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Regulation of Synaptic Transmission by Ambient Extracellular Glutamate

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Abstract

Many neuroscientists assume that ambient extracellular glutamate concentrations in the nervous system are biologically negligible under nonpathological conditions. This assumption is false. Hundreds of studies over several decades suggest that ambient extracellular glutamate levels in the intact mammalian brain are ~0.5 to ~5 μM . This has important implications. Glutamate receptors are desensitized by glutamate concentrations significantly lower than needed for receptor activation; 0.5 to 5 μM of glutamate is high enough to cause constitutive desensitization of most glutamate receptors. Therefore, most glutamate receptors in vivo may be constitutively desensitized, and ambient extracellular glutamate and receptor desensitization may be potent but generally unrecognized regulators of synaptic transmission. Unfortunately, the mechanisms regulating ambient extracellular glutamate and glutamate receptor desensitization remain poorly understood and understudied.

Keywords

Glutamate; Synaptic; Synapse; Glutamate receptor; Ambient extracellular glutamate

Many neuroscientists assume that the concentration of ambient extracellular glutamate bathing the nervous system is biologically negligible under nonpathological conditions. But is it? In this review, we will discuss what the concentration of ambient extracellular glutamate in the nervous system actually is and consider whether this is enough to affect brain function. We will conclude that ambient extracellular glutamate is likely a strong determinant of brain function. Unfortunately, the regulation and role of ambient extracellular glutamate remain understudied and poorly understood.

Glutamate is the most abundant amino acid in the diet, plays a central role in cellular metabolism, and is widely recognized as the principal neurotransmitter in the central nervous system. Low ambient extracellular glutamate therefore implies extremely efficient uptake and sequestration. All cells import and concentrate glutamate. For example, plasma glutamate is 30 to 100 μM , whereas red blood cell cytoplasmic glutamate is ~500 μM and muscle cell cytoplasmic glutamate is ~5000 μM (Henriksson 1991; Filho and others 1999; Small and Tauskela 2005). This represents a 10-fold to 100-fold gradient. Synaptic vesicle glutamate transporters in glutamatergic neurons produce a gradient that is similar in magnitude. For example, the concentration of glutamate in the cytoplasm of glutamatergic neurons is ~10,000 μM , and synaptic vesicles within these neurons have glutamate concentrations near 100,000 μM (Riveros and others 1986; Burger and others 1989; Clements and others 1992; Ottersen and others 1992; Shupliakov and others 1992; Danbolt 2001). If the glutamate gradient between neuronal cytoplasm and extracellular fluid were similar to the gradients achieved by other cell

types or synaptic vesicles, ambient extracellular glutamate would be $\sim 100 \mu\text{M}$. However, $100\text{-}\mu\text{M}$ ambient extracellular glutamate would clearly preclude intercellular glutamatergic transmission and trigger massive neurodegeneration. Therefore, the concentration of ambient extracellular glutamate in the nervous system must be lower. Bouvier and others (1992) originally calculated a lower limit of $0.6 \mu\text{M}$ for extracellular glutamate, based on the properties of glutamate uptake transporters. But this is a lower limit; the real concentration is likely to be different.

If we assume that ambient extracellular glutamate must be lower than the concentration known to trigger excitotoxicity and subsequent neurodegeneration, then ambient extracellular glutamate must be only a few micromolars, because excitotoxicity is known to occur at extracellular glutamate concentrations as low as 2 to $5 \mu\text{M}$ (Meldrum and Garthwaite 1990; Rosenberg and others 1992), with swelling and apoptosis predominating at $< 20 \mu\text{M}$ glutamate and fast necrosis at $> 100 \mu\text{M}$ glutamate (Cheung and others 1998). However, glutamate excitotoxicity is extremely sensitive to exacerbating conditions, including, in particular, alterations of neuronal metabolism (Greene and Greenamyre 1996; Fiskum 2000), and studies of glutamate excitotoxicity are typically performed in culture in which cell health is unlikely to be optimal. Therefore, *in vivo* ambient extracellular glutamate may be slightly higher than one might expect based on excitotoxicity studies. But how much higher?

Arguably the most straightforward way of measuring *in vivo* ambient extracellular glutamate is to extract biological fluid and analyze the glutamate concentration using standard analytical chemistry methods. Cerebrospinal fluid (CSF) is most easily extracted, and therefore, it has been most extensively analyzed. Most studies report that healthy subjects have CSF glutamate in the range of 3 to $10 \mu\text{M}$ (Tucci and others 1998; Danbolt 2001; Rainesalo and others 2004). However, glutamate levels in CSF might misrepresent perineuronal glutamate levels in the brain. Glutamate in CSF could be quite low compared to perisynaptic glutamate because of frequent synaptic release, which transiently raises synaptic glutamate to 1000 to $3000 \mu\text{M}$ (Clements and others 1992; Bergles and others 1999). On the other hand, glutamate in CSF might be quite high compared to perisynaptic glutamate because of localized glutamate uptake.

A number of techniques have been used to measure extracellular glutamate in brain tissue. Microdialysis is the most common sampling approach and yields estimates for extracellular glutamate that are typically in the range of 1 to $5 \mu\text{M}$ after correction for recovery (Herrera-Marschitz and others 1996; Miele and others 1996; Baker and others 2002). High recovery push-pull perfusion and direct sampling methods both sample from a more limited volume than microdialysis, which is useful for assays of localized synaptic activity. Push-pull perfusion measurements of extracellular glutamate are similar to those from micro-dialysis: $< 2 \mu\text{M}$ (Kottgoda and others 2002). Direct sampling typically yields lower numbers: $\sim 0.5 \mu\text{M}$ (Kennedy and others 2002). Electrochemical sensors capable of measuring glutamate *in situ* often report values much higher than other methods (Kulagina and others 1999; Oldenziel and others 2006). For example, Kulagina and others (1999) and Oldenziel and others (2006) measured extracellular glutamate concentrations *in vivo* from rat striatum using electrochemical microsensors and obtained values that averaged $29 \mu\text{M}$ and $18 \mu\text{M}$, respectively. Some of the discrepancy between these electrochemical measurements and other techniques may be caused by low electrochemical glutamate selectivity. Consistent with this, electrochemical microarray measurements combined with a background subtraction approach estimated rat striatal and frontal cortex extracellular glutamate at $\sim 2 \mu\text{M}$, within the range obtained by other techniques (Day and others 2006).

Thus, numerous studies and techniques suggest that ambient extracellular glutamate concentration in the resting/anesthetized mammalian brain is in the range of 0.5 to $5 \mu\text{M}$. Is this biologically negligible?

Ambient Extracellular Glutamate and Synaptic Transmission

There is evidence that extracellular glutamate controls several important neurological processes, including neuronal and glial cell differentiation and migration during development (LoTurco and others 1995; Nguyen and others 2001; Demarque and others 2002; Manent and others 2005; Manent and Represa 2007). Ambient extracellular glutamate also likely plays an important nonpathological role in control of glutamatergic synapse strength. Synapse strength essentially determines information flow in the brain, and activity-dependent changes in glutamatergic synapse strength are now recognized as the basis of learning and memory (Barco and others 2006; Whitlock and others 2006). Therefore, ambient extracellular glutamate may be an important unrecognized determinant of neuronal circuit function and plasticity.

The easiest way to imagine ambient extracellular glutamate affecting any biological process, including synaptic transmission, is via glutamate receptors. A typical initial thought is that ambient extracellular glutamate, if it does anything at all, might perpetually activate glutamate receptors. However, another possibility is that ambient extracellular glutamate might lead to constitutive desensitization of receptors, and therefore, suppression of glutamatergic signaling. Which actually occurs depends on the glutamate sensitivity of receptor activation and desensitization relative to the concentration of ambient extracellular glutamate. The exact glutamate sensitivity of activation and desensitization depends on glutamate receptor type.

There are two main types of glutamate receptor in the nervous system: ionotropic (pore-forming) glutamate receptors and metabotropic (G-protein coupled) glutamate receptors. Mammalian ionotropic glutamate receptors are functionally and molecularly differentiated into three subgroups based on agonist pharmacology and subunit composition: 1) N-methyl-D-aspartate (NMDA) receptors, 2) amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors, and 3) kainate receptors. As their names imply, NMDA receptors display particular sensitivity to NMDA, AMPA receptors display particular sensitivity to AMPA, and kainate receptors display particular sensitivity to kainate (Monaghan and Wenthold 1997). Differences between the three subtypes are attributed to the fact that, although all ionotropic glutamate receptors are thought to be tetrameric, each receptor subtype is assembled from a different set of subunit proteins. For example, NMDA receptors are assembled from NR1, NR2, or NR3 subunits, AMPA receptors are composed of various combinations of GluR1, GluR2, GluR3, and GluR4 subunits, and kainate receptors are composed of GluR5, GluR6, GluR7, KA1, and KA2 subunits (Monaghan and Wenthold 1997; Ozawa and others 1998; Kew and Kemp 2005). (It should be noted that glutamate receptors are also present in many nonneuronal cell types, in which the subunit composition and pharmacology are likely to differ [Gill and Pulido 2001, 2005; Verkhratsky and Kirchhoff 2007]). There are several different types of NR1, NR2, and NR3 subunits, each encoded by different genes, and alternative forms of each of the subunits mentioned above can be created by alternative splicing or posttranslational modification (Kohr 2006; Paoletti and Neyton 2007). Thus, there is significant diversity in the subunits that assemble to form receptors, and therefore, significant functional and cell biological diversity within each of the three ionotropic glutamate receptor subtype families. Presumably, an organism's ability to "mix and match" subunits in different cell types and in different stages of development allows it to "fine-tune" receptor properties.

Mammalian metabotropic glutamate receptors are also divided into three subfamilies, or "groups," based on molecular and pharmacological differences. Group I metabotropic glutamate receptors are formed from mGluR1 or mGluR5 subunits and often activate phospholipase C pathways. Group II and Group III metabotropic receptors are composed of mGluR2/mGluR3 or mGluR4/ mGluR6/mGluR7/mGluR8 subunits, respectively, and generally suppress adenylate cyclase activity (Pin and Duvoisin 1995; Kew and Kemp 2005; Ferraguti and Shigemoto 2006). As with ionotropic glutamate receptors, further diversity is

generated through alternative splicing and posttranslational modifications (Ferraguti and Shigemoto 2006).

Which glutamate receptors are likely to be activated by ambient extracellular glutamate? Fig. 1 summarizes glutamate dependence of various glutamate receptor subtypes and compares these data to the probable concentration of ambient extracellular glutamate. AMPA and kainate receptors mediate most of the fast excitatory neurotransmission in the CNS and require the highest concentrations of glutamate to activate. For example, native AMPA receptors in rat neurons are activated by glutamate concentrations between ~100 μ M and ~10,000 μ M, with an EC₅₀ (effective concentration required to induce a 50% effect) ~700 to ~1000 μ M (Jahn and others 1998; Dzubay and Jahr 1999; Pang and others 2002). Kainate receptors are activated at slightly lower glutamate concentrations, with EC₅₀s ~300 to ~800 μ M (Wilding and Huettner 1997; Paternain and others 1998; Bowie and others 2003). Thus, neither AMPA nor kainate receptors are likely to be activated by ambient extracellular glutamate. AMPA receptors heterologously expressed in oocytes can be activated by substantially lower glutamate concentrations: 20 to 2000 μ M (Coquelle and others 2000; Gong and others 2005), but this is still rather high relative to ambient extracellular glutamate and may represent anomalous pharmacology and/or gating caused by lack of modulatory accessory proteins or perfusion, because AMPA receptors heterologously expressed in human embryonic kidney (HEK) cells show glutamate dose-response curves similar to those from native receptors (Suzuki and others 2005).

Compared to that of AMPA and kainate receptors, NMDA receptor activation is much more sensitive to glutamate. In the presence of the co-agonist glycine, both native and recombinant NMDA receptors are activated by 0.5 to 50 μ M of glutamate, with most EC₅₀s ~2 to ~4 μ M (Patneau and Mayer 1990; Williams 1994; Nahum-Levy and others 2001), although single channel measurements from heterologously expressed receptors have yielded EC₅₀ values that are substantially lower: 0.45 μ M (Wyllie and others 1996). These EC₅₀s, compared to estimates of ambient extracellular glutamate concentration in vivo, suggest that a substantial fraction of NMDA receptors could be constitutively activated by ambient extracellular glutamate. Consistent with this, Sah and others (1989) showed that NMDA receptors in hippocampal CA1 pyramidal cells were constitutively activated and attributed this tonic activation to ambient extracellular glutamate. Tonic NMDA receptor activation appeared as membrane current “noise” in hippocampal slice recordings that was blockable with the NMDA receptor blocker APV (2-Amino-5-phosphonopentanoic acid; Sah and others 1989). This observation has been replicated (Le Meur and others 2007) and the source of ambient glutamate probed in more detail (see below). Tonic activation of NMDA receptors has also been noted in dentate gyrus granule cells and salamander retinal ganglion cells (Gottesman and Miller 2003). Tonic activation of NMDA receptors is thought to primarily affect cell excitability. Depolarization increases the size of tonic NMDA receptor currents because of release of voltage-dependent Mg²⁺ block, thereby increasing the tendency toward regenerative depolarization (Cavelier and others 2005).

Metabotropic glutamate receptors are also activated by relatively low concentrations of glutamate. The exact EC₅₀ for glutamate activation of mGluRs depends on receptor subtype. Measured EC₅₀s range from 0.02 μ M (for mGluR8) to 1000 μ M (for mGluR7), although most EC₅₀ values for most mGluR subtypes are ~10 μ M (for review, see Conn and Pin 1997). In principle, tonic activation of mGluRs could be either excitatory or inhibitory, depending on downstream effectors. Indeed, both excitatory and inhibitory effects have been noted and attributed to tonic activation of mGluRs. For example, activation of excitatory sodium/calcium exchange in baroreceptors has been attributed to tonic activation of group I mGluRs (Sekizawa and Bonham 2006). Tonic activation of group III mGluRs causes suppression of supraoptic nucleus spontaneous activity and also inhibition of hypocretin/orexin neurons (Boudaba and

others 2003; Acuna-Goycolea and others 2004). At the level of network function, tonic activation of mGluRs triggers 40 Hz (“gamma”) oscillations in hippocampal and neocortical slices (Whitington and others 1995; Bartos and others 2007), and these oscillations are thought to underlie “binding” of sensory stimuli (Singer 1993). Thus, although it is difficult to predict how tonic activation of mGluRs might affect brain function, it is likely that such effects are computationally important.

Perhaps more important than the possibility of tonic activation of glutamate receptors is the high likelihood that ambient extracellular glutamate causes constitutive receptor desensitization. Glutamate triggers both activation and desensitization of glutamate receptors. Activation is faster than desensitization, and thus, typically precedes desensitization when glutamate concentrations rise quickly. Thus, receptors are transiently activated in response to fast application of agonist. However, activation will not necessarily happen at all during slow increases in extracellular glutamate or in response to ambient extracellular glutamate. In ionotropic glutamate receptors, activation is not required for desensitization, which can occur from unopened channel states, and desensitization is much more sensitive to glutamate than activation. For example, the EC₅₀ for NMDA receptor desensitization in cultured hippocampal neurons is only 1 to 2 μM (Zorumski and others 1996; Nahum-Levy and others 2001). In one study, the EC₅₀ for glutamate “predesensitization” of NMDA receptors in excised outside-out nucleated patches from cultured embryonic mouse neurons was only 0.3 μM (Sather and others 1992). In that study, 80% of NMDA receptors were desensitized by 1 μM glutamate (Sather and others 1992). Native AMPA and kainate receptors have desensitization EC₅₀ values ~ 4 μM and ~ 3 to ~ 13 μM , respectively (Colquhoun and others 1992; Zorumski and others 1996; Wilding and Huettner 1997; Paternain and others 1998), making at least some constitutive desensitization of these receptor subtypes by ambient extracellular glutamate also likely. Thus, the most likely effect of ambient extracellular glutamate on glutamate receptors will be constitutive desensitization, rather than activation. Fig. 2 summarizes the glutamate dependence of steady-state deactivation for various types of ionotropic glutamate receptors (data for metabotropic glutamate receptors remain largely unavailable). As shown, a substantial fraction of all ionotropic glutamate receptor subtypes may be constitutively desensitized by ambient extracellular glutamate.

Metabotropic glutamate receptor desensitization involves phosphorylation, altered intracellular protein interactions, and receptor internalization (Ferguson 2001; Dhami and Ferguson 2006). In the case of ionotropic receptors, desensitization represents a shift to a very stable non-conducting protein conformation (Mayer and Armstrong 2004; Mayer 2005; Weston and others 2006), and recent evidence suggests that mobility and/or trafficking of desensitized ionotropic glutamate receptor proteins may also be altered (Tardin and others 2003; Priel and others 2006; Augustin and others 2007). In either case, desensitized receptors are functionally removed from the synapse. Consistent with the idea that steady-state glutamate receptor desensitization by ambient extracellular glutamate might suppress synaptic transmission in vivo, Zorumski and others (1996) showed that synaptic currents recorded from cultured rat hippocampal neurons were significantly decreased by relatively low concentrations of bath-applied glutamate. Specifically, the EC₅₀ of suppression for AMPA receptor-mediated synaptic currents was 3.8 μM glutamate, and for NMDA receptor-mediated synaptic currents, 1.3 μM glutamate (Zorumski and others 1996). Similarly, excitatory postsynaptic currents in cultured embryonic chick neurons can be reduced by about one-half after bath application of 10 μM glutamate (Trussell and Fischbach 1989). More recently, the number of functional synaptic glutamate receptors in *Drosophila* synapses was shown to be regulated by glutamate concentrations in a normal physiological range (Augustin and others 2007). Many other studies have shown that synaptic current sizes in glutamatergic synapses are increased after application of desensitization inhibitors, further strengthening the idea that glutamate receptor desensitization significantly limits synaptic transmission in vivo. However, AMPA receptor

desensitization is fast enough to limit peak synaptic responses, making it difficult to determine how much synaptic augmentation after desensitization inhibitors is caused by steady-state desensitization. Nevertheless, if ambient extracellular glutamate is 0.5 to 5 μM , as discussed above, the EC50 values for glutamate receptor desensitization strongly suggest that glutamatergic synaptic transmission strength in vivo might be less than one-half what it might otherwise be without steady-state desensitization (Fig. 2).

Why would the brain constitutively cripple synaptic transmission? One possibility is that constitutive receptor desensitization provides a means for regulating synaptic strength. Steady-state receptor desensitization by ambient extracellular glutamate is analogous to steady-state inactivation of voltage-gated channels by resting membrane potential. Steady-state inactivation of voltage-gated channels is an important regulator of membrane excitability in many different tissues. For example, approximately two-thirds of rat skeletal muscle voltage-gated sodium channels are inactivated at a resting potential of -90 mV (Ruff and others 1988; Featherstone and others 1996). Consequently, only one-third of muscle sodium channels are normally available for action potential generation. A similar situation is present in neurons, in which rest potential is typically more positive but so also is the voltage dependence of sodium channel steady-state inactivation (Pun and Gesteland 1991; Jung and others 1997; Ptak and others 2005; Aracri and others 2006). Because the voltage dependence of steady-state inactivation is so steep, the cell can rapidly, reversibly, and dramatically change the number of functionally available channels in the membrane without actually altering the amount of channel protein in the membrane. For example, membrane hyperpolarization would increase the fraction of available sodium channels within a few hundred milliseconds as channels recover from inactivation (Jung and others 1997). This would increase distance to threshold but also ultimately membrane excitability. Alternatively, channel phosphorylation can shift the voltage dependence of inactivation and therefore rapidly alter the number of functional channels, with consequent dramatic changes in cell excitability (Muramatsu and others 1994; Catterall 1999; Franceschetti and others 2000). If glutamatergic synapse strength is limited in vivo by steady-state receptor desensitization, it is easy to imagine that glutamatergic synapse strength could also be highly regulated by anything that changes the EC50 of desensitization or anything that changes levels of ambient extracellular glutamate. Presumably, steady-state receptor desensitization can be modified by mechanisms known to regulate glutamate binding and desensitization kinetics, such as phosphorylation, or interactions with allosteric regulatory proteins such as TARPs (transmembrane AMPA receptor regulatory proteins), which alter AMPA receptor desensitization (Raymond and others 1994; Tong and others 1995; Gereau and Heinemann 1998; Hatt 1999; Liao and others 2001; Priel and others 2005; Jackson and others 2006; Walker and others 2006; Tomita and others 2007). Nevertheless, despite intense interest in excitatory synaptic transmission and the detailed molecular mechanisms regulating it, there is relatively little known about modulation of glutamate receptor steady-state desensitization or regulation of ambient extracellular glutamate.

Regulation of Ambient Extracellular Glutamate

Ambient extracellular glutamate is the steady-state balance between glutamate secretion (which will increase ambient extracellular glutamate concentration) and glutamate uptake (which will decrease ambient extracellular glutamate). Glutamate secretion under nonpathological conditions is usually attributed only to fusion of synaptic vesicles in neurons—e.g., synaptic transmission. But glia also secrete numerous transmitters, including glutamate (Martin 1992; Vesce and others 1999; Montana and others 2006), suggesting that glia may be an important point source for ambient extracellular glutamate. Glutamate secretion in astrocytes in particular has been relatively well studied and involves calcium-dependent glutamate secretion mechanisms similar to those used by neurons (Montana and others 2006). However, ambient extracellular glutamate levels in the brain are largely calcium

independent and insensitive to tetrodotoxin (TTX; Timmerman and Westerink 1997; Jambaudon and others 1999; Shinohara and others 2000; Baker and others 2002; Fillenz 2005). In *Drosophila*, complete block of synaptic vesicle fusion does not affect suppression of ionotropic glutamate receptor function by ambient extracellular glutamate (Featherstone and others 2002). Therefore, ambient extracellular glutamate is likely mainly a product of nonvesicular glutamate release. Unfortunately, nonvesicular neurotransmitter release is still somewhat controversial, primarily because the molecular mechanisms remain poorly understood.

Nevertheless, there are numerous recognized ways that glutamate can be released nonvesicularly in the nervous system, including swelling-activated anion channels, gap junction hemi-channels, purinergic (P2X) receptors, and cystine-glutamate exchangers (Kimelberg and others 1990; Baker and others 2002; Parpura and others 2004). Although still understudied, there is excellent evidence that cystine glutamate exchange, in particular, is a strong regulator of ambient extracellular glutamate and behavior.

Cystine-glutamate exchangers mediate 1:1 exchange between extracellular cystine and intracellular glutamate (Sato and others 1999; Sato and others 2000; Kanai and Endou 2001; Kim and others 2001; Hosoya and others 2002; Sato and others 2002). Functional xc- system transporters are heterodimeric transmembrane proteins composed of one “heavy” 4F2hc subunit and one “light” xCT subunit (Sato and others 1999; Fernandez and others 2006). 4F2hc is an accessory subunit thought to regulate protein trafficking and is used by several different types of amino acid transporters. xCT is required for amino acid selectivity and transport and is found only in xc- system transporters (Sato and others 1999; Chillaron and others 2001; Wagner and others 2001; Verrey and others 2004; Palacin and others 2005).

Cystine-glutamate exchangers appear to be present in many types of cultured mammalian cell lines and were originally assumed to function purely as a cystine-uptake mechanism for glutathione synthesis during oxidative stress (Bannai and Ishii 1982; Bannai and others 1984; Christensen 1990). However, in neurons and glia, the xc- transport system is dispensable for glutathione synthesis but rate-limiting for nonvesicular export of glutamate (Baker and others 2002; Chung and others 2005). (Neurons and glia do not need to rely on system xc- for cystine uptake, because they contain large numbers of excitatory amino acid transport [EAAT] family proteins. EAATs are best known as sodium-dependent transporters for glutamate uptake, but EAATs also efficiently import cysteine—the reduced form of cystine used in glutathione synthesis [Chen and others 2000; Flynn and McBean 2000; Danbolt 2001; McBean 2002; Chen and Swanson 2003; Chung and others 2005]). Furthermore, mouse brain xCT is primarily expressed in meninges and circumventricular organs (Sato and others 2002; Burdo and others 2006), where it would largely contribute to the free glutamate content of CSF—but not general cystine homeostasis in brain cells. Consistent with the idea that cystine-glutamate transporters play a role in glutamate homeostasis, subsequent pharmacological studies suggest that cystine-glutamate transporters are an important source of extracellular glutamate in rat brains (Baker and others 2002; Baker and others 2003). Specifically, Baker and others (2002) showed a 60% drop in extrasynaptic glutamate levels in rat striatum after application of cystine-glutamate exchange antagonists, whereas blockade of voltage-dependent sodium and calcium channels had little or no effect. Baker and others (2002) also showed that cystine-glutamate exchange activity in the striatum was negatively regulated by metabotropic glutamate receptor activation via cAMP-dependent protein kinase, consistent with the idea that ambient extracellular glutamate levels are actively maintained by the brain (Baker and others 2002).

To verify these results genetically and pursue the possibility that cystine-glutamate exchange regulates ionotropic glutamate receptor function, Augustin and others (2007) identified and genetically deleted a *Drosophila* xCT gene. Consistent with the results from rat described by

Baker and others, ambient extracellular glutamate levels dropped to approximately one-half of normal in the *Drosophila* xCT mutants (Augustin and others 2007). Consistent with the idea that ambient extracellular glutamate controls steady-state glutamate receptor desensitization, the decrease in ambient extracellular glutamate in these *Drosophila* xCT mutants was also associated with a doubling of functional synaptic glutamate receptors (Augustin and others 2007). Thus, regulation of glutamate receptors by ambient extracellular glutamate and xCT-based transporters appears to be highly conserved.

If cystine-glutamate exchange regulates ambient extracellular glutamate and ambient extracellular glutamate modulates glutamatergic transmission as argued above, then cystine-glutamate exchange function should control behavior. It does. Baker and others (2003) showed that rats undergoing cocaine withdrawal had drops in ambient extracellular glutamate. Intracranial perfusion of cystine or systemic administration of N-acetylcysteine, which should increase cystine-glutamate exchange, normalized ambient extracellular glutamate levels and ameliorated cocaine-seeking behavior (Baker and others 2003). In the *Drosophila* xCT mutants described by Augustin and others (2007), the mutated xCT gene was named “genderblind” because mutants showed equal sexual preference for both male and female partners. Subsequent work verified that this genderblind phenotype was caused by abnormally strong glutamatergic transmission, such that normally aversive or neutral male pheromones became attractive to other males (Grosjean and others, submitted). Both studies are consistent with the idea that ambient extracellular glutamate, regulated prominently by cystine-glutamate exchangers, modulates complex behavior, and they support the larger idea that ambient extracellular glutamate spatially modulates glutamatergic circuit function in vivo (Fig. 3).

Problems and Future Directions

It is difficult to measure ambient extracellular glutamate in vivo. Indeed, a major criticism of analytical extracellular glutamate measurements is that sampling probes likely disrupt brain tissue, leading to spuriously high extracellular glutamate measurements. Our argument that ambient extracellular glutamate regulates synaptic transmission, although supported by experimental evidence, also assumes that perisynaptic glutamate levels are equivalent to ambient extracellular glutamate levels. But perisynaptic glutamate levels may be very different because of synaptic secretion of localized high affinity glutamate uptake. This problem needs to be overcome.

One possible solution is a genetically encoded optical probe for glutamate—perhaps a glutamate receptor whose fluorescence changes after agonist binding. Such a sensor was recently described by Namiki and others (2007). This sensor, termed EOS (for Glutamate [E] Optical Sensor) improves on previous enzyme-linked and green-fluorescent-protein-based probes (Nicholls and Sihra 1986; Okumoto and others 2005), which could not be used in vivo and/or had low sensitivity. EOS, generated from the glutamate-binding region of a GluR2 AMPA receptor, reliably shows relatively fast fluorescence changes in response to nanomolar concentrations of glutamate (Namiki and others 2007). Unfortunately, EOS fluorescence is generated by a fluorescent dye that must be bath applied and which then conjugates to the protein via introduced cystines. Thus, although a promising tool for cell culture and perhaps brain slice studies, EOS will not allow in vivo extracellular glutamate measurements. Recent insights into ionotropic glutamate receptor binding region structure and channel gating (Mayer and Armstrong 2004; Mayer 2005) will hopefully allow engineering of better optical glutamate biosensors. The possibility that the presence of glutamate biosensors disrupts ambient extracellular glutamate concentration by sequestering glutamate will also be something important to consider.

Better in vivo glutamate sensors will hopefully also provide a better understanding of ambient extracellular dynamics in normal and dysfunctional brain. Ambient extracellular glutamate rises substantially during acute pathological conditions such as seizure, ischemia, or fever (Benveniste and others 1984; Wahl and others 1994; Liu and others 1997; Timmerman and Westerink 1997; Tucci and others 1998; Sherwin 1999; Kovacs and others 2003; Rainesalo and others 2004; Nyitrai and others 2006). Victims of amyotrophic lateral sclerosis (ALS) also show consistent but etiologically poorly understood increases in CSF glutamate (Jerusalem and others 1996; Spreux-Varoquaux and others 2002; Ryberg and others 2003) that may be linked to defective glutamate uptake (Heath and Shaw 2002; Kanai and Hediger 2003; Sattler and Rothstein 2006). Ambient extracellular glutamate varies spatially and temporally in healthy brains (Juhász and others 1989; Juhász and others 1990; Juhász and others 1991; Castañeda and others 2004; Lena and others 2005). Circadian changes in ambient extracellular glutamate may be caused by circadian rhythms in glial glutamate uptake that are themselves regulated by melatonin (Adachi and others 2002). Changes in ambient extracellular glutamate and glial activity may also contribute to mood (Lee and others 2007). The possibility that ingestion of monosodium glutamate—a common food additive—can alter extracellular glutamate levels in certain regions of the brain has always been controversial and is now generally discounted (Monno and others 1995; Bogdanov and others 1996; Geha and others 2000).

Despite the progress mentioned above, the molecular mechanisms controlling ambient extracellular glutamate are poorly understood. Most interest in ambient extracellular glutamate is driven by interest in excitotoxicity and/or kinetics of glutamatergic neurotransmission at synapses. As a result, there is a wealth of information with regard to structure and function of glutamate uptake mechanisms, as reviewed extensively elsewhere (cf Anderson and Swanson 2000; Danbolt 2001; Sattler and Rothstein 2006; Beart and O'Shea 2007). However, there is relatively little knowledge regarding mechanisms for glutamate release besides synaptic transmission, which, as noted above, does not apparently contribute substantially to ambient extracellular glutamate. Under the assumption that ambient extracellular glutamate is a biologically important phenomenon, an important task for future research will be identification and characterization of yet-uncharacterized regulators of ambient extracellular glutamate, including pumps, channels, and buffer systems.

Finally, the biggest challenge posed by extracellular ambient glutamate is the possibility that we will never really understand brain computation in vivo without understanding the three-dimensional fluidic microenvironment surrounding each relevant synaptic connection. Perhaps the strongest determinant of glutamatergic synapse strength in vivo is not activity (e.g., LTP or long-term depression [LTD]) or other relatively well-studied phenomena (such as modulation of receptor trafficking), but rather, whether the synapse falls within the volume controlled by a specific ambient extracellular glutamate source. If so, an understanding of brain computation is unlikely to be obtained by experiments performed in cell culture or in slice preparations outside any native chemical milieu. We need a new approach. Of course, this is not a new problem. It has long been clear that the brain is a neurosecretory organ suffused with dozens or hundreds of signaling compounds. The addition of glutamate (or other fast neurotransmitters) to the list of neuromodulatory compounds circulating throughout the brain is not a fundamentally new challenge. Nevertheless, it is a challenge that has generally been ignored and that must eventually be faced if we are ever to truly understand brain function. We need to stop thinking of the brain as a wiring diagram but more like the autoendocrine organ that it truly is.

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Fig. 1.

Glutamate dependence of activation for various glutamate receptor subtypes, compared to the probable concentration of ambient extracellular glutamate. NMDA receptors (NMDAR) and metabotropic receptors (mGluR) are activated by relatively low concentrations of glutamate (1 to 20 μM), and thus have typically sigmoidal dose-response curves that are left-shifted compared to those from AMPA receptors (AMPA) and kainate receptors (KAR), which are activated only by glutamate concentrations of 100 to 2000 μM . If ambient extracellular glutamate is $\sim 2 \mu\text{M}$, then about 40% of NMDARs and 10% of mGluRs could be constitutively activated in vivo. There is experimental evidence that this is the case (see text), although the fraction of total receptors activated will also depend on the fraction of receptors that are constitutively desensitized (see Fig. 2).

Fig. 2.

Glutamate dependence of desensitization for various glutamate receptor subtypes, compared to the probable concentration of ambient extracellular glutamate. Compared to activation (Fig. 1), steady-state desensitization of ionotropic glutamate receptor occurs at much lower glutamate concentrations (0.1 to 10 μM). If ambient extracellular glutamate is $\sim 2 \mu\text{M}$, then one-half to three-quarters of glutamate receptors might be constitutively desensitized, and thus functionally silent, in vivo. However, slight changes in ambient extracellular glutamate concentration or dose-dependence of steady-state desensitization could have dramatic effects on glutamate receptor availability and synaptic strength.

Fig. 3.

Spatial variation in ambient extracellular glutamate concentration could differentially modulate neuronal circuit function. A hypothetical point source (asterisk) for ambient extracellular glutamate is present near the left center of this image. As a result, ambient extracellular glutamate concentration (green shading) is highest there but drops with distance. Four hypothetical glutamatergic neural circuits, each possibly leading to a different behavioral output, are represented by arrows and numbered 1 to 4. All else being equal, circuits 3 and 4 will be weaker than circuits 1 and 2 because of suppression of synapse strength by ambient extracellular glutamate. This will decrease the probability of behaviors triggered by circuits 3 and 4, compared to 1 and 2. Temporal variations in glutamate uptake (as described in the text) or ambient extracellular glutamate secretion could alter the relative strength of the circuits at different times. In this way, ambient extracellular glutamate could serve as a potent modulator of spatially “connected” circuits and regulate behavior.