Nicotine responses in hypersensitive and knockout α4 mice account for tolerance to both hypothermia and locomotor suppression in wild-type mice

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Nicotine responses in hypersensitive and knockout α4 mice account for tolerance to both hypothermia and locomotor suppression in wild-type mice. Physiol Genomics 31: 422–428, 2007. First published August 21, 2007; doi:10.1152/physiolgenomics.00063.2007.—Nicotinic receptors containing the α4 subunit (α4* nAChRs) have high sensitivity and are widely expressed in the central nervous system, yet their contributions to behavioral tolerance, a hallmark of nicotine dependence, are unclear. To evaluate the contribution of α4* and non-α4 nAChRs in the development of tolerance to hypothermia and locomotor suppression, knockout (KO), hypersensitive Leu9’Ala α4 knock-in, and wild-type (WT) mice received daily nicotine injections, and their behaviors were compared. Repeated selective activation of α4* nAChRs in Leu9’Ala mice produced profound tolerance to hypothermia over 7 days, whereas no tolerance was observed in α4 KO animals. The summed time course and temperature response (after appropriate normalizations) from these two mutant mouse strains resembled the time course of WT tolerance. In addition, daily selective activation of α4* nAChRs elicited locomotor activation in Leu9’Ala mice, but nicotine suppressed activity in α4 KO mice and this did not change with daily drug exposure. Again, appropriately combined responses from the two mutant strains resembled the biphasic nicotine-induced activity in WT animals. Thus, by analyzing nicotinic responses in two complementary mouse lines, one lacking α4* nAChRs, the other expressing hypersensitive α4* nAChRs, one can accurately separate non-α4 nAChR responses from α4 nAChR responses, and one can also account for WT tolerance to both hypothermia and locomotor suppression. Our study suggests a new paradigm for bridging the gap between genetic manipulation of a single receptor and whole animal behavioral studies and shows that activation of α4* nAChRs is both necessary and sufficient for the expression of tolerance.

NEURONAL nAChRs are cation-selective ligand-gated ion channels that are activated by the endogenous ligand, acetylcholine, as well as the naturally occurring alkaloid found in tobacco, nicotine. At present, 12 neuronal nicotinic acetylcholine receptor subunits have been identified (α2–10 and β2–4)(12). The majority of subunits form functional heteromeric pentamers, while a subset may form homomeric receptors. Thus, a myriad of nAChR subtypes may exist. Identification of nAChR subtypes specifically involved in dependence-related behaviors will give insights into the mechanism of nicotine dependence and will help to identify specific neural circuits that participate in addiction (17).

Previously, we have engineered and characterized a mouse line expressing a single point mutation in the M2 domain of the α4 nAChR subunit (3, 7, 16). This mutation, Leu9’Ala, renders α4* nAChRs hypersensitive to nicotine by ~50-fold compared with wild-type (WT) mice. In the absence of specific agonists, this “knock-in” Leu9’Ala mouse line allows for the selective activation of α4* receptors with small doses of nicotine that do not activate other nAChR subtypes. Leu9’Ala mice exhibit dependence-related behaviors including tolerance and sensitization when challenged with daily injections of nicotine at doses that have little effect in WT animals. However, locomotor and hypothermia tolerance profiles in Leu9’Ala mice differ from those of WT mice tested at 50-fold higher nicotine doses, indicating that α4* nAChR-mediated responses in WT mice are likely confounded by responses due to activation of other non-α4* nAChR subtypes. Indeed, nicotine doses required to elicit physiological responses in WT mice, and those doses that occur in smokers, activate multiple receptor subtypes (6, 19).

Mouse strains have also been developed that lack α4 nAChRs (9, 15). In this report, we utilize these two complementary mouse models to evaluate the role of α4* nAChRs in the response to nicotine. The Leu9’Ala line can be used to selectively activate α4* nAChRs with low doses of nicotine. The α4-null or knockout line (α4KO) allows us to determine the response to nicotine that is mediated by non-α4* nAChRs. We ask whether experimental data can be subjected to simple algorithms that account for the WT nicotine effects based on responses from these two mutant strains. A comparison of nicotine-mediated effects in these two lines does implicate α4* nAChRs in a hallmark of dependence: tolerance.

METHODS

Mice

All experiments were conducted in accordance with the guidelines for care and use of animals provided by the National Institutes of Health, as well as with an approved animal protocol from the California Institute of Technology animal care and use committee. Animals were kept on a standard 12-h light/dark cycle and given food and water ad libitum. To minimize variability in responses due to genetic background differences, mice from both Leu9’Ala and α4 KO lines were back-crossed at least eight generations to the C57BL/6J strain with the exception of animals used for Fig. 3, A and B, which were back-crossed at least three generations (8).

Telemetry Probes

Vital View PDT-4000 temperature and activity telemetric probes were used (Respironics). For implantation, mice were anesthetized with halothane, and a 1-cm incision was made at the back of the neck. Probes were inserted subcutaneously into the back. The incision was...
sealed with surgical glue, and the mice were allowed to recover for 48 h. Temperature and activity data were acquired at 30-s intervals using Vital View software and analyzed in Origin.

Nicotine Administration

Nicotine base was used in all behavioral experiments and administered through intraperitoneal injection with 1-ml syringes. Nicotine, mecamylamine, and SCH23390 were obtained from Sigma. Animals were injected daily with saline for at least 3 days prior to the start of each temperature and activity experiment, and average temperature changes for saline injections were <0.2°C. Preinjection with SCH23390 did not produce significant temperature changes compared with saline. All activity and body temperature measurements were recorded in the home cage.

Calculations

Predicted hypothermia tolerance profiles. We assume that, in WT mice, there are at least two components of nicotine-induced hypothermia and that these are mediated by at least two nAChR subtypes whose pathways are parallel and additive. We therefore write, $\Delta T_{WT} = \Delta T_A + \Delta T_N$, where $\Delta T_{WT}$ is the total change in body temperature for WT, $\Delta T_A$ is the change in body temperature due to activation of $\alpha^A$ nAChRs, and $\Delta T_N$ is the change in body temperature due to activation of non-$\alpha^A$ nAChRs. For Leu9'Ala mice injected with 0.015 mg/kg nicotine, $\Delta T_{Leu9'Ala} = \Delta T_A$, whereas for $\alpha^A$ KO mice injected with 2 mg/kg nicotine, $\Delta T_{\alpha^A KO} = \Delta T_N$. Thus, we predict that $\Delta T_{WT} = \Delta T_{Leu9'Ala} + \Delta T_{\alpha^A KO}$ for each daily exposure to nicotine.

To measure tolerance to nicotine-induced hypothermia, we injected Leu9'Ala, $\alpha^A$ KO, and WT mice with 0.015 mg/kg, 2 mg/kg nicotine, and 2 mg/kg nicotine ip, respectively, once daily over the course of 7 days. WT mice received 2 mg/kg nicotine for comparison because this dose would be expected to maximally activate high-sensitivity $\alpha^A$ nAChRs while also activating lower-sensitivity, non-$\alpha^A$ nAChRs, presumably activated by the same dose in $\alpha^A$ KO mice. Doses >2 mg/kg induce seizures in appreciable numbers of mice and, therefore, were not tested. This strategy could be confounded by compensatory or developmental changes in expression of other nAChR subtypes in Leu9'Ala or $\alpha^A$ KO mice. However, to date, radioligand binding and in situ hybridization analyses have revealed no such changes (3, 15, 16).

We measured the maximal change in temperature (usually negative) within 40 min after a nicotine injection on day $n$; usually this extremum occurred at ~25 min. We averaged these changes for all mice of each genotype to obtain $\Delta T(n)_A$, $\Delta T(n)_N$, $\Delta T(n)_{WT}$ on each day $n$. We then define

$$\Delta T(n)_A = \Delta T(n)_D [\Delta T(1)_A + \Delta T(1)_N]$$

$$\Delta T(n)_N = \Delta T(n)_D [\Delta T(1)_A + \Delta T(1)_N]$$

$$\Delta T(n)_{WT} = \Delta T(n)_{WT}/\Delta T(n)_{WT}.\$$

In most cases, $-1 < \Delta T(n)_N$, $\Delta T(n)_N$, $\Delta T(n)_{WT} < 0$. Thus, we predict WT activity after a nicotine injection on day $n$: $$\Delta T(n)_{WT} = \Delta T(n)_D + \Delta T(n)_N$$

Predicted locomotor profiles. We assume there are at least two nicotine-induced components that affect locomotor activity in WT mice, an $\alpha^A$ nAChR and non-$\alpha^A$ nAChR component. We write, $L = B_{WT}W = B_{WT}^{\alpha A}A$, where $L$ is total locomotor activity after nicotine exposure, and $B$ is the average baseline activity over 30 min immediately following a saline injection. $A$, $N$, and $W$ are positive dimensionless parameters. $A$ describes the effect of $\alpha^A$ nAChR activation on activity and equals unity for the KO strain. $N$ describes the non-$\alpha^A$ nAChR effect on activity and equals unity for nicotine doses too low to activate non-$\alpha^A$ receptors. The parameter $W$ is defined similarly for WT mice. Because activity is affected in opposite directions for $\alpha^A$ KO and Leu9'Ala strains but cannot have a negative value, multiplication is more appropriate than simple addition for combining the two processes, thus $W = AN$. For Leu9'Ala mice injected with 0.015 mg/kg nicotine $L_A = B_{\alpha A}A$. Then $A = L_A/B_{\alpha A}$. For $\alpha^A$ KO mice injected with 2 mg/kg nicotine, $L_N = B_2N$. Then, $N = T_\alpha/B_N$.

To measure locomotor effects of nicotine, we injected Leu9'Ala, $\alpha^A$ KO, and WT mice with 0.015 mg/kg, 2 mg/kg nicotine, and 2 mg/kg nicotine ip, respectively, once daily over the course of 6 days. We averaged total activity during 30 min immediately after each nicotine injection for all mice of each genotype to obtain $L(n)_A$, $L(n)_N$, and $L(n)_{WT}$ on each day, $n$. Then:

$$L_A(n) = B_{\alpha A}N(n)$$

$$L_A(n) = B_{\alpha A}A(n)$$

Thus, one predicts WT activity after a nicotine injection on day $n$:

$$L_{WT}(n) = B_{WT}A(n)N(n)$$

Statistical Tests

Significance in all experiments was determined by one-way ANOVA followed by post hoc analysis (Tukey test unless otherwise stated).

RESULTS

Hypothermia

Acute nicotine injection elicits hypothermia in mice (13). Figure 1A illustrates the dose-response relationships for nicotine-induced hypothermia in WT, Leu9'Ala, and $\alpha^A$ KO mice. Previously, we reported that Leu9'Ala exhibit a leftward-shifted dose-response relationship compared with WT (16). We repeat this result here, using Leu9'Ala mice on a congenic, C57BL/6J background. The congenic Leu9'Ala mice are ~50-fold more sensitive than WT to the hypothermic effects of nicotine, similar to the previously studied Leu9'Ala mutant line on a mixed genetic background.

In $\alpha^A$ KO mice, 0.5 mg/kg nicotine does not elicit hypothermia, and this is significantly different from the nicotine effect in WT animals (1.09 ± 0.07°C in $\alpha^A$ KO mice compared with -2.08 ± 0.39°C in WT, $P < 0.001$, $F_{1,14} = 97.4$, Fig. 1A). When challenged with higher doses of nicotine, 1 or 2 mg/kg, $\alpha^A$ KO mice do exhibit hypothermia although at significantly less levels than WT mice challenged with the same dose. It is not possible to provide ED$_{50}$ values because two of the dose-effect relations do not saturate at high nicotine doses; instead, we present nicotine doses that induced a 2°C decrease: 0.009, 0.4, and 1.7 mg/kg, for L9'A, WT, and $\alpha^A$, respectively.

We previously reported that in WT and Leu9'Ala mice, the noncompetitive nicotinic antagonist, mecamylamine, significantly blocked nicotine-induced hypothermia (16). We now report that mecamylamine also blocked nicotine-induced hypothermia in $\alpha^A$ KO mice. The body temperature of $\alpha^A$ KO mice challenged with 2 mg/kg nicotine 15 min after a saline injection decreased 2.74 ± 0.31°C, while body temperature dropped only 0.80 ± 0.11°C in $\alpha^A$ KO mice pretreated with 2 mg/kg mecamylamine ($P < 0.001$, $F_{1,9} = 29.4$, Fig. 1B). The peripheral nicotinic receptor antagonist, hexamethonium, did not significantly block nicotine induced hypothermia in either $\alpha^A$ KO or Leu9'Ala mice [1 mg/kg, data not shown, not significant (NS), $n = 6$ per genotype].
Direct and indirect dopamine agonists elicit hypothermia in rodents (1, 2), and previously, we found that Leu9-11032Ala mice express hypersensitive α4 nAChRs in midbrain dopaminergic neurons, suggesting that nicotine-induced hypothermia may have a dopamine-dependent component (16). The D1 antagonist SCH23390 significantly attenuates nicotine-induced hypothermia in Leu9-11032Ala animals (saline 0.03 mg/kg nicotine × 15 min after either a saline (Sal, left, n = 6) or mecamylamine (Mec, right, 2 mg/kg, n = 6) injection. C: temperature response in Leu9-11032Ala α4 KO mice elicited by injection of 2 mg/kg Nic ip 15 min after either a Sal (left, n = 6) or SCH23390 (right, 2 mg/kg, n = 6) injection. Data are expressed as means ± SE. Significance was measured via 1-way ANOVA (Tukey post hoc). **P < 0.01, ***P < 0.001.

Fig. 1. Expression and pharmacology of nicotine-induced hypothermia in Leu9-Ala, α4 knockout (KO) and wild-type (WT) mice. A: dose-response relation for WT (●), Leu9-Ala homozygous (▲) and α4 KO homozygous mice (○) challenged with nicotine (Nic). Each data point represents peak temperature drops from 4–10 animals, 15–40 min after the injection (see Ref. 16). B: temperature response in α4 KO mice elicited by injection of 2 mg/kg nicotine ip 15 min after either a saline (Sal, left, n = 5) or mecamylamine (Mec, right, 2 mg/kg, n = 6) injection. C: temperature response in Leu9-Ala α4 mice elicited by injection of 0.030 mg/kg Nic ip 15 min after either a Sal (left, n = 6) or SCH23390 (right, 2 mg/kg, n = 6) injection. D: temperature response in α4 KO mice elicited by injection of 2 mg/kg Nic ip 15 min after either a Sal (left, n = 6) or SCH23390 (right, 2 mg/kg, n = 6) injection. Data are expressed as means ± SE. Significance was measured via 1-way ANOVA (Tukey post hoc). **P < 0.01, ***P < 0.001 compared with 1st drug exposure.
mg/kg ip, respectively) on exposure day 1, 4, or 7. Similar to our previous findings, selective activation of Leu9/Ala nAChRs with 0.015 mg/kg nicotine is sufficient for the development of tolerance. After seven injections of nicotine over 7 days, nicotine-induced hypothermia is decreased by 78.8 ± 13.3% (Fig. 2B, P < 0.01, F1,12 = 11.8). To determine whether non-α4* nAChRs are also necessary for a component of tolerance, we injected α4 KO animals with 2 mg/kg nicotine daily for 7 days. The hypothermic response of α4 KO mice measured after 7 days of treatment was not significantly different than the response following the first injection; thus, tolerance did not develop (day 1 = −2.40 ± 0.26°C compared with day 7 = −2.28 ± 0.23°C, NS; Fig. 2, A and B). The right-hand axis of Fig. 2B gives the scale for the parameters defined in Eqs. 1 and 2).

Figure 2C illustrates the tolerance profile of WT in response to daily injections of 2 mg/kg nicotine. WT mice developed significant tolerance by the seventh injection (day 1: −4.74 ± 0.17°C compared with day 7: −2.11 ± 0.21°C, P < 0.001, F1,8 = 57.8). This level of tolerance differed from tolerance profiles in Leu9/Ala or α4 KO mice by themselves (WT % attenuation between days 1 and 7: 49 ± 4.8% compared with 78 ± 13.3% in Leu9/Ala and −4.9 ± 9.4% in α4 KO mice, P < 0.01, F2,14 = 14.2). The right-hand axis of Fig. 2C gives the scale for the parameter defined in Eq. 3.

Together, our data suggest that the nicotine-induced hypothermia in WT mice is determined by the combination of activating both α4* and non-α4* nAChRs but that α4* nAChRs alone are primarily responsible for the magnitude of tolerance that develops with continued nicotine exposure. To test this hypothesis, given by Eq. 4, we summed the tolerance profiles from Leu9/Ala and α4 KO to predict the tolerance profile in wild-type animals (Eq. 4). Figure 2D illustrates the normalized predicted tolerance profile compared with the actual tolerance profile from WT mice. The WT response was nearly identical to the predicted profile generated from the summed profiles of the two lines (Fig. 2D).

**Locomotor Activity**

Previously, we reported that selective activation of α4* nAChRs with nicotine in Leu9/Ala mice activates locomotor activity after multiple drug exposures. This effect is blocked by mecamylamine and also by the D1 antagonist SCH233900 (2). Figure 3 illustrates dose-response relationships between nicotine and changes in locomotor activity in WT (Fig. 3A) and Leu9/Ala mice (Fig. 3B). Selective activation of α4* nAChRs with 0.015 or 0.030 but not 0.01 or 0.0075 mg/kg nicotine in Leu9/Ala mice activates locomotion after multiple drug exposures. WT mice exhibit no significant changes in activity after exposures to low doses of nicotine that induce activity in Leu9/Ala mice (Fig. 3A, 0.015 mg/kg nicotine). However, with larger doses, nicotine does alter activity in a dose-dependent manner.

To determine a dose of nicotine that elicits an effect on locomotor activity in α4 KO mice, we injected mice acutely with 0.5, 1.0, or 2.0 mg/kg nicotine. Compared with saline and consistent with previous reports (9, 15), neither 0.5 nor 1.0 mg/kg nicotine had a significant effect on locomotor activity, whereas 2.0 mg/kg significantly suppressed activity similar to WT mice (P < 0.01, F1,14 = 15.9, Fig. 3C). In addition, this suppression of nicotine was significantly blocked by mecamylamine, indicating that the effect was mediated via nAChR activation (Fig. 3D).

Figure 4, A and B, depicts the pattern of locomotor activity for 30 min immediately after saline injection or after one or six injections at different doses. **Saline.** A: locomotor responses in WT mice after 1, 3, and 6 daily injections of 0.015, 0.5, 0.75, or 1.5 mg/kg nicotine. B: locomotor responses in Leu9/Ala homozygous mice after 1, 3, and 6 daily injections of 0.0075, 0.010, 0.015, or 0.030 mg/kg nicotine. In A and B, each data point represents 30 min of activity immediately following drug injection. *P < 0.05, ***P < 0.001 compared with saline (day 0). #P < 0.05 compared with first drug exposure in A and #P < 0.05 compared with 0.0075 mg/kg day 6 in B. C: locomotor response to acute injection of saline or nicotine (0.5, 1.0, or 2.0 mg/kg) in α4 KO mice. Each bar represents activity summed over 30 min immediately following injection.

**Nicotine.** A: locomotor responses in WT mice after 1, 3, and 6 daily injections of 0.015, 0.5, 0.75, or 1.5 mg/kg nicotine. B: locomotor responses in Leu9/Ala homozygous mice after 1, 3, and 6 daily injections of 0.0075, 0.010, 0.015, or 0.030 mg/kg nicotine. In A and B, each data point represents 30 min of activity immediately following drug injection. *P < 0.05, ***P < 0.001 compared with saline (day 0). #P < 0.05 compared with first drug exposure in A and #P < 0.05 compared with 0.0075 mg/kg day 6 in B. C: locomotor response to acute injection of saline or nicotine (0.5, 1.0, or 2.0 mg/kg) in α4 KO mice. Each bar represents activity summed over 30 min immediately following injection. D: locomotor responses in α4 KO mice injected with 2 mg/kg nicotine 15 min after an injection of either saline (left bar) or 2 mg/kg mecamylamine (middle bar). Mice that received mecamylamine prior to nicotine injection were injected with nicotine alone 24 h later (right bar). Data are expressed as means ± SE. Significance was measured via 1-way ANOVA (Tukey post hoc). Each bar represents 15 min activity immediately following nicotine injection. *P < 0.05, **P < 0.01.
single daily injections of nicotine in α4 KO and Leu9’Ala homozygotes. Summing this activity in the Leu9’Ala mice illustrates that selective activation of α4* nAChRs by 0.015 mg/kg nicotine produces significant locomotor activation (Fig. 4D, day 1: 452 ± 79 counts compared with day 6: 919 ± 68 counts, P < 0.001, F = 19.9). Conversely, a single injection of 2 mg/kg nicotine significantly suppresses activity in α4 KO mice (Fig. 4A, 759 ± 80 counts postsaline compared with 336 ± 61 counts post-2 mg/kg nicotine, P < 0.001, F = 17.76, Fig. 4C), and this activity suppression persists over the course of six daily injections (Fig. 4C). The right-hand axes of Fig. 4, C and D, present scales for the parameters N and A defined in Eqs. 5 and 6, respectively. We injected WT mice with 2 mg/kg nicotine once daily for 6 days. The first injection significantly suppressed locomotion (Fig. 4E). By the sixth injection, nicotine-induced locomotor activity was significantly increased compared with the first injection (day 1 = 0.193 ± 0.0318 compared with day 6 = 0.539 ± 0.132, P < 0.05, F = 6.53).

Figure 4F presents the prediction of Eq. 7: nicotine-induced locomotion factors caused by simultaneous selective activation of α4 nAChRs and activation of non-α4* nAChRs. Equation 7 predicts that, in WT mice, daily nicotine injection will initially suppress locomotion then increase activity with each successive injection. This prediction agrees well with the data of Fig. 4E.

**DISCUSSION**

This report introduces a new method to evaluate the contribution of a single receptor pathway to an observed drug-induced behavior. The method requires the existence of two mutant strains: the KO strain allows one to evaluate the response due to the drug’s effect on other receptors, and a hypersensitive strain (admittedly available in only rare instances) allows one to induce the response due to selective activation of the receptor itself. Furthermore, the hypersensitive strain must have the same expression of the receptor, at the cellular and subcellular level; the Leu9’Ala strain meets these requirements (3, 16). One then asks whether the behavior of the WT strain is the sum or product (as appropriate) of the behavior observed in the KO strain and that observed in the hypersensitive strain. One certainly expects such an analysis to apply well at the level of electrophysiological data on single cells, but the present paper shows that the analysis yields straightforward results when applied to physiological and be-
havioral responses at the whole animal level. It is important for such a straightforward analysis that all strains are on an isogenic background. To avoid confounds produced by genetic variability and to facilitate comparison between strains, both α4 KO and Leu9/Ala mice in the relevant experiments were back-crossed to C57BL/6J mice for at least eight generations.

Selective activation of α4* nAChRs with low doses of nicotine in Leu9/Ala mice causes hypothermia. At higher doses, nicotine also induces hypothermia in WT animals and, at still higher doses, in mice that do not express α4* nAChRs (Fig. 1A). While nicotine-induced hypothermia in both mutant strains is centrally mediated and sensitive to blockade by the non-competitive nicotinic receptor antagonist mecamylamine (Fig. 1B and Ref. 16), only α4-mediated hypothermia is blocked by a D1 antagonist; thus the two hypothermic responses are pharmacologically distinct (Fig. 1, C and D).

Based on these data, nicotine-induced hypothermia in WT mice is initiated by activation of at least two receptor subtypes: one containing α4 subunits and the other(s) containing non-α4 subunits. Note that the shift between knock-in and WT is much greater than the shift between WT and KO in nicotine-induced hypothermia; for instance, 1 mg/kg induces partial hypothermia in both WT and KO mice. The latter point implies that the α4-dependent component is not necessary for nicotine-induced hypothermia. On the other hand, the α4-dependent component displays tolerance in our experiments, while the α4-independent component does not. Interestingly, in β2 knockout mice nicotine-induced hypothermia responses resemble our data with α4 knockouts, consistent with the idea that hypothermia-controlling α4* nAChRs, like most α4* nAChRs, also contain the β2 subunit (11).

Previously, we found that daily selective activation of α4* nAChRs in Leu9/Ala mice elicited tolerance to nicotine-induced hypothermia. Nicotine responses were attenuated by >75% by the seventh daily exposure. However, when challenged with daily nicotine doses that produce hypothermia, WT animals develop tolerance to a significantly lesser extent than Leu9/Ala mice. Figure 2 illustrates that this arises from at least two different nicotinic receptor dependent mechanisms. The α4-mediated hypothermic component isolated in Leu9/Ala mice develops profound tolerance, whereas activation of the non-α4* nAChR component in α4 KO mice does not change with repetitive nicotine exposure. Summing these two independent responses predicts the WT tolerance response. Together, these data indicate that activation of α4* nAChRs is both necessary and sufficient for tolerance development of the hypothermic response.

In rats, single daily nicotine injections produce sensitization to the locomotor actions of nicotine (4, 5). However, in WT mice, acute nicotine initially suppresses activity (Figs. 3A, 4E). With successive daily exposures, mice develop modest tolerance to this suppression. Previous studies have shown that mice lacking β2* nAChR expression are less sensitive to nicotine-induced locomotor suppression at low doses of nicotine but equally sensitive at high doses (18). Similarly, we find that α4 KO animals are equally sensitive to locomotor suppression compared with WT animals at high nicotine doses (i.e., 2 mg/kg). Interestingly, selective activation of α4* nAChR in Leu9/Ala mice does not acutely suppress locomotor activity at the doses we have measured.

Here we show that the biphasic WT locomotor profile likely arises from at least two combined nicotine actions with opposite effects on locomotor activity. Activation of non-α4* nAChRs in α4 KO mice suppresses locomotor activity, and this effect persists with every daily exposure (Fig. 4, A and C). Selective activation of α4* nAChRs, on the other hand, produces time- and exposure-dependent increases in activity (Fig. 4, B and D). In WT mice, these two mechanisms converge: non-α4* nAChR activation initially suppresses activity, but, with continued daily activation of α4* nAChRs, activity increases, producing the observed locomotor patterns seen with repeated nicotine exposure.

Although this study does not directly explore mechanisms, the locomotor data, and their dependence on dopaminergic processes (16), are consistent with recent observations on α4* receptor changes during chronic nicotine at the cellular and circuit level in the midbrain (14). On day 1, nicotine (1.5–2 mg/kg) suppresses locomotion equally in WT and α4 KO mice, consistent with the idea that nicotine-induced locomotor suppression at high doses involves non-α4B* receptors (10, 18), although the responding neurons are unknown. Repeated nicotine applications would increase the number of functional α4* levels in some GABAAergic neurons (14). In response to nicotine, such upregulated α4* receptors would increase firing in the GABAAergic neurons, inhibiting the downstream neurons that produce locomotor suppression via non-α4B2 receptors; the latter receptors would not change sensitivity during repeated nicotine applications. Thus, tolerance to nicotine-induced locomotor suppression would occur in a circuit. That selective activation of α4* receptors produces no initial locomotor suppression but does eventually induce locomotor activation may indicate that there is, initially, also ongoing suppression of locomotor activity, in keeping with present concepts about the complementary roles of the direct and indirect pathways in the basal ganglia.

For our calculations, we elicited hypothermia and activity measurements with 2 mg/kg nicotine in α4 KO animals and 0.015 mg/kg in Leu9/Ala mice, doses that yield equivalent decreases in body temperature when acutely administered to drug-naive animals of each strain. Previously, we have found that nicotine-induced hypothermia accurately reflects nicotine sensitivity to a number of behavioral and physiological responses. However, it could be argued that the Leu9/Ala dose is near the plateau phase of the nicotinedehytempera dose-response relation for this strain and that tolerance development may be due to a ceiling effect. Such a ceiling mechanism is rendered unlikely by the observations that tolerance occurs at multiple hypothermia-inducing doses, including doses that do not saturate the dose-response relation (3). The combined α4 KO and Leu9/Ala responses accurately account for WT tolerance to both hypothermia and locomotor suppression elicited by 2 mg/kg nicotine, a dose chosen because it would likely activate a majority of high-sensitivity α4* nAChRs while also activating non-α4* nAChRs to the same extent as an equal dose in α4 KO mice.

Together, our data indicate that α4* nAChR activation is necessary and sufficient for the development of tolerance as measured by daily injections of nicotine.
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