Glutamate Transporters are Differentially Expressed in the Hippocampus During the Early Stages of One-day Spatial Learning Task

Ada I. Fraticelli-Torres, MS; Félix Matos-Ocasio, BS; Kenira J. Thompson, PhD

Introduction: Uptake of glutamate in the hippocampus by specialized transporters appears to be important for the prevention of glutamate-induced neurotoxicity. However, the role of these transporters in synaptic plasticity and learning is still unclear. We examined the expression pattern of glutamate transporters at different stages of spatial learning using a one-day (three blocks) version of the Morris Water Maze.

Methods: Male rats (Sprague Dawley, 3 months old) were divided into three groups (learner, swim control, or naïve control) and animals were sacrificed after the first, second, or third block of training. The hippocampi were immediately extracted and flash frozen for RNA analysis. Real time polymerase chain reaction was employed to examine the expression of glutamate transporter 1 (Glt-1), Glt1b, glutamate-aspartate transporter (GLAST) and excitatory amino acid carrier-1 (EAAC1) in whole hippocampi.

Results: EAAC1 and GLAST RNA were downregulated in the learner and swimmer groups (compared to naïve) after the first two blocks of training during the one-day protocol but EAAC1 returned to control levels by the end of the third block. GLAST levels were upregulated by the third block of training. Glt-1b expression was downregulated during the second block of training but returned to control by the third block.

Conclusions: The observed decreases in glutamate transporter expression may be important during the early stages of spatial learning as a possible mechanism to enhance glutamatergic availability during critical stages of learning. However, similar decreases in glutamate transporter expression in both the learner and swimmer groups indicate that the observed differences may be task-induced. Additional experiments are currently underway to examine this possibility. (*Ethn Dis.* 2010;20[Suppl 1]:S1-28–S1-32)

Key Words: Hippocampus, Glutamate Transporters, GLAST, EAAC1, Glt-1

From Department of Physiology and Pharmacology, Ponce School of Medicine.

Address correspondence and reprint requests to Kenira J. Thompson; Department of Physiology and Pharmacology; Ponce School of Medicine; 395 Zona Industrial Reparada; Ponce, PR 00716-2348; kthompson@psm.edu

INTRODUCTION

Glutamate is the main excitatory neurotransmitter in the hippocampus. The main mechanism for termination of glutamatergic transmission is uptake of glutamate by specialized transporters localized mainly to adjacent astrocytes. Within the hippocampus, at least three of the five glutamate transporters appear to be localized within the synapses and immediate peri-synaptic areas: glutamate transporter 1 (Glt-1); excitatory amino acid carrier-1 (EAAC1); and glutamate-aspartate transporter (GLAST).[1] The Glt-1 is the main transporter for glutamate clearance in the brain. It has two identified splice variants: Glt-1, expressed by both neurons and astrocytes; and Glt-1b, expressed by astrocytes.[2,3] The EAAC1 is mainly localized to neurons,[4,5] whereas GLAST is found mainly in astrocytes.[6,7] All three, GLAST, EAAC1 and Glt-1, are conserved among several species,[8] and are important for glutamate homeostasis.

Glutamate uptake by its transporters appears to be important for the prevention of glutamate-induced neurotoxicity.[9] More recently, a possible role in synaptic plasticity has been suggested. Changes in glutamate uptake and differential translocation of EAAC1 to the plasma membrane have occurred in the hippocampus after long-term potentiation (LTP) induction and contextual fear conditioning.[10] In addition, previous studies showed an increase in Glt1 expression after *in vivo* induction of LTP at the mossy fiber-cornus ammonius 3 (MF/CA3) area of the hippocampus in rats,[11] suggesting a possible role of this transporter in MF/CA3 plasticity. Because plasticity is a necessary process for learning and memory, this study, using the Morris water maze task, investigates whether modulation of glutamate transporters in the hippocampus occurs during the different stages of spatial learning.

METHODS

Animals and Behavior

Male Sprague Dawley (approx. 3 mo. old, 350–400g) rats were obtained from the Ponce School of Medicine Animal Care Facility and housed in pairs on a 12 hour light-dark cycle. Food and water was provided *ad libitum*. To reduce stress-mediated effects on behavior, animals were handled for one week before training. Each rat was randomly assigned to a learner (swim with platform), swimmer (swim without platform) or naïve (don't swim) group and trained in the water maze using a one-day protocol.[12] On training day, learner rats had to find a hidden transparent platform submerged 3 cm from the surface of the water of a 6 ft diameter pool. The rats underwent one, two or three blocks of training with one hour rest between blocks. In each block of training the learner rats underwent four 60 second trials (or until platform reached) with the platform located at the fourth quadrant. If after 60 sec the rat did not find the platform, it was gently directed towards it and allowed to sit for 15 sec on the platform before being removed from the pool, towel-dried and allowed to rest for 60 sec. After the last training trial, an additional trial (probe trial) was performed in which the rats were placed in the pool and allowed to swim for 60 sec without the platform. Swimmer rats swam without the platform for the duration of each trial (60 sec). Rats in the naïve group were not exposed to the water

maze and remained in their home cages for the duration of experiment. Each trial was recorded using a ceiling-mounted video camera connected to a computerized tracking/imaging analyzer system (HVS Image, Hampton, UK; Watermaze Software, Edinburgh, UK).

At the end of the last trial each rat was deeply anesthetized with sodium Nembutal solution (100 mg/kg; ip) and decapitated. The brain was removed and the hippocampi dissected out whole and flash frozen for further processing.

Tissue Processing and Real Time Polymerase Chain Reaction

RNA was isolated with Trizol Reagent as described by the manufacturer (Invitrogen, Carlsbad, CA). Then 1 μg of RNA from each sample was reversed transcribed using iScript cDNA Synthesis Kit (Bio-Rad Labs, Hercules, CA). A total of 3.75 ng of cDNA and 200 nM of primer were used for the real time (RT) polymerase chain reaction (PCR) using iQ SYBR Green Supermix (Bio-Rad Labs, Hercules, CA). The PCR and detection were accomplished in a iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad Labs, Hercules, CA). A dissociation curve protocol was performed at the end of the amplification following typical procedures. All amplifications were run in triplicate with a no-template control for each primer pair. The target gene expression levels was computed by comparative C_T method (against hipoxantine-guanine phosphoribosyl transferase[HPRT]) and the data transformed by the formula $2^{-\Delta Ct}$.

Primer Design

The sequence used for the HPRT gene primers were those described by Pennequin, et al.[13] Each of the reminding primer was designed after the known mRNA sequences published at the Nucleotide tool of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Prim-

ers for Glt1-a, Glt1-b, GLAST and EAAC1 were designed using BioEdit software (Ibis Bioscience, Carlsbad, CA) and care was used to prevent homology areas between the different glutamate transporter genes. The sequences and expected product sizes were as follows: (1) Glt1-a forward (F) 5′-TGTTGAG-GAAGAACCTTGGAAACGTG-3′, reverse (R) 5′-AGCATCTTTTACCA-TAAGATACGCTGG-3′; 96 bps (2) GLT1-b F 5′-CAATTTTCTTCACT-CT TTGGGCCAAG-3′, R 5′-TTGG-TCTACATATTTTTCTGACCC-TTCC-3′; 105 bps (3) GLAST F 5′-GACATTTCTCTCTAGGGGCAG-GCTG-3′, R 5′-GGTTTGGGAA-GAGTACCCAAGAAGG-3′; 187 bps (4) EAAC1 F 5′-AACTGTTAGGAT-TAGGTCATGGGTC TGG-3′, R 5′-AATTGGGCACTAGTTGACT-GGGTGG-3; 197 bps. (Annealing temperature = 61°C). Each primer was used for RT-PCR of hippocampal tissue to confirm the size of the products by running the resulting reaction mix on an agarose gel.

Statistics

Statistical analysis was performed using SigmaStat Software (Point Richmond, CA). One-Way ANOVA, One-Way Repeated measures ANOVA or *t*-tests were performed on particular data as indicated in the figure legends. Holm-Sidak test was performed as post-hoc analysis for each significantly different ANOVA.

RESULTS

Rats Learned the Location of the Platform During Training in the Morris Water Maze

With each block of training, rats in the learner group took progressively less time to reach the target platform (fig. 1A). In addition, by the end of block 2, learner rats preferred the target quadrant significantly more than swim control rats, whereas swim control rats

maintained the same preference across training.

EAAC1 and GLAST Expression Were Downregulated During Learning of the Morris Water Maze Task

Glutamate transporter expression of the learner group was compared to that of two controls, naïve and swim control. GLAST expression was decreased compared to naïve after the first and second block of training when memory acquisition is presumably taking place (Fig. 2A). A similar pattern was observed for EAAC1 (Fig. 2B), in which expression was decreased by the end of the first and second block. However, this down-regulation was also observed in the swim control group for both EAAC1 and GLAST, suggesting that the differences were task related. The levels of EAAC1 and GLAST RNA returned to control by the end of the third block.

Glt-1b Expression Was Downregulated During Learning

Glt-1b expression was also decreased compared to naïve control in both learner and swim control groups after the second block of training (Fig. 2D). Its expression returned to control levels by the third block of training. No differences were observed in Glt-1 expression levels (Fig. 2C).

DISCUSSION

Our results indicate a transient decrease in EAAC1 and GLAST glutamate transporter RNA expression during the early stages of a spatial learning task (20 min and 80 min after initiation of training). This decrease returned to baseline levels by the end of training (140 min). The transient decrease in expression suggests that regulation of transporter expression may be important during the acquisition phase of learning. By decreasing the expression of
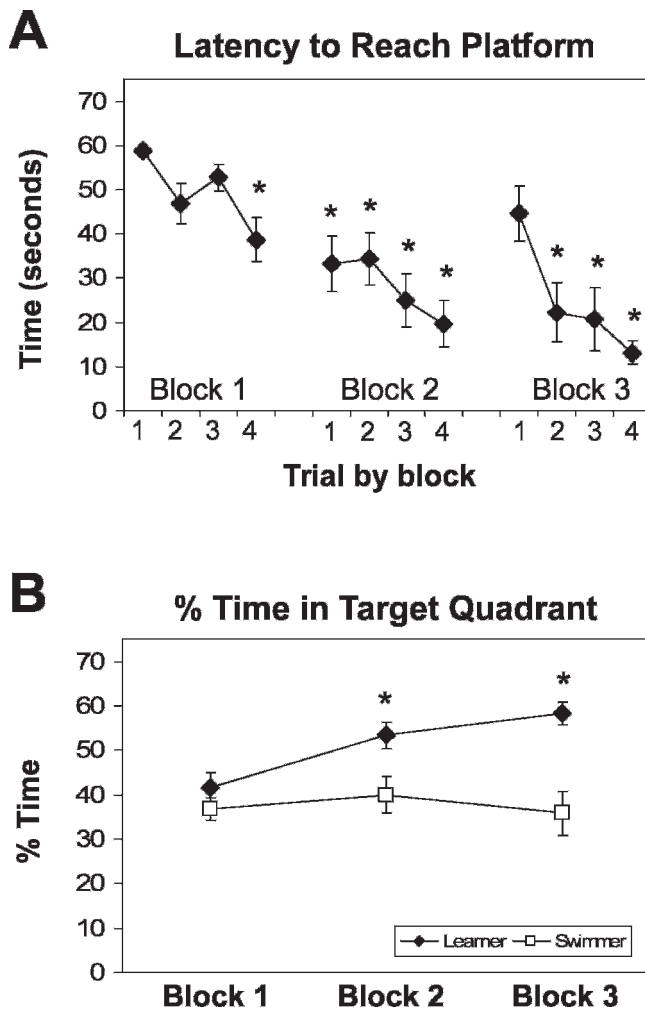
Fig 1. Rats learned the position of the platform. The latency to platform of learner rats progressively decreased within blocks and with each trial of training; *$P>.001$ vs B1T1 (A). By block 2, learner rats spent more time in the platform quadrant than the swimmer rats and than in other quadrants *$P>.05$ vs B1 and vs swimmer (B) during the probe trial (no platform trial)

the glutamate transporters at early stages, more glutamate becomes available to activate receptors such as NMDA and mGluRs, known to be necessary for the early stages of hippocampal LTP.[14–17] In fact, similar transient changes in expression of the NR1 subunit of the NMDA receptor were observed in the hippocampus 30 min but not 120 min after inhibitory-avoidance learning.[18] Also, changes occurred in RNA expression of glutamate receptor subunits and of the Glt1 transporter following learning and LTP.[19,20]

One limitation of our study is that while we observed changes in RNA expression of the transporters, we do not know if these changes are paralleled by changes in transporter protein expression or activity. However, our results support recent *in vitro* studies indicating that increased glutamate concentrations induce a cell-death independent down-regulation of GLAST and Glt-1 protein.[21] Additionally, increases in glutamate concentration in the ventral hippocampus occur during the acquisition phase of inhibitory avoidance learning,[22] further validating the importance

of glutamate modulation during early learning.

Our results indicate a decreased glutamate transporter expression after a spatial learning task, yet other studies have shown increases in glutamate transporter expression after hippocampal plasticity. For example, LTP induction in area CA1 of the hippocampus results in increased glutamate uptake and increased expression of EAAC1, and GLAST 30 min after induction.[10] Moreover, during contextual fear conditioning, increases in uptake and surface expression of EAAC1 were observed 24 hours after conditioning. Although differences in protocol (learning paradigm, and assessment of protein vs. RNA) could account for the contradicting results, it is also possible that the difference in time-point assessment and of learning phase is responsible for the variations. In fact, other studies that observed changes in glutamate uptake and expression during contextual fear conditioning examined changes 24 hours after conditioning (presumably after consolidation) and no post-training (acute) data is available.[10] Our study is the first to examine RNA levels immediately after training and during the acquisition phase of learning, thus contributing significantly to understanding the effects of acute modulation of glutamate transmission during early stages of learning. Currently, we are examining whether the transporter protein changes in parallel with RNA after Morris Water Maze learning.

Our work describes the time-dependent expression pattern of glutamate transporter RNA during early stages of spatial learning. Much evidence shows that a tight regulation of various molecular events and the timing of these events are important for the induction of synaptic plasticity, such as in the case of LTP. For example, inhibiting the NMDA receptor during LTP induction results in abolishment of LTP, but had no effect if the inhibitors were applied later (for review).[23] In
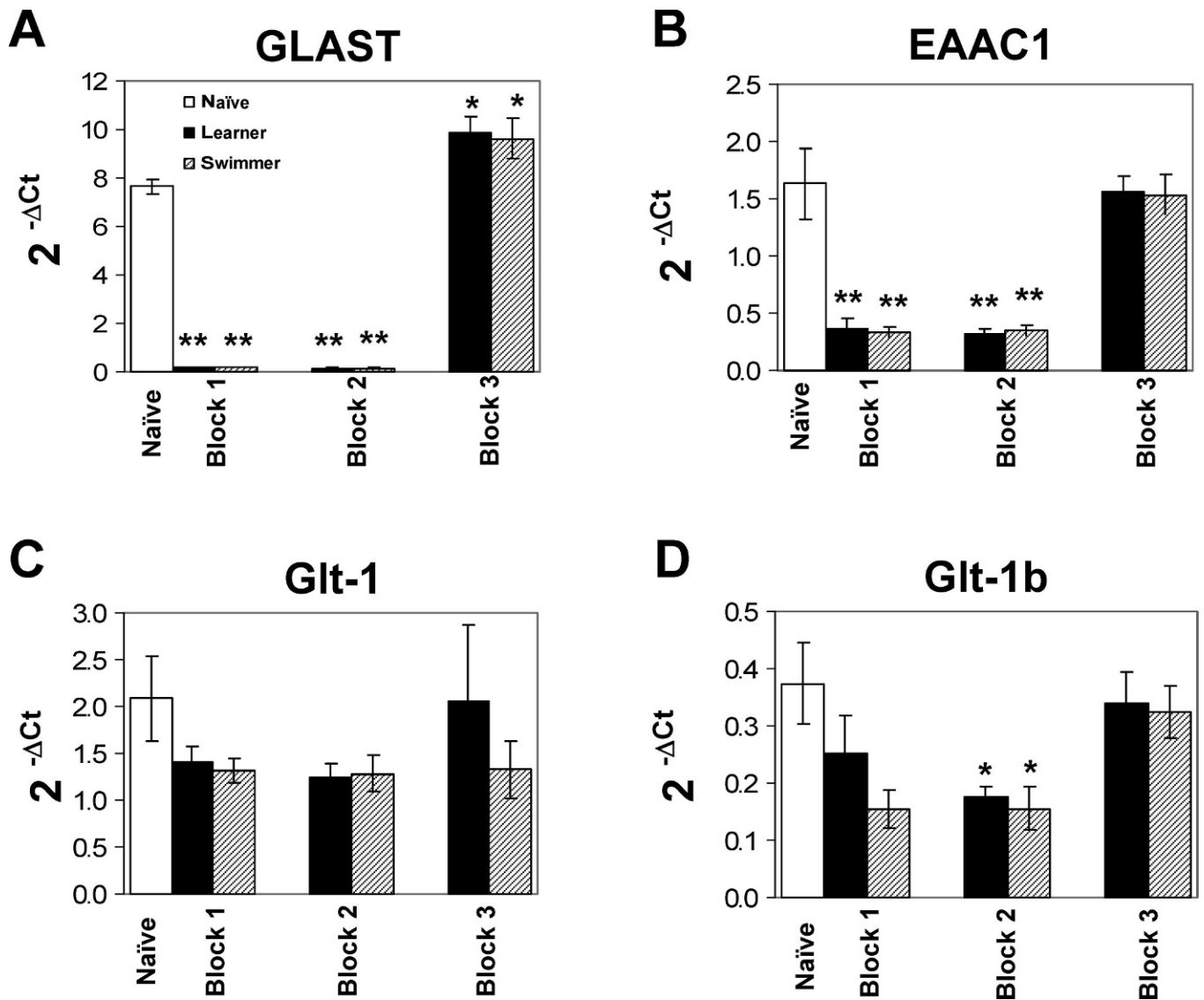
Fig 2. Glutamate transporter expression after water maze learning. RNA expression of GLAST (A) and EAAC1 (B) was down-regulated in learner and swimmer rats compared to naïve after the first and second block of training. GLAST expression was up-regulated by the third and last block. Glt-1 expression did not differ between groups (C) whereas Glt-1b was down-regulated by the second block but returned to control levels by the third block (D). ΔCt = cycle threshold of transporter gene − cycle threshold of HPRT housekeeping gene. *$P>.05$ vs naïve **$P>.001$ vs naïve

addition, both pre-synaptic changes such as increased probability of transmitter release and post-synaptic changes seem to occur during LTP.[24] Glutamate transporters regulate clearance of glutamate at synapses, and are modified in the hippocampus during LTP.[10] Our results indicate a clear alteration of glutamate transporter RNA expression during the early stages of a spatial learning task, thus supporting the idea

that regulation of glutamate concentrations is essential at all stages of spatial learning.

Our results also show decreased EAAC1 and GLAST expression following swimming (no learning), which implies that these changes are not due to spatial learning but are task related instead. One possibility is that the changes observed are due to the physical activity inherent to the task. In fact,

voluntary exercise enhances synaptic plasticity in the hippocampus, and improves performance in the water maze and consolidation of hippocampus-dependent memories.[25,26] In addition, environmental enrichment has effects similar to those of exercise on learning processes.[27,28] Due to the exposure to a novel task, handling, and visual cues in the room, exposure to the water maze may constitute environmen-

tal enrichment on its own. If a down-regulation of glutamate transporters is involved in improved learning and synaptic plasticity and exercise also results in such a reduction, it is possible that glutamate transporters may modulate the effects of exercise on learning. In that case, similar changes would be expected in glutamate transporter expression in both swimmer (no learner) and learner groups. Additional experiments in our lab are ongoing to determine whether the physical activity during the one-day water maze training protocol also results in enhancement of synaptic plasticity and learning.

Our findings provide important information regarding glutamate transporter modulation on hippocampal learning, which could be essential for the development of novel pharmacotherapies to treat hippocampal-related memory disorders, which are prevalent in Hispanic populations.

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