Positive allosteric activation of GABA<sub>A</sub> receptors bi-directionally modulates hippocampal glutamate plasticity and behaviour

Guofu Shen, Mahmoud S. Mohamed, Paromita Das and Elizabeth I. Tietz<sup>1</sup>

Neuroscience and Neurological Disorders Program, Department of Physiology and Pharmacology, University of Toledo College of Medicine, Health Science Campus, Toledo, OH 43614, U.S.A.

Abstract

Long-term BZ (benzodiazepine) anxiolytic therapy increases the risk of physical dependence manifested as withdrawal anxiety. BZ-induced potentiation of GABA<sub>A</sub>R (γ-aminobutyric acid type-A receptor) function by 1-week oral administration of FZP (flurazepam) bi-directionally modulates excitatory glutamatergic synaptic transmission in hippocampal CA1 neurons during drug withdrawal. Previous electrophysiological studies on acutely isolated and intact CA1 neurons, as well as immunofluorescence and post-embedding immunogold electron microscopy studies, suggest increased synaptic insertion of GluR (glutamate receptor) 2-lacking AMPARs (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors) in 2-day FZP-withdrawn rats. Preliminary studies indicated a similar increase in GluR1, then phospho-Ser<sup>831</sup>-GluR1, as well as CaMKII<sub>α</sub> (Ca<sup>2+</sup>/calmodulin-dependent protein kinase II), but not phospho-Thr<sup>286</sup>-CaMKII levels at the same time point. In our studies, whole-cell recordings in hippocampal slices revealed that AMPAR mEPSC (miniature EPSC (excitatory postsynaptic current)) amplitude was increased in 1-day FZP-withdrawn rats followed by an increase in estimated single-channel conductance in 2-day-FZP-withdrawn rats. Enhanced conductance was no longer observed in slices pre-incubated for 2 h in the CaMKII inhibitor KN-93, but not the inactive analogue KN-92. To evaluate whether CaMKII-mediated AMPA potentiation could occlude LTP (long-term potentiation), LTP was induced by TBS (theta burst stimulation) and recorded using whole-cell and extracellular techniques. LTP was induced in both groups, but only maintained for < 15 min in 2-day FZP-withdrawn rats. LTP was fully restored after 7-day withdrawal. Despite the lack of LTP maintenance, impairment of object recognition, place and context was not observed in 2-day-FZP-withdrawn rats. Since L-VGCC (L-type voltage-gated calcium channel) current density was doubled on drug withdrawal and up to 2 days, Ca<sup>2+</sup> entry through L-VGCCs and perhaps subsequently through Ca<sup>2+</sup>-permeable AMPARs are proposed to be responsible for enhanced CaMKII<sub>α</sub> levels and AMPAR potentiation. Mechanisms associated with several different models of activity-dependent plasticity may underlie BZ physical dependence.

Bi-directional modulation of glutamate receptors

BZs (benzodiazepines) have their anxiolytic, hypnotic and anticonvulsant actions through allosteric enhancement of GABA<sub>A</sub>R (γ-aminobutyric acid type-A receptor) function to increase GABA affinity and the frequency of Cl<sup>-</sup> channel opening [1,2]. Despite the relatively high therapeutic index of BZs, their long-term use can lead to tolerance and physical dependence and limit their clinical utility [3–5].

Although BZ tolerance is primarily related to GABA<sub>A</sub>R dysfunction [6], glutamatergic strength is bi-directionally regulated in relation to the progression of anxiety-like behaviour upon withdrawal from 1-week administration of the BZ, FZP (flurazepam). AMPAR (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor)-mediated mEPSC (miniature EPSC (excitatory postsynaptic current)) amplitude was increased without any effect on mEPSC frequency, kinetics or RMP (resting membrane potential) in hippocampal CA1 neurons from 1-day (15–30%) and 2-day (30–50%) FZP-withdrawn rats [7,8], while NMDAR (N-methyl-D-aspartate receptor) currents were decreased by 50% in 2-day-FZP-withdrawn rats [8,9]. There was a significant positive correlation between the potentiation of AMPAR current amplitude and anxiety-like behaviour measured in the elevated plus-maze in 1-day-FZP-withdrawn rats [10], but anxiety was not observed 2 days after cessation of treatment [8]. Expression of anxiety could be modulated by systemic injection of glutamate antagonists 24 h prior to behavioural testing and/or electrophysiological recording [8,10]. Pre-injection of the AMPA antagonist GYKI-52466 blocked potentiation of AMPAR currents and the appearance

Key words: α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor (AMPA receptor), benzodiazepine dependence, drug withdrawal, γ-aminobutyric acid type-A receptor (GABA<sub>A</sub>R), glutamatergic plasticity, N-methyl-D-aspartate receptor (NMDAR).

Abbreviations used: AMPAR, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor; BZ, benzodiazepine; CaMKII, Ca<sup>2+</sup>/calmodulin-dependent protein kinase II; EPSC, excitatory postsynaptic current; FZP, flurazepam; GABA<sub>A</sub>R, γ-aminobutyric acid type-A receptor; GluR, glutamate receptor; LTP, long-term potentiation; L-VGCC, L-type voltage-gated calcium channel; mEPSC, miniature EPSC; NMDAR, N-methyl-D-aspartate receptor; RMP, resting membrane potential; TBS, theta burst stimulation

<sup>1</sup>To whom correspondence should be addressed (email liz.tietz@utoledo.edu).
Figure 1 | CaMKII activity is required for AMPAR potentiation in 2-day FZP-withdrawn rats

(A) Representative traces show increased mean AMPAR-mediated mEPSC amplitude in 2-day-FZP-withdrawn rats, reversed by pre-incubation of hippocampal slices with the CaMKII inhibitor KN-93. The 30% increase in mEPSC amplitude (CON: −8.8 ± 0.5 pA, n = 9, compared with FZP: −11.4 ± 1.1 pA, n = 9) recorded in ACSF (artificial cerebrospinal fluid) was similar to that previously detected in neurons from 2-day-FZP-withdrawn rats [7,8]. Likewise, there was no effect on mEPSC frequency, rise-time and decay kinetics or RMP. (B) Non-stationary noise analysis revealed that the mean conductance in the FZP-withdrawn group was ~2-fold greater (14.7 ± 2.4 pS, n = 9 neurons; open circles/broken line) compared with the CON group (8.5 ± 0.7 pS, n = 10 neurons, closed circles/continuous line). Average mEPSC (C) amplitude or (D) conductance in CA1 neurons from CON (closed bars) and FZP-withdrawn (open bars) rats after 2 h incubation in ACSF plus 10 μM KN-93 or KN-92, an inactive analogue. The significant increase in mEPSC amplitude and conductance (**P < 0.01) in FZP-withdrawn neurons compared with CON neurons recorded in ACSF was blocked by pre-incubation with KN-93 (*P < 0.05, **P < 0.01), but not KN-92.

of anxiety. In contrast, latent anxiety-like behaviour was unmasked in 2-day FZP-withdrawn rats by systemic injection of the NMDA antagonist, MK-801, which prevented the reduction in NMDAR, but not AMPAR currents [8]. Collectively, these findings provide strong support for the hypothesis that BZ physical dependence, manifested as anxiety-like behaviour, is related to the modulation of hippocampal glutamatergic neurotransmission, and the decrease of NMDAR currents may serve as a natural break to alleviate anxiety.

Electrophysiological and immunohistochemical studies indicated that increased AMPAR-mediated neurotransmission was related to an increase in Ca2+-permeable GluR (glutamate receptor) 2-lacking AMPARs at CA1 neuron synapses [8,11,12]. Glutamate-elicited whole-cell current amplitude was increased 50%, reflected in an increase in glutamate efficacy in acutely isolated CA1 neurons, without a change in decay kinetics. Moreover, AMPAR-mediated currents in isolated neurons and mEPSCs in hippocampal slices showed a negative shift in rectification in the presence of spermine analogues, suggesting augmented membrane incorporation of GluR2-lacking AMPARs [11,13,14]. Confocal immunocytochemical studies and post-embedding immunogold electron microscopic studies confirmed an increase in GluR1, but not GluR2 subunits at CA1 neuron asymmetric synapses [11,12].

CaMKII (Ca2+/calmodulin-dependent protein kinase II)-mediated glutamate plasticity

The latter findings are very reminiscent of enhanced AMPA conductance after insertion of GluR1 homomeric AMPARs in recombinant neurons and other models of activity-dependent plasticity such as LTP (long-term potentiation) [15–17]. Therefore CaMKII-mediated signalling was investigated as a possible cellular mechanism for modulation of AMPAR potentiation [15,18]. The possibility that phosphorylation of GluR1 at Ser831 by CaMKII could lead to increased AMPAR single-channel conductance [19] was explored by estimating AMPAR single-channel conductance using non-stationary noise analysis in the presence or absence of CaMKII inhibitors. AMPAR single-channel conductance was significantly increased in 2-day FZP-withdrawn rats and was reversed after 2 h bath application of 10 μM KN-93, but not the inactive analogue KN-92 (Figure 1). Preliminary immunoblot studies of PSD...
Lack of LTP maintenance in CA1 neurons from 2-day FZP-withdrawn rats

LTP was induced by TBS of the Schaffer collateral pathway and measured using both whole-cell [A, C, eEPSC (evoked EPSC)] and extracellular [B, D, fEPSP (field excitatory postsynaptic potential)] techniques. No significant differences were found in the stimulus intensity used (CON: 0.50 ± 0.04 mV, n = 7, compared with FZP: 0.59 ± 0.06 mV, n = 7) to elicit 100–200 pA eEPSC responses or mean amplitude (CON: 159 ± 27.6 pA, compared with FZP: 175 ± 46.5 pA, n = 7) between control and FZP-withdrawn groups. Representative traces of (A) eEPSCs elicited at 15 s intervals and (B) fEPSPs elicited at 1–15 min intervals after TBS (ten bursts of four pulses at 100 Hz; 200 ms interburst interval) are superimposed over the baseline response, shown in grey. (C) eEPSCs and (D) fEPSPs and population spikes (results not shown) were recorded for 60 min. The degree of stimulus-induced potentiation was normalized to the baseline response. LTP was induced in CA1 neurons derived from both control rats (closed circles, eEPSCs, n = 7; fEPSP, n = 11) and 2-day FZP-withdrawn rats (open circles, eEPSCs, n = 7; fEPSP, n = 13), although it could be maintained only in the CON group. Population spike amplitude was also significantly enhanced in the 2-day-FZP-withdrawn group (CON: 4.5 ± 0.5 mV compared with FZP: 7.6 ± 0.7 mV, P = 0.001).

(postsynaptic density)-enriched subcellular fractions from CA1 minislices revealed increased expression of total GluR1 and phospho-Ser831-GluR1 proteins, along with increased levels of CaMKII, although not phospho-Thr286-CaMKII [20]. Importantly, preliminary findings further showed that only total GluR1 levels and AMPAR current amplitude but not phospho-Ser831-GluR1 or total or phospho-CaMKII levels were enhanced in 1-day FZP-withdrawn rats [21]. The time course of enhanced AMPA amplitude and conductance is consistent with increased synaptic incorporation and subsequent phosphorylation of GluR1 homomers [16] during BZ withdrawal.

Since CaMKII plays an important role in LTP, experiments were also conducted to evaluate whether enhanced CaMKII levels could occlude LTP [22]. LTP was induced by TBS (theta burst stimulation) in CA1 neurons from 2-day FZP-withdrawn rats and recorded using whole-cell or extracellular electrophysiological techniques. While early LTP induction was not occluded, LTP was not maintained for the 60 min recording period (Figure 2). In 7-day-FZP-withdrawn rats, when no significant AMPAR or GABAAR changes were observed in CA1 neurons [8,23], both LTP induction and maintenance were no longer impaired (results not shown). Whether memory impairment accompanied the lack of LTP maintenance was evaluated by rats’ intrinsic tendency to explore novel objects, places and contexts, measures sensitive to hippocampal damage [24]. Given the narrow time window in which AMPAR potentiation occurs during BZ withdrawal, this approach was useful as little training was required. Nevertheless, no effect on this form of memory was observed in 2-day-FZP-withdrawn rats using this paradigm (Figure 3). To evaluate whether
AMPAR potentiation was involved in the lack of LTP maintenance, rats were injected with GYKI-52466 (a GluR antagonist) or vehicle 24 h before LTP induction [8,10]. The results of these latter studies were inconclusive, as LTP in both control and FZP-withdrawn neurons was sensitive to prior vehicle injection (results not shown), perhaps related to the stress of injection [25]. Alternatively, the compensatory decrease of NMDAR during BZ withdrawal could be responsible for the lack of LTP maintenance since selective reduction of NR2B subunits impaired LTP expression [26]. Indeed, initial findings indicate a decrease in ifenprodil-sensitive NR2B-mediated currents along with a decrease in NR1 and NR2B, but not NR2A subunits at CA1 synapses of 2-day FZP-withdrawn rats [21,27].

Possible mechanisms of drug-induced plasticity

Findings to date suggest that CA1 neuron AMPAR potentiation during BZ withdrawal may involve a two-step process, GluR1 homomer incorporation followed by CaMKII-mediated Ser831-GluR1 phosphorylation [20]. As with models of activity-dependent plasticity such as LTP, CaMKII may play an important role in mediating AMPAR strength and CA1 neuron hyperexcitability in the hippocampus, an important locus within the neural circuits mediating anxiety [8,28,29]. As previously reported after tetanic stimulation [30], non-phospho-CaMKIIα levels were enhanced during BZ withdrawal, although phospho-Thr286-CaMKII levels were not elevated [30]. Whether phospho-Thr286-CaMKII may be an intermediary in CaMKII-mediated signalling during drug withdrawal remains a possibility. Alternatively, CaMKII activation may be long-lasting due to high levels of Ca2+/calmodulin [31]. In fact, L-VGCCs (L-type voltage-gated calcium channels), rather than NMDARs, may be responsible for initiating Ca2+-mediated signalling mechanisms associated with AMPAR potentiation, since prior injection with the L-VGCC antagonist nimodipine also prevented AMPAR potentiation and anxiety [10] consistent with a doubling of high-voltage-activated calcium channel current density in CA1 neurons immediately and up to 2 days after FZP withdrawal [32]. In contrast, CaMKII-mediated phosphorylation of GluR1 subunits may serve as a common final pathway for promoting both activity-dependent plasticity and drug-induced adaptations at CA1 pyramidal neuron synapses associated with BZ physical dependence.

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