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Localized injections of midazolam into the amygdala and hippocampus induce differential changes in anxiolytic-like motor activity in mice

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Abstract

Various strains of mice display a reliable increase in motor activity in response to benzodiazepines given at low to moderate doses. This hyperactivity has been described as both an anxiolytic-associated increase in exploratory activity and a nonspecific stimulant effect controlled by central neural mechanisms separate from those involved in the anxiolytic-like effects. The purpose of the current study was to investigate the neural circuitry underlying the hyperactivity effects of benzodiazepines in mice. Specifically, we examined the relationship between anxiety and motor activity after bilateral intra-amygdala or intra-hippocampal microinjections of the nonselective full benzodiazepine receptor agonist midazolam in C57BL/6 mice. Behavioral measures of anxiety and motor activity in open field were examined in mice given localized injections of 0, 2, 8 or 32 nmol of midazolam directed into the amygdala or hippocampus. Midazolam injected into the amygdala at the low dose produced an anxiolytic-like effect, as reflected by an increase in central open field activity. Higher doses injected into the amygdala produced a motor-depressant action, indicative of a drug-induced sedative effect. Infusions into the hippocampus produced a biphasic effect on motor activity with the two lower doses of midazolam producing a motor-stimulant action and the high dose producing a motor-depressant effect. Hippocampus injections produced no anxiolytic-like effects. The current findings demonstrate that injections of midazolam produced a regional dissociation of the anxiety-related and motor-related parameters and provide evidence that the stimulant and anxiolytic effects of benzodiazepines are independent phenomena regulated by different central mechanisms.

Keywords

amygdala; anxiolytic; benzodiazepine; hippocampus; locomotor activity; mouse

Introduction

Gamma-amino butyric acid (GABA) is the major inhibitory neurotransmitter in the brain. Benzodiazepines (BZs) enhance the inhibitory effects of GABA at GABA(A) receptors and are renowned for their anxiolytic capacity in humans and a number of rodent models of anxiety. In addition to their anxiolytic capacity, BZs also produce sedative/motor impairing effects (Prut and Belzung, 2003). In general, past studies that have used systemic injections of diazepam in rats indicate that the anxiolytic-like effects of BZ agonists occur at low to moderate doses (e.g. 0.5–5mg/kg), whereas high doses (e.g. >10 mg/kg) reliably decrease motor activity,

which is a commonly used index of the sedative effects of various drugs (Vogel and Vogel, 2002). Unlike rats, however, various strains of mice also display a reliable increase in motor activity in response to diazepam given at low to moderate doses (Dawson *et al.*, 1995; Kralic *et al.*, 2002; Prut and Belzung, 2003; Mi *et al.*, 2005). The hyperactivity seen in mice has often been described as an anxiolytic-associated increase in exploratory activity. Others, however, suggest that the stimulant effects of BZs are nonspecific and are caused by central neural mechanisms separate from those involved in the anxiolytic-like effects (Soderpalm *et al.*, 1991; Dawson *et al.*, 1995; Phillips and Shen, 1996). At present, the neural circuitry underlying the hyperactivity effects of BZs in mice is unknown.

Important insights into the neural mechanisms underlying the behavioral effects of BZs have been obtained by exploring the consequences of local injections into various brain structures (Menard and Treit, 1999). Owing in part to the strong evidence implicating their roles in anxiety and memory disorders, the amygdala and hippocampus have been primary investigative sites for the actions of BZs (Hevers and Luddens, 1998; Teuber *et al.*, 1999; Smith, 2001). Using rats, numerous studies have demonstrated the anxiolytic-like effects of locally administered BZ receptor ligands in the amygdala (Petersen *et al.*, 1985; Hodges *et al.*, 1987; Shibata *et al.*, 1989; Pesold and Treit, 1995; Zangrossi *et al.*, 1999) and hippocampus (Gonzalez *et al.*, 1998; Nazar *et al.*, 1999; Bailey *et al.*, 2002).

The purpose of the current study was to investigate the neural structures involved in the hyperactivity effects of BZs in mice. Specifically, we examined the relationship between anxiety and motor activity after bilateral intra-amygdala or intra-hippocampal microinjections of the nonselective full BZ receptor agonist midazolam (MDZ) in C57BL/6 mice.

Methods

Subjects

Adult (8–12 weeks of age) male C57BL/6J mice weighing 22–28 g (Jackson Laboratories, Bar Harbor, Maine, USA) were used in this study. C57BL/6 mice were selected for this study because this strain is the most widely used inbred strain in behavioral research and is commonly used as a background strain for the development of genetic knockout mice (e.g. Anagnostopoulos *et al.*, 2001; Belzung and Griebel, 2001; Bothe *et al.*, 2004).

Mice were housed in standard group cages (fewer than six per cage) and were given free access to food and water on a 12-h light/dark cycle (light on from 07.00 h). All experiments were performed during the light cycle and were approved by Emory University Institutional Review Board following principles and standards of animal care outlined by the NIH Institutional Animal Care and Use Committee (National Research Council, 1996). All efforts were made to minimize animal suffering and to reduce the number of animals used. Each animal was tested once.

Surgery

Mice were implanted with a single 6-mm stainless-steel guide cannula (26 gauge; Plastics One, Roanoke, Virginia, USA) under ketamine–medetomidine anesthesia (ketamine: 80 mg/kg; medetomidine: 1.0mg/kg). The guide cannula was fixed to the skull using dental acrylic and jeweler's screws. The following stereotaxic coordinates from bregma were used: amygdala; anteroposterior (AP)= − 1.4, mediolateral (ML)= ±3.5, dorsoventral (DV)= − 5.1; hippocampus; AP= − 1.6, ML= ±1.7, DV= − 2.5. Dorsoventral coordinates were measured from the skull surface with the internal cannula extending 2mm beyond the end of the guide cannula. Coordinates were based on the mouse brain atlas of Paxinos and Franklin (2001).

After surgery, mice were given post-surgical intraperitoneal injections of Antisedan 4.0 mg/kg; medetomidine reversing agent) and placed on a heated pad. After recovery from anesthesia, mice were given a narcotic analgesic (buprenorphine, 0.05 mg/kg, subcutaneous) and returned to home cages for 6 days of recovery before testing. During recovery, body weight, eating, and drinking were monitored daily. Mice were also handled daily for acclimation and inspection of cannula fixture. Of the 74 mice used in the study, 37 received cannula placement directed at the basolateral complex of the amygdala (AMYG) and 37 directed at the dorsal hippocampus (HIPPO).

Activity chambers

The effects of MDZ on spontaneous locomotor activity was measured using individual activity chambers (27.9 × 27.9 cm), constructed from clear polycarbonate and equipped with three 16-beam infrared arrays at the base of each chamber wall (MED Associates Inc., Model, OFAMS, St. Albans, Vermont, USA).

General procedure

Six days after surgery, mice were individually transported to the experimental room in standard cages and left undisturbed for 5 min before testing. Mice were gently restrained and received injections of either phosphate-buffered saline (PBS) vehicle (AMYG, $n=10$; HIPPO, $n=10$), 2 nmol of MDZ (AMYG, $n=9$; HIPPO, $n=9$), 8 nmol of MDZ (AMYG, $n=9$; HIPPO, $n=9$), or 32nmol of MDZ (AMYG, $n=9$; HIPPO, $n=9$). Injections were administered using an injection cannula (33 gauge cannula; Plastics One), which extended 2.0mm beyond the tip of the guide cannula. Pilot data indicated that the likelihood of hippocampal and amygdala damage was reduced when the injection cannula extended 2.0mm as opposed to a shorter extension (e.g. 0.5–1.0 mm). Drug was delivered manually with a 5- μ l Hamilton microsyringe attached to the injection cannula via polyethylene tubing (PE-10) and administration of a volume of 0.4 μ l/side was delivered over a period of 60 s by slowly turning the microsyringe plunger. Using the cannula system designed for mice (Plastics One), this manual delivery method resulted in more reliable delivery of the drug than attempts using an automated pump. The confirmation of successful infusion was obtained by monitoring the movement of a small air bubble in the tubing. After each infusion, the injection cannula was allowed to remain for 60 s. Mice were then placed back into a holding cage for 5 min to reduce stress associated with the injection procedure. Mice were then placed into activity chambers for a 30-min session. Between subjects, the chambers were thoroughly cleaned with a 50% ethanol–water mixture and allowed to dry for 15 min in the ventilated testing room.

At the end of the experiments, the animals were anesthetized with ketamine–metomidine, and 0.4 μ l of 1% Evan's blue dye was microinjected bilaterally into the brain according to the above-described procedure as a marker of the injection site. Five minutes after dye injections, mice were decapitated, and the brains were removed, frozen in dry ice, and stored at -80°C . Serial coronal sections (40 μ m) of brains were cut on a cryostat (Leica, Nussloch, Germany) at -20°C and mounted on gelatin-coated slides.

Behavioral analysis

The effects of MDZ on motor behavior were measured by examining the total ambulatory distance during the 30-min test session. In rodents, drug-induced changes in both the ambulatory distance and the counts represent a standard behavioral assay for testing the motor effects of drugs (Vogel and Vogel, 2002). The anxiolytic-like effects were evaluated by computing percentage of time mice spent in the central zone of the open field. An increase in the percentage of central zone time is indicative of an anxiolytic-like effect and best takes into account potential confounding changes in locomotive activity (Prut and Belzung, 2003). The central zone was defined as the central compartment of the floor centrally located 6 cm from

the perimeter of the chamber walls. All testing was conducted under standard room lighting. Data were analyzed with Activity Monitor software (MED Associates Inc.). Data were analyzed with two-way analyses of variance (ANOVAs) with dose (vehicle, 2, 8, 32 nmol) and location (AMYG, HIP) as between-subject variables. Significant main effects and interactions were evaluated with one-way ANOVAs, simple contrasts, and independent sample *t*-test with Bonferroni corrections. Analyses of total ambulatory distance (locomotion) and total ambulatory counts revealed near identical results; thus, only ambulatory distance was reported.

Drugs

The nonselective full BZ receptor agonist MDZ (Sigma Chemical, St Louis, Missouri, USA) was selected from available BZs because of its superior solubility and its common use in intracranial infusion studies. Previous rodent studies have reported anxiolytic effects using intraperitoneal MDZ administration (0.5–10 mg/kg; e.g. Pandossio and Brandao, 1999; Schenberg *et al.*, 2001; Carvalho *et al.*, 2005) and localized injections of MDZ into the hippocampus and amygdala (0.1–40 µg/side; for a review, see Menard and Treit, 1999). MDZ was dissolved in PBS verified to be within the normal physiologic pH levels (7.3–7.4). Mice received microinjections of either PBS vehicle alone (VEH), or different doses of MDZ (2, 8, or 32nmol, approximately 0.4–10 µg) at a volume of 0.4 µl/side. The doses and volume used were chosen on the basis of previous studies that have obtained behavioral effects in mice (Nunes-de-Souza *et al.*, 2000) and rats (Maciejak *et al.*, 2002; Yasoshima and Yamamoto, 2005).

Results

Histology

Of the 74 mice that received injections, five were excluded from statistical analysis because of extensive necrosis extending from the tip of the guide cannula or misplacement (AMYG, *n*=34; HIP, *n*=35). Inspection of the remaining AMYG brain sections revealed that dye injections were centered within the lateral and basolateral nuclei of the amygdala. In five AMYG brain sections, dye was also evident in adjacent areas including either the basomedial or central nuclei of the amygdala, perirhinal cortex, or caudate putamen. Dye in the HIP brain sections of animals was restricted to the dorsal hippocampus with highest concentration of staining in the dentate gyrus; however, weak staining in some mice was also seen in cortical tissue dorsal to the injection site. An evaluation of the standardized residuals by group revealed that the dependent variables of animals with spread of dye to collateral areas did not differ substantially from their respective group means (within-cells *z*s, <2.0). Representative photomicrographs of the dye-injected brain sections showing the typical site and spread of microinjections are seen in Fig. 1.

Total ambulatory distance

The overall ANOVA analysis revealed significant between- subject effects of injection location [$F(1,61)=28.92$, $P<0.01$] and MDZ dose [$F(3,61)=15.04$, $P<0.01$]. The location $\times$ dose interaction was also significant [$F(3,61)=14.88$, $P<0.01$], indicating that mice in the HIP and AMYG groups responded differently across dose levels.

To examine the location $\times$ dose interaction, AMYG and HIP groups were analyzed separately with one-way ANOVAs to compare responding across levels of dose. For the mice in the AMYG group, this analysis showed a reliable dose effect [$F(3,30)=10.70$, $P<0.01$]. A priori contrasts revealed no difference between vehicle and low dose of MDZ (2 nmol) [$t(30)=1.31$, NS]. Both medium and high doses (8 and 32 nmol), however, produced significantly decreased activity counts [$t(30)>3.41$, $P<0.01$]. As illustrated in Fig. 2, polynomial contrasts supported a linear dose–response effect of AMYG activity [$F(1,30)=32.05$, $P<0.01$]. The analysis of the

HIPP group also revealed a significant dose effect [$F(3,31)=10.06$, $P<0.01$]. Contrasts showed that MDZ injections on locomotor low and medium doses of MDZ injected into the hippocampus produced significant increases in activity when compared with vehicle animals [$t(31)>2.18$, $P<0.05$]. In contrast, mice that received the high dose of MDZ displayed less activity than vehicle animals [$t(31)=2.5$, $P<0.02$]. As illustrated in Fig. 2, polynomial contrasts supported a biphasic dose–response effect of HIPP MDZ injections on locomotor activity [$F(1,31)=26.60$, $P<0.01$].

Percentage of time in central zone

Time in the central zone compared with total time in the open-field apparatus was used as a measure of anxiolysis (Fig. 3). For percentage of time in the central zone, the overall analysis indicated reliable main effect of dose [$F(3,61)=15.35$, $P<0.01$] and a significant location $\times$ dose interaction [$F(3,61)=3.81$, $P<0.02$], indicating a differential effect of dose level in HIPP and AMYG groups of mice. Separate one-way ANOVAs comparing responding across levels of dose revealed significant effects for both AMYG and HIPP groups [$F(3,30)=24.88$, $P<0.01$ and $F(3,31)=3.77$, $P<0.02$, respectively]. For mice receiving amygdala injections, contrasts showed that mice that received the low dose of MDZ spent significantly more time in the central zone than vehicle controls [$t(30)=2.27$, $P<0.04$], indicative of an anxiolytic-like effect. Both medium and high doses of MDZ resulted in a decrease in central zone time [$t(30)<2.87$, $P<0.02$]. For mice receiving hippocampal injections, the high dose of MDZ resulted in a decrease in central zone time [$t(31)=2.30$, $P<0.02$]. No significant effect was seen at other dose levels.

Dissociation between amygdala and hippocampal effects

For the 2 nmol dose of MDZ, we evaluated behavior differences between AMYG and HIPP animals by performing independent sample t -test with Bonferroni corrections. For locomotor activity, this analysis revealed that hippocampal injected animals showed significantly more activity than animals with amygdala-specific injections [$t(14)=3.43$, $P<0.05$]. In contrast, amygdala injected animals spent significantly more time in the central zone than those receiving hippocampal injections [$t(14)=3.16$, $P<0.05$]. Figure 4 illustrates the locomotor and anxiolytic-like effects of localized amygdala or hippocampal injections of 2 nmol MDZ as a percentage of their respective vehicle animals.

Discussion

In the current study, we find differential activity and anxiolytic-like effects of the BZ MDZ injections directed at the dorsal hippocampal versus amygdala. MDZ injected into the basolateral complex of the amygdala produced a dose-dependent decrease in motor activity. Infusion into the hippocampus, however, had a biphasic effect on motor activity. The two lower doses of MDZ (2 and 8 nmol) produced a motor-stimulant action, as reflected by an increase in total motor activity. In contrast, injection of the high dose (32 nmol) produced a motor-depressant effect. An evaluation of the central zone activity in the open field revealed that only the low dose of MDZ injected into the amygdala produced an anxiolytic-like effect, as reflected by an increase in the percentage of time in the central zone.

The current findings demonstrate that bilateral injections of MDZ produced a regional dissociation of the anxiety-related and motor-related parameters at the low dose (2nmol). Application of MDZ into the amygdala increased percentage of central time without effect on locomotion activity. In contrast, injections into the hippocampus increased locomotion with no resulting effect on percentage of central time. These results provide evidence that in mice, the stimulant and anxiolytic-like effects of BZ in the open field may be independent phenomena regulated by different central mechanisms (Soderpalm *et al.*, 1991; Dawson *et al.*, 1995;

Phillips and Shen, 1996). Injection of higher doses, however, reduced locomotion and central time activity at both sites. The decrease in central time is likely secondary to the sedative/motor-depressant effects of high MDZ doses rather than an index of anxiogenic properties (Vogel and Vogel, 2002).

In rats, the anxiolytic-like effects of BZs infused into the amygdala have been demonstrated on a number of animal models of anxiety, including the elevated-plus maze (Green and Vale, 1992; Pesold and Treit, 1995), social interaction tests (Gonzalez *et al.*, 1996), conflict tests (Petersen *et al.*, 1985; Hodges *et al.*, 1987; Shibata *et al.*, 1989), defensive freezing (Helmstetter, 1993), fear induced analgesia (Fanselow and Kim, 1992), and conditioned avoidance (Harris and Westbrook, 1995). Using the elevated plus maze, Nunes-de-Souza *et al.* (2000) have also demonstrated anxiolytic-like effects after MDZ injections in the amygdala of Swiss albino mice. On the other hand, microinjections of BZ antagonists and inverse agonists into the amygdala induce anxiogenic-like behaviors (Hodges *et al.*, 1987; Da Cunha *et al.*, 1992), and infusions of antagonists can also prevent the anxiolytic-like effects of peripheral injections of BZ agonists (Petersen *et al.*, 1985; Sanders and Shekhar, 1995). While some evidence indicates that separable aspects of the anxiolytic-like effects of BZs involve different amygdala nuclei (Pesold and Treit, 1995), experiments that have used localized injections into either the central or basolateral nuclei of the amygdala have both shown stronger anxiolytic-like effects of BZs infused into the lateral-basolateral complex than into the central nucleus (Green and Vale, 1992; Pesold and Treit, 1995; Silva and Tomaz, 1995; Da Cunha *et al.*, 1999; Menard and Treit, 1999; McGaugh *et al.*, 2002). In the current study, histological analysis of microinjection sites indicated infusions were directed into the basolateral complex, but we cannot exclude the possible spread of MDZ to surrounding areas, including the central nucleus.

Injections of MDZ into the dorsal hippocampus in rats have been shown to produce an anxiolytic-like effect in the open field test (Stefanski *et al.*, 1993; Nazar *et al.*, 1999) and Vogel conflict test (Stefanski *et al.*, 1993; Plaznik *et al.*, 1994; Nazar *et al.*, 1999). The effective dose on these behavioral tests, however, is markedly different. In the open field, relatively low concentrations of MDZ (0.1 μ g) increase central time. In contrast, higher MDZ concentrations (10 μ g) disinhibit conflict behavior in the Vogel test. The lack of an anxiolytic effect in our study may be due to the higher dose range (2–32 nmol, approximately 0.4–10 μ g) when compared with past experiments. Interestingly, the dose of MDZ that produces an anxiolytic-like effect in the Vogel conflict test also significantly inhibits locomotor activity in the open field (Stefanski *et al.*, 1993; Nazar *et al.*, 1999). This latter finding is consistent with the sedative effect of hippocampal injections seen in this study.

Systemic administration of BZ agonists at high doses is known to decrease motor activity in rodents; however, relatively few studies have explicitly examined the role of hippocampal and amygdala BZ receptors on motor activity. In the amygdala, microinjection of drugs that inhibit GABA(A) receptor function increase locomotor activity (Turski *et al.*, 1985; Sanders and Shekhar, 1991), whereas GABA(A) receptor agonists have been shown to both increase (Helmstetter, 1993; Muller *et al.*, 1997) and decrease (Zarrindast *et al.*, 2004) locomotor activity. The current results clearly revealed a dose-dependent reduction after injections of MDZ into the amygdala. This effect may be due to direct projections from the amygdala to brainstem nuclei known to be involved in arousal and sleep control (Morrison *et al.*, 2000; Sanford *et al.*, 2002). With regard to the hippocampus, our results and past studies demonstrate that MDZ significantly inhibits locomotion in the open field at relatively high drug concentrations, that is, 20–30 nmol (Stefanski *et al.*, 1993; Nazar *et al.*, 1999).

Our data show that MDZ stimulated open field activity when injected at lower concentrations into the hippocampus. In mice, the increases in locomotor activity seen after low systemic doses may reflect the action of BZs on GABAergic circuits in the hippocampus. In rats,

increases in activity have been reported after or intra-hippocampal application of a number of drugs that potentially alter local GABA function. For example, pharmacological stimulation of the hippocampus by the GABA antagonist picrotoxin reliably induced locomotor hyperactivity (Flicker and Geyer, 1982; Plaznik *et al.*, 1989; Nazar *et al.*, 1999), possibly via direct stimulation of dopamine release in the nucleus accumbens (Bast and Feldon, 2003). Hyperactivity, however, has also been observed following local dorsal hippocampus injections of muscimol, the Na⁺ channel blocker tetrodotoxin, and the *N*-methyl-D-aspartate antagonist MK-801 (Zhang *et al.*, 2000; Bast and Feldon, 2003). The pattern of hyperactivity seen in the latter studies has been attributed to a deficit in habituation to the environment, consistent with the detrimental effects of dorsal hippocampal pharmacological inhibition and *N*-methyl-D-aspartate receptor blockade on spatial and contextual memory (Bast and Feldon, 2003). Given the variety of effects seen in rats, it is unclear whether our results represent a novel effect seen in both mice and rats or reflect a subtle genetic and/or phenotype difference between these two species.

To date, the results of studies in mice are most often interpreted on the basis of findings in rats. Researchers have an increasing awareness, however, that anatomical and behavioral differences between these two species may complicate and limit the use of rat studies to interpret findings in mice (Jardim *et al.*, 1999; Blanchard *et al.*, 2001; Asan *et al.*, 2005). Surprisingly, few studies have explicitly examined the effect of centrally administered BZ receptor ligands in mice. Given the growing use of genetically altered mice lines as a strategy for understanding GABA(A) function, this line of research will benefit from the continued investigation of the neural circuits underlying normal and drug-induced behaviors in mutated and normal mice.

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Fig. 1. Photomicrographs of coronal mouse brain sections and corresponding atlas plate (Paxinos and Franklin, 2001) indicating the typical site and spread of microinjections as assessed by stain injections. All doses of midazolam were injected at a volume of 0.4 μ l into the amygdala (a) or hippocampus (b).

Fig. 2.

Effects of intra-amygdala (AMYG) and intra-hippocampal (HIPP) microinjections of midazolam (MDZ) on spontaneous locomotor activity. Activity was assessed for 30 min in the open field 5 min after bilateral injections of vehicle (VEH), 2, 8, or 32 nmol of MDZ. All data are presented as means $\pm$ standard error of the mean. Asterisks indicate a significant difference from VEH.

Fig. 3. Effects of intra-amygdala (AMYG) and intra-hippocampal (HIPP) microinjections of midazolam (MDZ) on anxiety as assessed by percent central zone time. Central zone time was assessed for 30 min in the open field 5 min after bilateral injections of vehicle (VEH), 2, 8, or 32 nmol of MDZ. All data are presented as means $\pm$ standard error of the mean. Asterisks indicate a significant difference from VEH.

Fig. 4.

Comparisons of the locomotor and anxiolytic-like behaviors in mice given either intra-amygdala (AMYG) or intra-hippocampal (HIPP) microinjections of 2 nmol midazolam (MDZ). For ease of presentation, both total ambulatory distance (Locomotion) and anxiolytic-like effects (% Central) are shown as a percentage of their respective vehicle animals.