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BRIEF COMMUNICATION

The Effects of Long-Term, Low-Dose Diazepam Treatment on the Guinea Pig Righting Reflex and Medial Vestibular Nucleus Neuronal Activity

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HUTCHINSON, M. A., C. L. DARLINGTON AND P. F. SMITH. *The effects of long-term, low-dose diazepam treatment on the guinea pig righting reflex and medial vestibular nucleus neuronal activity.* PHARMACOL BIOCHEM BEHAV **50**(4) 665–669, 1995. – Guinea pigs received a 2 mg/kg IP injection of diazepam, or an equivalent volume of vehicle, daily for 28–60 days. To determine whether tolerance developed to the ataxic effects of diazepam on the righting reflex, daily righting reflex latency (RRL) measurements were made before and 20, 30, and 40 min following the diazepam or vehicle injection for 28 days. Analyses of the RRLs for individual animals indicated that a significant decrease in RRL over time (indicating tolerance) occurred in only one out of nine animals receiving diazepam and in none of the vehicle animals. Medial vestibular nucleus (MVN) neurons in brain stem slices from animals receiving chronic diazepam treatment had a significantly higher average firing rate than those from vehicle controls. These results suggest that: a) long-term treatment with single 2 mg/kg daily IP injections of diazepam does not result in tolerance to diazepam's ataxic effects on the righting reflex in the majority of animals; b) this form of diazepam treatment may, nonetheless, induce a hyperactivity of brain stem MVN neurons that may be consistent with the occurrence of a withdrawal syndrome.

Diazepam Tolerance Vestibular nucleus in vitro Righting reflex

IN MANY animal studies, administration of benzodiazepines (BDZs) for a period of 2–4 weeks has been shown to induce tolerance to their anticonvulsant (11,17), sedative (12), muscle relaxant, and ataxic effects (18,21). Whether, and to what extent, tolerance also develops to the anxiolytic effects of BDZs is more controversial [e.g., (3,31)].

In general, tolerance develops more easily when consistent blood plasma levels of the BDZ are maintained for long periods of time, e.g., using divided doses (12) or SC osmotic minipumps that permit continuous release (19). Nonetheless, tolerance to the effects of some BDZs, such as lorazepam and triazolam, has been obtained with single, daily injections, even using low doses i.e., less than 3 mg/kg/day (e.g., lorazepam, 0.125–0.5 mg/kg/day IP (6,7,15); triazolam, 0.075–0.25 mg/kg/day IP (6)). In contrast, few studies have demonstrated tolerance to diazepam when low doses are used in single, daily injections [e.g., 1 mg/kg/day IP (1)].

Diazepam has been reported to depress vestibular reflexes (20), probably via its action on $GABA_A$ receptors within the brain stem vestibular nucleus (26,28). The righting reflex is a fast postural reflex that is initiated by the vestibular labyrinth and generated by vestibular nucleus neuron projections to the spinal cord (30). In guinea pigs, diazepam has been demonstrated to increase the latency to generate a righting reflex in a

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dose-dependent manner (25,27); part of this effect is probably mediated by the action of diazepam on $GABA_A$ receptors in the vestibular nucleus. In previous studies, the latency to generate a righting reflex (righting reflex latency, RRL) has proven to be a sensitive measure of the ataxic effect of diazepam on this vestibular reflex, and one that is relatively simple to quantify (4,25,27). The aim of the present study was to determine whether tolerance develops to the ataxic effects of diazepam on the guinea pig righting reflex when single, daily injections of a low dose (2 mg/kg IP) of diazepam are administered for 28 days. This dose was chosen because pilot studies have shown that it results in increased RRLs without inducing the high levels of sedation seen at doses used in previous studies [e.g., 5 mg/kg (25)]. In the anxiolytic dose range used in humans, diazepam does not induce high levels of sedation (31). A second aim was to obtain a neural correlate for diazepam's effect on the righting reflex. For this purpose in vitro recordings were made from neurons in the medial vestibular nuclei (MVN) (2,5,28) [an area known to mediate the righting reflex (30)] of animals that continued to receive diazepam for 57–60 days.

METHOD

Data were obtained from 13 male pigmented guinea pigs (380–840 g) with both vestibular labyrinths intact. Nine animals received daily IP injections of 2 mg/kg diazepam in a volume of 0.4 ml/kg vehicle (Valium MM, Roche, NZ) for at least 28 days; four of these animals received diazepam injections for 57–60 days, at which time they were used for slice experiments. The remaining four animals received daily IP injections of an equivalent volume of vehicle solution for 44–47 days before being used for slice experiments. Injections were given within 2 h of the same time each day in the same environment, and the last diazepam or vehicle injection was administered 24–26 h prior to the slice experiment.

For all 13 animals, righting reflex latency (RRL) measurements were made every day for 28 days using an electronic device that was designed and built in the Department of Psychology, University of Otago (4). Four diazepam and four vehicle animals continued to have RRL measurements made in the same way until day 42. However, because in some diazepam-treated animals RRL was measured for only 28 days (see above), only RRL data up to day 28 were used for statistical analyses.

Briefly, the device used to measure RRLs consisted of a semicylindrical platform positioned on a 2 kg load cell. The load cell transduced changes in load during a righting reflex into a signal that was amplified and displayed as a waveform on a MacClassic computer screen via a MacLab data acquisition system (Analog Digital Instruments) (4). The MacLab unit sampled from the amplifier at 20 Hz, giving a measurement resolution of 0.05 s.

Animals were placed on the tolerometer platform in the supine position and as they generated a righting reflex, the MacLab Chart program displayed a trace on the Macintosh computer screen, which represented the load changes during the reflex manouvre. For each trace, cursors available in the Chart program were used to measure the difference (in seconds) between the placement of the animal on the platform in the supine position and the peak of the initial positive deflection (corresponding to the initiation of the righting reflex movement) (4). Traces from each RRL test were stored on disk for later analysis.

Animals in both the diazepam and vehicle groups had their RRLs measured daily, before and 20, 30, and 40 min following their injection. Three postinjection measurement times were used in an attempt to ensure that the RRL would be measured at the time when the diazepam exerted its maximal effect on the righting reflex. These three times were selected on the basis of pilot studies that showed that the maximal effect of 2 mg/kg diazepam occurred at different times on different days, but usually between 20 and 40 min postinjection. Pharmacokinetic data suggest that the peak blood plasma concentrations of diazepam are reached approximately 20 min following an IP injection in rats (13). The preinjection measurement was subtracted from each of the three postinjection measurements to control for daily fluctuations in RRL; for each animal, this resulted in three RRL differences for each of the 28 days of testing.

In preparation for the slice experiments, animals were anesthetized with 30 mg/kg IP Nembutal, decapitated, and the brain stem quickly removed to chilled artificial cerebrospinal fluid (ACSF) of approximately 4°C. Coronal slices containing the MVN (approximately 400–600 μm thick) were cut by hand with a chilled razor blade and the two MVN were dissected from the slice (2,28). The MVN could be identified easily during dissection by its proximity to the sulcus limitans (in the horizontal plane) and the IVth ventricle (in the coronal plane). The MVN slices were incubated in a standard immersion slice chamber at 28–30°C for 1–2 h prior to recording and superfused continuously with standard 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, Phenol red (0.05%), continuously bubbled with 95% O_2 and 5% CO_2 and maintained at a pH of 7.4. During recording, the chamber temperature was maintained at 35–37°C and the ACSF flow rate was 2 ml/min. The time from decapitation to immersion in the slice chamber was always less than 6 min (2,25,28).

Extracellular recordings were made from single, randomly sampled MVN neurons using glass micropipettes (3–5 MΩ impedance) filled with 2 M NaCl and Fast green FCF dye, to facilitate visualization of the electrode position. The electrode was moved through the slice using a Narishige nanostepper. Action potentials were amplified using a Dagan amplifier (low-pass filtered at 30 kHz), fed into a Grass audiomonitor, and displayed on an Iwatsu (DS-6411) digital storage oscilloscope. The discharge of MVN neurons in vitro is very regular (2,25,26,28) and, therefore, firing frequency was measured as the inverse of the interspike interval using the computational facilities of the oscilloscope (25). Only one slice was removed from each diazepam- or vehicle-treated animal, and an attempt was made to record an approximately equal number of neurons from the slices each group.

Previous studies have indicated that the acute effects of diazepam, at doses as low as 2 mg/kg IP, are highly variable between animals [e.g., 5.1–180.0 s (27)]. Therefore, rather than conducting group analyses, planned analyses were carried out for the individual diazepam-treated and vehicle-treated animals. The largest RRL difference of the three daily measurements was calculated for each animal for each day in the 28 days of testing. For each animal, the average maximal RRL differences for the first and last 14 days of treatment were calculated and compared using planned, two-tailed, paired t-tests (29). If the average RRL difference for the last 14 days of treatment was significantly less than that for the first 14 days, it was concluded that tolerance had developed.

The average firing rates of MVN neurons in slices from animals in the diazepam and vehicle groups were compared using a two-tailed, unpaired t-test (29). A χ^2 test was used to

compare the distributions of firing rates in the two groups of slices (29). The significance level was set at 0.05 for all statistical comparisons.

RESULTS

The effects of 2 mg/kg IP diazepam on the righting reflex were highly variable (Fig. 1). In some cases, the diazepam injection caused a large increase in RRL (e.g., >30 s; Fig. 1A),

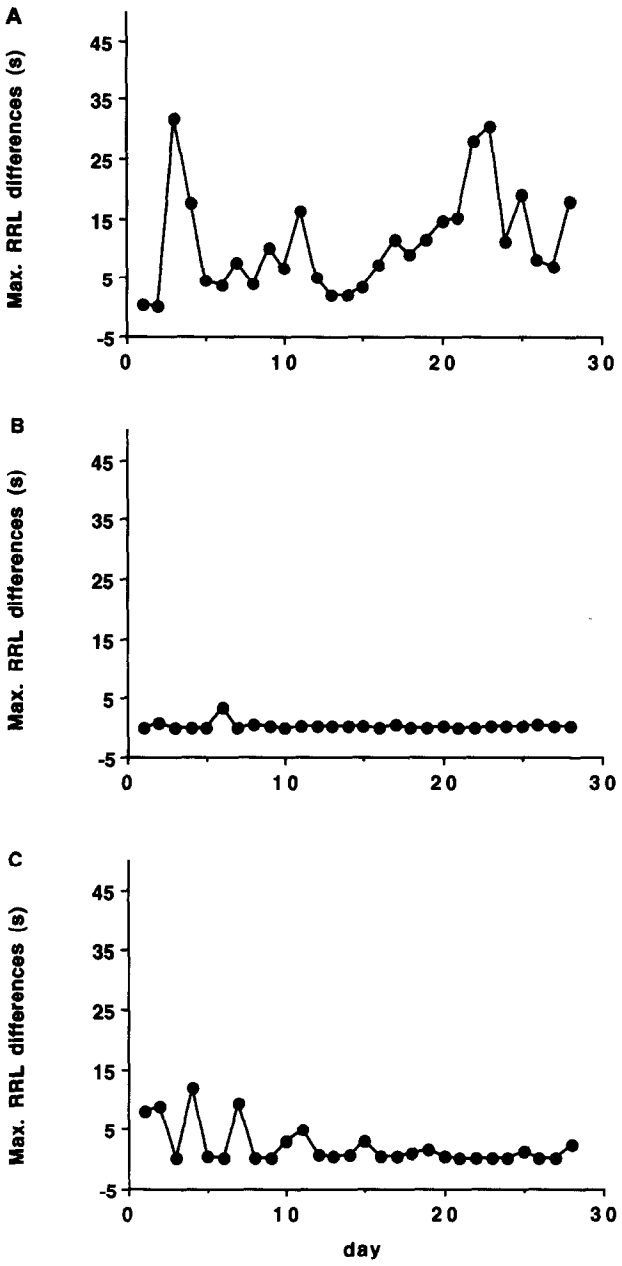

FIG. 1. Maximum righting reflex latency (RRL) differences (i.e., maximal postinjection latency minus preinjection latency, in seconds) for three guinea pigs that received 2 mg/kg/day diazepam IP for 28 days. (A) Example of an animal that showed large increases in RRL but that showed no clear signs of tolerance. (B) Example of an animal that showed no change in RRL. (C) Example of the animal that initially showed an increase in RRL but then developed tolerance.

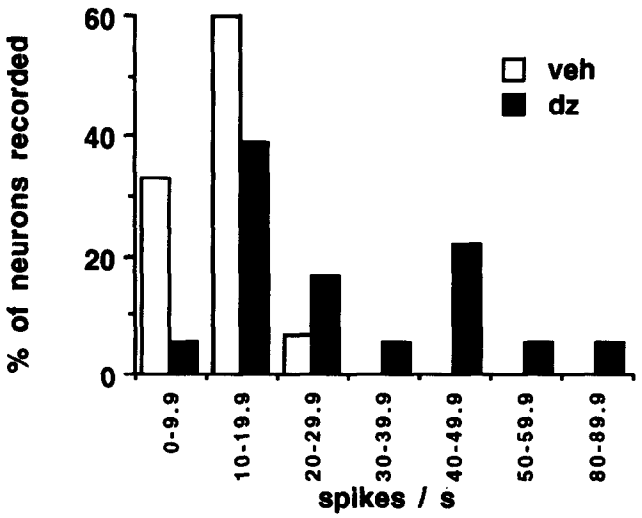

FIG. 2. Firing rate distributions for medial vestibular nucleus (MVN) neurons in slices from vehicle-injected animals (veh) and diazepam-injected animals (2 mg/kg/day IP, for up to 63 days, dz). The y axis shows the percentage of neurons that were recorded in each firing rate category.

in other cases, it had little effect (Fig. 1B). Although three out of nine diazepam-treated animals showed a trend toward shorter RRLs during the last 14 days of treatment, only one animal showed a reduction in RRL difference over time that was statistically significant ($p < 0.05$; Fig. 1C). None of the vehicle-treated animals showed significant changes in RRL over time, similar to previous studies (25,27).

The average firing rate for MVN neurons in slices from vehicle-treated animals (control slices) was 13.0 ± 5.3 (SD) spikes/s ($n = 15$ neurons), which was similar to the average firing rate that we have recorded for MVN neurons in slices from uninjected animals in previous studies [e.g., 13.7 ± 11.7 spikes/s, $n = 68$; (2)]. The average firing rate for MVN neurons in slices from diazepam-treated animals was significantly higher than for control slices (29.7 ± 19.9 spikes/s, $n = 18$, $p < 0.005$, unpaired t-test). When the average firing rate was calculated for each slice from each animal, similar overall means for the vehicle- and diazepam-treated groups were obtained (13.0 ± 1.2 and 27.5 ± 10.8 spikes/s, respectively). The distributions of the firing rates for neurons in the two groups of slices were also significantly different ($p < 0.001$, χ^2 test; Fig. 2). The firing rates of MVN neurons from the animal that showed behavioral tolerance to diazepam and MVN neurons from diazepam-treated animals that did not exhibit tolerance, were similar.

DISCUSSION

The present study demonstrated that, for the majority of guinea pigs, the administration of 2 mg/kg/day diazepam IP for 28 days did not result in the development of tolerance to the ataxic effects of diazepam on the righting reflex. Only three out of nine diazepam-treated animals showed any indication of tolerance, and in only one case was there a statistically significant reduction in RRL. These data are consistent with previous evidence that tolerance is difficult to induce with single daily injections of low doses (i.e., <3 mg/kg) of diazepam (1). It is possible that the individual differences in the

chronic effects of diazepam were due in part to pharmacokinetic factors; however, it has been suggested that different animals can have different sensitivities to the effects of BDZs on the CNS (8). We believe that it is unlikely that tolerance was not observed in most cases because RRL is an unreliable measure of ataxia; our previous studies have shown that, where tolerance to the ataxic effects of diazepam does develop, the RRL reflects this adaptation (25,27). Statistical analysis was restricted to the RRL data for the first 28 days of treatment (see the Method section); however, of the four diazepam-treated animals in which RRL was measured beyond day 28, only one showed any further decrease in RRL between day 28 and day 42. Therefore, for the majority of animals in which tolerance did not develop by day 28, no further signs of tolerance were observed with longer periods of treatment with 2 mg/kg/day diazepam.

The present study also demonstrated that treatment with 2 mg/kg/day diazepam for up to 60 days caused a large and significant increase in the average firing rate of MVN neurons in vitro (Fig. 2). The significance of this finding is unclear at present. Although pharmacokinetic data for the metabolism of diazepam in guinea pig are limited, the available data (14) suggest that the elimination half-life is approximately 2.4 h; therefore, it is possible that the MVN neuronal activity recorded in the present experiment was related to diazepam withdrawal. Nonetheless, these data suggest that long-term diazepam administration may produce adaptive changes in MVN neurons that result in the development of hyperactivity. These neuronal changes appear to develop irrespective of the development of behavioral tolerance and are of special interest considering the low diazepam dose used. Many previous studies describing neural changes induced by long-term BDZ administration have used high-dose regimes that bear little relationship to doses used in humans [e.g., 100–150 mg/kg/day flurazepam (32)]. However, some previous studies have reported that withdrawal syndromes can be induced with 2 mg/kg/day or less of diazepam (5), lorazepam (19,22), alprazolam (10,16) or clonazepam (23,24). In the present experiment, 24–26 h following the final diazepam injection, animals displayed behavioral hyperactivity similar to that observed in previous studies of diazepam withdrawal (5). Given the short half-life for diazepam in guinea pig (14), the possibility must also be considered that animals were experiencing some withdrawal symptoms between the daily diazepam injections, which were spaced 24 h apart ($\pm$ 2 h); this might account for some of the RRL variability between animals.

The duration of BDZ administration may be a critical factor in the development of neuronal hyperactivity within the MVN. In a previous study using guinea pigs (25), the majority of animals received a single, daily IP injection of 5 mg/kg diazepam for 3–4 weeks; however, no significant changes in MVN neuron resting activity in vitro were observed. Consequently, when using single, daily IP injections, administration of diazepam for longer than 4 weeks may be necessary to induce neuronal hyperactivity within the MVN. Future studies will need to address the mechanism(s) responsible for the hyperactivity of MVN neurons following long-term diazepam administration, by evaluating changes in their sensitivity to excitatory and inhibitory amino acid neurotransmitters, GABA in particular (9).

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REFERENCES

1. de Angelis, L.; File, S. E. Acute and chronic effects of three benzodiazepines in the social interaction anxiety test in mice. Psychopharmacology (Berlin) 64:127–129; 1979.
2. Darlington, C. L.; Smith, P. F.; Hubbard, J. I. Guinea pig medial vestibular nucleus neurons in vitro respond to ACTH-(4–10) at picomolar concentrations. Exp. Brain Res. 82:637–640; 1990.
3. Davis, M.; Gallager, D. W. Continuous slow release of low levels of diazepam produces tolerance to its depressant and anxiolytic effects on the startle reflex. Eur. J. Pharmacol. 150:23–33; 1988.
4. Dingwall, B.; Reeve, B.; Hutchinson, M.; Smith, P. F.; Darlington, C. L. The tolerometer: A fast, automated method for the measurement of righting reflex latency in chronic drug studies. J. Neurosci. Methods 48:111–114; 1993.
5. Eisenberg, R. M. Diazepam withdrawal as demonstrated by changes in plasma corticosterone: A role for the hippocampus. Life Sci. 40:817–825; 1987.
6. File, S. E. Rapid development of tolerance to the sedative effects of lorazepam and triazolam in rats. Psychopharmacology (Berlin) 73:240–245; 1981.
7. File, S. E. Recovery from lorazepam tolerance and the effects of a benzodiazepine antagonist (Ro 15-1788) on the development of tolerance. Psychopharmacology (Berlin) 77:284–288; 1982.
8. File, S. E.; Andrews, N.; Wu, P. Y.; Zharkovsky, A.; Zangrossi, H. Modification of chlordiazepoxide's behavioural and neurochemical effects by handling and plus-maze experience. Eur. J. Pharmacol. 218:9–14; 1992.
9. Gallager, D. W.; Lakoski, J. M.; Gonsalves, S. F.; Rauch, S. L. Chronic benzodiazepine treatment decreases postsynaptic GABA sensitivity. Nature 308:74–76; 1984.
10. Galpern, W. R.; Miller, L. G.; Greenblatt, D. J.; Shader, R. I. Differential effects of chronic lorazepam and alprazolam on benzodiazepine binding and $GABA_A$-receptor function. Br. J. Pharmacol. 101:839–842; 1990.
11. Gonsalves, S. F.; Gallager, D. W. Time course for development of anticonvulsant tolerance and GABAergic subsensitivity after chronic diazepam. Brain Res. 405:94–99; 1987.
12. Harro, J.; Lang, A.; Vasar, E. Long-term diazepam treatment produces changes in cholecystokinin receptor binding in rat brain. Eur. J. Pharmacol. 180:77–83; 1990.
13. Hironaka, T.; Fuchino, K.; Fujii, T. Absorption of diazepam and its transfer through the blood–brain barrier after intraperitoneal administration in the rat. J. Pharmacol. Exp. Ther. 229:809–815; 1984.
14. Klotz, U.; Antonin, K. H.; Bieck, P. R. Pharmacokinetics and plasma binding of diazepam in man, dog, rabbit, guinea pig and rat. J. Pharmacol. Exp. Ther. 199:67–73; 1976.
15. Lister, R. G.; File, S. E.; Greenblatt, D. J. Functional tolerance to lorazepam in the rat. Psychopharmacology (Berlin) 81:292–294; 1983.
16. Lopez, F.; Miller, L. G.; Greenblatt, D. J.; Chesley, S.; Schatzki, A.; Shader, R. I. Chronic administration of benzodiazepines. V. Rapid onset of behavioral and neurochemical alterations after discontinuation of alprazolam. Neuropharmacology 29:237–241; 1990.
17. Mana, M. J.; Kwon Kim, C.; Pinel, J. P. J. Tolerance to the anticonvulsant effects of carbamazepine, diazepam and sodium valproate in kindled rats. Pharmacol. Biochem. Behav. 41:109–113; 1991.

18. Matsubara, K.; Matsushita, A. Changes in ambulatory activities and muscle relaxation in rats after repeated doses of diazepam. Psychopharmacology (Berlin) 77:279–283; 1982.
19. Miller, L. G.; Greenblatt, D. J.; Beth Roy, R.; Summer, W. R.; Shader, R. I. Chronic benzodiazepine administration II. Discontinuation syndrome is associated with upregulation of γ-aminobutyric $acid_A$ receptor complex binding and function. J. Pharmacol. Exp. Ther. 246:177–181; 1988.
20. Padoan, S.; Korttila, K.; Magnusson, M.; Pyykko, I.; Schalen, L. Reduction of gain and time constant of vestibulo-ocular reflex in man induced by diazepam and thiopental. J. Vestib. Res. 1:97–104; 1990.
21. Rosenberg, H. C.; Chiu, T. H. Nature of functional tolerance produced by chronic flurazepam treatment in the cat. Eur. J. Pharmacol. 81:357–365; 1982.
22. Schatzki, A.; Lopez, F.; Greenblatt, D. J.; Shader, R. I.; Miller, L. G. Lorazepam discontinuation promotes "inverse agonist" effects of benzodiazepines. Br. J. Pharmacol. 98:451–454; 1989.
23. Scherkl, R.; Frey, H.-H. Physical dependence on clonazepam in dogs. Pharmacology 32:18–24; 1986.
24. Scherkl, R.; Scheuler, W.; Frey, H.-H. Anticonvulsant effect of clonazepam in the dog: Development of tolerance and physical dependence. Arch. Int. Pharmacodyn. 278:249–260; 1985.
25. Scott, S. J.; Smith, P. F.; Darlington, C. L. Quantification of the depressive effects of diazepam on the guinea pig righting reflex. Pharmacol. Biochem. Behav. 47:739–741; 1994.
26. Smith, P. F.; Darlington, C. L. Neurochemical mechanisms of recovery from peripheral vestibular lesions (vestibular compensation). Brain Res. Rev. 16:117–133; 1991.
27. Smith, P. F.; Darlington, C. L. Rapid tolerance to the depressive effects of diazepam on guinea pig motor control using divided doses. Pharmacol. Biochem. Behav. 48:535–538; 1994.
28. Smith, P. F.; Darlington, C. L.; Hubbard, J. I. Evidence for inhibitory amino acid receptors on guinea pig medial vestibular nucleus neurons in vitro. Neurosci. Lett. 121:244–246; 1991.
29. Snedecor, G. W.; Cochran, W. G. Statistical methods, 8th ed. Ames, IA: Iowa State University Press; 1989.
30. Wilson, V. J.; Melvill Jones, G. Mammalian vestibular physiology. New York: Plenum Press; 1979.
31. Woods, J. H.; Katz, J. L.; Winger, G. Benzodiazepines: Use, abuse and consequences. Pharmacol. Rev. 44:151–347; 1992.
32. Yu, O.; Chiu, T. H.; Rosenberg, H. C. Modulation of GABA-gated chloride ion flux in rat brain by acute and chronic benzodiazepine administration. J. Pharmacol. Exp. Ther. 246:107–113; 1988.