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## Functional Identification of Activity-Regulated, High-Affinity Glutamine Transport in Hippocampal Neurons Inhibited by Riluzole

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### Abstract

Glutamine (Gln) is considered the preferred precursor for the neurotransmitter pool of glutamate (Glu), the major excitatory transmitter in the mammalian CNS. Here, an activity-regulated, high-affinity Gln transport system is described in developing and mature neuron-enriched hippocampal cultures that is potently inhibited by riluzole ( $IC_{50}$  1.3  $\pm$  0.5  $\mu$ M), an anti-glutamatergic drug, and is blocked by low concentrations of 2-(methylamino)isobutyrate (MeAIB), a system A transport inhibitor.  $K^+$ -stimulated MeAIB transport displays an affinity ( $K_m$ ) for MeAIB of 37  $\pm$  1.2  $\mu$ M, saturates at  $\sim$ 200  $\mu$ M, is dependent on extracellular  $Ca^{2+}$ , and is blocked by inhibition of voltage gated  $Ca^{2+}$ -channels (VGCCs). Spontaneous MeAIB transport is also dependent on extracellular  $Ca^{2+}$  and VGCCs, but is also blocked by the  $Na^+$  channel blocker tetrodotoxin, by Glu receptor antagonists, and by GABA indicating its dependence on intact neural circuits driven by endogenous glutamatergic activity. The transport of MeAIB itself does not rely on  $Ca^{2+}$ , but on  $Na^+$  ions, and is pH-sensitive. Activity-regulated, riluzole-sensitive spontaneous and  $K^+$ -stimulated transport is minimal at 7–8 days *in vitro* (DIV), coordinantly induced during the next two weeks, and is maximally expressed by DIV>20; the known period for maturation of the Glu/Gln cycle and regulated presynaptic Glu release. Competition analyses with various amino acids indicate that Gln is the most likely physiological substrate. Activity-regulated Gln/MeAIB transport is not observed in astrocytes. The functional identification of activity-regulated, high-affinity, riluzole-sensitive Gln/MeAIB transport in hippocampal neurons may have important ramifications in the neurobiology of activity stimulated presynaptic Glu release, the Glu/Gln cycle between astrocytes and neurons, and neuronal Glu-induced excitotoxicity.

### Graphical abstract

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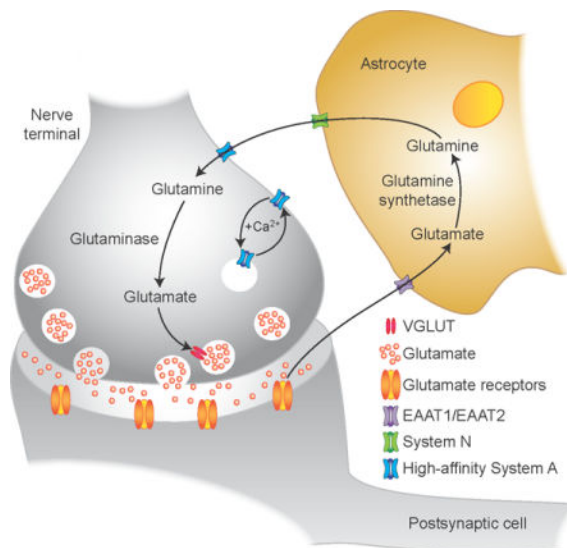
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**Author contribution:** J.D.E. conceived the study, performed the experiments, analyzed the data, and wrote the paper.



This report describes a  $\text{Ca}^{2+}$ -regulated ‘system A’ glutamine transport system in hippocampal neuron-enriched primary cultures that is dependent on neural activity in mature synapses, potentially inhibited by riluzole (a blocker of synaptic glutamate release), and that is up-regulated during the critical postnatal period of functional maturation of the glutamate/glutamine cycle between astrocytes and neurons and synaptic glutamate release. The novel high-affinity system A transporter described here may have physiological and pathological implications in understanding the neurobiology of excitotoxic synaptic glutamate release in acute and chronic neurodegenerative diseases.

## Keywords

glutamate/glutamine cycle; activity-dependent regulation; neurotransmitter cycling; neuronal glutamine transporter; excitotoxicity; neuroprotection

## INTRODUCTION

Excessive presynaptic Glu release is involved in several acute and chronic neurodegenerative diseases (Benveniste *et al.* 1984, Obrenovitch & Urenjak 1997, Puel *et al.* 1998, Dudek & Sutula 2007, Kostandy 2012, Dorsett *et al.* 2016). Riluzole is a benzothiazole anticonvulsant that was shown many years ago to be neuroprotective in numerous conditions of excessive synaptic Glu release including global and focal cerebral ischemia (Pratt *et al.* 1992, Doble 1996, Stutzmann 1997, Kanthasamy *et al.* 1999). Riluzole has received renewed attention as an anti-glutamatergic agent because of novel methods that increase its brain penetration (Verma *et al.* 2016), the reported beneficial effects of riluzole in animal models of Alzheimer’s disease (Hunsberger *et al.* 2015, Mokhtari *et al.* 2016, Pereira *et al.* 2016), and riluzole-derivatives with increased anti-convulsant properties (Coleman *et al.* 2015). Today, riluzole is registered under the trade name Rilutek for the treatment of amyotrophic lateral sclerosis (ALS). Riluzole exhibits various pharmacological activities, the most potent of which is inhibition neuronal  $\text{Na}^+$  channels at concentrations of 1–50  $\mu\text{M}$  (Benoit & Escande

1991, Herbert *et al.* 1994, Song *et al.* 1997, Stefani *et al.* 1997, Prakriya & Mennerick 2000, Spadoni *et al.* 2002), which would reduce neuronal circuit activity and Ca<sup>2+</sup>-dependent synaptic Glu release (Cheramy *et al.* 1992, Martin *et al.* 1993, Wang *et al.* 2004). Na<sup>+</sup>-dependent Gln import into synapses from astrocytes to replenish synaptic Glu stores released is a potential novel target to reduce excessive synaptic Glu release (Kanamori & Ross 2013, Tani *et al.* 2014, Marx *et al.* 2015). Direct functional evidence for activity-regulated Ca<sup>2+</sup>-dependent Gln transport in hippocampal neurons is, however, lacking.

The concept of Glu/Gln cycling for replenishment of neurotransmitter Glu pools originated from <sup>13</sup>C- and <sup>14</sup>C-NMR determinations *in vivo* (Sibson *et al.* 2001, Rae *et al.* 2003) and 'classic' biochemical experiments *in vitro* where it was shown that <sup>3</sup>H-Gln was a preferred precursor for synaptic Glu released in various neuronal preparations including the hippocampus (Hamberger *et al.* 1979, Reubi 1980). Activity-induced modulation of synaptic efficacy (Bacci *et al.* 2002, Armano *et al.* 2002) and glutamatergic epileptiform activity (Tani *et al.* 2010, Kanamori & Ross 2011) requires transport of Gln that is significantly reduced by inhibition of Gln synthesis in astrocytes or by application of MeAIB that blocks Gln import into neurons. MeAIB is a competitive and reversible inhibitor of the low affinity ( $K_m \sim 0.5$  mM) Na<sup>+</sup>-coupled neuronal Gln transporters SNAT1 and SNAT2 (Mackenzie & Erickson 2004). An activity-induced electrogenic Gln transporter has recently been reported that is inhibited by high concentrations (20 mM) of MeAIB (Billups *et al.* 2013) suggesting that activity-regulated Gln/MeAIB transport in the specialized calyx of Held synapse is mediated by SNAT1 or SNAT2 (Blot *et al.* 2009, Marx *et al.* 2015). While SNAT1 and SNAT2 are localized to CNS neurons they are not expressed in axon terminals in the cerebral cortex or hippocampus (Mackenzie *et al.* 2003, Conti & Melone 2006, Grewal *et al.* 2009, Jenstad *et al.* 2009), suggesting that an unidentified neuronal Gln/MeAIB transporter supports Glu transmission in the hippocampus.

Here, the hypothesis that a novel, high-affinity, Ca<sup>2+</sup>-regulated Gln/MeAIB transport system exists in hippocampal neurons is tested; such activity should be optimally regulatable by neuronal activity under physiologic levels of Gln (~30 μM) reported in extrasynaptic space (Kanamori & Ross 2013). I report that high-affinity, activity-regulated, Ca<sup>2+</sup>-dependent Gln/MeAIB transport exists in hippocampal neurons and that it is inhibited by riluzole, a drug that reduces synaptic release of Glu and Glu-induced excitotoxicity *in vitro* and *in vivo*. I also report that both spontaneous and K<sup>+</sup> depolarization-stimulated Gln/MeAIB transport are dramatically induced between 8 and 20 days *in vitro* (DIV), which is coordinately regulated with the postnatal maturation of the Glu/Gln cycle (Chowdhury *et al.* 2007, Hertz 2013) and synaptic vesicular Glu release in cortical and hippocampal neurons *in vitro* and *in vivo* (Bolshakov & Siegelbaum 1995, Wasling *et al.* 2004, Wilson *et al.* 2005, De Gois *et al.* 2005). The identification of activity-regulated, riluzole-sensitive, high-affinity neuronal Gln/MeAIB transport emphasizes the importance of this potential therapeutic target in conditions of excessive synaptic release of Glu in hippocampal neurons during development, in mature synapses, and in the aged. A preliminary report of these results has recently appeared (Erickson 2016)

## Materials & Methods

### Cell culture

Primary hippocampal neurons were prepared from embryonic day 19 Sprague Dawley rats (Envigo, Indianapolis, Indiana.RRID:RGD\_5508397) of both sexes (Brewer *et al.* 1993). All animal procedures were approved by the Institutional Animal Care and Use Committee at LSUHSC, and complied with the NIH guidelines. Pregnant dams were subjected to an overdose of inhalation of compressed CO<sub>2</sub> administered in an anesthesia chamber and then decapitated using a guillotine. All measures were taken to minimize pain, discomfort, or suffering of animals. Briefly, during dissection the hippocampi were collected in Hanks Balanced Salt Solution (without Ca<sup>2+</sup>) containing 10 mM MgCl<sub>2</sub>, 10 mM HEPES (pH 7.2), 0.2 mM glutamine, penicillin (100 U/ml) and streptomycin (100 mg/ml) (pen/strep) at 4°C. Hippocampi were then incubated in oxygenated Neurobasal medium (NB) without B27 (Invitrogen, Gaithersburg, MD) containing 0.1 mg/ml papain (Sigma, St. Louis, MO) and 0.2 mg/ml cysteine for 15 min at 37°C with shaking. The tissue was transferred to a 15 ml conical tube and allowed to settle and the medium then replaced twice with DMEM containing 10% (v/v) FBS. The tissue was then dissociated by trituration with fire-polished Pasteur pipettes in DMEM containing FBS, the cells were counted, diluted in DMEM containing 10% (v/v) FBS, 0.5 mM glutamax, and 25 µM Glu, and then seeded in six-well dishes (4 X 10<sup>5</sup> cells/well) precoated with 25 µg/ml poly-D-lysine (>300,000 MW, Sigma). 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. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> with the addition of 1 ml of NB medium supplemented with B27, 0.5 mM glutamax and pen/strep every third day of the first week, and every other day thereafter.

Astrocyte-enriched cultures were also prepared as secondary cultures from same plating of hippocampal cells as above, except that the cells were maintained in DMEM containing 10% FBS, instead of NB medium. In addition, after one week the cells were trypsinized to remove them from the bottom of the flask, and then split 1/10 in DMEM containing 10% FBS and transferred to a new flask. Once confluent, these cells were again split 1/10 in DMEM containing FBS and allowed to reach confluency. Cells were then dispersed (4 X 10<sup>5</sup>/well) in poly-D-lysine coated 6-well clusters for analysis. Astrocytes were treated with high K<sup>+</sup> depolarization (+/- Ca<sup>2+</sup>) and transport of <sup>14</sup>C-MeAIB (20 µM, 5 min) at 37°C was measured.

### Uptake studies

Uptake studies were performed using primary neuron-enriched hippocampal cultures at various days of maturation *in vitro* (DIV). Briefly, cells (most studies were performed at DIV 14) were first rinsed in buffer (3 ml) containing 125 mM NaCl, 4.8 mM KCl, 25 mM HEPES (pH 7.4), 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 2.5 mM MgSO<sub>4</sub>, 5.6 mM glucose, and 1 mM EGTA (N buffer), and then K<sup>+</sup>-stimulated (5 min) or spontaneous uptake experiments were performed. Following K<sup>+</sup>-stimulated or spontaneous Gln/MeAIB uptake the dishes were removed from the 37°C incubator and placed on ice, and the cells were then 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 determined by liquid scintillation counting (EcoScint, Nat. Diag.). Various controls (4°C, K<sup>+</sup> 4.8 mM, no Ca<sup>2+</sup> +EGTA, +MeAIB 5 mM) were appropriately included with each experiment as indicated.

For K<sup>+</sup>-stimulated uptake experiments, N buffer-rinsed cells were bathed (37° C, 5 min) in *a*) N buffer, *b*) N buffer (without EGTA) containing 1.2 mM CaCl<sub>2</sub>, *c*) high K<sup>+</sup> buffer containing 69.8 mM NaCl, 60 mM KCl, 25 mM HEPES (pH 7.4), 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 2.5 mM MgSO<sub>4</sub>, 5.6 mM glucose, and 1 mM EGTA, or *d*) high K<sup>+</sup>-buffer (without EGTA) containing 1.2 mM CaCl<sub>2</sub>. The solutions were removed and the cells rinsed in N buffer to chelate Ca<sup>2+</sup>. Uptake studies were then performed immediately in N buffer (37° C, 5 min) that contains 20 μM unlabeled Gln or MeAIB spiked with <sup>3</sup>H-Gln or <sup>14</sup>C-MeAIB (Perkin-Elmer; 2 μl/well), respectively. For kinetic analysis, various concentrations of unlabeled MeAIB (3.9 μM – 500 μM) were added and uptake was terminated at 3 min. Various amino acid substrate competitors (2 mM) were added directly to the radiolabeled <sup>14</sup>C-MeAIB solution and uptake was performed for 5 min. Amino acids tested included: alanine (ala), proline (pro), sarcosine, histidine (his), MeAIB, Gln, leucine (leu), glycine (gly), serine (ser), N,N'-dimethylglycine (N'-DMG), betaine, aminoisobutyric acid (AIB), 2-amino-2-norbornane-carboxylic acid (BCH), D-serine, arginine (arg), cyclo-D-serine, D-aspartate (D-asp), taurine, and γ-aminobutyric acid (GABA). In some experiments verapamil (ver, 20 μM) or tetrodotoxin (TTX, 1 μM) were present during K<sup>+</sup>-stimulation, but they were not present during the 5 min uptake period.

For spontaneous uptake experiments N buffer-rinsed cells were bathed (37° C, 5 or 15 min) in *a*) N buffer or *b*) N buffer (without EGTA, with 1.2 mM CaCl<sub>2</sub>) containing 20 μM unlabeled MeAIB spiked with <sup>14</sup>C-MeAIB (2 μL/well). Cells were pretreated with vehicle (DMSO or water) for control values or with verapamil (20 μM), α-methyl-4-carboxyphenylglycine (MCPG, 1 mM), 2-amino-5-phosphonopentanoic acid (AP5, 50 μM) together with 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]-quinoxaline-7-sulfonamide (NBQX, 10 μM), or TTX (1 μM) 2 min prior to addition of <sup>14</sup>C-MeAIB and these drugs were present during the uptake period. Riluzole (0.039 – 20 μM) and phenytoin (1.56 – 200 μM) dose-response inhibition experiments were performed by pre-incubating cells in Ca<sup>2+</sup>-containing medium with various concentrations of inhibitors (2 min) and then replacing the medium with the same medium (+ drug) containing 20 μM MeAIB.

## Data analysis

Uptake of radioactive amino acids was independently measured in culture wells and the final values were derived from at least three independent hippocampal neuronal preparations. Scintillation counts were converted into molar quantities by calculation of specific activity (dpm/pmol) of the radiolabeled/unlabeled Gln or MeAIB mixture used. Error bars represent the means +/- S.E. or means +/- S.D. (for kinetics) of the average values from independent experiments. Comparisons of mean values from 3–6 independent neuronal preparations were done by one-way ANOVA with pairwise comparisons between groups adjusted with Tukey-Kramer posthoc analysis. All nonlinear regression analyses were performed in Prism7 (GraphPad Software Inc. La Jolla, CA).

## RESULTS

### **K<sup>+</sup>-stimulated, Ca<sup>2+</sup>-dependent Gln transport exists in hippocampal neurons**

High K<sup>+</sup>-depolarization (56 mM) of axon terminals was the original biochemical method used to show that Gln was a preferred precursor for the Ca<sup>2+</sup>-dependent releasable pool of Glu from hippocampal neurons. An important question that still remains is whether such synaptic activation induces Ca<sup>2+</sup>-dependent Gln transport activity in hippocampal neurons? To examine this question neuron-enriched hippocampal primary cultures were subjected to brief (5 min) high K<sup>+</sup> depolarization (60 mM) in Ca<sup>2+</sup>-containing Krebs-Ringer solution, washed in Ca<sup>2+</sup>-free Krebs-Ringer solution containing EGTA (N buffer), and then <sup>3</sup>H-Gln (20 μM) transport was measured in N buffer over 5 min. Uptake in the absence of Ca<sup>2+</sup> or at 4°C was also examined for specificity and to control for non-specific effects. Low concentrations of Gln were used to minimize transport from low affinity neuronal Gln transporters, such as SNAT1 and SNAT2. We also examined a selective subpopulation of Gln transporters sensitive to inhibition by MeAIB (5 mM). MeAIB-inhibitable <sup>3</sup>H-Gln transport at low concentrations of Gln (20 μM) in the absence of Ca<sup>2+</sup> is low (<5% of total <sup>3</sup>H-Gln uptake) under Ca<sup>2+</sup>-free spontaneous conditions. However, MeAIB inhibitable and Ca<sup>2+</sup>-dependent <sup>3</sup>H-Gln uptake increases approximately 25% following a brief exposure to K<sup>+</sup>-depolarization (Fig. 1). MeAIB is a transported substrate of the 'classic' system A transporters (Christensen 1990) and is not metabolized, which renders it more stable than Gln. Therefore, <sup>14</sup>C-MeAIB (20 μM) was used in all subsequent experiments to identify, and characterize, a subpopulation of Gln/MeAIB transporters that relies on extracellular Ca<sup>2+</sup> ions for functional expression in a whole cell assay and that is stimulated by K<sup>+</sup>-depolarization.

### **K<sup>+</sup>-stimulated, Ca<sup>2+</sup>-dependent MeAIB transport exists in hippocampal neurons**

Here, the identification and characterization of K<sup>+</sup>-stimulated, Ca<sup>2+</sup>-dependent <sup>14</sup>C-MeAIB transport activity in primary cultures of hippocampal neurons is reported. K<sup>+</sup>-stimulated <sup>14</sup>C-MeAIB transport was measured in Ca<sup>2+</sup>-free Krebs buffer (N buffer, pH 7.4) in cells that have had brief (5 min) prior exposure to K<sup>+</sup>-depolarizing conditions (60 mM) in the presence or absence of Ca<sup>2+</sup>, and the cells are then rinsed in N buffer to remove Ca<sup>2+</sup>, which is also required for endocytosis (Hosoi *et al.* 2009). Under these conditions Ca<sup>2+</sup>-dependent <sup>14</sup>C-MeAIB transport increased ~10-fold by K<sup>+</sup>-depolarization (Fig. 2). K<sup>+</sup>-stimulated <sup>14</sup>C-MeAIB transport is eliminated by omission of exogenous Ca<sup>2+</sup> (+EGTA) or by inclusion of verapamil (20 μM), an inhibitor of P-type voltage-gated Ca<sup>2+</sup> channels, during the period of K<sup>+</sup>-depolarization (Fig. 2). The transport process itself is not dependent on Ca<sup>2+</sup> because <sup>14</sup>C-MeAIB uptake occurs in N buffer, which does not contain Ca<sup>2+</sup> (N buffer also contains EGTA to chelate residual Ca<sup>2+</sup> ions). Direct high K<sup>+</sup>-depolarization of axon terminals bypasses any dependence on intact neuronal circuitry; hence, K<sup>+</sup>-induced <sup>14</sup>C-MeAIB transport is not affected by tetrodotoxin (TTX, 1 μM) present during K<sup>+</sup>-stimulation. However, the transport process itself is dependent on the presence of Na<sup>+</sup>, as K<sup>+</sup>-stimulated Ca<sup>2+</sup>-dependent <sup>14</sup>C-MeAIB transport is not observed when Li<sup>+</sup> ions are substituted for Na<sup>+</sup> in the uptake buffer (Fig. 3). The dependence of <sup>14</sup>C-MeAIB transport on the pH of the uptake buffer was also examined and found to be modestly increased at pH 8.2 and decreased at pH 6.0 (Fig. 3).



Kinetics of  $K^+$ -induced,  $Ca^{2+}$ -dependent  $^{14}C$ -MeAIB transport activity differentiate this neuronal transport system from SNAT1- or SNAT2-mediated activity, which display much lower affinity ( $K_m \sim 0.5$  mM).  $K^+$ -induced,  $Ca^{2+}$ -dependent MeAIB transport in neurons displays an affinity ( $K_m$ ) for MeAIB of  $37 \pm 1.2$   $\mu$ M and saturates at 200  $\mu$ M (Fig. 4). The relative ability of various amino acid substrates at 2 mM to block  $K^+$ -induced/ $Ca^{2+}$ -dependent  $^{14}C$ -MeAIB transport, which is indicative of relative substrate affinity of the transporter, is shown in Fig. 5. Amino acids tested include alanine, proline, sarcosine, histidine, MeAIB, Gln, leucine, glycine, serine, N,N dimethylglycine (N,N-DMG), betaine, aminoisobutyric acid (AIB), 2-amino-2-norbornanecarboxylic acid (BCH), D-serine, arginine, cyclo-D-serine, D-aspartic acid, taurine, and  $\gamma$ -aminobutyric acid (GABA). The preferred substrate competitors alanine, proline, sarcosine, histidine, MeAIB and Gln displayed greater than 90% inhibition of  $^{14}C$ -MeAIB transport when present at 2 mM concentration (Fig. 5). Taurine, GABA, cyclo-D-serine, and D-aspartic acid do not interfere with  $K^+$ -stimulated transport of MeAIB. Activity-induced,  $Ca^{2+}$ -dependent MeAIB transport is not observed in cultured hippocampal astrocytes (Fig. 2). High  $K^+$  was used here to maximally depolarize axon terminals, which is reasonable for the initial screen to identify the substrates and inhibitors and kinetically characterize this novel high-affinity  $Ca^{2+}$ -dependent Gln/MeAIB transport system in primary hippocampal neurons.

### Spontaneous $Ca^{2+}$ -regulated MeAIB transport exists in hippocampal neurons

High  $K^+$ -stimulation (60mM) of axon terminals is not physiological. Does activity-regulated,  $Ca^{2+}$ -dependent Gln/MeAIB transport spontaneously exist under physiologic conditions in neuronal hippocampal cultures? To examine this question, neurons were incubated under physiological conditions in Krebs buffer with  $^{14}C$ -MeAIB (20  $\mu$ M; with or without  $Ca^{2+}$ ). Spontaneous transport of low concentrations of MeAIB requires exogenous  $Ca^{2+}$  (1.2 mM) and is blocked by inhibitors of VCGGs (verapamil 20  $\mu$ M) (Fig. 2, Fig. 6), like  $K^+$  stimulated Gln/MeAIB transport (Fig 1, 2). Spontaneous,  $Ca^{2+}$ -dependent  $^{14}C$ -MeAIB transport is blocked by TTX (1  $\mu$ M), an inhibitor of  $Na^+$  channels that are required for action-potential mediated  $Ca^{2+}$ -stimulated exocytosis of Glu and neural circuit activity (Fig. 6), unlike  $K^+$  stimulated transport. Spontaneous transport does not occur with  $Li^+$  ions substituted for  $Na^+$  (*not shown*). Transport is dependent on excitatory-driven neural circuit activity because it is blocked by inhibitors of AMPA and NMDA Glu receptors that mediate postsynaptic Glu transmission (Fig. 6). Spontaneous transport is not affected by MCPG (Fig. 6), a group I/II metabotropic Glu receptor inhibitor. Spontaneous MeAIB transport is inhibited by high concentrations (2 mM) of GABA (Fig. 6), which activates GABA receptors to increase inhibitory transmission and shunt Glu-driven neural circuit activity. Together, these results indicate that a  $Ca^{2+}$ -dependent Gln/MeAIB transporter exists spontaneously in hippocampal neuronal cultures and requires  $Na^+$ - and  $Ca^{2+}$ -dependent Glu transmission for functional activity.

Alanine, proline, sarcosine, histidine, MeAIB and Gln display greater than 90% inhibition of  $^{14}C$ -MeAIB transport when present at 2 mM concentration (Fig. 6), which indicates that they are also preferred potential substrates for spontaneous  $Ca^{2+}$ -dependent  $^{14}C$ -MeAIB transport, like they are for  $K^+$ -stimulated transport. Gln is present at much greater levels in extrasynaptic space *in vivo*, compared to all other amino acids. Taurine, an osmolyte and

regulator of  $\text{Ca}^{2+}$  homeostasis in neurons, does not interfere with  $^{14}\text{C}$ -MeAIB transport (Fig. 6). Together these results indicate that a  $\text{Ca}^{2+}$ -regulated, Gln-sensitive MeAIB transporter exists spontaneously in primary neuron-enriched hippocampal cultures.

### **Spontaneous, $\text{Ca}^{2+}$ -regulated MeAIB transport is potently inhibited by riluzole, a neuroprotective agent**

Riluzole is neuroprotective in conditions of excessive synaptic Glu release because it inhibits Glu release from synapses by unclear mechanisms. Does riluzole inhibit activity-regulated Gln/MeAIB import into hippocampal neurons? To examine this question, neurons were pre-incubated with riluzole or other potential anti-glutamatergic agents for 2 min in  $\text{Ca}^{2+}$ -containing Krebs buffer and then with same medium containing  $^{14}\text{C}$ -MeAIB (20  $\mu\text{M}$ ) for 15 min. Drugs examined are anti-glutamatergic agents used in epilepsy, global and focal ischemic stroke, ALS, Glu-induced hearing loss, that include riluzole, lamotrigine, phenytoin, carbamazepam, topiramate, ethosuximide, valproic acid, Gabapentin, zonosamide, and levetiracetam. At 20  $\mu\text{M}$  concentration, riluzole completely blocks spontaneous  $\text{Ca}^{2+}$ -dependent MeAIB transport while lamotrigine, phenytoin and toperimate were much less effective inhibiting approximately 54%, 25%, and 20% of control values, respectively. The relative inhibitory constant ( $\text{IC}_{50}$ ) for riluzole to inhibit spontaneous  $\text{Ca}^{2+}$ -dependent MeAIB transport is 1.3  $\pm$  0.1  $\mu\text{M}$  (Fig. 7). Phenytoin displays an  $\text{IC}_{50}$  value of 57  $\pm$  4  $\mu\text{M}$  to block activity-regulated,  $\text{Ca}^{2+}$ -dependent, high-affinity Gln/MeAIB transport in hippocampal neuronal cultures (Fig. 7), which is 50X less potent than riluzole. All other agents tested are ineffective at 20  $\mu\text{M}$  concentration. The relative potency values for riluzole and phenytoin described here are consistent with the reported differences between riluzole and phenytoin to provide neuroprotection against presynaptic Glu-induced excitotoxicity in acute and chronic neurodegenerative conditions.

### **$\text{K}^{+}$ -stimulated, and spontaneous $\text{Ca}^{2+}$ -regulated MeAIB transport is endogenously up-regulated during the 2<sup>nd</sup> and 3<sup>rd</sup> weeks of development in hippocampal neurons**

The Glu/Gln cycle and synaptic VGLUT1-mediated glutamatergic transmission develops in hippocampal and cortical pyramidal neurons during the 2<sup>nd</sup> and 3<sup>rd</sup> postnatal weeks *in vivo* and *in vitro*. Does  $\text{K}^{+}$ -stimulated and  $\text{Ca}^{2+}$ -dependent spontaneous transport of  $^{14}\text{C}$ -MeAIB develop in neuronal cultures along a similar time-line? To examine this question, neurons were incubated in Krebs buffer with  $\text{Ca}^{2+}$  for spontaneous conditions, or in  $\text{Ca}^{2+}$ -free conditions after brief  $\text{K}^{+}$ -depolarization with  $\text{Ca}^{2+}$  (*see* Experimental Procedures) across several postnatal developmental time points (DIV 8, 12, 14, 16, and 20). Spontaneous and  $\text{K}^{+}$  depolarization-induced,  $\text{Ca}^{2+}$ -dependent  $^{14}\text{C}$ -MeAIB transport is not present in DIV 8 cultures (Fig. 8). Both  $\text{K}^{+}$ -stimulated and spontaneously active  $^{14}\text{C}$ -MeAIB transport are dramatically and coordinately up-regulated during the 2<sup>nd</sup> and 3<sup>rd</sup> postnatal weeks (DIV 8–20). High-affinity,  $\text{Ca}^{2+}$ -dependent  $^{14}\text{C}$ -MeAIB transport is  $\text{K}^{+}$ -depolarization stimulated, spontaneously active, and is completely blocked by the presence of 5 mM MeAIB at all developmental ages. Collectively, these results demonstrate that activity dependent,  $\text{Ca}^{2+}$ -regulated Gln/MeAIB transport is developmentally up-regulated during the critical period of maturation of glutamatergic presynaptic strength and is maximally expressed in fully-differentiated, mature hippocampal neurons.



## DISCUSSION

This report describes, for the first time, a high-affinity,  $\text{Ca}^{2+}$ -regulated,  $\text{K}^{+}$ -stimulated, and spontaneously active Gln/MeAIB transport system in hippocampal neuron-enriched primary cultures that is regulated endogenously by neuronal activity in mature synapses, potentially inhibited by riluzole (a blocker of Glu release), and that is up-regulated during the critical postnatal period of functional maturation of the Glu/Gln cycle between astrocytes and neurons and synaptic Glu release. The novel Gln/MeAIB transport system described here may have physiological and pathological implications in understanding the neurobiology of excitotoxic synaptic Glu release in the hippocampus in acute and chronic neurodegenerative diseases.

Gln transport is robust in hippocampal neurons due to the multitude of Gln transporter subtypes (at least 14) that exist in the CNS that subserve multiple physiologic functions in many cellular compartments (Schiøth *et al.* 2013, Albrecht *et al.* 2007). However, only a small percentage (<5%) of the total amount of Gln transported in hippocampal neuronal culture (5 min at 20  $\mu\text{M}$   $^3\text{H}$ -Gln concentration) is sensitive to inhibition by MeAIB (5 mM), a selective blocker of system A transport (Christensen 1990, McGivan & Pastor-Anglada 1994). Yet,  $\text{Ca}^{2+}$ -dependent MeAIB-sensitive Gln transport activity can be stimulated by brief high  $\text{K}^{+}$ -depolarization of axon terminals suggesting that an activity-regulated  $\text{Ca}^{2+}$ -dependent Gln/MeAIB transporter exists in hippocampal neurons. The transport of MeAIB was therefore used here as a specific substrate to isolate and to further characterize this Gln transporter subpopulation, and because MeAIB is not metabolized, and so is more stable than Gln. As surmised,  $\text{Ca}^{2+}$ -dependent transport of MeAIB is greatly stimulated following brief  $\text{K}^{+}$ -depolarization.  $\text{K}^{+}$ -stimulated MeAIB transport is dependent on extracellular  $\text{Ca}^{2+}$ , and in the presence of 1.2 mM  $\text{Ca}^{2+}$ , MeAIB transport is blocked by inhibition of VGCC's (verapamil, 20  $\mu\text{M}$ ). This  $\text{K}^{+}$  depolarization-induced Gln/MeAIB transport system displays relatively high affinity for MeAIB (37  $\pm$  1.2  $\mu\text{M}$ ) and is maximally active at MeAIB concentrations of 200  $\mu\text{M}$ , unlike low-affinity ( $K_m \sim 0.5$  mM) neuronal Gln/MeAIB transporters SNAT1 or SNAT2 (Mackenzie & Erickson 2004). The novel, high affinity Gln transporter in hippocampal neurons described here would operate efficiently at low extrasynaptic levels of Gln ( $\sim 30$   $\mu\text{M}$ ) reported in the hippocampus *in vivo* (Kanamori & Ross 2013), which would be ideal for activity-dependent regulation of Gln cycling into synapses. While other precursors could contribute to regulating cytoplasmic Glu levels and vesicular Glu stores in cortical and hippocampal axon terminals (Hassel & Brathe 2000, Lieth *et al.* 2001, Kam & Nicoll 2007, Takeda *et al.* 2012), Gln is still considered the preferred precursor for loading the  $\text{Ca}^{2+}$ -dependent releasable pool of vesicular Glu (Leke & Schousboe 2016).

The present study challenges existing research paradigms that the known low-affinity neuronal Gln/MeAIB transporters SNAT1 or SNAT2 are involved in supplying Gln at synapses for Glu transmission (Chaudhry *et al.* 2002, Mackenzie & Erickson 2004, Conti & Melone 2006). A higher affinity Gln/MeAIB transport system that is regulated by neuronal activity, and inhibited by riluzole, is a potential new target to affect presynaptic Glu transmission in hippocampal neurons. MeAIB is a substrate-competitor for several other amino acid transporters as well, such as the lysosomal amino acid transporter LYAAT-1

(Sagne *et al.* 2001), the electrogenic H<sup>+</sup>-dependent amino acid cotransporter PAT2 (Boll *et al.* 2002), the synaptic vesicle enriched amino acid transporter NTT4/Rxt1 (Zaia & Reimer 2009), and a broad spectrum amino acid transporter SNAT8 (Hagglund *et al.* 2015). Gln uptake by neuronal SNAT7 and SNAT8 isoforms is weak in oocytes and they may also transport cationic and anionic amino acids (Hagglund *et al.* 2011, Hagglund *et al.* 2015). NTT4/Rxt1 is found in preparations enriched in synaptic vesicles (Masson *et al.* 1999) and recently was described to be a leu/pro/his/Gln/MeAIB transporter when molecularly engineered for expression at the plasma membrane (Zaia & Reimer 2009). The transporter responsible for activity-dependent, spontaneous and K<sup>+</sup>-stimulated, Ca<sup>2+</sup>-regulated neuronal <sup>14</sup>C-MeAIB transport may also, of course, be still an orphan or a misidentified Slc member.

Low levels of Ca<sup>2+</sup>-regulated Gln/MeAIB transport described in the present study could be a result of the intracellular sequestration of an inducible, Ca<sup>2+</sup>-dependent recycling of a high-affinity Gln/MeAIB transporter protein in excitatory synapses providing an important regulatory mechanism for activity regulated Glu transmission. Indeed, regulated cycling of transporters involved in the recycling of other neurotransmitters, such as acetylcholine and GABA, rely on cycling of intracellular pools of high-affinity choline (CHT1) and GABA (GAT1) transporters to the plasma membrane in nerve terminals (Deken *et al.* 2003, Ribeiro *et al.* 2007, Ferguson *et al.* 2003). SNAT2 is also regulated at the level of recycling of an intracellular pool of SNAT2 vesicles in many cell types as an osmolyte (Franchi-Gazzola *et al.* 2006) including neurons, but is not required for presynaptic Glu transmission in cortical neurons *in vitro* (Grewal *et al.* 2009). The dependence of spontaneous Ca<sup>2+</sup>-regulated MeAIB transport on Na<sup>+</sup> (TTX-inhibitable, Li<sup>+</sup> intolerant), Ca<sup>2+</sup> (VGCC-inhibitable), and postsynaptic Glu receptor function (NBQX/AP5-inhibitable) described here indicates the requirement for action potential stimulated and Glu-driven neural circuit activity. Blockade of endogenous synaptic Glu transmission by inclusion of GABA, to increase synaptic inhibition, also blocks spontaneous Ca<sup>2+</sup>-regulated MeAIB transport. Collectively these results support the presence of intracellular stores of an activity-regulated, high affinity Gln/MeAIB transporter protein in hippocampal neurons that exhibits Ca<sup>2+</sup>-dependent cycling to the plasma membrane for functional expression and that is inducible by neuronal activity.

Biochemical studies and <sup>13</sup>C-NMR determinations have similarly identified the time period during which the Glu/Gln cycle activity develops in the rat to between postnatal days 10 and 30 (Chowdhury *et al.* 2007, Hertz 2013). Presynaptic Glu release and Glu transmission also increases dramatically in pyramidal neurons during this time period *in vivo* and *in vitro* (Bolshakov & Siegelbaum 1995, Wasling *et al.* 2004, Wilson *et al.* 2005, De Gois *et al.* 2005). In the present study, spontaneous and K<sup>+</sup>-stimulated Gln/MeAIB transport is negligible in DIV 8 cultures but is maximal (>10-fold increase) by DIV 20. The developmental period selected for neuronal experiments to investigate the role for regulated Gln/MeAIB transport in excitatory transmission is therefore critical. Gln transport during this 'critical' period of postnatal development may contribute in mechanisms of synaptic growth and differentiation. Synaptic growth, re-organization and plasticity may require Gln because activity-dependent Gln transport could facilitate presynaptic Glu release that strengthens selective excitatory synaptic pathways in the brain. For instance, Glu-dependent secretion of BDNF sculpts cortical and hippocampal neural circuits during development (Lu

2003, Kuczewski *et al.* 2010, Park & Poo 2013), stimulates energy metabolism (Burkhalter *et al.* 2003) and regulates Gln/MeAIB-sensitive dendritic development (Burkhalter *et al.* 2007). Thus, activity-regulated synaptic Gln transport during development may facilitate proper establishment of synaptic Glu release in neural circuits for the development of stable neural networks.

The inhibition of spontaneous Gln/MeAIB transport by riluzole suggests that neural activity regulated Gln/MeAIB transport may be linked to Na<sup>+</sup>-dependent/action-potential driven and Ca<sup>2+</sup>-regulated synaptic release of Glu. Riluzole has been shown to inhibit synaptic Glu release (Martin *et al.* 1993, Kretschmer *et al.* 1998, Lingamaneni & Hemmings 1999, Wang *et al.* 2004). Neuroprotection by riluzole may occur by inhibiting Glu release in neurons *via* mechanisms that include inhibition of Na<sup>+</sup> channels (Benoit & Escande 1991, Martin *et al.* 1993, Herbert *et al.* 1994, Stefani *et al.* 1997, Prakriya & Mennerick 2000, Spadoni *et al.* 2002). Riluzole inhibition of Na<sup>+</sup> channels would reduce action-potential driven neuronal circuit activity and thus, synaptic Glu release that drives AMPA receptor mediated Glu activity and subsequent NMDA-mediated excitotoxicity. Phenytoin, an established Na<sup>+</sup> channel blocker, also blocks activity-regulated Gln/MeAIB transport, and both riluzole and phenytoin are neuroprotective in ischemia-induced neuronal death *in vivo* (Malgouris *et al.* 1989, Pratt *et al.* 1992, Taft *et al.* 1989, Ates *et al.* 2013). However, riluzole is more than 50X more potent than phenytoin to inhibit activity-regulated Gln/MeAIB transport, which agrees with its improved potency to prevent excitotoxic Glu-induced neuronal damage in the hippocampus (Ates *et al.* 2013). These results support the development of selective riluzole-derived drugs to target neural-regulated Gln/MeAIB transport activity for neuroprotection.

A recent quantitative analysis of the rates of release and resupply of vesicular Glu required to sustain synaptic Glu release confirms that maintaining the synaptic cytoplasmic Glu supply *via* Gln (or tricarboxylic acid cycle intermediates) released from glia is essential for continued Ca<sup>2+</sup>-dependent synaptic Glu release under spontaneous conditions, and under conditions of increased neural activity (Marx *et al.* 2015). Excessive and sustained synaptic release of Glu is an initial event that triggers Ca<sup>2+</sup>-dependent Glu-mediated cell death in postsynaptic neurons in various deleterious conditions including traumatic brain injury, peri-operative neuronal and cardiac stress, epilepsy, global and focal cerebral ischemia, mild Alzheimer's disease, noise-induced hearing loss, and in several *in vitro* models of neurodegenerative diseases including oxygen-glucose reperfusion injury (Bittigau & Ikonomidou 1997, Dodd 2002, Holmes 2002, Kostandy 2012, Dorsett *et al.* 2016, Mancini *et al.* 2016, Pereira *et al.* 2016). Riluzole is neuroprotective in all of these acute and chronic neurodegenerative conditions (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) by uncertain mechanisms. Riluzole is more effective as a neuroprotective agent when given prophylactically, which is in line with its presumed role to reduce synaptic Glu release and resultant Glu-induced excitotoxicity postsynaptically. The identification of Ca<sup>2+</sup>-regulated, riluzole-sensitive, high-affinity Gln/MeAIB transport in hippocampal neurons supports a role for an activity-regulated Gln transporter in regulating Glu release from synapses and resulting synaptic Glu-induced excitotoxicity.

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## The abbreviations used are

<b>Gln</b>	glutamine
<b>MeAIB</b>	2-(methylamino)isobutyrate
<b>VGCCs</b>	voltage gated calcium channels
<b>SNAT</b>	sodium-coupled neutral amino acid transporter
<b>BCH</b>	2-amino-2-norbornanecarboxylic acid
<b>AIB</b>	aminoisobutyric acid
<b>N<sup>'</sup>N-DMG</b>	N <sup>'</sup> N-dimethylglycine
<b>TTX</b>	tetrodotoxin
<b>Ver</b>	verapamil
<b>NBQX</b>	2,3-dioxo-6nitro-1,2,3,4-tetrahydrobenzo[f]-quinoxaline-7-sulfonamide
<b>AP5</b>	2-amino-5-phosphonopentanoic acid
<b>MCPG</b>	$\alpha$ -methyl-4-carboxyphenylglycine
<b>DIV</b>	days <i>in vitro</i>
<b>NTT4/Rxt1</b>	synaptic vesicle enriched amino acid transporter
<b>LYAAT-1</b>	lysosomal amino acid transporter
<b>EAAT</b>	excitatory amino acid transporter
<b>PAT2</b>	proton-dependent amino acid cotransporter
<b>CHT1</b>	high-affinity choline transporter
<b>GAT1</b>	GABA transporter
<b>VGLUT1</b>	vesicular Glu transporter
<b>NB</b>	Neurobasal medium

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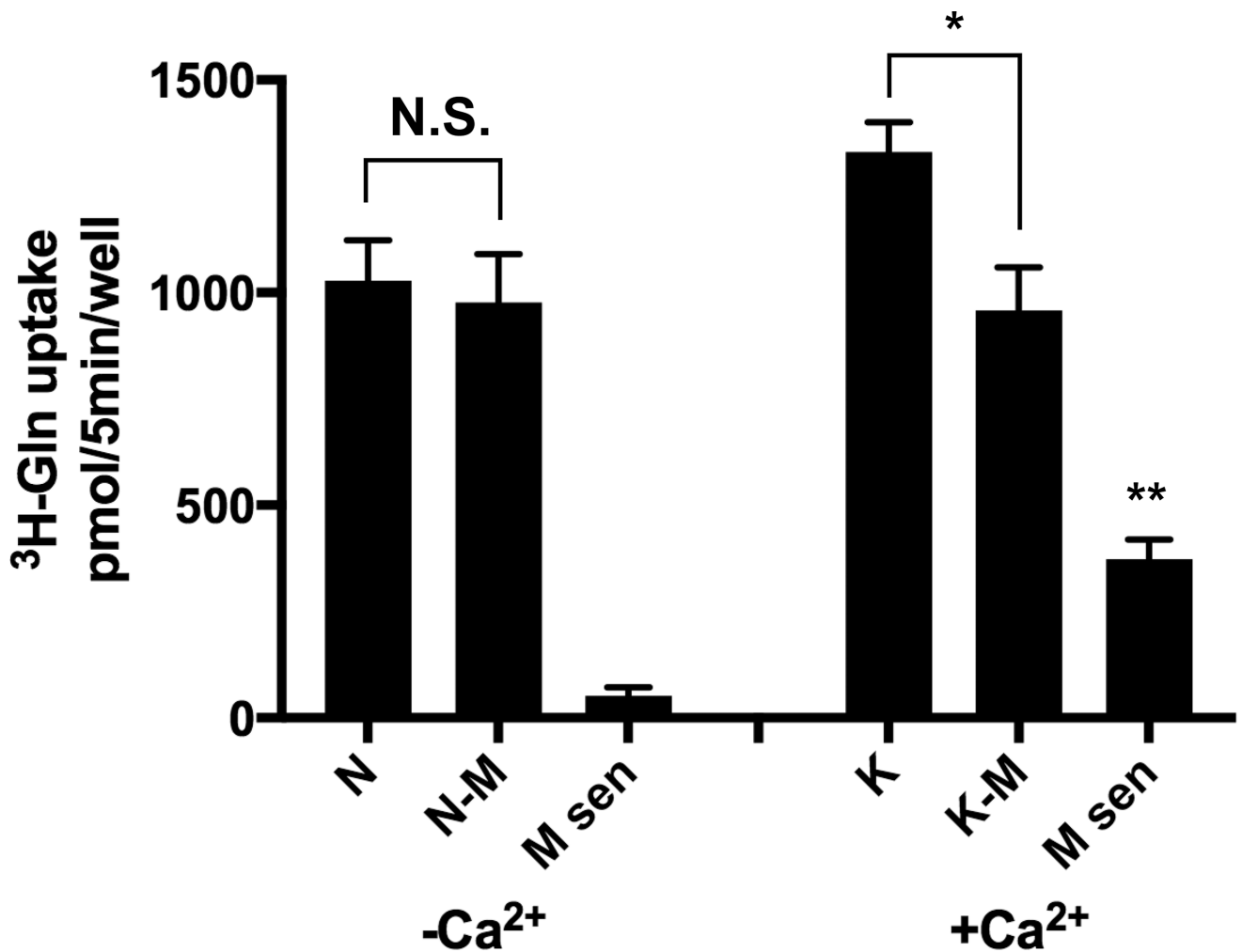
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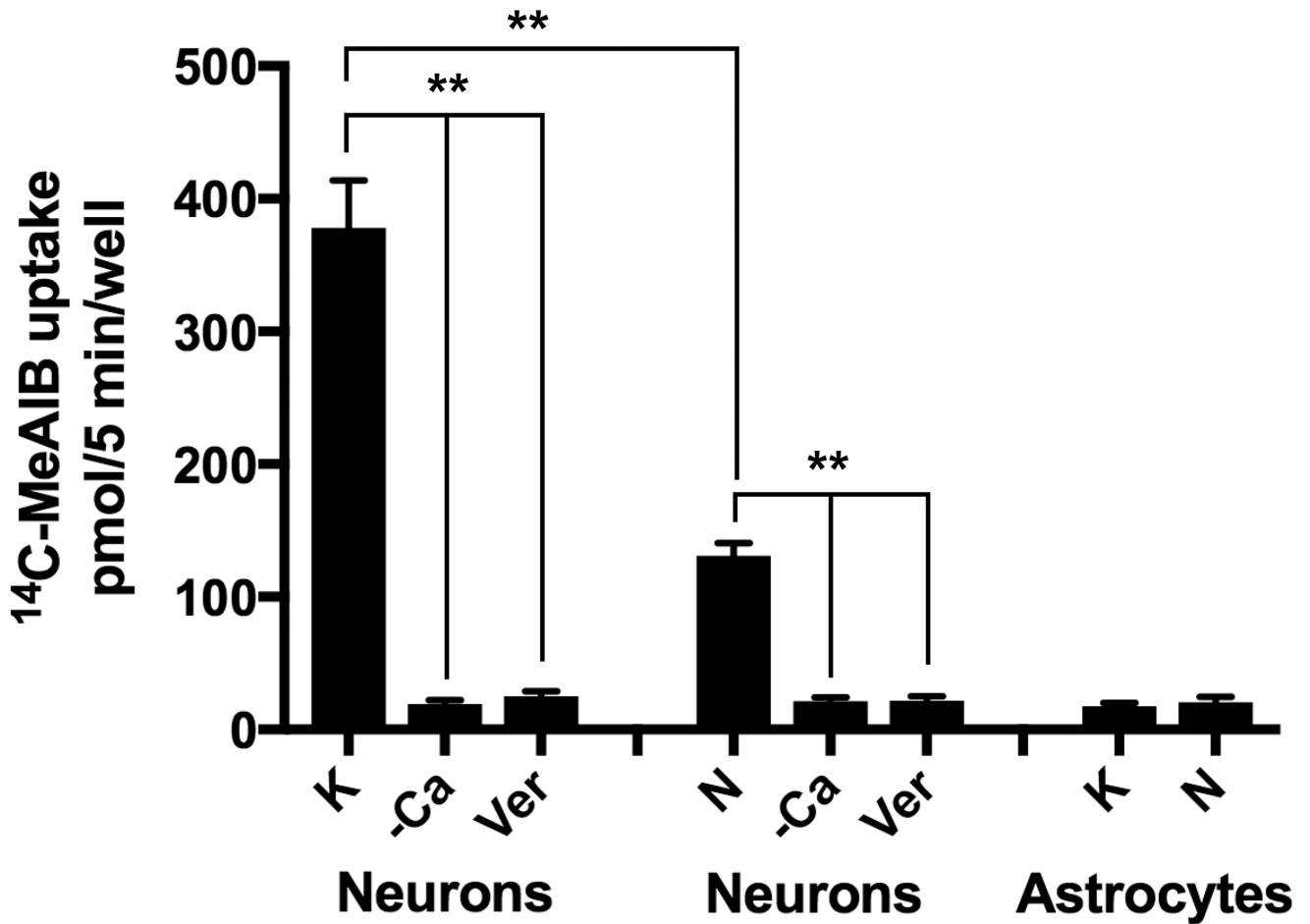
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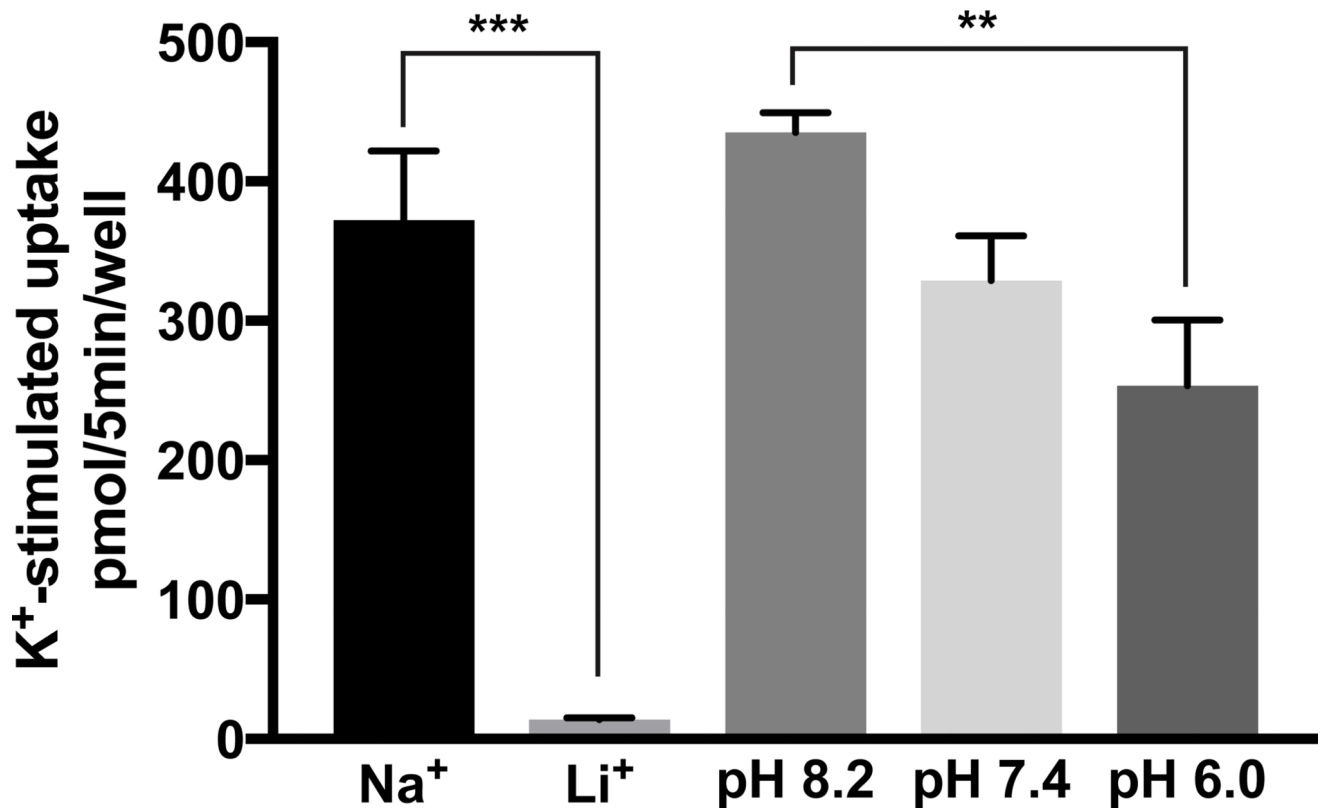
**Fig. 1. Ca<sup>2+</sup>-dependent <sup>3</sup>H-Gln transport in hippocampal neurons is stimulated by brief high K<sup>+</sup>-induced depolarization**

A relatively low concentration of <sup>3</sup>H-Gln (20 μM) in the uptake medium is used to isolate a high affinity K<sup>+</sup>-stimulated Gln transport system in cultured hippocampal neurons.

Abundant uptake of <sup>3</sup>H-Gln by neurons is observed following 5 min incubation in normal Krebs buffer without Ca<sup>2+</sup> (N buffer) but <sup>3</sup>H-Gln transport is not significantly affected by inclusion of MeAIB (5 mM). The uptake of <sup>3</sup>H-Gln increases ~25% by brief exposure (5 min) to high K<sup>+</sup>-depolarization (plus Ca<sup>2+</sup>) of axon terminals (K), which is blocked by inclusion of MeAIB (5 mM) in the uptake buffer (K-M). The MeAIB-sensitive portion of <sup>3</sup>H-Gln transport increases ~8X fold following K<sup>+</sup>-depolarization (M sen, +Ca<sup>2+</sup>). The transport of <sup>3</sup>H-Gln is conducted in N buffer at DIV 14. Uptake values obtained at 4°C were less than 5% and were subtracted. *N*, normal Krebs buffer without Ca<sup>2+</sup> and with EGTA (1 mM). *N-M*, Same buffer with MeAIB (5 mM). *K*, Krebs buffer containing Ca<sup>2+</sup> (1.2 mM) and with Na<sup>+</sup> replaced with high K<sup>+</sup> (60 mM, 5 min). *K-M*, Same K<sup>+</sup>-buffer with MeAIB (5 mM). *M-sen*, MeAIB-sensitive transport with same buffers. Data are the mean ± S.E. values for n=3 independent experiments.  $F_{5,18} = 59.4$ ,  $p < 0.01$  (main effect). \* $p = 0.04$  (K vs K-M), \*\* $p = 0.003$  ( $M_{\text{sen}+\text{Ca}}$  vs  $M_{\text{sen}-\text{Ca}}$ ), N.S. not significant.



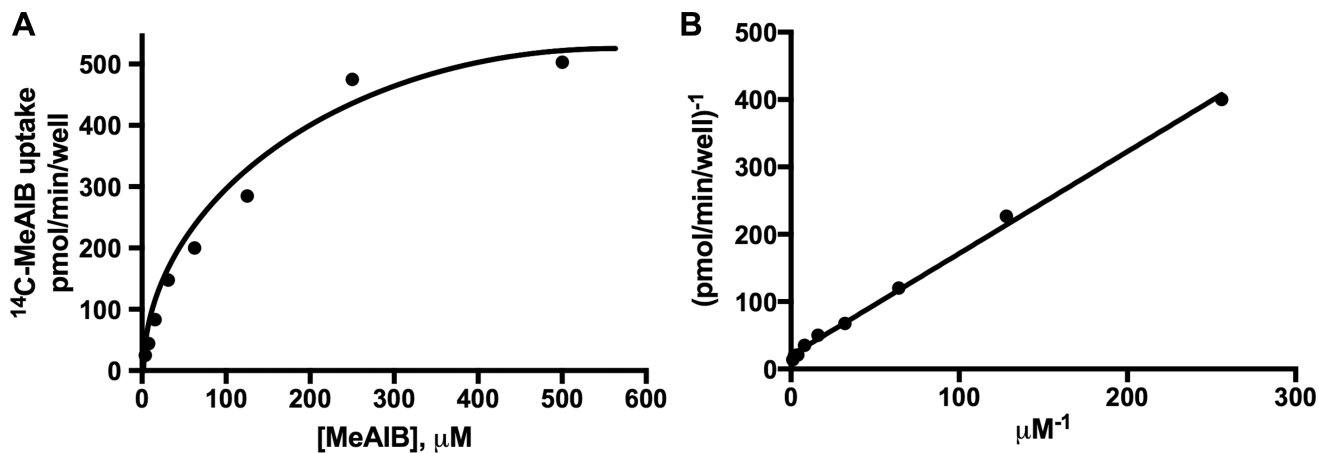
**Fig. 2. Ca<sup>2+</sup>-dependent <sup>14</sup>C-MeAIB transport is stimulated by brief high K<sup>+</sup>-induced depolarization and exists spontaneously in neurons, but not in astrocytes**  
 Hippocampal neurons (DIV 14) were first rinsed in N buffer that does not contain Ca<sup>2+</sup>, but EGTA (1 mM). For K<sup>+</sup>-stimulated uptake, cells are exposed to high K<sup>+</sup> (60 mM) (with or without 1.2 mM CaCl<sub>2</sub>) for 5 min at 37°C. Cells were then rinsed in N buffer and <sup>14</sup>C-MeAIB uptake is performed for 5 min in N buffer (37°C). For spontaneous uptake, cells are first rinsed in N buffer and then incubated (5 min, 37°C) in N buffer or in normal Krebs medium with Ca<sup>2+</sup> (1.2 mM) containing MeAIB (20 μM). Uptake values obtained at 4°C were less than 5% and were subtracted. *K<sup>+</sup>*, high K<sup>+</sup> depolarization. *N*, Krebs buffer without Ca<sup>2+</sup> and with EGTA. *-Ca*, 5 min pre-treatment in same buffers without Ca<sup>2+</sup> (with 1 mM EGTA). *Ver*, Verapamil (20 μM) is included during the 5 min K<sup>+</sup>-depolarization period in Ca<sup>2+</sup> containing high K<sup>+</sup> buffer. Astrocytes pretreated with high K<sup>+</sup> depolarization (plus Ca<sup>2+</sup>) do not transport significant amounts of <sup>14</sup>C-MeAIB (20 μM, 5 min, 37°C). Data are the mean ± S.E. for n=3 independent experiments.  $F_{7,24} = 25.5$ ,  $p < 0.01$  (main effect) \*\*  $p < 0.001$ .



**Fig. 3. Ca<sup>2+</sup>-dependent, high affinity <sup>14</sup>C-MeAIB transport in hippocampal neurons is dependent on Na<sup>+</sup> ions and is pH-sensitive**

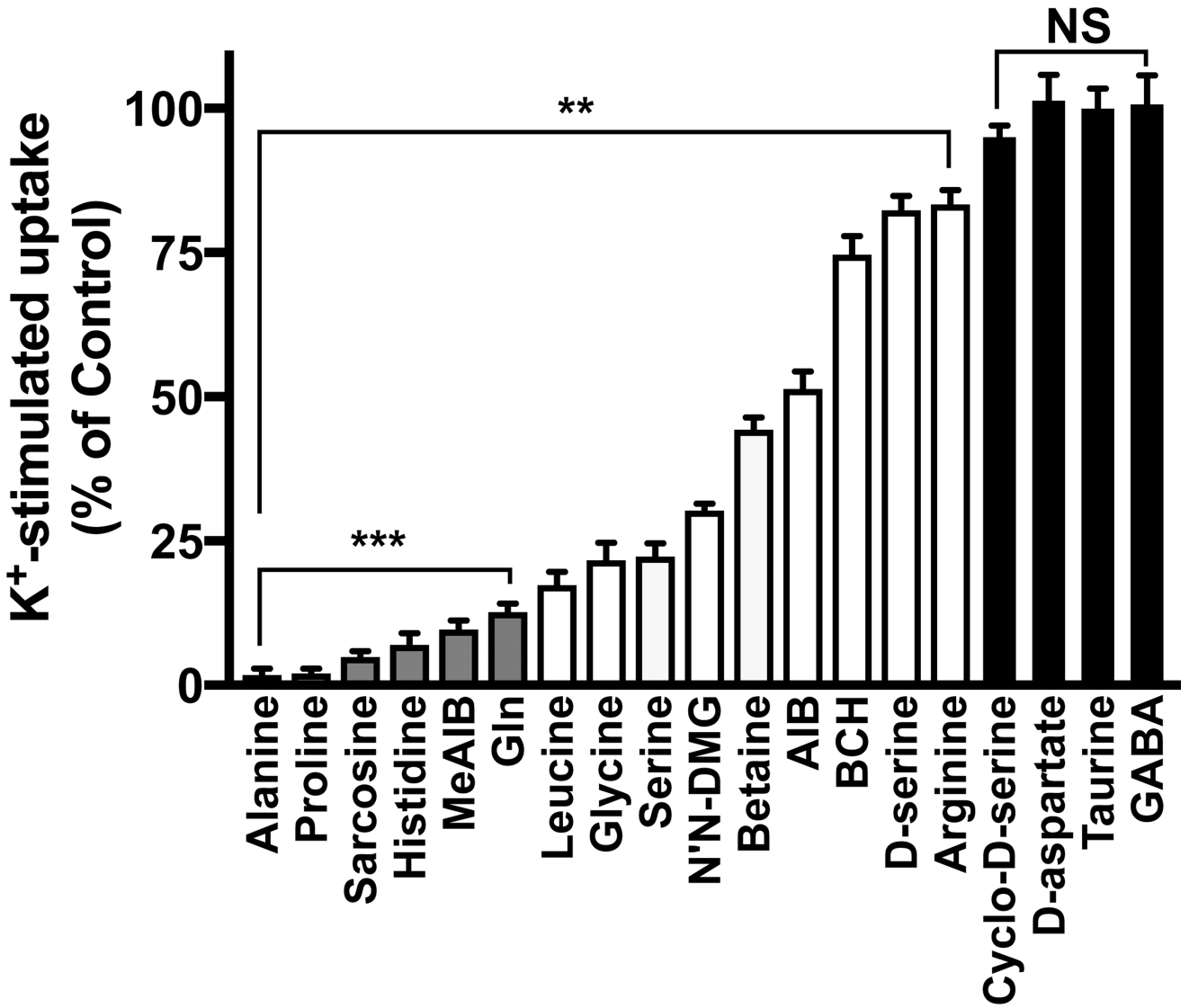
Cultures are exposed to high K<sup>+</sup> (60 mM) (plus 1.2 mM CaCl<sub>2</sub>) for 5 min at 37°C, then rinsed in N buffer, and <sup>14</sup>C-MeAIB (20 μM) uptake is performed (5 min, 37°C) in: *A*, N buffer or N buffer that contains 145 mM LiCl, instead of Na<sup>+</sup>, or *B*, N buffer at pH 8.2, 7.4, or 6.0. Uptake values obtained at 4°C were less than 5% and were subtracted. Data are the mean ± S.E. values for n=3 independent experiments.  $F_{4,18} = 73.1$ ,  $p < 0.01$  (main effect). \*\*  $p < 0.01$ .





**Fig. 4. Kinetic analysis of  $\text{Ca}^{2+}$ -dependent  $\text{K}^{+}$ -stimulated  $^{14}\text{C}$ -MeAIB transport in hippocampal neurons**

*A*, Saturation isotherm of initial uptake velocity (3 min) of  $^{14}\text{C}$ -MeAIB (3.9 – 500  $\mu\text{M}$ ) in hippocampal neuron-enriched primary cultures (DIV 14). *B*, Lineweaver-Burk analysis of initial uptake velocity. Cells are exposed to high  $\text{K}^{+}$  (60 mM) (with 1.2 mM  $\text{CaCl}_2$ ) for 5 min at  $37^\circ\text{C}$ , then rinsed in N buffer, and  $^{14}\text{C}$ -MeAIB uptake is performed in N buffer ( $37^\circ\text{C}$ ). Uptake values obtained at  $4^\circ\text{C}$  were less than 5% and were subtracted. The  $K_m$  and  $V_{\text{max}}$  value for MeAIB was calculated from the nonlinear regression of specific MeAIB uptake velocity *versus* the extracellular concentration of MeAIB. Data are from a representative experiment performed in duplicate, and  $K_m$  values are derived from  $n=4$  independent experiments.



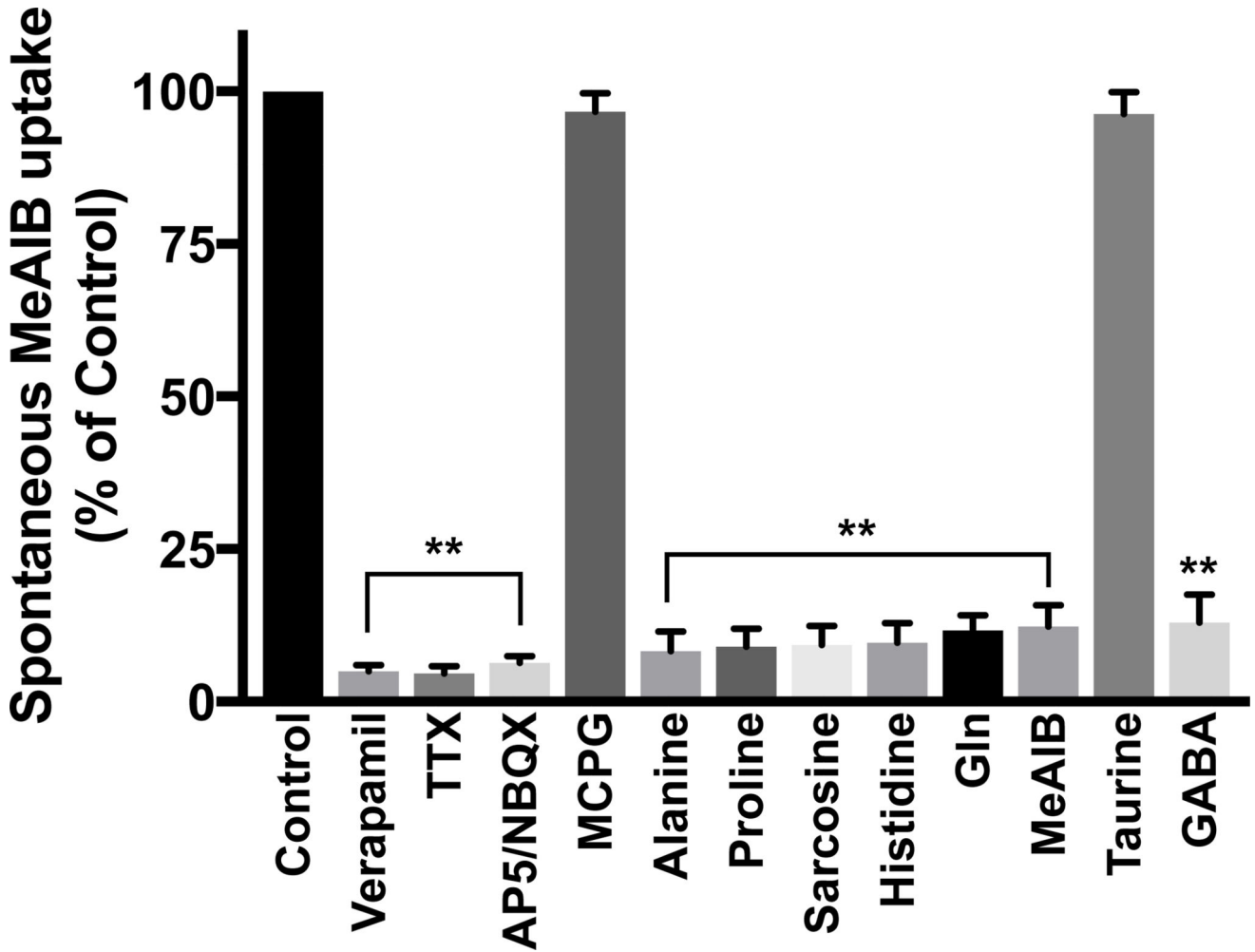
**Fig. 5. Substrate competition profiles for Ca<sup>2+</sup>-dependent, high affinity <sup>14</sup>C-MeAIB transport** Cultures are exposed to high K<sup>+</sup> (60 mM plus 1.2 mM CaCl<sub>2</sub>) for 5 min at 37°C, then are rinsed in N buffer, and <sup>14</sup>C-MeAIB (20 μM) uptake is performed in N buffer (5 min, 37°C). Transport is conducted in the absence or presence of various amino acid competitive substrates (2 mM). Uptake values obtained at 4°C were less than 5% and were subtracted. Subtracted data were normalized to <sup>14</sup>C-MeAIB uptake in the absence of unlabeled amino acids. N'N-DMG, N'N dimethylglycine. AIB, aminoisobutyric acid. BCH, 2-amino-2-norbornane-carboxylic acid. Cyclo-D-ser, cyclo-D-serine. Data are the mean +/- S.E. values from n=3-5 independent experiments. \*\**p* < 0.01, indicates significant differences from uptake in the absence of unlabeled substrate. \*\*\**p* < 0.01) indicate amino acids (ala, pro, sarcosine, his, MeAIB, Gln) that reduce <sup>14</sup>C-MeAIB transport by greater than 90%. N.S., not significant.

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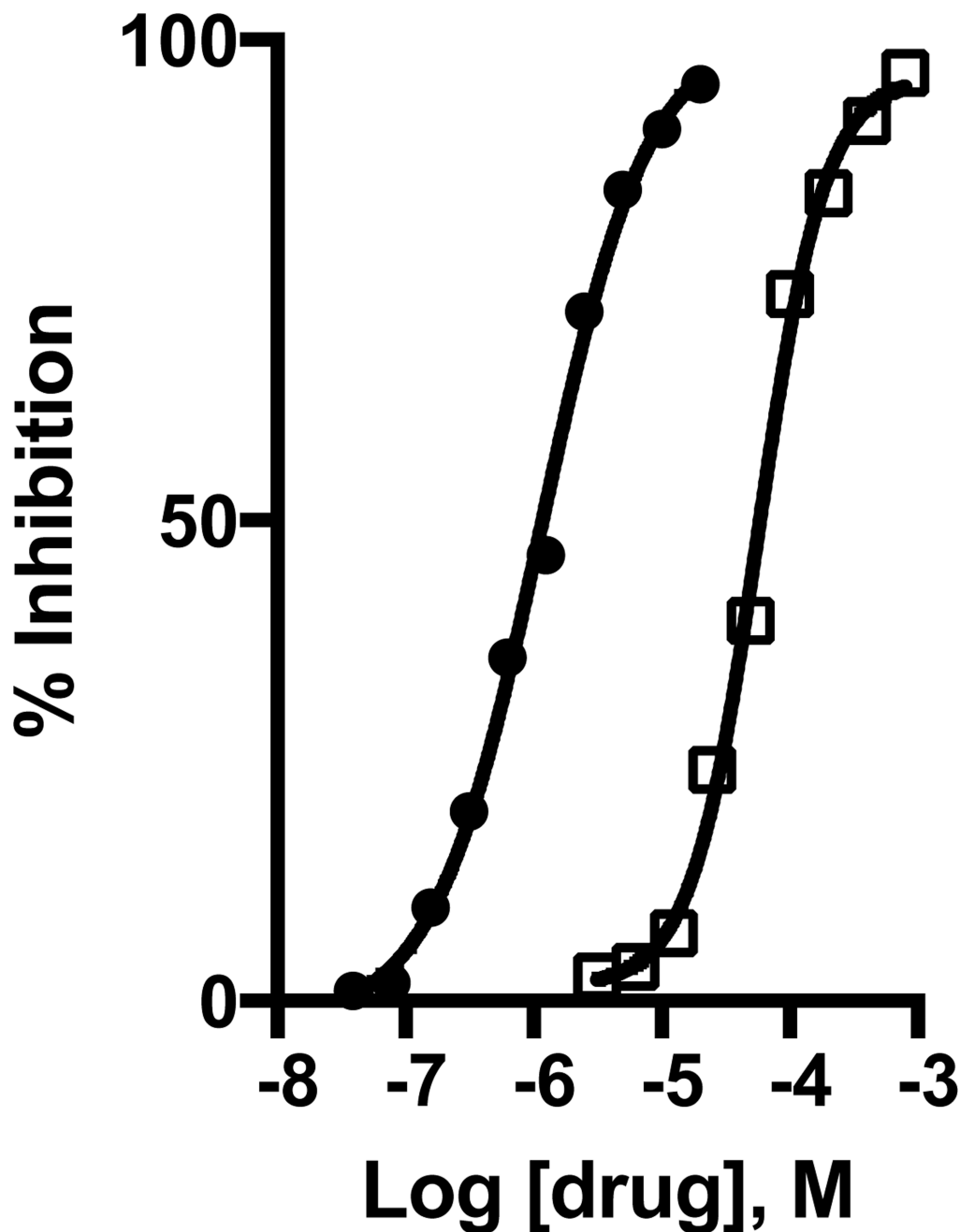
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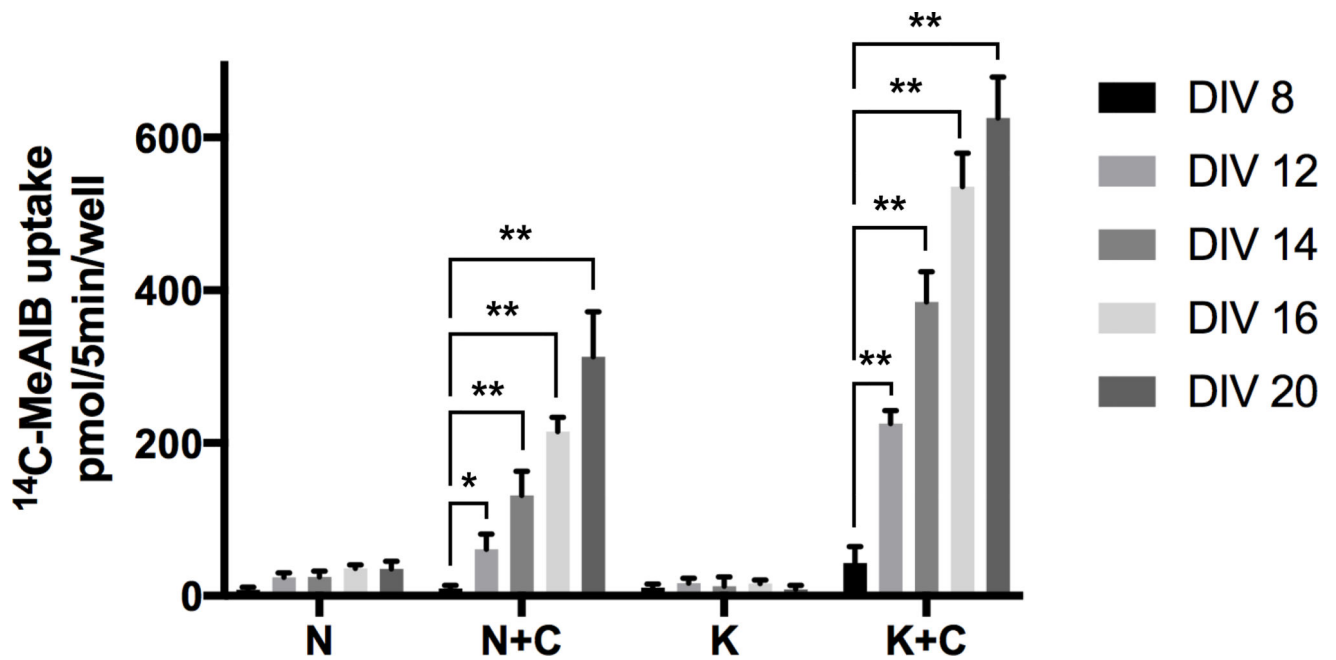
**Fig. 6. Spontaneous  $^{14}\text{C}$ -MeAIB transport requires extracellular  $\text{Ca}^{2+}$  and is blocked by inhibitors of VGCCs,  $\text{Na}^+$  channel activity, glutamatergic transmission *via* AMPA and NMDA receptors, and by GABA**

Cultures (DIV 14) were rinsed in N buffer and then preincubated in Krebs buffer with  $\text{Ca}^{2+}$  with or without inhibitors (2 min) and then in the same buffers containing  $^{14}\text{C}$ -MeAIB (20  $\mu\text{M}$ ) at  $37^\circ\text{C}$  (5 min). Uptake values obtained at  $4^\circ\text{C}$  were less than 5% and were subtracted. *Con*, control. *Ver*, verapamil 20  $\mu\text{M}$ , *A/N*, AP5, 50  $\mu\text{M}$  / NBQX 10  $\mu\text{M}$ , TTX, tetrodotoxin 1  $\mu\text{M}$ ; MCPG,  $\alpha$ -methyl-4-carboxyphenylglycine, 1 mM. Selected amino acids (2 mM) included with  $^{14}\text{C}$ -MeAIB during transport reveal that spontaneous  $^{14}\text{C}$ -MeAIB transport exhibits that same substrate preference to  $\text{K}^+$ -stimulated transport, except for GABA, which is inhibitory due to the blockade of excitatory glutamatergic transmission. Data are the mean  $\pm$  S.E. values from  $n=3$  independent experiments.  $F_{12,39} = 571$  (main effect),  $p < 0.0001$ . \*\* $p < 0.0001$ .



**Fig. 7. Spontaneous  $\text{Ca}^{2+}$ -regulated  $^{14}\text{C}$ -MeAIB transport is potently and preferentially blocked by riluzole, an anti-glutamatergic drug**

Spontaneous  $\text{Ca}^{2+}$ -dependent  $^{14}\text{C}$ -MeAIB transport (20  $\mu\text{M}$ ) is measured (15 min, 37°C) with and without various anti-glutamatergic drugs, following their brief (2 min) pre-incubation at 37°C. Spontaneous transport in  $\text{Ca}^{2+}$ -free Krebs buffer was measured in all independent experiments and is generally less than 15% of  $\text{Ca}^{2+}$ -containing control samples, was not affected by any of the drugs tested, and was subtracted as the negative background control. The  $\text{IC}_{50}$  value for riluzole (black circles) and phenytoin (open squares) inhibition of  $^{14}\text{C}$ -MeAIB transport was calculated from dose response curves. Data shown are from a representative experiment, and the experiment was repeated  $n=4$  times.



**Fig. 8. Developmental up-regulation of the expression of activity-dependent spontaneous and K<sup>+</sup>-stimulated Ca<sup>2+</sup> sensitive <sup>14</sup>C-MeAIB transport**

Spontaneous and K<sup>+</sup>-stimulated <sup>14</sup>C-MeAIB transport (20 μM) is measured (5 min, 37°C) in hippocampal neuron-enriched cultures at different days *in vitro* (DIV 8, 12, 14, 16, and 20). *N*, normal Krebs buffer without Ca<sup>2+</sup> and with EGTA. *N+C*, same buffer with Ca<sup>2+</sup> (1.2 mM) and without EGTA. *K*, Krebs buffer Na<sup>+</sup> replaced with high K<sup>+</sup> (60 mM) but without Ca<sup>2+</sup> and with EGTA. *K+C*, Same K<sup>+</sup> buffer with Ca<sup>2+</sup> and without EGTA. Ca<sup>2+</sup>-dependent spontaneous and K<sup>+</sup>-stimulated transport are controlled by inclusion of MeAIB (5 mM). Background levels at 4°C are consistently at <100 pmol/well. MeAIB (5 mM) subtracted data are the mean ± S.E. values from n=3 independent experiments. F<sub>9,72</sub> = 481, *p* < 0.0001 (main effect). \* *p* < 0.05, \*\* *p* < 0.001.