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Imaging the spread of reversible brain inactivations using fluorescent muscimol

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Abstract

Muscimol is a $GABA_A$-agonist that causes rapid and reversible suppression of neurophysiological activity. Interpretations of the effects of muscimol infusions into the brain have been limited because of uncertainty about spread of the drug around the injection site. To solve this problem, the present study explored the use of a fluorophore-conjugated muscimol molecule (FCM). Whole-cell recordings from horizontal brain slices demonstrated that bath-applied FCM acts like muscimol in reversibly suppressing excitatory synaptic transmission. Two types of *in vivo* experiments demonstrated that the behavioral effects of FCM infusion are similar to the behavioral effects of muscimol infusion. FCM infusion into the rat amygdala before fear conditioning impaired both cued and contextual freezing, which were tested 24 or 48 hr later. Normal fear conditioning occurred when these same rats were subsequently given phosphate buffered saline infusions. FCM infusion into the dorsomedial prefrontal cortex impaired accuracy during a delayed-response task. Histological analysis showed that the region of fluorescence was restricted to 0.5 to 1 mm from the injection site. Myelinated fiber tracts acted as diffusional barriers, thereby shaping the overall spread of fluorescence. The results suggest that FCM is indeed useful for exploring the function of small brain regions.

Keywords

$GABA_A$; Agonist; lesions; fear conditioning; amygdala; prefrontal cortex

1.0 Introduction

Reversible inactivations of small volumes of brain tissue have been used to explore fundamental questions in behavioral and systems neuroscience (Majchrzak and Di Scala,

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2000). Reversible inactivations are an important alternative to permanent brain lesions (Jarrard, 2002) in assessing structure-function relationships. The obvious advantage is that reversible brain inactivations allow within-subjects designs. One common disadvantage is that this method typically leaves considerable uncertainty about the size and shape of the volume of the drug-infused tissue.

Muscimol, a $GABA_A$-agonist (Beaumont et al., 1978), is commonly and productively used for reversible inactivations (Lomber, 1999; Martin and Ghez, 1999; Krupa and Thompson, 1997). However, the spatial extent of a muscimol infusion is indeterminate without the use a radioactive tracer ([^{3}H]muscimol; Martin, 1991; Edeline et al., 2002). Indirect estimates about the spread of muscimol have been obtained by measuring its anticipated functional consequences on glucose uptake (Martin, 1991), evoked field potentials (Wilensky et al., 2006), and multiunit activity levels (Arikan et al., 2002; Edeline et al., 2002). Practical considerations have precluded the routine use of either radioactive or functional techniques (but see Krupa and Thompson, 1997).

Here we evaluated the use of a fluorophore-conjugated muscimol molecule (FCM) for producing local and reversible brain inactivations. FCM was first used in conjunction with muscimol to assess the role of dorsomedial prefrontal cortex (dmPFC) in a delayed-response task (Narayanan and Laubach, 2006; Narayanan et al., 2006). Here, the evaluation of FCM made use of whole-cell recordings, well-characterized brain-behavior relationships, and optical quantification methods. The results show that FCM can serve as a stand-alone and practical substitute for muscimol.

2.0 Materials and Methods

2.1 Subjects

This study used a total of 15 rats, described for each experiment below. Procedures were approved by the Yale University Animal Care and Use Committees (whole-cell, acute infusion, and fear conditioning) and the John B. Pierce Laboratory (delayed-response task).

2.2 Drugs

Muscimol was acquired from Sigma-Aldrich. FCM (commercially designated as Muscimol-TMR-X) was acquired from Molecular Probes (product #M23400, www.molecularprobes.com). FCM is a conjugate of muscimol and the Bodipy® TMR-X fluorophore (covalent amide bonding). FCM is stable and will not dissociate into its constituent parts in solution. The Bodipy® TMR-X portion of FCM is highly lipophilic (personal communication, Molecular Probes, Technical Support, Carlsbad, CA). FCM has excitation and emission peaks, respectively, at 543 nm and 572 nm. Figure 1 shows the structures, formulas, and molecular weights of FCM and muscimol.

For both acute infusions and fear conditioning experiments, 1 mg of FCM was dissolved into 2 mL of 0.01 M phosphate buffered 0.9% saline (PBS), resulting in a 0.8 mM concentration. For the brain-slice experiments, this stock solution was then diluted in artificial cerebral spinal fluid (aCFS) to a final bath concentration of 10 μM. For the delayed-response experiments, 1 mg FCM was dissolved in 1 mL of 0.9% saline, resulting in a 1.6 mM concentration. FCM concentrations higher than 1 mg/ml are possible, using dimethyl sulfoxide (DMSO) as a solvent, but were not explored here.

2.3 Whole-Cell Recordings

Whole-cell recordings were performed in horizontal brain slices that included the medial temporal lobe. The methods have been described in extensive detail elsewhere (Faulkner and

Brown, 1999; Moyer and Brown, 1998; 2002; 2007). Briefly, male Sprague-Dawley rats (n = 3; 22 – 32 days) were deeply anesthetized with halothane and decapitated. The brain was quickly removed and placed in ice-cold oxygenated (95% O2/5%CO_2) sucrose-aCSF containing (in mM): 206 sucrose, 2.8 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 2 $MgSO_4$, 1.25 NaH_2PO_4, 26 $NaHCO_3$ and 10 D-glucose for about 3 min. Horizontal slices (300 – 400 μm) containing perirhinal cortex (PR; approx. −7.8 D/V) were cut at ~1° C using a temperature-controlled vibrotome (Vibrotome 3000, Vibrotome Company Inc., MO).

Slices were moved to an incubation chamber at room temperature (22 – 26° C) for at least an hour. The oxygenated incubation aCSF contained (in mM): 124 NaCl, 2.8 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 2 $MgSO_4$, 1.25 NaH_2PO_4, 26 $NaHCO_3$ and 10 D-glucose, pH 7.4, 295 mOsmol. Individual slices were transferred to a submerged-type recording chamber and perfused with oxygenated aCSF. Recordings were done at room temperature. PR was visualized using an upright microscope (Zeiss Axioskop) equipped with infrared-filtered light and differential interference contrast optics. A bipolar stimulating electrode was positioned into layer I of PR. The whole-cell recording pipette (3 – 5 MΩ) was directed to layer V of PR. The pipette solution contained (in mM): 110 K-gluconate, 10 HEPES, 1.0 EGTA, 20 KCL, 2.0 $MgCl_2$, 2.0 Na_2ATP, 0.25 Na_3GTP and 10 Dphosphocreatine (d-Tris), pH 7.4, 290 mOsmol (Moyer and Brown, 2007).

2.4 Surgery

Rats (described for specific experiments below) were anesthetized with injections (*i.p.*) of either a cocktail of ketamine (100 mg/kg) and diazepam (10 mg/kg), or a cocktail of ketamine (100 mg/kg) and xylazine (10 mg/kg). Anesthesia was maintained at surgical levels throughout surgeries with supplements of ketamine (30 mg/kg), approximately every hour. Under aseptic conditions, the scalp was retracted, and the skull was leveled between bregma and lambda. Craniotomies were made above the target cannula sites. A microsyringe or guide cannula (26-guage; Plastics One, Roanoke, VA) was lowered into the region of interest: basolateral nucleus of the amygdala (BLA; coordinates from bregma: −3.0 A/P, ± 5.0 M/L, −7.5, D/V); PR (coordinates from bregma: −4.5 A/P, − 6.8 M/L −7 D/V at 45° from the medial plane); or dmPFC (coordinates from bregma: −3.2 A/P, ±1.4 M/L, −3.7 D/V at 12° in the frontal plane). After guide cannula were positioned, craniotomies were sealed with cyanoacrylate (SloZap, Pacer Technologies, Rancho Cucamonga, CA) accelerated by ZipKicker (Pacer Technologies), and/or methyl methacrylate (i.e., dental cement; AM Systems, Port Angeles, WA). Rats were given buprenorphine subcutaneously (0.05 mg/kg) every 12 hours after surgery for analgesia for 36–48 hours and allowed to recover in their home cages.

2.5 Microsyringe infusions

Four male rats (Sprague-Dawley) weighing approximately 300 – 400g were used in the acute infusion experiments. A micromanipulator (Sutter Instrument Company, Novato, CA) was used to position the tip of a hypodermic microsyringe (26 gauge, non-coring, Hamilton Co., Reno, NV) into BLA, PR or dmPFC. Rats received one BLA and one PR injection, each in a different hemisphere. One of the four rats received an additional dmPFC infusion in the same hemisphere as the PR infusion. A motorized infusing apparatus (Stoelting, Wood Dale, IL), attached to the micromanipulator, delivered fluid at a rate of 0.05 μL/min. The injected volumes were 0.5 μL for BLA and dmPFC, and 1 μL for PR. The holes in the skull were covered with a thin layer of bone wax and the overlying skin was sutured. Rats were monitored and given analgesics as stated above. Subjects were allowed to recover for 24 hr and perfused.

2.6 Cannula infusions

Chronic Infusions of FCM were made with a 33-gauge injector cannula (Plastics One). The injector cannula extended 1 mm from the tip of the cannula guide when the BLA was targeted

and extended 0.2 mm from the cannula tip when the dmPFC was targeted. Rats were restrained by hand or lightly anesthetized using halothane. Injector cannula were inserted into the guide cannula. The infusion fluid (0.5 μl) was delivered into each site at a rate of 15 μpl/hr (0.25 μL/min; Martin and Ghez 1999) via a syringe infusion pump (PHD 2000 Infusion, Harvard Apparatus, Holliston, MA). The infusion fluid was PBS, saline (0.9%; Phoenix Scientific, St. Joseph, MO), muscimol (1 μg/μl), or FCM (0.5 –1 μg/μl), specified for each experiment below. The fluid was infused via polyethylene tubing (0.38 mm diameter; Intramedic, New York, NY) that was attached to the infusion cannula on one end and to a 10 μl Hamilton syringe (Hamilton) on the other end. Injections were confirmed by monitoring movement of fluid in the tubing via a small bubble. After infusions were complete, the infusion cannula was left in place for 2 –3 min. Behavioral studies began 30 –45 minutes after infusion. Previous physiological studies have suggested that the effects of muscimol begin almost immediately and are stable for many hours (Hikosaka and Wurtz, 1985; Krupa et al., 2004).

2.7 Histology

Histological analysis identified the locations of the cannula tips and quantified the spread of FCM. Subjects were given an overdose of sodium pentobarbital (50 mg/kg, i.p.) and perfused with 50 mL of 0.01 M phosphate buffered, 0.9% saline followed by 100 mL of 4% paraformaldehyde. Brains were removed and cryoprotected in 30% sucrose. Coronal sections (50 μm thickness) were acquired with a freezing microtome (AO860; Rankin Biomedical). FCM was visualized using a Zeiss M^2 Bio Fluorescence Microscope (Carl Zeiss, Thornwood, NY) that was equipped with a high-intensity lamp (Zeiss) and FITC and Texas Red filter sets (Zeiss). Images were acquired with a computer-assisted CCD camera (AxioCam HRc; Zeiss) imaging system (AxioVision CD 28; Zeiss). Exposure times were adjusted to maximize image quality between 2 and 8 seconds and did not affect the visualized area of fluorescence. Dark-field images of PBS-soaked slices allowed the visualization of fibers for gross histological localizations, in acute infusions and fear conditioning experiments. Alternatively, in the delayed-response task, slices were counterstained with a fluorescent-green Nissl (Neurotrace®; Molecular Probes). Images were reassembled (by direct insertion without manipulation into red and green color channels) using Adobe Photoshop (Adobe Systems, San Jose, CA). Measurements of drug spread were estimated from equally-spaced 50 μm sections. Red channels were loaded in MATLAB and image averages were computed based on custom scripts (delayed-response task).

2.8 Fear Conditioning

Four male Sprague-Dawley rats weighing approximately 300 –400g were used in the fear conditioning experiment. Rats were singly housed with *ad libitum* access to food and water. Rats were handled prior to and after surgical implantation of the guide cannula. The delay fear conditioning procedures used here were similar to those described in detail previously (Lindquist et al., 2004). Briefly, the conditional stimulus (CS) was a 22 kHz ultrasonic vocalization (USV; 7.712 s duration) pre-recorded from a conspecific (Anderson, 1954; Brudzynski, 2007; Allen et al., 2007; Furtak et al., 2007). The unconditional stimulus (US) was a 0.4 s, 0.8 mA foot shock delivered through a standard grid floor (Coulbourn Instruments). Rats were placed in the conditioning chamber and given 2 min to habituate to the experimental context. Next they were given 5 conditioning trials (ITI = 180 ± 30 s) in which the CS always coterminated the US.

Freezing served as the conditional response (CR; Blanchard and Blanchard, 1969). Behavior was recorded for offline analysis with a miniature IR-CCD camera (Circuit Specialists, Mesa, AZ) digitized and encoded in MPEG-2 format (WinTV-PVR, Hauppauge, NY). Video files were stored and viewed on a personal computer. Twenty-four and 48 hours after conditioning subjects were tested for CS-elicited freezing in a shifted context or context-elicited freezing in

the original context. The order of testing sessions was counterbalanced among animals. Freezing was scored manually by a research assistant blind to all experimental conditions.

The fear conditioning procedures were performed once with pre-training FCM infusions (0.5 μL) into BLA and then were repeated 72 hr later with PBS infusions (vehicle) into BLA (always in that order). See Table I for the behavioral and infusions schedule used in the fear conditioning experiments. A day after completion of all behavioral experiments, rats were given second infusion of FCM and perfused 40 –45 min later.

2.9 Delayed-Response Task

Four male Brown-Norway rats (5 –10 months old) were trained to perform a delayed-response task. Rats were motivated by water restriction, while food was available *ad libitum*. Rats received 10 –15 ml of water during each behavioral session as reward. Additional water (5–10 ml) was provided 1 –3 hours after each behavioral session in the home cage. Rats were maintained at ~90% of their free-access body weights during the course of these experiments and received one day of free access to water per week.

The apparatus and methods for the delayed-response task have been described extensively elsewhere (Naranayan et al., 2006; Naranayan and Laubach, 2006). Briefly, when the chamber light turned, a trial of the delayed-response task was initiated by the rat depressing a lever and ended when the lever was released. The delay period was 1.2 s (Figure 5A). A trigger stimulus was given on 50% of trials (8 kHz tone). Correct responses occurred when the rats successfully maintained a lever press for the full foreperiod duration and released the lever within a pre-defined response latency (within 600 ms after the end of the foreperiod). Each correct response in the task activated the water pump at 0.03 ml/s for 1–2 s, depending on the stage of training. Premature responses occurred when rats did not maintain a lever press for the full foreperiod, and late responses occurred when the lever was released after the response window. These latter two types of trials were followed by a timeout period, in which all experimental stimuli were extinguished for 4–8 s. Behavior was monitored throughout via a closed-circuit video camera. See Table II for the daily procedures in the delayed-response task infusion experiments.

Forty-five minutes prior to the last testing session, FCM (0.5 μl at a concentration 1 μg/μl) was bilaterally infused into dmPFC. Rats performed the reaction time task for 30 minutes, after which they were deeply anesthetized and transcardially perfused.

3.0 Results

3.1 Effects of FCM on Excitatory Postsynaptic Potentials and Input Resistance

Horizontal rat brain slices were used to verify that FCM, like unlabeled muscimol, does in fact suppress neuronal and synaptic function. Whole-cell recordings were made from neurons in layer V of PR. Synaptic responses were evoked in these neurons by stimulating layer I of PR. The complete experimental protocol was conducted on eleven healthy cells recorded from 3 rat pups. In each case, bath application FCM (10 μM in aCSF) caused an immediate and rapid suppression of excitatory synaptic transmission and a parallel reduction in the input resistance (R_N) of the postsynaptic neuron (Figure 2). In each of the eleven cells, the maximum effect occurred 15 –25 min after the start of the infusion (the 1st and 2nd time points that were measured). Excitatory postsynaptic potential (EPSP) amplitudes were reduced by 45 – 64% of their baseline levels. Recovery of R_N and EPSP amplitudes required 2 – 3 hours. Thus the results show that FCM, like muscimol, reversibly depresses neural function.

3.2 Asymmetrical spread of FCM

The spread of FCM was analyzed in four rats that received acute infusions of FCM into BLA, PR and/or dmPFC. Infusions were performed using a microsyringe. Rats recovered for 24 hr after the surgery, were deeply anesthetized and then given transcardiac perfusions. Brains were removed and incubated in 4% paraformaldehyde for 24 hr. The brains were cryoprotected in 30% sucrose for 72 hr. Slicing and imaging occurred immediately after the incubation periods.

FCM was visualized in coronal brain slices (50 μm thickness; Figure 3). Notably, infusions into different brain regions resulted in different diffusion gradients. Figure 3 highlights one such infusion in PR in which the spread of the infusion was impeded medial to the infusion site. This caused asymmetrical fluorescence in the medial-lateral axis. The deep cerebral white matter (dcw) appears to act as a diffusion barrier (Figure 3C). Restricted diffusion through white matter may be related to the lipophilic properties of the fluorophore portion of FCM. An asymmetrical spread was also observable in BLA and dmPFC infusions. Thus the shape of the affected tissue depends on the injection site.

3.3 Infusion of FCM into BLA impairs Fear Conditioning

The functional effect of FCM infusion was tested using a well-characterized brain-behavior relationship. Fear conditioning gives robust results that are known to depend upon normal functioning in the BLA (for review LeDoux; 2000; Maren, 2001). Histological analysis showed FCM to be well-localized within BLA in 3 of 4 rats (Figure 4C). One rat showed no evidence of fluorescence in brain slices and was thus excluded from the behavioral analysis. This animal showed robust cue and context conditioning. In the remaining rats, pre-training infusions of FCM resulted in little freezing to the 22 kHz USV (7%) or the conditioning context (9%). The FCM effect size (*d*; Cohen, 1988) on conditioning was very large for both cue conditioning ($d = 4.7$) and context conditioning ($d = 3.8$).

The inhibition of BLA was reversible. Re-conditioning 3 days later, with pre-training PBS (vehicle) infusions, resulted in robust levels of freezing to both the 22 kHz USV (60%) and the conditioning context (91%). A repeated-measures ANOVA revealed a main effect of Infusion Type (PBS versus FCM), $F_{(1,2)} = 36.288$, $p < 0.02$. The mean levels of freezing to the cue and context were not significantly different, $F_{(1,2)} = 0.966$, $p = 0.43$. There was no interaction between the Infusion and Stimulus Type, $F_{(1,2)} = 1.230$, $p = 0.38$. These results show that pre-training infusions of FCM into BLA reversibly block normal fear conditioning to both a cue and a context (Figure 4B), as is true for pre-training muscimol infusions into BLA (Helmstetter and Bellgowan, 1994; Muller et al., 1997; Wilensky et al., 2002; Wilensky et al., 2006).

3.4 Infusions of FCM into dmPFC in a Delayed-Response Task

We also assessed the behavioral effect of FCM infusions into dmPFC. This assessment used an instrumental task on which performance is known to be impaired by muscimol infusion into dmPFC (Narayanan et al., 2006; Narayanan and Laubach, 2006). Rats were trained to asymptotic performance over days. After asymptotic performance was achieved, saline infusions were made bilaterally into dmPFC. Following saline infusions, rats made correct responses on 53 ± 3% (mean ± SD) of trials. Twenty-four hours later, muscimol was infused into dmPFC. Following muscimol infusions, rats made correct responses on 18 ± 12% of trials. The performance decrease was significant (paired $t_{(3)} = 6.78$, $p < 0.01$). A repeated-measures ANOVA revealed that rats made more premature responses in muscimol sessions relative to saline sessions ($F(1,1143) = 110.43$, $p < 0.001$). After recovery, FCM was infused into dmPFC. Rats made correct responses on 29 ±11%, significantly less than in saline sessions (paired $t_{(3)} = 4.76$, $p < 0.02$; Figure 5B). Repeated-measures ANOVA revealed that rats made

significantly more premature response following FCM infusions than following saline sessions ($F(1,1190)= 21.88$, $p < 0.001$).

Successful performance was not significantly different between muscimol infusion sessions and FCM-infusion sessions (paired $t_{(3)} = 2.72$, $p > 0.05$). A repeated-measures ANOVAs revealed that rats made significantly more premature responses following muscimol infusions than after FCM infusions ($F_{(1,780)}= 26.74$, $p < 0.001$). This effect might reflect the fact that the drug concentration was higher for muscimol (8.8 mM) than for FCM (1.6 mM). These sizable concentration differences could affect the amount of inhibition at the infusion site as well as the spatial gradient of the inhibition. Monitoring of behavior prior to and during delayed-response performance revealed that dmPFC inactivations with FCM caused no gross changes in alertness, locomotion, stereotyped behavior, paw movements or activity levels.

Histology showed that all cannula placements were located in dmPFC. FCM was well-restricted to dmPFC, with the exception of a small amount of fluorescence that was sometimes evident around the guide cannula, near anterior cingulate cortex (Figure 5C). Further histological analysis quantified the average spread of FCM. An average fluorescence was computed from 8 slices taken from 4 animals (Figure 5D). Fluorescence was consistently observed ~0.5 mm from the cannula tip in the medial-lateral axis and ~0.7 mm in the dorsal-ventral axis.

4.0 Discussion

4.1 FCM reversibly depresses synaptic transmission

Whole-cell recordings in PR showed that bath application of FCM decreased the mean amplitude of EPSPs and also decreased RN (Figure 2). These results show that the desired neuropharmacological action persists after conjugating muscimol with a large fluorophore. Following washout of FCM from the bath, the recovery of neural function required hours (Figure 2). The slow reversal may result from the lipophilic properties of the Bodipy-TMR-X fluorophore®, as discussed below.

4.2 FCM spread is not always uniform

The spread of FCM can be irregularly shaped, depending on the microanatomy surrounding the infusion site. Figure 3 shows an example in which a 1 μL infusion of FCM into PR cortex resulted in asymmetrical spread. In this case, the diffusion of FCM was impeded by a large fiber bundle that runs along the medial border of PR. Such diffusion barriers can be productively used to restrict the drug to a region of interest. For two reasons, the spread of FCM from the injection site may be much more limited than the spread of muscimol. First, the molecular weight of FCM is approximately 6 times larger than the molecular weight of muscimol. Second, FCM may dissolve in lipid-rich myelinated fibers and cell membranes, which could also slow diffusion.

4.3 FCM infusion into BLA reversibly impairs fear conditioning

Fear conditioning is an ideal task for assessing FCM because much is known about the neurobiology of fear conditioning. In particular, the BLA is essential for normal fear conditioning. This has been shown using both permanent lesions and reversible inactivations (LeDoux, 2000; Maren, 2001). Pretraining infusions of BLA with FCM significantly impaired fear conditioning (Figure 4).

Infusions of FCM into BLA prior to fear conditioning resulted in almost no cued or contextual freezing during testing sessions, which occurred 24 and 48 hr later. Thus fear conditioning to a 22 kHz USV depends on the functioning of BLA at the time of acquisition. Three days later,

re-conditioning trials were given with pre-training vehicle infusions. Reconditioning resulted in normal levels of freezing to the cue and context (24 or 48 hr later), showing that the FCM inhibition is temporary. Similarly, previous experiments have shown that muscimol infusions into BLA temporarily impair fear conditioning (Helmstetter and Bellgowan, 1994; Muller et al., 1997; Wilensky et al., 2002; Wilensky et al., 2006). Thus both FCM and muscimol infusions into BLA reversibly impair fear conditioning.

4.4 dmPFC infusions of FCM and muscimol have similar effects

The use of FCM for reversible inactivation was further accessed during performance on a delayed-response task. In each subject, the effects of FCM were directly compared to the effects of muscimol. Both compounds impaired performance to nearly the same extent (Figure 5B). Performance recovered 24 hr later. Muscimol infusions resulted in greater number of premature responses compared to FCM infusions. This difference might be due to a smaller diffusion radius of FCM compared to muscimol.

4.5 Conclusions

A fluorescently-conjugated muscimol molecule was assessed for use in visualizing the spread of reversible brain inactivations. FCM was evaluated physiologically, behaviorally, and optically. We conclude that FCM is valuable for visualizing the spatial gradient of reversible brain inactivations. FCM is likely to find many productive uses in the fields of systems and behavioral neuroscience. By combining FCM infusions with single-unit recordings, it should be possible get insights into the relative contributions of one brain area to the firing patterns in another (see Krupa et al., 2004; Naranayan and Laubach, 2006).

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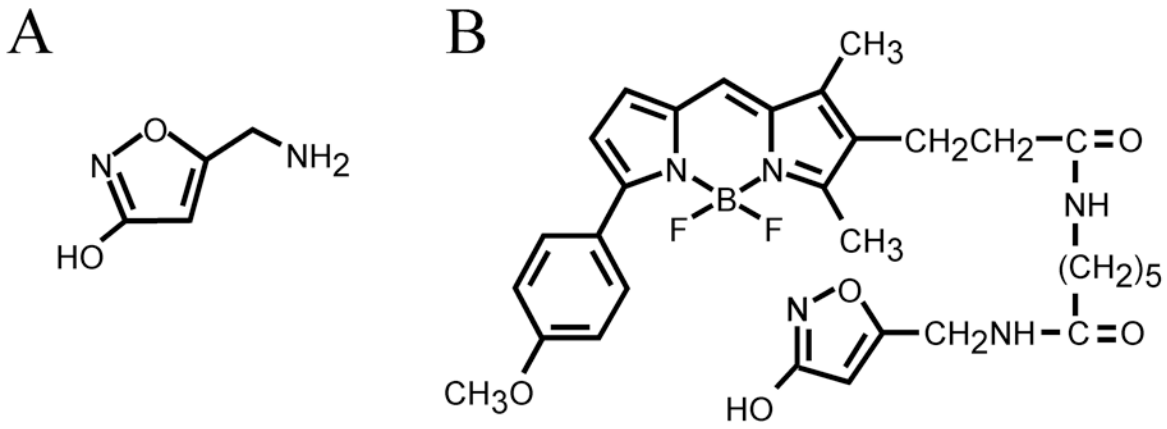

Figure 1.

Molecular structure of muscimol compared to fluorescent muscimol. A, Structure of muscimol. The molecular formula is $C_4H_6N_2O_2$. The molecular weight is 114.10. B, Structure of Bodipy-TMR-X®-conjugated muscimol (FCM). The molecular formula is $C_{31}H_{36}BF_2N_5O_5$. The molecular weight is 607.46. The structure of FCM was reproduced with permission from Molecular Probes (Molecular Probes, Carlsbad, CA).

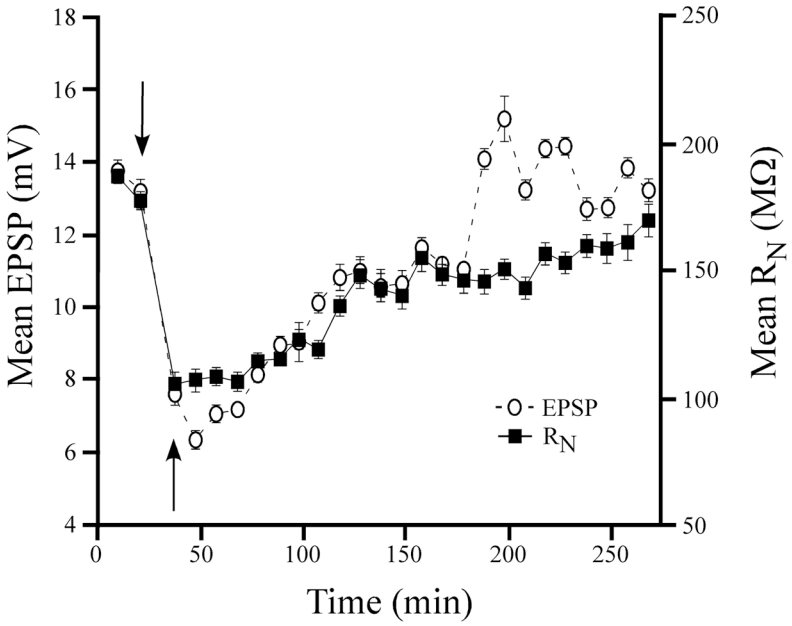

Figure 2.

Parallel changes in the excitatory postsynaptic potential (EPSP) amplitude and the input resistance (R_N) in a layer V perirhinal neuron. The left ordinate shows EPSP amplitudes, which are plotted as a function of time (open circles connected by a dashed line). The right ordinate shows concurrent input resistance (filled squares connected by a solid line). Each point is the average of ten measurements. Error bars are ± 1 SEM. FCM was bath applied at 20 min (downward arrow). Washout started at 35 min (upward arrow). Infusion with FCM caused a rapid and large reduction in the EPSP amplitudes and a parallel decline in the input resistance. Following washout (upward arrow), the input resistance and EPSP amplitude recovered over the time course of hours.

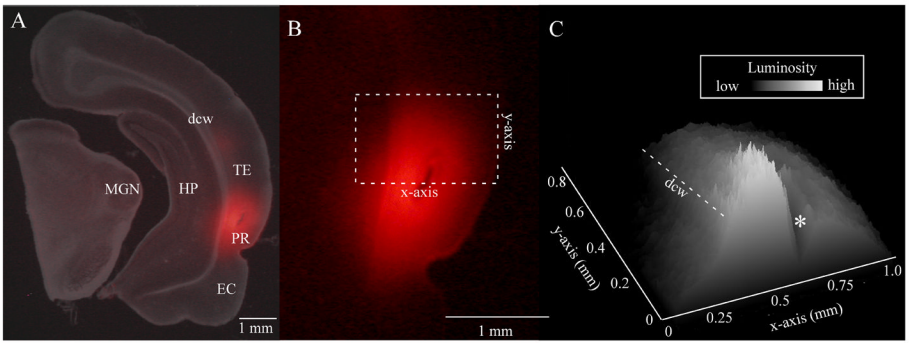

Figure 3.

Infusions of FCM can result in asymmetrical diffusion gradients around the injection site. The example shown here is a 1 μL infusion of FCM into area 36 of perirhinal cortex. A, A coronal slice of the right hemisphere at approximately −4.7 A/P, −6.8 M/L −6.7 D/V of the Paxinos and Watson atlas (1998). The image is an overlay of a dark field image of PBS-moist tissue and the FCM. B, A magnified image of the FCM spread. There is a hint that the spread of fluorescence was impeded by white matter. The checkered box indicates the area analyzed for luminosity. C, Quantification of the luminosity of the FCM. The asterisk to the right of the peak fluorescence indicates the location of damage from the infusion needle. The highest intensity of fluorescence is represented by the white peak at 0.5 mm (x-axis) by 0.0 − 0.2 (y-axis). The dashed line represents the approximate location of deep cerebral white matter medial to the infusion site. The white matter acted as a diffusion barrier, causing a rapid drop in luminosity. Abbreviations: dcw, deep cerebral white matter; MGM, medial geniculate nucleus of the thalamus; HP, hippocampus; TE, temporal cortex; PR, perirhinal cortex; and EC, entorhinal cortex.

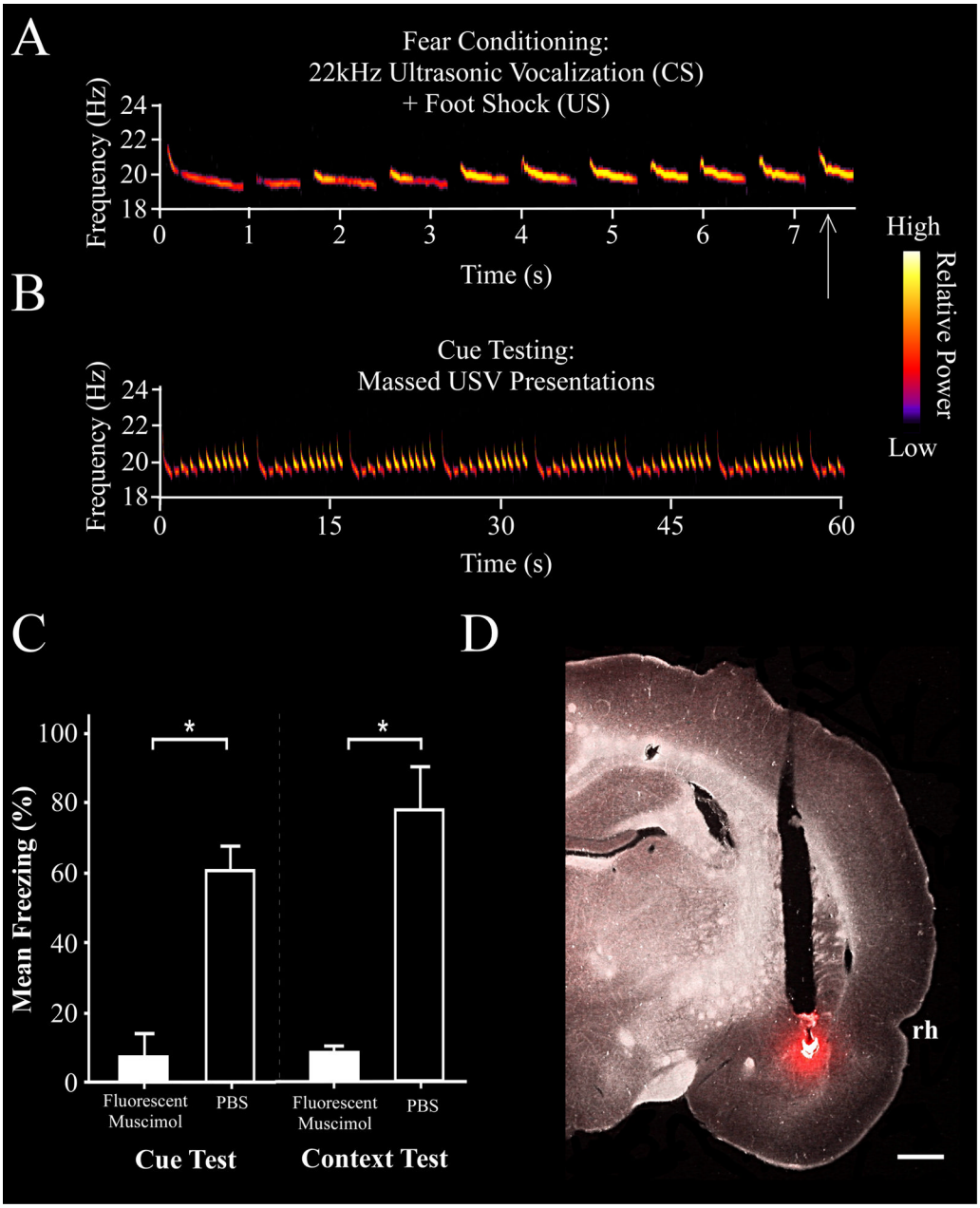

Figure 4.

Effect of pre-training infusions of fluorescent muscimol (FCM) into the basolateral nucleus of the amygdala (BLA) on cued and contextual fear conditioning (n = 3). A, Spectrogram of the cue, a 22 kHz ultrasonic vocalization (USV) that was prerecorded from a conspecific. The arrow indicates the time of a 0.4 s, 0.8 mA foot shock that coterminated with the cue. B, During testing, the USV was presented repeatedly for 360 s (60 s is shown). C, Conditioning in rats infused with FCM or vehicle (PBS). Cue conditioning was tested in a shifted context. FCM severely impaired the acquisition of cued and contextual fear conditioning, similar to what has been previously reported using muscimol. Normal fear conditioning occurred after PBS infusions. D, Image of FCM infusion into BLA. The red fluorescence is true color, overlaid with PBS-moist tissue for gross histological localization. The fluorescence is well-restricted to BLA. Calibration bar is 1 mm. Abbreviation: rh, rhinal sulcus.

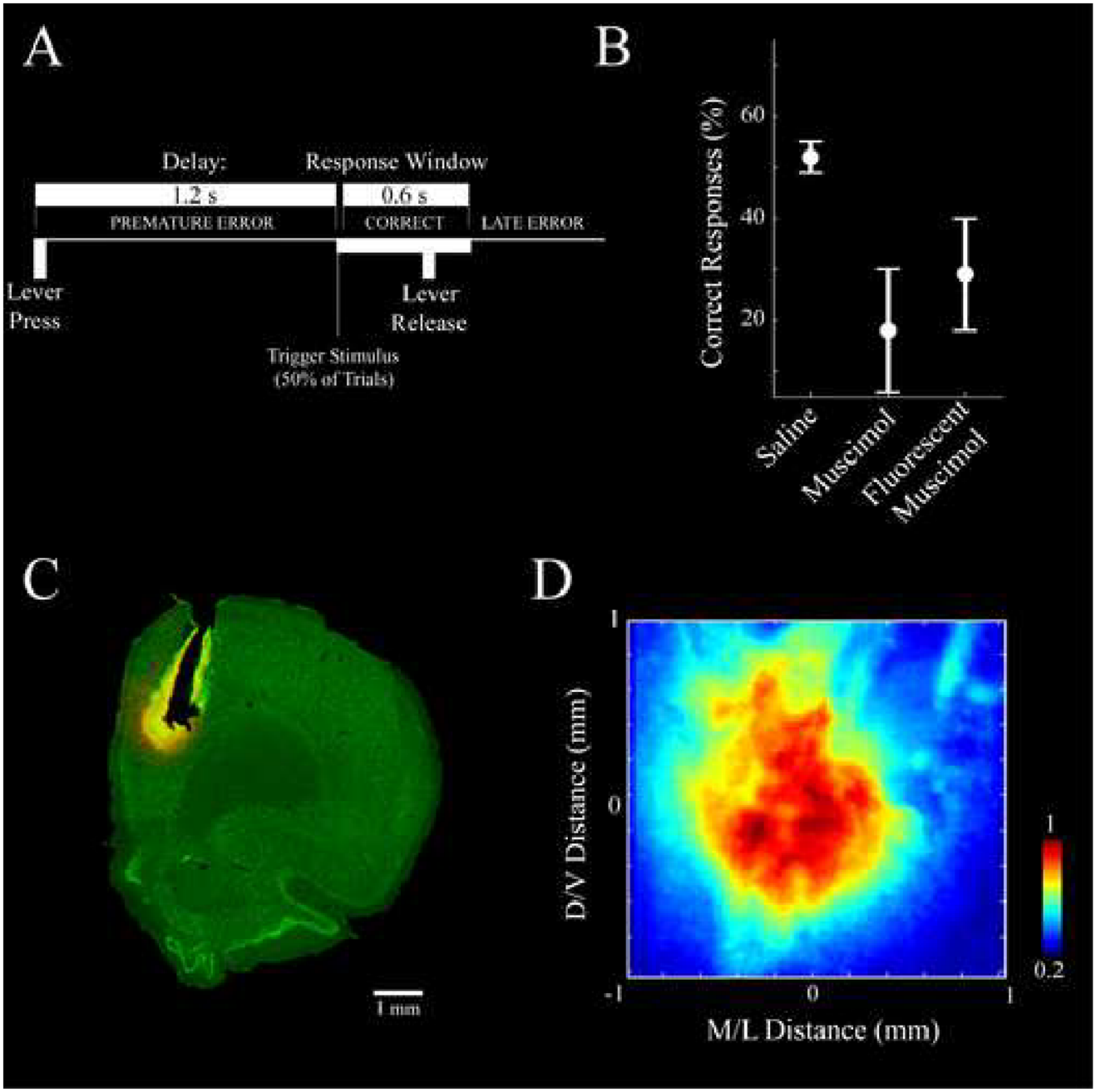

Figure 5.

Performance effects of FCM infusions into the dorsomedial prefrontal cortex (dmPFC) on a delayed-response task (n = 4). The coordinates used for the dmPFC are as follows: AP: +3.2, DV −3.7, ML ± 1.4. Infusions of both muscimol and FCM into dmPFC significantly decreased the number of correct trials. The majority of the errors were premature responses (Naranayan et al., 2006; Naranayan and Laubach, 2006). A, A schematic representation of the trials in the delayed-response task. B, The percentage of correct responses after infusion of saline, muscimol and FCM. C, A sample FCM infusion into the dmPFC. The slice is counterstained with a green fluorescent Nissl (Molecular Probes). D, A false-color plot of the spread of FCM averaged over 8 slices from 4 rats. The images are aligned to the highest-intensity fluorescence

in each slice. Fluorescence intensity is normalized within each slice. The spread of fluorescence was 0.5 – 1.0 mm around the injection site.

Table I

Daily fear conditioning procedures.

DAY	PROCEDURE
1	Surgery: Bilateral BLA cannula implantation
2 – 5	Recovery
6 – 10	Handling
11	Pre-training FCM infusions and 1st fear conditioning session
12	Testing Day 1 (Cue or Context)
13	Testing Day 2 (Cue or Context)
14	Pre-training PBS infusions and 2nd fear conditioning session
15	Testing Day 3 (Cue or Context)
16	Testing Day 4 (Cue or Context)
17 – 20	FCM Infusion, Perfusion, Histology and Imaging

Table II

Daily delayed-response task procedures.

DAY	PROCEDURE
1–3	Handling
4	Autoshaping
~5 – 7	Lever-press training
~7–14	Hold and release training in delayed-response task
15	Surgery: Bilateral dmPFC cannula implantation
16–22	Recovery
~22	Post-surgical reintroduction to delayed-response task
23	Pre-testing saline infusion in delayed-response performance
24	Pre-testing muscimol infusion in delayed-response performance
25	No infusion (recovery) in delayed-response performance
26	Pre-testing FCM infusion in delayed-response performance and perfusion
27–28	Histology and imaging