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The midbrain raphe nuclei mediate primary reinforcement via GABA_A receptors

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Abstract

Because rats learn to lever-press for brief electrical stimulation of the median and dorsal raphe nuclei (MRN and DRN, respectively), these brain sites have long been implicated in reward processes. However, it is not clear whether the MRN and DRN integrate reward-related signals or merely contain fibers of passage involved in reward processes. To shed light on this issue, the present study employed chemicals that selectively modulate neurotransmission, in particular the GABA_A receptor agonist muscimol. Rats quickly learned to lever-press for muscimol infusions (50 and 100 μ M) into the MRN or DRN. Muscimol was not self-administered when cannulae were placed just outside these nuclei. The reinforcing effects of muscimol appeared to be greater when the drug was administered into the MRN than into the DRN, as demonstrated by higher infusion rates and better response discrimination. These observations are consistent with the additional finding that muscimol administration into the MRN, but not the DRN, induced conditioned place preference. The reinforcing effects of muscimol administration into the MRN were blocked by coadministration of the GABA_A antagonist picrotoxin (100 μ M) and by pretreatment with the dopamine receptor antagonist SCH 23390 (0.025 mg/kg, i.p.). The present results suggest that median and dorsal raphe neurons presumably inhibited by muscimol via GABA_A receptors are involved in integration of primary reinforcement, and that median raphe neurons exert tonic inhibition over dopamine-dependent reward circuitry. The midbrain raphe nuclei may be involved in a variety of reward-related phenomena including drug addiction.

Keywords

conditioned place preference; intracranial self-administration; muscimol; rat; reward

Introduction

More than five decades ago, Olds & Milner (1954) discovered that rats readily learn to lever-press to receive brief electrical brain stimulation. This effect is now known as ‘brain stimulation reward’, and the associated behaviour as ‘intracranial self-stimulation’. Brain stimulation reward has given investigators an invaluable tool to study brain ‘reward’ systems. A large majority of self-stimulation research has focused on the lateral hypothalamic medial forebrain bundle, which contains ascending dopaminergic fibers of passage and supports fast and enduring self-stimulation. While the role of the median and dorsal raphe nuclei (MRN and DRN, respectively) in reward has not been as thoroughly investigated, stimulation of the MRN, DRN or their vicinity has been found to be as effective a reinforcer as stimulation of the medial forebrain bundle (Miliaressis *et al.*, 1975; Rompré & Miliaressis, 1985; Rompré & Boye,

1989). This finding suggests that the MRN and DRN are important elements of the brain reward circuitry.

Determining whether the MRN and DRN integrate reward-related signals or merely contain fibers of passage involved in reward signals is critical for understanding mechanisms of reward systems. Indeed, fibers of neurons containing acetylcholine and norepinephrine, which have been implicated in reward (Ikemoto & Wise, 2002; Olson *et al.*, 2006), run through the vicinity of these regions. On the other hand, Fletcher *et al.* (1993) showed that administration of the 5-HT_{1A} agonist 8-OH-DPAT into the MRN or DRN, which results in selective inhibition of serotonin synthesis and release in terminal regions, induces conditioned place preference, a measure of rewarding effects. In addition, selective injections of 8-OH-DPAT into the MRN facilitate intracranial self-stimulation at the lateral hypothalamic medial fore-brain bundle (Fletcher *et al.*, 1995). These studies suggest that selective inhibition of serotonergic neurons localized in the MRN and DRN is rewarding.

We sought to determine whether the MRN and DRN are in fact central to reward processes using intracranial self-administration procedures. Previous studies (Przewlocka *et al.*, 1979; Fink & Morgenstern, 1986; Wirtshafter & Klitenick, 1989) showed that muscimol administration into the MRN or DRN elicits heightened locomotion in rats. Such findings indicate possible rewarding effects of muscimol administration into these regions, because drug administrations or focal brain stimulations that are reinforcing tend to elicit exploratory behaviour in rats (Wise & Bozarth, 1987; Ikemoto & Panksepp, 1994). We first examined whether rats learn to self-administer muscimol into the MRN or DRN using a two-lever procedure. Although rats self-administered muscimol into these nuclei, rats failed to discriminate the lever that delivered muscimol infusions from the lever that did not. We then conducted additional experiments to examine possible rewarding effects of muscimol administration with a revised two-lever procedure with explicit cues and with a conditioned place preference procedure. We then examined the roles of GABA_A receptors and dopamine receptors in muscimol self-administration.

Materials and methods

The following experiments were conducted in two locations, experiment 1 in Indianapolis and experiments 2–6 in Baltimore. The two different testing sites resulted in some differences in materials and methods between experiment 1 and the rest. These differences, however, appear to be insignificant for the questions being investigated.

Subjects

Forty female Wistar rats (Harlan, Indianapolis, IN, USA) weighing 250–320 g at the time of surgery were used in experiment 1, and were individually housed and kept on a 12-h light–dark cycle (lights on at 09.00 h). Seventy-three male Wistar rats (Harlan, Dublin, VA, USA) weighing 280–350 g at the time of surgery were used in experiments 2–6, and were individually housed and kept on a reversed 12-h light–dark cycle (lights on at 21.00 h). Food and water were available *ad libitum* except during testing. Constant temperature and relative humidity were maintained in the colony and experimental rooms. The procedures were approved by the Animal Care and Use Committees of the institutions and were in accordance with National Institutes of Health guidelines.

Surgery

In experiment 1, a unilateral 22-gauge guide cannula was implanted in rats under halothane anaesthesia, aimed at the DRN, MRN and surrounding regions using the following coordinates (posterior distance from bregma, lateral distance from the midline and ventral distance from

skull surface were measured along the trajectory of the angled cannula). These respective coordinates (in mm), purposefully varied to survey in and around these nuclei, were: 6.8–8.0, 2.5–4.0, 5.0–6.9 with 20° for the DRN and its surrounding regions, and 6.8–8.0, 1.5–3.0, 7.8–8.4 with 10° for the MRN and its surrounding regions. While not in use, a 28-gauge stylet maintained the patency of the guide cannula. The stylet extended 0.5 mm beyond the tip of the guide. In experiments 2–6, rats were anaesthetized with sodium pentobarbital (31 mg/kg, i.p.) and chloral hydrate (142 mg/kg, i.p.) and implanted with a unilateral 24-gauge guide cannula that ended 1.0 mm above the target site. The target coordinates were 7.4, 1.6, 8.0 and 10° angle for the MRN, and 7.4, 2.6, 6.2 and 20° angle for the DRN. These stereotaxic surgeries were performed using the flat-skull method, in which bregma and lambda lie in the same coronal plane. Testing began 5–7 days after surgery.

Drugs

The GABA_A agonist muscimol hydrobromide and the GABA_A antagonist picrotoxin (Sigma Chemical Co., St Louis, MO, USA) were dissolved in an artificial cerebrospinal fluid consisting of (in mM) NaCl, 120.0; KCl, 4.8; KH₂PO₄, 1.2; MgSO₄, 1.2; NaHCO₃, 25.0; CaCl₂, 2.5; and d-glucose, 10.0 (experiment 1); or NaCl, 148; KCl, 2.7; CaCl₂, 1.2; and MgCl₂, 0.85 (experiments 2–6). When necessary, pH levels were adjusted to 7.3 ± 0.2 with NaOH or HCl. The D₁ receptor antagonist *R*(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrochloride (SCH 23390; Sigma) was dissolved in 0.9% saline.

Self-administration apparatus

Experiment 1 was conducted in standard operant chambers (Coulbourn Instruments, Allentown, PA, USA) equipped with two identical levers (35 mm wide, protruding 18 mm from the wall). The levers were 12 cm apart and 15 cm above a grid floor. The chamber was enclosed in a sound-attenuating box equipped with a ventilating fan. Delivery of infusions and monitoring of levers were controlled by a desktop computer equipped with an operant control system (L2T2 system; Coulbourn Instruments). Infusions were administered by the electrolytic microinfusion transducer (EMIT) system (Bozarth & Wise, 1980). Two platinum electrodes were placed in an infusate-filled cylinder container (28 mm in length × 6 mm in diameter) equipped with a 28-gauge injection cannula (Plastic One, Roanoke, VA, USA). The electrodes were connected via spring-protected cable and a swivel to a constant-current generator (MNC, Inc., Shreveport, LA, USA) which delivered 4–6 µA of quiescent current and 200 µA of infusion current between the electrodes. Electrodes were acclimatized to the infusate for several hours before being used for testing. Depression of the active lever delivered the infusion current for 5 s, which led to the rapid generation of H₂ gas, raised the pressure inside the airtight cylinder and, in turn, forced 100 nL of the infusate through the injection cannula, which protruded 1 mm beyond the tip of guide cannula.

Experiments 2, 3, 5 and 6 were conducted in standard operant chambers (Medical Associates, St Albans, VT, USA) equipped with two levers (45 mm wide, protruding 19 mm from the wall) and a cue light located just above each lever. The levers were 12 cm apart and 10 cm above a grid floor. The chamber was enclosed in a sound-attenuating box equipped with a ventilating fan. Each rat's 31-gauge injection cannula was connected by PE tubing to a micropump (Ikemoto & Sharpe, 2001) hanging a few millimeters above the rat's head. Each pump consisted of a miniature step motor and a small plastic reservoir and was connected to an electrical swivel. When activated, the motor advanced a shaft into the reservoir, displacing its infusate (75 nL) into the injection cannula.

Experiment 1: site- and concentration-dependent effects of muscimol self-administration

Each rat was placed individually in the operant chamber. To minimize air trapped at the tip of the injection cannula, an infusion current was delivered for 5 s as the injection cannula was inserted into the guide cannula. Depression of the 'active' lever resulted in the delivery of 100 nL infusate over a 5-s period followed by a time-out period (55 s), during which depression of the active lever produced no infusion. The time-out period was instituted to avoid continuous delivery of infusions, which may result in adverse drug effects. Depression of the 'inactive' lever had no programmed consequence. The assignment of left and right levers for active and inactive functions was counterbalanced among subjects and remained the same for each rat throughout the experiment. No cue was provided to signal the delivery or availability of infusions. No shaping technique was used to facilitate the acquisition of lever responses. The numbers of infusions and responses on the active and inactive levers were recorded, including lever presses during the time-out period. Over four sessions, each rat received four different concentrations of muscimol: 0, 25, 50 or 100 μM (i.e. 0, 2.5, 5 or 10 pmol or 0, 0.5, 1 or 2 ng per 100-nL infusion). The order of testing these concentrations was counterbalanced among rats. The numbers of responses on each lever and subsequent infusions were recorded. Sessions lasted 180 min or until the rats received a total of 40 infusions. The total number of possible infusions was limited to minimize possible adverse drug effects.

Experiment 2: two-lever discrimination effects

Each rat was placed in the operant chamber with two retractable levers. A response on the active lever resulted in a 5-s infusion (75 nL), turned on a tone, extinguished the light above the lever and retracted the active and inactive levers for 20 s. A response on the inactive lever retracted both the active and inactive levers for 20 s and resulted in no other programmed consequences. The assignment of left and right levers for active and inactive functions was counterbalanced among subjects and remained the same for each rat throughout the experiment. Each rat received vehicle in session 1 and 100 μM muscimol (i.e. 7.5 pmol or 1.5 ng per 75-nL infusion) in sessions 2–5. Sessions lasted 90 min or until the rats received a total of 60 infusions. Because we did not observe adverse effects of the drug in experiment 1, we made the total number of infusions greater (60) and timeout period shorter (20 s) in this experiment than experiment 1.

Experiment 3: replication of the failure to discriminate between two levers

Experimentally naïve rats were placed in the operant chamber with two retractable levers, and received 100 μM muscimol (75 nL) into the MRN over four sessions. The procedure of the first two sessions consisted of key features similar to those of experiment 1: a response on the active lever delivered an infusion of over 5 s followed by a 55-s time-out period, whereas a response on the inactive lever resulted in no programmed consequence. Responding on the levers did not result in lever retraction or provide any programmed cues for infusions. Sessions 3 and 4 used the same two-lever procedure described in experiment 2.

Experiment 4: conditioned place preference

The place-conditioning chamber (MED Associates) consisted of two compartments ($21 \times 21 \times 28 \text{ cm}^3$) and an area ($21 \times 21 \times 12.5 \text{ cm}^3$) connecting the compartments; a guillotine door separated each compartment from the connecting area. One compartment differed from the other in wall colour (black vs. white), floor type (net vs. grid) and lighting; the amount of light was modulated in each compartment so that rats did not prefer one compartment to the other prior to place conditioning. In session 1, experimentally naïve rats were placed individually in the chamber with guillotine doors open for 15 min without any treatment; each rat had access to both compartments, and the time spent in each compartment was recorded. In sessions 2–5, each rat was placed in a cylinder and received an intracranial injection (500 nL delivered over

60 s via a 31-gauge injection cannula extending 1 mm below the tip of the guide). An additional 30-s period elapsed before the injection cannula was removed. Each rat was then placed in one of the compartments with guillotine doors closed and remained there for 30 min; the placement into the two compartments was alternated over sessions. Muscimol (100 μ M) injections were paired with one compartment and vehicle injections with the other for the MRN and DRN muscimol groups. Vehicle injections were paired with both compartments for the MRN vehicle group. The order of drug treatments and the assignment of the compartments with drug treatments were counterbalanced among the subjects for each group. In session 6, the rats were placed individually in the chamber without an injection and given access to both compartments; the time spent in each compartment was recorded for 15 min. Sessions were separated by 24 h. Place preference scores were obtained by subtracting time spent in vehicle-paired compartment from time spent in drug-paired compartment. In order to obtain place preference scores in the vehicle group, one of the compartments was assigned as 'drug-paired' before obtaining data, even though the compartment was paired with vehicle for this group.

Experiment 5: GABA_A receptor mediation of muscimol self-administration

The subjects for this experiment were the same rats that completed experiment 2. Two of the 17 rats were eliminated because their self-administration rates of 100 μ M muscimol into median raphe nucleus were < 0.2 infusions per minute and therefore they were classified as nonresponders. Using the two-lever procedure described for experiment 2, the rats received vehicle, 100 μ M muscimol, and 100 μ M muscimol plus 100 μ M picrotoxin over three sessions. The order of testing these drugs was counterbalanced.

Experiment 6: dopamine-dependent effects of muscimol

The subjects for this experiment were the same rats that completed experiment 3. Two of the 13 rats were eliminated for the same reason mentioned for experiment 5 (see above). Results from two additional rats were eliminated from the data analysis because of low infusion number (< 3) when muscimol was substituted for vehicle alone; such low numbers prevented analysis of interinfusion intervals (see Results). Thirty minutes before the start of session 1, all rats were treated with 0.9% saline (1 mL/kg, i.p.), and received vehicle infusions. Thirty minutes before muscimol (100 μ M) self-administration in session 2, half of the rats received saline and the other half received SCH 23390 (0.025 mg/kg, i.p.). In session 3, pretreatments were reversed between the two groups and they received muscimol infusions.

Histology

Upon completion of experiment 1, all rats were killed by CO₂ inhalation and their brains were removed and frozen. The rats from experiments 2–6 were deeply anaesthetized with a mixture of sodium pentobarbital (31 mg/kg) and chloral hydrate (142 mg/kg), and their brains were removed and placed in 10% formalin. Frozen coronal sections (40 μ m) encompassing the cannula tracks were cut with a cryostat and stained with Cresyl Violet. Cannulae placements were confirmed by microscopic examination.

Statistical analyses

Detailed descriptions of statistical analyses are provided in figure legends. Newman–Keuls *post hoc* tests followed ANOVAs when significant interactions or main effects were found. Interinfusion interval data in experiment 6 were highly varied and, thus, were square-root-transformed to maintain homogeneity of variance.

Results

Experiment 1: site- and dose-dependent effects of muscimol self-administration

Rats quickly learned to self-administer muscimol when cannula placements were located within the DRN and MRN (Figs 1 and 2). When infusion sites were slightly lateral or dorsal to the DRN, or lateral or ventral to the MRN, rats did not self-administer muscimol. Based on the histological data, the cannula placements of each rat were classified into three groups: cannula tip (0.5 mm) in contact with the DRN, cannula tip in contact with the MRN, or cannula tip in the region outside the DRN and MRN. Figure 3A summarizes mean infusion levels as a function of concentrations of muscimol. Concentration-dependent effects of muscimol on infusions were found for cannula placements in the DRN and MRN, but not for the region outside of the DRN and MRN. Surprisingly, we did not find significant differences in the number of lever presses between active and inactive levers (Fig. 3B). In addition, the rats that obtained a significant number of muscimol infusions (> 20) into the MRN or DRN displayed hyperactivity, often accompanied by licking and nonaggressive biting (not quantified). These observations were consistent with previous findings of heightened locomotion induced by muscimol injections into the MRN and DRN (Przewlocka *et al.*, 1979; Fink & Morgenstern, 1986; Wirtshafter & Klitenick, 1989).

Experiment 2: two-lever discrimination effects

The arousal effects following repeated muscimol administration may have interfered with lever discrimination in experiment 1. To overcome arousal interference with lever discrimination, the present experiment implemented a 'penalty' for responding on the inactive lever that consisted of a retraction of both levers for a 20-s period in the absence of muscimol infusions; this 20-s timeout without reward would be punishing if rats expected to earn muscimol infusions. On the other hand, when the active lever was depressed both levers were also retracted for 20 s, but delivered 100 μ M muscimol accompanied by the extinction of a light cue above the lever and the presentation of a tone. Rats trained in this procedure learned to quickly discriminate between the two levers (Fig. 4A). In session 1, rats received vehicle infusions upon active-lever presses. As illustrated in event records of a representative rat (Fig. 4B), rats typically lever-pressed indiscriminately between the two levers during the first 40 min of the session and decreased responding thereafter. In sessions 2–5, they received 100 μ M muscimol into the MRN or DRN upon active-lever presses. Rats responded on the active lever more frequently than the inactive lever throughout the session (Fig. 4B).

Experiment 3: replication of the failure to discriminate between two levers

Many conditions of experiment 2 were different from those of experiment 1, including the sex of rats, testing chamber, testing length, etc. in addition to response contingencies. To confirm that response contingencies, instead of other factors, determined the successful lever discrimination in experiment 2, we examined whether rats fail to learn to discriminate between the two levers when response contingencies are those of experiment 1 while all other factors are identical to experiment 2. Rats failed to discriminate between the two levers when they obtained muscimol into the median raphe with the two levers available throughout testing and no explicit cues provided in sessions 1 and 2, whereas the rats learned to discriminate between the two levers when they received muscimol with the response contingencies of experiment 2 in sessions 3 and 4 (Fig. 5).

Experiment 4: conditioned place preference

The rewarding effects of muscimol into the MRN and DRN were also examined using a conditioned place preference procedure. The compartment paired with MRN muscimol was preferred over the one paired with vehicle ($n = 10$) (Fig. 6). The compartment paired with DRN

muscimol was not significantly preferred over the vehicle-paired compartment ($n = 8$). As expected, the compartment paired with vehicle was not preferred over another vehicle-paired compartment after conditioning ($n = 12$).

Experiment 5: GABA_A receptor mediation of muscimol self-administration

To determine whether the reinforcing effects of muscimol are mediated by GABA_A receptors, we examined the effects of coadministration of picrotoxin on muscimol self-administration into the MRN. Co-administration of picrotoxin diminished self-administration of muscimol (Fig. 7).

Experiment 6: dopamine-dependent effects of muscimol

Because dopamine has been implicated in the reinforcing effects of many drugs of abuse, we examined whether muscimol self-administration into the MRN is dependent on normal dopamine transmission. Pretreatment with the systemic dopamine antagonist SCH 23390 (0.025 mg/kg, i.p.) diminished self-administration of muscimol into the MRN (Fig. 8A). Self-administration rates of muscimol when rats were pretreated with the dopamine antagonist were similar to self-administration rates of vehicle when pretreated with saline. When rats were pretreated with the dopamine antagonist and received muscimol, they self-administered muscimol as if they received vehicle. They lever-pressed at normal rates in the beginning of the session but did not maintain lever-pressing throughout the session, suggesting an extinction-like responding. On the other hand, when they were pretreated with saline and earned muscimol, they maintained self-administration at similar rates throughout the session. These observations were confirmed by interinfusion-interval analysis in which we compared the first two interinfusion intervals and the last two interinfusion intervals as a function of treatment. As shown in Fig. 8B, the first two interinfusion intervals of the sessions with the three treatments did not differ from each other, while the last two interinfusion intervals of the sessions were significantly longer when the rats earned vehicle or when they earned muscimol with the dopamine antagonist pretreatment than when they earned muscimol with saline pretreatment.

Discussion

We found that rats quickly learn to self-administer muscimol into the MRN or DRN. Muscimol administration into the MRN or DRN was highly arousing, an effect that apparently made it difficult for rats to make selective responding. However, rats did selectively respond on the active lever when the access to levers was restricted and drug administration was accompanied by cues, suggesting that muscimol administration into these regions was reinforcing. MRN injections of muscimol also induced conditioned place preference, an effect consistent with the interpretation of positive reinforcement. The MRN appears to be more responsible for the reinforcing effects of muscimol than the DRN, as rats tended to deliver muscimol into the MRN at higher rates than the DRN and discriminated between the two levers better when muscimol was administered into the MRN than the DRN, and showed conditioned place preference after muscimol injections into the MRN but not the DRN. The reinforcing effects of muscimol into the MRN were mediated via GABA_A receptors, because coadministration of the GABA_A receptor antagonist picrotoxin diminished muscimol self-administration. In addition, muscimol's reinforcing effects depended on normal dopamine transmission because dopamine antagonist pretreatment diminished muscimol self-administration.

Arousal and primary reinforcement

Initial lack of lever discrimination for muscimol administration into the MRN or DRN appears to be best explained by the drug's arousing effects. Previous studies that employed similar self-administration procedures and different drugs yielded discrimination between the two levers.

Without the presentation of cues, rats discriminated between the active and inactive levers when the active lever was reinforced with the mixture of D₁ and D₂ agonists administered into the nucleus accumbens (Ikemoto *et al.*, 1997b), the cholinergic receptor agonist carbachol into the accumbens (Ikemoto *et al.*, 1998) or the GABA_A antagonist picrotoxin administered into the vicinity of the anterior ventral tegmental area (Ikemoto *et al.*, 1997a). We suspect that the arousing effects of muscimol administered into the MRN or DRN are more pronounced than those of these drugs, and that heightened levels of arousal induced by intraraphe muscimol disrupted lever-discrimination, although we cannot exclude other explanations such as a learning (discrimination) deficit due to side-effects of muscimol injections.

The arousing effects of drugs, which can interfere with lever discrimination, may be critical in primary reinforcement. Wise & Bozarth (1987) argued a close relationship between positive reinforcing effects and locomotor effects of drugs in rats. Arousal including locomotion is a common feature of most, if not all, drugs that have been shown to be self-administered into various brain regions in rats (for references, see the discussions of Ikemoto *et al.*, 2005, 2006). For example, although rats discriminate the active lever from the inactive lever, the administration of dopamine receptor agonists into the nucleus accumbens shell increases inactive-lever presses as a function of drug dose (Ikemoto *et al.*, 1997b), suggesting an arousal effect. Arousing effects are not limited to drug reinforcers. The opportunity to interact with natural rewards such as food and sexual mates can be highly arousing in some circumstances such as scheduled delivery of rewards (Staddon, 1977). Future research should investigate how arousal and its neuronal mechanisms are related to primary reinforcement of drugs and other reinforcers and their mechanisms.

Circuitry involved in muscimol reward triggered via the MRN and DRN

The ascending projections of the midbrain raphe nuclei are very extensive. The median raphe neurons project to the ventral midbrain, hypothalamus, thalamus, nucleus accumbens–olfactory tubercle complex and septohippocampal complex (Vertes & Martin, 1988); similarly, the dorsal raphe neurons project to the ventral midbrain, hypothalamus, thalamus, striatal complex and septohippocampal complex (Vertes, 1991). Because of the vast projection, it is not easy to pinpoint which pathways are involved in primary reinforcement. In addition, it is not clear whether serotonergic projections play any role in muscimol reward. As mentioned above, Fletcher *et al.* (1993 (1995) showed that the administration of 8-OH-DPAT, which selectively inhibits serotonergic neurons, into the MRN or DRN induces conditioned place preference, and intra-MRN administration of 8-OH-DPAT facilitates lateral hypothalamic stimulation reward. Therefore, the reinforcing effects of muscimol administration may have been mediated, at least in part, by the inhibition of serotonergic neurons. Indeed, microinjections of muscimol into the MRN or DRN, which elicit heightened locomotion, also decrease serotonin levels in some forebrain regions as measured by *in vivo* microdialysis (Shim *et al.*, 1997) or serotonin synthesis as measured *ex vivo* (Nishikawa & Scatton, 1985). However, 8-OH-DPAT injections into the MRN at doses that do not elicit heightened locomotion significantly decrease serotonin levels in the hippocampus (Shim *et al.*, 1997), suggesting that the arousing effects of MRN muscimol do not depend on its effects on serotonin. Indeed, selective lesions of serotonergic neurons by 5,7-dihydroxytryptamine or serotonin depletion by p-chloro-phenylalanine do not reduce heightened locomotion induced by intra-MRN muscimol (Paris & Lorens, 1987; Wirtshafter *et al.*, 1987). It has yet to be determined whether the findings on locomotion can be generalized to the rewarding effects of muscimol.

Nonserotonergic projection from the MRN to the ventral tegmental area and the nucleus accumbens–olfactory tubercle complex may control the mesolimbic dopamine system. The present study found that systemic dopamine antagonist treatments disrupt intra-MRN muscimol self-administration, suggesting that the rewarding effects of intra-MRN muscimol

are dependent on normal dopamine transmission. Indeed, intra-MRN muscimol appears to increase dopamine metabolism in the nucleus accumbens (Nishikawa *et al.*, 1986; Wirtshafter *et al.*, 1988). Interestingly, the depletion of serotonin by p-chloro-phenylamine does not influence increased dopamine metabolism in the nucleus accumbens following intra-MRN administration of muscimol (Wirtshafter & Trifunovic, 1992). Because the MRN sends its afferent projections to the ventral tegmental area and the nucleus accumbens–olfactory tubercle complex and because muscimol administration inhibits neurons, a reasonable hypothesis is that intra-MRN muscimol administration inhibits nonserotonergic neurons projecting to the mesolimbic dopamine system and, in turn, disinhibits the dopamine system, which is rewarding.

Another structure that may be involved in the rewarding effects of intraraphe muscimol is the supramammillary nucleus, which is localized just above the mammillary body and receives afferents from the MRN and DRN (Vertes, 1991; Vertes *et al.*, 1999; Kiss *et al.*, 2002). Recent studies have shown that this structure plays an important role in drug reinforcement. Rats readily learn to self-administer nicotine, the excitatory amino acid AMPA or GABA_A receptor antagonists into the supramammillary nucleus (Ikemoto *et al.*, 2004, 2006; Ikemoto, 2005). Interestingly, the rewarding effects of AMPA or GABA_A receptor antagonist administration into the supramammillary nucleus are blocked by systemic dopamine receptor antagonists, although it is not clear how the supramammillary nucleus is connected with the dopamine reward system. Thus, the projections from the MRN and DRN to the supramammillary nucleus may play an important role in the rewarding effects of intra-MRN or DRN muscimol.

Conclusions and implications

The present data suggest that midbrain raphe nuclei, particularly the MRN, participate in primary reinforcement. Brain stimulation reward elicited via the vicinity of the MRN and DRN may recruit selective stimulation of GABAergic fibers that results in release of GABA in these nuclei. Because electrical stimulation has the property to excite myelinated fibers at lower current levels than nonmyelinated fibers and possibly axons rather than cell bodies (Ranck, 1975), local projection neurons including serotonergic ones may be nonmyelinated (Azmitia & Gannon, 1983) while GABAergic input fibers may be myelinated. The present data also suggest that median raphe neurons containing GABA_A receptors tonically inhibit dopamine-dependent reward circuitry and imply that the MRN plays a significant role in a variety of reward-related behaviour including drug addiction; they also imply that the brain drug reinforcement circuitry is not limited to the mesocorticolimbic dopamine system.

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References

- Azmitia E, Gannon P. The ultrastructural localization of serotonin immunoreactivity in myelinated and unmyelinated axons within the medial forebrain bundle of rat and monkey. *J Neurosci* 1983;3:2083–2090. [PubMed: 6604793]
- Bozarth MA, Wise RA. Electrolytic microinfusion transducer system: An alternative method of intracranial drug application. *J Neurosci Meth* 1980;2:273–275.
- Fink H, Morgenstern R. Behavioral function of GABA in the median raphe nucleus. *Biomed Biochim Acta* 1986;45:531–538. [PubMed: 3707566]

- Fletcher PJ, Ming ZH, Higgins GA. Conditioned place preference induced by microinjection of 8-OH-DPAT into the dorsal or median raphe nucleus. *Psychopharmacology* 1993;113:31–36. [PubMed: 7862825]
- Fletcher PJ, Tampakeras M, Yeomans JS. Median raphe injections of 8-OH-DPAT lower frequency thresholds for lateral hypothalamic self-stimulation. *Pharmacol Biochem Behav* 1995;52:65–71. [PubMed: 7501680]
- Ikemoto S. The supramammillary nucleus mediates primary reinforcement via GABA (A) receptors. *Neuropsychopharmacology* 2005;30:1088–1095. [PubMed: 15689963]
- Ikemoto S, Glazier BS, Murphy JM, McBride WJ. Role of D₁ and D₂ receptors in the nucleus accumbens in mediating reward. *J Neurosci* 1997b;17:8580–8587. [PubMed: 9334429]
- Ikemoto S, Glazier BS, Murphy JM, McBride WJ. Rats self-administer carbachol directly into the nucleus accumbens. *Physiol Behav* 1998;63:811–814. [PubMed: 9618003]
- Ikemoto S, Murphy JM, McBride WJ. Self-infusion of GABA_A antagonists directly into the ventral tegmental area and adjacent regions. *Behav Neurosci* 1997a;111:369–380. [PubMed: 9106676]
- Ikemoto S, Panksepp J. The relationship between self-stimulation and sniffing in rats: does a common brain system mediate these behaviors? *Behav Brain Res* 1994;61:143–162. [PubMed: 8037862]
- Ikemoto S, Qin M, Liu ZH. The functional divide for primary reinforcement of D-amphetamine lies between the medial and lateral ventral striatum: Is the division of the accumbens core, shell and olfactory tubercle valid? *J Neurosci* 2005;25:5061–5065. [PubMed: 15901788]
- Ikemoto S, Qin M, Liu ZH. Primary reinforcing effects of nicotine are triggered from multiple regions both inside and outside the ventral tegmental area. *J Neurosci* 2006;26:723–730. [PubMed: 16421292]
- Ikemoto S, Sharpe LG. A head-attachable device for injecting nanoliter volumes of drug solutions into brain sites of freely moving rats. *J Neurosci Meth* 2001;110:135–140.
- Ikemoto S, Wise RA. Rewarding effects of the cholinergic agents carbachol and neostigmine in the posterior ventral tegmental area. *J Neurosci* 2002;22:9895–9904. [PubMed: 12427846]
- Ikemoto S, Witkin BM, Zangen A, Wise RA. Rewarding effects of AMPA administration into the supramammillary or posterior hypothalamic nuclei but not the ventral tegmental area. *J Neurosci* 2004;24:5758–5765. [PubMed: 15215298]
- Kiss J, Csaki A, Bokor H, Kocsis K, Kocsis B. Possible glutamatergic /aspartatergic projections to the supramammillary nucleus and their origins in the rat studied by selective [(3)H]D-aspartate labelling and immunocytochemistry. *Neuroscience* 2002;111:671–691. [PubMed: 12031353]
- Miliaressis E, Bouchard A, Jacobowitz DM. Strong positive reward in median raphe: specific inhibition by para-chlorophenylalanine. *Brain Res* 1975;98:194–201. [PubMed: 126107]
- Nishikawa T, Fage D, Scatton B. Evidence for, and nature of, the tonic inhibitory influence of habenulointerpeduncular pathways upon cerebral dopaminergic transmission in the rat. *Brain Res* 1986;373:324–336. [PubMed: 2424555]
- Nishikawa T, Scatton B. Inhibitory influence of GABA on central serotonergic transmission. Raphé nuclei as the neuroanatomical site of the GABAergic inhibition of cerebral serotonergic neurons. *Brain Res* 1985;331:91–103. [PubMed: 2985201]
- Olds J, Milner P. Positive reinforcement produced by electrical stimulation of septal area and other regions of rat brain. *J Comp Physiol Psychol* 1954;47:419–427. [PubMed: 13233369]
- Olson VG, Heusner CL, Bland RJ, During MJ, Weinshenker D, Palmiter RD. Role of noradrenergic signaling by the nucleus tractus solitarius in mediating opiate reward. *Science* 2006;311:1017–1020. [PubMed: 16484499]
- Paris JM, Lorens SA. Intra-median raphe infusions of muscimol and the substance P analogue DiMe-C7 produce hyperactivity: Role of serotonergic neurons. *Behav Brain Res* 1987;26:139–151. [PubMed: 2447909]
- Paxinos, G.; Watson, C. *The Rat Brain in Stereotaxic Coordinates*. 3. Academic Press; San Diego: 1997.
- Przewlocka B, Stala L, Scheel-Krüger J. Evidence that GABA in the nucleus dorsalis raphe induces stimulation of locomotor activity and eating behavior. *Life Sci* 1979;25:937–946. [PubMed: 513941]
- Ranck JB Jr. Which elements are excited in electrical stimulation of mammalian central nervous system: a review. *Brain Res* 1975;98:417–440. [PubMed: 1102064]

- Rompré PP, Boye S. Localization of reward-relevant neurons in the pontine tegmentum: a movable electrode mapping study. *Brain Res* 1989;496:295–302. [PubMed: 2804637]
- Rompré PP, Miliaressis E. Pontine and mesencephalic substrates of self-stimulation. *Brain Res* 1985;359:246–259. [PubMed: 4075148]
- Shim I, Javaid J, Wirtshafter D. Dissociation of hippocampal serotonin release and locomotor activity following pharmacological manipulations of the median raphe nucleus. *Behav Brain Res* 1997;89:191–198. [PubMed: 9475626]
- Staddon, JER. Schedule-induced behavior. In: Honig, WK.; Staddon, JER., editors. *Handbook of Operant Behavior*. Prentice Hall, Inc.; Englewood Cliffs, NJ: 1977. p. 125-152.
- Vertes RP. A PHA-L analysis of ascending projections of the dorsal raphe nucleus in the rat. *J Comp Neurol* 1991;313:643–668. [PubMed: 1783685]
- Vertes RP, Fortin WJ, Crane AM. Projections of the median raphe nucleus in the rat. *J Comp Neurol* 1999;407:555–582. [PubMed: 10235645]
- Vertes RP, Martin GF. Autoradiographic analysis of ascending projections from the pontine and mesencephalic reticular formation and the median raphe nucleus in the rat. *J Comp Neurol* 1988;275:511–541. [PubMed: 3192756]
- Wirtshafter D, Klitenick MA. Comparative studies of locomotor behavior following microinjections of muscimol into various sites in the paramedian tegmentum. *Pharmacol Biochem Behav* 1989;32:625–628. [PubMed: 2740418]
- Wirtshafter D, Trafunovic R. Nonserotonergic control of nucleus accumbens dopamine metabolism by the median raphe nucleus. *Pharmacol Biochem Behav* 1992;41:501–505. [PubMed: 1533937]
- Wirtshafter D, Klitenick MA, Asin KE. Evidence against serotonin involvement in the hyperactivity produced by injections of muscimol into the median raphe nucleus. *Pharmacol Biochem Behav* 1987;27:45–52. [PubMed: 2441421]
- Wirtshafter D, Klitenick MA, Asin KE. Is dopamine involved in the hyperactivity produced by injections of muscimol into the median raphe nucleus? *Pharmacol Biochem Behav* 1988;30:577–583. [PubMed: 3211966]
- Wise RA, Bozarth MA. A psychomotor stimulant theory of addiction. *Psychol Rev* 1987;94:469–492. [PubMed: 3317472]

Abbreviations

DRN

dorsal raphe nucleus

MRN

median raphe nucleus

SCH 23390

R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride

Fig. 1.

Tips of injection cannulas in experiment 1 are depicted. The numbers of infusions with 25, 50 and 100 μM muscimol solutions over three sessions were added for each subject, and the sums were classified into one of three levels: ≤ 30 (low), 31–60 (medium), and ≥ 61 (high) infusions. Tips of injection cannulae with infusion levels of low, medium, and high are, respectively, indicated by empty, shaded and solid rectangles. The number on the right of each coronal section indicates the distance from bregma in mm. The drawings are adapted and modified from Paxinos & Watson (1997).

Fig. 2. Representative coronal sections showing typical injection cannula placements. Arrows indicate the tips of injection cannulas. (A) MRN placement. (B) DRN placement.

Fig. 3. Self-administration of muscimol into the MRN, DRN and adjacent regions. Data are mean $\pm$ SEM. (A) A 3×4 mixed ANOVA was performed on infusions with site (MRN, $n = 8$; DRN, $n = 11$; region outside, $n = 21$) as a between-subjects factor and concentration (25, 50 and 100 μM) as a within-subjects factor. A significant site $\times$ concentration interaction was found: $F_{6,111} = 5.24$, $P < 0.0001$. * $P < 0.05$ vs. respective vehicle values. (B) Repeated-measures 2×4 ANOVAs on responses with lever (active vs. inactive) and concentration revealed significant concentration effects for MRN and DRN ($F_{3,21} = 4.93$, $P < 0.01$ and $F_{3,30} = 5.43$, $P < 0.01$, respectively), but no significant lever effect or lever $\times$ concentration interaction.

Fig. 4.

Lever discrimination for muscimol administration into the MRN or DRN. (A) Repeated-measures ANOVAs with two levers over five sessions revealed significant interactions: $F_{4,64} = 19.19$, $P < 0.01$ and $F_{4,48} = 8.15$, $P < 0.01$ for MRN ($n = 17$) and DRN ($n = 13$), respectively. * $P < 0.05$ vs. respective inactive lever-press values. (B) Event records show active- and inactive-lever presses when a rat received vehicle or 100 μ M muscimol into the MRN.

Fig. 5.

Two-lever discrimination depended on the consequences of responding. Rats ($n = 13$) received 100 μM muscimol into the MRN over four sessions, and were tested in sessions 1 and 2 with a similar procedure to that used in experiment 1 (i.e. unrestricted access to the levers and no explicit signal for infusions) and then in sessions 3 and 4 with the identical procedure used in experiment 2 (i.e. restricted access to the levers and explicit signals for infusions). A repeated-measures ANOVA with two levers over four sessions revealed a significant lever $\times$ session interaction: $F_{3,36} = 5.30$, $P < 0.01$. * $P < 0.05$ vs. respective inactive lever-press values.

Fig. 6.

Conditioned place preference induced by MRN administration of muscimol. Place preference scores (time spent in drug compartments – time spent in vehicle compartments) were analysed with a 2×2 mixed ANOVA with group (vehicle vs. muscimol) and conditioning (pre vs. post) and another 2×2 mixed ANOVA with group (MRN vs. DRN) and conditioning (pre vs. post). Significant interactions were found between treatment and prepost conditioning: $F_{1,20} = 7.02$, $P < 0.05$ and $F_{1,16} = 4.76$, $P < 0.05$ for vehicle vs. muscimol into the MRN and muscimol into the MRN vs. DRN, respectively. * $P < 0.05$ vs. preconditioning values.

Fig. 7.

Effects of coadministration of the GABA_A antagonist picrotoxin on self-administration of muscimol into the MRN. Rats ($n = 15$) received 100 μ M muscimol (MUS) plus 100 μ M picrotoxin (PIC), 100 μ M muscimol, and vehicle (VEH) over three sessions. A one-way repeated-measures ANOVA on infusion rate revealed a significant main effect: $F_{2,28} = 15.87$, $P < 0.01$. * $P < 0.01$ vs. the values of MUS + PIC or VEH.

Fig. 8.

Effects of dopamine antagonist pretreatment on muscimol self-administration into the MRN. Rats ($n = 9$) received muscimol (MUS) with the pretreatment of SCH 23390 (SCH; 0.025 mg / kg, i.p.), muscimol with the saline (SAL) pretreatment, and vehicle (VEH) with the saline pretreatment over three sessions. (A) A one-way repeated-measures ANOVA on infusion rate revealed a significant main effect: $F_{2,16} = 6.14$, $P < 0.05$. * $P < 0.05$ vs. the values of SCH + MUS or SAL + VEH. (B) The analysis on infusion intervals found that a significant interaction between the treatment and first – last interinfusion-interval factors: $F_{2,48} = 3.58$, $P < 0.05$, following a $3 \times 2 \times 2$ repeated-measures ANOVA with treatment, session phase (first vs. last),

and trial (2) on square-root interinfusion intervals. The first phases of all treatments did not differ from each other. $*P < 0.01$ vs. respective first phase.