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Intracranial self-stimulation induces Fos expression in GABA-ergic neurons in the rat mesopontine tegmentum

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Abbreviations: ACh, acetylcholine; ChAT, choline acetyltransferase; ICSS, intracranial self-stimulation; LDTg, laterodorsal tegmental nucleus; MFB, medial forebrain bundle; PBS, phosphate buffered saline solution; PPTg, pedunculo pontine tegmental nucleus; 5-HT, serotonin; TH, tyrosine hydroxylase; VTA, ventral tegmental area

Running title: Fos, ChAT and GABA in mesopontine tegmentum after ICSS

ABSTRACT

The cholinergic neurons which originate in the mesopontine tegmentum and innervate the midbrain ventral tegmental area have been proposed to play a key role in intracranial self-stimulation reward. This mesopontine area also contains GABA neurons. Detailed information is still lacking, however, about the relationship of cholinergic and GABA-ergic neurons in this region to self-stimulation reward. Therefore, using double immunostaining for Fos as a marker of neuronal activity and choline acetyltransferase as a marker of cholinergic neurons, or for Fos and GABA, we investigated whether self-stimulation of the medial forebrain bundle induces Fos expression within cholinergic and GABA-ergic neurons in two regions of the mesopontine tegmentum, i.e., pedunculopontine tegmental nucleus and laterodorsal tegmental nucleus. Self-stimulation of the medial forebrain bundle for one hour induced a large increase in the number of cells expressing Fos in both the pedunculopontine tegmental nucleus and laterodorsal tegmental nucleus, when compared to control brains. However, the self-stimulation-induced expression of Fos was restricted mostly to GABA-, but not choline acetyltransferase-, immunostained cells. We also examined, using microdialysis, whether self-stimulation increases acetylcholine efflux in the ventral tegmental area, a terminal region of the mesopontine tegmentum cholinergic pathway. One-hour of self-stimulation significantly increased acetylcholine efflux from this terminal area. The results indicate that intracranial self-stimulation of the medial forebrain bundle may increase acetylcholine release without affecting expression of Fos in cholinergic neurons, while the same stimulation may induce Fos expression in GABA-ergic neurons of the mesopontine tegmentum. GABA-ergic as well as cholinergic neurons in this area appear to be activated by self-stimulation reward in the medial forebrain bundle.

KEY WORDS:

Intracranial self-stimulation, Microdialysis, Fos, Acetylcholine, Choline acetyltransferase, GABA

Although mesoaccumbal dopamine is considered important in the mediation of intracranial self-stimulation (ICSS) behavior (Phillips and Fibiger, 1978; Fibiger and Phillips, 1987; Wise and Rompre, 1989), its role seems to be modulatory (Garris et al., 1999; Miliaressis et al., 1991; Zacharko and Anisman, 1991). Several recent studies have demonstrated that ICSS of the medial forebrain bundle (MFB) or ventral tegmental area (VTA) increases the expression of Fos, an anatomical marker of cellular activation, in many brain regions where catecholaminergic or serotonergic cell bodies are found (Arvanitogiannis et al., 1996, 1997; Flores et al., 1997; Hunt and McGregor, 1998; Nakahara et al., 1999). Furthermore, one preliminary but noteworthy report using a double labeling technique for Fos and tyrosine hydroxylase (TH), an indicator of catecholamine neurons, has revealed that most of the TH-immunoreactive cells in the VTA of self-stimulating rats did not express Fos (Hunt and McGregor, 1998). Recently, our quantitative analysis using a combined immunostaining method for Fos and serotonin (5-HT) or for Fos and TH extended this finding and proved that the percentage of Fos/TH double-labeled cells in association with ICSS was approximately 54% of all Fos-positive cells in the locus coeruleus and only 13% in the VTA, whereas 38% of Fos-labeled cells contained 5-HT in the dorsal raphe (Ishida et al., 2001). Interestingly, the same study also found that a relatively large portion (61-88%) of these Fos-positive cells were GABA-ergic neurons, which are co-distributed with monoaminergic neurons in the VTA, locus coeruleus and dorsal raphe (Ishida et al., 2001). Thus, it was suggested that the ICSS-induced expression of Fos protein was evidently different among the three cell populations, and that 5-HT, noradrenaline and GABA as well as dopamine may also play some role in the expression of ICSS behavior.

Acetylcholine (ACh) has been considered another important transmitter related to ICSS reward. It has been reported that the rewarding effects of ICSS are strongly inhibited or blocked by cholinergic antagonists infused in the VTA (Rada et al., 2000; Yeomans and Baptista, 1997), or by cholinergic agonists in the PPTg where muscarinic ACh receptors are inhibitory (Yeomans et al., 1993). Several lines of evidence have proposed that ICSS of the MFB directly activates non-dopaminergic, descending myelinated axons that connect monosynaptically and/or multisynaptically to dopaminergic cells of the VTA (Bielajew and

Shizgal, 1986; Gallistel et al., 1981; Yeomans 1989, 1995). One of the important multisynaptic pathways comes from cholinergic neurons of the mesopontine tegmentum (Yeomans, 1995; Yeomans et al., 1993). Thus, mesopontine cholinergic input can activate the ascending mesoaccumbal dopaminergic pathway, which is responsible for the initiation of ICSS behavior (Garris et al., 1999). Moreover, the mesopontine tegmentum contains many GABA-ergic neurons which are co-distributed with the cholinergic neurons (Ford et al., 1995; Honda and Semba, 1995; Jones, 1991b). However, the effects of ICSS on the Fos expression in cholinergic as well as GABA-ergic cells of the mesopontine tegmentum have not been reported.

In the present study, therefore, we used double immunostaining for Fos and choline acetyltransferase (ChAT) as an index of cholinergic neurons, or for Fos and GABA to clarify the neurotransmitter phenotype of neurons expressing Fos following ICSS of the MFB in two regions of the mesopontine tegmentum, i.e., pedunculopontine tegmental nucleus (PPTg) and laterodorsal tegmental nucleus (LDTg). Additionally, we also investigated, using microdialysis, whether ICSS affects ACh efflux in the VTA, a target region projecting from the cell bodies of cholinergic neurons in the PPTg and LDTg.

EXPERIMENTAL PROCEDURES

Subjects and surgery

All of the procedures for animal treatment and surgery were in accordance with the guidelines established by the *Institute for Experimental Animals of Hamamatsu University School of Medicine*, and were approved by the University committee for animal experiments.

Male Wistar rats (Japan SLC, Hamamatsu, Japan), weighing 230-250 g were used. The animals were individually housed in cages, which were maintained on a light-dark cycle (lights on from 7 A.M. to 7 P.M.) in a temperature controlled environment (23°C), with food and water continuously available. For the immunohistochemical experiment, the rats had monopolar electrodes bilaterally implanted in the MFB (3.8 mm posterior to bregma, 1.6 mm lateral from the midline and 8.0 mm ventral from the surface of the skull) of the brain under sodium pentobarbital (50 mg/kg, i.p.) using stereotaxic coordinates (Paxinos and Watson,

1982). The reference electrode, a 1.2 mm watch screw, was attached to the frontal bone. For the microdialysis experiment, the animals had an electrode and a guide cannula implanted, ipsilateral to the electrode, near the VTA (at a 12° angle with respect to the interaural line; 6.8 mm posterior to bregma, 1.0 mm lateral to the midline, and 6.0 mm ventral from the surface of the skull). The electrode or electrode/cannula assembly was secured with skull screws and dental cement, and then a dummy probe was placed inside the cannula. Electrodes consisted of a stainless steel wire (0.2 mm in diameter) coated with polyurethane, except for the tip. The cannula guide was made from the catheter taken from a 22G JELCO™ I.V. catheter placement unit (Johnson and Johnson Medical, Arlington, Texas) as described elsewhere (Nakahara et al., 1993). The dialysis probe, which was made from the 25G introducing needle taken from the same catheter unit, was concentric in design with a side-by-side inlet and outlet arrangement and a dialysis tubing. The effective area of the dialysis membrane (Cuprophane, 0.23 mm in outer diameter, 10 µm in wall thickness, M.W. cut-off: <35000) (Nikkiso, Tokyo, Japan) was 2 mm in length. The dummy probe was made from a JELCO™ I.V. stylet.

*ICSS training**

Following 5-7 days of post-operative recovery, the rats were placed in a 25.0 x 30.0 x 28.5 cm transparent acrylic box with a lever and trained to press the lever for the rewarding MFB stimulation. Each lever-press delivered a 0.3 sec train of biphasic, rectangular pulses with a pulse duration of 0.1 msec and a pulse frequency of 100 pulses/sec on a continuous reinforcement schedule. The current intensity was varied by the experimenter, and finally fixed at the value between 500 and 800 µA that sustained stable lever-pressing. The subjects showing stable lever-pressing with minimal motoric side-effects were chosen for further testing.

*Immunohistochemistry** For the immunohistochemical study, ICSS was estimated for each electrode on the training day, and one electrode which produced a more vigorous response than the other was used on the test day. The immunohistochemical experiment started at least 48 hr later, to wait for Fos expressed following the ICSS training to disappear. The experimental animals were placed in the ICSS box and were allowed to press the lever

for brain-stimulation reward for 1 hr. Two hours following the final ICSS, each rat received an overdose of sodium pentobarbital (100 mg/kg, i.p.) and was perfused transcardially first with 250 ml of cold saline followed by 500 ml of cold freshly prepared 3% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (pH 7.4), and finally 250 ml of 10% sucrose in 0.1 M phosphate buffer. The brains were removed quickly and immersed in 30% sucrose in 0.1 M phosphate buffer overnight for two nights at 4°C. The control animals were subjected to the same procedure, except that they received no stimulation on the test day. The brains were subsequently cut on a freezing microtome into 50 µm coronal sections.

Immunohistochemistry was performed as previously described (Ishida et al., 2000, 2001; Maloney et al., 1999; Nakahara et al., 1999). For the immunostaining of Fos protein, we used a rabbit polyclonal antibody raised against a peptide corresponding to human *c-fos* amino acid residues 3-16 (diluted 1:5000; Santa Cruz Biotechnology, Santa Cruz, CA). For the immunostaining of ChAT and GABA, a mouse anti-ChAT antiserum (1:3000; Chemicon International, Temecula, CA) and a mouse anti-GABA antiserum (1:500; Sigma, St. Louis, MO) were used, respectively. In this study, Fos was immunostained in the first place and ChAT or GABA in the second place. The sections, collected in phosphate buffered saline solution (PBS; pH 7.4), were processed first for Fos immunohistochemistry according to the instructions for use of streptavidin-biotin system (Histofine SAB-PO(R) kit, Nichirei, Tokyo, Japan). After incubation in 10% normal goat serum for 60 min at room temperature, the sections were incubated at 4°C overnight with the antibody to Fos. The sections were washed three times in PBS and incubated at room temperature for 60 min with a secondary biotinylated goat anti-rabbit IgG, washed three times in PBS, and further incubated at room temperature for 20 min with a streptavidin-peroxidase complex. After three washes in PBS, the reaction products of biotinylated goat anti-rabbit IgG and streptavidin-conjugated horseradish peroxidase were visualized using 0.01% diaminobenzidine tetrahydrochloride (Sigma) and 0.0003% hydrogen peroxide, and intensified by pretreatment with 0.125% cobalt chloride. This method induced a black reaction of Fos-positive cells. Next, after an overnight wash in PBS, the sections were again incubated at 4°C overnight with the antibody against ChAT or GABA. The tissues were processed using a similar procedure as described above,

except that the second reaction was visualized with the avidin-biotin kits (Vector Laboratories, Burlingame, CA) using diaminobenzidine tetrahydrochloride as a chromogen without cobalt enhancement. This approach produced a brown reaction of ChAT- or GABA-positive cells. Finally, the sections were mounted on gelatin-coated glass slides, air-dried, dehydrated, coverslipped and analyzed with light microscopy. There was no apparent cross-reactivity between the anti-Fos antibody and each of the anti-ChAT and anti-GABA antibodies. Omission of the primary antibodies from the immunocytochemical procedure eliminated Fos-, ChAT- or GABA-like immunoreactivity.

Cell counting was made from two to four sections/nucleus in each animal under 10 x magnification using a microscopic 0.25 x 0.25 mm² grid. The single- and double-immunostained cells were counted bilaterally in two regions, PPTg and LDTg. The cell counts were averaged per nucleus.

Microdialysis

The microdialysis collections started 2-3 days after the ICSS training. On the morning of the experiment, the dummy probe was replaced with the dialysis probe, which was secured to the guide cannula using sticky wax. The animals were then placed in the ICSS box from which the lever had been removed. The dialysis probe was perfused with a Ringer solution (138 mM NaCl, 2.4 mM KCl, 1.2 mM CaCl₂, at pH 6.5-7.0) containing 10 µM eserine (Wako Pure Chemical Industries, Osaka, Japan), an acetylcholine esterase inhibitor, at a flow rate of 2 µl/min. Measurement of steady-state levels of ACh was begun after a 180-min stabilization period. Three consecutive samples were collected at 20-min intervals in small plastic vials to determine steady-state (basal) levels. Subsequently, the rats were allowed to press the lever for brain-stimulation reward for 1 hr. Sample collections continued during and for 2 hr after ICSS. ACh levels in dialysates were analyzed by reverse-phase high-performance liquid chromatography (HPLC) with electrochemical detection. The HPLC system (BAC-300 system, EICOM, Kyoto, Japan) consisted of an EP-300 pump, an ECD-300 electrochemical detector with a WE-PT platinum working electrode, and a PowerChrom system (ADI, Sydney, Australia). The detection potential was set at +450 mV against an Ag/AgCl reference electrode. Choline and acetylcholine were separated on a reverse-phase

Eicompak AC-GEL column (4 μ m, 2 ϕ x 150 mm) and reacted on an Eicom AC-Enzymepak immobilized column (3 ϕ x 5 mm) containing acetylcholine esterase and choline oxidase. The flow rate was 150 μ l/min, and the sensitivity was set to 0.1 nA/V full scale. The mobile phase consisted of a 50 mM phosphate buffer (pH 8.2) containing 1.23 μ M sodium 1-decansulfonate and 13.4 μ M disodium ethylenediaminetetraacetic acid. Following the experiments, all animals were deeply anesthetized with an overdose of sodium pentobarbital and perfused intracardially with 10% formalin in saline. The brains were removed and placed in 10% formalin for more than 1 week. Brain tissues were frozen and coronally cut as 30 μ m slices and stained with cresyl violet for the verification of the tip location of cannulas and electrodes.

Statistics

To evaluate the effects of stimulation on single- and double-labeled cells in each nucleus, the bilateral average cell counts of the cells for the ICSS group were compared, using the Mann-Whitney U-test, to the corresponding average for the control group. To evaluate the unilateral effects of stimulation on each nucleus, the cell counts for each stimulated side was compared, using the Wilcoxon signed rank test, to the corresponding values for the unstimulated side in the ICSS group. The effect of ICSS on extracellular levels of ACh was analyzed using one-way repeated measures analysis of variance coupled with the least significant difference test for multiple comparisons. The level of significance was set at $p < 0.05$.

RESULTS

Fos expression associated with ICSS

In the two brain regions examined, ICSS of the MFB significantly increased the expression of Fos when compared with control brains. Moreover, both regions showed a significantly higher number of Fos-positive cells on the stimulated side than on the unstimulated side. However, the extent to which the MFB stimulation induced Fos expression depended on the individual region (Tables 1 and 2).

Co-localization of Fos expressed following ICSS with either ChAT or GABA

The neurotransmitter phenotype of the cells showing an increased expression of Fos was also examined. Even in control animals, there was a significant difference in the number of double-labeled neurons in the PPTg and LDTg with GABA-positive/Fos-positive cells being much larger than ChAT-positive/Fos-positive cells. This indicates that ChAT-labeled neurons in these nuclei did not express Fos as easily as GABA-labeled neurons, even at rest. ICSS caused a significant increase in the number of ChAT/Fos double-labeled cells in both hemispheres of the LDTg, but with no asymmetry (Table 1 and Fig. 1). In the PPTg, this increase was much higher than in the LDTg, but apparently did not reach significance because of the variability of the ICSS data. In contrast, significantly increased Fos expression was observed in many of the cells that were immunostained for GABA in both regions, with a difference in the number of GABA/Fos double-labeled cells between the stimulated and unstimulated side (Table 2 and Fig. 1). The mean proportion of co-localization for Fos and GABA in the stimulated side represented $52.6 \pm 0.16\%$ of all of the Fos-positive cells in the PPTg, and $44.4 \pm 0.16\%$ in the LDTg. Thus, a large portion of Fos-positive cells following ICSS contained GABA. However, a similar proportion of Fos-positive cells also contained GABA in the PPTg and LDTg in control brains, thus ICSS did not increase the percentages of GABA/Fos double-labeled cells with respect to the total number of Fos-positive cells in the two nuclei.

ACh efflux in the VTA during ICSS

In order to examine whether ICSS of the MFB affects the release of ACh in cholinergic terminal areas, we monitored, using microdialysis, ACh effluxes from the VTA. This area demonstrated a significant increase in ACh ($F_{11,44}=28.94$; $p<0.001$) effluxes during ICSS (Fig. 2). Thus, the result indicates that there was no relation between the expression of Fos in cholinergic cells of the mesopontine tegmentum and the release of ACh in its terminal area associated with ICSS.

As illustrated in Fig. 3, electrode tips were found within or near the MFB. Microdialysis probe tracts were also confirmed to be located near or inside the VTA.

DISCUSSION

The present immunohistochemical data demonstrated that ICSS of the MFB induces the expression of Fos in cholinergic cell body areas of the PPTg and LDTg, with ipsilateral predominance, a result that corresponds with findings in previous reports (Arvanitogiannis et al., 1997; Oda et al., 1999). Evidence from double-immunostaining for Fos and ChAT or for Fos and GABA revealed, however, that Fos expression induced by the stimulation is mostly confined to GABA-, but not ChAT-, immunostained cells which are co-distributed with cholinergic neurons in the above regions. Recently, such Fos expression in the PPTg and LDTg has also been obtained from another laboratory, using the dual-immunostaining of Fos and reduced nicotinic adenine dinucleotide phosphate-diaphorase as a marker of cholinergic neurons (Oda et al., 1999). In addition, consistent with recent evidence (Rada et al., 2000), our microdialysis finding confirmed that the rewarding MFB stimulation increases ACh efflux in the VTA.

Absence of Fos expression in cholinergic neurons

Surprisingly, many of the cholinergic cells in the PPTg and LDTg did not express Fos in response to ICSS. Recently, nicotine, a drug reward, has also been found to induce Fos expression in non-cholinergic, but not cholinergic, neurons of the PPTg and LDTg (Lanca et al., 2000). Nevertheless, in cats paradoxical sleep is reported to be associated with induction of Fos immunoreactivity in ChAT-positive cells of the mesopontine nucleus (Maloney et al., 1999). Thus, as the cholinergic neurons appear to possess the capability of expressing Fos, it is possible that one-hour of ICSS was not sufficient to induce Fos expression in cholinergic neurons of the PPTg or LDTg. Noradrenergic and serotonergic neurons, which are most active in animals that are awake (Aston-Jones and Bloom, 1981; McGinty and Harper, 1976), project to the mesopontine tegmentum (Honda and Semba, 1994; Jones, 1991a). These transmitters have been shown to inhibit cholinergic neurons of the LDTg (Koyama and Kayama, 1993; Leonard and Llinas, 1994; Luebke et al., 1992; Williams and Reiner, 1993). Our recent immunohistochemical study showed ICSS-induced significant increases in the number of double-labeled, TH-positive/Fos-positive cells in the locus coeruleus and in the number of double-labeled, 5-HT-positive/Fos-positive cells in the dorsal raphe (Ishida et al.,

2001). It is also possible, therefore, that metabolic activation of cholinergic neurons in the PPTg and LDTg was suppressed during ICSS, at least partially, through the excitation of noradrenergic and/or serotonergic neurons, thus resulting in very few cholinergic cells expressing Fos before and after ICSS. However, this possibility seems to be improbable, because previous (Rada et al., 2000) and present microdialysis studies clearly demonstrated that ICSS of the lateral hypothalamus increased ACh efflux in the VTA, a target region projecting from the cell bodies of the PPTg and LDTg cholinergic neurons. The increase in VTA ACh levels observed during ICSS strongly suggests activation of cholinergic cells in the mesopontine tegmentum. Fos is not necessarily expressed in every neuron that responds to a variety of physiological and pharmacological stimuli (Deutch et al., 1991; Dragunow and Faull, 1989; Sagar et al., 1988). Therefore, a lack of Fos induction in the cholinergic neurons following ICSS cannot be interpreted as an absence of activity of these neurons. Alternatively, it is most likely that cholinergic cells of the LDTg and PPTg did not require the mechanism involved in Fos expression for increasing ACh release under our ICSS situation. Such a possibility should be confirmed by detecting other transcription factors responsible for activation of ACh release associated with ICSS.

Terminal ACh release

Presynaptic regulation of the terminal release of neurotransmitters has been found, under stressful conditions, in the dopaminergic and serotonergic systems. Tail-pinch stress caused an increase in the 5-HT release of serotonergic terminals without enhancing the firing activity of serotonergic cells in the raphe (Reuter and Jacobs, 1996; Reuter et al., 1997). Furthermore, restraint stress increased extracellular levels of dihydroxyacetic acid, a major dopamine metabolite, in the nucleus accumbens, whereas the same stress did not increase Fos expression in the VTA dopaminergic neurons projecting to the nucleus accumbens (Deutch et al., 1991). Therefore, there still remains a possibility that terminal ACh release occurred through presynaptic mechanisms, independently of alterations in the cholinergic neuronal activity of self-stimulating animals. Presynaptic regulation of terminal release may be one of the common features of the central neurotransmitter system.

Co-expression of Fos and GABA

A previous study by Ford et al. (1995) reported that GABA-ergic cells were more numerous than ChAT-positive cells in the LDTg and PPTg. This finding appears to be incompatible with the present result showing that the proportion of ChAT- and GABA-positive cells was similar in these nuclei. However, this incompatibility might be due to different methods employed. There is a possibility that since Ford et al. used an antibody to glutamic acid decarboxylase (GAD) to detect GABA-ergic neurons, with the brains pretreated with colchicine to maximize levels of GAD within the cell bodies of neurons, they could not properly determine GABA-ergic neurons.

We demonstrated for the first time that ICSS increased Fos expression in GABA-ergic cells which are co-distributed with cholinergic cells in the PPTg and LDTg (Ford et al., 1995; Honda and Semba, 1995; Jones, 1991b). However, in control brains, the percentage of GABA/Fos double-labeled neurons was 41 to 64% of all of the Fos-positive cells, and this percentage was unchanged in the ICSS-treated brains. This indicates that ICSS increased just the total number of Fos-positive cells, but not the proportion of GABA/Fos double-labeled neurons in these brain regions. In addition, previous (Hunt and McGregor, 1998; Ishida et al., 2001) and present studies showed that Fos expression is very low in dopaminergic neurons of the VTA and cholinergic neurons of the PPTg and LDTg which are both important for brain-stimulation reward. It is likely, therefore, that spontaneous activity in dopaminergic and cholinergic neurons is under restraint because their activity has major effects on overall locomotion, cortical activation and reward (Sheel-Kruger and Willner, 1991; Yeomans, 1995). The GABA-ergic neurons, which restrain these dopaminergic and cholinergic neurons, appear to be even more important when dopaminergic and cholinergic neurons release their transmitters as they do during ICSS and other rewarding events. Further research is needed to determine a precise mechanism by which these Fos-expressing GABA-ergic cells influence neighboring cholinergic neurons.

Conclusions

In summary, the present findings show that Fos expression increased by ICSS of the MFB was limited mostly to GABA-ergic, but not cholinergic, neurons in the LDTg and PPTg, whereas the same stimulation enhanced extracellular ACh in the VTA. Thus, during ICSS,

cholinergic cells of the mesopontine tegmentum may excite but not require the mechanism involved in Fos expression for ACh release, while excitation of GABA-ergic neurons during this situation may need the Fos mechanism. Mesopontine GABA-ergic as well as cholinergic neurons may be an important component of the neurochemical mechanisms underlying ICSS reward.

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Table 1. Number of neurons labeled for ChAT or Fos and neurons containig both ChAT and Fos immunoreactivities

	control		ICSS	
	left (n)	right (n)	stimulated (n)	unstimulated (n)
PPTg				
ChAT [†]	15.8±2.4 (9)	15.2±1.9 (9)	19.0±2.0 (8)	20.4±2.4 (8)
Fos [‡]	1.7±0.4 (9)	2.0±0.7 (9)	10.7±1.7 ^{**x} (8)	3.5±1.1 (8)
ChAT/Fos [§]	0.1±0.1 (9)	0.2±0.1 (9)	0.8±0.4 (8)	0.5±0.4 (8)
LDTg				
ChAT [†]	39.6±3.8 (8)	39.4±3.6 (8)	51.2±4.4 (8)	48.4±3.7 (8)
Fos [‡]	9.0±1.9 (8)	8.3±1.3 (8)	21.6±3.2 ^{*x} (8)	16.9±2.8 (8)
ChAT/Fos [§]	0.4±0.3 (8)	0.5±0.3 (8)	1.3±0.4 [*] (8)	1.1±0.2 (8)

[†]ChAT cell numbers correspond to the mean±SEM of the total number of ChAT-positive cells counted unilaterally and averaged for two to four sections per nucleus.

[‡]Fos cell numbers correspond to the mean ±SEM of the total number of Fos-positive cells counted unilaterally and averaged for two to four sections per nucleus.

[§]ChAT/Fos cell numbers correspond to the mean±SEM of the total number of double-labeled cells counted unilaterally and averaged for two to four sections per nucleus.

^{*} $p < 0.05$, ^{**} $p < 0.01$; combined stimulated and unstimulated counts differ from the combined values in control group. ^x $p < 0.05$; differences between stimulated and unstimulated counts in ICSS group.

Table 2. Number of neurons labeled for GABA or Fos and neurons containig both GABA and Fos immunoreactivities

	control		ICSS	
	left (n)	right (n)	stimulated (n)	unstimulated (n)
PPTg				
GABA [†]	16.4±0.6 (8)	16.8±0.8 (8)	18.6±0.9 (9)	16.9±0.4 (9)
Fos [‡]	1.8±0.6 (8)	1.4±0.6 (8)	17.1±1.8 ^{***xx} (9)	8.8±1.0 (9)
GABA/Fos [§]	0.9±0.4 (8)	0.9±0.5 (8)	9.0±0.8 ^{***x} (9)	4.7±0.5 (9)
LDTg				
GABA [†]	28.3±1.3 (8)	29.5±1.9 (8)	32.0±1.8 (9)	32.9±1.6 (9)
c-Fos [‡]	8.3±1.4 (8)	8.4±1.2 (8)	25.7±1.7 ^{***xx} (9)	17.6±1.1 (9)
GABA/c-Fos [§]	3.4±0.6 (8)	3.8±0.6 (8)	11.4±0.8 ^{***x} (9)	8.4±0.8 (9)

[†]GABA cell numbers correspond to the mean±SEM of the total number of GABA-positive cells counted unilaterally and averaged for two to four sections per nucleus.

[‡]Fos cell numbers correspond to the mean ±SEM of the total number of Fos-positive cells counted unilaterally and averaged for two to four sections per nucleus.

[§]GABA/Fos cell numbers correspond to the mean±SEM of the total number of double-labeled cells counted unilaterally and averaged for two to four sections per nucleus.

^{***} $p < 0.001$; combined stimulated and unstimulated counts differ from the combined values in control group.

^x $p < 0.05$, ^{xx} $p < 0.01$; differences between stimulated and unstimulated counts in ICSS group.

FIGURE LEGENDS

Fig. 1. A and B: Photomicrographs of neurons immunoreactive for either Fos or ChAT in the mesopontine tegmentum of ICSS rats. Black and white arrowheads indicate single-labeled ChAT- and Fos-positive cells, respectively. C and D: Photomicrographs of neurons immunostained for both Fos and GABA in the mesopontine tegmentum of ICSS animals. White arrows indicate double-labeled cells. Black arrowheads indicate adjacent single-labeled GABA-positive cells. The black circle in the drawing indicates the location of areas where photomicrographs were taken. Scale bar, 20 μ m.

Fig. 2. Effect of ICSS at the MFB on the dialysate levels of ACh in the VTA during continuous perfusion of the Ringer solution with eserine (10 μ M). Inset displays ICSS rates every 20 min. Values are the mean $\pm$ SEM (n=5). During ICSS of the MFB, animals received a mean $\pm$ SEM of 1104.2 $\pm$ 103.2 trains of stimulations in the first 20-min ICSS session, 1298.8 $\pm$ 102.3 in the second 20 min session, and 1129.0 $\pm$ 53.8 in the third 20 min session. ** p <0.01 significant difference from basal levels.

Fig. 3. Coronal sections showing the site of the electrode placement (*upper*) and microdialysis membrane placement (*lower*). The solid and open circles indicate electrodes used in immunohistochemical and microdialysis experiments, respectively. The electrodes are all depicted on the left side of the brain for convenience sake. The numbers above each section indicate the distance (mm) posterior (-) to bregma. Drawings are adopted from the atlas of Paxinos and Watson (1982). *mfb*, medial forebrain bundle; *VTA*, ventral tegmental area.

Figure 1

Figure 2

Figure 3

Electrodes

Probes