Niflumic Acid Activates Additional Currents of the Human Glial l-Glutamate Transporter EAAT1 in a Substrate-Dependent Manner

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The astrocytic l-glutamate (l-Glu) transporter EAAT1 participates in the removal of l-Glu from the synaptic cleft and maintenance of non-toxic concentrations in the extracellular fluid. We have shown that niflumic acid (NFA), a non-steroidal anti-inflammatory drug (NSAIDs), alters l-Glu-induced EAAT1 currents in a voltage-dependent manner using the two-electrode voltage clamp technique in Xenopus oocytes expressing EAAT1. In this study, we characterised the effects of NFA on each type of ion-flux through EAAT1. NFA modulated currents induced by both l-Glu and l-aspartate (l-Asp) in a voltage-dependent manner. Ion-substitution experiments revealed that the activation of additional H+ conductance was involved in the modulation of currents induced by l-Asp and l-Glu, but Cl− was involved only with the l-Asp currents. NFA activated additional currents of EAAT1 in a substrate-dependent manner.

Key words astrocytic l-glutamate transporter; niflumic acid; additional conductance; EAAT1; voltage-dependent manner

Neuronal and astrocytic l-glutamate (l-Glu) transporters (EAATs) are the only significant machinery for the removal of l-Glu from the synaptic cleft and maintenance of non-toxic concentrations in the extracellular fluid.1,2 Along with controlling extracellular l-Glu concentrations, EAATs also play a role in the regulation of functional crosstalk between neurons and glial cells by modulating the ion flux. l-Glu is co-transported into a cell with 3 Na+ and 1 H+ by EAATs, followed by the counter-transport of 1 K+.3 l-Glu and Na+ binding to EAATs activates a non-stoichiometrically-coupled (uncoupled) Cl− conductance.4 The Na+ influx triggers functional metabolic crosstalk between neurons and astrocytes,4 and the uncoupled Cl− conductance dampens neuronal excitability.5,6

Niflumic acid [2-((3-(trifluoromethyl)phenyl)amino)-3-pyridinecarboxylic acid, NFA], a member of a class of non-steroidal anti-inflammatory drugs (NSAIDs), alters l-Glu-induced EAAT1 currents in a voltage-dependent manner using the two-electrode voltage clamp technique in Xenopus oocytes expressing EAAT1. In this study, we characterised the effects of NFA on each type of ion-flux through EAAT1. NFA modulated currents induced by both l-Glu and l-aspartate (l-Asp) in a voltage-dependent manner. Ion-substitution experiments revealed that the activation of additional H+ conductance was involved in the modulation of currents induced by l-Asp and l-Glu, but Cl− was involved only with the l-Asp currents. NFA activated additional currents of EAAT1 in a substrate-dependent manner.

MATERIALS AND METHODS

Expression of EAAT1 in Xenopus Oocytes All of the animals were treated in accordance with the guidelines for the Care and Use of Laboratory Animals of the Animal Research Committee of the National Institute of Health Sciences, Japan. A pcDNA3.1 plasmid containing the cDNA of the human glutamate transporter EAAT1 was obtained from Dr. Keiko Shimamoto (Suntory Institute for Bioorganic Research, Osaka, Japan). The plasmids containing the EAAT1 cDNA were linearized at a NotI (Toyobo, Osaka, Japan) site, and capped RNA was transcribed from the linearized cDNA construct with a bacteriophage T7 RNA polymerase (mMESSAGE mMACHINE; Ambion, Austin, TX, U.S.A.). Oocytes were collected from anesthetised Xenopus laevis. The isolated oocytes were then treated with collagenase (2 mg mL−1, type 1, Sigma, St. Louis, MO, U.S.A.), and capped mRNA was injected into either defolliculated stage V or VI oocytes. The oocytes were incubated for 2–7 d at 18°C in ND96 solution containing 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, and 5 mM N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES) (pH 7.5) supplemented with 0.01% gentamycin.

Electrophysiology Two-electrode voltage clamp recordings from EAAT1-expressing oocytes were performed at room temperature (25–27°C) using glass microelectrodes filled with 3 mM KCl solution (resistance=1–4 MΩ) and an Ag/AgCl pellet bath ground (EP2; World Precision Instruments, Sarasota, FL, U.S.A.). A bath-clamp amplifier (OC-725C; Warner Instruments, Hamden, CT, U.S.A.) was used with a Digidata 1320A interface (Axon Instruments, Foster City, CA, U.S.A.). The pClamp suite of programs (ver. 8.2; Axon Instruments) and the Clampfit data acquisition software were used to control stimulation parameters, and to acquire and analyse data. An Ag/AgCl pellets were used to avoid voltage errors associated with buffer changes. Oocytes were continuously superfused with ND96 solution. To adjust the extracellular H+ to various concentrations, the HEPES was replaced by either 2-(N-morpholino)ethanesulfonic acid (MES) (pH 5.5, 6.5) or [2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]-1-propanesulfonic acid (TAPS) (pH 8.5). For Na+ substitution experiments, Na+ was replaced by equimolar choline ions. For

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In the Cl⁻ substitution experiments, Cl⁻ was replaced by equimolar gluconate ions. Oocytes were bathed in an experimental chamber (0.5 mL) filled with ND96 solution and voltage-clamped at −50 mV. A 400 ms hyperpolarising voltage step to −120 mV was applied every 2 s to confirm clamp conditions and observe the voltage dependence of current responses. As

Fig. 1. Niflumic Acid (NFA) Modulated L-Asp-Induced Currents in Xenopus Oocytes Expressing EAAT1 in a Voltage-Dependent Manner

A: The traces of L-Asp (30 μM)-induced inward currents in either the absence or presence of NFA (1 mM) at −50 mV (bold line) and −120 mV (thin line). The oocytes were held at −50 mV and hyperpolarised to −120 mV for 400 ms every 2 s. B: Concentration–response relationships of the effects of NFA on L-Asp currents at −50 mV and −120 mV. The effects of NFA on the peak amplitude of the L-Asp current were examined by comparing the current in the presence of NFA to that recorded just prior to the drug treatment (control response) at each concentration. Data are analysed by paired t-test. Graph shows the summary of the results. Each column shows the averaged data normalized to the control (4–5 oocytes for each) at each concentration. At −50 mV, NFA inhibited the peak amplitudes of the L-Asp currents in a concentration-dependent manner, whereas NFA only slightly increased the peak amplitude of the L-Asp-induced currents at −120 mV. *p<0.05 vs. control. C: Representative current–voltage relationships for L-Asp (30 μM) in either the absence or presence of NFA (1 mM). The current–voltage relationships were obtained with a holding potential of −50 mV and implementation of 400-ms voltage jumps in 10 mV increments over the range from −120 mV to +40 mV. For the control, the current values at steady state were subtracted from those measured in the presence of L-Asp. For the NFA-treated group, the current values in the presence of NFA alone were subtracted from those in the presence of both NFA and L-Asp. NFA treatment produced a leftward shift of $E_{rev}$ (from 18.4±5.4 to −2.7±4.1 mV; n=10, p<0.05, paired t-test). D: The relationship between the effects and the holding potential. The current in the presence of NFA was normalized to that obtained just before the application of NFA. The effects of NFA were voltage-dependent (n=10).
a substrate, 30 µM of either L-Glu or L-Asp (half of the EC₅₀) was applied to the oocytes by superfusion at 0.2 mL·s⁻¹ of constant flow rate for 15 s with regular 30 s intervals. NFA was applied from 30 s before to 5 s after the end of the application of substrate. The current–voltage relationships for substrate transport were determined by subtracting the steady-state currents obtained with a holding potential of −50 mV either implementing 400 ms voltage jumps in 10 mV increments from −120 to +60 mV or implementing an 800 ms ramp pulse from −120 to +40 mV in the absence of substrate from the corresponding currents in the presence of substrate. The currents are normalised to the amplitude of the L-Asp or L-Glu currents generated at −100 mV (Figs. 2–8).

Preparation of the Compounds All chemicals were purchased from Wako (Tokyo, Japan) unless otherwise stated. NFA, TAPS, and MES were purchased from Sigma (St. Louis, MO, U.S.A.). L-Asp and L-Glu stock solutions (20 mM) were made in purified water (Millipore, Billerica, MA, U.S.A.). The NFA stock solution (300 mM) was made in dimethyl sulfoxide (DMSO) and dissolved in ND96 solution immediately prior to each experiment. The pH of every solution was adjusted to 7.5, and the final concentrations of the solvents were less than 1%.

Statistical Analysis All of the data are presented as the mean±S.E.M. p Values were obtained by statistical analysis, as noted in the figure legends.

RESULTS Effects of NFA on L-Asp-Induced Currents in Xenopus Oocytes Expressing EAAT1 We first examined the effects of NFA on the L-Asp-induced currents in Xenopus oocytes expressing EAAT1. The left trace in Fig. 1A represents the inward control current produced by L-Asp (30 µM) voltage clamped at −50 mV with 400 ms hyperpolarising voltage steps to −120 mV every 2 s. At −50 mV, NFA (300 µM) inhibited the peak amplitude of the L-Asp-induced currents in a concentration-dependent manner, whereas at 120 mV, NFA only slightly increased the peak amplitude of the L-Asp-induced currents (Figs. 1A, B). The currents are normalised to the amplitude of the L-Asp currents generated at −100 mV (Figs. 2–8).

Preparation of the Compounds All chemicals were purchased from Wako (Tokyo, Japan) unless otherwise stated. NFA, TAPS, and MES were purchased from Sigma (St. Louis, MO, U.S.A.). L-Asp and L-Glu stock solutions (20 mM) were made in purified water (Millipore, Billerica, MA, U.S.A.). The NFA stock solution (300 mM) was made in dimethyl sulfoxide (DMSO) and dissolved in ND96 solution immediately prior to each experiment. The pH of every solution was adjusted to 7.5, and the final concentrations of the solvents were less than 1%.

Statistical Analysis All of the data are presented as the mean±S.E.M. p Values were obtained by statistical analysis, as noted in the figure legends.
−96.5 mV (cross-over potential), indicating that NFA inhibited the L-Asp currents at potentials more positive than −96.5 mV and increased the currents at potentials more negative than −96.5 mV. The influence of NFA on the peak current amplitude was voltage-dependent (Fig. 1D). This voltage-dependent effect of NFA has also been observed with L-Glu current in our previous study.21)

**Involvement of Additional H⁺ Conductance** We examined whether any additional H⁺ conductance is involved in these voltage-dependent effect of NFA. The effects of NFA were examined when the extracellular pH was 7.5 and 5.5. Figures 2A-1 and B-1 show the average current–voltage relationships for the L-Asp current (30 µM) in either the absence (solid line) or presence (dotted line) of NFA (300 µM) at pH 7.5 (Fig. 2A-1) and pH 5.5 (Fig. 2B-1). At pH 7.5, the curve of the L-Asp current in the presence of NFA crossed the control curve at −84 mV, whereas the crossover potential was at +4 mV at pH 5.5. The L-Asp-gated NFA-induced conductance was obtained by subtracting the L-Asp current from the L-Asp current in the presence of NFA (Figs. 2A-2, B-2). As the extracellular H⁺ concentration increased, the $E_{rev}$ of the L-Asp-gated NFA-induced conductance (sub-$E_{rev}$) shifted toward the more positive membrane potential. The average shift of the sub-$E_{rev}$ gated by L-Asp was $-45.1 \pm 4.0$ mV per pH unit ($n=5$), which is consistent with previous reports for H⁺-selective channels,22,23 suggesting that NFA promotes additional H⁺ conductance. Regarding L-Glu currents (Fig. 3), as the extracellular H⁺ concentrations increased, the crossover potential shifted toward the more positive potential (Figs. 3A-1, B-1), and the sub-$E_{rev}$ also shifted toward a more positive membrane potential (Figs. 3A-2, B-2). The average shift of the sub-$E_{rev}$ gated by L-Glu changed $-36.6 \pm 4.6$ mV per pH unit ($n=5$), suggesting that NFA promotes additional H⁺ conductance in this case as well. There were no significant differences between the average shifts of the sub-$E_{rev}$ per pH unit gated by L-Asp and L-Glu (Student’s t-test, $p=0.2$).

**Involvement of Additional Na⁺ Conductance** We examined if the additional Na⁺ conductance is involved in these voltage-dependent effect of NFA. The current–voltage relationships for the L-Asp current in either the absence or presence of NFA were examined under various extracellular [Na⁺] by choline substitution. Figures 4A-1 and B-1 show the average current–voltage relationships for L-Asp in either the absence (solid line) or presence (dotted line) of NFA (300 µM) at normal [Na⁺] (96 mM) (Fig. 4A-1) and low [Na⁺] (24 mM) (Fig. 4B-1). Decreasing the extracellular [Na⁺] resulted in a loss of crossover between −120 mV and +40 mV, indicating that low extracellular [Na⁺] results in a loss of the voltage-dependent modulation of L-Asp currents by NFA. The L-Asp-gated NFA-induced conductance-voltage relationships displayed inward rectification with the sub-$E_{rev}$ at −58 mV (Fig. 4A-2) at normal [Na⁺], whereas no significant subtracted currents

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Fig. 3. Influence of Extracellular pH on L-Glu Currents in Either the Absence or Presence of NFA

A-1 and B-1: Average current–voltage relationships for L-Glu (30 µM) in either the absence (solid line) or presence (dotted line) of NFA (300 µM) at pH 7.5 (A-1) and pH 5.5 (B-1). A-2 and B-2: Average current–voltage relationships for the L-Glu-gated NFA-induced conductance at pH 7.5 (A-2) and pH 5.5 (B-2). The average shift of the sub-$E_{rev}$ was $-36.6 \pm 4.6$ mV per pH unit, which is consistent with that reported for H⁺-selective channels. Each point represents the mean value from 5 oocytes.
were observed at low [Na\(^+\)], suggesting that the modulation of L-Asp currents by NFA depends on the extracellular Na\(^+\) concentration. Comparing the sub-\(E_{rev}\) at normal [Na\(^+\)] (96mM) with that at middle-low [Na\(^+\)] (48mM), the average shift of the sub-\(E_{rev}\) gated by L-Asp was \(-1.1\pm12.4\) mV (\(n=3\)) per 10-fold change in [Na\(^+\)]. In the case of L-Glu, decreasing the extracellular [Na\(^+\)] also resulted in a loss of crossover between \(-120\) mV and \(+40\) mV (Figs. 5A-1, B-1). The sub-\(E_{rev}\) gated by L-Glu was \(-68\) mV at normal [Na\(^+\)], whereas no significant subtracted currents were observed at low [Na\(^+\)] (Figs. 5A-2, B-2), suggesting that the voltage-dependent modulation of L-Glu currents by NFA depends on the extracellular Na\(^+\) concentration. Comparing the sub-\(E_{rev}\) at normal [Na\(^+\)] with that at middle-low [Na\(^+\)], the average shift of the sub-\(E_{rev}\) gated by L-Glu was \(7.8\pm2.2\) mV (\(n=3\)) per 10-fold change in [Na\(^+\)]. This value was not significantly different from that for L-Asp (Student’s \(t\)-test, \(p=0.5\)).

**Involvement of Additional Cl\(^-\) Conductance** Finally, we examined the contribution of additional Cl\(^-\) conductance. The current–voltage relationships for the L-Asp current in either the absence or presence of NFA were examined under various extracellular [Cl\(^-\)] by gluconate substitution. Figures 6A-1 and B-1 show the average current–voltage relationships for L-Asp in either the absence (solid line) or presence (dotted line) of NFA (300 \(\mu\)M) at normal [Cl\(^-\)] (103mM) (Fig. 6A-1) and low [Cl\(^-\)] (30mM) (Fig. 6B-1). At low [Cl\(^-\)], the crossover potential shifted toward the more negative potential (from \(-61\) mV to \(-115\) mV) and the sub-\(E_{rev}\) also shifted toward the more negative membrane potential. The average shift of the sub-\(E_{rev}\) gated by L-Asp was \(94.9\pm6.7\) mV per 10-fold change in [Cl\(^-\)] (\(n=4\)) (Fig. 6C), indicating that Cl\(^-\) contributes to the L-Asp-gated NFA-induced conductance. Regarding L-Glu currents, no changes were observed in the crossover potential at low [Cl\(^-\)] (Figs. 7A-1, B-1) and a small shift of the sub-\(E_{rev}\) gated by L-Glu occurred at low [Cl\(^-\)] (\(n=4\)) (Fig. 7C), indicating that Cl\(^-\) contributes to the L-Glu-gated NFA-induced conductance. The significant difference in the additional Cl\(^-\) conductance between the L-Asp current and L-Glu current (Student’s \(t\)-test, \(p<0.05\)) suggests that mechanisms for the modulation of EAAT1 currents by NFA is substrate-dependent.

**DISCUSSION**

In this study, we observed that the additional conductances...
of EAAT1 were activated when substrates were transported in the presence of NFA. Furthermore, the ionic contribution to the additional conductances is substrate dependent. To our knowledge, this is the first report showing the existence of additional conductances of EAAT1.

Poulsen and Vandenberg reported that NFA induced additional $\text{H}^+$ and $\text{Cl}^-$ conductances in *Xenopus* oocytes expressing EAAT4, and these conductances were not thermodynamically coupled to the transport of substrates. 18) These conductances have been referred to as ‘slippage.’ 24) In our experiments using cultured astrocytes, 25) 300 $\mu$M of NFA significantly decreased the $\text{L-Glu}$ uptake in cultured astrocytes (data not shown). Membrane potential of cultured astrocytes is approximately $-74 \text{ mV}$.26) Because the crossover potential of the $\text{L-Glu}$ currents and NFA (300 $\mu$M)-gated $\text{L-Glu}$ currents was $-72.7\pm4.5 \text{ mV}$ ($n=12$) in the present study, it is suggested that NFA-gated conductance observed here is not thermodynamically coupled to substrate transport, *i.e.*, NFA induces EAAT1 slippage as well. Transport experiments in voltage-clamped oocytes are necessary to confirm whether additional conductances in EAAT1 are not thermodynamically coupled to the substrate transport.

Sacher *et al.* presented a ‘clutch’ mechanism for slippage via the mammalian and yeast metal-ion transporter DCT1.27) This mechanism could be explained in terms of two unique but interconnected ion pathways, one dominated by the ion utilized for driving the transport and the other by the transported metal ions. Loose coupling (namely clutching) between the driving force pathway and the metal ion transport pathway generates this observed slippage. Regarding EAAT1, in the presence of NFA, additional $\text{H}^+$ conductance may have arisen as a consequence of a subtle disruption to the ion binding sites, which compromises the coupling between the substrate transport pathway and the ion co-transport ($\text{Na}^+/\text{H}^+/\text{K}^+$) pathway. In support of this, a chimeric transporter generated with EAAT1 and EAAT2, whose junction site is in helical hairpin 2 and in close proximity to the substrate and $\text{Na}^+$ binding site, allows both $\text{Na}^+$ and $\text{K}^+$ to pass through the transporter in the absence of $\text{L-Glu}$.28) Interestingly, the ionic contribution is substrate-dependent, *i.e.*, the activation of additional $\text{H}^+$ conductance was involved in the modulation by NFA of both of $\text{L-Glu}$ currents and $\text{L-Asp}$ currents, whereas $\text{Cl}^-$ was only involved in the modulation of $\text{L-Asp}$ currents. Wadiche *et al.* reported that $\text{Cl}^-$ permeation properties of EAAT1 were substrate-dependent, *i.e.*, the uncoupled $\text{Cl}^-$ conductance per transport cycle gated by $\text{D-Asp}$ was greater than that gated by $\text{L-Glu}$.29) The mechanisms underlying the difference between $\text{L-Glu}$ and $\text{L-Asp}$ observed here may be related to the one that causes the greater $\text{Cl}^-$ by $\text{D-Asp}$. To elucidate the mechanisms, it is necessary to identify

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**Fig. 5.** Influence of Extracellular [Na⁺] on $\text{L-Glu}$-Gated NFA-Induced Conductance and the $\text{L-Glu}$ Currents in Either the Absence or Presence of NFA

A-1 and B-1: Average current–voltage relationships for $\text{L-Glu}$ (30 $\mu$M) in either the absence (solid line) or presence (dotted line) of NFA (300 $\mu$M) at normal [Na⁺] (96 mM) (A-1) and low [Na⁺] (24 mM) (B-1). A-2 and B-2: Average current–voltage relationships for the $\text{L-Glu}$-gated NFA-induced conductance at normal [Na⁺] (96 mM) (A-2) and low [Na⁺] (24 mM) (B-2). The average shift of the sub-$\text{E}_\text{rev}$ gated by $\text{L-Glu}$ was 7.8±2.2 mV ($n=3$) per 10-fold change in [Na⁺], which was statistically insignificant compared with that by $\text{L-Asp}$. Each point represents the mean from 5 oocytes.
the binding site of NFA on EAAT1, and the stoichiometric interaction among the substrates, EAAT1, and NFA.

Alterations in the glial intracellular pH can induce a variety of changes in cellular function, e.g., ionic currents, gap junction conductance, and enzymatic activities. For example, in Bergmann glial cells, which highly express EAAT1, electrical coupling via gap junctions has been shown to be modulated by altering intracellular pH. Activation of additional H⁺ conductance by NFA could be related to the effects of the drug in altering intracellular pH.

In conclusion, we observed substrate-dependent mechanisms for the modulation of EAAT1 currents by NFA. Activation of additional H⁺ conductance was involved in the NFA-induced modulation of EAAT1 currents by L-Asp and L-Glu. The Cl⁻ ion was only involved in the NFA-induced modulation of L-Asp currents.

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Fig. 6. Influence of Extracellular [Cl⁻] on L-Asp-Gated NFA-Induced Conductance and the L-Asp Currents in Either the Absence or Presence of NFA

The current–voltage relationships for the L-Asp current in either the absence or presence of NFA were examined under various extracellular [Cl⁻] by gluconate substitution. A-1 and B-1: Average current–voltage relationships for L-Asp (30 µM) in either the absence (solid line) or presence (dotted line) of NFA (300 µM) at normal [Cl⁻] (103 mM) (A-1) and low [Cl⁻] (30 mM) (B-1). A-2 and B-2: Average current–voltage relationships for the L-Asp-gated NFA-induced conductance at normal [Cl⁻] (103 mM) (A-2) and low [Cl⁻] (30 mM) (B-2). Each point represents the mean from 5 oocytes. C: Each point (filled circle) represents the mean sub-\( E_{rev} \) for L-Asp obtained in A-2 and B-2. Alterations in the extracellular Cl⁻ concentration caused average shifts of 94.9±6.7 mV per 10-fold change in [Cl⁻] \((n=4)\) in the sub-\( E_{rev} \),
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Fig. 7. Influence of Extracellular Cl− on L-Glu-Gated NFA-Induced Conductance and the L-Glu Currents in Either the Absence or Presence of NFA

A-1 and B-1: Average current–voltage relationships for L-Glu (30 µM) in either the absence (solid line) or presence (dotted line) of NFA (300 µM) at normal [Cl−] (103 mM) (A-1) and low [Cl−] (30 mM) (B-1). A-2 and B-2: Average current–voltage relationships for the L-Glu-gated NFA-induced conductance at normal [Cl−] (103 mM) (A-2) and low [Cl−] (30 mM) (B-2). Each point represents the mean from 4 oocytes. C: Each point (open circle) represents the mean sub-Erev for L-Glu obtained in A-2 and B-2. Alterations in the extracellular Cl− concentrations caused average shifts of 1.9±8.1 mV per 10-fold change in [Cl−] (n=4) in the sub-Erev, which is significantly different from that of L-Asp (Student’s t-test, p<0.05).
REFERENCES

1) Logan WJ, Snyder SH. Unique high affinity uptake systems for glycine, glutamic and aspartic acids in central nervous tissue of the rat. *Nature*, **234**, 597–599 (1971).

2) Zerangue N, Kavanaugh MP. Flux coupling in a neuronal glutamate transporter. *Nature*, **383**, 634–637 (1996).

3) Fairman WA, Vandenberg RJ, Arriza JL, Amara SG. An excitatory amino-acid transporter with properties of a ligand-gated chloride channel. *Nature*, **375**, 599–603 (1995).

4) Voutsinos-Porche B, Bonvento G, Tanaka K, Steiner P, Welker E, Chatton JY, Magistretti PJ, Pellerin L. Glial glutamate transporters mediate a functional metabolic crosstalk between neurons and astrocytes in the mouse developing cortex. *Neuron*, **37**, 275–286 (2003).

5) Veruki ML, Morkve SH, Hartveit E. Activation of a presynaptic glutamate transporter regulates synaptic transmission through electrical signaling. *Nat. Neurosci.*, **9**, 1388–1396 (2006).

6) Wersinger E, Schwab Y, Sahel JA, Rendon A, Pow DV, Picaud S, Roux MJ. The glutamate transporter EAAT5 works as a presynaptic receptor in mouse rod bipolar cells. *J. Physiol.*, **577**, 221–234 (2006).

7) White MM, Aylwin M. Niflumic and flufenamic acids are potent reversible blockers of Ca\(^{2+}\)-activated Cl\(^-\) channels in *Xenopus* oocytes. *Mol. Pharmacol.*, **37**, 720–724 (1990).

8) Scott-Ward TS, Li H, Schmidt A, Cai Z, Sheppard DN. Direct block of the cystic fibrosis transmembrane conductance regulator Cl\(^-\) channel by niflumic acid. *Mol. Membr. Biol.*, **21**, 27–38 (2004).

9) Ottolia M, Toro L. Potentiation of large conductance KCa channels by niflumic, flufenamic, and mefenamic acids. *Biophys. J.*, **67**, 2272–2279 (1994).

10) Busch AE, Herzer T, Wagner CA, Schmidt F, Raber G, Waldegger S, Lang F. Positive regulation by chloride channel blockers of IsK channels expressed in *Xenopus* oocytes. *Mol. Pharmacol.*, **46**, 750–753 (1994).

11) Wang HS, Dixon JE, McKinnon D. Unexpected and differential effects of Cl\(^-\) channel blockers on the Kv4.3 and Kv4.2 KC\(^+\) channels. Implications for the study of the Ito(2) current. *Circ. Res.*, **81**, 711–718 (1997).

12) Malykhina AP, Shoeb F, Akbarali HI. Fenamate-induced enhancement of heterologously expressed HERG currents in *Xenopus* oocytes. *Eur. J. Pharmacol.*, **452**, 269–277 (2002).

13) Peretz A, Degani N, Nachman R, Uziyel R, Gibor G, Shabat D, Attali B. Meclofenamic acid and diclofenac, novel templates of KCNO2/Q3 potassium channel openers, depress cortical neuron activity and exhibit anticonvulsant properties. *Mol. Pharmacol.*, **67**, 1053–1066 (2005).

14) Fernandez D, Sargent J, Sachse FB, Sanguinetti MC. Structural basis for ether-a-go-go-related gene K\(^v\) channel subtype-dependent activation by niflumic acid. *Mol. Pharmacol.*, **73**, 1159–1167 (2008).

15) Zwart R, Oortgiesen M, Vijverberg HP. Differential modulation of alpha 3 beta 2 and alpha 3 beta 4 neuronal nicotinic receptors expressed in *Xenopus* oocytes by flufenamic acid and niflumic acid. *J. Neurosci.*, **15**, 2168–2178 (1995).

16) Hu H, Tian J, Zha Y, Wang C, Xiao R, Herz JM, Wood JD, Zhu MX. Activation of TRPA1 channels by fenamate nonsteroidal anti-inflammatory drugs. *Pfugers Arch.*, **459**, 579–592 (2010).

17) Furuta A, Rothstein JD, Martin LJ. Glutamate transporter protein subtypes are expressed differentially during rat CNS development. *J. Neurosci.*, **17**, 8363–8375 (1997).

18) Poulsen MV, Vandenberg RJ. Niflumic acid modulates uncoupled substrate-gated conductances in the human glutamate transporter EAAT4. *J. Physiol.*, **534**, 159–167 (2001).

19) Arriza JL, Fairman WA, Wadiche JI, Murdoch GH, Kavanaug MP, Amara SG. Functional comparisons of three glutamate transporter subtypes cloned from human motor cortex. *J. Neurosci.*, **14**, 5559–5569 (1994).

20) Chaudhry FA, Lehre KP, van Looenek Campagne M, Ottersen OP, Danbolt NC, Storm-Mathisen J. Glutamate transporters in glial plasma membranes: highly differentiated localizations revealed by quantitative ultrastructural immunocytochemistry. *Neuron*, **15**, 711–720 (1995).

21) Takahashi K, Iihi-Nozawa R, Takeuchi K, Nakazawa K, Sato K. Two non-steroidal anti-inflammatory drugs, niflumic acid and diclofenac, inhibit the human glutamate transporter EAAT1 through different mechanisms. *J. Pharmacol. Sci.*, **112**, 113–117 (2010).

22) DeCoursey TE, Cherny VV. Voltage-activated hydrogen ion currents. *J. Membr. Biol.*, **141**, 203–223 (1994).

23) Fairman WA, Sonders MS, Murdoch GH, Amara SG. Arachidonic acid elicits a substrate-gated proton current associated with the glutamate transporter EAAT4. *Nat. Neurosci.*, **1**, 105–113 (1998).

24) Vandenberg RJ, Huang S, Ryan RM. Slips, leaks and channels in glutamate transporters. *Channels (Austin)*, **2**, 51–58 (2008).

25) Sato K, Matsuki N, Ohno Y, Nakazawa K. Estrogens inhibit l-glutamate uptake activity of astrocytes via membrane estrogen receptor alpha. *J. Neurochem.*, **86**, 1498–1505 (2003).

26) Nowak L, Ascher P, Berwald-Netter Y. Ionic channels in mouse astrocytes in culture. *J. Neurosci.*, **7**, 101–109 (1987).

27) Sacher A, Cohen A, Nelson N. Properties of the mammalian and yeast metal-ion transporters DCT1 and Smf1p expressed in *Xenopus laevis* oocytes. *J. Exp. Biol.*, **204**, 1053–1061 (2001).

28) Vandenberg RJ, Arriza JL, Amara SG, Kavanaug MP. Constitutive ion fluxes and substrate binding domains of human glutamate transporters. *J. Biol. Chem.*, **270**, 17668–17671 (1995).

29) Wadiche JI, Amara SG, Kavanaug MP. Ion fluxes associated with excitatory amino acid transport. *Neuron*, **15**, 721–728 (1995).

30) Deitmer JW, Rose CR. pH regulation and proton signalling by glial excitatory amino acid transport. *J. Exp. Biol.*, **204**, 1053–1061 (2001).

31) Moller T, Moller T, Neuhau J, Kettenmann H. Electrical coupling among Bergmann glial cells and its modulation by glutamate receptor activation. *Glia*, **17**, 274–284 (1996).