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Research report

Nicotinic receptor blockade decreases fos immunoreactivity within orexin/hypocretin-expressing neurons of nicotine-exposed rats

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HIGHLIGHTS

- Mecamylamine induces somatic events and anxiety in rats given chronic nicotine.
- Nicotinic receptor blockade decreases Fos-immunoreactivity of orexin neurons.
- Orexin receptors should be assessed as targets for developing smoking cessation aides.

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ABSTRACT

Tobacco smoking is the leading cause of preventable death in the United States. Nicotine is the principal psychoactive ingredient in tobacco that causes addiction. The structures governing nicotine addiction, including those underlying withdrawal, are still being explored. Nicotine withdrawal is characterized by negative affective and cognitive symptoms that enhance relapse susceptibility, and suppressed dopaminergic transmission from ventral tegmental area (VTA) to target structures underlies behavioral symptoms of nicotine withdrawal. Agonist and partial agonist therapies help 1 in 4 treatment-seeking smokers at one-year post-cessation, and new targets are needed to more effectively aid smokers attempting to quit. Hypothalamic orexin/hypocretin neurons send excitatory projections to dopamine (DA)-producing neurons of VTA and modulate mesoaccumbal DA release. The effects of nicotinic receptor blockade, which is commonly used to precipitate withdrawal, on orexin neurons remain poorly investigated and present an attractive target for intervention. The present study sought to investigate the effects of nicotinic receptor blockade on hypothalamic orexin neurons using mecamylamine to precipitate withdrawal in rats. Separate groups of rats were treated with either chronic nicotine or saline for 7-days at which point effects of mecamylamine or saline on somatic signs and anxiety-like behavior were assessed. Finally, tissue from rats was harvested for immunofluorescent analysis of Fos within orexin neurons. Results demonstrate that nicotinic receptor blockade leads to reduced orexin cell activity, as indicated by lowered Fos-immunoreactivity, and suggest that this underlying cellular activity may be associated with symptoms of nicotine withdrawal as effects were most prominently observed in rats given chronic nicotine. We conclude from this study that orexin transmission becomes suppressed in rats upon nicotinic receptor blockade, and that behavioral symptoms associated with nicotine withdrawal may be aided by intervention upon orexinergic transmission.

1. Introduction

Tobacco smoking is a pervasive problem in the United States which remains the leading cause of preventable death and costs $295 billion annually in health care, crime and lost productivity [1]. Nicotine is the constituent psychoactive compound in tobacco that causes addiction and acts in the central nervous system (CNS) by binding to pentameric nicotinic acetylcholine receptors (nAChRs) which are widely distributed across brain structures governing

Abbreviations: CNS, central nervous system; DA, dopamine; ir, immunoreactivity; nAChR, nicotinic acetylcholine receptor; NAcc, nucleus accumbens; OxA, orexin-A; Ox1R, orexin receptor subtype 1; Ox2R, orexin receptor subtype 2; PBS, phosphate-buffered saline; VTA, ventral tegmental area.

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reward, affect, learning and memory [2,3]. Chronic nicotine administration causes high-affinity nAChRs, predominantly containing the β_2 receptor subunit, to desensitize and upregulate [4–10]. Accordingly, smoking cessation leads to altered cholinergic signaling which underlies in part the behavioral symptomatology associated with nicotine withdrawal [e.g.,11–12]. Pharmacotherapeutic strategies have focused on agonist or partial-agonist therapy to mimic the physiological effects that nicotine would otherwise exert within the CNS. While adjuncts to smoking cessation, including nicotine replacement therapy and the $\alpha4\beta2$ nAChR partial agonist varenecline, have been shown to partially attenuate withdrawal symptom severity, abstinence rates in treatment-seeking smokers have been documented between around 25% at 1-year post-cessation [13–16; for review,17]. Thus, new targets are needed to better treat nicotine addiction and promote tobacco abstinence.

Acute nicotine administration causes transient elevations of dopamine (DA) within the nucleus accumbens (NAcc) which is believed to function as a reward signal [18–21]. The 'positive reinforcement' associated with acute nicotine administration is transient as chronic use leads to nAChR desensitization and upregulation [e.g.,22]. As a result, altered cholinergic signaling following chronic nicotine use leads to depleted mesolimbic DA activity believed to underlie the behavioral symptoms of nicotine withdrawal [23–24; for review,25]. Hypodopaminergic tone in VTA target structures, including NAcc, contributes to impaired brain reward function and mediates relapse propensity as subsequent drug consumption is thought to bring users to a homeostatic set point which itself has shifted following chronic drug use [for reviews,26–27]. Drug withdrawal leads to increased inhibitory GABAergic tone on midbrain DA-producing neurons in adult rats [28–32]. Thus, the basal excitability of DA-producing cells within ventral tegmental area (VTA), which densely innervates and transmits DA to target structures including NAcc, is attenuated during nicotine withdrawal, and accordingly, transmitter systems that excite DA-producing neurons provide excellent therapeutic targets.

Hypothalalmic orexin/hypocretin neurons innervate most levels of the CNS and signal through two predominantly excitatory G-protein coupled receptors [33–37]. Functionally, orexin signaling is critical for regulation of feeding, wakefulness and goal-directed behavior [38–40]. Prior anatomical work has demonstrated that orexinergic fibers form appositions with DA-producing neurons of the VTA [41–43]. Moreover, orexin peptides have been shown to increase spontaneous firing rates of DA-producing cells within VTA *ex vivo* [44,45]. Separate lines of work in cocaine self-administering animals has revealed that orexin receptor blockade occludes cocaine-evoked elevations in mesolimbic DA and leads to reduced drug-seeking motivation [38–40,46,47]. Collectively, these studies demonstrate that orexin peptides are excitatory and that orexin receptor antagonism can be used to decrease activity of DA-producing neurons of the VTA in response to rewards that are known to evoke phasic elevations in mesolimbic DA.

The present study was conducted to examine the effects of nicotinic receptor blockade on orexin cell activity using a precipitated nicotine withdrawal model. Results reveal that rats given both chronic nicotine and acute mecamylamine most prominently showed somatic signs (e.g., ptosis, gasps), anxiety-like behavior and a reduction of orexin cell activity as indicated by lower expression of the neuronal activity marker Fos. These results suggest that nicotinic receptor blockade leads to decreased release of orexin peptides, and that prior nicotine exposure enhances this effect. Suppressed orexin peptide release may in turn alter the physiology of target neuronal populations, including DAergic neurons of VTA. Future studies should elaborate on the mechanisms by which nicotine withdrawal alters orexinergic cell activity and explore how pharmacological alteration of orexin signaling during nicotine withdrawal effects the constellation of behaviors known to drive relapse.

2. Material and methods

2.1. Subjects and surgery

Adult, male Sprague-Dawley rats (Charles River Laboratories; Wilmington, MA) were used for the present experiment. Animals were received at Temple University's vivarium and acclimated for one week prior to surgery and were pair-housed throughout the experiment. Animals underwent mini-osmotic pump (Alzet [Model 2001]; Durect Corporation; Cupertino, CA) implantation surgery. Half of the animals received a vehicle (0.9% saline) and half received a liquid (−)-nicotine treatment (3.16 mg/kg/d, freebase; Sigma [N3876]; St. Louis, MO). Briefly, animals were anesthetized with isofluorane (5% induction, 2–3% maintenance). Animals were shaved along their mid-scapular region, and the area was sterilized with iodine and alcohol. Animals then received an injection of buprenorphine (0.05 mg/kg, s.c.) for peri-operative analgesia, and a dollop of ophthalmic ointment was placed on and around eyes of animal to retain moisture (Artificial Tears; Henry Schein; Dublin, OH). A small incision was made using sharp scissors and connective tissues were cleared to insert pump with the head of the mini-osmotic pump facing the caudal surface of the animal. Following pump insertion, the incision was secured using 9 mm surgical staples. All surgical and behavioral procedures were approved by Temple University's Institutional Animal Care and Use Committee.

2.2. Experimental procedures

Following mini-osmotic pump implantation surgery, animals remained in their home cages for 7 days. On the 7th day following mini-osmotic pump implantation surgery, animals were administered either mecamylamine hydrochloride (3.0 mg/kg, s.c.; Sigma [M9020]; St. Louis, MO), dissolved in 0.9% saline, or vehicle (0.9% saline). Doses of nicotine and mecamylamine were selected based in part on prior work showing an emergence of somatic and affective symptoms of nicotine withdrawal [48,49]. Immediately following acute injections, animals were placed in an open field arena for 30 min. Thus, treatment groups consisted of: (i) chronic saline and acute saline (Sal-Veh), (ii) chronic saline and acute mecamylamine (Sal-Mec), (iii) chronic nicotine and acute saline (Nic-Veh), and (iv) chronic nicotine and acute mecamylamine (Nic-Mec). Video recordings were taken to assess behavioral measures in the open field arena. Animals were assessed for somatic withdrawal events by an experimenter blind to treatment conditions and animals were given 1 point for an observation of the following: teeth chattering/chews, tail writhes/wags and gasps, shakes and tremors, ptosis (eye-drooping; scored as 1 point for every 1 min of observation), and miscellaneous events (e.g., yawning, scratching) [50]. A summated global withdrawal score was used for analysis. Animals were further assessed for locomotor activity using a custom Matlab program script. Lastly, an experimenter blind to treatment conditions scored percent time in the center of open field arena as an assessment of anxiety.

Between 90 and 120 min following acute injections of either saline or mecamylamine, animals were injected with Somnasol (sodium pentobarbital and phenytoin sodium solution; ∼200 mg/kg, i.p.; Henry Schein; Dublin, OH) and underwent transcardiac perfusion with 0.9% saline (∼50–100 mL) followed by 4% paraformaldehyde (∼100–150 mL). Whole brains were extracted and post-fixed in 4% paraformaldehyde at 4°C for 24 h. Following post-fixation, brains were moved to a 30% sucrose solution for 3–5 days. Brains were then flash frozen in 2-methyl butane over dry