Zn$^{2+}$ Inhibits the Anion Conductance of the Glutamate Transporter EAAT4*

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Running Title: Zn$^{2+}$ Modulation of Glutamate Transport

Keywords
Glutamate transporter, aspartate transporter, zinc, chloride

Abbreviations: EAAT, Excitatory Amino Acid Transporter

* This work was supported by the Australian National Health and Medical Research Council.
**SUMMARY**

Glutamate transport by the Excitatory Amino Acid Transporters (EAATs) is coupled to the co-transport of 3 Na\(^+\) ions and 1 H\(^+\) and the counter-transport of 1 K\(^+\) ion, which ensures that extracellular glutamate concentrations are maintained in the sub-micromolar range. In addition to the coupled ion fluxes, glutamate transport activates an uncoupled anion conductance that does not influence the rate or direction of transport, but may have the capacity to influence the excitability of the cell. Free Zn\(^{2+}\) ions are often co-localized with glutamate in the CNS and have the capacity to modulate the dynamics of excitatory neurotransmission. In this study we demonstrate that Zn\(^{2+}\) ions inhibit the uncoupled anion conductance and also reduce the affinity of L-aspartate for EAAT4. The molecular basis for this effect was investigated using site-directed mutagenesis. Two histidine residues in the extracellular loop between transmembrane domains three and four of EAAT4 appear to confer Zn\(^{2+}\) inhibition of the anion conductance.
INTRODUCTION

Glutamate is the predominant excitatory neurotransmitter in the mammalian brain and excitatory amino acid transporters (EAATs) serve the role of controlling extracellular glutamate concentrations to maintain normal neurotransmission. Five different glutamate transporters have been identified in humans, termed EAAT1 - 5 (1-3). The rat homologues of EAAT1-3 are termed GLAST1 (4), GLT1 (5) and EAAC1 (6) respectively. The stoichiometry of substrate ion flux-coupling has been determined for the EAAT3 and GLT1 subtypes where glutamate is co-transported with 3 Na⁺ and 1 H⁺ followed by the counter-transport of 1 K⁺ ion (7,8). Glutamate transporters also allow an uncoupled flux of anions through the transporter, which requires the presence of glutamate and Na⁺ ions but does not appear to influence the rate or direction of glutamate transport (2,9). The magnitude of the uncoupled anion flux relative to the glutamate-coupled ion fluxes varies with the different transporters, with the anion flux greatest for EAAT4 and EAAT5, followed by EAAT1 and then EAAT3 and EAAT2. Thus, electrophysiological measurements of glutamate transport have components derived from the ion coupled transport conductance and the uncoupled chloride conductance.

Zn²⁺ is found in a number of regions of the brain, with >90% bound to proteins such as various Zn²⁺-finger proteins. Chelatable Zn²⁺ is enriched in a number of regions of the brain, especially in the mossy fibers of the hippocampus where it is stored in synaptic
vesicles with glutamate (reviewed by (10)). Stimulation of the mossy fibers leads to release of Zn\(^{2+}\) (11) and Zn\(^{2+}\) has been shown to modulate the activity of a number of proteins within excitatory synapses, including the N-methyl-D-aspartate (NMDA) subtypes of glutamate receptors (12,13), Ca\(^{2+}\) channels (14) and also glutamate transporters (15,16). Spiridon et al., (15) demonstrated that Zn\(^{2+}\) inhibits glutamate transport currents, whilst stimulating the uncoupled chloride conductance associated with the transporters in Muller cells of the Salamander retina. In contrast to the Muller cells, Zn\(^{2+}\) inhibits the chloride conductance associated with glutamate transporters of the retinal cone cells (15). The predominant glutamate transporter in Muller cells shows most similarity to the human EAAT1 and in a previous study we demonstrated that Zn\(^{2+}\) inhibits the transport current of EAAT1, but had little effect on the uncoupled chloride flux associated with the transport process (16).

In many proteins, the side chains of histidine, cysteine, glutamate or aspartate residues coordinate the binding of Zn\(^{2+}\). In EAAT1, we established that the side chains of histidine 146 and histidine 154 form part of the Zn\(^{2+}\) binding site. Alignment of the amino acid sequences of EAAT1-5 in this region (Figure 1) shows that histidine 146 is conserved in EAAT1, 2, 4 and 5 and that the residue corresponding to histidine 154 of EAAT1 is divergent. In EAAT2, which is insensitive to Zn\(^{2+}\), a glycine residue corresponds to the second histidine residue of EAAT1 and mutation of this residue to
histidine generates a transporter that is inhibited by $\text{Zn}^{2+}$ at comparable $\text{Zn}^{2+}$ concentrations to that of EAAT1 (16). From these results we hypothesized that the chemical nature of the side chains of the residues at the two positions will determine the sensitivity to $\text{Zn}^{2+}$. In this study we have investigated the actions of $\text{Zn}^{2+}$ on the glutamate transporter EAAT4 and found that $\text{Zn}^{2+}$ increases the EC50 for L-aspartate activation of the chloride conductance and also decreases the extent of activation of the chloride conductance. Both of the $\text{Zn}^{2+}$ binding site histidine residues identified in EAAT1 are conserved in EAAT4 and mutations of these residues reduce $\text{Zn}^{2+}$ modulation of the anion conductance of the transporter.
EXPERIMENTAL PROCEDURES

Chemicals

All chemicals were obtained from SIGMA Chemical Co (Sydney, Australia) unless otherwise stated. TBOA was kindly supplied by Dr Keiko Shimamoto and was also obtained from Tocris. ZnCl$_2$ was diluted from a stock solution of 20 mM in frog ringer’s solution. The stock solution was stored at 4° C and was made fresh each week.

Expression of excitatory amino acid transporters in *Xenopus laevis* oocytes and electrophysiological recordings.

The wild type and mutant EAAT4 transporters subcloned in the pOTV plasmid (1) were linearized with BamH1 and cRNA transcribed from the cDNA construct with T7 RNA polymerase and capped with 5’7-methyl guanosine using the mMESSAGE mMACHINE kit (Ambion Inc., Austin, TX, U.S.A). Mutations in EAAT4 were generated using the Quickchange Site-Directed Mutagenesis kit from Stratagene and used according to the manufacturer’s instructions.

Oocytes were harvested from *X. laevis* as previously described (18) and 50 nl of cRNA was injected into defoliculated stage V *X. laevis* oocytes and incubated in standard frog ringers solution (ND96; 96 mM NaCl, 2mM KCl, 1mM MgCl$_2$, 1.8 mM CaCl$_2$, 5 mM HEPES pH7.55), supplemented with 2.5 mM sodium-pyruvate, 0.5 mM theophylline,
50µg/ml gentamicin. Two to eight days later, current recordings were made using the two electrode voltage clamp technique with a Geneclamp 500 (Axon Instruments, Foster City, CA) interfaced with an MacLab 2e chart recorder (ADInstruments, Sydney, Australia) using the Chart software and a Digidata 1200 (Axon Instruments) controlled by an IBM compatible computer using the pCLAMP software (version 7, Axon Instruments).

The current-voltage relationships for L-glutamate or L-aspartate transport were determined by subtraction of steady state current measurements in the absence of L-glutamate or L-aspartate, obtained during 300msec voltage pulses to potentials between -100 to +60mV in 10 mV steps, from corresponding current measurements in the presence of substrate. In recordings where the extracellular chloride concentration was altered, a 3M KCl agar bridge was used to connect the ground electrode to the bath solution to minimize offset potentials. The chloride-free buffer used contained 96 mM Nagluconate, 2 mM Kgluconate, 1.8 mM Ca(gluconate)₂, 1 mM Mg(gluconate)₂, 5 mM HEPES, pH 7.5. Zn²⁺ chelates L-glutamate and L-aspartate and the free L-glutamate and free Zn²⁺ concentrations were calculated according the method of Dawson et al.(19) and employed by Spiridon et al.,(15). In all figures and calculations free Zn²⁺ and L-glutamate or L-aspartate concentrations are used. The following protocol was used to isolate the effects of Zn²⁺ on the transport, and the transport-activated anion conductances of the transporters. The conductance elicited by L-glutamate (or L-
aspartate) was first measured and after washout of substrate, Zn\textsuperscript{2+} was applied and baseline conductance measurements made, followed by co-application of substrate with Zn\textsuperscript{2+} and the conductance measured again. After washout of Zn\textsuperscript{2+} and substrate, substrate alone was re-applied to ensure that the conductance measurements return to control values.

**Analysis of kinetic data.**

Analysis of kinetic data was carried out using the Kaleidagraph Software version 3.1. Substrate (L-glutamate and L-aspartate) dose responses were fitted by least squares as a function of current (I) to \( \frac{I}{I_{\text{max}}} = \frac{[S]}{(EC_{50} + [S])} \), where \( I_{\text{max}} \) is the maximal current and \( EC_{50} \) the concentration of substrate that generates a half maximal current and \( [S] \) is the substrate concentration. Dose dependent Zn\textsuperscript{2+} inhibition of substrate activated currents were fitted by least squares to

\[ I = I(\text{glu/asp}) - (I_{\text{max}}[\text{Zn}^2\text{+}] / (IC_{50} + [\text{Zn}^2\text{+}])) + R, \]

where \( I(\text{glu/asp}) \) is current due to L-glutamate or L-aspartate alone, \( I_{\text{max}} \) is the maximal current inhibited by Zn\textsuperscript{2+}, \( [\text{Zn}^2\text{+}] \) is the Zn\textsuperscript{2+} concentration, \( IC_{50} \) is the concentration of Zn\textsuperscript{2+} causing half maximal inhibition and \( R \) is the residual transport current at a maximal dose of Zn\textsuperscript{2+}. \( EC_{50} \), \( IC_{50} \) and \( I_{\text{max}} \) values presented in table 1.
RESULTS

Zn$^{2+}$ Inhibits the Anion Conductance of EAAT4.

Application of L-aspartate, or L-glutamate, to oocytes expressing EAAT4 generates a conductance that is carried predominantly by chloride ions (2). Co-application of 100 µM Zn$^{2+}$ with 100 µM L-aspartate to oocytes expressing EAAT4 causes a reduction in amplitude of the aspartate-evoked conductance compared to 100 µM L-aspartate alone with little or no change in reversal potential (Figure 2A). Whilst the reduction in current amplitude in the presence of Zn$^{2+}$ was apparent at both positive and negative membrane potentials, the reduction in current amplitude was greater at positive potentials. A slightly different result was obtained for L-glutamate-evoked currents. In the absence of Zn$^{2+}$, L-glutamate generates a conductance approximately 50% of the conductance evoked by L-aspartate (2), see Figure 2B). In the presence of 100 µM Zn$^{2+}$, the reduction in the L-glutamate-evoked conductance was only apparent at positive potentials with little or no change in conductance at negative potentials (Figure 2B). In subsequent experiments on EAAT4, and the various mutants of EAAT4, we have presented the results for the effects of Zn$^{2+}$ on L-aspartate-evoked conductances because the larger conductance changes are more reliably measured than for L-glutamate and the extent of Zn$^{2+}$ modulation of the currents is greater. The onset of inhibition by Zn$^{2+}$ was rapid and reversible with washout of Zn$^{2+}$ from the bath solution, which
suggests a direct interaction between Zn$^{2+}$ and the transporter.

Inhibition of substrate-evoked currents by Zn$^{2+}$ could be due to inhibition of the coupled transport conductance or inhibition of the uncoupled anion conductance or both conductances. Under the conditions of this experiment and assuming that the stoichiometry of ion flux coupling for EAAT4 is the same as for EAAT3 and GLT1, a net inward flux of L-glutamate or L-aspartate is expected at membrane potentials up to +60 mV and therefore any outward current at positive potentials is due to the uncoupled anion conductance. The reduction in outward current at positive membrane potentials suggests that Zn$^{2+}$ inhibits the anion conductance. If the extracellular chloride ions are replaced with the more permeant anions bromide, iodide or nitrate, significantly greater L-aspartate-evoked outward currents are observed, with a relative order of magnitude (and relative current amplitude at +60 mV) of NO$_3^-$ (27.3) > I$^-$ (8.3) > Br$^-$ (3.75) > Cl$^-$ (1.0). Although 100µM Zn$^{2+}$ reduced the anion conductance for all 4 anions, the degree of reduction varied, with the greatest reduction observed for iodide (76±2%, n=4), followed by chloride (66±3%, n=7) and bromide (66±7%, n=4) and with the smallest reduction observed for nitrate (48±4%, n=5) (Figure 3B). In each case there was no significant change in reversal potential measured in the presence and absence of 100 µM Zn$^{2+}$. These results are consistent with Zn$^{2+}$ inhibiting the uncoupled anion conductance and although there are variations in the extent of inhibition for different
anions, Zn$^{2+}$ does not alter the relative anion permeability.

To measure the effects of Zn$^{2+}$ on the ion-coupled transport conductance, oocytes expressing EAAT4 were incubated in a buffer in which chloride ions were completely replaced with the impermeant anion gluconate for >40 hours. This procedure has been reported to reduce the intracellular chloride concentration of the oocyte to <4mM (9) and allows transport conductances to be measured in the absence of a significant uncoupled anion conductance. Application of L-aspartate to oocytes in chloride-free buffer generates inward currents at potentials up to +60 mV, as opposed to an outward current measured under standard conditions. At ±60 mV, currents measured under both chloride-free and standard conditions were inward but differed in relative amplitude. The L-aspartate-evoked conductance measured under chloride-free conditions was 15 ± 3 nA (n=7) compared to 74 ± 8 nA (n=3, from the same batch of oocytes) under standard conditions, which confirms previous observations that under standard conditions a majority of the L-aspartate-evoked conductance is due to activation of a chloride conductance. Under chloride-free conditions, co-application of 100 µM Zn$^{2+}$ with 100 µM L-aspartate generated a similar conductance compared to 100 µM L-aspartate alone (Figure 3C), which suggests that Zn$^{2+}$ has no effect on the coupled transport component of the currents mediated by EAAT4. Thus, Zn$^{2+}$ inhibition of the substrate-gated conductance of EAAT4 is most likely to be due to inhibition of the uncoupled anion conductance.
In further experiments on EAAT4 and EAAT4 mutants we have not distinguished between the coupled transport and the uncoupled anion components of the conductance and have assumed that Zn$^{2+}$ has a selective effect on the uncoupled anion conductance. The EC$_{50}$ for L-aspartate activation of the chloride conductance measured in the presence of 100 µM Zn$^{2+}$ (5.1 ± 0.5 µM, n=7) was increased compared to the EC$_{50}$ measured in the absence of Zn$^{2+}$ (3.4 ± 0.5 µM, n=7, p=0.05 2-tailed t test), (Figure 4A), which suggests that Zn$^{2+}$ modulates the interaction between L-aspartate and EAAT4. At -100 mV, Zn$^{2+}$ caused a maximal inhibition of 58 ± 10 % (n=4), whereas at +60 mV a maximal dose of Zn$^{2+}$ inhibited the current by 81 ± 5% (n=4)(Figure 4B). The IC$_{50}$ values for Zn$^{2+}$ inhibition of the anion conductance also differed at the different membrane potentials. At -100 mV, the IC$_{50}$ was 86 ± 29 µM (n=4) and at +60 mV the IC$_{50}$ was 38 ± 10 µM (n=4).

**Mutations of Histidine 154 and Histidine 164 Abolish Zn$^{2+}$ Sensitivity of EAAT4.**

We have previously identified two histidine residues in the large extracellular loop between transmembrane domains 3 and 4 of EAAT1 that form part of the Zn$^{2+}$ binding site. Alignment of the amino acid sequences of EAAT4 with EAAT1 shows that both histidine residues are conserved between the two transporters. In the following experiments we have used site-directed mutagenesis to investigate whether the conserved
histidine residues also form the Zn\textsuperscript{2+} binding site on EAAT4 that mediates inhibition of the anion conductance.

The two histidine residues were mutated to alanine to remove the imidazole group that is thought to interact with Zn\textsuperscript{2+}. In addition the second histidine residue, at position 164, was changed to glutamate because a glutamate residue is found at this position of the glutamate transporter EAAT5. Application of L-aspartate to oocytes expressing the EAAT4 mutants, H154A, H164A, and H164E generated dose dependent conductances that reversed direction at similar membrane potentials to that of wild type EAAT4 (Table 1). This suggests that the mutations have not caused significant structural changes to the pore of the transporter. In contrast to wild type EAAT4, co-application of 100 µM Zn\textsuperscript{2+} with 100 µM L-aspartate to oocytes expressing the EAAT4 mutant, H154A, had no significant effect on the conductance compared to L-aspartate alone (Figure 5) or the EC50 for L-aspartate-evoked conductance. This suggests that the mutation has selectively disrupted Zn\textsuperscript{2+} modulation of the substrate-activated anion conductance. Similar results were also observed for the second site mutants H164A and H164E (Figure 5). Thus, histidines residues at positions 154 and 164 appear to influence Zn\textsuperscript{2+} affinity for EAAT4, which is analogous to the results observed for EAAT1 (16).

Cysteine residues have also been identified in other proteins as forming Zn\textsuperscript{2+} binding sites and as EAAT4 contains two cysteine residues we investigated whether either of
these two residues play a role in mediating the effects of Zn$^{2+}$ on EAAT4. Application of 100 µM L-aspartate to oocytes expressing the EAAT4 C194A and EAAT4 C400A mutants showed similar current voltage relationships as wild type EAAT4 and co-application of 100 µM Zn$^{2+}$ caused similar reductions in the conductance as observed for the wild type EAAT4. Thus, the C194A and C356A mutations do not appear to alter the functional properties of the transporter or the sensitivity to Zn$^{2+}$ and are unlikely to form part of the Zn$^{2+}$ binding site on EAAT4.

In most cells expressing EAAT4, but not in uninjected oocytes, application of Zn$^{2+}$ alone appears to block a constitutive conductance. This constitutive conductance could be an intrinsic property of EAAT4 or could be due to the expression of an endogenous oocyte protein as a consequence of over expression of the transporter. The following observations suggest that an endogenous oocyte ion channel mediates the constitutive conductance. First, if the constitutive conductance were an intrinsic property of the transporter it would be expected that there should be a correlation between the amplitude of the leak conductance and the amplitude of the anion conductance. The amplitude of the constitutive conductance was variable both between, and within, batches of oocytes. The amplitude of the leak conductance blocked by 100 µM Zn$^{2+}$ varied from 250% of the substrate activated anion conductance to <5% of the anion conductance. Second, application of the glutamate transport blocker TBOA to oocytes expressing EAAT4, at concentrations that inhibit the substrate-activated anion conductance, does not block the
constitutive conductance. Third, the amplitude of the Zn\textsuperscript{2+}-blocked leak conductance does not appear to influence any of the transporter-mediated functional properties, such as the EC\textsubscript{50} for Zn\textsuperscript{2+} inhibition of the anion conductance or the extent of inhibition. Fourth, the same variability in amplitude of the leak conductance observed for EAAT4 was also observed for the EAAT4 mutants, EAAT4 H154A, EAAT4 H164A, EAAT4 H164E, EAAT4 C194A and EAAT4 C356A. Finally, other researchers have also described various Zn\textsuperscript{2+} blocked conductances in oocytes (20) that could be responsible for the leak conductance in oocytes expressing EAAT4. Although we cannot completely rule out the possibility that the constitutive conductance, or some proportion of the conductance, is an intrinsic property of the transporter that functions independently of the transport function, the above observations make this interpretation unlikely.
DISCUSSION

**Zn\(^{2+}\) inhibition of the anion conductance of EAAT4**

Zn\(^{2+}\) is found throughout the brain and may modulate the actions of glutamate by influencing the activity of NMDA receptors (12,13), Ca\(^{2+}\) channels (14) and also glutamate transporters (15,16). The actions of Zn\(^{2+}\) are most clearly demonstrated in the mossy fibres of the hippocampus where Zn\(^{2+}\) is co-released with glutamate upon stimulation (11). Whilst there are a number of physiological and pathological implications of Zn\(^{2+}\) modulation of excitatory neurotransmission, Zn\(^{2+}\) may also be used to study potential mechanisms for modulation of various proteins, including glutamate transporters. In this study we have used Zn\(^{2+}\) as a molecular probe to identify the molecular basis for differential modulation of the coupled and uncoupled conductance states of the glutamate transporter EAAT4.

There are two distinct types of conductances associated with glutamate transporter function: a coupled flux of Na\(^{+}\), H\(^{+}\), K\(^{+}\) and glutamate ions (7,8); and an uncoupled anion conductance that requires the presence of L-glutamate and Na\(^{+}\) ions (2,9,21,22). The relative contributions of the two components vary with the different transporter subtypes and the effects of Zn\(^{2+}\) on these conductances also vary between transporter subtypes. In the case of EAAT1, we have previously demonstrated that Zn\(^{2+}\) inhibits the
current due to the coupled Na⁺, K⁺, H⁺ and glutamate fluxes with little, if any, effect on the uncoupled anion conductance, whilst application of Zn²⁺ to oocytes expressing EAAT2 or EAAT3, does not appear to modulate any of the transporter associated conductances, (16), Vandenberg unpublished observations). In the current study we have investigated the actions of Zn²⁺ on the uncoupled anion conductance and the coupled substrate transport conductance of the EAAT4 subtype.

We have demonstrated that Zn²⁺ inhibits the uncoupled anion conductance of EAAT4, and also causes a small, but significant, increase in L-aspartate EC₅₀. Although Zn²⁺ reduced the amplitude of the anion conductances of EAAT4, Zn²⁺ did not change the reversal potentials of the anion conductances when carried by chloride, bromide, iodide or nitrate ions. The lack of changes in anion permeability suggests that Zn²⁺ binds to a site on EAAT4 that is distinct from the pore region of the transporter. The IC₅₀ for Zn²⁺ inhibition of the anion conductance decreases with an increase in membrane potential from 86 µM at −100 mV to 38 µM at +60 mV and the extent of maximal inhibition of the anion conductance also changes from 58% at −100 mV to 81% at +60 mV. These observations could be explained if the time spent in transport mode compared to anion-conducting mode is also dependent on membrane potential. At positive membrane potentials, the anion-conducting mode may predominate and as Zn²⁺ selectively inhibits the anion conductance of the transporter, the measured effects of Zn²⁺ may be more
apparent at these membrane potentials.

**Molecular Basis for Differential Zn\textsuperscript{2+} Modulation of Glutamate Transporter Subtypes.**

We have previously demonstrated that two histidine residues within the extracellular loop between transmembrane domains 3 and 4 form part of the Zn\textsuperscript{2+} binding site on EAAT1. Mutations of either of these histidine residues to alanine do not alter the glutamate transport kinetics of EAAT1 but do diminish the effects of Zn\textsuperscript{2+} on EAAT1 and therefore these Zn\textsuperscript{2+} binding site residues are unlikely to form part of the pore through which glutamate, Na\textsuperscript{+}, K\textsuperscript{+}, H\textsuperscript{+} and possibly Cl\textsuperscript{-} ions pass during the transport process. We have now extended this work to include a description of the Zn\textsuperscript{2+} binding sites on the EAAT4 subtype of excitatory amino acid transporters.

Alignment of the amino acid sequences of the EAATs shows that the two histidine residues of EAAT1 that bind Zn\textsuperscript{2+} are conserved in EAAT4. Mutation of either of these histidine residues to alanine abolishes Zn\textsuperscript{2+} inhibition of the anion conductance of EAAT4 and also Zn\textsuperscript{2+} modulation of L-aspartate EC\textsubscript{50}, which demonstrates that Zn\textsuperscript{2+} interacts with EAAT4 at a similar site to that of EAAT1. Although the Zn\textsuperscript{2+} binding sites are similar on EAAT1 and EAAT4, the effects of Zn\textsuperscript{2+} are different. In the case of EAAT1, Zn\textsuperscript{2+} inhibits the coupled fluxes of L-glutamate, Na\textsuperscript{+}, K\textsuperscript{+} and H\textsuperscript{+} with minimal
effect on the anion conductance (16), whereas for EAAT4 Zn$^{2+}$ causes a small increase in EC$_{50}$ for L-aspartate with no change in the level of inhibition of L-aspartate transport and significant inhibition of the anion conductance. There are a number of possible explanations for these differences. Wadiche and Kavanaugh (23) have demonstrated that glutamate transporters do not simultaneously function as a coupled transporter and an anion channel, but rather the transporters are likely to switch between the two modes of function. As the chloride conductance dominates the combined coupled transport/uncoupled chloride channel conductance in the cases of EAAT4, it may be predicted that the time spent in the anion channel mode is significantly greater than the transporter mode compared to EAAT1. Thus, Zn$^{2+}$ modulates the dominant process - ie the anion conductances of EAAT4 and the coupled glutamate, Na$^+$, K$^+$, H$^+$ fluxes of EAAT1.

If we compare the results of the human glutamate transporters expressed in oocytes with that observed for the actions of Zn$^{2+}$ on glutamate transporters in the salamander retina there are number of similarities, but also some distinct differences. In Muller cells of the salamander retina the predominant transporter is homologous to EAAT1 and in these cells Zn$^{2+}$ inhibits glutamate transport currents, but in contrast to human EAAT1 Zn$^{2+}$ stimulates the uncoupled chloride conductance. Furthermore, the K$_{0.5}$ for Zn$^{2+}$ modulation of the Muller cell transporter is 0.66 µM, which is approximately 10-20 fold
less than that observed for EAAT1. In other Zn$^{2+}$ binding proteins the number of
coordinating residues roughly correlates with the affinity of Zn$^{2+}$. With 2 coordinating
histidine residues affinities range from 10 - 100 $\mu$M where as with 3 coordinating
residues affinities in the range of 0.01 - 1 $\mu$M have been observed (17). Thus, the higher
affinity of Zn$^{2+}$ for the salamander EAAT1 may be due to the presence of an additional
coordinating residue. If the amino acid sequences of the human and salamander EAAT1s
are compared in the putative Zn$^{2+}$ binding site region a number of subtle differences are
apparent which could explain the different effects of Zn$^{2+}$ (Figure 1). The salamander
EAAT1 contains an extra histidine residue between the two histidine residue conserved
between the human and salamander EAAT1s that could possibly influence the binding
affinity or could create different conformational changes when Zn$^{2+}$ is bound compared
to Zn$^{2+}$ binding to EAAT1 such that the different functional effects are created. The
differences could also arise because of the expression of other glutamate transporter
subtypes or accessory proteins (24-26) in the Muller cell with each subtype responding
differently to Zn$^{2+}$. In cone cells of the salamander retina, Zn$^{2+}$ inhibits the chloride
conductance associated with glutamate transporters of the retinal cone cells (27). The
predominant glutamate transporter in cone cells is homologous to human EAAT5, but in
oocytes expressing human EAAT5 Zn$^{2+}$ appears to stimulate the anion conductance
(Wendy Fairman and Susan Amara, personal communication).
The quaternary structure of glutamate transporters is poorly defined, but recent characterization of electron micrographs of *Xenopus laevis* oocyte membranes containing the EAAT3 transporters suggests that transporters may exist as homomultimers consisting of between 3-6 subunits, with 5 subunits the most favored option (28). Furthermore, it was suggested that the subunits may function as separate transporters, but the chloride channel function of the transporters may arise through the association of the subunits to form a central channel. The binding of substrate to each of the subunits may alter the association of the subunits to change the conformation of the central chloride channel and allow passage of chloride ions. If this functional model is correct then the actions of Zn$^{2+}$ offer a particularly interesting insight into the mechanisms for differentially modulating the dual roles of glutamate transporters. Thus, the functional role of Zn$^{2+}$ ions may be to modify the association between subunits so as to change the rate of conformational changes required for the different modes of function of the transporters.

**Possible Physiological Roles of Zn$^{2+}$ Modulation of Glutamate Transporters**

The concentrations of Zn$^{2+}$ required to modulate EAAT4 are within the reported concentration range of free extracellular Zn$^{2+}$ found in various regions of the brain. EAAT4 is expressed predominantly in Purkinje cells of the cerebellum, which also co-express EAAT3 (29,30). Whilst Zn$^{2+}$ is found in the cerebellum and numerous studies have investigated the effects of exogenously applied Zn$^{2+}$ on synaptic neurotransmission
in this region, the levels of free Zn$^{2+}$ found in regions accessible to the transporters under normal or pathological conditions are not well established (10). If present in sufficiently high concentrations, Zn$^{2+}$ would inhibit activation of the anion conductance of EAAT4, which may alter the excitability of the Purkinje cells. An alternative suggestion for the physiological role of Zn$^{2+}$ binding to glutamate transporters is that the functional consequences of the action (inhibition of transport or modulation of the chloride conductance) may not be of particular relevance and that the primary role of Zn$^{2+}$ binding to transporters may be to provide a mechanism to limit exposure of other synaptic proteins to the deleterious effects of Zn$^{2+}$ during pathological insults. In a number of brain regions glutamate transporters are expressed at very high levels in close proximity of the synapse (29,31,32), with one estimate being that the number of transporters in close proximity to the glutamate release site is greater than the number of glutamate molecules released from a single synaptic vesicle (32). Thus, when Zn$^{2+}$ concentrations are elevated, which may occur under various pathological conditions (33), transporters may provide a sink for excessive Zn$^{2+}$ and thereby limit exposure of other synaptic proteins, such as ionotropic glutamate receptors, which are unlikely to be present at the same density as the transporters. The binding of Zn$^{2+}$ to highly abundant glutamate transporters may also serve to limit the extent of Zn$^{2+}$ uptake, which can be excitotoxic to neurons (33).
Acknowledgements

We are grateful to Wendy Fairman, Dr Susan Amara and Dr Mark Connor for constructive critisms in the preparation of the manuscript and Dr Hue Tran, Suzanne Habjan and Kong Li for the maintenance of the *Xenopus laevis* colony and the isolation and preparation of oocytes.

References

Figures Legends

Figure 1. Alignment of the amino acid sequences of selected human and salamander glutamate transporters in the region of the putative Zn$^{2+}$ binding domain.
Figure 2. \( \text{Zn}^{2+} \) inhibits the substrate-activated conductance of EAAT4. Current voltage plots of substrate elicited conductances of EAAT4 measured in the presence of \( \text{Zn}^{2+} \) 
\( (I_{(100 \mu M \text{ substrate}+100 \mu M \text{ Zn}^{2+} \text{ in buffer})}-I_{(100 \mu M \text{ Zn}^{2+} \text{ in buffer})}) \) (circles) and of substrate alone 
\( (I_{(100 \mu M \text{ substrate in buffer})}-I_{(\text{buffer})}) \) (squares). L-aspartate elicited conductances (A) and L-glutamate elicited conductances (B) measured in standard ND96. The data presented in both A and B are normalized to the current elicited by 100 \( \mu M \) L-aspartate at \( \pm 100 \text{ mV} \) and represent mean currents \( \pm \text{ SEM} \) from 5 cells.

Figure 3. \( \text{Zn}^{2+} \) selectively inhibits the anion conductance of EAAT4. A. L-aspartate elicited conductances measured as in Figure 2, but in a buffer in which 96 mM NaCl was replaced with 96 mM NaI. The data represent the mean currents \( \pm \text{ SEM} \) from 4 cells and in for each cell the current measurements are normalized to the current due to 100 \( \mu M \) L-aspartate at 0 mV. B. The \% reduction in slope conductance (over the range 0 to +40 mV) due to 100 \( \mu M \) \( \text{Zn}^{2+} \) in which the 96 mM NaCl in the extracellular buffer was changed to 96 mM NaBr, NaI or NaNO$_3$. C. L-aspartate elicited conductances measured from oocytes that had been incubated in a chloride-free buffer (gluconate substituted for chloride) for >40 hours prior to recording. Recordings were then made in the same chloride-free buffer. Data represent the mean \( \pm \text{ SEM} \) current measurements from 5 cells.

Figure 4. \( \text{Zn}^{2+} \) is a non-competitive inhibitor of the anion conductance of EAAT4. A.
L-aspartate dose responses were measured in the presence of 100 μM Zn\textsuperscript{2+} (squares) and absence of Zn\textsuperscript{2+} (circles) and fit to the modified Michalis Menton equation (see methods). EC\textsubscript{50} values and % inhibition values are presented in Table 1.  

Zn\textsuperscript{2+} causes a dose dependent inhibition of L-aspartate elicited conductances in oocytes expressing EAAT4. 100 μM L-aspartate was co-applied with increasing doses of Zn\textsuperscript{2+} and the currents measured at −100 mV(squares) and +60 mV(circles). Current measurements were normalized to the current measured in absence of Zn\textsuperscript{2+} and fit to the modified Michaelis Menton equation for inhibition as described in the methods.  

C. IC\textsubscript{50} values for Zn\textsuperscript{2+} inhibition of the anion conductance are plotted for membrane potentials between −100 and +60 mV.

**Figure 5. Histidine mutations in EAAT4 at positions 154 and 164 abolish Zn\textsuperscript{2+} sensitivity of EAAT4.** Current – voltage plots for L-aspartate elicited responses in oocytes expressing the EAAT4 mutants H154A, H164A, H164E and C194A were measured in the absence of Zn\textsuperscript{2+} (I(100 μM L-aspartate in ND96)−I(ND96); squares) and the presence of Zn\textsuperscript{2+} (100 μM L-aspartate+100 μM Zn2+ in ND96) − I(100 μM Zn2+ in ND96); circles). Current measurements are normalized to the current elicited by L-aspartate at −100 mV. Data represent mean ± SEM.
Kinetic parameters for Zn\(^{2+}\) modulation of glutamate transporters EAAT4 and site-directed mutants

The kinetic parameters presented for EAAT4 and the EAAT4 mutants were calculated for L-aspartate elicited currents. The EC\(_{50}\) values are for L-aspartate dose responses measured at \(-100\ \text{mV}\). The IC\(_{50}\) values are for Zn\(^{2+}\) dose responses at the indicated membrane potentials and %Inhibition values are the maximal inhibition values at the indicated membrane potentials. Zn\(^{2+}\) did not change the reversal potential of the either the mutants or wild type transporters and the values obtained for the each of the mutants were not significantly different from wild type.

# EC\(_{50}\) calculated from transport current measurement made in the presence of 100 \(\mu\text{M}\) Zn\(^{2+}\).

* For the wild type EAAT4 and the C194A mutant the EC50 for L-aspartate activated conductances were significantly different measured in the presence and absence of 100 \(\mu\text{M}\) Zn\(^{2+}\)

… For the C194A mutant, the %inhibition is the value obtained for 100 \(\mu\text{M}\) Zn\(^{2+}\).

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<th>IC(_{50}) (+60mV) ((\mu\text{M}))</th>
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hEAAT2  HPGNPKLKKQLGPG
hEAAT3  KPGVT. QKVGEIAR
hEAAT4  HPGKGS. KEGLHRE
hEAAT5  HPGSAAQKETTEQS
sEAAT1  HPGKG. TEHMHREG
sEAAT5  HPGAAAQKEEHLGG

Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Zn^{2+} inhibits the anion conductance of the glutamate transporter EAAT4
Ann D. Mitrovic, Fiona Plesko and Robert J. Vandenberg

J. Biol. Chem. published online May 14, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M011318200

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