Rapid Report

C-terminal interactions modulate the affinity of GLAST glutamate transporters in salamander retinal glial cells

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1. Proteins that interact with the intracellular carboxy termini of neurotransmitter- and voltage-gated ion channels are known to control the subcellular localization of the channels, localize other proteins near those channels, and modulate channel activity. By contrast, little is known about the control of neurotransmitter transporter function by interacting proteins.

2. To competitively disrupt interactions of the C- and N-termini of the GLAST glutamate transporter with other proteins, we dialysed whole-cell patch-clamped retinal glia with peptides identical to the eight amino acids at the C- or N-termini of the transporter, and compared the effect on transporter-mediated currents with dialysis of scrambled versions of the same peptides.

3. Dialysis with the N-terminus peptide had no effect on the maximum glutamate-evoked current nor on the glutamate affinity of the transporter. Dialysis with the C-terminus peptide had no effect on the maximum current, but increased the affinity of the transporter for glutamate (compared with scrambled C-terminus peptide, and with N- and scrambled N-terminus peptides: K_m decreased from 16 to 11 μM)).

4. These data suggest that disruption of an interaction between an intracellular protein and the last eight amino acids of the GLAST C-terminus, which have some similarity to the PDZ binding domain of ion channel C-termini, increases the glutamate affinity of GLAST. Thus, the interacting protein decreases the affinity of GLAST transporters.

5. Removing the GLAST C-terminus interaction increases the transporter current by 40 % at low glutamate concentrations. Thus, this interaction may significantly slow the removal of low concentrations of glutamate from the extracellular space, and affect the kinetics of retinal cell light responses.

Interaction of voltage-gated K^+ channels, and of neurotransmitter receptors, with other proteins may control their subcellular localization and activity, as well as locating molecules such as enzymes and IP_3 receptors close to the channels or receptors (Kim *et al.* 1995; Kornau *et al.* 1995; Brenman *et al.* 1996; Dong *et al.* 1997; Horio *et al.* 1997; Tu *et al.* 1998; Garcia *et al.* 1998; Nishimune *et al.* 1998; Tezuka *et al.* 1999; Yamada *et al.* 1999). The last four to seven amino acids of the carboxy termini of many channels play a crucial role in these interactions (although more distant amino acids can also contribute: Bassand *et al.* 1999). The motif S/T-X-V/I, present at the C-terminus of some K^+ channels and NMDA receptor subunits, allows binding of membrane proteins to the best characterised (PDZ) class of scaffolding protein, including the proteins PSD-95 (post-synaptic density-95), Dlg (*Drosophila* discs-large), and ZO-1 (zona occludens-1).

By contrast little is known about how neurotransmitter transporters interact with other proteins. Yeast two-hybrid experiments have suggested proteins that may interact with glutamate transporters (Lin *et al.* 1998; Marie *et al.* 1999), but functional effects of these proteins have not yet been reported. Nevertheless, such interactions may, as for ion channels, play an important role in glutamate transporter function, perhaps generating specific subcellular locations of the transporters (Chaudhry *et al.* 1995), or controlling their insertion into the membrane under the control of signalling systems (Davis *et al.* 1998). Glutamate transporters have both their N- and C-termini on the intracellular side of the cell membrane (Grunewald *et al.* 1998), and the neuronal

EAAT5 transporter has a PDZ-binding motif at its C-terminus, suggesting that the C-terminus may be involved in protein–protein interactions (Arriza *et al.* 1997). The other glutamate transporters have C-termini which diverge from the strictly defined PDZ binding motif (see Discussion).

Here we use a strategy for detecting interaction of proteins with glutamate transporters which does not depend on identifying the interacting proteins. If peptides identical to the interacting part of a glutamate transporter are introduced into the cell, they should compete with the transporter for binding to the interacting protein (cf. Nishimune *et al.* 1998). If the interacting protein modulates transporter function, its displacement by the peptide should produce an alteration of glutamate transport. Using this approach we show for the first time that C-terminal interactions modulate a transporter's glutamate affinity.

METHODS

Glutamate transporter activity was monitored electrically, at 23–25 °C, in glial (Müller) cells isolated enzymatically from the retinae of aquatic tiger salamanders (killed by stunning followed by destruction of the brain) as described by Brew & Attwell (1987).

External solution contained (mM): NaCl, 105; KCl, 2·5; $CaCl_2$, 3; $MgCl_2$, 0·5; glucose, 15; Hepes, 5; $BaCl_2$ (to block inward rectifier K^+ channels and improve signal to noise ratio), 6; pH adjusted to 7·4 with NaOH. The pipette solution for whole-cell clamping contained (mM): KCl, 95; NaCl, 5; Hepes, 5; $MgCl_2$, 2; MgATP, 5; $CaCl_2$, 1; K_2EGTA, 5; pH set to 7 with KOH; salamander GLAST C- or N-terminal peptides or scrambled versions of them (see Results), 0·2; and peptidase inhibitors (to block peptide breakdown in the cell) bestatin, 0·01; pepstatin, 0·001; and leupeptin, 0·105. Pipette series resistance in whole-cell mode was around 4 MΩ, leading to series resistance voltage errors < 2 mV. Pipette junction potentials have been compensated for.

The time needed for peptides to diffuse into the cell, across an idealised barrier at the end of the pipette of width w and area A, can be calculated as follows. The rate of increase of peptide concentration C, in a cell of volume V is given by:

$$V\mathrm{d}C/\mathrm{d}t = DA(C_{\mathrm{pipette}} - C)/w, \quad (1)$$

where D is the diffusion coefficient, and C_{pipette} is the peptide concentration in the pipette. The pipette series resistance across the barrier is:

$$R_{\mathrm{s}} = \rho w/A, \quad (2)$$

where ρ is the resistivity of the pipette solution. From eqns (1) and (2),

$$V\mathrm{d}C/\mathrm{d}t = D\,(C_{\mathrm{pipette}} - C)\rho/R_{\mathrm{s}}.$$

Thus, C approaches C_{pipette} exponentially:

$$C = C_{\mathrm{pipette}}\,(1 - \mathrm{e}^{-t/\tau}),$$

with a time constant of $\tau = VR_{\mathrm{s}}/(D\rho)$. For peptides with a molecular weight near 900, we estimated $D = 2{\cdot}6 \times 10^{-10}$ m^2 s^{-1} by interpolating (on a log–log graph of D against molecular weight) literature values for sucrose (MW = 342, $D = 5{\cdot}2 \times 10^{-10}$ m^2 s^{-1}) and somatostatin (MW = 1638, $D = 1{\cdot}66 \times 10^{-10}$ m^2 s^{-1}). For $\rho = 0{\cdot}8$ Ωm, $R_{\mathrm{s}} = 4$ MΩ, and a Müller cell volume of $V = 10^{-14}$ m^3, the predicted equilibration time constant is around 190 s.

Peptides were obtained from Immune Systems Limited (Paignton UK), were stored under argon (to prevent oxidation) at −20 °C, and were made up freshly before experiments into pipette solution bubbled with argon. Experiments with C- and scrambled C-terminus peptides, or N- and scrambled N-terminus peptides, were carried out in an interleaved manner, with alternate cells clamped with pipette solution containing either the normal or scrambled peptide.

Data are presented as mean ± S.E.M. For glutamate dose–response data, a Michaelis-Menten curve, $[\mathrm{Glu}]_{\mathrm{o}}\,I_{\mathrm{max}}/([\mathrm{Glu}]_{\mathrm{o}} + K_{\mathrm{m}})$, where I_{max} is the maximum current and K_{m} is the equilibrium dissociation constant, was fitted to data from each cell (Brew & Attwell, 1987) using the non-linear curve fitting routine in SigmaPlot (SPSS Inc.), and the resulting parameter values were averaged across cells, after normalising the maximum current by cell capacitance to reduce variation due to differing cell size (Barbour *et al.* 1991). Dose–response data in Fig. 1*C* and *D* were calculated for each cell normalised to the current obtained with 100 μM glutamate, averaged across cells (for each peptide), and then the data for each peptide were scaled to have the same maximum current at saturating glutamate concentration (since the maximum current/capacitance was not significantly different for the different peptides).

RESULTS

Glutamate-evoked currents (Fig. 1*A* and *B*) in salamander retinal Müller glia are dominated by the activity of GLAST glutamate transporters, which take up extracellular glutamate released by retinal photoreceptors and bipolar cells (Brew & Attwell, 1987; Eliasof *et al.* 1998). These transporters generate a current due to the co-transport of 2 net positive charges with each glutamate taken up (Zerangue & Kavanaugh, 1996; Levy *et al.* 1998), and also a small current due to Cl^- flux through an anion conductance that the transporter gates (Eliasof & Jahr, 1996; Billups *et al.* 1996) which is suggested to be proportional to the rate of glutamate uptake (Otis & Jahr, 1998).

The sequence of the salamander GLAST transporter has been reported by Eliasof *et al.* (1998). We compared the effect on the glutamate-evoked current of dialysing cells with pipette solutions containing a peptide (200 μM) with the sequence of the C- or N-terminus of the transporter or scrambled versions of them (see below). For quantitative comparison, data were measured 10 min after entering whole-cell mode to allow the peptides to enter the cell and compete with endogenous GLAST for binding to interacting proteins. In similar experiments Nishimune *et al.* (1998) found that 15 min dialysis of much larger neurons was sufficient for internally applied peptides to act.

Figure 1*A* and *B* shows specimen data from cells dialysed with C- and scrambled C-terminus peptides (PIDSETKM and MDKISTPE, respectively). Mean glutamate dose–response curves from such experiments are shown in Fig. 1*C*. Applying 5 and 20 μM glutamate to cells dialysed with C-terminus peptide evoked currents that were a larger fraction of the current evoked by 100 μM glutamate than those in cells dialysed with scrambled C-terminus peptide ($P = 0{\cdot}0016$ for 5 μM and $P = 0{\cdot}034$ for 20 μM, 9 cells studied for each peptide, Student's 2-tailed t test). By

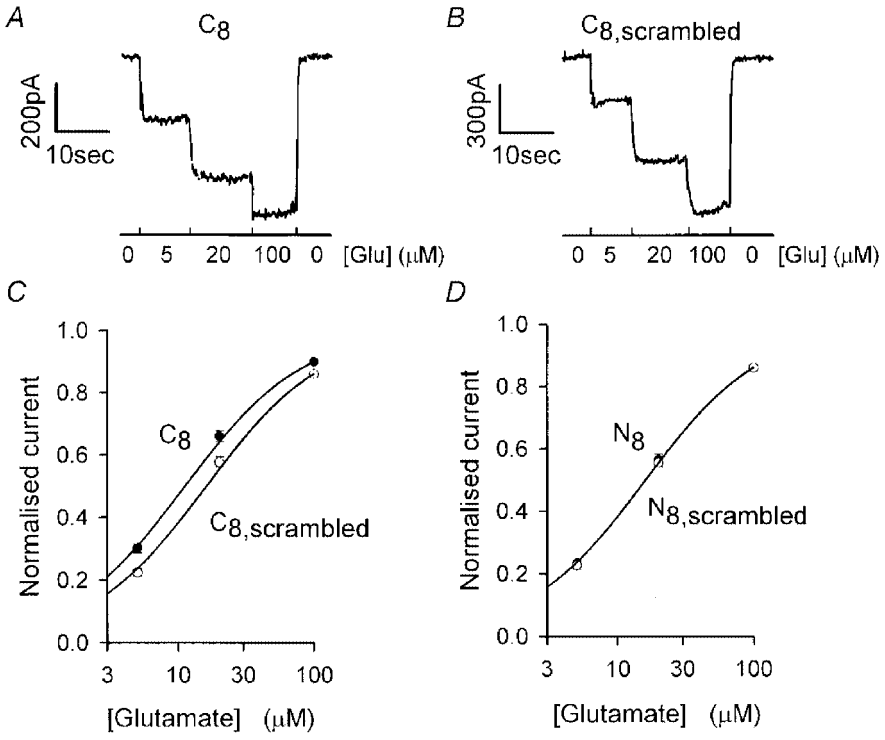

Figure 1. Effect of including 8 amino acid long GLAST C- and N-terminal peptides in the whole-cell pipette on glutamate-evoked currents

A, specimen data showing the current response of a Müller cell clamped to −63 mV with a pipette containing C-terminal peptide (last 8 amino acids: C_8), during superfusion of a solution containing 5, 20 and 100 μM glutamate (record obtained 10 min after going to whole-cell mode). *B*, specimen data (as in *A*) from a cell with the pipette solution containing scrambled C-terminal peptide ($C_{8,scrambled}$), normalised to the same current at 100 μM glutamate as for *A* for comparison. *C*, dose–response curves (mean current ± S.E.M., which is comparable to the symbol size) from data as in *A* and *B* (normalised and scaled as described in the Methods). Smooth curves are Michaelis-Menten curves with K_m values of 16·1 μM for the scrambled C-terminus peptide and 11·2 μM for the C-terminus peptide, i.e. the mean values in Fig. 2 obtained from the 9 cells studied for each peptide; extrapolated maximum current at high [glutamate] is 1 for both curves. *D*, dose–response data as in *C*, but for pipette solutions containing N-terminus (N_8) or scrambled N-terminus ($N_{8,scrambled}$) peptides. Smooth curve is the same as for the scrambled C-terminus peptide in *C*.

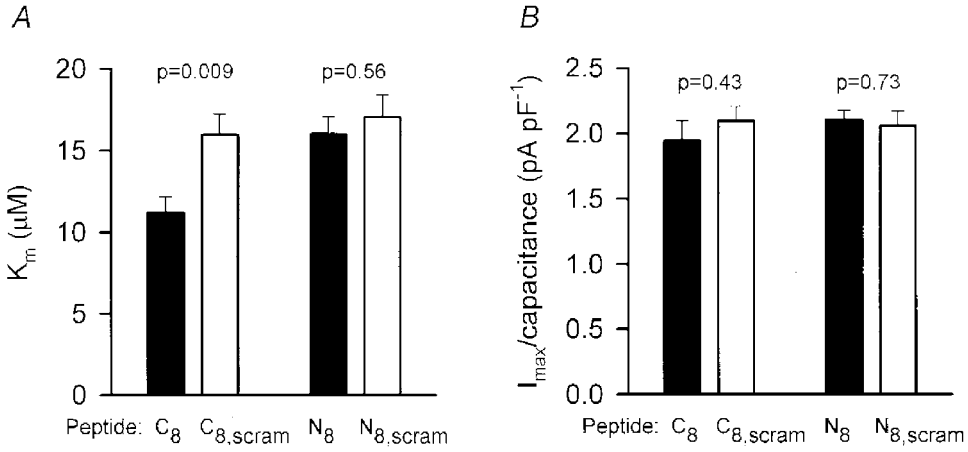

Figure 2. Effect of C- and N-terminal peptides on the K_m and I_{max} of uptake

Mean values (± S.E.M.) of K_m (*A*), and extrapolated maximum current at high [glutamate] normalised by cell capacitance (I_{max}/capacitance) (*B*), obtained from fitting Michaelis-Menten curves to dose–response data obtained as in Fig. 1, for pipettes filled with 8 amino acid peptides corresponding to the GLAST C- and N-termini (C_8, N_8), and scrambled versions of these peptides ($C_{8,scram}$, $N_{8,scram}$). *P* values shown are for 2-tailed *t* tests comparing data for normal and scrambled peptides.

contrast, fractional responses to 5 and 20 μM glutamate in cells dialysed for 10 min with N- and scrambled N-terminal peptides (MTKSNGED and ENMDKGST, respectively) were not significantly different (Fig. 1*D*; $P = 0{\cdot}62$ for 5 μM and $P = 0{\cdot}65$ for 20 μM, 8–9 cells studied for each peptide).

Fitting a Michaelis-Menten curve to the dose–response data from each cell showed that the K_m for glutamate activating a transporter current was not significantly different for intracellular dialysis with the N-, scrambled N- and scrambled C-terminus peptides, but was significantly reduced for the C-terminus peptide (Fig. 2*A*). The extrapolated maximum transporter current at high glutamate concentration (normalised by cell capacitance, a measure of cell membrane area) was not significantly different for all the peptides studied (Fig. 2*B*). Thus, dialysis with the C-terminus peptide specifically increases the glutamate affinity. Approximately 80 % of this affinity change had occurred 2 min after going to whole-cell mode (the earliest time at which we could measure the dose–response curve), suggesting that the values used for pipette peptide concentration and series resistance rapidly resulted in a sufficient concentration of peptide being achieved in the cell to displace endogenous proteins that bind to the GLAST C-terminus.

The voltage dependence of the transporter current evoked by 100 μM glutamate was not significantly different when cells were dialysed with C- or scrambled C-terminus peptides (current at +20 mV/current at −80 mV was $0{\cdot}129 \pm 0{\cdot}011$ for C- and $0{\cdot}134 \pm 0{\cdot}014$ for scrambled C-terminus peptide, 7 cells each, $P = 0{\cdot}78$), suggesting no effect on rate limiting steps of the carrier cycle involving transport of charge through the membrane field.

DISCUSSION

Dialysis of Müller cells with a peptide identical to the C-terminus of GLAST increases the affinity of GLAST for glutamate (compared with dialysis with scrambled C-terminus, N-terminus, or scrambled N-terminus peptides). Since this peptide should compete with endogenous GLAST C-terminus for binding to other proteins, the interacting proteins must normally decrease the affinity of GLAST for glutamate.

A shift of K_m from 16·1 to 11·2 μM (Fig. 2*A*) implies a 40 % increase in transport current at low glutamate concentrations, which may significantly alter the time course of the extracellular glutamate concentration during the tail of a synaptic current. Signal transmission from bipolar cells to ganglion cells in the retina is profoundly affected by slowing the rate of glutamate uptake into Müller cells (Higgs & Lukasiewicz, 1999), with the ganglion cell response becoming greatly prolonged. Thus, the interaction we have characterised may play a role in controlling the temporal response characteristics of the retinal output. Conceivably the interaction may be removed under certain conditions, speeding retinal response kinetics.

The salamander (and human) GLAST C-terminus ends with the amino acids ETKM, which is similar to the sequence ESXV at the C-terminus of NMDA NR2 subunits. This suggests that the endogenous protein being displaced by dialysis with GLAST C-terminus peptide is likely to be in the same PDZ family as the PSD-95 protein which binds to NMDA receptors (although we cannot rule out the existence of other interacting molecules, nor the possibility that the GLAST C-terminus actually binds to part of the GLAST molecule itself as occurs for the N-termini of potassium channels: Hoshi *et al.* 1990). Two other members of the glutamate transporter family have related C-termini, the retinal EAAT5 ending with ETNV and the Purkinje cell EAAT4 ending with ESAM, which suggests that they may also have their affinity modulated by proteins interacting with their C-terminus. By contrast, the two other glutamate transporters, i.e. the commonest splice variant of the most abundant glial transporter GLT-1, and the neuronal EAAC1, have very different C-termini (KREK and TSQF, respectively), suggesting that they may interact with a different class of protein.

The cytoplasmic C-terminus of GLAST is unlikely to be directly involved in binding extracellular glutamate, indeed there is some evidence that the glutamate binding site is in the transporter's membrane pore loop region near residues 400–440 (Zhang & Kanner, 1999; Seal & Amara, 1998). However, chimaeric transporters in which the C-terminus of EAAT1 (the human equivalent of GLAST) is replaced by that of the lower affinity transporter EAAT2 show a decrease in glutamate affinity (Mitrovic *et al.* 1998), suggesting that the properties of the transporter C-terminus can modulate glutamate affinity, as is also suggested by our data. It will be interesting to see whether proteins identified as interacting with glutamate transporters (Lin *et al.* 1998; Marie *et al.* 1999) alter the glutamate affinity, as expected from our data, and whether these interactions are modulated to adjust the properties of the transporters to different situations.

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