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REVIEW

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Entering a new era of quantifying glutamate clearance in health and disease

Kyle J. Brymer | Jocelyn R. Barnes | Matthew P. Parsons

Faculty of Medicine, Division of Biomedical Sciences, Memorial University of Newfoundland, St. John's, NL, Canada

Correspondence
Matthew P. Parsons, Faculty of Medicine, Division of Biomedical Sciences, Memorial University of Newfoundland, St. John's, NL A1B 3V6, Canada.
Email: matthew.parsons@med.mun.ca

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Abstract

Glutamate transporter proteins, expressed on both neurons and glia, serve as the main gatekeepers that dictate the spatial and temporal actions of extracellular glutamate. Glutamate is essential to the function of the healthy brain yet paradoxically contributes to the toxicity associated with many neurodegenerative diseases. Rapid transporter-mediated glutamate uptake, primarily occurring at astrocytic processes, tightens the efficiency of excitatory network activity and prevents toxic glutamate build-up in the extracellular space. Glutamate transporter dysfunction is thought to underlie myriad central nervous system (CNS) diseases including Alzheimer and Huntington disease. Over the past few decades, techniques such as biochemical uptake assays and electrophysiological recordings of transporter currents from individual astrocytes have revealed the remarkable ability of the CNS to efficiently clear extracellular glutamate. In more recent years, the rapidly evolving glutamate-sensing "sniffers" now allow researchers to visualize real-time glutamate transients on a millisecond time scale with single synapse spatial resolution in defined cell populations. As we transition to an increased reliance on optical-based methods of glutamate visualization and quantification, it is of utmost importance to understand not only the advantages that glutamate biosensors bring to the table but also the associated caveats and their implications for data interpretation. In this review, we summarize the strengths and limitations of the commonly used methods to quantify glutamate uptake. We then discuss what these techniques, when viewed as a complementary whole, have told us about the brain's ability to regulate glutamate levels, in both health and in the context of neurodegenerative disease.

KEYWORDS
astrocyte, biosensor, glutamate, glutamate transporter, neurotransmission, plasticity

1 | INTRODUCTION

Glutamate is the brain's primary excitatory neurotransmitter and is essential for rapid excitatory synaptic neurotransmission, neuronal survival, and synaptic plasticity. Paradoxically, glutamate can also act as a toxic chemical, and enhanced glutamate-induced excitotoxicity is heavily implicated in numerous central nervous system (CNS) diseases including Alzheimer disease (AD) and Huntington disease (HD), among others (Parsons & Raymond, 2014). As glutamate is not metabolized in the extracellular space, the CNS is equipped with highly efficient excitatory amino acid transporters (EAATs) that rapidly clear extracellular glutamate following neural activity. The majority of EAATs are located on astrocytes, with lower expression levels identified in neurons (Danbolt, 2001; Holmseth et al., 2012;

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Lehre & Danbolt, 1998). EAATs promote a high signal-to-noise ratio during synaptic activity and limit the toxic effects associated with excessive extracellular glutamate accumulation (Danbolt, 2001). Until recently, the study of transporter-mediated glutamate uptake was primarily performed in various biochemical uptake assay preparations, in addition to work from several laboratories utilizing real-time electrophysiological recordings of synaptically activated transporter currents (STCs) from astrocytes in acute brain slices (Bergles & Jahr, 1997; Brew & Attwell, 1987). However, with the recent increase in the number, specificity, speed, and accessibility of intensity-based glutamate sensing fluorescence reporters (iGluSnFRs) (Coates et al., 2020; Helassa et al., 2018; Marvin et al., 2013, 2018; Wu et al., 2018), the study of glutamate dynamics may indeed transition primarily to light-based imaging of glutamate transients in real time. As we prepare to embark on an inevitable new era in the quantification of glutamate dynamics, we felt it was an appropriate time to review the different methods used to study glutamate clearance, and how they can be viewed as complementary rather than competing techniques. In doing so, we also discuss what these techniques, when viewed as a whole, have told us about glutamate regulation in the CNS in both health and disease.

Significance

Glutamate, a chemical found in the brain, is essential for rapid communication between brain cells. Unfortunately, too much glutamate can be toxic to brain tissue, and glutamate toxicity has been implicated in numerous diseases of the brain. Many different experimental methods have been used by researchers to understand of how the brain regulates glutamate levels, and how those mechanisms go awry in brain disease. In this review, we discuss the advantages and limitations of the different techniques used to quantify glutamate levels, and what they have told us so far about glutamate regulation in both health and disease.

2 | GLUTAMATE TRANSPORTERS: SUBTYPES AND BASIC PROPERTIES

Following release, glutamate is rapidly removed from the extracellular space by Na^+-dependent high-affinity EAATs. Five EAATs (EAAT1-5) have been identified and are expressed on neurons and astrocytes throughout the nervous system. EAAT1 (also known as GLAST) and EAAT2 (also known as GLT-1) are predominantly astrocytic (Chaudhry et al., 1995; Lehre et al., 1995), although a small proportion (approximately 10%) of EAAT2 can also be found in axon terminals (Furness et al., 2008; Rimmele & Rosenberg, 2016). EAAT3 is primarily localized at perisynaptic sites in dendrites (He et al., 2000; Rothstein et al., 1994). While some EAAT3 expression has been previously reported in glial cells (Conti et al., 1998; Kugler & Schmitt, 1999), no EAAT3 expression could be detected outside neurons when EAAT3 antibodies were validated in knockout mice (Bjørn-Yoshimoto & Underhill, 2016; Holmseth et al., 2012). Thus, EAAT3 appears to be exclusively neuronal and exists at a relatively low density; approximately 100 times lower than the density of EAAT2 in the hippocampus (Holmseth et al., 2012). EAAT4 is also a neuronal transporter but is primarily located at somatodendritic locations in cerebellar Purkinje neurons (Furuta et al., 1997). EAAT5 is expressed in the retina, and is thought to serve more as a glutamate-gated Cl^- channel than as an effective means of glutamate uptake (Arriza et al., 1997; Schneider et al., 2014). In general, EAATs are expressed at extremely high concentrations throughout the brain. For example, in hippocampal CA1 stratum radiatum, the number of glutamate transporters is estimated to exceed 15,000 per μm^3 of tissue, with even higher densities observed in the cerebellar molecular layer (Lehre & Danbolt, 1998). Glutamate transport through EAATs is powered by Na^+ and K^+ ion gradients, and each full transport cycle consists of the co-transport of one glutamate molecule with three Na^+ and one H^+ ions into the cytosol, followed by the counter-transport of one K^+ ion out to the extracellular space (Zerangue & Kavanaugh, 1996). Thus, glutamate tran

sport is associated with a net positive current influx, a feature that has been cleverly exploited by patch-clamp recordings to generate measures of the time course of extracellular glutamate transients with unprecedented temporal resolution. These STCs are discussed in more detail later in this review.

Of the different EAATs, EAAT2 appears to be the most important for CNS function. It is estimated that EAAT2 accounts for over 1% of the total tissue protein in the hippocampus (Lehre & Danbolt, 1998). Primarily thought of as an astrocytic transporter, it is now known that a small but non-negligible percentage of EAAT2 protein is found in axon terminals. Presynaptically localized EAAT2 has been detected in the hippocampus (Chen et al., 2004; Furness et al., 2008), cortex (Melone et al., 2009), and striatum (Petr et al., 2013), and provides a putative means through which the CNS could bypass the canonical glutamate–glutamine cycle (Hertz, 2013) and directly recover glutamate into axon terminals following neural activity (Zhou et al., 2019). In addition, presynaptic EAAT2 can act as a source of glutamate for energy metabolism and biosynthesis in excitatory presynaptic terminals (McNair et al., 2019). EAAT2, particularly astrocytic EAAT2, is believed to be responsible for the large majority of glutamate clearance in the brain (Danbolt, 2001; Otis & Kavanaugh, 2000; Vandenberg & Ryan, 2013), although additional transporters can indeed make substantial contributions to overall uptake rates (Pinky et al., 2018; Romanos et al., 2019). Global knockout of EAAT2 results in lethal seizures (Tanaka et al., 1997), an effect that is mimicked by selective deletion of EAAT2 from astrocytes but not neurons (Petr et al., 2015). While the consequences of losing other EAAT subtypes are not as severe, knockout of EAAT1 results in motor deficits and exacerbated damage following acute cerebellar injury (Watase et al., 1998), and EAAT3 knockout mice exhibit altered locomotor behavior, increased anxiety-like behavior, glutathione deficiency, and brain atrophy (Aoyama et al., 2006; Bellini et al., 2018; Peghini et al., 1997). Antisense oligonucleotide-mediated

knockdown of individual glutamate transporters also demonstrated that the consequences of losing the primarily glial transporters EAAT1 or EAAT2 are more severe than the consequences of losing neuronal EAAT3 (Rothstein et al., 1996).

Excitatory synapses are oftentimes apposed by EAAT-expressing astrocytic processes in a tripartite synaptic configuration (Perea et al., 2009). A recent elegant imaging approach to quantify astrocyte–neuron proximity demonstrated that even different excitatory synaptic projections to the same region can exhibit differences in glial coverage (Octeau et al., 2018). Glial proximity can alter the spatiotemporal dynamics of glutamate by promoting rapid uptake through membrane EAATs. Furthermore, by altering the morphology of the perisynaptic extracellular environment, the mere physical presence of astrocyte membranes in close proximity to release sites impacts glutamate diffusion dynamics (Rusakov & Kullmann, 1998). By controlling not only the time course of extracellular glutamate but also the spatial localization of its effects, EAATs control the subtypes and subcellular localization of glutamate receptors that are activated following glutamate release. It is worth noting that while the tripartite synapse is commonly depicted in textbook and manuscript schematics, excitatory synapses are extremely heterogeneous and many are not directly apposed by astrocytic processes. In fact, in CA1 stratum radiatum, serial electron microscopy demonstrates that more than 40% of synapses do not have an astrocyte in close apposition (Ventura & Harris, 1999). Thus, EAAT control over the spatiotemporal dynamics of glutamate is highly complex and is likely to dramatically differ not only from one brain region to the next (Pinky et al., 2018), but even from one synapse to the next within a given region (Dvorzhak et al., 2019 ; Octeau et al., 2018).

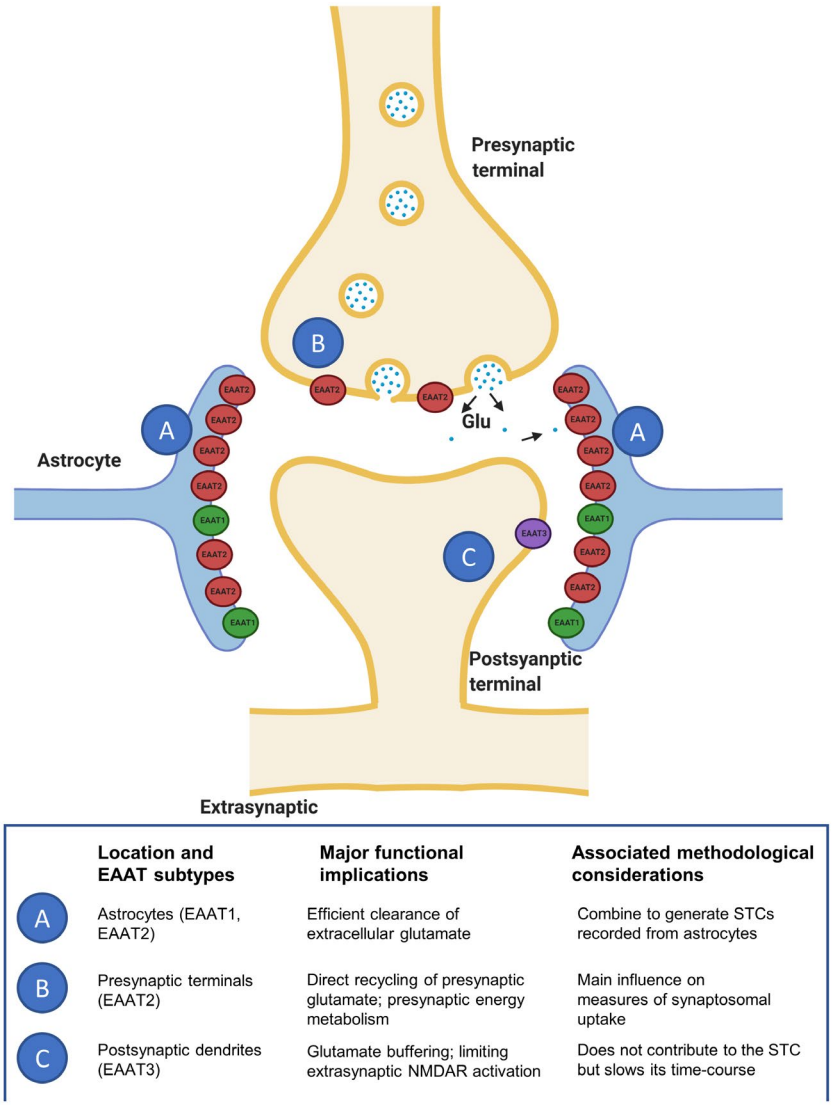
3 | QUANTIFYING GLUTAMATE TRANSPORTER FUNCTION: METHODOLOGICAL CONSIDERATIONS

The spatiotemporal profile of extracellular glutamate depends on numerous factors including the location of the release source, the amount and duration of glutamate released during neural activity, the tortuosity of the adjacent extracellular space, and the subcellular localization and density of nearby EAATs, to name a few. Given the complexities of EAAT control over glutamate dynamics, it is not surprising that no single method is unanimously viewed as the gold standard to quantify glutamate uptake. The following sections will describe commonly used techniques to measure glutamate uptake/clearance. As many laboratories continue to use one or several of the following techniques, generating a mutual understanding of the advantages and the caveats of each technique, and how they can be used to complement each other, is of importance.

3.1 | Synaptosomes and biochemical uptake assays

Much of our current understanding of EAAT function, both basally and following a plethora of experimental manipulations in both health and disease, stems from studies using biochemical uptake assays. Biochemical uptake assays quantify the uptake of a bulk concentration of extracellular radiolabeled glutamate over a time scale of minutes, and can be performed on a variety of preparations including brain slices, cell cultures, synaptosomes, and solubilized transporters reconstituted in artificial cell membranes. Synaptosomal preparations in particular have been widely used for decades to study glutamate uptake. The term “synaptosome" can be traced back to the 1960s (Whittaker et al., 1964), with synaptosome use for the study of glutamate uptake first commencing a decade later (Levi & Raiteri, 1973). Synaptosomes are commonly called “pinched-off nerve endings” due to the way they are processed, and generally contain the presynaptic terminal and presynaptic vesicles, as well as the postsynaptic membrane and the postsynaptic density (Bai & Witzmann, 2007). However, the amount of glial membrane within a synaptosome preparation can vary from laboratory to laboratory (Danbolt, 2001). In addition, fragments of glial cells may have a lower probability of resealing following homogenization compared to nerve terminals, thereby creating an scenario whereby neuronal compartments have a higher chance of achieving successful glutamate uptake compared to astrocytes (Danbolt et al., 2016). Indeed, it was elegantly demonstrated that the quantification of glutamate uptake in synaptosome preparations overemphasizes the contribution from nerve terminal transporters while diminishing the contribution from glial transporters (Petr et al., 2015). The authors conditionally knocked out EAAT2 in either astrocytes or neurons and found increased mortality and seizures in the astrocyte-specific EAAT2 knockouts and a very mild phenotype in the neuron-specific EAAT2 knockouts. Loss of neuronal EAAT2 did not significantly reduce overall EAAT2 protein levels quantified by Western blot, although uptake capacity in synaptosomes was reduced by over 50%. On the other hand, astrocyte-specific EAAT2 knockdown decreased total EAAT2 expression levels by approximately 80% yet had no significant effect on synaptosome uptake capacity (Petr et al., 2015). Similar effects were observed in a separate study that used a different EAAT2 conditional knockout mouse line and examined uptake in synaptosome preparations obtained from the hippocampus, cortex, thalamus, and striatum (Zhou et al., 2019).

The spatiotemporal dynamics of extracellular glutamate are determined by both glutamate uptake and diffusion. Not all synapses exhibit tripartite morphology (Ventura & Harris, 1999), glutamate transporters exhibit different regional and subcellular expression patterns (Holmseth et al., 2012; Lehre & Danbolt, 1998; Rothstein et al., 1994), and the tortuosity of the extracellular space can vary in a region- and activity-dependent manner (Bernardinelli et al., 2014; Hrabětová, 2005; Perez-Alvarez et al., 2014). Thus, biochemical uptake assays, regardless of the tissue preparation, represent powerful approaches to quantify bulk exogenous glutamate uptake over a time scale of minutes. This may be particularly useful when the research question is primarily focused on determining how a specific experimental manipulation affects overall uptake capacity. Indeed, overall uptake capacity, as measure through biochemical uptake assays, often correlates with EAAT expression levels; experimental



	Location and EAAT subtypes	Major functional implications	Associated methodological considerations
A	Astrocytes (EAAT1, EAAT2)	Efficient clearance of extracellular glutamate	Combine to generate STCs recorded from astrocytes
B	Presynaptic terminals (EAAT2)	Direct recycling of presynaptic glutamate; presynaptic energy metabolism	Main influence on measures of synaptosomal uptake
C	Postsynaptic dendrites (EAAT3)	Glutamate buffering; limiting extrasynaptic NMDAR activation	Does not contribute to the STC but slows its time-course

FIGURE 1 The fate of synaptically released glutamate. Schematic tripartite synapse depicting relative expression levels and subcellular localization of EAAT1 (green), EAAT2 (red), and EAAT3 (purple). Relative amounts and the subcellular location depicted are a rough approximation based on the values reviewed in Danbolt et al. (2016). When glutamate is released into the synaptic cleft, it can be taken up by astrocytes through EAAT1 or EAAT2 (depicted by a). Much of this glutamate is then processed by the canonical astrocyte glutamate–glutamine cycle, providing glutamine back to the neuron for subsequent glutamate production. Glutamate uptake into astrocytes can also be metabolized for use in the tricarboxylic acid cycle. Synaptically released glutamate can also be taken up directly by nerve terminals through EAAT2 (depicted by b), where it could potentially be used for subsequent release or for local energy metabolism (Danbolt et al., 2016; McNair et al., 2019). Synaptically released glutamate may also be taken up directly into postsynaptic dendrites through EAAC3 (depicted by c), although evidence suggests that EAAT3 may primarily buffer glutamate, thereby limiting extrasynaptic receptor activation and delaying glutamate uptake by astrocytic EAAT1 and EAAT2 (Scimemi et al., 2009). Figure was created with BioRender.com [Color figure can be viewed at wileyonlinelibrary.com]

manipulations that increase or decrease overall EAAT protein expression typically increase or decrease uptake capacity in biochemical assays, respectively. However, it is also important to note that due to the exogenous application of glutamate, all transporters will be exposed to the same glutamate concentration. Contrast this to intact brain tissue, where the concentration of glutamate that reaches a given transporter will depend on a variety of factors including the transporter's subcellular localization with respect to the localized site of glutamate release. When interpreting data obtained specifically from synaptosome preparations, one must consider that the result is dominated by the overall uptake capacity of neurons over astrocytes. Lastly, post-translational modifications such as phosphorylation and palmitoylation are known to affect EAAT function (Casado et al., 1993; Huang et al., 2010), and such modifications may not be accurately captured in synaptosome preparations. Therefore, in order to gain a more thorough understanding of CNS control over extracellular glutamate dynamics, additional techniques are required where glutamate release occurs endogenously and where the synaptic and glial microenvironments and EAAT localization are preserved as much as possible (Figure 1). This is particularly true if we expect to

understand glutamate dynamics on a millisecond time scale and at a subcellular spatial resolution. One essential technique that has been used to explore glutamate uptake *in situ* for decades is the electrophysiological recording of STCs (Bergles & Jahr, 1997).

3.2 | STC recordings from astrocytes

Until recently, STCs have been used as the exclusive technique to quantify glutamate uptake on a millisecond time scale, in real time and in response to endogenous synaptic glutamate release. As mentioned above, glutamate uptake is associated with a net inward current that can be recorded by patch-clamping single astrocytes. Synaptic activity, evoked by electrical stimulation, results in STCs that are sensitive to DL-threo-β-Benzyloxyaspartic acid (TBOA), a commonly used non-selective glutamate transporter blocker (Shimamoto et al., 1998). STC recordings began in the 1980s, with seminal work completed by the laboratories of David Attwell (Brew & Attwell, 1987) and Craig Jahr (Bergles & Jahr, 1997). STCs are most commonly recorded in the hippocampus, within the stratum radiatum following Schaffer collateral stimulation, but have also been recorded elsewhere including the cortex (Armbruster et al., 2016; Hanson et al., 2015; Romanos et al., 2019) and striatum (Goubard et al., 2011; Parsons et al., 2016). Glutamate uncaging has also been used to evoke transporter currents (Scimemi et al., 2009), and while the uncaging approach relies on exogenous glutamate as the source, it circumvents the need to disrupt the tissue with a stimulating electrode to stimulate dense bundles of excitatory axons. Uncaging-evoked transporter currents may also be of great value to those interested in quantifying uptake in brain regions that are not characterized by highly dense and well-organized excitatory connections, as is found in the hippocampus. While typically recorded from astrocytes, TBOA-sensitive transporter currents have also been detected in other cell types, including calyx of Held nerve terminals (Palmer et al., 2003) and cerebellar Purkinje cells (Wadiche et al., 2006). It is worth noting that transporter currents could not be detected in hippocampal CA1 pyramidal neurons, suggesting that EAAT3 expression at neuronal surfaces is too low to detect a clean electrophysiological signal (Holmseth et al., 2012). The decay kinetics of astrocytic STCs can vary among brain regions and development stages, but are typically associated with decay tau values of 20 milliseconds or less (Armbruster et al., 2016; Diamond, 2005; Parsons et al., 2016). Such measurements are associated with electrotonic filtering that slows the STC time course; deconvolving this filter yields a time constant value of approximately 1 millisecond in the adult stratum radiatum (Diamond, 2005). At present, no other technique can match the temporal resolution obtained through STCs.

As the processes from a single astrocyte can potentially encompass tens of thousands of excitatory synapses (Bushong et al., 2002; Chai et al., 2017), it is impossible to determine both the number and location of the release sites that contribute to a given STC. Considering the low membrane resistance characteristic of astrocytes, the STC is likely to be dominated by uptake occurring at EAATs on proximal astrocytic processes. High stimulus intensities are commonly used to evoke a clear STC, indicating that smaller glutamate transients are likely to go undetected with this approach. Furthermore, STCs recorded from astrocytes are restricted to reporting the temporal dynamics of glutamate in the microenvironment of the recorded astrocyte, which may differ from the temporal dynamics of the glutamate microenvironment at individual pre- and postsynaptic release sites. Nonetheless, STC recordings represent an extremely powerful technique to study glutamate uptake, and they provide the best temporal resolution of the methods currently available.

3.3 | Glutamate biosensors

In recent years, genetically encoded indicators of glutamate have become widely available to the neuroscience community, enabling real-time quantification of glutamate transients from neurons, glia, or even specific neuronal subtypes based on Cre-driven expression. iGluSnFR (Marvin et al., 2013) and more recent variants (Helassa et al., 2018; Marvin et al., 2018) are now being used by many laboratories throughout the world to further increase our understanding of extracellular glutamate regulation in health and disease. iGluSnFR was created by combining *Escherichia coli* GltI, which undergoes conformational change upon glutamate binding, and a circularly permutated green fluorescent protein (GFP). The resulting protein, iGluSnFR, embeds within the plasma membrane with its glutamate binding domain facing the extracellular surface. Upon glutamate binding, the conformational change increases the emission intensity of the GFP; thus, extracellular glutamate levels are monitored by quantifying relative changes in GFP intensity during neural activity. iGluSnFR exhibits kinetics on a millisecond time scale and can detect glutamate actions at individual spines or presynaptic boutons (Dürst et al., 2019; Helassa et al., 2018; Marvin et al., 2013).

Since the original description of iGluSnFr (Marvin et al., 2013), new variants are continuously being developed. These successive iterations extend the versatility of these glutamate biosensors. For example, a recent iGluSnFR variant, termed $iGlu_u$ ("u" for ultrafast), significantly improved upon the speed of original iGluSnFR. Two-photon imaging with high-frequency line or spiral scans permit fluorescence sampling with sufficient temporal and spatial resolutions to detect discrete $iGlu_u$ fluorescent events at single presynaptic boutons in response to activity patterns upwards of 100 Hz (Helassa et al., 2018). In addition to improving the speed of iGluSnFR, newer variants also increase its stability and affinity, while providing a wider range of emission wavelengths (e.g., blue, cyan, green, yellow) to improve compatibility with additional biosensors. Some of the new variants, known as SF-iGluSnFR (S72A, A184V, and A184S), are more thermodynamically stable and have increased soluble protein expression (Marvin et al., 2018). These SF-iGluSnFR variants have higher signal-to-noise ratios, allowing for improved transient detection in single spines compared to original iGluSnFR. In addition,

variants $iGlu_h$, $iGlu_m$ and $iGlu_l$ were recently designed to cover a wide affinity range, with Kd values ranging from low micromolar to tens of millimolar (Coates et al., 2020). Additional versions have been designed with a yellow-light excitable red fluorescent protein, termed R-iGluSnFR and R^{ncp}-iGluSnFR (Wu et al., 2018). Overall, the iGluSnFRs have been employed to measure glutamate in numerous preparations including cell culture, organotypic slice culture, acute slice, and *in vivo*. They have been used to visualize extracellular glutamate in a variety of regions including the retina, olfactory bulb, cortex, hippocampus, striatum, globus pallidus and brainstem, and in a wide range of species including mice, rats, zebrafish, ferrets, and cats (Table 1).

While tempting to think of iGluSnFR transients as a direct report of the true extracellular glutamate dynamics, the mere presence of iGluSnFR introduces artificial glutamate binding sites that will compete with endogenous binding sites including glutamate receptors and the EAATs themselves. Thus, an evoked iGluSnFR transient represents glutamate that binds and unbinds iGluSnFR before diffusing away from the imaging region of interest or before being transported through nearby EAATs. In fact, simply expressing iGluSnFR in astrocytes slows the time course of the STC (Armbruster et al., 2020). Thus, iGluSnFR expression artificially slows the time course of extracellular glutamate, and iGluSnFR and variants will always introduce additional binding sites to the system under study. As mentioned in an essential paper on glutamate uptake published 15 years ago, the ideal measurement of glutamate "requires a fast sensor that does not affect synaptically released transmitter before its uptake" (Diamond, 2005). By design, iGluSnFR and variants buffer synaptically released glutamate before its uptake, and therefore can never represent the true time course of extracellular glutamate. Keeping this caveat in mind, iGluSnFRs do respond extremely well to pharmacological inhibition of EAATs and can serve as a sensitive measure of relative changes in glutamate clearance capacity between different experimental parameters (Armbruster et al., 2016; Parsons et al., 2016; Pinky et al., 2018). iGluSnFR expression can also be directed to different cell types, and using a sophisticated imaging system, glutamate clearance can even be quantified at distinct subcellular microenvironments on a millisecond time scale.

The techniques described above, from the first foray into measuring glutamate uptake with biochemical uptake assays, to the cutting-edge imaging techniques available now, have resulted in the foundation of our vast, yet still incomplete, understanding of glutamate uptake. While each of the aforementioned techniques are not without their limitations, they can be viewed as complementary, rather than competing, techniques. A summary of the advantages and limitations of these techniques is shown in Table 2 and a schematic overview of each is shown in Figure 2. In the following sections, we will discuss how these techniques have combined to increase our understanding of extracellular glutamate regulation in health and in disease.

4 | GLUTAMATE UPTAKE IN THE HEALTHY BRAIN

To fully understand how improper handling of extracellular glutamate may contribute to CNS disease, we must first gain a more complete understanding of how glutamate dynamics are regulated in the healthy brain. In this section, we will discuss what biochemical uptake assays, STCs, and iGluSnFRs have told us about glutamate regulation in healthy brain tissue, including the contributions of EAAT

TABLE 1 Applications of iGluSnFR and its variants across different brain regions and different species. Articles included were compiled by a PubMed-indexed search with "iGluSnFR" and "Brain" included in the title, abstract, and/or keywords

Species	Region	Publications
Mouse	Retina	Marvin et al. (2013, 2018), Borghuis et al. (2014), Poleg-Polsky and Diamond (2016)
	Olfactory bulb	Brunert et al. (2016)
	Cortex	Armbruster et al. (2016), Hefendehl et al. (2016), Hikima et al. (2016), Xie et al. (2016), McGirr et al. (2017), Rakers et al. (2017), Eles et al. (2018), Marvin et al. (2018), Pinky et al. (2018), Romanos et al. (2019)
	Hippocampus	Marvin et al. (2013, 2018), Jensen et al. (2017), Balakrishnan and Mironov (2018), Pinky et al. (2018), Dürst et al. (2019), Barnes et al. (2020), Wilkie et al. (2020)
	Striatum	Parsons et al. (2016), Koch et al. (2018), Pinky et al. (2018)
	Globus pallidus	Cui et al. (2016)
	Cerebellum	Marvin et al. (2018)
Rat	Hippocampus	Soares et al. (2017), Soares et al. (2019), Helassa et al. (2018), Marvin et al. (2018), Wu et al. (2018)
Zebrafish	Retina	MacDonald et al. (2017)mac
	Brainstem	Marvin et al. (2013), Tabor et al. (2018)
Ferret	Cortex	Marvin et al. (2018)
Cat	Cortex	O'Herron et al. (2016)

TABLE 2 Comparison of the strengths and limitations of commonly-used techniques available to quantify glutamate uptake. iGluSnFR imaging provides the best spatial resolution, as glutamate transients can be detected at subcellular localizations such as individual spines or presynaptic varicosities. STCs report the summation of transporter currents in the recorded astrocyte, while uptake assays are largely devoid of subcellular synaptic structure. STC recordings provide the best temporal resolution, particularly when deconvolution methods are used to extract the effects of electrotonic filtering (Diamond, 2005). While slower, iGluSnFR imaging also offers millisecond time scale responses, with newer iGluSnFR variants improving upon the original's kinetics. Physiological relevance is relatively low in synaptosome uptake assays, as these preparations tend to overestimate neuronal glutamate uptake at the expense of astrocytic glutamate uptake (Petr et al., 2015). iGluSnFR relies on the introduction of an artificial glutamate binding site to the region of interest, and STC recordings are largely limited to acute slice or culture preparations and are influenced to some degree by filtering properties of the recorded astrocyte. iGluSnFR imaging provides the best control over cell-type specificity, as expression can be directed toward specific cells of interest. Lastly, of the three methods, iGluSnFR imaging is the only one suitable for *in vivo* experiments

	Glutamate source	Spatial resolution	Temporal resolution	Physiological relevance	Cell-type specificity	*In vivo* compatibility?
Synaptosome uptake assays	Exogenous	+	+	+	+	No
Astrocyte STC recordings	Synaptic	++	+++	++	++	No
iGluSnFR and variants	Synaptic	+++	++	++	+++	Yes

Note: + moderate performance; ++ good performance; +++ excellent performance.

subtypes to overall clearance rates as well as observed regional differences in uptake capacity. In addition, this section will discuss how EAATs exert control over synaptic plasticity and how glutamate uptake itself is subject to both short- and long-term activity-dependent modification.

4.1 | Contribution of EAAT subtypes to glutamate clearance rates

Mice lacking the EAAT2 protein are characterized by lethal seizures and a shortened life-span (Tanaka et al., 1997). Furthermore, glutamate uptake capacity, measured in synaptosomes obtained from cortical tissue, is reduced by over 90% in EAAT2 knockout mice (Tanaka et al., 1997). Thus, there is little doubt that EAAT2 is an essential transporter, and it is commonly cited that EAAT2 is responsible for approximately 90% of glutamate uptake in the forebrain (e.g., see Carter et al., 2019; Fontana, 2015; Kim et al., 2011; Peterson & Binder, 2020; Takahashi et al., 2015; Rose et al., 2018).

What do other techniques tell us about the contribution of different EAATs to overall uptake capacity? As mentioned above, STC recordings reflect the inward current associated with glutamate transport into astrocytes. As astrocytes do not express EAAT3 (Rothstein et al., 1994), the STC is produced by ion flux through EAAT1 and EAAT2. Saturating concentrations of DHK, a commonly used EAAT2-selective antagonist, can double the decay constant of the STC, confirming an essential role for EAAT2 to glutamate clearance rates. However, the STC cannot establish how slow glutamate clearance would become if all EAAT subtypes were inhibited, as complete EAAT inhibition blocks the STC itself. Interestingly, a saturating concentration of DHK reduces the size of the STC but not by the commonly cited amount of 90%–95%—a value that is largely derived from experiments in synaptosomes (Tanaka et al., 1997). A saturating concentration of DHK inhibits the STC by about 30% in the hippocampus (Bergles & Jahr, 1997), with another report estimating that approximately one third of the STC is dependent on EAAT1, with two thirds being dependent on EAAT2 (Scimemi et al., 2009, 2013). In the striatum, DHK reduces the STC by a little more than 30% (Goubard et al., 2011).

Using iGluSnFR, it is possible to visualize the consequences of selective EAAT2 inhibition and compare it to the effect of complete transporter inhibition. The decay of iGluSnFR transients evoked in brain slices increases significantly more following non-selective EAAT inhibition with TBOA compared to the effect of DHK alone. In fact, the DHK-induced slowing of the iGluSnFR transient only reaches about 20% of the slowing induced by TBOA (Armbruster et al., 2016; Parsons et al., 2016; Pinky et al., 2018). Based on the dense expression of EAAT proteins in the brain, perhaps it should not be surprising that even a reserve of a minority of functional transporters, regardless of subtype, can still clear glutamate rather effectively. A recent study using iGluSnFR demonstrated that while a saturating concentration of DHK almost doubled the decay constant of evoked iGluSnFR signals in the barrel cortex, it had no significant effect on iGluSnFR signals evoked with a single pulse or a train of pulses at 50 Hz in the anterior cingulate cortex (Romanos et al., 2019), suggesting that even different regions within the cortex are likely to exhibit a differential reliance on glutamate transporter subtypes. These results do not diminish the importance of EAAT2; rather they highlight the important yet perhaps overlooked contributions of non-EAAT2 transporters to glutamate uptake in the CNS.

4.2 | Regional differences in glutamate clearance

Recent work in our laboratory has also used iGluSnFR to reveal regional differences in the brain's ability to clear glutamate (Pinky

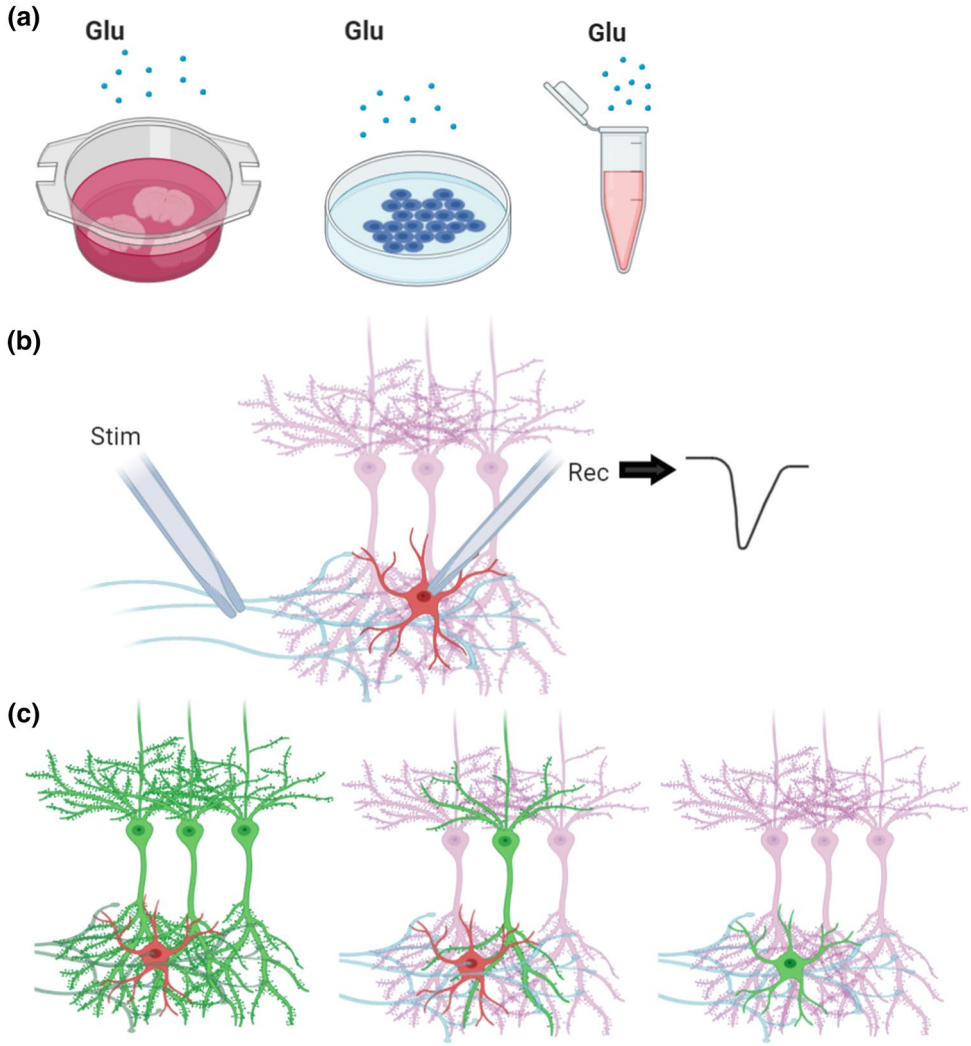


FIGURE 2 Schematic overview of common methods used to quantify glutamate uptake. (a) Biochemical uptake assays can be performed on a variety of preparations, including brain slices (left), synaptosomes (center) and tissue homogenates (right). In all cases, the technique relies on the application of exogenous radiolabeled glutamate on the time scale of minutes. (b) A patch-clamp recording pipette (rec) is used to record synaptically activated transporter currents (STCs), typically from a single astrocyte. Electrical stimulation (stim) is generally used to evoke the glutamate release that generates the STC, although glutamate uncaging can also be used as a source. The resulting STC occurs on a millisecond time scale and represents the glutamate uptake response in the microenvironment of the recorded astrocyte. (c) iGluSnFR (depicted by green fill) can be expressed by a neuronal-specific promoter such as synapsin (left). Sparse labeling (middle) can be achieved by single-cell electroporation, gene gun delivery or diluted injections of AAV-Cre with a Cre-dependent iGluSnFR. iGluSnFR can also be expressed under the control of an astrocyte-specific promoter such as GFAP (right). Once expression is generated, iGluSnFR transients can be evoked by various means including (but not limited to) electrical stimulation, sensory stimulation or glutamate uncaging. Depending on the imaging system, responses can be viewed at the population level or at the level of individual dendritic spines or presynaptic boutons. Figure was created with BioRender.com [Color figure can be viewed at wileyonlinelibrary.com]

et al., 2018). Using wild-type C57BL/6 mice, we compared glutamate clearance rates in the hippocampus, cortex, and striatum. The hippocampus was the most efficient in clearing glutamate released into the extracellular space during synaptic activity, with intermediate rates observed in the cortex, and slowest rates in the striatum (Pinky et al., 2018). Interestingly, independent of glutamate uptake, diffusion rates were also faster within the hippocampus which is likely due to regional differences in tortuosity (i.e., barriers to diffusion) of the extracellular space (Hrabětová, 2005; Pinky et al., 2018). Results from both iGluSnFR and STC studies agree that in the hippocampus, glutamate transporters do not become overwhelmed by brief periods of high-frequency neural activity up to 10 pulses at 100 Hz (Diamond & Jahr, 2000; Pinky et al., 2018). In agreement, astrocyte processes are more closely apposed to postsynaptic densities in the hippocampus than they are in the striatum, and the hippocampus also contains a higher percentage of excitatory synapses directly apposed by an astrocyte process (Chai et al., 2017). Similarly, STC decay constants are slower in the cortex and striatum compared to the hippocampus (Goubard et al., 2011; Hanson et al., 2015; Parsons et al., 2016). These regional differences emphasize the complexity of glutamate uptake throughout the brain and showcase some of the advantages of *in situ* preparations to the study of glutamate dynamics.

4.3 | EAAT control over synaptic plasticity

Despite its essential role in excitatory neurotransmission, glutamate has the potential to be neurotoxic (Choi et al., 1987). In addition to preventing excitotoxicity, EAATs serve as gatekeepers that dictate the subcellular localization of pre- and postsynaptic glutamate receptors activated during synaptic neurotransmission. For example, poor uptake can over-activate glutamate receptors, including extrasynaptically located NMDARs (exNMDARs) that have been associated with synapse-weakening and cell death signaling pathways (Hardingham & Bading, 2010; Hardingham et al., 2002; Parsons & Raymond, 2014). As the initiation of many forms of synaptic plasticity relies on the activation of a specific type of glutamate receptor residing at specific subcellular locations, it is not surprising that EAAT manipulations can have profound effects on activity-dependent synaptic plasticity.

The neuronal transporter EAAT3 plays an important role in synaptic plasticity by buffering synaptically released glutamate, thereby limiting its spatial spread following release (Scimemi et al., 2009). In EAAT3 knockout mice, the STC decay does not slow down as one may expect to occur following the complete loss of one EAAT subtype; in fact, the STC decay becomes significantly faster. As the STC is comprised of current flux through EAAT1 and EAAT2 only, the faster STC in EAAT3 knockout mice suggests the presence of neuronal EAAT3 acts as a buffer that slows the rate at which synaptically released glutamate reaches astrocytic membranes. Glutamate buffering by EAAT3 also limits the amount of glutamate that spills over onto GluN2B-containing exNMDARs, and knocking out EAAT3 impairs hippocampal long-term potentiation (LTP) due to overactivation of GluN2B-containing NMDARs (Scimemi et al., 2009). Thus, it appears that the presence of EAAT3 facilitates LTP by limiting both glutamate spillover and GluN2B-containing NMDAR overactivation.

Recently, our laboratory used iGluSnFR to monitor extracellular glutamate transients during theta burst stimulation, a commonly used LTP induction protocol (Barnes et al., 2020). We found that non-selective EAAT inhibition by TBOA resulted in a concentration-dependent increase in the size and decay constant of iGluSnFR signals, as well as a concentration-dependent inhibition of hippocampal LTP. Interestingly, blocking EAAT2 with 100 μM DHK (K_i = 24 μM) had no effect on hippocampal LTP despite having clear effects on iGluSnFR transients, increasing their size and slowing their decay. Thus, LTP is much more sensitive to non-selective EAAT inhibition compared to selective EAAT2 inhibition, despite similar effects on extracellular glutamate dynamics. We found that the LTP impairment induced by TBOA was due to calcium overload from the triggering of L-type voltage-gated calcium channels. Thus, EAATs exert tight spatiotemporal control over not only glutamate receptor activity but also subsequent activation of voltage-sensitive ion channels (Barnes et al., 2020).

Impairing glutamate uptake can have detrimental effects on synaptic plasticity, but is the converse true? If rapid glutamate uptake is necessary to achieve safe and efficient synaptic communication, while minimizing toxicity and maximizing the potential for plasticity, are there any consequences to increasing glutamate uptake capacity? Excessive uptake could conceivably reduce the efficiency of synaptic neurotransmission if glutamate is transported before it has a chance to bind to glutamate receptors. For example, postsynaptic metabotropic glutamate receptors (mGluRs) are commonly found at peri- and extrasynaptic sites (Baude et al., 1993; Luján et al., 1996); thus, at least some degree of glutamate spillover is often required to activate mGluRs. Ceftriaxone, a drug commonly used to increase EAAT2 expression, impairs mGluR-dependent long-term depression at mossy fibre-CA3 synapses (Omrani et al., 2009). A specific amount of glutamate spillover is also required to achieve normal bidirectional spike timing-dependent plasticity at corticostriatal synapses; increasing EAAT2 expression with ceftriaxone abolishes both NMDAR-dependent LTP and endocannabinoid-dependent LTD (Valtcheva & Venance, 2016). These data indicate that a delicate balance of uptake must be achieved throughout the CNS, as potentially dire consequences may arise from insufficient as well as excessive uptake (Figure 3).

4.4 | Activity-dependent alterations in glutamate uptake

Over two decades ago, two separate studies used STC recordings to quantify the amount of presynaptic glutamate release, with the goal of determining whether NMDAR-dependent LTP at CA3–CA1 synapses was expressed pre- and/or postsynaptically. In other words, was the sustained increase in synaptic strength following this form of plasticity dependent, at least in part, on long-term increases in presynaptic release probability? Both papers convincingly demonstrated that while high-frequency stimulation induced a long-lasting increase in postsynaptic responses to Schaffer collateral stimulation, STCs recorded from astrocytes were unaffected. However, these studies used the STC size to quantify putative changes in the amount of glutamate release and did not focus on the time course of the STC. So, while the STC has been reported to be unchanged during hippocampal LTP (Diamond et al., 1998; Lüscher et al., 1998), additional work has strongly suggested that neural activity patterns promoting synaptic plasticity also induce long-term changes in EAAT expression and function. A few years later, it was shown that high frequency stimulation (HFS) of the Schaffer collateral pathway increased glutamate uptake in hippocampal slices 30 min later (Levenson et al., 2002). To quantify glutamate uptake capacity, hippocampal slices were incubated in oxygenated artificial cerebrospinal fluid containing exogenous radiolabeled glutamate for 7.5 min. Interestingly, the observed increase in uptake capacity 30 min following HFS was the result of a NMDAR-dependent increase in the cytosol-to-membrane translocation of the neuronal transporter EAAT3 (Levenson et al., 2002). As STCs carry ion flux through EAAT1 and EAAT2, but not EAAT3, this provides a putative explanation for why previous studies found no effect of HFS on STC size (Diamond et al., 1998; Lüscher et al., 1998). The HFS-induced increase in EAAT3 returns to basal

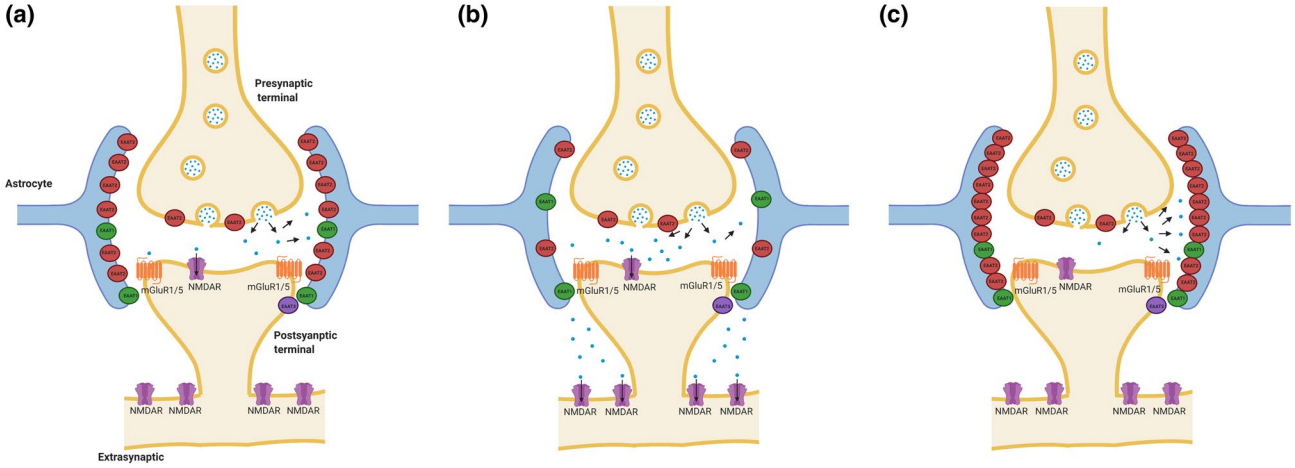


FIGURE 3 The Goldilocks principle applied to glutamate uptake. Tripartite synapses highlighting (a) optimal glutamate uptake, (b) too little glutamate uptake, and (c) excessive glutamate uptake. Optimal glutamate uptake results in glutamate being efficiently cleared from the synaptic cleft, while also facilitating synaptic neurotransmission by allowing glutamate sufficient access to glutamate receptors, namely those located synaptically and perisynaptically (a). The consequences of too little glutamate uptake include an increase in glutamate spillover outside the synaptic cleft which can lead to the overactivation of extrasynaptic NMDARs (b). Excessive glutamate uptake can impair synaptic plasticity, for example by reducing mGluR1/5 activation (Omrani et al., 2009). Figure was created with BioRender.com [Color figure can be viewed at wileyonlinelibrary.com]

levels by 3 hr post-HFS; however, at this time, a NMDAR-dependent increase in EAAT2 is observed (Pita-Almenar et al., 2006). Thus, plasticity-inducing activity patterns at Schaffer collateral pathway results in an initial increase in EAAT3, followed by a delayed and longer-lasting increase in EAAT2. Indeed, a clear increase in the size of astrocyte transporter currents evoked by glutamate uncaging is observed 3 hr, but not 30 min, following HFS (Pita-Almenar et al., 2012). The early increase in EAAT3 is dependent on PKC whereas the late and sustained increase in EAAT2 is dependent on PKA (Levenson et al., 2002; Pita-Almenar et al., 2006), and it is possible that such changes play an important role in the hippocampus' ability to code and store information (Pita-Almenar et al., 2012). As EAAT3 has been shown to buffer synaptically released glutamate (Scimemi et al., 2009), the early increase in EAAT3 expression following HFS may help limit the amount of glutamate spillover to extrasynaptic sites during the early stages of synapse strengthening. The sustained increase in EAAT2 may be a protective mechanism to help avoid over-excitation and excitotoxic insults following the establishment of LTP. Further studies are required to realize the full physiological relevance of these plastic changes in EAAT expression and function.

In addition to the documented changes minutes and hours following HFS, recent work using iGluSnFR and STCs has clearly demonstrated that EAAT-dependent glutamate uptake can be modulated rapidly and reversibly by neural activity (Armbruster et al., 2016; Parsons et al., 2016; Pinky et al., 2018; Romanos et al., 2019). In various brain regions including the hippocampus, cortex, and striatum, increasing the duration of neural activity results in a clear slowing of the iGluSnFR and STC decay. Further investigation into this phenomenon convincingly demonstrated that both the frequency and duration of neural activity could dramatically influence the rate of uptake of synaptically released glutamate, although such effects are short-lived and uptake rates return to baseline within 50 milliseconds of inactivity (Armbruster et al., 2016).

How does neural activity modulate glutamate clearance on such a rapid time scale? At present, the mechanisms underlying this effect are not fully understood, but it is clear that activity-dependent slowing of glutamate uptake is dependent on the duration and frequency of neural activity, rather than on the amount of glutamate that is released (Armbruster et al., 2016; Pinky et al., 2018). The most likely explanation is that sustained neural activity depolarizes nearby astrocyte processes, thereby reducing the driving force for glutamate through astrocytic EAATs. However, it is likely that the full explanation is more complicated than simple astrocytic depolarization, as a recent report demonstrated clear regional differences in the activity-dependent modulation of glutamate uptake. Increasing the frequency of neural activity from 50 to 100 Hz slows iGluSnFR and STC measures of glutamate uptake in the barrel cortex but accelerates these same measures in the anterior cingulate cortex. Interestingly, the frequency-dependent acceleration of glutamate uptake in the anterior cingulate cortex is sufficient to limit NMDAR activation (Romanos et al., 2019). Such opposing effects of activity on uptake rates cannot be explained by a single global mechanism involving astrocyte depolarization. In sum, emerging data convincingly demonstrate that the frequency and duration of neural activity evokes short-term plastic effects on glutamate uptake capacity. Further studies employing *in situ* techniques like STCs and iGluSnFR imaging are required to gain a more complete understanding of the underlying mechanisms, regional differences, and consequences of such activity-dependent modifications of synaptic neurotransmission.

5 | GLUTAMATE UPTAKE IN NEURODEGENERATIVE DISEASE

Neurodegenerative diseases are characterized by the gradual deterioration of vulnerable brain tissue over years to decades, dramatically reducing quality of life and typically shortening life-span by a number of years (Dugger & Dickson, 2017). Rates of neurodegenerative diseases in the population are increasing at an alarming rate; however, current treatments for most neurodegenerative diseases focus on managing the symptoms and do not treat the underlying pathology. Therefore, pathways/mechanisms underlying these diseases must be identified to aid in treatment. Reduced glutamate transporter expression and/or function is frequently reported among neurodegenerative diseases, including AD (Masliah et al., 1996; Scott et al., 2011), HD (Hassel et al., 2008; Huang et al., 2010; Liévens et al., 2001), amyotrophic lateral sclerosis (Rothstein et al., 1995), and others. For the remainder of this review, we will focus the discussion on two neurodegenerative diseases, HD and AD, to discuss what the different methodological approaches to quantifying glutamate uptake have told us about glutamate dynamics in these diseases.

5.1 | Huntington disease

HD is an autosomal dominant neurodegenerative disease caused by a repeat expansion in the huntingtin gene on chromosome 4, resulting in the production of a polyglutamine-expanded mutant huntingtin protein (mHTT). The presence of mHTT, in combination with reduced expression of the non-expanded/non-pathological huntingtin protein, results in a clinical triad of motor, cognitive, and psychiatric symptoms. HD is generally fatal within approximately 15 years of symptom onset.

Glutamate toxicity, particularly in the striatum, is a major contributor to the pathophysiology of HD (Raymond et al., 2011). While increased toxicity can be explained at least partially by a mislocalization of NMDARs to extrasynaptic sites (Okamoto et al., 2009; Milnerwood et al., 2010), there has also been a plethora of support for a glutamate uptake deficit in the HD brain. Reduced expression and/or post-translational modification of EAAT2, as well as reduced glutamate uptake capacity in synaptosome preparations, have been observed in various mouse models of HD (Behrens et al., 2002; Faideau et al., 2010; Huang et al., 2010; Liévens et al., 2001; Parsons et al., 2016; Shin et al., 2005).

However, in 2013, it was demonstrated that further reducing EAAT2 expression did not exacerbate the HD-like phenotype in the R6/2 mouse model of HD (Petr et al., 2013). In this paper, the authors generated heterozygous EAAT2 knockout mice that carried the R6/2 transgene and found that disease progression was no different than in R6/2 mice with two functional copies of EAAT2. They confirmed that EAAT2 expression was decreased in the brains of R6/2 mice at late disease stages, but that further reduction of EAAT in the crossed mice had no additional effect. They also found that glutamate uptake into striatal and cortical synaptosome preparations was similar between R6/2 mice and wild-type controls. From these findings, the authors concluded that alterations in EAAT2 expression and function are unlikely to be a significant modifier of HD progression (Petr et al., 2013).

A few years later, the first iGluSnFR experiments were performed in the striatum of YAC128 and R6/2 HD mice and no evidence of a functional uptake deficit was observed at the population level (i.e., low-magnification widefield imaging) in acute brain slices, despite a clear uptake deficit being detected in synaptosome preparations (Parsons et al., 2016). In fact, there was some evidence of accelerated clearance rates in the R6/2 model, a result that was demonstrated by both iGluSnFR and STC recordings in the striatum (Parsons et al., 2016). Similarly, the decay of evoked NMDAR-mediated currents has been shown to be either unchanged (Milnerwood et al., 2010) or accelerated (Parievsky et al., 2017) in the striatum of HD mice. More recently, when brain slices were exposed to exogenous radiolabeled glutamate for 60 min, striatal glutamate uptake measurements were not significantly different between WT and R6/2 mice, although reduced uptake was observed in R6/2 cortical tissue (Skotte et al., 2018). The results of these studies question whether slow glutamate uptake contributes to HD pathogenesis.

A possible solution to this discrepancy may have come from a recent study that took iGluSnFR imaging in HD to the level of individual release sites (Dvorzhak et al., 2019). For this study, the $iGlu_u$ variant was used to increase the temporal resolution of the fluorescent signals. Perhaps more importantly, the authors imaged glutamate transients from single cortico-striatal varicosities. Using the Q175 knock-in model of HD, they found that approximately 60% of varicosities observed exhibited decay kinetics within the rage of those found from wild-type control mice. However, 40% of varicosities exhibited $iGlu_u$ decay constants above 15 milliseconds, which was their cutoff for a "functional" synapse based on the data distribution from their wild-type animals. Thus, this elegant experimental design provides a compelling argument that glutamate uptake may be compromised at a subset of cortico-striatal synapses. It is possible that dysfunction in only a subset of synapses will go undetected with methods reliant on bulk stimulation of a population of synapses, including STC recordings and widefield iGluSnFR imaging with large regions of interest (Parsons et al., 2016). Together, these results suggest that while there is unlikely to be a global uptake deficit at all excitatory synapses in the HD striatum, it is likely that a subpopulation of synapses is affected later in disease progression. Interestingly, the number of close astrocyte contacts decreases for corticostriatal synapses and increases for thalamostriatal synapses in the R6/2 mouse model of HD (Octeau et al., 2018). It is currently unknown what percentage of release sites is affected overall, and at what stage of disease progression such synapse-specific abnormalities begin to emerge.

While the precise extent and consequences of uptake deficiencies in HD remain to be seen, it is well-documented that astrocytes—which mediate the bulk of glutamate uptake—are indeed dysfunctional in HD. In multiple mouse models of HD, astrocytes

were shown to express low levels of Kir4.1 potassium channels, and were depolarized compared to astrocytes from WT mice. Viral delivery of Kir4.1 not only restored the astrocyte resting membrane potential in HD mice, it also attenuated the motor phenotype (Tong et al., 2014). Although glutamate uptake was not assessed in this particular study, astrocytic depolarization alone would be predicted to reduce the driving force for glutamate uptake. Furthermore, restricting N-terminal mHTT expression to astrocytes was sufficient to drive a HD-like phenotype in mice, suggesting that mHTT-induced astrocyte dysfunction indeed contributes to disease pathogenesis. In fact, mHTT expression in astrocytes reduced EAAT2 expression by sequestering the transcription factor SP1 (Bradford et al., 2009). In this study, glutamate uptake was quantified by exposing acute brain slices containing the cortex and striatum to exogenous radiolabeled glutamate for 15 min. They found that DHK-sensitive (i.e., EAAT2 dependent) uptake was reduced in slices obtained from the astrocyte-mHTT mice. However, similar to the striatal results from the Skotte et al. (2018) study mentioned above, total uptake was not significantly reduced. Thus, it is possible that transporters other than EAAT2 may play a compensatory role. In addition, it is likely that that the phenotype that results from astrocyte-specific expression of mHTT is caused by perturbations to many of the essential functions of astrocytes, not just uptake. The contribution of astrocyte dysfunction to HD pathogenesis has received excellent coverage in recent reviews (Khakh et al., 2017; Wilton & Stevens, 2020).

In sum, whether slow glutamate uptake makes an essential contribution to HD progression, and whether global restoration of EAAT function is a valuable therapeutic strategy for the treatment of HD remains to be seen. Using iGluSnFR, we recently observed exaggerated accumulation of extracellular glutamate in the hippocampus of the Q175FDN mouse model of HD, but this was not due to reduced EAAT2 expression and was not ameliorated by ceftriaxone treatment (Wilkie et al., 2020). If we take the viewpoint that only a subset of synapses in the HD brain is characterized by slower transporter-mediated clearance rates, would a drug that results in a widespread increase in EAAT expression be beneficial or detrimental? Recall in our earlier discussion that for certain forms of plasticity, a certain amount of spillover is required to reach the desired receptors and that EAAT overexpression may impair certain forms of synaptic plasticity.

5.2 | Alzheimer disease

AD is an age-related neurodegenerative disease, which typically manifests as profound deficits in declarative memory, behavior, and personality changes. Familial AD can arise from mutations in genes encoding the amyloid precursor protein or the Aβ-generating proteases presenilin 1 and presenilin 2 (Chévez-Gutiérrez et al., 2012; Karch et al., 2014; Mullan et al., 1992). Mutations in these genes can all enhance levels of toxic Aβ fragments (e.g., $A\beta_{1\text{-}42}$), lending strong support to an involvement of Aβ toxicity in AD pathogenesis. Numerous other AD-related genes have now been identified, many of which play key roles in synaptic function (Karch et al., 2014; Kunkle et al., 2019). Tau aggregates (neurofibrillary tangles) also accumulate throughout the brain in AD, and together with Aβ plaques constitute the main neuropathological hallmarks of AD. The main initiator of AD pathology is still under debate, and is reviewed extensively elsewhere (Kametani & Hasegawa, 2018; Selkoe & Hardy, 2016).

Over two decades ago, Beckstrøm and colleagues (Beckstrøm et al., 1999) found no significant difference in EAAT1 or EAAT2 expression in post-mortem brain tissue (cingulate and inferior temporal gyri) from AD patients compared to controls. Similarly, a more recent study found no decrease in EAAT2 levels in the prefrontal cortex in patients with varying degrees of dementia (Poirel et al., 2018). In contrast, Li and colleagues found evidence for reduced EAAT2 protein in the AD prefrontal cortex (Li et al., 1997). How can these seemingly contradictory results of EAAT2 expression in AD tissue be explained? It should be noted that EAAT proteolysis occurs rapidly, and the stability of EAAT expression can vary dramatically in the hours after death and is also dependent on the epitope recognized by the EAAT antibody (Beckstrøm et al., 1999). Another complicating factor is the evidence for EAAT2 splice variants in the context of AD; AD brains have been shown to have significantly higher levels of EAAT2Δ7 and EAAT2Δ9, variants that significantly reduce EAAT2's uptake efficiency and may be expressed at the expense of the wild-type form of EAAT2 (Scott et al., 2011).

What have animal models told us about glutamate transporter function in AD? Various lines of evidence suggest that toxic Aβ oligomers may cause EAAT2 dysfunction in AD due in part to their toxic effect on astrocytes. Application of oligomeric $A\beta_{1\text{-}42}$ to cultured astrocytes reduces EAAT2 expression by approximately 25% within 24 hr (Abdul et al., 2009). Another study found that just 30 min of $A\beta_{1\text{-}42}$ was sufficient to reduce surface expression of EAAT2 by 30%–40% (Scimemi et al., 2013). Consistent with the reduced EAAT expression, Aβ fragments have been shown to impair glutamate uptake as measured in biochemical uptake assays (Harris et al., 1996; Matos et al., 2008; Parpura-Gill et al., 1997). Reduced EAAT expression and function has been reported in the $APP_{Sw,Ind}$ model that overexpresses human APP with mutations linked to familial forms of AD (Takahashi, Kong, et al., 2015). Interestingly, when $APP_{Sw,Ind}$ mice were crossed with transgenic mice overexpressing EAAT2, EAAT2 levels and biochemically measured EAAT2-dependent glutamate uptake was restored to WT levels (Takahashi, Kong, et al., 2015). Furthermore, the same study found that this cross also enhanced survival, improved measures of cognitive function, and reduced Aβ plaque formation. Pharmacologically increasing EAAT2 expression with the small molecule, LDN/OSU- 0212320, evoked similar beneficial effects in $APP_{Sw,Ind}$ mice (Takahashi, Kong, et al., 2015). Similarly, pharmacological upregulation of EAAT2 with ceftriaxone (Rothstein et al., 2005) increases hippocampal expression of the synaptic proteins synaptophysin and PSD-95, and improves cognitive function in the 3 × Tg mouse model of AD (Zumkehr et al., 2015). When EAAT2 is further reduced in AD mice, achieved by generating AβPPswe/PS1ΔE9 mice lacking one EAAT2 allele, cognitive impairments are exacerbated (Mookherjee et al., 2011). In an elegant experimental

design, EAAT2 deletion in astrocytes, but not neurons, accelerated age-related cognitive decline. Moreover, astrocyte-specific deletion of EAAT2 altered hippocampal expression of a variety of genes involved in the innate and adaptive immune response, similar to changes observed in human AD (Sharma et al., 2019).

Scimemi and colleagues (2013) recorded STCs from hippocampal astrocytes before and after $A\beta_{1\text{-}42}$ application in wild-type mice and found that the time course of glutamate clearance was significantly slower after $A\beta_{1\text{-}42}$ treatment, suggesting that $A\beta_{1\text{-}42}$ rapidly impairs astrocytic glutamate transport. Moreover, in the presence of the EAAT2 antagonist, DHK, $A\beta_{1\text{-}42}$ failed to prolong glutamate clearance suggesting that the $A\beta_{1\text{-}42}$-induced slowing of glutamate clearance was due to its effects on EAAT2. Interestingly, while cell surface expression of EAAT2 was significantly reduced by $A\beta_{1\text{-}42}$ treatment, total EAAT2 levels were unaltered. Both cell surface and total levels of EAAT1 were unaffected by $A\beta_{1\text{-}42}$ (Scimemi et al., 2013). These results suggest that $A\beta_{1\text{-}42}$ may slow the clearance of synaptically released glutamate by disrupting EAAT2 localization near the astrocytic plasma membrane. The detrimental effect of $A\beta_{1\text{-}42}$ on EAAT2 localization and function was prevented by reducing oxidative stress using Trolox, a vitamin E derivative (Scimemi et al., 2013), supporting earlier work that demonstrated a link between Aβ, the lipid peroxidation product hydroxy-2-nonenal and EAAT2 (Lauderback et al., 2001).

Not all studies agree that glutamate uptake is impaired in mouse models of AD or by exposure to toxic fragments of Aβ. For example, no evidence of impaired glutamate uptake was recently reported in the hippocampus of the 5xFAD model of AD (Andersen et al., 2021). In this study, the authors exposed cortical and hippocampal brain slices to exogenous radiolabeled glutamate for 60 min. With this approach, it cannot be concluded that the rapid uptake of synaptically released glutamate is undisturbed in AD mice. Their data demonstrate that when exogenous glutamate is applied for 60 min, 5xFAD tissue absorbs the same total amount of glutamate as wild-type tissue. Interestingly, almost two decades age, it was reported that Aβ application to cultured cortical astrocytes can actually *increase* glutamate uptake activity (Ikegaya et al., 2002). In this study, exogenous radiolabeled glutamate was applied to cultured astrocytes for 7 min, and the Aβ-induced increase in uptake capacity was due to an increase in EAAT1 expression at the cell surface. Oddly, a high concentration of the EAAT2 inhibitor DHK had no effect whatsoever on their measurements of glutamate uptake and EAAT2 was undetectable by Western blot, suggesting that EAAT2 expression was negligible in their cultures.

The aforementioned studies, when considered as a whole, suggest that any glutamate uptake deficits in AD are complex and cannot simply be explained as a global reduction in uptake. A study using iGluSnFR in the cortex of APPPS1 mice elegantly reflects the complexity of uptake dysregulation in AD (Hefendehl et al., 2016). Interestingly, wide-field glutamate imaging demonstrated no differences in iGluSnFR signals in the cortex. Therefore, the authors next turned to evoked iGluSnFR signals using *in vivo* two-photon imaging. While still at the population level (i.e., not at individual synapses), this approach resulted in sufficient resolution and optical sectioning required to categorize iGluSnFR responses based on their proximity to Aβ plaques. They found that iGluSnFR decay times were slower and EAAT2 expression was lower in brain tissue surrounding Aβ plaques. In regions where Aβ plaques were not present, glutamate clearance was similar to WT mice. Ceftriaxone partially restored glutamate dynamics, suggesting that the impaired glutamate dynamics near Aβ plaques was due, at least in part, to EAAT2 dysfunction (Hefendehl et al., 2016). More recently, SF-iGluSnFR was used to demonstrate that hippocampal hyperactivity characteristic of AD may be due to an Aβ-dependent impairment of transporter-mediated glutamate uptake (Zott et al., 2019). In all, ample evidence exists demonstrating a negative effect of Aβ on glutamate uptake. The reduced uptake efficiency in AD may be directly relevant to the cognitive symptoms of the disease, as the deficits in hippocampal synaptic plasticity induced by oligomeric fragments of Aβ have been reported to result from an Aβ-mediated spillover of glutamate that results in excessive activation of GluN2B-containing NMDARs (Li et al., 2009, 2011).

While toxic Aβ oligomers appear to be sufficient to negatively impact EAAT expression and function, hyperphosphorylated tau and neurofibrillary tangles (NFTs) also play critical roles in AD pathology. Relative to Aβ, our understanding of the impact of the various forms of tau on glutamate uptake is in its infancy. Nonetheless, transgenic mice that express human tau protein in astrocytes exhibit age-dependent tau hyperphosphorylation (Forman et al., 2005) and reduced levels of EAAT1 and EAAT2 in the brainstem and spinal cord, as well as reduced synaptosomal measures of glutamate uptake in these regions (Dabir et al., 2006). Overexpression of TauP301L has been shown to significantly increase glutamate clearance time as measured by ceramic-based microelectrode arrays implanted in CA1, and this was correlated with decreased performance on the Barnes maze (Hunsberger et al., 2015). Similarly, transgenic mice expressing full-length human tau with the A152T mutation also exhibit tau hyperphosphorylation in the hippocampus in addition to elevated levels of extracellular glutamate. The latter was ameliorated by ceftriaxone treatment, suggesting a role of EAAT2 (Decker et al., 2016). The findings highlighted above demonstrate that in addition to toxic Aβ fragments, hyperphosphorylated tau is also heavily implicated in impaired glutamate uptake.

Given the evidence supporting altered glutamate uptake in AD, it is perhaps not surprising that astrocyte dysfunction is increasingly recognized as a hallmark of the disease. Reactive astrocytes are observed in post-mortem AD tissue, particularly in close proximity to amyloid plaques and neurofibrillary tangles (Serrano-Pozo et al., 2011). EAAT2 expression negatively correlates with astrogliosis in AD; that is, as GFAP expression increases EAAT2 expression decreases (Simpson et al., 2010). In addition to the Aβ-induced slowing of STCs described above (Scimemi et al., 2013), it was also demonstrated that Aβ application can increase glutamate release from astrocytes (Talantova et al., 2013). Thus, evidence exists to suggest that Aβ can promote extracellular glutamate accumulation both through enhancing gliotransmission and slowing uptake. Recently, astrocytes in AD have been suggested to take on an "A1" phenotype

in response to cytokines released from microglia. These A1 astrocytes lose many of the known beneficial functions of astrocytes, and instead adopt a neurotoxic role (Liddelow et al., 2017). Similar to HD, the loss of numerous astrocyte functions in addition to uptake appears to play a large role in AD pathogenesis. The overall importance of astrocyte dysfunction to AD progression has been recently reviewed elsewhere (Acosta et al., 2017; Carter et al., 2019).

6 | SUMMARY

Biochemical uptake assays have provided strong evidence for a glutamate uptake impairment in both AD and HD. Novel techniques to visualize glutamate dynamics in real time (e.g., iGluSnFR) now demonstrate that the regulation of glutamate dynamics in health and disease is incredibly complex, and varies in both an activity- and region-dependent manner. EAAT manipulations can profoundly affect synaptic plasticity, and glutamate uptake itself is regulated by both short-term and long-term plasticity mechanisms. Optical measurements of glutamate uptake are beginning to reveal that glutamate dynamics at individual synapses, and their perturbation in disease, can be quite heterogeneous. Demonstrations of selective impairment of glutamate uptake in the vicinity of Aβ plaques in AD (Hefendehl et al., 2016), and in a subset of cortico-striatal presynaptic terminals in HD (Dvorzhak et al., 2019), provide excellent examples of how optical-based approaches can expand upon the findings generated from biochemical uptake assays and STC recordings. It is important to note that these glutamate uptake techniques should not be viewed as competing, but rather as complementary. When used in combination, these techniques elucidate a more comprehensive view of glutamate uptake than either can achieve alone. Similar to iGluSnFR and its variants, numerous genetically encoded voltage indicators (GEVIs) have become available, permitting the visualization of membrane potential changes at a subcellular level (Chamberland et al., 2017). By combining iGluSnFR and GEVI imaging, we can gain a better understanding of how localized alterations in glutamate dynamics can impact membrane potential at the subcellular level. Excitingly, novel techniques to image other neurotransmitter systems in real time are continuously being developed. For example, in addition to glutamate, sensors for GABA (iGABASnFR, (Marvin et al., 2019)), dopamine ($GRAB_{DA}$, (Sun et al., 2018) and dLight, (Patriarchi et al., 2018)), and norepinephrine ($GRAB_{NE}$, (Feng et al., 2019)) have been created and characterized, and are all currently available to the general research community through Addgene. With biochemical approaches, electrophysiology and high-speed imaging of specific biosensors, we are now equipped with complementary tools that will enable the study of neurotransmitter systems with extraordinary precision. Much excitement lies ahead as these approaches continue to be employed in creative ways to further our understanding of neurotransmission, both in health and disease.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

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ORCID

Kyle J. Brymer https://orcid.org/0000-0003-4929-9042
Matthew P. Parsons https://orcid.org/0000-0001-9772-1549

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SUPPORTING INFORMATION

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