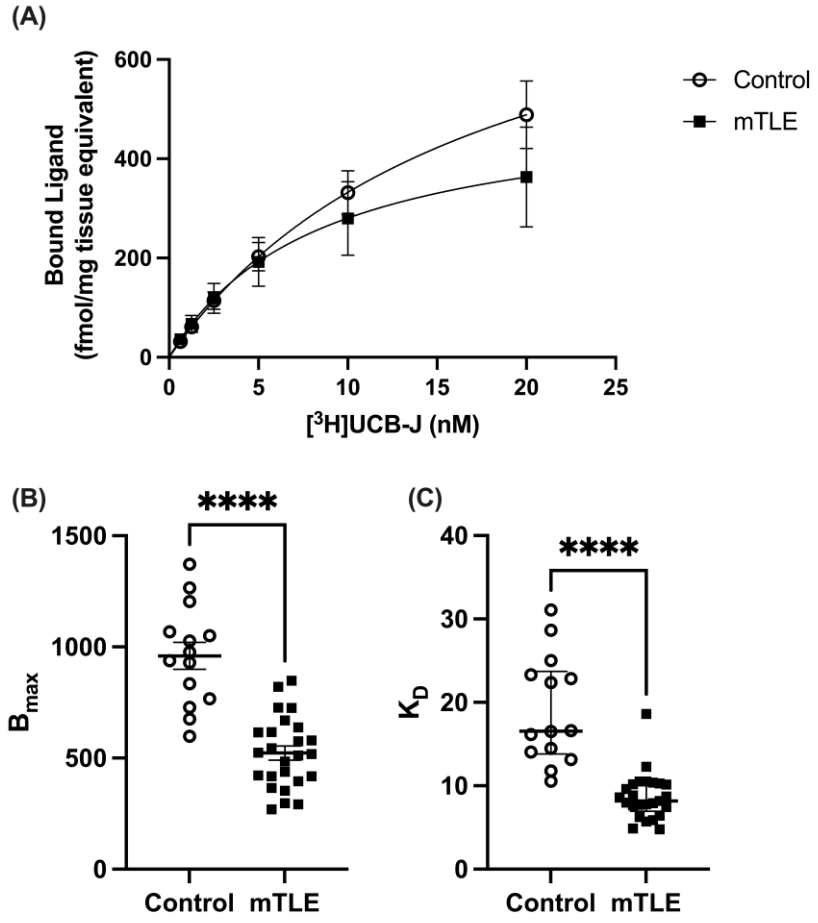

Figure 2: Reductions in B_{max} and K_D in the Epileptogenic Hippocampus

(A) Autoradiographic saturation binding curves constructed from total and non-specific binding data. Displayed are the mean specific binding curves for mesial temporal lobe epilepsy (mTLE) patients and non-epilepsy controls. Error bars represent standard deviation. (B) B_{max} values are reduced in the affected hippocampus of mTLE patients compared to controls. Values are represented as mean ± SEM (C) K_D values are reduced in the affected hippocampus of mTLE patients compared to controls. Values are represented as median ± IQR. **** represents $p < 0.0001$.

Table 2: Clinical Characteristics of Mesial Temporal Lobe Epilepsy Patients

ID	Gender	Epilepsy onset (years)	MRI diagnosis	EEG interictal/ictal	Age at surgery	Surgery	Duration of epilepsy (years)	ASM at surgery	Pathology	Engel class	Follow-up: years from (last) surgery	Frequency of FIAS/month*	History of FS
1	M	5	LHA	L/L	40	L CAH	35	DPH, LTG, TPM	MTS	Ia	3	3.5	Yes
2	M	53	BHA (R>L)	B/ B R>L	59	R CAH	6	OXC, LTG	no definite abnormality	Ia	9	7	No
3	M	2	LHA	L/L	38	L CAH	36	CBZ, CLB	MTS	III	14	3	No
4	F	12	LHA	L/L	58	L CAH	46	CBZ, CLB, LTG	MTS	Ia	7	2	No
5	M	–	RHA	B / R	53	R CAH	–	CBZ, TPM	MTS	II	13	–	No
6	M	3	LT pole resection LHA	None/L	51	L CAH	48	DPH, LEV, OXC, CLB	MTS	IV	3	3	No
7	F	32	LHA	B L>R / L	46	L CAH	14	OXC	MTS	Ia	13	1	Yes
8	F	–	normal	R/R	46	R ATL	–	CBZ, CLB	no definite abnormality	Ia	13	–	No
9	F	16	LHA	L/L	65	L CAH	49	LTG, DPH, TPM, CLB	no definite abnormality	Ia	7	4	No
10	F	6	LHA	L/L	29	L CAH	23	PB	no definite abnormality	Ia	0.5	4	No
11	F	35	RHA	R/R	38	R CAH	3	CBZ, CLB	MTS	Ia	12	2	No
12	M	18	RHA + BFT encephalomalacia	R/none	28	R CAH	10	CBZ, CLB	MTS	II	12	–	No
13	M	28	normal	R/R	32	R ATL	4	LTG	no definite abnormality	Ia	13	3.5	No

14	M	16	RHA	R/R	18	R CAH	2	GAB, CLB	MTS	Ib	12	8	No
15	F	30	LHA	B/B (SEEG: B/L)	43	L SAH	13	TPM, LTG	MTS	II	2	6	No
16	M	22	normal	R/R	32	R ATL	10	CBZ, CLB	MTS	Ia	–	1	No
17	F	58	RHA	R/R	60	R CAH	2	CBZ, CLB	MTS	Ia	2	2.5	No
18	M	44	BHA L>R	L/L	51	L SAH	7	CBZ, CLB, LEV	MTS	Ia	4.5	4.5	Yes
19	M	5	RHA	R	55	R ATL	50	OXC, LEV, CLB	MTS	Ia	6	9	No
20	F	8	L HMF LF encephalocele	L/L (SEEG: L/L)	39	L CAH	31	LEV, LCM	gliosis	Ia	6	6	No
21	F	40	LHA	L	42	L SAH	2	CBZ, CLB	gliosis	II	2	16	No
22	F	13	RHA	R	29	R SAH	16	LTG, CLB	MTS	Ia	9	2	No
23	M	13	BHA L>R + LFT encephalomalacia	L	67	L CAH	54	CBZ, LEV	gliosis	Ia	7	1	No
24	F	4	RHA	R	36	R ATL	32	CBZ	MTS	Ia	6	2.5	No
25	F	16	RHA	R	66	R CAH	50	CBZ, CLB	MTS	Ia	1.5	2.5	No

Legends: M: male, F: female, MRI: magnetic resonance imaging, B: bilateral, L: left, R: right, H: hippocampal, HA: hippocampal atrophy, HMF: hippocampal malformation/malrotation, T: temporal, F: frontal, EEG: electroencephalography, SEEG: stereo EEG, NA: not available, CAH: Cortico-Amygdalo-Hippocampectomy, SAH: Selective Amygdalo-Hippocampectomy, ATL: anterior temporal lobe resection (including amygdala and hippocampus), CBZ: carbamazepine, CLB: clobazam, DPH: phenytoin, LEV: levetiracetam, LTG: lamotrigine, OXC: oxcarbazepine, LCM: Lacosamide, PB: phenobarbital, TPM: topiramate, MTS: mesial temporal sclerosis, FCD III: focal cortical dysplasia type III, *Focal impaired awareness seizures (FIAS) per month in the year prior to surgery, FS: febrile seizures

Correlating [^{3}H]UCB-J Saturation Parameters with mTLE Clinical Characteristics

Correlation analyses revealed that B_{max} was significantly correlated with age at epilepsy onset (Spearman r = 0.5418, p = 0.0076**) and duration of epilepsy (Spearman r = -0.5740, p = 0.0042**) (Figures 3A-B). Correlations between B_{max} and frequency of seizures/age at surgery were both non-significant (p > 0.05) (Figures 3C-D). There were no significant differences in B_{max} between patients who had taken LEV prior to surgery and those who had not, patients who achieved seizure freedom and those who did not, patients whose resected tissue was obtained from the left vs. right hemisphere, and patients whose pathologic diagnosis of the resected specimen indicated no definite abnormality vs. patients with evidence of MTS or gliosis (Figure 3E-H).

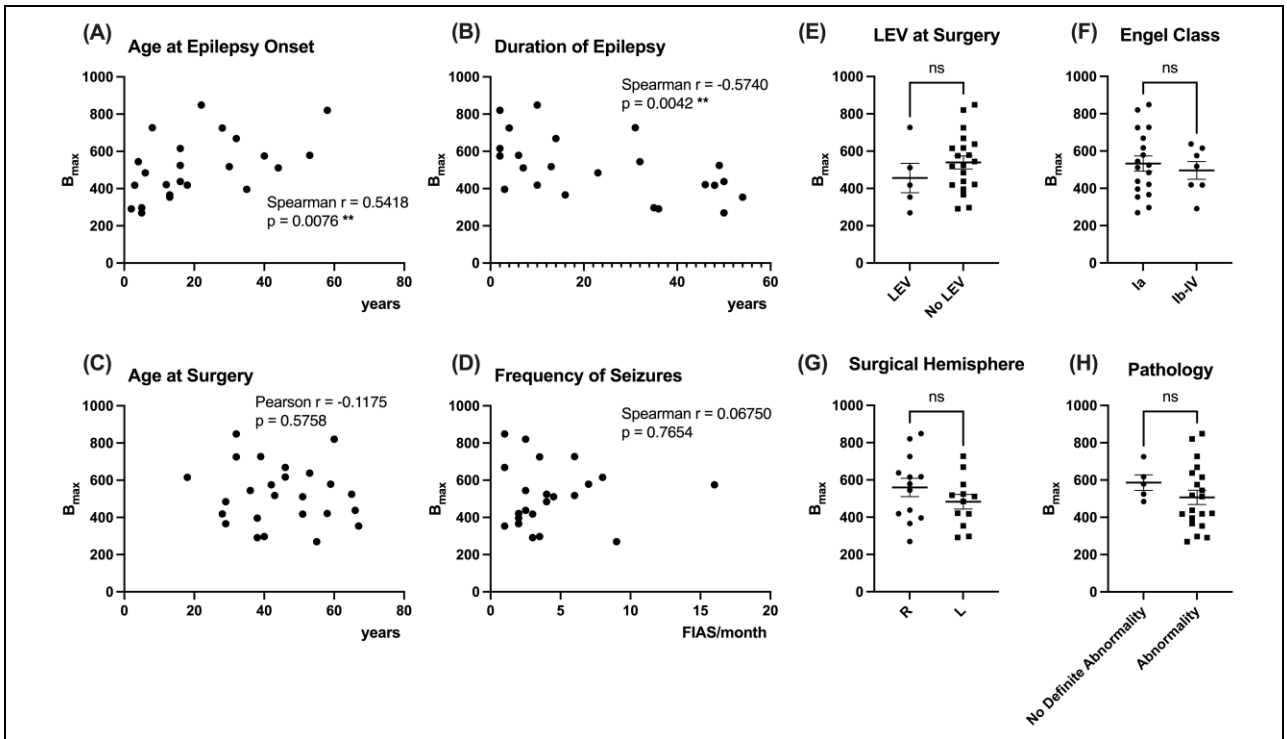

Figure 3: Analysis of B_{max} and mTLE Clinical Characteristics

Correlations between [^{3}H]UCB-J B_{max} (in fmol/mg) and age at epilepsy onset (A), duration of epilepsy (B), age at surgery (C), and frequency of seizures (focal impaired awareness seizures, FIAS, per month in the year prior to surgery) (D) are presented. (E) B_{max} values were not significantly different between mesial temporal lobe epilepsy (mTLE) patients who were treated with levetiracetam (LEV) prior to surgery compared to those who had not been

treated with LEV. Values are represented as mean ± SEM. (F) B_{max} values were not significantly different between mTLE patients who achieved seizure freedom following surgery (Engel Ia) compared to patients who did not achieve seizure freedom (Engel Ib-IV). Values are represented as mean ± SEM. (G) B_{max} values were not significantly different between mTLE patients whose surgical specimen came from the right (R) or left (L) hemisphere. Values are represented as mean ± SEM. (H) B_{max} values were not significantly different between mTLE patients whose specimen displayed no definite abnormality compared to patients whose specimen displayed an abnormality (mesial temporal sclerosis or gliosis). Values are represented as mean ± SEM. ** represents $p < 0.01$, ns represents a non-significant comparison.

The correlation between K_D and duration of epilepsy was also significant (Spearman r = -0.6071, p = 0.0021**) (Figure 4B). K_D was uncorrelated with age at epilepsy onset, age at surgery, and frequency of seizures (Figures 4A, C-D). There were also no significant differences in K_D between patients who had taken LEV prior to surgery and those who had not, patients who achieved seizure freedom and those who did not, patients whose resected tissue was obtained from the left vs. right hemisphere, and patients whose pathologic diagnosis of the resected specimen indicated no definite abnormality vs. patients with evidence of MTS or gliosis (Figure 4E-H).

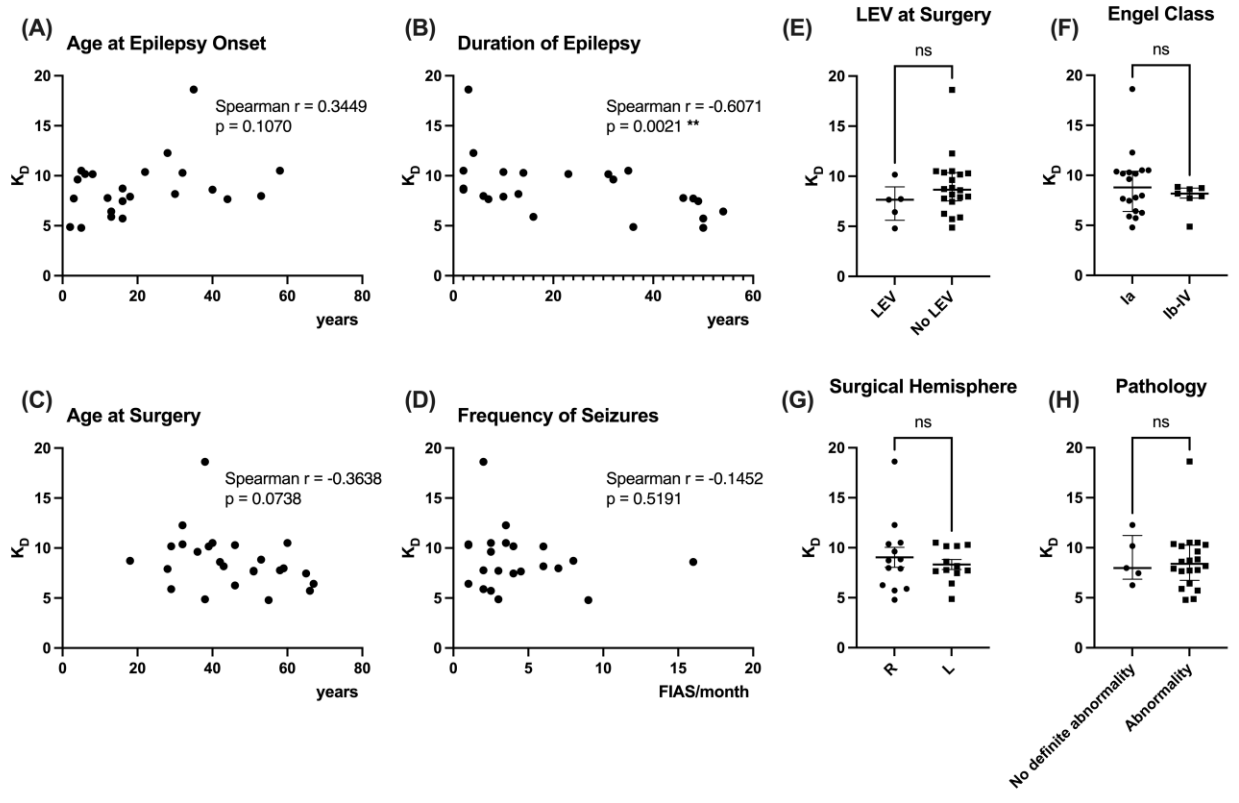

Figure 4: Analysis of K_D and mTLE Clinical Characteristics

Correlations between [^{3}H]UCB-J K_D and age at epilepsy onset (A), duration of epilepsy (B), age at surgery (C), and frequency of seizures (focal impaired awareness seizures, FIAS, per month in the year prior to surgery) (D) are presented. (E) K_D values were not significantly different between mesial temporal lobe epilepsy (mTLE) patients who were treated with levetiracetam (LEV) prior to surgery compared to those who had not been treated with LEV. Values are represented as median with interquartile range. (F) K_D values were not significantly different between mTLE patients who achieved seizure freedom following surgery (Engel Ia) compared to patients who did not achieve seizure freedom (Engel Ib-IV). Values are represented as median with interquartile range. (G) K_D values were not significantly different between mTLE patients whose surgical specimen came from the right (R) or left (L) hemisphere. Values are represented as mean ± SEM. (H) K_D values were not significantly different between mTLE patients whose specimen displayed no definite abnormality compared to patients whose specimen displayed an abnormality (mesial temporal sclerosis or gliosis). Values are represented as median with interquartile range. ** represents $p < 0.01$, ns represents a non-significant comparison.

mGluR5 Expression Correlates with SV2A Density

Correlation of receptor density (B_{max}) from the present [^{3}H]UCB-J and prior [^{3}H]ABP688 autoradiography (from Zimmermann et al. (2022)) revealed a significant positive correlation between mGluR5 density and SV2A density in the hippocampus of mTLE patients: Pearson r = 0.5912; p 0.0260, n = 14 (Figure 5).

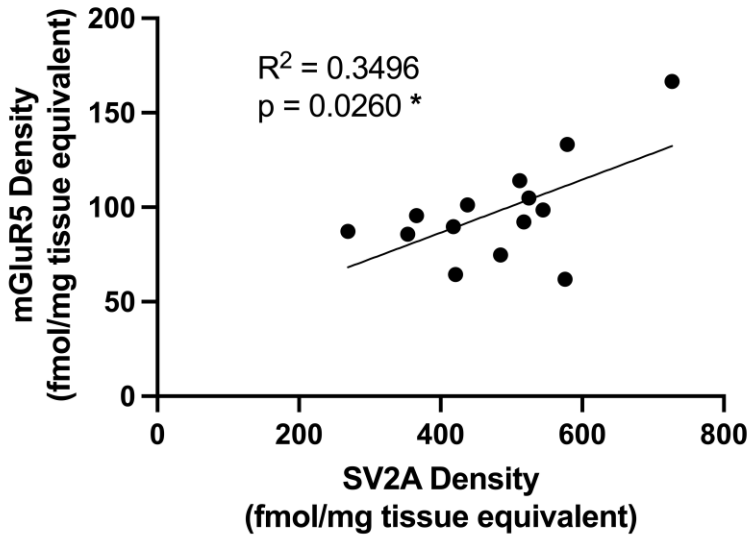

Figure 5: Correlation between SV2A and mGluR5 Density

The correlation between synaptic vesicle glycoprotein 2A (SV2A) receptor density as determined by [^{3}H]UCB-J autoradiography and metabotropic glutamate receptor type 5 (mGluR5) receptor density as determined by [^{3}H]ABP688 autoradiography is shown. * represents $p < 0.05$.

Characterizing [^{3}H]UCB-J Binding in FCD

Descriptive analysis of the 6 FCD patients (shown in Table 3) revealed a mean B_{max} value of 659.9 ± 169.9 fmol/mg tissue equivalent (mean ± SD). The median K_D was 11.81 (IQR= 23.20-7.972) (Figure 6).

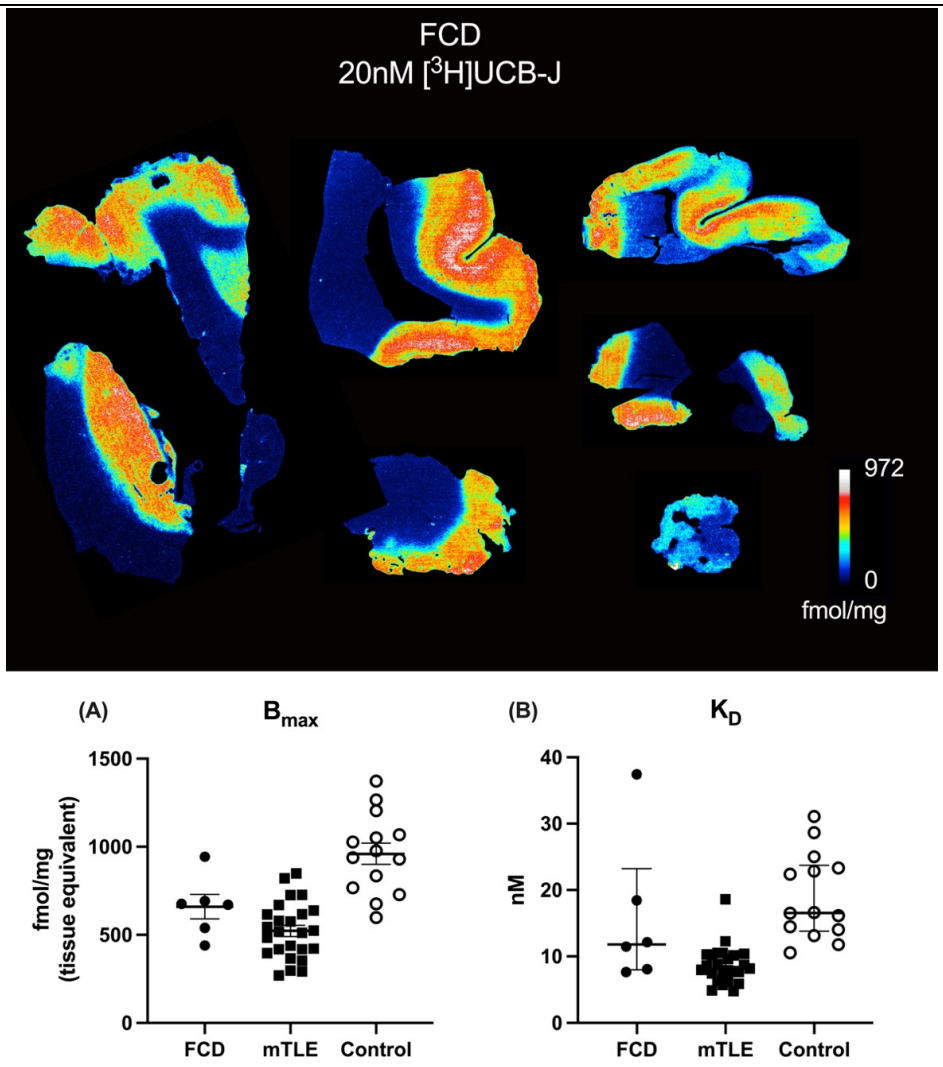

Figure 6: [^{3}H]UCB-J Binding in the Dysplastic Cortex of FCD patients

Top: Autoradiography with 20nM [^{3}H]UCB-J in the lesioned cortex of 6 focal cortical dysplasia (FCD) patients. *Bottom*: (A) B_{max} values in the surgically-extracted lesion of FCD patients, and in the hippocampus of mTLE patients and non-epilepsy controls. Values are represented as mean ± SEM. (B) K_D values in the surgically-extracted lesion of FCD patients, and in the hippocampus of mTLE patients and non-epilepsy controls. Values are represented as median ± IQR.

Table 3: Characteristics of Focal Cortical Dysplasia Patients

ID (P code)	Gender	Age at Surgery (years)	Resected Region	Diagnosis
1	M	55	Parietal	FCD
2	M	41	SMA	FCD
3	M	33	Orbitofrontal anterior insula	Hemimegaloencephaly, FCD
4	F	22	R-SMA	FCD
5	F	29	Basal Frontal Encephalo Cortex	FCD in the context of an encephalocele
6	M	30	Frontopolar cortex	FCD

Legends: M: male, F: female; R: Right; SMA: supplementary motor area; FCD: focal cortical dysplasia

Discussion

The present study utilized gold-standard saturation autoradiographic techniques to quantify SV2A density and binding affinity in the hippocampus of drug-resistant mTLE patients and healthy controls, as well as in the epileptogenic cortex of FCD patients. Our analysis revealed a significant reduction (45.5%) in SV2A density in the epileptogenic hippocampi of mTLE patients.

SV2A expression under normal conditions has been shown to correlate highly with the gold-standard immunohistochemical marker of synaptic density, synaptophysin, leading [^{11}C]UCB-J to be proposed as a general marker of synaptic density (Finnema et al., 2016). Therefore, the observed reductions in B_{max} in the present study may be reflective of synaptic loss within the hippocampus of mTLE patients, consistent with reports from previous studies (Alonso-Nanclares et al., 2011; Finnema et al., 2016; Finnema et al., 2020; Looney et al., 1999).

However, these reductions may also indicate a mTLE-associated specific loss of SV2A, independent of general synapse loss. Decreased SV2A expression or mutations of the SV2A gene have been associated with an epileptic phenotype in animal models of mTLE and in humans (Kaminski et al., 2009; Rossi et al., 2022; van Vliet et al., 2009). Therefore, *the observed reduction in SV2A density may reflect an mTLE-associated downregulation of SV2A that contributes to epileptogenesis*. Alternately, due to SV2A's expression on synaptic vesicles, the observed reductions in [^{3}H]UCB-J binding may signify a *reduction in synaptic vesicles rather than specific SV2A downregulation*.

Furthermore, we demonstrate for the first time to the best of our knowledge, an increase in binding affinity of [^{3}H]UCB-J for SV2A in the hippocampus of mTLE patients. The affinity of a particular ligand for its protein target is sensitive to the conformational state of the target (van den

Noort et al., 2021). Therefore, alterations to binding affinity can often be attributed to changes to a protein's conformational state.

The observed decrease in K_D in our study may be explained by a conformational change to hippocampal SV2A that results in increased binding affinity in mTLE patients. Interestingly, levetiracetam (LEV), a drug that binds to SV2A and is capable of displacing [^{11}C]UCB-J, has demonstrated a potent antiepileptic effect (De Smedt et al., 2007; Lynch et al., 2004). In addition, the antiepileptic efficacy of LEV and its derivatives correlates with their binding affinity for SV2A (Lynch et al., 2004). There was a subset of patients in our mTLE cohort who had been treated with LEV at time of surgery. We have demonstrated that presurgical treatment with LEV did not have an impact on either B_{max} or K_D values in our study, which supports our inclusion of these patients in the present study and is also consistent with a previous study showing that LEV binding does not cause large-scale conformational changes to SV2A (Lynch et al., 2008).

Some proteins will undergo conformational changes to restore homeostasis in response to specific input signals (Ha & Loh, 2012). The observed increase in [^{3}H]UCB-J-SV2A binding affinity may therefore reflect a hyperexcitability-induced conformational change to SV2A meant to elicit an endogenous neuroprotective effect in mTLE patients by increasing binding of an unknown endogenous ligand. Conversely, depending on the location and function of the [^{3}H]UCB-J binding site, increased affinity for SV2A may be linked to a conformational change that is associated with altered modulation of SV2A function, which could contribute to epileptogenesis.

Uncovering the structure of SV2A and its binding sites will be a crucial step in determining the possible effects of this change in binding affinity. Nevertheless, ***the changes to binding affinity suggest an SV2A-specific alteration is occurring in the hippocampus of mTLE patients,***

regardless of whether the observed reductions in SV2A density are attributable to general synaptic loss, specific SV2A downregulation, or loss of synaptic vesicles.

We have also demonstrated that [^{3}H]UCB-J B_{max} correlates with duration of epilepsy in our mTLE group. As the duration of epilepsy increases, B_{max} decreases, which may reflect an increase in synaptic changes as the disease persists. Several studies have suggested that changes within the epileptogenic zone in mTLE become gradually more severe over time as seizures continue, which is consistent with our findings (Bartolomei et al., 2008; Bonilha et al., 2006). We have also observed a positive correlation between age at epilepsy onset and [^{3}H]UCB-J B_{max}, which may indicate that individuals with more severe SV2A reductions are prone to an earlier epilepsy onset. However, we also observed in our study a negative correlation between age at epilepsy onset and duration of epilepsy within the mTLE group. Therefore, we cannot exclude the possibility that the correlation between age at epilepsy onset and B_{max} may be explained by individuals with an earlier onset in our cohort tending to have a longer duration of epilepsy prior to surgery, and therefore lower B_{max} values.

Surprisingly, we have also observed a significant negative correlation between K_D and duration of epilepsy in our mTLE group. We are unaware of any pre-existing literature indicating that dissociations constants are susceptible to disease duration. However, our findings suggest that K_D (and by extension, binding affinity of [^{3}H]UCB-J to SV2A) may be altered as epilepsy duration increases. There is currently some evidence that binding affinities can be altered by protein post-translational modifications (PTMs) or protonation (Schönichen et al., 2013; Su et al., 2017; Sun et al., 2010). It is therefore possible that the correlation between K_D values and epilepsy duration may be due to seizure-induced changes to the cellular environment or regulation of PTMs of the SV2A protein that elicit an increase in [^{3}H]UCB-J binding affinity. However, considerably more

research into the ways that binding affinity can be modified as a function of disease duration is required to reliably postulate the possible causes of the observed correlation between [^{3}H]UCB-J-SV2A binding affinity and epilepsy duration in mTLE patients.

We did not observe any significant differences in either B_{max} or K_D values between patients who became seizure-free versus those who did not following the surgical resection of their epileptogenic hippocampus, implying that neither SV2A density nor affinity in the resected tissue can predict post-surgical outcome in mTLE. We also did not observe any difference in B_{max} or K_D between mTLE patients with evidence of MTS or gliosis compared to patients with no definite abnormality, which suggests that the observed SV2A alterations are not tied to either MTS or gliosis as an underlying pathology. Similarly, we did not observe any lateralization of B_{max} or K_D reductions in our mTLE patients.

In addition, we have demonstrated that there exists a relationship between availability of SV2A and mGluR5 in the epileptogenic hippocampus. Both SV2A and mGluR5 are expressed at synapses, therefore, their correlated decrease in mTLE patients may reflect general synaptic loss resulting from aberrant synaptic activity (Löscher et al., 2016; Niswender & Conn, 2010). Conversely, reductions in expression or availability of one of the two proteins may lead to altered expression or availability of the other. For example, decreased SV2A expression may lead to changes in neurotransmitter release, which could in turn affect mGluR5 availability, or altered mGluR5 availability could affect signalling pathways involved in regulation of SV2A expression. Whether the relationship between SV2A and mGluR5 is causal or incidental to some other mechanism remains to be elucidated.

We also present the first quantitative characterization of [^{3}H]UCB-J binding in the dysplastic cortex of FCD patients. However, our study was limited by a lack of suitable non-

dysplastic cortical specimens (control specimens within the same anatomical region) with which to assess possible presence or magnitude of differences in SV2A density or binding affinity associated with FCD. Despite the lack of an adequate control group, we do note that the mean B_{max} in the epileptogenic cortex of FCD patients is closer in magnitude to the mean B_{max} in the hippocampus of mTLE patients (659.9 fmol/mg versus 522.7 fmol/mg, respectively) than to that of healthy control hippocampi (959.7 fmol/mg). Similarly, the median K_D value in FCD patients (11.81 nM) was closer to that in mTLE hippocampi (8.173 nM) than in control hippocampi (16.56 nM) (Figure 6). Whether these findings are due to differences in SV2A expression and binding affinity between cortical and hippocampal regions, or due to a shared alteration to synaptic density in mTLE and FCD remains to be clarified. Interestingly, a recent study examining [^{3}H]UCB-J binding in the temporal neocortex of drug-resistant TLE patients who underwent temporal lobe resection surgery, including 2 patients who demonstrated signs of FCD, showed reduced [^{3}H]UCB-J binding, suggesting that SV2A alterations in epilepsy may not be specific to the hippocampus (Pazarlar et al., 2022).

Limitations

Due to incomplete visibility of all hippocampal subregions in the mTLE surgical specimens, it was not possible to investigate regional hippocampal differences in SV2A density and binding affinity. SV2A immunocytochemistry has previously shown that in patients with hippocampal sclerosis, decreases in SV2A expression, while generally present throughout the hippocampus, demonstrate some regional specificity (van Vliet et al., 2009). A larger selection of specimens from patients who underwent an *en bloc* anterior temporal resection involving complete removal of the hippocampus may enable a reliable regional analysis in the future.

Furthermore, we could not obtain non-epilepsy cortical specimens within the same anatomical regions as the FCD specimens. Therefore, it was not possible to explore potential FCD-associated changes to SV2A density and affinity. Although reduced SV2A expression has been demonstrated *in vitro* through immunohistochemistry and Western blotting (Toering et al., 2009), and *in vivo* through PET with 18F-SynVesT-1 (Y. Tang et al., 2022), whether these reductions can similarly be detected using [^{3}H]UCB-J is unclear. The greater similarity between mTLE and FCD saturation binding parameters in this study suggests this reduction may also be present in FCD, however, this cannot be conclusively determined in the absence of an anatomically-suitable non-epilepsy comparison group. Similarly, whether SV2A also undergoes an FCD-associated binding affinity-altering change in protein conformation can only be determined *via* comparison with an adequate control group.

Future Directions

A future study should aim to investigate region-specific differences in hippocampal [^{3}H]UCB-J binding between mTLE patients and non-epilepsy controls to corroborate the previously reported regional SV2A expression differences (van Vliet et al., 2009).

The underlying cause of mGluR5 abnormalities in mTLE has not yet been discovered, however, the link between mGluR5 and SV2A density observed in our study suggests an association between mTLE-associated glutamatergic and structural changes. The discovery of this relationship between SV2A and mGluR5 expression merits further investigation into the possible ways that these two systems could be interconnected under normal conditions, and how they may be intertwined in the pathophysiology of mTLE.

In addition, a future study comparing SV2A density and binding affinity in FCD patients and healthy non-epilepsy individuals would help determine if FCD is also associated with decreases in SV2A density and/or increased [^{3}H]UCB-J-SV2A binding affinity, similar to mTLE, which would imply SV2A alterations as a general epileptogenic feature rather one specific to mTLE.

Conclusion

Reduced synaptic density in the epileptogenic hippocampus of mTLE patients has been shown *in vivo* through the use of PET with [^{11}C]UCB-J. We demonstrate in this study that this reduction can similarly be found *in vitro*, supporting alterations to structural components of synapses as an underlying feature of mTLE and providing *in vitro* validation for the observed *in vivo* reductions. We have proposed that the reduced [^{3}H]UCB-J binding may reflect synaptic loss, specific SV2A downregulation, or a loss of synaptic vesicles.

We have also identified an alteration to the binding affinity of [^{3}H]UCB-J for SV2A in the mTLE hippocampus, which we have suggested reflects alterations to the conformation of the SV2A protein linked to the pathophysiology of mTLE.

The concurrent reduction in [^{3}H]UCB-J binding and increase in binding affinity of [^{3}H]UCB-J for SV2A may represent two mechanisms through which SV2A function is altered in mTLE. Loss of SV2A may lead to reduced SV2A activity, whereas a potential conformational change to the protein may lead to abnormal function of SV2A. Given SV2A's proposed role as a modulator of neurotransmitter release, abnormalities in its activity or function may contribute to abnormal neuronal signalling.

We have also proposed that the observed correlations between duration of epilepsy and B_{max} and K_D in our study suggest that progressive synaptic changes may occur as the duration of epilepsy increases. The correlation between B_{max} and age at epilepsy onset could indicate that SV2A levels influence the age at epilepsy onset, or this correlation may be the result of the association between the age at epilepsy onset and duration of epilepsy in our mTLE cohort.

Furthermore, we have shown an association between the expression of SV2A and mGluR5 within the epileptogenic hippocampus. Uncovering the underpinnings of this association will be an interesting next step in epilepsy research.

We have also presented the first quantitative characterization of [^{3}H]UCB-J binding within the lesion of FCD patients. Although our study lacked a control group within the same anatomical region with which to assess the presence and/or magnitude of binding changes, we nevertheless observed that the saturation binding parameters (B_{max} and K_D) within the lesion of FCD patients bore greater similarities to those observed in the mTLE hippocampus than to those observed in the non-epilepsy hippocampus and could reflect either regional differences in SV2A properties between hippocampal and cortical regions, or a shared epileptogenic mechanism in mTLE and FCD.

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