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Nicotinic Receptor Blockade Decreases Fos Immunoreactivity within Orexin/Hypocretin-Expressing Neurons of Nicotine-Exposed Rats

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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

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symptoms associated with nicotine withdrawal may be aided by intervention upon orexinergic transmission.

Keywords

Addiction; Fos; Hypocretin; Hypothalamus; Mecamylamine; Nicotine Withdrawal; Orexin

1.0: Introduction¹

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 reward, affect, learning and memory [2–3]. Chronic nicotine administration causes high-affinity¹ 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 $\alpha_4\beta_2$ nAChR partial agonist varenicline, have been shown to partially attenuate withdrawal symptom severity, abstinence rates in treatment-seeking smokers have been documented between around 25% at 1-year postcessation [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]. 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

¹Abbreviations.

Central Nervous System – CNS;
Dopamine – DA;
Immunoreactivity – ir;
Nicotinic Acetylcholine Receptor – nAChR;
Nucleus Accumbens – NAcc;
Orexin-A – OxA;
Orexin Receptor, Subtype 1 – Ox1R;
Orexin Receptor, Subtype 2 – Ox2R;
Phosphate-Buffered Saline – PBS;
Ventral tegmental area – VTA.

target structures including NAcc, is attenuated during nicotine withdrawal, and accordingly, transmitter systems that excite DA-producing neurons provide excellent therapeutic targets.

Hypothalamic 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.

2.0: 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 isoflurane (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 miniosmotic 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 mecamlamine 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 mecamlamine 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 minutes. Thus, treatment groups consisted of: (i) chronic saline and acute saline (Sal-Veh), (ii) chronic saline and acute mecamlamine (Sal-Mec), (iii) chronic nicotine and acute saline (Nic-Veh), and (iv) chronic nicotine and acute mecamlamine (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 minute 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 minutes following acute injections of either saline or mecamlamine, 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 hours. 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 ice for 20 seconds. Finally, brains were mounted in a cryostat (Leica Biosystems [CM3050 S]; Buffalo Grove, IL), and 30 µM coronal sections were taken throughout the hypothalamus and stored in cryoprotectant at –20° C. A 1-in-3 selection was used to estimate counts for immunofluorescent analysis.

2.3: Immunofluorescence

Tissue sections extending throughout hypothalamus (approximate coordinates from stereotaxic atlas: –0.92 mm [anterior extent] to –5.20 mm [posterior extent], relative to bregma; Figure 2A, [51]) were washed six times for 10 minutes each in cold 0.1M phosphate-buffered saline (PBS). Sections were then blocked in 5% normal donkey serum (VWR International [89347–872]; Bridgeport, NJ) in PBS containing 0.3% Triton X-100 (Acros Organics [9002-931]; Geel, Belgium; hereafter named “PBS+”) followed by a 72-hour incubation in primary antibody solution (polyclonal rabbit anti-Fos [1:1,000; SC-52, Santa Cruz Biotechnology, Inc.; Dallas, TX] in 1.5% normal donkey serum in PBS+) at 4° C. For the final 24 hours of primary antibody incubation, polyclonal goat anti-orexinA (OxA) [1:2,000; SC-8070, Santa Cruz Biotechnology, Inc.; Dallas, TX] was added.

Following primary antibody incubation, tissue sections were rinsed six times for 10 minutes each in cold PBS and thereafter incubated in secondary antibody solution (donkey anti-rabbit IgG 555 conjugate [Alexa Fluor (A-31572); Life Technologies; Eugene, OR] and donkey anti-goat IgG 488 conjugate [Alexa Fluor (A-11055); Life Technologies; Eugene, OR] in 1.5% normal donkey serum in PBS+) for 3 hours at room temperature. Tissue sections were then washed three times for 10 minutes each in cold PBS, mounted on SuperFrost Plus microscope slides (Fisher Scientific [12-550-15]), allowed to dry for 2 hours and coverslipped with ProLong Gold antifade mounting reagent (Molecular Probes, Life Technologies [P36930]; Eugene, OR). An experimenter blind to treatment conditions captured images using a Coolsnap EZ CCD camera (Photometrics; Tucson, AZ) connected to an Eclipse 80i fluorescent microscope (Nikon Instruments, Inc.; Melville, NY). Double-labeled ($\text{OxA}^-/\text{Fos}^-$) and single-labeled ($\text{OxA}^-/\text{Fos}^-$) cells in hypothalamus were counted, and % $\text{OxA}^+/\text{Fos}^+$ cells was used as a dependent measure for all analyses. No significant differences were detected between hemispheres, and thus the % $\text{OxA}^+/\text{Fos}^+$ dependent measure reflects an average of pooled sections between hemispheres for each subject.

2.4: Statistical analysis

For all behavioral and cellular analyses, one-way ANOVAs examining Treatment Group (i.e. Sal-Veh, Sal-Mec, Nic-Veh, Nic-Mec) were used. Final sample sizes for behavioral analyses ranged from $n=4-6$. Tissue sections with incomplete bilateral hypothalamus were omitted from analyses, and estimated cell counts were generated from tissue sample sizes of 3–10 per animal, omitting subjects with fewer viable bilateral tissue sections. *A priori* Bonferroni-corrected contrasts were conducted comparing experimental groups to the Sal-Veh control group, final sample sizes $n=4-6$, and familywise α was maintained at 0.05 for all analyses.

3.0: Results

3.1: Nicotinic receptor blockade induces somatic withdrawal events and anxiety-like behavior

A summary table of somatic withdrawal events by Treatment Group can be found in Table 1. A one-way ANOVA examining somatic withdrawal events revealed a significant main effect of Treatment Group [$F(3, 15) = 10.285, p < 0.01$]. Planned contrasts revealed significantly greater somatic withdrawal events in the Nic-Mec group compared to the Sal-Veh control group [$|t(8)| = 4.478, p < 0.01$]. Somatic withdrawal events from the Sal-Mec group was not significantly greater than the Sal-Veh control group [$|t(6)| = 1.904, \text{n.s.}$].

A one-way ANOVA examining locomotor activity by Treatment Group was not significant [$F(3, 15) = 1.526, \text{n.s.}$; Figure 1A]. However, a one-way ANOVA examining % time in center of open field arena, used as an index of anxiety, revealed a significant main effect of Treatment Group [$F(3, 15) = 3.858, p < 0.05$; Figure 1B]. Planned contrasts revealed a significant reduction in % time in center of open field arena in the Nic-Mec group compared to the Sal-Veh control group [$|t(8)| = 3.286, p < 0.05$].

3.2: Nicotinic receptor blockade decreases Fos-immunoreactivity within orexin neurons

A one-way ANOVA examining Fos-immunoreactive (ir) orexin cells within hypothalamus revealed a significant main effect of Treatment Group [$F(3, 15) = 8.032, p < 0.01$; Figure 2B]. Planned contrasts revealed reduced Fos-ir orexin cells from Sal-Mec animals compared to Sal-Veh control group [$t(6) = 2.703, p < 0.05$] and an enhanced reduction of Fos-ir orexin cells within hypothalamus from Nic-Mec animals compared to the Sal-Veh control group [$t(8) = 4.826, p < 0.001$]. A summary of cell counts for all groups is contained within Table 2.

4.0: Discussion

Results from the present study indicate that nicotinic receptor blockade via acute single-dose mecamylamine reduced Fos-immunoreactivity of orexin/hypocretin-expressing neurons of the hypothalamus in rats. Notably, mecamylamine-induced suppression of Fos-immunoreactivity was most prominent in rats administered with chronic nicotine. Behavioral studies revealed that mecamylamine generally decreased locomotor activity when rats were placed in a novel, open field arena. Further, rats that received chronic nicotine and acute mecamylamine showed a significant reduction in the % of time spent within the center compartment of an open field arena, indicating an increase in anxiety-like behavior. Collectively, these results support that hypothalamic orexin/hypocretin transmission may be involved in mediating the state experienced during nicotine withdrawal.

Previous studies found that acute nicotine increases Fos-immunoreactivity of hypothalamic orexin neurons [52–53]. In support that nicotine directly excites orexin neurons, high levels of excitatory nAChR mRNA have been observed through *in situ* hybridization within rodent [54–55] and of human post-mortem [56] hypothalamus. Recently, it was shown that orexin neurons increase spontaneous firing following application of nicotine or the endogenous nAChR ligand acetylcholine *in vitro* [57], suggesting that nAChR signaling directly drives orexinergic cell activity. It is well established through rodent and human imaging studies that chronic nicotine desensitizes and upregulates high-affinity nAChRs [e.g., 58–59], which has been suggested to be part of a compensatory mechanism to functionally decrease otherwise excessive nicotine-induced cellular excitability [e.g., 11–12]. Accordingly, nicotine or partial nAChR agonists function in part to restore physiological homeostasis and attenuate behavioral symptoms of nicotine withdrawal in smokers [14, 60–65; for reviews, 17 and 66]. Taken together, prior studies and results from the present study demonstrate that: (i) acute nicotine increases orexin activity in part through nAChRs on orexin neurons, (ii) chronic nicotine desensitizes and upregulates nAChRs, and (iii) nicotinic receptor blockade leads to decreased orexinergic activity in nicotine-exposed rats.

Interestingly, a significant effect of acute mecamylamine was found on reducing Fos-ir within orexinergic somata. Early work from Pasumarthi, Reznikov and Fadel (2006) found that acute mecamylamine attenuated Fos induction within orexinergic somata following an acute nicotine injection, however no effects of mecamylamine alone were observed. A separate line of work found that pretreatment with mecamylamine reduced nicotine-induced elevations of Fos-ir within medium spiny neurons of the NAcc [67]. These studies support a modulatory role of mecamylamine on nicotine-induced alterations in Fos-ir within multiple

neuronal populations. Thus, the finding that a relatively high-dose of mecamylamine has an effect on orexinergic activity is not altogether surprising. Importantly, both behavioral and molecular findings clearly indicate that mecamylamine elicits exacerbated effects following chronic nicotine administration.

Nicotine withdrawal produces physical and negative affective symptoms, including anxiety and depression-like behavior, and multiple transmitter systems have been implicated in producing withdrawal-associated behaviors that ultimately drive relapse. Results from the present study suggest that orexin signaling may contribute in part to the behavioral symptoms experienced during nicotine withdrawal as was observed upon blocking nicotinic receptors with mecamylamine. A preliminary correlation analysis associating % time in the center of an open field arena (i.e. anxiety index) and Fos-ir within orexinergic somata revealed a positive trend from the Nic-Mec group, in that increased anxiety-like behavior associated with reduced orexinergic Fos-ir ($r = 0.67$; not shown). It should be noted that a novel, open field arena can induce a stress response in rodents and perhaps exacerbate effects of an acute pharmacological agent on neuronal Fos-immunoreactivity. In the current design, all rats went through an identical procedure and effects on Fos-ir were only observed from mecamylamine-treated groups. Thus, it is likely that mecamylamine itself is sufficient to alter orexin neuronal activity, and that an additive effect occurs in subjects receiving chronic nicotine. However, the possibility that stress circuits interact with cholinergic transmission within hypothalamus and ultimately underlie Fosir alterations cannot be discounted. Prior reports reveal that mecamylamine does not produce negative affect in rats given chronic saline but does reduce brain reward function in nicotine-treated rats [49]. Thus, mecamylamine itself likely mediates a subset of but not all behaviors emerging from nicotinic receptor blockade as is commonly used to precipitate nicotine withdrawal in rodent models.

An alternative explanation is grounded in the fact that orexin neurons sparsely transmit orexin peptide alone. Orexin neurons co-transmit the inhibitory endogenous kappa opioid receptor (KOR) ligand dynorphin in ~95% of orexinergic somata [40, 68]. The role of endogenous KOR signaling has been discussed as an underlying mechanism in the emergence of affective distress during drug withdrawal, such as producing increased brain reward thresholds in electrically self-stimulating animals which is indicative of a deficit of reward function [48, 69; for review, 70]. Dynorphin peptide release becomes elevated following nicotine administration [71], and these alterations in central dynorphin parallel deficits in brain reward function during nicotine withdrawal [72; for review, 73]. Thus, based on evidence demonstrating co-transmission of orexin and dynorphin peptides, future studies should aim to uncover the opposing and/or synergistic role of these peptides in the underlying behavioral symptomatology of nicotine withdrawal.

Modulating orexin signaling has received attention in the context of treating substance use disorders [74; for review, 75]. Orexin antagonists have been shown to reduce motivation to self-administer nicotine, cocaine and heroin in rats [40; 76–77]. Some studies reveal a therapeutic role of selective OX1R antagonists in treating behavioral symptoms associated with drug withdrawal and in reducing stress-induced reinstatement of drug-seeking [e.g., 78–81]. Recently, OX1Rs were found to form complexes with receptors for corticotropin-

releasing hormone, and these complexes may constitute the mechanism whereby stress evokes drug-seeking behavior in rats with a history of drug dependence [82]. Thus, our finding that nicotinic receptor blockade produces significant reductions in orexin Fos-ir was surprising when compared against the aforementioned studies demonstrating a therapeutic role of orexin receptor antagonism in rodent models of drug addiction. As Fos-ir assesses pre-synaptic neuronal activity, future studies can be performed to more holistically assess pre- and post-synaptic events related to orexin transmission in models of drug withdrawal.

Interestingly, chronic nicotine administration has been shown to lead to a downregulation of OX₁Rs in rat hypothalamus, and this altered hypothalamic signaling has been suggested to underlie withdrawal-associated behavioral changes including altered appetite [83]. A large body of work has uncovered functional significance of orexinergic innervation of DAergic VTA neurons in regulating drug-seeking motivation for cocaine in rodent self-administration models [38–42, 46; for review, 75]. Thus, orexin signaling appears to be critical for modulating the motivation to retrieve salient/palatable reinforcers, such as high-fat foods or drugs of abuse, and that altered orexin signaling following chronic drug administration underlies the emergence of behavioral symptoms such as anhedonia and negative affect.

5.0: Conclusions

Results from the present study extend our understanding of the neurobiology of nicotine withdrawal. A functional role of orexin transmission can be ascribed to the motivational and reinforcing characteristics of nicotine. Likewise, prior research reveals that chronic nicotine leads to desensitization and upregulation of central nAChRs, and that removal of nicotine (i.e. during withdrawal) leads to altered cellular physiology of neuronal populations known to be innervated by cholinergic transmission. Results from the present study implicate hypoactivity of orexin neurons as potential mediators of withdrawal-associated behaviors as nicotinic receptor blockade evoked somatic signs, anxiety-like behavior and a concordant reduction of Fos-ir within orexin neurons of nicotine-exposed rats. Based on extensive co-transmission with the inhibitory peptide dynorphin, future studies should attempt to detect specific roles of each peptide on target structures from hypothalamic afferents in the pathophysiology of nicotine addiction. Finally, future studies should delineate orexinergic pathways affected by nicotine withdrawal to uncover sites for therapeutic intervention.

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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.

Figure 1.

(A) Mean velocity (cm/min) $\pm$ *S.E.M.* by Treatment Group across time. (B) Mean % time in center of open field arena $\pm$ *S.E.M.* by Treatment Group. * $p < 0.05$, ** $p < 0.01$ compared to Sal-Veh control group, $n=4-6$. (C) Representative tracks of Sal-Veh (green) and Nic-Mec (purple) animal recorded from open field arena and analyzed with Matlab program. Dashed-line indicates inner border of open field arena which was used to define center/surround for analysis in (B).

Figure 2.

(A) Atlas images of hypothalamic area used for immunofluorescent analyses adapted from [51]. (B) Vertical dot plot of individual data and mean % OxA⁺/Fos⁺ cells in hypothalamus by Treatment Group, n=4–6. (C) Representative photomicrographs of immunofluorescent analyses by Treatment Group. Green signal indicates orexin-A immunoreactivity, and red coloring indicates Fos immunoreactivity. Shaded region represents hypothalamic area containing orexin neurons that was included in analysis. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to Sal-Veh control group. IIIv—third ventricle, BLA—basolateral nucleus of the amygdala, fx—columns of the fornix, mtt—mammillothalamic tract, opt—optic tract, Thal—thalamus, tp—thalamic peduncle, ZI—zona incerta.

Table 1

Summary table of mean values from assessment of somatic withdrawal events by Treatment Group $\pm$ *S.E.M.* The last column, Global Withdrawal Score, is shaded grey and was used for analyses. * $p < 0.05$, ** $p < 0.01$ compared to Sal-Veh control group. Finals N's=4–6.

Treatment Group	Somatic Withdrawal Events					
	Teeth Chattering, Chews	Tail Writhe/Wags and Gasps	Shakes and Tremors	Ptois	Misc.	Global Withdrawal Score
Sal-Veh	1.00 $\pm$ 0.58	0.25 $\pm$ 0.25	1.00 $\pm$ 1.00	0.00 $\pm$ 0.00	1.00 $\pm$ 0.41	3.25 $\pm$ 2.02
Sal-Mec	1.25 $\pm$ 0.63	0.25 $\pm$ 0.25	0.75 $\pm$ 0.75	4.50 $\pm$ 0.87	2.75 $\pm$ 1.80	9.50 $\pm$ 3.97
Nic-Veh	0.00 $\pm$ 0.00	0.60 $\pm$ 0.40	0.80 $\pm$ 0.49	0.80 $\pm$ 0.80	0.80 $\pm$ 0.37	3.00 $\pm$ 0.71
Nic-Mec	1.17 $\pm$ 0.65	5.67 $\pm$ 1.50	0.50 $\pm$ 0.22	7.00 $\pm$ 0.52	2.33 $\pm$ 0.49	16.67 $\pm$ 1.58**

Table 2

Summary table of estimated cell counts ($\text{Fos}^-/\text{OxA}^+$, $\text{Fos}^-/\text{OxA}^+$, total OxA^+).

Treatment Group	Estimated Cell Counts		
	$\text{Fos}^+/\text{OxA}^+$	$\text{Fos}^-/\text{OxA}^+$	Total OxA^+
Sal-Veh	703 ± 93	559 ± 101	1261 ± 193
Sal-Mec	332 ± 31	539 ± 91	871 ± 88
Nic-Veh	463 ± 43	607 ± 84	1070 ± 102
Nic-Mec	353 ± 84	790 ± 67	1143 ± 132