Selective c-fos Induction and Decreased Dopamine Release in the Central Nucleus of Amygdala in Rats Displaying a Mecamylamine-Precipitated Nicotine Withdrawal Syndrome

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KEY WORDS: central amygdala; microdialysis; Fos immunohistochemistry; immediate early genes

ABSTRACT In the present study the neuronal expression of Fos, the protein product of c-fos, was used to study changes in neuronal activity in nerve terminal regions of the ascending dopaminergic system during nicotine withdrawal. Rats were infused for 14 days with nicotine (9 mg/kg/day nicotine hydrogen tartrate) via minipumps, whereas control animals carried empty pumps. Withdrawal was induced by the nicotinic receptor (nAChR) antagonist mecamylamine (1 mg/kg, s.c.). The behavior of each animal was observed after mecamylamine injection and subsequently its brain was processed for Fos-like immunoreactivity. Following mecamylamine, the score of abstinence signs increased in the nicotine-treated rats as compared to controls. The number of Fos-positive nuclei was substantially increased in the central nucleus of amygdala (CNA) in animals undergoing mecamylamine-precipitated withdrawal, whereas no significant changes in c-fos expression were observed in the basolateral amygdaloid nucleus, the core and the shell of the nucleus accumbens, the dorsolateral striatum, or the medial prefrontal cortex. Since there are indications of involvement of amygdaloid dopaminergic neurotransmission in anxiety—a core symptom of withdrawal from dependence-producing drugs—in a second experiment utilizing microdialysis we examined whether nicotine withdrawal affects dopaminergic neurotransmission in the CNA. Following mecamylamine injection, dopamine (DA) significantly decreased in nicotine-treated animals compared with controls. These results indicate that the mecamylamine-precipitated nicotine withdrawal reaction is accompanied by a selective induction of c-fos and a concurrent decrease in DA release in the CNA, which may have a bearing on symptoms such as anxiety and distress, which frequently are associated with the nicotine abstinence reaction in humans. Synapse 35:15–25, 2000.

INTRODUCTION

Nicotine can induce dependence in man as well as in experimental animals. Cessation of chronic nicotine exposure or, in rodents, administration of a nicotinic receptor (nAChR) antagonist, induces a nicotine withdrawal reaction, the somatic component of which is exhibited in various behavioral symptoms (Hildebrand et al., 1997, 1998a; Hughes et al., 1991, 1994). Several lines of experimental evidence indicate that stimulation of the mesolimbic dopaminergic system is of great importance for the reinforcing and dependence-producing properties of nicotine (Clarke, 1990; Corrigall, 1991). However, the anatomical sites mediating the nicotine withdrawal reaction remain, as yet, largely unknown and thus warrant further investigation. Re-
ently, we have shown that a nicotine withdrawal syndrome including gasps, teeth chatter, yawns, and reduced locomotor activity, as well as a concomitant reduction of dopamine (DA) release in the nucleus accumbens (NAC), can be elicited in the rat by an intrategmental injection of mecamylamine (Hildebrand et al., 1998b). This indicates that nAChRs in the ventral tegmental area (VTA) subserve a critical role for some of the behavioral and biochemical manifestations of the nicotine withdrawal reaction. However, the postsynaptic consequences of nicotine withdrawal within the nerve terminal regions of the mesolimbic dopaminergic projections have not been well characterized so far.

One way to evaluate the involvement of postsynaptic neurons in the nicotine withdrawal reaction is to examine their expression of immediate early genes, such as c-fos, by means of in situ hybridization of their mRNAs or by immunohistochemistry of the protein they encode. Several immediate early genes can apparently serve as markers for neuronal activity and may be activated by various stimuli. These genes encode proteins that function as transcription factors to couple short-term, cell-surface events to long-term, genomic changes in response to extracellular stimuli (Chaudhuri, 1997; Hughes and Dragunow, 1995; Morgan and Curran, 1991). Changes in the induction of c-fos have been extensively studied and c-fos has been shown to be rapidly expressed in neurons in response to a variety of physiological and pharmacological challenges, such as rewarding brain stimulation, nociceptive stimulation, kindling, activation of various receptors, and administration of several psychotropic drugs (for a recent review see, Herrera and Robertson, 1996). Systemic or intrategmental administration as well as self-administration of nicotine has been shown to increase Fos-like immunoreactivity in several brain regions, including some which receive dopaminergic innervation from the VTA (Matta et al., 1993; Nisell et al., 1997; Pagliusi et al., 1996; Panagis et al., 1996; Pang et al., 1993; Ren and Sagar, 1992; Salminen et al., 1996). However, the tentative immediate early gene induction following nicotine withdrawal has not been investigated to date.

The present study was designed to gain a better understanding of the neuroanatomical pathways involved in nicotine withdrawal by determining Fos-like immunoreactivity in various brain regions known to receive dopaminergic projections from the VTA. Our histochemical analysis demonstrated that nicotine withdrawal increases the number of Fos-positive nuclei selectively in the central nucleus of amygdala (CNA), indicating altered postsynaptic neuronal function. Interestingly, cannabinoid withdrawal was also recently demonstrated to induce the c-fos proto-oncogene in the CNA (Rodríguez de Fonseca et al., 1997). The amygdala seems to be important not only in reward processes and positive emotion (see for review, e.g., Bardo, 1998; Koob et al., 1998), but also in the regulation of negative emotional behavior (e.g., conditioned and unconditioned fear, defense, stress reactions, and anxiety), especially with regard to autonomic and somatic responses (for review, see Bohus et al., 1996; Davis et al., 1994). Notably, a negative affective syndrome including anxiety, dysphoria, and depression is considered to represent a common feature of withdrawal from many drugs of abuse, including nicotine (Costall et al., 1989; Hughes et al., 1991, 1994; Markou et al., 1998). Interestingly, the CNA has reciprocal connections with the VTA and is rich in DA (Bernard et al., 1993; Fallon and Ciofi, 1992; Freedman and Cassell, 1994; Gonzales and Cheselet, 1990). Thus, it appears likely that changes in dopaminergic neurotransmission in the CNA may play a role in mediating the stress-related symptoms of nicotine withdrawal. In fact, it has been shown that DA receptor antagonists, when injected into the CNA, tend to worsen stress ulcers (Glavin, 1992; Ray et al., 1988), and that DA metabolism within the amygdala is affected by several types of stress (Beaulieu et al., 1987; Coco et al., 1992). Based on the above findings and considerations, as well as a recent study by Eaton et al. (1996) showing that an enhanced dopaminergic neurotransmission through either DA-D1 or DA-D2 receptors results in an increase in c-fos expression in the CNA, we examined in a second experiment, utilizing in vivo microdialysis, whether nicotine withdrawal is accompanied by alterations in DA output in the CNA.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 250–300 g at the beginning of the experiments were used. Before surgery, they were housed in groups, four to five animals per cage, under standard laboratory conditions and maintained on a 12-h light/dark cycle (lights on at 06:00) with ad libitum access to food and water.

Chronic nicotine treatment

Rats were implanted subcutaneously (s.c.) under brief ether anesthesia with Alzet osmotic minipumps (model 2ML2) which were either empty (control animals) or contained nicotine hydrogen tartrate (Sigma, St. Louis, MO). Nicotine was dissolved in saline and the pH was adjusted to 7.20–7.40 with NaOH. The concentration of nicotine was adjusted to compensate for differences in the body weight of the subjects; the average-weight rat received a dose of approximately 9 mg/kg/day nicotine hydrogen tartrate for 14 days. Prior to implantation, each pump was primed by being placed in a saline (0.9% NaCl) solution kept at 37°C for 4 h. The minipumps used in control animals were “recycled” after thorough cleaning and disinfection, whereas the nicotine-containing pumps were used only once. Following implantation, the animals were housed...
Individually. Alzet minipumps have been widely used for chronic, continuous administration of nicotine as well as various other substances. Thus, the stability of nicotine in the minipumps and the achieved steady-state concentration in rat plasma have previously been shown to be both reliable and reproducible (Benwell et al., 1994, 1995). In preliminary experiments we have also found that Alzet minipumps containing nicotine at the concentration mentioned above and implanted for 7 or 14 days yield serum concentrations of nicotine of about 30–45 ng/ml (Hildebrand et al., in preparation).

**Measurement of behavioral signs during nicotine withdrawal**

Before the behavioral measurements, animals were acclimatized to the experimental setting by being gently handled, weighed, injected s.c. with saline, and habituated to the behavioral testing cage (made of transparent Plexiglas, 35 × 35 × 40 cm) for 1 h on 2 subsequent days, i.e., on days 12 and 13. On day 14, rats were injected s.c. with either saline (1 ml/kg) or mecamylamine (Sigma; 1 mg/kg), immediately transferred to the Plexiglas cage and the frequency of signs such as gasps, shakes, teeth chatter, ptosis, and yawns were assessed for 30 min; these signs have previously been shown to increase in response to a mecamylamine challenge injection in nicotine-treated rats (Hildebrand et al., 1997, 1998a). The maximal score for ptosis was 30. The behavioral observations were performed under “blind” conditions, i.e., by an experimenter who was unaware of the chronic treatment and the challenge injection the animals had received.

**Fos immunohistochemistry**

Fos immunohistochemistry was performed as described by Chergui et al. (1996) and Panagis et al. (1997). Brains from eight animals were processed at a time, each time counterbalanced across treatment groups. Two hours after the induction of the withdrawal reaction by the mecamylamine injection, i.e., 90 min after the conclusion of the behavioral observations, the animals were deeply anesthetized with pentobarbital (100 mg/kg, i.p.) and transcardially perfused with 150 ml saline followed by 300 ml of 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). Subsequently, the brains were removed, postfixed for 2 h in paraformaldehyde, and placed overnight in 15% sucrose in PBS. The brains were then cut in 30 µm coronal sections on a microtome. The sections were washed in PBS (2 × 15 min) and placed in 0.3% H2O2 in PBS (10 min) and then washed in PBS (3 × 20 min) before being incubated for 48 h in PBS containing 0.02% Na Azide, 2% normal rabbit serum, and sheep anti-Fos primary antibody diluted 1:2,000 (sheep polyclonal antibody, Cambridge Research Biochemicals, Wilmington, DE, OA-11–824). The sections were then washed in PBS (3 × 20 min) and incubated for 1 h in PBS containing 2% normal rabbit serum and a biotinylated rabbit anti-sheep secondary antibody (Vector Laboratories, Burlingame, CA) diluted 1:200. After washing in PBS (3 × 20 min) and incubated for 1 h in PBS containing avidin-biotinylated-horseradish peroxidase complex (1:100, Vector Laboratories), the sections were washed again in PBS (2 × 15 min), then rinsed for 10 min in 0.2 M Na acetate buffer. The immunohistochemical reaction was visualized using a glucose oxidase 3,3-diaminobenzidine-nickel method terminated by washing in PBS. The sections were mounted on gelatin-coated slides, dehydrated, and coverslipped for microscopic observation.

The number of Fos-positive nuclei was counted in sections cut: +3.5 mm for the medial prefrontal cortex (mPFC), +1.7 mm for the core and the shell of the NAC, +1.2 mm for the striatum (STR), -2.3 to -2.8 mm for the CNA (see Fig. 4) and -2.56 to -2.80 mm for the basolateral nucleus of amygdala (BLA), relative to bregma (Paxinos and Watson, 1986). The distribution of Fos-like immunoreactivity was examined by light microscopy with the assistance of an image analysis system (Biocom). Counting was computer-aided using Biocom’s Histo software. The average density and area of a Fos-like positive cell nucleus was determined and the grain counting program was used to discriminate target cells, i.e., Fos-positive nuclei, from background based on relative size and density. Cells were counted in a rectangle of 600 × 445 mm bilaterally in three sections from each region at 125× magnification, and an average value of all six measurements was calculated for each region. Given that immunostaining was performed in sections from animals from all treatment groups within each run, possible differences in staining sensitivity between runs were controlled for.

**Brain surgery and microdialysis experiments**

On day 12 following Alzet pump implantation, rats were anesthetized with pentobarbital (60 mg/kg, i.p.) and mounted in a stereotaxic frame (David Kopf). Atropine sulfate (0.6 mg/kg, i.p.) was also injected to reduce bronchial secretion. A vertical, concentric probe of our own production (consisting of stainless steel inlet and outlet tubings, PE-20 and capillary tubings, and dialysis membrane) was subsequently implanted in the CNA. Coordinates (incisor bar set at -3.3 mm) measured from bregma were: AP: -2.56, ML: -4.2, DV: -8.45 mm (see Fig. 4; Paxinos and Watson, 1986). The microdialysis probe was introduced into the brain and fixed to the skull with stainless steel screws and dental cement. The active surface length of the semipermeable dialysis membrane (copolymer of acrylonitrile and sodium methallyl sulfonate, i.d. = 0.24 mm, 40,000 Da
molecular weight cutoff, Hospal AN69, Filtral 10) was 1.0 mm. On the day prior to the dialysis experiment, rats were transported from their home cage to the dialysis room for habituation.

Microdialysis was performed in freely moving rats approximately 48 h after probe implantation (i.e., day 14 after Alzet pump implantation) in the same type of cages as those in which rats were kept after brain surgery. The dialysis probes were perfused with a physiological salt solution (Apoteksbolaget, Sweden) containing 147 mM NaCl, 3.0 mM KCl, 1.3 mM CaCl₂, 1.0 mM MgCl₂, and 1.0 mM sodium phosphate (pH 7.4) at a rate of 2.5 µl/min by means of a microperfusion pump (Harvard Apparatus, Dover, MA). A sample of the perfusate was collected, loaded into the injector (Valco Instruments, Houston, TX) and automatically injected into the analytical system every 30 min. The injector was directed by a computer using the Turbochrom 4 software (Perkin Elmer, Oak Brook, IL). The extracellular concentrations of DA, dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) were determined by high-performance liquid chromatography with electrochemical detection (HPLC-ED) as previously described (Nomikos et al., 1989, 1994; Hildebrand et al., 1998a). We used a mobile phase (pH 4.0) containing 4.5 g Na acetate, 3.7 mg Na₂EDTA, 100 mg octanesulfonic acid and 90 ml HPLC-methanol per 1,000 ml mobile phase. It was delivered by an HPLC pump (Pharmacia, Uppsala, Sweden, LKB HPLC pump 2150) at 0.8 ml/min. Electrochemical detection was accomplished using a coulometric detector (ESA Coulochem II) connected with a conditioning cell (ESA model 5021) and with a high sensitivity analytical cell (ESA model 5011) that allowed amine detection by the sequential oxidation and reduction of the microdialysis samples. Chromatograms were simultaneously collected on a two-pen chart recorder (Kipp and Zonnen). On the experimental day, after a stable (less than 10% variation among three consecutive DA samples) outflow of DA, DOPAC, and HVA had been established, the animals first received an injection of saline (0.9% NaCl, 1.0 ml/kg, s.c.) followed 2 h later (i.e., after four 30-min samples) by an injection of mecamylamine hydrochloride (Sigma; 1.0 mg/kg, s.c.) and after another 3 h (i.e., six 30-min samples) the experiment was terminated. At the end of the experiment, a few randomly chosen animals were perfused in the central amygdaloid nucleus with a Ca²⁺-free perfusion solution to confirm that the DA assessed was of neuronal origin. In all cases, DA levels decreased substantially, i.e., more than 75% below basal values.

Rats were sacrificed upon termination of the experiment and the brains were fixed in a solution of 10% formalin in 25% sucrose and subsequently sliced (50 µm) on a microtome and stained with neutral red in order to allow microscopic verification of probe placement. Only the data from rats with probes located within the CNA according to the stereotaxic atlas (Paxinos and Watson 1986) were used for subsequent calculations.

Data analysis and statistics

Behavioral and histochemical experiments

Statistical significance of the results was determined from raw data and evaluated by two-way (treatment × challenge) analysis of variance (ANOVA) followed by the Newman-Keuls test for multiple comparisons, where appropriate. A P-value < 0.05 was considered significant. Pearson r correlation tests were also conducted in order to detect putative correlations between the behavioral (individual categories or total signs) and the histochemical (number of Fos-positive nuclei in each region) data after saline or mecamylamine challenge in control and nicotine-treated rats.

Biochemical experiments

The basal values of DA and its metabolites in the two treatment groups were evaluated by Student's t-test. The biochemical data were calculated as percent changes from baseline levels according to the following scheme: first, the average of the two samples preceding the saline injection was defined as 100%; this baseline was then used for the subsequent four samples. The sample labeled B1 in Figure 5 was the last sample before the saline injection. Second, the average of the two samples immediately preceding the mecamylamine injection was defined as 100% and used as a baseline for the following six post-mecamylamine samples. The sample labeled B2 in Figure 5 was the last sample before the mecamylamine injection. Thus, the biochemical data were evaluated in two sets, i.e., one set included the results of the last baseline sample together with the subsequent four samples after saline injection, whereas the second set included data of the last sample before and all samples after mecamylamine injection. This design was used to compensate for small changes in the dialysate levels of DA and its metabolites following saline injection. Statistical significance was determined by using two-way (treatment × time) ANOVA with one repeated-measures variable (time) or one-way ANOVA with one repeated-measures variable (time) followed by the Newman-Keuls test, where appropriate. A P-value < 0.05 was considered significant.

Results

Behavioral experiments: mecamylamine-precipitated nicotine withdrawal

Figure 1 shows overall and individual categories of withdrawal signs precipitated by an s.c. injection of either mecamylamine or saline in nicotine-treated and in nicotine-naïve animals. Mecamylamine injection significantly increased overall abstinence signs as well as teeth chatter and yawns. Statistical evaluation of the
data revealed significant treatment \([F(1,26) = 6.07, P < 0.05] \) \([F(1,26) = 5.64, P < 0.05] \) \([F(1,26) = 4.55, P < 0.05] \), challenge \([F(1,26) = 12.48, P < 0.01] \) \([F(1,26) = 9.11, P < 0.01] \) \([F(1,26) = 6.36, P < 0.05] \), and interaction \([F(1,26) = 5.03, P < 0.05] \) \([F(1,26) = 6.61, P < 0.05] \) \([F(1,26) = 8.47, P < 0.01] \) effects, respectively. Post-hoc analysis showed that after mecamylamine injection there was a significant increase in the number of withdrawal signs as compared to the number of signs after saline injection in the nicotine-treated group \((P < 0.01)\); also, there was a significant increase in the number of signs following mecamylamine injection in the nicotine-treated animals when compared to the number of signs after mecamylamine injection in the nicotine-naive animals \((P < 0.01)\).

**Histochemical experiments: distribution of Fos-like immunoreactivity**

The mecamylamine-precipitated nicotine withdrawal syndrome is associated with an increase in Fos-like immunoreactivity selectively in the CNA (Figs. 2, 3, Table I). An overall two-way ANOVA revealed significant treatment \([F(1,26) = 13.74, P < 0.01] \), challenge \([F(1,26) = 17.17, P < 0.001] \), and interaction \([F(1,26) = 11.56, P < 0.01] \) effects. Post-hoc comparisons revealed that a mecamylamine injection in nicotine-treated animals resulted in a significant increase \((P < 0.001)\) in the number of Fos-immunoreactive nuclei in the CNA as compared to the other groups (Fig. 2, Table I). The number of Fos-positive nuclei was not significantly
affected in the other areas examined (mPFC, the core and shell of the NAC, the STR, and the BLA, see Table I). There was no statistically significant correlation between the various withdrawal signs or the overall score and the number of Fos-positive nuclei in the CNA in any of the groups studied; there was, however, a statistically significant (P < 0.05) correlation between the overall score, the number of teeth chatters and yawns, and the number of Fos-positive nuclei in the CNA (r = 0.46, r = 0.45 and r = 0.42, respectively), when all groups were included in the correlation matrix.

### Biochemical experiments: extracellular concentrations of DA and its metabolites in the CNA

Statistical analysis revealed that basal dialysate concentrations of DA did not differ significantly between the nicotine-treated (mean ± SEM: 0.29 ± 0.12...
fmol/min, n = 6) and the control animals (mean ± SEM: 0.34 ± 0.08 fmol/min, n = 8). Mecamylamine, but not saline, injection in the nicotine-treated, but not in the nicotine-naive, animals resulted in a decrease in DA release in the CNA (maximal reduction 32%; Fig. 5). Statistical evaluation of the data after mecamylamine injection revealed significant treatment [F(1,12) = 18.58, P < 0.01] and interaction [F(6,72) = 2.79, P < 0.05] effects. Post-hoc comparisons demonstrated that mecamylamine significantly reduced DA output during samples 3, 4, and 6 (P < 0.05-0.001) as compared to nicotine-naive animals, and during samples 4 and 6 (P < 0.05) as compared to the baseline sample. Similarly to DA, concentrations of DOPAC and HVA were also reduced in the CNA after mecamylamine injection in the nicotine-treated, but not in control animals, although these effects were slightly less pronounced (maximum decrease about 20%; data not shown).

DISCUSSION

The major finding of this study is that mecamylamine-precipitated nicotine withdrawal in the rat results not only in an increase in abstinence signs, such as teeth chatter and yawns, but also in a region-specific activation of c-fos and a decrease in DA release in the CNA.

A common behavioral consequence of withdrawal from various drugs of abuse is negative affective and motivational states that in humans are characterized by irritability, restlessness, dysphoria, depression, anhedonia, and anxiety (Gawin, 1991; Gawin and Kleber, 1986; Jaffe, 1990; Jasinski et al., 1985; Markou et al., 1998), and in animals by reward deficits and anxiety-related behavior (Bhattacharya et al., 1982, 1995; Costall et al., 1989, 1990; Markou and Koob, 1991; Schulteis et al., 1994, 1995; Stinus et al., 1990). Specifically, nicotine withdrawal in humans produces several behavioral changes, including irritability, anxiety, impaired concentration, insomnia, restlessness, impatience, hostility, depression, and craving for nicotine (Glassman, 1993; Hughes et al., 1991). It has been demonstrated that nicotine withdrawal in experimental animals mimics anxiety states (Emmett-Oglesby et al., 1990) and results in increased thigmotaxis as well as anxiety-like behavior in the elevated plus-maze.
Furthermore, DSM-IV includes anxiety as one of the nicotine withdrawal symptoms in man (APA, 1994) and clinical studies report increased anxiety following smoking cessation (Hughes et al., 1994). The results of the present experimental study are in agreement with other studies reporting that the CNA is a critical component of brain networks mediating emotional behavior, fear, and anxiety. Thus, lesions of this brain region have been reported to reduce experimental anxiety-like behavior in the rat (Kopchia et al., 1992; Möller et al., 1997). Moreover, c-fos expression was observed in the CNA when rats were subjected to stress (Honkaniemi et al., 1992) or an anxiety provoking environment in the Vogel test (Möller et al., 1994). Interestingly, in the recent study by Rodríguez de Fonseca et al. (1997) it was shown that cannabinoid withdrawal results in an increase in c-fos expression in the same subregion of the extended amygdala. Other reports, utilizing electrophysiological methods, c-fos mRNA measurements, or immunohistochemistry for Fos, have demonstrated increased cellular activity in the amygdala during withdrawal from ethanol (Knapp et al., 1998), as well as from morphine (Freedman and Aghajanian, 1985; Hayward et al., 1990; Stornetta et al., 1993). Moreover, amygdala has been reported to be involved in the aversive stimulus effects and various other signs of opiate withdrawal (Calvino et al., 1979; Stinus et al., 1990).

The induction of c-fos in the case of nicotine withdrawal was restricted to the CNA, in contrast to results obtained with other drugs of abuse, such as morphine and cannabis, where increased c-fos expression was observed also in other brain regions innervated by the VTA, i.e., the mPFC, the NAC, and the STR (Beckman et al., 1995; Chahl et al., 1996; Druhan et al., 1996; Hayward et al., 1990; Matsumoto et al., 1993; Rasmussen et al., 1995; Rodríguez de Fonseca et al., 1997). The differences in the spatial expression of c-fos between withdrawal from nicotine and withdrawal from other dependence-producing drugs may reflect the well-known differences in the pharmacological and behavioral profiles of these drugs. Smokers frequently report that smoking exerts a calming effect when they are exposed to stressful environmental stimuli and it has been shown that smoking increases under such conditions (Frith, 1971; Gilbert, 1979; Pomerleau, 1986; Pomerleau and Pomerleau, 1987). Findings from animal studies also indicate that nicotine indeed produces a significant anxiolytic effect (Brioni et al., 1993; Costall et al., 1989). Therefore, it is conceivable that nicotine may affect anxiety-like behaviors by interfering with neuronal mechanisms within the amygdaloid complex, and that nicotine withdrawal, which is anxiogenic per se, is associated with enhanced neuronal activity, as reflected by the increase in c-fos expression, within this brain region.

In the present study, the increased Fos-like immunoreactivity in the amygdala observed in nicotine-withdrawn rats was specifically restricted to the CNA. For comparison, the effects on c-fos expression were also studied in the BLA. Interestingly, we did not detect any differences in c-fos expression in this subregion of the amygdaloid complex. It has been suggested that the BLA plays a different role than the CNA in the mediation of anxiety (Bohus et al., 1996; LeDoux et al., 1990;
Maren, 1996). Thus, the BLA seems to be more important for integrating uni- and polymodal sensory information in order to extract their aversive and anxiogenic content, whereas the CNA rather is considered necessary for the expression of behavioral and autonomic fear (Hitchcock and Davis, 1991; Miserendino et al., 1990).

The neurochemical identity of the cell bodies exhibiting increased Fos-like immunoreactivity in the CNA is presently unknown. According to Davis et al. (1994), the VTA, the locus coeruleus, and the dorsolateral tegmental area receive projections from the amygdaloid complex and through activation of dopaminergic, noradrenergic, and cholinergic neurons the CNA may generate behavioral and EEG arousal as well as increased vigilance. On the other hand, the CNA represents an anatomical target of the VTA efferents and is also rich in DA (Bernard et al., 1993; Freedman and Cassel, 1994). Furthermore, it has been shown that chemical stimulation of the VTA increases DA metabolism in the amygdala (Hagan et al., 1990), whereas lower DA levels in the amygdala appear as a consequence of lesioning the VTA (Oades and Halliday, 1987). Interestingly, DA has even been suggested to act as an endogenous anxiety signal within the amygdaloid complex (Beaulieu et al., 1987; Coco et al., 1992; Glavin, 1992; Ray et al., 1988). The reduced DA output in the CNA during nicotine withdrawal, observed in the present study, may be interpreted as a consequence of an altered dopaminergic activity in the VTA. Indeed, recent findings from our laboratory point to a significant role of the VTA in the behavioral and biochemical manifestations of nicotine withdrawal syndrome (Hildebrand et al., 1998b). Moreover, in anesthetized rats undergoing mecamylamine-precipitated withdrawal we have observed a marked decrease in firing in a substantial proportion of the DA cells in the VTA (Hildebrand et al., in preparation). The inhibited neuronal activity of this subset of DA cells within the VTA may be related to the reported reduction in DA levels in the CNA observed in the present study. Interestingly, a decrease in DA levels and an increase in c-fos expression in another part of the extended amygdala, the NAC, has been reported following morphine withdrawal (Acquas and Di Chiara, 1992; Druhan et al., 1996; Rossetti et al., 1992). In that case, it was suggested that a reduction in dopaminergic transmission, e.g., through D2 receptors, may be causally related to c-fos induction, given that in the striatal complex c-fos expression is inhibited through D2 receptor stimulation (see Le Moine et al., 1997). In the CNA, however, an enhanced dopaminergic neurotransmission through either D1 or D2 receptors results in an increase in c-fos expression (Eaton et al., 1996). Thus, the observed c-fos induction in the CNA in response to nicotine withdrawal is probably not related to the attenuated DA output. Nevertheless, it remains to be tested whether the reduced DA output in the CNA plays a role in mediating some behavioral symptoms of the nicotine withdrawal reaction, the reported anxiety, and the reward deficits observed in other studies. If this is the case, treatment that reverses this deficit in dopaminergic neurotransmission may be useful in ameliorating symptoms of nicotine withdrawal in man such as anxiety and distress.

ACKNOWLEDGMENTS

We thank Mrs. Anna Malmerfelt and Mrs. Annika Olsson for excellent technical assistance.

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