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# CNS 7056

## A Novel Ultra-short-acting Benzodiazepine

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***Background:*** **A new benzodiazepine derivative, CNS 7056, has been developed to permit a superior sedative profile to current agents, *i.e.*, more predictable fast onset, short duration of sedative action, and rapid recovery profile. This goal has been achieved by rendering the compound susceptible to metabolism *via* esterases. The authors now report on the profile of CNS 7056 *in vitro* and *in vivo*.**

***Methods:*** **The affinity of CNS 7056 and its carboxylic acid metabolite, CNS 7054, for benzodiazepine receptors and their selectivity profiles were evaluated using radioligand binding. The activity of CNS 7056 and midazolam at subtypes ($\alpha_1\beta_2\gamma_2$, $\alpha_2\beta_2\gamma_2$, $\alpha_3\beta_2\gamma_2$, $\alpha_5\beta_2\gamma_2$) of the γ-aminobutyric acid type A ($GABA_A$) receptor was evaluated using the whole cell patch clamp technique. The activity of CNS 7056 at brain benzodiazepine receptors *in vivo* was measured in rats using extracellular electrophysiology in the substantia nigra pars reticulata. The sedative profile was measured in rodents using the loss of righting reflex test.**

***Results:*** **CNS 7056 bound to brain benzodiazepine sites with high affinity. The carboxylic acid metabolite, CNS 7054, showed around 300 times lower affinity. CNS 7056 and CNS 7054 (10 μM) showed no affinity for a range of other receptors. CNS 7056 enhanced GABA currents in cells stably transfected with subtypes of the $GABA_A$ receptor. CNS 7056, like midazolam and other classic benzodiazepines, did not show clear selectivity between subtypes of the $GABA_A$ receptor. CNS 7056 (intravenous) caused a dose-dependent inhibition of substantia nigra pars reticulata neuronal firing and recovery to baseline firing rates was reached rapidly. CNS 7056 (intravenous) induced loss of the righting reflex in rodents. The duration of loss of righting reflex was short (< 10 min) and was inhibited by pretreatment with flumazenil.**

***Conclusions:*** **CNS 7065 is a high-affinity and selective ligand for the benzodiazepine site on the $GABA_A$ receptor. CNS 7056 does not show selectivity between $GABA_A$ receptor subtypes. CNS 7056 is a potent sedative in rodents with a short duration of action. Inhibition of substantia nigra pars reticulata firing and the inhibition of the effects of CNS 7056 by flumazenil show that it acts at the brain benzodiazepine receptor.**

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BENZODIAZEPINES act at a specific site on the γ-aminobutyric acid type A ($GABA_A$) receptor to enhance the action of the inhibitory neurotransmitter, GABA.[1] Benzodiazepines act by a positive allosteric modulation of the receptor–chloride channel complex. Under most circumstances, benzodiazepines increase chloride ion flux, causing membrane hyperpolarization and inhibition of neuronal activity. Compounds acting at this site, such as diazepam, have been widely used to induce anxiolysis, amnesia, and sedation. Another agent acting at this site, midazolam, was introduced to the market in the early 1980s as the first water-soluble benzodiazepine.[2] Midazolam has gained widespread use as an intravenous agent to provide sedation for short procedures and in intensive care. However, the recovery from sedation induced by midazolam can be prolonged because of the production of active metabolites[2] and a reliance on the liver enzyme cytochrome P450 3A4 for metabolism.[3] The latter means that drug–drug interactions can be problematic,[3] as is its administration to patients with compromised liver function.[4]

In this report, we describe a new benzodiazepine derivative, CNS 7056, which has been developed to provide an intravenous agent with a predictable fast-onset, short duration of action and rapid recovery profile. The strategy for developing this agent emanated from the development of the ultra-short-acting opioid analgesic remifentanil.[5,6] Remifentanil is a carboxylic acid ester that is broken down rapidly in the body by esterases to an inactive carboxylic acid metabolite. This model has been translated to a benzodiazepine scaffold to provide a range of ester derivatives.[7,8] The carboxylic ester, CNS 7056, which is broken down by esterases to the metabolite, CNS 7054 (fig. 1), is the lead compound of this series. We now report on the radioligand binding profile, electrophysiologic profile at subtypes of the $GABA_A$ receptor, and *in vivo* sedative activity of CNS 7056.

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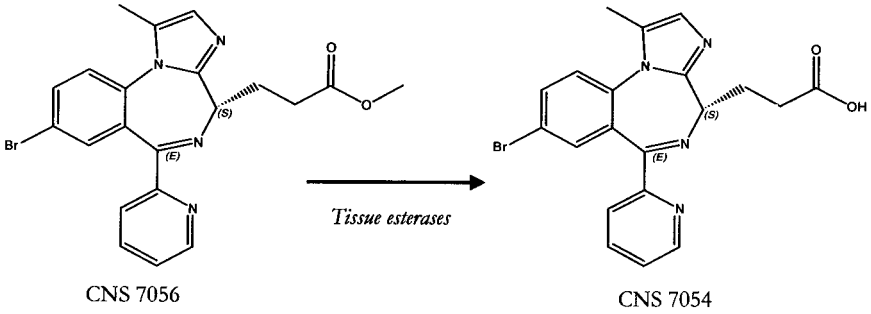


**Fig. 1. CNS 7056 and its carboxylic acid metabolite, CNS 7054.**

## Materials and Methods

Animal studies were conducted with adherence to all relevant regulations, including institutional animal care and use committee approval (GlaxoSmithKline, Research Triangle Park, NC; Northeastern University, Bouve College of Health Sciences, Boston, MA) or government licensure (Porsolt and Partners Pharmacology Research Laboratory, Le Genest-Saint-Isle, France).

### *Radioligand Binding Studies*

Human cerebral cortex membranes, Yucatan micropig cerebral cortex membranes, and rat (Sprague-Dawley) whole brain membranes (minus cerebellum) were obtained from Analytical Biologic Services Inc. (Wilmington, DE). Membranes were prepared using the method of Marangos and Martino.[9] Stock membrane was diluted with assay buffer (50 mM Tris hydrochloride, pH 7.4, 150 mM NaCl at 4°C) to a final concentration of 15 μg (protein) per well. Membranes and compounds were allowed to incubate for 90 min with [$^3$H]flunitrazepam (2.5 nM) at 4°C (final volume 200 μl). Binding was terminated by filtering the contents of each well through GF/B Unifilter 96-well microplates. Plates were washed with eight times the well volume using ice-cold Tris HCl buffer, pH 7.4. Radioactivity was measured using a plate-based scintillation counter.

The potential for undesirable side effects was assessed by determining the affinity of CNS 7056 and CNS 7054 (10 μM) for a range (40) of receptors, enzymes, and ion channels (additional information regarding this is available on the ANESTHESIOLOGY Web site at http://www.anesthesiology.org).

### *Modulatory Effects on Recombinant GABA Currents Recorded from Stably Transfected Cells*

Stably transfected Ltk cells were generated at BSys GmbH (Witterswil, Switzerland). Cells were transfected, using a pcDNA3 vector containing the dexamethasone promoter system, with the following $GABA_A$ receptor subtypes (rat): $\alpha_1\beta_2\gamma_2$, $\alpha_2\beta_2\gamma_2$, $\alpha_3\beta_2\gamma_2$, $\alpha_5\beta_2$ (the $\gamma_{2S}$ subunit was used) using Invitrogen's (Carlsbad, CA) standard protocol (catalog No. K2780-01). Cells were maintained in Dulbecco's modified eagle medium and nutrient mixture F-12 (Dulbecco's modified eagle medium/F-12 1X, liquid, with GlutaMax I; Gibco-BRL, Gaithersburg, MD) supplemented with 9% foetal bovine serum (Gibco-BRL) and 0.9% penicillin–streptomycin solution (Gibco-BRL). Cells were ready for electrophysiologic experiments 24–48 h after induction of expression with dexamethasone. Stable integration was confirmed functionally: $\beta/\gamma$ constructs alone are not active; $\alpha/\beta$ are active but without benzodiazepine action; $\alpha/\beta/\gamma$ exhibit full $GABA_A$ characteristics. The transfection level was determined using a green fluorescent protein reporter gene. Stable transfection was determined by the existence of resistant clones.

The bath solution for electrophysiologic experiments consisted of 137 mM NaCl, 4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM D-glucose. The pH of the bath solution was adjusted to 7.4 using NaOH. The pipette solution consisted of 130 mM KCl, 1 mM $MgCl_2$, 5 mM Mg-ATP, 10 mM HEPES, and 5 mM EGTA. The pH of the pipette solution was adjusted to 7.2 using KOH.

Culture dishes (35 mm), upon which cells were seeded at a density allowing single cells to be recorded, were placed on the dish holder of the microscope and continuously perfused (at approximately 1 ml/min) with the bath solution. All solutions applied to cells including the pipette solution were maintained at room temperature (approximately 25°C). After formation of a gigaohm seal between the patch electrodes and individual cells (pipette resistance range 2.0–7.0 MΩ; seal resistance range > 410 MΩ), the cell membrane across the pipette tip was ruptured to assure electrical access to the cell interior (whole cell patch configuration). The cells were voltage clamped at a holding potential of −80 mV. Compensation procedures for series resistance and capacitance were applied if required. As soon as a stable seal could be established, GABA inward currents were measured upon rapid application of 2 μM GABA (corresponding to approximately $EC_5$) to patch clamped cells. Once control recordings were accomplished, cells were perfused with a coapplication of 2 μM GABA and CNS 7056 (0.1–10 μM) or midazolam (0.03–3 μM). A cumulative dose–response analysis was evaluated per cell (five test compound concentrations). The degree of current modulation (*i.e.*, increase over control) was calculated as a percentage of the control response.

*Techniques for Extracellular Single-unit Recording*

Male Sprague-Dawley rats (Taconic, Germantown, NY) ranging in weight from 225 to 350 g were anesthetized with chloral hydrate (400 mg/kg intraperitoneal) and placed in a stereotaxic instrument. Additional doses of chloral hydrate were administered through a lateral tail vein as needed throughout the experiment. Body temperature was monitored and maintained at 36°–38°C using a rectal probe and feedback-controlled heating pad.

The skull was surgically exposed, and a small burr hole was drilled at a location 2.0 mm lateral to lambda and 3.0 mm anterior to the lambdoid suture. An electrode was lowered through the hole to the level of the substantia nigra using a Narashige microdrive. Electrodes were made from 2.0-mm glass capillary tubing pulled on a Narashige PE-2 microelectrode puller. The electrodes were filled with a 1% solution of pontamine sky blue in 2 M NaCl (used for later marking the location of the electrode tip in brain), and the tip was broken back to a 1- to 2-$\mu$m diameter under microscopic control to achieve an impedance of 3–6 M$\Omega$.

Standard extracellular recording methods were used to monitor neuronal activity.[10–12] Briefly, electrical signals were passed through a high-input impedance amplifier and then passed to an amplitude/time discriminator, which was set so that each spike triggered a rate counter. The amplified signal was displayed on an oscilloscope and sent to an audio monitor. Firing rates were summed over successive 10-s intervals.

Upon conclusion of each experiment, recording sites were marked by passing a 20-$\mu$A current through the electrode for 15–20 min. The rats were then perfused using 10% buffered formalin solution, and the brain was removed and sectioned. Histologic examination revealed that the pontamine blue spots marking the locations of recorded cells were within the substantia nigra pars reticulata (SNpr), within the following stereotaxic boundaries (according to the atlas of Konig and Klippel[13]): anterior, 1,760 to 2,580 $\mu$m; lateral, 2.0 to 2.4 mm; ventral, $-1.5$ to $-2.5$.

CNS 7056 (0.9% wt/vol saline solution; 0.025, 0.25, or 2 mg/ml, depending on required dose) was administered intravenously by rapid bolus through a lateral tail vein, and the effects on firing of SNpr neurons were determined. A stable 3- to 5-min period of baseline firing was recorded before the first dose of drug, and then drug injections were given at 1-min intervals in increasing increments such that each dose doubled the previously administered cumulative dose (without correcting for any metabolism of the drug). Drug administration was continued up to a total of 8 mg/kg. After the last dose of drug was given, monitoring of neuronal activity was continued until the cell returned to its baseline firing rate. For each neuron, the firing rate after each dose was averaged and compared with the mean baseline rate before drug administration to generate a percentage change in firing at each dose. Seven dose–response curves were generated, and mean responses at each dose were plotted. The time from administration of the last dose of drug (maximal inhibition of firing) to the time when firing recovered to $\pm$10% of the original baseline rate was also determined.

*Rat Loss of Righting Reflex*

Male Wistar rats weighing 300–450 g were used (one drug treatment only per animal). Animals were placed in plastic restrainers, and the test compound was administered intravenously *via* the tail vein. Animals were immediately removed from the restrainer, and the time to onset of loss of righting reflex (LRR) was recorded. LRR was defined as the loss of an animal's ability to right itself when placed in a supine position. After LRR, animals were laid on their backs. Once an animal was able to right itself for the first time, it was placed on its back once again. If the animal was able to right itself two more (consecutive) times, it was determined that the animal had recovered from LRR.

For animals demonstrating LRR, reflex responses (corneal, Pinna, and Haffner) were measured. The Pinna reflex was measured by placing a fine wire in the animal's ear canal. The reflex was considered to be present if the animal shook its head. The corneal reflex was measured by touching the eye of the animal with a fine wire. The reflex was considered to be present if the animal blinked. The analgesic (Haffner) reflex was measured by pinching a rear hind foot (using the experimenter's thumb and forefinger). This reflex was considered to be present if the animal withdrew its foot.

CNS 7056 was prepared in a 0.9% wt/vol saline solution. A dose of 25 mg/kg was administered by rapid bolus *via* the lateral tail vein to six rats (10 mg/ml). A marketed injectable formulation of midazolam (Versed®; Hoffmann-La Roche, Nutley, NJ; 5 mg/ml) was used. A dose of 25 mg/kg was administered to six rats again *via* the lateral tail vein.

*Loss of Righting Reflex Test in the Mouse*

Mice, placed in individual acrylic glass cages (20 $\times$ 10 $\times$ 10 cm), were injected with either vehicle (saline solution) or test substance by the intravenous route (lateral tail vein; doses adjusted to 10 ml/kg). The latency to LRR and the duration of LRR were recorded. The righting reflex test was performed as soon as the animals appeared sedated, at approximately 20- to 30-s intervals. Once the righting reflex was absent, LRR was measured by testing for the return of the righting reflex approximately every 20–30 s thereafter. Eight mice were studied per group, and drugs were administered intravenously by rapid bolus. Two studies were conducted. The first evaluated LRR induction by CNS 7056 (15–30 mg/kg intravenous in 0.9% wt/vol saline), CNS 7054 (30–100

mg/kg intravenous in 0.9% wt/vol saline), or midazolam (15–50 mg/kg intravenous; Laboratoire Aguettant, Lyon, France; 5 mg/ml). The second experiment evaluated the impact of pretreatment with flumazenil (20 mg/kg intraperitoneal, 0.9% saline; 15 min before administration of CNS 7056) on the effects of CNS 7056 (30 mg/kg intravenous).

*Statistical Analysis*

Data are presented as mean ± SEM. The number of separate experiments is as indicated. Software programs used for curve fitting were RoboSage (a proprietary Microsoft Excel add-in; binding studies), Kaleidograph (Synergy Software, Reading, PA; *in vitro* electrophysiology studies), and GraphPad Prism (Graphpad Software, San Diego, CA; *in vivo* electrophysiology studies). For radioligand binding studies, pKi values were determined using the method of Cheng and Prusoff.[14] For the mouse LRR study comparing pretreatment with flumazenil with vehicle pretreatment, data were analyzed using the Mann–Whitney U test.

## Results

*Radioligand Binding*

In the concentration range tested ($10^{-4}$ to $10^{-11}$ M), CNS 7056 and CNS 7054 competed for [$^3$H]flunitrazepam (2.5 nM) binding to tissue homogenates from human, rat, and pig brain (table 1 and fig. 2). In each species, CNS 7056 showed higher affinity. Hill coefficients for both compounds were close to unity in each of the tissues (not shown). The separation between the affinity of CNS 7056 and its carboxylic acid metabolite, CNS 7054, ranged from 410-fold in human brain tissue to 320-fold in rat brain tissue (table 1 and fig. 2).

Selectivity studies (additional information regarding this is available on the ANESTHESIOLOGY Web site at http://www.anesthesiology.org) revealed that CNS 7056 (10 μM) showed no detectable affinity at any site tested other than the $GABA_A$ benzodiazepine receptor. Sites tested included adenosine, adrenergic, cholecystokinin, dopamine, endothelin, glutamate, muscarinic, nicotinic, opiate, and serotonin receptors and $Ca^{2+}$, $K^+$, and $Na^+$ ion channels. The carboxylic acid metabolite, CNS 7054 (10 μM), showed no detectable affinity at any site tested.

**Table 1. Affinities of CNS 7056 and CNS 7054 to Compete for [$^3$H]Flunitrazepam (2.5 nM) Binding to Brain Homogenates**

| | CNS 7056 pKi | CNS 7054 pKi | Ratio |
|---|---|---|---|
| Human | 7.53 ± 0.25 | 4.91 ± 0.37 | 410-fold |
| Rat | 7.50 ± 0.21 | 4.99 ± 0.11 | 320-fold |
| Yucatan micropig | 7.56 ± 0.22 | 5.05 ± 0.08 | 326-fold |

Results are mean ± SD of 4–8 separate experiments. Experiments were conducted in triplicate. Competition curves were analyzed using the proprietary Microsoft Excel add-in RoboSage. pKi values were calculated using the Cheng-Prusoff equation.[14] Mean Hill coefficients were not different from unity.

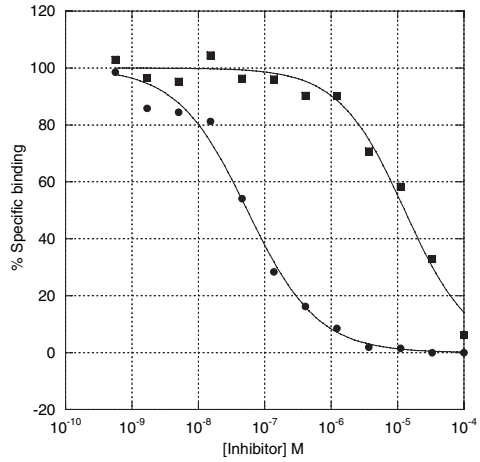


**Fig. 2. Competition for [$^3$H]flunitrazepam (2.5 nM) binding to homogenates of human brain by CNS 7056 (*circles*) and CNS 7054 (*squares*). Data are means of triplicate determinations from a single representative experiment.**

*Modulatory Effects on Recombinant GABA Currents Recorded from Stably Transfected Cells*

Both CNS 7056 (0.1–10 μM) and midazolam (0.03–3 μM) enhanced GABA (2 μM) currents in stably transfected Ltk cells (fig. 3 and table 2). Midazolam was slightly more potent than CNS 7056 on each subtype, but neither compound showed clear selectivity between the subtypes examined (table 2). The maximal effects of midazolam and CNS 7056 were similar at subtypes containing the $\alpha_1$, $\alpha_2$, and $\alpha_3$ subunits. CNS 7056 seemed to have a slightly higher maximal effect than midazolam at the $GABA_A$ subtype expressing the $\alpha_5$ subunit.

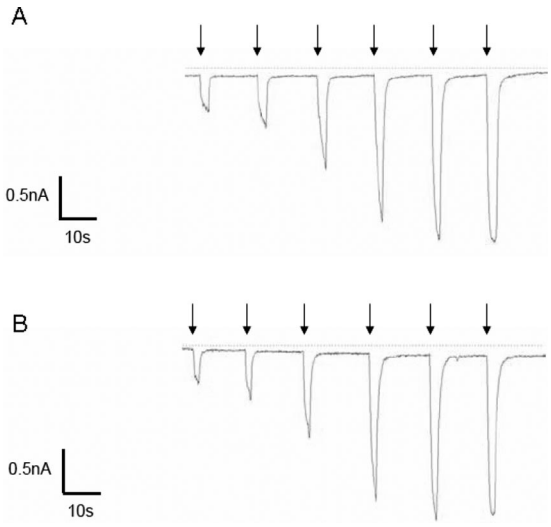


**Fig. 3. Representative current traces for the effects of CNS 7056 (*A*; 0, 0.1, 0.3, 1, 3, 10 μM) and midazolam (*B*; 0, 0.03, 0.1, 0.3, 1, 3 μM) on γ-aminobutyric acid (GABA) stimulated currents in cells stably transfected with complementary DNA encoding the $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor subtype. *Arrows* indicate the addition of GABA (2 μM).**

**Table 2. Enhancement of GABA Currents (2 μM) by CNS 7056 (0.1–10 μM) and Midazolam (0.03–3 μM) in Ltk Cells Stably Transfected with $GABA_A$ Receptor Subtypes, Assessed Using the Whole Cell Patch Clamp Technique**

| | CNS 7056 | | Midazolam | |
|---|---|---|---|---|
| Subtype | Emax, % | $pEC_{50}$ | Emax, % | $pEC_{50}$ |
| $\alpha_1\beta_2\gamma_2$ | 375 ± 173 | 6.44 ± 0.10 | 300 ± 140 | 6.81 ± 0.13 |
| $\alpha_2\beta_2\gamma_2$ | 186 ± 88 | 6.18 ± 0.32 | 196 ± 175 | 6.60 ± 0.13 |
| $\alpha_3\beta_2\gamma_2$ | 210 ± 54 | 6.04 ± 0.13 | 301 ± 52 | 6.53 ± 0.14 |
| $\alpha_5\beta_2\gamma_2$ | 289 ± 28 | 5.86 ± 0.16 | 104 ± 57 | 6.93 ± 0.07 |

Results are mean ± SD, n = 3–7. Five concentrations of test drug were used in each individual experiment. Data were analyzed by curve fitting with the computer program Kaleidograph to the following equation: Y = Bottom + (Top − Bottom)/(1 + 10^(LogEC50 − X) * Hill Slope)). The value for the bottom of the curve was fixed to zero.

Emax = maximum enhancement of the γ-aminobutyric acid (GABA)–induced current as a percentage; $pEC_{50}$ = negative log of the $EC_{50}$ value.

*Substantia Nigra Electrophysiology*

Neurons recorded in these studies were located in the SNpr. SNpr neurons displayed smooth, sharp, biphasic action potentials with an average duration of less than 1 ms and firing rates between 10–40 spikes/s. Intravenous administration of increasing doses of CNS 7056 caused dose-related decreases in SNpr neuronal firing. The cumulative log dose–response curves for the inhibition of firing are shown in figure 4. The curves reveal that firing was suppressed to around 20% of baseline levels. The $ED_{50}$ for inhibition of SNpr firing by CNS 7056 was 0.83 mg/kg (95% confidence interval, 0.74–0.94; n = 7). The time course of recovery of neuronal firing after the highest dose of CNS 7056 is shown in figure 5. Recovery to baseline firing rates was achieved within approximately 7 min.

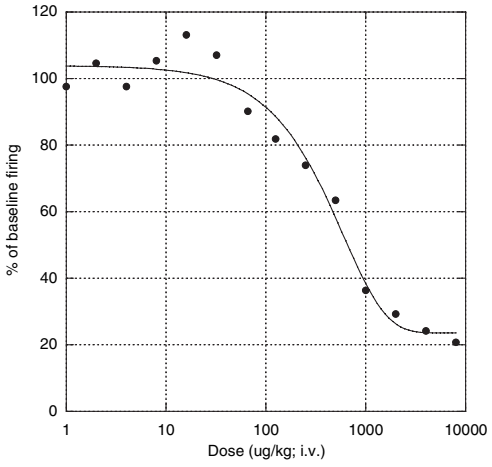


**Fig. 4. Inhibition of substantia nigra pars reticulata cell firing in the anesthetized rat by intravenous CNS 7056. Results are the mean of seven separate experiments. SDs are omitted for the sake of clarity, but are all less than 45% of baseline firing. Standard extracellular recording methods were used to monitor neuronal activity.[10–12] A stable 3- to 5-min period of baseline firing was recorded before the first dose of drug, and then drug injections were given at 1-min intervals in increasing increments such that each dose doubled the previously administered cumulative dose. For each neuron, the firing rate after each dose was averaged and compared with the mean baseline rate before drug administration to generate a percentage change in firing at each dose.**

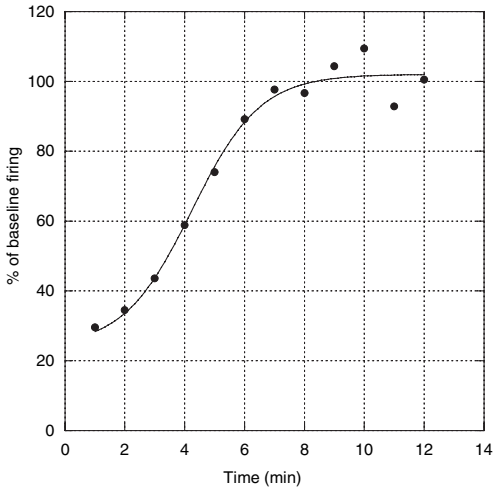


**Fig. 5. Recovery of substantia nigra pars reticulata cell firing in anesthetized rats after intravenous administration of CNS 7056 (8 mg/kg). Results are the mean of seven separate experiments. SDs are omitted for the sake of clarity, but are all less than 42% of baseline firing. Standard extracellular recording methods were used to monitor neuronal activity.[10–12] A stable 3- to 5-min period of baseline firing was recorded before the first dose of drug. After administration of the 8-mg/kg dose of CNS 7056, firing was monitored for an additional period of 10–12 min to determine the time course of recovery. For each neuron, the firing rate in each 1-min interval during recovery was averaged and compared with the mean baseline rate before administration to generate a percentage change in firing.**

*Rat Loss of Righting Reflex*

Intravenous administration of both midazolam (25 mg/kg) and CNS 7056 (25 mg/kg) brought about an immediate loss of the righting reflex in rats (table 3). Recovery from the LRR was observed more rapidly in rats treated with CNS 7056 than rats treated with midazolam (table 3). When tested during LRR, none of the rats receiving CNS 7056 exhibited a Haffner, corneal, or Pinna reflex. Two of the six rats treated with midazolam exhibited the Haffner reflex. None exhibited the corneal or Pinna reflex.

*Mouse Loss of Righting Reflex*

Intravenous administration of CNS 7056 (15–30 mg/kg; n = 8) brought about a rapid LRR in mice (table 3). The duration of LRR was short (1.1–3.6 min). Similar studies with midazolam showed a steep dose–response curve and longer duration of LRR (49–65 min; table 3). No LRR was observed in animals treated with CNS 7054 (30–100 mg/kg; table 3).

In a separate study, pretreatment with flumazenil (20 mg/kg intraperitoneal) significantly reduced the LRR duration induced by CNS 7056 (30 mg/kg; 0.7 ± 0.7 *vs.* 8.4 ± 2.6 min; $P = 0.013$; Mann–Whitney U test) in mice.

**Table 3. Effect of CNS 7056, Midazolam, and CNS 7054 in the LRR Test in Rodents**

| Treatment (mg/kg) | Species | Number of Animals with LRR/Total Animals | Latency to LRR, min* | LRR Duration, min |
|---|---|---|---|---|
| Vehicle | Mouse | 0/8 | — | — |
| CNS 7056 (15) | Mouse | 0/8 | — | — |
| CNS 7056 (20) | Mouse | 2/8 | — | 1.6 ± 3.4 |
| CNS 7056 (25) | Mouse | 4/8 | 0.6 ± 0.3 | 1.1 ± 2.0 |
| CNS 7056 (30) | Mouse | 6/8 | 1.2 ± 0.5 | 3.6 ± 5.0 |
| CNS 7054 (30) | Mouse | 0/8 | — | — |
| CNS 7054 (50) | Mouse | 0/8 | — | — |
| CNS 7054 (70) | Mouse | 0/8 | — | — |
| CNS 7054 (100) | Mouse | 0/8 | — | — |
| Midazolam (20) | Mouse | 0/8 | — | — |
| Midazolam (30) | Mouse | 0/8 | — | — |
| Midazolam (40) | Mouse | 7/8 | 2.0 ± 1.0 | 49.3 ± 32.0 |
| Midazolam (50) | Mouse | 6/8 | 1.2 ± 0.7 | 65.1 ± 40.4 |
| CNS 7056 (25) | Rat | 6/6 | 0 ± 0 | 9.6 ± 0.19 |
| Midazolam (25) | Rat | 6/6 | 0 ± 0 | 24.6 ± 6.1 |

The loss of righting reflex (LRR) test was performed as soon as the animals appeared sedated, at approximately 20- to 30-s intervals. Once the righting reflex was absent, duration of LRR was measured by testing for the return of the righting reflex approximately every 20–30 s thereafter. Drug or vehicle (saline) was administered as a bolus *via* the lateral tail vein. Values are mean ± SD.

* Latency was only calculated if three or more animals exhibited LRR.

## Discussion

Competition studies using the benzodiazepine site ligand [$^3$H]flunitrazepam show that CNS 7056 is a high-affinity benzodiazepine ligand acting at the $GABA_A$ receptor. Cross-species comparisons show that a similar high affinity was observed using homogenates of human, rat, or pig brain. The carboxylic acid metabolite, CNS 7054, revealed a much lower affinity in all three species. The separation in affinity between CNS 7056 and CNS 7054 was between 320-fold and 410-fold, depending on the species, with the highest separation being seen in human brain tissue. Selectivity studies showed that CNS 7056 is highly selective for the $GABA_A$ receptor and that CNS 7054 has no measurable affinity for any off-target site tested (additional information regarding this is available on the ANESTHESIOLOGY Web site at http://www.anesthesiology.org).

Perhaps the biggest breakthrough in recent years in understanding the actions of GABA and agents that modify the GABA receptors has come with the identification of the subunits that make up the $GABA_A$ receptor subtypes and the subsequent development of knock-out and knock-in mice (for reviews, see Barnard *et al.*,[15] Mohler,[16] and Mohler *et al.*[17]). The latter have provided remarkable insights into the role of the subtypes of the $GABA_A$ receptor in the various actions of benzodiazepine ligands. We evaluated the effects of CNS 7056 and midazolam at the four main $GABA_A$ receptor subtypes ($\alpha_1\beta_2\gamma_2$, $\alpha_2\beta_2\gamma_2$, $\alpha_3\beta_2\gamma_2$, $\alpha_5\beta_2\gamma_2$) that mediate the effects of benzodiazepines using whole cell patch clamp. CNS 7056, like midazolam, enhanced GABA currents. Like most classic benzodiazepine ligands,[15–17] CNS 7056 and midazolam showed little difference in $EC_{50}$ between the four subtypes. That said, CNS 7056 seemed to be slightly more potent at the $\alpha_1$-containing subtype, which is thought to mediate the sedative effects of benzodiazepines.[16,17] CNS 7056 also seemed to have a slightly higher maximal effect than midazolam at the $\alpha_5\beta_2\gamma_2$ subtype. The implications of this are unknown but may be minimal because $GABA_A$ receptor subtypes expressing the $\alpha_5$ subunit constitute less than 5% of brain $GABA_A$ receptors.[16,17]

Substantia nigra pars reticulata neurons of the rat brain have been shown to receive a prominent GABAergic innervation by striatonigral afferents and have been shown to be potently and reliably inhibited by intravenous administration of GABA or GABA agonists.[18,19] Similarly, benzodiazepines such as diazepam and flurazepam have been shown to inhibit SNpr cell firing, and this effect can be reversed by the benzodiazepine antagonist flumazenil.[20] *In situ* hybridization and immunohistochemical studies show the selective expression of the $\alpha_1$, $\beta_2$, and $\gamma_2$ subunits of the $GABA_A$ receptor in the rat SNpr.[21–23] CNS 7056 also potently inhibited the firing of these neurons. However, whereas the maximal inhibition of SNpr firing by these older (diazepam and flurazepam) benzodiazepines reached only 40–50% of baseline firing rates,[20] CNS 7056 achieved an 80% inhibition of firing. Therefore, comparison with literature data indicates that CNS 7056 exhibits greater efficacy in the electrophysiologic system, although a direct comparison is required to confirm this. The mechanisms by which CNS 7056 might induce a greater inhibition of firing are not clear, although actions of benzodiazepines at other allosteric sites on the $GABA_A$ receptor have been described.[24] Recovery from the inhibition was also rapid, with a return to baseline firing levels after approximately 7 min. These recoveries are much more rapid than those observed in previous studies with diazepam and flurazepam, where 30–50 min was required for firing to recover to predrug baseline firing rates.[20]

CNS 7056 induced rapid sedation in rats and mice, as

measured by LRR. The duration of LRR was short (< 10 min). In mice, there was a dose relation to the number of animals in which LRR was observed, the latency to onset of LRR, and the duration of LRR. The onset time was similar to that observed with midazolam. The duration of LRR induced by midazolam in both rats and mice was clearly longer than that observed with CNS 7056. Comparison with literature data shows that, over the dose range tested, CNS 7056 has a similar duration of LRR to that seen in mice with propofol (10–40 mg/kg intravenous; 2–15 min) or etomidate (5–15 mg/kg intravenous; 10–50 min).[25] No LRR was seen after intravenous administration of the carboxylic acid metabolite of CNS 7056, CNS 7054, at doses up to 100 mg/kg.

The $ED_{50}$ to inhibit firing in the SNpr of rats was 0.83 mg/kg. This was lower than the $ED_{50}$ to induce LRR in mice (approximately 25 mg/kg). The reasons for this discrepancy are not clear but could be because the nigral firing is more sensitive to inhibition by benzodiazepines or simply due to species differences (mouse *vs.* rat).

Flumazenil is an antagonist of benzodiazepine ligands; as such, it is a useful agent to overcome overdoses of benzodiazepines or to reverse their effects, *e.g.*, after a short diagnostic procedure. In our studies, as anticipated, pretreatment of mice with flumazenil blocked the LRR induced by CNS 7056. In this part of the mouse study, a longer LRR was observed for CNS 7056 than in the dose–response part. We believe that this difference is due to the steep dose–response curve observed and interexperimental variability.

We conclude that CNS 7065 is a high-affinity and selective ligand for the benzodiazepine site on the $GABA_A$ receptor. It enhances GABA currents in cells stably transfected with subtypes of the $GABA_A$ receptor and, like midazolam and other classic benzodiazepines, shows similar activity at the four subtypes tested ($\alpha_1\beta_2\gamma_2$, $\alpha_2\beta_2\gamma_2$, $\alpha_3\beta_2\gamma_2$, $\alpha_5\beta_2\gamma_2$). CNS 7056 is a potent sedative in rodents, with a short duration of action. Inhibition of SNpr firing and inhibition of the sedative effects of CNS 7056 by flumazenil show that its site of action is the central benzodiazepine receptor. Further investigation of CNS 7056 to explore its utility as a short-acting sedative and intravenous anesthetic are clearly justified. Preliminary studies to evaluate the *in vitro* metabolic profile and the *in vivo* pharmacokinetic profile of CNS 7056 have already been conducted and have been published in abstract form.[26,27]

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