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Lasting synaptic changes underlie attention deficits caused by nicotine exposure during adolescence

Danielle S Counotte[1], Natalia A Goriounova[2], Ka Wan Li[1], Maarten Loos[1], Roel C van der Schors[1], Dustin Schetters[3], Anton NM Schoffelmeer[3], August B Smit[1], Huibert D Mansvelder[2*], Tommy Pattij[3*], Sabine Spijker[1*§]

[1]Molecular and Cellular Neurobiology, [2]Integrative Neurophysiology, Center for Neurogenomics & Cognitive Research (CNCR), VU University, and [3]Anatomy and Neurosciences, VU University medical center, Neuroscience Campus, Amsterdam, The Netherlands.

[*]Authors contributed equally
[§]Correspondence should be addressed to SS (sabine.spijker@cncr.vu.nl).

Abstract

Tobacco smoking and nicotine exposure during adolescence interfere with prefrontal cortex (PFC) development and lead to cognitive impairments in later life. The molecular and cellular underpinning of these consequences remains elusive. We find that adolescent nicotine exposure induces lasting attentional disturbances and reduces mGluR2 protein and function on presynaptic terminals of PFC glutamatergic synapses. Restoring mGluR2 activity in vivo by local infusion of a group II mGluR agonist in adult rats that received adolescent nicotine rescued attentional disturbances.

Increased risk-taking and reckless behavior of adolescents has been linked to late development of brain areas involved in executive cognitive functioning such as the prefrontal cortex (PFC)[1], which shows dynamic changes in grey and white matter proceeding late into adolescence. Adolescence also marks a period of increased vulnerability to initiation and subsequent abuse of drugs including tobacco smoking[2]. Nicotine exposure during adolescence interferes with PFC development[3] and has long-lasting consequences for cognitive performance[4-6]. In line with human epidemiological data, nicotine exposure in adolescent rats has long-term cognitive consequences. Indeed, most adult smokers start their habit before the age of 19 years, and more than 70% of adolescents report to have tried a cigarette at least once[7]. Nicotine, acting on nicotinic acetylcholine receptors, hampers PFC function and may interfere with PFC maturation, altering structure and function that persist into adulthood. In adolescent smokers, PFC activity, working-memory and attention are reduced[4, 5]. In later life, behavioral disturbances and mental health problems are strongly correlated with adolescent nicotine use[6]. What mechanisms underlie these long-term consequences of nicotine exposure during adolescence is unclear.

To uncover molecular and cellular mechanisms underlying long-term cognitive disturbances resulting from adolescent nicotine exposure, we exposed adolescent rats to nicotine and assessed visuospatial attention, protein expression and synaptic physiology in PFC during adulthood. Between postnatal day (PND) 28 and 50, rats show typical adolescent-like behaviors, such as peer-directed and risk-taking behavior and altered sensitivity to drugs of abuse[8]. Similar to findings from human epidemiological data, adolescent nicotine exposure in rats has long-term effects on attentional processing[9]. The 5-choice serial reaction time task (5-CSRTT) is the most widely employed translational paradigm that has tremendously contributed to our understanding of the neural correlates of divided and sustained attention and impulsive action[10, 11]. Nicotine treatment during adolescence (PND 34–43) increased impulsive behavior ($p=0.042$), and impaired measures of attention (7%, $p=0.015$) in adulthood following five weeks of abstinence[9] (**Fig. 1a**). The latter is comparable to the decrease (3–4%) in visuospatial attention observed in male adolescent smokers[4, 5]. Increasing attentional load augmented the difference between these groups (**Supplementary Fig.**

1). In contrast, adult nicotine exposure (PND 60-69) did not have these long-term consequences. Nicotine treatment had no effect on locomotor behavior, body weight, learning or motivation (**Supplementary Figs. 2** and **3**). Thus, in rats nicotine exposure during adolescence has profound impact specifically on attention and impulsive behavior later in life.

Visuospatial attention in the 5-CSRTT strongly depends on the integrity of the medial PFC (mPFC)[12]. Synaptic connectivity and dynamics of neuronal interactions in mPFC underlie attentional processing. To investigate whether long-term molecular changes were induced in synaptic connectivity in mPFC by adolescent nicotine exposure, we performed iTRAQ-based quantitative proteomics of synaptic membrane fractions of rat mPFC. Synaptic protein levels from animals exposed to either nicotine or saline during adolescence (PND 34–43) were quantified one day after nicotine exposure ended (PND 44), as well as after five weeks of abstinence (PND 78). From 297 unique proteins reliably quantified at these two time points (**Supplementary Fig. 4**), nine synaptic proteins were significantly affected by age and pretreatment (**Supplementary Table 1**), indicating that adolescent nicotine exposure altered the developmental expression profile of these proteins. Post-hoc analyses revealed three significantly regulated proteins five weeks after nicotine pre-treatment in adolescents (PDN78) of which only mGluR2 could be confirmed by immunoblotting (p=0.048; **Fig. 1b** and **Supplementary Fig. 4**). In contrast, nicotine exposure during adulthood (PND 60–69) did not alter synaptic protein levels of mGluR2, neither after one day, nor after five weeks of abstinence. These findings show that in mPFC, synaptic metabotropic glutamate receptor protein levels are altered specifically by adolescent and not adult nicotine exposure. We next studied functional consequences of down-regulation of synaptic mGluR2 protein levels after adolescent nicotine exposure for mPFC glutamatergic synaptic function in adulthood. Activation of mGluR2s in developing somatosensory cortex suppresses glutamatergic transmission[13]. Similarly, in adult mPFC, activation of mGluRs by the group II agonist LY379268 (5 μM) strongly reduced extracellular evoked excitatory postsynaptic currents (eEPSCs) in whole-cell recordings from mPFC layer V pyramidal neurons (**Fig. 1c**). These mGluR2 receptors were most likely localized on presynaptic glutamatergic terminals, since LY379268 increased paired-pulse ratio of eEPSCs and reduced miniature EPSC amplitudes recorded in the presence of TTX (1 μM) (**Supplementary Fig. 6**). In pyramidal neurons from PND 78 animals, five weeks after adolescent nicotine exposure, mGluR2/3 activation reduced eEPSC amplitudes to a lesser extent than in those from saline-treated animals (p=0.013) (**Fig. 1c,d**). Baseline EPSCs were unaltered by nicotine exposure (**Supplementary Fig. 7**). The reduced mGluR-mediated synaptic inhibition in nicotine exposed animals (**Fig. 1c-d**; p=0.0006, **Supplementary Fig. 7b**) is in line with reduced synaptic mGluR2 protein expression in the mPFC of these animals (**Fig. 1b**). In adult nicotine-treated animals, eEPSC amplitude reduction by LY379268 was not different from their saline-treated controls, in line with unaltered synaptic mGluR2 protein levels.

Synaptic group II mGluRs have been implicated in short-term plasticity of glutamatergic synapses in cortical areas, and short-term depression and facilitation strongly shape information transfer in cortical networks. We tested whether reduction in synaptic mGluR2 levels following adolescent nicotine exposure affected short-term plasticity of glutamatergic synapses in adult mPFC (**Fig. 2**). Five weeks abstinence following adolescent nicotine exposure resulted in less depression of mPFC glutamatergic synapses compared with their saline-treated controls (**Fig. 2a,b**; $p<0.001$). This corroborated the observed reduced synaptic mGluR2 protein levels in adolescent nicotine-treated animals. Nicotine exposure during adulthood (PND 60-69) did not reduce synaptic depression (PND 104), but instead resulted in a slight increase of depression at short intervals (**Fig. 2b**; $p=0.033$). The group II mGluR antagonist MPPG (100-200 μM) reduced short-term depression ((**Fig. 2c**; $p=0.012$). The group II mGluR agonist LY379268 strongly reduced the amplitude of the first eEPSC in the train by 80% ($p<0.001$; **Supplementary Fig. 8**), confirming that mGluR2s are involved in synaptic depression at these synapses. Thus, adolescent nicotine exposure resulted in reduced synaptic mGluR2 signaling and short-term plasticity on mPFC output layer V pyramidal neurons after five weeks of abstinence, and may explain reduced mPFC functioning and disturbances in attention.

To test whether mGluR2 signaling in the mPFC is involved in attention, we infused the type II antagonist MPPG into the mPFC of control animals. Decreasing mGluR2 activity in these animals selectively reduced attentional performance (**Fig. 3a**; $p=0.018$; **Supplementary Table 2**), showing the importance of mPFC mGluR2 function in attention. Next, to determine whether reduced synaptic mGluR2 protein levels in the mPFC can indeed explain the observed decrements in attention after adolescent nicotine exposure, we increased mGluR2 activity *in vivo* by infusing LY379268 into the mPFC during behavioral testing (**Fig. 3b,d**). Adolescent nicotine exposure reduced attention (*cf.* **Fig. 1**, **Supplementary Fig. 9, 10** and **Supplementary Table 3**), and LY379268 selectively improved attention in nicotine-treated animals in a dose-dependent manner (**Fig. 3b**; $p=0.033$). This indicates that augmenting mGluR2 activity in adult animals previously exposed to nicotine during adolescence restores attention performance. Infusion of LY379268 in mPFC did not ameliorate impulsivity in these animals (**Fig. 3c**, **Supplementary Table 4**). In saline-treated animals, LY379268 did not affect attention performance (**Fig. 3c)**, but increased impulsivity (**Fig. 3d**; $p=0.018$) and MPPG did not affect impulsivity (**Fig. 3c)**, indicating that mGluR2 activity affects attention and impulsivity differentially and emphasizing that these behavioral domains are under differential control by the mPFC[11,14].

This study uncovers molecular and cellular changes induced by adolescent nicotine exposure that result in cognitive disturbances in adult life and reveals that a lasting down-regulation of mGluR2 on presynaptic terminals of glutamatergic synapses in PFC persists into adulthood causing disturbances in attention. Decreased mGluR2 functionality reduced short-term plasticity of

glutamatergic inputs to PFC output layer V pyramidal neurons and thereby most likely alters information transfer in active networks underlying attention[15]. Restoring mGluR2 activity *in vivo* in the PFC of adult animals exposed to nicotine during adolescence remediated the attention deficit.

Our findings stress that nicotine impacts the brain in different ways during adolescence. Although nicotine has acute behavioral effects on attention in adult rats[10], the adult mPFC does not seem to suffer from lasting consequences from nicotine exposure. Cognitive performance, synaptic mGluR2 protein levels and glutamatergic synaptic depression were all unaffected by nicotine exposure during adulthood. Thus, this clearly pinpoints adolescence as a period of increased vulnerability for the effects of nicotine. Not only from a behavioral, but also from a molecular point of view, the adolescent brain is more susceptible to consequences of nicotinic receptor activation. What sequence of molecular events ties nicotine exposure to altered synaptic mGluR2 protein levels, without increasing mGluR2 gene expression (data not shown), is as yet unknown. Nicotine easily penetrates the blood-brain barrier at concentrations experienced by smokers and readily binds to nAChRs in PFC to enhance glutamatergic and GABAergic synaptic transmission. Perhaps elevated mGluR2 levels following nicotine exposure at the end of adolescence compensate for nicotine's actions and inhibit neurotransmitter release. Regardless, altered glutamatergic synaptic transmission in mPFC plays a key role in attention and impulsivity and altered mGluR2 levels have been linked to several brain disorders. The sustained molecular and synaptic changes that result from nicotine exposure during adolescence and alter cognitive performance during adulthood may prompt us to reconsider our views on the aetiology of attention deficits.

Supplementary Information is linked to the online version of the paper.

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Author contributions

DSC and NAG contributed equally to the experiments in this paper. DSC, KWL, ABS, SS designed the molecular experiments. NAG, HDM designed the physiological experiments. DSC, ANMS, SS, TP, designed the behavioral experiments. DSC, RCvdS executed the molecular experiments. NAG executed physiological experiments. DSC, DS executed behavioral experiments. DSC, ML, SS analyzed molecular experiments. NAG, HDM analyzed physiological

experiments. DSC, TP analyzed behavioral experiments. DSC, HDM, TP, SS wrote the manuscript.

Figure legends

Figure 1 Adolescent nicotine exposure affects measures of attentional performance, mGluR2 levels and function on the long term. (**a**) Visuospatial divided and sustained attention (accuracy; left) indicated by percentage of correct stimulus detections (average 5 baseline sessions, see methods), and impulsive behavior indicated by number of prematurely expressed responses before stimulus onset (right) measured 5 weeks after adolescent (n=11) or adult (n=11) nicotine exposure. (**b**) Quantification of immunoblot analysis of synaptic mGluR2 expression (n=8). (**c**) Time course of eEPSC amplitude reduction by LY379268 in adolescent nicotine (black, n=25) or saline exposed animals (gray, n=24), per cell each data point is an average of 7 eEPSCs. Insets: example eEPSC traces in control (a) and in presence of LY379268 (b). Right panel: average of last 10 responses in presence of LY379268. (**d**) mGluR2/3-dependent inhibition of eEPSC amplitudes is different due to adolescent nicotine treatment (saline, n=39; nicotine, n=47, $F(3,128)=6.2$; $p=0.0006$), with no difference in adult treated animals (saline, n=22; nicotine, n=26). Mean±SEM is presented. $^{**}p<0.01$, $^{*}p<0.05$. All experiments were approved (ethical committee VU University Amsterdam).

Figure 2 Short-term depression in mPFC is reduced 5 weeks after nicotine exposure during adolescence. (**a**,**d**) Example of short-term plasticity recorded from a layer V pyramidal neuron after extracellular stimulation in layer II/III of adolescent nicotine- or saline-exposed animals. (**b**) Summary of short-term depression during 10 stimuli (25–200 ms intervals), measured in adolescent-treated (left panel, $F_{(1,137)}=21.6$ $p<0.001$, saline n=10-26, nicotine n=10-23) and adult-treated animals (right panel $F_{(1,255)}=4.57$ $p=0.033$; saline n=24, nicotine n=31). Average of last 3 responses in the train was normalized to the first one for analysis (**c**) mGluR group II/III antagonist MPPG reduces short-term plasticity in mPFC layer V pyramidal neurons ($F_{(2,124)}=7.03$ $p=0.012$; control n=11, MPPG 100 µM n=10-11, MPPG 200 µM n=6). Mean±SEM is presented.

Figure 3 Intra-mPFC infusion of mGluR2/3 agonist LY379268 reverses long-term attentional disturbances in adolescent nicotine-exposed animals. (**a**) Infusion of the group II antagonist MPPG decreased divided and sustained attention (accuracy) in control animals. (**b**) LY379268 normalized the nicotine-induced disturbances in divided and sustained attention (accuracy) in adolescent nicotine-exposed animals (dose $F_{(1,10)}=4.08$, $p=0.033$), with no effect on adolescent saline-exposed animals (dose $F_{(1,10)}=1.71$, n.s.). (**c,d**) Impulsive behavior was not affected in control animals by MPPG (c), nor in adolescent nicotine-exposed animals by LY379268, but was increased in saline-exposed animals (dose $F_{(1,10)}=4.98$, $p=0.018$). Mean±SEM is presented. $^{*}p<0.05$.

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Supplementary Information Titles

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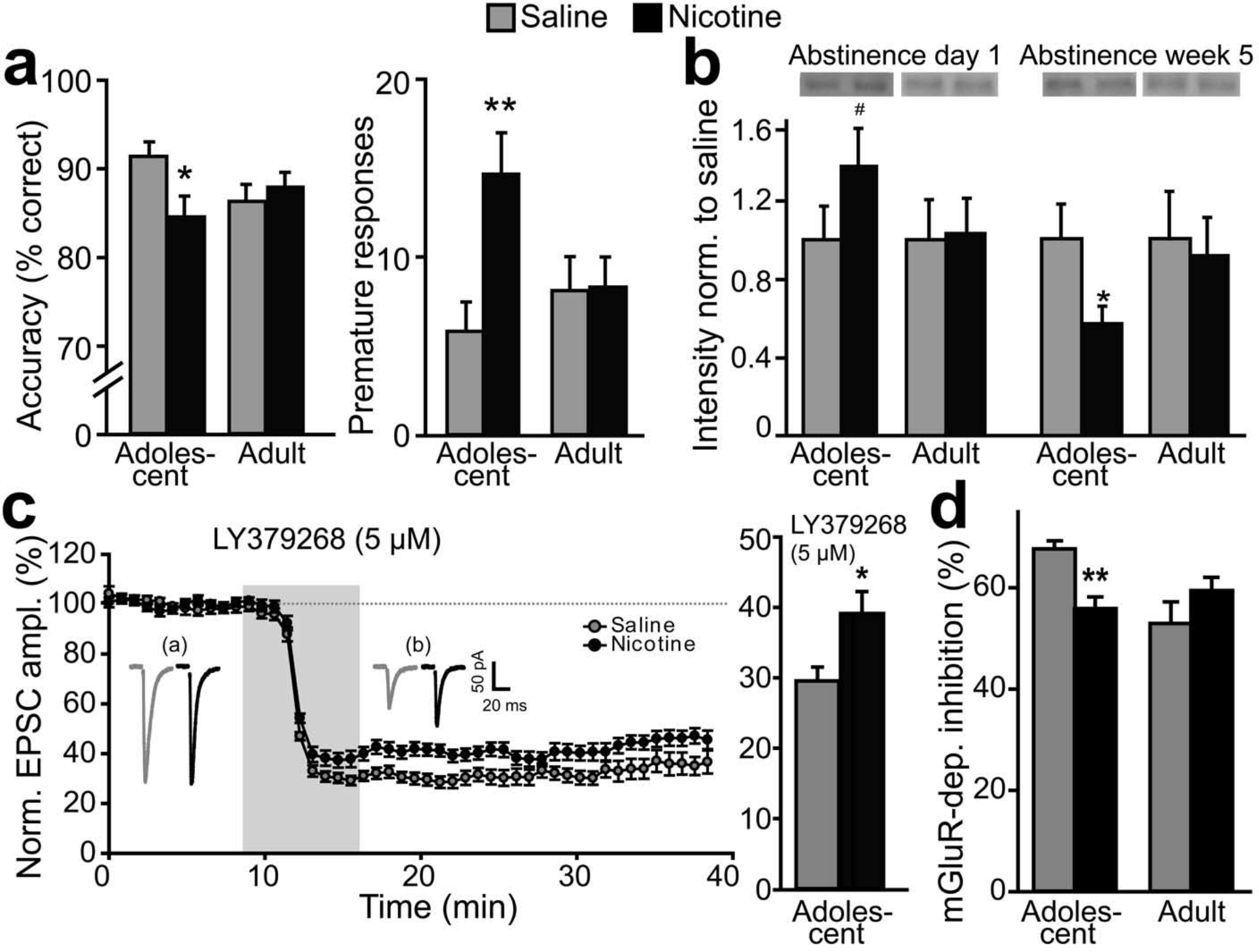
Journal: Nature Neuroscience

Article Title:	Lasting synaptic changes underlie attention deficits caused by nicotine exposure during adolescence
Corresponding Author:	Sabine Spijker

Supplementary Item & Number **(add rows as necessary)**	Title or Caption
Supplementary Figure 1	Manipulation of task parameters in the 5-CSRTT following adult and adolescent nicotine exposure.
Supplementary Figure 2	Locomotor activity and bodyweight are not differentially affected by adolescent nicotine exposure.
Supplementary Figure 3	No learning defects in 5-CSRTT acquisition due to adolescent nicotine exposure.
Supplementary Figure 4	iTRAQ proteomics and immunoblotting for adolescent nicotine-induced changes.
Supplementary Figure 5	Adolescent nicotine exposure does not alter AMPA-R/NMDA-R current ratios.
Supplementary Figure 6	mGluR2 modulates EPSCs through a presynaptic action.
Supplementary Figure 7	Nicotine-exposure during adolescence does not alter baseline EPSCs in adult animals.
Supplementary Figure 8	Activation of mGluR2/3 by agonist LY379268 (5 µM) reduces short-term plasticity in pyramidal neurons in layer V of the mPFC.
Supplementary Figure 9	Performance in the 5-CSRTT over five baseline sessions before and after cannulae implantation.
Supplementary Figure 10	Performance in the 5-CSRTT before, during and after intracranial infusions of LY379268.
Supplementary Table 1	Protein changes following one day and five weeks of adolescent nicotine exposure.
Supplementary Table 2	Behavioral parameters in the 5-CSRTT after intracranial infusion of 0.5 and 2.0 nmol/side MPPG in the mPFC.
Supplementary Table 3	Behavioral parameters in the 5-CSRTT averaged over 5 baseline sessions.
Supplementary Table 4	Behavioral parameters in the 5-CSRTT after intracranial infusion of LY379268 in the mPFC.
Supplementary Results & Discussion, Methods, and References	[NO DESCRIPTIVE TITLE NECESSARY]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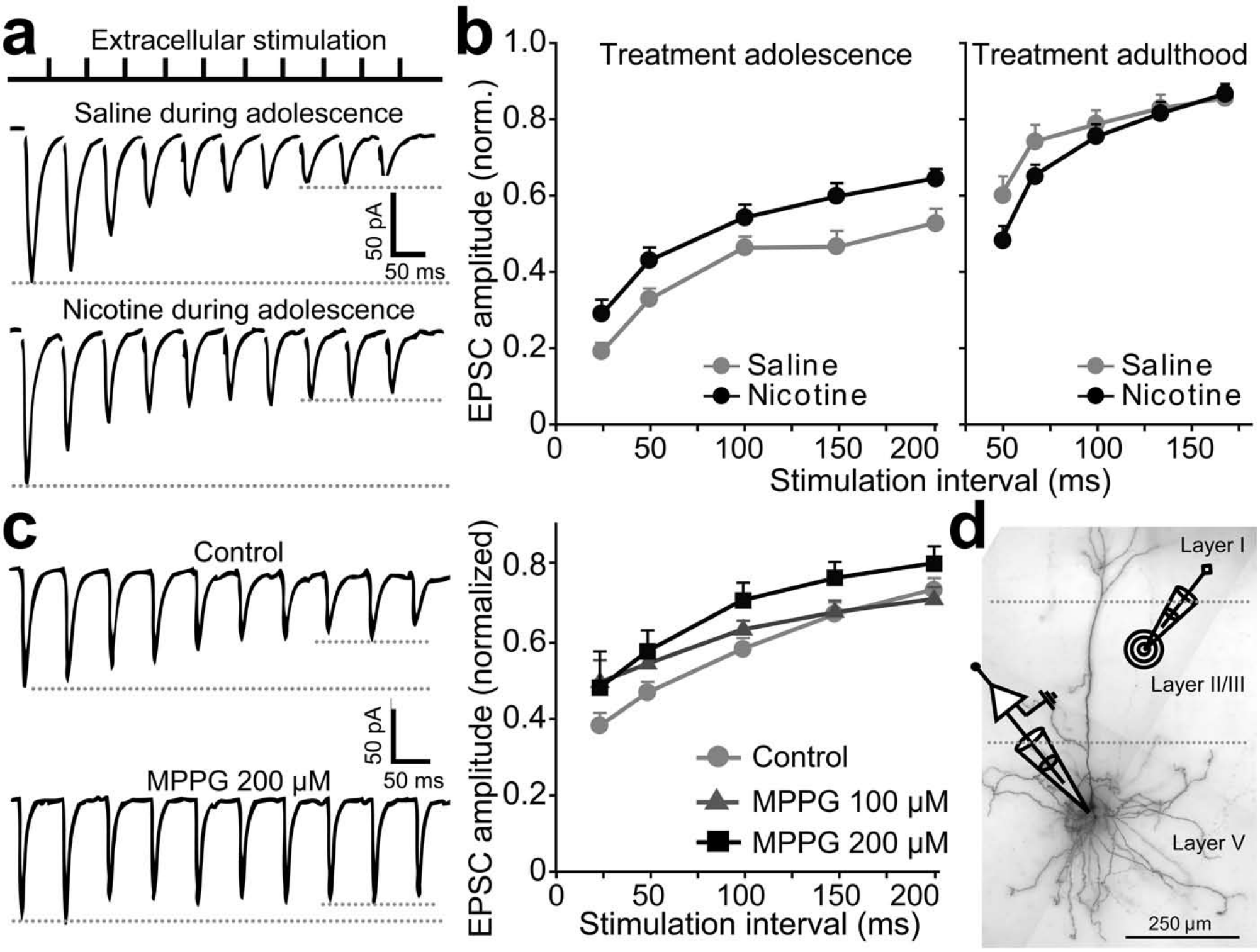
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Control
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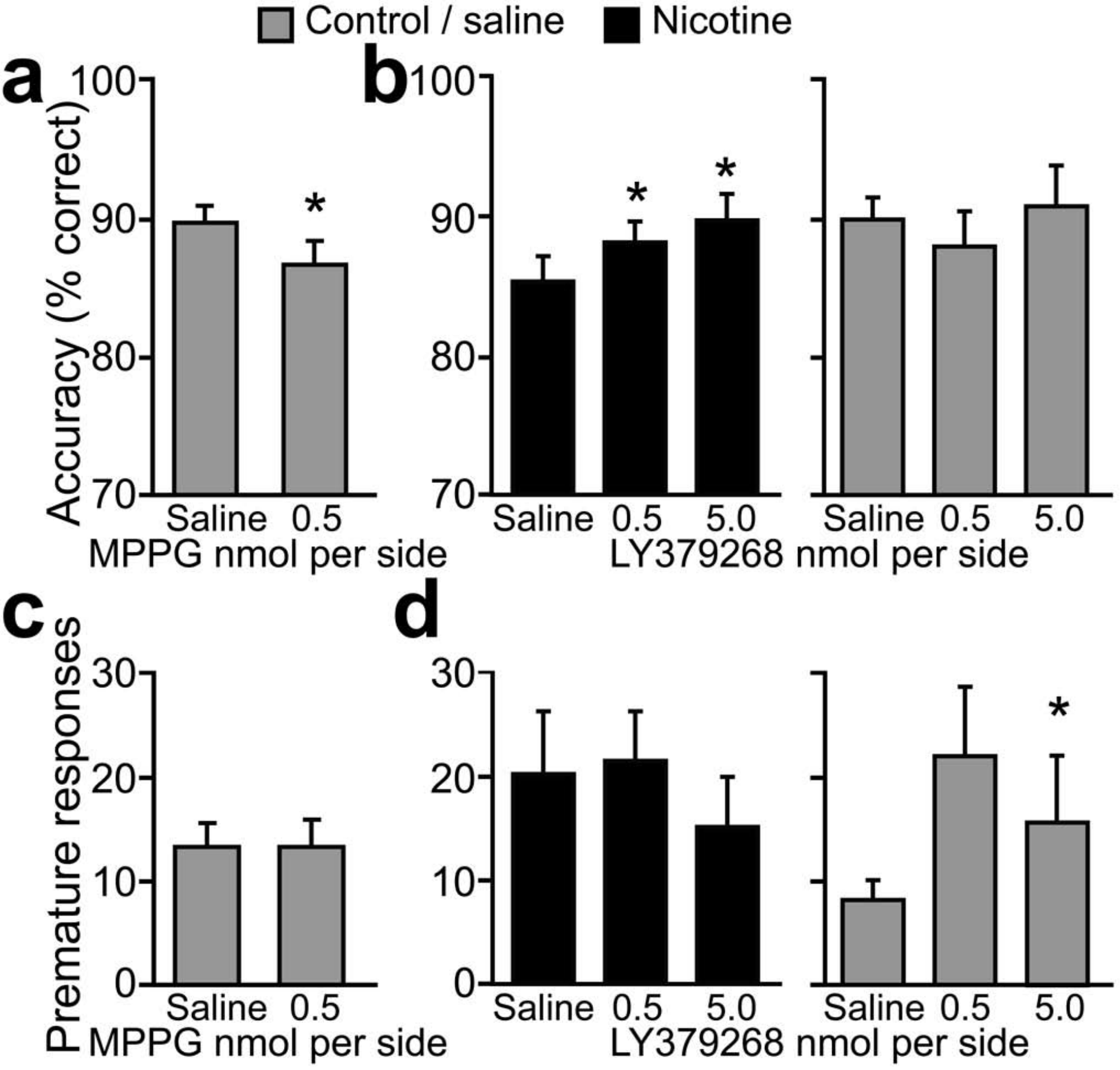
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