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Ca^{2+}-regulated expression of high affinity methylaminoisobutryic acid transport in hippocampal neurons inhibited by riluzole and novel neuroprotective aminothiazoles

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ABSTRACT

High affinity methylaminoisobutyric acid(MeAIB)/glutamine(Gln) transport activity regulated by neuronal firing occurs at the plasma membrane in mature rat hippocampal neuron-enriched cultures. Spontaneous Ca^{2+}-regulated transport activity was similarly inhibited by riluzole, a benzothiazole anticonvulsant agent, and by novel naphthalenyl substituted aminothiazole derivatives such as SKA-378. Here, we report that spontaneous transport activity is stimulated by 4-aminopyridine (4-AP) and that phorbol-myristate acetate (PMA) increases high K^+ stimulated transport activity that is inhibited by staurosporine. 4-AP-stimulated spontaneous and PMA-stimulated high K^+-induced transport is not present at 7 days *in vitro* (DIV) and is maximal by DIV~21. The relative affinity for MeAIB is similar for spontaneous and high K^+-stimulated transport ($K_m \sim 50$ μM) suggesting that a single transporter is involved. While riluzole and SKA-378 inhibit spontaneous transport with equal potency ($IC_{50} \sim 1$ μM), they exhibit decreased (~3-5 X) potency for 4-AP-stimulated spontaneous transport. Interestingly, high K^+-stimulated MeAIB transport displays lower and differential sensitivity to the two compounds. SKA-378-related halogenated derivatives of SKA-75 (SKA-219, SKA-377 and SKA-375) preferentially inhibit high K^+-induced expression of MeAIB transport activity at the plasma membrane ($IC_{50} < 25$ μM), compared to SKA-75 and riluzole ($IC_{50} > 100$ μM). Ca^{2+}-dependent spontaneous and high K^+-stimulated MeAIB transport activity is blocked by ω-conotoxin MVIIC, ω-agatoxin IVA, ω-agatoxin TK ($IC_{50} \sim 500$ nM) or cadmium ion ($IC_{50} \sim 20$ μM) demonstrating that P/Q-type Ca_V channels that are required for activity-regulated presynaptic vesicular glutamate (Glu) release are also required for high-affinity MeAIB transport expression at the plasma membrane. We suggest that neural activity driven and Ca^{2+} dependent trafficking of the high affinity MeAIB transporter to the plasma membrane is a unique target to understand mechanisms of Glu/Gln recycling in synapses and acute neuroprotection against excitotoxic presynaptic Glu induced neural injury.

1. Introduction

Spontaneous activity-regulated high affinity methylaminoisobutyric acid (MeAIB)/glutamine (Gln) transport activity that is potently inhibited by riluzole has been described in mature hippocampal neurons *in vitro* (Erickson, 2017). We previously screened two chemical libraries of riluzole-related compounds (Sankaranarayanan et al. 2009; Shim et al. 2019) and have identified naphthalenyl aminothiazoles derivatives that similarly block activity regulated spontaneous transport activity but are structurally unique (Kyllo et al. 2023). We then synthesized novel chlorinated naphthalenyl aminothiazole compounds that retained potency to inhibit this spontaneous MeAIB transport system in mature hippocampal neurons in dissociated cultures (Kyllo et al. 2023). Our purpose was to identify novel and potent compounds that target unique sites and that provide neuroprotection *in vivo*. We also previously showed that riluzole and novel chlorinated napthalenyl aminothiazole derivates such as SKA-378 that we have developed prevent acute hippocampal neural injury in the kainic acid model of temporal lobe epilepsy (TLE) (Kyllo et al. 2023). Riluzole is also neuroprotective in a number of other animal models (Malgouris et al. 1989; Pratt et al. 1992; Bae et al. 2000; Ruel et al. 2005; Heurteaux et al. 2006; Hunsberger et al. 2015; Pereira et al. 2016; Verma et al. 2016). Riluzole has multiple targets that might contribute to its neuroprotective properties with various degrees ($IC_{50} \sim 1$–500 μM) of potency. Riluzole's actions may

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include the inhibition of Na^+ channel activity (Herbert et al. 1994; Song et al. 1997; Stefani et al. 1997; Prakriya and Mennerick, 2000; Spadoni et al. 2002); K^+ and Ca^{2+} channel activity (Zona et al. 2002; Duprat et al. 2000; Huang et al. 1997) protein kinase activity (Noh et al. 2000), and Glu receptor activity (Debono et al. 1993; Zona et al. 2002). Riluzole has also been reported to stimulate small-conductance Ca^{2+}-activated K^+ channels (Sankaranarayanan et al. 2009; Grunnet et al. 2001) and the activity of synaptic Glu clearance transporters (Fumagalli et al. 2008) located on perisynaptic astrocytes that are critically important to resist direct Glu excitotoxicity (Sheldon and Robinson, 2007). The most potent activity of riluzole that has been reported is the inhibition of spontaneous activity-regulated, high affinity MeAIB/Gln transport in mature hippocampal neurons in this *in vitro* preparation with an IC_{50} ~1 μM (Erickson, 2017). We previously showed that riluzole and SKA-378 block spontaneous MeAIB transport activity non-competitively and therefore by an indirect mechanism (Kyllo et al., 2023). We previously showed that inhibition of the Na^+ channel subtype $Na_V1.6$ by 4-alphaTTX blocks activity regulated spontaneous MeAIB transport (Kyllo et al. 2023) and therefore $Na_V1.6$ activity likely drives neural activity-regulated spontaneous expression of the MeAIB transport activity described here in mature neurons. Voltage-gated Ca^{2+}-channels are also required for Ca^{2+}-dependent exocytosis of synaptic vesicles in axon terminals (Catterall, 2011) and both spontaneous and high K^+-induced MeAIB transport activity are dependent on extracellular Ca^{2+} ion for Ca^{2+} entry into the presynaptic nerve terminal (Erickson, 2017; Kyllo et al. 2023). The transport of MeAIB by the transporter itself does not depend on extracellular Ca^{2+} ion because it is removed from the Krebs buffer after high K^+ stimulation and then the ^{14}C-MeAIB uptake assay itself does not include Ca^{2+} ion (Erickson, 2017). Here, we show that the highest MeAIB transport activity (pmol/min) was observed after PMA-pretreatment and high K^+-stimulation of exocytosis of vesicular membrane transporter proteins to the plasma membrane. Our work suggests that novel aminothiazole derivatives such as SKA-378 may help to identify novel molecular targets underlying its acute neuroprotective properties (Kyllo et al., 2023).

Methylaminoisobutyric acid (MeAIB) is considered to be a selective substrate for a neutral Na^+ dependent amino acid transport system originally defined as system A that prefers alanine described years ago to distinguish it from system L exchange transport activity that prefers leucine in most cells (Christensen, 1990). System A transporters in the SLC38 gene family are called SNATs for Sodium-coupled Neutral Amino acid Transporters that enable Na^+-dependent concentration of substrate across the plasma membrane into most cells (Mackenzie and Erickson, 2004). SNAT1 and SNAT2 are low affinity transporters (K_m ~ 0.3–2 mM) (Varoqui et al. 2000; Yao et al. 2000; Reimer et al. 2000; Chaudhry et al. 2002b) and are preferentially expressed in neuronal cell bodies and proximal neuron dendrites in the brain (Mackenzie et al. 2003; Melone et al. 2004, 2006). Gln is the most abundant amino acid in the central spinal and ventricular fluids (Gjessing et al. 1972; McGale et al. 1977) and may also be the most abundant neutral amino acid substrate in extrasynaptic space (Kanamori & Ross, 2004, 2011, 2013). Gln is thought to be an important precursor for the synthesis of both Glu and γ-aminobutyric acid (GABA), the major excitatory and inhibitory neurotransmitters in the brain (Albrecht et al. 2007). Glu and GABA neurotransmission is thought to rely, in part, on Gln as the recycling of released neurotransmitter (*esp.* Glu) through astrocytes and conversion to Gln *via* a Glu/Gln shuttle has been reported in hippocampal and cortical neurons (Walls et al. 2015; Chaudhry et al. 2002a; Conti and Melone, 2006). The major pathway for clearance of Glu from the synaptic cleft after exocytoxic Glu release is *via* high affinity Glu transporters localized on perisynaptic astrocytes (Rothstein et al. 1996; Amara and Fontana, 2002; Sun et al. 2014). Glu is inactivated in astrocytes to Gln (Norenberg and Martinez-Hernandez, 1979) which then can be safely transferred to neurons and converted to Glu in axon terminals by phosphate-activated glutaminase (Kvamme et al. 2001; Masson et al. 2006). The mechanism of Gln transport into axon terminals for neurotransmitter synthesis is presently not known (Leke and Schousboe, 2016). Glu neurotransmitter biosynthesis can also occur by other precursors such as α-ketoglutarate and alanine or other tricarboxylic metabolic intermediates (Peng et al. 1993; Schousboe et al. 1997; Takeda et al. 2012) and GABA released from axons can be taken back up in axon terminals by high affinity GABA reuptake transporters (Deken et al. 2003). SNAT1 and SNAT2 have not been reported to exist in axon terminals (Mackenzie et al. 2003; Melone et al. 2004, 2006) and thus are unlikely to mediate Glu transmission directly. This suggests that an unidentified neuronal transporter mediates activity-regulated high affinity MeAIB/Gln transport in mature hippocampal neurons. Synaptic terminal expression of the machinery required for any direct influence on the presynaptic changes in Glu or GABA levels in the cytoplasm available for vesicular sequestration and exocytotic release is critical for presynaptic neurotransmission (Marx et al. 2015; Voglmaier and Edwards, 2007; Pietrancosta et al. 2020). Our results suggest that riluzole and SKA-378 are useful tools to dissect selective mechanisms involved in activity-stimulated cycling of high affinity MeAIB/Gln transport activity to the plasma membrane in mature hippocampal neurons.

2. Methods

2.1. Primary neuron-enriched hippocampal culture

Primary hippocampal neurons were prepared from embryonic day 19 Sprague Dawley rats of both sexes as described with minor modifications (Erickson, 2017; Kyllo et al. 2023). Pregnant dams were subjected to an overdose of compressed CO_2 administered in an anesthesia chamber and then decapitated using a guillotine. During dissection, the hippocampi from the embyros were collected in Hanks Balanced Salt Solution (without Ca^{2+}) containing 10 mM $MgCl_2$, 10 mM HEPES (pH 7.2), 0.5 mM Gln, penicillin (100 U/ml) and streptomycin (100 μg/ml) (pen/strep) on ice. Hippocampi were then incubated in oxygenated Neurobasal medium (NB) without B27 (Invitrogen) containing 0.25 mg/ml papain (Sigma-Aldrich) and 0.25 mg/ml cysteine for 18 min at 37 °C with shaking. The tissue was transferred to a 15 ml conical tube containing DMEM plus 10% (v/v) fetal bovine serum (FBS) and allowed to settle. The medium was removed and the tissue was dissociated in same medium (~1 ml) by trituration with fire-polished Pasteur pipettes, diluted in DMEM containing 10% (v/v) FBS, 0.5 mM glutamax, and 25 μM Glu, and then seeded in six-well Costar clusters (2 dissociated hippocampi/dish) precoated with 25 μg/ml poly-D-lysine (>300,000 MW, Sigma-Aldrich). Hippocampi from all rats were pooled and then subdivided accordingly. After 2 h at 37 °C, the medium was removed and replaced with NB medium supplemented with B27, 1% (v/v) FBS, 0.5 mM glutamax, 25 μM Glu, and pen/strep. Neurons were fed by addition of 1 ml of NB medium supplemented with B27, 0.5 mM glutamax and pen/strep (without FBS) twice a week for 3 weeks. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Spontaneous MeAIB transport assay in hippocampal neuronal culture

Uptake studies were performed using primary neuron-enriched hippocampal cultures at 19–21 days of maturation *in vitro* (DIV), unless the developmental expression was assessed (DIV 7 and DIV 14). Spontaneous ^{14}C-MeAIB (23.5 μM) transport in neural cultures was examined in the presence of 2.5 mM Ca^{2+} for 15 min as previously described (Erickson, 2017; Kyllo et al. 2023). Here, we examined whether 4-aminopyridine (4-AP) could enhance spontaneous ^{14}C-MeAIB transport activity. Cells were first rinsed in buffer (2 ml) containing 125 mM NaCl, 4.8 mM KCl, 25 mM HEPES (pH 7.4), 1.2 mM KH_2PO_4, 2.5 mM $MgSO_4$, 5.6 mM glucose, and 1 mM EGTA (N buffer) at room temperature. The medium was then replaced with same buffer but without EGTA and containing 2.5 mM $CaCl_2$ and pre-incubated for 5 min (± inhibitors) at 37 °C. For determination of the effect of the activator

4-aminopyridine (4-AP), this compound was added together with ^{14}C-MeAIB. For determination of the effect of inhibitors of excitatory transmission, the cultures were preincubated for 5 min with various concentrations of the inhibitor and then replaced with the same buffer (+inhibitor) containing ^{14}C-MeAIB. Untreated neuronal cultures and cultures treated with TTX (1 μM), verapamil (25 μM), 2,3-dioxo-6nitro-1,2,3,4-tetrahydrobenzo[f]-quinoxaline-7-sulfonamide (NBQX, 10 μM) together with 2-amino-5-phosphonopentanoic acid (D,L-AP5, 50 μM), GABA (2 mM), or uptake in the absence of Ca^{2+} ions were initially examined to isolate the neural activity-regulated MeAIB/Gln transport activity. Values at 4 °C were minimal (<1%). The n values indicated representative data from independent neuronal cultures.

2.3. High K^+-induced MeAIB transport activity in hippocampal neuronal culture

Uptake studies were performed using primary neuron-enriched hippocampal cultures at 19–21 DIV, unless the developmental expression was examined (DIV 7 and DIV 14). Here, we examined whether phorbol 12-myristate 13-acetate (PMA) could further enhance a brief high KCl (60 mM, 5 min) – stimulation of ^{14}C-MeAIB transport activity (Erickson, 2017). Cells were first rinsed in N buffer (2 ml) at room temperature. These cells were then bathed (37 °C, 5 min) in N buffer or spontaneous buffer described above that contains Ca^{2+} (2.5 mM) to allow for a brief pre-incubation of potential inhibitors (*e.g.,* staurosporine). Then, cultures were incubated (10 min, 37 °C) with N buffer, spontaneous buffer alone, or spontaneous buffer that contains PMA (50 nM) ± staurosporine (200 nM) or a PMA derivative (4-alpha PMA, 2 μM) that is inactive. After this period, the medium was removed and high KCl (60 mM) buffer (without EGTA) containing 2.5 mM $CaCl_2$ was added and the cultures incubated at 37 °C for 5 min. The solution was then removed and the cells rinsed in N buffer to replace the high KCl medium and chelate Ca^{2+} ions. Uptake studies were then performed immediately in N buffer (37 °C, 5 min) that contains 20 μM unlabeled MeAIB and 3.4 μM ^{14}C-MeAIB (PerkinElmer; 2 μl/ml/well). For competition analysis, the cultures were pretreated with riluzole or various novel aminothiazole derivatives in Ca^{2+}-containing spontaneous buffer (5 min) and then they were also added to the high K^+ (60 mM) medium. The direct assay for high K^+-induced transport activity here occurs in Ca^{2+}-free medium and so ^{14}C-MeAIB transport activity itself does not require Ca^{2+}. Values at 4 °C were minimal (<1%). The n values indicated representative data from independent neuronal cultures.

2.4. MeAIB transport inhibition by riluzole and aminothiazole derivatives of SKA-75

Riluzole, SKA-75 and various halogenated aminothiazole derivatives of SKA-75 (SKA-378, SKA-219, SKA-377 and SKA-375) were tested for their relative inhibitory potency (IC_{50}) in the four assays described above. We examined the potency to inhibit spontaneous *versus* 4-AP stimulated spontaneous ^{14}C-MeAIB transport activity over 15 min using six concentrations of these compounds. The highest concentration used represents ~90% inhibition of the transport activity in a 6-well tissue cluster: spontaneous alone (20–0.625 μM), except SKA75 (40–1.25 μM), and spontaneous +4-AP (40–1.25 μM). We also examined potency of these compounds to inhibit high K^+-induced transport activity (5 min assay) and the impact of a 10 min preincubation with PMA stimulation on high K^+-stimulated ^{14}C-MeAIB transport activity. The six concentrations of compounds used in the high K^+-stimulated assays were higher in order to represent 90% inhibition of the transport activity in a single 6-well plate as well. These include during PMA treatment only or only during high K^+ stimulation for all compounds (100–3.12 μM) except for SKA-75 and riluzole (400–12.5 μM). A 5 min pre-incubation period with drug (or buffer±Ca^{2+} alone) was also included in these experiments so as to determine the Ca^{2+}-regulated transport activity. Experiments conducted in the absence of Ca^{2+} served as negative controls and were subtracted.

2.5. Kinetic analysis

We measure the rate (K_m) and the total capacity (V_{max}) of the MeAIB transport system for high K^+-stimulated uptake by using the concentration range of unlabeled MeAIB (9.375, 18.75, 37.5, 75, 150, and 300 μM) added together with 3.4 μM ^{14}C-labeled MeAIB for uptake. The affinity and capacity of the spontaneous activity-regulated MeAIB transport system was recently reported (Kyllo et al. 2023). Transport activity in the absence of Ca^{2+} in these experiments is also measured at each MeAIB concentration to be subtracted from the total uptake values to isolate this Ca^{2+}-regulated transport activity. Thus, these measurements determine the participation of a Ca^{2+}regulated transporter for high affinity MeAIB uptake at the plasma membrane. The kinetic effect of SKA-378 was measured in the high K^+-stimulated transport assay using a concentration (25 μM) of SKA-378 that produced ½ maximal (V_{max}) inhibition of ^{14}C-MeAIB uptake. SKA-378 was present only during the high K^+ exposure period (with 5 min pretreatment, +Ca^{2+}) and then ^{14}C-MeAIB transport (5 min) is measured in Ca^{2+}-free medium after a brief rinse in N buffer.

2.6. CaV antagonists

We tested several Ca_V antagonists to verify that high K^+-stimulated MeAIB transport activity is dependent on presynaptic exocytotic Glu release. Verapamil and nifedipine were obtained from Sigma. All peptide antagonists (ω-conotoxin MVIIC, ω-conotoxin GVIA, ω-conotoxin SVIB, ω-agatoxin TK, ω-agatoxin IVA, and SNX-482) were from Alomone labs (Jerusalem, Israel). We performed concentration-response curves for verapamil and nifedipine (25–0.78 μM), and for ω-conotoxin MVIIC (2.5–0.078 μM) and Cd^{2+} (100–3.125 μM). We also examined the % inhibition values for ω-agatoxin TK and ω-agatoxin IVA at 500 nM, and ω-conotoxin GVIA, ω-conotoxin SVIB, SNX-482 at 1 μM, or nickel ion (Ni^{2+}) at 100 μM. The Ca_V inhibitors were added during a 5 min pretreatment in normal Krebs buffer and then during the high K^+ (60 mM) exposure during 5 min. The cultures were then rinsed in N buffer and uptake of ^{14}C-MeAIB (5 min) was then started in N buffer. Inhibition of spontaneous transport activity was also examined with a 5 min pretreatment followed by a 15 min period of ^{14}C-MeAIB uptake with MVIIC (1.25–0.039 μM) or Cd^{2+} (50–1.56 μM) in Ca^{2+}-containing (2.5 mM) Krebs buffer.

Following the uptake of ^{14}C-MeAIB under spontaneous or high K^+-stimulated transport conditions, the cultures were removed from the 37 °C incubator, placed on ice, and washed with ice-cold N buffer (2.5 ml). SDS (1%, 2 ml) was then added to solubilize the cells and the amount of radioactivity was determined by liquid scintillation counting using EcoScint (National Diagnostics).

2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism software version 7.0 (GraphPad Software, Inc). Active compounds were used for detailed structure activity relationship (SAR) by analyzing their concentration-response and inhibitory potency (IC_{50} values; ± SEM). Experimental values (n) were derived from independent neuronal culture preparations. Data were analyzed by repeated analysis of variance (ANOVA), with Dunnett's procedures to correct for multiple comparisons. A value of $p < 0.05$ was regarded as statistically significant.

3. Results

3.1. Novel functional properties of activity-regulated, high affinity MeAIB/Gln transport in mature hippocampal neuron-enriched dissociated culture

We here further evaluated the previously reported functional properties of activity-regulated spontaneous and K^+-stimulated MeAIB/Gln transport in neuron-enriched hippocampal cultures (Erickson, 2017; Kyllo et al. 2023). To determine if activity-regulated MeAIB transport in mature DIV 19–21 neuronal networks *in vitro* can be stimulated by means other than a brief 5 min exposure to high KCl (60 mM), and in a more physiological manner, 4-aminopyridine (4-AP) was utilized to increase spontaneous Ca^{2+}-dependent presynaptic neurotransmission. 4-AP is known to prevent neuronal repolarization *via* K^+ channel inhibition during spontaneous network activity prolonging depolarization and thereby stimulating neural activity. The likely target of 4-AP at the concentrations used here are the Kv1 family of K^+ channels, possibly the Kv1.5 subtype (Alvina and Khodakhah, 2010). Spontaneous and 4-AP-stimulated ^{14}C-MeAIB transport activity is performed in a Krebs based medium that contains Ca^{2+} (2.5 mM) for 15 min at 37 °C. 4-AP stimulates Ca^{2+}-regulated spontaneous ^{14}C-MeAIB transport activity (Fig. 1A). First, a concentration-response was performed to identify the optimal concentration of 4-AP to use and it was revealed that 200 μM maximally stimulated activity (Fig. 1A); doses up to 2 mM did not stimulate activity further (*not shown*). 4-AP stimulates spontaneous Ca^{2+}-regulated MeAIB transport in mature neurons by 1.6 ± 0.1 fold (n = 4–6) as spontaneous transport alone is (484 ± 27 pmol/well) and 4-AP stimulated is (781 ± 39 pmol/well). The effect of various agents that can block 4-AP stimulated neural activity is also shown in Fig. 1A. Na^+ channel blockade by tetrodotoxin (1 μM) or Ca^{2+} channel blockade by verapamil (25 μM) eliminate >90% of total MeAIB transport activity. The combination of NBQX (10 μM) and APV (50 μM) that blocks post-synaptic AMPA and NMDA Glu receptors similarly reduce activity. GABA, the major inhibitory neurotransmitter in hippocampal cultures, at 2 mM, also blocks transport activity to the same extent. Removal of Ca^{2+} ion likewise, blocks neural activity regulated high affinity MeAIB transport activity. Previously, it was reported that these treatments block spontaneous neural-activity regulated MeAIB transport activity (Erickson, 2017). Thus, spontaneous ^{14}C-MeAIB transport activity (±4-AP) is reduced >90% by agents that inhibit Glu-mediated spontaneous excitatory activity in these mature neuronal networks. Together, these results support the concept that spontaneous neuronal network activity in mature hippocampal dissociated cultures is required for expression of a presynaptic, Ca^{2+} regulated, high affinity MeAIB transporter at the plasma membrane.

To determine if high KCl (60 mM; 5 min) depolarization stimulated MeAIB transport activity (Erickson, 2017) can be further induced, the effects of PMA that is known to increase the availability of synaptic vesicles for depolarization-induced exocytosis in hippocampal neurons was examined (Fig. 1B). First, a concentration-response effect was performed to identify the optimal concentration of PMA to use and it was revealed that 50 nM maximally stimulated activity (Fig. 1B); doses up to 2 μM did not stimulate activity further (*not shown*). Pretreatment of mature neuronal cultures with PMA for 10 min was performed prior to the brief 5 min high KCl exposure and the direct assay for ^{14}C-MeAIB

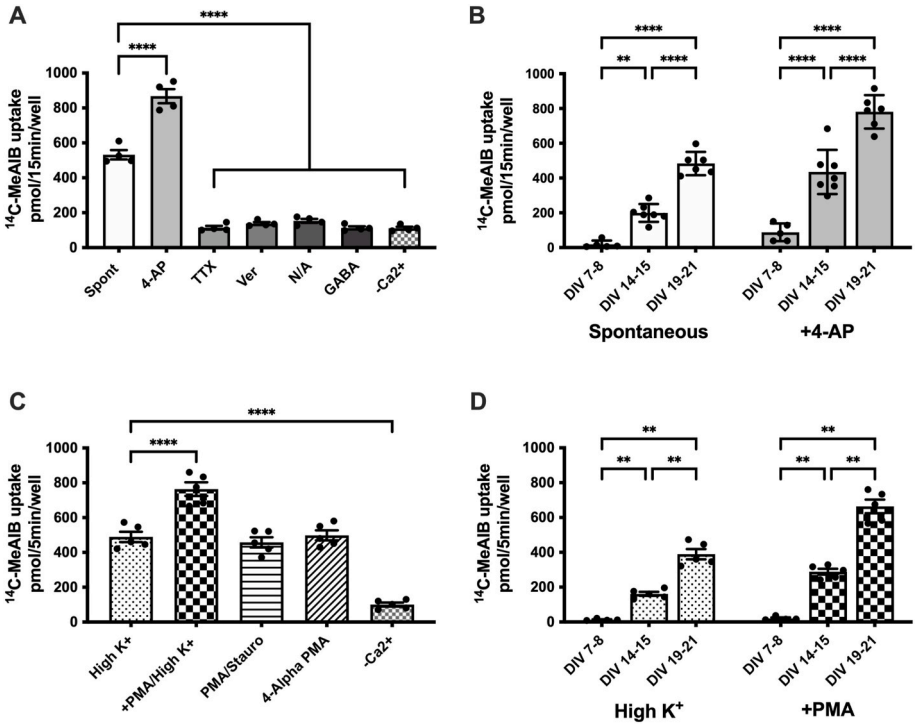

Fig. 1. *Spontaneous and high K $^+$ -stimulated expression of high affinity MeAIB transport activity at the plasma membrane in hippocampal neuronal cultures.* ***A,*** 4-AP stimulates spontaneous (Spont) transport activity (200 μM, 15 min) and is dependent on neural network activity: TTX (1 μM), Ver (25 μM), N/A – NBQX (10 μM)/AP5 (50 μM), GABA (2 mM). Spontaneous expression of transport activity requires external Ca^{2+} ions (-Ca^{2+}). Statistical values are compared to spontaneous transport activity alone. ***B,*** Developmental increase in 4-AP stimulated spontaneous transport is the same as spontaneous transport alone and occurs during the 2nd and 3rd post dissection (E19) weeks *in vitro*. Background values (-Ca^{2+}) were subtracted. ***C,*** Pretreatment with PMA (50 nM, 10 min) stimulates expression of transport activity after high K^+ exposure (60 mM, 5 min) that is selectively blocked by staurosporine (stauro, 200 nM). The inactive 4-alphaPMA does not stimulate transport. High K^+-expression of transport activity also requires external Ca^{2+} ion (-Ca^{2+}). Statistical values are compared to high K^+-stimulated transport alone. ***D,*** Developmental increase for PMA stimulation of transport activity after high K^+ exposure occurs is the same as high K^+ stimulation alone and occurs during the 2nd and 3rd post dissection weeks *in vitro*. Background values (-Ca^{2+}) were subtracted. A value of $p < 0.05$ is regarded as statistically significant (**p < 0.01, ****$p < 0.0001$).

transport was then performed in cultures after removal of high K^+ ion and Ca^{2+} ions (*see* Methods). PMA stimulates high KCl induced MeAIB transport in mature neurons by 1.7 ± 0.05 fold (n = 5) as high K^+ stimulation alone is 389 ± 30 pmol/well and PMA stimulated high K^+ induced levels are 664 ± 38 pmol/well. Treatment with staurosporine (200 nM), a general inhibitor of protein kinase activity, inhibits only the PMA-stimulated portion of transport activity (Fig. 1B); higher concentrations of staurosporine (2 μM) do not further block activity (*not shown*). The inactive PMA derivative 4-alpha PMA (2 μM) did not stimulate high K^+-stimulated MeAIB transport activity (Fig. 1B). Removal of Ca^{2+} ion prevents all MeAIB transport activity described here.

The expression of high-affinity spontaneous and high K^+-stimulated MeAIB/Gln transport is developmentally regulated in hippocampal neurons (Erickson, 2017). The developmental expression of 4-AP stimulated spontaneous ^{14}C-MeAIB transport activity and PMA-stimulated high KCl-depolarization induced transport activity was examined here also during the 2nd and 3rd weeks in neuron-enriched hippocampal cultures (Fig. 1C). Ca^{2+} regulated spontaneous MeAIB transport is minimal in DIV 7–8 cultures (18 ± 9 pmol, n = 5) but is greatly enhanced during the next two weeks with DIV 14–15 levels increasing (208 ± 14 pmol, n = 7) and maximal levels at DIV 19–21 (484 ± 27 pmol, n = 6). 4-AP stimulated spontaneous MeAIB transport activity is also minimal in DIV 7–8 cultures (88 ± 15 pmol, n = 5) but is greatly enhanced during the next two weeks with DIV 14–15 levels increasing (435 ± 48 pmol, n = 7) and maximal levels exhibited at DIV 19–21 (781 ± 39 pmol, n = 6). High K^+-induced MeAIB transport activity is also minimal in DIV 7–8 cultures (13 ± 3 pmol, n = 4) but is greatly enhanced during the next two weeks with DIV 14–16 levels increasing (162 ± 11 pmol, n = 4) and maximal levels exhibited at DIV 19–21 (389 ± 30 pmol, n = 5) (Fig. 1D). PMA-stimulation of high K^+-induced MeAIB transport activity is also minimal in DIV 7–8 cultures (21 ± 5 pmol, n = 4) but is greatly enhanced during the next two weeks with DIV 14–16 levels increasing (289 ± 17 pmol, n = 4) and maximal levels exhibited at DIV 19–21 (664 ± 38 pmol, n = 5) (Fig. 1D).

3.2. *Differential inhibition of neural activity regulated spontaneous and high K^+-stimulated MeAIB transport in mature hippocampal neurons by riluzole and SKA-378*

The potency of riluzole, SKA-75 and the several brominated and chlorinated derivatives of SKA-75 (Fig. 2) to inhibit spontaneous high-affinity ^{14}C-MeAIB transport was compared to the potency of these compounds to inhibit high K^+-stimulated ^{14}C-MeAIB transport activity in mature hippocampal neuron-enriched cultures (Fig. 3). Compounds were assessed during spontaneous transport or 4-AP stimulated spontaneous activity (15 min), and also during the high K^+ stimulation period (5 min) or just during the 10 min pre-incubation with PMA alone before high K^+-stimulation. The relative order of potency of all of these compounds to inhibit high-affinity MeAIB transport was the same in all four assays with spontaneous > 4-AP stimulated spontaneous > high K^+ ~ PMA-stimulated high K^+ transport (Fig. 3, Table 1). The potency of SKA-75 to inhibit both spontaneous and high K^+ stimulated transport activity is lower compared to the halogenated derivatives (Fig. 3, Table 1). The potency of SKA-378, SKA-219, SKA-377, and SKA-375 were the highest in all four assays described. The IC_{50} of SKA-378 to inhibit spontaneous transport alone did not differ from riluzole (~1 μM) (Fig. 3, Table 1). The potency of riluzole and all aminothiazole compounds examined to inhibit 4-AP stimulated spontaneous transport decreased by ~3-5X fold (Fig. 3, Table 1). Higher concentrations of SKA-75 and riluzole were needed to inhibit high K^+-stimulated transport (IC_{50} values ~100 μM) (Table 1) and when present only during the PMA treatment (10 min) riluzole and SKA-75 were even less potent (IC_{50} ~150 μM). Higher concentrations of SKA-378 and similar chlorinated (SKA-377) and brominated (SKA-219, SKA-375) derivatives were required to inhibit high K^+-stimulated transport but all of these halogenated aminothiazoles exhibited greatly reduced IC_{50} values compared to SKA-75 and riluzole (Table 1). The halogenated SKA-75 compounds retained their relative higher affinities (IC_{50} ~20 μM) when present only during the PMA treatment when compared to high K^+-stimulation alone (Fig. 3, Table 1). The potency of all compounds is decreased by K^+ depolarization stimulated transport activity induced by 4-AP or high K^+ treatment alone (Fig. 3, Table 1). We find that the chlorinated SKA-75 derivatives are similarly as potent as the brominated ones in all assays described here (Fig. 3, Table 1).

We have previously determined that riluzole and SKA-378 do not compete at the substrate binding site of the high-affinity MeAIB transporter because inhibition of spontaneous neural activity-regulated transport of MeAIB by these compounds could not be overcome by saturating (~300 μM) levels of cold MeAIB (Kyllo et al. 2023). We therefore determined whether the inhibition of high K^+-stimulation of MeAIB transport by SKA-378 is competitive with MeAIB or if inhibition occurs *via* an indirect mechanism, as well (Fig. 4). We included SKA-378 (25 μM) only during the high K^+ stimulation period (5 min), which is near its IC_{50} value for inhibition of high K^+-stimulated MeAIB transport activity (Table 1). We then determined the K_m and the V_{max} of MeAIB transport in Ca^{2+}-free medium (5 min) using MeAIB concentrations (303.4–12.775 μM) as described (*see* Methods). We find that SKA-378 is an indirect inhibitor of K^+-stimulated high-affinity MeAIB transport activity as the affinity (K_m) of the transporter for MeAIB is not affected

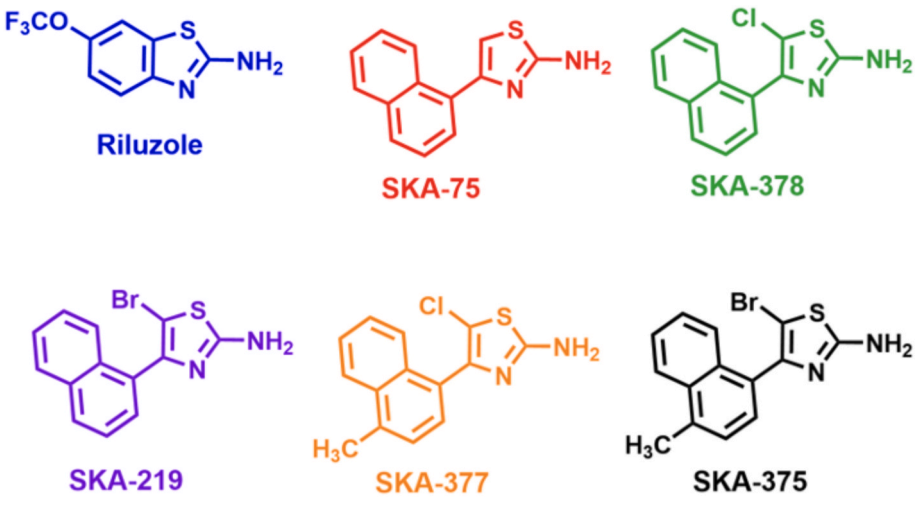

Fig. 2. Chemical structures of riluzole, SKA-75, SKA-378, SKA-219, SKA-377 and SKA-375.

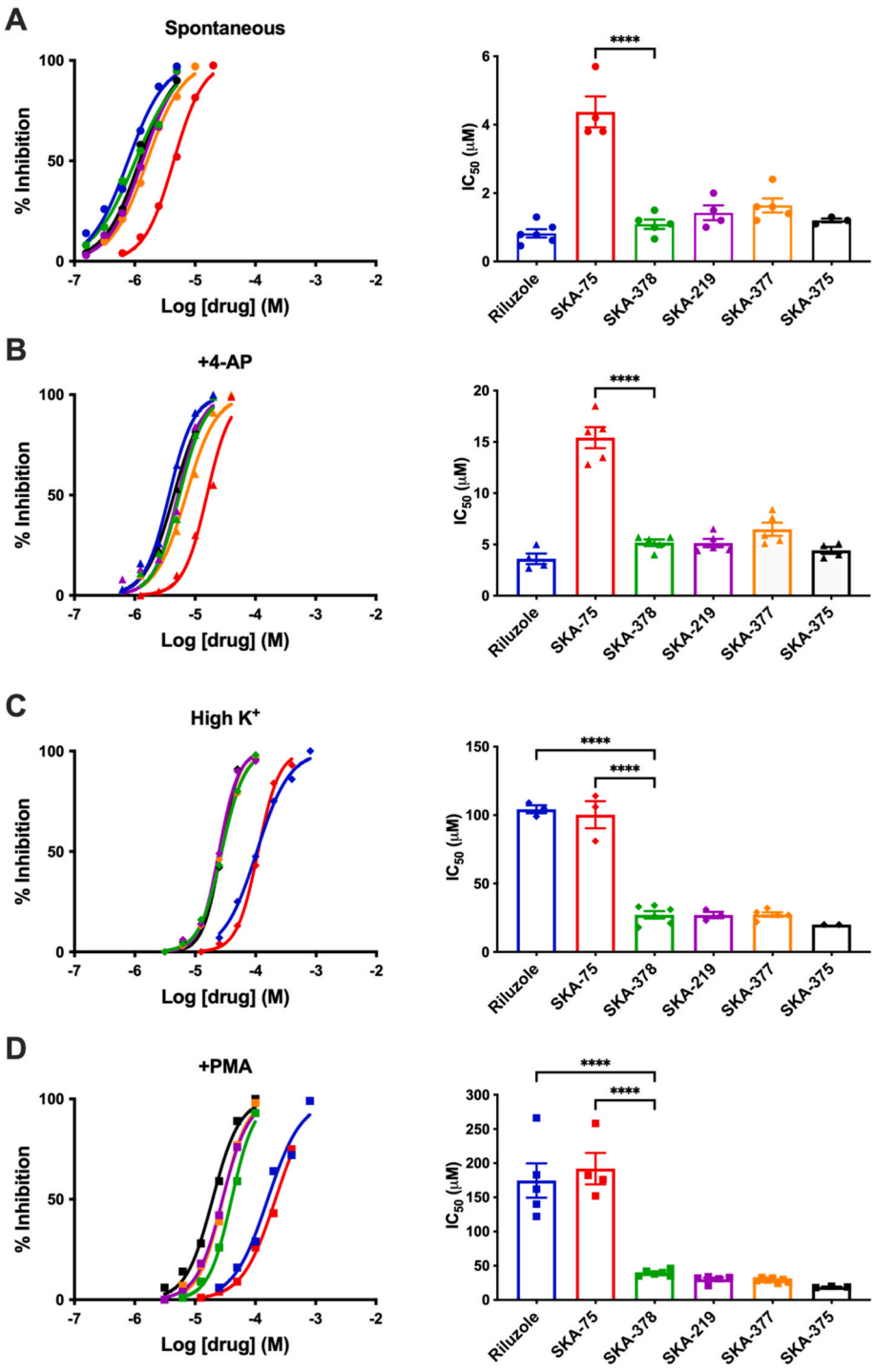

Fig. 3. *Potency of riluzole, SKA-75 and halogenated derivatives of SKA-75 on spontaneous and high K^+ expression of MeAIB transport activity. A,* Concentration response curves reveal that SKA-378 (*green*), SKA-219 (*purple*), SKA-377 (*orange*), and SKA-375 (*black*) similarly inhibit spontaneous high affinity MeAIB transport activity similar to riluzole (*blue*). The curve is shifted to the right for SKA-75 (*red*), yet the IC_{50} value is still in the low μM range. ***B,*** Concentration response curves for all compounds are shifted to the right in the presence of 4-AP (200 μM) with IC_{50} values 3-5X greater than for spontaneous transport alone. ***C,*** Concentration response curves for all compounds are further shifted to the right (5-50X) with PMA but now riluzole joins SKA-75 as least potent. ***D,*** Following high K^+-exposure and stimulation with PMA, concentration response curves show that all halogenated SKA-75 derivatives are more potent than SKA-75 or riluzole. All IC_{50} values in *A-D* are compared to SKA-378. A value of $p < 0.05$ is regarded as statistically significant (****$p < 0.0001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(control: 49 ± 5 μM, SKA-378: 53 ± 6 μM; n = 2) while the maximal velocity of MeAIB transport activity (V_{max},V) is reduced by ~50% in the presence of SKA-378 (Fig. 4). Our results indicate that halogenated derivatives of SKA-75 target a site more potently than riluzole that inhibits Ca^{2+}-dependent high K^+-stimulated expression of MeAIB transport activity to the plasma membrane in mature hippocampal neurons (see Fig. 5).

Table 1
IC_{50} values of riluzole, SKA-75 and novel active halogenated derivatives of SKA-75 on high affinity ^{14}C- MeAIB transport activity in mature hippocampal neurons.

Compounds	Spontaneous	Spontaneous +4-AP	High K^+-stimulated	PMA only
Riluzole	0.86 ± 0.02 (5)	3.6 ± 0.5 (4)	104 ± 2.9 (3)	152 ± 13 (5)
SKA-75	4.5 ± 0.45 (4)	15.4 ± 1.3 (4)	100 ± 10 (3)	170 ± 16 (4)
SKA-378	1.1 ± 0.17 (4)	5.2 ± 0.3 (5)	25 ± 2.7 (5)	32 ± 1.4 (6)
SKA-219	1.6 ± 0.23 (4)	5.1 ± 0.4 (5)	25 ± 2.3 (3)	30 ± 2.4 (5)
SKA-377	1.7 ± 0.26 (4)	6.4 ± 1 (5)	26 ± 1.6 (3)	29 ± 1.2 (7)
SKA-375	1.2 ± 0.09 (3)	4.4 ± 0.3 (4)	21 ± 1 (3)	19 ± 0.6 (3)

3.3. Inhibition of high K^+-stimulated and spontaneous MeAIB transport in mature hippocampal neuronal cultures by antagonists of voltage-gated Ca^{2+} channels (Ca_V)

We evaluated the Ca^{2+}-regulated mechanism driving high K^+ stimulation of high affinity MeAIB transport activity at the plasma membrane by including various inhibitors of voltage-gated Ca^{2+} channels (Ca_V) during the high KCl (60 mM, 5 min) exposure period (+5 min pretreatment in normal K^+ buffer (5 mM) containing 2.5 mM Ca^{2+}. These inhibitors included: verapamil (Ver), nifedipine (Nif), ω-conotoxin GVIA (GVIA), ω-conotoxin SVIB (SVIB), ω-conotoxin MVIIC (MVIIC), ω-agatoxin TK (TK), ω-agatoxin IVA (IVA), SNX-482 (482), nickel (Ni^{2+}) and cadmium (Cd^{2+}) ions. The most effective Ca_V inhibitor to block Ca^{2+}-dependent expression of high K^+-stimulated activity is the P/Q-type blocker MVIIC (IC_{50} = 551 ± 98 nM, n = 4). The P/Q blockers IVA and TK inhibited 65% and 53%, respectively of MeAIB transport activity at 500 nM. The L-type Ca_V antagonist verapamil (IC_{50} = 6 ± 1.3 μM, n = 3) and nifedipine (IC_{50} = ~12 μM, n = 2) were less potent to block high K^+-stimulated expression of the transport activity. The N-type antagonists ω-conotoxin GVIA and ω-conotoxin SVIB inhibited <15% at 1 μM. Cd^{2+} was an effective blocker (IC_{50} = 17 ± 3 μM, n = 5) (Fig. 4) but Ni^{2+} was ineffective at 100 μM (*not shown*). MVIIC (IC_{50} = 321 ± 96, n = 4) and Cd^{2+} (IC_{50} = 10 ± 4.6, n = 4) were also potent blockers of spontaneous neural activity-regulated MeAIB transport activity (Fig. 4). Other Ca^{2+} channel antagonists were not examined further in spontaneous transport assays. Our results indicate that Ca_V inhibitors that are known to inhibit Glu exocytotic release from vesicles in presynaptic excitatory neuronal synapses block the expression of high-affinity MeAIB transport activity to the plasma membrane.

4. Discussion

The *in vitro* preparations of hippocampal/cortical neurons used to study basic and developmental mechanisms of Glu and GABA neurotransmission maintain many of the important features of neural network activity, in general, as they develop a stable balance of excitatory *vs.* inhibitory transmission for neural circuit stability and neuronal survival. Neuronal maturation of excitatory/inhibitory presynaptic release of Glu and GABA in hippocampal circuits occurs after 3 weeks *in vitro* and after 3 postnatal weeks *in vivo* in rats because the major vesicular transporters that mediate presynaptic excitatory and inhibitory transmission in these neurons (*i.e.,* VGLUT1 and VGAT/VIAAT) are up-regulated during this period (Minelli et al. 2003a, 2003b; Boulland et al. 2004; De Gois et al. 2005), homeostatically regulated in mature neurons by long-term (>24h) changes in activity (Wilson et al. 2005; De Gois et al. 2005; Hartman et al. 2006; Wierenga et al. 2006, Han and Stevens, 2009; Erickson et al. 2006), and they are required to support presynaptic glutamate and GABA transmission (Takamori et al. 2000; Sudhof, 1995,

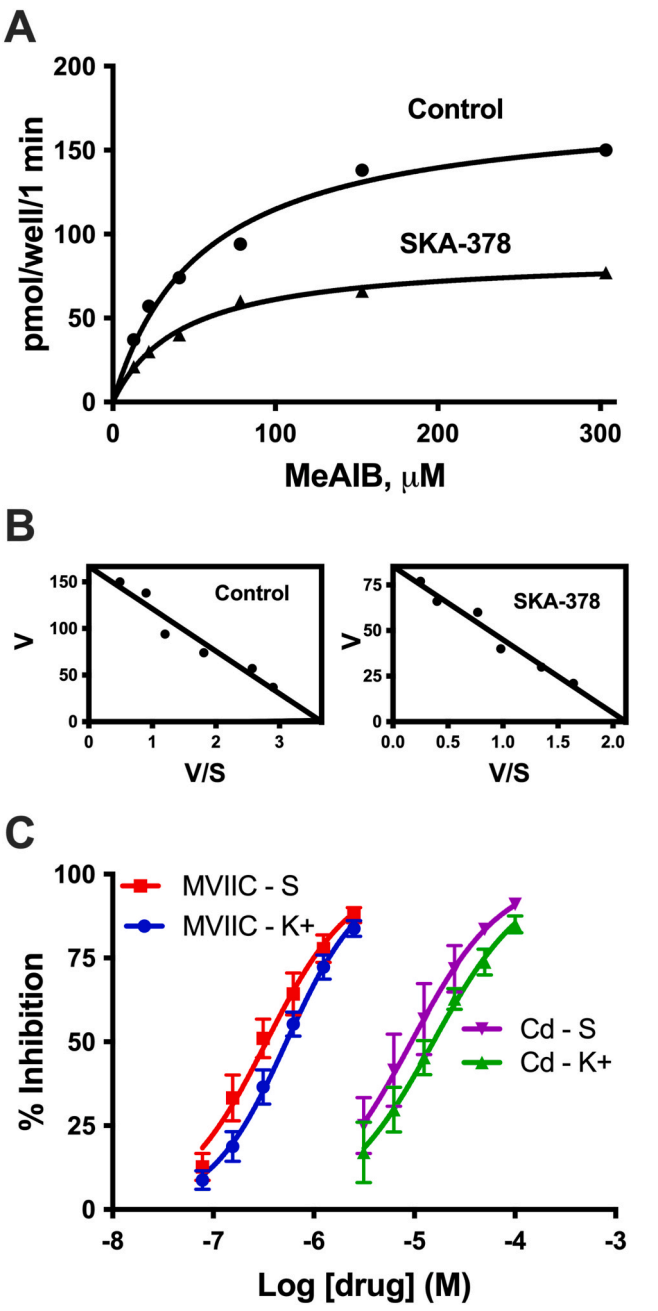

Fig. 4. Non-competitive inhibition by SKA-378 of Ca^{2+}-dependent high K^+ stimulation of high affinity MeAIB transport activity. ***A,*** Michaelis-Menten kinetics of MeAIB uptake in the presence of SKA-378 (25 μM). Background was measured in the absence of Ca^{2+} ions at each MeAIB concentration and was subtracted. Representative kinetic experiment is shown and was repeated twice. ***B,*** Eadie-Hofstee scatchard analysis shows that the -slope (K_m) is not affected by SKA-378 while the initial velocity (V, V_{max}) of transport is reduced by ~50% by SKA-378. ***C,*** Concentration response curves for inhibition of spontaneous and high K^+ stimulated transport by P/Q calcium channel (Ca_V) antagonists ω-conotoxin MVIIC (MVIIC) and cadmium ions (Cd^{2+}). MVIIC or Cd^{2+} were present only during the high K^+-depolarization period to block expression of MeAIB transport activity to the plasma membrane. Background uptake was determined by omitting Ca^{2+} ion during spontaneous uptake or during the high K^+-stimulation period and were subtracted. The potency of MVIIC to inhibit spontaneous *vs.* high K^+-stimulated transport was not statistically different (p > 0.99). The potency of Cd^{2+} to inhibit spontaneous *vs.* high K^+-stimulated transport was not statistically different (p = 0.27). Values are results from 3 independent neuronal cultures.

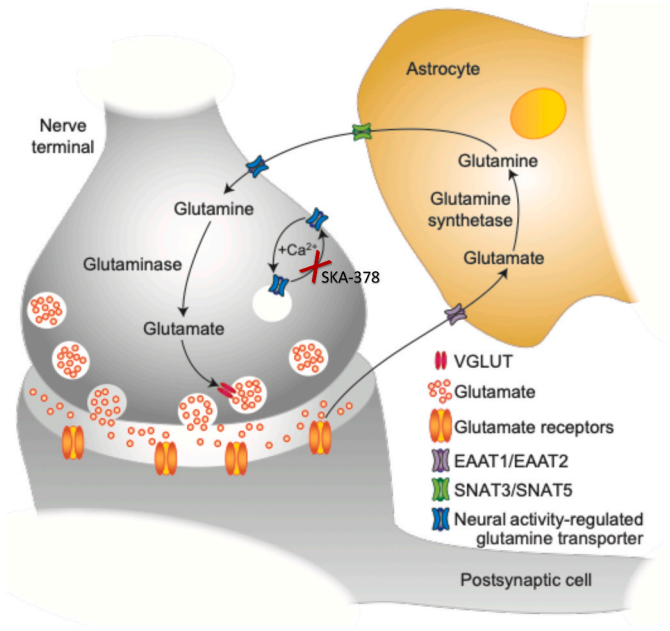

Fig. 5. Model of the Glu/Gln cycle between neurons and astrocytes that could recycle Glu for neurotransmission indicates where the neural activity regulated MeAIB/Gln transport system we describe further here operates (*blue*) that supports the concept of a Ca^{2+}-dependent pathway for cycling of this high affinity transporter under spontaneous and by high K^+ stimulation in synaptic terminals for exocytotic release. Our data indicate that SKA-378 targets an unidentified site (*X*) to inhibit Ca^{2+}-dependent exocytosis and recycling of MeAIB transport activity to the plasma membrane in hippocampal neurons >5X more potently than riluzole. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2012; Wojcik et al. 2006). Neurotransmitters and synaptic vesicles are recycled in nerve terminals in a coordinate and activity-regulated way, but the mechanisms of Glu recycling in excitatory synapses are still not clear.

A neural activity regulated Gln transport system in mature hippocampal neuronal cultures that displays relative high affinity for MeAIB has recently been described (Erickson, 2017) compared to the low affinity system A MeAIB neuronal transporters previously reported (Mackenzie and Erickson, 2004). Glu-mediated excitatory activity in mature hippocampal neuronal networks drives spontaneous and 4-AP stimulation of activity-regulated ^{14}C-MeAIB transport. Indeed, inclusion of GABA to increase inhibitory neurotransmission shuts down transport activity, altogether. Postsynaptic Glu receptor blockade (NBQX/AP5) also blocks Ca^{2+} regulated transport activity. Our results further confirm that a Ca^{2+} regulated high-affinity MeAIB transport system develops during the period of functional maturation of Glu neurotransmission and Glu/Gln recycling between hippocampal neurons and astrocytes. In spontaneous active cultures, Ca^{2+}-dependent MeAIB transport is blocked by inhibitors of presynaptic excitatory neurotransmission such as TTX and verapamil that inhibit voltage-gated Na^+ and Ca^{2+} channels, respectively. Brief high KCl exposure (60 mM) results in Ca^{2+} dependent exocytosis of most available synaptic vesicles in the releasable pool (Hamberger et al. 1979; Reubi, 1980). Extracellular Ca^{2+} ion is required for spontaneous neural activity-regulated MeAIB transport activity and is also required during the high K^+ stimulation period. The affinity of the transport activity for MeAIB (K_m ~ 50 μM) is roughly the same for spontaneous and high K^+-stimulated activity suggesting that a single transporter molecule is responsible for both spontaneous neural activity-regulated and high K^+-stimulated transport activities. While, the identity of the high affinity MeAIB transporter in hippocampal neurons is presently not known, spontaneous *versus* high K^+ stimulated MeAIB transport activity likely involves differential recycling mechanisms of intracellular vesicles that harbor the high-affinity MeAIB transporter protein for expression to the plasma membrane.

Kinetic analysis of SKA-378 and riluzole's blockade of neural activity-regulated spontaneous MeAIB transport in neurons *in vitro* indicates that inhibition occurs *via* a non-competitive, indirect mechanism (Kyllo et al. 2023). Here, we show that blockade of high K^+ induced MeAIB transport activity by SKA-378 is also non-competitive by Eadie Hofstee analysis of Michaelis-Menten kinetic data. We suggest that riluzole and SKA-378 target a site involved in the trafficking of the high affinity MeAIB transporter to the plasma membrane in axon terminals of hippocampal excitatory synapses. Spontaneous MeAIB transport relies on neural activity for expression that is dependent on the influx of external Ca^{2+} ions for presynaptic Glu release that drives excitatory network activity. High K^+-stimulated MeAIB transport also relies on extacellular Ca^{2+} influx during the high K^+-stimulation period for vesicle exocytosis of membrane proteins to the plasma membrane. However, the high-affinity MeAIB transporter itself is not dependent on Ca^{2+} ion as all experiments after high K^+-stimulation of exocytosis of vesicular membrane proteins to the plasma membrane are performed in Ca^{2+}-free (N) buffer. Interestingly, a brief (10 min) treatment with PMA stimulates high K^+-induced transport activity at the plasma membrane. We suspect that this is because PMA increases the availability of vesicles in the releasable pool of vesicles for high K^+-stimulated exocytotic Glu release from axon terminals (Stevens and Sullivan, 1998). The relationship between spontaneous and high K^+-induced MeAIB transport activity likely involves a single high-affinity MeAIB transporter that cycles between intracellular and plasma membrane compartments in a Ca^{2+}-regulated way. All halogenated derivatives of SKA-75 (SKA-378, SKA-219, SKA-377 and SKA-375) exhibit a ~5X increased potency to inhibit high K^+-induced MeAIB transport compared to riluzole and SKA-75. Our results suggest that SKA-378 targets a site that interferes with the Ca^{2+}-regulated exocytosis of vesicles that harbor the high-affinity MeAIB transporter more potently than riluzole. Recent studies suggest that riluzole attenuates the efficacy of Glu transmission by interfering with the size of the readily releasable Glu pool (Lazarevic et al. 2018). Riluzole inhibited PMA-stimulated exocytotic release of Glu from cortical neurons (IC_{50} ~ 100 μM) and this effect was correlated with the inhibition of protein kinase C-dependent Munc18-1 phosphorylation (Lazarevic et al. 2018), which is known to regulate synaptic vesicle recycling (Lai et al. 2007). PMA may also interact with other proteins involved in the exocytotic/endocytotic trafficking of the high-affinity MeAIB transporter in axon terminals. While it is not established yet that the high affinity MeAIB transporter resides on vesicles in presynaptic terminals, SKA-378 might act on a similar site as riluzole to block Glu release but with greater potency as described here. We believe that our novel halogenated aminothiazole derivatives of riluzole may interfere with pathways involved in the recycling pools of vesicles that reach the plasma membrane in a Ca^{2+}-dependent and neural activity-regulated way. The absolute dependence of high affinity MeAIB transport expression on the plasma membrane for Ca^{2+} suggests that voltage-gated Ca^{2+} channels are required.

Our evaluation of the Ca_V channels involved here indicates that P/Q-type Ca_V channels (Ca 2.2) that have been reported to be coupled to Glu release from presynaptic nerve terminals (Turner et al. 1992; Takahashi and Momiyama, 1993; Wheeler et al. 1994) contribute to the regulation of the level of this high-affinity MeAIB transporter protein at the plasma membrane for both spontaneous and high K^+-stimulated MeAIB transport activity. The P/Q-type Ca_V channel (Ca 2.2) antagonist ω-conotoxin MVIIC (Sato et al. 2000; Nimmrich and Gross, 2012) was an effective blocker (IC_{50} ~ 500 nM) of K^+-stimulation of MeAIB transport activity. K^+-induced release of Glu from brain synaptosomes can be blocked by ω-agatoxin-VIA (Turner et al. 1992), which is a blocker of P-type Ca^{2+} channels (Mintz et al. 1992a, 1992b). We find that ω-agatoxin-VIA and ω-agatoxin TK similarly block MeAIB transport activity at 500 nM. The L-type channel antagonist verapamil and nifedipine were much less

potent with IC_{50} values > 5 μM. The N-type (Ca_V 2.1) channel blocker ω-conotoxin-GVIA and ω-conotoxin SVIB were not effective at 1 μM. The R-type (Ca 2.3) Ca_V inhibitor SNX-482 (1 μM) and Ni^{2+} (100 μM) also had no effect. However, Cd^{2+} was an effective blocker (IC_{50} = 5 μM). MVIIC and Cd^{2+} similarly also block spontaneous activity-regulated MeAIB transport activity. Our results indicate that P/Q Ca_V channel activity significantly contributes to driving both neural activity-regulated spontaneous and high K^+ stimulated high affinity MeAIB transport activity at the plasma membrane in mature hippocampal synapses in dissociated culture.

In conclusion, the differential inhibition by riluzole and SKA-378 of spontaneous MeAIB transport activity and high K^+-stimulated MeAIB transport activity may help to dissect the mechanisms that are involved in acute neuroprotection in the hippocampus by SKA-378 and riluzole following kainic acid-induced seizure activity. Both riluzole and SKA-378 inhibit spontaneous neural-activity regulated transport activity more potently than high K^+-stimulated activity. While riluzole and SKA-378 are equipotent to inhibit spontaneous transport activity, SKA-378 is a ~5X more potent blocker of high K^+-stimulated MeAIB transport activity than riluzole. Kinetic analysis of the effects of these compounds on spontaneous and high K^+ assays indicates that they affect only the V_{max} of transport and therefore inhibit the MeAIB transport activity by indirect mechanisms; although these compounds could still act on the transporter at site other than the substrate recognition site. Previous results indicated that $Na_V1.6$ channel activity drives spontaneous neural-activity regulated MeAIB transport in hippocampal neurons (Kyllo et al. 2023). Here, we have shown that P/Q-type Ca_V channel activity drives both spontaneous transport activity and high K^+-stimulated MeAIB transport activity. The differential potencies of our compounds between spontaneous and high K^+ stimulation indicate that both riluzole and our novel aminothiazole derivatives such as SKA-378 should be further used as templates for compounds that specifically target sites important for neuroprotection in vulnerable hippocampal/limbic neurons. We suggest that neural activity driven Ca^{2+} dependent cycling of this high affinity MeAIB transporter from intracellular stores to the plasma membrane could be a unique target of our aminothiazole derivatives and tools to understand mechanisms that are involved in Glu/Gln recycling in synapses and that may be important to prevent acute neural injury in the hippocampus that occurs following epileptic seizure activity, and possibly other conditions of Ca^{2+}-regulated presynaptic excitotoxicity in limbic neural circuits in the brain.

CRediT authorship contribution statement

Jeffrey D. Erickson: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition. **Thomas Kyllo:** Formal analysis. **Heike Wulff:** Resources, Writing – review & editing.

Declaration of competing interest

The authors have no conflict of interests.

Data availability

Data will be made available on request.

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