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Age-related changes in nicotine response of cholinergic and non-cholinergic laterodorsal tegmental neurons: Implications for the heightened adolescent susceptibility to nicotine addiction

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ABSTRACT

The younger an individual starts smoking, the greater the likelihood that addiction to nicotine will develop, suggesting that neurobiological responses vary across age to the addictive component of cigarettes. Cholinergic neurons of the laterodorsal tegmental nucleus (LDT) are importantly involved in the development of addiction, however, the effects of nicotine on LDT neuronal excitability across ontogeny are unknown. Nicotinic effects on LDT cells across different age groups were examined using calcium imaging and whole-cell patch clamping. Within the youngest age group (P7–P15), nicotine induced larger intracellular calcium transients and inward currents. Nicotine induced a greater number of excitatory synaptic currents in the youngest animals, whereas larger amplitude inhibitory synaptic events were induced in cells from the oldest animals (P15–P34). Nicotine increased neuronal firing of cholinergic cells to a greater degree in younger animals, possibly linked to development associated differences found in nicotinic effects on action potential shape and afterhyperpolarization. We conclude that in addition to age-associated alterations of several properties expected to affect resting cell excitability, parameters affecting cell excitability are altered by nicotine differentially across ontogeny. Taken together, our data suggest that nicotine induces a larger excitatory response in cholinergic LDT neurons from the youngest animals, which could result in a greater excitatory output from these cells to target regions involved in development of addiction. Such output would be expected to be promotive of addiction; therefore, ontogenetic differences in nicotine-mediated increases in the excitability of the LDT could contribute to the differential susceptibility to nicotine addiction seen across age.

1. Introduction

The single largest cause of preventable death in the world is directly linked to tobacco smoking. Smoking-related diseases claim an estimated five million people each year and this figure will likely continue to grow with the increasing spread of tobacco into developing countries (Mackay and Crofton, 1996). Abstinence from smoking is hindered by development of drug dependency to nicotine, the psychobiologically-relevant compound in tobacco-containing products. Conventional medical guidelines (Diagnostic and Statistical Manual of Mental Disorders, 5th Edition (DSM-V)) suggest that individuals must exceed a certain threshold of nicotine exposure before they can become addicted (American Psychiatric Association, 2013). Non-habitual use of nicotine in individuals with exposures below this threshold is believed to be due to a variety of psychosocial reasons distinct from neurobiological addiction. However, evidence-based clinical medicine indicates that individuals can become addicted with a much lower exposure than that suggested by the DSM-V. Indicating that neuroadaptations have occurred even in low exposure individuals, abstinence from nicotine is associated with signs of withdrawal and craving (DiFranza et al., 2011; Shiffman et al., 1995). Especially worrisome is the apparently increased proclivity of adolescences to addict to drugs of abuse. Adolescents have been shown to exhibit symptoms of addiction to nicotine within a few weeks of limited exposure such as two cigarettes a week, and withdrawal symptoms can

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appear in adolescents just two days after their first cigarette (DiFranza, 2008; DiFranza et al., 2000; Scragg et al., 2008). Although it is unknown whether adolescents perceive more reward from nicotine exposure than do older individuals, one possible mechanism, which is not mutually exclusive from other processes underlying their enhanced sensitivity to development of signs of addiction (Doremus-Fitzwater et al., 2010), is that nicotine is more excitatory in the young within the neurobiological reward circuitry involved in assigning a positive valiance to environmental stimuli.

The laterodorsal tegmental nucleus (LDT) is a pontine nucleus, comprised of distinct populations of cholinergic, GABAergic and glutamate containing cells, as well as neurons which co-localize ACh and GABA (Jia et al., 2003; Mieda et al., 2011; Wang and Morales, 2009). Long studied for it's role in arousal, this nucleus has recently been identified as an important component in the neurocircuitry underlying addiction-related processes (Dautan et al., 2014; Forster and Blaha, 2000; Lammel et al., 2012; Lodge and Grace, 2006; Nelson et al., 2007). The LDT sends cholinergic and glutamatergic projections to the midbrain ventral tegmental area (VTA) with a large proportion of this input synapsing on dopamine (DA)-containing cells (Omelchenko and Sesack, 2006) within this nucleus. Large effluxes of DA from VTA cells at terminals within the nucleus accumbens (NAcc) are believed to signal relevancy of behaviorally-motivating stimuli, such as drugs of abuse. Cholinergic input to the VTA, probably arising in large part from the LDT (Omelchenko and Sesack, 2005, 2006) is predominately excitatory (Mameli-Engvall et al., 2006; Melis et al., 2013), acting at nicotinic acetylcholine receptors (nAChRs) and muscarinic acetylcholine receptors (mAChRs) demonstrated on DA-containing cells (Miller and Blaha, 2005; Pidoplichko et al., 2004). Supporting the conclusion that the LDT-VTA pathway is functionally important, inactivation of the LDT prohibits the DA-containing VTA neurons from burst firing (Lodge and Grace, 2006), a firing pattern required for behaviorally relevant release of DA from these cells (Floresco et al., 2003; Grace and Onn, 1989). Furthermore, optogenetic stimulation of the LDT-VTA pathway is sufficient to induce addictive-related behavior in mice, indicating that stimulation of LDT neurons can prompt reward, possibly involving glutamate release in the VTA (Lammel et al., 2012). These and other studies (Forster and Blaha, 2000) indicate that the LDT plays an important role in addiction processes. Accordingly, it is important to understand how drugs of abuse, such as nicotine, affect cholinergic and glutamatergic cellular activity within the LDT, which would be expected to affect output to target midbrain reward-related nuclei.

Based on the clinical data showing a heightened sensitivity of adolescents to addict to nicotine and the role of the LDT in the reward circuitry, we hypothesized that actions of nicotine on cholinergic LDT neurons vary across ontogeny, resulting in a differential response to nicotine in younger individuals from responses elicited in LDT neurons from older animals. Although we have previously shown that nicotine and other nAChR agonists have strong actions on the membrane excitability of cholinergic and non-cholinergic LDT neurons via both pre- and postsynaptic mechanisms (Ishibashi et al., 2009), we did not examine age-related differential effects of this drug on LDT cellular activity. Accordingly, in the present study, we examined our hypothesis of an age-related difference in nicotinic actions using *in vitro* calcium imaging and patch clamp electrophysiology to compare nicotine-induced actions on cholinergic and non-cholinergic LDT neurons across different ranges of postnatal ages. Additionally, we examined ontogenetic changes in nicotine-induced synaptic input to cholinergic and non-cholinergic LDT neurons. Taken together, our data indicate that age-associated nicotinic activation of cholinergic LDT neurons could result in a greater excitation of DA-containing VTA cells in younger animals and, in conjunction with known direct activation by nicotine of DA-VTA neurons, contribute to the processes of assigning reward to this drug of abuse differentially across ontogeny.

2. Methods

2.1. Animals

These studies were performed in mice across different developmental stages. The gestational period of a mouse is roughly equivalent to the first and second trimesters of human development and the first week of postnatal life corresponds to the third trimester of human gestation (Bayer et al., 1993; Dobbing and Sands, 1979). All animal studies complied with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and with Danish laws regulating experiments on animals. After determining that efforts to reduce the number of animals used, to explore alternatives to animal experiments and to minimize animal suffering had been made, the animal studies were permitted by the Animal Welfare Committee, appointed by the Danish Ministry of Justice.

2.2. Tissue preparation

NMRI (Taconic, Denmark or Harlan, The Netherlands) or C57 mice (Taconic, New York) (age 7–34 days) were anaesthetized with isofluorane and decapitated. A block of the brain containing the LDT was extracted into ice-cold artificial cerebrospinal fluid (ACSF) (in mM: NaCl 124, KCl 5, Na_2HPO_4 1.2, $CaCl_2$ 2.7, $MgSO_4$ (anhydrous) 1.2, Dextrose 10, $NaHCO_3$ 26, oxygenated in 95% oxygen/5% carbogen) and subsequently mounted into the cutting chamber of a Leica vibrotome (VT1200S). Coronal slices (250 μM) were cut in ice-cold oxygenated ACSF and subsequently incubated for 15 min at 37 °C. Recordings were conducted at room temperature.

2.3. Calcium imaging

As a high yield, rapid screen for nicotine-induced cellular activity across different developmental ages, we used "bulk-loading" calcium imaging which allows monitoring of large numbers of cells (Fig. 1 A) without complete change of the cytoplasm, including second messengers that may be important in the mediation of responses following drug exposures. Since this is a high yield method, we were able to monitor adequate numbers of cells to allow examination of age-related changes in nicotine-induced intracellular calcium changes across three relatively narrow age groups. To this end, we incubated slices from three different age groups (group A, postnatal day (P)8–P10; group B, P11–P15; group C, P16–P21) with the calcium indicator dye, fura 2-AM (15 μM in DMSO; Molecular probes, Invitrogen, Denmark), which crosses cellular membranes and following de-esterification, becomes trapped intracellularly (Tsien, 1981). Within the LDT, this calcium binding dye allows measurements of changes in intracellular calcium concentrations as, when excited by the appropriate wavelength, the dye alters its emission spectrum when calcium is bound (Kohlmeier et al., 2004). Brain slices were incubated in oxygenated dye at 31 °C for 10 min plus 1 min for every day of age. Using this method of dye loading, the quality of the fura 2-AM loading of LDT neurons decreases dramatically after three weeks of postnatal age. Therefore, only NMRI mice aged P7–P21 days were utilized. After the appropriate time in the dye solution, the slice was placed in the recording chamber and rinsed for 30 min to remove excess dye before optical recordings began. The LDT was located using a 4× objective and neurons were visualized under differential interference contrast optics with a 40× water immersion objective (NA 0.8, Olympus, Germany), mounted on a fixed stage microscope (Olympus BX50WI, Germany). Neurons were visualized and fluorescent measurements were made using a frame-transfer, cooled 12 bit CCD camera system (Sensicam, PCO Instruments, Germany) controlled by TILL-VISION software (Till Photonics, Germany) and an xenon light source (Osram, Germany). Initially, a high-resolution, full frame image was acquired. Regions of interest (ROI) within this full frame image were selected to encompass dye loaded cells. For data collection, each pixel within this ROI was summed according to binning parameters best selected to balance the spatial and temporal resolution (2 × 2). Fura 2-AM is a ratiometric dye and therefore fluorescence changes were measured using excitation at two different wavelengths (340 and 380 nm) and values obtained were ratioed ($F = F_{340}/F_{380}$) following subtraction of autofluorescence, which was determined from a ROI selected in the field where dye-filled cells were not apparent at any level of focus. Temporal and spatial changes are presented using the equation dF/F. In this equation, the difference in fluorescence at rest is subtracted from the maximum change in fluorescence (dF), which is then divided by the fluorescence at rest (F) with upward going deflections indirectly indicating rises in calcium. Exposure times for each wavelength were selected so that the brightest pixel in the field was between 7 and 10% of the upper limit of the dynamic range which reduced bleaching of the dye and phototoxicity due to overexposure. Dye loading was found to vary from slice to slice, as well as from cell to cell. To determine whether drug-induced changes were due to poorer loading in older animals, the baseline fluorescence of both wavelengths was examined and found to differ across the three age groups with a higher intensity in the older groups (340 nm group A, $n = 427$, $F = 106.8 \pm 2.2$; group B, $n = 396$, $F = 142.4 \pm 2.6$; group C, $n = 340$, $F = 131.7 \pm 2.6$; and 380 nm group A, $n = 387$, $F = 139 \pm 3.3$; group B, $n = 390$, $F = 182 \pm 3.3$; group C, $n = 339$, $F = 165.6 \pm 3.6$; one