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ORIGINAL ARTICLE

Prenatal nicotine is associated with reduced AMPA and NMDA receptor-mediated rises in calcium within the laterodorsal tegmentum: a pontine nucleus involved in addiction processes

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Despite huge efforts from public sectors to educate society as to the deleterious physiological consequences of smoking while pregnant, 12–25% of all babies worldwide are born to mothers who smoked during their pregnancies. Chief among the negative legacies bestowed to the exposed individual is an enhanced proclivity postnatally to addict to drugs of abuse, which suggests that the drug exposure during gestation changed the developing brain in such a way that biased it towards addiction. Glutamate signalling has been shown to be altered by prenatal nicotine exposure (PNE) and glutamate is the major excitatory neurotransmitter within the laterodorsal tegmental nucleus (LDT), which is a brainstem region importantly involved in responding to motivational stimuli and critical in development of drug addiction-associated behaviours, however, it is unknown whether PNE alters glutamate signalling within this nucleus. Accordingly, we used calcium imaging, to evaluate AMPA and NMDA receptor-mediated calcium responses in LDT brain slices from control and PNE mice. We also investigated whether the positive AMPA receptor modulator cyclothiazide (CYZ) had differential actions on calcium in the LDT following PNE. Our data indicated that PNE significantly decreased AMPA receptor-mediated calcium responses, and altered the neuronal calcium response to consecutive NMDA applications within the LDT. Furthermore, CYZ strongly potentiated AMPA-induced responses, however, this action was significantly reduced in the LDT of PNE mice when compared with enhancements in responses in control LDT cells. Immunohistochemical processing confirmed that calcium imaging recordings were obtained from the LDT nucleus as determined by presence of cholinergic neurons. Our results contribute to the body of evidence suggesting that neurobiological changes are induced if gestation is accompanied by nicotine exposure. We conclude that in light of the role played by the LDT in motivated behaviour, the cellular changes in the LDT induced by exposures to nicotine prenatally, when combined with alterations in other reward-related regions, could contribute to the increased susceptibility to smoking observed in the offspring.

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Introduction

Within the western world, nearly one quarter of all pregnant mothers smoke while pregnant,[1–3] and in particular regions, that number has been reported to be even higher.[4–7] This abysmal statistic has not altered much within the last decade suggesting a plateau, which indicates that the population of prenatally exposed [prenatal nicotine exposure (PNE)] individuals is not likely to reduce with currently ongoing attempts via public service education to reduce maternal smoking. Complicating the issue of prenatal exposures, in an attempt to abstain from smoking while pregnant, many women use nicotine patches to reduce cravings. Unfortunately, while not definitively established, data suggest that this method of exposure to nicotine may actually be more harmful to the developing brain than smoking,[2,8] as use of the patch while pregnant ensures that the developing foetus is exposed to a constant high level of nicotine. Studies suggest that nicotine is responsible for poor outcomes[9–11] associated with smoking while pregnant and use of the patch may be associated with similar risks and lead to negative physical and behavioural consequences. Among many adverse health outcomes, individuals exposed prenatally to nicotine demonstrate an increased risk of addicting to drugs of abuse later in life.[12–15] Although environmental issues could certainly contribute to the transgenerational risk, studies show that rates of development of dependency to drugs of abuse are higher in individuals whose mothers smoked during pregnancy when compared with rates of dependency of those whose mothers smoked before and after, but not during, pregnancy.[16] These and other studies indicate that it is not simply the pro-smoke environment that is leading to the high rates of smoking in those prenatally exposed but that other factors are involved. Further, while the increased prevalence of nicotine use in this population could be casual and does not have to be due to dependency, data indicate maternal smoking during pregnancy is associated with an acceleration of the progression from experimentation to daily smoking and later, dependence.[17–19] Taken together, these studies indicate that nicotine serves

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as a developmental teratogen and is altering the normal development of the brain. This alteration likely affects regions of the brain known to be critical in assigning environmental stimuli saliency, resulting in an altered assignment of reward more likely to result in dependent behaviours.

The laterodorsal tegmental nucleus (LDT), which is comprised of a mixed population of cholinergic, GABAergic and glutamatergic neurons has recently been recognized as playing an important role in the assignment of reward to motivational stimuli, like drugs of abuse and more natural stimuli, like food and water[20–23](for review,[24]). The LDT provides a major excitatory pathway to the regions of the brain classically known to play a role in reward.[20,25–27] One common feature of drugs of abuse is their ability to activate reward-related dopamine (DA)-containing neurons of the ventral tegmental area (VTA) to release high levels of DA to target regions within the nucleus accumbuns (NAc). The greater the DA efflux, the greater the likelihood the stimulation will be reinforced and repeated and eventually become pathological.[28–30] Output from the LDT to the VTA critically controls the excitability of VTA DA-containing neurons allowing the behaviourally relevant firing pattern necessary to generate high DA efflux in the NAc.[21–23,31,32] While antatomical, physiological and behavioural data indicate that it is the cholinergic projection from the LDT that is functionally significant in this action[22,23,25,26,33] and the LDT provides the major excitatory cholinergic innervation of both the VTA and NAc,[27] findings from a recent study[20] implicated a role of a glutamatergic LDT–VTA pathway in controlling VTA functioning. Optogenetic stimulation of the LDT–VTA pathway *in vivo* resulted in drug dependency-associated behaviours in absence of a triggering drug, which based on *in vitro* cellular findings was inferred to be due to glutamate mechanisms originating from the LDT.[20] Regardless of the neuronal phenotype, neurons of the LDT are functionally important in processes involved in assignment of stimulus saliency and reward expectation via projections to limbic structures involved in motivated processes.

Exposure to nicotine during the prenatal period has been shown to alter the trajectory of neuronal development and functioning where it has been studied. As nAChRs are present even before neuralation,[34,35] it is no surprise that their inappropriate activation by exogenous application of nicotine is associated with substantive changes in developmental processes affecting outcome of serotonergic, cholinergic, noradrenergic, dopaminergic and glutamatergic transmission within regions of the brain important in reward discrimination (for review[2,3,36]). Abnormalities in development of these systems would be expected to contribute to alterations in postnatal processing of environmental stimuli. However, changes induced in neuronal development or function within the LDT have not been well studied. We have shown that PNE is associated with altered excitability of cholinergic LDT neurons, which influences their postnatal response to nicotinic agents.[37] Glutamate provides a major excitatory influence within the LDT and evidence for presence of subunits for glutamate receptors on cholinergic and non-cholinergic LDT neurons has been presented.[38–40] Glutamate input sources from projection neurons outside the LDT, which includes terminals originating from the medial prefrontal cortex (mPFC), allowing an indirect control of the VTA by the mPFC via a glutamatergic relay in the LDT.[38,39,41] Glutamate transmission in the LDT also sources locally from a population of glutamate-containing neurons neighboring the cholinergic cells within the LDT.[42] As alterations in glutamate signalling by PNE have been detected within the hippocampus and medulla, and found to be due to changes in ionotropic glutamate receptors,[43–46] it was of interest to examine whether PNE leads to alterations in glutamate functioning within the LDT. Using calcium imaging in brain slices from mice prenatally exposed to nicotine, we found evidence for reductions in functioning of AMPA and NMDA receptors within the LDT. In addition, we provide evidence for the first time that the cognitive-enhancer AMPAkine, cyclothiazide (CYZ), which potentiates the AMPA receptor, has actions on neurons of the LDT and these actions are altered in PNE animals. Given the role of the LDT in the brain circuit important in stimulus saliency and reward, differences detected in AMPA and NMDA receptor signalling within this nucleus are hypothesized to contribute to the heightened addiction liability upon postnatal exposure to drugs of abuse in those exposed to nicotine prenatally. It is hoped that better understanding of the specific mechanisms underlying the heightened risk for addiction to nicotine in this vulnerable population will lead to informing design of a rational strategy for developing new pharmacological aids to treat addiction, or even prevent its emergence, in these susceptible individuals.

Methods

Animals

All animal studies were conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) as well as with Danish legislations. The Animal Welfare Committee, appointed by the Danish Ministry of Justice, approved the animal study after determining that efforts to diminish and explore alternatives to animal experiments and to minimize animal suffering had been made.

Experiments were conducted on brain slices from PNE animals or controls. PNE was achieved using an oral exposure model. Pregnant NMRI mice (Harlan) were exposed to nicotine via addition of nicotine to their drinking water. The concentration of nicotine utilized (Sigma-Aldrich; 300 μg/ml, free base) in the dam's drinking water were derived from studies in rodents indicating that maternal nicotine exposure via this method introduces a significant distribution of nicotine in the offspring[47] and studies showing that the resulting plasma concentration of nicotine induced both dependence and tolerance in mice indicating exposure of the foetuses to nicotine *in utero*.[48] Saccharin (Sigma-Aldrich; 2% w/v) was added to water as a sweetener to mask the bitter taste of nicotine.

The population of control animals included naïve mice and animals that had been exposed to saccharin during gestation. Data were pooled from these two groups after determination

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that no difference between naïve and saccharin animals in calcium responses upon application of agonists was detected. Litter size, sex distribution, birth weight and water intake were tested in both naïve, saccharine and nicotine mice to rule out any potential differences in these variables that could bias our results. A random check of the water intake indicated that saccharine mice were drinking twice the amount compared with nicotine fed, however, water consumption in all three groups was within normal limits reported for other studies on mice[49] and similar water restrictions seen in other studies using this model were not believed to explain noted neural actions of nicotine[50] or alcohol,[51] and were therefore not considered to be of significant issue in the present study. After pregnancy, animals continued to be single housed in an open cage system in a temperature of 22 ± 2°C, with a humidity of 36–58%. The light within the room was on a 12:12 light:dark cycle with light on between 6 am and 6 pm. Animals had *ad libitum* access to water and food during the entire duration of the experiments. After birth, the drinking water was switched to plain tap water for all groups. Animals sourced from different mothers and from multiple litters in order to avoid the contribution of litter effects to the study.

Brain slice preparation

Mice between the ages of postnatal day (PND) 7–22 were decapitated while under general anaesthesia with isoflurane (Isoba® Vet. 100%). A block of the brain containing the LDT was rapidly removed and submerged in ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM) 124 NaCl, 5 KCl, 1.2 Na_2HPO_4, 2.7 $CaCl_2$, 1.2 $MgSO_4$, 10 glucose and 26 $NaHCO_3$ at pH 7.4 and saturated with carbogen (95% O_2/5% CO_2). Subsequently the tissue was glued to the cutting platform in a coronal plane, supported by an agar block and re-submerged in ice-cold oxygenated ACSF. Finally, the brainstem was carefully sectioned at 250 µm using a Leica vibratome (VT1200S, cutting speed 5-10 mm/s).

LDT slices were identified by known landmarks, that is, by locating the visible pericentral part of the dorsal tegmental nucleus (DTgP) as while the LDT itself is not so easily visible, it is known to be located lateral to the DTgP areas. Correct inclusion of the LDT within the imaged areas was confirmed *post hoc* by use of immunohistochemistry optimized to detect cholinergic neurons, which are used to delineate the periphery of the LDT. Slices containing the LDT were incubated at 37°C for 15 min and then stored at room temperature for at least 1 h before further procedures were conducted.

Calcium imaging

LDT containing brain slices were loaded with the ratiometric fluorescent dye FURA-2-acetoxymethylester (FURA-2, AM) in accordance with known guidelines.[52] To this end, slices were incubated for 10 min plus 1 min for every PND at 32°C in a small volume ACSF equilibrated with carbogen and containing 15 µM FURA-2, AM freshly prepared from a stock solution of 3.3 mM prepared in DMSO. Although inclusion of glia cells in the study cannot be ruled out, this loading protocol has been reported to load neurons in the LDT[53] and cells for this study were selected for inclusion on basis of size and presence of processes, which were dendritic in appearance. During and after incubation with FURA-2, AM the tissue was exposed to a minimum of light to avoid photo bleaching. Slices were rinsed in the chamber for at least 20 min before commencement of drug application experiments to ensure FURA-2, AM de-esterification, wash out of dye debris and temperature equilibration.

Recordings were obtained from slices submerged in a recording chamber at room temperature, perfused at 0.5–1 ml/min with continuously oxygenated ACSF. The LDT was first localized on an Olympus microscope (BX-51, Japan) with a 4× objective (Plan N, Olympus, Japan) by bright field illumination and landmarks. Individual cells loaded with FURA-2, AM were then imaged using a 40× water immersion lens (LUMPlanF N, Olympus). Optical recordings were made using a cooled CCD camera system (12 bit Sensicam, PCO imaging, Germany) controlled by imaging software (LA Acquisition, TILL Vision, Germany). The ratiometric recordings were obtained by software-controlled, rapid switching of the excitation wavelength between 340 and 380 nm and appropriate monitoring of the emission spectra using a Chroma FURA-2 filter set and a Xenon Bulb (Osram, Germany). FURA-2 bound to free Ca^{2+} ions emits a greater fluorescence when exited at 340 nm than at 380 nm, hence a rise in $F_{340\,nm}/F_{380\,nm}$ ratio corresponds to a rise in free calcium. Images were binned at 2 × 2 and image pairs were collected at 1000–2000 ms intervals. Regions of interest (ROIs) were selected to encompass filled cells, which appeared to be neurons based on size and presence of dendritic processes, and exposure times at the two wavelengths were adjusted to give a maximum fluorescence intensity of ~10% of the maximum range so as to reduce the possibility of aliasing the fluorescent signal. A region of the field devoid of cells was also monitored as an indicator of background fluorescence. During the recordings, responses were monitored, and following recordings, offline analysis was conducted with a software analysis tool available within LA Acquisition (OA, Till Vision). The ratiometric measure of change in fluorescence is shown on graphs as dR/R, where R is the average fluorescence ratio ($F_{340}/F_{380\,nm}$) within a single ROI following subtraction of background fluorescence. dR/R is the ratio change following subtraction of the average R measured before drug application and hence, this value represents a relative expression of change in calcium and is not meant to report absolute values. Ratio measurements were not converted to absolute values of calcium concentrations due to several well-known uncertainties associated with FURA-2 imaging within brain slices, as discussed in Connor and Cormier.[54] dR/R ratios in all figures are presented such that upgoing traces represent rises in intracellular calcium.

Drugs

50 mM NMDA (Sigma-Aldrich), 1 mM AMPA (Sigma-Aldrich), 1.5 mM DNQX (Sigma-Aldrich) and 50 mM AP5 (Sigma-Aldrich) stock solutions were made from solid drugs

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dissolved in distilled water and frozen in appropriate aliquots to be diluted in ACSF at the final concentrations. Stock solutions of 30 mM CYZ (Tocris) were made in DMSO and diluted in ACSF at a ratio of 1:1000, which is a concentration of solvent that does not exert actions on LDT cells.[53] Drugs acting at glutamate receptors, AMPA, NMDA, DNQX, and AP5, and CYZ were applied using an automatic multi-barrel system (ALA Scientific Instruments, VC3 8,8 channel valve commander with 50 ml syringes). Barrel flow (~2.5 ml/min), equal chamber distribution and wash out time (~5 min) were tested before experiments by monitoring the flow pattern of phenyl red. Preliminary experiments indicated that application of 50 μM NMDA and 10 μM AMPA for 30 s using the multi-barrel system gave rise to consistent calcium responses with a duration >2 min. A total of 15 μM DNQX and/or 50 μM AP5 (for > 5 min) were applied before NMDA or AMPA to ensure block of AMPA, and NMDA, receptors, respectively. 30 μM CYZ was both pre- (for > 5 min) and co-applied when testing its effect on AMPA-induced calcium responses.

Immunohistochemistry

To confirm that imaging had been conducted in the LDT, we used immunohisochemistry to identify neuronal nitric oxide synthase (nNOS)-positive cells within the slice. Presence of nNOS has been shown to be a reliable marker of cholinergic LDT neurons, and the pattern of cholinergic cells and their periphery, serves to identify the boundaries of this nucleus. In contrast to detection of markers of acetylcholine synthesis or transport, detection of nNOS is robust in brain slices from which imaging has been conducted and kept alive for extended periods of time in ACSF.[55] To process slices for detection of cholinergic neurons, following calcium imaging, slices were incubated with 4% w/v paraformaldehyde (PFA) for 24 h before being stored in 30% w/v glucose solution in order to stop the fixation induced by PFA and to promote cryoprotection. Slices were then kept at 5°C until commencement of further procedures. Slices of 250 μm were re-sectioned at 40 μm on a cryostat (Leica CM3050 S, Germany). Re-sectioned slices were then exposed to a primary nNOS rabbit-antibody with subsequent incubation in an appropriate secondary (Alexa Flour® 488 goat anti-rabbit IgG, Molecular Probes®). Slices were then mounted on glass slips, and dehydrated. Inclusion of the LDT in slices from which recordings were conducted and data presented was confirmed by presence of nNOS positive cells in a pattern typical of the LDT.[56]

Data processing and analysis

Recordings were excluded if movement of the slice disabled capture of single neurons within appropriate sized ROIs for ⩾2 min or major changes in focus across the data collection occurred. In addition, single ROIs were excluded if dye debris crossed the ROI, which induced larger artefacts in the fluorescence (F). Responses were verified and distinguished from potential artefacts by frequently assuring that the changes in fluorescence ratio R ($= F_{340\,nm}/F_{380\,nm}$) were reflected in the single channel recordings at 340 and 380 nm. Data sets were analysed using either IGOR Pro (WaveMetrics, Inc., USA) or Prism 5 for Mac OS X (GraphPad Software, Inc., version 5.0d), both including appropriate built-in tools to quantify the response variables rise time (time from peak onset to peak maximum), length (time from peak maximum to 50% decay), and amplitude (difference between baseline and peak maximum). As in other studies using calcium imaging within the LDT,[53,55] the profile of responses varied. For purposes of analysis, these profiles were categorized into three categories based on the kinetic profile as was defined and done in previous work: (1) plateau-like responses, (2) smooth spiker and (3) oscillatory, and quantification of response variables presented was based on analysis of the cells responding with the plateau kinetic pattern. Data is presented as the mean amplitude ($\Delta R/R$, %) ±standard error of the mean (S.E.M.) of the plateau-like responses, with the n-value indicating the number of cells exhibiting this response, unless otherwise stated. The number of cells exhibiting quantifiable plateau-like responses was on average 10 cells per slice. Data were collected across multiple litters and while most often only one LDT slice was obtained from each animal, in some cases, two slices containing the LDT could be obtained. All figures were prepared using IGOR or Prism.

Statistics

Statistical analysis presented in this report was generated using IGOR and Prism. In particular for comparison of means,

Fig. 1. AMPA induces smaller calcium responses in laterodorsal tegmental nucleus (LDT) neurons from prenatally exposed animals (PNE) compared with controls (Con). (a1) Representative coronal brain slice under 4× optics showing the region of the brain including the LDT and with indications of the known landmarks for the region, that is, the adjacent dark oval-shaped dorsal tegmentum nucleus (DTgP). (a2) Representative close up (40× optics) view of LDT neurons successfully loaded with FURA-2, AM, a fluorophore, which changes its fluorescence when bound to calcium. Regions of interest (ROIs) are drawn around the cells as shown by the boxes to monitor changes in fluorescence (dR/R) within these regions induced by drug. The background was chosen as a region devoid of filled cells (green dashed line box). Scale bar indicates 20 μm. (b) AMPA-induced responses from PNE (red) and control cells (black). Data from first application of 10 μm (b1, b2) or 1 μM (b3) AMPA are presented. (c) Cumulative fractions of the dR/R response from individual cells in control (black) and PNE conditions (red) applied 10 μM (c1) or 1 μM (c2) AMPA indicating that the response size distributions were significantly different between PNE and control conditions (K–S test, $n = 384$, $P = 0.0040$), indicating a reduced AMPAR-mediated calcium flux in slices obtained from prenatally nicotine-exposed animals. (d) Histograms showing mean response amplitudes ± S.E.M. from first application (R1) of either 10 μm (d1) or 1 μM (d2) AMPA both indicate that in LDT cells from PNE animals, responses were significantly decreased compared with responses in control slices (student's t-test, $n_{10\,\mu M} = 384$, $P = 0.0007$; d1 & $n_{1\,\mu M} = 45$, $P = 0.0085$; d2).

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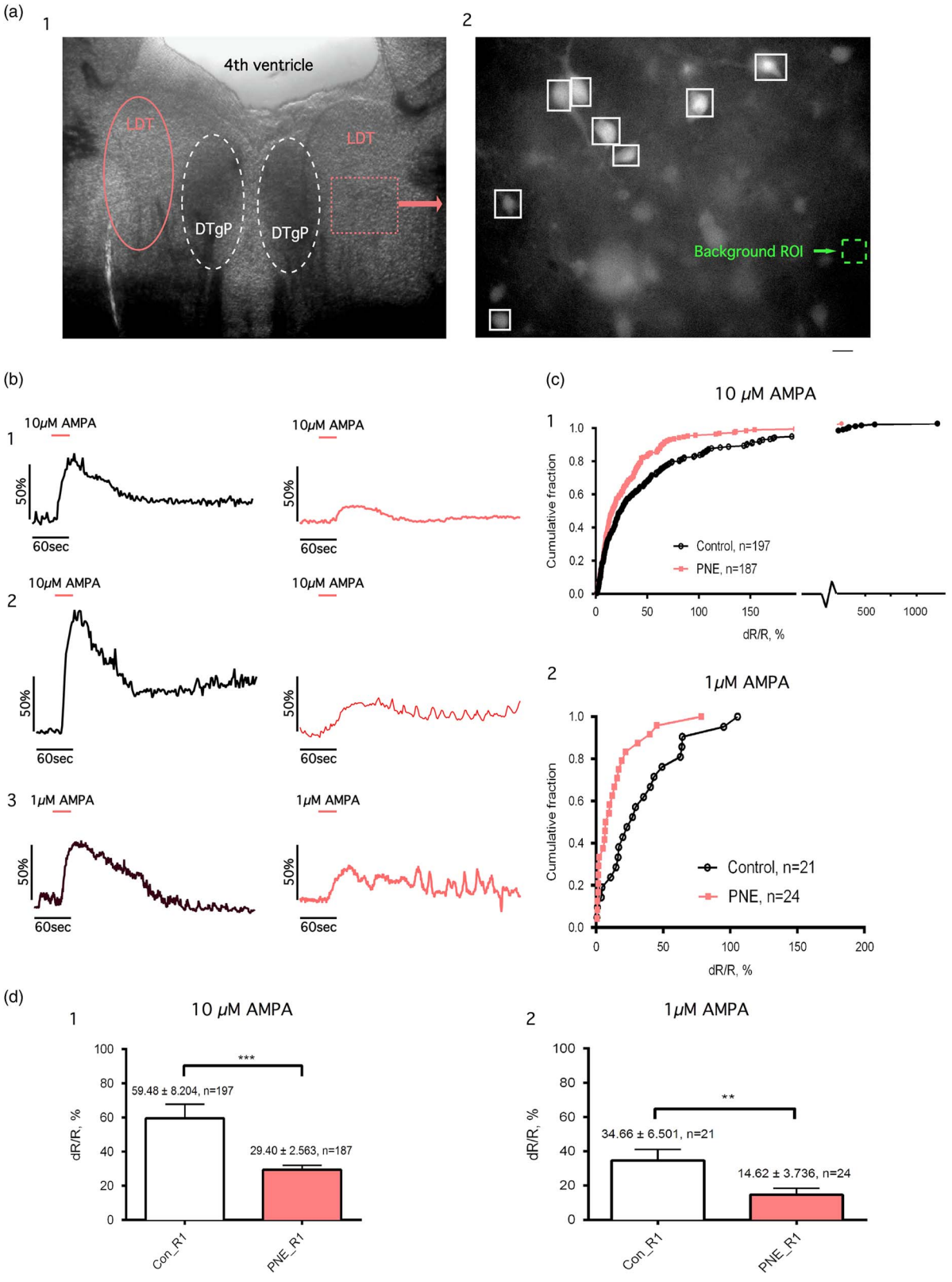
student's paired and unpaired *t*-test in Prism was used. When appropriate, data were evaluated using a one-way ANOVA, including Bartlett's pre-test (for equal variances) and Tukey's post-test (comparing all pairs of columns) in Prism. A χ^2-test with Yates correction, available at the online GraphPad support web page was utilized for non-parametric evaluations. Finally,



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IGOR was used to perform Kolmogorov–Smirnov (K–S) test for comparison of response size distributions. Statistical significance was determined as $P < 0.05$, which is indicted by a single asterisk in figures, however, in some cases, multiple asterisks are utilized in graphs to indicate when *P*-values were even lower than this value: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$.

Results

AMPA induces smaller rises in calcium in PNE animals

Application of 10 μM AMPA for 30 s induced significantly smaller calcium responses in LDT cells from PNE animals when compared with rises induced in these cells in controls (student's *t*-test, $n = 384$, $P = 0.0007$; Fig. 1a, 1b and 1d). On average, the amplitude of the AMPA-induced calcium rise for the PNE group was about half that observed in controls. Before pooling data from the two control subgroups, that is, naïve and saccharin, responses in these groups were compared and amplitudes were found not to be significantly different (student's *t*-test, $n_{\text{naïve}} = 144$, $n_{\text{saccharin}} = 53$, $P = 0.1994$). Smaller responses in the PNE group were confirmed by analysis of the cumulative distribution of the response size, which clearly indicated a significantly greater fraction of larger AMPA-mediated calcium responses in LDT cells from controls, when compared with the distribution of the amplitude of responses in the LDT from PNE animals (K–S test, $n = 384$, $P = 0.0007$; Fig. 1c and 1d). Significantly smaller responses in the PNE group were replicated using a lower concentration of AMPA (Fig. 1b and 1d). Interestingly, while the 10 μM AMPA dose was chosen over 1 μM for further experiments as the higher concentration induced larger amplitude responses, this effect was only significant for responses elicited in PNE animals (student's *t*-test, $n_{1\,\mu M} = 24$, $n_{10\,\mu M} = 187$, $P = 0.0439$), and was not found to be the case in recordings from controls ($n_{1\,\mu M} = 21$, $n_{10\,\mu M} = 197$, $P = 0.3271$), further indicating differences in AMPA responsitivity induced by the PNE treatment. Repeat applications of AMPA were able to elicit responses; however, differences upon multiple applications were apparent between control and PNE animals. The second application to AMPA was significantly decreased in the LDT of control animals, whereas it was not significantly altered in cells of the LDT from PNEs (Fig. 2a). Further, the mean second to first response ratio for responses obtained in PNE slices was significantly different from that in control slices (student's *t*-test, $n = 107$, $P = 0.0235$; Fig. 2c). While the second to first response ratio was <1 in control slices, this ratio was >1 in PNE animals. Therefore, we conclude that consecutive applications of AMPA resulted in a decrease in the response amplitude in control animals that was not present in the PNE group (student's paired *t*-test, $n_{\text{con}} = 54$, $P_{\text{con}} = 0.0010$, $n_{\text{PNE}} = 53$, $P_{\text{PNE}} = 0.0828$; Fig. 2b). A subset of the data was analysed for differences in other response kinetic variables, namely rise time and duration of the response. Our data indicated that responses from control slices had a significantly shorter rise time (student's *t*-test, $n = 103$, $P < 0.0001$; data not shown) and exhibited a faster decay rate ($n = 83$, $P < 0.0001$; data not shown) than responses obtained in PNE slices. No significant difference in latency to onset was observed between groups ($n = 103$, $P = 0.3328$; data not shown). Taken together, our results suggest that PNE reduces AMPA-induced calcium responses in LDT neurons and alters the mechanisms determining response size upon second exposure to AMPA in these cells as well, which suggests the possibility that gestational nicotine changes AMPA receptors in such a way that mechanisms involved in receptor desensitisation are altered.

Nicotine alters the neural calcium response to consecutive NMDA applications

Calcium rises induced by a 30-s application of 50 μM NMDA were found to not be different across groups; however, upon second application a significant increase of response size was observed in the control group, which was not an effect observed in cells from the PNE group (student's paired *t*-test, $n_{\text{con}} = 53$, $p_{\text{con}} = 0.0147$, $n_{\text{PNE}} = 24$, $p_{\text{PNE}} = 0.5687$; Fig. 3a, 3b and 3c). Hence, a significant difference in second to first response ratio was observed (student's *t*-test, $n_{\text{con}} = 53$, $n_{\text{PNE}} = 24$, $P = 0.0012$; Fig. 3d). A subset of the data were evaluated for differences in other response kinetic variables; however, no significant differences between the control and PNE groups were observed in rise time (student's *t*-test, $n = 53$, $P = 0.7600$; data not shown) or duration of response ($n = 43$, $P = 0.6758$; data not shown). In summary, whereas no difference between the two groups were observed in the amplitude of the calcium responses induced by first NMDA application, the second to first response amplitude ratio were significantly different among the groups. These data suggest that PNE affects the functioning of NMDA receptors in the LDT. Further, as responses to first applications were similar across treatment groups, these data support the conclusion that differences seen in responses to AMPA were not a function of PNE alteration in cell health inducing non-specific differences, but rather, likely due to specific changes in activity involving ionotropic glutamate receptors.

Similarities and differences between AMPA- and NMDA-induced calcium responses

The data collected monitoring calcium responses elicited by the first application of 10 μM AMPA indicated that PNE resulted in an approximately two-fold decrease in the calcium response in LDT cells (Table 1). Contrary to this finding, responses to first applications of NMDA in LDT neurons from PNE animals were not significantly different from response amplitudes observed in controls. Furthermore, whereas second responses to AMPA were significantly decreased in slices obtained from control animals, second responses to AMPA observed in PNE slices were not significantly different from first responses. In contrast, second responses to NMDA were larger in control conditions, whereas this activity-dependent enhancement was not noted in second NMDA

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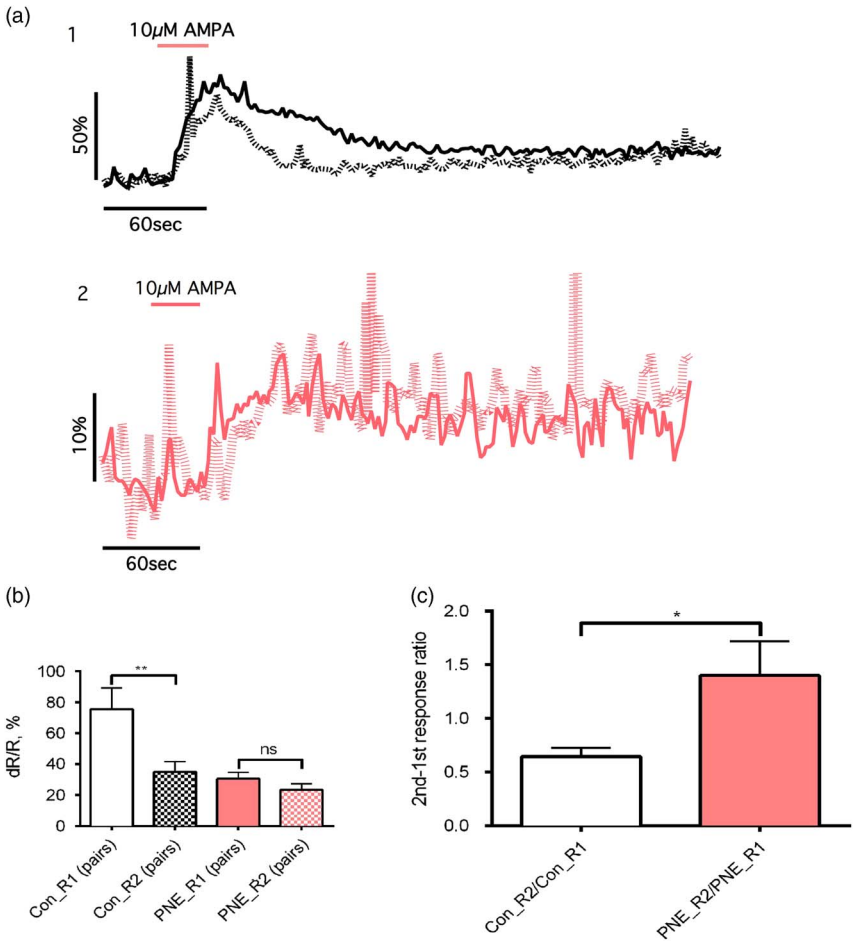

Fig. 2. Responses to AMPA were significantly attenuated upon repeat applications in control laterodorsal tegmental nucleus (LDT) cells (Con), but were not significantly reduced in cells from prenatally exposed (PNE) animals. (a) Single cell example of AMPA-induced responses in a control (black; a1) and PNE (red; a2) slice indicating the decrease in response size from first application (full line) to second application (dotted line) in control slices, but not in PNE slices. Note different scale bars on *y*-axes. (b) Response amplitude means ± S.E.M. from PNE (red) and control cells (Con; black/white), responding to both by first (R1) and second (R2) AMPA application (pairs). For the population of recorded cells, response amplitudes elicited by second applications of AMPA were significantly reduced in control slices (student's paired *t*-test, $n = 54$, $P = 0.0010$), however, while the mean amplitude of responses from first and second AMPA application suggested a decrease upon repeat application, a significant attenuation in repeat responses was not present in cells from slices from PNE's ($n = 53$, $P = 0.0828$). (c) This contrast was reflected in a significant second to first response ratio difference between responses in PNE and control data (student's *t*-test, $n_{con} = 54$, $n_{PNE} = 53$, $P = 0.0235$).

responses in cells from PNE animals. In summary, when data using AMPA and NMDA receptor agonists are compared, they emphasize the differences induced by PNE of actions mediated by these two ionotropic glutamate receptors. Taken together, we hypothesize that PNE affects mechanisms underlying AMPA and NMDA receptor desensitisation and activity-dependent enhancement, respectively, and actions extend to altering the kinetics of AMPA responses in PNE animals, an effect not noted in controls or in responses mediated by the NMDA receptor.

CYZ significantly potentiates the neural calcium response to AMPA

The development of compounds that modify the activity of AMPA receptors allowed us to further investigate the suggested differences in AMPA responses obtained between PNE and control LDT cells. Accordingly, we evaluated the potential differential effect of the ampakine CYZ, a positive allosteric modulator working at the AMPAR. Pre-application of 30 μM CYZ reversed the observed decrease in response to consecutive applications of 10 μM AMPA seen in control animals. In fact, CYZ significantly potentiated the effect of AMPA in both groups, however, the degree of potentiation differed across treatment groups by an order of magnitude of 6.7 and 2.5 in control and PNE slices, respectively (student's paired *t*-test, $n_{con} = 72$, $p_{con} < 0.0001$, $n_{PNE} = 60$, $p_{PNE} < 0.0001$; Fig. 4a and 4b). Hence, the response ratio in control slices was significantly larger than that observed in PNE slices (student's *t*-test, $n_{con} = 72$ and $n_{PNE} = 60$, $P = 0.0245$; Fig. 4b).

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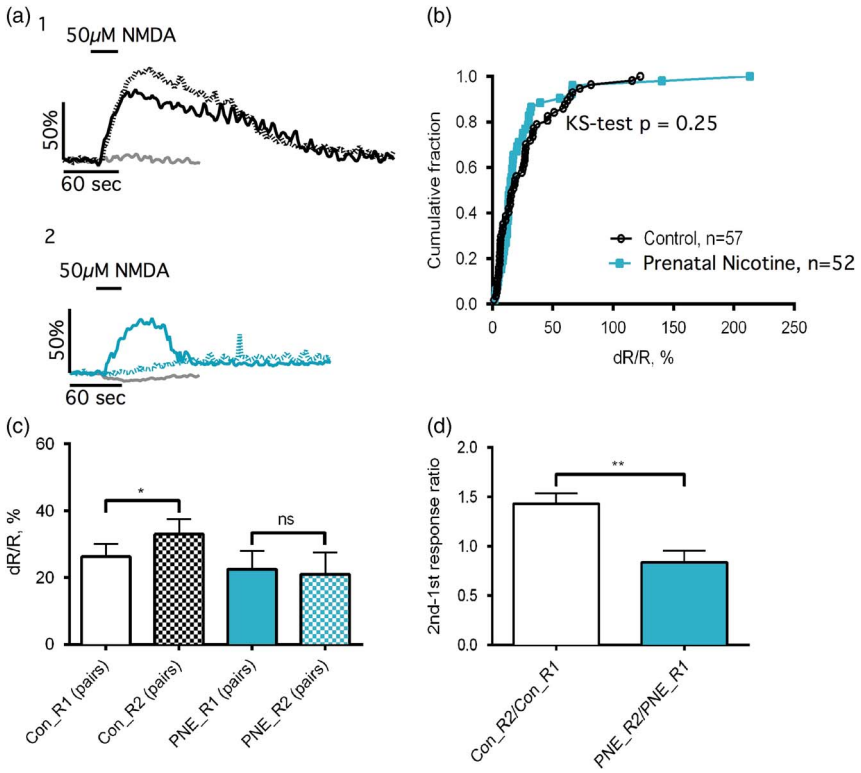

Fig. 3. First time applications of NMDA-induced calcium rises that were not significantly different between laterodorsal tegmental nucleus (LDT) cells in prenatal nicotine exposed (PNE) and control slices. (a) Single cell trace examples from first (full line) and second (dotted line) NMDA application in control slice (black; a1) and a PNE slice (blue; a2), and ACSF test run in both slices (grey traces). Whereas most second responses (R2) in the control group showed an increase compared with first response (R1), some responses in the PNE group decreased, and on average, no difference was detected between first and second applications in the prenatally exposed animals. (b) Examination of the cumulative distributions of the response size amplitude revealed there were not significant differences across groups for this parameter (K–S test, $n = 109$, $P = 0.25$). (c) Response amplitudes are presented as mean ± S.E.M. No significant differences between the means of Con_R1, Con_R2, PNE_R1 and PNE_R2 were observed (one-way ANOVA with Tukey's post-test, $P > 0.05$, data not shown). However, when comparing data from cells responding to both first and second NMDA application (pairs) the response size was significantly increased upon second NMDA application in the control (student's paired *t*-test, $n = 53$, $P = 0.0147$) an effect that was not noted in the prenatally exposed group ($n = 24$, $P = 0.5687$). (d) The second to first response ratio was significantly different for the two groups as show in the histogram summarising data collected from a population of cells in which first and second applications of NMDA were tested ($n = 77$, $P = 0.0012$).

Table 1. *Data from cells responding to both first and second drug application (pairs) are presented as mean ± S.E.M.*

		Response size (%)		
Drug applied	Group	First application	Second application	Second to first response ratio
AMPA	Control	$76 \pm 14^{(A),(B)}$	$35 \pm 7^{(B)}$	$0.6 \pm 0.1^{(D)}$
	PNE	$30 \pm 4^{(A)}$	23 ± 4	1.4 ± 0.3
NMDA	Control	$26 \pm 4^{(C)}$	$33 \pm 5^{(C)}$	$1.4 \pm 0.1^{(D)}$
	PNE	23 ± 5	21 ± 7	0.8 ± 0.1

PNE, prenatal nicotine exposure.

Summary of statistics: (A) First response to AMPA in control compared with PNE slices were significantly different (student's *t*-test, $n = 384$, $P = 0.0007$). (B) Response size was significantly decreased upon second AMPA application in control slices (student's paired *t*-test, $n = 54$, $P = 0.0010$). (C) Response size was significantly increased upon second NMDA application in control slices (student's paired *t*-test, $n = 53$, $P = 0.0147$). (D) AMPA control response ratio is significantly different from NMDA control response ratio (Student's *t*-test, $n = 106$, $P < 0.0001$).

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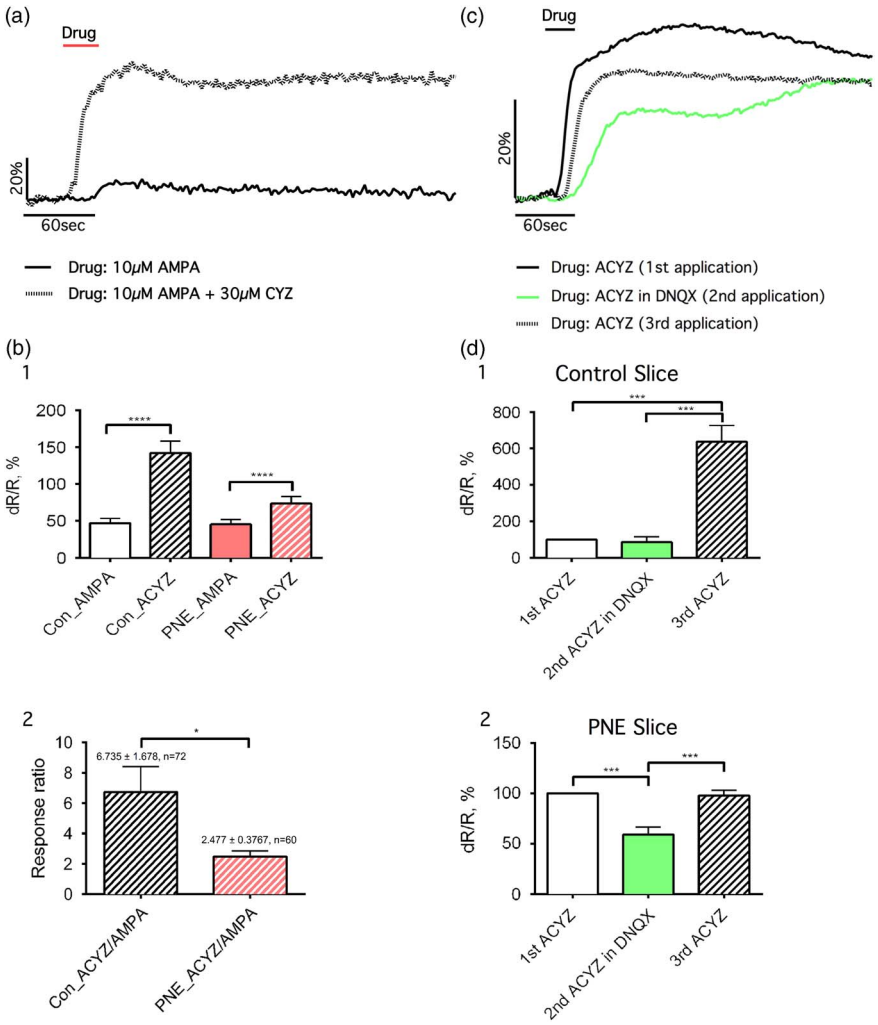

Fig. 4. An AMPAkine potentiated the actions of AMPA on inducing calcium rises in control and prenatally exposed cells; however, this potentiation was significantly greater in control cells. (a) Single cell example from a control slice showing the potentiating effect of the AMPAkine cyclothiazide (CYZ) on the calcium response elicited by a 30-s application of 10 μM AMPA to a laterodorsal tegmental nucleus (LDT) neuron. This is the first time this cellular action of an AMPAkine within the LDT has been reported. Analyses was made as to whether any differences in CYZ response existed between naïve and saccharine animals and as there was no significant difference detected ($P = 0.5795$), data from these two groups were pooled for this study. (b) CYZ significantly potentiated the effect of AMPA in both control (Con) and prenatally exposed (PNE) slices (student's paired *t*-test, $n_{con} = 72$, $n_{PNE} = 60$, $^{****}P < 0.0001$), however, the degree of potentiation was significantly smaller in the PNE cells when compared with that elicited in controls ($^{*}P = 0.0245$). Data are shown as mean ± S.E.M. and only data from complete data sets are included, that is, when a quantifiable plateau-like response were obtained upon both first application, consisting of pure 10 μM AMPA, and 3rd application, consisting of 10 μM AMPA co-applied with 30 μM CYZ (abbreviated ACYZ). Second application consisted of 10 min 30 μM CYZ pre-application. Effects of CYZ, which has been shown to reverse response decrease upon consecutive AMPA applications where it has been studied, were specific to actions at the AMPA receptor in the LDT as the AMPA receptor antagonist DNQX inhibited the effect of a 30-s 10 μM AMPA application in the presence of 30 μM CYZ. (c) Shown is a single cell example of response inhibition caused by the presence of 15 mM DNQX. (d) Population data showing that in both control (d1) and PNE (d2) cells, enhancements of AMPA-induced calcium rises were reduced in presence of DNQX. Response amplitudes from each cell were normalized to first response induced by ACYZ and found to be significantly reduced as indicated by the asterisks (one-way repeated measures ANOVA with Tukey's post-test, $^{***}P < 0.001$). Recovery of response upon a third application of AMPA following washout of DNQX was possible in some cases (3rd ACYZ).

To test whether the effect of CYZ was in fact induced through activation of AMPARs, the responses following 5 min pre-application of the AMPA receptor antagonist, DNQX (15 mM) were examined. The data indicated that the receptor antagonist significantly decreases the potentiating effect of CYZ on AMPA-induced responses in cells from both control and PNE groups, and that the inhibition is reversed following wash (one-way ANOVA, $P < 0.001$; Fig. 4c and 4d)

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suggesting that actions of CYZ are specific to potentiation of AMPA receptors.

In addition, it was observed that application of 30 μM CYZ in isolation could induce rises in calcium in both PNE and control slices, which is consistent with previously demonstrated ongoing glutamate transmission occurring within the LDT in this brain slice preparation.[42] However, significantly fewer cells in PNE slices (12%) compared with cells in control slices (33%) exhibited increasing calcium levels as a response to CYZ application (χ^2 with Yates correction, $n = 219$, $p = 0.0005$; Table 2). A significantly higher percentage of cells failed to respond to CYZ alone in the LDT of PNE animals (46%) when compared with the percentage of cells that exhibited no response to this AMPAkine in control LDT (27%; Table 2). The remaining cells responded to CYZ with decreases in calcium, however, there were no significant differences in this response between PNE and control animals. In summary, our results indicate that CYZ strongly potentiated the effect of AMPA at the AMPA receptor and that the degree of potentiation was smaller in slices from PNE mice than in slices from controls. Furthermore, a larger percentage of cells in control slices exhibited a rise in calcium as a response to CYZ application when compared with cells in PNE slices. Thereby, these data support our other findings indicating that PNE changes AMPA receptor-mediated activity within the LDT.

Table 2. *Application of 30 μM CYZ in isolation induced calcium responses in LDT neurons*

	Group	
Activity (% of total cells exhibiting the activity)	Control (%)	PNE (%)
Ca^{2+} increase	33	12
Ca^{2+} decrease	29	32
No change in Ca^{2+}	27	46

CYZ, cyclothiazide; LDT, laterodorsal tegmental nucleus.

Summarized are the response type distributions for the nicotine and control groups. A significantly larger proportion of cells responded to CYZ application with an increase in calcium in the control group when compared with the proportion of cells responding in the prenatally exposed group (χ^2 with Yates correction, $n_{con} = 119$, $n_{PNE} = 100$, $P = 0.0005$).

Immunohistochemistry

As in previous studies,[56,57] we utilized a marker of cholinergic neurons, nNOS[58] to confirm that data sourced from cells within the LDT. Staining for nNOS within a subpopulation of slices utilized in this study ($n = 13$), revealed that in all cases, recordings were conducted within the boundaries of the LDT (Fig. 5a and 5b). Upon gross examination, there did not appear to be any differences in nNOS staining across control

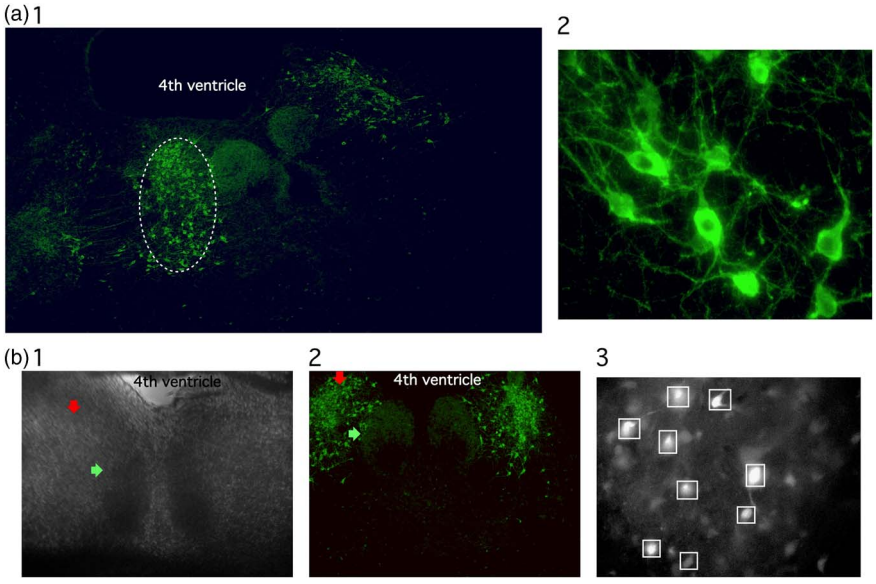

Fig. 5. Calcium imaging was conducted within the region of the laterodorsal tegmental nucleus (LDT). (a1) Low gain fluorescent image of a sample coronal brain slice used in the present study in which presence of the bilateral LDT can be seen. Neuronal nitric oxide synthase (nNOS)-positive cells are shown in green, and the anatomical limits of the LDT can be defined by the extent of nNOS positive cells (dashed circle on left LDT). (a2) High gain (40×) detail of the LDT in another slice, indicating that gross morphology can be appreciated at this level of magnification. (b1) Another coronal slice from which data for this study were collected viewed under bright field optics. (b2) The same slice viewed under fluorescence after processing for nNOS immunohistochemsitry. This procedure was used to confirm that recordings were conducted from the LDT. (b3) High gain fluorescent image of the FURA-2, AM cells within the LDT shown in b1 and b2. Regions of Interest (ROIs) are drawn around cell bodies to indicate neurons from which recordings were conducted.

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and PNE conditions. When taken together with our earlier report showing that Fura-2, AM loads neurons within the LDT (Kohlmeier *et al.*),[24] we conclude that alterations in AMPA and NMDA receptor-mediated signalling associated with exposure to nicotine during gestation alters calcium rises in neurons of the LDT, but it does not appear to alter gross morphology of these cells. Further experiments will need to be conducted with more detailed histological analysis to determine whether PNE is associated with alterations in dendritic morphology within the LDT as it has been shown to occur in other brain regions where this has been studied.[59,60]

Discussion

Summary

The motivation for the current study was to determine whether gestational nicotine exposure affects glutamatergic neurotransmission mediated by AMPA and NMDA receptors in the LDT, a brain region thought to be involved in the neurobiology underlying reward-related processing of salient, motivated stimuli. Using calcium imaging, we compared calcium rises induced by AMPA and NMDA receptor agonists in the LDT in brain slices obtained from mice exposed during the gestational period to nicotine to those from naïve and control mice. Taken together, our data indicate that prenatal exposure to nicotine is altering the functioning of AMPA and NMDA receptor-mediated signalling within the LDT. Our data suggest that early-life exposure to nicotine induces a reduction in glutamate signalling, resulting in a lower neuronal response to glutamatergic input. Reduced glutamatergic responsiveness would be expected to play a role in alterations of behaviours associated with PNE and known to be controlled by the LDT.

Caveats

Our study was conducted using pharmacological agents that specifically activate NMDA and AMPA receptors, and these receptors have been shown to be present on postsynaptic LDT cells. mRNA for glutamate receptor subunits has been detected in the LDT,[38,39] and electrophysiological whole cell patch clamp studies from our lab and others have indicated functional presence of these receptors in this nucleus and that activation of these receptors results in rises in calcium.[40,42] While both AMPA and NMDA receptors are calcium permeable, the present study did not specifically examine whether calcium sourced via an increase in the calcium conductance of these glutamate receptors. Calcium influx can occur directly via these ionotropic receptors, or source secondarily to depolarisation of the cell sufficient to activate voltage-operated calcium channels or via mobilisation of secondary messengers involved in release of calcium from intracellular stores.[61] As we were primarily interested in determining the functional outcome of activation of glutamate receptors, our study did not include blockade of these secondary sources. Therefore, we make no conclusions that the calcium rises elicited by the glutamate receptor-acting drugs, AMPA or NMDA, were mediated by flux of ions directly through these receptors. While PNE-induced changes could occur to alter conductances of voltage-operated calcium permeable channels or could result in changes in the intracellular stores decreasing their susceptibility to activation, we do conclude from our data, regardless of mechanism, that exposure to nicotine prenatally induced alterations in calcium rises initiated by AMPA and NMDA receptor stimulation, and it would be expected that this functional change would have consequences for processes in which the LDT is involved.

Another caveat of our study is that with the bulk load calcium imaging technique, the phenotype of individual responding neurons cannot be identified. This technique offered several attractive features: (1) with the method utilized, neurons, specifically, within the LDT have been shown to be loaded with the calcium-binding ratiometric dye;[53] (2) we were able to monitor AMPA- and NMDA-induced responses in large numbers of neurons at the same time; and (3) we were able to monitor responses to glutamate receptor activation without disturbing the intracellular contents upon which responses may have been dependent. However, the LDT contains several neuronal cell types, that is, GABAergic, glutamatergic and cholinergic[62] with some cholinergic cells co-localising GABA[63,64] and the disadvantage of the calcium imaging technique employed is that we were not able to distinguish between these LDT cell types and correlate responses with a particular neuronal population sorted by neurotransmitter content. In future, now that changes suggestive of PNE-induced alterations in receptor functioning have been detected, using more invasive, lower yield, methods such as patch clamping, it would be interesting to determine whether differences in glutamate receptor responses exist following early-life nicotine exposure are found within a specific LDT cell phenotype, especially in light of the different roles played by subpopulations of LDT cells.[24]

Our study was conducted to determine whether PNE introduces alterations in glutamate receptor-induced intercellular calcium. As the first study of this type in the LDT, we examined the presence of this alteration across a range of PNDs up to 3 weeks postnatal. However, dynamic changes in neurotransmitter systems occur during this time, including changes in the glutamate system.[43–45,65] Therefore, it would be interesting in future studies to examine whether differential responses exist from week to week within the range of ages studied here and further, whether differences during this time are still present, or altered in the adult.

Finally, the oral administration model was utilized to assay the gestational effects of nicotine on glutamate receptor functioning within the LDT. While we did not measure levels of the nicotine metabolite, cotinine, in the foetus to assay levels of drug exposure, nor monitor addictive behaviours in the mother to verify psychobiological effects or evidence of drug exposure by alterations in behaviour, this method of nicotine administration has been shown by other studies to result in nicotine

distribution to the foetus, as well as engendering dependence and tolerance in the mother, suggesting the foetus is exposed to behaviourally relevant nicotine concentrations.[47,48,66] Although perhaps less of a factor than nicotine exposure via constant-infusion osmotic pumps, one common criticism of the chronic oral administration nicotine exposure model is that this method of nicotine exposure creates a constant plasma level of nicotine, which does not recapitulate the human use pattern of regular cigarette smokers that results in varying plasma levels across the day with a decrease during the night.[67] Interestingly, however, as the majority of fluid consumption in rodents occurs during the active portion of the circadian cycle,[47] the oral consumption method could be expected to result in intermittent plasma nicotine levels better mimicking daytime smoking than delivery of nicotine via implanted osmotic pumps. Further, an important advantage of the oral administration model is that it is expected to be non-stressful, unlike other nicotine application methods such as daily injections. As stress has been shown to induce synaptic plasticity changes similar to that of addiction,[68] a potentially stressful nicotine administration model could complicate interpretations, that is, whether neurobiological changes in offspring were induced by nicotine treatment or maternal stress. However, when considering the effects of gestational nicotine exposure, the low clearance of the placenta suggest that the method of administration might not be as critical. Nicotine and its metabolites accumulate in the foetal circulation and fluids at higher levels than in the mother's blood,[69,70] and can be present significantly beyond the last exposure of the mother.[71] Hence, the foetus would not be expected to experience the same amount of fluctuation in plasma nicotine levels as the smoking mother. Taken together, the oral nicotine administration model used in the current study is expected to correlate sufficiently with the nicotine exposure expected from maternal smoking in humans. Regardless, no animal model can completely mimic smoking behaviour in humans and it should therefore always be taken as a caveat that the model chosen might determine the level of changes observed, if not the type of changes induced. For example, a study that compared two animal models of nicotine exposure indicated that, while the quality of the changes was not affected by method of exposure, that is, both methods induced region-specific changes in nAChR subunit composition, the degree of alteration depended on the administration method employed.[72]

PNE-induced altered AMPAR-mediated responses

Our findings suggest the possibility that early-life exposure to nicotine changes the numbers or properties, such as receptor desensitisation, of glutamate ionotropic receptors in the LDT. Ours is not the first study to examine changes that would be expected to impact on glutamate transmission mediated by AMPA receptors, which are altered by early-life exposure to nicotine. Others have reported that, following nicotine exposure during the 1st and 2nd week of gestation, a significant upregulation of GluA1, an AMPAR subunit, on PND 1 and a significant reduced expression of GluA2, another AMPAR subunit, as well as an overall significant decrease in AMPARs on PND 63 occur in rat hippocampus.[43] In another study, following initiation of nicotine exposure during the 1st gestational week, GluA1 levels were reduced at 3 weeks of age in the pups,[44] as well as levels of the vesicular transporter for glutamate.[44] Immunohistochemical studies demonstrated that levels of GluA2/3 were decreased in medullary respiratory neurons in the 1st postnatal week following PNE, which began in the second half of the 1st gestational week.[46] The potential cellular mechanisms underlying changes affecting AMPAR, as well as NMDAR, subunit expression in animals prenatally exposed to nicotine have been examined. The expression of the calcium/calmodulin-dependent protein-kinase II alpha (CaMKIIα), which mediates phosphorylation and subsequent activation of AMPARs, and the presence of the post-synaptic density protein 95 (PSD95) responsible for stabilising AMPARs and assembling signal transduction complexes, were analysed in hippocampi from exposed and non-exposed rats. An upregulation in the hippocampus of both these proteins on PND 1 extending to PND 14 for CaMKIIα was noted, which could partly explain the observed upregulation in AMPAR GluA1 subunit expression seen in the same study.[43] However, under different exposure protocols, reduced levels of PSD95 were found in the hippocampus following early-life nicotine, raising the possibility that the expression profile is age dependent, since in this study animals were examined at PND 21.[44]

Examination of alteration of currents mediated by AMPA receptors induced by PNE has been conducted within several nuclei. PNE was associated with a reduction in AMPA-mediated currents that was also reflected in a lower threshold for induction of long-term potentiation (LTP), which is an AMPA receptor-dependent event.[44,73] Interestingly, while whole cell currents mediated by AMPA receptors were not found to be reduced in medullary nuclei, a reduction in function controlled by these nuclei and mediated by AMPA receptors was detected,[46] suggesting to the authors that the kinetics of the conductance through the receptor had altered, perhaps reflecting a change in subunit composition. Alterations in subunit expression of AMPA receptors associated with PNE have never been investigated in the LDT but such drug-induced changes are a possibility based on our findings of smaller calcium rises and the alteration in kinetics of these rises in LDT cells from prenatally exposed animals. The calcium permeability of AMPARs has been shown to depend on their subunit composition; in fact the presence of GluA2 has been linked to low Ca^{2+} permeability.[74,75] Therefore, it is tempting to speculate based on our findings of reduced AMPA-stimulated calcium rises that prenatal nicotine is altering the ratio of receptors containing the GluA2 subunit so that the decreased AMPA-mediated calcium responses seen in the LDT of slices from prenatally nicotine-treated animals are the result of a shift from GluA2-lacking to GluA2-containing AMPARs. However, countering this interpretation are findings that prenatal cocaine exposure (gestational day 11–18) delays maturation of glutamatergic neurotransmission in the VTA, including the age-dependant switch from GluA2-lacking to

GluA2-containing AMPARs.[76,77] Since GluA2-containing AMPARs have been linked to decreased calcium permeability, such an action would result in increased calcium permeability of AMPARs in the prenatally cocaine exposed at early age, a mechanism, which would not explain the decreases in AMPA-induced calcium rises seen in the present study with prenatally nicotine exposed animals. However, different outcomes in subunit expression for AMPA receptors could occur based on nuclei where these changes are induced, and actions on alterations of subunit compositions may be specific to the individual drug to which the foetus has been exposed. Taken together with other studies showing that PNE induces functional changes in AMPA-mediated excitability and alterations in subunit expression for AMPA receptors, which could impact on calcium rises subsequent to AMPA receptor stimulation, our data are consistent with the interpretation that the observed decrease in AMPA-mediated calcium response in LDT neurons in gestationally nicotine-exposed slices is due to changes in subunit composition or delayed changes in subunit composition linked to neuronal maturation, caused by the PNE. Therefore, we suggest that the observed decrease in AMPA-mediated calcium responses in LDT slices from PNE animals is caused by changes in AMPAR quantity, subunit composition and/or calcium permeability. However, future experiments will have to be conducted to directly examine the mechanisms underlying reduced AMPAR-mediated calcium responses in the LDT. And since alterations seen could include, or be independent of, changes in AMPA receptors, which are not highly permeable to calcium, design of experiments examining changes in this population of receptors should also be conducted since they play a vital role in postnatal membrane excitability.

PNE-induced altered NMDAR-mediated responses

An activity-dependent enhancement of calcium mediated by NMDA receptors within the LDT noted in control animals is a novel finding. Although we did not observe differences in the calcium responses evoked by first application of NMDA in slices from prenatal nicotine-exposed animals compared with controls, we did see a significant difference in second to first response ratio. Our data suggests that PNE not only affects the AMPARs in LDT neurons, but also the NMDARs. Hence, PNE affects two important players of glutamatergic neurotransmission. But the way by which prenatal nicotine's influence is manifested in outcome following stimulation of these receptors is quite different. Previous studies of subunit expression within the hippocampus revealed a significant upregulation of NMDAR subunits GluN1, GluN2a and GluN2d on PND 1 and a decreased expression of subunits GluN1 and GluN2c on PND 63 in prenatally nicotine-exposed rats when compared with controls.[43] As it is likely that at least a population of NMDA responses sourced from GluN1-containing receptors, as mRNA for this subtype has been shown in the LDT,[39] strikingly, no significant differences were reported for this NMDA receptor subtype at PND 14, which is the age similar to that of the mice in our current study. Hence, the difference in calcium response observed upon second NMDA application could be due to activation of other mechanisms than any related to the subunit composition, or numbers, of the NMDAR. However, neurodevelopmental differences between rats and mice suggest that events observed at a certain postnatal period in rats may in fact not correlate with the exact same period of time in mice[78] and neuronal region-specific changes in NMDA receptors likely occur. The activity-dependent enhancement of responses to second applications of NMDA observed in control slices is reminiscent of synaptic LTP,[68] however, that NMDA receptor-dependent process is mediated by activity-dependent rises in AMPA receptor functioning, which was not seen in the present study. While rises in calcium have been shown to inhibit currents through NMDA receptors,[79–81] calcium entering through initial activation of the NMDA receptor could be expected to result in production of protein lipase C and protein kinase C with subsequent activation of Src, which has been shown to potentiate NMDA responses.[82–85] Other kinases, including, protein kinase A has also been shown to influence the calcium permeability of NMDA receptors.[86] While the mechanisms of the enhanced response to repeated NMDA application in control animals was not elucidated, clearly gestational exposure to nicotine resulted in alterations in processes underlying activity-dependent enhancement of NMDA receptor-mediated responses. These alterations could include PNE-induced differences in the numbers and/or kinetic characteristics of these receptors, or changes in the intracellular signalling processes activated by initial NMDA receptor activation, which are known to enhance or depress NMDA receptor function upon subsequent excitation. Future experiments will be needed to elucidate the specific mechanisms underlying the alteration in NMDA receptor function following PNE.

AMPAkines

To further study the differential AMPA-mediated calcium responses observed in prenatally exposed and control slices, we continued our investigations including the ampakine CYZ, a positive allosteric modulator of the AMPAR currently of great interest due to its cognitive enhancing properties. We showed that CYZ not only highly potentiated the effect of first applications of AMPA, but it also reversed the observed decrease in AMPA response upon consecutive applications. Therefore, while it was not the focus of this study, we have shown that the potentiating effect of CYZ established for other brain regions such as the hippocampus[87–89] and the nucleus accumbens,[90] extends to the LDT. Collaborating our finding that AMPA responses are altered by gestational nicotine, CYZ resulted in a larger magnitude of potentiation in control compared with PNE cells. Moreover, our experiments showed that a smaller proportion of cells in PNE slices responded with rises in calcium in response to CYZ application alone. Since we showed using DNQX that actions of CYZ were specific to potentiation

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of AMPA receptors, these data provide further evidence that gestational nicotine alters AMPA receptor cellular actions. CYZ has been shown to almost completely eliminate desensitisation of flip, one of the two splice variants of the AMPARs, whereas it only slows the process for flop, the other receptor variant.[74] It is therefore tempting to speculate that the reduced potentiating effect of CYZ observed in nicotine slices, may be due to a down regulation of the flip variant of AMPAR. The hypothesis that cholinergic activation during the prenatal period could alter splicing events, is supported by findings in primary neuronal cultures showing that alterations in neuronal excitability lead to post-transcriptional alterations resulting in reductions in mRNA for the flip variant.[91] Nevertheless, while the underlying molecular mechanism is unclear, when taken together, our experiments with the ampakine CYZ showed that its potentiating effect is decreased in nicotine slices compared with controls. Hence, they further support our standing hypothesis that PNE induces changes at the AMPAR, possibly decreasing glutamatergic neurotransmission in the LDT.

Functional significance of altered glutamate transmission in the LDT associated with PNE

From our pharmacological study, we conclude that PNE results in decreased glutamatergic neuroactivity in the LDT present at a postnatal early developmental age. We hypothesize that these changes found in young mice can be extrapolated to adolescent humans born of mothers who smoked during pregnancy and propose in line with current anatomical and physiological models of addiction, that the functional consequences of this change may underlie, in part, the increased susceptibility to addiction observed in prenatally exposed individuals. Activity within the LDT is importantly involved in processing of natural motivational stimuli considered healthy such as food, as well as in the processing of reward of stimuli that are maladaptive, such as drugs of abuse (for review see,[24]). Excitation of LDT–VTA pathways is considered critical to behaviourally relevant firing of DA–VTA neurons leading to neurobiological signal of reward for both natural and drug rewards.[21,31] Accordingly, the degree of excitation of LDT neurons would be expected to have a large impact on output from the LDT to midbrain targets and glutamate has been shown to play a major role in excitability of neurons of the LDT. Hypoglutamatergic tone in the LDT would be expected to result in a lower level of activity in the LDT, leading to a reduction of output from terminals to target regions, such as the VTA. Accordingly, prenatal exposure to nicotine could be associated with a hypoaroused LDT, resulting in reduced reward associated with natural reinforcers. This is analogous to suggestions that proclivity to addict to drugs of abuse is associated with a hypodopaminergic brain leading to an increased pleasurable sensation upon experimentation with hedonic substances and thereby heightened risk of developing dependence.[92–94] Accordingly, nicotine or use of other drugs of abuse, which activate the LDT greatly[95,96] could alter the relative subjective experience of reward by drugs of abuse leading to an increased pleasurable sensation in the hypoexcited brain and increased likelihood of smoking continuation and potentially nicotine addiction. Further, smoking in prenatally nicotine-exposed individuals could be interpreted as self-medication in an attempt to achieve a normal activity level of the circuit. Studies showing an increased frequency of smoking in schizophrenics have led to the suggestion of a potential similar phenomenon of nicotine self-medication in this disorder.[97] In summary, while the human situation is likely to be complicated by factors of environmental influences, as well as possible contributions from epigenetic processes induced by pre-conception exposures to nicotine or other drugs of abuse (for review, see[98]), we believe our findings of reduced glutamate activation within the LDT induced by PNE suggest a neurobiological mechanism, which contributes to the observed increased susceptibility to smoking, and use of other drugs of abuse, detected in individuals prenatally exposed to nicotine.

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Conflicts of Interest

The authors disclose that they have no conflicts of interest with respect to this manuscript.

Ethical Standards

All animal studies were conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) as well as with Danish legislations. The Animal Welfare Committee, appointed by the Danish Ministry of Justice, approved the animal study after determining that efforts to diminish and explore alternatives to animal experiments and to minimize animal suffering had been made.

References

1. Slotkin T. Consensus on postnatal deficits: comparability of human and animal findings. *Ann N Y Acad Sci*. 1998; 846, 153–157.
2. Slotkin TA. Nicotine and the adolescent brain: insights from an animal model. *Neurotoxicol Teratol*. 2002; 24, 369–384.
3. Slotkin TA. Cholinergic systems in brain development and disruption by neurotoxicants: nicotine, environmental tobacco smoke, organophosphates. *Toxicol Appl Pharmacol*. 2004; 198, 132–151.

4. Arria AM, Derauf C, Lagasse LL, *et al*. Methamphetamine and other substance use during pregnancy: preliminary estimates from the infant development, environment, and lifestyle (ideal) study. *Matern Child Health J*. 2006; 10, 293–302.
5. Martin JA, Hamilton BE, Ventura SJ, Menacker F, Park MM. Births: final data for 2000. *Natl Vital Stat Rep*. 2002; 50, 1–101.
6. Martin JA, Hamilton BE, Ventura SJ, Menacker F, Park MM. Births: final data for 2001. *Natl Vital Stat Rep*. 2002; 51, 1–102.
7. Nelson DE, Giovino GA, Shopland DR, *et al*. Trends in cigarette smoking among US adolescents, 1974 through 1991. *Am J Public Health*. 1995; 85, 34–40.
8. Blood-Siegfried J, Rende EK. The long-term effects of prenatal nicotine exposure on neurologic development. *J Midwifery Womens Health*. 2010; 55, 143–152.
9. Slotkin TA, Orband-Miller L, Queen KL, Whitmore WL, Seidler FJ. Effects of prenatal nicotine exposure on biochemical development of rat brain regions: maternal drug infusions via osmotic minipumps. *J Pharmacol Exp Ther*. 1987; 240, 602–611.
10. Navarro HA, Seidler FJ, Eylers JP, *et al*. Effects of prenatal nicotine exposure on development of central and peripheral cholinergic neurotransmitter systems. evidence for cholinergic trophic influences in developing brain. *J Pharmacol Exp Ther*. 1989; 251, 894–900.
11. Navarro HA, Seidler FJ, Schwartz RD, *et al*. Prenatal exposure to nicotine impairs nervous system development at a dose which does not affect viability or growth. *Brain Res Bull*. 1989; 23, 187–192.
12. Kandel DB, Wu P, Davies M. Maternal smoking during pregnancy and smoking by adolescent daughters. *Am J Public Health*. 1994; 84, 1407–1413.
13. Cornelius MD, Leech SL, Goldschmidt L, Day NL. Prenatal tobacco exposure: is it a risk factor for early tobacco experimentation? *Nicotine Tob Res*. 2000; 2, 45–52.
14. Cornelius MD, Goldschmidt L, Day NL. Prenatal cigarette smoking: long-term effects on young adult behavior problems and smoking behavior. *Neurotoxicol Teratol*. 2012; 34, 554–559.
15. Goldschmidt L, Cornelius MD, Day NL. Prenatal cigarette smoke exposure and early initiation of multiple substance use. *Nicotine Tob Res*. 2012; 14, 694–702.
16. Al Mamun A, O'Callaghan FV, Alati R, *et al*. Does maternal smoking during pregnancy predict the smoking patterns of young adult offspring? A birth cohort study. *Tob Control*. 2006; 15, 452–457.
17. Buka SL, Shenassa ED, Niaura R. Elevated risk of tobacco dependence among offspring of mothers who smoked during pregnancy: a 30-year prospective study. *Am J Psychiatry*. 2003; 160, 1978–1984.
18. Shenassa ED, McCaffery JM, Swan GE, *et al*. Intergenerational transmission of tobacco use and dependence: a transdisciplinary perspective. *Nicotine Tob Res*. 2003; 5(Suppl. 1), S55–S69.
19. Oncken C, McKee S, Krishnan-Sarin S, O'Malley S, Mazure C. Gender effects of reported in utero tobacco exposure on smoking initiation, progression and nicotine dependence in adult offspring. *Nicotine Tob Res*. 2004; 6, 829–833.
20. Lammel S, Lim BK, Ran C, *et al*. Input-specific control of reward and aversion in the ventral tegmental area. *Nature*. 2012; 491, 212–217.
21. Lodge DJ, Grace AA. The laterodorsal tegmentum is essential for burst firing of ventral tegmental area dopamine neurons. *Proc Natl Acad Sci U S A*. 2006; 103, 5167–5172.
22. Forster GL, Blaha CD. Laterodorsal tegmental stimulation elicits dopamine efflux in the rat nucleus accumbens by activation of acetylcholine and glutamate receptors in the ventral tegmental area. *Eur J Neurosci*. 2000; 12, 3596–3604.
23. Forster GL, Falcon AJ, Miller AD, Heruc GA, Blaha CD. Effects of laterodorsal tegmentum excitotoxic lesions on behavioral and dopamine responses evoked by morphine and d-amphetamine. *Neuroscience*. 2002; 114, 817–823.
24. Kohlmeier KA. Off the beaten path: drug addiction and the pontine laterodorsal tegmentum. *ISRN Neurosci*. 2013; 2013, 24pp.
25. Omelchenko N, Sesack SR. Laterodorsal tegmental projections to identified cell populations in the rat ventral tegmental area. *J Comp Neurol*. 2005; 483, 217–235.
26. Omelchenko N, Sesack SR. Cholinergic axons in the rat ventral tegmental area synapse preferentially onto mesoaccumbens dopamine neurons. *J Comp Neurol*. 2006; 494, 863–875.
27. Dautan D, Huerta-Ocampo I, Witten IB, *et al*. A major external source of cholinergic innervation of the striatum and nucleus accumbens originates in the brainstem. *J Neurosci*. 2014; 34, 4509–4518.
28. Drevets WC, Gautier C, Price JC, *et al*. Amphetamine-induced dopamine release in human ventral striatum correlates with euphoria. *Biol Psychiatry*. 2001; 49, 81–96.
29. Pontieri FE, Tanda G, Di Chiara G. Intravenous cocaine, morphine, and amphetamine preferentially increase extracellular dopamine in the 'shell' as compared with the 'core' of the rat nucleus accumbens. *Proc Natl Acad Sci U S A*. 1995; 92, 12304–12308.
30. Pontieri FE, Tanda G, Orzi F, Di Chiara G. Effects of nicotine on the nucleus accumbens and similarity to those of addictive drugs. *Nature*. 1996; 382, 255–257.
31. Grace AA, Floresco SB, Goto Y, Lodge DJ. Regulation of firing of dopaminergic neurons and control of goal-directed behaviors. *Trends Neurosci*. 2007; 30, 220–227.
32. Maskos U. The cholinergic mesopontine tegmentum is a relatively neglected nicotinic master modulator of the dopaminergic system: relevance to drugs of abuse and pathology. *Br J Pharmacol*. 2008; 153(Suppl. 1), S438–S445.
33. Mameli-Engvall M, Evrard A, Pons S, *et al*. Hierarchical control of dopamine neuron-firing patterns by nicotinic receptors. *Neuron*. 2006; 50, 911–921.
34. Atluri P, Fleck MW, Shen Q, *et al*. Functional nicotinic acetylcholine receptor expression in stem and progenitor cells of the early embryonic mouse cerebral cortex. *Dev Biol*. 2001; 240, 143–156.
35. Schneider AS, Atluri P, Shen Q, *et al*. Functional nicotinic acetylcholine receptor expression on stem and progenitor cells of the early embryonic nervous system. *Ann N Y Acad Sci*. 2002; 971, 135–138.
36. Kohlmeier KA. Nicotine during pregnancy-changes induced in neurotransmission which could heighten proclivity to addict and induce maladaptive control of attention. *J Devel Orig Health Dis.* 2014; in press, doi:10.1017/S2040174414000531.

37. Christensen MH, Nielsen ML, Kohlmeier KA. Electrophysiological changes in laterodorsal tegmental neurons associated with prenatal nicotine exposure: implications for heightened susceptibility to addict to drugs of abuse. *J Devel Orig Health Dis.* 2014; E-pub 23 October 2014; doi:10.1017/S204017441400049X.
38. Inglis WL, Semba K. Discriminable excitotoxic effects of ibotenic acid, AMPA, NMDA and quinolinic acid in the rat laterodorsal tegmental nucleus. *Brain Res*. 1997; 755, 17–27.
39. Inglis WL, Semba K. Colocalization of ionotropic glutamate receptor subunits with NADPH-diaphorase-containing neurons in the rat mesopontine tegmentum. *J Comp Neurol*. 1996; 368, 17–32.
40. Sanchez RM, Surkis A, Leonard CS. Voltage-clamp analysis and computer simulation of a novel cesium-resistant A-current in guinea pig laterodorsal tegmental neurons. *J Neurophysiol*. 1998; 79, 3111–3126.
41. Sesack SR, Deutch AY, Roth RH, Bunney BS. Topographical organization of the efferent projections of the medial prefrontal cortex in the rat: an anterograde tract-tracing study with *Phaseolus vulgaris* leucoagglutinin. *J Comp Neurol*. 1989; 290, 213–242.
42. Kohlmeier KA, Ishibashi M, Wess J, Bickford ME, Leonard CS. Knockouts reveal overlapping functions of M(2) and M(4) muscarinic receptors and evidence for a local glutamatergic circuit within the laterodorsal tegmental nucleus. *J Neurophysiol*. 2012; 108, 2751–2766.
43. Wang H, Davila-Garcia MI, Yarl W, Gondre-Lewis MC. Gestational nicotine exposure regulates expression of AMPA and NMDA receptors and their signaling apparatus in developing and adult rat hippocampus. *Neuroscience*. 2011; 188, 168–181.
44. Parameshwaran K, Buabeid MA, Karuppagounder SS, *et al*. Developmental nicotine exposure induced alterations in behavior and glutamate receptor function in hippocampus. *Cell Mol Life Sci*. 2012; 69, 829–841.
45. Parameshwaran K, Buabeid MA, Bhattacharya S, *et al*. Long term alterations in synaptic physiology, expression of beta2 nicotinic receptors and ERK1/2 signaling in the hippocampus of rats with prenatal nicotine exposure. *Neurobiol Learn Mem*. 2013; 106, 102–111.
46. Jaiswal SJ, Pilarski JQ, Harrison CM, Fregosi RF. Developmental nicotine exposure alters AMPA neurotransmission in the hypoglossal motor nucleus and pre-Botzinger complex of neonatal rats. *J Neurosci*. 2013; 33, 2616–2625.
47. Pauly JR, Sparks JA, Hauser KF, Pauly TH. In utero nicotine exposure causes persistent, gender-dependant changes in locomotor activity and sensitivity to nicotine in C57Bl/6 mice. *Int J Dev Neurosci*. 2004; 22, 329–337.
48. Matta SG, *et al*. Guidelines on nicotine dose selection for in vivo research. *Psychopharmacology (Berl)*. 2007; 190, 269–319.
49. Bachmanov AA, Reed DR, Beauchamp GK, Tordoff MG. Food intake, water intake, and drinking spout side preference of 28 mouse strains. *Behav Genet*. 2002; 32, 435–443.
50. Schneider T, Bizarro L, Asherson PJ, Stolerman IP. Gestational exposure to nicotine in drinking water: teratogenic effects and methodological issues. *Behav Pharmacol*. 2010; 21, 206–216.
51. Leichter J, Lee M. Does dehydration contribute to retarded fetal growth in rats exposed to alcohol during gestation? *Life Sci*. 1984; 35, 2105–2111.
52. Yuste RF, Lanni F, Konnerth A. *Imaging Neurons: A Laboratory Manual*. 2000. ColSpring Harbor Laboratory Press: Cold Spring Harbor, New York.
53. Kohlmeier KA, Inoue T, Leonard CS. Hypocretin/orexin peptide signaling in the ascending arousal system: elevation of intracellular calcium in the mouse dorsal raphe and laterodorsal tegmentum. *J Neurophysiol*. 2004; 92, 221–235.
54. Connor JA, Cormier RJ. Cumulative effects of glutamate microstimulation on Ca(2+) responses of CA1 hippocampal pyramidal neurons in slice. *J Neurophysiol*. 2000; 83, 90–98.
55. Kohlmeier KA, Vardar B, Christensen MH. Gamma-hydroxybutyric acid induces actions via the GABA receptor in arousal and motor control-related nuclei: implications for therapeutic actions in behavioral state disorders. *Neuroscience*. 2013; 248C, 261–277.
56. Kohlmeier KA, Leonard CS. Transmitter modulation of spike-evoked calcium transients in arousal related neurons: muscarinic inhibition of SNX-482-sensitive calcium influx. *Eur J Neurosci*. 2006; 23, 1151–1162.
57. Kohlmeier KA, Watanabe S, Tyler CJ, Burlet S, Leonard CS. Dual orexin actions on dorsal raphe and laterodorsal tegmentum neurons: noisy cation current activation and selective enhancement of Ca2+ transients mediated by L-type calcium channels. *J Neurophysiol*. 2008; 100, 2265–2281.
58. Vincent SR, Kimura H. Histochemical mapping of nitric oxide synthase in the rat brain. *Neuroscience*. 1992; 46, 755–784.
59. Mychasiuk R, Muhammad A, Carroll C, Kolb B. Does prenatal nicotine exposure alter the brain's response to nicotine in adolescence? A neuroanatomical analysis. *Eur J Neurosci*. 2013; 38, 2491–2503.
60. Mychasiuk R, Muhammad A, Gibb R, Kolb B. Long-term alterations to dendritic morphology and spine density associated with prenatal exposure to nicotine. *Brain Res*. 2013; 1499, 53–60.
61. Berridge MJ. Neuronal calcium signaling. *Neuron*. 1998; 21, 13–26.
62. Wang HL, Morales M. Pedunculopontine and laterodorsal tegmental nuclei contain distinct populations of cholinergic, glutamatergic and GABAergic neurons in the rat. *Eur J Neurosci*. 2009; 29, 340–358.
63. Jia HG, Yamuy J, Sampogna S, Morales FR, Chase MH. Colocalization of gamma-aminobutyric acid and acetylcholine in neurons in the laterodorsal and pedunculopontine tegmental nuclei in the cat: a light and electron microscopic study. *Brain Res*. 2003; 992, 205–219.
64. Mieda M, Hasegawa E, Kisanuki YY, *et al*. Differential roles of orexin receptor-1 and -2 in the regulation of non-REM and REM sleep. *J Neurosci*. 2011; 31, 6518–6526.
65. Wang H, Gondre-Lewis MC. Prenatal nicotine and maternal deprivation stress de-regulate the development of CA1, CA3, and dentate gyrus neurons in hippocampus of infant rats. *PLoS One*. 2013; 8, e65517.
66. Berlin I, Heilbronner C, Georgieu S, Meier C, Spreux-Varoquaux O. Newborns' cord blood plasma cotinine concentrations are similar to that of their delivering smoking mothers. *Drug Alcohol Depend*. 2010; 107, 250–252.
67. Rang HP. *Rang and Dale's Pharmacology*. 2007. Elsevier: Edinburgh, Philadelphia.
68. Kauer JA, Malenka RC. Synaptic plasticity and addiction. *Nat Rev Neurosci*. 2007; 8, 844–858.

69. Lambers DS, Clark KE. The maternal and fetal physiologic effects of nicotine. *Semin Perinatol*. 1996; 20, 115–126.
70. Jauniaux E, Gulbis B, Acharya G, Thiry P, Rodeck C. Maternal tobacco exposure and cotinine levels in fetal fluids in the first half of pregnancy. *Obstet Gynecol*. 1999; 93, 25–29.
71. Marin SJ, Christensen RD, Baer VL, Clark CJ, McMillin GA. Nicotine and metabolites in paired umbilical cord tissue and meconium specimens. *Ther Drug Monit*. 2011; 33, 80–85.
72. Moretti M, Mugnaini M, Tessari M, *et al*. A comparative study of the effects of the intravenous self-administration or subcutaneous minipump infusion of nicotine on the expression of brain neuronal nicotinic receptor subtypes. *Mol Pharmacol*. 2010; 78, 287–296.
73. Vaglenova J, Parameshwaran K, Suppiramaniam V, *et al*. Long-lasting teratogenic effects of nicotine on cognition: gender specificity and role of AMPA receptor function. *Neurobiol Learn Mem*. 2008; 90, 527–536.
74. Traynelis SF, Wollmuth LP, McBain CJ, *et al*. Glutamate receptor ion channels: structure, regulation, and function. *Pharmacol Rev*. 2010; 62, 405–496.
75. Liu SJ, Savtchouk I. Ca(2+) permeable AMPA receptors switch allegiances: mechanisms and consequences. *J Physiol*. 2012; 590, 13–20.
76. Bellone C, Mameli M, Luscher C. In utero exposure to cocaine delays postnatal synaptic maturation of glutamatergic transmission in the VTA. *Nat Neurosci*. 2011; 14, 1439–1446.
77. Mameli M, Bellone C, Brown MT, Luscher C. Cocaine inverts rules for synaptic plasticity of glutamate transmission in the ventral tegmental area. *Nat Neurosci*. 2011; 14, 414–416.
78. Clancy B, Darlington RB, Finlay BL. Translating developmental time across mammalian species. *Neuroscience*. 2001; 105, 7–17.
79. Legendre P, Rosenmund C, Westbrook GL. Inactivation of NMDA channels in cultured hippocampal neurons by intracellular calcium. *J Neurosci*. 1993; 13, 674–684.
80. Lieberman DN, Mody I. Regulation of NMDA channel function by endogenous Ca(2+)-dependent phosphatase. *Nature*. 1994; 369, 235–239.
81. Rosenmund C, Feltz A, Westbrook GL. Calcium-dependent inactivation of synaptic NMDA receptors in hippocampal neurons. *J Neurophysiol*. 1995; 73, 427–430.
82. Benquet P, Gee CE, Gerber U. Two distinct signaling pathways upregulate NMDA receptor responses via two distinct metabotropic glutamate receptor subtypes. *J Neurosci*. 2002; 22, 9679–9686.
83. Salter MW. Src, N-methyl-D-aspartate (NMDA) receptors, and synaptic plasticity. *Biochem Pharmacol*. 1998; 56, 789–798.
84. Lu WY, Xiong ZG, Lei S, *et al*. G-protein-coupled receptors act via protein kinase C and Src to regulate NMDA receptors. *Nat Neurosci*. 1999; 2, 331–338.
85. Huang Y, Lu W, Ali DW, *et al*. CAKbeta/Pyk2 kinase is a signaling link for induction of long-term potentiation in CA1 hippocampus. *Neuron*. 2001; 29, 485–496.
86. Skeberdis VA, Chevaleyre V, Lau CG, *et al*. Protein kinase A regulates calcium permeability of NMDA receptors. *Nat Neurosci*. 2006; 9, 501–510.
87. Desai MA, Burnett JP, Ornstein PL, Schoepp DD. Cyclothiazide acts at a site on the alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor complex that does not recognize competitive or noncompetitive AMPA receptor antagonists. *J Pharmacol Exp Ther*. 1995; 272, 38–43.
88. Yamada KA, Tang CM. Benzothiadiazides inhibit rapid glutamate receptor desensitization and enhance glutamatergic synaptic currents. *J Neurosci*. 1993; 13, 3904–3915.
89. Donevan SD, Rogawski MA. Allosteric regulation of alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionate receptors by thiocyanate and cyclothiazide at a common modulatory site distinct from that of 2,3-benzodiazepines. *Neuroscience*. 1998; 87, 615–629.
90. Grilli M, Summa M, Salamone A, *et al*. In vitro exposure to nicotine induces endocytosis of presynaptic AMPA receptors modulating dopamine release in rat nucleus accumbens nerve terminals. *Neuropharmacology*. 2012; 63, 916–926.
91. Orlandi C, La Via L, Bonini D, *et al*. AMPA receptor regulation at the mRNA and protein level in rat primary cortical cultures. *PLoS One* 2011; 6, e25350.
92. Volkow ND, Fowler JS, Wang GJ, Swanson JM. Dopamine in drug abuse and addiction: results from imaging studies and treatment implications. *Mol Psychiatry*. 2004; 9, 557–569.
93. Volkow ND, Li TK. Drug addiction: the neurobiology of behaviour gone awry. *Nat Rev Neurosci*. 2004; 5, 963–970.
94. Melis M, Spiga S, Diana M. The dopamine hypothesis of drug addiction: hypodopaminergic state. *Int Rev Neurobiol*. 2005; 63, 101–154.
95. Ishibashi M, Leonard CS, Kohlmeier KA. Nicotinic activation of laterodorsal tegmental neurons: implications for addiction to nicotine. *Neuropsychopharmacology*. 2009; 34, 2529–2547.
96. Christensen MH, Ishibashi M, Nielsen ML, Leonard CS, Kohlmeier KA. Age-related changes in nicotine response of cholinergic and non-cholinergic laterodorsal tegmental neurons: implications for the heightened adolescent susceptibility to nicotine addiction. *Neuropharmacology*. 2014; 85, 263–283.
97. de Leon J, Dadvand M, Canuso C, *et al*. Schizophrenia and smoking: an epidemiological survey in a state hospital. *Am J Psychiatry*. 1995; 152, 453–455.
98. Vassoler FM, Byrnes EM, Pierce RC. The impact of exposure to addictive drugs on future generations: physiological and behavioral effects. *Neuropharmacology*. 2014; 76, 269–275.