

Fig. 1. Dorsal root ganglion neurons were voltage-clamped at a holding potential of -80 mV using a Multiclamp 700B amplifier and the signals digitized using a Digidata 1440A (Axon Instruments, Sunnyvale, CA). The signals were sampled at 5 kHz and filtered at 1 kHz and the data analyzed offline using Clampfit 10 analysis software. To evoke responses, the neurons were stimulated by 100 ms applications of 1 mM acetylcholine every 2 min using a Picospritzer at 6 p.s.i. (General Valve Corporation, Pine Brook, NJ). After a steady response baseline was achieved, the neurons were exposed to various toxin antagonists. (A) Representative current traces before and after exposure to 200 nM α -Ctx Arib[V11L; V16D]; (B) control response before exposure to antagonists and after application of α -Ctx Arib[V11L; V16D] followed by 500 nM α -Ctx PnIA; C, control response followed by the application of a cocktail of antagonists (200 nM α -Ctx Arib[V11L; V16D], 500 nM α -Ctx PnIA, and 1 μ M dihydro- β -erythroidine), to isolate responses mediated by $\alpha 3\beta 4^*$ and $\alpha 6\beta 4^*$ nAChRs, followed by 200 nM α -Ctx MII[E11A] then 10 μ M α -Ctx AulB. Toxins were applied sequentially and additively. Scale bars apply to A, B, and C.

addition of α -Ctx MII[E11A], which under these conditions selectively blocks $\alpha 6\beta 4^*$ vs. $\alpha 3\beta 4^*$ nAChRs, further inhibited $36.1 \pm 5.6\%$ ($n = 8$) of the remaining response providing evidence for expression of $\alpha 6\beta 4^*$ receptors (Fig. 1C). Finally, the remaining response was inhibited by $76.9 \pm 5.2\%$ ($n = 5$) upon addition of α -Ctx AulB indicating the expression of $\alpha 3\beta 4^*$ receptors (Fig. 1C). These results taken together with PCR data support the conclusions of other investigators that DRG neurons express multiple nAChR subtypes and to our knowledge, this is the first report of $\alpha 6\beta 4^*$ nAChRs in DRG.

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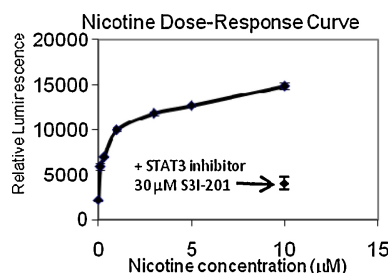
Investigations into nicotinic Stat3 signaling using a luciferase reporter plasmid

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Although better known as ion channels, nicotinic $\alpha 7$ receptors in cells involved in inflammation or the immune system also signal through the Jak/Stat pathway when activated by nicotine or other agonists [1]. We recently showed that $\alpha 4\beta 2$ receptors also signal in part through Jak/Stat [2], and this signaling pathway may be involved in nicotine's neuro-protective and anti-inflammatory actions. In order to investigate nicotine-driven Jak/Stat signaling, we generated a custom reporter plasmid consisting of a Stat3 promoter driving a secreted luciferase reporter gene. Nicotine has no effect on luciferase expression in SH-EP1 and GH4C1

cells transfected with the reporter plasmid but lacking nicotinic receptors. In contrast, co-transfection with $\alpha 7$ in GH4-C1 cells allows dose-dependent nicotine-driven Stat3 signaling that is blocked by 30 μ M Jak inhibitor AG-490 or 30 μ M Stat3 inhibitor S3I-201. In order to see nicotine-driven $\alpha 7$ -dependent Stat3 signaling in SH-EP1 cells, transfection of $\alpha 7$ with reporter plasmid requires co-transfection with the chaperone Ric3, which allows $\alpha 7$ receptors to traffic to the cell surface. We are investigating the effect of various agonists and antagonists on the effects of $\alpha 7$ -mediated Stat3 signaling measured using this novel reporter plasmid. Future experiments will correlate nicotine-driven Stat3 signaling measured by secreted luciferase activity in cells expressing various nicotinic receptor subtypes with phosphorylated Stat3 levels measured by Western blotting.



References

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2.1

Section 2. In vivo pharmacology and clinical studies

Developmental nicotine exposure and the $\alpha 5$ nicotinic acetylcholine receptor

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Maternal smoking during pregnancy can expose the developing fetus to high concentrations of nicotine and has been linked with deficits in attention later in life. Layer VI pyramidal neurons of the medial prefrontal cortex (mPFC) may be a target for the teratogenic effects of nicotine because they are directly excited by nicotinic acetylcholine receptor (nAChR) stimulation during development, are a major source of feedback projections from the mPFC to the thalamus, and are believed to play an important role in attention. We sought to test this hypothesis by exposing mice to either nicotine tartrate (200 μ g/mL calculated as nicotine free base) or tartaric acid control via maternal drinking water throughout gestation and up to weaning on postnatal day 21. Since the nAChR $\alpha 5$ subunit plays a critical role in the normal nicotinic response in these neurons, we tested its contribution to the developmental effects of nicotine by performing this study in both wild type (WT) and $\alpha 5$ subunit knockout ($\alpha 5^{-/-}$) mice. We found a striking interaction between developmental nicotine exposure and $\alpha 5$ genotype during the third week of postnatal life, where the ability of both acetylcholine (in the presence of atropine to block muscarinic receptors) and nicotine to stimulate layer VI neurons was increased by developmental nicotine exposure only in $\alpha 5^{-/-}$ mice. In WT mice, by contrast, the $\alpha 5$ subunit appears to protect the nicotinic response in layer VI neurons from being changed by exposure

to nicotine in development. The interaction between developmental nicotine exposure and $\alpha 5$ genotype occurred in the absence of changes to neuronal membrane properties. Since nicotinic stimulation can influence neuronal growth and maturation, ongoing experiments are investigating the effects of developmental nicotine exposure on mPFC layer VI neuron morphology. Moreover, since developmental nicotine-induced attention deficits can persist beyond developmental periods, we are also testing the effects of developmental nicotine exposure on mPFC layer VI neuron function and morphology in fully mature, adult mice.

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$\alpha 6^*$ nAChR expression and function in brain areas influencing DA transmission probed with $\alpha 6$ -GFP transgenic mice

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Midbrain dopamine neurons serve critical functions in mediating arousal, motivation, motor control, and reward learning. The nicotinic cholinergic system, acting via $\beta 2^*$ and $\alpha 7^*$ nAChRs, is an important regulator of DA transmission. Nicotinic ACh receptors containing the $\alpha 6$ subunit are expressed in a few select brain areas, including midbrain DA neurons, noradrenergic neurons of the locus coeruleus, and glutamatergic retinal ganglion cells. To better understand the regional and subcellular expression pattern of $\alpha 6$ -containing nAChRs, we created and studied transgenic mice expressing a variant $\alpha 6$ subunit with GFP fused in-frame in the M3-M4 intracellular loop. $\alpha 6$ -GFP receptors functioned normally *in vitro* in cultured cells, as well as *in vivo* in synaptosomal DA release experiments. In $\alpha 6$ -GFP transgenic mice, $\alpha 6$ nAChR expression in the brain largely matched previous studies using radiolabeled α -conotoxin MII or mRNA *in situ* hybridization. Surprisingly, we also found $\alpha 6$ subunit expression in selected neuronal cell bodies in medial habenula, interpeduncular nucleus, and superior colliculus. MHb neurons expressing $\alpha 6$ subunits were located in the medial aspect of the MHb adjacent to the ventricle, which were completely distinct from $\alpha 4$ -subunit containing MHb neurons located on the lateral aspect of the MHb. We also noted specific presynaptic and postsynaptic $\alpha 6$ expression in the ventral IPN. In the visual system, $\alpha 6$ subunits were strongly expressed in most retinal ganglion cells, and were weakly expressed in some neurons in dLGN and visual cortex. In superior colliculus, part of the extended basal ganglia critical for relaying short-latency salience signals to midbrain DA neurons, we found strong $\alpha 6$ expression in retinal axons, along with postsynaptic $\alpha 6$ expression in a fraction of SC GABAergic interneurons. In patch clamp recordings from mice expressing hypersensitive $\alpha 6$ subunits, we recorded $\alpha 6$ -dependent, presynaptic and/or postsynaptic nicotinic responses in SC neurons. Together, these electrophysiological results demonstrate that $\alpha 6^*$ nAChRs are uniquely situated to mediate cholinergic modulation of glutamate and GABA release in SC. More globally, our results from these studies support the emerging hypothesis that $\alpha 6^*$ nAChRs, via their expression in key salience centers such as MHb/IPN, superior colliculus, locus coeruleus, and midbrain DA