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# Cell autonomy, receptor autonomy, and thermodynamics in nicotine receptor up-regulation

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## ABSTRACT

Chronic nicotine exposure, in smokers or in experimental rodents administered nicotine, produces elevated levels of nicotinic acetylcholine receptors in several brain regions. However, there are few data on up-regulation of receptors in specific neuronal subtypes. We tested whether functional up-regulation of nicotinic responses occurs in cultured GABAergic neurons of the ventral midbrain. Fura-2 measurements of nicotinic responses were made on ventral midbrain neurons from knock-in mice heterozygous for the  $\alpha 4$ -M2 domain Leu9'Ala mutation, which confers nicotine hypersensitivity. Chronic nicotine exposure at a concentration (10 nM for 3 days) that activates only the hypersensitive  $\alpha 4^*$  (Leu9'Ala) receptors, but not wild-type receptors, resulted in significant potentiation of ACh (100  $\mu$ M)-elicited responses. Experiments were also performed on midbrain neuronal cultures heterozygous for the  $\alpha 4^*$  (Leu9'Ala) mutation as well as for a GFP protein fused to a GABA transporter that reliably reveals GABAergic neurons. In cultures chronically treated with 10 nM nicotine, there was significantly increased  $\alpha 4^*$  nicotinic-induced  $\text{Ca}^{2+}$  influx elicited by low concentration of ACh (3  $\mu$ M). Furthermore, chronic exposure to the competitive antagonist dihydro- $\beta$ -erythroidine, but not to the noncompetitive antagonist mecamylamine, induced up-regulation of ACh elicited nicotinic responses. These results suggest that occupation of  $\alpha 4^*$  nicotinic receptor binding site(s), at the interface between two subunits, is sufficient to promote assembly and/or up-regulation of functional receptors in GABAergic neurons. Up-regulation in neurons is both "cell-autonomous", occurring at the cell itself, and "receptor autonomous", occurring at the receptor itself, and may be a thermodynamic necessity of ligand-protein interactions.

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## 1. Introduction

Nicotine is the most widely abused drug, afflicting over one billion people worldwide. The primary targets of nicotine are neuronal nicotinic acetylcholine receptors (nAChRs), of which there are 12 different subunits that combine in a variety of stoichiometries to form pentameric ligand-gated cation channels [1]. Most  $\alpha 4$  subunit containing ( $\alpha 4^*$ ) receptors also contain the  $\beta 2$  subunit ( $\beta 2^*$ ) [2], although other subunits are

probably present in specific neuronal types. This so-called  $\alpha 4\beta 2^*$  nAChR receptor family comprises the most prevalent high-sensitivity nAChRs in the brain [3]; that is, nicotine at the sub-micromolar concentrations found in tobacco smoke binds to, and activates,  $\alpha 4\beta 2$  receptors more than other subtypes.

Activation of  $\alpha 4\beta 2^*$  nicotinic receptor subunits is necessary and sufficient for tolerance, sensitization and reward behavior [4–6]. Nearly a quarter-century ago, pioneering studies *in vivo* using radio-ligand binding showed that chronic nicotine

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administered to rodents results in elevated levels of nicotinic receptors [7,8]. Up-regulation of  $\alpha 4\beta 2^*$  receptors with chronic nicotine also occurs *in vitro* in transfected clonal cell lines [9–13] and in primary neuronal cultures [14]. Furthermore, the mechanism of receptor up-regulation is apparently due to posttranscriptional mechanisms since nAChR mRNA expression levels are unaltered with chronic nicotine [12,13,15]. Although the mechanism of receptor up-regulation is unclear, several models have been proposed. Chronic nicotine might increase receptor assembly or decrease receptor degradation [9,14,16]; act as a pharmacological chaperone to facilitate assembly of subunits [10]; facilitate subunit protein folding into the correct conformation that would allow for subunit assembly [17]; decrease turnover rate of the receptor [18]; induce a conformational switch into a more easily activated high-affinity receptors [19,20]; or facilitate maturation of nicotinic receptors in the endoplasmic reticulum of the secretory pathway [21].

We previously found that chronic nicotine can functionally up-regulate nicotinic responses in cultured ventral midbrain neurons [14]. In a subsequent publication using cultured midbrain neurons from the  $\alpha 4$  (Leu9/Ala) mice, we showed that nicotine at a low enough concentration to interact selectively with  $\alpha 4^*$  receptors was sufficient to up-regulate nicotinic responses [5]. In the present study, we examined the influence of chronic nicotine in  $\alpha 4$  (Leu9/Ala) midbrain neuronal cultures in only one subtype of neurons—GABAergic neurons.

## 2. Methods

### 2.1. Calcium imaging in cultured ventral midbrain neurons

Changes in  $[Ca^{2+}]_i$  were measured in ventral midbrain neuronal cultures using the ratiometric fura-2 method [5,14,22]. Ventral midbrain tissue (including the SN and VTA) obtained from embryonic day 14 embryos was digested with 1 mg/ml papain, and cells were mechanically separated by gentle pipetting. Cells were plated onto 35 mm dishes with glass coverslip bottoms in Neurobasal culture medium containing 2% B27 supplement, 0.5 mM Glutamax, and 5% horse serum, all obtained from Invitrogen (Carlsbad, CA). Cell cultures were kept in a temperature-, humidity-, and  $CO_2/O_2$ -controlled incubator. Measurements were made at 28–35 days in culture. For chronic drug application, (–)-nicotine hydrogen tartrate (10 nM), dihydro- $\beta$ -erythroidine hydrobromide (DH $\beta$ E, 10  $\mu$ M), mecamylamine hydrochloride (MEC, 10  $\mu$ M), or control solution (cell culture media) were incubated in the culture dishes for 3 days with replacement of fresh drug solution at each day of incubation. The chronic drug or control solution was removed  $\sim$ 1 h before the imaging experiments. Dishes were rinsed thoroughly twice with extracellular solution and during this 1 h period, cells were loaded with 1.25  $\mu$ M fura-2 AM (Invitrogen) in a 0.01% pluronic acid solution for 30 min at room temperature ( $22 \pm 2^\circ C$ ) and in the dark. During experiments, cultures were continually perfused with an extracellular solution (in mM): 150 NaCl, 10 HEPES, 10 glucose, 4KCl, 2CaCl<sub>2</sub>, and

2MgCl<sub>2</sub>. Various channel blockers to suppress nonspecific responses were included in the extracellular solution (in  $\mu$ M): 0.5 TTX (voltage-gated Na<sup>+</sup> channels), 0.5 atropine (muscarinic receptors), 10 CNQX (AMPA and kainate receptors), 20 bicuculline (GABA<sub>A</sub> receptors), 50 AP-5 (NMDA receptors) and 0.01 methyllycaconitine (MLA,  $\alpha 7$  receptors). Ascorbic acid at 1 mM was also added to the extracellular solution to protect neurons from photodamage. Imaging was performed with an inverted fluorescence microscope (IX71, Olympus, Melville, NY) using a 40 $\times$  oil-immersed objective (UApo/340, 1.35 numerical aperture). To generate ratiometric estimates of  $[Ca^{2+}]_i$ , pairs of images were obtained with alternate excitation wavelengths of 340/380 nm and emission wavelength of 510 nm. A U-tube connected to a pipette, placed  $\sim$ 0.5 mm away from the targeted cell population, was used to deliver nicotine. Nicotinic receptors desensitize at agonist concentrations too low to cause appreciable activation; therefore, between applications, negative pressure was maintained on the U-tube to prevent nicotine from leaking out of the pipette. Nicotine was applied onto cultured cells when solution flow in the U-tube was momentarily (2 s) reversed by stopping outflow with a computer-controlled pinch valve. Images were captured with a 16-bit CCD camera (Cascade 650, Photometrics, Tucson, AZ) and analyzed with Slidebook 4.1 imaging software (Intelligent Imaging Innovations, Denver, CO).

### 2.2. Spectral confocal imaging

GAT1-GFP fluorescence images were collected from an inverted confocal microscope equipped with spectrally resolved multiple detector systems (C1si, Nikon, Tokyo, Japan). Each pixel of the X–Y image has complete spectral emission data, comprising the lambda stack [23–25]. Spectral images were taken, with excitation at 440 nm by a modulated diode laser at 20% power, over  $512 \times 512$  pixels at 12-bit intensity resolution using a 60 $\times$  plan apochromat water (1.2 NA) objective. The pinhole was set to a nominal diameter of 61.3  $\mu$ m. Lambda stacks of images were acquired at wavelengths between 479 and 634 nm, at 5 nm steps.

In all cases, detector gains and laser attenuators were adjusted to lie within the linear range of the detector.

### 2.3. Statistical analysis

All values are reported as mean  $\pm$  S.E.M. and the number of experiments was also reported. Significant differences (at  $p < 0.05$ ) were determined between two groups using a *t*-test for continuous data meeting parametric assumptions of normality and equal variance. Otherwise the Mann–Whitney rank sum test was used for nonparametric data. For comparisons between three groups, significant difference (at  $p < 0.05$ ) were determined using a one-way analysis of variance for continuous data meeting parametric assumptions of normality and equal variance. Otherwise the Kruskal–Wallis one-way analysis of variance on ranks was performed with Dunn's method of pairwise post hoc analysis. Degrees of freedom (d.f.) were reported for results of statistical analyses.

### 3. Results

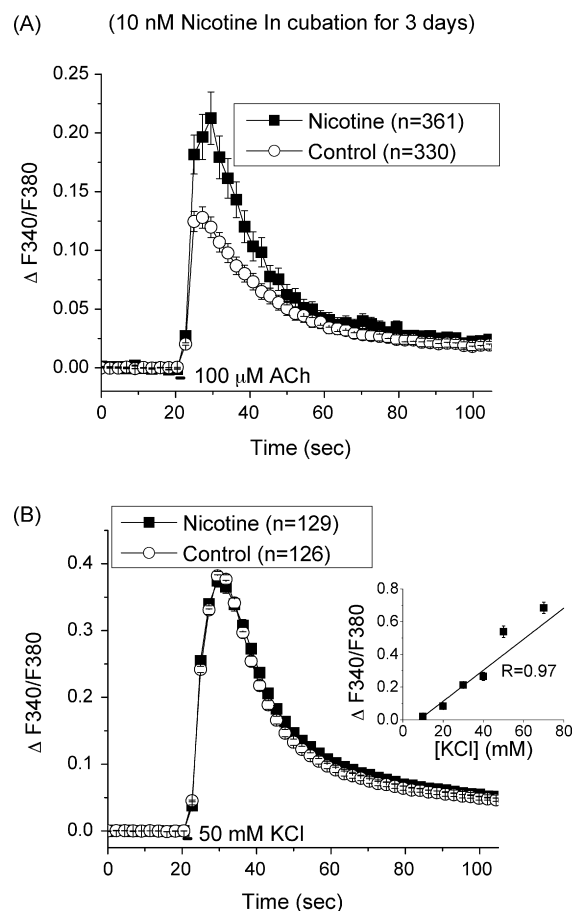
#### 3.1. Interaction of chronic nicotine with $\alpha 4^*$ receptors in cultured ventral midbrain neurons is sufficient for up-regulation of nicotinic responses

We examined whether selective interaction of chronic nicotine with  $\alpha 4^*$  nAChRs in cultured ventral midbrain neurons is sufficient to induce up-regulation of functional nicotinic  $\text{Ca}^{2+}$  flux through nicotinic receptors using fura-2 measurements. We used cultured neurons from brain tissue of knock-in mice heterozygous for the  $\alpha 4$  (Leu9/Ala) mutation [5,22]. The Leu9/Ala mutation is near the gate (second transmembrane region) of the channel and renders the receptor 40- to 50-fold more sensitive to ACh or nicotine [5,22]. Appreciable nAChR signals appear in midbrain cultures after a period of several weeks. Therefore, imaging was performed on neurons 4–5 weeks in culture.

Either saline (control) or a low chronic dose of nicotine (10 nM for 3 days) was applied to the dishes of the cultured ventral midbrain neurons. Nicotine at 10 nM does not activate wild-type  $\alpha 4\beta 2$  nicotinic receptors, does activate (<7% maximal response)  $\alpha 4$  (L9/A)  $\beta 2$  nAChRs, and falls within a “window” that does not completely desensitize receptors [22]. Nicotine was washed from the dish of cultured neurons for at least 1 h. When we tested nicotinic responses elicited by 2 s application of 100  $\mu\text{M}$  ACh (activates 60% and 100% of wild-type and hypersensitive receptors, respectively), we found a significant up-regulation ( $66 \pm 7\%$ ) of peak fura-2 measured calcium fluxes in ventral midbrain neurons from chronic nicotine ( $0.21 \pm 0.02$ ) as compared to chronic saline ( $0.13 \pm 0.01$ ; t-test,  $p < 0.001$ , d.f. = 689; Fig. 1A).

These results support the idea that selective interaction of chronic nicotine with the  $\alpha 4^*$  (Leu9/Ala) receptors is sufficient for up-regulation of nicotinic responses in ventral midbrain neurons, and confirmed previous experiments on cultures from knock-in mice with hypersensitive  $\alpha 4^*$ -Leu9/Ala receptors [5].

One potential confounding issue may be that the up-regulated fura-2 responses could reflect calcium influx through up-regulated voltage-gated calcium channels, which activate subsequent to membrane depolarization following activation of nicotinic receptors. Various channel blockers to suppress nonspecific responses were included in the extracellular solution: TTX (voltage-gated  $\text{Na}^+$  channels), atropine (muscarinic receptors), CNQX (AMPA and kainate receptors), bicuculline ( $\text{GABA}_A$  receptors), AP-5 (NMDA receptors) and methyllycaconitine (MLA,  $\alpha 7$  receptors). In previous studies of whole-cell patch-clamp recordings of  $\alpha 4\beta 2$  nAChRs, toxins and pharmacological inhibitors of a variety of voltage-gated calcium channels directly blocked  $\alpha 4\beta 2$  nAChRs with similar or higher affinity [22], vitiating their use in these experiments. Therefore, we activated voltage-gated  $\text{Ca}^{2+}$  channels by depolarizing the cultured neurons in the presence of the various channel blockers in the extracellular solution; we found no significant difference in fura-2 measured  $\text{Ca}^{2+}$  influx in neurons chronically treated with nicotine (10 nM for 3 days) versus saline treated controls ( $p = 0.5$ , t-test, d.f. = 253). Thus, we ruled out that the up-regulated nicotinic responses with chronic nicotine was due to secondary activation of voltage-gated calcium channels.



**Fig. 1 – Fura-2 recordings of cultured ventral midbrain neurons after chronic exposure to nicotine.** Fura-2 imaging was performed on ventral midbrain cultures from embryos heterozygous for the hypersensitive  $\alpha 4$  (Leu9/Ala) mutation. (A) Time course of fura-2-recorded  $\text{Ca}^{2+}$  fluxes, showing that midbrain neurons previously exposed to chronic nicotine (10 nM for 3 days) had greater ACh (100  $\mu\text{M}$  for 2 s) induced nicotinic responses than control neurons. (B) Since voltage-gated calcium channel blockers could not be used because they also target nicotinic receptors [22], in control experiments fura-2 recordings in cultured neurons were conducted during membrane depolarization with 50 mM KCl (2 s). There was no difference in the KCl-elicited  $\text{Ca}^{2+}$  influx between control and chronic nicotine treated neurons, indicating that functional voltage-gated calcium channel responses are not up-regulated. Therefore, the functional up-regulation in (A) is due directly to nicotinic receptor activity. Inset shows a plot of fura-2 responses over various KCl concentrations, showing that the responses do not saturate at 50 mM KCl. Fura-2 measurements were taken in the presence of ion channel and receptor blockers: atropine, AP-5, CNQX, bicuculline, TTX, and MLA.

Thus, in ventral midbrain neurons chronically treated with nicotine the functional up-regulation of fura-2 measurements was, in fact, due to functional up-regulation of  $\text{Ca}^{2+}$  flux through up-regulated nicotinic receptors rather than

indirectly due to voltage-gated calcium channels in ventral midbrain neurons.

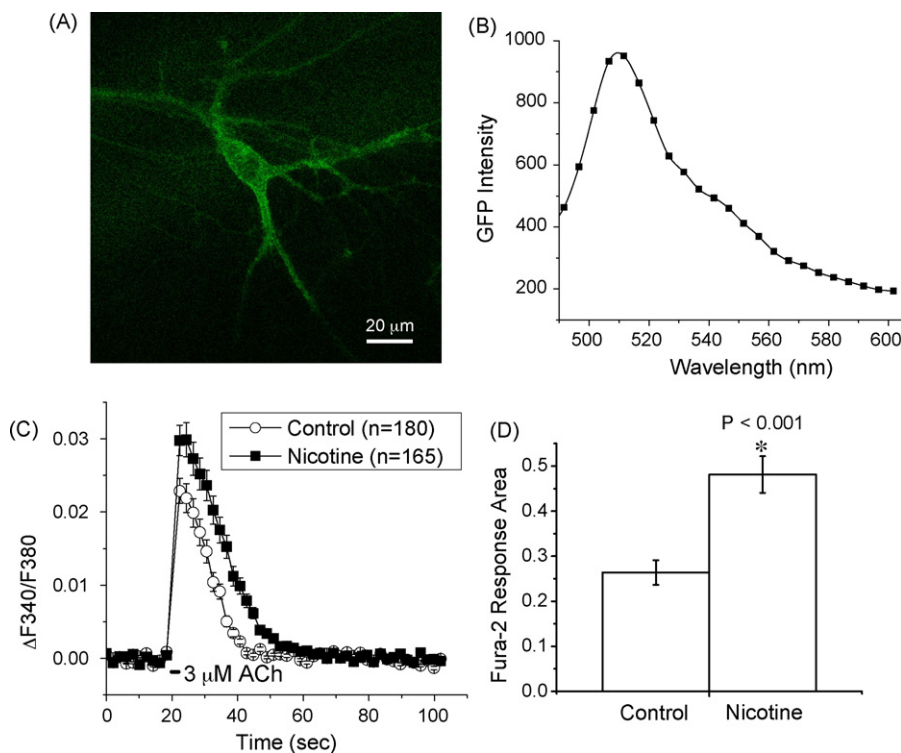
### 3.2. Interaction of chronic nicotine with $\alpha 4^*$ receptors in cultured GABAergic neurons is sufficient for up-regulation of $\alpha 4^*$ responses

To begin exploring the mechanism of functional up-regulation in GABAergic neurons of the ventral midbrain, we further tested whether ventral midbrain GABAergic neurons, in particular, responded to chronic nicotine with up-regulated functional  $\alpha 4^*$  receptors in a cell culture system using fura-2 measurements. Previous measurements with this system showed that chronic incubation with 1  $\mu$ M nicotine functionally up-regulated nicotine responses with pharmacology suggestive of  $\alpha 4\beta 2^*$  receptors. Also, in cultured neurons transfected with fluorescent  $\alpha 4\beta 2^*$  receptors, chronic incubation with 1  $\mu$ M nicotine numerically up-regulated receptors as measured by fluorescence of the subunits [14].

In the present study, we tested whether ventral midbrain GABAergic neurons, in particular, responded to chronic nicotine with up-regulated functional  $\alpha 4^*$  receptors. To distinguish living GABAergic mouse neurons in culture, we chose a knock-in strain containing a GFP moiety in the GAT1 GABA transporter, because (1) rigorous electrophysiological tests on this strain

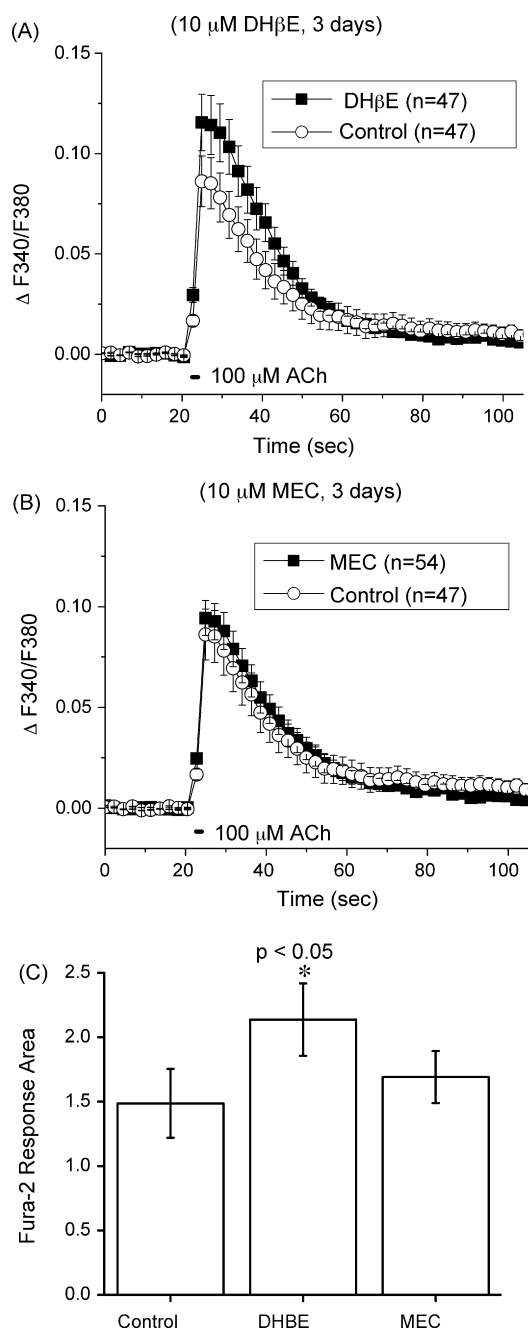
showed that the set of fluorescent neurons equals the set of GABAergic neurons; (2) the GAT1-GFP strain is maintained on a mixed Sv129-C57BL6 background similar to the  $\alpha 4$ -YFP strain; (3) the GAT1-GFP strain has normal electrophysiological, behavioral, and reproductive characteristics [26]. We bred GAT1-GFP mice with wild-type mice. In pilot experiments on the midbrain cultures from embryonic mice, we found that the cultures contained >95% GABAergic neurons. These observations that nearly all the neurons are GABAergic, combined with previous experiments showing up-regulation of functional  $\alpha 4\beta 2$  receptors transfected into neurons [14], provided proof that chronic nicotine up-regulates functional  $\alpha 4^*$  receptors in nearly pure cultures of ventral midbrain GABAergic neurons. Contact with additional neuronal types, such as their typical pre-synaptic input cells, is not necessary for nicotine-induced up-regulation in cultured GABAergic neurons.

The availability of the  $\alpha 4^*$  hypersensitive strain allowed for extending these results to test two additional points: whether activation of  $\alpha 4^*$  receptors (without concurrent activation of other nAChRs) on GABAergic neurons is sufficient for functional up-regulation of these  $\alpha 4^*$  receptors; and whether the previous pharmacological evidence for  $\alpha 4$  responses could be supplanted by more direct isolation of  $\alpha 4^*$  responses. The  $\alpha 4$ -L9'A strain has been well-characterized electrophysiologically, neurochemically, and behaviorally; it has only a modestly decreased



**Fig. 2 – Fura-2 recordings of cultured GABAergic ventral midbrain neurons after chronic exposure to nicotine.** Fura-2 imaging was performed on ventral midbrain cultures from embryos heterozygous for the hypersensitive  $\alpha 4$  (Leu9/Ala) mutation and the GAT1-GFP mutation [26], which allowed us to visually identify GABAergic neurons. (A) A z-projection of spectrally unmixed confocal images of a cultured  $\alpha 4$  (Leu9/Ala) Het neuron expressing GAT1-GFP. (B) A spectrum of the neuron showing characteristic GFP emission with peak emission at  $\sim 510$  nm. (C) Time course of fura-2-recorded  $\text{Ca}^{2+}$  fluxes showing that GAT1-GFP+ neurons in chronic nicotine cultures (10 nM for 3 days) had greater ACh (3  $\mu$ M for 2 s) induced responses than control neurons. (D) Bar graph showing that the area of the ACh induced fura-2 responses in nicotine treated neurons was also significantly augmented over control. Fura-2 measurements were taken in the presence of ion channel and receptor blockers: atropine, AP-5, CNQX, bicuculline, TTX, and MLA.





**Fig. 3 – Fura-2 recordings of cultured ventral midbrain neurons after chronic exposure to nicotinic antagonists.** Fura-2 imaging was performed on ventral midbrain cultures from embryos heterozygous for the hypersensitive  $\alpha 4$  (Leu9/Ala) mutation. (A) Time course of fura-2-recorded  $\text{Ca}^{2+}$  fluxes showing that cultured midbrain neurons in chronic DH $\beta$ E (10  $\mu\text{M}$  for 3 days), had greater ACh (100  $\mu\text{M}$  for 2 s) induced responses than control neurons. (B) Time course of fura-2-recorded  $\text{Ca}^{2+}$  fluxes showing that midbrain neuronal cultures in chronic noncompetitive antagonist, mecamylamine (10  $\mu\text{M}$  for 3 days), had no significant difference in ACh (100  $\mu\text{M}$  for 2 s) induced responses as compared to control neurons. (C) Bar graph showing that the area of the ACh induced fura-2 responses in DH $\beta$ E treated neurons was significantly augmented over control but not for mecamylamine (MEC)

number of  $\alpha 4\beta 2^*$  receptors [5,22]. We bred homozygous GAT1-GFP mice with homozygous  $\alpha 4\text{-L9'A}$  mice (from a colony that is also maintained on a mixed Sv129/C57BL6 background), producing embryonic mice heterozygous for both the hypersensitive  $\alpha 4\text{L9'A}$  knock-in allele [5] and the GAT1-GFP knock-in allele [26]. We exposed the embryonic cultures to a low concentration of nicotine (10 nM) for 3 days, so that only the hypersensitive  $\alpha 4^*$  receptors were chronically activated [5]. Then, by measuring  $\text{Ca}^{2+}$  flux in fluorescent neurons containing hypersensitive  $\alpha 4\text{L9'A}^*$  nAChRs, we were confident that a low ACh dose (3  $\mu\text{M}$  for 2 s) activated primarily the  $\alpha 4^*$  nAChRs on GABAergic neurons. We found that chronic incubation in nicotine for 3 days resulted in a  $30 \pm 9\%$  and  $82 \pm 15\%$  increase in peak and area of ACh-induced  $\text{Ca}^{2+}$  response ( $p < 0.002$  and  $p < 0.001$ , t-test, d.f. = 343, respectively) as compared to control (Fig. 2).

Because the experiments allowed us both to activate chronically, and then to test, only  $\alpha 4$  responses in a well-characterized neuronal culture from ventral midbrain, we conclude that  $\alpha 4^*$  receptor activating concentrations of nicotine in GABAergic neurons are sufficient for functional up-regulation of these  $\alpha 4^*$  receptors.

### 3.3. Chronic competitive antagonism with DH $\beta$ E but not MEC up-regulates $\alpha 4^*$ responses

Because nicotinic antagonists can also up-regulate  $\alpha 4^*$  nicotinic responses in HEK293 cell lines [9,19], we also examined the effects of chronic exposure of two antagonists on the nicotinic responses in cultured ventral midbrain neurons from knock-in mice with hypersensitive Leu9/Ala  $\alpha 4^*$  receptors. Fura-2 responses elicited by 2 s application of 100  $\mu\text{M}$  ACh were significantly larger ( $34 \pm 4\%$ ) in neurons treated for 3 days) with the nicotinic receptor competitive antagonist dihydro- $\beta$ -erythroidine (DH $\beta$ E), 10  $\mu\text{M}$  ( $0.12 \pm 0.01$  versus  $0.09 \pm 0.01$  in DH $\beta$ E versus control, respectively,  $p = 0.037$ , t-test, d.f. = 92)(Fig. 3A). However, there was no significant difference in the fura-2 measured nicotinic responses in neurons incubated with the noncompetitive antagonist mecamylamine (10  $\mu\text{M}$ , 3 days) ( $0.09 \pm 0.01$  versus  $0.09 \pm 0.01$  in mecamylamine versus control, respectively,  $p = 0.6$ , t-test, d.f. = 99).

Previous studies in cell lines [9–11] showed that chronic administration of the competitive antagonist DH $\beta$ E functionally up-regulates  $\alpha 4^*$  nicotinic responses, with no effect of the noncompetitive antagonist mecamylamine. The results from this study extend this result to GABAergic ventral midbrain neurons.

## 4. Discussion

### 4.1. Up-regulation is “cell autonomous” and “receptor autonomous”

In the present study we have extended the results of previous studies examining up-regulation of  $\alpha 4^*$  nAChRs in ventral

treated neurons. Fura-2 measurements were taken in the presence of ion channel and receptor blockers: atropine, AP-5, CNQX, bicuculline, TTX, and MLA.

midbrain neurons from wild-type mice using nicotinic receptor pharmacology [14] and from the gain-of-function  $\alpha 4$  (Leu9'Ala)\* knock-in mice [5]. We present data showing that selective activation of  $\alpha 4^*$  nAChRs is sufficient for  $\alpha 4^*$  receptor up-regulation in almost pure cultures of GABAergic ventral midbrain neurons. We term this result “cell autonomy”, by analogy with the classical genetic concept of a protein whose mutation produces a phenotype in the cell that expresses the protein, without the need for interactions with other cells.

Cultured midbrain neurons have yielded extensive information about  $\alpha 4^*$  receptor up-regulation. In previous studies on cultured midbrain neurons from wild-type mice, chronic nicotine up-regulated functional responses with pharmacology appropriate to  $\alpha 4^*$  receptors [14]. Also, transfected fluorescent  $\alpha 4^*$  subunits were up-regulated at the protein level [14]. Experiments with cultures from  $\alpha 4$ -hypersensitive mice then showed that up-regulation occurred in chronic nicotine concentrations (10 nM) that activated only  $\alpha 4^*$  receptors [5]. In the present experiments, we add the two facts that functional up-regulation occurs in homotypic GABAergic cultures lacking their typical presynaptic partners and that functional up-regulation occurs when tested with ACh concentrations that activate only the  $\alpha 4$  receptors. It can thus be concluded that activation of  $\alpha 4^*$  receptors on GABAergic neurons is sufficient for numerical up-regulation of functional  $\alpha 4^*$  receptors on these same GABAergic neurons. We note that because desensitization occurs with a high probability following channel opening, we also allow the possibility that up-regulation arises more strongly from desensitization than from activation. We term this result “receptor autonomy”, because activation of no other nicotinic receptors appears necessary within the cell-autonomous process that leads to up-regulation.

The mechanism of up-regulation of nicotinic receptors remains unsettled. “Cell autonomy” and “Receptor autonomy” are certainly consistent with the data from clonal cell lines transfected with nAChRs, that nicotine acts as a “pharmacological chaperone” or “maturational enhancer” for  $\alpha 4\beta 2^*$  receptors [10,21,27]. Nicotinic receptor up-regulation could also arise from mechanisms that regulate receptor turnover [10], from proteins that govern assembly and trafficking [9,20,21,28–31], or from additional factors that regulate desensitization [32]. Another potential mechanism of nAChR up-regulation may be due to signal transduction mechanisms initiated by calcium influx through the receptors. One strong counterargument to the Ca influx hypothesis is that the competitive antagonist DH $\beta$ E also induces functional up-regulation (Fig. 3). Interestingly, the noncompetitive antagonist, mecamylamine, did not produce any functional up-regulation (Fig. 3), consistent with a previous study performed in HEK293 cells [9]; this point is discussed in the next section. Furthermore, human  $\alpha 4$  harboring a Ser 247 Phe mutation (at the M2 6' position) renders the channel nonfunctional in HEK tsA201 cells and yet chronic nicotine induces robust up-regulation of the receptor in this clonal cell line [10]. This point also argues against a calcium signaling mechanism of nicotinic receptor up-regulation.

In the future, it will be vital to examine changes in specific nAChR subunit expression in specific neuronal types *in vivo*. Such information will allow us to better understand how

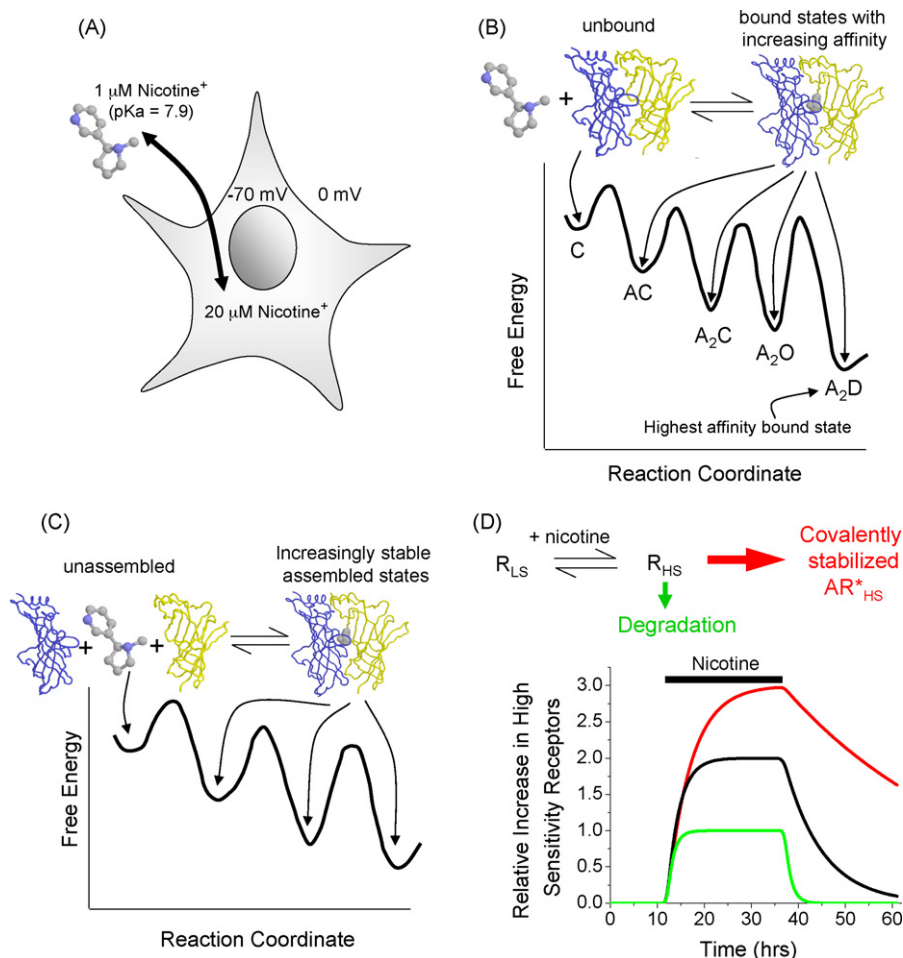
altered nicotinic receptor expression in specific neuronal circuits will alter their functional output and ultimately lead us to a better understanding how chronic nicotine use develops into an addiction.

#### 4.2. Thermodynamic aspects of the pharmacological chaperone concept at $\alpha 4\beta 2$ receptors

Recent papers provide incisive discussions about the mechanism of up-regulation [9,13,19,21,27,33,34]. That up-regulation is both “cell-autonomous” and “receptor-autonomous”, proceeds post-translationally [12,13,15,18,19], occurs before insertion in the plasma membrane [9,21,27], and does not involve an obvious Ca signaling pathway (present data) allows one to consider relatively straightforward concepts in up-regulation. Even in biochemistry, thermodynamics rules the fate of molecular complexes, if kinetic barriers permit. It is therefore useful to ask whether, and to what extent, up-regulation is determined by the energetics of ligand binding and by chemical potentials that are proportional to  $\log(\text{concentration})$ . However, we do not distinguish between entropy and enthalpy when referring to the thermodynamics of nicotine–receptor interactions [35–38]. In the context of drug–receptor binding, thermodynamics provides that the drug–receptor interaction eventually reaches its most stable state (again, kinetic barriers permitting) (Fig. 4). First, we note that a drug's overall affinity for a receptor includes contributions from the possibly complex conformational states that occur only (or predominantly) if the drug is bound. In other words, if a drug-bound receptor isomerizes to form an open channel, then channel opening usually “locks” the agonist onto the receptor [39]; and the overall dissociation constant is lowered by the presence of all the open states (Fig. 4B). Indeed, if desensitization follows binding and is at least as likely as opening, then desensitization also significantly further locks the agonist onto the receptor and decreases the overall dissociation constant of the drug–receptor complex (Fig. 4B).

If a drug is present at a concentration comparable to, or higher than, the overall dissociation constant for the high-affinity interaction, then the presence of the drug leads to a significant fraction of high-affinity complexes; if, on the other hand, the drug is present at a concentration lower than the overall binding constant, the equilibrium is shifted only slightly. We now add a kinetic argument: in most straightforward kinetic schemes, the stable states are likely to form, in the presence of ligand, somewhat faster but on the same time scales as they disappear when ligand is removed. If formation of the stable states requires minutes to hours, we expect the stabilization to last at least as long [12,13]. This stabilization of high-affinity or high-sensitivity states, which outlasts the presence of the ligand, is a form of up-regulation that can be analyzed without reference to detailed cell biological mechanisms. Indeed, much of up-regulation may simply be stabilization. According to this reasoning, it is not surprising that nicotine, when present at the sub-micromolar concentrations in the brain of smokers, most strongly up-regulates  $\alpha 4\beta 2^*$  receptors; for  $\alpha 4\beta 2^*$  receptors have higher overall affinity than most other nAChRs.

That nicotine binds at some subunit interfaces of a pentamer provides another level of stabilization [16,19,40].



**Fig. 4 – Thermodynamics of nicotinic receptor up-regulation.** (A) Since nicotine is cell-permeant in its deprotonated form and a weak base (pK<sub>a</sub> = 7.9), it accumulates within cells according to the membrane potential—roughly 20-fold at  $-70\ \text{mV}$ . Thus, intracellular nicotine can interact with nicotinic receptor subunits in compartments where they are being assembled and transported. The concentration of nicotine accumulated inside the neurons is 100- to 1000-fold greater than the dissociation constant for the receptors and therefore can bind to a majority of the intracellular pool of receptors. (B) When nicotine binds to the interface of nicotinic receptor subunits (modified from the AChBP structure from Celie et al. [55]), the bound complex has a lower total free energy than the unbound state, and the complex eventually finds the bound state of highest affinity (lowest total free energy) [56]. C, closed; A, agonist; O, open; and D, desensitized. (C) Nicotine helps stabilize a full pentamer in the assembled state by binding to the interfaces of the nicotinic receptor subunits. (D) The concentration of nicotine found in the bloodstream of smokers selectively up-regulates the high sensitivity (HS) over the low sensitivity (LS) subpopulation of nicotinic receptors since the EC<sub>50</sub>s of the low and high subpopulations are  $\sim 1\ \mu\text{M}$  and  $\sim 100\ \mu\text{M}$ , respectively. This situation, which summarizes the concepts of panels (A–C), is denoted by the black line. In addition, the assembled receptor may be diverted away from the degradative pathway (green line). Additional interactions with other proteins or covalent modifications may result in further “covalent stabilization” (red line), resulting in additional nicotinic receptor up-regulation. Since receptor synthesis and assembly occur over hours, we expect that the turnover rate should be at least as long.

Because nicotine binds to both  $\alpha$  and  $\beta$  subunits simultaneously, it stabilizes the ternary complex,  $\alpha$ -nicotine- $\beta$ . Nicotine therefore helps to stabilize a full pentamer in an assembled state [14] (Fig. 4C).

It is now well accepted that  $\alpha 4 \beta 2$  receptors exist in a mixed population whose EC<sub>50</sub> values differ by 50- to 100-fold [14,19,41,42] ( $\sim 1\ \mu\text{M}$  and  $\sim 100\ \mu\text{M}$ ). The reasoning given in the previous paragraph implies that micromolar nicotine concentrations preferentially stabilize, and therefore preferentially up-regulate the high-affinity but not the low-affinity

form of the pentamer. The physical difference between the states is not yet clear. It could be an isomerization [19,20]. Alternatively, several papers suggest that the two forms differ in subunit composition: the high- and low-sensitivity forms would consist of the  $\alpha 4_2 \beta 2_3$  and  $\alpha 4_3 \beta 2_2$  pentamers, respectively [43–45]. The physical basis of the distinction is indeed important because the nature of the difference may determine the barriers to interconversion; for instance, if the subunit composition gives rise to the difference, interconversion is unlikely to happen after the endoplasmic reticulum; and if

stoichiometry is involved, then available subunit ratios also influence the outcome [18]. However, thermodynamic arguments about stabilization by nicotine do not require one to know this difference. Nicotine at concentrations  $\sim 1 \mu\text{M}$  must stabilize the high-sensitivity form 50 to 100 times more than the low-affinity form, corresponding to  $(RT/F)\ln(100) = 2.6 \text{ kcal/mol}$ . The additional stabilization provided by desensitization might change these values; there are inconsistent observations about the relative desensitization properties of the two forms.

In this view, up-regulation by stabilization of functional, high-sensitivity nicotinic receptors by nicotine is a thermodynamic necessity. The process is probably enhanced by the fact that nicotine is cell-permeant and can therefore interact with receptors when they are being synthesized and transported [9,21], not simply after stable insertion into the cell membrane. Furthermore, because nicotine, like most alkaloids, is a weak base, it accumulates within cells according to the membrane potential—roughly 20-fold at  $-70 \text{ mV}$  [46] (Fig. 4A). The endoplasmic reticulum probably has little transmembrane potential, allowing the nicotine concentration that interacts with nascent receptors to reach values roughly equal to that of the cytoplasm. It is not surprising that nicotine exhibits an  $\text{EC}_{50}$  of  $<100 \text{ nM}$  for up-regulation in clonal cell lines transfected with  $\alpha 4$  and  $\beta 2$  subunits [27]. Nicotinic receptors are also up-regulated by impermeant agonists including simple tetramethylammonium and carbachol, showing that surface interactions alone may provide a sufficient thermodynamic driving force; however full up-regulation by impermeant agonists does require rather high concentrations ( $0.1\text{--}1 \text{ mM}$ ) [21,33,47].

According to these considerations, one expects any drug to up-regulate nicotinic receptors by stabilizing them if (a) it binds at a subunit interface, (b) has an  $\text{EC}_{50}$  or overall dissociation constant in the micromolar range, and (c) permeates cell membranes. The competitive antagonist DH $\beta$ E is therefore not a surprising up-regulator in the present and previous experiments [9,19,21]. One thermodynamic distinction between agonist and antagonist binding to neuronal nicotinic receptors is that agonist binding is both enthalpy and entropy driven, while antagonist binding is completely entropy driven [35].

Channel blockers typically bind on the axis of the channel pore and therefore touch all subunits; if such binding stabilizes the assembled state of the receptor, it too might up-regulate. “Foot-in-the-door” open-channel blockers like QX-222 at muscle nicotinic receptors strongly stabilize agonist binding [48]; therefore the most dramatic effects of channel blockers might be to enhance agonist-induced up-regulation when the blocker is applied simultaneously with agonist. However, there are few examples of such well-defined actions for neuronal AChRs. Neither hexamethonium [49] nor chlorisondamine [50] are foot-in-the door blockers at high-sensitivity  $\alpha 4\beta 2$  receptors; it is not surprising that the latter has no effect on nicotine-induced up-regulation in animals [51]. Also unsurprisingly, the channel blockers hexamethonium, mecamylamine, or decamethonium by themselves do not produce appreciable up-regulation [9,33].

Biochemical specificity sometimes exceeds that afforded by free energy differences alone. Proofreading processes in

DNA replication and in protein synthesis reduce the error rate far below that expected from free energy differences. “Pharmacological chaperones” might accomplish an analogous task by allowing integral membrane proteins to escape retention and degradation by quality control systems in endoplasmic reticulum [52]. Typically these proofreading mechanisms use high-energy phosphate bonds to lock in a structure even after the thermodynamic driving force has changed to disfavor the structure. Perhaps another class of proteins exists to modulate the purely thermodynamic specificity of Cys-loop receptor assembly. If so, one expects to find that such a protein binds at subunit interfaces. Considering the structural similarity between snake venom toxins (which bind at the interface) and the lynx/SLURP family [53,54], the lynx/SLURP proteins would be good candidates.

In conclusion, thermodynamic arguments, modified by kinetics and compartmentalization, can explain much of the phenomenology associated with nicotine-induced up-regulation of  $\alpha 4\beta 2$  receptors. However, several apparently contradictory results, particularly with other drugs, remain unexplained by thermodynamics alone and require more detailed mechanistic explanations.

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