Previous data show that nicotinic receptors in the ventral tegmental area are of importance for the nicotine withdrawal syndrome. Here, we have investigated the specific role of \( \alpha_7 \) nicotinic receptors in the ventral tegmental area for the neurobiological and behavioral consequences of nicotine withdrawal. Rats were exposed to nicotine for 14 days via s.c. osmotic minipumps. Bilateral intrategmental injections of the selective \( \alpha_7 \) nicotinic receptor antagonist methyllycaconitine reduced locomotion in the nicotine-treated rats, but not in control animals. Unilateral intrategmental injection of methyllycaconitine reduced dopamine output in the ipsilateral nucleus accumbens of nicotine-treated rats, but not in controls. Our results indicate that \( \alpha_7 \) nicotinic receptors in the ventral tegmental area are involved in the nicotine withdrawal syndrome.

Key words: Dopamine; \( \alpha_7 \) subunit; Locomotor activity; Methyllycaconitine; Microdialysis; Nucleus accumbens

Introduction

Chronic administration of nicotine both in humans and in animals induces dependence, the physical component of which is exhibited in various behavioral symptoms after cessation of chronic nicotine exposure or systemic administration of a nicotinic receptor antagonist [1,2]. Previous evidence indicates that stimulation of the mesolimbic dopaminergic system is of great importance for the reinforcing and dependence-producing properties of nicotine [3,4]. Thus, several effects of nicotine in the brain are believed to be mediated via stimulation of nicotinic receptors, which are located on both cell bodies and terminals of the dopamine neurons [5]. However, several studies indicate that nicotinic receptors within the somatodendritic region of the mesolimbic dopaminergic system, i.e., the ventral tegmental area, rather than the nerve terminal area, i.e., the nucleus accumbens, are of major importance for nicotine’s stimulatory actions on behavior and dopamine release in the nucleus accumbens [6,7]. Moreover, recent studies from our laboratory demonstrate that the ventral tegmental area plays a significant role both in the behavioral and biochemical aspects of the nicotine withdrawal reaction [8]. Thus, intrategmental injections of the nicotinic receptor antagonist mecamylamine in animals treated chronically with nicotine elicited typical withdrawal signs, such as gasps, teeth chatter, yawns and hypolocomotion, as well as a concomitant reduction in dopamine release in the nucleus accumbens. Several subtypes of nicotinic receptors are expressed in the brain, and it is therefore important to assess which of the nicotinic receptor subtypes that mediate the above mentioned effects of nicotine. Experimental studies indicate the existence of \( \alpha_7 \) subunits of the nicotinic receptor in the ventral tegmental area [9,10], and we have recently shown that the \( \alpha_7 \)-specific nicotinic receptor antagonist methyllycaconitine when infused into the ventral tegmental area attenuates both the nicotine- and food-induced accumbal dopamine release [11]. However, it is not yet known whether the same receptor conformation is involved also in the nicotine withdrawal syndrome. Accordingly, the present study was undertaken to investigate the putative role of \( \alpha_7 \) nicotinic receptors in the ventral tegmental area for the generation of some of the behavioral and biochemical effects of nicotine withdrawal. Rats were chronically treated with nicotine as previously described [2,8,12] and methyllycaconitine was locally injected into the ventral tegmental area. In these animals locomotor activity in an open field was studied and, separately, in freely moving animals the extracellular concentrations of dopamine and its metabolites dihydroxyphenylacetic acid and homovanillic acid in the nucleus accumbens were assessed by means of in vivo microdialysis.
Materials and Methods

Animals, surgery and induction of nicotine dependence: Male Wistar rats, weighing 280–330 g at the time of surgery, were anesthetized with pentobarbital (60 mg/kg, i.p.) and stereotactically implanted with guide canulae (25 gauge) into the ventral tegmental area. Coordinates were 5.3 mm posterior to bregma, 0.7 mm lateral to the midline, and 7.2 mm below the brain surface (i.e. the dura mater) according to the anatomical atlas of the rat brain [13]. The tips of the guide canulae were located 1.0 mm above the actual injection sites. The guide canulae were subsequently fixed to the skull with anchoring screws and dental cement. Copper wire pieces were inserted into the guide canulae to prevent obstruction. The rats that were used for microdialysis experiments were implanted with a unilateral cannula, whereas the rats used in the behavioral experiments were implanted bilaterally. Following stereotaxic implantation of the canulae in the ventral tegmental area the animals were implanted s.c. with Alzet osmotic minipumps (model 2ML2) containing nicotine hydrogen tartrate. 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, and the average-weighted rat received a dose of ~9 mg/kg/day nicotine hydrogen tartrate for 14 days. Before implantation, each pump was primed for 4 h in 37°C physiological saline (0.9% NaCl). This specific schedule of chronic nicotine administration has previously been shown to result in the appearance of several abstinence signs, such as teeth chatters, gasps and yawns when animals were injected with mecamylamine either systemically [2,12] or intrategmentally [8]. Rats in the control groups were implanted with empty minipumps. In the microdialysis experiments the animals were stereotaxically implanted in the ipsilateral nucleus accumbens (coordinates: +1.6 mm from bregma, −1.2 mm lateral to midline, −8.2 mm from the skull surface) with a vertical concentric microdialysis probe of own production. The active surface length of the dialysis membrane was 2.25 mm beginning −0.5 mm from the tip of the probe.

Intrategmental injections: For intrategmental injections, an injection cannula constructed from a 31 gauge stainless-steel tubing was inserted into the guide cannula, protruding 1 mm into the ventral tegmental area. A volume of 0.5 μl saline or methyllycaconitine (RBI) dissolved in saline was injected either unilaterally (microdialysis experiments) or bilaterally (behavioral experiments) into the ventral tegmental area of the hand-restrained animal during 1 min. The injection cannula was then left in place for an additional 1 min after the injection, in order to allow sufficient diffusion of the drug. The day prior to the experiment, during habituation, the injection cannula was inserted into the guide canulae for 1 min without any actual injection. This sham insertion probably caused a small brain tissue lesion similar to that produced by an actual injection, thus interference of putative lesion-induced effects with drug-induced effects on biochemistry or behavior on the experimental day was in all probability minimal.

Measurements of locomotor activity: The locomotor activity of the animals was monitored in a computer-assisted square open field (68 × 68 × 45 cm), equipped with two rows of eight photocells, along two adjacent sides, that were placed 4.0 and 12.5 cm above the floor. The open-field was enclosed in a dark, ventilated and sound-attenuating box. Locomotor activity was tested as previously described [8]. Photobeam interruptions were detected and recorded by a computer. The following quantitative variables were calculated: total locomotor activity (all photobeams interruptions in the lower rows); peripheral activity (all interruptions of photobeams spaced next to the wall in the lower rows); forward locomotion (successive interruptions of photocells in the lower rows with the animal moving in the same direction) and rearing (total number of photobeam interruptions in the upper rows). The percentages of peripheral activity and forward locomotion counted to horizontal activity counts were also calculated to assess the pattern, i.e. the qualitative aspects, of locomotion. Thus, the percentages of peripheral to horizontal activity counts and forward locomotion to horizontal activity counts are indicative of the spatial distribution of movements and perseverance of forward locomotion, respectively [8]. Locomotor activity was assessed for 30 min on 4 consecutive days. The day before the first observation, rats were habituated to the open field for 30 min. Prior to habituation each rat received an intrategmental sham insertion, as described above. The first experimental session was 11 days and the last 14 days, respectively, after the onset of nicotine infusion, or implantation of a sham pump. Each animal received three doses of methyllycaconitine (1.0, 3.0 and 9.0 μg per side) dissolved in saline as well as saline alone. The sequence of injections was counterbalanced with respect to order.

Microdialysis experiments: The day prior to microdialysis experiments, rats were transported from their home cage to the microdialysis room for habituation. During habituation each rat was restrained and received an intrategmental sham inser-
tion (see above). Microdialysis experiments were performed in freely moving rats 14 days after implantation of the minipump, and approximately 48 h subsequent to intracraniac surgery. The microdialysis probes were perfused with a physiological salt solution containing 147 mM NaCl, 3.0 mM KCl, 1.3 mM CaCl₂, 1.0 mM MgCl₂, 1.0 mM sodium phosphate (pH 7.4) at a rate of 2.5 μl/min by means of a microperfusion pump (Harvard Apparatus). A sample of the perfusate was collected, loaded directly into the sample loop of the injector (Valco Instruments Co.) and automatically injected into the analytical system every 15 min. The loading and injecting modes of the injector were computer-directed (Turbochrom 4 software, Perkin Elmer). Extracellular concentrations of dopamine, dihydroxyphenylacetic acid and homovanillic acid were determined by high-performance liquid chromatography with electrochemical detection (HPLC-ED) as described previously [2,8,12]. The mobile phase was delivered by an HPLC pump (Pharmacia 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 day of the experiment, after a stable outflow of dopamine, dihydroxyphenylacetic acid and homovanillic acid had been established, the animals received first an intrategmental injection of 0.5 μl saline followed 2 h later (i.e. after eight 15 min samples) by an injection of 0.5 μl saline containing 3.0 μg methyllycaconitine, and after another 4 h (i.e. sixteen 15 min samples) the experiment was terminated. At the end of the experiment a few randomly chosen animals were perfused in the nucleus accumbens with either a Ca²⁺-free perfusion solution or 2 μM tetrodotoxin in the perfusion solution to confirm that the dopamine assessed was indeed of neuronal origin. In all cases, dopamine levels decreased substantially, i.e. >75% below basal values.

Histological verification: After completion of the experiments, the animals were sacrificed under deep anaesthesia and their brains removed and fixed in a solution of 10% formalin in 25% sucrose. Each brain was subsequently sliced on a microtome and stained with neutral red in order to allow microscopical verification of cannula and probe placement. Only data from animals with cannulae and probes located within the ventral tegmental area and nucleus accumbens, respectively, were used for subsequent calculations. In the few nicotine-treated rats that were excluded due to cannula placement outside of the ventral tegmental area there were no effects of the methyllycaconitine injections on any of the behavioral or biochemical parameters studied.

Data analysis: The basal values of dopamine and its metabolites in the two treatment groups were evaluated by Student’s t-test. Raw data were used for the statistical evaluation of the behavioral measurements, whereas 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 eight samples. The sample labeled B1 in the figures is the last sample before saline injection. Second, the average of the two samples immediately preceding the methyllycaconitine injection was defined as 100% and used as a baseline for the following 16 post-methyllycaconitine samples. The sample labeled B2 in the figures is the last sample before methyllycaconitine 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 eight samples after saline injection, whereas the second set included data of the last sample before and all samples after methyllycaconitine injection. This design was used to compensate for the slight changes, which were seen in the dialysate levels of dopamine and its metabolites following saline injection. Statistical significance was determined by using two-way (treatment x time) analysis of variance (ANOVA) with one repeated measures variable (time) or one-way ANOVA with one repeated measures variable (time) followed by the Newman-Keuls test, when appropriate. A value of p < 0.05 was considered to be significant.

Results

Behavioral experiments: locomotor activity in an open field: Figure 1 shows the effects of an intrategmental injection of saline (0.5 μl/side) or methyllycaconitine (1.0, 3.0 and 9.0 μg/0.5 μl/side) on various aspects of locomotor behavior in animals treated chronically with nicotine and in control animals. Methyllycaconitine injection significantly decreased total locomotor activity (Fig. 1A), forward locomotion (Fig. 1B) and rearing (Fig. 1C) in animals treated chronically with nicotine. Post-hoc comparisons revealed that these effects were statistically significant for all the doses tested (p < 0.05–0.001) compared with the score after saline injections. Moreover, total locomotor activity and for-
ward locomotion in the nicotine-treated animals, following all doses of methyllycaconitine, was significantly lower than in the control animals. Methyllycaconitine injections significantly decreased the ratio forward locomotion/horizontal activity in animals treated chronically with nicotine (data not shown), indicating that the pattern of locomotion was also affected. A two-way ANOVA also revealed a significant interaction effect on the ratio peripheral activity/horizontal activity (data not shown); post hoc comparisons, however, did not indicate differences between the various groups although there was a tendency for an increase in this ratio after methyllycaconitine injections in chronically nicotine-treated animals.

**Biochemical experiments: extracellular concentrations of dopamine, dihydroxyphenylacetic acid and homovanillic acid in the nucleus accumbens:** Statistical analysis revealed that basal dialysate concentrations of dopamine and its metabolites did not differ significantly between the animals treated chronically with nicotine and the control animals. The mean (± s.e.m.) basal values of dopamine, dihydroxyphenylacetic acid and homovanillic acid were 5.6 ± 1.4 and 3.6 ± 0.9, 958 ± 150 and 841 ± 85, 467 ± 40 and 386 ± 37 for the group receiving chronic nicotine (n = 6) and the control group (n = 8), respectively. Intrategmental injection of methyllycaconitine, but not saline, in the nicotine-treated animals decreased accumbal dopamine release (maximal reduction 38%; Fig. 2A), which was statistically significant (p < 0.05-0.001) as compared to baseline (B2) during almost the entire postinjection interval (samples 5–15), as well as when compared with the control animals during samples 4–11 (p < 0.01–0.001). In chronically nicotine-treated animals, the reduced dopamine output after methyllycaconitine injection was also followed by a significant decrease in the extracellular levels of dihydroxyphenylacetic acid and homovanillic acid, compared with control animals; this difference was significant in the second sample and in samples 10–12 for dihydroxyphenylacetic acid (Fig. 2B; p < 0.05) and in samples 4–6 and 8–12 for homovanillic acid (Fig. 2C; p < 0.05–0.001).

During the microdialysis experiments, two independent observers noted increases in some of the behavioral signs of nicotine withdrawal, i.e. gasps, teeth chatter and yawns, after intrategmental injection of methyllycaconitine in animals treated chronically with nicotine, although these observations were not systematically analyzed.

**Discussion**

The major finding of the present study is that administration of the α7 nicotinic receptor antagonist methyllycaconitine into the ventral tegmental area of rats treated chronically with nicotine produces some of the core behavioral symptoms of the
nicotine withdrawal syndrome, in particular a significant reduction in spontaneous locomotor activity. Furthermore, in rats displaying this withdrawal reaction we observed a significant decrease in the extracellular levels of dopamine and its metabolites in the nucleus accumbens. Several investigations have previously used methyllycaconitine in order to examine behavioral effects of nicotine in vivo [11,14]. The doses of methyllycaconitine used in the present study are within the low range of the doses previously used, e.g. in a recent study in which methyllycaconitine was microinjected into the ventral hippocampus [14]. Consequently, the results of this study point to the conclusion that nicotinic receptors composed of α7 subunits within the ventral tegmental area are indeed involved in the nicotine withdrawal reaction, since methyllycaconitine is considered a selective antagonist for α7 nicotinic receptors [15–17].

Recent observations [18,19] indicate that α7 receptors are presynaptically located and that they modulate neurotransmission in several brain areas. Thus, a major role of α7 nicotinic receptors may be to facilitate release of several neurotransmitters, which in turn generate behavioral effects. Within this context, we have proposed that nicotinic receptors composed of α7 subunits may be specifically localized on glutamatergic afferents to the ventral tegmental area, and that stimulation of these α7 nicotinic receptors results in an increase in glutamate release, which in turn results in stimulation of excitatory amino acid receptors on dopamine neurons within the ventral tegmental area, [11] although some recent in vitro electrophysiological data may appear at variance with this interpretation (P. Grillner et al., personal communication). At any rate, our previous and present data so far indicate that α7 nicotinic receptors within the ventral tegmental area contribute to both the acute effects of nicotine on the mesolimbic dopamine system, which seems critically involved in the reinforcing and dependence-producing actions of nicotine, as well as to the abstinence syndrome. Chronic nicotine infusion to rodents, in doses that may be relevant to smoking, as well as smoking itself, causes upregulation of the number of brain nicotinic receptors as well as desensitization of some nicotinic receptor-mediated central effects in the rat, such as the stimulatory effect of nicotine on accumbal dopamine release and on locomotion. It is, however, presently unclear whether all subtypes of nicotinic receptors in all parts of the brain respond in the same manner to chronic nicotine exposure [20,21]. Thus, whether specific changes in α7 nicotinic receptor function, i.e. desensitization and/or upregulation, within the ventral tegmental area contribute to the presently observed behavioral and biochemical effects of nicotine withdrawal remains to be determined.

The present findings are generally consistent with the results of previous studies that have demon-
strated a reduction in accumbal dopamine output following withdrawal from nicotine [12] as well as from several other addictive drugs, such as amphetamine, cocaine, ethanol and morphine [22], and this phenomenon is very likely to contribute to some of the symptoms generally encountered in these withdrawal reactions in man, such as dysphoria and lack of motivation [1]. The degree of locomotor activity in animals subjected to withdrawal has been hypothesized to serve as an index of the so-called `affective symptoms' of drug withdrawal [23,24]. Moreover, a recent study demonstrated dramatically increased reward thresholds in nicotine withdrawn rats [25]. In view of the crucial role of the mesolimbic dopamine system for incentive behavior these phenomena may consequently be causally related to reduced accumbal dopamine output and, as judged by the present results, involve α7 nicotinic receptors in the ventral tegmental area.

Conclusion

Our previous work has demonstrated that α7 nicotinic receptors in the ventral tegmental area mediate, to a significant extent, both the systemic nicotine- and the food-induced dopamine release in the nucleus accumbens. The present data indicate that nicotinic receptors composed of α7 subunits within the ventral tegmental area are also involved in the generation of the nicotine withdrawal reaction.

References


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