

GABA Uptake via GABA Transporter-1 Modulates GABAergic Transmission in the Immature Hippocampus

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GABA uptake limits GABA actions during synaptic responses when the density of active release sites is high or multiple axons are synchronously activated. GABA transporter-1 (GAT-1) is the main neuronal GABA transporter subtype and is already expressed in the early postnatal rat hippocampus. However, previous studies have demonstrated little functional role for the transporter during this developmental period. We used whole-cell voltage-clamp and field-potential recordings in hippocampal slices of neonatal rats (postnatal day 4–5) to study whether GAT-1 plays a role in GABAergic transmission during spontaneous population oscillations, which are seen as “giant depolarizing potentials” (GDPs) in intracellular recordings. We show that the GDP-associated GABAergic current observed in CA3 pyramidal neurons is strongly enhanced by the GAT-1-specific blocker NO-711 (1-[2-[[[(diphenylmethylene)imino]oxy]ethyl]-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid hydrochloride]). Our results indicate a novel role for GAT-1 in the control of endogenous activity of the immature hippocampus.

Key words: GAT-1; development; hippocampus; network; activity; GDP

Introduction

Na⁺-dependent uptake regulates the ambient level of GABA in the interstitial space in brain tissue (Frahm et al., 2001; Nusser and Mody, 2002; Semyanov et al., 2003). In the mature brain, blocking GABA uptake pharmacologically (Thompson and Gähwiler, 1992) or by genetically disrupting the expression of GABA transporter-1 (GAT-1) (Jensen et al., 2003) has little effect on spontaneous GABA_A-mediated IPSCs (sIPSCs) or miniature GABA_A-mediated IPSCs (Nusser and Mody, 2002; Overstreet and Westbrook, 2003). However, where the density of active release sites is high, unitary IPSCs elicited by single presynaptic action potentials are prolonged (Overstreet and Westbrook, 2003). Moreover, evoked IPSCs as a result of stimulation of multiple axons and consequent GABA spillover are limited by cellular uptake (Thompson and Gähwiler, 1992; Isaacson et al., 1993; Roepstorff and Lambert, 1994).

GAT-1 is the main neuronal GABA-transporter subtype (Guastella et al., 1990) and is abundant in presynaptic GABAergic terminals (Minelli et al., 1995). Although it is expressed already at birth (Yan et al., 1997; Frahm and Draguhn, 2001), there is little evidence for any functional role for GAT-1 in neuronal signaling during the early postnatal period in the rat hippocampus (Draguhn and Heinemann, 1996; Caillard et al., 1998; Sabau et al., 1999; Demarque et al., 2002).

A salient feature of endogenous neuronal activity in the immature hippocampus is the presence of spontaneous population

oscillations, which were originally observed in intracellular voltage recordings in pyramidal neurons and termed “giant depolarizing potentials” (GDPs) (Ben-Ari et al., 1989; Leinekugel et al., 2002). This type of synchronous network event is thought to be involved in the maturation of neuronal circuits (Feller, 1999; Penn and Shatz, 1999; Katz and Crowley, 2002). In this work, we show that although it has no or very little effect on single sIPSCs in early postnatal hippocampal slices, blocking GAT-1 leads to a marked enhancement of GABA_A receptor (GABA_AR)-mediated transmission during GDPs.

Materials and Methods

Postnatal day 4–5 Wistar rat pups were decapitated, and brains were quickly removed and dissected in cold (0–4°C), oxygenated (95% O₂–5% CO₂) standard solution, containing (in mM): 124 NaCl, 3.0 KCl, 2.0 CaCl₂, 25 NaHCO₃, 1.1 NaH₂PO₄, 1.3 MgSO₄, and 10 D-glucose, pH 7.4 at 32°C. For whole-cell recordings, coronal brain slices (350 μm) were cut with a vibrating blade microtome (VT1000S; Leica, Nussloch, Germany), bisected into two hemispheric components, and allowed to recover for at least 1 hr at 32°C before use.

Individual slices were transferred into a submersion-type recording chamber, perfused (rate, 3–4 ml/min) with oxygenated (32–33°C) standard solution, and anchored with platinum wires on the bottom of the chamber. Axopatch 200B (Axon Instruments, Union City, CA) and EPC-10 (HEKA Elektronik, Lambrecht, Germany) amplifiers were used for voltage-clamp recordings in whole-cell mode. Patch pipettes had resistances of 5–7 MΩ when filled with (in mM): 140 Cs-methanesulfonate, 2 MgCl₂, and 10 HEPES, pH 7.2 with CsOH. Series resistance and whole-cell capacitance were estimated by compensating for the fast current transients associated with 5–10 msec, 5 mV voltage steps. Series resistance [15.6 ± 0.9 MΩ (mean ± SEM)] was left uncompensated or was compensated by 60–70% with a lag of 6–9 μsec. In voltage-clamp recordings, the holding potential was 0 mV when corrected using a calculated 13 mV liquid junction potential. Assuming a 0.3 permeability ratio for bicarbonate versus Cl⁻, the calculated reversal

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potential of GABA_A-mediated responses ($E_{\text{GABA-A}}$) is approximately -75 mV under the present conditions (Kaila et al., 1993). Using infrared video microscopy (Stuart et al., 1993), CA3 pyramidal neurons were selected for recordings on the basis of the orientation of soma in the pyramidal layer and with the apical dendrite pointing toward the stratum lucidum. A bipolar electrode was placed in the stratum lucidum at a distance of 100–300 μm from the recording site to deliver stimuli (100 μsec , 15–50 V) in trains (9–30 pulses, 10 Hz) at 90 sec intervals. Field-potential (fp) recordings were performed with a glass capillary electrode (tip diameter, ~ 10 μm) filled with 150 mM NaCl.

2,3-Dihydroxy-6-nitro-7-sulfonyl-benzo[*f*]quinoxaline (NBQX), D,L-AP-5, (2S)-3-[[[(15S)-1-(3,4-dichlorophenyl)ethyl]amino-2-hydroxypropyl]phenylmethyl]phosphinic acid (CGP), tetrodotoxin (TTX), and bicuculline methobromide (bic) were from Tocris Cookson (Bristol, UK). 1-[2-[[[(diphenylmethylene)imino]oxy]ethyl]-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid hydrochloride (NO-711) was from Sigma (St. Louis, MO).

Data were stored on tape and low-pass filtered at 2 kHz before digitization at 5 kHz for analyses with Clampfit 8.0 (Axon Instruments) and Strathclyde Electrophysiology Windows Whole Cell Program and Windows Electrophysiology Disk Recorder (WinEDR) (Dr. John Dempster, University of Strathclyde, Glasgow, UK) programs. For analysis of spontaneous IPSCs, events during inter-GDP intervals with a peak amplitude of >6 pA were detected with an algorithm in WinEDR (Strathclyde Electrophysiology Software). sIPSCs that appeared to consist of more than one event were rejected. These amounted to $\sim 35\%$ of the total number originally detected, with no significant difference between control and NO-711 application. For averaging, signals were aligned by the rising phase. Averaged sIPSCs were fitted with two exponential functions, and weighted decay time constants (τ) were calculated using the equation $\tau = A_1\tau_1 + A_2\tau_2$, where A_i is the relative amplitude and τ_i is the time constant of each component (Overstreet and Westbrook, 2003). The fp GDPs were aligned by the onset of the fast negative phase for averaging. Data are expressed as mean \pm SEM. Quantitative comparisons were based on paired Student's *t* tests, for which *p* values of <0.05 were considered to be statistically significant.

Results

A stimulus train protocol (9 pulses, 10 Hz) was used to trigger synchronous firing of interneurons and to promote synaptic release of GABA. NBQX (10 μM), D,L-AP-5 (40 μM), and CGP (1 μM) were applied to exclude effects involving ionotropic glutamate and GABA_B receptors. The cells were clamped at a holding potential of 0 mV, whereas calculated $E_{\text{GABA-A}}$ is approximately -75 mV (see Materials and Methods). In addition to IPSCs triggered by individual stimulus pulses, the train of stimuli induced a gradually increasing, outwardly directed baseline current followed by a slow decay (Fig. 1A). The mean time of decay to 30% from the instantaneous level of the slow current at the time of the last stimulus was 630 ± 130 msec. The mean total charge transfer after the last pulse in the stimulus train was 47 ± 20 pC ($n = 6$). The train-evoked slow outward current was greatly enhanced by bath application of the GAT-1-specific inhibitor NO-711 (2.5 μM), with its duration increasing to $350 \pm 40\%$ ($p = 0.0015$) and total charge transfer increasing to $260 \pm 59\%$ ($p = 0.044$) from the control value ($n = 6$) (Fig. 1A,B). The IPSCs and the slow outward current were blocked by 10 μM bicuculline, confirming that both were mediated by GABA_A receptors ($n = 3$) (Fig. 1A). Additional experiments were performed in extracellular solution containing 0.5 mM Ca^{2+} and 5 mM Mg^{2+} (Berry and Pentreath, 1976) in the presence of the glutamate- and GABA_B-receptor blockers to reduce possible self-sustaining excitatory effects within the interneuronal network. However, a train of stimuli (30 pulses, 10 Hz) again induced a current that was prolonged by bath application of 2.5 μM NO-711 (Fig. 1C). A 1 μM concentration of TTX blocked the stimulus-induced currents ($n = 3$), in-

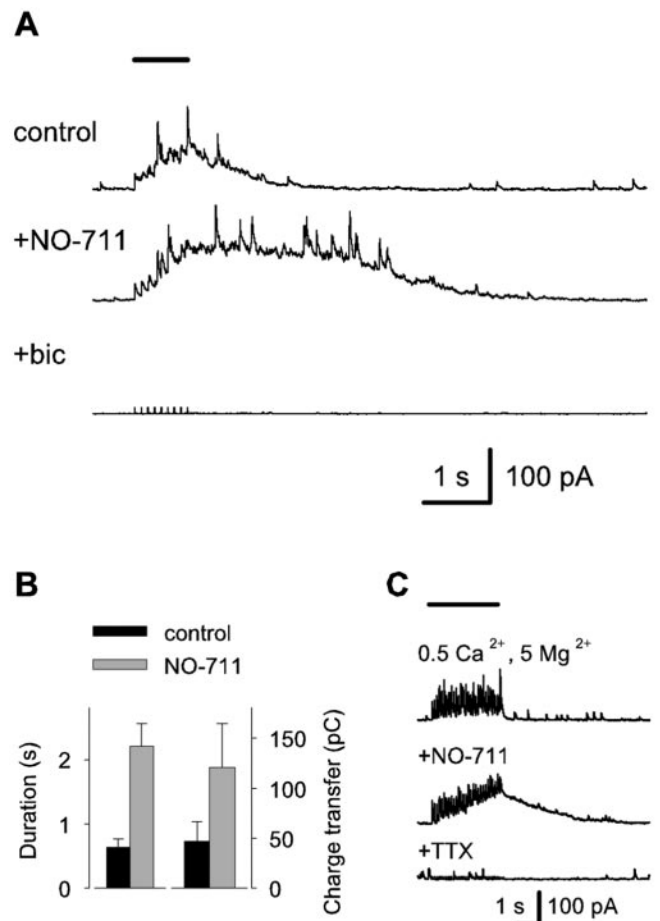


Figure 1. Blocking GAT-1 enhances stimulus train-induced slow GABA_A-mediated currents in neonatal CA3 neurons. *A*, Specimen voltage-clamp recordings at a holding potential of 0 mV with a low chloride pipette filling solution showing currents evoked by a train of stimuli (9 pulses, 10 Hz) indicated by the horizontal bar shown at the top (stimulus artifacts are truncated). All recordings were done in the presence of 10 μM NBQX, 40 μM D,L-AP-5, and 1 μM CGP. NO-711 was applied at 2.5 μM , and bic was applied at 10 μM . *B*, Bar graph showing the duration (defined as time of decay to 30% from the instantaneous level) of the slow current at the time of the last stimulus pulse and the mean total charge transfer after the last pulse ($n = 6$ recordings). Events occurring >4 min after the start of NO-711 application were included in the quantification. *C*, Voltage-clamp recordings showing currents evoked by a train of stimuli (30 pulses, 10 Hz; horizontal bar) in a solution containing 0.5 mM Ca^{2+} plus 5 mM Mg^{2+} (top trace), with 2.5 μM NO-711 (middle trace), and subsequent addition of 1 μM TTX (bottom trace).

dicating that they are dependent on action potential firing and not on nonvesicular GABA release (cf. Demarque et al., 2002).

Next, we investigated whether GABA uptake would be involved in endogenous network activity, which is characteristic of the neonate hippocampus (see Introduction and Ben-Ari et al., 1989; Leinekugel et al., 2002). In the absence of the glutamate- and GABA_B-receptor blockers, voltage-clamp recordings showed spontaneous bursts of outwardly directed GABAergic currents (Fig. 2A) that will be called GDPs (Ben-Ari et al., 1989). The mean duration (time between currents at 10% of the peak amplitude during event onset and decay) of these events was 620 ± 60 msec, and the total charge transfer was 69 ± 21 pC ($n = 6$) (Fig. 2B). Bath application of 2.5 μM NO-711 increased GDP duration to $230 \pm 27\%$ ($p = 0.005$) and total charge transfer to $187 \pm 20\%$ ($p = 0.007$) of the control level ($n = 6$) (Fig. 2A,B). Blocking GAT-1 preferentially exerted its effect on the decaying phase of GDPs. The 90–10% decay time in NO-711 was enhanced to

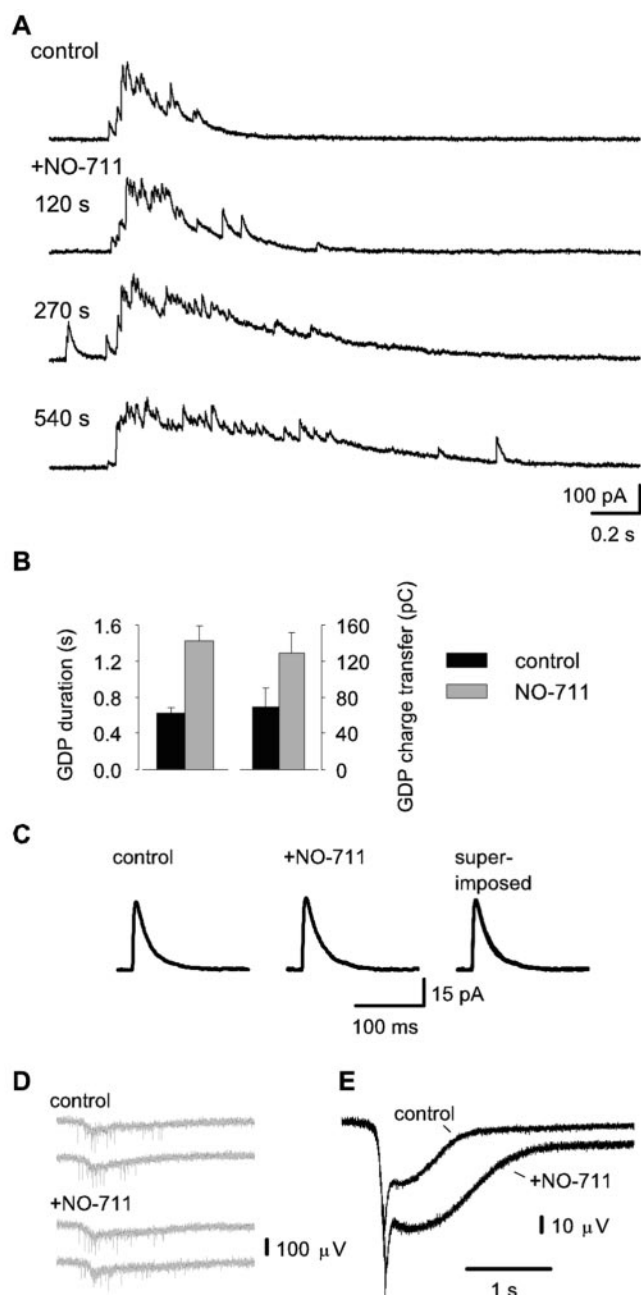


Figure 2. GAT-1 regulates GABAergic transmission during GDPs. *A*, Voltage-clamp recordings showing spontaneous bursts of GABA_AR-mediated outward currents under standard conditions and the effect of 2.5 μ M NO-711. *B*, Bar graph showing GDP duration and the associated total charge transfer from six recordings. Events occurring >4 min after the start of NO-711 application were included in the quantification. *C*, NO-711 has no detectable effect on sIPSCs. Averaged recordings of 46 sIPSCs in control and 36 recordings in the presence of 2.5 μ M NO-711. *D*, Specimen traces showing spontaneous fp responses associated with GDPs in control and in the presence of 2.5 μ M NO-711. *E*, Averaged recordings of 33 spontaneous fp responses both in control and in the presence of 2.5 μ M NO-711

237 \pm 25% versus control (462 \pm 76 msec; p = 0.003), whereas the 10–90% rise time was not statistically different from control.

In accordance with previous results in mature tissue (Thompson and Gähwiler, 1992; Nusser and Mody, 2002; Overstreet and Westbrook, 2003), 2.5 μ M NO-711 had no statistically significant effect on the mean total charge transfer (1.1 \pm 0.07 pC in control and 1.1 \pm 0.02 pC in NO-711), peak amplitude (37 \pm 5.6 pA in control and 33 \pm 4.8 pA in NO-711), or weighted decay τ (23.8 \pm

2.9 msec in control and 27 \pm 4.2 msec in NO-711) of averaged sIPSCs (330 and 325 single sIPSCs in control and in the presence of NO-711, respectively, from six recordings) (Fig. 2*C*). The average sIPSC frequency during inter-GDP intervals under control conditions (2.5 \pm 0.8 Hz) was not different from that seen in the presence of 2.5 μ M NO-711 (2.0 \pm 0.6 Hz).

In agreement with the intracellular recordings (Fig. 2*A*), 2.5 μ M NO-711 had no detectable effect on the initial fast phase of the spontaneous fp responses associated with GDPs, but it enhanced the mean amplitude of their slower phase by a factor of 1.3 \pm 0.1 (28 \pm 7 μ V in control; p = 0.05; 336 and 193 single fp GDPs in control and in the presence of NO-711, respectively, from five recordings) (Fig. 2*D,E*). In addition, a marked increase in the duration of the GDP-associated fp responses was seen, with a mean time to 90% decay of 4.0 \pm 1.3 and 2.3 \pm 0.9 sec in the presence and absence of NO-711, respectively (p = 0.03). However, the increase in the field response was not accompanied by any clear change in the rate of unit spiking (Fig. 2*D*). Finally, the field recordings indicated a decrease in GDP frequency in NO-711 to 51 \pm 16% of the value in control (0.022 \pm 0.006 Hz; p = 0.036). The decrease in GDP frequency was not studied further, but it may well reflect an increase in the time needed to restore neuronal Cl⁻ gradients after the enhanced dissipation (and a consequent reduction in the driving force for excitatory GABA responses) that is likely to take place during the NO-711-mediated increase in GDP amplitude and duration (cf. Chub and O'Donovan, 2001).

Discussion

The present data indicate that GAT-1 modulates synaptic transmission under physiological conditions in the early postnatal rat hippocampus. In particular, GAT-1 operation limits the duration of extracellular GABA transients during synchronous pyramidal and interneuronal firing associated with GDPs, which constitute the main type of electrophysiological activity in the immature hippocampus both *in vitro* and *in vivo* (Ben-Ari et al., 1989; Leinekugel et al., 2002).

In the work by Draguhn and Heinemann (1996), IPSCs elicited by single stimuli in the early postnatal dentate gyrus were not affected by the GABA uptake blocker tiagabine. The authors suggested that the relatively large extracellular space characteristic of immature brain tissue, permitting effective net diffusion, is responsible for clearing GABA from synaptic clefts. Our results are not in direct contradiction with this idea. However, a dramatic effect of blocking GABA uptake was observed after more intense synaptic activity.

In mature neurons, a decrease in peak IPSC amplitude has been observed after inhibition of GAT-1 and has been explained by receptor desensitization (Overstreet et al., 2000). The lack of a statistically significant effect with NO-711 on sIPSC amplitude in the present study is in agreement with the smaller sensitivity of GABA_A receptors in immature neurons to desensitization (Cherubini et al., 1990).

The GAT-1 protein is primarily located in presynaptic GABAergic terminals (Minelli et al., 1995), but during early postnatal development of the rat hippocampus, it is seen also in somata of interneurons (Yan et al., 1997). In addition, GAT-1 mRNA has been detected in principal neurons (Frahm and Draguhn, 2001). The stoichiometry of GAT-1 in the uptake mode has been suggested to be 1 GABA:2 Na⁺:1 Cl⁻ (Cammack et al., 1994). Although the exact ionic milieu surrounding GAT-1 is not known, the relatively high intracellular chloride concentration of immature neurons (Kakazu et al., 1999; Kuner and Augustine,

2000; Payne et al., 2003) could potentially inhibit the uptake mode. However, we observed an increase in the GABA_AR-mediated currents in response to GAT-1 block, which suggests that under physiological conditions, it operates in net uptake mode in immature tissue as well.

The results as a whole support the idea that GAT-1 prevents GABA spillover to extrasynaptic sites and cross-talk between synapses (Thompson and Gähwiler, 1992; Isaacson et al., 1993; Barbour and Hausser, 1997) during intense interneuronal firing that is associated with early network activity in the immature hippocampus, and probably in other brain structures as well. Given the key role of GABA in the generation of immature-type spontaneous network activity (Owens and Kriegstein, 2002), the dramatic influence of blocking GAT-1 on GDPs suggests that GABA uptake may be intimately involved in activity-dependent neuronal wiring. More specifically, GABA uptake might promote independence of GABAergic synapses (Overstreet and Westbrook, 2003) in developing brain structures.

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