NEUROCHEMISTRY OF L-GLUTAMATE TRANSPORT IN THE CNS: A REVIEW OF THIRTY YEARS OF PROGRESS

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The review highlights the landmark studies leading from the discovery and initial characterization of the Na^+-dependent "high affinity" uptake in the mammalian brain to the cloning of individual transporters and the subsequent expansion of the field into the realm of molecular biology. When the data and hypotheses from 1970's are confronted with the recent developments in the field, we can conclude that the suggestions made nearly thirty years ago were essentially correct: the uptake, mediated by an active transport into neurons and glial cells, serves to control the extracellular concentrations of L-glutamate and prevents the neurotoxicity. The modern techniques of molecular biology may have provided additional data on the nature and location of the transporters but the classical neurochemical approach, using structural analogues of glutamate designed as specific inhibitors or substrates for glutamate transport, has been crucial for the investigations of particular roles that glutamate transport might play in health and disease. Analysis of recent structure/activity data presented in this review has yielded a novel insight into the pharmacological characteristics of L-glutamate transport, suggesting existence of additional heterogeneity in the system, beyond that so far discovered by molecular genetics. More compounds that specifically interact with individual glutamate transporters are urgently needed for more detailed investigations of neurochemical characteristics of glutamatergic transport and its integration into the glutamatergic synapses in the central nervous system. A review with 162 references.
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Before Cloning: From Synaptosomes and Brain Slices to the Molecular Mechanisms of Neurodegeneration

It has been thirty years since Solomon Snyder's group[1] discovered that homogenates of mammalian brain tissue accumulated L-[^{3}H]glutamate by a specific uptake system that could be characterized as Na^+-dependent and "high affinity". In other words, the uptake was saturable with respect to substrate (L-glutamate) concentrations, with an apparent $K_m < 50$ μM, or, about one to two orders of magnitude lower than the values of K_m that had been reported from a majority of amino acid uptake studies performed in various experimental models up to that time. L-Glutamate appeared to be accumulated against a concentration gradient and this implied the presence of an active, energy-requiring, transport mechanism. The thermodynamical aspects of the process were less well understood but it seemed most probable that L-glutamate would be translocated across the cytoplasmic membrane together with Na^+ thus utilising the transmembrane ionic gradient that was continuously regenerated by (Na^+, K^+)-dependent ATPase.

The preparations used in Logan's and Snyder's studies were homogenates of brain tissue enriched in "synaptosomes", or, subcellular particles originating from pinched-off nerve endings[2,3]. Accordingly, the strong presence of the Na^+-dependent high affinity uptake of glutamate in such material was interpreted as implying that the transport system(s) mediating the uptake was/were located in synaptic nerve terminals[4]. It seemed, therefore, natural to think of the process as being intimately involved in the synaptic function probably by limiting the temporal and spatial "spread" of the transmitter ("mopping up"; for a review, see ref.[5]), assuring that the synaptic currents would be "fast-on, fast-off" and the synaptic transmission would stay sharp and crisp. Another possible function of the transport could be to continuously replenish the presynaptic stores of L-glutamate, thus "recycling" the neurotransmitter and reducing the demand for its *de novo* synthesis.

The role of L-glutamate as an excitatory transmitter in the central nervous system (CNS) had not yet been fully appreciated in the early 1970's and the arguments in favour of the synaptic location and synaptic function of the Na^+-dependent "high affinity" uptake of L-glutamate were not easily accepted (for a review of the early history of amino acid neurotransmitters, see ref.[5]). Moreover, synaptosomal preparations usually contained material of non-neuronal origin[6,7] and the Na^+-dependent "high affinity" uptake of L-glutamate was also found in preparations containing no synapses and/or no neurons[8–12] (for a review, see ref.[13]). Results of those studies, considered

in isolation, could have made the putative role of glutamate uptake in the central nervous physiology quite difficult to evaluate. However, a broader approach to the problem was being developed. Using techniques earlier applied to the studies of high affinity uptake of the inhibitory neurotransmitters γ-aminobutyrate and glycine[14,15], high affinity uptake of L-[^{3}H]glutamate was studied in tissue slices, or, more specifically, in "mini-slices" of brain, *i.e.*, small prisms of rat cerebral cortex. Rather than focusing directly on the cellular location of the transport system(s), or, on the nature of hypothetical membrane-bound proteins ("transporters") mediating the translocation of glutamate from extracellular space into the cytosol, the aim of these experiments was to characterise glutamate uptake in terms of substrate specificity, ionic dependence and structural requirements of the substrate-binding site. One of the early achievements of these, essentially neurochemical, studies made it possible to refute a hypothesis about the role of ion-dependent electrogenic transport in the generation of L-glutamate-evoked neuronal depolarization[16–18]. This result was considered very important at the time, since it helped to put a clear distinction between glutamate transport and the receptors mediating the excitatory effects of glutamate on the neuronal membranes (for a review, see ref.[5]).

Most of the information originating from the neurochemical studies was indispensable in the process of developing glutamate transport-specific inhibitors and substrates[19–25] (Fig. 1) but its impact extended well beyond the field of neurochemistry. Thus one of the earliest-identified transport-preferring glutamate analogues, *threo*-3-hydroxyaspartate[26] was found to potentiate excitatory actions of externally applied L-glutamate at spinal neurons of anaesthetised animals[27]. This finding provided clear experimental demonstration of the ability of the Na^+-dependent high affinity transport to control excess concentrations of L-glutamate *in vivo*. High doses of L-glutamate could be toxic in the CNS ("excitotoxocity"[28,29]) and, therefore, the failure to control normal glutamate excitation because of inadequate glutamate transport could lead to neurotoxicity and the loss of neurons in particular pathological states[30,31]. This hypothesis was first formulated in concrete terms with respect to a specific disease only around 1990 but neurological disorders now suspected as probably or possibly involving deficient glutamate transport extend from the amyotrophic lateral sclerosis (ALS; motor neuron disease) to the dementia of Alzheimer type and include such diverse conditions as schizophrenia, brain tumours, cerebral ischaemia and the sequelae of head trauma as well as hereditary DNA repair disorders such as Cockayne syndrome[32–41]. Moreover, recent evidence suggests that a deficiency in glutamate transport may cause significant disturbances of

brain metabolism and further exacerbate the neurotoxic effects of increased extracellular L-glutamate by reducing the energy supply of neurons[42]. This finding further extends the range of mechanisms by which the changes in glutamate transport can contribute to the demise of neurons in the CNS. Again, the use of glutamate analogues specifically developed as transport-selective inhibitors has been absolutely essential in testing this hypothesis[42].

Extensive use of structural analogues of glutamate in the characterization studies of glutamate uptake in 1970's and 1980's uncovered subtle variations in the properties of glutamate transport among brain regions[43,44] and, especially, among the variety of cell culture preparations containing neurons and glial cells usually isolated from immature mammalian brains and used extensively in the studies of L-glutamate transport[45]. This heterogeneity of the system strongly signalled the presence of multiple transport

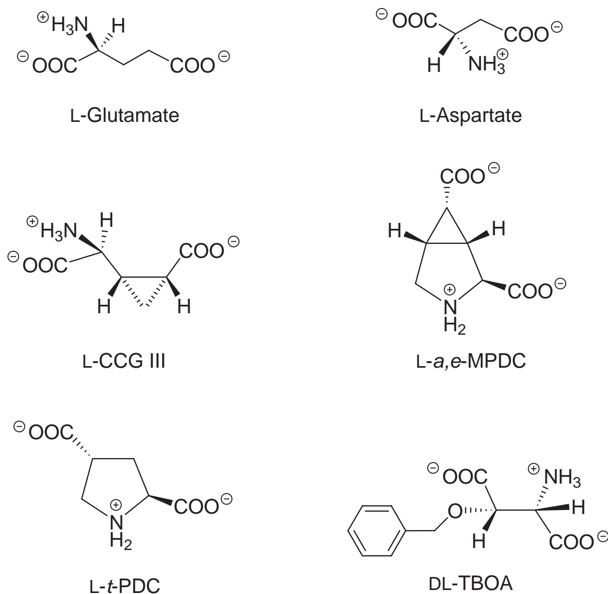

FIG. 1
Structures of some typical substrate/inhibitors of the Na^+-dependent glutamate transport in brain (abbreviations are explained in the legend of Table II). Synthetic L-CCG III has been used in neurochemical studies[116,120,122,123,157,158] as a synthetic compound[23,159] but it had been shown earlier to exist, presumably in racemic mixtures, as a natural compound[160]. Other analogues are purely synthetic[22–25]. TBOA stands for 3-(benzyloxy)-DL-aspartate, for the meaning of the other abbreviations see legend of Table II

mechanisms, probably in the form of several distinct "transporter" molecules mediating the Na^+-dependent, "high affinity" uptake in the CNS. Several such transporters (*cf.* Table I for the list and nomenclature) have been subsequently cloned and characterised[46–50] (see reviews[51–54] for further details).

After Cloning: Nature and Location of Glutamate Transport in Brain Tissue

Identification and sequencing of the individual transporter molecules made it possible to prepare specific antibodies and to study the location of the transporters at cellular and subcellular level. Thus, for example, the immunoreactivity (IR) corresponding to EAAT1 (GLAST) was present mostly in the cerebellar cortex while EAAT2 (GLT-1) IR was strongly expressed in the forebrain[55–59] as well. Both EAAT1 and EAAT2 IR's were found in the cytoplasmic membranes of astrocytes, but the IR corresponding to EAAT3 (EAAC1) was located preferentially in neurons. Only Purkinje cells of the cerebellar cortex have so far been reported to contain significant amounts of EAAT4 IR while EAAT5 IR has been found almost exclusively in the ret-

TABLE I
Glutamate transporters cloned from the central nervous system

Transporter	Principal location	References
EAAT1 (GLAST)	cerebellar cortex, Bergman glia	46, 51, 59
EAAT2 (GLT-1)	forebrain, astrocytes	47, 51, 55
EAAT3 (EAAC1)	neurons of the CNS	48, 51, 59
EAAT4	cerebellar cortex, Purkinje cells	49, 53, 59
EAAT5	retina	50, 54, 59

The transporters are listed in the order of EAAT numbers. EAAT stands for "Excitatory Amino Acid Transporter". The EAAT nomenclature system is used for the transporters cloned from human brain tissue, the designations in the parenthesis (*e.g.* GLAST, GLutamate and ASpartate Transporter) were given to the transporters first cloned from rat brain (GLAST and GLT-1) or from the rabbit intestine (EAAC1). Other abbreviations are sometimes used, *e.g.* sEAAT1 – 5 for the EAAT homologues and their splice variants (such as sEAAT5A or sEAAT5B) found in the retinae of tiger salamanders, *Ambystoma tigrinum*[154] or DipEAAT1, a variant of EAAT1 cloned from the nervous system of *Diploptera punctata*[144] and many more.

ina (reviews[51,53,54]). The simple scheme of glial v. neuronal localization of glutamate transporters, particularly with respect to EAAT1 and EAAT2, does not have universal validity though and there are many exceptions, especially in the immature brain cells[60–67]. In fact, it seems that L-glutamate transport has a specific role in the brain ontogeny, perhaps as a factor exerting fine control over the extracellular concentrations of L-glutamate that might, in turn, act as an important signalling factor regulating the differentiation and survival of newly formed neurons.

In addition, there is a number of splice variants of individual transporters that could be region- or cell-specific and may further add to the variations in the characteristics of glutamate transport from one region to another or from the adult to the developing brain.

Glutamate Transporters: Subtype of Ion Channels?

Glutamate transporters in the mammalian CNS share amino acid sequences with members of a family of proteins that have been known to exist in membranes of bacterial, archaeal and eukaryotic cells[68]. Among the characteristic features of glutamate transporters are at least eight membrane-spanning regions, some of them forming loop-pore structures similar to those usually found in ion channels[69]. In fact, almost all Na^+-dependent glutamate transporters function as chloride-selective channels. This characteristic is most prominently developed in EAAT4 and EAAT5 while it is almost non-existent in GLT-1 (for a recent review see ref.[59]). The permeability to chloride ions is triggered off by L-glutamate but it is thermodynamically independent from the amino acid transport *i.e.* the flux follows the chloride concentration gradient, needs no metabolic energy but it does not drive the transport of L-glutamate. Increased permeability to chloride ions would tend to hyperpolarize neuronal membranes and contribute to transmembrane currents and, indeed, participation of glutamate transporters in the generation of synaptic currents has been demonstrated on Purkinje cells in the cerebellar cortex where the chloride ion-permeable EAAT4 is known to be expressed[70,71].

Precise nature of the substrate-binding sites on the transporter proteins and exact mechanisms by which they are involved in the transport of L-glutamate have remained, to a large extent, conjectural. The substrate molecule is subject to stringent structural requirements[11,13,18] and, furthermore, the transport is strongly influenced by the presence of small ions, especially Na^+ (refs[4,13,18]). The process can be particularized in terms of at least three

distinct phases. The first event is the recognition and capture (specific "binding") of the substrate. This is followed by an interaction between the occupied "binding site" (or additional such sites along which the substrate might move) and Na^+, resulting in a conformational change and translocation of the substrate across the membrane. The third, final, step comprises the recovery of the transporter *i.e.* dissociation of the substrate inside the cell (K^+-dependent event) and repositioning of the protein molecule within the membrane so that it is ready to receive L-glutamate once again. The complex nature of the process may explain rather slow frequency of transporter "cycling" observed in some studies[72]. The binding of the substrate has, indeed, been shown to be a distinct process[73], possibly with a specific physiological – if, perhaps, "paradoxical" – role (for a review and further details, see ref.[59]). The necessary conformational change, thought to be caused by Na^+, is in line with general characteristics of proteins participating in amino acid transport[74]. The hypothetical mechanism, as outlined above, is, therefore, based on concrete experimental observations[73–75].

The rational design of compounds that could specifically modify the function of glutamate transport would be greatly helped by more precise knowledge of the molecular mechanisms underlying the sequence of events. Can it, at least, be specified what parts of the transporter molecules contribute to each particular phase of the process? GLT-1 seems to have been most extensively studied in this regard and several amino acid positions have been considered as important for its function. Thus glutamate 404 has been shown to be essential for the normal transport of L-glutamate (but the point-mutation had only a small effect on the transport of L- and D-aspartate) yet it did not seem to impair the initial binding step[76]. Furthermore, it interfered with the interaction between the transporter and K^+, while the effect of Na^+ was not significantly influenced[77]. Moreover, changes to nearby serine residues 440 and 443 reduced the sensitivity to the nontransportable inhibitor dihydrokainate[78]. These mutations were located within the pore-loop-like structure that may or may not directly contribute to the initial recognition and binding of L-glutamate but seems to be important for the interaction with Na^+ that provides the driving force for the transport[79]. This observation could, therefore, mean that not only the initial recognition of the substrate but also the latter part of the transport process (coupling with Na^+ accompanied by conformational change) puts particular constraints on the structural characteristics of the substrate and these may be different from those of the initial binding site. Systematic use of glutamate and aspartate analogues combined with molecular modelling,

as has been done, for example, with metabotropic glutamate receptors[80], would be of great benefit in the further explorations of functional components of the transporter molecules.

Energetics of L-Glutamate Transport – What Role Does it Play in Brain Ischaemia?

Ionic gradients, especially those of Na^+, have been known for some time to be the main driving force for the transport of L-glutamate[1,4]. The exact stoichiometry of the process is, therefore, of considerable interest. Initial studies in brain slices and glial cell cultures suggested simple one-to-one ratio during the cotransport of L-glutamate and Na^+ (refs[11,18]) but later studies[81,82] indicated complex relationships that could be only in part explained by the multiplicity of glutamate transporters (see ref.[83] for the discussion and earlier references). The stoichiometry of the transport by GLT-1, the most abundant glutamate transporter in the forebrain[59], was estimated to be three Na^+ and one H^+ cotransported with one glutamate anion, accompanied by countertransport of one K^+ (ref.[84]). Therefore, it has been suggested that, if the above relationship holds, the extracellular concentrations of L-glutamate would be significantly increased under conditions of high concentration of K^+ that may occur during cerebral ischaemia[84,85].

Existence of such mechanisms could have far reaching implications for the therapies of stroke. Application of L-glutamate transport inhibitors to the rat striatum or cerebral cortex under ischaemic conditions reduced the efflux of L-glutamate by only about half, though[86,87], even when a potent non-transportable inhibitor[87] was used. Efflux of taurine, the transport of which would not have been inhibited, was reduced by about the same amount[87]. Perhaps the Ca^{2+}-dependent release of L-glutamate from the presynaptic compartment also significantly contributes to the efflux of amino acids under ischaemic conditions[88] and mere inhibition of L-glutamate transport may not adequately reduce the extracellular glutamate. Furthermore, can a theoretical model – based on properties of GLT-1 that might perhaps be relevant in the ischaemic forebrain[85] where GLT-1 is the most abundant L-glutamate transporter[59] – be automatically transposed into, say, the cerebellar cortex where the dominant L-glutamate transporter is GLAST (ref.[59])? Increased external concentration of K^+ did not result in the reversal of L-glutamate transport in cultured astrocytes expressing GLAST (refs[89,90]) but, even if this finding reflected the characteristics of GLAST-expressing Bergman glia *in vivo*, it would appear to have little or no

relevance for the pathology of ischaemia since there seems to be no evidence of strokes causing less damage in the cerebellum than in the rest of the brain!

Some recent studies have implied that L-glutamate transport is closely associated with energy metabolism. For example, inhibition of L-glutamate transport *in vivo* has been shown to result in neurodegeneration only when the energy metabolism is inhibited at the same time[91] and the local increase in the utilization of glucose in response to the stimulation of rat sensory cortex has been found to be reduced after an administration of antisense oligonucleotides targeted against GLAST (ref.[92]). Direct links between L-glutamate transport and oxidative metabolism have also been proposed[93]. Such findings and suggestions have to be taken into account when considering the possible role(s) of L-glutamate transport (and, indeed, the very role of increased extracellular L-glutamate[94]) in central nervous tissue during ischaemia. Finally, it may be possible to demonstrate the release of L-glutamate by the reversal of transport in an experimental model, but how much of L-glutamate would really be released by this mechanism in the brain tissue where L-glutamate may be rapidly metabolised, possibly as soon as it is taken up by glial cells[95,96]?

Binding of Radioligands to Glutamate Transporters Studied by Autoradiography *in vitro*

Immunocytochemistry provides data on the location and distribution of IR but has only a limited potential to study the biochemical or pharmacological characteristics of the protein molecules *in situ*. An alternative approach, using the Na^+-dependent binding of ^3H-labelled transporter-specific ligands to the substrate-binding sites on the glutamate transporters in sections of brain tissue and visualising it by the autoradiography on ^3H-sensitive films[97–99], can do both: one can quantify the number of bound radioligand molecules by densitometric analysis and, since the method is based on labelling the sections *in vitro*, it is possible to expose the binding sites to a variety of conditions or pharmacological agents in a controlled environment and obtain quantitative data on the regional abundance of the binding sites, their ion requirements, pharmacology and other characteristics as well, all at the same time. As L-[^3H]glutamate was thought to be unsuitable as a radioligand in such experiments – having been shown to interact, in similar studies, with the ligand-binding sites on several glutamate receptors[98,99] – the technique, when introduced some time ago[100], used

D-[^{3}H]aspartate. In theory, L-[^{3}H]glutamate bound to the receptors could be "dissected" pharmacologically from L-[^{3}H]glutamate labelling the glutamate transporters by employing selected non-radioactive glutamate receptor-specific ligands that could suppress the binding of L-[^{3}H]glutamate to the receptor sites. However, even if the particular receptor ligands were known, from other studies, to have negligible affinity for the glutamate uptake, the presence of such compounds, some of which could be structurally very similar to glutamate, would not be desirable in an experimental system designed to study, among other things, subtle aspects of the structural specificity of substrate-binding sites on the transporters.

D-[^{3}H]Aspartate had already been known to possess high affinity for glutamate uptake and to be a very weak agonist at glutamate receptors[18]. Both of these characteristics – and the belief that it was neither normally present in brain nor being handled as a natural substrate by brain enzymes – made D-aspartate appear to be a near-ideal tool for studies of the Na^{+}-dependent high affinity glutamate transport in the central nervous tissue. For example, D-[^{3}H]aspartate had been used as a "non-metabolisable" radioligand in studies labelling the glutamate-accumulating structures in brain *in vivo*[101]. However, doubts about the nature of the binding sites labelled by D-[^{3}H]aspartate in thaw-mounted brain sections *in vitro*, particularly in the hippocampus, were expressed[102–105]. Some modifications were introduced into the autoradiographic technique[103] but it was also suggested that D-[^{3}H]aspartate, indeed, may not have been the ideal radioligand for the studies of glutamate transport sites and that the L-enantiomer should be used in the autoradiographic studies, where the potential metabolic conversion of the ligand was of little importance because the experiments were carried out at low temperatures[13,106]. Another study, however, concluded that there was no difference between D- and L-[^{3}H]aspartate Na^{+}-dependent binding to the thaw-mounted brain sections *in vitro*[107].

It might be of interest to note that, more recently, an enzyme capable of processing D-aspartate has been detected in the CNS (ref.[108]) and D-aspartate was found to be present, possibly even synthesised, in several mammalian tissues, including brain[109,110].

Structural Requirements of Glutamate Transport: "Stereoselective Anomaly"

The claims that there was no difference between the characteristics of D- and L-[^{3}H]aspartate as radioligands labelling substrate binding sites on

glutamate transporters in brain sections[107] would seem to be in line with data from most of the other similar studies[11,18,45]. This characteristic of L-glutamate transport was first noticed in the early experiments using brain slices when the high affinity uptake of L-[^{3}H]glutamate was found to be "inhibited" approximately equally well by both D- and L-aspartate[18]. L-[^{3}H]Aspartate was subsequently used as an alternative radiolabelled substrate[18,26] and uptake of D-[^{14}C]aspartate by brain slices was also investigated[111]. It was somewhat surprising then to find that D-glutamate did not interact with L-[^{3}H]glutamate uptake[18]. This particular set of traits of the Na^+-dependent glutamate transport – strong preference for L- over D-glutamate but virtually no differentiation between the enantiomers of aspartate – was also found in other experimental models[112,113] and was termed "stereospecific anomaly" by Christensen[112]. Christensen suggested a simple explanation for the phenomenon: enantiomers of aspartate, bound to the transporter either in "alpha" or "beta" positions[112], would be handled with equal efficiency[112]. "Alpha" or "beta" refers to the position of the amino group in the molecule of aspartate. It is, of course, always at C2 (L- and D-aspartate are both classified as α-amino acids) but one can imagine L-aspartate bound to the transporter site being replaced by D-aspartate in such a way that C4 assumes the position of C1 and *vice versa*. The sense in which the pharmacophores (two negative charges at C1 and C4 carboxyls and a positive charge on the amino group[13]) are ordered would remain the same as for L-aspartate, only the amino group would now be at an apparent "C3" or "beta" position. The main conjecture of the theory is that, while the amino group in "beta" position would be tolerated very well (D- and L-aspartate would have about equal affinity for the site), "gamma" position produced by analogously rotated molecule of D-glutamate would not be accepted. However, similar considerations applied to the molecule of *threo*-3-hydroxyaspartate would predict large difference between the affinities of its two enantiomers because the OH group would be placed at an apparent "alpha" or "C2" position and substitutions at C2 are poorly tolerated[13,114]. Differences of varying magnitude between D- and L-*threo*-3-hydroxyaspartate as inhibitors of, respectively, L-[^{3}H]glutamate uptake[113] or L-[^{3}H]aspartate binding[115] have been noted but they are often very small[26]. Also, 3-aminoglutarate ("β-glutamate") does not always strongly interact with L-[^{3}H]glutamate uptake[18,113]. Christensen's hypothesis is, in general, difficult to test[113] and an alternative proposal that is based on molecular modelling and provides a better explanation of a specific set of experimental data has been put forward[113,116].

Substrate selectivity and structural specificity of glutamate transport has been studied extensively both in preparations derived directly from brain tissue and in individual transporters expressed in *Xenopus laevis* oocytes[11,17,18,45,117–121] (for reviews, see refs[13,121]). It has also been studied in cultured cell lines originating from healthy brain tissue or, more often, from tumours[45,122] and, also, in cultured cells of non-neural origin, sometimes transfected with, or, known to spontaneously express, particular glutamate transporters[113,123,124]. Based on these and many previous[13,18] studies, it is possible to conclude that the essential features of a "good" ligand for the substrate-binding sites on glutamate transporters are two negatively and one positively ionisable group (separated by optimum distances, as in a partly folded conformation of L-glutamate molecule) as well as absence of substituents that could sterically hinder the approach of the molecule to the binding site. Substituents on C3 of the 4C-carbon chain (aspartate-like molecules) or on C3 and C4 of the 5C-chain (glutamate-like structures), are, however, tolerated[18,24–26,113–117]. Several highly transporter-specific ligands based on conformationally restricted glutamate-like and aspartate-like structures have been synthesised[19–25] (Fig. 1). Such compounds are essential for the detailed studies of the structural requirements of the binding sites[113,117] and could also lead to the design and synthesis of substrates/inhibitors that would be highly selective towards individual glutamate transporters[119,123,125]. Moreover, many of the most recent conclusions have been based on structure–activity relationships studied on transporter proteins expressed in *Xenopus laevis* oocytes[121]. How can the data from the heterologously expressed transporters be translated to the context of brain tissue? Or, can we identify regional variations in the substrate selectivity of glutamate transport that could reflect the specific composition of the system in terms of individual transporters?

Regional Variations in the Substrate Selectivity of Glutamate Transport in Brain

Quantitative autoradiography of L-[^{3}H]aspartate binding in sections of rat brain has produced a large and homogeneous set of data that might help us to answer the question raised at the end of the previous section. In order to illustrate the significance of the data obtained with this experimental approach, let us first discuss two findings related to L-glutamate transport in the cerebellum.

First observation is that of the density of L-[^{3}H]aspartate binding being higher in the cerebellar cortex that in any other part of the brain, including the cerebral neocortex[115,116]. This is in contrast to the results obtained when uptake of L-[^{3}H]glutamate by synaptosomal fractions was studied[44,117]. On the first approach, the explanation might seem trivial. While in the autoradiograms only the strongly labelled thin layer of the cortical grey matter was densitometrically evaluated, the synaptosomal preparations may have included fragments of the white medullary centre of the cerebellum, *i.e.* structures originating from nerve fibres that would contain few if any glutamate transport sites[115]. This would tend to "dilute" the observed uptake in synaptosomes but in the autoradiographical studies only the densely labelled grey matter of the cerebellar cortex was evaluated. Indeed, when the radioactivity in the thaw-mounted sections was measured by scintillation counting, the L-[^{3}H]aspartate binding in the cerebellum appeared marginally smaller than that in the forebrain[116].

Granule cells of the cerebellum are the most numerous neurons in brain (about 10^{11} in the human cerebellar cortex, aproximately half of all neurons in the brain) and each has been estimated to form, *via* parallel fibres, about 500 glutamatergic "cross-over" synapses with the dendritic trees of Purkinje cells, in addition to making many synapses with basket, Golgi type II and outer stellate cells[126]. This represents an exceptionally dense agglomeration of excitatory synapses, particularly when it is realized that almost all of them are confined to the rather thin uppermost (molecular) layer of the cerebellar cortex. If there is a correlation between the amount of traffic in synaptic glutamate and the potency of glutamate transport, as suggested by some studies[127] (but see also ref.[105]), the high density of L-[^{3}H]aspartate binding in the cerebellar cortex would be easily accounted for.

Second finding is that of 2-aminoadipate inhibiting the L-glutamate transport in the cerebellum more strongly than that in the forebrain regions, observed when uptake of suitable radioligands by synaptosomal fractions was used as an experimental model[44,128,129]. In contrast, DL-2-aminoadipate had virtually no effect on L-[^{3}H]aspartate binding, studied by quantitative autoradiography, in any part of the brain. 2-Aminoadipate may be a marker for the glutamate–cystine exchanger[130] but it seems improbable that this would explain the results, because L-[^{3}H]glutamate uptake by synaptosomes is Na^+-dependent[44,117] whereas glutamate–cystine exchanger is not[130]. The answer may come from the recent literature on the characteristics of the individual glutamate transporters in the cerebellum.

GLAST is the predominant transporter in the cerebellar cortex (for a review see ref.[59]) and neither the uptake of D-[^{3}H]aspartate by Bergman glia, cultured from chick cerebellum and shown to express an avian variant of GLAST, nor the uptake of L-[^{3}H]glutamate by human fibroblasts (where GLAST appears to be the principal glutamate transporter[113]) were strongly inhibited by 2-aminoadipate[131,132]. Furthermore, 2-aminoadipate inhibited uptake of neither L-[^{3}H]glutamate nor D-[^{3}H]aspartate *via* EAAC1, a glutamate transporter that could be thought, because it is located in neurons[59], to make a significant contribution to the uptake of L-glutamate by synaptosomes. In reality, neither EAAC1 nor any other known glutamate transporter have ever been conclusively proven to exist in nerve endings despite the presence there of a Na^+-dependent, "high-affinity", *threo*-3-hydroxyaspartate-sensitive uptake of D-aspartate (distinct from the "low affinity" glutamate uptake by synaptic vesicles that is Na^+-independent[133,134] and may[135] or may not[133,134] accept aspartate[133,134]) demonstrated by immunocytochemical studies[59]. Do we, then, have to consider unknown characteristics of a hypothetical transporter, not yet cloned, in order to explain the unusual pharmacology of glutamate uptake by cerebellar synaptosomes? In fact, it has been reported that the function of a known glutamate transporter, namely EAAT4, found almost exclusively in the Purkinje cells of the cerebellum, is sensitive to 2-aminoadipate[49]. Purkinje neurons are much less numerous than the granule cells but are among the largest structures in the CNS both as perikarya (up to 80 μm in diameter) and in terms of dense networks of multiply-branched dendrites expanding across the thickness of the molecular layer[126]. These dendrites are known to contain EAAT4 in their membranes, particularly in the dendritic spines adjacent to the synapses[59]. Thus there seems to be sufficient amount of material in the cerebellar tissue to supply "synaptosomal" fractions with structures rich in EAAT4. In other words, if a significant proportion of "synaptosomal" fractions, prepared from the cerebellar cortex, is actually formed by fragments of dendrites (particularly pinched-off dendritic spines) and perikarya originating from Purkinje cells, the effect of 2-aminoadipate could be explained. In contrast, 2-aminoadipate would have no effect on the L-[^{3}H]aspartate binding observed in brain sections which have been neither homogenized nor fractionated and have retained at least their gross anatomical structure. Any binding of L-[^{3}H]aspartate to EAAT4 would, of course, be overwhelmed by the binding to GLAST that is very strongly expressed in surrounding Bergman glia[59] (about an order of magnitude more strongly than EAAT4 in the dendrites of Purkinje cells, see ref.[59], for a review and references) and that is known to handle glutamate in a 2-aminoadipate insensitive man-

ner[113,131,132]. Thus the data on the distribution and characteristics of L-glutamate transporters obtained in a variety of studies can be reconciled both with the inhibition of L-glutamate uptake by 2-aminoadipate observed in synaptosomal fractions and with the lack of effect of 2-aminoadipate on the binding of L-[^{3}H]aspartate observed in brain section, *i.e.* when using whole tissue with glutamate transporters *in situ*. The only major untested assumptions are the contamination of "synaptosomal" fractions by fragments of Purkinje dendrites and, perhaps, the Bergman glia of the cerebellar cortex generating "gliosomes"[6,7] to a lesser degree than the astrocytes of the forebrain.

The data on L-[^{3}H]aspartate binding are summarised in Table II. It is clear that the rules outlined in the previous section are followed in all three regions of the rat brain (Table II). Regional differences in structural requirements of the binding sites, if there are any, seem small. The data from Table II are presented again in Fig. 2 but in a form modified to emphasize both similarities and differences among the three regions. Specifically, IC_{50}'s (concentrations of test compound causing 50% inhibition) were converted to their reciprocal values and plotted *versus* the range of test compounds sequentially numbered, as in Table II, in the order of their potencies as inhibitors of L-[^{3}H]aspartate binding in the cerebral neocortex. The part of each graph where $1/IC_{50} \approx 0$ illustrates the large proportion of molecules with structures often very similar to that of glutamate that have little or no affinity for the transporters. They also include several important ligands for glutamate receptors, such as kainate, CGS 19755, TZG, AIDA, and the broad-spectrum agonist at the metabotropic glutamate receptors ACPD. Also, as discussed above, 2-aminoadipate, a substrate selective for the Na^+-independent glutamate transport[130], is inactive. Three compounds clearly distinguish between the L-[^{3}H]aspartate binding sites in the cerebellum and those in the cerebral neocortex and hippocampus. All of them show preference for the binding sites in the two forebrain structures (Fig. 2, Table II). L-*trans*-Pyrrolidine-2,4-dicarboxylate (L-*t*-PDC), a conformationally restricted analogue of L-glutamate[21,121], is approximately 10 times more potent inhibitor of L-[^{3}H]aspartate binding in the forebrain than in the cerebellar cortex. D-*threo*-3-Hydroxyaspartate (D-*t*-3-OHA)[26] has the ratio of IC_{50}'s in the cerebellar cortex to those in the forebrain equal to about six while D-aspartate displays the ratio of IC_{50}'s about four to five (Table II, Fig. 2). It is the effects of D-aspartate that are most intriguing.

In addition to the large amount of evidence favouring about equal potency of L-glutamate, L-aspartate and D-aspartate (discussed above[11,18,45,121]) as ligands and substrates for L-glutamate transport systems, there are data

TABLE II
Analogues of glutamate and aspartate as inhibitors of L-[^{3}H]aspartate binding in the CNS

	Neocortex	Hippocampus	Cerebellar cortex	ref.
1. D-Aspartate	0.3 ± 0.1	0.3 ± 0.0	1.4 ± 0.2	137
2. L-Aspartate	0.4 ± 0.0	0.4 ± 0.1	0.4 ± 0.0	137
3. L-CCG III	0.9 ± 0.2	1.3 ± 0.2	1.8 ± 0.2	116
4. L-*t*-3OHA	1.4 ± 0.3	1.4 ± 0.3	1.8 ± 0.3	116
5. L-*t*-PDC	2.3 ± 0.3	2.2 ± 0.3	22.3 ± 4.0	115
6. D-*t*-3OHA	2.6 ± 0.3	2.5 ± 0.3	15.0 ± 2.2	115
7. (2*S*,4*R*)-4-Methylglutamate	3.2 ± 0.4	1.7 ± 0.2	4.9 ± 0.7	116
8. L-Serine-*O*-sulfate	14.3 ± 2.2	13.0 ± 1.5	13.6 ± 1.5	115, 137
10. L-Glutamate	18.3 ± 2.6	18.7 ± 3.1	7.5 ± 0.5	Fig. 3
11. L-*a,e*-MPDC	36 ± 4	51 ± 7	96 ± 10	116
12. *cis*-ABDA	53 ± 10	34 ± 7	65 ± 14	116
13. L-CCG IV	(100)	(100)	(100)	116
14. Dihydrokainate	(200)	(200)	inactive	115
15. Kainate	(200)	(200)	inactive	115
16. *trans*-ABDA	inactive	inactive	inactive	116
17. DL-2-Aminoadipate	inactive	inactive	inactive	115
18. DL-3-Aminoadipate	inactive	inactive	inactive	115
19. (1*S*,3*S*)-ACPD	inactive	inactive	inactive	116
20. (*R*,*S*)-AIDA	inactive	inactive	inactive	116
21. (Tetrazol-5-yl)glycine	inactive	inactive	inactive	116
22. CGS 19755	inactive	inactive	inactive	116
23. L-SOP	inactive	inactive	inactive	162
24. L-ODAP	inactive	inactive	inactive	162

The values are concentrations (μM) causing 50% inhibition (IC_{50} ± SEM, values rounded off to the first decimal point) of L-[^{3}H]aspartate (20 nM) binding to thaw-mounted horizontal sections of fresh-frozen rat brain. References, in parentheses, are listed in the last column. Abbreviations: L-CCG III (2*S*,1′*S*,2′*R*)-2-(carboxycyclopropyl)glycine; L-CCG IV (2*S*,1′*R*,2′*S*)-2-(carboxycyclopropyl)glycine; L-*t*-PDC, L-*trans*-pyrrolidine- 2,4-dicarboxylate (*trans*-L-proline-4-carboxylate); L-*a,e*-MPDC, L-*anti,endo*-3,4-methanopyrrolidine dicarboxylate (3,4-(carboxymethano)-L-proline); ABDA, 1-aminocyclobutane-1,3-dicarboxylate; L-ODAP, 3-(oxalylamino)-L-alanine (also known as OXDAPRO, "β-*N*-oxalyl-L-α,β-diaminopropionate"; neurotoxin linked to neurolathyrism[156]); L-SOP, L-serine-*O*-phosphate; ACPD, 1-aminocyclopentane-1,3-dicarboxylate; AIDA, 1-aminoindane-1,5-dicarboxylate, CGS 19755, *cis*-4-(phosphonomethyl)piperidine- 2-carboxylate. The values in parentheses are approximate estimations from inhibitions observed at four inhibitor concentrations *i.e.* the values were not computed. "Inactive" signifies that 50% inhibition was not reached at 200 μM concentration.

in the literature suggesting that L-glutamate, D- and L-aspartate may actually differ from each other in their interactions with glutamate transport, particularly, when the radioligand uptake is studied separately in various brain regions[128,136]. In fact L-*t*-PDC, D-*t*-3-OHA and D-aspartate, the same compounds that differentiate between the L-[^{3}H]aspartate binding in the forebrain and the cerebellar cortex, respectively, display also the greatest regional variations in the experiments using L-[^{3}H]glutamate and L-[^{3}H]aspartate uptake in synaptosomal preparations as an experimental model[128,136]. The results of those studies are, by their nature, difficult to compare directly with the set of data from the L-[^{3}H]aspartate binding experiments[115,116,137] (see also the discussion at the beginning of this section). None of the substrates used in those experiments showed more than two-fold difference between their affinities for L-[^{3}H]glutamate and L-[^{3}H]aspartate uptake[136]. This contrasts with much greater differences between the affinities of the three compounds for L-[^{3}H]aspartate binding in, respectively, forebrain and cerebellar regions[113,137] (Fig. 2, Table II). None of the transporters known to exist in the cerebellar cortex (EAAT1/GLAST,

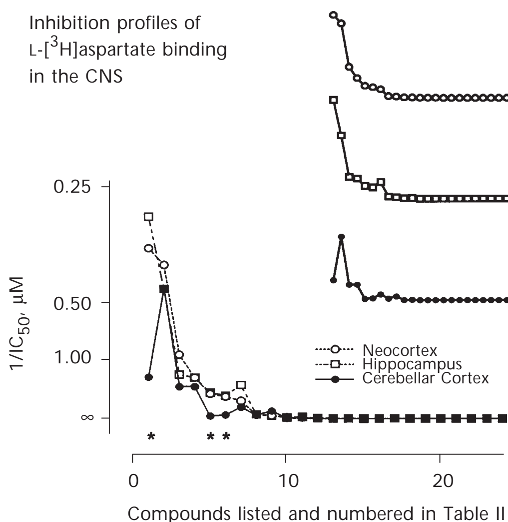

FIG. 2
Reciprocal values of IC_{50}'s (μM^{-1}) are plotted *versus* compounds listed and numbered in Table II. Thus constructed "inhibition profiles" are shown again separately to further demonstrate their overall similarities and differences. Asterisks mark IC_{50} values in the cerebellar cortex that are significantly different from those obtained in the two forebrain regions, at $P < 0.01$, D-aspartate, L-*t*-PDC, D-*t*-3OHA, *cf.* Table II

EAAT2/GLT-1, EAAT3/EAAC1, and EAAT4) have been shown to differentiate between D- and L-aspartate and there is no evidence that EAAT5 that might favour L- over D-aspartate[50] has significant presence outside retina.

Yet, there are data in the literature that support the findings obtained in the L-[^{3}H]aspartate binding studies. Gordon and Balazs[43] using homogenates of 8-day old rat cerebella reported that the uptake of L-[^{3}H]aspartate had about five times higher affinity (K_m = 1.15 μM) than the uptake of D-[^{3}H]aspartate (K_m = 5.43 μM). The difference was statistically significant at $P < 0.02$ (ref.[43]). The authors argued that, in their preparations, astrocytes accounted for almost all of the uptake, though they tested this hypothesis by using L-[^{3}H]glutamate – not L-[^{3}H]aspartate or D-[^{3}H]aspartate – as a substrate. Interestingly, when they put the cerebellar astrocytes into culture, K_m of uptake increased about ten-fold[43]. L-[^{3}H]Glutamate uptake by cultured astrocytes, used for many years as one of the standard models for studying glutamate transport, did not differentiate between L- and D-aspartate[45,138]. Thus it is possible that the glutamate transporters in cultured astrocytes differed from those in the intact brain and did not faithfully reproduce in every detail the characteristics of L-glutamate transport *in vivo*.

There are not many studies in the literature that compare D- and L-[^{3}H]aspartate binding to the thaw-mounted sections of frozen brain, using quantitative autoradiographic methods[137]. Both ligands seem to produce very similar results, the binding is sodium dependent[100,116] and it is inhibited by increasing concentrations of L-glutamate (Fig. 3). When the autoradiograms are represented as a three-dimensional "landscape", it is apparent that, in the cerebellar cortex, the binding of L-[^{3}H]aspartate is greater than the binding of D-[^{3}H]aspartate (Fig. 4). This is supported by densitometric analysis: while in the forebrain the specific binding of L-[^{3}H]aspartate is 1.2 to 2.3 times higher than that of D-[^{3}H]aspartate, in the cerebellar cortex the ratio is 2.2 to 3.2 (ref.[137]). Also, D-[^{3}H]aspartate binding in the cerebellar cortex , but not in the forebrain, is more susceptible to the inhibition by L-SOS than the corresponding binding of L-[^{3}H]aspartate. The difference in IC_{50} values is about 7- to 8-fold[137] and the difference is highly statistically significant ($P < 0.001$, ref.[137]).

There are several known factors with potential influence on the structural selectivities and substrate specificities of glutamate transport. The presence of such factors and/or the magnitude of their hypothetical effects may vary widely from one experimental model to another; therefore, their existence has to be kept in mind when interpreting the results of L-[^{3}H]aspartate and

D-[^{3}H]aspartate binding studies and trying to make meaningful comparisons with the data obtained in alternative experimental models.

For example, the structure of the binding site can be modified allosterically either when the transporter molecules bind to each other[139,140] or when they bind to transporter-associated proteins[141,142]. The clustering of transporters or the presence of the transporter-associated proteins may vary

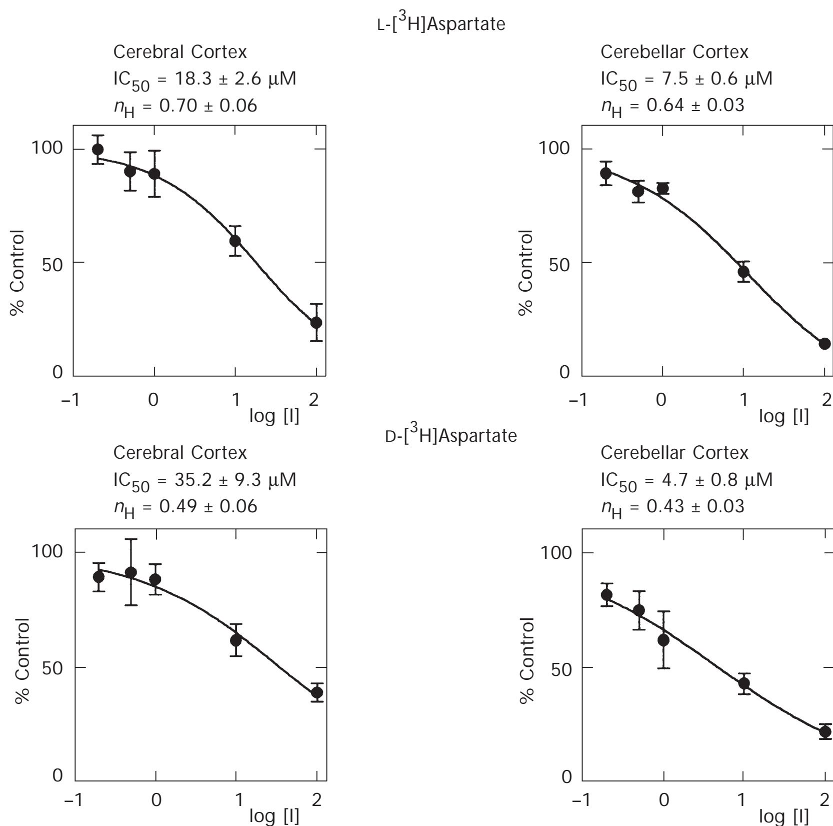

FIG. 3
Inhibition of L- and D-[^{3}H]aspartate binding (both radioligands at 20 nM) by L-glutamate. The points are means ± S.D. of five or six values. The difference between the values of IC_{50} *vs* D-[^{3}H]aspartate in the cerebral cortex and the cerebellar cortex is statistically significant ($P < 0.01$, ANOVA, Tukey–Kramer test). The lines were fitted and the values of IC_{50} were computed by a non-linear regression (GraphPad Prism Software, San Diego, CA, U.S.A.) using equations and criteria described in detail elsewhere[161]

according to the conditions specific for the model used in the experiments and neither the culture systems nor the expression of glutamate transporters in *Xenopus laevis* oocytes may have reproduced all traits of the environment that normally exist in the context of brain tissue. Also, keeping the cells in culture may favour appearance of transporters and/or splice variants that are not strongly expressed *in vivo*.

Another possibility is that the structural specificity of the substrate-binding site is very fragile and may be changed by freezing and thawing of the tissue. The structural specificity of EAAT1 (GLAST) can be, in fact, changed even by such subtle manipulation as varying membrane potential *in vitro*[143]. One would have to further postulate, however, that the changes brought about by freezing and thawing are very small and affect only the stereospecificity *versus* aspartate and its close analogues *t*-3-OHA and L-*t*-PDC and are confined to the cerebellar cortex. This does not seem very probable.

Finally, there is a possibility that the cerebellar cortex contains an as yet unidentified glutamate transporter. Its characteristics would be, except for the stereospecificity of the binding site, very similar to those of the known transporters. In fact, a protein molecule fitting the description – a variant of EAAT1 that prefers L- over D-aspartate – has recently been cloned from an insect nervous system[144]. The presence of such transporter in the mamma-

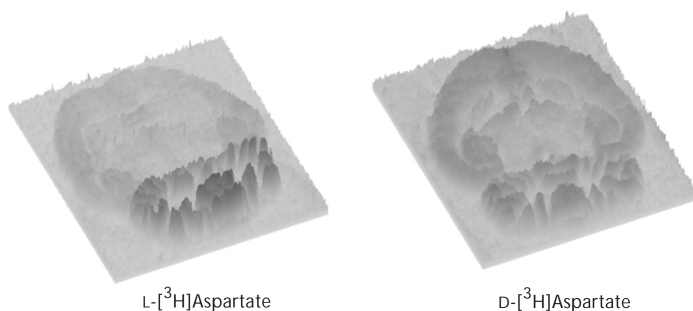

FIG. 4
"Three-dimensional landscape" images, sometimes referred to as "surface" plots, were created by Scion Image Software (NIH Image for Windows, Beta 4.0.2) from autoradiograms obtained after two-week long and five-week long exposures of thaw-mounted horizontal sections of rat brain, labelled by L- or D-[^{3}H]aspartate, against tritium-sensitive films (Amersham Hyperfilm-^{3}H). Total density of the autoradiograms, not converted into "specific binding" of the ligands, was included. See Killinger *et al.*[115] for a photograph and more detailed structure of a similar autoradiogram

lian brain would provide perhaps the most straightforward explanation of the characteristics of L-glutamate transport revealed by the Na^+-dependent binding of L-[^{3}H]aspartate observed in the autoradiographic studies.

In summary, the regional variations in the substrate selectivity of [^{3}H]L-aspartate binding in rat brain clearly point to L-glutamate transport in the cerebellar cortex being different from that in the forebrain. Given the known properties, abundance and distribution of the glutamate transporters identified to date in the cerebellar cortex, and considering the excellent preservation of brain structures in the L-[^{3}H]aspartate binding experiments it would seem natural to accept the lesser sensitivity of L-glutamate transport to the inhibitions by L-*t*-PDC and D-*t*-3OHA, as well as the ability to differentiate between L- and D-aspartate, as the most typical characteristics of the L-glutamate transport in the rat cerebellum.

Overview and Conclusions

Looking back over the three decades that have passed since the first observations and characterizations of the "high affinity uptake of L-glutamate" in brain tissue[1,4,17,18], one might state with some satisfaction that the initial conclusions – based on little more than intuition and common sense but supported by sound neurochemical data – turned out to be broadly correct: glutamate transport controls extracellular levels of glutamate[145,146] and it is intimately involved in the function of glutamatergic synapses[147,148]. Ironically, the hypothesis that the electrogenic transport of L-glutamate forms the basis of the synaptic currents associated with the glutamatergic neurotransmission – so strongly refuted thirty years ago[16,17] – has been making partial comeback, if only at a few special types of synapses[70,71].

In the near future, the relationship between deficient glutamate transport and neurodegenerative diseases will probably continue to be the major focus of interest for both clinical neurologists and basic neuroscientists[149]. Regulation of the expression of glutamate transporters will obviously be at the forefront of research efforts[40,150–152] but attention should also be paid to the relationship between faulty glutamate transport and the disturbances of the brain metabolism[91,93], particularly with regard to the metabolic trafficking between neurons and astrocytes[42]. The field would much benefit if medicinal chemists could produce transporter-selective ligands that could be used as pharmacological tools to elucidate the specific contributions of individual glutamate transporters to the function of the central nervous system[119,123,125]. More comprehensive models of homeostasis and information

processing in the central nervous system, integrating molecular mechanisms with the neuroanatomy and neurophysiology[153], would come a step closer.

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