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Involvement of α6 nicotinic receptor subunit in nicotine-elicited locomotion, demonstrated by *in vivo* antisense oligonucleotide infusion

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ENHANCED locomotion in a habituated environment is a well documented effect of nicotine mediated by the mesotelencephalic dopaminergic system. The nicotinic receptor subunit α6 is, among other subunits, strongly expressed in the dopaminergic neurons of the mesencephalon. To examine the functional role of this subunit, we inhibited its expression *in vivo* using antisense oligonucleotides. *In vitro* treatments of embryonic mesencephalic neuron cultures demonstrated that the α6 antisense oligonucleotides caused a marked decrease in the level of α6 subunit protein. *In vivo*, 1 week infusion of α6 antisense oligonucleotides by osmotic mini-pump reduced the effect of nicotine on locomotor activity in habituated environment by 70%. These data support the notion that the effects of nicotine on the dopaminergic system involve α6 subunit containing nAChRs. *NeuroReport* 10:2497–2501 © 1999 Lippincott Williams & Wilkins.

Key words: Acetylcholine; Cholinergic behavior; Dopaminergic system

Introduction

Neuronal nicotinic receptors (nAChRs) are involved in numerous brain functions, including the control of voluntary motion, the modulation of sensory inputs and memory processes [1,2]. Nicotine injections differentially modulate locomotor activity depending on the experimental paradigm [3]. In a new environment, nicotine at a relatively high dose provokes an inhibition of exploratory behavior and, as a consequence, of total locomotion. Conversely, in a known environment, nicotine at relatively low doses, as well as other nicotinic agonists, elicits an increase in global locomotion [4–6]. This latter effect is blocked by destruction of meso-telencephalic dopaminergic nerve terminals or cell bodies [6].

nAChRs are abundant in the dopaminergic neurons of the mesencephalon. At least α3, α4, α5, α6 and β2, β3 subunits [7] and to some extent α7 [8] are expressed in dopaminergic neurons where they may co-assemble to form multiple receptor subtypes. The β2 subunit has been shown to be necessary for nicotine-elicited dopamine (DA) release and reinforcement, as evaluated in a self-administration paradigm [9]. Electrophysiological recordings further showed that β2-containing nAChRs are present in the soma of dopaminergic cells [9]. These nAChRs are also thought to include the α4 subunit [10,11]. nAChRs present on dopaminergic nerve terminals display a different pharmacology, and have been proposed to contain the α3 subunit [12–15]. However, it has recently been shown that ventral mesencephalon is relatively poor in α3 subunit mRNA but rich in α6 subunit RNA and protein [7,16], a subunit with strong sequence similarities to the α3 subunit. Moreover, a 6-OHDA lesion causes an alteration of α6 but not α3 expression, which could mean that traces of α3 are located in non-dopaminergic cells [17]. It was therefore hypothesized that a nAChR oligomer containing the

α6 subunit contributes, at least in part, to the effects of nicotine on the mesostriatal dopaminergic pathway [7].

The aim of the present study is to analyze the possible contribution of α6 subunit containing nAChRs on nicotine-elicited locomotion, a behaviour related to the activation of the mesostriatal dopaminergic system.

Materials and Methods

Antibody generation: The peptide sequence HKSSEIAPGKRLSQQPAQWV, beginning at amino acid 399 of the preprotein, was selected in the variable portion of the rat α6 subunit cytoplasmic domain [18]. This sequence shows no similarities with any of the other nicotinic subunits. After synthesis, the peptide was coupled to bovine thyroglobulin (TG, Sigma T1001) with 0.2% glutaraldehyde. Rabbit immunizations were performed by Eurogentec (Belgium). Antibodies were purified on peptides coupled to active ester agarose Affi-Gel 10 (Bio-Rad 1536099, CA, USA), dialysed, concentrated and stored at −20°C in 50% glycerol.

Western blotting: Cells were homogenized at 0.1 mg ml^{-1} in homogenization buffer (50 mmol l^{-1} Tris–HCl pH6.8, 100 mmol l^{-1} DTT, 2% SDS, 0.1% pyronin Y, 10% glycerol). After electrophoresis, proteins were transferred to nitrocellulose membrane (ECL, Amersham). The membrane was incubated for 30 min in Tris sodium Triton X-100 (TNT), 5% powder milk, overnight in the same buffer with the primary antibody (3 μg ml^{-1}). Blots were then washed three times for 10 min in TNT, incubated for 1 h in TNT with a biotinylated anti-rabbit antibody (Amersham RPN 1004, 1/500), washed three times for 10 min each in TNT, incubated for 1 h in TNT with a streptavidin–horseradish peroxidase complex (Amersham RPN 1051, 1/250), and finally washed three times for 10 min in TNT and twice in water. The labeling was revealed using the ECL system (Amersham 2106). The band intensities were quantified with an image analyzer (Vidas, Kontron, Germany).

Oligodeoxynucleotides (ODNs): ODNs were chosen according to the classical features considered for antisense experiments. Two antisense ODNs and one nonsense ODNs were retained for experiments (AS3, 5′-GCCAACTCAAAGTGCACC-3′ (AUG + 179); AS4, 5′-TGTGGAAACCGAAGCTGT-3′ (AUG−56); and NS2, 5′-TACTCGCGGCGCGAAAAG-3′). *In vitro* studies were conducted with ODNs possessing three modified nucleotides at each end. The ODNs used for *in vivo* experiments possessed only four modified nucleotides at the 3′ end. AS4, which was used in the *in vivo* experiments, was directed against the 5′ region of the rat α6 subunit mRNA (5′ to the translation initiation site). Its reverse complement shows no sequence similarity with any other nAChR subunit cDNA.

Preparation of cell cultures: A monolayer of glial cells was prepared from postnatal rat brain (PO–2). The ventral mesencephalic region was dissected in HBSS (Gibco 24020-091) and the cells dissociated in HBSS, 0.025% Trypsin, EDTA at 37°C. The reaction was stopped by adding 1 vol plating medium (PM: DMEM Gibco 31885-023, 10% fetal calf serum, 10% horse serum) and 1 vol FCS and the cells centrifuged at 200 × ***g***. The supernatant was removed, 2 ml PM added and the resuspended cells were filtered through a 70 μm cell Strainer (Gibco). After another centrifugation–homogenization–filtration cycle the cells were diluted to 100 000/ml PM and plated in 5 μg ml^{-1} polyornithine-coated dishes (Nunc). The next day, the medium was removed and 2 ml feeding medium (FM: DMEM, 5% horse serum) was added. The medium was changed every 2 days, increasing the ratio of glial/neuronal cells. One week after plating, the ventral mesencephalon of 15-day-old embryos was dissected by the same method and plated on the glial layer. Neurons were differentiated for 2 days. After 4 days FM was removed and replaced with DMEM, N2 supplement [19] and fibronectin (10 μg ml^{-1}), with or without ODNs (0.5–2 μmol l^{-1}). Half the medium was changed and the mix was replaced (medium plus antisense ODNs, medium plus nonsense ODNs or medium only) every 2 days.

In vivo administration of ODNs: *In vivo* experiments were performed in 200 g male Sprague–Dawley rats. ODNs (2–5 μg μl^{-1}) were infused by osmotic mini-pumps (Alzet model 2001) at the rate of 1 μl h^{-1} for 7 days. Mini-pumps were placed under the dorsal skin of the animals and linked to an intraventricular cannula (Alzet brain infusion kit). The cannulae were placed in the lateral ventricle (coordinates: lateral 1.9 mm, caudal 1.2 mm to Bregma, 3.5 mm deep) and maintained on the skull with cyanacrylate paste and dental cement (SS White). During surgery animals were deeply anesthetized with sodium pentobarbital.

Studies of nicotine-elicited locomotion: On the seventh day of ODN infusion, rats were placed in transparent cages crossed with eight infrared beams. After 1 h animals received (−)-nicotine bi-tartrate (Sigma) or saline solution by i.p. injection. The locomotion-stimulating effect of nicotine was calcu-

lated as the ratio H/N where H represents the beam breaks during the habituated period (second hour) and N represents for the beams breaks during the non-habituated period (first hour), or by the normalized formula:

$$\frac{\frac{H}{N} - \left\langle \frac{H_s}{N_s} \right\rangle}{\left\langle \frac{H_n}{N_n} \right\rangle - \left\langle \frac{H_s}{N_s} \right\rangle}$$

where H_s and N_s are relative to the saline injected animals in the absence of ODNs and H_n and N_n are relative to the animals injected with 0.21 mg kg^{-1} free-base nicotine in the absence of ODNs.

Results

Western-blot of fetal mesencephalic neurons with anti-α6 subunit antibodies showed a band of ~ 60 kDa, which is close to the predicted mol. wt (53.3 kDa) of α6 protein. The slightly lower mobility could be due to glycosylation. The intensity of the band increased 4-fold from day 6 to day 8 of culture. The ODNs were applied on day 4 of neuronal culture and the intensity of α6 subunit immunoreactivity was evaluated on days 6 and 8. On day 6, no significant change in α6 subunit immunoreactivity was detected with any treatment. Instead, on day 8, treatment of fetal mesencephalic neurons with antisense ODN (AS4) resulted in a marked and significant decrease in the intensity of α6 protein, measured by Western blot. Treatment with another antisense ODN (AS3), as well as with a nonsense ODN, showed no significant differences compared with the control cultures (without ODN treatment; Fig. 1).

The effect of AS4 was dose-dependent. Treatment with 0.5 μmol l^{-1} did not result in a significant decrease in α6 subunit immunoreactivity. By contrast, 1, 2 and 5 μmol l^{-1} AS4 provoked decreases in α6 subunit immunoreactivity of 20%, 55% ($p < 0.01$) and 35% ($p < 0.05$), respectively (not shown).

In order to determine whether ODN infusions had any effect on the activity of meso-telencephalic dopaminergic pathways, we measured nicotine-elicited locomotion in animals habituated to the test environment. Rats placed in a new cage demonstrated high locomotion activity (non-habituated period, N). This activity decreased exponentially as the animals became used to the novel environment. After 1 h a resting state was reached (habituated period, H). (−)-Nicotine or saline was injected i.p. 1 h after placement in the cage. While animals injected with saline showed a short-lasting increase (due to the handling and the injection), nicotine-

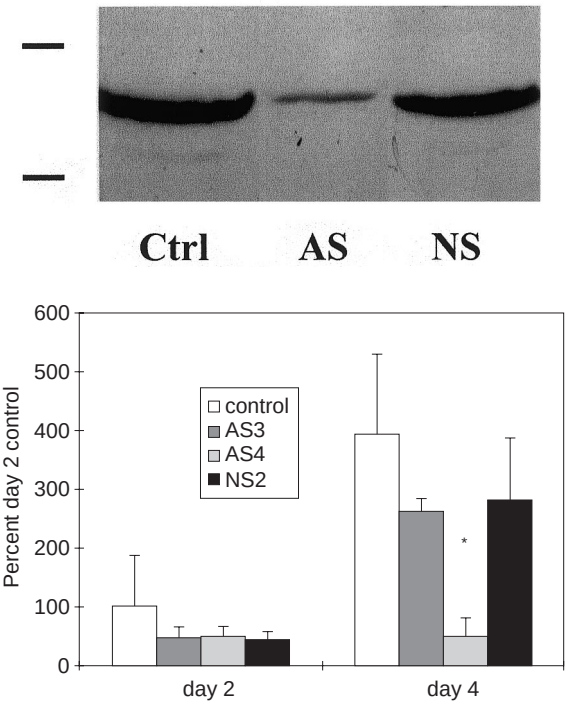

FIG. 1. Effects of oligonucleotide treatments on α6 protein in cultures of mesencephalic neurons. (**A**) ECL autoradiogram of a representative Western blot, showing the effect of antisense (AS) and nonsense (NS) ODNs on cultured mesencephalic neurons. (**B**) Quantification of the changes in the α6 band. Values are mean ± s.e.m., $n = 4$ for each treatment. Statistical analysis according to one-way ANOVA, $p < 0.05$ *vs* control culture.

elicited sustained locomotor activity lasted about 1 h. The effect was dose-dependent, with an EC_{50} of about 0.18 mg kg^{-1} (−)-nicotine free base.

After 7 days of ODN or vehicle infusion via mini pump, rats were placed in the cages and their locomotion was measured. No significant differences were found during the non-habituated period between the sham-operated animals, and animals infused with nonsense or antisense ODNs. At the beginning of the habituated period the animals were injected with 0.21 mg kg^{-1} (−)-nicotine free base. Like the control animals, both rats infused with vehicle and nonsense ODN showed a marked nicotine-elicited increase in locomotion. However, rats infused with antisense ODN showed only a small increase in activity during the habituated period when injected with nicotine (Fig. 2). When the data were expressed as (ratio habituated/non-habituated) a highly significant difference ($p < 0.01$, one-way ANOVA) was found between antisense- and nonsense-infused animals.

When the results were normalized and expressed as modification of nicotine effect, the antisense treatment was found to suppress 70% of the nicotine effect. Moreover, the temporal analysis of locomotor activity at treatment reduced the sustained nicotine-elicited increase in locomotion

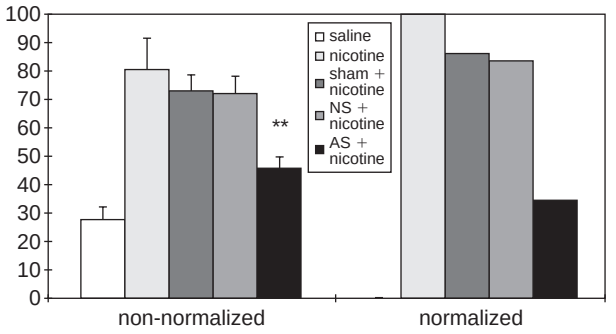

FIG. 2. Effects of antisense (AS) and nonsense (NS) ODNs on nicotine-elicited locomotion. The left part of the graph presents the raw data (ratio habituated/non-habituated). Values are mean ± s.e.m., $n=4$ for non-operated rats and $n=7$ for operated rats. Statistical analysis according to one-way ANOVA, ** $p<0.01$, AS *vs* NS). In the right part, the values are normalized in order to attribute the value 0 to the non-operated rats injected with saline and 100 to the non-operated rats injected with 0.21 mg kg^{-1} (−)-nicotine free base (see Materials and Methods for details).

whereas it did not modify the brief post-injection increase in activity, which is possibly induced by stress (Fig. 3).

Discussion

The continuous infusion of ODNs by the mean of an osmotic mini-pump linked to an intra-ventricular cannula can maintain high concentrations of ODN in the CSF for several days and cause its relatively homogeneous diffusion in the brain [20]. We used this technique to infuse antisense ODNs directed against the nicotinic subunit α6. The dissection of substantia nigra pars compacta is difficult. Since the dopaminergic cells are scattered, the resulting tissue is contaminated with variable amount of non-dopaminergic tissue. Moreover, we do not know the respective α6 content of striatum and ventral mesencephalon. It was therefore not possible to use Western blot to quantify the amount of α6 protein *in vivo*. Indeed, none of anti-α6 antibodies we have generated so far worked in immuno-cytochemical experiments. It was therefore impossible to quantify the extent of α6 inhibition by AS4 *in vivo*. This ODN, however, dose-dependently inhibited α6 expression in mesencephalic cultures, which is the standard control in this type of experiment (see for instance the reference paper on NMDAR1 [21]).

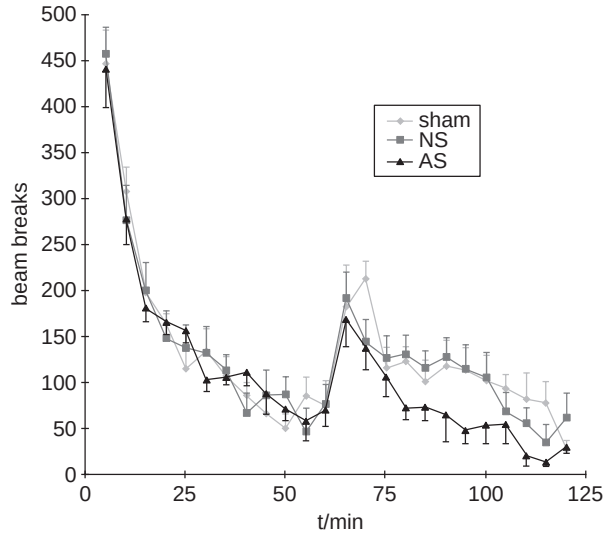

FIG. 3. Time course of the effects of oligonucleotide treatments on the nicotine elicited locomotor activity. Nicotine was injected after 60 min. Note that between 15 min and 50 min after nicotine injection there is a marked option between antisense (AS) and nonsense (NS) or sham treatments. Each point represents the mean ± s.e.m. of seven animals.

This study shows that the increase in locomotor activity elicited by nicotine is mediated at least in part by an α6 subunit containing nAChR. On the basis of pharmacological studies of nicotine-stimulated DA release in striatal synaptosomal preparations, it was initially suggested that a nAChR composed of α3β4 subunits mediated this effect [12–15]. However, no β4 subunit mRNA and little α3 subunit mRNA can be detected in mesencephalic dopaminergic neurons [7]. In addition, experiments performed in mice lacking the β2 subunit have shown that this subunit is a necessary component of the nAChR isoform(s) which mediate(s) nicotine-induced DA release [9]. Experiments on striatal slices showed that DA release is inhibited by 100 μmol l^{-1} neuronal bungarotoxin [13], a concentration which blocks the α3β2 currents but not α4β2 currents [22]. More recent experiments showed that the conotoxin MII, which is specific for α3β2 in reconstituted systems [23], inhibits DA release [14,15] in striatal slices as well as in synaptosomes. Overall, these observations brought up the suggestion that an α3β2 receptor plays an important role in DA release in striatum [14], although the relevance of α4β2-containing receptors could not be completely ruled out [24].

The coding sequences of α3 and α6 subunits display an overall 59.5% nucleotide identity. However, the differences are mainly located in portions of the sequence encoding the signal peptide and the variable part of the cytoplasmic domain. In the portion encoding the cysteine loop, identity increases to 90%. Since the α6 subunit is expressed at much higher levels than the α3 subunit in the catecholaminergic nuclei [7], previous *in situ* hybridization studies [25] might have detected α3 as well as α6 subunit mRNAs with probes directed against the entire α3 subunit cDNA.

The mature amino-terminal domains of the α3 and α6 subunits show 67.3% residue identity. In addition, 30% of the differences represent conservative amino acid substitutions. In the ACh binding

site segments there is only one replacement, α3S→α6T. In view of the difficulty of expressing the α6 subunit *in vitro*, the pharmacology of α6-containing nAChRs remains almost unknown, although the structural evidence discussed here suggests that it must be very similar to that of α3-containing nAChRs. The pharmacological evidence of nAChR-elicited release of striatal DA previously attributed to α3-containing nAChRs may in reality apply to α6-containing nAChRs.

Since the α6 subunit mRNA is more abundant than that of the α3 subunit, we have proposed that nicotine-elicited DA release is possibly mediated through α6β2-containing receptors [7].

Conclusion

Recent *in situ* hybridization experiments showed that mesencephalic dopaminergic neurons express very high levels of α6, about 20 times higher than α3 subunit mRNA. It was therefore proposed that α6β2 rather than α3β2-containing receptors had an important role in nicotine-induced dopamine release in this neuronal system [7]. Present data indeed show that α6-containing nAChRs are involved in mediating the effect of nicotine on locomotion in habituated environments. As discussed in the introduction, this effect of nicotine is primarily due to an action on dopaminergic mesostriatal neurons. These data therefore support the hypothesis that the α6 subunit containing nAChRs present on dopaminergic neurons are activated by pharmacological doses of nicotine and may contribute to some of the effects of this drug on motor behavior.

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