Pergamon

0306-4522(94)00558-3

Neuroscience Vol. 66, No. 1, pp. 87–99, 1995
Elsevier Science Ltd

0306-4522/95 $9.50 + 0.00

REDUCTION OF GABA-MEDIATED INHIBITORY POSTSYNAPTIC POTENTIALS IN HIPPOCAMPAL CA1 PYRAMIDAL NEURONS FOLLOWING ORAL FLURAZEPAM ADMINISTRATION

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Abstract—Oral administration of the benzodiazepine, flurazepam, for one week results in tolerance *in vivo* and *in vitro* and in a reduction in recurrent and feedforward inhibition *in vitro* in the CA1 pyramidal cell region of hippocampus. In the present study CA1 pyramidal cells were examined intracellularly *in vitro* in rat hippocampal slices (500 μm) from rats sacrificed two or seven days after cessation of oral flurazepam treatment. Following drug treatment, the membrane characteristics of CA1 pyramidal cells were not significantly different from control neurons. $GABA_A$-mediated, early inhibitory postsynaptic potentials were significantly reduced in amplitude (60%) in pyramidal neurons from rats killed two days, but not in those killed seven days, after the end of drug administration. The decrease in early inhibitory postsynaptic potential amplitude was observed using just-subthreshold, threshold and supramaximal orthodromic stimulation as well as following antidromic activation. The magnitude of the decrease in the early inhibitory postsynaptic potential amplitude was similar in the presence of the $GABA_B$ antagonist, CGP 35348, and could not be attributed to differences in the strength of afferent stimulation between flurazepam-treated and control groups. The size of the $GABA_B$-mediated, late inhibitory postsynaptic potentials was also significantly decreased (45%) in comparison to control cells. Reversal potentials for both the early (−72 mV) and late (−92 mV) hyperpolarizations were not significantly different between groups. Following high intensity orthodromic stimulation, in the presence of an intracellular sodium channel blocker (QX-314) which also blocks the $GABA_B$-mediated late hyperpolarization, a bicuculline-sensitive late depolarizing potential was unmasked in neurons from FZP-treated rats, but never from control cells. Excitatory postsynaptic potential amplitude was significantly increased in flurazepam-treated neurons and the threshold for the synaptically-evoked action potential was significantly increased. Following depolarizing current injection, the duration and frequency of pyramidal cell discharges and the action potential threshold were not altered by oral flurazepam treatment. The amplitude of the fast afterhyperpolarization was also not changed. Overall, the findings indicate an impairment of transmission at GABAergic synapses onto hippocampal CA1 pyramidal cell neurons after chronic benzodiazepine treatment at a time when rats are tolerant to the anticonvulsant effects of the benzodiazepines *in vivo*.

Prolonged benzodiazepine administration in rats invariably results in functional tolerance, a reduced sensitivity of the CNS to benzodiazepine actions.[23,41] Benzodiazepines, which bind to the benzodiazepine recognition site on the $GABA_A$ receptor, potentiate $GABA_A$ receptor function by increasing the frequency of chloride channel opening.[23] The involvement of the $GABA_A$ receptor and decreased GABA function in mediating benzodiazepine tolerance is well established,[21] however the synaptic mechanisms underlying benzodiazepine tolerance remain unknown. The hippocampus, an important site of benzodiazepine actions,[23] is one of those brain areas in which the $GABA_A$ receptor and GABA function is regulated following chronic benzodiazepine treatment.[12,13,34,52,54,61–64] Recently, using an extracellular electrophysiological approach, we demonstrated that both recurrent[61] and feedforward[64] paired-pulse inhibition were significantly reduced in the CA1 region of hippocampus in rats killed two days, but not seven days, after one week flurazepam treatment. These findings were consistent with an impairment in endogenous GABA function in hippocampus of chronic benzodiazepine treated rats which coincided with the occurrence of benzodiazepine anticonvulsant tolerance *in vivo*.[42–44]

This study was designed as an initial investigation of the intracellular electrophysiological characteristics of hippocampal CA1 pyramidal neurons in *in vitro* hippocampal slices obtained from rats killed two days and seven days following continuous oral administration of flurazepam for one week. We examined the hypothesis that the amplitude of the GABA-mediated, early inhibitory postsynaptic potential (IPSP) is reduced in the CA1 region of

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Abbreviations: AHP, afterhyperpolarization; AP, action potential; EPSP, excitatory postsynaptic potential; IPSP, inhibitory postsynaptic potential; RMP, resting membrane potential; ACSF, artificial cerebrospinal fluid; THIP, tetrahydroisoxazolopyridinol.

hippocampus of chronic flurazepam-treated rats two days, but not seven days, after ending drug administration. EPSP–IPSP sequences were elicited by orthodromic, Schaffer collateral stimulation and antidromic stimulation via the alveus. Early, $GABA_A$-mediated IPSPs were also pharmacologically isolated in the presence of a $GABA_B$ receptor antagonist. In order to block the postspike afterhyperpolarization in some neurons, an intracellular Na^+ channel blocker was included in the micropipette. Pyramidal cell excitability was evaluated following flurazepam treatment by examining the threshold, frequency and duration of pyramidal cell discharges elicited by depolarizing current injection.

EXPERIMENTAL PROCEDURES

Chronic benzodiazepine treatment

Using behavioral methods[41,44,53] and both *in vivo*[56] and *in vitro*[56,61,62] electrophysiological techniques, continuous oral flurazepam administration for one week has been shown to result in benzodiazepine tolerance. Rats exposed to flurazepam for one week show no signs of intoxication, other overt behavioral effects, or spontaneous withdrawal signs.[53]

Male, Sprague–Dawley rats (Harlan, Haslett, MI) (185–225 g initial weight, 50–55 days of age) were offered flurazepam dihydrochloride (pH 6.3) for seven days (100 mg/kg × 3 days; 150 mg/kg × 4 days) in their drinking water in a 0.02% saccharin vehicle used to disguise the bitter taste. Drug treatment was preceded by a two day adaptation period during which rats received the drug vehicle. Following the flurazepam treatment rats received 0.02% saccharin water for two days or seven days until killed for electrophysiological study of hippocampal CA1 pyramidal cells. Rats were killed at these time-points after one week flurazepam treatment since they were previously shown to be tolerant to benzodiazepine anticonvulsant actions up to four days, but not seven days, after cessation of flurazepam administration.[43] Further, using a sensitive radioreceptor assay, residual benzodiazepine metabolites were not detected in hippocampus two days after ending treatment[61] and thus would not confound electrophysiological findings. As in previous studies,[53,61] only rats which consumed a weekly average ≥ 100 mg/kg flurazepam day (138.3 ± 2.1 mg/kg/day, $n = 16$) were included in the study. Pair-handled controls ($n = 17$) received the drug vehicle for the entire experimental period. The experimenter was not informed of the rats treatment histories until after the data analysis.

Slice preparation and electrophysiological recording

Hippocampal slices (500 μM) were cut on a Vibratome (Pelco 101, Ted Pella, Inc.) in ice-cold oxygenated (95% O_2/5% CO_2) artificial cerebral spinal fluid [ACSF (mM): 126.0 NaCl, 3.0 KCl, 1.5 $MgCl_2$, 2.4 $CaCl_2$, 1.2 NaH_2PO_4, 25.9 $NaHCO_3$, 10 D-glucose] then maintained at room temperature in the continuously oxygenated buffer. After a minimum of 1 h, one slice was placed in the recording chamber between two nylon nets and superfused with oxygenated ACSF (1.5 ml/min; 33 ± 1°C). Intracellular recordings were made from the pyramidal cell layer of the CA1 region with borosilicate glass, fiber-filled micropipettes (Sutter, 1.2 mm O.D.) pulled on a Brown–Flaming micropipette puller (P80 or P97, Sutter) and filled with 3 M potassium acetate (impedance: 60–140 MΩ). In additional neurons from control rats and rats sacrificed two days after the end of flurazepam treatment, 100 μM QX-314 was included in the micropipette.

Recording and stimulating electrodes were positioned visually using a micropositioner and the overlying net as guides for controlled placement. For orthodromic activation, the stimulating electrode was placed in the stratum radiatum, 300 μm below the pyramidal cell layer and 700–800 μm distal toward the fimbria.[14] For antidromic activation electrodes were placed in the alveus towards the subiculum. Synaptic potentials were evoked ≥ 30 s intervals with a 0.1 ms, 50–450 μA pulse from a concentric (75 μm), bipolar, tungsten stimulating electrode coupled to a Master-8 Stimulator and Iso-Flex constant current stimulus isolation unit (A.M.P.I.). Signals were amplified with an Axoclamp 2A amplifier (Axon Insturments, Inc.), with an active bridge circuit for current injection, and displayed on a storage oscilloscope (Tektronix). Signals were simultaneously converted with a PC-Mate Labmaster AD converter (Tecmar, Inc.) and recorded on computer disk with pCLAMP 5.5 software (Axon Instruments, Inc.) for subsequent analysis.

The IPSPs elicited by stimulus intensities above threshold are contaminated by postspike afterhyperpolarizations (AHPs).[30,33] One method used to avoid this contamination was to apply a stimulus intensity which elicited an EPSP just-subthreshold for an action potential (AP). The subthreshold stimulus intensity used was empirically determined from a series of stimulations and defined as the stimulus intensity in the threshold range which did not elicit an AP on 50% of 4–6 trials. IPSP amplitudes were also measured following an AP elicited by a threshold or a 2 × threshold stimulus intensity. In another group of neurons the AHP contamination due to AP generation was eliminated by including QX-314 in the micropipette. Use of QX-314 had the added advantage of blocking the $GABA_B$, K^+ channel-mediated, late hyperpolarization.[36] All synaptic potentials were measured from the prestimulus baseline to their peak (Fig. 2). Postsynaptic potential amplitudes, latency to peak and duration were also measured after threshold and suprathreshold (2 × threshold) stimulation. Values reported represent averages of 3–5 responses per cell at each stimulus intensity.

The reversal potentials of the early and late IPSPs elicited by suprathreshold stimulation were measured at the peak of the hyperpolarization at a fixed latency for each cell (Fig. 3). The reversal of the early IPSP was calculated by linear regression from hyperpolarizations between −60 and −80 mV. Reversal potentials for the late IPSP were extrapolated since it was difficult to reverse the late hyperpolarization.[36]

Neurons accepted for inclusion into the study met several criteria: large stable resting membrane potentials (RMP) (range: −58 to −72 mV), input resistances >15 MΩ (−0.6 to +0.3 nA, 150 ms pulse duration) and AP amplitude >60 mV (range: 60–93 mV). Since orthodromic EPSP-IPSP sequences are voltage-dependent,[11,31] the membrane potential was maintained at −60 mV throughout the remainder of the experiment by injection of current via the recording electrode.

Pyramidal cell discharges were elicited by a 500 ms, +0.6 nA depolarizing current pulse. Following injection, AP threshold, frequency and total train duration were measured. AP frequency was determined over the total train duration. The fast AHP was measured from baseline to its peak. Slow AHPs, which are dependent on the number of APs generated,[33] were not measured since AP frequency varied between cells of each group.

The majority of neurons were recorded during superfusion with ACSF. In some flurazepam-treated cells, killed two days after drug treatment, and their matched control neurons, the $GABA_A$-mediated IPSP was pharmacologically isolated in the presence of the $GABA_B$ antagonist, CGP 35348 (25–50 μM). Other neurons from rats killed two days after ending treatment were recorded with electrodes containing QX-314 and were superfused with ACSF with

or without bicuculline (2.5–100 μM). Since bicuculline increases CA3 pyramidal cell excitability resulting in direct activation of CA1 pyramidal cells,[59] the portion of the hippocampal slice containing the CA3 region was removed in the latter slices with a knife cut.

Data collected under each stimulation protocol were analysed separately by multivariate analysis of variance. Pairwise comparisons of individual means were made with orthogonal contrasts. The level of significance was set at $P \leqslant 0.05$ according to the modified method of Bonferroni corresponding to the number of multiple comparisons made.[24] For example, for four or fewer comparisons $P \leqslant 0.05$. For five or six comparisons the significance level was set at $P \leqslant 0.04$ or $P \leqslant 0.03$, respectively.

Materials

Buffer chemicals were purchased from Sigma Chemical Co. (St Louis, MO) or Fisher Scientific Co. (Pittsburgh, PA). Flurazepam HCl was from Research Biochemicals International (Natick, MA). QX-314 was obtained from Alomone Labs (Jerusalem, Israel). CGP 35348 was kindly provided by Dr M. F. Pozza, CIBA-GEIGY, Ltd (Basel, Switzerland).

RESULTS

Intrinsic characteristics

Neurons from flurazepam-treated rats killed two days or seven days after the end of drug administration showed no significant differences from control neurons in their RMP, input resistance, time constant or AP amplitude (Table 1). The trend toward a decrease in the membrane time constant in flurazepam-treated neurons was not significant ($P = 0.30$). The passive membrane characterisitcs of these rat CA1 pyramidal neurons was similar to those reported by others.[57]

Neuronal excitability

The characteristic response of both treated and control neurons observed following depolarizing current injection was an initial fast AP volley, which showed adaptation, followed by a silent period (Fig. 1). AP threshold, measured following synaptic activation, was significantly higher in flurazepam-treated neurons killed two days, but not seven days, after the end of drug administration (Table 1). There were no significant differences between groups in the AP threshold elicited by current injection. In rats killed two days after treatment, there was a trend toward an increase in the response of flurazepam-treated neurons to intracellular current injection, however in comparison to control neurons, there was no significant difference in the average AP frequency (flurazepam-treated: 31.5 ± 4.0 Hz; control 24.1 ± 2.7 Hz, $P = 0.13$) or the total train duration (flurazepam-treated: 300.6 ± 45.7 ms; control 269.2 ± 40.5 ms, $P = 0.60$). Measurement of the amplitude of the fast AHP indicated no difference between groups (flurazepam-treated: 5.6 ± 0.9 mV; control 5.4 ± 0.8 mV, $P = 0.90$).

Synaptic potentials

Potassium acetate-filled micropipettes. The amplitude and duration of inhibitory synaptic potentials recorded in control neurons following synaptic activation were similar to those reported by others (Fig. 2; Table 2).[36,37] There was a trend ($P = 0.06$) toward a decrease (25%) in the amplitude of both early and late IPSPs in neurons from the older control rats, i.e. those matched with neurons from rats killed seven days (65–70 days of age) after the end of oral flurazepam treatment in comparison to those killed two days (60–65 days of age) after cessation of drug administration. IPSPs elicited by antidromic stimulation were significantly smaller in the CA1 region from older control rats ($P < 0.01$; Table 2). The decreased IPSP amplitude in older, as compared to younger, control rats parallels the findings of Dunwiddie[18] of a gradual decrease in GABA inhibition in rats beyond 30 days of age.

In flurazepam-treated cells from rats killed two days after treatment, there was a significant decrease in the amplitude of the early IPSP elicited by subthreshold stimulation (Fig. 2; Table 2; $P < 0.01$). The peak of the $GABA_A$-mediated IPSP was also

Table 1. Properties of hippocampal CA1 pyramidal cells from rats killed 2 days and 7 days after cessation of oral flurazepam treatment

	Time after flurazepam treatment			
	2 Days		7 Days	
	Control ($n = 17$)	Flurazepam-treated ($n = 16$)	Control ($n = 8$)	Flurazepam-treated ($n = 8$)
Membrane characteristics				
Resting potential (mV)	-66.5 ± 0.9	-66.1 ± 0.9	-65.4 ± 1.6	-68.3 ± 1.3
Input resistance (MΩ)	46.7 ± 6.8	39.3 ± 5.1	55.1 ± 3.5	54.0 ± 4.0
Time constant (ms)	12.4 ± 1.2	9.7 ± 0.9	14.7 ± 1.9	12.7 ± 1.7
AP amplitude (mV)	74.1 ± 2.5	73.1 ± 1.9	70.4 ± 2.6	70.4 ± 2.7
AP threshold (mV)				
Current injection	-55.1 ± 0.6	-55.7 ± 1.0	-52.8 ± 1.8	-52.5 ± 1.0
Synaptic activation	-55.4 ± 0.7	-52.5 ± 0.6*	-55.0 ± 0.6	-54.9 ± 0.6
Reversal potentials	($n = 10$)	($n = 10$)		
Early IPSP (mV)	-73.1 ± 1.4	-71.9 ± 1.9	-75.0 ± 1.1	-75.2 ± 2.5
Late IPSP (mV)	-90.8 ± 1.2	-93.2 ± 2.1	-92.0 ± 2.4	-97.4 ± 3.1

*Asterisk denotes significant difference between control and flurazepam-treated neurons ($P = 0.01$). Values represent the mean ± S.E.M.

delayed in cells from these flurazepam-treated slices ($P = 0.02$). In these neurons the duration of the early hyperpolarization could not be reliably measured due to the overlap of the late IPSP.[36] The postsynaptic responses following threshold and suprathreshold stimulation (data not shown) were also significantly different between these groups ($P < 0.01$) and were comparable in magnitude to those seen following antidromic stimulation (Table 2). In *in vitro* studies carried out at room temperature ($22 \pm 1°C$),[63] significant ($P < 0.01$) changes of a similar magnitude and direction were observed in the amplitudes of the EPSP (9.0 ± 1.3 vs 4.9 ± 0.7 mV) and the early (1.8 ± 0.4 vs 6.0 ± 0.9 mV) and late (2.7 ± 0.3 vs 5.0 ± 0.5 mV) IPSPs in cells from flurazepam-treated rats killed two days after the end of treatment ($n = 14$ neurons/9 rats) in comparison to those from control rats ($n = 11$ neurons/6 rats).

In neurons from rats killed two days after the end of oral flurazepam treatment, there were no significant differences between groups in the current intensities used to elicit postsynaptic potentials

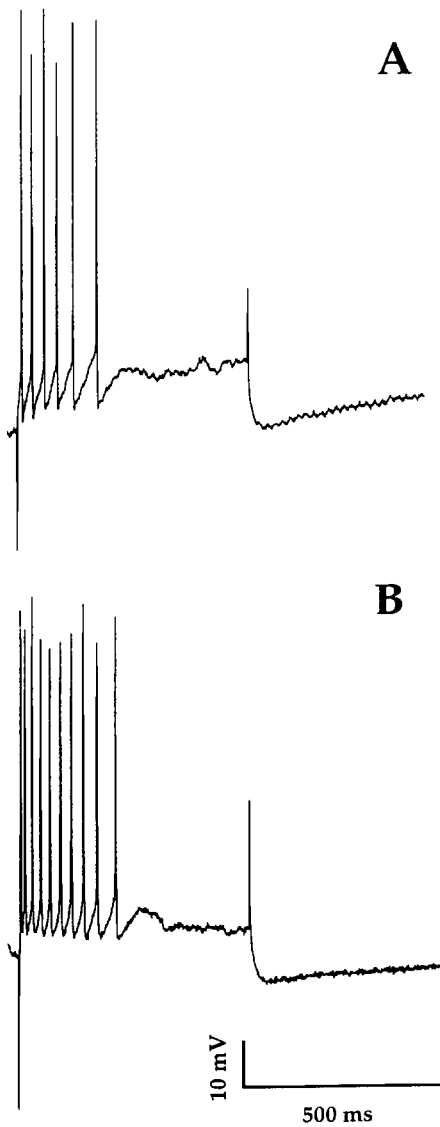

Fig. 1. Representative response of (A) a control (RMP = −65 mV, 37.0 Hz, 162.0 ms) and (B) a flurazepam-treated (RMP = −60 mV, 43.5 Hz, 207.0 ms) neuron to injection of a +0.6 nA, 500 ms current pulse. There were no significant differences in action potential frequency or the duration of the AP train between groups.

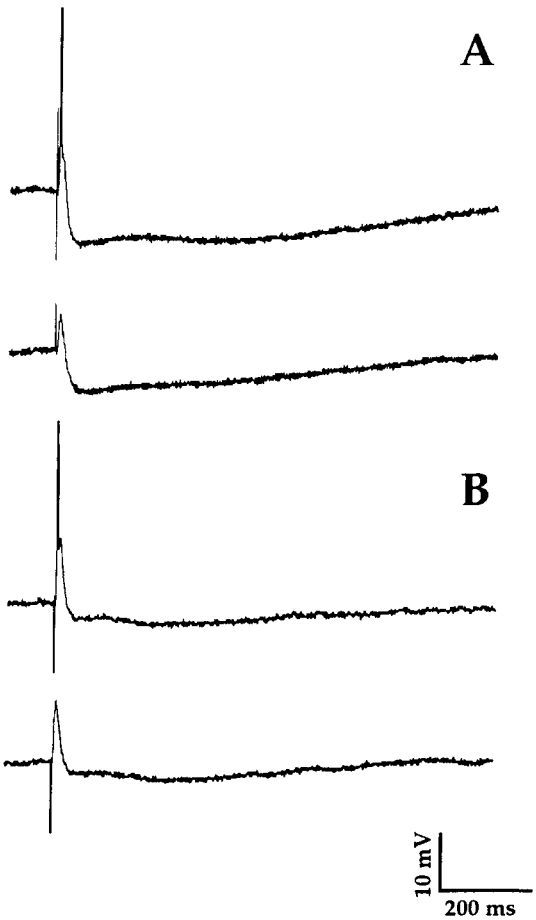

Fig. 2. Representative intracellular recordings from (A) a control hippocampal CA1 pyramidal neuron (RMP = −70 mV) and (B) a neuron (RMP = −66 mV) from a rat sacrificed two days after ending one week oral flurazepam administration. An orthodromic stimulation of the Schaffer collateral pathway just-subthreshold for an action potential elicited (lower trace) an EPSP (control, 4.9 mV; flurazepam-treated = 9.8 mV) followed by an early control, 6.8 mV; flurazepam-treated = 2.0 mV) and a late (control, 5.9 mV; flurazepam-treated = 2.5 mV) hyperpolarization. Suprathreshold stimulation (2 × threshold) resulted in AP generation (control, 88.4 mV; flurazepam-treated = 76.4 mV), truncated in the upper traces. Following suprathreshold stimulation there was a decrease in the amplitude of the early (control, 9.8 mV; flurazepam-treated = 2.4 mV) and the late (control, 8.1 mV; flurazepam-treated = 3.7 mV) IPSP. At each stimulus intensity the magnitude of the decrease in the early and the late IPSPs for these neurons, averaged 70% and 55%, respectively. Membrane potential was held at −60 mV during all recordings.

Table 2. Amplitude and latency of synaptic potentials recorded in hippocampal CA1 pyramidal cells from rats killed two days and seven days after cessation of oral flurazepam treatment

Stimulus intensity and location	EPSP peak mV	Early IPSP peak mV	Early IPSP peak ms	Late IPSP peak mV	Late IPSP peak ms
Two days after flurazepam treatment					
Subthreshold orthodromic					
Control ($n = 10$)	4.6 ± 0.5	6.8 ± 0.6	56.4 ± 5.2	6.4 ± 0.5	225.4 ± 14.0
Treated ($n = 10$)	9.7 ± 0.4*	2.2 ± 0.4*	73.9 ± 5.1*	2.9 ± 0.4*	215.8 ± 10.8
Suprathreshold antidromic					
Control ($n = 10$)		8.6 ± 1.3	47.2 ± 8.0	6.5 ± 0.9	214.5 ± 30.8
Treated ($n = 7$)		3.2 ± 0.7*	49.4 ± 5.2	3.4 ± 0.6*	198.5 ± 9.7
Seven days after flurazepam administration					
Subthreshold orthodromic					
Control ($n = 8$)	4.6 ± 0.4	5.1 ± 1.0	64.8 ± 6.8	4.6 ± 0.6	372.9 ± 32.3
Treated ($n = 8$)	4.7 ± 0.4	5.2 ± 1.0	66.1 ± 10.3	4.7 ± 0.6	317.4 ± 27.1
Suprathreshold antidromic					
Control ($n = 7$)		5.4 ± 1.1	59.0 ± 4.2	4.8 ± 0.9	325.4 ± 29.5
Treated ($n = 7$)		4.8 ± 0.8	59.6 ± 4.9	4.0 ± 0.5	314.5 ± 28.8

*Asterisks denote significant differences between control and flurazepam-treated neurons ($P \leq 0.03$–0.05). Values represent the mean ± S.E.M.

using any stimulation paradigm (e.g. subthreshold orthodromic stimulus intensity: flurazepam-treated, 270.5 ± 72.9 μA; control, 319.5 ± 76.6, $P = 0.63$). There was no correlation between stimulus intensity and IPSP amplitude (r = −0.07 vs 0.01) or between stimulus intensity and IPSP latency (r = 0.32 vs 0.40) in either drug-treated or control neurons. The relationship between EPSP amplitude and early IPSP amplitude in these treated and control cells is shown in Fig. 3.

The late IPSP was also significantly decreased in amplitude (Fig. 2; Table 2; $P < 0.01$) in rats killed two days after ending flurazepam treatment. The time to peak of the late IPSP was not significantly different between groups ($P = 0.57$), however both the total duration (flurazepam-treated: 539.8 ± 54.6 ms; control: 725.7 ± 51.5 ms) and the half-decay time

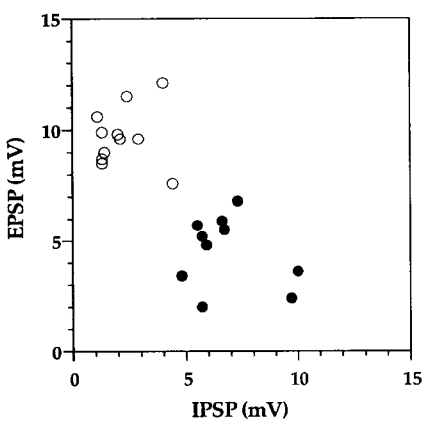

Fig. 3. Relationship between EPSP amplitude (mV) and IPSP amplitude (mV) for all control (●) and flurazepam-treated neurons (○) from rats sacrificed two days after the end of drug administration recorded in ACSF (33°C). There was no overlap between groups in the EPSP-IPSP sequences recorded. The separation between groups reflects the effect of decreased IPSP amplitude to increased EPSP amplitude.

(subthreshold-flurazepam-treated: 372.3 ± 29.3 ms; control: 510.0 ± 35.3 ms) of the $GABA_B$-mediated hyperpolarization were significantly decreased following chronic flurazepam-treatment ($P < 0.01$).

The size of the EPSP in CA1 cells from treated rats, killed two days after treatment cessation, was significantly increased in comparison to control neurons (Fig. 1; Table 2, $P < 0.01$). There was no significant difference in the rise time of the EPSP between groups (flurazepam-treated: 1.8 ± 0.1 mV/ms; control: 1.4 ± 0.2 mV/ms, $P = 0.21$).

There were no significant differences in the amplitude, latency to peak or duration of either the EPSP or the early or late IPSPs in neurons from rats killed seven days after the end of flurazepam treatment in comparison to their matched controls (Table 2).

Reversal potentials. There were no significant differences in the reversal potentials for the peak, early IPSP or late IPSP between groups (Table 1; Fig. 4). The values obtained for the reversal potentials for the early and late hyperpolarizations were comparable to those obtained by others and are consistent with those of Cl^- and K^+ channels, respectively.[11,48,49]

CGP 35348 superfusion. CGP 35348 (25–50 μM) completely blocked the $GABA_B$-mediated late IPSP in CA1 pyramidal cells. In neurons from rats killed two days after the end of oral flurazepam administration (6 neurons/4 rats), recorded in the presence of 25 and 50 μM CGP 35348 (Fig. 5; Table 3), there was a significant decrease ($P = 0.04$) in the amplitude of the $GABA_A$-mediated IPSP in comparison to cells from matched control rats ($n = 7$ neurons/4 rats). The increase in IPSP amplitude in flurazepam-treated neurons during CGP 35348 superfusion was a relatively consistent finding, but this trend was not significant ($P = 0.25$). As with neurons recorded during ACSF superfusion (Table 2), there was a significant increase ($P \leq 0.1$) in EPSP amplitude

elicited by subthreshold stimulation in flurazepam-treated neurons both prior to and during CGP 35348 superfusion (Table 3).

QX-314-filled micropipettes. QX-314 was included in the micropipette during some recording sessions to block AP generation, and thus AHPs.[30,33] Since QX-314 also blocked the late, K^+-mediated hyperpolarization[36] a "pure" $GABA_A$-mediated IPSP was also recorded. As previously reported,[10] QX-314 (100 μM) decreased the RMP of pyramidal neurons somewhat (control: -60.4 ± 2.8 mV, $n = 7$ neurons/6 rats; flurazepam-treated: -54.2 ± 4.2 mV, $n = 9$ neurons/7 rats) but the RMP of cells was not significantly different between groups ($P = 0.20$). Following cell penetration with QX-314-containing micropipettes, the AP and late IPSP were typically blocked within 10–45 min. A few cells required up to 2 h to achieve blockade. Though the relationship was not

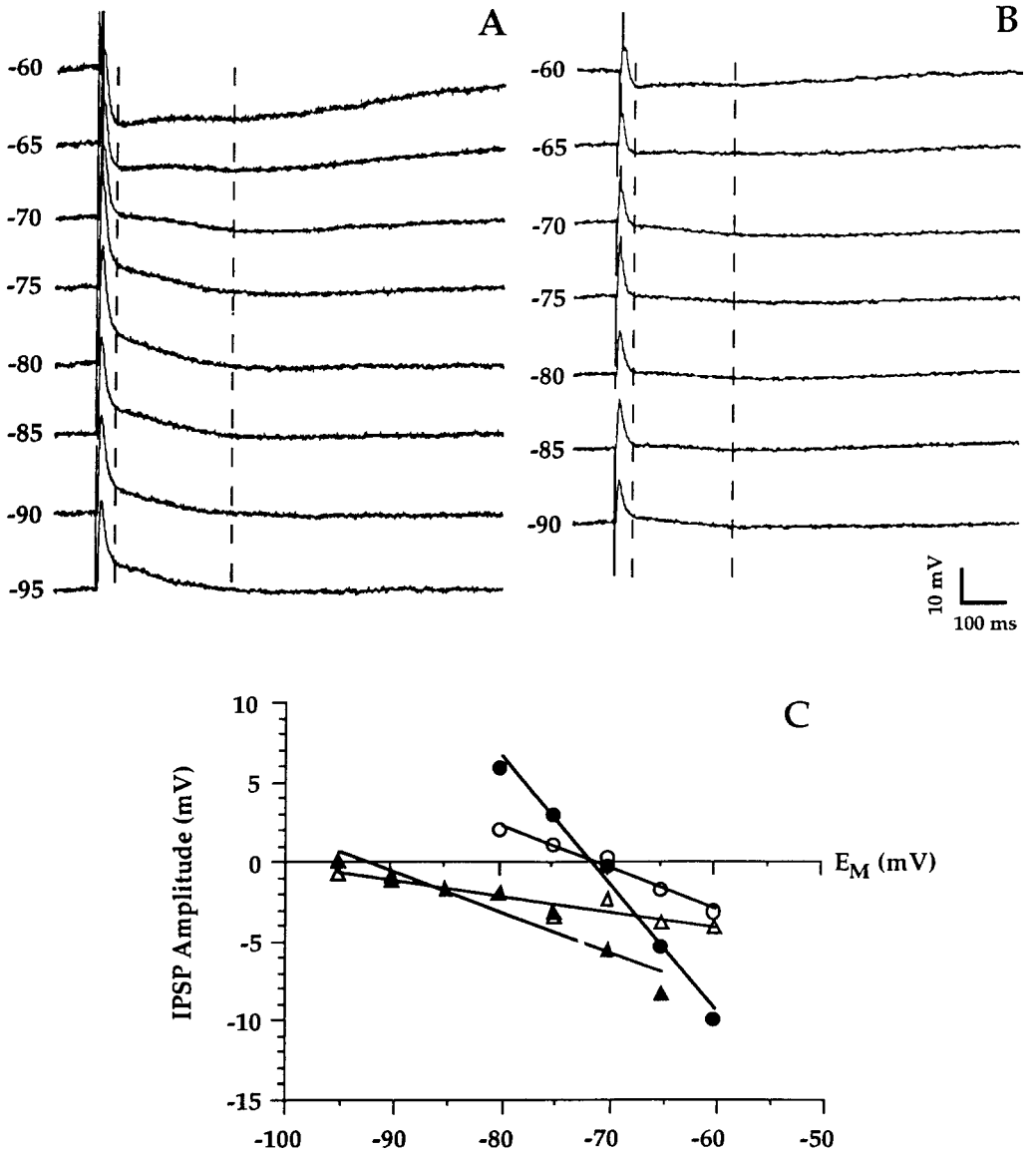

Fig. 4. Example of reversal potential determinations in the (A) control and (B) flurazepam-treated neurons shown in Fig. 1. Reversal potentials were determined at a fixed latency at the peak of the early and late hyperpolarizations (dashed lines). For the early IPSP the reversal potential was determined by linear regression for responses between -60 and -80 mV. As shown in panel C, the reversal potential for the early IPSP (●) for this control neuron was -72 mV. The early IPSP reversal in the flurazepam-treated neuron (○) was also calculated to be -72 mV. As has been reported, the late hyperpolarization could not easily be reversed and therefore was calculated by extrapolation. For the late hyperpolarization, the control (△) and flurazepam-treated (△) reversal potentials were -90 and -96 mV, respectively. The increase in the negative slope of the regression line for the flurazepam-treated neuron represents a decrease in the amplitude of the early and late hyperpolarizations in comparison to the control neuron. Mean reversal potentials for each group are reported in Table 1.

empirically determined, the onset of QX-314 action appeared positively correlated with micropipette resistance.[10] Maximal $GABA_A$-mediated IPSPs recorded following QX-314 blockade were significantly decreased in CA1 neurons from rats killed two days after the end of oral flurazepam administration (control: 11.2 ± 1.2 mV; flurazepam-treated: 6.3 ± 1.1 mV, $P < 0.01$) in the absence of a difference in the amplitude of the associated EPSP (control: 15.2 ± 3.0 mV; flurazepam-treated: 12.8 ± 1.6 mV, $P = 0.44$). The maximal EPSP amplitude recorded in the same cells was also not significantly different between groups (control: 24.0 ± 4.0 mV; flurazepam-treated; 18.1 ± 3.8 mV, $P = 0.25$) but the associated $GABA_A$-mediated IPSP was significantly reduced (control: 9.1 ± 0.8 mV; flurazepam-treated: 4.3 ± 1.2 mV, $P < 0.01$). There was no difference in the current intensity used to elicit maximal EPSPs in this group of cells (control: 175.0 ± 33.3 μA; flurazepam-treated: 169.4 ± 40.0 μA, $P = 0.73$).

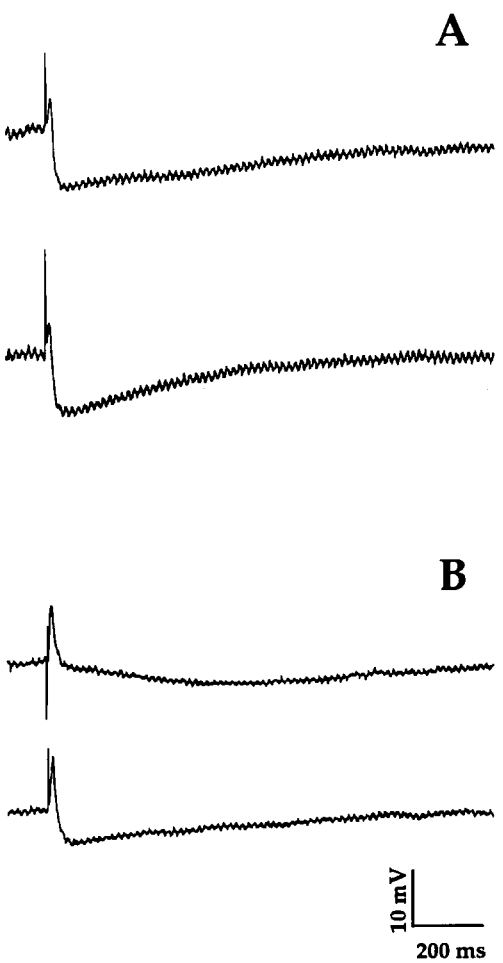

Fig. 5. Representative intracellular recordings before and after superfusion with the $GABA_B$ antagonist CGP 35348 (50 μM) from (A) a control hippocampal CA1 pyramidal neuron (RMP = −72 mV) and (B) a neuron (RMP = −64 mV) from a rat sacrificed two days after ending oral flurazepam treatment. An orthodromic stimulation just-subthreshold for an action potential (upper trace) elicited an EPSP (control: 5.6 mV; flurazepam-treated: 10.3 mV) followed by an early (control: 10.5 mV; flurazepam-treated: 1.2 mV) and a late (control: 9.0 mV; flurazepam-treated: 4.0 mV) hyperpolarization. Following CGP 35348 superfusion (lower trace) the late hyperpolarization was abolished and the difference in early IPSP amplitude between groups persisted (control: 11.47 mV; flurazepam-treated: 6.4 mV). The increase in the early IPSP amplitude during CGP 35348 superfusion, though relatively common, was not significant. All responses were recorded at −60 mV.

In neurons recorded with QX-314 electrodes, a depolarizing event was unmasked in flurazepam-treated neurons at high stimulus intensities, i.e. requiring an EPSP amplitude $\geqslant 11.7$ mV. A depolarizing potential was seen in 10 or 12 neurons from seven flurazepam-treated rats killed two days after the end of treatment. The two neurons in which a depolarizing potential was not elicited upon orthodromic stimulation had atypically, high-amplitude IPSPs, 8.1 and 10.3 mV, respectively, and were from the same flurazepam-treated rat. No depolarizing events were observed in neurons from rats killed seven days after cessation of drug treatment ($n = 5$ neurons/5 rats) nor in any of the 10 neurons sampled from six control rats.

The peak of the depolarizing event (131.7 ± 29.6 ms, $n = 7$) was typically later than that of the early IPSP. However, the amplitude, latency and duration of the depolarization varied with stimulus intensity and on some occasions fused with the EPSP (Fig. 6). The amplitude of the depolarization increased as the neuronal membrane was hyperpolarized by current injection. The reversal potential of the depolarization was difficult to determine since it was contaminated by the early IPSP. It was calculated by regression analysis to be -53 ± 10.8 mV ($n = 4$), in the same range as previously reported.[3,51] In neurons ($n = 5$) superfused with bicuculline, both the early IPSP and the late depolarization were reduced in amplitude. Both events could be completely blocked with 50–100 μM bicuculline (data not shown).

DISCUSSION

Recurrent and feedforward inhibition[2,5,26–29,45] in the CA1 region of the hippocampus are mediated by activation of several classes[26,28,45,47] of GABA-immunoreactive[22] interneurons which induce hyperpolarization of pyramidal cells.[26,27,29] The early hyperpolarization evoked in pyramidal cells by activation of GABAergic interneurons is reduced following application of penicillin and $GABA_A$ antagonists.[2,11,15,32,48] The late hyperpolarization is abolished by $GABA_B$ antagonists.[11,19] In CA1 pyramidal cells recorded in *in vitro* hippocampal slices from oral flurazepam-treated rats, there was a significant reduction in the amplitude of both GABA-mediated early and late IPSPs, using all three stimulus paradigms. A comparison of the average percent change in amplitude of the early and late IPSPs (Table 2) suggests that the fast, $GABA_A$-

Table 3. Synaptic potential amplitudes recorded during superfusion of ACSF or 50 μM CGP 35348 in hippocampal CA1 pyramidal cells from rats killed two days after cessation of oral flurazepam treatment

Stimulus intensity and location	EPSP peak (mV) ACSF	EPSP peak (mV) CGP 35348	Early IPSP peak (mV) ACSF	Early IPSP peak (mV) CGP 35348
Subthreshold orthodromic				
Control ($n = 7$)	5.3 ± 0.3	4.8 ± 0.2	9.9 ± 0.9	9.6 ± 0.9
Treated ($n = 6$)	7.8 ± 0.8*	7.5 ± 0.8*	3.1 ± 1.0*	5.6 ± 1.8*

*Asterisks denote significant differences between control and flurazepam-treated neurons ($P \leqslant 0.01$–0.04). Values represent the mean $\pm$ S.E.M.

mediated IPSP was affected to a greater extent (−60%) by this chronic benzodiazepine treatment than the slower $GABA_B$-mediated response (−45%). Early IPSP amplitude was also significantly depressed in flurazepam treated neurons in the presence of QX-314 when action potentials and the late hyperpolarization were blocked. A similar decrease was observed in the amplitude of the "pure" $GABA_A$-mediated IPSP recorded in the presence of CGP 35348. Synaptic potentials were modified two days after the end of chronic flurazepam treatment, at a time when rats are tolerant to benzodiazepine anticonvulsant actions *in vivo*, but not seven days after the end of treatment, when rats are no longer tolerant.[43] The reduction in IPSP amplitude in the CA1 region of hippocampus following oral flurazepam administration provides a basis for our previous findings of a decrease in recurrent[61] and feedforward[64]

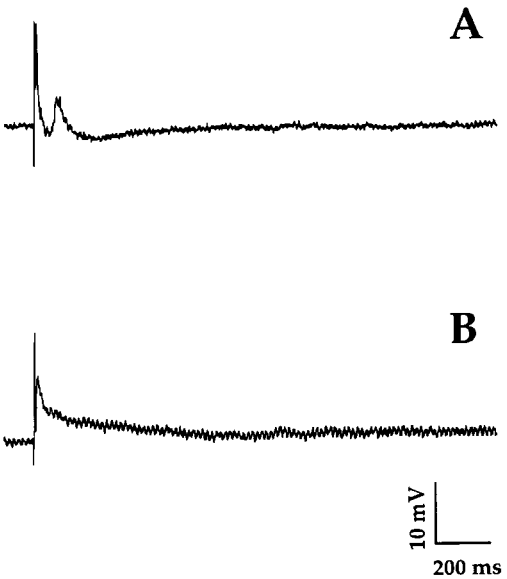

Fig. 6. Representative depolarizing potentials recorded in two flurazepam-treated CA1 pyramidal neurons. Depolarizing potentials were only detected in flurazepam-treated cells from rats sacrificed two days after the end of drug administration. Neurons were recorded with K^+ acetate-filled micropipettes containing 100 μM QX-314. In addition to blocking AP generation, inclusion of QX-314 abolished the late hyperpolarization. (A) A depolarizing potential which required an 18 mV EPSP to be elicited. (B) A depolarizing potential fused with an EPSP.

paired-pulse inhibition after chronic benzodiazepine administration and suggests that GABA-mediated inhibition is significantly impaired at GABAergic synapses onto CA1 pyramidal cells of benzodiazepine tolerant rats. The temporal correlation between the occurrence of benzodiazepine anticonvulsant tolerance *in vivo* and changes in GABAergic inhibition in the CA1 region of hippocampus *in vitro*, suggests that a reduction in the effectiveness of GABAergic transmission plays a significant role in the establishment of benzodiazepine tolerance.

When comparing postsynaptic potentials between cells subjected to different treatments it is important to demonstrate that the differences measured are not simply due to differences in the location or intensity of afferent stimulation. Dingledine *et al.*,[17] demonstrated that, as stimulating electrodes are moved distally from a pyramidal cell neuron, intracellular EPSP amplitude increased as IPSP amplitude increased or remained unchanged. If EPSP-IPSP responses between treated and control neurons were simply due to random, rather than systematic, electrode placements along the Schaffer collateral pathway axis, a random, overlapping pattern of EPSP-IPSP responses would be expected. Figure 3 illustrates no overlap between the amplitude of EPSP-IPSP sequences recorded in flurazepam-treated and control neurons. EPSP-IPSPs amplitudes have also been reported to vary proportionately with stimulus intensity.[17,46] If differences in stimulus intensity between groups were related to differences in early IPSP amplitude the distribution of treated neuron responses would be expected to fall about the same line as control responses. Figure 3 also illustrates that this was not the case. Rather, EPSP amplitude increased as a function of decreased IPSP amplitude in flurazepam-treated neurons when compared to responses in control cells. Further, when afferent stimulation was maximal, in neurons recorded with QX-314-containing micropipettes, significant differences in IPSP amplitude, of a similar magnitude, were still detected between flurazepam-treated and control groups. Thus, the decrease in transmission at inhibitory synapses onto hippocampal CA1 pyramidal neurons was not likely due to differences in afferent stimulation.

A few possible, not mutually exclusive, synaptic mechanisms may mediate the decrease in GABA-

mediated inhibition following chronic benzodiazepine administration. First, it is possible that the intrinsic activity or excitability of GABAergic interneurons in hippocampus is decreased after chronic flurazepam administration. Nonetheless, both early and late monosynaptic IPSPs, evoked by direct stimulation of GABAergic interneurons in the presence of excitatory amino acid antagonists,[11,32] are decreased to a similar degree in CA1 neurons of flurazepam-treated rats.[65] This more recent finding would suggest that additional presynaptic mechanisms may also play a role to decrease inhibition at GABAergic synapses in the benzodiazepine tolerant hippocampus. For example, presynaptic GABA release may be decreased independent of changes in GABAergic interneuron activity.

The reduction in early IPSP amplitude may also, at least in part, be due to an attenuation of the postsynaptic response at the $GABA_A$ receptor. Regionally localized modifications at the $GABA_A$ receptor, including changes in the hippocampus, have been reported after different chronic benzodiazepine treatment regimens. These alterations have included changes in the affinity, number and/or coupling of GABA and benzodiazepine binding sites.[21,34,54] Consistent with reports of postsynaptic modifications of the $GABA_A$ receptor during chronic benzodiazepine treatment, our laboratory reported a significant decrease in the potency, but not efficacy, of superfused GABA, $GABA_A$ agonists and diazepam to inhibit CA1 evoked responses in hippocampal slices from flurazepam-treated rats.[62] GABA agonist potency has been directly correlated with the average duration of Cl^- channel opening.[4] Subsensitivity to $GABA_A$ agonists following chronic benzodiazepine administration could therefore be due to altered Cl-channel kinetics.[48] On the other hand, the absence of a difference in the reversal potentials for the early hyperpolarization between groups (Table1; Fig. 3) suggests that a change in the driving force for Cl^- may not be responsible for the decreased size of $GABA_A$-mediated IPSP in flurazepam-treated rats. An alteration in the phosphorylation state of the $GABA_A$ receptor is one possible mechanism which could account for a change in GABA receptor kinetics.[50] Another possible mechanism which could relate to a change in GABA receptor subsensitivity is a change in the expression of the genes encoding the subunits of the $GABA_A$ receptor. A change or rearrangement in the composition of various $GABA_A$ receptor subunits ($\alpha_{1\text{-}6}$, $\beta_{1\text{-}3}$, $\gamma_{1\text{-}2}$) during flurazepam exposure could result in a decrease in receptor affinity[21,34,54] thus modifying the effectiveness of GABA transmission following benzodiazepine treatment. Using *in situ* hybridization techniques, our laboratory recently confirmed a discretely localized decrease in α_1, but not α_5 or γ_2, subunit mRNA expression in CA1 pyramidal cells two days after cessation of flurazepam treatment.[52] Since the α_1 subunit protein has been localized to apical and basal pyramidal cell dendrites[51] the latter findings suggest that, if a change in subunit composition relates to a change in GABAergic function, that a reduction in dendritic $GABA_A$-mediated inhibition may be a minimal requirement for the reduction in early IPSP amplitude following chronic benzodiazepine treatment.

Whether different or overlapping populations of GABA neurons mediate early and late IPSPs has not been firmly established. However, a subpopulation of feedforward interneurons in stratum lacunosum-moleculare have been identified which may mediate the late hyperpolarization.[27] A significant reduction in the size of the late, $GABA_B$-mediated IPSP[7,19,37] was found in CA1 cells from benzodiazepine-treated rats which could not be attributed to a change in the K^+ reversal potential (Table 1; Fig. 2). The extent to which the early, $GABA_A$-mediated IPSP contributed to the reduction in the late IPSP is difficult to assess since the offset of the early IPSP and the onset of the late IPSP overlap,[36] i.e. the size of the late IPSP may be somewhat underestimated in treated rats relative to control neurons. However, results of studies using monosynaptic stimulation have shown that $GABA_B$-mediated IPSPs, recorded in the presence of picrotoxin, are significantly reduced in amplitude following chronic flurazepam treatment.[65]

The available evidence does not yet allow us to distinguish whether similar or independent mechanisms are responsible for the decrease in both the $GABA_A$- and $GABA_B$-mediated inhibitory responses. Since $GABA_B$ receptors are not, like $GABA_A$ receptors, coupled to the benzodiazepine receptor-linked Cl^- channel,[4,23] chronic benzodiazepine treatment would not be predicted to modulate postsynaptic $GABA_B$ receptor function. This was illustrated in our previous extracellular study i.e., there was no change in the ability of the $GABA_B$ agonist, baclofen, to suppress CA1 evoked responses in hippocampal slices of one week flurazepam-treated rats.[62] A reduction in GABA release following flurazepam treatment would be one mechanism consistent with a reduction in the magnitude of both the early and late GABA-mediated IPSPs. However, studies of endogenous KCl-evoked GABA release in superfused hippocampal slices from chronic flurazepam-treated rats did not reveal reductions in GABA release.[55] Whole-cell voltage clamp studies of spontaneous and evoked IPSCs in CA1 pyramidal neurons will be critical to elucidating the mechanisms underlying the decrease in the magnitude of both GABA-mediated IPSPs, to determine whether reduced GABAergic inhibition following chronic benzodiazepine treatment is a result of decreased pre- or postsynaptic function, or both.

In addition to mediating hyperpolarization in CA1 pyramidal cell soma and dendrites, GABA has been shown to induce a "late" depolarization in pyramidal cell dendrites which occurs between the early and the late hyperpolarizations.[1,3,38,40,51,60] The late GABA-

mediated depolarization has been shown to be inhibitory.[1,40,60] Experimental techniques which increase dendritic GABA concentration such as local GABA application, blockade of GABA uptake, superfusion with 4-aminopyridine and high intensity stimulation, reliably induce a $GABA_A$-mediated inhibitory depolarization.[1,3,38,40,51,60] The depolarizing response is more sensitive to bicuculline than the $GABA_A$-mediated hyperpolarization whereas the hyperpolarization is more sensitive to the $GABA_A$ agonist tetrahydroisoxazolopyridinol (THIP). Based on such electrophysiological and pharmacological evidence, Alger and Nicoll[1] suggested that the depolarizing event was mediated by extrasynaptic $GABA_A$ receptors. Subpopulations of $GABA_A$ receptors have been identified in somal and dendritic layers of the CA1 region[39] and recent *in situ* hybridization[52,58] and immunohistochemical[6,20] studies support the possibility of multiple $GABA_A$ receptor subtypes localized in various layers of the CA1 region. Based on these findings, we recently proposed[52] that multiple subtypes of native dendritic $GABA_A$ receptors, assembled from different $GABA_A$ receptor subunits, may be responsible for the biphasic response to GABA in the CA1 region. Thus a $GABA_A$ receptor subtype may mediate the depolarizing event, perhaps with a lower affinity for GABA and THIP and a higher affinity for bicuculline. We have also proposed that $GABA_A$ receptor subunit composition is modified by chronic benzodiazepine treatment and, as noted above, have demonstrated differential regulation of $GABA_A$ receptor subunit mRNA expression in the hippocampus following one week oral flurazepam administration.[52] Thus $GABA_A$ receptor subtypes could be modulated differentially after chronic benzodiazepine treatment. A depolarizing event of variable latency and duration, similar to that previously described in CA1 pyramidal cells, was unmasked in flurazepam-treated slices during high-intensity stimulation using QX-314-filled electrodes (Fig. 6). Reduction of the hyperpolarizing IPSP during intense synaptic stimulation has been reported to unmask a depolarizing potential in CA3 pyramidal cells.[51] Thus, the appearance of the depolarizing event in the treated neuron may have been related to the reduction in the size of the GABA-mediated hyperpolarizing IPSPs following oral flurazepam administration since it was not seen in neurons from control rats and was not observed in two flurazepam-treated neurons with less typical, high-amplitude early IPSPs. However, the converse may be true, i.e. the depolarizing event may have attenuated hyperpolarizing IPSP amplitudes in flurazepam-treated rats. The benzodiazepine sensitivity of the depolarizing response to GABA is unclear. Alger and Nicoll[1] reported that the GABA-mediated hyperpolarizing response was more sensitive to diazepam than the depolarizing potential. Blaxter and Cottrell[8] reported variable responses of the hyperpolarizing and depolarizing potentials to diazepam and flurazepam. Whether the $GABA_A$-mediated depolarizing potential is modified by chronic benzodiazepine treatment will require further study.

Another finding of this study was the significant increase in the amplitude of the just-subthreshold EPSP and the significant increase in the action potential threshold following synaptic activation in flurazepam-treated as compared to control neurons. Several factors might have contributed to the increased EPSP amplitude in flurazepam-treated cells. One factor was the reduction in early IPSP amplitude. At the peak of the EPSP, feedforward and recurrent inhibitory conductances predominate.[17,31] Further, application of penicillin and $GABA_A$ receptor antagonists onto CA1 pyramidal cells was reported to increase intracellular EPSP amplitude.[14,15,31,32] The level of synaptic inhibition has been shown to regulate the late, NMDA-mediated component of the EPSP[17] with low frequency synaptic activation near resting potential (-60 mV).[9] Thus decreased GABA-mediated inhibition associated with chronic flurazepam administration might have unmasked the NMDA component, increasing peak EPSP amplitudes. Another possibility is the presence of the $GABA_A$-mediated, late inhibitory depolarization which may have contributed to the apparent size of the EPSP and the increase in action potential threshold following synaptic activation. Since there was no difference in action potential threshold measured following current injection a mechanism other than a change in the intrinsic Na^+ channel conductance responsible for the action potential must have been responsible for the apparent change in action potential threshold following synaptic activation. Since the inhibitory depolarization shunts excitatory inputs to pyramidal cell dendrites,[3] it could also have modified action potential threshold following synaptic activation. Attempts to measure peak EPSP conductance to evaluate the possible contribution of the depolarizing event were unsuccessful due to the inability to synchronize the short hyperpolarizing pulse with the variable EPSP peak and due to the contamination of the hyperpolarizing pulse with the current injection artifact. In the absence of a selective inhibitor of the depolarizing event it was also not possible to directly test whether the depolarization contributed to the altered AP threshold.

In protocols designed to simulate acute benzodiazepine withdrawal effects, prolonged *in vivo* or *in vitro* exposure of rats to high doses of clonazepam resulted in a decrease in the duration of the slow AHP, in a higher likelihood of spontaneous bursting, and a decrease in action potential threshold without a change in the magnitude of GABA-mediated IPSPs.[12,13] Consistent with the absence of behavioral evidence of withdrawal in rats administered oral flurazepam for one week,[53] and as noted previously,[61] evidence of hyperexcitability of CA1 neurons two days after cessation of one week oral flurazepam

treatment was not strong. There was no change in AP threshold elicited by current injection and no change in action potential frequency. A trend was seen in chronic flurazepam-treated cells towards an increase in the duration of the AP train (Fig. 1). The period of accommodation following the current-induced train of action potentials is primarily a function of the slow AHP,[33] nonetheless blocking GABA inhibition with the $GABA_A$ antagonist, bicuculline, increases burst duration.[37] Thus, the small increase in current-induced activation of flurazepam-treated cells was probably primarily related to a decrease in GABA-mediated inhibition and was more likely related to tolerance than to withdrawal phenomena.

In summary, oral flurazepam administration for one week resulted in a significant reduction in GABA-mediated inhibition in CA1 pyramidal cells in rats which are tolerant to benzodiazepine anti-convulsant actions *in vivo*.[42–44] These findings extend previous work in *in vitro* hippocampal slices of benzodiazepine tolerant rats[61–65] and provide a foundation for future systematic investigations of CA1 pyramidal cell function following chronic benzodiazepine administration which can provide a better understanding of the synaptic mechanisms related to benzodiazepine tolerance.

Acknowledgements—The authors would like to thank W. C. Ferencak III, Drs M-Y. Shi, C. Ren and H. Tan for their technical assistance. We would like to acknowledge the contribution of Dr Timothy J. Teyler and Dr Carl R. Lupica as consultants during the early phases of this work. We also thank Dr Xiaoguang Huang for his helpful suggestions and for critically reading the final manuscript. Supported by a NIDA grant (RO1-DA04075) and a Research Scientist Development Award (KO2-DA00180) to E.I.T. from NIDA.

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(*Accepted* 7 *October* 1994)