Published in final edited form as:

Anesth Analg. 2014 June ; 118(6): 1293–1300. doi:10.1213/ANE.0000000000000201.

Benzodiazepine Site Agonists Differentially Alter Acetylcholine Release in Rat Amygdala

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Abstract

Background—Agonist binding at the benzodiazepine site of γ -aminobutyric acid type A receptors diminishes anxiety and insomnia by actions in the amygdala. The neurochemical effects of benzodiazepine-site agonists remain incompletely understood. Cholinergic neurotransmission modulates amygdala function, and in this study we tested the hypothesis that benzodiazepine-site agonists alter acetylcholine (ACh) release in the amygdala.

Methods—Microdialysis and high performance liquid chromatography quantified ACh release in the amygdala of Sprague-Dawley rats ($n=33$). ACh was measured before and after IV administration (3 mg/kg) of midazolam or eszopiclone, with and without anesthesia. ACh in isoflurane-anesthetized rats during dialysis with Ringer's solution(control) was compared to ACh release during dialysis with Ringer's solution containing (100 μ M) midazolam, diazepam, eszopiclone, or zolpidem.

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Results—In unanesthetized rats, ACh in the amygdala was decreased by IV midazolam (–51.1%; $P=0.0029$; 95% CI= –73.0% to –29.2%) and eszopiclone (–39.6%; $P=0.0222$; 95% CI= –69.8% to –9.3%). In anesthetized rats, ACh in the amygdala was decreased by IV administration of midazolam (–46.2%; $P=0.0041$; 95% CI= –67.9% to –24.5%) and eszopiclone (–34.0%; $P=0.0009$; 95% CI= –44.7% to –23.3%), and increased by amygdala delivery of diazepam (43.2%; $P=0.0434$; 95% CI= 2.1% to 84.3%), and eszopiclone (222.2%; $P=0.0159$; 95% CI= 68.5% to 375.8%).

Conclusions—ACh release in the amygdala was decreased by IV delivery of midazolam and eszopiclone. Dialysis delivery directly into the amygdala caused either increased (eszopiclone and diazepam) or likely no significant change (midazolam and zolpidem) in ACh release. These contrasting effects of delivery route on ACh release support the interpretation that systemically administered midazolam and eszopiclone decrease ACh release in the amygdala by acting on neuronal systems outside of the amygdala.

Introduction

The amygdaloid nuclear complex, located bilaterally in the temporal lobes, contributes to the regulation of fear and anxiety, learning and memory, sleep, and autonomic control.¹⁻³ These traits and behavioral states make amygdaloid function relevant for anesthesia care.⁴⁻⁶ Patients who experience greater anxiety have higher pain scores,⁷ and women who report less anxiety before elective hysterectomy experience less postoperative pain.⁸ Patients who receive midazolam preoperatively have less discomfort during the postoperative period⁹ and premedication with midazolam improves patient satisfaction.¹⁰ In healthy volunteers, sleep disruption enhanced pain perception¹¹ and pain relief provided by level I analgesics¹² can be exceeded by pain relief produced by slow wave sleep.¹³

The amygdaloid nuclear complex is comprised of at least 10 subnuclei.^{14,15} The central nucleus of the amygdala (CeA) contributes to the regulation sleep and wakefulness via cholinergic mechanisms.¹⁶ The benzodiazepine (BZ) site agonist eszopiclone, indicated for treatment of insomnia, decreases acetylcholine (ACh) release in sleep-promoting regions of the pontine reticular formation.¹⁷ These relationships encouraged us to test the two-tailed hypothesis that the BZ site agonists midazolam, diazepam, eszopiclone, and zolpidem alter ACh release in and around the CeA region of the amygdaloid nuclear complex.

Methods

Animals

All procedures using animals were approved by the University of Michigan Committee on Use and Care of Animals, and complied with the *Guide for the Care and Use of Laboratory Animals* (8th Edition, National Academies of Sciences Press, Washington, DC, 2011). Adult, male Sprague-Dawley rats ($n=33$) were purchased from Charles River Laboratories, Inc. (Wilmington, MA). Rats were housed in a temperature-controlled room on a 12 h light:12 h dark cycle with food and water available *ad libitum*.

Quantifying ACh Levels in the Amygdala

High performance liquid chromatography with electrochemical detection (Bioanalytical Systems, West Lafayette, IN) was used to measure ACh release (pmol/12.5 min) in the amygdala. Chromatograms were digitized and quantified using Chromgraph Software (Bioanalytical Systems, West Lafayette, IN) in reference to a 7-point standard curve ranging from 0.05 to 1.0 pmol of ACh. CMA/11 microdialysis probes (CMA Microdialysis, Stockholm, Sweden) had a cuprophane membrane of 1 mm in length and 0.24 mm in diameter, and a molecular cutoff of 6,000 Daltons. The probes were perfused continuously with Ringer's solution (147 mM NaCl, 2.4 mM CaCl₂, 4.0 mM KCl, 10 μM neostigmine, pH 6.0 ± 0.2). Perfusion flow rate was held constant at 2.0 mL/min by a CMA/400 syringe pump (CMA Microdialysis, Stockholm, Sweden). Before and after every experiment, the percent recovery of ACh by each dialysis probe was quantified *in vitro* to ensure that measured changes in ACh were not an artifact of intra-experimental changes in probe membrane function. As described previously¹⁷⁻²¹ the physical characteristics of the CMA/11 probe membranes are such that they delivered approximately 5% of the drug concentration used to perfuse the probes. Thus, for the present study that used a drug concentration of 100 μM, it can be estimated that the concentration of drug delivered by dialysis to the amygdala was about 5 μM.

Experimental Design

All sedative/hypnotics used in this study are BZ site agonists. Midazolam and diazepam are benzodiazepines. Eszopiclone and zolpidem are cyclopyrrolones (i.e., non-benzodiazepines) that bind to the BZ site on γ-aminobutyric acid type A (GABA_A) receptors. Three experimental approaches were used (Fig 1). First, ACh release was measured in the amygdala of unanesthetized rats before and after IV administration of BZ site agonists (Fig. 1A). Second, ACh release was quantified after IV administration of BZ site agonists to rats that were anesthetized with isoflurane (Fig. 1B). Third, ACh release was measured while reverse dialysis was used to deliver BZ site agonists into the amygdala of isoflurane-anesthetized rats (Fig. 1C).

Study 1. Quantifying ACh release in the amygdala of unanesthetized rats before and after intravenous administration of BZ site agonists—

At least one week before the experiment, rats (n=9) were anesthetized with isoflurane and the percent of delivered isoflurane was monitored continuously by spectrometry (CardiCap^{TM/5}, Datex-Ohmeda, Louisville, CO). A rectal thermistor was placed to measure core body temperature, which was maintained between 36°C and 38°C. A chronic jugular-vein catheter (Micro-Renathane tubing, MRE-040, Braintree Scientific, MA) was implanted to provide a route for systemic drug administration. An injection port (8I313000BM10, Plastics One, Roanoke, VA) was attached to the exterior end of the catheter between the scapulae (Fig. 1A) and sealed with a cap (8IC313DCCACC, Plastics One). After placement of the catheter, the rat was positioned in a Kopf Model 962 stereotaxic frame (David Kopf Instruments, Tujunga, CA) with a Kopf model 920 rat adaptor and rat anesthesia mask (Kopf model 906). A craniotomy was performed and a CMA/11 guide cannula was implanted at 2.3 mm posterior to bregma, 4.0 mm lateral to midline, and 3.5 mm above the amygdala (CeA), according to a rat brain atlas.²² Three stainless steel anchor screws (MPX-00800-02-C, Small Parts Inc.,

Miami Lakes, FL) were inserted into the skull. The guide cannula was fixed to the skull with dental acrylic (Lang's Jet Acrylic, Lang Dental Mfg. Co., Wheeling, WV).

During the recovery period, rats were handled daily and conditioned to being placed in a Plexiglas recording chamber (Raturn, Bioanalytical Systems, West Lafayette, IN). Rats had free access to food and water. On the day of the experiment, a microdialysis probe was inserted into the CeA guide cannula. The probe was perfused continuously with Ringer's solution (2 $\mu\text{L}/\text{min}$). A syringe containing either midazolam or eszopiclone was connected to the jugular vein catheter with a 55 cm length of Micro-Renathane tubing. This tubing made it possible to perform drug injections without handling the animals. Rats were kept awake during collection of the first five dialysis samples (12.5 min/sample) before drug administration. This procedure ensured all rats were in the same state of consciousness before systemic drug delivery, and these samples provided control levels of ACh. Next, saline, midazolam (3 mg/kg), or eszopiclone (3 mg/kg) was administered IV at a rate of 200 $\mu\text{L}/\text{min}$ and five additional dialysis samples were collected. At the end of every experiment the microdialysis probe was removed, the connector for the jugular vein catheter connector was disconnected, and the animal was returned to its home cage.

Study 2. Quantifying ACh release in the amygdala of isoflurane-anesthetized rats before and after intravenous administration of BZ site agonists—

Rats ($n=6$) were anesthetized with 4% isoflurane in 100% oxygen. Delivered isoflurane concentration then was reduced to 2.5 %. A catheter (Micro-Renathane tubing, MRE-040, Braintree Scientific, MA) was inserted into one jugular vein (Fig. 1B). After placement of the catheter, the rat was positioned in a stereotaxic frame. A craniotomy was performed and a CMA/11 microdialysis probe was aimed for the CeA (2.3 mm posterior to bregma, 4.0 mm lateral to midline, and 8.5 mm below the skull surface) according to a rat brain atlas.²² After stereotaxic positioning of the microdialysis probe, delivered isoflurane concentration was decreased to 1.5 % and maintained at this level for the remainder of the experiment.

The microdialysis probe was perfused continuously with Ringer's solution (2 $\mu\text{L}/\text{min}$) during the course of sample collection. Five microdialysis samples (each 25 μL) were collected from the amygdala before and after IV administration (200 $\mu\text{L}/\text{min}$) of midazolam (3 mg/kg) and eszopiclone (3 mg/kg). After collection of the last dialysis sample, the jugular vein catheter and the microdialysis probe were removed and all incisions were closed. Isoflurane delivery was discontinued and the animal recovered before being returned to its home cage.

Study 3. Quantifying ACh release in the amygdala during microdialysis delivery of BZ site agonists to the amygdala of isoflurane-anesthetized rats—

Each rat ($n=18$) was anesthetized, placed in a stereotaxic frame, and a microdialysis probe was aimed for the amygdala as described above (Fig. 2B). Five microdialysis samples were collected during dialysis with Ringer's solution (control). A CMA/110 liquid switch was then activated to perfuse the probe with Ringer's solution alone, or Ringer's solution containing midazolam (100 μM , Hospira, Lake Forest, IL), diazepam (100 μM , Sigma-Aldrich, St. Louis, MO), eszopiclone (100 μM , Toronto Research Chemicals, Toronto, Canada), zolpidem (100 μM , Sigma-Aldrich, St. Louis, MO), or tetrodotoxin (TTX, 1 μM).

Five microdialysis samples were obtained during dialysis administration of BZ site agonists. This within-subjects design was used to test only one drug per rat (Fig. 2C). After collection of the last sample, the dialysis probe was removed from the brain, the scalp was closed, and delivery of isoflurane was discontinued.

Histological Confirmation of Dialysis Sites

Three to seven days after each experiment, rats were deeply anesthetized and decapitated. Brains were removed, frozen, and sectioned coronally at a thickness of 40 μm . Serial sections were slide-mounted, dried, fixed with paraformaldehyde vapor at 80°C, and stained with cresyl violet. Tissue sections were compared to a rat brain atlas²² in order to localize microdialysis sites.

Statistical Analyses

In consultation with a statistician at the University of Michigan Center for Statistical Consultation and Research, data were analyzed with Statistical Analysis System v9.3, (SAS Institute, Inc., Cary, NC) software. First, each animal's ACh values were calculated as percent change from the mean of the animal's control values. These values appeared to be relatively symmetrically distributed and required no further transformation. Second, a linear mixed model was fitted in which the percentage ACh release was the dependent variable and treatment (control versus drug) was used as the independent variable. A random effect (i.e., random intercept) was also included for each animal to consider the fact that each animal provided multiple, possibly correlated ACh samples for each treatment. A P-value < 0.05 was considered statistically significant. Residuals were assessed for normality. Figures are shown as mean percent change + SD. Results of statistical tests comparing the effects on ACh release of each drug to its control are reported with mean percentage difference, P-value, and 95% confidence interval (CI). These CIs consider the correlation among observations on the same animal.

Results

For each animal, measures of ACh were included in the analyses only if histological results (Fig. 2) confirmed that those measures were obtained from the amygdaloid nuclear complex. Figure 2A shows coronal brain sections that were modified from an atlas²² by adding colored bars to indicate the location of each dialysis membrane. A more detailed view (Fig. 2B) identifies specific nuclei^{14,15} comprising the amygdaloid nuclear complex. Figure 2C schematically illustrates the anterior-to-posterior span of the dialysis sites in the amygdaloid nuclear complex. A representative cresyl violet-stained section used to confirm microdialysis probe placement within the amygdaloid nuclear complex is shown in Figure 2D.

Drug-induced changes in neurotransmitter levels can result from alterations in neurotransmitter synthesis, degradation, or release. Dialysis delivery of the sodium channel blocker TTX is a standard technique for inferring action potential-dependent release of a measured transmitter.^{23,24} Before quantifying the effects of the BZ site agonists, the amygdala of rats ($n = 3$) was dialyzed with Ringer's solution containing TTX (1 μM). The

results (no figure presented) revealed that compared to ACh measured during microdialysis with Ringer's solution (control), TTX caused a significant decrease of -39.7% ($P = 0.0158$, $95\% \text{ CI} = -67.0\% \text{ to } -12.3\%$) in ACh release. The finding that TTX caused an approximately 40% decrease in ACh release in rat amygdala can be compared to TTX causing a 55% decrease in ACh release measured in mouse prefrontal cortex,²³ and TTX causing a 58% decrease in ACh release within the pontine reticular formation of mice.²⁴ The TTX-induced decrease of ACh in rats supports the interpretation that a portion of the present microdialysis measures reflects action potential-dependent ACh release in the amygdala.

Additional control experiments were essential for confirming that ACh release changed as a function of administered BZ site agonists. Therefore, two additional control studies were performed to ensure that ACh release measured from the amygdala was stable throughout the 125 min interval required to collect microdialysis samples. One study followed the design shown in Fig. 1C. The results (not illustrated) revealed that compared to ACh release measured during the first 62.5 min of dialysis with Ringer's solution, there was no significant difference in ACh release during the subsequent 62.5 min of dialysis with Ringer's solution (mean difference = 8.3% ; $P = 0.5509$; $95\% \text{ CI} = -27.1\% \text{ to } 43.7\%$; $n = 3$ rats). A similar series of control experiments, performed according to the design illustrated by Fig. 1B, revealed that IV administration of saline to unanesthetized rats caused a nonsignificant change -16.4% ($P = 0.1192$; $95\% \text{ CI} = -39.4\% \text{ to } 6.6\%$) in ACh release ($n = 3$ animals, data not shown). These control experiments verified that measures of ACh were stable and free of procedural confounds. The foregoing control experiments provided a basis for the results summarized by Figures 3, 4, and 5.

Study 1 ($n = 3$ animals per drug) was designed to quantify ACh release in the amygdala before and after BZ site agonists were administered systemically to intact, unanesthetized rats. The results demonstrate that IV administration of midazolam (Fig. 3A) caused a significant ($P = 0.0029$; $95\% \text{ CI} = -73.0\% \text{ to } -29.2\%$) decrease of -51.1% in ACh release. Likewise, ACh release in the amygdala was significantly ($P = 0.0222$; $95\% \text{ CI} = -69.8\% \text{ to } -9.3\%$) decreased (-39.6%) by eszopiclone (Fig. 3B).

The results from Study 2 ($n = 3$ animals per drug) show ACh release in the amygdala of isoflurane-anesthetized rats before (Ringer's solution) and after IV administration of midazolam (Fig. 4A) or eszopiclone (Fig. 4B). ACh release was significantly ($P = 0.0041$; $95\% \text{ CI} = -67.9\% \text{ to } -24.5\%$) decreased by midazolam (-46.2%) and significantly ($P = 0.0009$; $95\% \text{ CI} = -44.7\% \text{ to } -23.3\%$) decreased by eszopiclone (-34.0%).

Study 3 ($n = 3$ animals per drug) quantified the effects of delivering BZ site agonists directly into the amygdala. Microdialysis delivery of midazolam (Fig. 5A) into the amygdala of isoflurane-anesthetized rats nonsignificantly ($P = 0.0814$; $95\% \text{ CI} = -2.6\% \text{ to } 29.0\%$) changed ACh release within the amygdala by 13.2% . Figure 5B shows that dialysis delivery of diazepam to the amygdala caused a significant ($P = 0.0434$; $95\% \text{ CI} = 2.0\% \text{ to } 84.3\%$) increase of 43.2% in ACh release. Figure 5C illustrates that microdialysis delivery of eszopiclone to the amygdala also significantly ($P = 0.0159$; $95\% \text{ CI} = 68.5\% \text{ to } 375.8\%$) increased ACh release in the amygdala by 222.2% . There was no significant ($P = 0.2674$;

95% CI = -18.6% to 50.9%) difference (16.1%) in ACh release comparing zolpidem to control (Fig. 5D).

Discussion

The anxiolytic and sedating properties of the BZ site agonists used in this study are well documented. The present study quantified the effects of these BZ site agonists on cholinergic neurotransmission in the CeA region of the amygdala. The results are discussed in relation to two novel findings. 1) When administered via microdialysis into the amygdala, eszopiclone and diazepam significantly increased ACh release in the amygdala, whereas midazolam and zolpidem did not significantly alter ACh release in the amygdala. 2) In contrast, IV administration of midazolam and eszopiclone caused a significant decrease in ACh release within the amygdala of isoflurane-anesthetized rats and of rats that were not anesthetized. The finding that ACh release in the amygdala was decreased by systemic delivery of midazolam and eszopiclone, but increased by amygdala delivery, indicates that these sedative/hypnotics decrease ACh release in the amygdala by altering the excitability of extra-amygdaloid neurons.

Acetylcholine and Amygdaloid Modulation of Sleep and Wakefulness

Cholinergic neurotransmission contributes to normal amygdaloid function.^{2,14-16,25,26} The amygdala is richly endowed with cholinergic terminals²⁷ many of which originate from neurons in the basal forebrain.²⁸ Amygdala neurons are depolarized by muscarinic receptor agonists²⁹ and application of ACh to the amygdala increases neuronal discharge.³⁰ Normally, neuronal discharge in the amygdala is relatively quiescent³¹ and activation of the amygdala increases neurotransmitter release.^{2,32} Additional structure/function data emphasize the relevance of amygdala ACh levels for human amygdaloid function. For example, the human amygdala contains acetylcholinesterase,³³ and the neurotoxic acetylcholinesterase inhibitor soman increases firing rate in the amygdala to an extent that results in seizures.³⁴ Others have measured ACh in the amygdala during a learning task and found that increased ACh release was indicative of amygdala activation.³⁵

The effects of midazolam on sleep and electroencephalographic power are to increase a non-rapid eye movement sleep-like state, decrease rapid eye movement sleep, decrease electroencephalographic delta (0.5 to 4 Hz) power, and increase electroencephalographic sigma (11 to 16 Hz) power.³⁶ Human brain imaging data show that the BZ site agonist triazolam, indicated for treatment of insomnia, causes deactivation of the amygdala.³⁷ Eszopiclone and zolpidem were included in the present study because they are also used for treatment of insomnia, a disorder in which the amygdala fails to deactivate during sleep.¹ No previous studies, however, have compared the effects of midazolam, diazepam, eszopiclone, and zolpidem on ACh release in the amygdala.

Midazolam and Eszopiclone Decrease ACh Release in the Amygdala

Midazolam consistently has been reported to produce an anxiolytic effect when administered into the basolateral amygdala.³⁸⁻⁴¹ In addition, intraperitoneal administration of eszopiclone causes anxiolysis.⁴² These findings fit with the concept that BZ site agonists produce their

desired clinical actions by enhancing GABAergic inhibition, resulting in diminished neuronal excitability within the amygdala. This interpretative view of a mechanism of action of BZ site agonists also is consistent with the present finding that systemically administering midazolam and eszopiclone caused a significant decrease in ACh release in the amygdala of unanesthetized (Fig. 3) and anesthetized (Fig. 4) rats.

How is one to interpret the finding (Fig. 5) that the effects of BZ site agonist on ACh release in the amygdala vary as a function of systemic versus amygdala drug delivery? The Figure 5 results support and extend previous neurochemical studies comparing the effect of diazepam, eszopiclone, and zolpidem on ACh release in medial regions of the pontine reticular formation,¹⁷ a brain region known to regulate states of sleep and anesthesia.^{43,44} Unlike the present findings in amygdala (Fig. 5), microdialysis delivery of zolpidem and eszopiclone into the pontine reticular formation caused a concentration-dependent increase in ACh release in the pontine reticular formation.¹⁷ Similar to the present results with systemic drug administration (Figs. 3 and 4), IV administration of eszopiclone decreased ACh release in the pontine reticular formation of anesthetized and unanesthetized rats.¹⁷

Comparing results obtained from the amygdala (Figs. 3, 4, and 5) with data from the pontine reticular formation¹⁷ permits two conclusions. First, the contrasting effects on ACh release of local versus systemic drug delivery emphasize the need for the multiple experimental approaches illustrated by Figure 1. Microdialysis drug delivery to specific brain regions is a powerful tool for identifying receptor subtypes modulating transmitter release⁴⁵ and for elucidating autoreceptor function.²³ It is clear, however, that the results from studies using only microdialysis drug delivery do not permit strong inferences regarding the brain sites through which systemically administered drugs alter neurotransmitter levels. A drug delivered by microdialysis to a specific brain region may cause the same change in neurotransmitter levels that are caused by systemic delivery of that drug. In such a case, the principle of parsimony supports the conclusion that the brain region dialyzed has been identified as one site of action for the systemically administered drug.^{17,46,47} For example, comparing the effects on neurotransmitter release caused by local versus systemic delivery of opioids has successfully identified the substantia innominata of the basal forebrain as one region through which IV opioids decrease cortical ACh release.⁴⁶ In the present study the increase in ACh release caused by microdialysis delivery of diazepam and eszopiclone to the amygdala (Fig. 5) is contrary to the fact that ACh promotes behavioral and electrographic arousal, and contrary to the sedating actions of diazepam and eszopiclone. Thus, the present results (Figs., 3, 4, and 5) support an alternative conclusion that midazolam and eszopiclone decrease ACh release in the amygdala by acting on neuronal systems outside of the amygdala.

Limitations and Future Directions

Microdialysis probes were aimed for the CeA region but some dialysis sites were located in other amygdaloid nuclei due to the limited spatial resolution of microdialysis probes (Fig. 2). Therefore, it is not possible to attribute the present ACh measures to any specific subregion of the amygdala. Many studies already have shown that administering sedative/hypnotics into the amygdala causes sedation and anxiolysis.³⁸⁻⁴¹ In addition, sleep is

increased by microinjecting cholinomimetics into the amygdala.^{16,48} To fill existing gaps in knowledge, and to satisfy the mandate for nonduplicative research, the present study focused on amygdala neurochemistry. The emphasis on changes in amygdala ACh release caused by BZ site agonists was stimulated by the recent discovery that the relationship between GABAergic and cholinergic neurotransmission modulates levels of behavioral arousal.⁴⁹ The present focus on ACh should not be interpreted, however, to imply primacy of cholinergic function in the amygdala. Multiple endogenous neurotransmitter systems (e.g., GABAergic, glutamatergic, noradrenergic, and histaminergic) are involved in regulating neuronal excitability within the amygdala. Unlike our previous studies characterizing ACh release as a function of drug concentration,^{17,45} the present experiments were limited to one concentration for each of the four BZ site agonists. The present study identifying concentrations of each drug that altered ACh release is an essential first step needed to justify future concentration-response studies.

We are aware of no data concerning the relative affinity or efficacy of midazolam for different alpha subtypes in any brain region. Understanding the GABA_A receptor subtypes through which BZ site agonists alter the physiological and behavioral traits characteristic of sedation and sleep has the potential to help develop future BZ site agonists with highly specific actions. For example, studies using transgenic mice already have succeeded in demonstrating that the phenotypes of sedation and electroencephalographic power altered by diazepam are mediated by different alpha subtypes of the GABA_A receptor.⁵⁰ Activation of GABA_A receptors containing the alpha-1 subunit mediate sedation, while alpha-2 containing GABA_A receptors mediate anxiolysis.⁵¹

In conclusion, ACh release in the amygdala was decreased by systemic administration of midazolam and eszopiclone, and increased by direct administration of these drugs into the amygdala. This differential response indicates that these sedative/hypnotics change ACh release in the amygdala by altering the excitability of extra-amygdaloid neurons. The present results encourage future efforts to characterize the neuronal networks through which BZ site agonists alter the regulation of behavioral arousal by the amygdala. Cholinergic transmission in the pontine reticular formation plays a key role in regulating states of anesthesia and sleep.^{43,44} BZ site agonists alter ACh release in the pontine reticular formation¹⁷ and neurons in the amygdala provide excitatory input to the pontine reticular formation.⁵² Continuing to derive a detailed understanding of the brain regions and neurotransmitters through which BZ site agonists cause sedation and sleep will advance what has been described as a new horizon for anesthesiology.⁵³

Acknowledgments

Funding: Supported by grants HL65272 (RL) and MH45361 (HAB) from the National Institutes of Health, Bethesda, MD, and by the Department of Anesthesiology, University of Michigan, Ann Arbor, MI

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A. Study 1: Intravenous administration of BZ site agonists to non-anesthetized rat

B. Study 2: Intravenous administration of BZ site agonists to anesthetized rat

C. Study 3: Microdialysis delivery of BZ site agonists to the amygdala of anesthetized rat

Figure 1. Schematic time lines illustrating procedures for each of the three studies. Boxes describe the sequence of manipulations performed during each experiment. Green boxes indicate when dialysis samples containing acetylcholine (ACh) (the dependent measure) were obtained. **A.** Rats were implanted with a chronic jugular vein cannula and a microdialysis guide cannula. Intact, unanesthetized rats were used to measure ACh release in the amygdala before and after administration of benzodiazepine (BZ) site agonists via the previously implanted jugular vein cannula. **B.** Measurements of ACh release in the amygdala (Amyg) from

isoflurane-anesthetized rats were obtained while drugs were delivered via an acute, jugular vein catheter (JVC). **C.** Microdialysis delivery of BZ site agonists directly to the amygdala during isoflurane anesthesia.

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Figure 2.

Histologic confirmation of dialysis sites. **A.** The coronal plates were modified from a rat brain atlas²² to illustrate the location of microdialysis sites within the amygdala. The size of the dialysis membrane is indicated by the cylinders drawn to scale, relative to the brain. The color-coding of the cylinders corresponds to the experimental conditions (green = Study 1, red = Study 2, blue = Study 3, gray = tetrodotoxin (TTX) and the Ringer's and saline control experiments). The colors green, red, and blue are used in Figs. 3, 4, and 5, respectively, to identify which microdialysis sites contributed to the ACh release data. The number within each coronal diagram indicates mm posterior to bregma. **B.** Enlarged view of the different subnuclei comprising the amygdala nuclear complex. **C.** Vertical lines on a sagittal diagram of the rat brain²² depict the anterior-to-posterior range of the microdialysis sites within the amygdala. **D.** A cresyl violet-stained coronal brain section shows a typical microdialysis site in the amygdala. The arrow marks the most ventral position of the dialysis membrane. The section was localized at approximately 2.28 mm posterior to bregma. Abbreviations: AA: anterior amygdaloid area; ACo: anterior cortical amygdaloid nucleus; BLA: basolateral amygdaloid nucleus, anterior part; BMA: basomedial amygdaloid nucleus, anterior part; CeA: central amygdaloid nucleus, anterior; Cx1,2,3: cortex-amygdala transition zone, layers

1 through 3; I: intercalated nuclei of the amygdala; MeAD: medial amygdaloid nucleus, anterior dorsal.

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Study 1: BZ site agonists iv, no anesthesia

Figure 3.

Acetylcholine (ACh) release in the amygdala was decreased by intravenous administration of benzodiazepine (BZ) site agonists to unanesthetized rats. **A.** Midazolam caused a significant decrease (–51.1%) in ACh release in the amygdala. **B.** Eszopiclone caused a significant decrease (–39.6%) in ACh release. For each drug, the asterisk denotes a significant decrease in ACh compared to ACh release before (Pre-) drug administration.

Study 2: BZ site agonists iv, with anesthesia

Figure 4.

Intravenous administration of benzodiazepine (BZ) site agonists to anesthetized rat decreased acetylcholine (ACh) release. **A.** Midazolam decreased (–46.2%) ACh release in the amygdala when administered to anesthetized rats via an acute intravenous catheter. **B.** Using a similar experimental design, intravenous eszopiclone administration decreased (–34.0%) ACh release. For both midazolam and eszopiclone, the asterisk indicates a significant decrease in ACh compared to control levels of ACh release before (Pre-) drug administration.

Study 3: BZ site agonists via microdialysis, with anesthesia

Figure 5.

Benzodiazepine (BZ) site agonists delivered via microdialysis to the amygdala of anesthetized rats altered acetylcholine (ACh). Amygdala ACh release was not altered by **A.** midazolam (13.2%) and **D.** zolpidem (16.1%) but was significantly increased by **B.** diazepam (43.2 %), and **C.** eszopiclone (222.2%). Asterisks indicate significant differences in ACh release caused by BZ site agonists compared to ACh levels measured during dialysis with Ringer's (control).