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Tolerance to the ataxic effects of diazepam in guinea pig is not associated with a reduced sensitivity of $GABA_A$ receptors in the vestibular nucleus

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Abstract

Some studies have suggested that drug tolerance observed following repeated benzodiazepine exposure may be associated with the development of a subsensitivity to γ-aminobutyric acid (GABA) in dorsal raphe and hippocampal neurons. In other areas such as the substantia nigra such subsensitivity has not been found. The aim of the present study was to determine whether tolerance develops to the ataxic effects of diazepam on the righting reflex following low (i.e. 2 mg/kg i.p.), multiple daily doses and, if so, whether it is correlated with the development of a subsensitivity of medial vestibular nucleus neurons to the selective $GABA_A$ receptor agonist, isoguvacine. Guinea pigs which received i.p. vehicle injections three times daily for 5 days, or single daily doses of 2 or 6 mg/kg diazepam, showed increased righting reflex latencies in response to a 6 mg/kg diazepam challenge dose. However, guinea pigs which received 2 mg/kg diazepam i.p., three times daily for 5 days, exhibited minimal or no ataxia when given the same diazepam challenge dose, indicating the development of tolerance. Brain stem slices including the medial vestibular nucleus were removed from guinea pigs which had received the same diazepam and vehicle three times daily injection schedules, and recordings were made from single neurons during superfusion of isoguvacine. Although medial vestibular nucleus neurons from animals which received chronic diazepam administration showed smaller decreases in firing rate in response to 10^{-8} M isoguvacine, the difference was not statistically significant compared to neurons from animals which received vehicle treatment or acute diazepam treatment. Resting activity was also similar between the diazepam and vehicle groups, in contrast to a previous study which had shown hyperexcitability in medial vestibular nucleus cells from animals which had received single daily injections for up to 60 days. These results suggest that, in contrast to studies which have employed single daily doses, tolerance to the ataxic effects of diazepam on the righting reflex occurs rapidly with divided daily doses. However, this tolerance is not correlated with significant changes in the sensitivity of $GABA_A$ receptors on medial vestibular nucleus neurons.

Keywords: Medial vestibular nucleus; Benzodiazepine tolerance; GABA receptor; Isoguvacine

1. Introduction

'Drug tolerance', or the decrease in drug effect which occurs with repeated administration, is of interest both for its clinical significance and because it represents a basic form of neural plasticity (see File, 1985; Woods et al., 1992; Shader and Greenblatt, 1993, for reviews). In the case of benzodiazepines, tolerance to their anticonvulsant, sedative, muscle relaxant and ataxic effects is known to develop within approximately 2–4 weeks of continuous administration (e.g. Matsubara and Matsushita, 1982; Rosenberg and Chiu, 1982; Gonsalves and Gallager, 1987; Harro et al., 1990; Mana et al., 1991; for a review see Smith and Darlington, 1994b). Since the majority of studies suggest that these forms of tolerance are not due to pharmacokinetic changes (e.g. Lister et al., 1983; Tyma et al., 1984; Löscher and Schwark, 1985; Gallager et al., 1985; Davis and Gallager, 1988; Gonsalves and Gallager, 1988), a large part of the observed tolerance is thought to be due to plasticity in the central nervous system (CNS) ('pharmacodynamic' or 'functional' tolerance; see File, 1985, for a review).

A number of studies have demonstrated that chronic diazepam administration results in a subsensitivity of dorsal raphe neurons to GABA (Gallager et al., 1984, 1985; Gonsalves and Gallager, 1985, 1987, 1988; Wilson and

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Gallager, 1988; Hernandez et al., 1989). Similarly, chronic flurazepam administration has been shown to cause a reduction in the ability of the selective $GABA_A$ receptor agonist, isoguvacine, to inhibit CA1-evoked field potentials in the hippocampus (Xie and Tietz, 1992). Although GABAergic subsensitivity in the dorsal raphe and hippocampus may account for the development of some forms of tolerance, not all areas of the CNS show similar changes. For example, chronic diazepam treatment did not result in a subsensitivity of substantia nigra pars reticulata neurons to GABA (however, the ability of benzodiazepines to increase the effects of GABA was reduced; Wilson and Gallager, 1987, 1989).

Because of the relative simplicity of the vestibular reflexes and their direct relationship to activity in the brain stem vestibular nuclei, we have used the vestibular system to examine the effects of chronic diazepam treatment (Dingwall et al., 1993; Scott et al., 1994; Smith and Darlington, 1994a; Hutchinson et al., 1995a). Although substantial tolerance to the ataxic effects of diazepam on the righting reflex does not develop when single daily injections are delivered (e.g. Scott et al., 1994; Hutchinson et al., 1995a), administering diazepam in divided daily doses results in the rapid development of tolerance (Smith and Darlington, 1994a) (presumably, this is due to the maintenance of higher blood plasma levels of diazepam over longer periods of time with multiple daily doses, leading to more continuous benzodiazepine receptor occupation; e.g. Davis and Gallager, 1988). While it has been demonstrated that tolerance to the ataxic effects of diazepam can develop within 3 days when it is delivered in relatively large daily doses (i.e. 6 mg/kg, three times daily; Smith and Darlington, 1994a), at present it is unclear whether this rapid form of tolerance might be related to the development of a GABAergic subsensitivity in vestibular nucleus neurons which contribute to the righting reflex. It is also not known whether the hyperexcitability of medial vestibular nucleus (MVN) neurons which has been shown following long periods of benzodiazepine administration, is seen with rapid tolerance (Hutchinson et al., 1995a). Such adaptive changes could potentially lead to long-term abnormalities in vestibular reflexes (e.g. Blair and Gavin, 1979; Padoan et al., 1990), particularly during a withdrawal syndrome elicited by discontinuation of the benzodiazepine. Diazepam is sometimes used to alleviate vertigo and dizziness (McCabe et al., 1973; Bernstein et al., 1973; see Baloh, 1994, for a recent review) and has been shown to reduce the firing rate of medial vestibular nucleus neurons (Sekitani et al., 1971; Ryu and McCabe, 1974), probably via the benzodiazepine recognition site on the $GABA_A$ receptor complex. Therefore, the chronic effects of diazepam on the vestibular reflexes are of potential clinical importance.

The aims of the present study were: (1) to determine whether rapid tolerance develops to the ataxic effects of diazepam on the righting reflex when divided, low (i.e. 2 mg/kg) doses are used; (2) to determine whether the development of tolerance is related to changes in the sensitivity of medial vestibular nucleus neurons to a $GABA_A$ receptor agonist.

2. Materials and methods

Labyrinthine-intact guinea pigs ($n = 37$) were housed in pairs in cages in an animal holding room with a 12 h light-dark cycle; food and water were available ad libitum. 24 animals (250–360 g) were used in the behavioural study; a further 13 animals (290–370 g) were used in the electrophysiological study.

2.1. Behavioural study

The objective of the behavioural study was to determine whether 2 mg/kg diazepam, injected i.p. three times daily (i.e. a total daily dose of 6 mg/kg), would result in tolerance to the effects of diazepam on the righting reflex over 5 days. Righting reflex latency was defined as the time taken to complete a righting reflex, that is, the time from when the animal was placed in the supine position until the prone position was reached (Smith and Darlington, 1994a). Because a 2 mg/kg i.p. diazepam dose has been shown to have variable effects on righting reflex latency (Hutchinson et al., 1995a), righting reflex latency was tested on day 6 following a 6 mg/kg i.p. challenge dose of diazepam.

The animals were randomly divided into four groups ($n = 6$ in each), receiving the following injection schedules: (1) 2 mg/kg i.p. diazepam (Valium 10, Roche, New Zealand), three times per day (approximately 8–9 a.m., 12–1 p.m. and 5–6 p.m.) for 5 days; (2) 0.4 ml/kg i.p. vehicle, three times per day, for 5 days (as for group 1); (3) 2 mg/kg i.p. diazepam once per day for 5 days; (4) 6 mg/kg i.p. diazepam once per day for 5 days. For the animals injected three times daily, the first and last injections were given at around mid-day on days 1 and 5, giving a total of 13 injections. All injections were administered in a volume of 0.4 ml/kg i.p. The vehicle consisted of 89% saline, 8% ethanol and 3% sodium benzoate. On day 6, a single challenge dose of 6 mg/kg i.p. diazepam was administered to all animals 30 min prior to the measurement of righting reflex latency (see below). Prior to the beginning of the experiment, all animals were tested for the presence of a normal righting reflex latency. The animals used in this study had a mean righting reflex latency of 0.58 s (± 0.19 (S.D.), $n = 24$), which was within the normal range (Smith and Darlington, 1994a). While the purpose of group 1 was to determine the effect of divided daily doses of diazepam (2 mg/kg, three times daily) on righting reflex latency, the other three groups were used as controls. Group 2 (vehicle, three times daily) controlled for the effects of handling, injection per se, and

exposure to the measurement apparatus and laboratory conditions. Group 3 (2 mg/kg diazepam, once daily) controlled for the effects of single daily administration at the same dose. Group 4 (6 mg/kg diazepam, once daily) controlled for the effects of the total amount of diazepam that group 1 received each day (i.e. 3 × 2 mg/kg).

Righting reflex latency was measured using a custom-made electronic device ('tolerometer') which has been described in detail previously (Dingwall et al., 1993). Briefly, the tolerometer consisted of a 2 kg load cell connected to a strain gauge amplifier. During righting reflex tests, the guinea pig was placed in the supine position on a platform above the load cell. During the righting reflex, the load changes were transduced into an electrical signal which was amplified and displayed on a Macintosh Classic computer using a MacLab data acquisition system; righting reflex latencies were measured using cursors available in the Chart program (Fig. 1A). With a 100 Hz sampling frequency, the resolution of the system in measuring latency was 0.05 s.

2.2. *In vitro electrophysiological study*

The purpose of the in vitro electrophysiological study was to determine the effects of the 2 mg/kg three times daily diazepam injection schedule used in the behavioural study, on the sensitivity of medial vestibular nucleus neurons to the selective $GABA_A$ receptor agonist, isoguvacine (Johnston, 1992).

The 13 guinea pigs were randomly divided into the following three groups: (1) 2 mg/kg diazepam i.p., three times daily for 5 days (as for the behavioural study) ($n = 5$ animals); (2) vehicle injection i.p., three times daily for 5 days (as for the behavioural study) ($n = 4$); (3) a single i.p. 2 mg/kg diazepam injection 1 day prior to the slice experiment ('acute diazepam condition'; $n = 4$). This third group was designed to control for the acute effects of a diazepam injection the day prior to the slice experiment, irrespective of whether diazepam had been administered on the previous 4 days. The results of these electrophysiological experiments were compared with those from a previous study in which brain stem slices from drug- and handling-naive guinea pigs were used ($n = 16$ animals; data from Hutchinson et al., 1995b). In all conditions, the last diazepam or vehicle injection was 20–22 h prior to the slice experiment.

Prior to decapitation, animals were anaesthetized with ether. The brain stem was rapidly dissected and submerged in 4°C artificial cerebrospinal fluid (ACSF, see below). Coronal slices containing the medial vestibular nucleus, approximately 500 μm thick, were cut using a Stoelting tissue chopper. Slices were incubated in an immersion slice chamber at 29°C for 2–3 h prior to recording and were continuously superfused with ACSF (in mM): NaCl (126.0), KCl (5.0), KH_2PO_4 (1.25), $MgSO_4$ (1.3), $NaHCO_3$ (26.0), glucose (10.0), $CaCl_2$ (2.5). The ACSF was continuously bubbled with 95% O_2 and 5% CO_2 and maintained at a pH of approximately 7.4. The time from decapitation until immersion of the brain stem in the chilled ACSF was always less than 3 min; the slice was always settled in the slice chamber in less than 15 min (Hutchinson et al., 1995b; see Darlington et al., 1995, for a review).

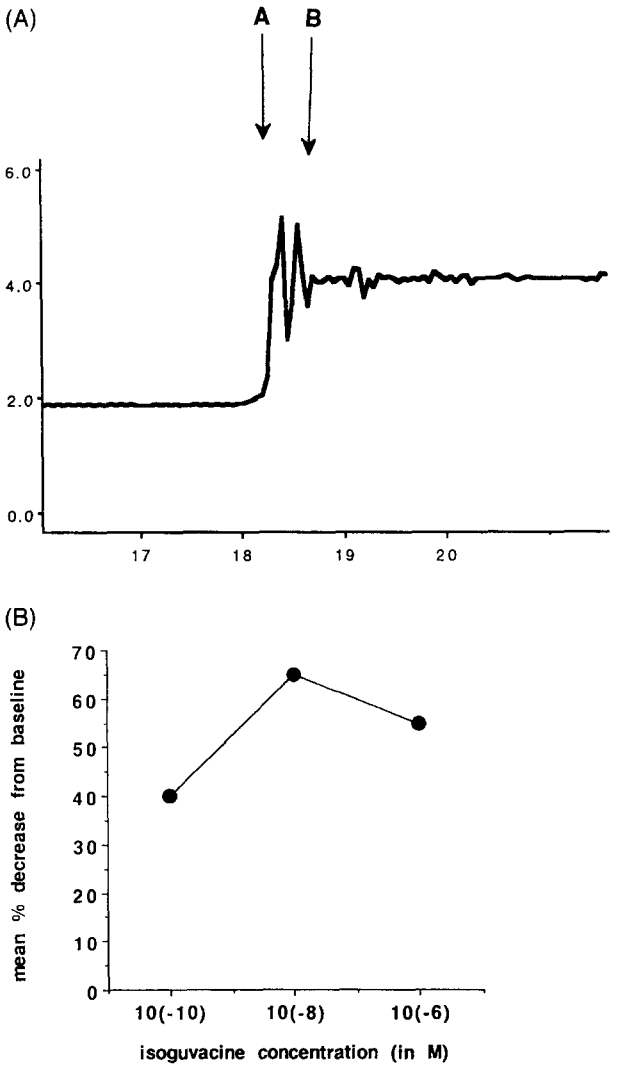

Fig. 1. A: example of a righting reflex trace obtained using the tolerometer system described. The initial upward deflection (arrow A) represents the placement of the animal in the supine position. The high frequency, large amplitude 'spikes' which follow during the next second represent the righting reflex manouvre; this is followed by a relatively stable baseline when the animal reaches the prone position (arrow B). The x axis represents time (in s). The y axis represents amplitude (in mV; the latter are arbitrary units since all measurements are in the time domain). B: mean %decrease in firing rate from baseline for medial vestibular nucleus (MVN) neurons in slices from drug-naive guinea pigs in response to three concentrations of isoguvacine. This graph demonstrates that 10^{-8} M isoguvacine is the optimal concentration for activating $GABA_A$ receptors on medial vestibular nucleus neurons (data from Hutchinson et al., 1995b).

During recording, the chamber temperature was maintained at 34–36°C. ACSF flow rate was maintained at 2.0–2.5 ml/min with a chamber turnover time of approximately 2 min.

Action potentials from single medial vestibular nucleus neurons were recorded extracellularly using glass micropipettes filled with 2 M NaCl (impedance 2–6 MΩ) and green dye, to facilitate visualization of the electrode tip when placing the electrode; the medial vestibular nucleus was easily identified by its proximity to the IVth ventricle. The electrode was advanced through the slice in 1–2 μm steps using a Narishige nanostepper. Signals were amplified ($\times 10$) using a Dagan 8100-1 amplifier (low-pass filtered at 30 kHz), displayed on an Iwatsu digital storage oscilloscope and monitored using a Grass audiomonitor. For the purposes of analysing action potential waveforms, signals were sampled from the Dagan amplifier by a MacLab data acquisition system (40–100 kHz sampling frequency) and displayed on a Macintosh LC II computer using a Chart program. In order to analyse action potential frequency, signals from the Dagan amplifier were fed into a MacLab bioamplifier (filtered at 0.3 Hz and 2.5 kHz, with a notch filter set at 50 Hz) and displayed on the second channel of the oscilloscope. Action potentials from a single neuron were isolated using a window discriminator and the action potential frequency was converted to a voltage using a custom-made frequency-to-voltage converter. A voltage proportional to action potential frequency (accuracy $\pm$ 0.5 Hz) was displayed on the LC II computer using the MacLab data acquisition system. Histograms of action potential frequency over time could be displayed and analysed using the Chart program.

The selective $GABA_A$ receptor agonist, isoguvacine (RBI, NJ), was dissolved in ACSF and applied to the slice by superfusion (see Johnston, 1992, for a review). From a previous study (Hutchinson et al., 1995b), it was determined that 10^{-8} M was the optimal concentration of isoguvacine to activate $GABA_A$ receptors on medial vestibular nucleus neurons (Fig. 1B); therefore, this isoguvacine concentration was used in the present slice studies. For each neuron, stable baseline resting activity was recorded for approximately 2–4 min while superfusing with ACSF, then the drug solution was turned on for 4 min (i.e. twice the turnover time), followed by a return to the control solution. Firing frequency was considered to have increased or decreased when a change of greater than or equal to 20% of baseline occurred, with a minimum change of 1 Hz (i.e. twice the measurement error). This conservative criterion has proven useful to exclude small changes in firing rate caused by extraneous variables, particularly for neurons with low firing rates (e.g. Hutchinson et al., 1995b). Only neurons which showed reversible changes in firing frequency were analysed, in order to eliminate the possibility that a change was due to cell damage.

2.3. Statistical analysis

The righting reflex latency data were analysed using a Fisher Exact test (Siegel, 1956). For the purposes of this test, a retarded righting reflex was defined as one which was more than 3 standard deviations slower than the mean righting reflex latency, based on the righting reflex latency measurements made for all animals before the injection schedules began (see above). This meant that animals taking longer than 1.15 s to complete a righting reflex were classified as having a retarded righting reflex. Using this criterion, none of the animals measured before the experiment began would have been misclassified. Using the Fisher Exact test, the proportion of animals exhibiting a retarded righting reflex in the four groups was analysed (Siegel, 1956). This test was used in preference to a parametric test because it is highly unusual for a guinea pig to take longer than 1 s to generate a righting reflex (Smith and Darlington, 1994a) and when it takes longer than this for the animal to right itself, it could be argued that the movement is no longer a 'reflex'.

For the neuronal data, average resting activity and the average magnitude of the decrease in firing rate in the three conditions (vehicle, diazepam chronic, diazepam acute) were compared using planned, 2-tailed t tests (i.e. planned on the basis of previous studies by Gallager et al. (1984)). The proportion of cells showing a decrease, increase or no change in response to isoguvacine was analysed using a χ^2 test (Snedecor and Cochran, 1989). The significance level was set at 0.05 for all comparisons.

3. Results

3.1. Behavioural study

All animals in the vehicle, 2 mg/kg diazepam once daily and 6 mg/kg diazepam once daily groups showed retarded righting reflexes (according to the criteria explained above) in response to the 6 mg/kg challenge dose of diazepam (range 1.6–180 s). However, animals in the 2 mg/kg diazepam three times daily group showed either no effect (4/6 animals) or a small increase in righting reflex latency (2/6 animals) in response to the diazepam challenge (range 0.65–4.6 s). These differences were shown to be significant ($P < 0.01$, Fisher Exact test), indicating the development of tolerance in the 2 mg/kg diazepam, three times daily group. Mean righting reflex latencies (in s) for the four groups in the behavioural study are shown in Fig. 2.

3.2. In vitro electrophysiological study

Most neurons in slices from vehicle animals showed the regular discharge frequency which has been shown by previous studies to be characteristic of medial vestibular nucleus neurons in vitro (mean resting discharge rate: 17.1 $\pm$ 11.9 Hz (S.D.), $n = 38$ neurons). Of those neurons which showed a decrease in firing in response to isoguvacine, some showed a monophasic decrease, while others showed a biphasic decrease (initial increase, followed by a

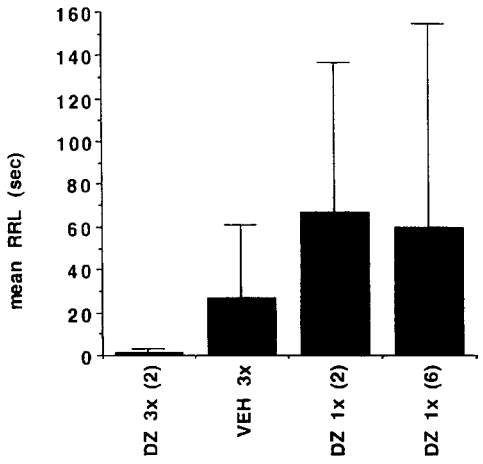

Fig. 2. Mean righting reflex latency (RRL, in s) for guinea pigs which received diazepam, 2 mg/kg i.p., three times daily for 5 days (DZ 3×(2)); vehicle i.p., three times daily for 5 days (VEH 3×); diazepam, 2 mg/kg i.p., once daily for 5 days (DZ 1×(2)); or diazepam, 6 mg/kg i.p., once daily for 5 days (DZ 1×(6)) ($n = 6$ animals in each group). In each case, a challenge dose of 6 mg/kg i.p. was administered on day 6, 30 min prior to testing. The filled columns represent means, the bars ± 1 S.D.

larger decrease (Hutchinson et al., 1995b)). In Table 1, the results for cells which showed decreases have been reported separately as monophasic and biphasic responses, as well as together, to allow comparison of the two response categories. In the text, all analyses of decreases refer to the two groups together, as the functional significance of the different response types is unclear at present.

MVN neurons in slices from chronic diazepam animals showed an average decrease in discharge from baseline of −44.0% (S.D. = 22.7%, $n = 11$). The magnitude of this decrease was similar to neurons in the acute diazepam group (−48.2 ± 31.4%, $n = 5$) and less than for neurons from the vehicle group (−61.0 ± 32.2%, $n = 10$); however, the difference was not statistically significant ($P > 0.05$, 2-tailed t tests; Fig. 3A, Table 1).

The percentages of medial vestibular nucleus neurons in the diazepam (chronic and acute) and vehicle groups responding to isoguvacine are shown in Table 1. The number of neurons exhibiting decreases (as opposed to increases or no change in firing rate) in response to isoguvacine, changed significantly between the three groups ($P < 0.05$, $\chi^2 = 6.37$, df = 2; Table 1), apparently due to fewer cells responding to isoguvacine in the acute diazepam group. The proportion of cells showing decreases in naive, vehicle and chronic diazepam groups respectively, was 48%, 50% and 58%. However, only 21% of cells tested in the acute diazepam group responded with a decrease in firing rate.

Mean resting activities for neurons which were drug tested were as follows: vehicle group, 18.2 Hz (S.D. = 12.3, $n = 20$); chronic diazepam group, 14.5 Hz (S.D. = 10.4, $n = 19$); and acute diazepam, 18.0 Hz (S.D. = 9.1, $n = 23$). There were no significant differences in resting activity between these groups ($P > 0.05$, two-tailed t tests; Fig. 3B). Resting activities in these groups were also compara-

Table 1
Neurons showing a decrease in firing rate: comparison of monophasic and biphasic responses

	Naive	Vehicle	Acute DZ	Chronic DZ
Proportion of neurons with each response type				
Decrease				
(a) monophasic	32% (6/19)	20% (4/20)	17% (4/23)	21% (4/19)
(b) biphasic	16% (3/19)	30% (6/20)	4% (1/23)	37% (7/19)
No response	53% (10/19)	45% (9/20)	78% (18/23)	42% (8/19)
Increase	0%	5% (1/20)	0%	0%
Mean % decrease in firing rate				
Monophasic	−54.0% ($n = 6$)	−50.0% ($n = 4$)	−47.5% ($n = 4$)	−43.0% ($n = 4$)
(S.D.)	28.0	31.9	36.2	10.0
Biphasic	−91.0% ($n = 3$)	−68.0% ($n = 6$)	−51.0% ($n = 1$)	−45.0% ($n = 7$)
(S.D.)	15.6	33.0	0	28.5
Total	−66.0% ($n = 9$)	−61.0% ($n = 10$)	−48.2% ($n = 5$)	−44.0% ($n = 11$)
(S.D.)	30.0	32.2	31.4	22.7
Resting activity (spikes / s)				
Neurons drug tested				
Mean	16.5	18.2	18.0	14.5
(S.D.)	14.6	12.3	9.1	10.4
n	19	20	23	19
All neurons				
Mean	13.9	17.1	16.4	13.9
(S.D.)	12.1	11.9	8.6	12.2
n	63	38	30	34

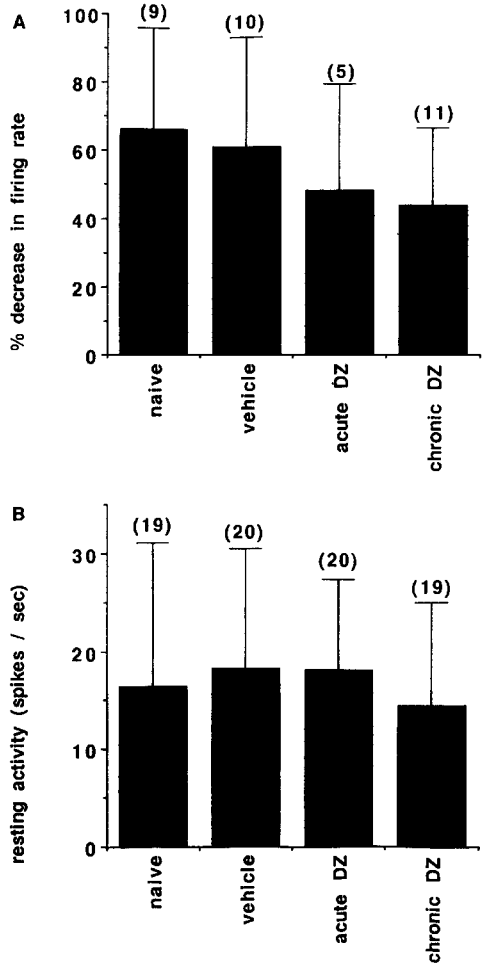

Fig. 3. A: mean decrease in firing rate, expressed as a percentage change from baseline firing rate, for medial vestibular nucleus neurons recorded in brainstem slices from guinea pigs which received no injection (naive, $n = 9$ neurons; data from Hutchinson et al. (1995b) shown for comparison); the vehicle i.p., three times daily for 5 days (vehicle; $n = 10$); diazepam, 2 mg/kg i.p., one injection the day prior to the slice experiment (acute DZ; $n = 5$); or diazepam, 2 mg/kg i.p., three times daily for 5 days (chronic DZ; $n = 11$). The filled columns represent means, the bars ± 1 S.D. B: mean resting activity for medial vestibular nucleus neurons in the above four groups ($n = 19$, 20, 20 and 19 neurons, respectively). The filled columns represent means, the bars ± 1 S.D.

ble to those found in non-handled, drug-naive animals in a previous study (16.5 Hz, S.D. = 14.6, $n = 19$ (Hutchinson et al., 1995b); see Table 1, Fig. 3B).

4. Discussion

The results of the present experiment confirm that tolerance to the ataxic effects of diazepam on the righting reflex can develop rapidly (within 5 days) when the drug is administered in divided doses. Animals which received vehicle injections, or 2 or 6 mg/kg diazepam once daily, showed retarded righting reflexes following a 6 mg/kg diazepam challenge dose on day 6; while animals which received 2 mg/kg diazepam three times daily, generated righting reflexes which were either normal or only slightly retarded. These results are consistent with those from a previous study in which a higher dose of diazepam, 6 mg/kg i.p., was administered three times daily for 5 days (Smith and Darlington, 1994a), and demonstrate that tolerance can also develop rapidly using much lower doses (i.e. 2 mg/kg). The use of a challenge dose proved to be a useful method for examining the effects of repeated administration of a low dose of diazepam (2 mg/kg). This is because such low doses have high within animal variability in ataxic effect on righting reflex latency over the course of chronic treatment, making measurement of tolerance difficult (Hutchinson et al., 1995a). The results of the behavioural study are generally consistent with the view put forward by Davis and Gallager (1988) that maintaining blood plasma levels of diazepam for longer periods of time (in the present case, using divided doses) results in a faster development of tolerance. This may be particularly true in lower mammalian species in which the elimination half-lives of benzodiazepines are very short; for example, the half-life of diazepam in guinea pig is approximately 2.4 h (Klotz et al., 1976).

The results from the in vitro electrophysiological study suggest that the chronic diazepam treatment used does not have any significant effects on the sensitivity of medial vestibular nucleus $GABA_A$ receptors to a $GABA_A$ receptor agonist. There was no significant difference, in isoguvacine-mediated inhibition of medial vestibular nucleus neuron firing rate, in the chronic diazepam group when compared to the naive, vehicle and acute diazepam groups. It is therefore unlikely that the tolerance demonstrated in the behavioural experiment was due to GABAergic subsensitivity in the medial vestibular nucleus. It is possible that GABAergic subsensitivity in another part of the CNS was responsible; or alternatively, another mechanism may underlie the development of tolerance, such as a reduction of the functional coupling between the benzodiazepine and GABA binding sites on the $GABA_A$ receptor (Yu et al., 1988; Tietz et al., 1989; Roca et al., 1990; Allan et al., 1992; Li et al., 1993). A pharmacokinetic explanation for the observed tolerance cannot be excluded on the basis of our current evidence; however, this explanation seems unlikely given the evidence from previous studies (e.g. Lister et al., 1983; Tyma et al., 1984; Löscher and Schwark, 1985; Gallager et al., 1985; Davis and Gallager, 1988; Gonsalves and Gallager, 1988).

We must also acknowledge the possibility that the $GABA_A$ receptor sensitivity that we observed in medial vestibular nucleus neurons was at least partially related to a withdrawal syndrome. Since the half-life of diazepam is so short in guinea pig (i.e. 2.4 h (Klotz et al., 1976)), the fact that brain stem slices were removed 20–22 h following the final diazepam injection may mean that neuronal activity in the medial vestibular nucleus was a better representation of 'withdrawal related to dependence' rather than 'tolerance'. However, in the case of the benzodi-

azepines, the hyperexcitability observed during withdrawal may, in some cases, be causally related to the development of tolerance; therefore, a distinction between the neuronal changes relating to tolerance and those relating to withdrawal, is difficult to make and it would be expected that the two are related (see Smith and Darlington, 1994b for a review). In any case, since our results suggest no significant change in $GABA_A$ receptor sensitivity in the medial vestibular nucleus following this particular diazepam injection paradigm, our data suggest that neither tolerance nor withdrawal results in significant $GABA_A$ receptor changes in the medial vestibular nucleus.

The finding that decreased GABA sensitivity does not develop in the medial vestibular nucleus in parallel with the development of behavioural tolerance is inconsistent with evidence from the hippocampus (Xie and Tietz, 1991, Xie and Tietz, 1992), dorsal raphe (Gallager et al., 1984, 1985; Gonsalves and Gallager, 1985, 1987, 1988; Wilson and Gallager, 1988; Hernandez et al., 1989) and spinal cord (Sher et al., 1983), which has suggested that chronic diazepam administration results in the development of subsensitivity of $GABA_A$ receptors to GABA. On the other hand, the present results are consistent with studies which have shown that substantia nigra neurons do not develop a subsensitivity to GABA following chronic diazepam treatment (Wilson and Gallager, 1987; Wilson and Gallager, 1989). It seems likely that, while some areas of the CNS exhibit marked changes in GABAergic sensitivity as a result of chronic benzodiazepine administration, others do not (Marley and Gallager, 1989).

A finding of interest was the significantly lower proportion of cells which responded to isoguvacine following an acute treatment with diazepam, compared to cells from chronic vehicle-treated, chronic diazepam-treated, or untreated animals. The reason for this change is unclear at present but a transient down-regulation of GABA binding sites, or reduced coupling between the GABA site and the Cl^- ion channel, are possible explanations which could be examined. This finding highlights the complex differences in the action of benzodiazepines on the CNS when given acutely versus chronically.

The results of the present study also add to our increasing understanding of $GABA_A$ receptors in the medial vestibular nucleus. It is generally believed that type II medial vestibular nucleus interneurons (receiving excitatory input from contralateral type I medial vestibular nucleus neurons) release GABA which inhibits ipsilateral type I neurons via $GABA_A$ receptors (e.g. Furuya et al., 1992). Binding studies have confirmed that $GABA_A$ receptors within the medial vestibular nucleus have benzodiazepine binding sites (see Hutchinson et al., 1995b for a review). The results of in vivo electrophysiological studies are consistent with this conclusion (Sekitani et al., 1971; Ryu and McCabe, 1974). Our results, using the selective $GABA_A$ receptor agonist, isoguvacine, are consistent with previous in vitro studies which indicate that many medial vestibular nucleus neurons have $GABA_A$ receptors which are modulated by benzodiazepines (see Darlington et al., 1995 for a review).

In conclusion, the present study clearly demonstrated the development of tolerance to the ataxic effects of diazepam on the righting reflex following small, divided daily doses. This is in contrast to previous studies which have failed to find tolerance with single daily injections, even over much longer periods of treatment (Hutchinson et al., 1995a). It was also found that this tolerance did not correlate with a decrease in sensitivity of medial vestibular nucleus neurons to a $GABA_A$ receptor agonist, as has been demonstrated in some brain regions. Tolerance to the ataxic side effects of benzodiazepines is beneficial during benzodiazepine therapy for anxiety disorders (Woods et al., 1992) or vertigo (Baloh, 1994) in humans. The implication of the present study is that this form of tolerance may be achieved most rapidly by using divided doses (or longer-acting compounds, which also result in more continuous receptor occupation), without causing a change in $GABA_A$ receptor sensitivity to endogenous GABA which might ultimately be detrimental to vestibular reflex function.

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