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Pertussis Toxin Attenuates Intracranial Morphine Self-Administration

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SELF, D. W. AND L. STEIN. *Pertussis toxin attenuates intracranial morphine self-administration.* PHARMACOL BIOCHEM BEHAV **46**(3) 689–695, 1993.—Mu and delta opioid receptor subtypes are thought to mediate the reinforcing actions of opioids. Since these opioid receptors use pertussis toxin (PTX)-sensitive inhibitory G-proteins for signal transduction, we determined whether PTX would block the opioid reinforcement signals produced by intrahippocampal or intraventral tegmental area (VTA) injections of morphine in rats. Hippocampal PTX pretreatment prevented the acquisition of intrahippocampal morphine self-administration. Similarly, in rats previously trained to self-administer morphine in the VTA, PTX injections in the VTA abolished morphine self-administration behavior, while sparing behavior reinforced by food pellets. This result suggested that the toxin did not interfere generally with motor capacity but rather acted selectively to block morphine reinforcement. Inactivated PTX did not reduce VTA morphine self-administration, thus demonstrating that PTX blockade of opioid reinforcement is primarily due to enzymatic inactivation of inhibitory G-proteins. All these findings are consistent with the hypothesis that inhibitory G-proteins in the hippocampus and VTA mediate the reinforcing effects of opioid drugs.

Self-administration Pertussin toxin Reward or reinforcement Opiate or opioid Hippocampus Ventral tegmental area Guanine nucleotide binding or G-protein

OPIOID drugs of abuse are thought to produce their reinforcing effects by an activation of mu and/or delta opioid receptors in reward-relevant brain regions [for a review, see (30)]. This conclusion is supported by experimental data from three reward paradigms. Selective agonists acting at mu and delta receptors supported self-administration behavior (5,40), enhanced the reinforcing value of subthreshold electrical brain stimulation (15,20), and clearly induced a conditioned place preference (3,25,31). Conversely, central application of mu- or delta-selective antagonists effectively blocked both intracranial opioid self-administration (34) and opioid-induced conditioned place preference (3,31). On the other hand, the kappa selective agonist U50,488 was not self-administered (36), and it failed to facilitate brain stimulation reinforcement (20) or produce a place preference (2, 4,25).

Mu and delta opioid receptors are known to modulate brain cell activity and inhibit cyclic AMP formation by pertussis toxin (PTX)-sensitive mechanisms (1,12,13,16). PTX irreversibly antagonizes opioid receptor signal transduction by a PTX-catalyzed ADP-ribosylation (inactivation) of inhibitory guanine nucleotide binding proteins G_i and G_o [for a review, see (17)]. Injection of PTX in the cerebral ventricles or in certain brain regions blocks morphine analgesia, an indication that central G_i/G_o-proteins play a role in opioid antinociception (6,11,18,27,29). We used a similar approach—direct injection of PTX in the same brain sites that support intracranial morphine self-administration—in an attempt to demonstrate involvement of G_i/G_o-proteins in opioid reinforcement. One advantage of this brain-injection approach is that the generalized toxicity of PTX is lessened because the toxin is largely confined to the brain region where the morphine is self-administered.

Rats will learn to self-administer morphine directly into the hippocampal CA3 region (34) and the ventral tegmental area (VTA) (9). Our experiments utilized an electrolytic microinfusion transducer system (EMIT) system to deliver nanoliter volumes of drug solutions into relatively discrete brain regions (8). The system employs electrolysis of H_2O within a sealed reservoir to produce hydrogen and oxygen gas bubbles; these bubbles expel nanoliter volumes of drug solution through an injection cannula directly into the brain of freely moving rats. Here, the effects of PTX pretreatments on both the acquisition of intrahippocampal morphine self-administration and on the maintenance of intra-VTA morphine self-administration were investigated.

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METHOD

Subjects

Naive male Sprague-Dawley rats (Charles Rivers) initially weighing 270-300 g were used for these experiments. The animals were individually housed following surgery, fed ad lib, and kept on a 12L : 12D cycle (lights on at 7:00 a.m.).

Surgery

Under equithesin anesthesia (3.33 ml/kg, IP), rats were stereotaxically implanted with a unilateral guide cannula (22 ga, Plastics One, Roanoke, VA) aimed at either the dorsal CA3 region of the hippocampus or the VTA. Coordinates for surgery were: (CA3) −4.0 mm AP from bregma, 4.0 mm LAT, 3.1 mm ventral to dura; and (VTA) −5.0 mm from bregma, 0.8 mm LAT, 7.3 mm ventral to dura (28). Between tests, dummy cannulas extending 0.1–0.2 mm beyond the guide cannula tip were placed in guide cannulas to maintain their patency. Animals were allowed at least 1 week to recover from the surgery before testing.

Apparatus

The injection cannula (28 ga, Plastics One) was precut to extend 0.5 mm beyond the guide cannula tip. The injection cannula reservoir was filled with drug solution and secured to an electrode/connector assembly (Plastics One). Parafilm was wrapped tightly around the reservoir-electrode joint to ensure an air-tight seal. The injection cannula was inserted into the implanted guide cannula and secured. The animal was then placed in a sound-attenuating experimental chamber (27 × 25 × 30 cm) containing two nose-poke holes. Responses at the nose-poke holes were detected by photoelectric cells. The active nose-poke hole was indicated by a white cue light located just above the hole. A nose-poke response at the active hole activated the infusion of 100 nl (± 10 nl) of drug solution over 5 s; concurrently with the infusion, a tone was sounded to provide secondary reinforcement. The injection period was followed by a 30-s time-out period during which responses had no programmed consequences, and the cue light was extinguished. The "inactive" (nonreinforced) nose-poke hole was located on the same wall 7 cm adjacent to the active hole. A daily limit of 40 injections/8-h session was established to reduce the chances of brain damage due to excessive injection volumes. The system was controlled by a NOVA 4 computer (Data General) and Interact® I/O panel (BRS/LVE, Lehigh, PA).

Procedure

Experiment 1. The effects of PTX on the acquisition of intracranial morphine self-administration were evaluated in CA3-implanted animals. Training was conducted during the light cycle in three 8-h test sessions spaced 2 or 3 days apart. One rat failed to respond at the nose-poke hole and was eliminated from the study, and two rats were discarded for inaccurate cannula placements. PTX pretreatments were injected in the CA3 region via the self-administration guide cannula 2–3 days prior to both the first and third self-administration acquisition tests. Two PTX injections were given to ensure that the effect of the toxin would persist over all three acquisition tests. Each PTX injection was administered to restrained animals under light methoxyurethane anesthesia; the 28-ga injection cannula extended 0.5 mm beyond the tip of the guide cannula, and was connected by polyethylene tubing to a 1-μl syringe (Unimetrics, Shorewood, IL). Each 100-ng dose of PTX was dissolved in 0.5 μl phosphate-buffered saline and was slowly injected in 100-nl increments over 5 min. Control animals were similarly injected with the buffered saline vehicle.

Six of the 13 CA3-implanted animals were simultaneously tested for PTX-induced changes in motor performance. Spontaneous locomotor activity in a 1 × 1 m open field was tested 4–5 days following the initial PTX or vehicle pretreatment and within 1–2 days of the second self-administration test. The open field apparatus was divided into 25 sections, and the number of crossings in a 10-min test period was determined by an observer blind to the experimental condition.

Experiment 2. VTA morphine self-administration is more stable and reliable than hippocampal morphine self-administration. Thus, the effects of PTX on previously established morphine self-administration were studied in VTA-implanted animals. Tests were conducted during the animal's dark cycle, which also increases the stability of baselines. All animals in this experiment first had to exhibit reliable VTA morphine self-administration (an average of 20 self-injections per test session over three test sessions). When baselines had stabilized, a single 500-ng PTX injection (1 μl slowly injected over 5 min) was applied via the VTA guide cannula. After allowing 2 days for the toxin to produce its effects, morphine self-administration tests were again administered with 2–3 days between each test. Control animals were treated identically except that heat-inactivated PTX was substituted for active toxin.

Six of the VTA-implanted animals were tested concurrently for possible PTX-induced changes in response competence. These animals previously had received lever-press training with food pellets (45 mg, Bioserv) as the reinforcer. Immediately following self-administration testing, these animals were food deprived for 24 h, and the number of food-reinforced lever presses in a 10-min test period was recorded. The animals

TABLE 1

OBSERVATION OF VARIOUS BEHAVIORAL EFFECTS OF PERTUSSIS TOXIN AFTER INJECTIONS INTO THE CA3 REGION OF DORSAL HIPPOCAMPUS

Condition*	*N*	Wt. Gain (g) (±SEM)	Activity† (±SEM)	Grooming	Seizures	Deaths
Vehicle	3	55 ± 4	232 ± 42	yes	None	None
PTX (0.1 μg)	3	57 ± 10	245 ± 41	yes	None	None
PTX (1.0 μg)	6	wt. loss	NT	no	6/6	3

*Two injections were given 3 or 6 days apart.

†Activity was scored in an open field for 10 min 4 days after the first injection (NT = not tested).

were then fed ad lib for at least 24 h prior to the next self-administration test session. Food reinforcement rates before and after PTX injections were compared.

Drugs

Morphine sulfate was dissolved in Ringer's solution (10 pmol/100 nl injection) for hippocampal experiments. To minimize pH changes with the higher morphine concentrations used in VTA experiments (300 pmol/100 nl), morphine sulfate was dissolved in phosphate-buffered saline (308 mOs, pH 7.4), containing 1.9 mM $NaH_2PO_4 \cdot H_20$, 8.1 mM $Na_2HPO_4 \cdot 7H_20$, 2.4 mM KCl, 137.6 mM NaCl. Pertussis toxin (salt free, List Biological Labs, Inc., Campbell, CA) was reconstituted and stored in phosphate-buffered saline as a stock solution at 0.5 $\mu g/\mu l$.

Histology

At the end of experiments, animals were injected with chloral hydrate anesthesia (5 ml/kg 0.526 M in 0.9% saline, IP) and transcardially perfused with 0.9% saline followed by 10% formalin in saline. Brains were dissected, frozen, and sliced in 40-μm coronal sections. The sections were placed on gelatin-coated slides, stained with cresyl violet, and examined for cannula placement. Only animals with accurate placements were retained for data analysis.

Data Analysis

Daily self-administration totals from the hippocampal acquisition experiments were analyzed by two-factor ANOVA (group × test session) with repeated measures on test session. Individual mean comparisons were conducted with Newman-Keuls' post hoc analysis. For VTA maintenance experiments, daily self-administration scores were summed for the three consecutive test sessions prior to and following PTX and averaged for each treatment group; these 3-day means before and after PTX pretreatment were analyzed by two-way ANOVA (group × pre/post) and pre vs. post mean comparisons were made with paired *t*-tests. An alpha level of 0.05 or less was considered statistically significant. Food reinforced responses were analyzed similarly.

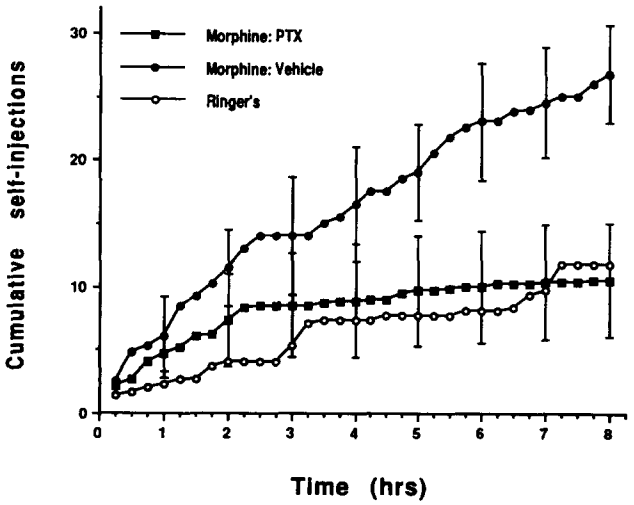

FIG. 1. Effects of pertussis toxin (PTX) pretreatment on acquisition of intrahippocampal morphine self-administration on the first test day. Data points show the mean ± SEM cumulative self-injections over the 8-h test session. Six rats self-administering morphine sulfate (10 pmol/100 nl injection) were pretreated intrahippocampally with PTX (Morphine: PTX). Four rats self-administering morphine were pretreated with the PTX vehicle (Morphine: Vehicle). A second control group received no hippocampal pretreatment and was reinforced only with Ringer's solution (n = 3).

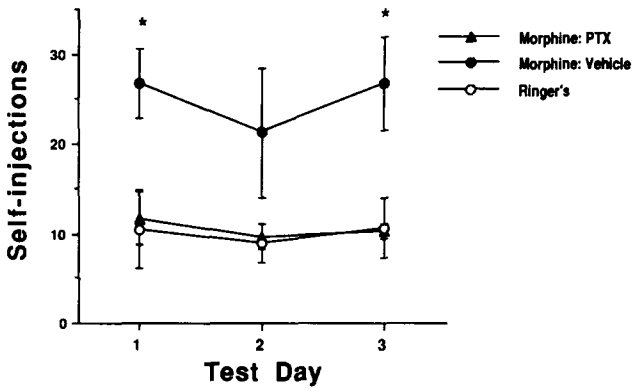

FIG. 2. Effects of pretreatment with PTX (100 ng) on acquisition of morphine self-administration in the CA3 region of hippocampus for each of three test sessions. Data are expressed as the mean ± SEM self-administration totals for each test sessions. Groups consisted of animals self-administering morphine (10 pmol/100 nl injection) and pretreated with PTX (Morphine + PTX) or vehicle (Morphine + Vehicle), and controls self-administering Ringer's solution. *Signifies significant differences from both Morphine + PTX, and Ringer's groups ($p < 0.05$, Newman-Keuls).

RESULTS

Table 1 shows various effects of two dose levels of PTX injected directly into the CA3 region of the hippocampus. Tonic-clonic type seizure activity developed in all animals pretreated with the high PTX dose (two 1-μg injections spaced 6–7 days apart); these animals also exhibited a lack of grooming and suffered marked weight loss. Three of the six high-dose animals died 3–5 days after the second injection. On the other hand, no obvious behavioral deficits were noted when the injected dose of PTX was reduced by an order of magnitude to 0.1 μg. In addition, the low-dose group had similar scores to vehicle-pretreated controls in open field tests for spontaneous locomotor activity. Accordingly, the low PTX dose was used to test the toxin's effects on the acquisition of intrahippocampal morphine self-administration.

Figure 1 shows acquisition curves of morphine (or Ringer's solution) self-administration in CA3-implanted animals on the first day of testing in Experiment 1. Initially, both PTX- and vehicle-treated groups self-administered morphine at a higher rate than the control group self-administering Ringer's solution, but the PTX-treated group ceased morphine self-administration after only a few hours, and responded at the same low rate as the group self-administering Ringer's solution for the rest of the test session. On the other hand, rats pretreated with the PTX vehicle rapidly learned to self-administer morphine and responded at a higher rate than the other groups did for most of the test session. The initially high response rate in the PTX-treated groups suggests that the PTX treatment did not impair the animals' ability to respond, but may have reduced the reinforcing efficacy of the morphine injec-

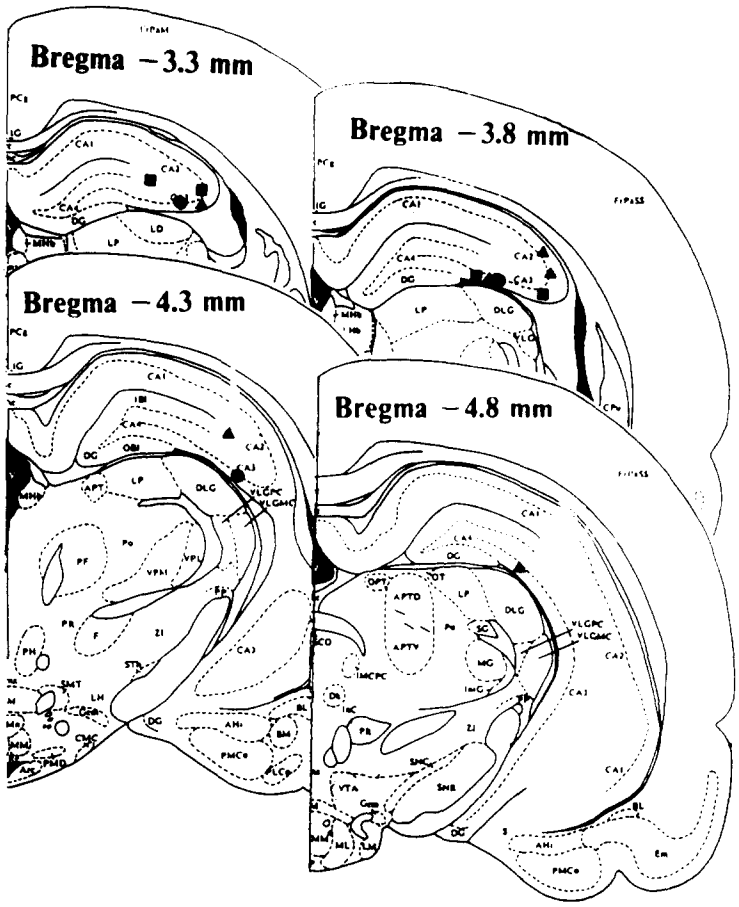

FIG. 3. Localization of injection cannula tips in the CA3 region of dorsal hippocampus for the 13 animals used in data analysis. The three groups are indicated as follows: Ringer's (circles), Morphine + Vehicle (squares), and Morphine + PTX (triangles).

tions enough to prevent acquisition of self-administration over 3 days of acquisition testing (Fig. 2); again, the vehicle-pretreated group took a greater number of morphine injections than either the PTX-pretreated morphine group or the control group self-administering Ringer's solution, $F(2, 10) = 5.181$, $p = 0.029$. Responding at the inactive (nonreinforced) nose-poke hole was similar in all three groups and declined over the three test sessions (not shown). Thirteen of the 15 CA3-implanted animals were found to have accurate cannula placements in the CA3 region of the dorsal hippocampus. Two animals with cannula placements in the lateral ventricle were eliminated from the data analysis. Locations of the injection cannula tips of the 13 animals used in the experiment are shown in Fig. 3.

In Experiment 2, VTA-implanted animals were first trained to reliably self-administer morphine sulfate (300 pmol/100 nl injection). A significant effect of treatment was found for the pre- vs. posttreatments, $F(1, 18) = 39.045$, $p < 0.001$, and the group × pre/post interaction also was significant, $F(2, 18) = 4.972$, $p = 0.019$. Substitution of phosphate-buffered saline for the morphine reinforcement led to diminished self-administration, from 77.5 ± 9.47 responses in three morphine sessions to 36.7 ± 6.90 responses in three saline sessions, $t(5) = 10.877$, $p < 0.001$ (Fig. 4). Pretreatment with 500 ng PTX similarly reduced morphine self-administration from 61.1 ± 11.5 responses in three pre-PTX sessions to 43.5 ± 11.8 responses in three post-PTX sessions, $t(10) = 3.174$, $p = 0.01$. Six of the PTX-treated animals were concurrently tested on alternate days for response competence at a food-reinforced lever. PTX did not significantly affect food-reinforced lever-pressing rates (222.3 ± 19.1 responses in three pre-PTX sessions and 197.0 ± 27.5 responses in three post-PTX sessions), $t(5) = 1.424$, $p = 0.214$. Thus, morphine self-administration was significantly reduced by PTX at the same time that food-reinforced responding was not. Moreover, these rats exhibited a full recovery from the PTX effects 2 weeks after the PTX treatment. Thus, in these six animals, PTX reduced morphine self-administration to 69.4 ± 13.2% of their pre-PTX baselines during their first week of testing, but morphine self-administration had returned to 108.5 ± 18.9% of baseline by the second week (not shown). Figure 4 also shows that animals pretreated with heat-inactivated PTX self-administered morphine at rates similar to their baseline self-administration rates (68.3 ± 17.8 responses in three preinactivated PTX sessions vs. 62.3 ± 20.9 responses in three postinactivated PTX sessions), $t(3) = 0.399$, $p = 0.717$.

Three of the 15 rats used in Experiment 2 had to be dis-

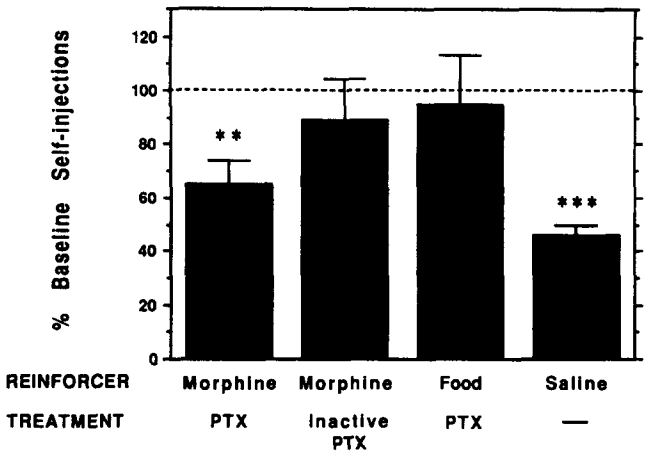

FIG. 4. Effects of pretreatment with 500 ng PTX (n = 11) on the maintenance of VTA morphine self-administration (300 pmol/100 nl injection) or food-reinforced behavior (n = 6). Controls show the effects on VTA self-administration of pretreatment with inactive PTX (n = 4) or substitution of saline for morphine (n = 6). Data are expressed as the mean ± SEM of percent baseline responding (see text). *Signifies p = 0.002; **$p < 0.001$ (paired t-test) when compared to baseline responding.

carded. One animal with a correctly placed VTA cannula failed to demonstrate reliable morphine self-administration; a second rat with a misplaced cannula approximately 1 mm lateral and 1 mm dorsal to the target site also did not self-administer morphine above saline levels, and a third rat dislodged an accurately placed guide cannula before the experiment was completed. The cannula placements of the remaining 12 animals used in the data analysis are shown Fig. 5.

DISCUSSION

Pretreatment with intrahippocampal PTX prevented the acquisition of morphine self-administration in the CA3 region of the hippocampus. Similarly, intra-VTA PTX reduced morphine self-administration to the low levels maintained by saline reinforcement in animals previously trained to self-administer morphine into the VTA. The toxin produced no obvious motor or performance deficits at the doses employed in the self-administration experiments. Indeed, in the VTA-treated animals, PTX selectively reduced nose-poke responses for morphine injections while failing to reduce higher rates of lever pressing for food reinforcement. Since significant effects of PTX on motor performance can thus be excluded, the reduction in morphine self-administration produced by the toxin is more reasonably explained by blockade of morphine reinforcement. Furthermore, the full recovery of baseline self-administration rates suggests that the PTX effects probably resulted from a transient and functional disruption of opioid reinforcement processes, rather than from a permanent lesion of "reinforcement-relevant" neurons.

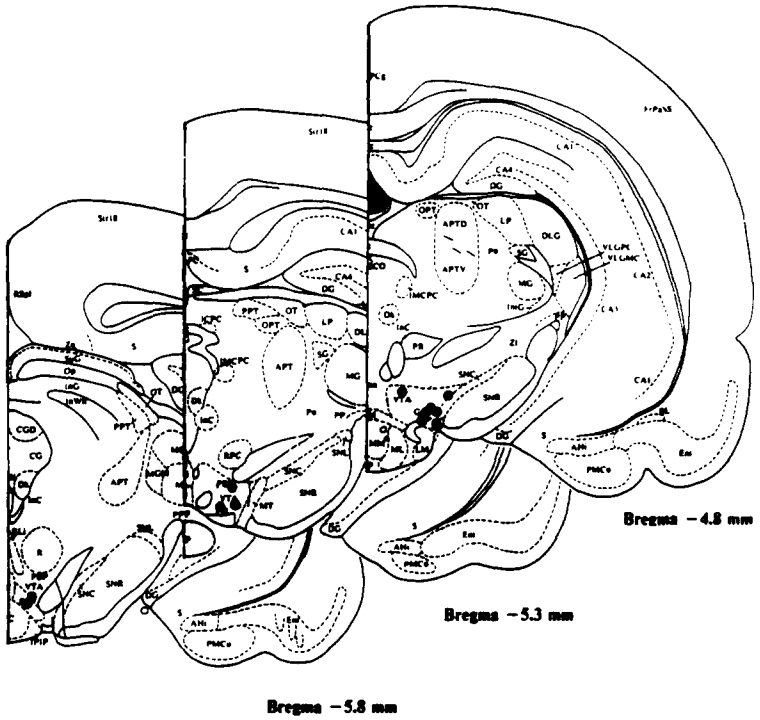

FIG. 5. Localization of injection cannula tips in the VTA region for the 12 animals used in the data analysis. All animals exhibited reliable morphine self-administration behavior prior to the PTX treatments.

The present studies utilized the minimal dose of morphine that effectively supported self-administration behavior based on pilot studies. In this regard, the hippocampus displayed greater sensitivity than the VTA to the reinforcing effects of morphine, as exemplified by a thirtyfold difference in effective dose between the two regions. This difference may be due to the higher density of mu opioid receptors in the hippocampus relative to the VTA (24). However, increasing the self-administered dose of morphine had little or no effect on self-administration rates in either brain area.

The few animals with misplaced guide cannulas did not acquire self-administration behavior, consistent with the idea that the morphine injections acted primarily at the targeted CA3 and VTA sites rather than elsewhere. This suggestion is supported by earlier reports that opioid self-administration in the CA3 (34) and the VTA (7) has some anatomical specificity. Similarly, it seems unlikely that PTX produced its effects in nontargeted brain regions since there is evidence that intracerebral PTX injections remain highly localized (37). Moreover, widespread diffusion of intracerebral PTX injections is more likely to have produced generalized toxicity and to have reduced response rates for food reinforcement, but this was not the case.

Inactivated PTX failed to alter morphine self-administration; thus, enzymatic activity is required for PTX to produce its reinforcement-blocking effects. Since G_i- and G_o-proteins are the only known substrates for PTX in the brain (26,33), it is likely that inactivation of these proteins was responsible for the attenuation of morphine's action at the injected brain site. If so, our results can be taken to indicate that G_i- and/or G_o-mediated pathways are involved in opioid reinforcement.

Morphine exhibits a marked preference for mu opioid receptors (22). Stevens et al. (34) found that dynorphin A self-administration in the hippocampus was attenuated by coinfusion with a mu-selective but not delta- or kappa-selective antagonists, and a preliminary report suggests that mu- and delta-selective agonists are self-administered directly in the VTA (14). These results suggest that morphine reinforcement can be produced by activation of mu opioid receptors in the hippocampus, and both mu and delta receptors in the VTA. The present findings with PTX are consistent with this interpretation, since both mu and delta receptor-mediated responses are blocked by PTX (12). This interpretation is additionally supported by recent evidence that PTX pretreatment prevents the acquisition of the conditioned place preferences induced by mu and delta opioid agonists (35). Kappa-mediated opioid responses, which are not blocked by PTX in rat brain slices (38), also do not mediate opioid reinforcement (30).

It might be argued that our results with PTX could be due to disruption of nonopioid neurotransmission in the hippocampus and VTA, rather than to specific impairment of opioid signal transduction. For example, opioids are thought to produce their reinforcing effects in the VTA via activation or disinhibition of VTA dopamine neurons (21,23). Dopamine neurons possess autoreceptors that are blocked by PTX (19), and chronic blockade of these autoreceptors can result in depolarization inactivation of dopamine neurons (10,39); hence, PTX could indirectly block morphine self-administration by blocking dopamine autoreceptors rather than by directly blocking opioid receptor transduction. However, the same PTX dose used in our studies has been reported to enhance cocaine's facilitation of dopamine release from VTA neurons (32), an observation inconsistent with a strong PTX-induced inactivation of VTA dopamine neurons. Similarly, in the hippocampus, PTX injections are reported to block the electrophysiological effects of mu and delta opioid agonists without affecting the functional integrity of downstream pathways (16). These findings support the notion that PTX attenuated opioid reinforcement by specific blockade of opioid receptor-mediated processes in the VTA and hippocampus.

In conclusion, local injections of PTX attenuated intracranial morphine self-administration in both the hippocampal CA3 region and the VTA. Control experiments indicated that the PTX-induced attenuation probably involved an enzymatic inactivation of G_i- and G_o-proteins, and was produced by reward-related and not performance-related changes. These findings support the idea that G_i- and/or G_o-proteins in the hippocampus and VTA mediate the reinforcing effects of opioid drugs.

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