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Original article

Orexin type-2 receptor blockade prevents the nicotine-induced excitation of nucleus accumbens core neurons in rats: An electrophysiological perspective

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ABSTRACT

Background: The nucleus accumbens core (NAcc) expresses both orexin and nicotinic acetylcholine receptors (nAChRs). Orexin is among important neurotransmitters, which regulates addictive properties of drugs of abuse including nicotine. The role of orexin-2 receptor (OX2R) in the regulation of NAcc neural activity in response to nicotine has not yet been studied. Hence, in this study, we examined whether the OX2R antagonist (TCS-OX2-29) can adjust the effects of nicotine on electrical activity of NAcc neurons, in urethane-anesthetized rats, using the single unit recording.

Methods: Neuronal firing of NAcc was recorded for 15 min, then TCS-OX2-29 (OX2R-antagonist; 1, 3 and 10 ng/rat) or DMSO were microinjected into NAcc, just 5 min before subcutaneous (*sc*) administration of nicotine (0.5 mg/kg) or saline. The spontaneous firing activity was recorded for 70 min, after nicotine injection.

Results: The results demonstrated that nicotine significantly excites the NAcc neurons and interestingly, the administration of TCS-OX2-29 (3 and 10 ng/rat) into the NAcc, inhibited nicotine-induced increases of NAcc neuronal responses. Furthermore, administration of TCS-OX2-29 (10 ng/rat), just 5 min before *sc* administration of saline instead of nicotine, did not significantly alter the neuronal responses, compared to the saline-control group.

Conclusion: Our results showed that, although OX2R blockade alone did not affect neuronal activity in the NAcc, it was able to prevent the exciting effects of nicotine on NAcc neuronal activity. Therefore, we proposed that orexin has a potential modulator effect, in response to nicotine.

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Introduction

Nicotine, the key psychoactive factor of tobacco leaves, is responsible for cigarette addiction [1]. Nicotine excites meso-corticolimbic dopaminergic neurons firing, by affecting nicotinic acetylcholine receptors (nAChRs) [2], and finally leading to dopamine (DA) release from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) and the prefrontal cortex (PFC) [3,4]. These have a critical role in reinforcing properties of drugs of abuse, including nicotine [5].

The orexins/hypocretins neurons (release orexin A and B) send projections to the areas, involved in reward processing and drug addiction, such as the NAc [6–8]. Their corresponding receptors (orexin-1 receptor (OX1R) and orexin-2 receptor (OX2R)) are G-protein coupled receptor [9]. Both receptors have been reported to be expressed in the NAc [7]. Orexin system regulates multiple brain functions, such as sleep/wakefulness, arousal, pain, autonomic function, feeding behaviors, metabolism, cognition, natural and drug rewards, as well as opioids dependence and withdrawal [10–16]. Studies have shown that orexinergic system has an important role in mediating the addictive behavior [8,15,17], including the addictive response to nicotine; it has been demonstrated that OX1R blockade in the insular cortex, or systemic administration of OX1/2R antagonist, reduced the nicotine self-administration [18,19].

Although, most of the studies have focused on OX1R [15,16,18], some other studies have shown that OX2R may also play an important role in reward. For example, long-lasting cocaine administration increased OX2R protein expression in the NAc [20]. Furthermore, OX2R blockade attenuated ethanol- [21] and morphine-induced CPP [22], and decreased heroin self-administration in rats [23]. Recently, it has been demonstrated that OX2R blockade in the VTA attenuated nicotine-induced conditioned place preference (CPP) [24]. Therefore, considering

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that a few studies have been performed on the role of OX2R in the reward circuits, in particular in response to nicotine, the aim of this study was to determine the effect of OX2R blockade on the activity of the nucleus accumbens core (NAcc) neurons, in response to systemic administration of nicotine, using the single unit recording.

Materials and methods

Animals and surgical procedure

Experiments were executed on adult male Wistar rats (240–290 g), obtained from the Pasteur Institute; Tehran, Iran. The Ethic Committee of Animal Use of Isfahan University of Medical Sciences approved the study, and all trials were performed in accordance with the guidelines for Animal Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23), revised in 2011. Animals were deeply anesthetized with intraperitoneal (*ip*) injections of Urethane (1.2 g/kg and supplementary doses were given as required; Sigma-Aldrich, Germany), and were placed in a stereotaxic apparatus (Stoelting, USA). The skull was exposed and a hole was made for the insertion of a two-barreled micropipette in the NAcc (AP: + 2.16 mm; ML: 1.4 mm; DV: 6–7.4 mm) [25].

Experimental designs

We investigated dose responses to TCS-OX2-29 (1, 3 and 10 ng/rat in 0.5 μl 10% DMSO; Tocris Bio-science, Bristol, UK), as an OX2R antagonist on the neuronal discharge rate of NAcc neurons, in response to 0.5 mg/kg, *sc* injection of nicotine hydrogen tartrate (Sigma-Aldrich, Germany). In addition, we measured the neuronal response of NAcc neurons to the maximum dose of TCS-OX2-29 (10 ng/rat), just 5 min before *sc* administration of saline instead of nicotine. The recordings were executed extracellularly on NAc neurons of intact rats, using a single unit recording technique.

Drugs administrations

A two-barreled micropipette (one pipette for drug microinjection and the other for extracellular action potentials recording) was gently guided into the NAcc, using a manual microdrive, until the best spike activity was distinguished with a signal-to-noise ratio of more than two, isolated from the background noise. Drug was microinjected into the NAcc, using a manual pressure injector and the recording was performed, using a fine tip (1–3 μm) glass microelectrode, filled with 2 M NaCl solution. Extracellular action potentials were amplified 10,000 times, filtered at 0.3–3 kHz, and displayed continuously on an oscilloscope. Then, single-unit firings were digitized at 50 kHz sampling rate and 12-bit voltage resolution. The spike rates were counted and displayed online in time bins of 1000 ms, over the complete recording period, using a commercial analog to digital data acquisition system. Data analysis was performed, using the associated software, eLab (Science Beam Institute, Iran).

The NAc neurons exhibited slow firing rate, between 0.5 and 6.0 Hz [25,26]. When, steady firing rate was identified, a baseline recording was conducted for about 15 min, then, the drug was microinjected into the NAcc. Subsequently, after 5 min, nicotine was infused *sc* and the activity of the neurons was carried on for about 70 min. Control groups received the same volume of saline and DMSO, instead of nicotine and the test drug, respectively.

Histological verifications

After completion of each experiment, in order to mark the place of electrodes, the animals were euthanized with an overdose of urethane and perfused transcardially with 100 ml of normal saline, followed by 100 ml of 10% buffered formalin, then decapitated, the brains were dissected, and placed in a 10% formalin for 72 h. Finally, the brains were sliced coronally in 55 μm sections, through the electrode locations, and the sites of the electrodes were verified,

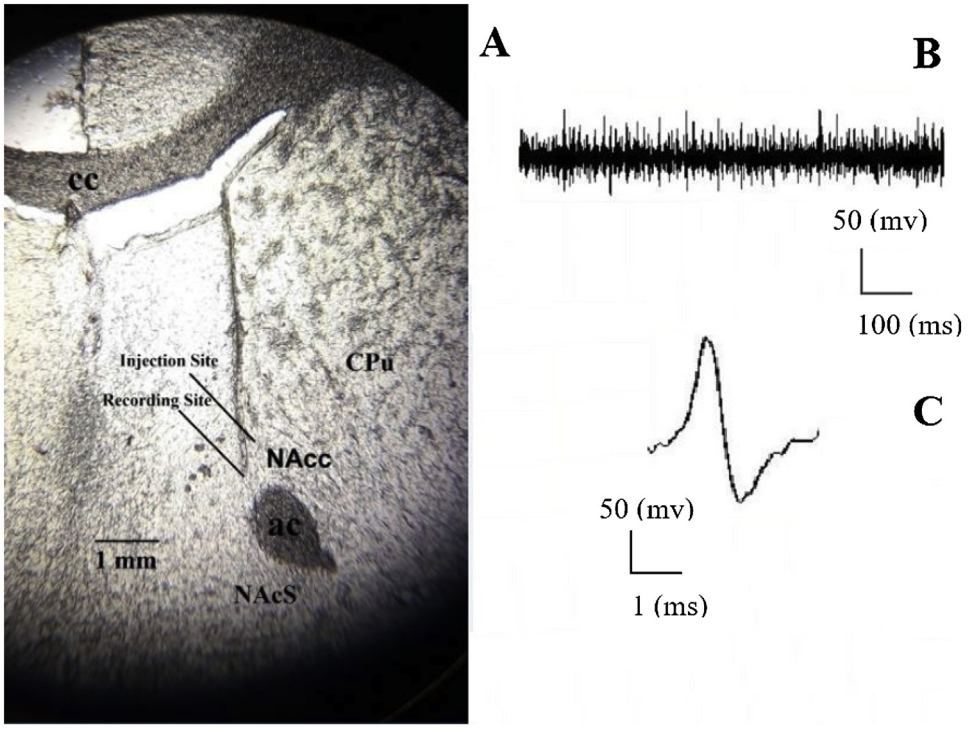

Fig. 1. (a) A representative image, displaying the recording and microinjection site in the NAcc; corpus callosum (cc), caudate putamen (CPu); NAc core (NAcc) and shell (NAcS), and anterior commissure (ac). (b) A representative pattern of baseline neuronal electrical activity recorded from the NAcc. (c) An expanded waveform of a spike recorded from a NAcc neuron.

according to the stereotaxic atlas of Paxinos and Watson [27] (Fig. 1).

Statistical analysis

The pre-injection spontaneous firing rate over 15-minute period was defined, as the average firing rate (in spikes per second). An increase/decrease of firing rates beyond the mean ± two-fold of the SD of the baseline firing rate, for five consecutive minutes, was considered as an excitatory/inhibitory response, respectively [28]. We used the percentage of increase/decrease in firing rate multiplied by the duration of excitation/inhibition (percent × duration in minutes) [28]. The data were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's *post-*

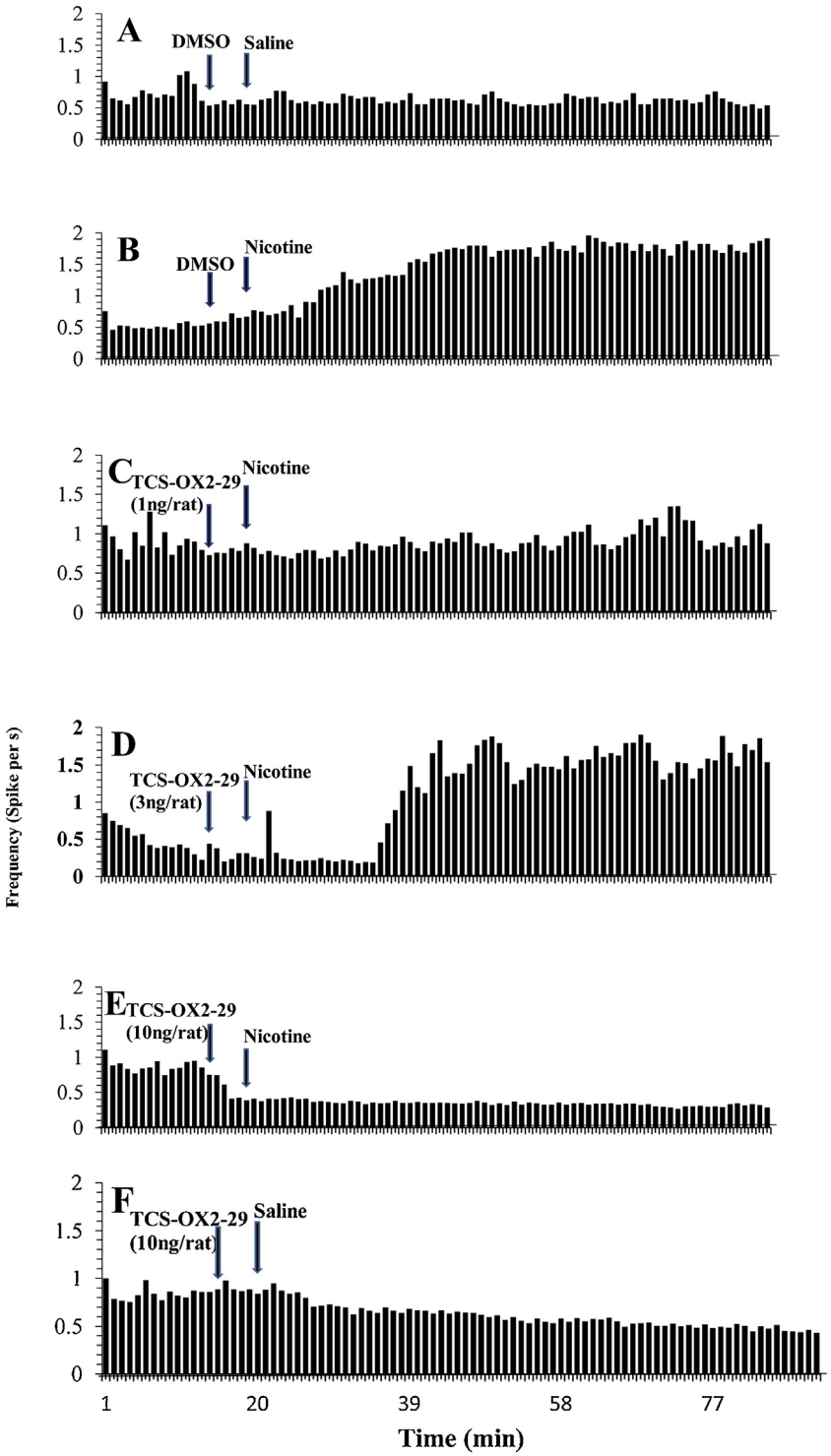

Fig. 2. Histograms representing spike frequency of the entire recording (90 min) of all neurons. Subcutaneous injection of saline could not affect firing frequency (a); but nicotine increased the firing rate (b); the 1 ng (c), 3 ng (d), and 10 ng (e) doses of TCS-OX2-29 could prevent the nicotine-induced excitation on a dose-dependent manner. The microinjection of 10 ng TCS-OX2-29, just 5 min before *sc* administration of saline instead of nicotine did not significantly alter the neuronal response, compared to the saline control group (f).

hoc test for multiple comparisons, and the χ^2 test for comparing cells with excitatory or inhibitory responses, between different groups. Data are expressed as mean ± SEM, and statistical significance was set at $p < 0.05$.

Results

Subcutaneous injection of nicotine excited the majority of NAcc neurons

In the first set of experiment, after the stabilization period (20 min) and baseline recording (15-min), DMSO was microinjected into the NAcc and 5 min later, nicotine (0.5 mg/kg *sc*) was administered. Subcutaneous injection of nicotine (0.5 mg/kg *sc*) in eight rats increased the spike frequency per seconds in the majority of neurons' 9/12 (75%), decreased just 1/12 (8.33%) neuron firing activity, and 2/12 (16.66%) neuron remained unchanged (Fig. 2b). On the other hand, saline (instead of nicotine) in eight rats, had no effect on 6/11 neurons (54.54%), while reducing neuronal firing of 3/11 (27.27%) neurons, and also excited just 2/11 (18.18%) neuron (Fig. 2a).

A χ^2 test, for comparing the difference in the proportion of neurons with excitatory responses to those with inhibitory or no response, revealed a significantly higher proportion of excited neurons in the nicotine group, compared to the saline group [χ^2 (1) = 7.425, N = 23, $p = 0.009$] (Table 1).

Intra-NAcc microinjection of TCS-OX2-29 prevented nicotine-induced excitation of NAcc neurons

In the second set of the experiment, to find out the effect of TCS-OX2-29 on the NAcc neuronal firing, after stabilization period (at least 20 min) and 15 min of baseline recording, different doses of TCS-OX2-29 (1, 3 and 10 ng/rat) were microinjected into the NAcc, just 5 min prior to the administration of nicotine (0.5 mg/kg *sc*). The different response rates of neurons, in the nicotine + TCS-OX2-29 (1 ng) group (10 rats) were 3/13 neurons (23.07%) inhibition, 8/13 neurons (61.53%) excitation, and 2/13 neurons (15.38%) with no response (Fig. 2c); in the nicotine + TCS-OX2-29 (3 ng) group (14 rats) were 11/20 neurons (55%) inhibition, 4/20 neurons (20%) excitation, and 5/20 neurons (25%) with no response (Fig. 2d); in the nicotine + TCS-OX2-29 (10 ng) group (14 rats) were 12/24 neurons (50%) inhibition, 3/24 neurons (12.5%) excitation, and 9/24 neurons (37.5%) with no response (Fig. 2e). Moreover, administration of maximum dose (10 ng/rat) of TCS-OX2-29 (14 rats), into the NAcc, just 5 min before *sc* administration of saline instead of nicotine, decreased the firing rate of majority of neurons' 13/19 (68.42%), increased the spike frequency of just 2/19 (10.52%) neurons, and caused no response in 4/19 (21.05%) of the single-unit responses (Fig. 2f).

The proportion of neurons with excitatory response, to those with inhibitory or no response, between the nicotine control group and nicotine + TCS-OX2-29 (1 ng) group was not significant [χ^2 (1) = 520, N = 25, $p = 0.387$], but a significant response has been found between the nicotine control group and the nicotine+TCS-OX2-29 (3 ng) group [χ^2 (1) = 9.406, N = 32, $p = 0.003$], and also between the nicotine control group and the nicotine+TCS-OX2-29 (10 ng) group [χ^2 (1) = 14.062, N = 36, $p = 0.000$] (Table 1).

The χ^2 test also showed a significant difference in the proportion of neurons with inhibitory response, to those with excitatory or no response, between saline + DMSO group and saline + TCS-OX2-29 (10 ng) group [χ^2 (1) = 4.739, N = 30, $p = 0.035$] (Table 1).

Statistical analysis for comparison of the percentage of change, in the neuronal firing rates between the groups showed that nicotine increased neuronal firing, with respect to the saline group, also, orexin antagonist could adjust the excitatory effect of nicotine [F (5, 98) = 5.092; $p < 0.001$] (Fig. 3). Furthermore, statistical analysis revealed that microinjection of the maximum dose of TCS-OX2-29 (10 ng), just 5 min before *sc* administration of saline

Table 1
Data from the Chi Square test analyses.

Groups	Saline + DMSO	Nicotine + DMSO
Saline + DMSO	_	$p = 0.009$
Nicotine + DMSO	$p = 0.009$	_
Nicotine + TCS-OX2-29 (1 ng)	_	$p = 0.387$
Nicotine + TCS-OX2-29 (3 ng)	_	$p = 0.003$
Nicotine + TCS-OX2-29 (10 ng)	_	$p = 0.000$
Saline + TCS-OX2-29 (10 ng)	$p = 0.035$	_

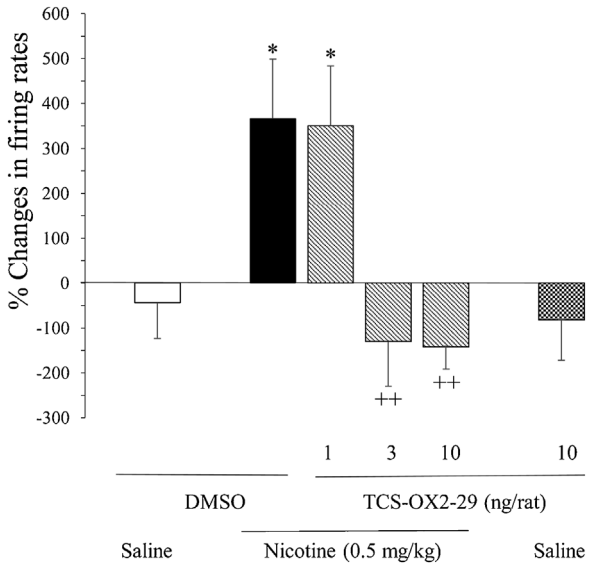

Fig. 3. The effect of OX2R blockade on the percentage of increase/decrease activity of the NAcc single neurons in response to nicotine. Data are expressed as mean ± SEM, $^*p < 0.05$ different from the saline-control group, $++p < 0.01$ different from the nicotine-control group.

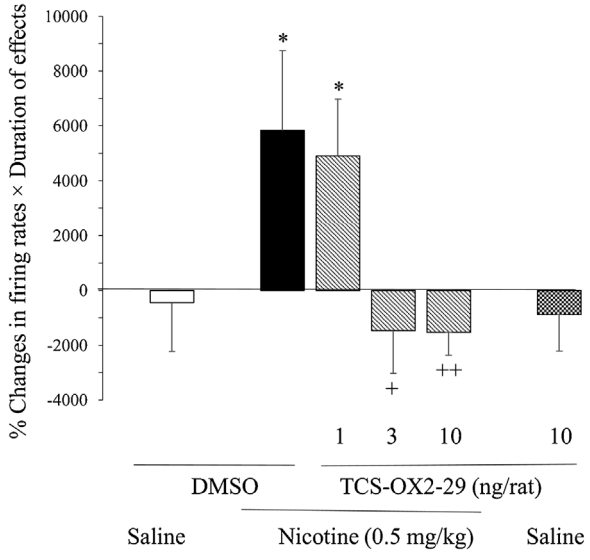

Fig. 4. The effect of OX2R blockade on neuronal responses of the NAcc single neurons in response to nicotine, when the duration of the inhibition was taken into account. Data are expressed as mean ± SEM $^*p < 0.05$ different from the saline-control group, $+p < 0.05$ and $++p < 0.01$ different from the nicotine-control group.

instead of nicotine, into the NAcc, did not change the neurons' response, compared to the DMSO + saline control group.

In addition, when the duration of the excitatory and inhibitory responses (regardless of inactive neurons) was taken into account, nicotine increased neuronal firing, in comparison with the saline group, and orexin antagonist could modify the excitatory effect of nicotine [The one-way ANOVA; followed by Tukey test [$F(5, 70) = 3.291$; $p < 0.01$] (Fig. 4). In the nicotine + TCS-OX2-29 (3 ng) group, antagonist could block the nicotine effect for about 20 min, whereas in the nicotine + TCS-OX2-29 (10 ng) group, blockade lasted until the end of the recording. Moreover, statistical analysis showed that the maximum dose of saline + TCS-OX2-29 did not alter the neurons firing, relative to the saline + DMSO control group.

Discussion

Studies have shown that orexinergic system probably has a modulatory role in the addictive response to nicotine [18,19,24,28]. While, the expression of both Ox1R and Ox2R have been reported in the NAc [7], but Ox1R levels are very low [29] and orexin's actions have been attributed to Ox2R binding [7,30], reviewed by Sharf et al. [6]; therefore, in the present study, we sought to test the role of OX2R on the NAcc neuronal firing, in response to nicotine.

Our data showed that nicotine (0.5 mg/kg) increases the spike frequency of the majority of neurons in the NAcc, presenting a sensitive response to nicotine in this region. This dose of nicotine is the same as previous behavioral studies [24,31–33], showing a dose of 0.5 mg/kg nicotine, produces reliable CPP in male Wistar rats. Also, in a single electrophysiological investigation, this dose of nicotine inhibited fimbria-induced excitation of normally inactive NAc neurons, in anesthetized rats [34].

The NAc neurons exhibited slow firing rate, between 0.5 and 6.0 Hz [25,26], and our data confirm this low firing rate. Electrophysiological studies have shown that systemic administration of nicotine, increases the cell firing of the VTA DA neurons [35]. Some electrophysiological and microdialysis studies have revealed that nicotine stimulates the DA cell firing by local action in the VTA [36–39]. Systemic nicotine-induced DA release in the NAc was reduced by 50%, via blocking the nAChRs in the NAc [40], indicating that the NAc nAChRs play a role in the nicotine-induced release of the mesolimbic DA. Actually, a well-designed balance between excitatory and inhibitory inputs to the VTA DA neurons, results in the rewarding outcome of nicotine, by increasing glutamatergic excitation and decreasing GABAergic inhibition onto DA neurons [41]. Consequent activation of the VTA cholinergic receptors regulates DA release into the NAc, for an additional reinforcing effect [42]. Kleijn et al. found that the NAc shell could be more sensitive to nicotine than the VTA, in increasing the accumbal DA release [35]. Therefore, nicotine can affect NAc neurons directly by affecting NAc nAChRs or indirectly, *via* nicotine-induced release of the mesolimbic DA into NAc.

As a second result, we observed that OX2R blockade in the saline-treated rats, changed the proportion of neurons responses, but did not affect the percentage of change with respect to saline + DMSO group. However, OX2R blockade prevented the exciting effects of nicotine on NAcc neuronal activity, and a dose of 3 ng of the antagonist could block the nicotine effect for about 20 min, while a dose of 10 ng blocking effect lasted until the end of the recording.

It has been demonstrated that activation of nicotinic acetylcholine receptors in the NAcc is essential for drug reinforcement [43]. The vast majority of cells of the NAc (90–95%) has been made of GABA-containing medium spiny neurons (MSNs), and the remaining cells are cholinergic and GABAergic interneurons [44]. The nAChRs in the NAc have been shown to modulate the activity of the GABAergic MSNs [45]. In addition, studies have shown that orexin stimulates GABAergic neurons in most regions of the brain; it had excitatory effects on GABAergic neurons in the pars reticulate of substantia nigra [46]. In the VTA, orexin excited the firing response of a diverse subpopulation of DA neurons, and increased GABA cells firing rate [47]. Moreover, it has been reported that orexin excites arcuate nucleus GABAergic neurons [48].

In addition, it has been observed that selective endogenous nAChR activation in the NAc, elicits DA release *via* the action of glutamatergic receptor on DA terminals [49,50]. It has been shown that DA receptors blockade on the NAc MSNs weakened dopamine-dependent reward [51]. Dopamine and glutamate inputs can influence each other [52,53] and NAc MSNs express both DA and glutamate receptors [49,50]. Glutamate inputs from some brain areas, such as the prefrontal cortex (PFC), amygdala (AMG), and hippocampus [43–45] project on and activate MSNs of NAc, and blockade of glutamate receptors causes a reduction in the excitability of NAc MSNs [54,55], and affects excitatory postsynaptic potentials [55,56]. There is an anatomical relationship between orexin and dopamine neurons in the medial PFC, AMG, and NAc [57]. Thus, it seems that OX2R blockade probably inhibits the dopamine-dependent nicotine excitation of NAc neurons by influencing the glutamate and dopamine receptors of MSNs.

Previously, studies have shown that cocaine administration increases OX2R protein expression in the NAc [20], and also, OX2R blockade in the VTA attenuated nicotine-induced CPP [24], thus, it can be expected that orexin will affect neuronal responses in the NAcc *via* OX2R. However, in the present study, we observed that OX2R blockade by itself had no effects on neuronal activity in the NAcc, therefore, it can be deduced that OX2R instead of direct effects on neuronal activity, it modifies evoked responses by other factors such as nicotine, through influencing on nAChR or other neurotransmitter systems like dopamine, glutamate or GABA.

In conclusion, it seems that orexin by itself probably had no effects on basal neuronal activity in the NAcc, nevertheless, its baseline action is necessary, and can modulate responses to nicotine probably by affecting nicotinic receptors or other downstream systems. However, this potential interaction needs further investigation to clarify the fundamental mechanism of orexin system action on NAcc neurons.

Declarations of interest

None.

Acknowledgements

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