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Research report

Activation of immediate early genes by nicotine after chronic neonatal nicotine exposure in brain areas involved in stress and anxiety responses

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ABSTRACT

Maternal smoking has negative long-term consequences on affective behaviors, and in rodents, chronic neonatal nicotine exposure (CNN) results in increased anxiety. In rat pups, acute nicotine stimulation activates brain regions associated with stress and anxiety, but chronic nicotine exposure could desensitize of nicotinic acetylcholine receptors, the molecular target of nicotine. Here, we determined whether CNN affected neuronal activation by an acute nicotine challenge. Using in situ hybridization, we analyzed mRNA expression of the immediate-early genes (IEGs) c-Fos, Arc, Egr-1 and Npas4, which are markers for neuronal activation and implicated in synaptic plasticity. Following CNN (6 mg/kg/day) or control treatment from postnatal day (P)1 to P7, an acute i.p. nicotine (0.7 mg/kg) or saline injection (control) was administered on P8, and brains collected after 30 min. In drug-naive pups, acute nicotine stimulated IEGs expression specifically in brain areas associated with innate anxiety including the paraventricular hypothalamic nucleus, central nucleus of the amygdala (CeA), and locus coeruleus (LC). Following CNN, acute nicotine stimulated IEG expression in all three areas, but activation was significantly reduced in the LC (c-Fos, Egr-1, Npas4), and CeA (c-Fos). Notably, nicotine-induced Npas4 expression was greatly diminished in the LC, which may affect inhibitory synapse formation in noradrenergic neurons. Thus, after CNN, neurons located in areas associated with anxiety brain circuitry maintained responsiveness to nicotine, but tolerance differentially developed to nicotine. In the developing brain, repeated activation by nicotine of areas related to limbic pathways could alter circuit connectivity and increase responsiveness to stress and anxiety later in life.

1. Introduction

Maternal smoking or gestational exposure to nicotine has adverse behavioral consequences in the offspring (Abbott and Winzer-Serhan, 2012; Winzer-Serhan, 2008). In rodents, one of the most consistently reported long-term negative outcomes of developmental exposure to nicotine is an increase in innate anxiety-like behavior (Vaglenova et al., 2004, Huang et al., 2007, Eppolito et al., 2010, Lee et al., 2016), which may create a predisposition to anxiety-related disorders later in life. The underlying mechanisms for this long-lasting effect are unclear. However, during critical developmental periods, repeated activation of neuronal nicotinic acetylcholine receptors (nAChRs) by nicotine could alter functional connectivity in stress- and anxiety-related brain areas and pathways, resulting in enhanced anxious responses to environmental stressors (Leonardo and Hen, 2008).

Several interconnected brain areas are involved in the control of stress and anxiety-like behaviors. In particular, the amygdala, the locus coeruleus (LC) noradrenergic system, and the hypothala mic–pituitaryadrenal (HPA) system are key components in the central control of stress and anxiety. As part of the limbic system, the amygdala represents the central component involved in the processing of emotions (Adhikari, 2014), and the central nucleus (CeA) integrates emotionally relevant information into behavioral and physiological responses (Fox et al., 2015, Lebow and Chen, 2016). The LC is the major central noradrenergic nucleus and coordinates behavioral and autonomic responses related to anxiety, fear and stress (Berridge and Waterhouse, 2003). The hypothalamic paraventricular nucleus (PVN) regulates activity of the HPA axis and connects the brain to the neuroendocrine stress response system (Herman and Tasker, 2016). Dysregulation of these brain areas results in increased risks for anxiety- and stress-related disorders (Gilpin et al., 2015, McCall et al., 2017, Myers et al., 2017).

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In neonatal rat pups, an acute administration of nicotine activates these key areas, as demonstrated by the robust transcriptional activation of the immediate early genes (IEGs), c-Fos and Arc, in neurons residing in the CeA, bed nucleus of stria terminals (BST) and the PVN in neonatal rat pups (Schmitt et al., 2008). However, it is well established that chronic nicotine exposure causes desensitization of nAChRs, resulting in functional tolerance to nicotine (Giniatullin et al. 2005, Fenster et al., 1997). Thus, receptor desensitization may reduce activation of nAChRs by the endogenous ligand acetylcholine and exogenous agonists including nicotine. This mechanism could protect the developing brain from potentially detrimental effects of repeated nAChR activation, and reduce stimulation of key brain areas linked to stress and anxiety. To address this question, neonatal rat pups were chronically treated with nicotine, and then acutely challenged with nicotine. The transcription factor c-Fos is rapidly induced in response to cellular activity and regulates expression of multiple target genes (Morgan and Curran, 1991; Sheng and Greenberg, 1990). Therefore, induction of c-Fos was used as an indicator of neuronal activation in the key areas, CeA, PVN and LC. We also determined activation of other IEGs that are rapidly induced by neuronal activity including Arc (activity-regulated cytoskeletal associated protein, also known as Arg3.1), Egr-1 (early-growth response gene 1, also known as NGFI-a, and Zif-268) (Okuno, 2011), and Npas4 (neuronal PAS domain protein 4) (Lin et al., 2008, Spiegel et al., 2014). These IEGs are specifically linked to neuronal plasticity, long-term potentiation and synapse formation (Ploski et al., 2011, Minatohara et al. 2016, Jones et al., 2001), and alterations in synaptic connectivity could be an underlying mechanism that leads to long-term changes in emotionality following developmental nicotine exposure. Here, we present evidence that nicotine maintains the ability to activate neuronal ensembles in nuclei of the central anxiety-associated circuitry after chronic nicotine exposure, although, area-specific tolerance to nicotine's effects differentially developed for the induction of IEGs.

2. Results

Induction of IEGs, in particular c-Fos, is often used as a surrogate measure for neuronal activation, although, neuronal activation is not necessarily followed by specific induction of neuronal activity markers such as c-Fos, Egr1, and Arc. In addition, there is expression of IEGs in the absence of a specific stimulus. In particular, Arc and Egr-1 exhibited widespread mRNA expression in the P8 rat brain in saline treated animals, whereas mRNA expression levels for c-Fos and Npas4 were low. For c-Fos, basal mRNA expression was detected in few brain areas, most prominently in the piriform cortex, paraventricular thalamic nucleus (PV), and the inferior colliculus, and in scattered cells located in the hippocampus, basal ganglia, amygdala, thalamus, hypothalamus, brainstem and cerebellum. In response to an acute nicotine challenge, c-Fos mRNA induction was detected in the CeA, PVN, and LC, cortical subplate, medial habenula (MHb), thalamic nuclei, and scattered cells in different brain structures. Since the focus of this study was on the CeA, PVN and the LC, brain areas that are part of the central stress- and anxiety circuitry and the neuroendocrine stress response system, we analyzed these areas in more detail.

2.1. Expression of IEGs in the central nucleus of the amygdala (CeA)

In the CeA, expression of c-Fos mRNA was low after an acute injection with saline in either chronically control- or nicotine-treated groups (AS/CC, AS/CN) indicating that neither the daily handling and intubation procedure nor the chronic exposure to nicotine caused increased neuronal reactivity to an acute saline injection in P8 rat pups (Fig. 1). In contrast, in both the CC- and CN-treated groups, an acute injection of nicotine resulted in robust induction of c-Fos mRNA in the CeA as compared to the respective saline treated groups. Quantification of the hybridization signals showed that an acute nicotine injection resulted in an 8.72- and 4.76-fold increase in c-Fos mRNA expression levels over saline in chronic control- and chronic nicotine-pre-treated rat pups, respectively (AS/CC = 53.0 ± 4.3, AS/CN = 74.2 ± 24.5, AN/CC = 462.1 ± 41.8, AN/CN = 353.1 ± 23.5 nCi/g, Fig. 1, Table 1). Statistical analysis revealed that pre-treatment with chronic nicotine significantly attenuated acute nicotine-induced c-Fos expression in the CeA in AN/CN-compared to AN/CC-treated animals ($F = 4.84$, $p < 0.01$), suggesting that the chronic nicotine treatment prior to the acute nicotine injection, reduced nicotine-induced neuronal activation indicated by diminished c-Fos mRNA activation.

Induction of the other IEGs, Arc, Egr-1 and Npas4, exhibited a similar response pattern as seen for c-Fos in the CeA. After a saline injection, expression levels in the CeA were low for all three IEGs, whereas an acute nicotine injection caused rapid induction of Arc, Egr-1 and Npas4 mRNA. For Arc mRNA expression, an acute nicotine injection resulted in a 7.47- and 6.0-fold increase over saline in control- and nicotine-treated rat pups, respectively (AS/CC = 231.5 ± 37.2, AS/CN = 280.9 ± 32.9, AN/CC = 1729.6 ± 241.8, AN/CN = 1682.4 ± 276.3 nCi/g). For Egr-1 mRNA expression, an acute nicotine injection resulted in a 7.62- and 7.70-fold increase over saline in control- and nicotine-pre-treated rat pups, respectively (AS/CC = 238.5 ± 53.7, AS/CN = 187.7 ± 52.7, AN/CC = 1816.6 ± 308.9, AN/CN = 1445.9 ± 310.9 nCi/g). For Npas4 mRNA expression, an acute nicotine injection resulted in a 5.67- and 6.38-fold increase over saline in control- and nicotine-pre-treated rat pups, respectively (AS/CC = 40.7 ± 5.6, AS/CN = 30.9 ± 3.2, AN/CC = 230.6 ± 22.5, AN/CN = 197.1 ± 14.5 nCi/g, Fig. 1, Table 1). Statistical analysis revealed that, in contrast to c-Fos, acute nicotine induce similar levels of Arc, Erg-1 and Npas4 mRNA expression in the CeA of rat pups from CC- and CN-treated groups (Arc: $F = 0.427$, $p = 0.86$; Egr-1: $F = 1.5506$, $p = 0.264$; Npas4: $F = 2.619$, $p = 0.09$). Thus, after chronic nicotine exposure, a subsequent acute nicotine challenge elicits a response which is sufficient to induce neuronal activity that drives activation of at least a subset of IEGs.

2.2. Expression of IEGs in the hypothalamic paraventricular nucleus (PVN)

In the PVN, after an acute saline injection, expression of c-Fos mRNA was low in both chronic control- or nicotine-treated groups (AS/CC, AS/CN) (Fig. 2). In contrast, an acute nicotine injection resulted in robust induction of c-Fos mRNA in both CC- and CN-treated pups, in PVN neurons, and caused a 3.95- and 2.70-fold increase in c-Fos mRNA expression in AN/CC- and AN/CN treatment groups, respectively, when compared saline (AS/CC = 43.1 ± 6.3, AS/CN = 56.7 ± 16.9, AN/CC = 170.1 ± 17.7, AN/CN = 152.9 ± 23.2 nCi/g, Fig. 2, Table 1). Statistical analysis revealed that in the PVN c-Fos expression was similarly induced by acute nicotine in CC- and CN-treated animals ($F = 0.945$, $p = 0.519$) suggesting that prior chronic nicotine treatment did not result in substantially altered nicotine-induced neuronal activation.

In contrast to the CeA, in the PVN, expression of the IEGs Arc and Npas4 was low in all treatment groups, and showed no or very low responses to an acute nicotine injection (Fig. 2). Low levels of Egr-1 mRNA expression were detected in saline injected animals in both CC- and CN-treated pups, and similar to c-Fos, Egr-1 mRNA expression exhibited a robust nicotine-induced response (Fig. 2, Table 1). For Egr-1, acute nicotine resulted in a 5.06- and 3.62-fold increase over saline in CC- and CN-groups, respectively (AS/CC = 88.2 ± 24.4, AS/CN = 103.1 ± 31.7, AN/CC = 446.3 ± 27.9, AN/CN = 372.9 ± 74.6 nCi/g, Fig. 2). Statistical analysis revealed that