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

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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 underpinnings of these consequences remain elusive. We found that adolescent nicotine exposure induced lasting attentional disturbances and reduced 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 nicotine as adolescents 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, including the PFC[1], which shows dynamic changes in gray 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]. Consistent 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 that they have tried a cigarette at least once[7]. Nicotine, acting on nicotinic acetylcholine receptors, hampers PFC function and may interfere with PFC maturation, inducing alterations in 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]. The mechanisms that underlie these long-term consequences of nicotine exposure during adolescence remain unclear.

To determine the 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 28 and 50 (P28 and P50), 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 procedure and has tremendously contributed to our understanding of the neural correlates of divided and sustained attention and impulsive action[10,11]. Nicotine treatment during adolescence (P34–43) increased impulsive behavior ($P = 0.042$) and impaired measures of attention (7%, $P = 0.015$) in adulthood after 5 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 Results**, **Supplementary Discussion** and **Supplementary Fig. 1**). In contrast, adult nicotine exposure (P60–69) 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, nicotine exposure in rats during adolescence has a profound effect 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 isobaric tag for relative and absolute quantification—based quantitative proteomics of synaptic membrane fractions of rat mPFC. Synaptic protein levels from animals exposed to either nicotine or saline during adolescence (P34–43) were quantified 1 d after nicotine exposure ended (P44), as well as after 5 weeks of abstinence (P78). From 297 unique proteins reliably quantified at these two time points (**Supplementary Fig. 4**), nine synaptic proteins were significantly affected by age and pretreatment ($P < 0.05$; **Supplementary Table 1**), indicating that adolescent nicotine exposure altered the developmental expression profile of these proteins. *Post hoc* analyses revealed three significantly regulated proteins 5 weeks after nicotine pre-treatment in adolescents (P78) of which only mGluR2 could be confirmed by immunoblotting ($P = 0.048$; **Fig. 1b** and **Supplementary Figs. 4** and **5**). In contrast, nicotine exposure during adulthood (P60–69) did not alter synaptic protein levels of mGluR2 after 1 d or after 5 weeks of abstinence. These findings indicate that synaptic metabotropic glutamate receptor protein levels in mPFC are altered specifically by adolescent and not adult nicotine exposure. We next studied the functional consequences of downregulation of synaptic mGluR2 protein levels after adolescent nicotine exposure for mPFC glutamatergic synaptic function in

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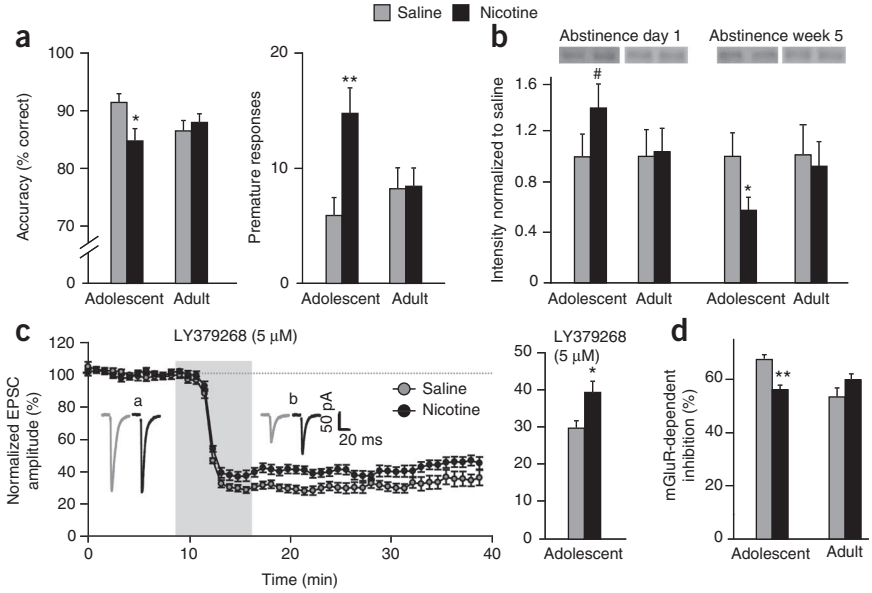

Figure 1 Adolescent nicotine exposure affects measures of attentional performance, mGluR2 levels and long-term function on the long term. (**a**) Visuospatial divided and sustained attention is (accuracy, left) indicated by the percentage of correct stimulus detections (average of five baseline sessions; **Supplementary Methods**) and impulsive behavior is indicated by the number of prematurely expressed responses before stimulus onset (right) measured 5 weeks after adolescent ($n = 11$) or adult ($n = 11$) nicotine exposure. $^{*}P < 0.05$, $^{**}P < 0.01$. (**b**) Quantification of immunoblot analysis of synaptic mGluR2 expression ($n = 8$). $^{\#}P < 0.1$. (**c**) Time course of eEPSC amplitude reduction by LY379268 in adolescent nicotine- (black, $n = 25$) or saline-exposed rats (gray, $n = 24$); each data point is an average of seven eEPSCs. Insets, example eEPSC traces in control (a) and in the presence of LY379268 (b). Right, average of the last ten responses in the presence of LY379268. (**d**) mGluR2/3-dependent inhibition of eEPSC amplitudes was different as a result of adolescent nicotine treatment (saline, $n = 39$; nicotine, $n = 47$; $F_{3,128} = 6.2$, $P = 0.0006$), with no difference in rats treated as adults (saline, $n = 22$; nicotine, $n = 26$). Data represent mean ± s.e.m. All experiments were approved by the ethics committee of VU University Amsterdam.

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, as LY379268 increased the paired-pulse ratio of eEPSCs and reduced miniature EPSC amplitudes recorded in the presence of tetrodotoxin (1 μM) (**Supplementary Fig. 6**). In pyramidal neurons from P78 rats, 5 weeks after adolescent nicotine exposure, mGluR2/3 activation reduced eEPSC amplitudes to a lesser extent than in those from saline-treated rats ($P = 0.013$; **Fig. 1c**,**d**). Baseline EPSCs were unaltered by nicotine exposure (**Supplementary Fig. 7**). The reduced mGluR-mediated synaptic inhibition that we observed in nicotine-exposed animals ($P = 0.0006$; **Fig. 1c**,**d** and **Supplementary Fig. 7b**) is consistent with the reduced synaptic mGluR2 protein expression in the mPFC of these animals (**Fig. 1b**). In adult nicotine-treated rats, eEPSC amplitude reduction by LY379268 was not different from their saline-treated controls, consistent with the 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 of abstinence following adolescent nicotine exposure resulted in less depression of mPFC glutamatergic synapses than was seen in the saline-treated controls ($P < 0.001$; **Fig. 2a**,**b**). This corroborated the observed reduced synaptic mGluR2 protein levels in adolescent nicotine-treated animals. Nicotine exposure during adulthood (P60–69) did not reduce synaptic depression (P104), but instead resulted in a slight increase of depression at short intervals ($P = 0.033$; **Fig. 2b**). The group II mGluR antagonist MPPG (100–200 μM) reduced

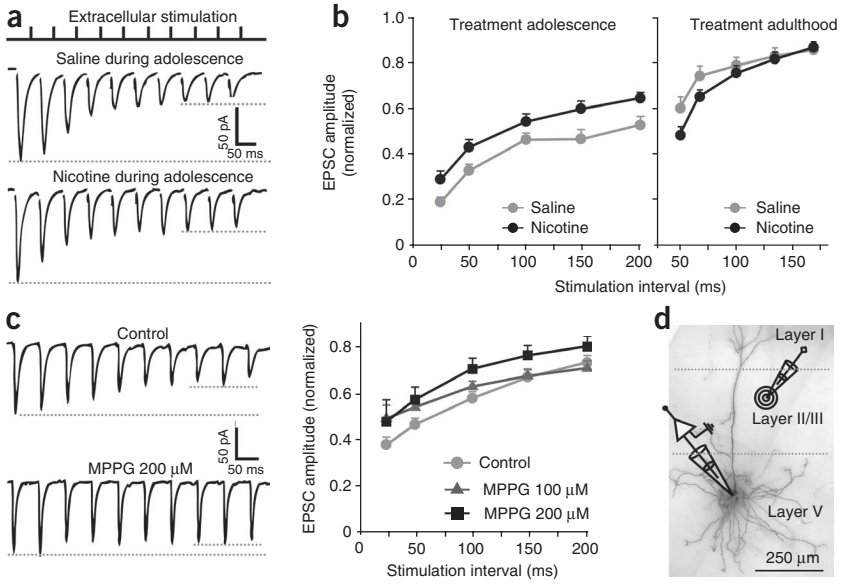

Figure 2 Short-term depression in mPFC is reduced 5 weeks after nicotine exposure during adolescence. (**a**,**d**) Example of short-term plasticity recorded from a layer V pyramidal neuron after extracellular stimulation in layer II/III of adolescent nicotine- or saline-exposed animals. (**b**) Summary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

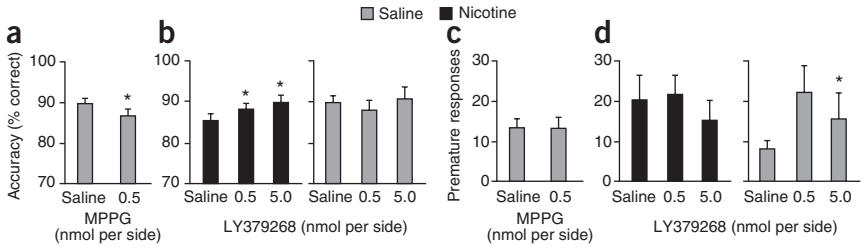

Figure 3 Intra-mPFC infusion of mGluR2/3 agonist LY379268 reverses long-term attentional disturbances in rats exposed to nicotine as adolescents. (**a**) Infusion of the group II antagonist MPPG decreased divided and sustained attention (accuracy) in control animals. $^*P < 0.05$. (**b**) LY379268 normalized the nicotine-induced disturbances in divided and sustained attention (accuracy) in rats exposed to nicotine as adolescents (dose, $F_{1,10} = 4.08$, $P = 0.033$), with no effect on rats exposed to saline as adolescents. (**c**,**d**) Impulsive behavior was not affected in control rats by MPPG (**c**) or in rats exposed to nicotine as adolescents by LY379268 (**d**), but was increased in saline-exposed rats (dose, $F_{1,10} = 4.98$, $P = 0.018$). Data represent mean ± s.e.m.

short-term depression ($P = 0.012$; **Fig. 2c**). 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 results in reduced synaptic mGluR2 signaling and short-term plasticity on mPFC output layer V pyramidal neurons after 5 weeks of abstinence, which may explain the 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 rats (**Fig. 3**). Decreasing mGluR2 activity in these animals selectively reduced attentional performance ($P = 0.018$; **Fig. 3a** and **Supplementary Table 2**), indicating that mGluR2 in mPFC is important for 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 (**Fig. 1**, **Supplementary Figs. 9** and **10** and **Supplementary Table 3**) and LY379268 selectively improved attention in nicotine-treated animals in a dose-dependent manner ($P = 0.033$; **Fig. 3b**). 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** and **Supplementary Table 4**). In saline-treated rats, LY379268 did not affect attention performance (**Fig. 3c**), but it did increase impulsivity ($P = 0.018$; **Fig. 3d**), 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].

Our results uncover molecular and cellular changes induced by adolescent nicotine exposure that result in cognitive disturbances in adult life and reveals that a lasting downregulation 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 altered information transfer in active networks underlying attention[15]. Restoring mGluR2 activity *in vivo* in the PFC of adult rats exposed to nicotine during adolescence remediated the attention deficit.

Our findings stress that nicotine affects 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. 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. The sequence of molecular events that ties nicotine exposure to altered synaptic mGluR2 protein levels, without increasing mGluR2 gene expression (data not shown), remains unknown. Nicotine easily penetrates the blood-brain barrier at concentrations experienced by smokers and readily binds to nicotinic acetylcholine receptors 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 is important for 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 etiology of attention deficits.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

D.S.C. and N.A.G. contributed equally to the experiments in this paper. D.S.C., K.W.L., A.B.S. and S.S. designed the molecular experiments. N.A.G. and H.D.M. designed the physiological experiments. D.S.C., A.N.M.S., S.S. and T.P. designed the behavioral experiments. D.S.C. and R.C.v.d.S. executed the molecular experiments. N.A.G. executed physiological experiments. D.S.C. and D.S. executed behavioral experiments. D.S.C., M.L. and S.S. analyzed molecular experiments. N.A.G. and H.D.M. analyzed physiological experiments. D.S.C. and T.P. analyzed behavioral experiments. D.S.C., H.D.M., T.P. and S.S. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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