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Chapter 7

THE DORSAL RAPHE NUCLEUS NEURONS AS TARGETS FOR THE PRESYNAPTIC EFFECTS OF NICOTINE

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ABSTRACT

The dorsal raphe nucleus (DRN) contains the largest group of serotonergic (5-HT) neurons in the brain and provides most of the 5-HT innervation of the forebrain.

Two types of nicotinic acetylcholine receptors (nAChRs) were identified in 5-HT DRN neurons: the heteromeric α4β2 and the homomeric α7. Previous clinical studies from our group demonstrated that nicotine applied as patches in patients with major depression produces antidepressant effects. To determine if this effect depends on nicotine-induced 5-HT release from the DRN, experimental studies were conducted in rat midbrain slices using the whole patch clamp technique. Nicotine, applied in perfusion or locally into the DRN, increased the firing frequency of ~70% 5-HT DRN neurons, an effect dependent on increases presynaptic glutamate and noradrenaline release. In the remaining 30% of 5-HT DRN nicotine induced a decrease in firing rate

associated with a presynaptic increase in serotonin and GABA release. The nicotine-induced increase in glutamate and serotonin release was dependent on presynaptic α4β2 nAChRs, whereas the increase in noradrenaline and GABA release was mediated by presynaptic α7 nAChRs. These data suggest that small concentrations of nicotine produce long lasting excitatory effects through glutamate release, as well as inhibitory effects through 5-HT release. High doses of nicotine will produce additional stimulatory and inhibitory effects through noradrenaline and GABA release respectively.

NICOTINIC ACETYLCHOLINE RECEPTORS (NACHRS)

Nicotinic acetylcholine receptors **(**nAChRs) belong to the family of ligand-gated ionic channels, which also includes $GABA_A$, $GABA_C$, glutamate, $5\text{-}HT_3$, glycine and ATP-gated channels. The first nAChRs discovered were postsynaptic, located in the neuromuscular junction, autonomic ganglia and spinal cord. The stimulation of these nAChRs produced Na^+ and Ca^{2+}-dependent fast excitatory responses. Later on, a variety of neuronal nAChRs were discovered in the central nervous system (CNS), located both in neuronal somata and axon terminals. Surprisingly, in most of cases, the stimulation of CNS nAChRs did not produce the fast excitatory responses previously reported with peripheral nAChRs, but only an increase in the release of various neurotransmitters. It was concluded that CNS nAChRs have mainly a presynaptic location and their role is to modulate the release of various neurotransmitters (Wonnacott, 1997). This opinion was reinforced by two observations. First, neuronal nAChRs are more permeable to Ca^{2+} than to Na^+; as a consequence, presynaptic nAChRs may induce neurotransmitter exocytosis by increasing intracellular Ca^{2+} as a sole effect or by activating voltage-gated Ca^{2+} channels too (McGehee and Role, 1995; Wonnacott, 1997). Second, in most CNS neurons nicotine induces neurotransmitter release in the presence of tetrodotoxin (TTX), i.e., independently of action potential generation (Wonnacott, 1997).

Neuronal nAChRs are pentameric structures formed of α and non-α (also termed β) subunits. Nicotinic neuronal AChRs can be classified in homomeric, formed out a single type of subunit and heteromeric, which contain combinations of α and β subunits. To the date nine types of α and three types of β subunits were isolated. The combination of these subunits may generate, theoretically, more than1000 different types of nAChRs. However two types of neuronal nAChRs were more frequently identified in the CNS: the

homomeric α7 [$(\alpha7)_5$] and the heteromeric α4β2 [$(\alpha4)_2(\beta4)_3$] (Flores et al., 1992; Paterson and Nordberg, 2000). Agonist binding sites of nAChRs are located at the interface between α subunits and a neighboring subunit. nAChRs' pharmacological and biophysical profiles depend on their subunit composition. For example, α7 nAChRs are highly permeable to calcium, desensitize rapidly and present low affinity for nicotine, whereas the $(alpha4)_2(beta2)_3$ nAChRs present a much lower permeability for Ca^{2+}, desensitize slower and exhibit a higher affinity for nicotine. Identification of nAChRs subtypes may be achieved through molecular biology, electrophysiological and biochemical techniques (Gotti et al., 2009). It is not always possible to determine the complete subunit composition of nAChRs. Therefore, a possibly unknown subunit in the composition of a nAChRs is suggested by an asterisk ("*"). For example, $\alpha3\beta4^*$ indicates a nAChR known to contain α3 and β4 subunits but also additional, unknown subunits. If both the subunit composition and the subunit stoichiometry are known, the number of each subunit in a pentamer is indicated by subscript numbers ((for example the $(\alpha4)_2(\beta2)_3$ nAChR contains two α4 subunits and three β2 subunits)).

THE DORSAL RAPHE NUCLEUS (DRN)

DRN is located in the midline of the brainstem, between the oculomotor nucleus and the middle region of the pons. It contains the largest population of 5-HT neurons in the brain (~ 160,000 in humans and ~ 12,000 in rats). Most 5-HT DRN neurons are located in the rostral division of the nucleus, in the dorsal (DRD), ventral (DRV), interfascicular (DRI) and ventro-lateral subdivisions (Baker et al., 1992) (Figure 1). 5-HT DRN neurons project preferentially towards the forebrain (Olivier, 2015) and are involved in the regulation of a large number of brain functions such as: sensory processing, cognitive control, emotions, learning and memory, mood, sleep, anxiety, autonomic control, motor activity, feeding, body temperature, aggressive behavior, sexual activity and stress (Olivier, 2015). The serotonergic system contains at least 14 different classes of receptors ($5\text{-}HT_{1A, 1B, 1D, 1E, 1F; 2A, 2B, 2C, 3, 4, 5A, 5B, 6 and 7}$) and a serotonin transporter (SERT). With the exception of the $5\text{-}HT_3$ receptor, which is ionotropic, all the other 5-HT receptors are metabotropic, coupled to G proteins. $5\text{-}HT_{1A}$ and $5\text{-}HT_{1B}$ receptors function both as inhibitory autoreceptors and heteroreceptors (Artigas, 2013).

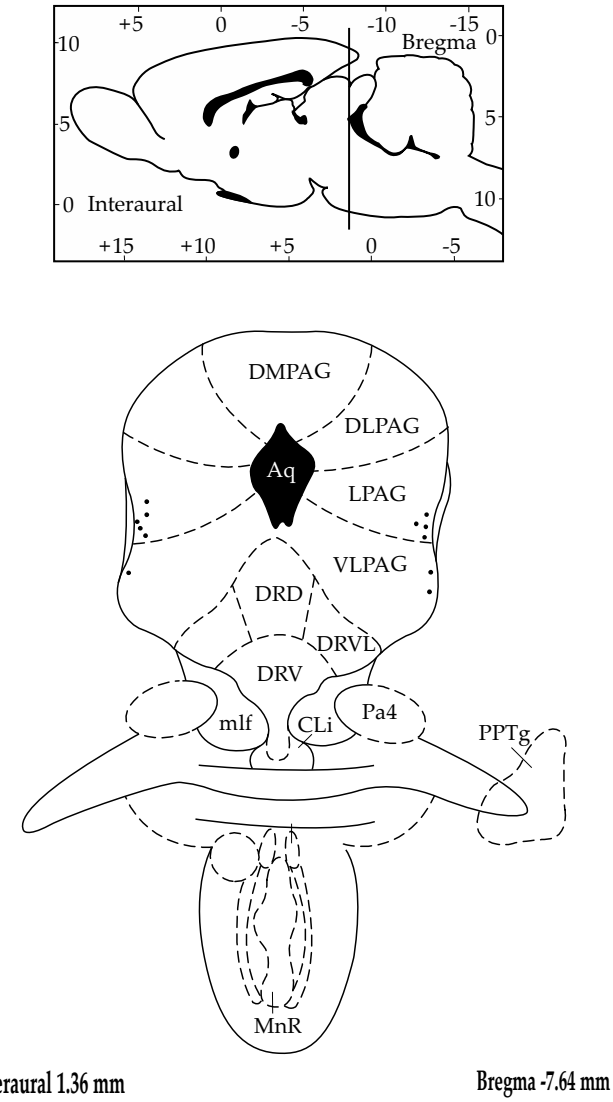

Figure 1. Anatomy of the dorsal neucleus (DRN). In a coronal rat midbrain slice, the DRN is located between the aqueduct (Aq) and the medial longitudinal fasciculus (mlf). It contains various subdivisions as the dorsal (DRD), ventrolateral (DRVL) and the ventral (DRV) ones. Other anatomical elements presented: CLi=caudal linear nucleus of the raphe; DMPAG=the dorsomedial periaqueductal grey; DLPAG=dorsolateral periaqueductal grey; LPAG=lateral periaqueductal grey; VLPAG=ventrolateral periaqueductal grey; PA4=paratrochlear nucleus; PPTg=pedunculopontine tegmental nucleus; MnR=median raphe nucleus. Bregma -7.64 (modified from Paxinos y Watson, 1996, with permission).

PREMISES OF THE PRESENTED STUDIES

Experiments performed in cats (Vazquez et al., 1996) demonstrated that transdermal nicotine suppresses the ponto-geniculo-occipital waves of the rapid eye movement (REM) sleep. Since the same effect can be obtained through electrical stimulation of the DRN, it may be assumed that nicotine induces the stimulation of 5-HT DRN neurons that project to laterodorsal and pedunculopontine tegmental cholinergic nuclei, generators of the REM sleep. Moreover, clinical studies performed by our group (Salín-Pascual and Drucker-Colín, 1998) demonstrated that nicotine applied as parches improves the mood of non-smoking patients with major depression. This effect may also depend on an increase in 5-HT release from the DRN, since 5-HT is a potent endogenous antidepressant agent (Blier, 2013).

The presence of nAChRs containing α4 and α7 nAChR subunits in the DRN of rats was demonstrated by Bitner et al., (2000) and by Bitner and Nikkel (2002) respectively, using immunohistochemical techniques and was confirmed in a later study by Cucchiaro and Commons (2003). It was assumed that these α subunits belong to α4β2 nAChRs and α7 nAChRs, because these nAChRs are more frequently expressed in the CNS.

NICOTINE-INDUCED α7 nACHRS DEPENDENT NORADRENALINE RELEASE IN THE DRN

In the study of Li et al., (1998), performed in rat midbrain slices using the whole-cell patch clamp technique, nicotinic agonists ((acetylcholine 30μM, nicotine 20 μM and 1,1-dimethyl-4-phenylpiperazinium (DMPP) 15 μM)) elicited depolarization in the majority (~60%) of 5-HT DRN neurons (Figure 2). This depolarization was mimicked by the α_1 receptor agonist phenylephrine, blocked by the α_1 receptor antagonist prazosin and prolonged by the noradrenaline (NE) uptake inhibitor nisoxetine. In control conditions the depolarizing response was blocked by mecamylamine (MEC, 50 μM), which indicates the nicotinic mediation of the response, and by the specific antagonist of the α7 nAChRs methyllycaconitine (MLA, 100 nM). According to the authors, these data suggest that nicotinic agonists induce noradrenaline release in the DRN by acting on presynaptic α7 nAChRs of noradrenergic varicosities originated in locus coeruleus. At its turn noradrenaline acts on postsynaptic α_1 receptor of 5-HT neurons producing depolarizing effects. An

interesting experimental data is that tetrodotoxin (TTX, 0.6 µM) blocked DMPP induced depolarization. The existence of a nicotine – dependent excitatory effect on dorsal raphe 5-HT neurons was confirmed by the study of Mihailescu et al., (1998), using extracellular recordings in rat midbrain slices. In this study bath administration of nicotine (10-300 µM) increased both the firing rate of 67.5% 5-HT DRN neurons by 10-90% and DRN 5-HT levels by 2-7 times. These stimulatory effects of nicotine were blocked by MEC (1-20 µM). Interestingly MEC induced its own stimulatory effects on 5-HT DRN neuron firing rate and 5-HT release.

NICOTINE-INDUCED SEROTONIN RELEASE IN THE DRN

The study of Li et al., (1998) indicated that after blocking the α_1 receptors with prazosin (1 µM), DMPP produced a hyperpolarizing effect which was mimicked by 5-HT, antagonized by pindobind (a blocker of 5-HT_{1A} receptors) but was unaffected by TTX or low Ca^{2+} and high Mg^{2+}. It was concluded that nicotinic agonists induce 5-HT release from 5-HT DRN neuron dendrites or axon collaterals. The Ca^{2+} independence of this hyperpolarizing effect was explained through activation of the inositol phospholipid turnover in 5-HT neurons. Alternatively it was proposed that the low Ca^{2+} high Mg^{2+} solution used in the experiments reduced but did not suppress the Ca^{2+} influx through nAChRs; this residual low Ca^{2+} influx may have been sufficient to induce 5-HT release.

In the study of Frias et al., (2012) performed in midbrain slices of adrenalectomized rats implanted with 70 mg corticosterone pellets, the prevalence of inhibitory responses of 5-HT DRN neurons to nicotine (78.6%) was overwhelmingly higher than in sham-operated animals in which only excitatory effects were observed. The inhibitory effect of nicotine (1 µM) observed in adrenalectomized rats was blocked, as previously reported in brain slices of normal animals (Mihailescu et al., 2002), by the 5-HT_{1A} receptor antagonist WAY100635 (25 nM) and prevented by the specific α4β2 nAChR antagonist dihydro-β-erythroidine hydrobromide (DHβE, 100 nM) (Figure 3).

Combined, the results of these experiments suggest that nicotine-induced 5-HT release in the DRN is mediated by somatodendritic α4β2 nAChR.

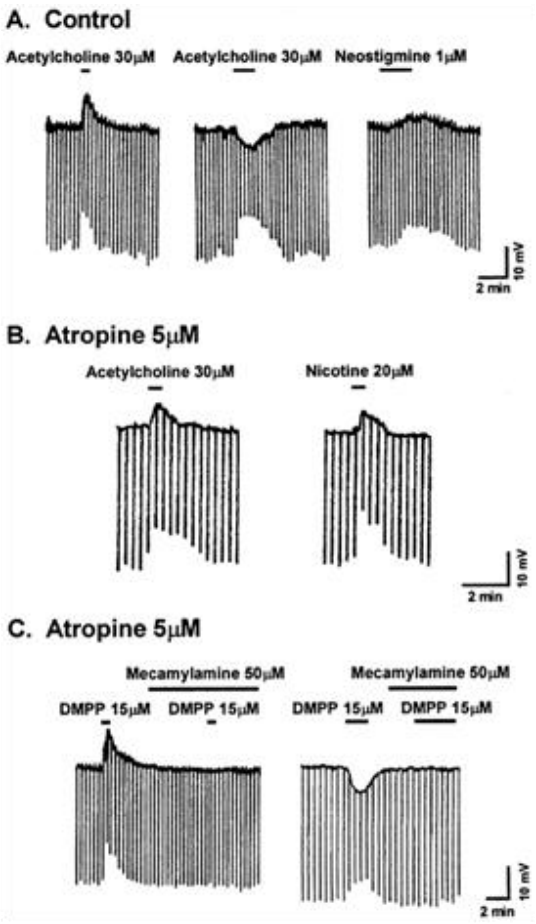

Figure 2. Acetylcholine activates nicotinic responses in dorsal raphe neurons. A, Three voltage traces show typical depolarizing and hyperpolarizing membrane potential responses to acetylcholine and a depolarizing response to neostigmine from three different neurons. All responses are associated with a decrease in input resistance, measured with intra- cellular current injection (600 msec in duration; 50 pA in amplitude; downward deflections in all traces). B, In the presence of the muscarinic antagonist atropine, acetylcholine and nicotine both induce a membrane depolarization, suggesting activation of nicotinic receptors. C, The role of nicotinic receptors in the cholinergic responses is supported further by blockade of both depolarizing and hyperpolarizing responses to the nicotinic agonist DMPP by the nicotinic antagonist mecamylamine in two different neurons in the presence of atropine. The depolarizing response recovers by 50% after a 10 min wash in control medium, and the hyperpolarizing response recovers by 90% after a 6 min wash. From: Li et al, J Neurosci 1998 18:1904-1912, with permission.

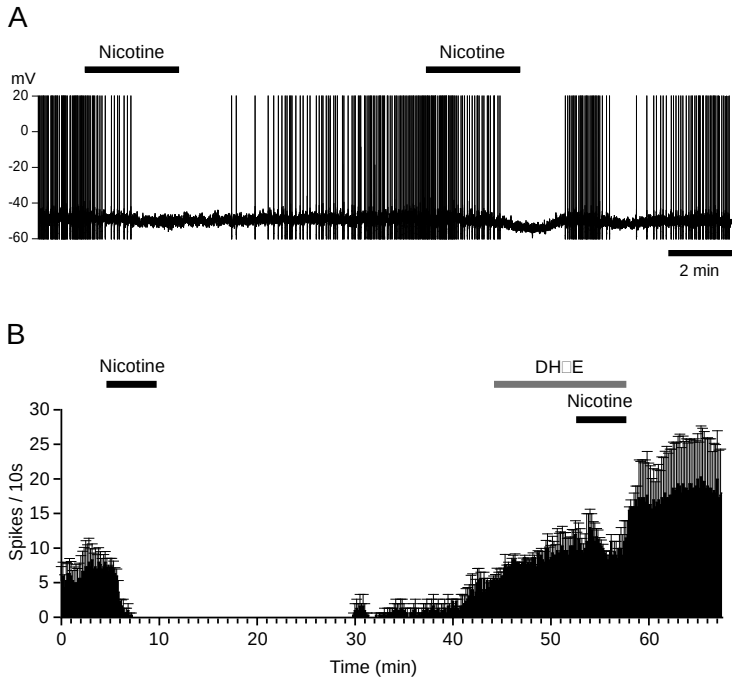

Figure 3 Dihydro-β-erythroidine (DHβE, 100 nM), a selective antagonist at α4β2 nAChRs, reduces the inhibitory effects of nicotine on 5-HT DRN neurons obtained from Adx+CSR rats (adrenalectomized rats subcutaneously implanted with 75 mg corticosterone pellets). (A) Brief (3 min), repeated administrations of nicotine (1μM) produced reproducible inhibitory effects on the firing of an identified 5-HT DRN neurons obtained from an Adx + CSR rats. (B) Frequency histogram showing that prolonged (8 min) administration of nicotine (1 μM) produces a long-lasting inhibition of 5-HT DRN neuron firing. After recovery, administration of DHβE 100 (nM) converted the inhibitory effect of nicotine (1 μM) into a stimulatory one. The results are presented as means ± SEM (n = 5). From: Frías-Dominguez et al., Brain Res Bull. 2013, 98:10-22, with permission.

NICOTINE-INDUCED GLUTAMATE RELEASE IN THE DRN

Nicotine produces excitatory effects in 70-80% of 5-HT DRN neurons (Li et al, 1998; Mihailescu et al., 1998, 2002; Chang et al., 2014), an effect explained originally only by a presynaptic intra-raphe release of noradrenaline (Li et al, 1998). However the stimulation of DRN 5-HT neurons by nicotine also occurred in presence of high concentrations of the α1-adrenoreceptor agonist phenylephrine (50 μM) (Mihailescu et al., 1998). 5-HT DRN neurons

receive glutamatergic afferents from the laterodorsal and pedunculopontine tegmental nuclei (Wolf and Butcher, 1989), cerebral cortex and glutamatergic DRN interneurons (Celada et al., 2011). Since one of the most common effects of nicotine is to produce a presynaptic release of glutamate (McGehee et al., 1995) we investigated if the stimulatory effect of nicotine on 5-HT DRN neurons depends on glutamate release (Garduño et al., 2012). Experiments were performed in midbrain slices obtained from young Wistar rats. The activity of DRN neurons was recorded using the whole-cell current and voltage clamp techniques. Neurons were identified as serotonergic in line, using electrophysiological criteria (Aghajanian and Vandermaelen, 1982) and off line using immunohistochemical techniques. Bath administration of nicotine (0.3-1μM) increased the frequency but not the amplitude of spontaneous excitatory postsynaptic currents (sEPSCs) by ~88% in 16 out of 18 5-HT DRN neurons tested. This effect was mimicked by exogenous acetylcholine (ACh, 1 mM) and by eserine (an inhibitor of acetylcholine esterase, 10 μM) and was suppressed by CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), an antagonist of glutamate AMPA/kainic acid receptors, 10 μM). Nicotine-induced glutamate release was not affected by pre-treatment with TTX (500 nM), a blocker of voltage-gated Na^+ channels, which strongly suggests a presynaptic effect of nicotine but was blocked by a mixture of voltage-gated calcium channel (VGCC) blockers. An interesting observation resulting fro this study is that the increase in firing rate of 5-HT DRN neurons produced by nicotine persisted 10-20 minutes after washing the drug, which suggests that a single administration of nicotine induces of a long-term potentiation of glutamate release in the DRN.

Nicotine-induced glutamate release was mediated by α4β2 nAChRs since it was not blocked by methyllycaconitine (MLA 100 nM), a specific antagonist of α7 nAChRs (Figure 4A and 4B) but was blocked by the specific antagonist of α4β2 nAChRs DHβE (100 nM) (Figure 4C and 4D). The Ca^{2+} influx induced by activation of presynaptic α4β2 nAChRs was not sufficient to induce glutamate release and but opened, through depolarization, VGCC of axon terminals. This phenomenon was previously described by Dajas Bailador et al., (2002).

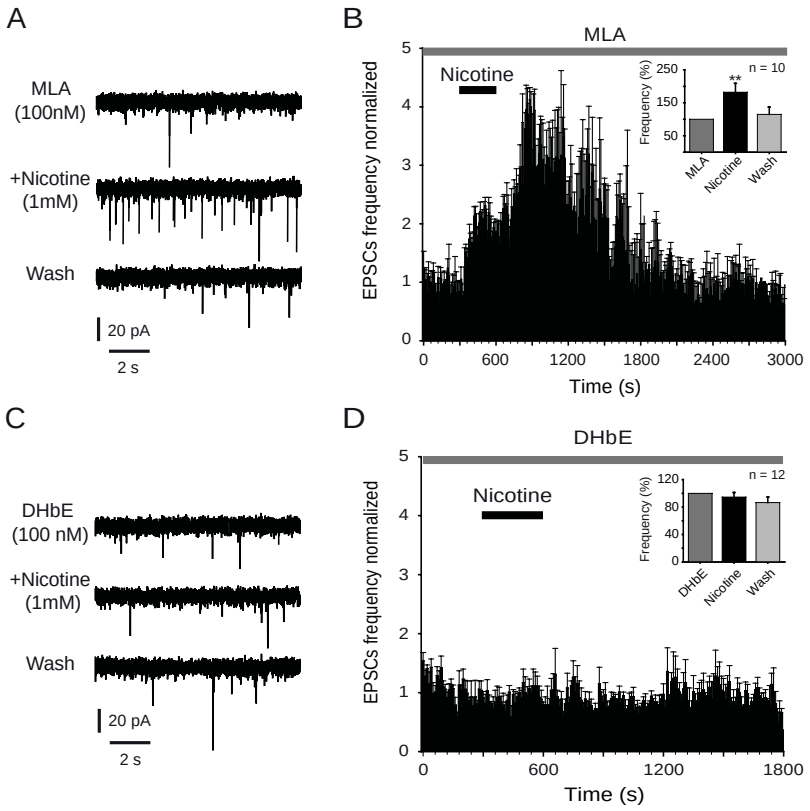

Figure 4 Nicotinic effects are performed through β2-containing nAChRs. *A*, Traces showing sEPSCs recorded from a 5-HT-positive neuron in the presence of MLA (100nM, top), MLA plus nicotine (1 µM, middle) and nicotine washout (bottom). *B*, Time-frequency histograms from 10 identified 5-HT neurons tested with nicotine (1 µM) in the presence of MLA. The inset shows the normalized sEPSCs frequency. *C*, Traces showing sEPSCs recorded from 5-HT positive neuron in the presence of DHβE (100 nM, top), DHβE plus nicotine (1 µM, middle) and nicotine washout (bottom). *D*, Time-frequency histogram from 12 identified 5-HT neurons tested with nicotine (1 µM) in the presence of DHβE. The inset shows the normalized sEPSCs frequency. In all the experiments nicotine was applied after a 10 min pretreatment with DHβE or MLA. From Garduño et al., J. Neurosci, 2012; 32:15148-15157, with permission.

NICOTINE-INDUCED GABA RELEASE

5-HT DRN neurons receive GABAergic afferents from various areas of the brain such as: lateral and posterior hypothalamus, lateral preoptic area,

ventral pontine periaqueductal gray, substantia nigra, ventral tegmental area (Gervasoni et al., 2000) and rostromedial tegmental nucleus (Lavezzi et al., 2012; Sego et al., 2014). DRN contains a large population of GABAergic neurons located especially in the lateral wings of the DRN (Gervasoni et al., 2000), which project both to 5-HT DRN neurons and other areas the brain. GABA released from these afferents inhibits directly the activity of 5-HT DRN neurons by means of $GABA_A$ and $GABA_B$ receptors of 5-HT DRN neurons (Tao et al, 1996). In addition, recent studies demonstrated that GABA modulates glutamatergic synaptic transmission through presynaptic mechanisms (Soiza-Reilly et al., 2013). This last regulatory mechanism has two components: a $GABA_A$-mediated facilitation and a $GABA_B$–mediated inhibition of glutamate release (Soiza-Reilly et al., 2013).

Locally released GABA, tonically inhibits the activity of 5-HT DRN neurons since bicuculline, a $GABA_A$ receptor antagonist, increased the firing rate of 5-HT DRN neurons of rat midbrain slices (Mihailescu et al., 2002). A similar effect was observed when bicuculline was administered to unanesthetized rats during slow wave and paradoxical sleep (Gervasoni et al., 2000). It was proposed that the decrease in 5-HT DRN neuron firing rate during sleep depends on an increase in local GABA release (Nitz and Siegel 1997; Gervasoni et al., 2000).

Immunohistochemical studies by Bitner and Nikkel (2002) demonstrated the presence of α7 subunit-containing nAChRs in putative GABAergic cell of the DRN. The data issuing from the study of Bitner and Nikkel did not allow however determining neither the neuronal location (somato-dendritic, axon terminal) nor the functionality of the above-mentioned α7 nAChRs. Therefore we performed a detailed study concerning the effects of nicotine on GABAergic activity in the DRN (Hernandez-Vazquez et al., 2014).

The experiments were performed in coronal midbrain slices obtained from young (postnatal days 18-21) Wistar rats. GABAergic spontaneous inhibitory postsinaptic currents (sIPSCs) were recorded from post hoc-identified 5-HT DRN neurons using the whole cell voltage-clamp technique. Administration of nicotine (1 μM) increased sIPSC frequency in 72% of identified 5-HT DRN neurons (Figure 5A). This effect was not mediated by α4β2 nAChRs, since RJR-2403 (100 nM), a specific nAChRs agonist did not reproduce it and DHβE, a specific α4β2 nAChR antagonist, did not block it. The effect was mimicked by the selective agonist of α7 nAChR, PNU-282987 (100 nM), blocked by MLA (20 nM), and MEC (50 μM) (Figure 5D) and exacerbated by the positive allosteric modulator of the same receptor, PNU-120596 (10 μM).

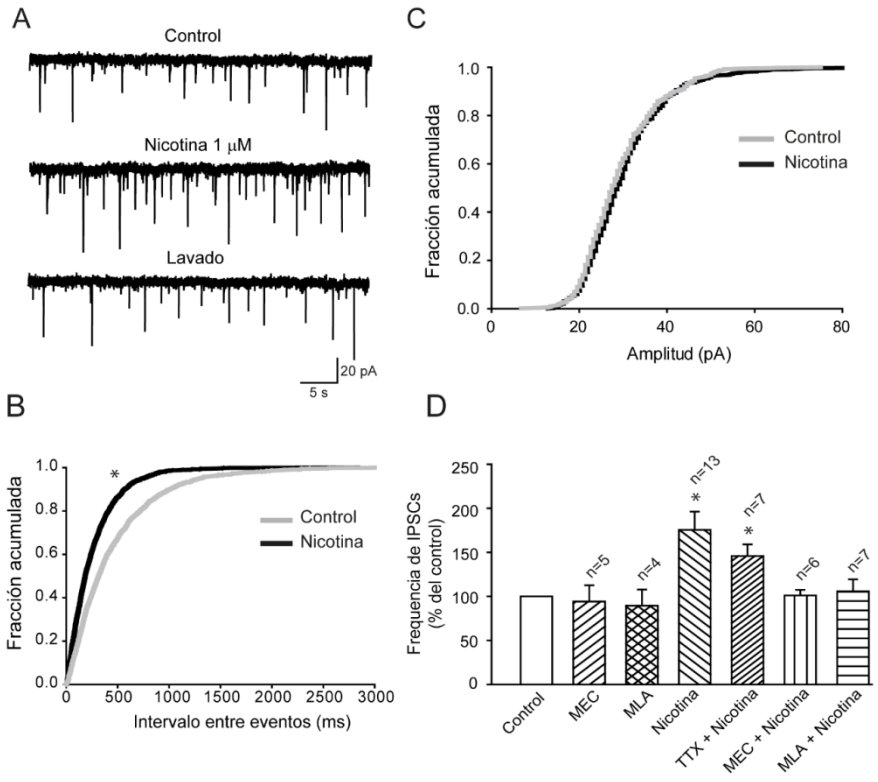

Figure 5. Nicotine increases GABAergic sIPSC frequency in the majority of 5-HT DRN neurons. *A*, *top*: control recording of sIPSCs in the presence of CNQX (10 μM) and APV (50 μM). *Middle*: in the same neuron, nicotine (1 μM) increases GABAergic sIPSC frequency. *Bottom*: after washout of nicotine, sIPSC frequency returned to basal values. *B* and *C*: cumulative probability distributions of frequency (*B*) and amplitude events (*C*) from the neuron in *A* showing that nicotine increases the frequency but not the amplitude of GABAergic sIPSCs. *D*: summary of the results showing the increases of sIPSC frequency with nicotine observed in 72% of 5-HT DRN neurons ("responder cells"). Mecamylamine (MEC; 50 μM) and methyllycaconitine (MLA; 20 nM) did not alter sIPSC frequency in basal conditions but blocked the stimulatory effect of nicotine. Data are expressed as % of control group. *Significant difference vs. control ($P < 0.05$). From: Hernandez-Vazquez et al., J Neurophysiol., 2014; 112(12):3154-63, with permission.

Therefore, the nAChR involved in nicotine-induced sIPSCs was the homomeric α7. TTX (1 μM) did not change significantly (Figure 5D) the nicotine-induced increase in sIPSCs frequency, which indicates the presynaptic location of α7 nAChR on GABAergic neurons. SIPSCs' frequency was independent on voltage-gated calcium channels (VGCC) but

was dependent on Ca^{2+}-induced Ca^{2+} release (CICR). This last finding reinforces the idea of α7 nAChR mediation of nicotine-induced GABA release since α7 nAChRs possess the highest Ca^{2+} permeability and their activation at presynaptic sites is sufficient to induce by itself neurotransmitter release (Dajas-Bailador et al., 2002, Dickinson et al, 2008).

In conclusion, nicotine-induced GABA release depends on presynaptic α7 nAChR, does not require activation of voltage-gated Ca^{2+} channels but involves calcium-induced calcium release.

DISCUSSION

Analyzing the results of the above-presented studies several conclusions and hypothesis may be withdrawn:

1) Since the nicotine-induced glutamate, serotonin and GABA releases in the DRN are not affected by TTX, they are very likely mediated by presynaptic nAChRs.
2) The stimulation of both α7 and α4β2 presynaptic nAChRs induces intra-raphe release of two neurotransmitters with opposed effects on 5-HT DRN neurons. Thus, the stimulation of α7 nAChRs induces the release of noradrenaline, which excites 5-HT DRN neurons and of GABA, which inhibits the same neurons. Likewise, α4β2 nAChRs induce the release of both glutamate, which stimulates 5-HT DRN neurons and 5-HT which inhibits 5-HT DRN neurons.
3) Based of nAChRs affinities for nicotine it may be assumed that low nicotine concentrations will stimulate mainly α4β2 nAChRs and generate excitatory effects through glutamate release and inhibitory effects through 5-HT release. The intensity of nicotine excitatory effects depends in this case on the density of DRN glutamatergic terminals expressing α4β2 nAChRs, whereas the intensity of 5-HT-dependent inhibitory effects depends on the amount of 5-HT released by nicotine and on the sensitivity of 5-HT_{1A} autoreceptors.
4) It is expectable that nicotine-induced intra-raphe GABA and noradrenaline release reach their maximum with higher concentrations of nicotine, since α7 nAChRs have a lower affinity for nicotine.
5) A single nicotine administration is capable of inducing a long-term potentiation of glutamate release; however this phenomenon could not

be observed for nicotine-induced serotonin, noradrenaline or GABA releases.

The main problem with these conclusions and hypothesis is that all experiments were performed in midbrain slices, in which most of afferent DRN connections were eliminated. It is likely that nicotine effects depending on the increase or decrease in firing rate of neurons that project to the DRN were omitted.

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