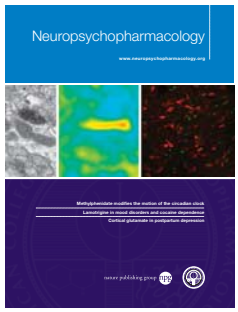

Neuregulin 3 Signaling Mediates Nicotine-Dependent Synaptic Plasticity in the Orbitofrontal Cortex and Cognition

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Neuregulin 3 signaling mediates nicotine-dependent synaptic plasticity in the orbitofrontal cortex and cognition

Running Title: NRG3 and nicotine in OFC function

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Abstract

Neuregulin 3 (*NRG3*) and ErbB4 have been linked to nicotine addiction; however, the neuronal mechanisms and behavioral consequences of NRG3-ErbB4 sensitivity to nicotine remain elusive. Recent literature suggests that relapse to smoking is due to a lack of impulsive control, which is thought to be due to altered functioning within the orbitofrontal cortex (OFC). Therefore, we examined circuitry changes within this structure following nicotine application. We report that nicotine controls synaptic plasticity in the OFC through NRG3/ErbB4-dependent regulation of GABAergic inhibition. We observed that both nicotine and NRG3 facilitated the conversion of long-term potentiation into long-term depression at cortical layer 3/5 synapses. Induction of long-term depression by nicotine relied on nicotinic receptor activation and key regulators of NRG3 signaling: (1) release of intracellular calcium, (2) activation of the BACE1 beta-secretase, and (3) ErbB4 receptor activation. Nicotine-induced synaptic plasticity was also associated with accumulation of intracellular GABA and was completely blocked by $GABA_A$/$GABA_B$ antagonists. To test whether these mechanisms underlie OFC-dependent behavior, we evaluated the effects of nicotine in the go/no-go task. Nicotine-impaired stimulus discrimination in this task was rescued by pharmacologic disruption of the NRG3 receptor, ErbB4. Together, our data indicate that nicotine–induced synaptic plasticity in the OFC and cognitive changes depend on NRG3-ErbB4 signaling. We propose that nicotine activation of this pathway may contribute to nicotine addiction, particularly in individuals with genetic variation in *NRG3*.

Key words: NRG3; ErbB4; nicotine; synaptic plasticity; OFC; go/no-go; nicotine withdrawal;

Introduction

Tobacco smoking is the leading cause of preventable death in the world, responsible for approximately 6 million deaths each year (2016). Despite the health risks, approximately 18% of the general U.S. population smokes (Jamal *et al*, 2014). Several pharmacotherapies are currently available for nicotine dependence; however, relapse is common and smoking cessation is successful in less than 5% of quit attempts (Nides, 2008). These high rates of relapse to smoking may be due to genetic factors. Heritability of nicotine dependence is estimated at 40% to 75% (Rose JE, 2009) and genome-wide association studies have identified a number of associated risk alleles. Genes encoding a member of the epidermal growth factor family, neuregulin 3 (NRG3), and its receptor, ErbB4, have been recently linked to smoking cessation outcomes (Loukola *et al*, 2014; Turner *et al*, 2014). These genes are of particular interest due to their therapeutic potential, with downstream targets under therapeutic development for several neuropsychiatric disorders. For example, NRG3-ErbB4 signaling is shown to regulate activity of the protein PI3K (Krivosheya *et al*, 2008), which is being examined as a potential target for treatment of schizophrenia (Papaleo *et al*, 2016).

Smoking has been consistently shown to reduce the thickness of gray matter volume in the OFC, a brain region involved in decision making and processing of reward-related information (Kuhn *et al*, 2010; Li *et al*, 2015; Tremblay and Schultz, 2000; Volkow and Fowler, 2000). Acute nicotine increases blood oxygen level dependent (BOLD) fMRI signal in the striato-thalamo-orbitofrontal circuit (Bruijnzeel *et al*, 2015) and the behavioral effects of nicotine exposure on this circuit have been extensively studied (Ashare *et al*, 2014). Neuronal mechanisms underlying such effects, however, are not readily ascertained since nicotine facilitates release of multiple neurotransmitters, including glutamate, GABA, and dopamine,

which may result in facilitation as well as suppression of neuronal output (Alkondon *et al*, 2000; Ji and Dani, 2000; Ji *et al*, 2001; Turner, 2004; Wang *et al*, 2006). Nicotine has been reported to enhance long-term potentiation (LTP) in a number of brain regions, including hippocampus (Nakauchi and Sumikawa, 2012), amygdala (Huang *et al*, 2008), and ventral tegmental area (Mansvelder and McGehee, 2000). Few studies have evaluated the effects of nicotine on plasticity in frontocortical areas, although one report showed that nicotine raised the threshold for LTP induction in the prefrontal cortex by enhancing GABAergic transmission (Couey *et al*, 2007). Although GABAergic interneurons throughout the brain are enriched with ErbB4 receptors (Bean *et al*, 2014; Loos *et al*, 2014), ErbB4 receptor interaction with nicotinic signals has yet to be examined in the context of synaptic plasticity in the OFC.

We, and others, have previously shown that transcriptional regulation of NRG3-ErbB4 signaling regulates behavioral symptoms of anxiety induced by nicotine withdrawal (Bi *et al*, 2015; Turner *et al*, 2014). This is consistent with epidemiological studies suggesting that nicotine dependence increases the risk of anxiety disorders and panic attacks (Bruijnzeel, 2012), although tobacco smoking has also been reported to exert anxiolytic effects that may underlie the addiction potential of nicotine (Gilbert *et al*, 1989; Torres and O'Dell, 2016). Frontocortical activation, including the OFC, has been implicated in processing anxiety and anxiety-dependent cognitive traits of attention and impulsivity, all of which are strongly dysregulated in nicotine dependence (Gold *et al*, 2015; Rauch *et al*, 2007; Ursu and Carter, 2009). To evaluate these behaviors in rodents, we used a go/no-go discrimination task that allows for discrimination between measures of attention and impulsivity in mice, as a behavioral read-out of the link between nicotine and NRG3 signaling. To mechanistically understand how nicotine and NRG3 interact to influence OFC functionality, we utilized electrophysiological and molecular

approaches with the overall goal of identifying a putative pathway by which nicotine triggers NRG3/ErbB4 signaling resulting in nicotine-induced behaviors. Understanding of the mechanics of this pathway and its connection with nicotine has clinical potential, particularly in individuals with genetic risk variations in NRG3 that have been found associated with smoking cessation outcomes.

Materials and methods

Animals and housing

Male B6/129F1 and NRG3^{ska} (Jackson Laboratories; 6-10 weeks of age; 20–25g) were used in both the electrophysiology and molecular experiments. NRG3^{ska} mice arose as a spontaneous mutation, which was later identified as a microsatellite repeat within intron 7 of the Nrg3 gene; strain of origin is A/J, which also possess this mutation. In previous experiments, the B6/129 F1 mice have been successfully utilized as a control line for these animals by providing widely comparable mice that can be used for testing, show hybrid vigor, and do not display genetic drift (Turner *et al*, 2013). Both B6/129F1 and NRG3^{ska} subjects were group-housed in a colony room with *ad libitum* food and water access and were maintained on a 12-h light/dark cycle, with lights on at 0700 hours in accordance with the University of South Carolina Animal Care and Use Committee. For behavioral studies, C57BL/6J (Jackson Laboratories, 6-10 weeks; 20-25g) mice were employed due to their widespread use as controls in previous go/no-go and attentional experiments (Cole *et al*, 2015; D'more *et al*, 2013; Ortega *et al*, 2013) and their close relation to the C57/129 F1 hybrids. The C57BL/6J mice were housed individually with *ad libitum* food but progressively water-restricted to 5 min of water per day in addition to sweetened water received as a reward for each correct response (see below). All behavioral training and testing took place 7

days/week between 9:00 and 16:00 h in accordance with the Animal Care and Use Committee of Temple University.

Drugs

The following compounds and drugs were used in this study: (-)-Nicotine hydrogen tartrate (MP Biomedicals, Santa Ana, CA); NRG3 (Sino Biological, Beijing, China); ErbB4 inhibitor, afatinib (LC Laboratories, Woburn, MA); $GABA_A$ receptor antagonist, picrotoxin (Acros Organics, NJ); $GABA_B$ receptor antagonist, CGP55845; NMDA antagonist, DL-AP5; α7 nicotinic acetylcholine receptor (nAChR) antagonist, α-Bungarotoxin; α4β2 nAChR antagonist, Dihydro-β-erythroidine hydrobromide (DHβE); intracellular Ca^{2+} release blocker, ruthenium red (all from Tocris Bioscience, San Diego, CA); β-secretase (BACE1) inhibitor, LY2811376 (Selleck Chemicals, Houston, TX). All drugs were prepared as concentrated stock solutions in water, except afatinib, picrotoxin and LY2811376 stock concentrations, which were dissolved in 100% ethanol, and CGP55845 stock concentration, which was dissolved in DMSO. Final ethanol and DMSO concentration did not exceed 2%. Nicotine was prepared fresh on each test day and its pH was adjusted to 7.0-7.2 using 1N NaOH. For behavioral studies, nicotine was dissolved in physiological saline and administered intraperitoneally (i.p.) at 0.1 mg/kg expressed as base. Afatinib was dissolved in physiological saline containing 10% DMSO and 100 mM Captisol (Captisol, La Jolla, CA) and administered i.p. at 10 mg/kg, as previously described (Turner *et al*, 2014).

OFC Slices

Mice were sacrificed and their brains removed and sectioned into 300 μm-thick coronal slices containing OFC with a Vibratome (VT1200, Leica Biosystems, Buffalo Grove, IL) in an ice-cold artificial cerebrospinal fluid solution (ACSF), in which NaCl was replaced by an equiosmolar concentration of sucrose. ACSF consisted of 130 mM NaCl, 3 mM KCl, 1.25 mM NaH_2PO_4, 26

mM $NaHCO_3$, 10 mM glucose, 1 mM $MgCl_2$, and 2 mM $CaCl_2$ (pH 7.2–7.4 when saturated with 95% O_2/5% CO_2). Slices were incubated in ACSF at 32–34°C for 30 min and kept at 22–25°C thereafter, until transfer to the recording chamber. For experiments with ErbB4 or BACE1 inhibition, slices were maintained in ACSF supplemented with afatinib (10 μM) or LY2811376 (25 μM) for 1 hr prior to recording. The osmolarity of all solutions was 305–315 mOsm. Slices were viewed under an upright microscope (Olympus BX51WI, Olympus, Center Valley, PA) with a 10x objective.

Electrophysiology

The recording chamber was continuously perfused (1–2 ml/min) with oxygenated ACSF heated to 32±1 °C using an automatic temperature controller (Warner Instruments, Hamden, CT). Recording pipettes were pulled from borosilicate glass capillaries (World Precision Instruments, Sarasota, FL) to a resistance of 1–2 MΩ when filled with ACSF. Stimulation and recording electrodes were both positioned in layer 3/5 of OFC. Field excitatory post-synaptic potentials (fEPSPs) were evoked at current intensity eliciting 50-60% of the maximal response amplitude, and delivered at 0.05 Hz, through a bipolar tungsten electrode. Stable baseline responses (less than 5% variability) were acquired for at least 10 min prior to drug application or induction of LTP. LTP was induced by a train of 100 pulse (50 Hz) stimuli repeated three times at 10 second intervals using the minimal current required to elicit a maximal fEPSP response. Other, more typical, LTP protocols (e.g. 100 Hz stimulation) did not elicit a reliable LTP (data not shown). Following LTP induction, fEPSPs were acquired for 60 min at 0.05 Hz at baseline stimulus intensity. All recordings were conducted with a Multi-Clamp700B amplifier (Molecular Devices, Sunnyvale, CA). Currents were high-pass filtered at 2 kHz and digitized at 20 kHz using a Digidata 1550 acquisition board and pClamp10 software (Molecular Devices). Recorded data were analyzed with Clampfit (Molecular Devices).

RT-PCR

mRNA was performed on OFC from naïve B6129F1 wild-type and NRG3^{ska} (n=8/group) mice. After sacrifice, OFC were dissected out with tissue punch. Half of the samples were used for mRNA analysis; the other halves were used for protein analysis as previously described (Turner *et al*, 2014). For RT-PCR experiment, first, RNA was isolated using the RNeasy mini kit (Qiagen, Valencia, CA). Then cDNA was synthesized and SYBR-green RT-PCR reactions were assembled using Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA) along with 5 μM primers (final concentration). The mRNA levels of NRG1, NRG3, ErbB3, ErbB4, α4, β2 and α7 nAChR genes were normalized to the housekeeping gene, hypoxanthine-guanine phosphoribosyltransferase (HPRT). Primer sequences are available upon request.

Western blots

Naïve B6129F1 wild-type and NRG3^{ska} mice (n=8/group), or wild-type mice received acute saline or nicotine (0.1 mg/kg) injections (i.p.) 20 min before sacrifice (n=7/group). OFC was dissected out using tissue punches (2 mm diameter) and homogenized in 200 μl ice-cold extraction buffer containing 50mM Tris, 1mM EGTA, 1mM EDTA, 1% SDS, and 1mM PMSF (pH 7.4). Protein concentrations were determined using a BCA assay, with bovine serum albumin as the standard. Prior to loading, 6x SDS Sample buffer (50mM Tris, 2.5% SDS, 36% glycerol, 0.03% bromophenol blue and 1M DTT) was added to each sample, which were then boiled for 5 min. Equivalent amounts of protein (30 μg) were resolved in Any kD TGX precast protein gels (Bio-Rad, Hercules, CA) and transferred to nitrocellulose membranes. Membranes were first incubated with LI-COR blocking buffer (LI-COR, Lincoln, NE) for 1h at RT, then probed with primary antibodies NRG3 (1:1000, Abgent, San Diego CA), ErbB4 (1:2,500, a gift from Dr. Andres Buonanno at NIH), p-PI3K p85-Tyr458, PI3K p85 (all 1:1,000; Cell signaling,

Beverly, MA), β-tubulin (1:2,000, BD biosciences, San Jose, CA) and GAPDH (1:4,000, Santa Cruz, Dallas, TX) overnight at 4 °C. After washing in PBS, the blots were incubated in correspondent fluorescent secondary antibodies (1:10,000 or 1:20,000, LI-COR, Lincoln, NE) in LI-COR blocking buffer for 2h at RT. Membranes were then washed and immunolabeling signals were detected using Odyssey SA system (LI-COR, Lincoln, NE). Densitometry measurements were performed using the Image Studio software (LI-COR, Lincoln, NE). Protein levels of NRG3 or ErbB4 were normalized to GAPDH or β-tubulin, and protein levels of p-PI3K (p85-Tyr458) were normalized to total PI3K (p85) protein.

[^{3}H]GABA release and uptake assay

Mice were sacrificed and frontal cortex dissected out by hand and chopped into ~0.2 mm cubes using razor blades. Tissue was evenly divided and pre-incubated in oxygenated ACSF for 30 min at 37°C. Aminooxyacetic acid (0.1 mM), a GABA transaminase inhibitor, and β-alanine (1 mM), a glial GABA uptake inhibitor, were present in all solutions.

For GABA release, tissue was incubated in 50 nM [^{3}H]GABA (PerkinElmer Life Sciences, Waltham, MA; 30 Ci/mmol) for 30 min at 37°C. After a triple wash with ACSF, basal [^{3}H]GABA release was measured for 15 minutes in fresh 3 ml ACSF medium every 5 min. Nicotine-evoked [^{3}H]GABA release, was measured following a 15 minute incubation in ACSF containing 0, 1 or 100 μM nicotine. Depolarization-evoked [^{3}H]GABA release was measured during incubation in ACSF containing 20 mM KCl for 30 min. At the end of all release assays, brain tissue was lysed by incubation in 0.2 N HCl for 45 min. The medium was collected and residual [^{3}H]GABA levels measured. The sum of basal, evoked, and residual radiation levels was assumed to represent 100% of total [^{3}H]GABA content.

For GABA uptake, tissue was incubated in ACSF in the presence or absence of nicotine (1 μM) for 30 min at 37°C. Then, ACSF containing 400 nM [^{3}H]GABA was added for 2 min at either 37°C (total [^{3}H]GABA uptake) or 0°C (nonspecific [^{3}H]GABA uptake). To terminate the assay, slices were washed three times with ice-cold ACSF. Tissue was lysed by incubation with NaOH (0.1N) for 30 min. Total protein concentrations of the samples were measured using a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA). Specific [^{3}H]GABA uptake was determined by subtracting the non-specific from the total [^{3}H]GABA uptake and normalized to the total protein concentration.

Operant chambers

Mouse operant conditioning chambers (MED Associates; St. Albans, VT, USA) were equipped with a standard grid floor and house light (28V, 100mA), a central reward port attached to a fluid dipper, and two ultra-sensitive retractable levers positioned on the left and right sides of the chamber with large cue lights (2.5cm; 28V, 100mV) above each lever. All events, including light presentation, lever operations, and reward delivery, were controlled by a SmrtCtrl interface running MED-PC IV software on a Dell PC (Optiplex 960). All operant boxes were equipped with a video tracking system consisting of HD cameras (960H/700TVL) connected to an 8-channel HD Analog DVR system (Q-See, Anaheim, CA)

Go/no-go visual discrimination task

The operant go/no-go visual discrimination task was designed to assess attention and impulse control under conditions of response conflict in mice. This procedure was adapted from operant tasks previously used (Gubner *et al*, 2010; Harrison *et al*, 1999; Kolokotroni *et al*, 2011; McDonald *et al*, 1998). Briefly, water-restricted animals were autoshaped on a fixed-ratio 2 (FR 2) schedule of reinforcement to lever press for water sweetened with a calorie-free sweetener

saccharin (0.066%; 10 µL). The concentration of saccharin was based on previous studies assessing instrumental and consummatory behaviors in mice (Ben Hamida *et al*, 2013; Olive *et al*, 2003; Ortega *et al*, 2013). After attaining at least 30 lever press responses within a 30-min session for three consecutive days, animals were advanced to a pre-training phase as described previously (Cole *et al*, 2015; D'Amore *et al*, 2013; Ortega *et al*, 2013). During this phase, the animals were required to press the active lever within 10 s to get the reward. To control for any novelty effects associated with the visual stimulus during the go/no-go phase of training (see description below), trials were randomly associated with unpredictably occurring visual stimuli (presented only in 50% of trials) that involved illumination of the panel light for 10s above the lever during the pre-training phase.

Mice that attained the pre-training criterion (30 rewards and ≤10% omissions for three consecutive days) were moved to the go/no-go phase where they were required to discriminate between response cues (go trials) and withholding cues (no-go trials), respectively. A session began with illumination of the house light followed by 48 trials (24 go and 24 no-go trials) with an ITI of 9 ± 3s. Go trials consisted of the presentation of a continuous illumination of a 7 s cue light from either the left or the right panel, followed by the presentation of both levers 2 s later. Levers were presented for 5 s and co-terminated with the cue light. Two lever press responses (FR 2) on the cued lever were scored as a "hit" and were followed by reward (sweetened water) delivery. Failure to respond on the go trial resulted in a timeout phase during which the house light was extinguished for 10 s, and which counted as an "omission error". No-go trials consisted of a flashing (2 Hz) cue light from either the right or left panel. As with the go trials, both levers were presented 2s later and co-terminated with the flashing cue after 5 s. Two responses on any lever during the no-go trials resulted in a timeout phase (no reward). These responses represented

commission errors and were labeled as "false alarms". To obtain a reward during the no-go trials animals were required to withhold lever responding to cue presentations. Presentation of continuous or flashing cues was pseudorandomized in each session. Single responses on any lever during all trial types were not scored and allowed the animal an opportunity to either withhold or continue lever pressing. Two lever presses on a non-cued lever in either go or no-go trials were scored as never-reinforced errors. Animals were considered to have achieved performance criterion after three consecutive days of ≥70% correct responding on all trials.

After attaining performance criterion, each mouse underwent 4 tests in a counterbalanced order: vehicle + saline (veh + sal), saline + afatinib (sal + afat), vehicle + nicotine (veh + nic), and nicotine + afatinib (nic + afat). Afatinib (10 mg/kg) or vehicle was given 20 min prior to behavioral testing. Acute nicotine (0.36 mg/kg) or saline was injected 10 min prior to behavioral testing sessions. Between tests, mice underwent at least 5-days of washout period during which no injections were given, but behavior monitored to make sure that performance criterion was maintained/re-established.

The number of hits, false alarms, omission errors, never-reinforced errors, and response latencies were obtained for each test. Data for go and no-go trial performance was expressed as the probability of hits (p(hits)), calculated as percent of correct responses in go trials and probability of false alarms (p(fa)), calculated as percent of incorrect responding in no-go trials. Sensitivity index (SI) served as a measure of attention and discriminability, and was calculated using the formula $[p(hit) - p(fa)]/[2(p(hit) + p(fa))] - [p(hit) + p(fa)]^2$ as described previously (McDonald *et al*, 1998; McGaughy and Sarter, 1995). The ratio of number of rewards earned to the total number of responses in the go trials served as a measure of efficiency (Gubner *et al*, 2010).

Statistics for murine experiments

One-way ANOVAs with Bonferroni's post-hoc tests or Student's t-tests were used to analyze electrophysiology data. Student's t-tests were used to analyze protein expression and GABA uptake. Repeated one-way ANOVAs with Fisher's LSD post-hoc tests were used to analyze GABA release and all behavioral data. All data are presented as the mean±SEM and α was set at $p<0.05$.

Results

Nicotine and NRG3 modulate long-term plasticity in the OFC

We began by evaluating the effects of nicotine and NRG3 on long-term synaptic plasticity in the OFC. Under control conditions, a 50 Hz stimulation protocol resulted in 15% potentiation of fEPSPs evoked in OFC layer 3/5 (Fig. 1A/E) that lasted up to 60 minutes of recording. In the presence of nicotine (1 μM), we observed a pronounced conversion of this long-term potentiation (LTP) to long-term depression (LTD). Nicotine reduced fEPSP amplitudes to about 60% of baseline values (Fig. 1A/E). Application of NRG3 (2 nM) had a very similar effect at steady-state levels, although short-term depression during the first 5 minutes after the induction protocol was less pronounced than after nicotine (Fig. 1A/E). LTP induced in control conditions could be completely blocked by the NMDA receptor antagonist, DL-AP5 (50 μM) with short-term effects similar to nicotine and NRG3 (Fig. 1B). However, DL-AP5 did not block either nicotine- or NRG3-induced conversion of LTP to LTD (Fig. 1B). These results indicate that acute application of either nicotine or NRG3 can strongly regulate the direction of long-term plasticity in the OFC and that this regulation is independent of NMDA receptor activation.

Nicotine activates NRG3-ErbB4 signaling

Some behavioral and molecular effects of nicotine have been shown to rely upon NRG3 signaling (Turner *et al*, 2014). Therefore, to ascertain that NRG3 effects are mediated by activation of its ErbB4 receptor in the OFC, we pre-incubated slices with ErbB4 antagonist afatinib (10 µM, 1 hr incubation). Results showed that afatinib attenuated the short-term effect and completely occluded the steady-state effect of nicotine on LTP in the OFC ($F_{(9,67)}$=7.70, p<0.0001; post hoc: p<0.0001; Fig. 1C/E). To further strengthen the link between nicotine and NRG3 signaling, we examined nicotine-induced plasticity in NRG3 scaramanga mice ($NRG3^{ska}$). These mice possess a naturally occurring microsatellite repeat in exon 7 of NRG3, resulting in reduced expression of NRG3 protein (mRNA levels failed to reach statistical significance), but elevated expression of ErbB4 mRNA (protein levels failed to reach statistical significance) in the OFC (Fig. 2A/B). Similar to the results seen with afatinib, nicotine-induced conversion of LTP was absent in $NRG3^{ska}$ mice ($F_{(9,67)}$=7.70, p<0.0001; post hoc: p<0.001; Fig. 1C/E), confirming the involvement of NRG3 (despite of unchanged ErbB4 protein levels) in nicotine action. This indicates that in mice with reduced NRG3 expression, nicotine was unable to elicit significant signaling via NRG3 to the ErbB4 receptor, despite normal ErbB4 protein levels. These results together suggest that nicotine regulates long-term plasticity in the OFC via activation of the NRG3-ErbB4 signaling pathway. These results are supported by the lack of compensatory regulation of NRG1, ErbB3, or nAChR subunits in the OFC of $NRG3^{ska}$ mice (Fig. 2C/D).

NRG3 is a membrane-anchored protein that is released into extracellular space by cytosolic Ca^{2+}-dependent BACE1-mediated cleavage (Hu *et al*, 2008; Ma *et al*, 2007). Nicotine is believed to mediate Ca^{2+} influx through activation of homomeric α7 and heteromeric α4β2* nicotinic receptor (nAChR) subtypes, both of which are enriched in the frontal cortex (Dickinson *et al*, 2008; Poorthuis *et al*, 2013; Rousseau *et al*, 2005). Therefore, we tested the effects of α7

nAChR antagonist, α-bungarotoxin (αBTX, 100 μM) and α4β2* antagonist, dihydro-β-erythroidine (DHβE, 1 μM) on nicotine-induced synaptic plasticity. Both antagonists attenuated nicotine-induced LTD with a slightly stronger effect of DHβE, indicating that both receptor subtypes are involved in nicotine effects on OFC plasticity (Fig. 1D/E). We investigated whether nicotine regulation of OFC plasticity depended on release of Ca^{2+} from intracellular stores (Dickinson *et al*, 2008; Rousseau *et al*, 2005). Following incubation with a ryanodine receptor blocker, ruthenium red (20 μM), we observed a significant attenuation, but not a complete rescue, of nicotine-induced plasticity, indicating involvement of both Ca^{2+} influx through nAChRs and Ca^{2+}-dependent Ca^{2+} release from ryanodine receptor-sensitive stores (Fig. 1D/E). Finally, we investigated whether extracellular nicotine triggers Ca^{2+}-dependent BACE1 activity, a required enzymatic step in NRG3 cleavage. Pre-incubation of OFC slices with BACE1 inhibitor, LY2811376 (25 μM) significantly attenuated, but did not completely block, nicotine-induced plasticity (Fig. 1D/E).

Nicotine/NRG3/ErbB4-mediated plasticity depends on GABA signaling

ErbB4 receptors are expressed by GABAergic interneurons throughout the brain (Bean *et al*, 2014), and may modulate GABAergic activity via PI3-kinase signaling (Hatakeyama *et al*, 2003). We confirmed that nicotine treatment activates PI3 kinase in the OFC (t_{12}=2.53, $p<0.05$; Fig. 3A) and examined whether nicotine exposure affects GABA uptake or release levels. Our results showed that application of nicotine (1 μM or 100 μM) did not alter release of [^{3}H]GABA at baseline or following potassium-induced depolarization (Fig. 3B). On the other hand, GABA uptake was significantly increased upon nicotine application (t_8=2.87, $p<0.05$; Fig. 3C), likely resulting in elevated vesicular GABA content (Ortinski *et al*, 2006). To evaluate the contribution of GABAergic inhibition on nicotine-induced plasticity, we pre-incubated OFC slices with

picrotoxin (100 μM) and CGP55845 (1 μM), $GABA_A$ and $GABA_B$ antagonists, respectively. Blockade of GABA receptor-mediated signaling completely restored LTP observed under control conditions (t_{12}=7.13, p<0.0001; Fig. 3D/E). We conclude that nAChR activation in the OFC stimulates NRG3/ErbB4 signaling to promote an increase in GABAergic signaling and conversion of OFC LTP to LTD.

NRG3-ErbB4 signaling leads to deficits in attentional control

Attention and impulse control deficits are hallmark features of nicotine addiction (Baker *et al*, 2012). To examine whether dysregulated NRG3-ErbB4 signaling may underlie these phenotypes, we evaluated the effects of ErbB4 inhibition during nicotine administration on cognitive flexibility using a go/no-go discrimination task. This task has been shown to evaluate both attention and impulsivity controlled by the larger striato-thalamo-orbitofrontal circuit (Eagle and Baunez, 2010; Tremblay *et al*, 2000). We found a significant treatment effect on sensitivity index (SI), a cumulative measure of attention and discriminability (see Methods). Nicotine administered 20 minutes prior to behavioral testing significantly reduced SI, an effect that was rescued by administration of afatinib ($F_{(3,27)}$=8.03, p<0.001; Fig. 4A). This finding indicated that acute nicotine impaired stimulus discrimination via ErbB4 signaling. Further analysis conducted by trial type indicated that the effect on SI was primarily driven by go trial performance (p(hits)), which, similarly to SI, was reduced by nicotine and rescued by afatinib ($F_{(3,27)}$=4.47, p<0.01; Fig. 4B). The incidence of never-reinforced responses to levers not associated with a visual stimulus in this well-practiced task was very low (not shown) and reduction in p(hits) was due primarily to withholding a response on a go-trial (i.e. omission errors), an indicator of attentional bias (see Discussion). Omission errors were significantly increased in the nicotine+vehicle treated group, but not in the nicotine+afatinib treated animals (F(3,27)=4.04, p=0.017; Fig.4C). Video analysis

showed unchanged reward port visits (Sal+Veh: 0.30±0.15; Sal+Afat: 0.20±0.13; Nic±Veh: 0.40±0.16; Nic±Afat: 0.10±0.10), indicating that the omission errors represented withholding of responses on go trials, rather than a general loss of interest in reward. None of the drug treatments affected p(fa), a measure of impulse control ($F_{(3,27)}$=1.08 , p=0.38; Fig. 4D). Lastly, we did not observe any changes in the efficiency ($F_{(3,27)}$=1.0, p=0.41; Fig. 4E) or response latencies for hits and false alarms (both p>0.300; Fig. 4F), indicating that omission error changes following nicotine were not due to general motor impairment. These findings indicate that nicotine-mediated ErbB4 signaling interferes with attentional control, but not impulsivity-associated processes.

Discussion

The OFC regulates impulsivity, affective value of reinforcers and emotion-attention interactions (Hartikainen, 2003; Kringelbach, 2005; Torregrossa *et al*, 2008) with implications for the study of substance use disorders (Schoenbaum *et al*, 2016), including nicotine dependence. Previous studies reported that nicotine self-administration in rodents alters synaptic morphology in the OFC (Vazquez-Sanroman *et al*, 2016), while tobacco smokers display both morphological and functional connectivity changes within this region (Claus *et al*, 2013; Li *et al*, 2015). Our study provides the first evidence that nAChR activity gates the direction of long-term plasticity in the OFC via NRG3-ErbB4 signaling. Nicotine mediated conversion of LTP to LTD represents a form of "metaplasticity" that may contribute to the NRG3/ErbB4 dependent behavioral effects of nicotine in the OFC, as we show in the go/no go task.

Nicotine signaling through nAChRs has divergent effects on neuronal plasticity. For example, previous studies show that both α4β2* and α7 nAChR activation can either potentiate (Lagostena *et al*, 2008; Tang and Dani, 2009; Welsby *et al*, 2009) or prevent (Alkondon and

Albuquerque, 2001; Alkondon *et al*, 1997; Ji *et al*, 2001) LTP induction in hippocampus, with variable effects attributed to activation of specific interneuron populations. We observe that nicotinic stimulation in the OFC not only prevents induction of LTP, but also changes the sign of plasticity to result in a profound LTD (Fig 1A/E). While α4β2* and α7 nAChRs are expressed in the OFC (Cloke and Winters, 2015; Mendez *et al*, 2013), their specific localization is not clear. In the PFC, α4β2* and α7 nAChRs have been shown to be expressed by both GABAergic interneurons as well as pyramidal neurons (Poorthuis *et al*, 2013). Our studies with nAChR subtype selective antagonists confirmed that both α4β2* and α7 nAChRs contribute to nicotine-induced effects on synaptic plasticity in the OFC.

Activation of α7 or α4β2* nAChRs increases intracellular Ca^{2+} concentration, although by different mechanisms (Dickinson *et al*, 2008; Rousseau *et al*, 2005). Our pharmacological experiments indicate that nicotine-mediated effects on OFC plasticity involve release of Ca^{2+} from intracellular stores. While Ca^{2+} can regulate many synaptic processes, Ca^{2+}-induced activation of BACE1 has been shown to dramatically impact both LTP expression and LTP-associated behaviors (Ma *et al*, 2007). Our data suggest that BACE1 activation by Ca^{2+} derived from intracellular stores and Ca^{2+}-permeable nAChRs promotes conversion of OFC LTP to LTD by nicotine (Fig 1D/E). While we demonstrate that BACE1 activation triggers NRG3 signaling, the identity of other molecular targets downstream of BACE1 signaling remains undefined.

Separate studies have demonstrated that ErbB4 expression is prominently localized to OFC GABAergic interneurons (Bean *et al*, 2014) and that nicotine regulation of synaptic plasticity in the prefrontal cortex involves enhanced GABAergic transmission (Couey *et al*, 2007). Our results suggest a model by which nAChR signaling induces NRG3 cleavage via BACE1, to promote activation of ErbB4 receptors located on GABAergic interneurons with

effects on long-term synaptic plasticity (Fig. 5). The contrast between complete inhibition of nicotine effects by ErbB4 and partial inhibition by BACE1 antagonist supports the evidence that mechanisms other than proteolytic cleavage of NRG3 by BACE1 can promote ErbB4 activation by nicotine (Vullhorst *et al*, 2015). Antagonists of both nAChRs and ErbB4 receptors eliminated nicotine-associated depression of fEPSPs in the OFC, but were unable to restore synaptic potentiation. Full recovery of LTP was observed only after treatment with GABA receptor antagonists, suggesting that nicotine-dependent plasticity in the OFC involves both ErbB4-dependent and ErbB4-independent components. The latter may reflect contribution of GABA released in response to interneuron depolarization by presynaptic nAChRs.

Nicotine has been shown to increase both spontaneous and evoked GABA release using whole-cell recordings (Bianchi *et al*, 1995; Kofalvi *et al*, 2000). While we saw a significant increase in [^{3}H]GABA uptake following nicotine application (Fig. 3C), we report no change in either basal or KCl-induced GABA release after nicotine application using [^{3}H]GABA release assays (Fig. 3B). This discrepancy could be due to differences in temporal resolution of the two approaches. For example, [^{3}H]-based analyses report pooled neurotransmitter concentrations over time scales that are substantially longer than those involved in electrophysiological responses. Alternatively, [^{3}H]GABA release assays may be more effective at capturing fluctuations in ambient, but not synaptic GABA concentrations reported by electrophysiological assays. In our experiments, increased [^{3}H]GABA uptake is likely to result in increased vesicular GABA content and increased synaptic, but not ambient GABA concentrations.

In conjunction with alterations in OFC plasticity, we find evidence that nicotine-induced activation of the NRG3/ErbB4 pathway impacts behavioral performance relevant to OFC-mediated executive function. Acute nicotine impaired measures of attention, but not impulse

control/response inhibition in the go/no-go discrimination task and this effect could be reversed by pharmacologic inhibition of ErbB4 signaling. The effects of acute nicotine on impulsive responding that we observe are inconsistent with studies that report increased behavioral disinhibition and those that report negligible nicotine effects (Day *et al*, 2007; Hahn *et al*, 2003; Kirshenbaum *et al*, 2011; Kolokotroni *et al*, 2011; Stolerman *et al*, 2000). This could be due to differences in nicotine dose, specific behavioral procedures used (5-choice serial reaction time task, differential reinforcement of low rates of behavior, go/no-go procedure, stop-signal task), and the species involved. Additionally, effects of acute nicotine could be more prominent for some aspects of impulsivity such as impulsive choice behavior (Kolokotroni *et al*, 2011), but not others, such as impulsive action evaluated by the go/no-go task that we employed.

Reduced SI observed with acute nicotine in our study was primarily associated with reduced hits and high omission errors during go-trials. Because in a well-practiced task, it is rare that animals will respond to a lever not associated with a visual stimulus (see Results; the incidence of never-reinforced errors was very low), these data indicate that acute nicotine affected the animals' optimal decision strategy to perform the task. Omission errors in a go/no-go task reflect high attention to losses (Yechiam *et al*, 2006). Therefore, nicotine treatment may increase bias towards the blinking cue (no-go trial) to avoid punishment, resulting in adoption of a more conservative strategy of withholding responses on go trials leading to higher omission errors. Anxiety is commonly associated with cognitive bias and affects cognitive strategies to make optimal choices (Hartley and Phelps, 2012). Thus, it is plausible that attentional bias towards no-go trials might have occurred due to anxiogenic effects of acute nicotine while amelioration of cognitive deficits by afatinib occurred due to the interruption of ErbB4-mediated anxiogenesis. Previous studies from our lab have shown that transcriptional regulation of NRG3-

ErbB4 signaling regulates behavioral symptoms of anxiety induced by nicotine withdrawal (Turner *et al*, 2014). This is consistent with epidemiological studies suggesting that nicotine dependence increases the risk of anxiety disorders and panic attacks (Bruijnzeel, 2012). Frontocortical activation, including OFC, has been implicated in processing anxiety and anxiety-dependent cognitive traits of attention and impulsivity, all of which are strongly dysregulated in nicotine dependence (Gold *et al*, 2015; Rauch *et al*, 2007; Ursu *et al*, 2009). Additionally, patients with OFC damage display deficits in attentional control specifically in response to emotional stimuli (Hartikainen *et al*, 2012; Maki-Marttunen *et al*, 2017) . Thus, nicotine-induced alterations in OFC neuronal plasticity together with activity in associated corticolimbic structures (e.g. hippocampus, amygdala) that are engaged in affective decision processes, might contribute to anxiety-related cognitive bias towards no-go signals. A potential limitation of this study is that afatinib was given systemically and this may have produced pharmacological inhibition of ErbB4 receptors throughout the brain. Therefore, we could not rule out the possibility that NRG3 signaling in brain regions other than OFC might also have contributed to the observed behavioral effects. Further studies employing experimental approaches to regionally target ErbB4 receptors are warranted to establish that nicotine-induced alterations in executive functions are causally linked to modulation of NRG3 signaling in the OFC.

Elevated levels of NRG3 have previously been shown to impact affective behaviors in mice (Turner et al 2014). Interestingly, increased NRG3 expression due to genetic variation was recently observed in patients with affective disorders (Paterson 2017). These increased NRG3 protein levels in human prefrontal cortex have been attributed to the intronic *NRG3* single nucleotide polymorphism (SNP) rs10728842 positioned proximally to an alternative promoter and transcription start site (Kao *et al*, 2010). This same SNP has also been shown to correlate

with significantly reduced human prefrontal cortex functionality (Tost et al 2014) and associate with affective disorders involving frontal cortical function (Paterson et al 2017).

In summary, we find that aberrant OFC synaptic plasticity and dysregulated attentional control processes in the presence of nicotine depend on nicotine interactions with NRG3/ErbB4 pathway. Nicotine dependence and withdrawal symptoms include increased neurocognitive bias and elevated anxiety,that have been linked to OFC dysfunction (McClernon *et al*, 2015; O'Driscoll *et al*, 2014). Therefore, we propose that increased NRG3-ErbB4 signaling in the OFC in carriers of the *NRG3* risk allele may result in dysregulated attentional control processes, perhaps leading to increased rates of nicotine dependence, particularly in individuals co-morbid for psychiatric illnesses linked to genetic variation in *NRG3*. Thus, targeting the NRG3 pathway may represent a new direction for development of therapeutics for nicotine dependence.

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Figure Legends

Fig. 1. Nicotine effects on OFC neuroplasticity rely on NRG3/ErbB4 signaling. (**A**) A stimulation protocol, inducing LTP in control (ACSF) conditions results in LTD in the presence of NRG3 (2 nM) or nicotine (1 μM) (ACSF: n=12 slices, 5 mice; NRG3: n=6 slices, 3 mice; nicotine: n=7 slices, 3 mice). (**B**) DL-AP5 (50 μM) blocks LTP in normal ACSF but not LTD in ACSF supplemented by NRG3 (2 nM) or nicotine (1 μM) (AP5: n=7 slices, 3 mice; Nicotine+AP5: n=6 slices, 3 mice; NRG3+AP5: n=8 slices, 3 mice). (**C**) ErbB4 inhibitor afatinib (10 μM) attenuated both nicotine- and neuregulin induced LTD (NRG3+afatinib: n=6 slices, 3 mice; nicotine+afatinib: n=7 slices, 3 mice). Nicotine-induced LTD is also attenuated in a strain of $NRG3^{ska}$ mice that express reduced NRG3 levels (Nicotine ($NRG3^{ska}$): n=7 slices, 3 mice). (**D**) Nicotine-induced LTD was attenuated by α7 nAChRs antagonist, αBTX (100 μM); α4β2*, antagonist, DHβE (1 μM); ryanodine-sensitive Ca^{2+} release blocker, ruthenium red (20 μM); or BACE1 inhibitor, LY2811376 (25 μM) (Nicotine+ αBTX: n=7 slices, 3 mice; Nicotine+DHβE: n=6 slices, 3 mice; Nicotine+ruthenium red: n=8 slices, 3 mice; Nicotine+LY2811376: n=9 slices, 4 mice). (**E**) fEPSP amplitudes in the last 10 minutes of recordings for treatment groups in A-D. Data are presented as mean±SEM. Data are analyzed by one-way ANOVAs followed by Bonferroni's post-hoc tests, or Student's t-test. * $p<0.05$ vs. ACSF; $^{\&}$ $p<0.05$ vs. NRG3; $^{\#}$ $p<0.05$ vs. nicotine.

Figure 2. Molecular changes in the OFC of $NRG3^{ska}$ mice. (**A**) $NRG3^{ska}$ mice have reduced expression of NRG3 protein in the OFC compared to wild type controls. (**B**) ErbB4 mRNA is increased in the OFC of $NRG3^{ska}$ mice. (**C**) NRG1 and ErbB3 mRNA levels are similar in WT and $NRG3^{ska}$ mice. (**D**) The expression of nAChRs mRNA is unchanged in the $NRG3^{ska}$ mice.

Protein levels of NRG3 or ErbB4 were normalized to GAPDH or β-tubulin, then normalized to WT controls. mRNA levels are normalized to WT controls. Data are presented as mean±SEM. n=8 for both WT and $NRG3^{ska}$ mice. Data are analyzed by Student's t-tests. * $p<0.05$ compared to WT.

Figure 3. Nicotine regulates GABAergic transmission downstream of ErbB4 signaling in the OFC. (**A**) Acute nicotine treatment (0.1 mg/kg, i.p.) increases the phosphorylation of ErbB4 target, PI3K p85 at Tyr458 (n=7 per group). (**B**) Nicotine (1 μM or 100 μM) fails to alter basal or depolarization-induced GABA release in frontal cortex (n=2-3 per group). (**C**) Nicotine (1 μM) significantly increases GABA uptake (n=9). (**D**, **E**) Co-application of $GABA_AR$ antagonist, picrotoxin (100 μM) and $GABA_BR$ antagonist, CGP55845 (1 μM) completely blocks nicotine-induced conversion of LTP (n=7 slices, 4 mice). Data are presented as mean±SEM. Data are analyzed by Student's t-tests. * $p<0.05$ vs. saline or ACSF; $^{\#}$ $p<0.05$ vs. nicotine.

Figure 4. Nicotine-induced impairments in the go/no-go task are sensitive to ErbB4 antagonism. (**A**) Afatinib rescues nicotine-induced deficits in sensitivity index (SI). (**B**) Afatinib rescues correct responding on go-trials [percent hits: p(hits)]. (**C**) Afatinib rescues nicotine-induced increase in omission errors. (**D**) Incorrect responding on no-go trials [percent false alarms: p(fa)] is unchanged following nicotine and afatinib treatments. (**E**) Nicotine and afatinib treatments do not affect the ratio of number of rewards earned to the total number of responses in the go trials (Efficiency). (**F**) Response latencies for hits and false alarms remain steady between groups. In all panels, n=10 per treatment condition. Data are presented as mean±SEM. Data are

analyzed by one-way repeated measures ANOVAs followed by LSD post-hocs. * $p<0.05$ vs. veh + sal.

Figure 5. A proposed model for nicotine-induced synaptic plasticity in the OFC. Nicotine activates Ca^{2+}-permeable nAChRs, and Ca^{2+}-sensitive ryanodine receptors (RyR). Increased intracellular Ca^{2+} enhances the proteolytic activity of BACE1, which directly cleaves NRG3. NRG3 binds to ErbB4 and increases GABA release. Nicotine also increases GABA uptake, presumably via an ErbB4-independent pathway. Increased vesicular GABA coupled with ErbB4-facilitated GABA release decreases excitability of post-synaptic pyramidal neurons and promotes LTD.