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Disinhibition reduces extracellular glutamine and elevates extracellular glutamate in rat hippocampus in vivo

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Abstract

Disinhibition was induced in the hippocampal CA1/CA3 region of normal adult rats by unilateral perfusion of the GABA_AR antagonist, 4-[6-imino-3-(4-methoxyphenyl)pyridazin-1-yl]butanoic acid hydrobromide (gabazine), or a GABA_BR antagonist, *p*-(3-aminopropyl)-*p*-diethoxymethylphosphinic acid (CGP 35348), through a microdialysis probe. Effects of disinhibition on EEG recordings and the concentrations of extracellular glutamate (GLU_{ECF}), the major excitatory neurotransmitter, and of extracellular glutamine (GLN_{ECF}), its precursor, were examined bilaterally in freely-behaving rats. Unilateral perfusion of 10 μ M gabazine in artificial CSF of normal electrolyte composition for 34 min induced epileptiform discharges which represent synchronized glutamatergic population bursts, not only in the gabazine-perfused ipsilateral hippocampus, but also in the aCSF-perfused contralateral hippocampus. The concentration of GLU_{ECF} remained unchanged, but the concentration of its precursor, GLN_{ECF}, decreased to $73 \pm 4\%$ ($n = 5$) of the baseline during frequent epileptiform discharges, not only in the ipsilateral, but also in the contralateral hippocampus, where the change can be attributed to recurrent epileptiform discharges per se, with recovery to 95% of baseline when epileptiform discharges diminished.

The blockade of GABA_BR, by CGP 35348 perfusion in the ipsilateral hippocampus for 30 min, induced bilateral Na⁺ spikes in extracellular recording. These can reasonably be attributed to somatic and dendritic action potentials and are indicative of synchronized excitatory activity. This disinhibition induced, in both hippocampi, (a) transient 1.6 to 2.4- fold elevation of GLU_{ECF} which correlated with the number of Na⁺ spike cluster events and (b) concomitant reduction of GLN_{ECF} to ~70%.

Intracellular GLN concentration was measured in the hippocampal CA1/CA3 region sampled by microdialysis in separate groups of rats by snap-freezing the brain after 25 min of gabazine perfusion or 20 min of CGP perfusion when *extracellular* GLN (GLN_{ECF}) was 60-70% of the pre-perfusion level. These intracellular GLN concentrations in the disinhibited hippocampi showed no statistically significant difference from the untreated control. This result strongly suggests that the observed decrease of GLN_{ECF} is not due to reduced glutamine synthesis or decrease in the rate of

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efflux of GLN to ECF. This strengthens the likelihood that reduced GLN_{ECF} reflects increased GLN uptake into neurons to sustain enhanced GLU flux during excitatory population bursts in disinhibited hippocampus. The results are consistent with the emerging concept that neuronal uptake of GLN_{ECF} plays a major role in sustaining epileptiform activities in the kainate-induced model of temporal-lobe epilepsy.

Keywords

Extracellular glutamine; extracellular glutamate; disinhibition; rat hippocampus; $GABA_A/GABA_B$ receptor antagonists; epileptiform discharge

1. INTRODUCTION

An imbalance between excitatory and inhibitory neurotransmission is a widely accepted candidate mechanism for epileptogenesis (reviewed by (Trevelyan and Schevon, 2013)). Epileptic seizures, according to well-supported theory, are caused by glutamate excitotoxicity when the major excitatory neurotransmitter glutamate is released into the extracellular fluid (ECF) faster than it is taken up into glia, leading to overstimulation of glutamate receptors (Bradford, 1995; During and Spencer, 1993). The CA3 region of the hippocampus (HC), which is highly populated with pyramidal glutamatergic neurons with recurrent networks, is especially susceptible to synchronized excitatory population bursts that result in glutamate excitotoxicity. CA3 pyramidal neurons innervate the dendrites of CA1 pyramidal cells. Normally, excitation of these glutamatergic neurons is under control of GABAergic inhibitory interneurons (Chrobak and Buzsáki, 1996). The axon terminals of GABAergic neurons target and inhibit the somatodendritic region and the axon initial segment of the pyramidal cells (Lovett-Barron et al., 2012; Ylinen et al., 1995). GABA, acting on ionotropic $GABA_A$ receptor on glutamatergic neurons, mediates fast inhibitory post-synaptic potentials via Cl^- influx, which results in hyperpolarization of the postsynaptic pyramidal cells and an increase in the threshold for firing. GABA can also act on metabotropic $GABA_B$ receptors. $GABA_B$ receptor activation in the hippocampal CA1/CA3 synaptic circuits is predominantly inhibitory because of the inhibition of glutamate release via presynaptic heteroreceptors (Biermann et al., 2010). Thus, pyramidal neurons are subject to two antagonistic polarizations; dendritic excitation from glutamatergic neurons and somatic/perisomatic inhibition from GABAergic interneurons. When inhibitory control is weakened or lost (disinhibition), massive depolarization of the target pyramidal neurons occurs, resulting in epileptiform discharges.

Our previous studies on the metabolic and pathophysiological bases of glutamate excitotoxicity showed that GLN_{ECF} , which upon take into neurons, serves as the precursor of the metabolic and neurotransmitter pools of GLU, is significantly reduced in response to electrographic seizures in kainate-induced rat model of temporal lobe epilepsy (Kanamori and Ross, 2011). This novel finding raised an intriguing possibility that neuronal uptake of GLN_{ECF} is accelerated during epileptic seizures to replenish the neurotransmitter pool of glutamate. To examine this hypothesis, the present study investigates the effects of glutamatergic population bursts induced by disinhibition on GLU_{ECF} and GLN_{ECF} .

Disinhibition was induced in normal adult rat hippocampus (HC) by unilateral perfusion of (a) the ionotropic GABA_AR antagonist, gabazine (Mienville and Vicini, 1987), or (b) the metabotropic GABA_BR antagonist CGP 35348 (Olpe et al., 1990). GABA_A antagonism induced epileptiform discharges and GABA_B antagonism induced complex Na⁺ spike clusters, not only in the treated ipsilateral, but also in the contralateral HC by transmission of the neuronal activity through the commissural fibers. Hence, in the aCSF-perfused contralateral HC, changes in GLU_{ECF} and GLN_{ECF} can reasonably be attributed to the occurrence of glutamatergic population bursts per se, without additional complex effects of perfusion as in the ipsilateral HC. The results show that GLN_{ECF} is significantly reduced in both disinhibition paradigms, while GLU_{ECF} is transiently elevated with GABA_BR blockade. Collectively, these results strongly suggest an important role for GLN_{ECF} in sustaining high flux of neurotransmitter glutamate during excitatory population bursts in disinhibited hippocampus.

2. MATERIAL AND METHODS

2.1. Implantation of EEG electrodes and microdialysis guide cannula

All studies were approved by the HMRI Institutional Animal Care and Use Committee in conformance with the NIH Guide for the Care and Use of Laboratory Animals. Adult male Wistar rats (275–400 g) were anesthetized with ketamine/xylazine (100/5.2 mg/kg wt) and placed on stereotaxic instrument. The EEG recording electrode consisted of a pair of stainless steel wires (0.125 mm in diameter, 20 mm in length and tips 0.5 mm apart) that were terminated with a pair of sockets (Plastics One, Roanoke, VA, USA). The grounding electrode was single wire of the same dimension. The recording electrode was attached (with Loctite 401) to a microdialysis guide cannula fitted with a stylet (Bioanalytical systems, West Lafayette, IN, USA), so that the electrode tips were 1.7 mm below the end of the guide cannula. This EEG electrode/microdialysis guide cannula complex was implanted bilaterally at coordinates of AP = −5.6 mm and L = ± 4.4 mm, and V = 5.4 mm for the electrode and 3.7 mm for the guide cannula. As shown in Fig. 1 inset, this places the electrode tips in the CA3 region, and the end of the microdialysis guide cannula in the CA1 region, within ~0.5 mm of the dentate gyrus. The grounding electrode was fixed to the parietal bone with an anchor screw. The sockets (one from the grounding electrode and two pairs from the recording electrodes) were inserted into the bottom contacts of a 6-pin plastic pedestal which was then cemented to the skull and capped.

2.2. EEG/microdialysis studies

Several days after the surgery, the rat was briefly anesthetized with pentobarbital (40 mg/kg wt), and the electrode contacts on the skull were connected to a 90-cm cable which is mesh-covered on proximal end and equipped with solder lugs on the distal end for connection to a 4-channel amplifier (A-M systems, Carlsborg, WA, USA) for bilateral recording. For microdialysis, the guide stylet was replaced with a 2-mm microdialysis probe (Fig. 1 inset). The probe spans CA1-CA3 region, with the bottom of the probe in the CA3 region and the top of the probe in the CA1 region, and collects extracellular fluid from hippocampal tissue ~700 μm in diameter and 2 mm in length. The rat was placed in RATTN (BioAnalytical Systems) with its collar attached to a balance arm, and the EEG cable and the inlet and

outlet dialysis tubings passed through an opening in its sensor. This allows for EEG recording and microdialysis in freely-behaving rats (Kanamori and Ross, 2013).

EEG recordings were taken broad-band with low- and high-frequency cutoff filters at 0.1 Hz and 10 KHz, impedance of 3 M Ω at 1 KHz, and sampled at 10 KHz/channel with a gain of 10K. Data were acquired and processed with DATAPAC 2K2 software (Run Technology, Mission Viejo, CA, USA). For microdialysis, the probe was perfused at a rate of 2 μ L/min with aCSF containing the following equivalents of electrolytes (mM); 150 Na⁺, 3.0 K⁺, 1.4 Ca²⁺, 0.8 Mg²⁺, 1 PO₄³⁻ and 155 Cl⁻ at pH 7.4. A 3-h stabilization period preceded dialysate collection. The rat usually awoke within 1 h of probe insertion, i.e. about 2 h before the start of dialysate collection. After collection of basal dialysates for 1.5 h, perfusion of gabazine or CGP was started, as described below. Accordingly, the interval between pentobarbital injection and the start of EEG recordings was about 30 min (allowing for time to connect EEG cables and microdialysis tubings), but only recordings from awake rat are shown in results. The interval between pentobarbital injection and the start of gabazine or CGP perfusion in awake rats was about 4.5 h (1 h till waking, additional 2 h for dialysate stabilization and 1.5 h for basal dialysate collection).

2.3. Perfusion of GABA_AR and GABA_BR antagonists

2.3.1. Perfusion protocol in subgroup I—In this group of rats, the antagonists were perfused as follows. Gabazine (SR95531, Sigma Aldrich; 10 μ M in aCSF at pH 7.4) was perfused into the ipsilateral (right) hippocampus through the microdialysis probe for 34 $\pm$ 2 min (n = 5) followed by co-perfusion with 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine hydrochloride (GYKI 52466, an AMPA receptor antagonist; 100 μ M in aCSF, pH 7.4) for 54 $\pm$ 2 min followed by 90-min washout with aCSF. The effect of co-perfusion with GYKI 52466 and of washout with aCSF on the frequency of incidence of epileptiform discharges was analyzed as follows: the number of epileptiform discharges during 34-min perfusion with gabazine was normalized to the number/30 min and set to 100% in each rat. Then the number of epileptiform discharges occurring in subsequent periods, i.e. the last 30 min of (a) the 54-min co-perfusion with GYKI 52466 and (b) the 90-min washout, was expressed as a percentage of the normalized number with data expressed as mean $\pm$ SE for n = 5 rats. Effluxing dialysates were collected and analysed for GLU_{ECF} and GLN_{ECF}.

CGP 35348 (abbreviated to CGP hereafter; Tocris Bioscience, purchased from R & D Systems, Minneapolis, Minnesota) was dissolved in aCSF and pH adjusted to 7.4. CGP was sequentially perfused through the microdialysis probe at 0.1 mM for 30 min followed by washout with aCSF for 62 min, then at 0.32 mM for 30 min followed by 62 min washout, then at 1 mM for 30 min followed by 30-60 min washout. Different doses were used to examine whether EEG characteristics or the concentrations of GLU_{ECF} and GLN_{ECF} depended on the dose. The completeness of washout was monitored by following the time-course of disappearance of CGP in the effluxing dialysate of the ipsilateral HC. In both gabazine and CGP experiments, the contralateral (left) HC was perfused with aCSF only. Dialysates, collected every 3 or 5 min during gabazine or CGP perfusion and every 10 min

thereafter, were stored at -20°C until analyses. In untreated controls, dialysates were collected every 10-15 min.

2.3.2. Perfusion protocol in subgroup II—In another group of rats (subgroup II), the experiment was stopped after perfusion of $10\text{ }\mu\text{M}$ gabazine for 25 min or of 0.1 mM or 1 mM CGP for 20 min (details in Sections 3.1.2 and 3.2.4) to freeze the brain for analysis of intrahippocampal GLN and GLU (Section 2.6). These shorter perfusion times were used in subgroup II because experiments in subgroup I showed that GLU_{ECF} elevation was significant (Section 3.2.2) and GLN_{ECF} decreased to the lowest level (Sections 3.1.2 and 3.2.4) at these time points. Hence, intrahippocampal GLN and GLU were examined at these time points.

2.4. Verification of electrode/cannula placement

After each microdialysis experiment, the rat was anesthetized and the brain snap-frozen and stored in liquid nitrogen. The locations of the microdialysis probe and the electrodes were confirmed in each rat as follows. The probe was removed and the guide stylet reinserted. The cement fixing the guide stylet and the EEG electrodes to the skull could be lifted from the skull of the anesthetized rat by inserting a flat spatula between the cement and the nasal bone. Accordingly, the cement with the electrodes and the guide cannula still attached could be examined for vertical coordinates in every rat. The lateral coordinates, too, could be confirmed from the distance between the right and left electrodes and the location of the burr holes on the exposed skull. The confirmed coordinates of the microdialysis probe and the EEG electrode (mean $\pm$ SE) are shown in Section 3.2.7.

2.5. HPLC assay

GLU_{ECF} , GLN_{ECF} and CGP were quantified by HPLC, using pre-column derivatization with ortho-phthaldehyde (OPA) and 2-mercaptoethanol, separation on a reverse-phase C_{18} column ($250 \times 4.6\text{ mm}$) by gradient elution with 50 mM sodium phosphate buffer ($\text{pH } 5.38$) and methanol, followed by fluorometric detection (Kanamori and Ross, 2011). The analytical column was protected with a guard column ($45 \times 4.6\text{ mm}$) which was changed after every ~ 30 injections to ensure good peak resolution. The time-course of change in GLU_{ECF} and GLN_{ECF} during perfusion (indicated by time T) was analyzed by expressing the concentration at T as percentage of the pre-perfusion concentration in each rat, then taking mean $\pm$ SE for 5 rats. When CGP was perfused at 1 mM concentration following washout with aCSF, the concentration during the last 5 min of washout (when CGP in the efflux dialysate was $< 1\%$ of the initial value), was set to 100% and the concentration at T expressed as percentage of that concentration.

2.6. Tissue concentrations of GLN and GLU in the hippocampal region sampled by microdialysis

The brain snap-frozen in liquid nitrogen after 25 min of gabazine perfusion or 20 min of CPG perfusion (Subgroup II) was placed in a large rat brain slicer (Zivic instruments, Pittsburgh, PA, U.S.A.) and 2-mm coronal slices were prepared at -10°C . Hippocampal tissue with coordinates of $\text{AP} = -4.8$ to -6.8 mm , $\text{L} = \pm 4.0$ to 6.0 mm and $\text{V} = 3.0$ to 6.0 mm and visually identified as hippocampal formation, was sectioned bilaterally, placed in a

pre-cooled pre-weighed vial and immediately frozen in liquid nitrogen. This tissue contains the CA1 and CA3 regions of the hippocampus where gabazine or CPG was perfused (ipsilateral) and dialysates collected. For extraction of brain metabolites, each tissue was weighed, suspended in 75 μ L of 5 mM phosphate buffer at 4 °C and homogenized by sonication. After addition of perchloric acid to a final concentration of 7% (v/v), the homogenate was centrifuged at 12,850g for 10 min. The supernatant was neutralized with KOH (0.2 M to 2 M). After precipitating KClO₄, the supernatant containing brain metabolites was assayed for GLU and GLN by HPLC.

2.7. Statistical analyses

Repeated-measures ANOVA with Tukey HSD post hoc test (significant difference at $p < 0.05$ is indicated by * and at $p < 0.01$ by **) was used to examine whether the mean value of GLU_{ECF} or of GLN_{ECF} during perfusion differs significantly from the pre-perfusion concentration (Fig. 2) or from non-perfused controls (Figs. 4-5).

3. RESULTS

3.1. Disinhibition by GABA_AR blockade

3.1.1. Epileptiform discharges are induced in adult rat brain in vivo at physiologically normal electrolyte concentrations—Fig. 1 shows representative EEG recordings from the HC of a freely-behaving rat. In the ipsilateral HC, the perfusate was changed from (A) aCSF (baseline) to (B) gabazine, (C) gabazine + GYKI 52466, and (D) aCSF. The contralateral HC was perfused with aCSF only. Upon gabazine perfusion (Fig. 1B), epileptiform discharges appeared synchronously in both HC at 8.2 ± 0.53 min ($n = 5$) after the onset of perfusion. A typical wave in the epileptiform discharge (small open arrow and expanded in plot 1) had a linewidth at half-height of ~ 70 ms and an amplitude of ~ 0.6 mV. In this rat, epileptiform discharges initially occurred every ~ 9 s, then less frequently, and after a quiescent period, there was a burst of activity (Box) with waves occurring at a maximum frequency of every 0.5 to 1 sec (expanded plot 2 below large open arrow). The duration and amplitude of the epileptiform waves, observed in five rats of this study, are summarized in Table 1. The frequency of incidence of the epileptiform discharge during 30 min of gabazine perfusion was determined as described in Section 2.3.1. and includes the number of waves occurring during the burst of activity as seen in the expanded plot 2.

To examine whether gabazine-induced epileptiform discharges were mediated by fast neurotransmission through the AMPA receptor, GYKI 52466 (an antagonist of this receptor), was co-perfused with gabazine in the ipsilateral HC (Fig. 1C). The frequency of incidence of epileptiform discharges decreased significantly in both HC. Upon washout with aCSF (Fig. 1D), the epileptiform discharges became infrequent with the amplitude clearly decreased in the contralateral HC. As shown in Table 1, the number of epileptiform discharges occurring during gabazine perfusion was 187 ± 4.6 ($n = 5$) per 30 min. With this number considered 100%, epileptiform discharges occurring during the last 30 min of co-perfusion with GYKI 52466 decreased to 64 ± 8 % and, during the last 30 min of washout, to 24 ± 4 %.

Fig. 1 inset shows intrahippocampal locations of the EEG recording electrode and the microdialysis probe. The electrode tip is in CA3, while the microdialysis probe, with its 2-mm membrane for dialysate collection shown by the open rectangle, is in CA1/CA3 region.

3.1.2. Epileptiform discharges induced no change in GLU_{ECF} but a significant decrease in GLN_{ECF} —Basal dialysate GLU concentration was 1.2 ± 0.48 and 1.5 ± 0.62 μM ($n = 5$) in the contralateral and ipsilateral HC respectively and showed no statistically significant change in response to epileptiform discharges. By contrast, GLN_{ECF} showed significant changes, as a function of the frequency of incidence of epileptiform discharges, as described below. The basal dialysate GLN concentration was 23.2 ± 3.3 and 23.6 ± 2.7 μM ($n = 5$) in the contralateral and ipsilateral HC respectively. Before the start of gabazine perfusion, GLN_{ECF} collected at 5, 65 and 85 min after the stabilization period (Section 2.2.) varied less than $\pm 5\%$ in both HC. Accordingly, GLN_{ECF} concentration measured at 5 min before the start of gabazine perfusion was set to 100% in each rat and GLN_{ECF} at subsequent time points were expressed as percentage of that concentration. Fig. 2A shows the time-course of GLN_{ECF} (mean $\pm$ SE $n = 5$) in the aCSF-perfused *contralateral* HC where epileptiform discharges occurred synchronously with those in the ipsilateral HC. The rationale for focusing on this HC first is that the observed changes in GLN_{ECF} can reasonably be attributed to the incidence of epileptiform discharges per se (without possible additional effects from perfused gabazine and GYKI 52466). In Fig. 2, the perfusion periods are shown at the top, with the mean frequency of incidence of epileptiform discharges (%) during each period below. GLN_{ECF} in the contralateral HC (black diamonds) decreased to $73 \pm 4\%$ ($n = 5$; significantly different from baseline $p < 0.01$) during frequent epileptiform discharges induced by gabazine delivered to the ipsilateral HC. Also shown are GLN_{ECF} concentrations (open diamonds) in a separate group of rats ($n = 4$) where the brain was snap-frozen after 25 min of gabazine perfusion for measurement of intrahippocampal GLN concentration (Section 3.2.6). The time course of decrease is very similar, and significantly different from pre-perfusion level (see caption for details). In the rats in which microdialysis was continued, GLN_{ECF} gradually recovered to $81 \pm 6\%$ when epileptiform discharge became less frequent as a result of co-perfusion of the AMPA receptor antagonist which reduced fast glutamatergic neurotransmission. GLN_{ECF} recovered to $95 \pm 4\%$ when epileptiform discharges became infrequent and lower in amplitude after perfusate washout with aCSF. This final restored value differs significantly from the minimum value with $p < 0.05$. In summary this experiment shows that GLN_{ECF} decreases significantly in response to frequent occurrence of epileptiform discharges that represent synchronized bursts of glutamatergic neuron firing, and is restored toward basal levels when epileptiform discharges become rare.

Fig. 2B shows the corresponding time-course of GLN_{ECF} in the ipsilateral (treated) HC. GLN_{ECF} decreased to $76 \pm 5\%$ (black square) during frequent epileptiform discharges, significantly less than basal levels ($p < 0.01$) and is similar to that observed in the contralateral HC. GLN_{ECF} (open square) measured in a separate group of rats for snap-freezing of the brain after 25-min of perfusion shows similar decrease. In this HC, GLN_{ECF} decreased further to $\sim 60\%$ during co-perfusion of gabazine and GYKI 52466 and remained low after the perfusate was replaced by aCSF. These results strongly suggest that local

(ipsilateral) GYKI-induced inhibition of glutamatergic neurotransmission through the AMPA receptor contributed to changes in GLN_{ECF} in addition to changes resulting from occurrence of epileptiform discharges. The important findings are that (1) the effect of epileptiform discharges per se on GLN_{ECF} is clearly observed in the contralateral HC and (2) the timing and the extent of GLN_{ECF} decrease correlate with the frequency of incidence of epileptiform discharges.

3.2. Disinhibition by GABA_BR blockade

3.2.1. Na⁺ spikes—Fig. 3 shows bilateral EEG recordings from the hippocampal CA3 region of an awake freely-behaving rat, during ipsilateral perfusion of CGP, a GABA_BR antagonist. The top panel shows, from the left, recording during baseline pre-perfusion period, during 0.1 mM CGP perfusion, during post-washout pre-perfusion period and during 1 mM CGP perfusion, with a time scale of 1 s to show typical durations of the spike cluster events. The complex spike clusters occurred not only in the CGP-perfused ipsilateral HC but also synchronously in aCSF-perfused contralateral HC. Each complex spike event represents an extracellular recording of action potentials derived from both soma and dendrites of pyramidal neurons. The middle panel shows corresponding recordings from another rat plotted with a shorter time scale; the trace in the box is expanded in the bottom panel to show single spikes, each with a linewidth of ~0.3-0.5 ms at half-height which is characteristic of a Na⁺ spike. Table 2 summarizes the duration of a single spike, the mean duration of complex spike events, the range of amplitudes, and the total number of spike cluster events/20 min that occurred in individual rats during unilateral perfusion of 0.1 mM or 1 mM CGP. (Results for 0.32 mM were intermediate between results generated by 0.1 and 1 mM CGP perfusion and are not shown).

3.2.2. GLU_{ECF} elevation during disinhibition—Fig. 4A (top panel) shows an HPLC chromatogram of dialysate from the ipsilateral HC at 6 min after the start of 0.1 mM CGP perfusion. Well-resolved peaks for GLU_{ECF} , GLN_{ECF} and CGP are observed. The middle panel shows a chromatogram from the contralateral HC during 0.1 mM CGP perfusion of the same study. Absence of the CGP peak shows that CGP does not diffuse through the ventricle to the contralateral HC. GLU_{ECF} is significantly elevated and GLN_{ECF} reduced compared to pre-perfusion chromatogram (bottom panel). Fig. 4B shows the time-course of GLU_{ECF} in the ipsilateral HC during perfusion of 0.1 mM CGP (perfusion period shown at the top), compared to that of aCSF-perfused control HC. The perfusion time is referred to as T hereafter. T is the same as the microdialysis time t in Figs. 4B and C and differs only in Fig. 4D (see Figure caption). At $T = 6.5$ min, GLU_{ECF} was elevated to 237 ± 59 % (mean $\pm$ SE, $n = 6$), and decreased to near pre-perfusion level by $T = 25$ min. This 2.4-fold elevation of GLU_{ECF} is significantly different ($p < 0.05$) from that of aCSF-perfused control ($n = 6$), although SE is large due to inter-animal variation. The elevation suggests that GABA_BR blockade induced glutamate release into ECF at a rate faster than its uptake. Importantly, this is the first observation of GLU_{ECF} elevation in vivo in an intact hippocampus induced by GABA_BR blockade. Such elevation of GLU_{ECF} can trigger synchronized excitatory drive in the CA1/CA3 pyramidal neurons, resulting in the large number of observed action potentials derived from soma and dendrites (as seen in Fig. 3).

When perfusate CGP concentration was increased to 1 mM, the large excess of CGP in the effluxing dialysate (~ 800 fold the conc. of GLU_{ECF}) presented a degradation risk to the analytical column even though it was protected by a guard column. For this reason, dialysates from the ipsilateral HC during perfusion of 1 mM CGP were not analyzed by HPLC, and instead we focused on dialysates from the aCSF-perfused contralateral HC, to examine the effects of glutamatergic population bursts on GLU_{ECF} and GLN_{ECF} .

Fig. 4C shows the time-course of GLU_{ECF} in the aCSF-perfused contralateral HC during 30-min perfusion of 0.1 mM CGP to the ipsilateral HC. At $T = 7$ min, the 2-fold elevation of GLU_{ECF} is associated with large inter-animal variation. At $T = 16$ min, the 1.6 fold elevation ($n = 6$) is significantly different from the untreated control ($p < 0.05$). Fig. 4D shows the time-course of GLU_{ECF} in the contralateral HC during perfusion of 1 mM CGP in the ipsilateral HC (duration shown at the top). The 1.7-fold elevation at $T = 4.5$ min and 1.8 fold elevation at $T = 17$ min are significantly different from the control ($p < 0.05$ and 0.01 respectively). The percentage increase of GLU_{ECF} in the contralateral HC was similar whether 0.1 mM or 1 mM CGP was perfused in the opposite HC. These results strongly suggest that synchronized glutamatergic population bursts transmitted from the perfused HC to the opposite side through the commissural fibers caused release of neurotransmitter glutamate to ECF at a rate faster than its uptake, resulting in elevated GLU_{ECF} in the contralateral HC.

3.2.3. GLU_{ECF} elevation correlates with the number of Na^+ spike cluster events

—Fig. 4E plots the maximum GLU_{ECF} elevation in the ipsilateral HC (black triangles) vs the number of Na^+ spike cluster events/20 min (Table 2) observed in individual rats during perfusion of 0.1 mM CGP. The 20-min time interval was chosen because GLU_{ECF} elevation occurred during this period (Fig. 4B, C and D). Rats with the largest number of complex Na^+ spike cluster events also showed extensive (up to 500%) elevation of GLU_{ECF} (scale on left coordinate). Also shown is the percentage elevation of GLU_{ECF} vs the number of Na^+ spike cluster events in the contralateral HC when CGP was perfused in the opposite hippocampus at 0.1 mM (open triangle: Contralateral I) or 1 mM (black square; Contralateral II). The maximum percentage elevation of GLU_{ECF} in the contralateral HC (scale on the right coordinate) is 218%, which is less than that in the ipsilateral hippocampus. At 0.1 mM CGP concentration, there is scatter in the trend of GLU_{ECF} increase, but at 1 mM, the percentage increase in GLU_{ECF} correlates with the number of Na^+ spike cluster events.

3.2.4. GLN_{ECF} decreases during disinhibition—As shown in Fig. 5A, during perfusion of 0.1 mM CGP, GLN_{ECF} in the ipsilateral HC (black diamonds) decreased to $67 \pm 9\%$ at $T = 14$ min and was $74 \pm 7\%$ at $T = 26$ min; both significantly different from those in the untreated control ($p < 0.01$). Similar decrease in GLN_{ECF} (open diamonds) was shown by rats in which the brain was snap-frozen at the end of 20 min perfusion for analysis of intracellular GLN (Section 3.2.6). Fig. 5B shows the corresponding time-course in the aCSF-perfused contralateral HC. GLN_{ECF} decreased to $83 \pm 5\%$ at $T = 5$ min, to $68 \pm 4\%$ by $T = 16$ min, and recovered some to $80 \pm 4\%$ by 25 min, all significantly different from the control ($p < 0.01$). Similar time-course was found in rats whose brain was frozen after 20

min. Fig. 5C shows the time-course for the aCSF-perfused contralateral HC during perfusion of 1 mM CGP in the ipsilateral HC (perfusion duration shown at the top). GLN_{ECF} decreased significantly to $82 \pm 7\%$ at $T = 7$ min, and to $63 \pm 5\%$ at 16 min ($p < 0.05$ and 0.01 respectively) and was $72 \pm 12\%$ at 26 min. Again, the time-course up to $T = 15$ min was very similar in the group of rats which were sacrificed at 20 min for measurement of intracellular GLN. The percentage decrease of GLN_{ECF} in the contralateral HC was similar whether 0.1 mM or 1 mM CGP was perfused in the opposite HC.

3.2.5. GLN_{ECF} tends to decrease when GLU_{ECF} is elevated—Fig. 5D plots GLU_{ECF} elevation (bottom abscissa) in the ipsilateral HC (black triangle) vs GLN_{ECF} decrease in individual rats during perfusion of 0.1 mM CGP. Rats with high elevation of GLU_{ECF} show large decrease in GLN_{ECF} , although there is some scatter. The figure also shows GLU_{ECF} elevation in the contralateral HC (scale on top abscissa) vs GLN_{ECF} decrease during perfusion of 0.1 mM (Contralateral I: open triangle) and of 1 mM CGP (contralateral II: black square) in the opposite HC. At 1 mM concentration, there is a tendency for GLN_{ECF} to decrease significantly when GLU_{ECF} elevation is > 1.75 fold.

3.2.6. Intra-hippocampal GLN concentration—Fig. 6 shows the tissue concentration of GLN, measured in the hippocampal CA1/CA3 region (mean tissue weight: 12.0 ± 0.94 mg) in the brain snap-frozen when GLN_{ECF} was 60-70% of the pre-perfusion concentration, viz after 25 min of gabazine perfusion (Fig. 2) or after 20 min of 0.1 mM or 1 mM CGP perfusion (Fig. 5A-C) in treated rats. The *intrahippocampal* GLN concentration was 4.8 ± 0.36 mM in the aCSF-perfused hippocampus of control rats, 4.78 ± 0.8 mM in the gabazine-perfused ipsilateral hippocampus, 4.4 ± 0.27 mM in the corresponding contralateral hippocampus, 4.6 ± 0.72 mM in the ipsilateral hippocampus after 20 min of 0.1 mM CGP perfusion, 4.7 ± 0.4 in the corresponding contralateral hippocampus and 4.7 ± 0.59 mM in the contralateral hippocampus after 20 min of 1 mM CGP perfusion in the opposite hippocampus. There was no statistically significant difference among these mean values. The time-courses of decrease in *extracellular* GLN concentration measured by microdialysis in this group of rats show clear decrease (Fig. 2A (open diamond) and 2B (open square) and Figs. 5A, 5B and 5C (open diamonds)). The results clearly show that *intracellular* GLN concentration in the relevant hippocampal region remains unchanged, during significant decrease in GLN_{ECF} . Fig. 6 also shows *intrahippocampal* GLU concentrations in the brains of these treated vs control rats. There was no statistically significant difference among the groups.

3.2.7. Location of the EEG electrodes and the microdialysis probes—Table 3 shows the coordinates of the EEG electrodes and the microdialysis probes, expressed as the mean $\pm$ SE for the controls and for the ipsi- and contralateral hippocampi of each group of treated rats, as confirmed in each rat after the microdialysis experiment (Section 2.4). The results are shown separately for rats that underwent continuous microdialysis (Subgroup I) and for those (Subgroup II) in which it was terminated, after the period specified in the perfusate column, to freeze the brain for measurement of *intrahippocampal* GLN. As seen in Table 3, the confirmed electrode and the probe locations in the gabazine- and CGP-treated rats were virtually the same as in control, differing by less than 0.2 mm in the lateral and

0.36 mm in the vertical coordinates of the electrode and less than 0.23 mm in probe location. Hence, the recording electrode was in CA3 and the microdialysis probe was in the CA1/CA3 region (Fig. 1 inset) in all the rats reported in this study. Accordingly, the differences in the time-course of GLU_{ECF} and GLN_{ECF} described above are due to the effects of GABA_{A} R or GABA_{B} R antagonism and the resulting glutamatergic population bursts, not due to variation in the electrode/probe location.

4. DISCUSSION

4.1. In vivo observation of epileptiform discharges in disinhibited adult rat hippocampus

This work shows that unilateral perfusion of gabazine (10 μM) in the CA1/CA3 region of HC induced bilateral epileptiform discharges detectable by EEG electrodes in the CA3 region in freely-behaving rats. The dose was based on a previous report that local intrastriatal perfusion of 10 μM gabazine in monkey brain for 20 min induced prominent spike-wave activity without behavioral changes or other EEG abnormalities (Darbin et al., 2006). The wave patterns in vivo (Fig. 1) resemble the pattern reported in hippocampal slices superfused with 1 μM gabazine (Ellender et al., 2010). As described by (Karlócai et al., 2014), these gabazine-induced epileptiform discharges (termed “interictal events” by these authors) differ from physiological sharp waves observed during slow-wave sleep or awake immobility in freely-behaving rats (Buzsáki, 1986), although the term “sharp waves” are often used by clinicians to refer to pathological transient events observed in epileptic patients.

The origin of gabazine-induced epileptiform discharges can reasonably be attributed to the following sequence of events (Buzsáki, 1986; Karlócai, et al., 2014; Sullivan et al., 2011). Hippocampal pyramidal neurons are subject to two antagonistic polarizations, dendritic excitation from glutamatergic neurons and somatic/perisomatic inhibition from GABAergic interneurons (Section 1). Upon GABA_{A} R blockade by gabazine, the GABAergic inhibition of the post-synaptic pyramidal neurons is weakened, and the excitatory drive from glutamatergic neurons with recurrent networks causes massive depolarization of the pyramidal cells, resulting in the observed synchronized population bursts. Because the CA3 pyramidal neurons also target the dendrites of CA1 pyramidal cells of the contralateral HC through the commissural fibers (Suzuki and Smith, 1987), glutamatergic population bursts are transmitted to, and appear as epileptiform discharges in, the contralateral HC as well.

In the present work, gabazine was dissolved in aCSF of normal electrolyte composition, in contrast to the high- K^+ /low- Mg^{2+} aCSF used for microinjection of bicuculline or gabazine in a previous report (Isaev et al., 2005). Our results show that epileptiform discharges can be reliably induced in the hippocampus of freely-behaving adult rat using physiologically normal electrolyte perfusate to deliver gabazine through the microdialysis probe. Upon co-perfusion of GYKI 52466 with gabazine, the frequency of incidence of epileptiform discharges decreased substantially (Fig. 1C and Table 1). The result is in qualitative agreement with the previous finding in hippocampal slices that gabazine-induced epileptiform discharges are abolished upon addition of this antagonist of AMPA receptor (Ellender, et al., 2010). The present result suggests that the epileptiform discharges observed in vivo are also mediated by fast neurotransmission through the AMPA receptor.

4.2. GABA_BR blockade – origin of Na⁺ spikes

This study shows that unilateral perfusion of the GABA_BR antagonist CGP in CA1/CA3 region induced complex spike bursts in both HC (Fig. 3). A representative single spike had duration of ~0.3-0.5 ms at half-height, resembling the duration of a spontaneous Na⁺ spike (0.3-0.5 ms at half height) observed in vivo in the CA1 and CA3 regions of freely-behaving rats during awake immobility or slow wave sleep (Buzsáki et al., 1996). As shown in the bottom panel of Fig. 3, the polarity of Na⁺ spikes in the contralateral HC differs from that in the ipsilateral because of time-dependent changes in neuronal transmission through the commissural pathway. The observed Na⁺ spikes represent field potentials generated close to the soma of firing neurons as well as apical dendritic Na⁺ spikes back-propagating from axon hillocks. Support for the contribution from dendrites comes from previous studies: (a) this back-propagation is under inhibitory control (Buzsáki, et al., 1996), and (b) inhibition of back-propagation of population antidromic spikes into dendrite of CA1 pyramidal neurons is partially relieved by intracerebroventricular injection of GABA_BR antagonist (CGP 35348) (Leung and Peloquin, 2006).

4.3. GLU_{ECF} elevation in disinhibited hippocampus

This study shows that, upon blockade of GABA_BR, GLU_{ECF} was elevated 1.6 to 2.4 fold during CGP perfusion (Fig. 4B). A probable mechanism of GLU elevation in the disinhibited ipsilateral HC is that CGP blocked the presynaptic GABA_{B(1a,2)}} heteroreceptor located on glutamatergic neurons in CA1/CA3 synaptic circuit (Biermann, et al., 2010; Vigot et al., 2006). Normally, GABA binding to this receptor inhibits vesicular release of GLU. CGP 35348 is a potent antagonist of this presynaptic glutamate heteroreceptor, with the IC₅₀ value (drug concentration causing half-maximal antagonism) of 9 μM (Lanza et al., 1993) to 30 μM (Olpe, et al., 1990). The result is in accord with the report by Vigot et al. (2006) that mice lacking GABA_B1a subunit show decreased number of silent synapses, which is likely to be a consequence of uncontrolled glutamate release due to the constitutive absence of GABA_{B(1a,2)}} heteroreceptor.

CGP concentrations of 0.1 to 1 mM used in this study were based on previous reports that (a) intrahippocampal infusion of 20 - 30 μM CGP permitted study of the effect of GABA_BR blockade on theta and gamma rhythms in freely behaving rats (Leung and Shen, 2007), and (b) perfusion of 0.3 - 1 mM CGP in Mg²⁺-free medium evoked increases of extracellular guanosine 3',5'-cyclic monophosphate (cGMP) without causing behavioral seizures (Fedele et al., 1997). The present study is the first in vivo demonstration that perfusion of 0.1 mM CGP in the hippocampal CA3/CA1 region is sufficient to block the presynaptic GABA_{B(1a,2)}} heteroreceptor resulting in GLU_{ECF} elevation (Fig. 4B). Other possible effects of GABA_BR blockade include (a) effect on presynaptic GABA autoreceptor, (b) antagonism of postsynaptic membrane hyperpolarization and the late inhibitory postsynaptic potential in pyramidal neurons (Olpe, et al., 1990) and (c) elevation of cGMP mediated by the glutamate receptor/NO synthetase/guanylyl cyclase pathway (Fedele, et al., 1997). In the present study, the effect on presynaptic GABA autoreceptor is likely to be small, because CGP 35348 is a weak blocker of GABA autoreceptor (Lanza, et al., 1993). Also, elevation of cGMP is likely to be quite minor because CGP 35348 was delivered in aCSF containing normal (0.8 mM) Mg²⁺. A previous study showed that, in the presence of 1 mM Mg²⁺, cGMP elevation was at

most ~125%, compared to 175% in Mg^{2+} free medium (Fedele, et al., 1997). By contrast, CGP-mediated antagonism of membrane hyperpolarization in pyramidal neurons can contribute to the occurrence of Na^+ spike clusters and to excitability of glutamatergic neurons.

The observed elevation of GLU_{ECF} can, in turn, trigger synchronized excitatory drive in the ipsilateral CA1/CA3 pyramidal neurons and, upon transmission through the commissural fibers, induce synchronized population bursts in the contralateral HC as well. This scenario is supported by the observed bilateral synchrony of the Na^+ spikes (Fig. 3) and significant GLU_{ECF} elevation in the contralateral HC as well (Fig. 4C and 4D), although the maximum GLU_{ECF} elevation in the contralateral HC (2.18 fold) is smaller than that in the ipsilateral hippocampus (5-fold) (Fig. 4E). Evidence in support of this sequence of events includes the report that spontaneous complex-spike bursts occurred in association with population discharges of CA3-CA1 pyramidal cells during slow-wave sleep or immobility in freely behaving rats (Buzsáki, et al., 1996).

In gabazine-perfused HC, no significant change in GLU_{ECF} was detected, raising a question as to why epileptiform discharges did not induce GLU_{ECF} elevation. To the best of our knowledge, the effect of gabazine-induced disinhibition on hippocampal GLU_{ECF} has not been studied in vivo. For another GABA_AR antagonist, bicuculline, it was reported that maximal elevation of hippocampal GLU_{ECF} ($46 \pm 5\%$) occurred 40 min after the start of 100 μM bicuculline perfusion (Rowley et al., 1995). The IC_{50} of bicuculline is ~10 fold higher than that of gabazine (Baumann et al., 2003). Accordingly, it is likely that perfusion of 10 μM gabazine for 34 min in the context of this study was ineffective in raising GLU_{ECF} . Alternatively, EAAT2-mediated uptake of GLU_{ECF} into glia was rapid enough for its clearance during this period. Further studies are needed to fully explain the mechanism.

4.4. Disinhibition reduces GLN_{ECF} but not intracellular GLN

This study shows, for the first time in vivo, that GLN_{ECF} , the precursor of the excitatory neurotransmitter GLU, shows significant decrease during disinhibition induced by GABA_AR blockade (Fig. 2) or GABA_BR blockade (Fig. 5A-C). This GLN decrease occurred in both HC. However, GLN_{ECF} reduction in the aCSF-perfused contralateral HC is particularly significant, because this can reasonably be attributed to glutamatergic population bursts per se, as indicated by the occurrence of epileptiform discharges (Fig. 1) or the Na^+ spikes (Fig. 3), in the absence of additional possible effects of antagonist perfusion.

Decrease in GLN_{ECF} can be caused by decreased synthesis of GLN by glia-specific glutamine synthetase. This, in turn, can cause decreased efflux of glutamine to ECF mediated by SNAT 3, because the rate of GLN efflux depends on the GLN_{glia}/GLN_{ECF} ratio (Chaudhry et al., 1999) and decreases upon reduction of glial GLN concentration. These points are illustrated in Fig. 7 which shows schematically the major metabolic pathways of GLU and GLN and their transport pathways between synaptic vesicles, ECF, glia and the neuron, according to a suggested model of GLU/GLN cycle (Hertz, 1979). Our result in Fig. 6 shows that GLN concentrations in the relevant hippocampal region of the treated rats are the same as in control after 25 min of gabazine perfusion or 20 min of CGP perfusion.

Tissue concentration of GLN in the hippocampus represents predominantly (>99%) intracellular concentration and immunocytochemical studies suggest that at least 80% of intracellular GLN is in glia (Kanamori and Ross, 1997; Nagelhus, et al., 1996; Ottersen, et al., 1992). Hence, our result in Fig. 6 strongly suggests that disinhibition did not cause decreased GLN synthesis nor concomitant reduction of its efflux to ECF under our experimental conditions. This strengthens the likelihood that the observed decrease in GLN_{ECF} reflects its increased uptake into neurons, mediated by SNAT1/SNAT2 (Fig. 7), during enhanced neuronal activity as demonstrated by the epileptiform discharges (Fig. 1) and the Na^+ spike clusters (Fig. 3).

4.5. GLN_{ECF} as a source of neurotransmitter GLU in disinhibited hippocampus

Our results show that GLN_{ECF} was significantly reduced (Fig. 5) during the first 20 min of CGP perfusion when GLU_{ECF} was elevated (Fig. 4) and that maximum GLU_{ECF} elevation correlated with the number of Na^+ spike cluster events (Fig. 4E). These spike clusters can reasonably be attributed, at least in part, to dendritic excitation of pyramidal neurons (Section 4.2). Hence, our result suggests that the observed decrease in GLN_{ECF} , which can reasonably be attributed to its increased neuronal uptake (Section 4.4), is associated with enhanced flux of neurotransmitter GLU in vivo. Reports from other laboratories support this concept. SNAT2, which mediates neuronal uptake of GLN_{ECF} (Fig. 7), is highly enriched in somatodendritic compartments of CA1 pyramidal neurons; SNAT2-containing dendrites accumulate high levels of GLN and production of GLU from GLN can be accelerated in response to enhanced neuronal activity (Jenstad et al., 2009). A major role for GLN_{ECF} in sustaining enhanced neuronal activity has been proposed on the basis of studies of epileptiform discharges in hippocampal (Bacci et al., 2002), injured cortical (Tani et al., 2007) and disinhibited neocortical (Tani et al., 2010) slices. Noteworthy is a recent study (Tani et al., 2014) that addresses the controversial issue of whether GLN_{ECF} is important (a) for neurotransmitter generation (Chaudhry et al., 2002; Jenstad, et al., 2009) or (b) is the precursor of the metabolic, but not the neurotransmitter, pool of GLU (Grewal et al., 2009; Melone et al., 2006). Specifically, Tani, et al. (2014), using isolated nerve terminals in brain slice, provided evidence for utilization of GLN by neurons for synthesis of synaptically released GLU. Taken together, the results support the concept that GLN_{ECF} , taken up into somatodendritic region of pyramidal neurons by SNAT2, can be an important source of neurotransmitter GLU during enhanced neuronal activity.

Our present result is consistent with our previous study (Kanamori and Ross, 2011) which showed that GLN_{ECF} decreases in response to the occurrence of electrographic seizures in the chronic kainate-induced rat model of temporal-lobe epilepsy. Furthermore, electrographic seizures are significantly reduced upon inhibition of neuronal uptake of GLN_{ECF} by perfusion of 2-(methylamino)isobutyrate in vivo in this model (Kanamori and Ross, 2013). Collectively, the data strongly suggest that, in freely-behaving rats too, GLN_{ECF} plays an important role in replenishing the neurotransmitter pool of GLU when its flux through the neuron-ECF-glia cycle (Fig. 7) is enhanced by disinhibition as shown here, or as a result of spontaneous electrographic seizures. Thus, neuronal uptake of GLN_{ECF} can be one of key regulatory sites at which excitatory and inhibitory neurotransmissions are modulated by fluctuations in the supply of the precursor.

4.6. Summary

1. Unilateral hippocampal perfusion of GABA_AR antagonist, gabazine, induced bilateral occurrence of synchronous epileptiform discharges and that of GABA_BR antagonist, CGP 35348, induced Na⁺ spikes, in freely-behaving rats.
2. The changes in GLU_{ECF} and GLN_{ECF} were studied in the aCSF-perfused contralateral hippocampus, where the effects on concentrations of GLU_{ECF} and GLN_{ECF} can reasonably be attributed to epileptiform discharges or Na⁺ spikes *per se*.
3. GLU_{ECF}, unchanged by gabazine perfusion, was significantly elevated upon blockade of GABA_B presynaptic heteroreceptor, and the elevation correlated with the number of Na⁺ spike cluster events.
4. GLN_{ECF} decreased significantly upon disinhibition induced by GABA_AR or GABA_BR antagonists.
5. Intracellular GLN concentration in the hippocampal region sampled by microdialysis was unchanged in the treated rats compared to the control, suggesting that neither GLN synthesis nor the rate of efflux of GLN to ECF was altered by disinhibition.
6. The results strongly suggest that the observed decrease of GLN_{ECF} *in vivo* reflects enhanced uptake into neurons when its flux is accelerated by glutamatergic population bursts induced by disinhibition, and are consistent with our previous findings on the role of GLN_{ECF} in sustaining epileptiform activities in the kainate-induced rat model of temporal lobe epilepsy.

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Abbreviations

CGP	CGP 35348
ECF	extracellular fluid
GABA_AR	GABA _A receptor
GABA_BR	GABA _B receptor
GLN_{ECF}	extracellular glutamine
GLU_{ECF}	extracellular glutamate
HC	hippocampus

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Highlights

- Disinhibition was induced in rat hippocampus in vivo by GABA_AR or GABA_BR blockade.
- Unilateral blockade induced bilateral epileptiform discharges or Na⁺ spikes.
- GABA_BR-mediated disinhibition caused bilateral elevation of extracellular glutamate.
- Extracellular, but not intracellular, glutamine decreased significantly.
- Extracellular glutamine is a major source of neurotransmitter glutamate in vivo.

Fig. 1.

EEG recordings from the ipsilateral (treated) and the contralateral hippocampus of an awake adult rat during (A) baseline period, (B) perfusion of 10 μ M gabazine, (C) 10 μ M gabazine + 100 μ M GYKI 52466 (AMPA receptor antagonist), and (D) aCSF in the ipsilateral HC. Each trace shows a 15-min recording. Epileptiform discharges occur synchronously in both HC, although no active drug was perfused in the contralateral HC (aCSF only). A wave in the epileptiform discharge (small open arrow) in Fig. 1B is expanded in plot 1. A burst of epileptiform discharges (in box) is expanded in plot 2. The inset shows intrahippocampal locations of the EEG recording electrode and the microdialysis probe in a coronal map of rat brain at AP = -5.6 mm. Note that the electrode tip is in CA3 and the microdialysis probe, with the open rectangle showing its 2-mm membrane for dialysate collection, in the CA1/CA3 region. REC: EEG recorder.

Fig. 2.

The time-course of GLN_{ECF} in the CA1/CA3 region of freely-behaving rats, expressed as the mean ± SE (*n* = 5), and as percentage of the basal GLN_{ECF} concentration at 5 min before the start of gabazine (GBZ) perfusion. **A.** Contralateral HC. The perfusates and the number of epileptiform discharge in percentage shown at the top. GLN_{ECF} (black diamond) decreases significantly during frequent epileptiform discharge (***p* < 0.01 vs the basal) and recovers when epileptiform discharges become rare (**p* < 0.05 compared to the minimum). GLN_{ECF} (open diamond) in brain snap-frozen after 25 min of perfusion shows similar

decrease and is significantly different from preperfusion level with ** $p < 0.01$ or * $p < 0.05$. **B.** Ipsilateral HC: GLN_{ECF} (black square) decreases (** $p < 0.01$ vs the basal) and remains low. GLN_{ECF} (open square) in brain snap-frozen after 25 min of perfusion.

Fig. 3.

Complex Na^+ spike clusters observed bilaterally upon unilateral perfusion of CGP 35348 in freely-behaving rats. Top panel : From the left: recording during baseline pre-perfusion period, during 0.1 mM CGP perfusion, during post-washout pre-perfusion period and during 1 mM CGP perfusion. The time scale is 1 s to show representative durations of the cluster events. Middle panel: Corresponding recordings from another rat plotted with a shorter time scale; trace in the box is expanded (bottom panel) to show single spikes with a linewidth of $\sim 0.3\text{-}0.5$ ms at half-height which is characteristic of Na^+ spikes.

Fig. 4.

A. HPLC chromatograms of dialysates from (top) the ipsilateral HC during 0.1 mM CGP perfusion showing GLU_{ECF}, GLN_{ECF} and CGP; (middle) the aCSF-perfused contralateral HC during 0.1 mM CGP perfusion in the opposite HC, showing elevated GLU_{ECF} and reduced GLN_{ECF} compared to pre-perfusion dialysate (bottom). All other peaks are extracellular metabolites containing a primary amine except for the peak at 27.4 min which was present in a mixture of OPA and HPLC-grade water and does not originate from dialysates. **B.** The time-course of GLU_{ECF} (mean $\pm$ SE for $n = 5$; filled triangle) during

perfusion of 0.1 mM CGP in the ipsilateral HC vs non-treated controls ($n = 5$, open square). For simplicity, microdialysis time t is set to coincide with the start of perfusion time T although t is preceded by 3-h stabilization period (Section 2.2.); **C.** The time-course in the contralateral HC. **D.** The time-course of GLU_{ECF} in the contralateral HC during perfusion of 1 mM CGP in the ipsilateral HC for duration T as shown at the top. Microdialysis time t is longer here, because of intervening washout periods (Section 2.3.1). In **B**, **C** and **D**, * indicates significant elevation relative to control, with $p < 0.05$, and ** with $p < 0.01$. **E.** Maximum percentage elevation of GLU_{ECF} vs the number of Na^+ spike cluster events per 20 min in the ipsilateral HC (left coordinate) and in contralateral HC (right coordinate) of individual rats (Section 3.2.3). Contralateral I refers to data obtained with 0.1 mM CGP perfusion in the opposite hippocampus, and Contralateral II to that with 1 mM CGP (Note that, in the latter, data points from two rats overlap at $x = 1$).

Fig. 5.

A. The time course of GLN_{ECF} (mean $\pm$ SE for $n = 5$; black diamond) vs non-treated control ($n = 5$; open square) in the ipsilateral HC during perfusion of 0.1 mM CGP; **B:** the corresponding time-course in the contralateral HC. **C:** Time course in the contralateral HC during perfusion of 1 mM CGP in the ipsilateral HC for duration *T* as shown at the top. GLN_{ECF} is significantly reduced with $p < 0.01$ (**) or $p < 0.05$ (*). Open diamonds in A-C shows the time-courses of GLN_{ECF} in rats ($n = 4$) with the same treatment but sacrificed after 20 min to measure intracellular GLN. **D.** GLN_{ECF} decrease vs GLU_{ECF} elevation in the

ipsilateral HC (bottom abscissa; black triangle) and in the contralateral HC (top abscissa) during perfusion of 0.1 mM (open triangle) and 1 mM CGP (black square) in the opposite HC.

Fig. 6.

Tissue concentrations of glutamine and glutamate, expressed in mM as the mean $\pm$ SE for $n = 4$, in the hippocampal region sampled by microdialysis (mean weight: 12.0 ± 0.94 mg). The hippocampal tissue was obtained from the brain snap-frozen after 25 min of gabazine perfusion or 20 min of CGP perfusion vs control. Abbreviations: GBZ ipsi, gabazine-perfused ipsilateral hippocampus; contra, the corresponding contralateral hippocampus; CGP 0.1 mM ipsi, ipsilateral hippocampus after 0.1 mM CGP perfusion; contra,

corresponding contralateral hippocampus. CGP 1 mM contra, contralateral hippocampus after 1 mM CGP perfusion in the opposite hippocampus.

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Fig. 7.

A schematic diagram showing major metabolic pathways of GLU and GLN and their transport pathways between synaptic vesicles, extracellular fluid (ECF), glia and the neuron, according to a suggested model of GLU/GLN cycle (Section 4.4.). EAAT2, excitatory amino acid transporter subtype 2; GLNase, glutaminase; GLU_{NT} , excitatory neurotransmitter GLU; GS, glutamine synthetase; SNAT1, 2, 3, sodium-coupled neutral amino acid transporter subtypes 1, 2 and 3; (SNAT1, SNAT2 and SNAT3 are also called SAT1, SAT2 and SN1 respectively, but the new nomenclature is used in the text for consistency) (adapted from Kanamori and Ross, 2011).

Table 1

Duration, amplitude and frequency of incidence of epileptiform discharges in the contralateral hippocampus after ipsilateral gabazine (+ GYKI 52466) perfusion.

		Perfusate in the ipsilateral HC
Duration	50-120 ms (5)	gabazine
Amplitude	0.2 - 1.2 mV (3) 0.2 - >1.6 mV (2) ^a	gabazine
Frequency of incidence/30 min	187 ± 4.6 (5)	gabazine
Percentage of incidence/30 min	100	gabazine
	64 ± 8% (5) ^b	gabazine + GYKI 52466
	24 ± 4 % (5) ^b	aCSF

Number of rats for each value are shown in parenthesis.

^a In two of five rats, the maximum amplitude of epileptiform discharges exceeded 1.6 mV which is off-scale under the experimental condition.

^b In the last 30 min of 54-min co-perfusion of gabazine + GYKI 52466 and of 90-min washout with aCSF (see Section 2.3).

Table 2

Duration and amplitude of (a) a single Na⁺ spike and (b) spike cluster events observed during 20-min of CGP perfusion (top) and the number of spike events in individual rat (bottom).

Duration			Amplitude (mV)
Single spike - duration at half height (msec)	Spike cluster event - mean duration of an event (sec)		
	0.1 mM CGP	1 mM CGP	
~ 0.3-0.5 ms	4.2 ± 0.42 s (n = 77)	5.2 ± 0.45 s (n = 77)	0.05-1.3 mV

Rat	The number of Na ⁺ spike cluster events/20 min	
	0.1 mM CGP ipsi and contralateral	1 mM CGP contralateral
R1195	31	12
R1197	2	7
R1201	8	29
R1207	12	1
R1209	17	1
R1213	7	27
Total	77	77

Table 3

The coordinates of the EEG electrodes and the microdialysis probes in rats of each experimental group measured after the in vivo experiments as described in Material and Methods (Section 2.4). The data are expressed as the mean $\pm$ SEM for the number of rats shown in parenthesis. See text 3.2.7 for details.

Experimental group	Hippocampus	Perfusate	Microdialysis	Coordinates (mm)				
				EEG electrode			Microdialysis probe	
				AP	L	V	V (top)	V (bottom)
Control (4)	Ipsi- and contralateral	aCSF	Continuous	-5.6	4.3 $\pm$ 0.06	5.22 $\pm$ 0.10	3.65 $\pm$ 0.09	5.65 $\pm$ 0.09
GABA _A R blockade: Subgroup I (5)	Ipsilateral	GBZ, GBZ + GYKI, aCSF	"	-5.6	4.2 $\pm$ 0.14	5.2 $\pm$ 0.09	3.36 $\pm$ 0.18	5.36 $\pm$ 0.18
Subgroup I (5)	Contralateral	aCSF	"	-5.6	4.2 $\pm$ 0.14	5.4 $\pm$ 0.06	3.86 $\pm$ 0.1	5.86 $\pm$ 0.1
Subgroup II (4)	Ipsilateral	GBZ (25 min)	Terminated for freezing the brain	-5.6	4.5	5.1 $\pm$ 0.05	3.37 $\pm$ 0.12	5.37 $\pm$ 0.12
Subgroup II (4)	Contralateral	aCSF (")	"	-5.6	4.5	5.3 $\pm$ 0.12	3.42 $\pm$ 0.16	5.42 $\pm$ 0.16
GABA _A R blockade: Subgroup I (6)	Ipsilateral	CGP (0.1,0.32,1 mM)	Continuous	-5.6	4.3 $\pm$ 0.1	5.35 $\pm$ 0.07	3.78 $\pm$ 0.06	5.78 $\pm$ 0.06
Subgroup I (6)	Contralateral	aCSF	"	-5.6	4.3 $\pm$ 0.1	5.58 $\pm$ 0.17	3.88 $\pm$ 0.1	5.88 $\pm$ 0.1
Subgroup II (4)	Ipsilateral	CGP 0.1 mM (20 min)	Terminated for freezing the brain	-5.6	4.45 $\pm$ 0.03	5.57 $\pm$ 0.15	3.72 $\pm$ 0.07	5.72 $\pm$ 0.07
Subgroup II (4)	Contralateral	aCSF (")	"	-5.6	4.45 $\pm$ 0.03	5.42 $\pm$ 0.07	3.78 $\pm$ 0.1	5.78 $\pm$ 0.1
Subgroup II (4)	Ipsilateral	CGP 1mM (20 min)	"	-5.6	4.47 $\pm$ 0.03	5.27 $\pm$ 0.12	3.8	5.8
Subgroup II (4)	Contralateral	aCSF (")	"	-5.6	4.47 $\pm$ 0.03	5.43 $\pm$ 0.28	3.76 $\pm$ 0.14	5.76 $\pm$ 0.14