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Effect of Acute Nicotine on Fos Protein Expression in Rat Brain During Chronic Nicotine and Its Withdrawal

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SALMINEN, O., T. SEPPÄ, H. GÄDDNÄS AND L. AHTEE. *Effect of acute nicotine on Fos protein expression in rat brain during chronic nicotine and its withdrawal.* PHARMACOL BIOCHEM BEHAV **66**(1) 87–93, 2000.—To study the cholinergic regulation of hypothalamic paraventricular (PVN) and supraoptic (SON) nuclei and interpeduncular nucleus (IPN) we investigated the effects of acute nicotine (0.5 mg/kg, SC, 60 min) on Fos-like immunostaining (IS) during chronic nicotine and its withdrawal in rats. Nicotine or saline was infused to rats via osmotic minipumps (4 mg/kg/day) for 7 days; on the seventh day, the minipumps were removed surgically. In control rats, acute nicotine increased Fos IS significantly in all three brain areas studied. On the seventh day of nicotine infusion this effect partially persisted in IPN but was abolished in PVN and SON. After 72-h withdrawal nicotine-induced elevation of Fos IS was similar to that of control rats in all three areas. The observed attenuation of the response to acute nicotine during constant nicotine infusion in PVN and SON may be attributable to the desensitization of nicotinic acetylcholine receptors (nAChRs) mediating the effects of nicotine in these areas or in their input areas. IPN is connected to midbrain limbic system, so in agreement with our earlier observations, it seems that limbic nicotinic receptors do not very readily desensitize during chronic nicotine infusion. These findings support the suggestions that there are differences in the level of desensitization of nAChRs. © 2000 Elsevier Science Inc.

Nicotine Constant infusion Fos protein Desensitization Nicotinic acetylcholine receptors

IMMEDIATE early genes (IEG) are rapidly and transiently expressed in response to a variety of cellular stimuli, and the protein products of these genes act as transcription factors that regulate the expression of a wide variety of other cellular genes (16,19). The prototypical IEG is c-*fos*, which is expressed at very low levels in unstimulated brain, but which is rapidly induced by a large variety of stimuli (10,11,18). Induction of c-*fos* has been used extensively to map brain regions activated in response to many stimuli (13,34), including nicotine, the major addictive component of tobacco. Acute administration of nicotine induces the expression of Fos protein, encoded by IEG c-*fos*, in numerous brain regions including the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus (23,35) and interpeduncular nucleus [IPN; (29,32,35)].

Nicotine indirectly stimulates the release of corticotropin-releasing hormone (CRH) from the parvocellular region of the PVN (pcPVN) by acting on brainstem noradrenergic structures in a dose dependent and regionally selective manner (23,24). The CRH neurons located in pcPVN send their axons to the portal capillaries in the median eminence and subsequently stimulate adrenocorticotrophin (ACTH) release from the anterior pituitary (46). Nicotine is well known to elevate systemic vasopressin levels (2,52), which, in addition to regulating fluid and electrolyte balance (45), also potentiates the effects of CRH on ACTH release (31). Large (magnocellular) neurons, whose perikarya are located principally within the SON, PVN, and accessory nuclei of the hypothalamus synthesize vasopressin and oxytocin (44). Cholinergic innervation mediates the vasopressin release within SON (1). The cholinergic habenulo–interpeduncular pathway, i.e., the neurons sending their axons from medial habenula to IPN, possesses high density of ^{3}H-nicotine binding sites (7,20), that are either pre- or postsynaptic nicotinic acetylcholine receptors (nAChRs) (6,27). It has been reported that IPN partly mediates the nico-

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tinic depression of locomotor activity, and is involved in dampening the nicotinic arousal mechanisms (17). In humans, nicotine's effects have been found to depend on the baseline level of arousal at the time individual uses tobacco (33).

Previously, we investigated the effects of chronic nicotine infusion and withdrawal to the nicotine-induced Fos protein expression in various dopaminergic target areas to determine whether nicotine's actions could be detected in postsynaptic neurons at the level of IEGs (36). In this study, we extended the investigations of chronic nicotine and its withdrawal to the nicotine-induced Fos expression in PVN, SON, and IPN, which nuclei as described above seem to be also involved in nicotine's behavioural and hormonal effects.

METHOD

Animals

Male Wistar rats (body weight 200–300 g at the beginning of experiments) bred locally in the Laboratory Animal Center, University of Helsinki, were divided randomly into nicotine-receiving and control animals. The rats had free access to food and water and were housed singly after surgery. The lights were on from 0600–1800 h, and the ambient temperature was kept at 20–22°C. The experimental animals were maintained in accordance with the internationally accepted principles, and the experimental setup was approved by the Committee for Animal Experiments of the Faculty of Science of the University of Helsinki.

Drugs

Osmotic minipumps (Alzet 2001) contained 0.9% NaCl (saline) or nicotine hydrogen tartrate (Sigma Chemicals, St. Louis, MO) solutions. For injections (0.1 ml/100 g) (−)-nicotine base (Fluka, Buchs, Switzerland) was diluted with saline and pH of the final solution was adjusted to 7.0–7.4 with 0.05 M HCl prepared in saline. The osmotic minipumps were implanted under 3.5% halothane anaesthesia. When perfusing the rats intracardially they were anaesthetized with sodium pentobarbital (Orion Pharma, Espoo, Finland).

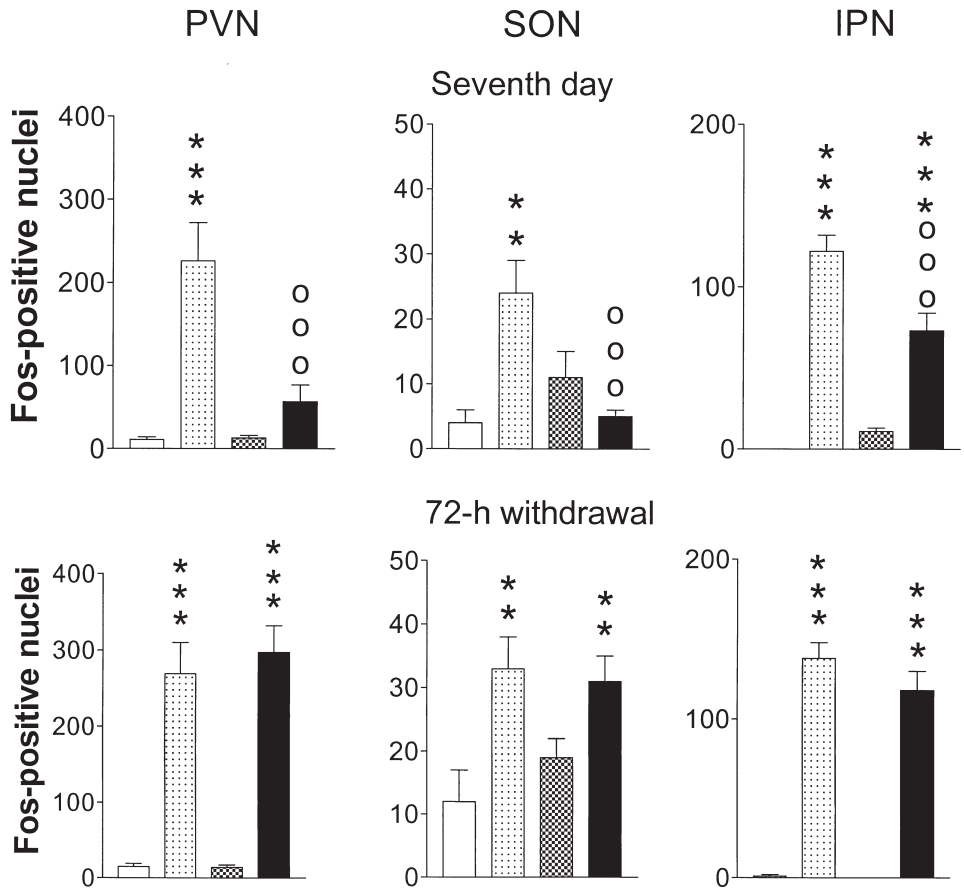

FIG. 1. The effect of an acute nicotine challenge (0.5 mg/kg, SC, 60 min) on Fos immunostaining in rat brain areas on the seventh day of chronic nicotine infusion (4 mg/kg/day) with the minipumps still in place and at 72 h after removal of the minipumps. Given are the mean numbers of Fos-positive nuclei (columns) ± SEM (vertical bars) of four to seven observations. $^{**}p < 0.01$, $^{***}p < 0.001$ compared with control rats given acutely saline SC, $^{ooo}p < 0.001$ compared with the control rats given acute nicotine. White columns, control rats + acute saline; dotted columns, control rats + acute nicotine; checkered columns, chronic nicotine + acute saline; black columns, chronic nicotine + acute nicotine. PVN, hypothalamic paraventricular nucleus; SON, supraoptic nucleus; IPN, interpeduncular nucleus.

Treatments

Osmotic minipumps containing either saline or nicotine solution were implanted subcutaneously to rats. Nicotine was infused at a dose of 4 mg/kg/day for 7 days. The dose refers to the base. Withdrawal was induced by removing the minipumps surgically on the seventh day of chronic treatment. The rats received saline (SC) or acute nicotine (0.5 mg/kg SC) 1 h before intracardial perfusion on the seventh day with the minipumps still in place or at 72 h after removal of the minipumps.

Tissue Preparation

At 1 h after acute saline or nicotine injection the rats were anaesthetized with pentobarbital (100 mg/kg IP) and intracardial perfusion was performed. Rats were perfused with 0.9% phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer pH 7.4. The brains were postfixed with the same fixative for 4 h at the room temperature after perfusion. The brains were immersed in a 20% sucrose solution at 4°C until used. The sections (40 μm) were cut on a cryostat.

Immunohistochemistry

The sections were first incubated in 2% normal rabbit serum (NRS, Vector Laboratories, Burlingame, CA; in PBS + 0.5% Tween 20 + 0.2% NRS) for 60 min to block nonspecific staining. The sections were then incubated in primary Fos antibody (OA-11-824, Genosys Biotechnologies Inc., Cambridge, UK) diluted in 1:1000 (experiments during chronic nicotine) or 1:2000 (withdrawal experiments) in PBS (in 0.5% Tween 20 + 4% NRS) for 72 h at 4°C. The antibody used was sheep polyclonal antibody to Fos oncoproteins to a synthetic peptide Met-Phe-Ser-Gly-Phe-Asn-Ala-Asp-Tyr-Glu-Ala-Ser-Ser-Ser-Arg-Cys, selected from a conserved region of mouse and human c-*fos* (47). The sections were processed with the avidin–biotin method (Vectastain Kit, Vector Laboratories, Burlingame, CA) with diaminobenzidine (Sigma Chemicals, St. Louis, MO) as the chromagen. The sections were then mounted on gelatine/chrome alumcoated slides, air dried, dehydrated through graded ethanols to xylene, and coverslipped with DePex (BDH Laboratory Supplies, Poole, UK). Controls for the immunostaining, which included omission of either primary or secondary antibody, demonstrated no Fos immunostaining.

Data Handling and Statistics

The Fos-positive nuclei were counted with a 10× objective with the assistance of a LEICA QWin image analysis system on selected brain areas within a rectangular area of 480 × 360 μm. The atlas of Paxinos and Watson (30) was used to identify the brain areas studied: the hypothalamic paraventricular nucleus (PVN), supraoptic nucleus (SON), and interpeduncular nucleus (IPN). A group mean (± SEM) was determined from the counts of four to seven rats in each treatment group. Data were analyzed by two-way ANOVA. If there were significant chronic × acute nicotine interactions ($p < 0.1$) the analysis was continued by comparing appropriate cell means with linear contrasts. Results were considered significant at $p < 0.05$.

RESULTS

The Hypothalamic Areas: Paraventricular and Supraoptic Nuclei

As shown in Figs. 1, 2, and 3, on the seventh day of chronic nicotine infusion or at 72 h after removal of the nicotine-

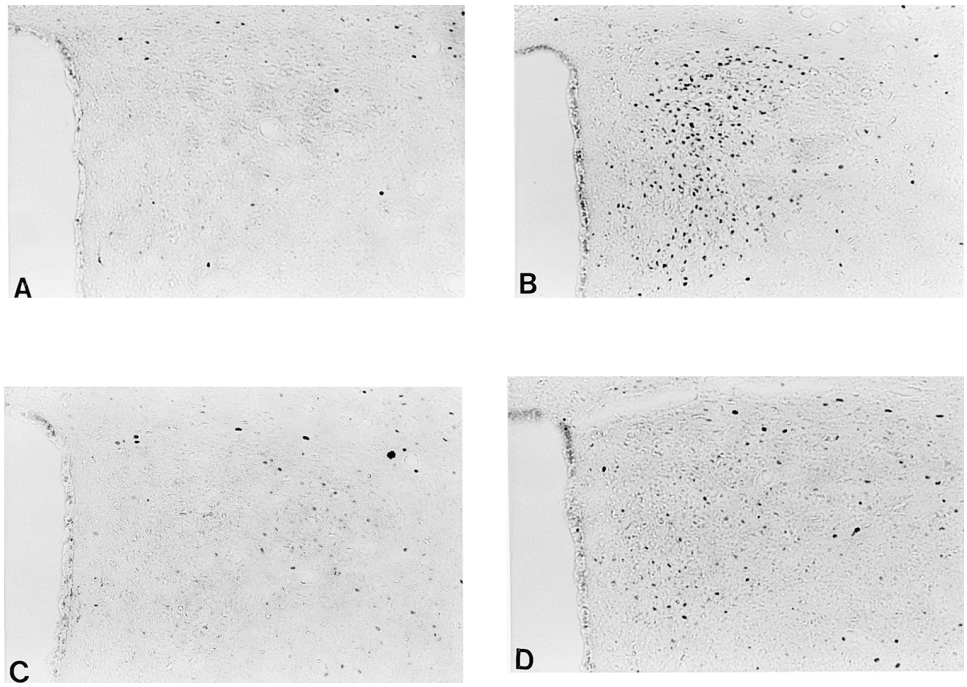

FIG. 2. Immunohistochemical localization of Fos protein expression in the hypothalamic paraventricular nucleus (PVN). (A) Control rat + acute saline. (B) Control rat + acute nicotine (0.5 mg/kg SC). (C) Chronic nicotine (4 mg/kg/day, 7 days) + acute saline. (D) chronic nicotine + acute nicotine.

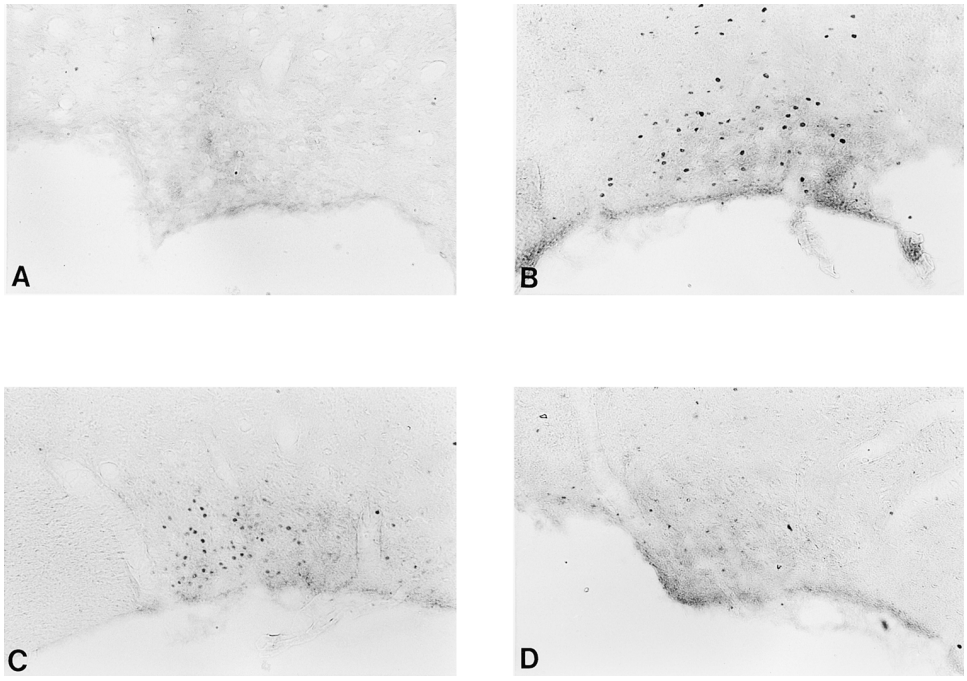

FIG. 3. Immunohistochemical localization of Fos protein expression in the hypothalamic supraoptic nucleus (SON). (A) Control rat + acute saline. (B) Control rat + acute nicotine (0.5 mg/kg SC). (C) Chronic nicotine (4 mg/kg/day, 7 days) + acute saline. (D) Chronic nicotine + acute nicotine.

releasing minipumps, the numbers of Fos-positive nuclei did not differ in the nicotine-infused and saline-infused control rats either in PVN and SON. Thus, neither chronic nicotine infusion nor its withdrawal affected the Fos protein expression.

In saline-infused control rats acute nicotine (0.5 mg/kg SC 60 min) increased significantly the number of Fos-positive nuclei in PVN and SON. However, on the seventh day of the chronic nicotine infusion, acute nicotine did not increase the number of Fos-positive nuclei in either hypothalamic nucleus studied. At 72 h after removal of the nicotine-releasing minipumps, the nicotine-induced increases of Fos expression in the nicotine-infused rats were similar to those of the saline-infused rats both in PVN and SON.

The Interpeduncular Nucleus

On the seventh day of chronic nicotine infusion or at 72 h after removal of the nicotine-releasing minipumps, the numbers of Fos-positive nuclei in IPN of the nicotine-infused and saline-infused control rats were similar (Figs. 1 and 4). Thus, chronic nicotine infusion or its withdrawal did not alter the number of Fos-positive nuclei.

In saline-infused control rats acute nicotine (0.5 mg/kg SC 60 min) increased the Fos immunostaining significantly. On the seventh day of chronic nicotine infusion acute nicotine increased Fos immunostaining, which was significantly less, however, than in saline-infused control rats. After 72-h withdrawal acute nicotine increased the number of Fos-positive nuclei in the nicotine-treated rats significantly and to the same degree as in the control rats.

DISCUSSION

In this study, the postsynaptic effects of nicotine in hypothalamic brain areas, supraoptic, and paraventricular nuclei, as estimated by Fos protein expression, were abolished on the seventh day of nicotine infusion. In the interpeduncular nucleus, the attenuation of Fos expression was clear, although acute nicotine still significantly increased the Fos IS during chronic nicotine. Nicotine's effects on Fos expression were restored after 72 h withdrawal in all above brain areas.

We found that Fos protein expression in response to acute nicotine was abolished in PVN and SON on the seventh day of nicotine infusion. This phenomenon could be caused by the desensitization of nAChRs located in these hypothalamic areas or in the regions regulating the functions of these areas. We previously used the same chronic nicotine administration protocol, which elevates the plasma nicotine concentrations approximately to those found in the plasma of heavy smokers (36), and found a similar abolishment of Fos expression in response to acute nicotine in dorsomedial caudate-putamen, a dopaminergic area.

Although nicotine is a potent activator of the HPA axis, as manifested by an increase in serum ACTH and corticosterone levels (3–5,8,50), nicotine does not directly act in the PVN. Systemic or ICV administration of nicotine induces noradrenaline (NA) release in the PVN in conscious rats (24,39) and subsequent ACTH secretion (2,21,22). Mecamylamine, given directly into the PVN of rats, did not antagonize the nicotine-induced elevation of extracellular NA concentrations as measured by *in vivo* microdialysis (15). In contrast, nicotine-induced NA release was blocked when mecamylamine was administered IP or directly into the fourth ventricle. Thus, systemic nicotine was concluded to act on nAChRs in the brain stem catecholaminergic regions rather than on presynaptic nAChRs to elicit NA release from axon terminals (25). Nicotine induces both Fos protein expression (23,35) and c-*fos* mRNA response (40) in PVN. The c-*fos* mRNA response desensitized when two intermittent IP nicotine injec-

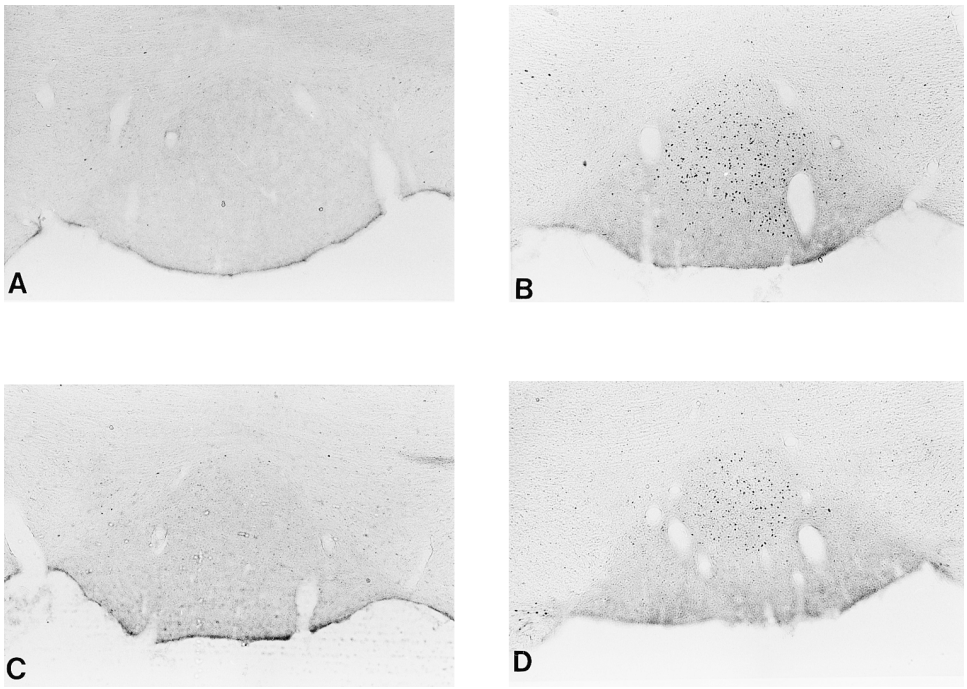

FIG. 4. Immunohistochemical localization of Fos protein expression in the interpeduncular nucleus (IPN). (A) Control rat + acute saline. (B) Control rat + acute nicotine (0.5 mg/kg SC). (C) Chronic nicotine (4 mg/kg/day, 7 days) + acute saline. (D) Chronic nicotine + acute nicotine.

tions were given at 2-h intervals (40). Sharp and Beyer (38) demonstrated that by repeating nicotine administration nicotine-induced release of ACTH was almost totally abolished. Also, nicotine-induced NA release in PVN was reduced by 50–60% when two nicotine injections were given at 100-min intervals either IP or directly into the fourth ventricle (24,39). These effects were concluded to result from desensitization of nAChRs. Our results show that the nAChRs mediating the effects of nicotine in PVN seem to be desensitized or tolerant during chronic nicotine infusion, as estimated by the Fos protein expression. This method does not reveal the actual location of nAChR desensitization, whether it occurs in NA-releasing neurons originating from brain stem or within PVN.

In SON, microinfusion of nicotine induces Fos protein expression mainly in the magnocellular vasopressin-releasing neurons (41). Shioda et al. (42) have localized both nAChR α4 subunit mRNA expression and immunoreactivity in the magnocellular neurons in SON. Nicotine seems to activate vasopressin-releasing neurons via nAChRs composed of α4 subunits and also linked to stimulation of cyclic AMP-protein kinase A-regulated Ca^{2+}-signaling pathway (43). On the other hand, nAChR α7 subunit mRNA has been detected in SON (37), and acetylcholine-evoked voltage clamp current in SON was abolished by nAChR α7 subunit selective antagonist methyllycaconitine but not by dihydro-β-erythroidine, an α4β2 nAChR subunit selective antagonist (51). In our study, the nAChRs mediating the nicotine-induced Fos protein expression in SON seem to be desensitized during chronic nicotine infusion. Both α4 and even more readily α7 nAChR subunit containing nicotinic receptors are reported to desensitize during nicotine exposure (14).

The IPN strongly expresses mRNAs for α2, α3, α4, α5, α7, β2, and β4 nAChR subunits (12,37,48,49). Also, in a quantitative *in vivo* ^{3}H-nicotine binding study the density of nicotinic binding sites in IPN was one of the highest of any brain region assayed (20). In addition to the cholinergic habenulo–interpeduncular pathway, IPN appears to occupy a central position on the midbrain limbic system (26). Most of the connections of the IPN are related to the neural circuits of the limbic system and its midbrain connections. For example, a direct pathway has been demonstrated from IPN to the hippocampus, the main integrative center of the limbic system (9). Thus, IPN is one of centers in the network of connections that convey and modulate the input from the limbic forebrain regions to the limbic midbrain area (9), and is involved in avoidance and emotional behavior (26) and also in arousal (17). In our study, the attenuation of Fos IS in IPN was clear but not as total as in PVN and SON, because acute nicotine still significantly increased the Fos IS during chronic nicotine infusion. This correlates quite well with our earlier observations (36) that in rats infused chronically with nicotine, acute nicotine still to some degree increased the Fos IS in nucleus accumbens, and furthermore, no decrease of nicotine-induced Fos IS was found in cingular cortex and central nucleus of amygdala of these rats, indicating that limbic nAChRs do not very readily desensitize during chronic nicotine treatment.

Our results are in agreement with the experiments of Pagliusi et al. 1996 (28). They studied Fos mapping in rats trained for nicotine self-administration for several weeks and found that Fos IS in SON, PVN, and IPN of rats trained to self-administer nicotine at least 10 days did not differ from that of control rats, suggesting tolerance to the acute effects of nicotine.

In conclusion, our results suggest that the levels of desensitization of nAChRs in various brain areas differ. Furthermore, after a 72-h withdrawal nicotine's effects on Fos protein expression were restored, showing that the desensitization of nAChRs is a transient and reversible phenomenon. The nAChRs in hypothalamic areas, PVN and SON, or in their input areas

regulating nicotine's effects on hormone secretion seem to be desensitized more easily than the nAChRs in limbic areas controlling behavior such as in IPN. Thus, there seems to be variations in the functional states and/or in the subunit combinations of nAChRs mediating nicotine's effects in brain.

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