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Reversible inactivation of the insular cortex by tetrodotoxin produces retrograde and anterograde amnesia for inhibitory avoidance and spatial learning

(aversively motivated learning/caudate nucleus/frontal cortex/gustatory neocortex)

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ABSTRACT **Tetrodotoxin (TTX; a voltage-sensitive sodium channel blocker) was microinjected bilaterally into the insular (IC), frontal (FC), or parietal (PC) cortex or the ventral caudate nucleus of rats either before or after they were trained in an inhibitory avoidance task. When administered either before or after training, injections of TTX into the IC impaired performance on a 48-hr retention test. Injections of TTX into the PC also impaired retention when administered before training. One week later, rats with cannulae in the IC, FC, and PC received microinjections of TTX either before or after training in a water maze (Morris) spatial learning task and retention was tested 24 hr later. TTX impaired retention when administered to the IC either before or after training. These findings indicate that a functionally intact IC during and after training in these tasks appears to be essential for the storage of long-term memory.**

The insular cortex (IC), or visceral neocortex, is known to be involved in visceral reactions and stress (1). The IC receives taste and visceral information from the thalamus (2–4) and sends direct projections to the nucleus of the tractus solitarius (the first-order relay for visceral information) (1). The IC also has connections with limbic structures, including the amygdala, the mediodorsal nucleus of the thalamus, and the medial prefrontal cortex (1, 5). Moreover, the IC receives afferent projections from limbic and primary visceral inputs (1, 5). It is well established that the IC is involved in taste/visceral-related memory. Lesions of the IC region in adult rats impair acquisition and retention of conditioned taste aversion (2, 3, 6, 7). Recent findings showing that *N*-methyl-D-aspartate-induced lesions of the IC disrupt the acquisition of inhibitory avoidance tasks indicated that the IC is also involved in exteronociceptive-based learning (8).

In the experiments reported here, tetrodotoxin (TTX; a reversible sodium-channel-dependent activity blocker) (9, 10) was microinjected bilaterally into specific cortical areas to produce reversible inactivation either before or after training in two tasks—inhibitory avoidance and water maze spatial learning. Retention tests were administered 1 or 2 days after training. The findings indicate that TTX microinjected into the IC produces anterograde as well as retrograde memory impairment in both learning tasks.

MATERIALS AND METHODS

Surgical, Histological, and Injection Procedures. Male Sprague–Dawley rats (220–250 g) from Charles River Breeding Laboratories were individually housed and maintained on a 12:12 hr light/dark cycle, with food and water available ad libitum. Behavioral training and testing were conducted during the light portion of the cycle (10:00 a.m. to 2:00 p.m.). One week after arrival, the rats were implanted bilaterally with 15-mm 23-gauge stainless steel cannulae under anesthesia (ketamine hydrochloride, 100 mg/kg i.p., atropine at 0.4 mg/kg; and xylazine at 5.0 mg/kg i.p. as preanesthetics). The tips of the cannulae were aimed 1 mm above the IC (AP = +2.0; ML = ±4.5; DV = −5.0; n = 12), frontal cortex (FC; AP = +2.0; ML = ±2.0; DV = −0.5; n = 9), and parietal cortex (PC; AP = +2.0; ML = ±4.0; DV = −1.5; n = 9) or ventral caudate nucleus (CN; AP = +2.5; ML = ±2.0; DV = −4.5; n = 11) (11) so that the injection needle tips (which protruded 1 mm from the tip of the cannula) reached the injection targets (Ap, anterior posterior; ML, medial lateral; DV, dorsal ventral). Behavioral procedures were begun 1 week after the surgery.

On completion of the experiments, the animals were sacrificed and perfused with 10% formalin; their brains were excised and stained with cresyl violet and examined under a microscope to determine cannula placements. The tip of the cannulae in the IC group was located in the border of the degranular and agranular IC and in some cases targeted the claustrum. For the PC, the tips were located in the PC area 1 (somatosensory cortex). The tip of the cannulae aimed at the FC was located in areas 1 and 2. The CN tips were located between the nucleus accumbens and the ventral part of the striatum, according to Paxinos and Watson (11).

TTX (Sigma; 6.0 ng/μl in citrate/phosphate buffer) or a citrate/phosphate buffer solution was bilaterally administered through 30-gauge injection needles connected to a Hamilton syringe (12). A 0.5-μl injection of TTX or buffer solution was delivered (0.75 μl/min) to each of the two cannulae simultaneously by an automated syringe pump. The needles were retained in the cannulae for an additional 30 s after the injections were completed.

Behavioral Procedures. The animals were trained in a trough-shaped stainless steel straight-alley inhibitory avoidance (passive avoidance) apparatus (13). A starting compartment illuminated by a tensor lamp was separated from a darkened compartment by a vertically sliding door. Half of the rats (multitrial inhibitory avoidance) received TTX injections and, starting 5 min later, were trained to inhibit entry into the illuminated compartment. Each rat was placed in the starting compartment facing the door leading to the dark compartment. When the rat turned away from the door, the door was opened and a timer was started. When the rat stepped into the dark compartment a foot shock (0.35 mA; 60 Hz) was delivered through the floor plates and remained on until the rat escaped into the starting compartment. Escape latencies were typically <3 s. The rat was retained in the apparatus and received a foot shock each time it reentered the

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Abbreviations: TTX, tetrodotoxin; IC, insular cortex; FC, frontal cortex; PC, parietal cortex; CN, ventral caudate nucleus.

dark compartment. Training was terminated when the rat remained in the starting compartment for 60 consecutive seconds. The number of trials (entries into the dark alley) was recorded. On the retention test 48 hr later, the rat was placed in the starting compartment, as in the training session, and the latency to enter the dark compartment (maximum of 600 s) was recorded.

The remaining half of the rats were given a single training trial (one-trial inhibitory avoidance). They were trained as described above except that they were removed from the apparatus after entering the dark compartment and receiving a foot shock (0.35 mA; 60 Hz; 0.7 s). Bilateral microinjections of either TTX or buffer solutions were administered immediately after the training, and retention was tested 48 hr later as described above.

One week later, the animals in the IC, PC, and FC groups received microinjections of TTX either before or after training in a water maze task (modified from ref. 14). The apparatus was a galvanized-steel water tank 1.83 m in diameter and 0.58 m in height containing a rectangular clear Plexiglas platform (12 × 14 cm) 19 cm in height (i.e., its surface was 1 cm below water level) located in the center of one of the quadrants of the tank. The platform remained in the same position throughout training. At the beginning of training, each animal was placed on the platform for 20 s and then participated in five consecutive trials. On each trial, the rat was placed in the water facing the wall of the tank at one of the four starting positions. The starting position was changed quasi-randomly across trials. The rat was allowed to swim until it located and climbed onto the platform (maximum of 90 s), where it remained for 20 s. If the rat failed to find the platform in 90 s, it was gently guided to it. Twenty-four hours later, each rat received a single free-swim probe trial; the rat was placed in the tank, as in the training trial, but with the platform removed, and was allowed to swim for 60 s. The performance of the rat was recorded by a video camera located above the tank. The videotapes were analyzed (by observers blind to the treatment received) to determine the time spent in each quadrant of the tank, the number of times the rat crossed the place where the platform had been located during training, and the number of times the rat crossed the equivalent portion of each of the other quadrants.

Data obtained in the inhibitory avoidance task were analyzed by analysis of variance (ANOVA) and Mann–Whitney U tests. Data obtained in the water maze are expressed as mean number of crossings and were analyzed with pairwise comparison ANOVAs for the TTX and the respective buffer groups. In all cases, P values of <0.05 were considered significant. The experiments were conducted blind with respect to the drug treatments. In all experiments, each group consisted of 10–14 animals.

RESULTS

Inhibitory Avoidance. Fig. 1 summarizes the effects of microinjections of TTX, administered before training, on acquisition and retention in the multitrial inhibitory avoidance task. As shown in Fig. 1 *Upper*, TTX affected acquisition only when administered to the IC. In comparison with the IC buffer controls, the TTX/IC group required a significantly greater number of trials to reach the learning criterion ($P < 0.05$). The acquisition performance of the remaining TTX groups did not differ from that of their respective controls. As is shown in Fig. 1 *Lower*, TTX injected into the IC, as well as the PC, before training impaired retention in the 48-hr test ($P < 0.05$ compared with the buffer controls). TTX injections administered to the FC and CN before training did not affect retention.

The effects of TTX administered after training in the one-trial inhibitory avoidance task are shown in Fig. 2. The

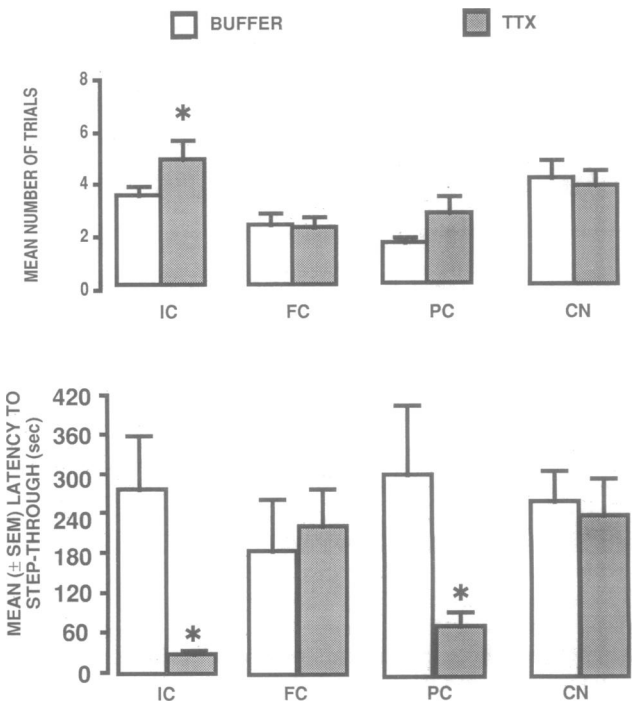

FIG. 1. Effects of pretraining injections of TTX on acquisition and retention in the multitrial inhibitory avoidance task. (*Upper*) Pretraining injection of TTX into the IC increased the number of trials necessary to reach criterion as compared to the IC buffer group (*, $P < 0.05$). (*Lower*) Pretraining injections of TTX into the IC and PC disrupted retention (*, $P < 0.05$ as compared to its respective buffer control).

retention latencies of rats given posttraining injections of TTX into the IC were significantly lower than those of the buffer controls ($P < 0.05$). Furthermore, as the retention latencies of the group given TTX injections into the IC were longer than the initial training latencies ($P = 0.05$), the TTX did not completely block inhibitory avoidance learning. TTX administered to the FC, PC, or CN after training did not significantly affect retention.

The performance of the rats during the training session in the water maze indicated that all groups learned to escape from the water. There were no significant differences among the groups in escape latencies on the five acquisition trials. Fig. 3 summarizes the mean number of crossings of the four quadrants of the tank on the free-swim probe trial. As can be seen, IC rats given TTX either before or after training crossed the target quadrant less frequently on the retention test probe trial, in comparison with buffer controls ($P < 0.05$). The

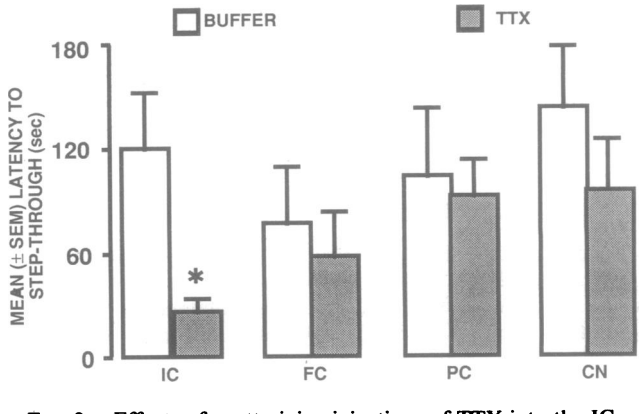

FIG. 2. Effects of posttraining injections of TTX into the IC on retention in the one-trial inhibitory avoidance task (*, $P < 0.05$).

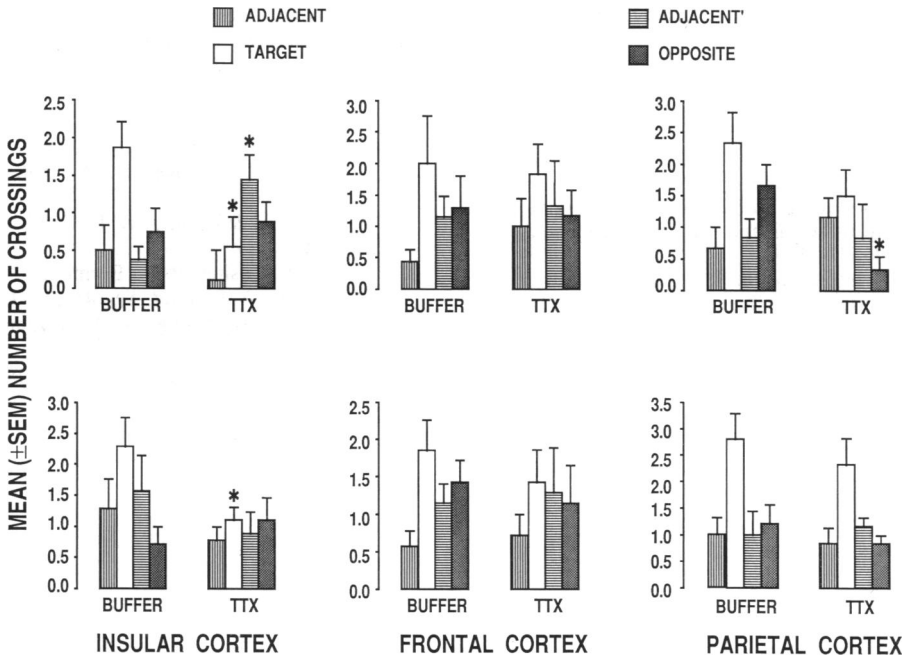

FIG. 3. Effects of TTX on retention of the water maze spatial task. (*Upper*) Pretraining injections into the IC significantly reduced the number of crossings of the target quadrant (*, $P < 0.05$ as compared with the IC buffer control). (*Lower*) Posttraining injections of TTX into the IC significantly reduced the number of crossings of the target quadrant when compared with the IC-injected buffer control.

group given TTX in the IC prior to training showed a significant increase in the number of crossings of one adjacent quadrant. In the other groups, the number of crossings of the target quadrant was greater than that of adjacent quadrants, indicating that the animals in those groups retained the information acquired during training.

DISCUSSION

These findings indicate that in two training tasks, retention is impaired by TTX microinjected into the IC either before or after training. TTX injected into the IC prior to training impaired, but did not prevent, acquisition of the inhibitory avoidance task and did not disrupt acquisition of the water maze task. In the inhibitory avoidance tasks, the TTX and control animals did not differ in mean latency to enter the dark compartment when first placed in the apparatus. Thus, the effects of TTX on performance in this task do not appear to be due to a nonspecific influence on response latency.

It is well documented that retrograde amnesia can be induced by electrical stimulation of the cortex or induction of spreading cortical depression (15–17). However, as such treatments produce widespread changes in cortical functioning, the findings have not contributed to an understanding of the locus of cortical regions involved in memory. In contrast, TTX effects on brain activity are fairly localized. Local injection of TTX into brain tissue decreases 2-deoxyglucose uptake within only 1 mm from the loci of injection (12, 18). Our findings clearly indicate that the TTX effects on retention were localized in the IC. Injections administered posttraining to adjacent PC or FC were ineffective. These findings are consistent with other evidence indicating that memory consolidation is not affected by injections of local anesthetics into the PC (19).

As was noted, TTX injected into the parietal cortex before training also significantly impaired retention of the inhibitory avoidance response. These findings are similar to those of Fukuchi *et al.* (20), who reported that administration of a cholinergic receptor irreversible antagonist (propylbenzylcholine mustard) to the PC and frontoparietal cortex impaired retention when applied before but not after a single inhibitory avoidance training trial (20). In addition, large ablation of PC or posterior cingulate areas has been reported to impair spatial learning in a Morris water maze (21). Microinjections of leupeptin into the FC before training have been found to disrupt spatial learning (22). However, to our knowledge there have been no previous reports of retrograde amnesia induced by drugs administered to localized cortical regions.

Studies of the role of the IC in cognitive processes have, to date, been limited to experiments examining either taste/visceral memorial representation (2, 3, 6, 23) or temporal pattern discrimination (24, 25). Our findings indicating that the IC is also involved in inhibitory avoidance as well as spatial learning tasks are consistent with evidence showing that the IC is a multimodal brain area for the perception of temporal patterns of various classes of sensory stimuli (24, 25).

In summary, the results of these experiments indicate that TTX produces retrograde and anterograde memory impairment in inhibitory avoidance and spatial learning tasks and suggest that functional integrity of the IC is necessary for the consolidation of memory in these tasks.

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1. van der Kooy, D. L., Koda, L. Y., McGinty, J. F., Gerfen, C. R. & Bloom, F. E. (1984) *J. Comp. Neurol.* **224,** 1–24.
2. Kiefer, S. W. (1985) *Ann. N.Y. Acad. Sci.* **443,** 100–109.
3. Lasiter, P. S. (1982) *Physiol. Psychol.* **10,** 377–383.
4. Norgren, R. & Wolf, G. (1975) *Brain Res.* **92,** 123–129.
5. Krushel, L. A. & van der Kooy, D. L. (1988) *J. Comp. Neurol.* **270,** 39–54.
6. Bermudez-Rattoni, F., Fernandez, J., Sanchez, M. A., Aguilar-Roblero, R. & Drucker-Colin, R. (1987) *Brain Res.* **416,** 147–152.
7. Bermudez-Rattoni, F., Sanchez, M. A. & Prado-Alcala, R. A. (1989) in *Aversion Avoidance and Anxiety*, eds. Archer, T. & Lars-Goran, N. (Erlbaum, Hillsdale, NJ).

8. Bermudez-Rattoni, F. & McGaugh, J. L. (1991) *Brain Res.*, in press.
9. Narahashi, T. (1972) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **31,** 1124–1132.
10. Rothfeld, J. M., Harlan, R. E., Shivers, B. D. & Pfaff, D. W. (1986) *Pharmacol. Biochem. Behav.* **25,** 857–863.
11. Paxinos, G. & Watson, C. (1986) *The Rat Brain in Stereotaxic Coordinates* (Academic, Orlando, FL).
12. Cahill, L. (1989) Dissertation (Univ. of California, Irvine).
13. McGaugh, J. L., Introini-Collison, I. B. & Nagahara, A. H. (1988) *Brain Res.* **446,** 37–49.
14. Decker, M. W., Introini-Collison, I. B. & McGaugh, J. L. (1989) *Psychobiology* **17,** 270–275.
15. Spanis, C. W. & Squire, L. R. (1987) *Behav. Neural Biol.* **48,** 237–245.
16. Gold, P. E., Macri, J. & McGaugh, J. L. (1973) *Science* **179,** 1343–1345.
17. Buresova, O. & Bures, J. (1985) *Brain Res.* **344,** 365–368.
18. Myers, R. D. (1966) *Physiol. Behav.* **1,** 171–174.
19. Perez-Ruiz, C. & Prado-Alcala, R. A. (1989) *Brain Res. Bull.* **22,** 599–603.
20. Fukuchi, I., Susumu, K., Nakahiro, M., Uchida, S., Ishida, R. & Yoshida, S. (1987) *Brain Res.* **400,** 53–61.
21. DiMattia, B. D. & Kesner, R. (1988) *Behav. Neurosci.* **102,** 471–480.
22. Deyo, R. A. & Conner, R. L. (1989) *Behav. Neural Biol.* **52,** 213–221.
23. Garcia, J., Lasiter, P. S., Bermudez-Rattoni, F. & Deems, D. (1985) *Ann. N.Y. Acad. Sci.* **443,** 8–20.
24. Colavita, F. B., Szeligo, F. V. & Zimmer, S. D. (1974) *Brain Res.* **79,** 153–156.
25. Colavita, F. B. & Weisberg, D. H. (1979) *Physiol. Behav.* **22,** 827–830.