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Reversible Disruption of Lordosis Via Midbrain Infusions of Procaine and Tetrodotoxin

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ROTHFELD, J. M., R. E. HARLAN, B. D. SHIVERS AND D. W. PFAFF. *Reversible disruption of lordosis via midbrain infusions of procaine and tetrodotoxin.* PHARMACOL BIOCHEM BEHAV 25(4) 857–863, 1986.—Behavioral effects of bilateral intracranial infusions of tetrodotoxin (1, 3.3 or 10 ng/rat), 50% procaine (2 μl/rat) or phosphate-buffered saline (PBS-2 μl/rat) into the dorsal midbrain of conscious, lightly-restrained female rats were evaluated. High levels of lordotic responsiveness were induced in ovariectomized animals treated with estradiol (E_2) capsules or subcutaneous injections of estradiol benzoate (EB) followed by progesterone (P). The effect of each of the 3 infusates on lordosis was determined using manual stimulation and lordosis quotient determinations. In addition, the vocalization by an animal during lordosis measurements, paw withdrawal to pinch, righting reflex latency and recognition of a platform edge were also monitored. Within 2 minutes following procaine or tetrodotoxin (TTX) infusions in E_2 implanted rats, lordotic responsiveness declined sharply. Whereas procaine-treated animals returned to control levels of responsiveness within 20 minutes, TTX infusions induced a more prolonged depression of lordosis lasting up to 8 hours. Infusions of PBS had no effect on any of the behaviors. In a separate group of animals treated with either E_2 or EB+P and infused with 10 ng TTX the time course of the decline in lordotic responsiveness was identical for both steroid treatments. Paw withdrawal was unaffected by TTX while all other measured behaviors were disrupted along the same time course as lordosis. Collectively the above results implicate the requirement of sodium-dependent neuronal activity within dorsal midbrain for the maintenance of the lordosis reflex, along with other behavioral responses influenced by this brain region.

Lordosis reflex Sexual receptivity Midbrain central gray Estrogen Tetrodotoxin

THE existence of a hypothalamic-mesencephalic circuit involved in the mediation of lordosis behavior in the rat has been established through behavioral neuroanatomical and electrophysiological studies [27, 29, 33, 43]. Estrogen-concentrating cells within the medial basal hypothalamus, some of which have projections to the dorsal midbrain central gray (MCG) provide the central locus for the estrogen-dependent nature of lordosis [8, 30, 33, 34]. The induction and maintenance of sexual receptivity are believed to include estrogenic effects on neuronal discharge within the medial basal hypothalamus as well as estrogenic influences on the synthesis of neurosecretory peptides and proteins involved in the expression of lordosis [15, 20, 34, 37]. Moreover, the results of such investigations support the idea that hypothalamic transduction of steroid input is essential for the activation of midbrain circuitry which is involved in the potentiation of brainstem and spinal efferents necessary for the activation of the spinal musculature utilized in eliciting the lordosis reflex [33].

Action potentials of hypothalamic origin are required for the expression of lordosis [17]. Electrical stimulation of the ventromedial nucleus (VMN) of the hypothalamus or dorsal MCG is capable of facilitating the behavior in estrogen-primed rats [41]. These observations imply the need for neuronal activity within the midbrain as well. To examine the involvement of sodium channel-dependent neuronal activity in the MCG we infused tetrodotoxin (TTX) or 50% procaine into the dorsal MCG and assessed their ability to disrupt the motor patterns specific to lordosis (dorsiflexion of the vertebral column), as well as other nonspecific motor activity. Furthermore, the induction of lordosis via both estradiol (E_2) implants, as well as a successive treatment schedule of estradiol benzoate (EB) followed by progesterone (P) allowed for a comparison of these two commonly used treatment regimes with respect to the effect of TTX.

METHOD

Female rats (Charles River) weighing 220–260 g were housed in air conditioned quarters on a 12 hr light/12 hr dark photoperiod (lights off at 1200) and fed food and water ad lib. Four days after their arrival animals were ovariectomized under Metophane (Pitman-Moore Inc.), and following a one week recovery were implanted stereotaxically with bilateral

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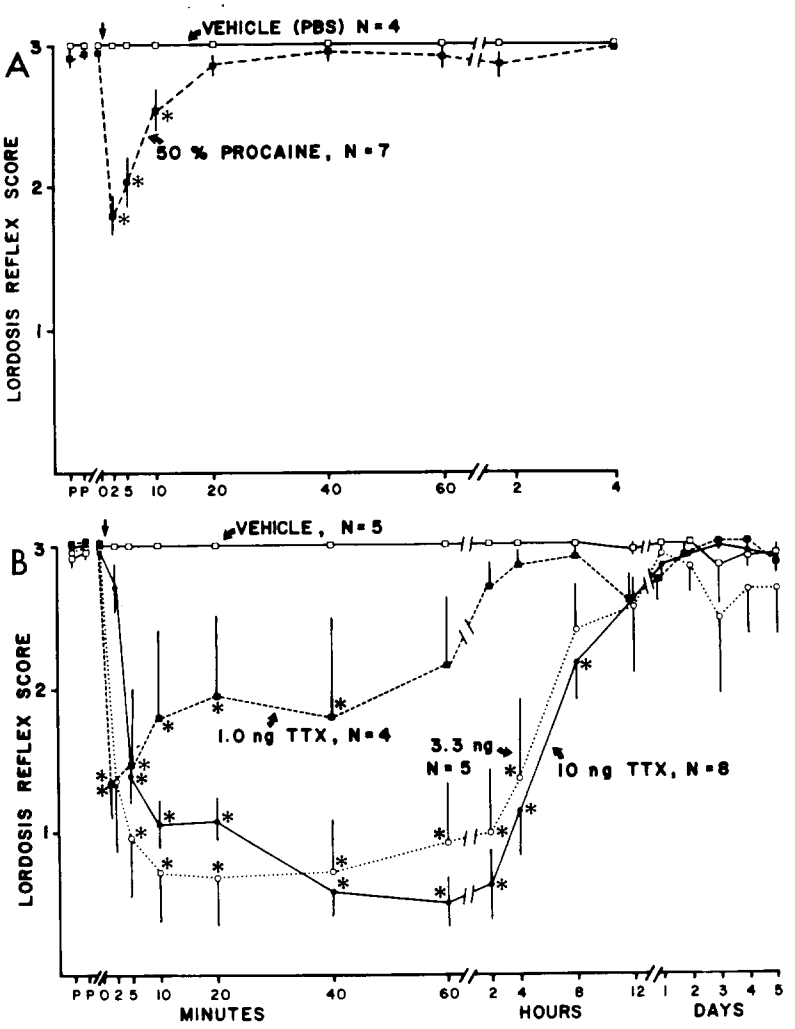

FIG. 1. Time course for the effect of (A) 50% procaine or (B) varying doses of tetrodotoxin (TTX) on the lordosis reflex initiated using estradiol implants. Preinfusion (arrows) scores were determined 10–45 minutes prior to infusions (time=0). *$p<0.05$ for TTX or procaine versus phosphate-buffered saline (PBS) infusions at each time point.

guide cannulae (22-gauge) fitted with a removable, blank internal guide (Plastic Products, Roanoke, VA) extending approximately 0.5 mm below the outer guide. Animals were anesthetized with chloropent (IP) and placed in a stereotaxic frame. Upon exposure of the cranium and removal of bone the bilateral cannulae (having a 2.5 mm separation between tips) were implanted using the following coordinates: 1.3 mm rostral to lambda and 4.5 mm below the dura, lowered at a 10° angle directed rostrally; cannulae were then cemented in place to small metal guide screws previously anchored in the skull. At the time of the stereotaxic surgery half of the animals were implanted subcutaneously with 5 mm silastic capsules (Dow Corning, 1.47 mm inner diameter, 1.96 mm outer diameter) containing crystalline estradiol (E_2). Following a recovery period of one week behavioral testing was begun.

Behavioral Testing

Those animals not receiving an E_2 implant were primed with 2.5 μg EB suspended in 0.2 ml sesame oil (subcutaneous injection) for two consecutive days followed 48 hours after the second estradiol injection by 2 mg P in 0.2 ml sesame oil. Animals treated in this manner were infused 6 hours following the P injection (1200 hr) and were then tested as described below.

The lordotic responsiveness of rats was determined using the manual stimulation technique, and in five animals receptivity was measured using a stud male. Manual stimulation was delivered as described previously [33]. In brief, rats were placed in a small cage and the lordosis reflex was determined through the application of manual cutaneous stimuli. Such stimuli consisted of stroking (4 times) the hind flanks using the thumb and forefinger, immediately followed by applying pressure to the perineum and tail base region. Using this technique lordosis reflex strength was rated on a numerical scale from 0 (no dorsiflexion of the vertebral column) to 3 (maximal dorsiflexion). For each time point tested the average of five scores obtained by manual cutaneous

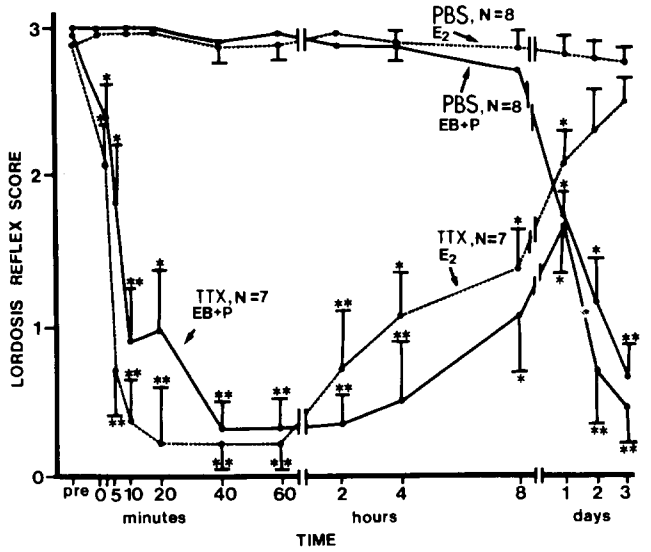

FIG. 2. Time course for the effect of tetrodotoxin (TTX) and phosphate-buffered saline (PBS) infusions on the lordosis reflex initiated using estradiol (E_2) implants or estradiol benzoate + progesterone (EB+P) treatment. Preinfusion (arrows) reflex scores were determined as in Fig. 1. $*p<0.05$, $**p<0.005$ for TTX versus PBS within each steroid treatment group at each time point.

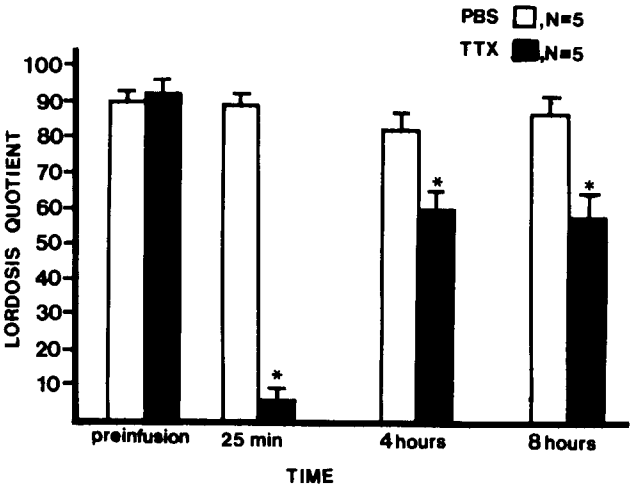

FIG. 3. Determination of lordosis quotients (L.Q.) in EB+P primed animals following phosphate-buffered saline (PBS) or tetrodotoxin (TTX) infusions. Preinfusion L.Q. were determined 10–45 minutes prior to infusions $*p<0.05$ for TTX infusions across the time interval tested and for PBS versus TTX at each time interval.

stimulation, delivered several seconds apart, was recorded as the reflex score for a particular animal. For behavior tests using males, an individual female was placed in a rectangular glass-walled mating area containing a stud male and was allowed to receive 10 mounts with pelvic thrusts. The lordosis quotient (L.Q.) for each female was expressed as the ratio of the number of lordosis responses to the number of mounts×100. Both manual stimulation and mating tests were performed during the dark phase of the daily light cycle under 25 Watt red light illumination maintained approximately 2–3 meters from the experimental area. All vocalizations by females during manual stimulation and mating tests were noted.

In addition to the behavioral tests described above, all animals (infused with TTX) were monitored immediately following lordosis testing for hindpaw withdrawal to pinch and righting reflex latency following placement of the animal on its dorsum. Another behavioral criterion scored was the recognition of a platform edge, included to examine the animal's ability to contain its exploratory locomotion within the confines of a limited table top surface (0.5×0.5 meter) during a 2 minute period. An animal displaying no such recognition would walk off the edge of a table (having a 1 meter drop) without hesitation. Intact recognition was characterized by exploration up to the edge and walking along the two perpendicular sides, displaying a clear awareness of the boundary defined by the edges.

Infusions

Midbrain infusions of TTX, procaine or phosphate buffered saline (PBS) were carried out in conscious lightly-restrained animals. Prior to the actual infusion the inner dummy (blank internal guide) cannulae were removed, cleaned and reinserted into the outer cannula, thereby assuring that clotted blood or tissue was not obstructing the guide cannulae. Following this, the inner dummy was again removed and a double inner cannula connected to 2 separate Hamilton microsyringes via PE (PE20) tubing was inserted to the same depth as the dummy. The simultaneous bilateral infusion of 1 μl (2 μl total) of TTX, 50% procaine or PBS into each cannula was completed over a 2 minute interval. The inner cannula was then left in place an additional minute to allow complete transfer of the perfusate. Following the replacement of the dummy cannula the lordosis reflex was immediately scored using the manual stimulation technique (2 minutes post infusion) and thereafter at 5 min, 10 min, 20 min, 40 min, 1 hour, 2 hour, 4 hour, 8 hour, 1, 2 and 3 days. In tests using stud males L.Q.'s were determined prior to infusion and at 20 min, 4 and 8 hours following it.

For the first portion of the study, lordotic responsiveness was determined in separate groups of E_2 implanted rats following infusions of 1.0, 3.3 or 10 ng TTX or 50% procaine diluted in PBS. In the second experiment (using different subjects) 10 ng infusions of TTX were administered to two separate groups of rats made receptive using E_2 implants or EB+P treatment. The lordosis reflex of all animals in these two groups were tested using manual stimulation. One week following the initial manual stimulation test, animals in the EB+P group were infused with 10 ng TTX and retested with males.

Histology

Following all behavioral tests animals were administered an overdose of Nembutal and transcardially perfused with Mirsky's fixative (National Diagnostics). Frozen sections through the cannula tracts were cut at 30 μm, mounted on slides and stained with cresyl violet. The anatomical locations of infusion sites were than plotted [32].

Statistics

Differences in lordosis reflex scores for the E_2 implanted

TABLE 1

SUMMARY OF BEHAVIORAL RESPONSES FOLLOWING THE INFUSION OF 10 ng OF TETRODOTOXIN (TTX) WITHIN THE DORSAL MIDBRAIN

	Steroid Treatment: Estrogen + Progesterone		Estrogen Implant	
	TTX	Saline	TTX	Saline
Number of animals displaying a loss in vocalization during manual stimulation*	5/7	0/8	6/7	0/8
Number of animals failing to display leg withdrawal in response to paw pinch	0/7	0/8	0/7	0/8
Number of animals displaying an impaired righting reflex response	7/7	0/8	7/7	0/8
Number of animals which repeatedly walked off an elevated platform edge†	6/7	0/8	7/7	0/8

*Infusion treatment (PBS vs. TTX) and loss of vocalization were significantly related ($p<0.005$).
†Infusion treatment (PBS vs. TTX) and loss of edge recognition were significantly related ($p<0.005$).

and EB+P primed animals with respect to their corresponding PBS controls (at each time point) were detected using the Mann-Whitney test. The above analysis was also applied to the L.Q. data obtained in EB+P treated animals. Comparisons across time periods for the L.Q. determinations were made using the Kruskal Wallis test and Dunn's multiple comparison test. All other behavioral data were evaluated using the Chi Square test of independence [7].

RESULTS

The effects of procaine and TTX infusions differed with regard to their alterations of the lordosis reflex. Within 2 minutes following procaine infusion, lordotic responsiveness, as determined by manual stimulation, decreased from a pretreatment score of 2.94±0.06 (SEM) to a minimum of 1.8±0.1. At 20 minutes post infusion procaine-treated animals had returned to control levels of responsiveness (Fig. 1A). In contrast TTX infusions induced a more prolonged depression of lordotic responsiveness. Increasing the infusion dose of TTX from 1 to 3.3 to 10 ng/rat resulted in a more complete inhibition of the reflex as well as prolonging the duration of this effect (Fig. 1B).

In a separate experiment, infusion of 10 ng of TTX into groups of rats having silastic implants of E_2 as well as those primed with EB+P induced a sharp inhibition of the lordosis reflex as determined by manual stimulation (Fig. 2). For both treatments a significant decline in lordosis occurred within 5 minutes following administration of TTX. The time course of this inhibition was similar in the two groups. In each case the greatest degree of inhibition occurred between 5 minutes and 2 hours post infusion. Similarly, the initial recovery from the effects of the TTX was the same in each case, from 4 hours to 1 day. Within two days the lordotic responsiveness of the implanted group returned to the same level as PBS infused animals. In contrast EB+P treated animals recovered to a maximum reflex score of 1.7 at 1 day which then steadily declined at the same rate as PBS-infused animals treated with EB+P.

Changes in lordotic responsiveness following TTX infusions as measured by L.Q. determinations were similar to those recorded using manual stimulation (Fig. 3). At 20–25 minutes post infusion the L.Q. was sharply reduced. In tests conducted at this time interval, females were capable of locomotor activity but were sluggish. Upon being mounted, females did not display avoidance behavior at any time. In the preinfusion L.Q. tests all five animals displayed proceptive behaviors (i.e., ear wiggling, hopping and darting); whereas at the 20 minute post-infusion determination these behaviors were completely absent. During the 4 and 8 hour tests proceptive behaviors were present but highly reduced in quantity.

In Table 1, the response profiles of E_2 implanted and EB+P-primed animals (along with respective controls) to 4 additional behavioral tests are listed. During manual stimulation conducted prior to infusions, all animals tested vocalized. However, following TTX administration 71% of the EB+P group and 85% of the E implant group did not vocalize in response to manual cutaneous stimulation. Chi Square analysis revealed a significant ($p<0.005$) relationship between infusion treatment (TTX or PBS) and the presence or absence of a vocalization response. Loss of vocalization occurred at 5 minutes post infusion and returned in all animals during the 8 hr–1 day lordosis determinations. In contrast, all PBS infused animals consistently vocalized at each testing interval. At no time did females tested with males vocalize. With respect to paw withdrawal, all but 2 animals in all instances displayed a similar response latency to pinching of the hindlimb. Monitoring of the righting reflex revealed an increase in the time taken to return to the upright position in 100% of all animals given TTX. This impairment was present up to the 8 hour test period and returned to control levels at the 1 day test.

At 40 min to 1 hour following TTX infusion, 100% of E_2-implanted and 85% of EB+P-primed animals displayed a severe impairment in their ability to remain on an elevated surface without walking off the side. Animals disregarded the edge of a table and would step right off without any

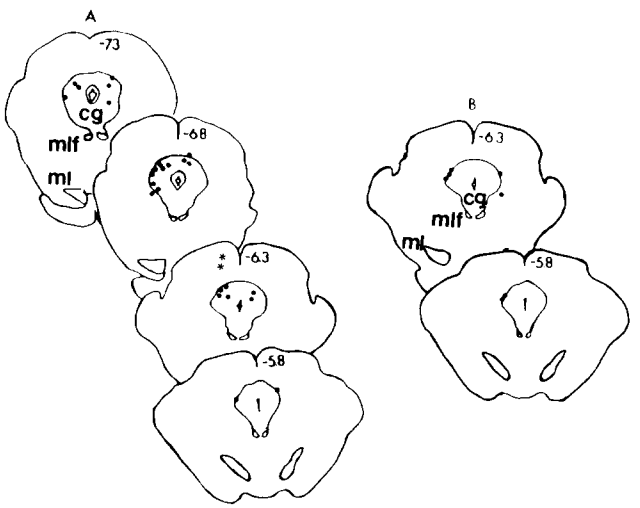

FIG. 4. Anatomical localization of bilateral cannula implants in (A) tetrodotoxin and (B) 50% procaine infused animals. Vehicle (PBS) infusion sites overlapped those diagrammed in A and B. Only one implantation site per animal for each bilateral cannula is indicated due to the symmetry (within 500 μm) of the two cannulas within each animal. The two placements which did not elicit inhibition are indicated (*). Drawings and anatomical coordinates (shown relative to bregma) follow the atlas of Paxinos and Watson [32]. Abbreviations: CG: central grey, ml: medial lemniscus, mlf: medial longitudinal fasciculus.

hesitation in their forward locomotion. Even after stepping off an edge and falling 2 feet before being caught, rats would continue to ignore the boundaries of the surface when replaced on it 3 additional times. Recovery from this deficit was seen in the majority of cases at the 8 hour test period. At no time did any PBS-infused animal display this behavior, but instead would move along the boundaries of the table repeatedly orienting toward the void beyond the edge and sniffing the air. As in the vocalization determination, table-edge response and infusion treatment were dependent ($p<0.005$).

Histology

Histological examination confirmed the placement of cannula to be within the dorsal portion of the midbrain (Fig. 4). In two additional cases (both TTX treated) cannula placement was outside the MCG. In these instances cannula tips were localized within the superior colliculus. For the above two animals, lordosis reflex scores did not decline as seen in the remaining TTX treated rats, but instead displayed slight fluctuations from corresponding controls. The results from these 2 rats were excluded from the data analysis.

DISCUSSION

Infusion of either the sodium channel blocker TTX or the local anesthetic procaine in the MCG were effective in suppressing lordosis behavior. Whereas the onset of this inhibition occurred within 2 minutes for both of these compounds, the magnitude of the inhibition of lordosis were greater for TTX than procaine. In contrast intrahypothalamic infusions of TTX within the VMN were reported to take 40 minutes before a significant depression of the reflex was noted, while infusion of 50% procaine had no effect on lordotic responsiveness [17].

The differential effectiveness of procaine and TTX infusions within the MCG possibly reflects the differing time course of their inactivation. Degradation of procaine via endogenous cholinesterases may serve to reduce the effective concentration of the drug. In contrast the binding of the guanidinium moiety of the TTX molecule to the exposed sodium pore of the membrane may result in TTX being less susceptible to enzymatic degradation than procaine [23,31].

Furthermore, the effectiveness of procaine in inhibiting lordosis when infused into the MCG, but not the VMN may be a function of differences in the neuronal discharge frequencies in these two regions. The procaine block of sodium channel activity results from the binding of the anesthetic to a receptor located within the channel [21]. Access to this site is therefore a function of accessibility to the channel pore as regulated by the discharge frequency of a neuron. The higher spontaneous discharge rates of lordosis-relevant units in MCG over those in VMN may partially account for the effectiveness of procaine in inhibiting lordosis when infused into the former region [5,42].

As well as providing excitatory inputs to the MCG, local neuronal circuits within the ventromedial hypothalamus having inhibitory influences on lordosis also exist [25]. While the presence of lordosis-facilitatory neural projections from the VMN to the dorsal MCG is documented [33], the significance of inhibitory circuits as well as their possible influence on other regions involved in lordosis modulation (e.g., MCG, medullary reticular formation) are unknown. The existence of both facilitatory and inhibitory influences may partially account for the differential lordosis-inhibiting effects of procaine and TTX infusions within these two areas [25]. The latency of onset for the inhibition of lordosis was twenty-fold shorter following TTX infusions in MCG than for similar infusions in VMN. This finding may be a reflection of TTX's influence on discrete components of the (inhibitory and excitatory) lordosis-regulating axis asymmetrically situated within medial basal hypothalamic and mesencephalic regions.

That TTX infusions in the dorsal MCG were able to inhibit lordosis supports the need for sodium-mediated currents within dorsal midbrain regions. On the presumption that estrogen-induced proteins originating from the VMN [28, 34, 35] are transported to and subsequently help to activate lordosis-relevant MCG sites; reduction of electrical activity in VMN might not have caused the cessation of lordosis until the depletion of these factors had occurred. Loss of postsynaptic activity in the midbrain would have a more immediate behavioral effect. To date the positive identification of lordosis inducing peptide(s) has not been completed, although there are several putative factors [18, 19, 38, 44, 45].

The induction of sexual receptivity in the ovariectomized rat has been achieved through either estrogen implants or the sequential administration of estrogen followed by progesterone [4]. Differences in the mechanism of action by which these two treatment schemes induce lordosis have been noted [13,46]. Despite such differences in the modulation of lordosis by these treatments, the estrogenic activation of receptivity may occur through its progesterone-like effects expressed within certain brain regions responsive to progesterone [24]. In the present study the time course of the inhibition of lordosis following TTX infusions was similar in

both estrogen implanted and estrogen-progesterone primed animals. This observation implies that the primary site of action for the steroidal potentiation of lordosis most probably resides at more rostral centers probably within the preoptic area and VMN of the hypothalamus [5,42].

Studies utilizing stimulation, lesioning and infusion techniques have confirmed the involvement of the dorsal MCG in the control of lordosis [18, 38, 40, 41, 44, 45]. Effective infusion sites in the present study were also found to be within this region. In two instances in which cannula implants were dorsal to the MCG, TTX did not block lordosis. Infusion of TTX into control brain sites not involved in controlling lordosis has also been reported to have no effect on the behavior [17]. These observations demonstrate that the inhibition of lordosis occurs as a result of the blockade of sodium channel activity within brain regions specifically involved in the regulation of lordosis. The specificity of the blocking action of TTX on sodium channels leading to the inhibition of sodium-dependent action potentials is well established [21, 31, 39] and therefore supports the idea that sodium currents within the MCG which give rise to neuronal discharge are necessary for the expression of lordosis.

Validation of the time course of action for inhibition of lordosis as measured using manual stimulation was confirmed by lordosis quotient determinations made with male rats. The largest decline in L.Q.'s occurred at a time when manual stimulation scores were at their nadir, and the onset of recovery from TTX was observed in a similar time frame for both testing procedures. In the course of conducting L. Q. determinations prior to and following TTX infusions it was found that despite the general decrease in the female's motor activity, stud males continued vigorously to pursue and execute mounts with thrusting. Although it was not determined in the present case, the localized effect of TTX within the MCG may disrupt neural mechanisms involved in the expression of the lordosis reflex while sparing other distinct brain regions involved in the activation of motivational aspects of sexual behavior [6, 9, 36, 47, 48]. Using an appropriate experimental design, the reversible effect of TTX on neural activity may aid in examining the interaction of these two factors in the expression of reproductive behavior. The fact that proceptive behaviors were lost following TTX infusions and began to return 4–8 hours post-infusion does not necessarily imply the overlap of systems controlling receptivity and proceptive behaviors. Rather this may merely reflect the proximity of distinct efferent pathways of these behavioral systems, in the course of their projections through the midbrain [36, 47, 49].

Anatomical [1–3, 10, 29] and behavioral [11, 12, 37, 48, 49] studies of the MCG have provided information regarding both the cytoarchitectural and functional organization of this region. The richness of afferent and efferent connections of the MCG [1, 10, 29] implies that the disruption of sodium-dependent unit activity in the dorsal MCG can affect specific functions correlated with this region. From a behavioral perspective it has been shown that the MCG is involved in mediating a broad range of functions including: nociceptive responsiveness [11,12], sexual behavior [18, 33, 38, 44, 45] escape behavior [12,26] and vocalization [14,22]. Behavioral deficits observed following TTX infusions into the MCG reflect this fact. In the course of manual stimulation delivered prior to infusions, all females tested vocalized. Following the TTX infusion, this response subsided along approximately the same time course as the inhibition of lordosis. Similarly the disruption of the righting reflex and the disregard for elevated platform heights implies that the TTX inhibition of sodium-based multiunit activity results in a temporary, reversible disruption of the functional integrity of the dorsal MCG. These results suggest that the inhibition of lordosis via TTX infusions is not merely a secondary effect brought on by the disruption of nonspecific sensorimotor deficits.

The specificity of midbrain infusions of TTX on the inhibition of lordosis is supported by several findings. Electrical stimulation, lesioning and infusion studies have shown the dorsal midbrain to be involved in the control of lordosis [18, 33, 38, 40, 41]. Furthermore, the disruption and subsequent recovery of vocalization, edge avoidance and righting responses for individual animals did not occur along the same time course as the inhibition and recovery of the lordosis reflex. Decreasing amounts of TTX infused into the midbrain yielded a progressive decrease in the degree of the lordosis reflex. This dose-dependent effect of TTX on the inhibition of lordosis was not paralleled by a similar change in time course for the other behaviors examined. The above observations support the idea that the TTX-induced inhibition of lordosis was not secondary to some other unrelated behavioral deficit produced by the TTX.

The inhibition of lordosis, along with the disruption of other sensorimotor qualities associated with the MCG, demonstrate the involvement of sodium-dependent neuronal discharge in mediating these behaviors.

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