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Neuroanatomical Circuitry Mediating the Sensory Impact of Nicotine in the Central Nervous System

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Abstract

Direct actions of nicotine in the CNS appear to be essential for its reinforcing properties. However, activation of nicotinic acetylcholine receptors (nAChRs) on afferent sensory nerve fibers are important components of addiction to, and withdrawal from, cigarette smoking. The present study was to identify the neuroanatomical substrates activated by the peripheral actions of nicotine and to determine whether these sites overlap brain structures stimulated by direct actions of nicotine. Mouse brains were examined by immunohistochemistry for c-Fos protein after intraperitoneal injection of either nicotine (NIC, 30 and 40 μg/kg) and/or nicotine pyrrolidine methiodide (NIC-PM, 20 and 30 μg/kg). NIC-PM induced c-Fos immunoreactivity (IR) at multiple brain sites. In the brainstem, c-Fos IR was detected in locus coeruleus, laterodorsal tegmental nucleus and pedunculotegmental nucleus. In the midbrain, c-Fos IR was observed in areas overlapping the ventral tegmental area (VTA) which includes paranigral nucleus, parainterfascicular nucleus, parabrachial pigmental area and rostral VTA. Other structures of the nicotine brain-reward circuitry activated by NIC-PM included hypothalamus, paraventricular thalamic nucleus, lateral habenular nucleus, hippocampus, amygdala, accumbens nucleus, piriform cortex, angular insular cortex, anterior olfactory nucleus, lateral septal nucleus, bed nucleus of stria terminalis, cingulate and medial prefrontal cortex, olfactory tubercle, medial and lateral orbital cortex. Nicotine, acting through central and peripheral nAChRs, produced c-Fos IR in areas that overlapped NIC-PM induced c-Fos expressing sites. These neuroanatomical data are the first to demonstrate that the CNS structures which are the direct targets of nicotine are also anatomical substrates for the peripheral sensory impact of nicotine.

Keywords

Nicotine; Nicotine pyrrolidine methiodide; Addiction; Sensory nerve fibers

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INTRODUCTION

The prevalence of tobacco smoking has not changed significantly over the past several years. In the United States alone, about 45 million people smoke tobacco (Joel et al., 2012). According to the Centers for Disease Control and Prevention (CDC), 75% of smokers would like to quit because of the known health risks associated with nicotine. Despite the availability of numerous nicotine addiction treatment strategies, less than 5% of individuals who try to quit remain smoke-free after one year (Yilmazel Ucar et al., 2014). Upon inhalation, nicotine, the major addictive component of tobacco smoke, passes into the bloodstream and, within seconds, crosses the blood-brain barrier to enter the brain parenchyma (Berridge et al., 2010; Rose et al., 2010). Once in the brain, nicotine activates various nicotinic acetylcholine receptors (nAChRs) located throughout the central nervous system (CNS) and modulates the activity of virtually all the major neurotransmitters via pre and/or postsynaptic mechanisms (Wonnacott et al., 2006; Dani and Bertrand, 2007; Albuquerque et al., 2009). Most studies evaluating the neurobiological mechanisms of nicotine addiction have focused on the direct effects of nicotine on the CNS and on the mesocorticolimbic pathways which include dopaminergic neurons of the ventral tegmental area (VTA) and its projections to nucleus accumbens (Acb) and medial prefrontal cortex (MPFC) (Schultz et al., 1997; Wise, 2009; Schultz, 2010; Ikemoto, 2010; De Biasi and Dani, 2011). However, activation of various CNS sites could result from interactions of nicotine with nAChRs abundantly expressed on epithelial cells of airways and afferent sensory nerve fibers (Gu et al., 2008; Rose et al., 1999; Alimohamadi and Silver, 2000; Dehkordi et al., 2009, 2010). Nicotine stimulation of peripheral sensory nerve fibers is important for mediating the sensory impact of nicotine such as taste, aroma and respiratory tract sensation and the associated perceptions are thought to be critically important for smoking satisfaction (Rose et al., 1984, 1985, 1999, 2006; Westman et al., 1996; Palmatier et al., 2006; Yerger and McCandless, 2011). Other drug-related sensory modalities associated with cigarette smoking such as visual, auditory and tactile or haptic sensations are also thought to be relevant in drug addiction (Schneider et al., 2001; Filbery et al., 2008; 2009; Janes et al., 2010; Seo et al., 2011; Claus et al., 2011; Yalachkov et al., 2013). Consistent with this idea, we have previously demonstrated that blockade of the sensory afferents in the upper respiratory tract by *local* anesthetics makes human smoking much less rewarding (Rose et al., 1985). Nicotine associated with cigarette smoking has a direct effect on the nAChRs located at the central nervous system (CNS), but it also stimulates peripheral nAChRs. Thus, the objectives of the present study were twofold: (1) To explore the brain regions which are activated by intraperitoneal (i.p.) injection of a peripherally-acting nicotine analog, nicotine pyrrolidine methiodide (NIC-PM) that does not cross the blood-brain barrier (Gillis and Lewis, 1956; Aceto et al., 1983; Lenoir et al., 2013) and (2) To determine whether the brain sites activated by NIC-PM overlap those activated by intraperitoneal (i.p.) administration of nicotine hydrogen tartrate, a form of nicotine that does cross the blood-brain barrier.

MATERIALS AND METHODS

Adult (2–3 month-old) CD-1 mice weighing 20–25 g were used. All procedures including the anesthesia and surgery were approved by the Institutional Animal Care and Use

Committee (IACUC) of Howard University. All efforts were made to minimize the number of animals used and their suffering. Animals (N=20) were housed at a room temperature 22–24°C with water and food freely available. To reduce the nonspecific effects of handling and experimental environment, animals were handled daily and exposed to the same conditions as during the actual experiments. Following an adaptation period of 3–4 d, the mice were treated by i.p. injection of saline (control), nicotine hydrogen tartrate salt (NIC, Sigma–Aldrich, Saint Louis, MO) and/or nicotine pyrrolidine methiodide (NIC-PM, Toronto Research Chemicals Company), the latter a quaternary nicotine analog which does not penetrate the blood-brain barrier (Gillis and Lewis, 1956; Aceto et al., 1983; Lenoir et al., 2013). The NIC dose (30 and 40 μg/kg) used in the present study is within the range reported to be optimal for maintaining intravenous self-administration of nicotine in rats (Cox et al., 1984; Donny et al., 1995) and comparable to the dose delivered during the smoking of one or two cigarettes in humans (Rose and Corrigall, 1977). NIC–PM (20 and 30 μg/kg) was given at a dose that is equimolar to nicotine. The 20 μg/kg of NIC-PM produced very little c-Fos activation in the brain. Thus, only the data obtained with 30 μg/kg NIC-PM were reported herein. Both forms of nicotine were dissolved in saline and injected i.p. Two h after i.p. injection of the saline (control), NIC and/or the NIC-PM, the mice were anesthetized with 5% isoflurane and were perfused transcardially with saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at pH 7.4. After perfusion, the brains were postfixed in 4% paraformaldehyde for one h and then cryoprotected in a 30% sucrose solution for a minimum of 2 d. Transverse sections of the brain were cut at 40 μm using a Bright OTF Cryostat (Hacker Instruments and Industries) and were stored in 0.5% sodium azide in 0.1 M PB (pH 7.4). Immunohistochemical procedures were performed using free floating sections as follows: Briefly, 1-in-5 series of brain sections extending from bregma −5.41 mm to bregma 2.33 mm (Paxinos and Franklin 2013) were rinsed three times in 0.1 M phosphate buffered saline (PBS) at pH 7.4. Nonspecific binding was blocked by incubating the tissues overnight in loading buffer containing 2% normal donkey serum (NDS, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and 0.3% Triton X-100. Tissues were then washed and incubated with rabbit anti- c-Fos antibody (1:5000; Cat # PC38, Millipore Corporation Temecula, CA) in 2% NDS and 0.3% Triton X-100 in PBS at 4°C for 48 h. The sections were then incubated in Alexa Fluor 594 donkey anti-rabbit secondary antibody (1:100; Jackson ImmunoResearch Laboratories Inc) in 0.1 M PBS for 2½ h. After washing in PBS, sections were rinsed in PBS, mounted and cover-slipped using Vecta Shield (Vector Laboratories Inc., CA) antifade mounting media.

Controls for each experiment were performed to determine whether the primary or the secondary antibodies produced false-positive results. The controls involved omission of the primary and/or secondary antisera to eliminate the corresponding specific labeling. Nonspecific activation of c-Fos was assessed by evaluating the CNS expression of c-Fos in animals receiving i.p. injection of normal physiological saline (NS).

Data Analysis

High resolution fluorescent images were acquired using Nikon (Nikon Instruments, Melville, NY) and Olympus (Olympus AX70, Olympus America) microscopes equipped with the adequate filter systems to observe the red fluorescence. 1-in-5 series of brain

sections extending from bregma −5.41 mm to bregma 2.33 mm (Paxinos and Franklin, 2013) were identified. NIC and NIC-PM induced c-Fos-expressing structures in representative brain regions were identified. The sections exhibiting the exact same anatomical structures for both the NIC and the NIC-PM treated groups were compared and analyzed. Images from all the brain regions of interest were captured at 4×, 10× and 20× magnification and minor adjustments of brightness and contrast were made using Adobe Photoshop CS3

Cell counting

Semi-quantitative estimate of the numbers of NS-, NIC-, NIC-PM-, c-Fos activated cells in VTA, Acb and PFC was performed using a NIC dose (40 μg/kg) that was approximately equimolar to NIC-PM (30 μg/kg). The counting of c-Fos immunoreactive cells was performed by an individual blinded to the treatment. Four 40 μm sections through each of the aforementioned structures were selected for each group (N=4) and well-defined areas based on anatomical landmarks were chosen for analysis. In VTA, the number of c-Fos IR cells in the sections selected between bregma −3.15 mm and bregma −2.79 mm were counted. In Acb, the number of c-Fos IR cells in the sections selected between bregma 0.61 mm and bregma 1.41 mm were counted. In PFC, the number of c-Fos IR cells in the sections selected between bregma 0.85 mm and bregma 1.21 mm were counted. The c-fos IR cells were counted in a single plane and overlapping cells were counted if they were distinguishable by a staining intensity greater than that of the background. The data were expressed as mean ± standard error. Statistical analysis was performed using one-way analysis of variance (ANOVA) to compare the effects of **NS,** NIC and NIC-PM in the aforementioned areas. The significance level was set at $P \leq 0.05$.

RESULTS

Figure 1 is a schematic diagram of representative brain regions showing NIC-PM induced c-Fos expression at multiple brain sites. The pattern of NIC–PM induced c-Fos expression was similar to that of NIC induced c-Fos expression and areas activated by the peripherally-acting analog of nicotine, often overlapped those which were stimulated by NIC (Figures 2 and 3). Overlap between the central and peripheral effects of i.p. NIC was a limiting factor which did not permit identification of the specific cells activated by the direct effects of nicotine on the CNS. Consequently, these data represent site-specific activations by NIC and NIC-PM and do not represent the specific cell groups activated by these compounds.

Figures 2 and 3 present the results of representative experiments demonstrating NIC and NIC-PM induced c-Fos activated cells in various structures of the mesocorticolimbic system. In the VTA (Figures 2 and 3), c-Fos expressing cells were scattered at sites medial to substantia nigra (SN) and medial lemniscus (ml) and in regions corresponding to paranigral nucleus (PN), parainterfascicular nucleus (PIF) and interpeduncular nucleus rostral (IPR). More rostrally, c-Fos IR cells were detected in rostral VTA (VTAR) and parabrachial pigmental area (PBP), as well as in areas that overlap the retromammillary nucleus (RM), interfascicular nucleus (IF) and rostral linear nucleus (RLi) (Figures 2 and 3). NIC and NIC-PM also produced intense activation of cells in medial prefrontal cortex

(MPFC). Activated cells were also observed in the core and shell components of nucleus accumbens (AcbC, AcbSh). In addition to the mesocorticolimbic pathways, multiple other CNS sites were activated by both NIC and NIC-PM.

Figure 4 demonstrates NIC-PM induced c-Fos immunoreactivity in representative brain regions. At the pontine medullary junction, c-Fos IR cells were detected at areas corresponding to locus coeruleus (LC), laterodorsal tegmental nucleus (LDTg) and pedunculotegmental nucleus (PTg). More rostrally, intense c-Fos expression was seen in pontine nucleus (Pn), periaqueductal gray (PAG), dorsal raphe nucleus (DRN), superior colliculus (SC) and entorhinal cortex (Ent) (Figures 1 and 4). Scattered c-Fos expression was also observed in areas that overlap the anterior and ventral tegmental nucleus (ATg, VTg). In addition to the VTA, other midbrain regions exhibiting c-Fos immunoreactivity were the dentate gyrus (DG), C1, C2, and C3 areas of hippocampus, amygdala (Amyg) and red nucleus (RN) (Figures 1 and 4). NIC-PM also produced intense stimulation of visual and somatosensory cortex (VC, SC) as well as various areas of cingulate cortex including medial prefrontal areas; i.e., prelimbic cortex (A32) and infralimbic cortex (A25). Activated cells were also observed in areas overlapping different nuclei of hypothalamus (HP) including arcuate hypothalamic nucleus (Arc), dorsomedial hypothalamic nucleus (DMN), anterior hypothalamic nucleus (AHN), paraventricular hypothalamic nucleus (PVN), medial preoptic nucleus (MPO), as well as lateral and posterior hypothalamic areas (LH, PH). Other areas strongly stimulated by NIC-PM included paraventricular thalamic nuclei (PVT), lateral habenular nucleus (LHb), lateral septal nucleus (LS), bed nucleus of stria terminalis (BST), nucleus of ventral limb of diagonal band (VDB), piroform cortex (Pir), angular insular cortex (AI), medial and lateral orbital cortex (MO, LO), anterior olfactory nucleus (AON) and olfactory tubercle (Tu).

Semi-quantitative estimation of the number of c-Fos activated cells in VTA, Acb and PFC demonstrated significantly greater activation by NIC than by NIC-PM (Figure 5). The number of c-Fos activated cells was also significantly greater for the NIC and NIC-PM treatments compared to the NS control ((P<0.001). At VTA, the number of NIC activated cells was 189.0 ± 16.4 vs. 142.5 ± 16.2 for NIC-PM (P=0.05). At Acb, the number of NIC activated cells was 192.3 ± 14.4 vs. 78.8 ± 9.5 for NIC-PM (P=0.03) and at PFC, the number of NIC activated cells was 93.3 ± 1.9 vs. 73.3 ± 4.6 for NIC-PM (P<0.001).

DISCUSSION

The present study demonstrates that NIC-PM, a peripherally-acting nicotine analog, acts through nAChRs present on afferents of somatic and visceral sensory nerve fibers, to stimulate cells at multiple sites throughout the brain. These include cells in brain regions overlapping the mesocorticolimbic reward pathways, and in areas implicated in tobacco-related arousal, cognition, memory, stress, and interoceptive awareness (Panagis et al., 2000; Kirouac et al., 2005; Parsons et al., 2006; Levin et al., 2006; Guillem et al., 2011; Verdejo-Garciaa et al., 2012; Flawin and Winder, 2013; Ramirez et al., 2014). The findings that virtually all the brain areas stimulated by nicotine actions on peripherally- and centrally-located nAChRs, were also activated by NIC-PM, suggests that the sensory impact of nicotine may be critical in mediating its direct CNS effects. These anatomical data are

supported by previous studies demonstrating that hexamethonium, a peripherally-acting nicotine antagonist, drastically decreased EEG and EMG responses to nicotine (Lenoir and Kiyatkin, 2011; Lenoir et al., 2013). The immediate EEG responses to nicotine were also strongly inhibited during urethane anesthesia (Lenoir and Kiyatkin, 2011). Importance of the sensory impact of nicotine has also been demonstrated in our previous human studies showing that blockade of sensory afferents in the upper respiratory tract by local anesthetics and by peripheral nicotinic receptor antagonists makes human smoking much less rewarding (Rose et al., 1985, 1999).

Numerous other studies have used methiodides of nicotine as probes to distinguish the central and peripheral actions of nicotine (Domino, 1965; Geller et al., 1971; Shechter and Rosecrans, 1972; Thompson et al., 1972; Tang and Kiyatkin, 2011; Lenoir and Kiyatkin, 2011; Lenoir et al., 2013). The present study using NIC-PM to differentiate the peripheral and central actions of nicotine, is based on the assumption that NIC-PM, which is a highly polar molecule, does not cross the blood-brain barrier and that NIC-PM has nicotine-like properties. This assumption is based on previous studies in mice demonstrating that radiolabeled NIC-PM penetrates the brain poorly (Aceto et al., 1983). The inability of NIC-PM to enter the brain has also been confirmed using highly-sensitive mass spectrometric methods (Lenoir et al., 2013). NIC-PM has also been shown to have potent nicotine-like properties (Gillis and Lewis, 1956). NIC and NIC-PM were both found to be equipotent in their pressor effects in cats (Barlow and Dobson, 1955), dogs (Larson and Haig, 1943) and monkeys (Zuo et al., 2009). Furthermore, CNS administration of NIC-PM in mice and rats is reported to produce antinociceptive effects comparable to those of nicotine (Aceto et al., 1983). Therefore, based on these studies, we assume that NIC-PM mimics the peripheral effects of NIC and that the c-Fos activation following NIC-PM administration, in the present study, is primarily due to interactions of nicotine with peripheral nAChRs.

The peripheral actions of nicotine are also believed to act as interoceptive cues capable of eliciting nicotine-like physiological and neural responses after repeated nicotine exposure. This notion is based on animal studies demonstrating that peripheral sensory stimuli associated with NIC-PM produce weak responses in drug naïve conditions. However, the same stimuli produce strong physiological and neural effects when injected after animals have nicotine experience (Lenoir et al., 2013). Numerous other studies have shown that pairing between exteroceptive environmental and/or peripheral interoceptive stimuli and a natural reinforcer (e.g., nicotine) results in classical conditioning and plays an important role in drug addiction (Razran, 1961; Gauvin et al., 1993; Bernstein and Koh 2007; Verdejo-Garcia et al., 2012). Importance of the sensory impact of nicotine has also been documented by studies demonstrating that damage to the insular cortex, a critical neural hub of interoceptive awareness, can lead to disruption of smoking behavior (Naqvi et al., 2007). In the present study, NIC and NIC-PM activated cells in cingulate, insular, olfactory (Pir AON and Tu) and orbitofrontal cortexes. These areas are implicated in processing conscious emotion via representation of body interoceptive states (Naqvi et al., 2007; Naqvi and Bechara, 2010; Kutlu et al., 2013).

NIC and NIC-PM Induced c-Fos Activation in VTA

In the present study, we observed a mild c-Fos response to NIC and/or NIC-PM in areas overlapping VTA, an important center for reward-seeking behavior (Schultz et al., 1997; Wise, 2009; Schultz, 2010; Ikemoto, 2010; De Biasi and Dani, 2011). However, VTA is known to receive excitatory synaptic inputs from a wide range of structures including various brainstem nuclei (Phillipson, 1979; Mejías-Aponte et al., 2009), PAG (Geisler et al. 2007; Moreira et al., 2009), MPFC (Takahata and Moghaddam, 2003; Kalivas et al., 2009), hypothalamus (Kempadoo et al., 2013), BST (Georges and Aston-Jones, 2001, 2002), LS (Talishinsky et al., 2012), Acb (Usuda et al., 1998) and SC (Comoli et al., 2003). All of these structures were shown to be activated by both NIC and NIC-PM. Intense activation of cells at MFPC by NIC and /or NIC-PM confirms numerous other studies regarding the importance of this cortical control center in induction of burst activity in VTA (Gariano and Groves, 1988; Tong et al., 1996; Nisell et al., 1997; Takahata and Moghaddam, 2003; Kalivas, 2009). Dopaminergic neurons of VTA are known to receive direct and indirect glutamatergic innervations from MPFC and enhancement of this pathway underlies drug addiction (Takahata and Moghaddam, 2003; Kalivas, 2009). PAG, the third largest subcortical source of glutamate input to VTA (Geisler et al. 2007) was also strongly stimulated by NIC and NIC-PM. The importance of this structure in reward and addictive properties of nicotine is not known. However, PAG is thought to supply VTA neurons with information important for processing nociceptive signals, defensive and stress behaviors (Moreira et al., 2009).

NIC and NIC-PM Induced c-Fos Activation of Brainstem Nuclei

The brainstem structures known to project to VTA and also activated by both NIC and NIC-PM were LDTg, PTg, LC and SC (Phillipson, 1979; Mejías-Aponte et al, 2009). Of these structures, LDTg and PTg comprise cholinergic, glutamatergic and GABAergic neurons and these three transmitters are expressed in the projections to VTA (Hallanger and Wainer, 1988; Oakman et al., 1995; Maskos, 2008; Ishibashi et al, 2009). The involvement of LDTg/PTg in nicotine addiction has been demonstrated in previous studies wherein a normally functioning LDTg was reported to be necessary for the burst firing of dopaminergic VTA neurons (Lodge and Grace, 2006) and that PTg lesions reduce nicotine self-administration behaviors (Lanca et al. 2000; Picciotto and Corrigall, 2002). VTA neurons are also shown to be modulated by adrenergic agents and LC, the main source of norepinephrine in the brain, was also found to be activated by NIC and NIC-PM (Phillipson, 1979; Mejías-Aponte et al., 2009). The exact role of LC in the nicotine reward and addiction circuitry is not known. However, based on its known functions, LC may provide VTA with signals related to arousal, cognition and stress associated with nicotine use (Samuels and Szabadi, 2008; Okere and Waterhouse, 2013). SC is another brainstem structure activated by NIC and NIC-PM. SC relays information about visual stimuli to VTA dopaminergic neurons (Comoli et. al., 2003; Overton et. al., 2014). The importance of Pn, another brainstem nucleus with intensive c-Fos IR after administering NIC and NIC-PM, in reward and addiction properties of nicotine is not clear. However, Pn is known to be critical in eyeblink conditioning in an associative learning paradigm (Villarreal and Steinmetz, 2005) and nicotine is widely believed to convey its reinforcing properties upon tobacco-related stimuli through associative learning (Gould and Davis, 2008).

NIC and NIC-PM Induced c-Fos Activation of Hypothalamic and Thalamic Nuclei

Among the hypothalamic and thalamic structures that were activated by NIC and NIC-PM, the LH, PVN, PVT and LHb are particularly important because of their roles in nicotine addiction (Kirouac et al., 2005; Parsons et al., 2006; Balcita-Pedicino, 2011; Martin-Fardon and Boutrel, 2012; Mahler et al., 2012). Orexin/hypocretin neurons of LH project to reward-associated brain regions such as Acb and VTA and these projections are important in relaying interoceptive-related signals to VTA (Peyron et al., 1998; Fadel and Deutch, 2002; Harris et al., 2005). Orexin/hypocretin neurons of PVN also play roles in the reinforcing and aversive properties of nicotine, as well as in the aversive properties of nicotine withdrawal (Maler et al., 2012). Of the thalamic nuclei, PVT contributes to the effects of nicotine on arousal and cognition and receives major orexin/hypocretin projections from LH (Kirouac et al., 2005; Parsons et al., 2006; Martin-Fardon and Boutrel, 2012). LHb, an epithalamic nucleus activated by NIC and NIC-PM, is thought to be a component of an anti-reward circuitry which inhibits VTA neurons via GABAergic projections to rostromedial tegmental nucleus (Balcita-Pedicino, 2011).

NIC and NIC-PM Induced c-Fos Activation of Other Limbic Structures

Other brain structures in the neurocircuitry of addiction which were activated by NIC and NIC-PM include hippocampus, LS and BST (Kenney and Gould, 2008; Luo et al., 2011; Sartor and Aston-Jones, 2012; Falvin and Winder, 2013). Nicotine addiction is considered to be a disorder of learning and memory and the hippocampus is a critical center for learning and memory (Gould, 2006; Gould and Leach, 2014; Ramirez et al., 2014). Nicotine alters various forms of hippocampus-dependent learning and memory through various long-lasting effects on hippocampal synaptic function (Gould, 2006; Gould and Leach, 2014). The hippocampus also receives input from midbrain dopaminergic cells and, in turn, projects to the prefrontal cortex and ventral striatum, areas involved in impulse control, decision-making, and reward evaluation (Kelley, 2004; Jones and Bonci, 2005). Intense c-Fos IR was also seen in BST, a component of the extended amygdala (ExtA). This nucleus plays critical roles in responses to stress, anxiety and reward (Falvin and Winder, 2013). BST projects to, and exerts a strong excitatory influence on the firing of dopaminergic neurons within VTA (Georges and Aston-Jones, 2001, 2002) and these projections appear to be necessary for learning to associate drug rewards with specific environmental cues (Dumont et al., 2005). LS, a nucleus associated with stress and drug addiction, was another structure exhibiting intense c-Fos IR (Olds & Milner, 1954; Sheehan et al., 2004; Luo et al., 2011; Sartor and Aston-Jones, 2012). That LS is part of the reward network is supported by previous studies on selfstimulation (Olds & Milner, 1954). LS has strong projections to LH-orexin neurons of the hypothalamus and to the midbrain regions (Risold and Swanson, 1997; Yoshida et al., 2006; Sartor and Aston-Jones., 2012 Talishinsky el al., 2012). Furthermore, LS has been associated with processing contextual information and sending this information to other brain regions for expression of drug reward-related behaviors (Luo et al., 2011; Sartor and Aston-Jones., 2012).

Conclusion

The present study provides the first neuroanatomical data demonstrating that peripherally-mediated sensory effects of nicotine are capable of eliciting neuronal activation at multiple levels of the drug addiction-reward circuitry in the mouse brain. The areas activated by NIC-PM overlapped those stimulated by NIC. Consistent with nicotine's actions on both central and peripheral nAChRs, semi-quantitative analysis demonstrated that a significantly greater number of cells in VTA, Acb and PFC were activated by nicotine than by its peripherally-acting analog NIC-PM. The widespread and overlapping activation of multiple brain areas by both nicotine and its peripherally-acting analog implies that the neurotransmitters and brain circuitry mediating the effects of nicotine on the addiction-reward pathways are much more diverse and complex than previously thought. Since these peripherally mediated effects always precede the direct central action of nicotine, they may condition (prepare) the brain for yet-to-come important reward signaling. The conjunction of anatomical sites where peripheral actions of nicotine overlap with sites known to be important for reinforcement may encourage conditioned associations between sensory cues and the reinforcing effects of nicotine, thereby promoting addiction.

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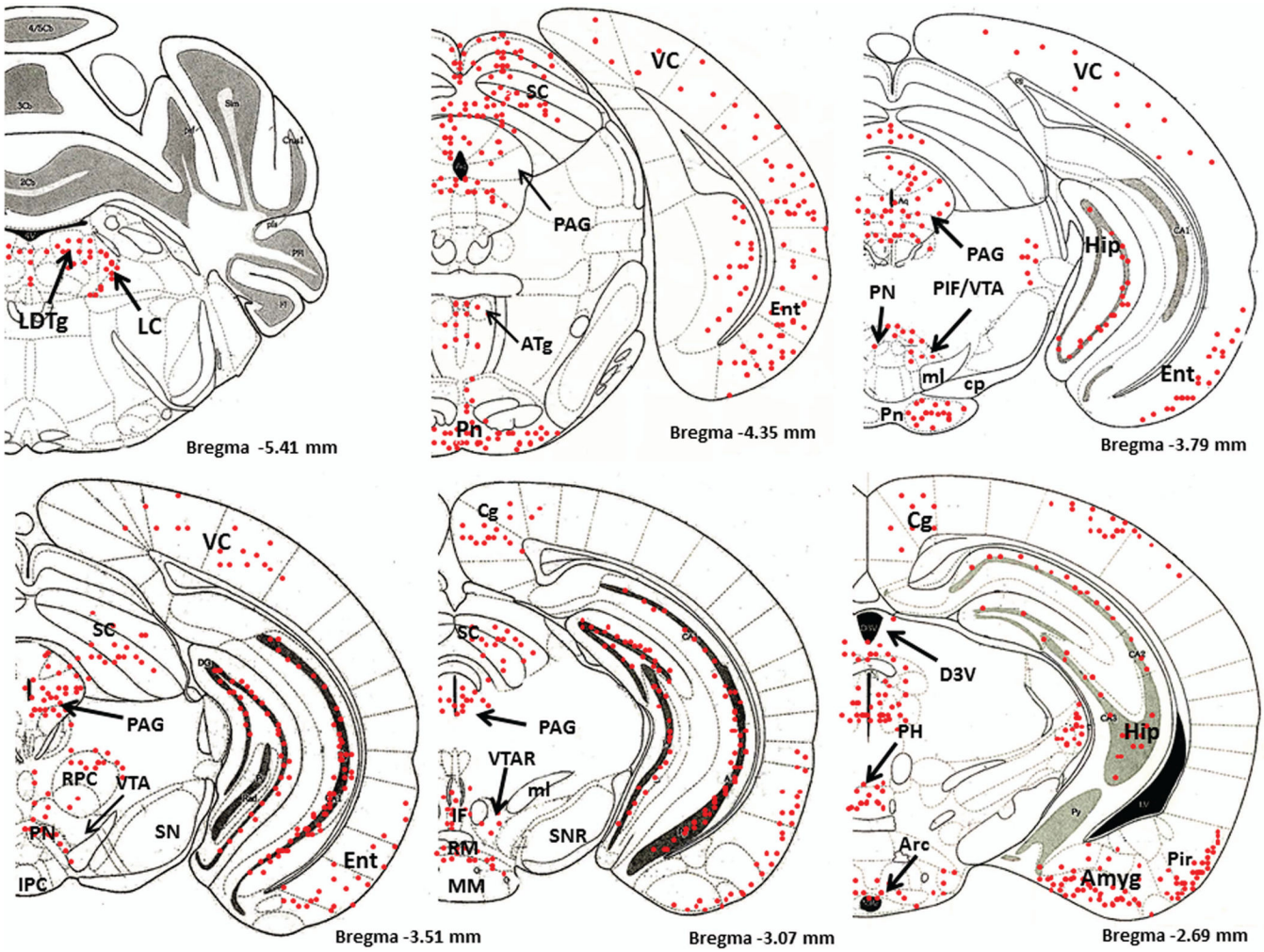
ABBREVIATIONS

Aca	Anterior Commissure, Anterior Part
AcbC	Accumbens Nucleus, Core Region
AcbSh	Accumbens Nucleus, Shell Region
AI	Angular Insular Cortex
AIP	Agranular Insular Cortex, Posterior Part
Amyg	Amygdala
AO	Anterior Olfactory Nucleus
APir	Amygdalopiriform Transition Area
Arc	Arcuate Nucleus
ArcM	Arcuate Hypothalamic Nucleus

ATg	Anterior Tegmental Nucleus
Cg	Cingulate cortex
cp	Cerebral Peduncle
CPu	Caudate Putamen (Striatum)
DRN	Dorsal raphe nucleus
D3V	Dorsal 3rd Ventricle
Ent	Entorhinal Cortex
f	Fornix
fmi	Forceps Minor of the Corpus Callosum
fr	Fasciculus Retroflexus
Hip	Hippocampus
HP	Hypothalamus
IP	Interpeduncular Nucleus
IPC	Interpeduncular Nucleus
LC	Locus Coeruleus
LDTg	Laterodorsal Tegmental Nucleus
LHb	Lateral Habenular Nucleus
LS	Lateral Septal Nucleus
LV	Lateral Ventricle
ml	Medial Lemniscus
MM	Medial Mamillary Nucleus
MO	Medial Orbital Cortex
MPFC	Medial Prefrontal Cortex
MPO	Medical Preoptic Nucleus
NS	Normal physiological saline
OC	Orbital Cortex
ON	Olfactory Nucleus
PAG	Periaqueductal Gray
PH	Posterior Hypothalamic Nucleus
PIF	Parainterfascicular Nucleus of the Ventral Tegmental Area
Pir	Piriform Cortex
PN	Paranigral Nucleus of the Ventral Tegmental Area

Pn	Pontine Nuclei
PSC	Primary Somatosensory Cortex
PVT	Paraventricular Thalamic Nucleus
PVN	Paraventricular hypothalamic nucleus
Py	Pyramidal Cell Layer of the Hippocampus
RPC	Red Nucleus, Parvicellular Part
SC	Superior Colliculus
SNR	Substantia Nigra
VC	Visual Cortex
VDB	Nucleus of the Vertical Limb of the Diagonal Band
VTA	Ventral Tegmental Area
3V	3rd Ventricle



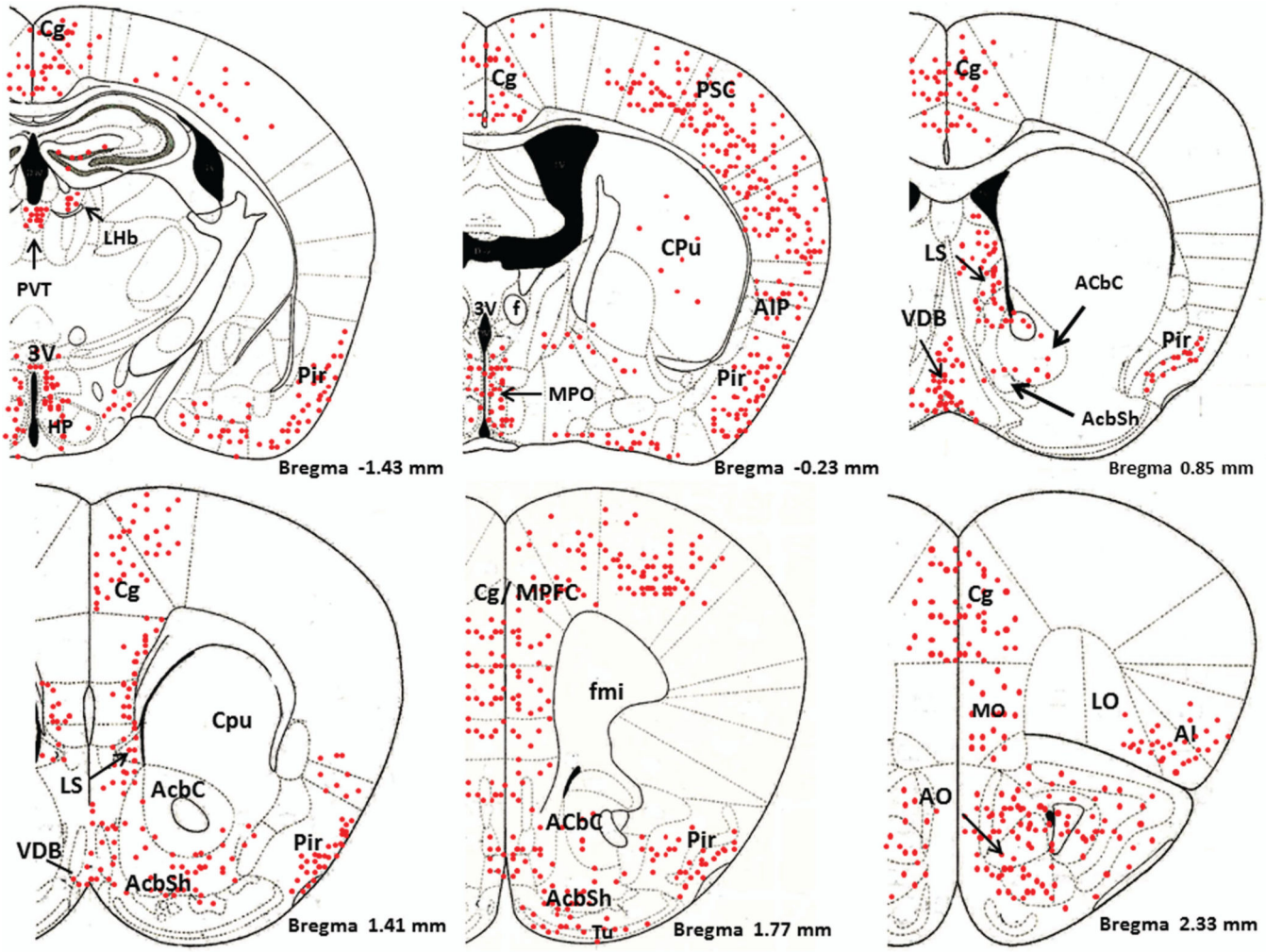
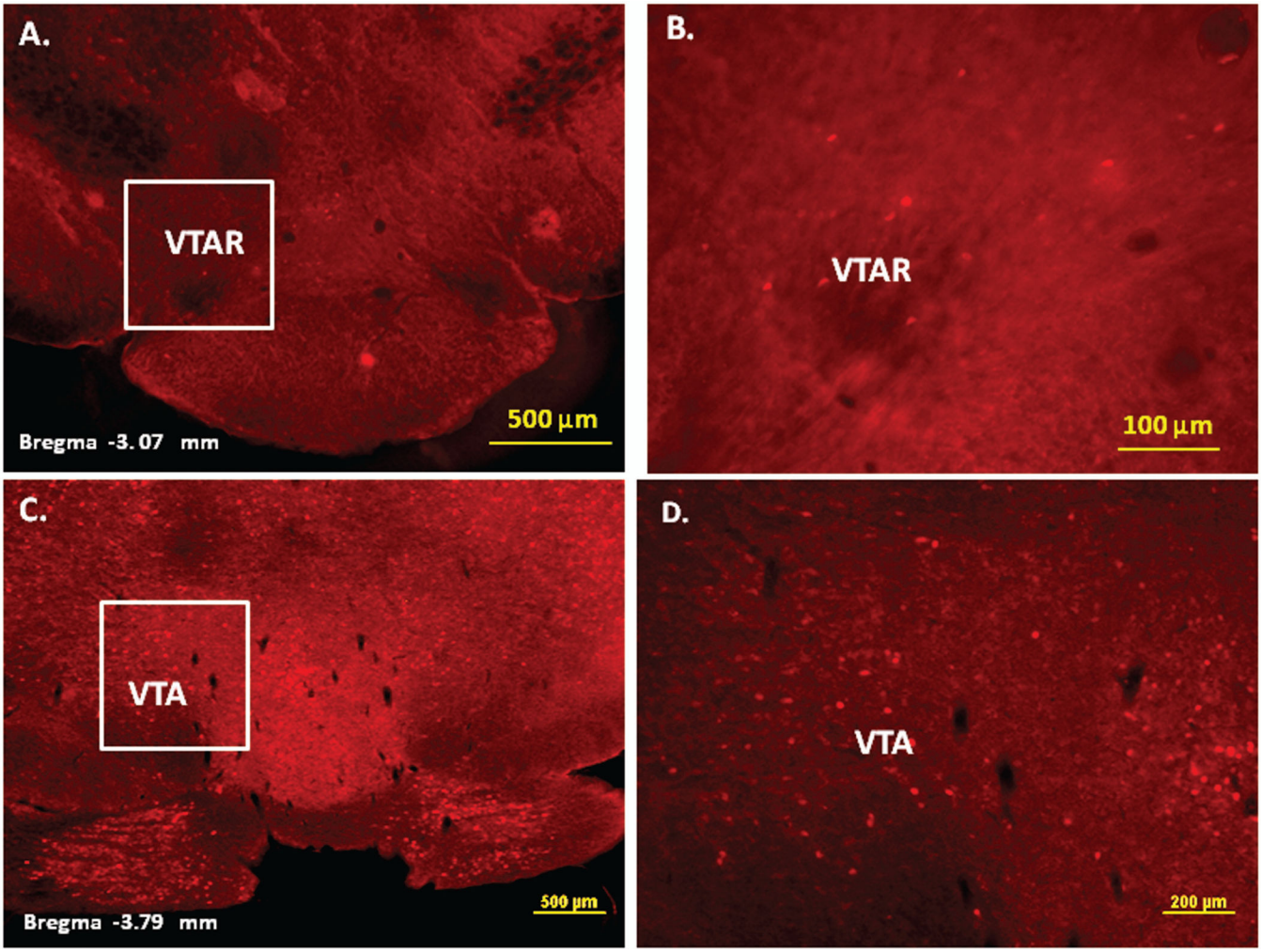


Figure 1.

Schematic diagrams of representative brain regions demonstrating the sites of c-Fos activated cells in the mouse brain following intraperitoneal injection of the peripherally-acting nicotine analog, nicotine pyrrolidine methiodide (NIC-PM, 30 μg/kg). NIC-PM induced c-Fos immunoreactivity at multiple sites extending from bregma −5.41 mm to bregma 2.33 mm. The sites activated by NIC were qualitatively identical to those activated by NIC-PM. The red-colored dots represent the relative intensity of site-specific c-Fos activation and do not represent the exact number of activated cells. For abbreviations, see “abbreviations”



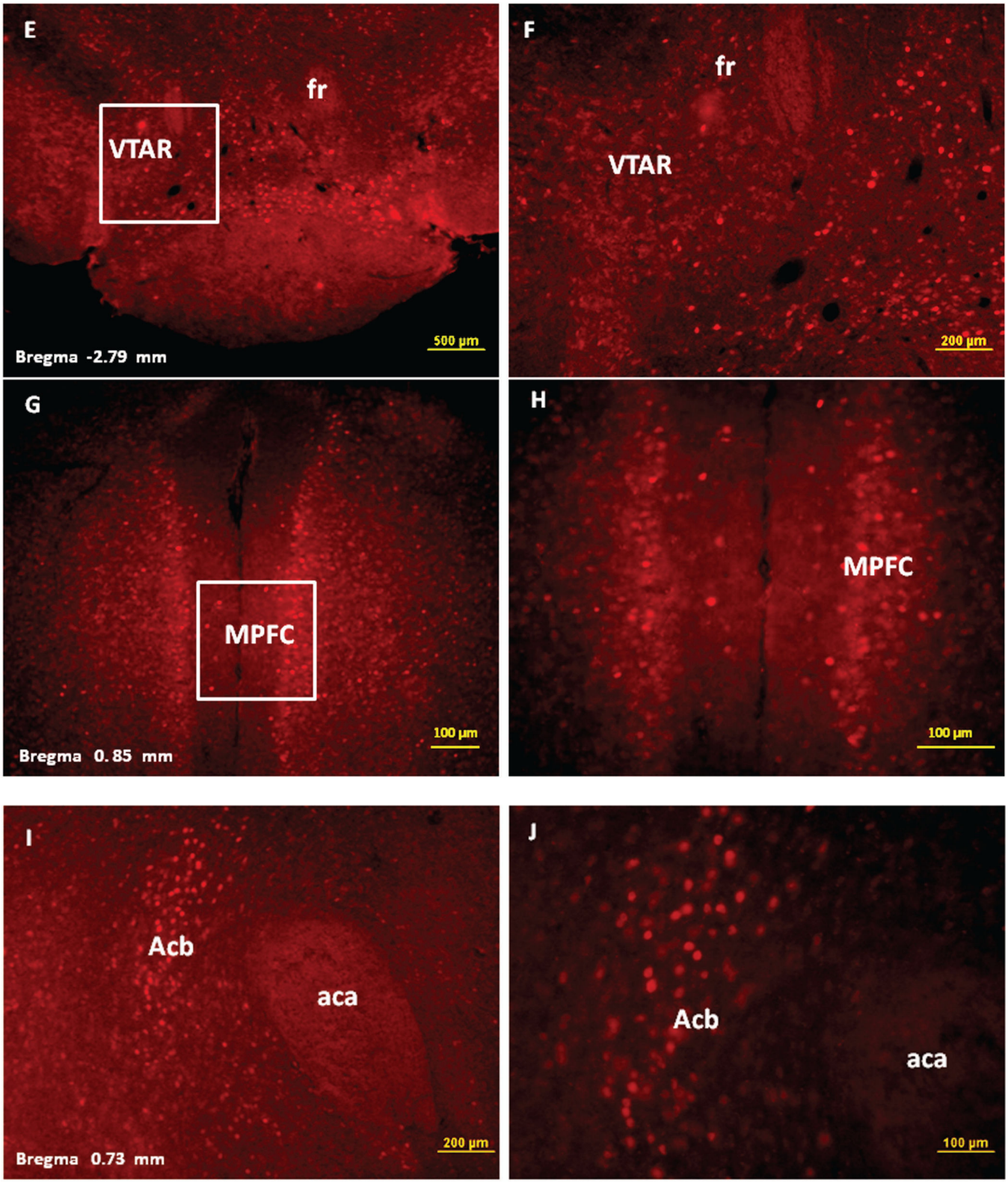
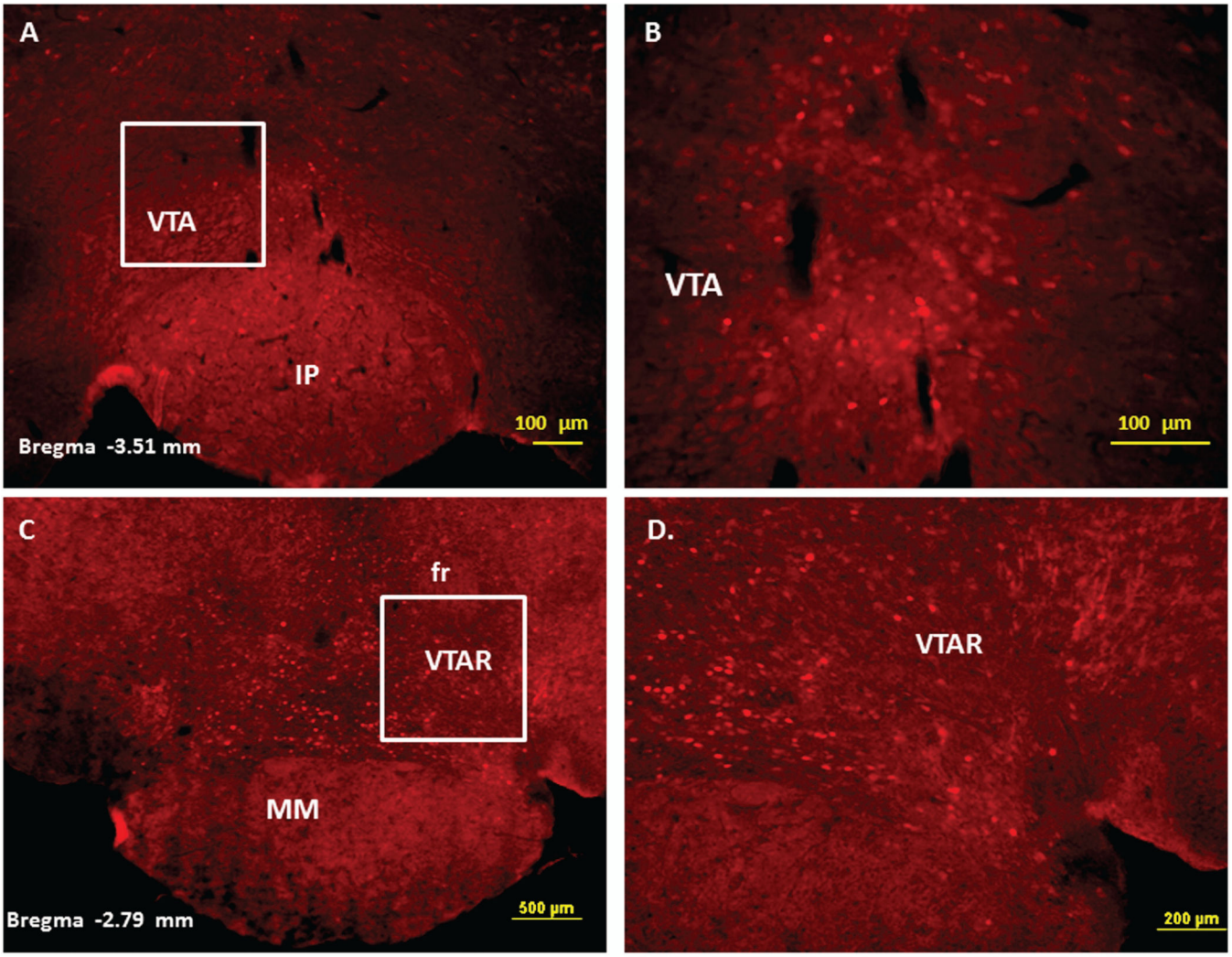


Figure 2.

Immunofluorescence staining demonstrating the sites of c-Fos activated cells of the mesocorticolimbic system in the mouse brain following intraperitoneal injection of the peripherally-acting nicotine analog, nicotine pyrrolidine methiodide (NIC-PM, 30 μg/kg). Panels A and B: Control data demonstrating c-Fos immunoreactivity observed following intraperitoneal injection of physiological saline (vehicle) at a representative site. Panels C-F: c-Fos immunoreactive cells in the ventral tegmental area (VTA). Panels G and H: c-Fos immunoreactive cells in medial prefrontal cortex (MPFC). Panels I and J: c-Fos immunoreactive cells in nucleus accumbens (Acb).



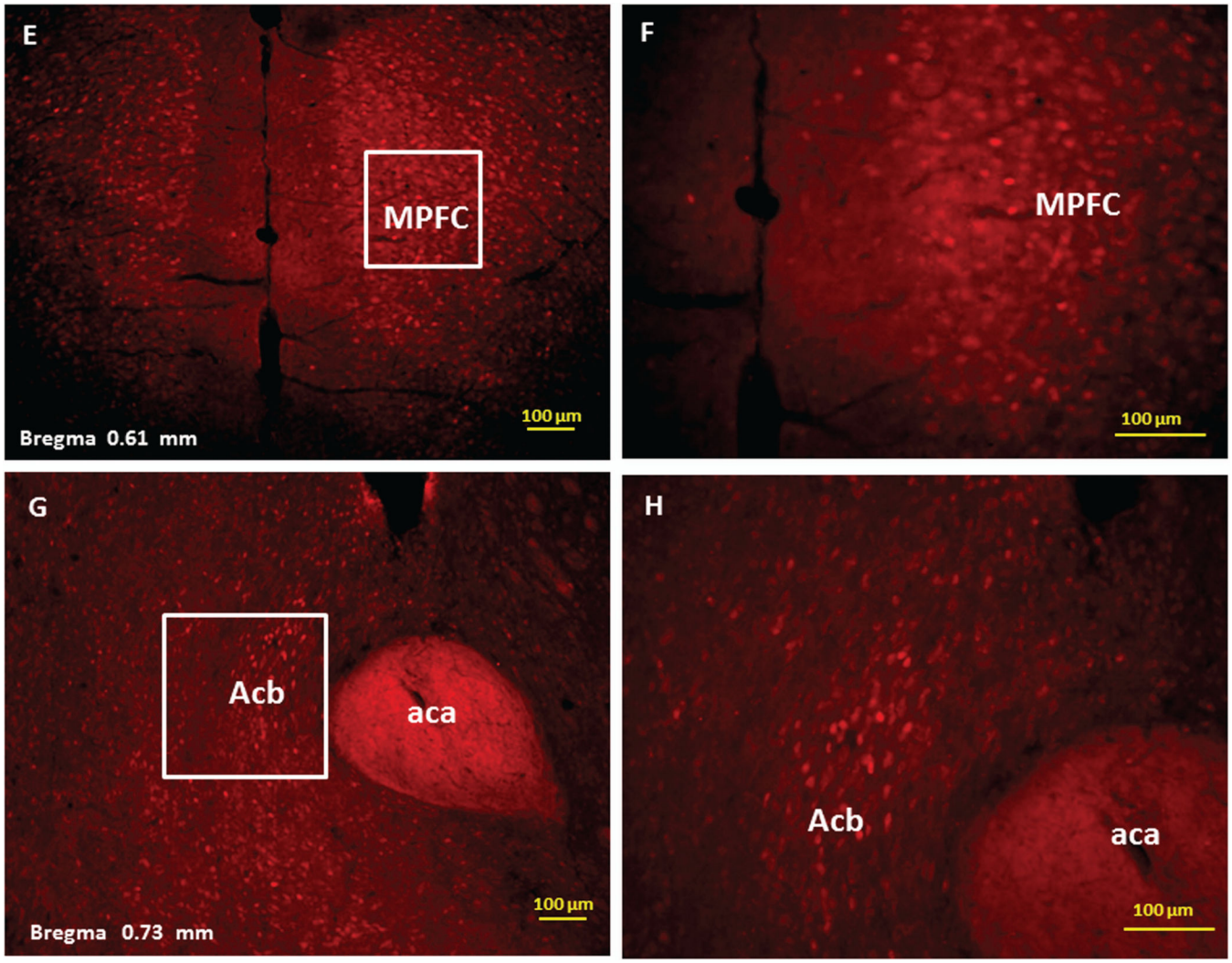


Figure 3.

Immunofluorescence staining demonstrating the sites of c-Fos activated cells of the mesocorticolimbic system in the mouse brain following intraperitoneal injection of nicotine hydrogen tartrate (NIC, 30 µg/kg). Panels A–D: c-Fos immunoreactive cells in the ventral tegmental area (VTA). Panels E and F: c-Fos immunoreactive cells in medial prefrontal cortex (MPFC). Panels G and H: c-Fos immunoreactive cells in nucleus accumbens (Acb).

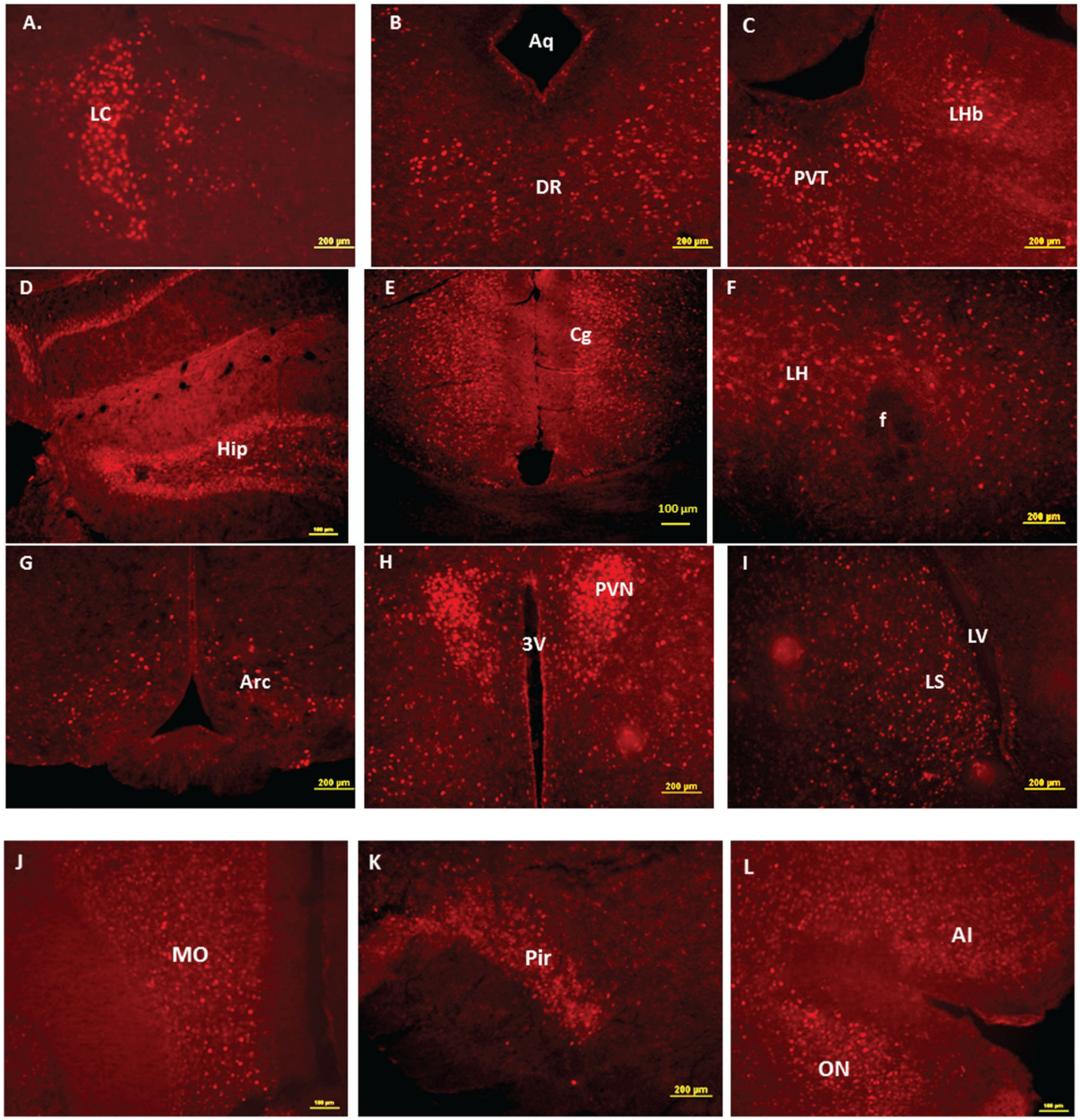


Figure 4.

Immunofluorescence staining in the mouse brain demonstrating representative c-Fos activated regions following intraperitoneal injection of the peripherally-acting nicotine analog, nicotine pyrrolidine methiodide (NIC-PM, 30 µg/kg). A: Locus coeruleus (LC). B: dorsal raphe nucleus (DRN). C: Paraventricular thalamic nucleus (PVT) and lateral habenular nucleus (LHb). D: Hippocampus (Hip). E: Cingulate cortex (Cg). F: Lateral hypothalamus (LH). G: Arcuate hypothalamic nucleus (Arc). H: Paraventricular

hypothalamic nucleus (PVN). I: Lateral septal nucleus (LS). J: Medial orbital cortex (MO). K: Piriform cortex (Pir). L: Angular insular cortex (AI) and olfactory nucleus (ON). The data obtained for the NIC treatment at the above representative sites were qualitatively identical to those obtained for the NIC-PM treatment.

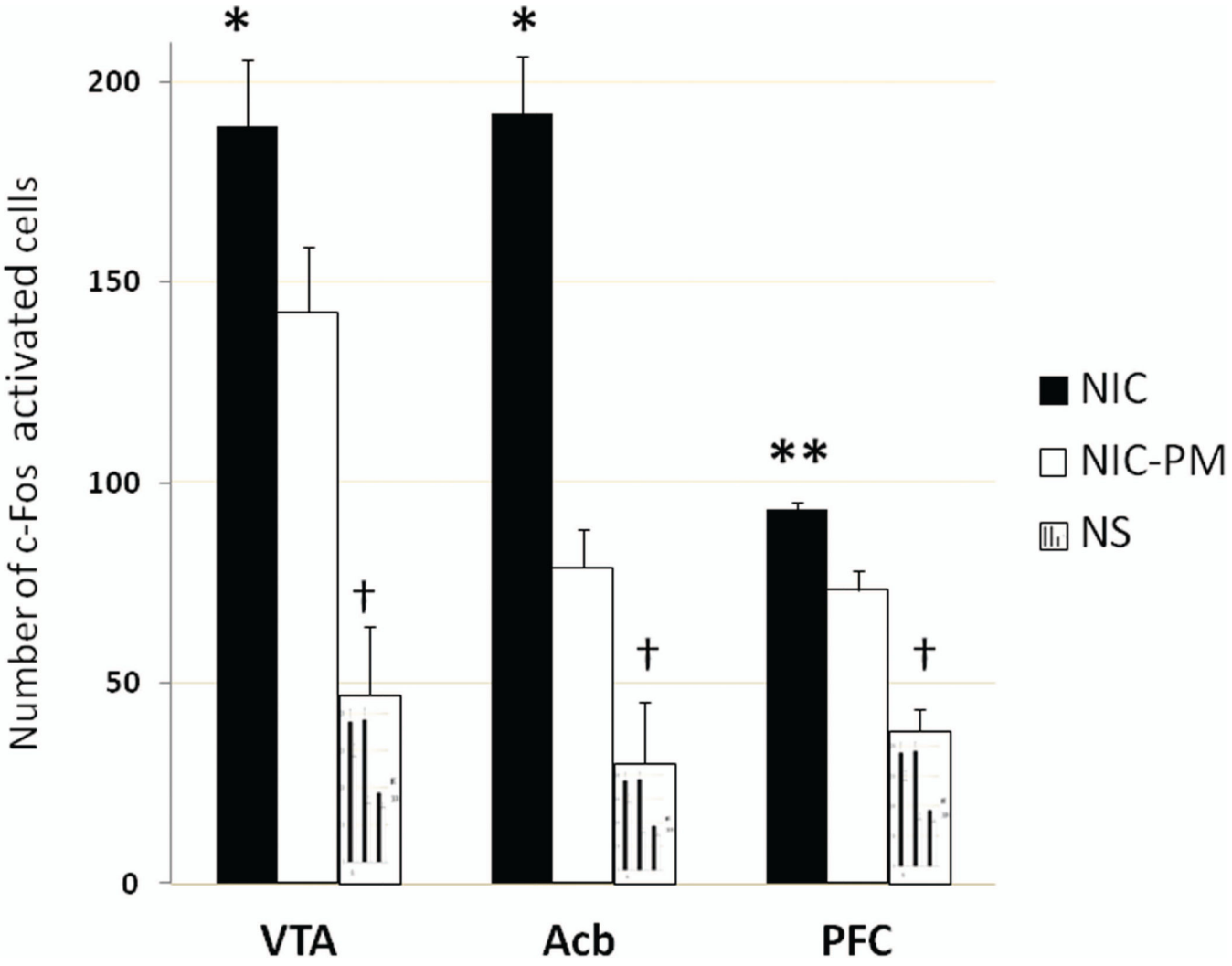


Figure 5.

Nicotine-induced activation of c-Fos at three representative sites in the mouse brain. Bars represent the mean (± standard error) number of c-Fos activated cells counted in ventral tegmental area (VTA), nucleus accumbens (Acb) and prefrontal cortex (PFC). c-Fos immunoreactivity was assessed following intraperitoneal administration of normal physiological saline (NS, control), nicotine hydrogen tartrate salt (NIC) or its peripherally-acting analog nicotine pyrrolidine methiodide (NIC-PM). Differences between the NS control and NIC or NIC-PM treatments were significant at †$P<0.001$ and between NIC and NIC-PM were significant at *$P\leq0.05$, **$P<0.001$.