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Research report

Role of bicarbonate ion in mediating decreased synaptic conductance in benzodiazepine tolerant hippocampal CA1 pyramidal neurons ☆

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Abstract

Chronic flurazepam treatment substantially impairs the function of GABAergic synapses on hippocampal CA1 pyramidal cells. Previous findings included a significant decrease in the synaptic and unitary conductance of CA1 pyramidal neuron $GABA_A$ receptor channels and the appearance of a $GABA_A$-receptor mediated depolarizing potential. To investigate the ionic basis of the decreased conductance, whole-cell voltage-clamp techniques were used to record evoked, $GABA_A$ receptor-mediated IPSCs carried by HCO_3^-–Cl^- or Cl^- alone. Hippocampal slices were prepared from rats administered flurazepam orally for 1 week, 2 days after ending drug treatment. Slices were superfused with HCO_3^-–aCSF or with HEPES–aCSF (without HCO_3^-) plus 50 μM APV and 10 μM DNQX. The micropipette contained 130 mM CsCl and 1 μM QX-314. $GABA_A$ receptors located on pyramidal cell somata or dendrites were activated monosynaptically by maximal stimulation of GABAergic terminals at the stratum oriens–pyramidale (SO–SP) or stratum lacunosum–molecular (S-L–M) border, respectively. In HCO_3^-–aCSF, there was a significant reduction in synaptic-conductance in flurazepam-treated neurons following both SO–SP (control: 1058 pS, flurazepam: 226 pS, $P<0.01$) and S-L–M (control 998 pS, flurazepam: 179 pS, $P<0.01$) stimulation, as well as the total charge transfer, indicating a decreased HCO_3^-–Cl^- flux. In HEPES–aCSF, the synaptic conductance and total charge transfer, and thus Cl^- flux, was unchanged in flurazepam-treated neurons (SO–SP: control 588 pS, flurazepam: 580 pS, $P>0.05$; S-L–M: control 595 pS, flurazepam: 527 pS, $P>0.05$). Taken together, these findings suggest that a reduction in HCO_3^- flux may play a prominent role in mediating the action of GABA and that a loss of HCO_3^- conductance may significantly contribute to impaired $GABA_A$ receptor function after chronic benzodiazepine treatment.

Theme: Neurotransmitters, modulators, transporters and receptors

Topic: $GABA_A$ receptors

Keywords: $GABA_A$ receptor; Flurazepam; Tolerance; Hippocampus; Chloride flux; Depolarizing potential

1. Introduction

The mammalian $GABA_A$ receptor is a pentameric protein, composed of subunits derived from several gene families with multiple variants (α1–6, β1–3, γ1–3, δ, ϵ and π) [10,18,36], which comprise an anion channel. The anion channel is differentially permeable to a range of anions including Cl^- and bicarbonate (HCO_3^-) ions [4,13]. The stoichiometry of the native $GABA_A$ receptor channel is still not precisely known. Nonetheless, studies of recombinant receptors have suggested that 2α, 2β and 1γ subunit may be the preferred combination at the synapse [8,62]. Studies in heterologous systems have demonstrated that the conductance of the $GABA_A$ receptor channel can vary as a function of changes in subunit composition [2,63]. The diversity of $GABA_A$ receptor subunit mRNAs [65] and proteins [16,53], particularly within the hippocampus, is consistent with the existence of multiple hippocampal $GABA_A$ receptor subtypes and the functional heterogeneity of GABA-mediated responses in this brain region [1,3,40,29,42,49,60].

In addition to the GABA binding site, the $GABA_A$ receptor complex also contains several allosteric binding sites for clinically important drugs including barbiturates

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and benzodiazepines. Benzodiazepines potentiate $GABA_A$ receptor function by increasing the frequency of channel opening [32,47] and at high concentrations may directly increase Cl^- conductance [12]. The capacity of the benzodiazepines to potentiate GABA-mediated inhibitory function relates to their potent anticonvulsant actions. Nonetheless, the clinical use of the benzodiazepines for treating epilepsies has been limited because of the rapid development of functional tolerance to their anticonvulsant actions [21,48]. The importance of $GABA_A$ receptor dysfunction to benzodiazepine functional tolerance has been well documented using several experimental approaches (for review see [23]).

Based on the findings of molecular studies from our laboratory and others [9,20,24,28,58,59,73,74], a change in $GABA_A$ receptor subunit composition was proposed as one mechanism associated with an impairment of $GABA_A$ receptor-mediated inhibitory function following prolonged benzodiazepine administration. In the CA1 region of hippocampus, the downregulation of specific (α1 and β3) $GABA_A$ receptor subunit mRNAs [58] and proteins [9] was accompanied by a decrease in $GABA_A$ receptor-mediated inhibition, i.e. a reduction in IPSP [70,71] and IPSC [44,72] amplitude. In fact, $GABA_A$ receptor-mediated estimated channel conductance was also reduced in CA1 pyramidal cells following chronic flurazepam treatment [44,72].

Another significant finding in our hippocampal benzodiazepine tolerance model was the unmasking of a $GABA_A$ receptor-mediated depolarizing potential in CA1 pyramidal cells following prolonged benzodiazepine administration [69]. Applied acutely, benzodiazepines and barbiturates have been shown to potentiate GABA-mediated depolarizing responses [1,37]. In contrast, the depolarizing response unmasked following chronic flurazepam administration was apparent at a time when benzodiazepine metabolites were no longer present in the hippocampus. Moreover, a change in IPSP reversal potential suggested a use-dependent Cl^- accumulation in benzodiazepine-treated CA1 pyramidal neurons [71].

Numerous mechanisms have been proposed to underlie the appearance of the depolarizing potential following repeated $GABA_A$ receptor activation, including a reversed electrochemical Cl^- gradient between pyramidal cell somata vs. dendrites [38], a HCO_3^--dependent, transient change in extracellular $[K^+]$ [26] or the differential re-establishment of Cl^- and HCO_3^- gradients following repeated $GABA_A$ receptor activation [55,56]. Compatible with each of these possible mechanisms, Alger and Nicoll [1] and subsequently Lambert et al. [31] proposed that the depolarizing potential was due to activation of $GABA_A$ receptor subtypes with different ionic selectivities. Whether single [37] or multiple [42] GABAergic interneuron populations may mediate the depolarizing potential remains speculative.

Regulation of specific CA1 pyramidal cell $GABA_A$ receptor subunits following prolonged benzodiazepine administration [9,58] implied that a change in $GABA_A$ receptor subunit composition may alter channel conductance and be reflected in a change in Cl^-–HCO_3^- flux. To test this hypothesis, whole-cell techniques were used in in vitro hippocampal slices to evaluate the current–voltage relationship of GABA-mediated evoked responses in CA1 pyramidal neurons of 1-week flurazepam-treated or control rats in HCO_3^-–aCSF or HEPES–aCSF. Estimates of slope conductance were made following localized, monosynaptic activation of somatic or dendritic $GABA_A$ receptors. Our findings indicated that the $GABA_A$ receptor-mediated current carried by HCO_3^-, but not that carried by Cl^-, was significantly modified after chronic benzodiazepine treatment. These findings suggest a change in the HCO_3^-–Cl^- permeability ratio of the channel during chronic benzodiazepine administration.

2. Materials and methods

2.1. Chronic benzodiazepine treatment

Rats administered the benzodiazepine, flurazepam orally for 1 week develop tolerance to the anticonvulsant actions of the benzodiazepines in vivo [48] and to their ability to potentiate GABA-mediated responses in CA1 pyramidal cells in vitro [67,72]. Following a 2-day 0.02% saccharin water adaptation period, flurazepam (100 mg/kg×3 days; 150 mg/kg×4 days) was given to adult male, Sprague–Dawley rats (185–225 g initial weight) in a saccharin vehicle as their only source of drinking water. The concentration of flurazepam was adjusted based on the rat's body weight and fluid consumption. All rats consumed a criterion dose of a weekly average of 100 mg/kg/day. Control rats were pair-handled and only offered saccharin water. Both control and flurazepam-treated rats were killed 2 days after ending treatment for preparation of hippocampal slices. Due to the rapid metabolism of flurazepam in rats, flurazepam and its active metabolites were not detectable in the hippocampus 2 days after ending treatment [66] so that the electrophysiological measurements were not affected by residual benzodiazepines. The experimenter was blind to the rats' treatment history until the data analysis was completed.

2.2. Slice preparation

The preparation of in vitro hippocampal slices from brains of flurazepam-treated and control rats, as well as whole-cell patch recordings from CA1 pyramidal cells, were carried out in either bicarbonate-containing artificial cerebral spinal fluid (HCO_3^-–aCSF) or bicarbonate-free buffer (HEPES–aCSF). HCO_3^-–aCSF contained [in mM]: NaCl, 126; KCl, 3.0; $MgCl_2$, 1.5; $CaCl_2$, 2.4; $NaHPO_4$, 1.2; D-glucose, 11.0; and $NaHCO_3$, 25.9. HCO_3^-–aCSF

was gassed with 95% O_2 and 5% CO_2 to a final pH of 7.4 (312–316 mOsm). In nominally HCO_3^-–CO_2-free buffer the $NaHCO_3$ in the aCSF was replaced by 26 mM HEPES (312 mOsm), the pH was adjusted to 7.4 with NaOH and the buffer gassed with 100% O_2. For brain slice preparation, the rats were decapitated using approved techniques, and their brains were quickly removed and placed in ice-cold, pregassed HCO_3^-–aCSF or HEPES–aCSF. The left hippocampus was gently dissected, glued onto a 4% agar block and immediately submersed in ice-cold HCO_3^-–aCSF or HEPES–aCSF, respectively. Using a vibratome (Pelco 101, Ted Pella, Redding, CA, USA), transverse slices (500 μm) were cut from the middle portion of the hippocampus. The slices were kept in either HCO_3^-–aCSF or HEPES–aCSF and continuously gassed at room temperature for 1 h prior to recording.

2.3. Electrophysiological recordings and analysis

Whole-cell recordings were made in CA1 pyramidal cells in either HCO_3^-–aCSF or HEPES–aCSF perfused at a constant rate of 1.5 ml/min at room temperature. Patch-pipettes were made from non-filamented, borosilicate capillaries (1.5 mm O.D., Sutter. Novato, CA, USA) with a two-stage pull programmed on a Flaming–Brown puller (P-97, Sutter) to a tip diameter of about 1 μm (4–7 MΩ). The internal solution contained [in mM]: CsCl 130, EGTA 1.0, $CaCl_2$ 0.5, $MgCl_2$ 2.0, ATP 2.0, HEPES 10.0, QX-314 2.0, pH adjusted to 7.2. Chloride-loading was shown to reverse the use-dependent shift in E_{Cl-} due to prolonged GABA receptor activation [33,72]. Cs^+ was used to block the K^+ current stimulated by postsynaptic $GABA_B$ receptor activation. QX-314, a lidocaine derivative, was used to block sodium channels and thus the spontaneous firing of CA1 neurons [34,71]. CA1 neurons were patched using 'blind' techniques. After gigaohm seal formation and cell rupture, electrical signals were low-pass filtered at 10 KHz, amplified 10× with an Axoclamp 2A amplifier (Axon Instruments Foster City, CA, USA) and voltage-clamped to the desired membrane potential in continuous mode (cSEVC). For mIPSC recordings, the amplified DC currents were offset and further amplified 100× (FL4, 4 pole Bessel Filter, Dagan, Minneapolis, MN, USA). The electrical signals were displayed using PCLAMP 6.0 software interfaced with a Digidata 1200 A/D converter (Axon Instruments) and stored on a PC computer and storage tape for later analysis.

Evoked $GABA_A$ receptor-mediated IPSCs were isolated in CA1 neurons in the presence of excitatory amino acid (EAA) antagonists, APV (50 μM) and DNQX (10 μM) in HCO_3^-–aCSF or HEPES–aCSF. To evoke IPSCs monosynaptically a bipolar tungsten stimulating electrode was placed in either the SO–SP or S-L–M, <0.5 mm from the recording site. Constant-current pulses (0.1 ms in duration) were generated from a Master-8 stimulator (A.M.P.I., Jerusalem, Israel) coupled to an Iso-Flex constant current stimulus isolation unit (A.M.P.I.). The intensity of stimulation was gradually increased until IPSC amplitude reached its maximum value. The interval between stimulations was a minimum of 30 s. Using the stimulus intensity which elicited an IPSC of maximal amplitude, the membrane potential was clamped from $V_h = -70$ to $+70$ mV and IPSCs averaged from the 2–3 responses at each membrane potential were recorded at 30-s intervals. Analysis of evoked IPSCs was carried out using PCLAMP 6.0 software. The peak amplitude of IPSCs was measured from the baseline immediately prior to stimulation. Values reported represent averages of 2–3 current responses at each V_h (−70 to +70 mV). To more accurately measure $GABA_A$ channel properties, synaptic conductance was estimated from the slope of the best-fit line to the current–voltage responses derived from individual cells. To reduce the contribution of inwardly rectifying Cl^- conductances activated by CIC-2 or CIC-3 channels to the $GABA_A$ receptor-mediated conductance only responses generated at $V_h = -70$ to 0 mV were used to calculate slope conductance [51,55,56]. The mean slope was compared between control and flurazepam treated-cells by Student's *t*-test. The area under the IPSC was used to estimate the total charge transfer. The slope of the charge transfer curve was used to estimate total charge transfer across all membrane holding potentials.

$GABA_A$-receptor-mediated mIPSCs were recorded in HEPES–aCSF for comparison to the results of previous mIPSC recordings in HCO_3^-–aCSF [72]. Miniature inward currents were recorded in the presence of 1 μM tetrodotoxin (TTX), 50 μM APV and 10 μM DNQX. Events were digitized at 20 KHz and low-pass filtered at 10 KHz, amplified 10× with an Axoclamp 2A amplifier (Axon Instruments) and voltage-clamped in continuous mode (cSEVC). The amplified DC currents were offset and then further amplified 100× (FL4, 4 pole Bessel Filter, Dagan). If series resistance, as monitored by the holding current, varied by >80% the recording was abandoned. To ensure that the relatively smaller events in FZP-treated neurons were reliably detected and to avoid voltage-clamp errors, mIPSCs were recorded at $V_h = -70$ and −90 mV. mIPSCs were analyzed off-line using Strathclyde CDR and SCAN software (J. Dempster, University of Strathclyde, Glasgow, UK) from 5-min segments of data acquired from each neuron under each holding potential. Detection threshold for individual mIPSC events was set just above the maximum recording system background noise. Data from individual neurons was omitted if the background noise exceeded ±2.0 pA or if event duration was <3 ms. For statistical comparisons using Student's *t*-test ($P \leq 0.05$), averaged mIPSC amplitude and decay (τ) were compared in CA1 neurons from control and FZP-treated rats. Relative cumulative frequency distributions of mIPSC am-

plitude and inter-event interval were also calculated for control and FZP-treated neurons. The Kolmogorov–Smirnoff (K–S) test was used to compare the cumulative probability distributions of amplitude and frequency and were considered different if $P<0.01$ [72].

2.4. Drug application and drug solutions

APV (D,L-2-amino-5 phosphonovaleric acid) and DNQX (6,7-dinitroquinoxaline-2,3-done) were made as a stock solution (100× the final concentration) and added to the perfusate to their final concentration with a syringe pump at a rate of l.5 μl/min. APV and DNQX were purchased from Research Biochemicals (Natick, MA, USA). QX-314 was from Alamone Labs. (Jerusalem, Israel). Buffer chemicals were from Sigma (St. Louis, MO, USA) or Fisher (Pittsburgh, PA, USA).

3. Results

3.1. $GABA_A$ receptor-mediated evoked IPSCs in HCO_3^-–aCSF

3.1.1. Stratum oriens–pyramidale stimulation

In the presence of EAA antagonists, IPSCs were evoked monosynaptically in hippocampal CA1 neurons by stimulation of interneuron terminals at the SO–SP border. Of the several populations of GABAergic interneurons anatomically and physiologically identified in CA1 region of hippocampus [6,15,30] SO–SP stimulation likely directly activates both GABAergic basket cell and stratum oriens interneuron cell bodies or their axons in or near the cell layer [31,34]. Representative evoked IPSCs recorded in HCO_3^-–aCSF are shown in Fig. 1A. We previously reported that evoked IPSC amplitude and thus $GABA_A$ receptor-mediated synaptic conductance was significantly

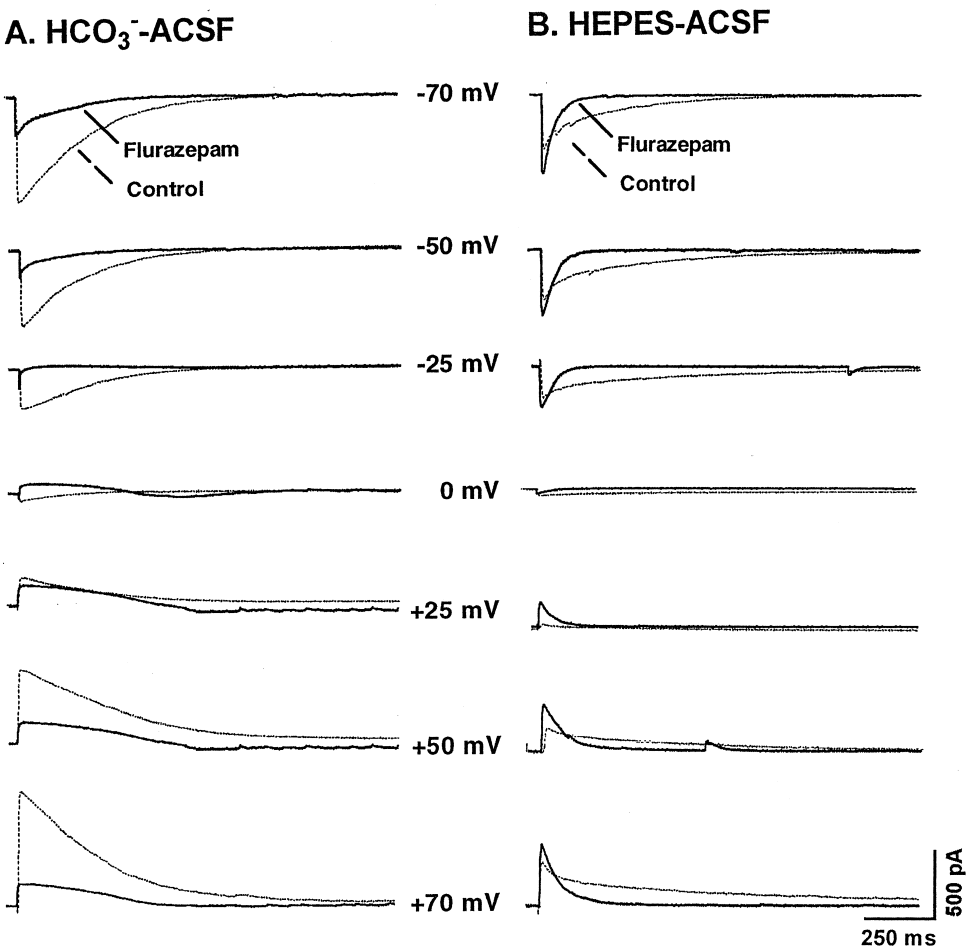

Fig. 1. IPSCs evoked by st. oriens stimulation in (A) HCO_3^-–aCSF or (B) HEPES–aCSF. Representative traces of monosynaptically evoked IPSCs from control and flurazepam-treated CA1 pyramidal neurons at membrane holding potentials from −70 to +70 mV. Isolated $GABA_A$ receptor mediated IPSCs were evoked in CA1 pyramidal cells by stimulation at the st. oriens–st. pyramidale border. (A) Evoked IPSCs recorded in HCO_3^-–aCSF. The peak amplitude of IPSCs was reduced in CA1 pyramidal cells derived from rats killed 2 days after the oral flurazepam treatment was stopped (solid trace) when compared to cells from pair-handled control rats (dashed trace). (B) When IPSCs were recorded in HEPES–aCSF, there were no significant differences in the amplitude of IPSCs evoked in flurazepam-treated CA1 neurons (solid trace) as compared to control neurons (dashed trace).

reduced in flurazepam-treated cells recorded in HCO_3^-–aCSF [72]. Under the identical experimental conditions these findings were replicated (Fig. 2A, SO–SP) in a smaller number of flurazepam-treated and control neurons. Mean evoked IPSC amplitude measured in control CA1 neurons were compared to those from flurazepam-treated neurons at each membrane potential ($V_h = -70$ to $+70$ mV) (Fig. 2A). IPSC decay was fit with a monophasic or biphasic function. The results of the monophasic fit are shown to simplify comparison between groups under each buffer condition (Table 1). The mean reversal potentials (E_{IPSC}) derived from the current–voltage responses of individual cells from both control and flurazepam-treated groups was near the E_{Cl^-/HCO_3^-} ($+.04$ mV) calculated from the Goldman–Hodgkin–Katz (GHK) current equation ($E_{IPSC} = 58 \log P_{Cl^-}[Cl^-]_i + P_{HCO_3^-}[HCO_3^-]_i / P_{Cl-}[Cl^-]_o + P_{HCO_3^-}[HCO_3^-]_o$) [19] assuming a $P_{HCO_3^-}/P_{Cl^-}$ permeability ratio of 0.25 [4,56], and were not significantly different between groups (Table 2). Synaptic conductance, estimated from the slope of the *I–V* relationship, was nearly five-fold lower in flurazepam-treated neurons when compared to control neurons (Table 2; Fig. 2A). The total charge transfer, estimated by calculating the area under the evoked IPSC, was also significantly ($P < 0.003$) reduced in flurazepam-treated cells when compared to control cells (Fig. 3A, SO–SP). This finding suggested that $GABA_A$

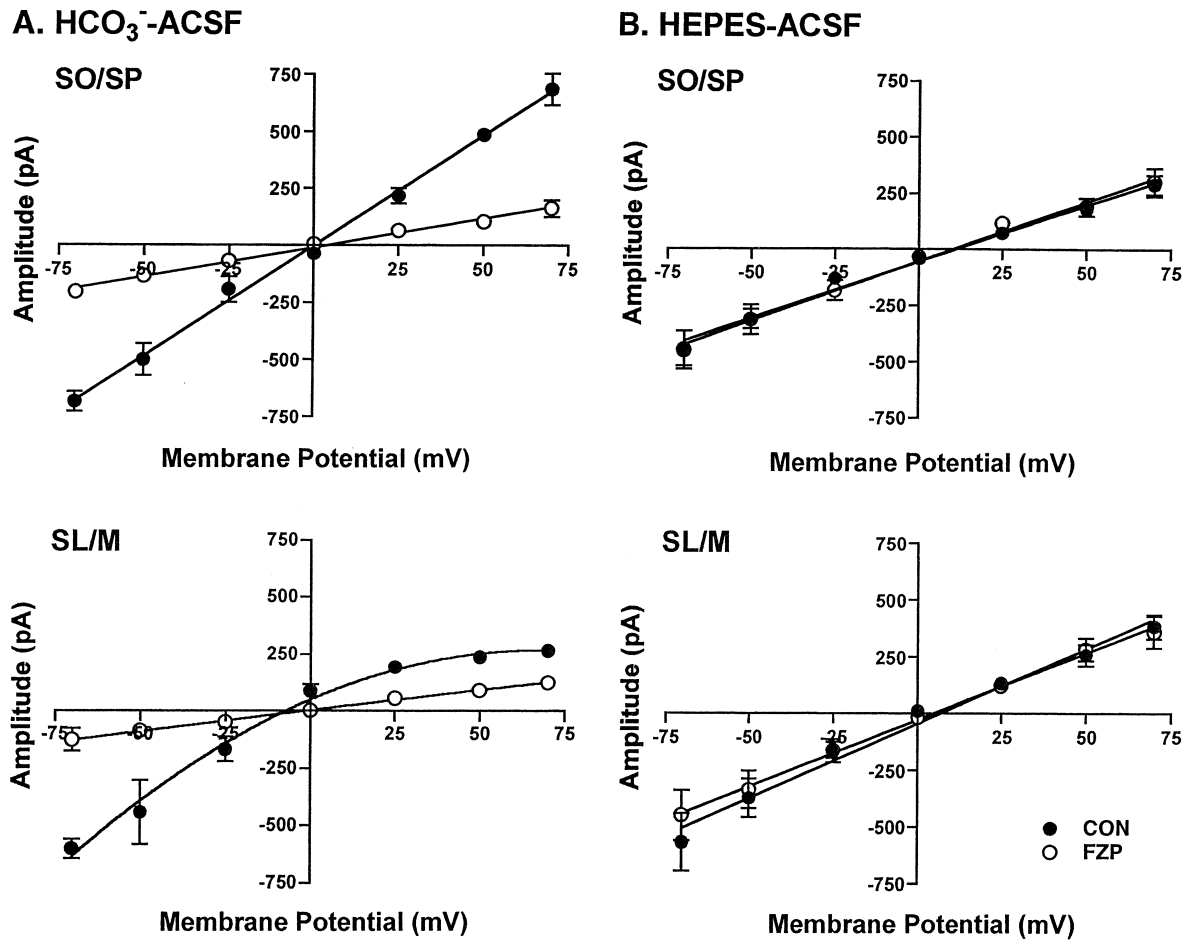

Fig. 2. Effect of 1-week flurazepam treatment on the evoked IPSCs *I–V* relationship. CA1 pyramidal neurons were voltage-clamped from -70 to $+70$ mV. All recording were carried out in the presence of excitatory amino acid antagonists APV (50 μM) and DNQX (10 μM). 130 mM CsCl and 1 μM QX-314 were included in the whole-cell pipette. (A) *I–V* relationship derived from control (closed circles) and flurazepam-treated (open circles) CA1 pyramidal cells recorded in HCO_3^-–aCSF following monosynaptic stimulation at the st. oriens–st. pyramidale (SO–SP) border (top) or the st. lacunosum–moleculare (S-L–M) (bottom). In control cells following SO–SP stimulation the *I–V* relationship was best fit with a linear function. Following S-L–M stimulation, the *I–V* relationship was best described by non-linear function indicating inward rectification. There was a significant decrease in the slope of the *I–V* relationship in flurazepam-treated cells following both SO–SP and S-L–M stimulation, indicating a reduction in synaptic conductance (see Table 2). (B) The *I–V* curves from control and flurazepam-treated cells recorded in HEPES–aCSF were best fit with a linear function. As shown in Table 2, the synaptic conductance estimated from the slope of *I–V* relationship derived from cells recorded in HEPES–aCSF was not significantly different between control and flurazepam-treated CA1 neurons following SO–SP or S-L–M stimulation.

Table 1
Amplitude and decay kinetics of CA1 pyramidal cell evoked IPSCs at $V_h = -70$ mV[a]

	Stimulation site				
	SO–SP			S-L–M	
	Amplitude (pA)	τ (ms)		Amplitude (pA)	τ (ms)
HCO_3–aCSF					
Control ($n=20$)	−691.3±87.7	217.1±32.1	Control ($n=6$)	−599.7±42.4	581.6±157.8
Flurazepam ($n=17$)	−199.1±2.5	299.1±39.8	Flurazepam ($n=7$)	−125.1±49.9	315.2±59.1
P value	≤0.001*	0.11	P value	0.001*	0.06
HEPES–aCSF					
Control ($n=12$)	−442.2±77.6	310.6±51.6	Control ($n=9$)	−566.6±125.9	255.5±36.4
Flurazepam ($n=16$)	−448.3±84.0	153.3±18.3	Flurazepam ($n=14$)	−448.4±109.8	174.9±32.5
P value	0.96	0.003*	P value	0.48	0.11

[a] SO–SP: st. oriens–st. pyramidale; S-L–M: st. lacunosum–molecular. Values represent mean±S.E.M.; asterisks denote significant difference at $P \leq 0.05$.

receptor mediated currents, carried by both Cl^- and HCO_3^-, were modified after chronic benzodiazepine treatment.

3.1.2. Stratum lacunosum–moleculare stimulation

By stimulating interneuron cell bodies and terminals in the S-L– layer, $GABA_A$ receptors primarily located in distal apical dendrites of pyramidal cells were activated [31,34]. Maximal IPSC amplitude in CA1 pyramidal cells was compared in hippocampal slices from control and flurazepam-treated rats following S-L–M stimulation. The *I–V* relationship derived from control neurons was best-fit by a non-linear sigmoidal curve and indicated inward rectification, whereas that from flurazepam-treated neurons was best fit by a linear function (though synaptic conductance was calculated from $V_h = -70$ to 0 mV, see above). The mean reversal potential for the control and for the flurazepam-treated group of neurons were not significantly different (Table 2). The slope of the *I–V* curve, i.e., synaptic conductance, was significantly, 5-fold, lower in flurazepam-treated neurons when compared to control neurons (Table 2; Fig. 2A, S-L–M). The total charge transfer was also significantly decreased in the flurazepam-treated, in comparison to the control cells (Fig. 3A, S-L–M). Together with the above findings following stimulation of interneuron terminals at the SO–SP border, we concluded that both somatic and dendritic $GABA_A$ receptor-mediated currents, when carried by both Cl^- and HCO_3^-, were reduced after chronic benzodiazepine treatment.

3.2. $GABA_A$ receptor-mediated evoked IPSCs in HEPES–aCSF

3.2.1. Stratum oriens–pyramidale stimulation

In another group of control and flurazepam-treated cells, $GABA_A$ receptor-mediated IPSCs resulting from stimulation of interneuron terminals in the SO–SP layer were recorded in HEPES–aCSF, i.e., HCO_3^- free buffer, so that Cl^- was the primary anion passing through the $GABA_A$ receptor channel (Fig. 1B). In the absence of HCO_3^- the GHK equation predicts a 2.0-mV shift in the reversal potential; the mean reversal potential for individual control and flurazepam-treated neurons were both positively shifted, ~2–5 mV, and were not significantly different

Table 2
Slope conductance and reversal potential of CA1 pyramidal cell IPSCs evoked by SO–SP or S-L–M stimulation[a]

	SO–SP			S-L–M	
	Slope (pS)	E_{IPSC}		Slope (pS)	E_{IPSC}
HCO_3–aCSF					
Control ($n=3$)	1058.0±61.1	−0.2±1.3	Control ($n=7$)	998.0±39.2	−7.8±2.5
Flurazepam ($n=4$)	225.5±32.1	0.9±3.2	Flurazepam ($n=8$)	178.5±8.5	−3.1±5.0
P value	0.005*	0.86	P value	0.001*	0.38
HEPES–aCSF					
Control ($n=8$)	588.4±77.6	5.4±3.2	Control ($n=9$)	595.3±13.9	3.4±2.4
Flurazepam ($n=10$)	579.9±19.7	2.8±3.3	Flurazepam ($n=14$)	526.7±14.2	0.9±3.5
P value	0.18	0.59	P value	0.74	0.35

[a] SO–SP: st. oriens–st. pyramidale; S-L–M: st. lacunosum–moleculare. Values represent mean±S.E.M.; asterisks denote significant difference at $P \leq 0.05$.

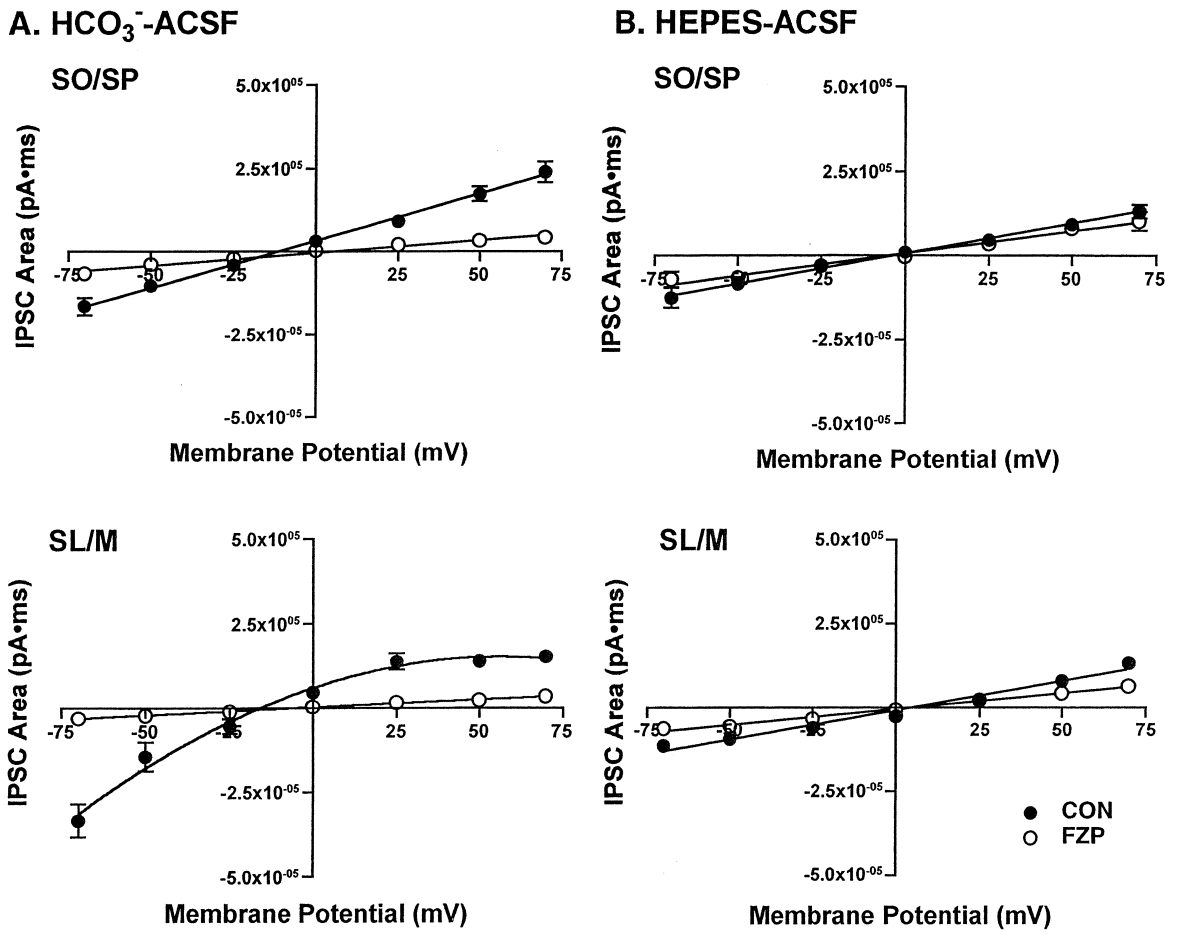

Fig. 3. Effect of 1-week flurazepam treatment the total charge transfer during evoked IPSCs. The area under evoked IPSCs was measured to estimate the total charge transfer due to activation of GABAergic terminals. Data was derived from those cells shown in Fig. 2. (A) The total IPSC area from control and flurazepam-treated CA1 neurons recorded in HCO_3^-–aCSF following monosynaptic stimulation at the st. oriens–st. pyramidale (SO–SP) border (top) or the st. lacunosum–moleculare (S-L–M) (bottom). Similar to the *I–V* relationship shown in Fig. 3, the total charge transfer curve derived from control cells was best fit by linear function following SO–SP stimulation whereas the curve was non-linear following S-L–M stimulation. Following both SO–SP and S-L–M, there was a significant reduction in charge transfer in flurazepam-treated as compared to control cells. (B) The total charge transfer during evoked IPSCs from control and flurazepam-treated cells recorded in HEPES–aCSF. The charge transfer, best described as a linear function was not significantly different between control and flurazepam-treated CA1 neurons following SO–SP or S-L–M stimulation.

(Table 2). The synaptic conductance estimated from control neurons recorded in HEPES–aCSF (588 pS) was ~50% of that recorded in HCO_3^-–aCSF (1058 pS), suggesting that HCO_3^- flux plays a significant role in mediating $GABA_A$ receptor currents (Table 2). The slope of the *I–V* relationship was not significantly different between control and flurazepam-treated neurons, indicating no difference in synaptic, $GABA_A$ receptor-mediated conductance between groups (Table 2; Fig. 2B, SO–SP). The total charge transfer following S-L–M stimulation was not significantly ($P<0.18$) decreased in flurazepam-treated, in comparison to the control cells (Fig. 3B, SO–SP). This result suggested that the primarily somatic, $GABA_A$ receptor-mediated current carried by Cl^- was not changed following chronic benzodiazepine treatment.

3.2.2. Stratum lacunosum–moleculare stimulation

Isolated IPSCs evoked in CA1 pyramidal neurons following S-L–M stimulation were also recorded in HEPES–aCSF. The voltage sensitivity of the control response in HEPES–aCSF was similar to that in HCO_3^-–aCSF. There were no significant differences between the mean reversal potentials between groups of individual control and flurazepam-treated cells (Table 2). In control cells the mean synaptic conductance recorded following S-L–M stimulation was equivalent to that recorded following SO–SP stimulation and the slope of the *I–V* relationship recorded was not significantly different between control and flurazepam-treated neurons (Table 2; Fig. 2B, S-L–M). The total charge transfer was also not significantly ($P>1.40$) decreased in the flurazepam-treated, in

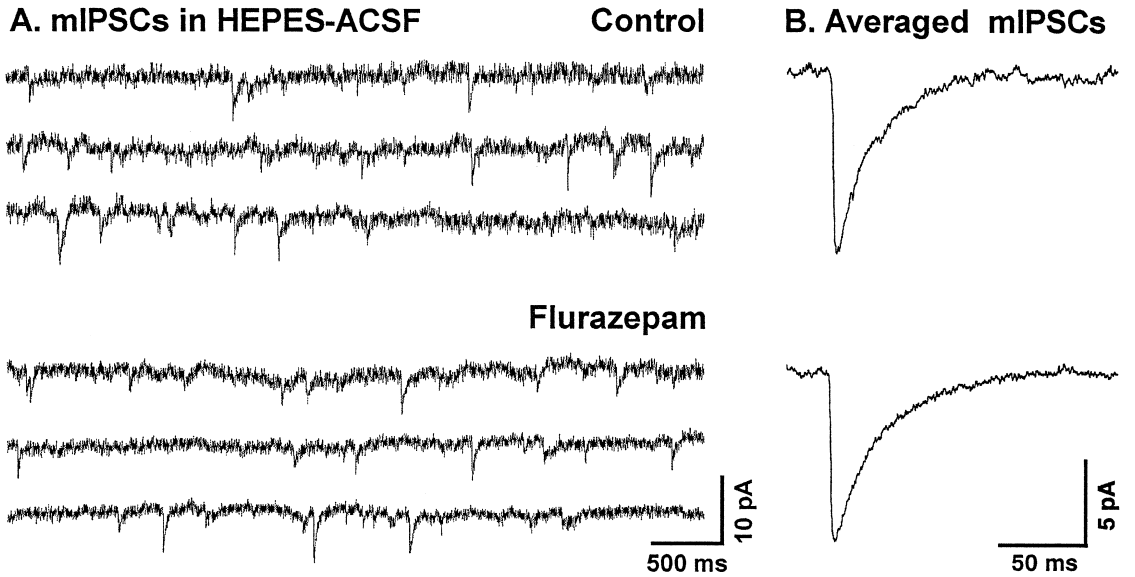

Fig. 4. Effect of 1-week flurazepam treatment on CA1 pyramidal cell mIPSCs recorded in HEPES–aCSF. (A) Representative traces of mIPSCs recorded for 5 min during 1 μM TTX superfusion from a control (top) and a flurazepam-treated (bottom) CA1 pyramidal neuron ($V_h = -70$ mV). As shown in Table 3, there was no change in the amplitude or decay of mIPSCs recorded from flurazepam-treated neurons as compared to control neurons in HEPES–aCSF. (B) Averaged mIPSCs from the same control (top) and flurazepam-treated neuron (bottom) as shown in (A).

comparison to control cells (Fig. 3B, S-L–M). Taken together, these findings suggested that both dendritic and somatic $GABA_A$ receptor-mediated Cl^- currents were not modified as a result of chronic benzodiazepine treatment.

3.3. $GABA_A$-receptor-mediated miniature (m)IPSCs in HEPES–aCSF

In addition to a decreased synaptic conductance, a 5-fold decrease in single-channel conductance (estimated by non-stationary fluctuation analysis) was noted in flurazepam-treated cells (5 pS) as compared to control CA1 neurons (27 pS) recorded in HCO_3^-–aCSF, associated with a 50% reduction in CA1 pyramidal cell mIPSC amplitude [72]. In the present experiment, $GABA_A$-receptor-mediated mIPSCs were recorded in HEPES–aCSF using whole-cell techniques as previously described [72]. Using the internal solution described above, spontaneous, inward currents were recorded in the presence of 1 μM tetrodotoxin (TTX), 50 μM APV and 10 μM DNQX at $V_h = -70$ and -90 mV (Fig. 4). The mIPSC amplitude of flurazepam-treated neurons recorded in HCO_3^-–aCSF was 40% of the control neuron response [61,72]. These historical data are presented in parentheses in Table 3 for comparison to the present findings. Similar to evoked IPSCs recorded in HEPES–aCSF, mIPSC amplitude in control neurons was smaller (−8.3 pA) than that recorded previously in HCO_3^-–aCSF (−26 pA). Moreover, when recorded in HEPES–aCSF, there was no significant difference between control and flurazepam-treated neuron mIPSC amplitude or decay at either holding potential (Table 3). There were no significant differences ($P > 0.01$) between control and FZP-treated neurons recorded in HEPES–aCSF in the distributions of either mIPSC inter-event interval or am-

Table 3
CA1 pyramidal cell mIPSC amplitude and kinetics in HEPES– (and HCO_3–aCSF)[a]

	Membrane potential			
	$V_h = -70$ mV		$V_h = -90$ mV	
	Amplitude (pA)	τ (ms)	Amplitude (pA)	τ (ms)
Control ($n=4$)	−8.3±1.1 (−25.6)	28.6±3.5 (25.1)	−10.5±1.5	19.8±1.7
Flurazepam ($n=4$)	−8.3±1.5 (−10.4)	26.2±4.1 (26.9)	−10.6±1.8	24.1±3.8
P value	0.96 (<0.01*)	0.63 (0.53)	0.95	0.27

[a] Values represent mean±S.E.M.; asterisks denote significant difference at $P \leq 0.05$; values in parentheses at $V_h = -70$ mV represent historical data from control ($n=9$) and flurazepam-treated ($n=13$) CA1 neurons recorded in HCO_3–aCSF [72].

plitude. These data imply that changes in Cl^- flux may not play a major role in the reduction in $GABA_A$ receptor-mediated conductance following 1-week flurazepam administration.

4. Discussion

Previous studies have reported that both synaptic and unitary $GABA_A$ receptor-mediated conductance are substantially reduced in CA1 pyramidal cells as a function of chronic benzodiazepine administration [44,72]. Since HCO_3^- flux plays an important role in the use-dependent appearance of $GABA_A$ receptor-mediated depolarizing potentials in CA1 pyramidal cells [55,56,25,26] we hypothesized that a change in HCO_3^-, as well as Cl^-, flux may underlie alterations in channel conductance following 1-week oral flurazepam administration [70]. To test this hypothesis synaptic conductance was evaluated in CA1 pyramidal cells from flurazepam-treated and control rats, both in HCO_3^-–aCSF and HEPES–aCSF. The major, somewhat surprising finding of the present study was that the amplitude of IPSCs in CA1 pyramidal cells from flurazepam-treated rats was significantly smaller when HCO_3^- was the major charge carrier, but not when the Cl^- ion predominates. Moreover, only a very small, non-significant change in peak IPSC amplitude and total charge transfer could be detected in CA1 neurons following 1-week flurazepam administration, when Cl^- was the predominant ionic species. Overall, these findings suggest that the HCO_3^- may play a more prominent role than previously considered in determining E_{GABA} during the normal function of GABAergic synapses (see also [25]) and in mediating the $GABA_A$ receptor dysfunction resulting from chronic benzodiazepine treatment.

When comparing the $GABA_A$ receptor-mediated slope conductance between control neurons in bicarbonate-containing (HCO_3^-–aCSF) vs. bicarbonate-free (HEPES–aCSF) buffer, the contribution of HCO_3^- to the slope conductance was much larger than expected based on ratio of $[HCO_3^-]/[Cl^-]$ (24 mM/130 mM) and the relative permeability ratio (0.25) of these ions in hippocampal CA1 neurons [55,56]. That is, when HCO_3^- was removed from bath, the slope conductance would be calculated to be reduced by about 5%, rather than the observed 50% decrease. This discrepancy may be related to the relatively large conductance measured in control neurons bathed in HCO_3^-–aCSF due to the maximal stimulation required to compare evoke IPSCs across experimental groups. In HCO_3^-–aCSF recording conditions, although the recorded neuron was loaded with Cl^-, the $[Cl^-]_{i/o}$ in interneurons is still quite asymmetric. Thus, in HCO_3^-–aCSF, maximal stimulation may have resulted in a polysynaptic, $GABA_A$ receptor-mediated depolarization of interneurons [26] resulting in an increased amount of GABA release. In HEPES–aCSF, such an excitatory effect on interneurons would have been eliminated due to absence of HCO_3^-. This interpretation also receives support from the finding that the frequency of mIPSCs recorded in HEPES−aCSF was unaffected between experimental groups.

As was found previously [72], synaptic conductance in chronic benzodiazepine treated neurons was reduced nearly five-fold in HCO_3^-–aCSF in comparison to control neurons, following maximal stimulation of GABAergic interneuron terminals. Using non-stationary fluctuation analysis of mIPSCs recorded in HCO_3^-–aCSF, the single channel conductance of synaptic, CA1 pyramidal cell $GABA_A$ receptors was previously calculated to be 27 pS ($N=46$) generally consistent with findings in CA3 pyramidal cells (30 pS) [45] and hippocampal (20 pS) [11] and cerebellar (28 pS) [5] granule cells. Using the measured slope conductance in control neurons (1058 pS) and the previously determined channel number ($N=46$), the channel conductance in the present experiment can be estimated to be ~23 pS, although the channel number determined using fluctuation analysis may underestimate the numbers of synaptic, and perhaps extrasynaptic, receptors activated by intense monosynaptic stimulation [5,39]. Non-stationary fluctuation analysis of mIPSCs from CA1 neurons of 1-week flurazepam-treated rats indicated a significant reduction in estimated $GABA_A$ receptor channel conductance to 5 pS. Estimated channel number remained unchanged ($N=50$) [72]. The single-channel conductance estimated from the synaptic conductance measured in flurazepam-treated neurons in the present experiment was also estimated to be ~5 pS. Since neither the amount of GABA in the terminal nor GABA release processes were altered by flurazepam treatment [72], the apparent reduction in synaptic, $GABA_A$ receptor-mediated conductance recorded in flurazepam-treated neurons was likely due to a change in the properties of the postsynaptic $GABA_A$ receptor (and the excitability of interneurons). Nonetheless, the reduction in synaptic conductance may or may not represent a true reduction in single-channel conductance. For example, it is possible that a change in channel gating or in other channel properties may have resulted in this apparent change in synaptic conductance, a question which can only be addressed by single-channel recording experiments.

Although localized monosynaptic stimulation of GABAergic terminals in somatic and dendritic layers cannot ensure that the combination of $GABA_A$ receptors activated was either primarily somatic or dendritic, the *I–V* curves generated in the two areas suggested that different combinations of $GABA_A$ receptors may have been activated by SO–SP and S-L–M stimulation. The outward rectification of the *I–V* curve generated following stimulation of terminals at the SO–SP border was characteristic of CA1 pyramidal cells [7,17,60] (Fig. 2). Inward rectification of the *I–V* curve was noted in control neurons following stimulation of terminals in the S-L–M layer in HCO_3^-–aCSF, but not HEPES–aCSF. In addition to the

possibility that Cl^- preferentially accumulated in pyramidal cell dendrites following maximal stimulation at more positive potentials, reducing the Cl^- driving force [56,57], there are at least a few other explanations for this finding. For example, though Cs^+ was used in the recording pipette, in the absence of a $GABA_B$ receptor blocker, one possibility is that a $Ba^{2+}(Cs^+)$-insensitive $GABA_B$-receptor-mediated, inwardly rectifying K^+ conductance contributed to the currents measured at positive holding potentials [43]. Williams and Lacaille [64] reported that a population of S-L–M interneurons activated a population of Ba^{2+}-resistant $GABA_B$-mediated IPSPs. However, Ba^{2+} almost completely blocked $GABA_B$ receptor-mediated K^+ conductances elicited synaptically by electrical stimulation [43]. An alternate K^+ conductance, e.g. generated by the K^+–Cl^- exchanger, may contribute to the current measurement [25,26]. The lack of inward rectification in HEPES–aCSF suggested a HCO_3^--dependence of the inward rectification, supporting the latter possibility [26]. Despite the inward rectification, synaptic conductance was also significantly reduced in flurazepam-treated vs. control neurons following S-L–M stimulation in HCO_3^-–aCSF, but not in HEPES–aCSF.

In the present experiment, in which intracellular and extracellular anion concentrations and membrane potential were experimentally controlled, the reduction in synaptic conductance may largely be explained by a reduction in HCO_3^- permeability. Ion permeability is an intrinsic property of the channel. Dependent on the experimental conditions and the cells under study, the HCO_3^-/Cl^- permeability ratio varies from 0.18–0.44 [4,13,27]. In comparison to Cl^-, HCO_3^- has a larger Stokes diameter and an asymmetrical charge distribution, both factors contributing to the lower permeability of HCO_3^- through the $GABA_A$ receptor channel under normal physiological conditions [4,13]. It is unlikely that the channel pore size per se of the channel was altered by chronic benzodiazepine treatment. Nonetheless, the effective pore size could be altered by a change in the conformation of the receptor as a result of change in agonist binding or, for example, by a change in the phosphorylation state of the receptor [35], though direct evidence for this possibility has not emerged. In addition, there may be some unexpected ion–ion interactions in the $GABA_A$ ionophore, so that the HCO_3^- has a much larger effect on the observed conductance than would be expected based on the relative permeability ratio. On the other hand, a change in $GABA_A$ receptor subunit composition could lead to a change in the amino acid residues within the TM2 membrane spanning region of the heteromeric protein and thus the positive charge distribution within the pore, altering HCO_3^- permeability [14,52]. Receptor stoichiometry and subunit composition has been shown to modify the channel properties of recombinant receptor systems. For example, subunit composition has been shown to affect the main conductance level and the gating kinetics of $GABA_A$ receptor channels [35,50,63]. However, the contribution of subunit composition to HCO_3^- permeability is unexplored. Nonetheless, $GABA_A$ receptors in the CA1 region may be remodeled following 1-week flurazepam treatment. The evidence includes localized decreases in $\alpha1$ and $\beta3$ subtype mRNA and protein expression, increases in the levels of $\beta2$ mRNA levels [9,58] and an upregulation of $\alpha4$ and $\alpha5$ subunit proteins [S. Chen, E.I. Tietz, unpublished observations] in the CA1 region of the hippocampus. If such changes in subunit composition also occurred in postsynaptic $GABA_A$ receptors on interneurons, the balance of excitatory/inhibitory inputs onto interneurons might be altered, affecting tonic GABA release. In fact, a selective decrease in the levels of the $\alpha1$ subunit mRNA was detected in a subpopulation of interneurons in the CA1 region located at the str. oriens–str. pyramidale border [58]. In contrast to our current finding in HEPES–aCSF, the frequency of spontaneous but not miniature IPSCs was decreased in FZP-treated cells recorded in bicarbonate-containing buffer, suggesting that interneuron activity under may indeed be altered following chronic benzodiazepine treatment. Thus, it is conceivable that a change in $GABA_A$ receptor subunit composition as a function of chronic benzodiazepine treatment may underlie a change in HCO_3^-, but not Cl^-, permeability, as well as a change in interneuron excitability.

Although the findings of the present study and previous studies do not directly address the ionic basis for the $GABA_A$ receptor-mediated depolarizing potential in CA1 pyramidal cells which arises as a result of 1-week flurazepam treatment, they do suggest that Cl^- accumulation and an altered HCO_3^- flux may play a central role. Though the mechanism(s) underlying the appearance of a $GABA_A$ receptor-mediated depolarizing potential are controversial, a consistent feature of the available models is the prominent role of HCO_3^- flux in facilitating the depolarizing response [26,42,55,56]. A network activity-dependent increase in extracellular $[K^+]$ can contribute to the amplitude and duration of the depolarizing potential [26], but likely by decreasing the KCl transport rate, facilitating an accumulation of Cl^-, a condition sufficient to explain the response [56]. The positive shift of the E_{IPSP} in flurazepam-treated neurons in intracellular experiments had suggested that the depolarizing response unmasked was related to excess Cl^- accumulation following repeated activation of the $GABA_A$ receptor during prolonged benzodiazepine administration, in conjunction with high intensity stimulation [70,71]. Based on the assumptions of the Cl^- gradient hypothesis, the absence of a depolarizing potential in flurazepam-treated neurons recorded in HEPES–aCSF was anticipated [56]. Depolarizing potentials were also not apparent following flurazepam treatment under the current whole-cell recording conditions in HCO_3^-–aCSF in which intracellular Cl^- concentration was equalized between experimental groups (see [33]). Although HCO_3^- was available under some experimental

conditions, Cl^- was equilibrated under all conditions suggesting that at least intracellular Cl^- accumulation was central to the appearance of the depolarizing potential after chronic benzodiazepine treatment (see also [54,42]).

A change in HCO_3^- permeability might result from a change in subunit composition as a result of 1-week flurazepam administration and provide a basis for the depolarizing potential. The role of $GABA_A$ receptor heterogeneity in mediating the depolarizing potential in naive CA1 pyramidal cells is a topic of current debate [25,26,37,56]. The idea that $GABA_A$ receptor channels with different ion selectivities may mediate depolarizing potentials originated with Alger and Nicoll [1], who noted the differential sensitivity of hyperpolarizing and depolarizing responses to $GABA_A$ receptor agonists and to allosteric modulators, i.e. barbiturates and benzodiazepines. The heterogeneity of $GABA_A$ receptors on hippocampal neurons is now well established and has been related to the functional heterogeneity of hippocampal responses [1,3,29,40,41,49,60]. Whether distinct $GABA_A$ receptor subtypes may mediate depolarizing responses in dendrites with a relatively higher permeability to HCO_3^- remains unknown [25,42]. To the contrary, Staley and Proctor [56] recently reported that HCO_3^--mediated current amplitude was invariant across the distal dendrites of CA1 pyramidal cells arguing against $GABA_A$ receptor subtypes with differential HCO_3^- permeability. Although differentially HCO_3^--permeable receptor subtypes may not exist in naïve animals, a change in $GABA_A$ receptor subunit composition after chronic benzodiazepine treatment might have contributed to the altered HCO_3^- permeability and the appearance of depolarizing event in CA1 pyramidal neurons from 1-week flurazepam treated rats.

At the current level of analysis, i.e. using whole-cell recording and voltage-clamp techniques, it was established that a the amplitude of IPSCs in CA1 pyramidal cells from flurazepam-treated rats, hence synaptic conductance, was significantly smaller when HCO_3^- was the major charge carrier, but not when the Cl^- ion was the primary charge carrier. It would be of interest to determine whether the lack of change in GABA-mediated [$^{36}Cl^-$] flux in brain synaptoneurosomes derived from flurazepam-treated rat brains or cortical cultures, assayed in HEPES–aCSF [22,68] is consistent with the absence of a change in Cl^--mediated synaptic conductance. Moreover, it will be important to establish the mechanism for the decreased HCO_3^- conductance and the similarities and dissimilarities among the mechanisms generating depolarizing potentials in naïve vs. chronic benzodiazepine-treated CA1 pyramidal neurons. Taken together, the findings suggest that HCO_3^- may play a very prominent role in mediating the actions of GABA at $GABA_A$ receptor synapses and that a loss of HCO_3^- conductance may contribute significantly to $GABA_A$ receptor dysfunction following 1-week flurazepam treatment and thus to benzodiazepine tolerance.

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