Polyamine regulation of ion channel assembly and implications for nicotinic acetylcholine receptor pharmacology

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Small molecule polyamines are abundant in all life forms and participate in diverse aspects of cell growth and differentiation. Spermidine/spermine acetyltransferase (SAT1) is the rate-limiting enzyme in polyamine catabolism and a primary genetic risk factor for suicidality. Here, using genome-wide screening, we find that SAT1 selectively controls nicotinic acetylcholine receptor (nAChR) biogenesis. SAT1 specifically augments assembly of nAChRs containing α7 or α4β2, but not α6 subunits. Polyamines are classically studied as regulators of ion channel gating that engage the nAChR channel pore. In contrast, we find polyamine effects on assembly involve the nAChR cytosolic loop. Neurological studies link brain polyamines with neurodegenerative conditions. Our pharmacological and transgenic animal studies find that reducing polyamines enhances cortical neuron nAChR expression and augments nicotine-mediated neuroprotection. Taken together, we describe a most unexpected role for polyamines in regulating ion channel assembly, which provides a new avenue for nAChR neuropharmacology.
**Results**

**Genome-wide screening identifies SAT1 as an enhancer of nAChR.** To identify novel regulators of α4β2 receptors, we co-transfected human embryonic kidney 293T cells (HEK293T) with plasmids encoding α4 and β2 subunits along with individual constructs from a 5943-cDNA clone library from the Broad Institute. Transfected cells were stimulated with nicotine (100 μM) and intracellular Ca2+ was quantified with a fluorescence imaging plate reader (FLIPR) (Fig. 1a). As previously shown, α4β2 alone produced a small nicotine-evoked Ca2+ signal, and co-transfection with NACHO significantly enhanced this (Fig. 1a). High throughput screening identified a single clone that profoundly augmented the nicotine-induced Ca2+ response—to levels much higher than NACHO; this clone encoded spermidine/spermine N1-acetyltransferase (SAT1) (Fig. 1a).

We evaluated the effect of SAT1 on other nAChRs and other Cys-loop receptors both by FLIPR and electrophysiology. Consistent with our screening results, SAT1 dramatically increased ACh-evoked currents from α4β2 (Fig. 1c, d). SAT1 also synergized with NACHO to further enhance the α4β2-mediated Ca2+ influx (Fig. 1b, d), suggesting that SAT1 and NACHO employ different mechanisms. Whereas SAT1 alone did not rescue homomeric α7 function, SAT1 powerfully synergized with NACHO to increase α7-mediated currents (Fig. 1b–d). In contrast, SAT1 had no significant effect on α6β4 or 5-HT3A receptor function (Fig. 1b–d).

SAT1, a small cystolic protein, is the rate-limiting enzyme for polyamine catabolism (Supplementary Fig. 1). Together with polyamine oxidase, SAT1 acetylates higher-order polyamines converting them to inactive forms that are transported out of cells. By lowering polyamine levels, SAT1 effects on nAChRs could reflect disinhibition, as polyamines can negatively affect nAChR gating. This seemed unlikely for two reasons. First, containing nAChRs are more sensitive to polyamine inhibition of gating than are α7 whereas we find the opposite sensitivity to SAT1 in our functional assays (Fig. 1b–d). Second, polyamines do not block nAChRs at the hyperpolarized membrane potentials we used for patch clamp studies. As an alternative mechanism, we asked whether SAT1 increases nAChR surface expression. To assess this, we utilized extracellular HA-tagged subunits that allow detection of surface receptors without disrupting channel function. Strikingly, SAT1 boosted surface levels of both α4β2 and α7 (Fig. 2a, b). SAT1 also augmented surface trafficking atop the effects of the α7 protein chaperones Ric-3, Bcl-XL, and Mcl-1, as well as the α7 chemical chaperone/orthosteric antagonist, methyllycaconitine (MLA) (Supplementary Fig. 2a, b) implying that receptor upregulation by SAT1 involves a mechanism distinct from any previously described.

SAT1 promotes assembly of nAChRs by catalyzing polyamines. To determine whether effects on nAChRs involve the catalytic activity of SAT1, we constructed a Tyr140Phe mutant that abolishes SAT1 enzyme activity. This mutant SAT1 (mutSAT1) did not change surface expression of α4β2 or α7 receptors in the presence or absence of NACHO (Fig. 2a, b). Ornithine decarboxylase 1 (ODC1) is the rate-limiting enzyme for polyamine synthesis (Supplementary Fig. 1), and difluoromethylornithine (DFMO) is an ODC1 active site inhibitor that depletes cellular polyamines. Like SAT1 co-transfection, DFMO pretreatment augmented surface expression of α4β2 and synergized with NACHO to further enhance α4β2 and α7 surface receptors (Fig. 2a, b). Pursuing the opposite tactic, we found that co-transfection with the polyamine biosynthetic enzymes ODC1 and adenosylmethionine decarboxylase 1 (AMID1) diminished nAChR surface staining (Supplementary Fig. 3a, b). Neither catabolic (SAT1 co-transfection or DFMO pretreatment) nor anabolic (ODC1 and AMID1 co-transfection) polyamine manipulations affected surface expression of the 5-HT3A receptor (Fig. 2a, b; Supplementary Fig. 3a, b).

**Neuronal nicotinic acetylcholine receptor (nAChR) ion channels mediate behavioral, cognitive and autonomic effects of acetylcholine and addictive properties of nicotine.** The nAChR family comprises nine alpha (α2-10) and three beta (β2-4) subunits that combine to form an array of pentameric cation channels throughout the brain and peripheral nervous systems. The major nACh receptors in brain are α7 homomers, α4β2 heteromers, and α6-containing heteromers. These receptors are targets of numerous approved and experimental medicines for diverse conditions including Alzheimer’s disease, Parkinson’s disease, and neuropathic pain.

Whereas nAChRs are compelling drug targets, progress is hampered because most receptor subtypes do not functionally express in the non-neuronal cell lines used for screening. We previously identified NACHO, a four-pass transmembrane protein that serves as a client-specific chaperone for assembly of most neuronal nAChRs. In brain, NACHO is essential for function of α7 receptors, and additional proteins, Ric-3 and certain Bcl-2 family members, synergize with NACHO to enhance α7 assembly and surface trafficking. NACHO also promotes assembly of α4β2 receptors, though Ric-3 and Bcl-2 proteins have minimal impact on α4β2.

Here, we sought factors that conspire with NACHO to enhance function of α4β2 receptors, the most abundant nAChR subtype in brain. These neuronal α4β2 receptors mediate diverse physiological actions of acetylcholine and underlie nicotine dependence. Genome-wide cDNA screening for protein enhancers of neuronal nAChRs identified a four-pass transmembrane protein that serves as a client-specific chaperone for assembly of most neuronal nAChRs. In brain, NACHO is essential for function of α7 receptors, and additional proteins, Ric-3 and certain Bcl-2 family members, synergize with NACHO to enhance α7 assembly and surface trafficking. NACHO also promotes assembly of α4β2 receptors, though Ric-3 and Bcl-2 proteins have minimal impact on α4β2.

Polyamines are abundant polycations, including spermidine and spermine, that play multiple roles in cell growth, differentiation, and survival. The interplay between their synthesis by ornithine decarboxylase-1 (ODC1) and their degradation by SAT1 controls polyamine levels. ODC1 is amongst the most dynamically regulated of all human proteins, and ODC1 is a drug target in oncology and infectious disease. SAT1 transcription is also highly-regulated, and its acetylation of polyamines promotes their cellular export. Interestingly, numerous large genomic studies link polymorphisms in SAT1 with suicidal behavior.

In neurons, polyamines play important roles in synaptic transmission by conferring inward rectification to certain potassium channels, AMPA receptors and nACh receptors. Polyamines also participate in the pathogenesis of neurodegenerative disorders and the excitoxicity associated with cerebral ischemia.

We now find that polyamines also control assembly of neuronal α4β2 and α7 receptors. By contrast, polyamines do not modulate assembly of α6β4 nAChRs, AMPA receptors or any other ion channel tested. Whereas polyamines classically regulate channel gating by occluding the ion pore, polyamine regulation of nAChR assembly instead relies on negatively charged residues within the α4 or α7 cytosolic loop. Neuropharmacology studies using wild-type and NACHO knockout mice show that lowering polyamine levels selectively upregulates cerebrocortical α4β2 and α7 levels and enhances the neuroprotective properties of nicotine. These studies identify an unexpected role for polyamines in controlling ion channel biogenesis and suggest new strategies in neuropharmacology.
We asked next whether polyamines influence nAChR assembly, which can be probed with orthosteric ligands, such as [3H]epibatidine, that only bind at the interface between folded subunits. Remarkably, either SAT1 or DFMO enhanced [3H]epibatidine, that only bind at the interface between folded subunits. As expected, when co-transfected with β2 channels in Xenopus laevis oocytes, Loss of negative charge in the transmembrane domain 2 (TM2) corresponding to Glu247 in α4 reduces calcium permeability through the mutant receptor.

We next mutated Trp156 (Fig. 3e) in the α4 ligand-binding domain. As expected, when co-transfected with β2, this α4W156A mutant was functionally inactive and did not bind to [3H]epibatidine (Fig. 3f, g). By contrast, this mutant showed typical surface staining enhancement upon co-transfection with SAT1 cDNA (Fig. 3h, i) indicating that polyamine regulation is independent of agonist binding.

To identify nAChR regions responsible for regulation, we generated chimeras of α4 with α6, as surface expression of the latter is not regulated by polyamines (Fig. 4a). We co-transfected HEK293T cells in 384-well plates were co-transfected with α4-expressing and β2-expressing plasmids along with an individual cDNA (black traces) from a human ORF collection (Broad Institute). Cells were stimulated with 100 μM nicotine (Nic). The highest response occurred in the well containing SAT1 (red trace) and was twice the α4β2 + NACHO response (gray trace), which served as positive control. b Quantification (mean ± SD) of maximum Ca2+ signal upon agonist stimulation from HEK293T cells transfected as indicated (n = 6). Co-transfection of SAT1 significantly enhances Ca2+ signal in α4β2 (p < 1e-4), α4β2 + NACHO (p < 1e-4) and α7 + NACHO (p = 0.002) cells. For all α6β4 conditions, cells were co-transfected with plasmids encoding accessory BARP and SULT2B13. c Representative whole-cell current responses elicited from HEK cells co-transfected with GFP and cDNAs as indicated. d Summary graphs of agonist-evoked peak currents (mean ± SEM) from HEK293T cells transfected with indicated cDNA combinations. Similar to maximum Ca2+ signal, co-transfection of SAT1 boosts evoked currents mediated by α4β2 (p < 1e-4), α4β2 + NACHO (p < 1e-4), α7 + NACHO (p < 1e-4) but not α6β4 (p = 0.98) or 5-HT3A (p = 0.73). Numbers indicate number of transfected cells that were analyzed and were pooled from three independent cultures. **p < 0.01, ***p < 0.001. One-way ANOVA between the groups for α4β2 and α7. Mann-Whitney U test versus control for α6β4 and 5-HT3A. Source data for panel b and d are provided as a Source Data file.

Polyamines regulate nAChR assembly via their cytosolic loop. Certain neuronal nAChRs display blunted ion flow at depolarized potentials. This inward rectification is mediated by polyamine binding to pore-lining glutamate residues in the nAChR transmembrane domain 2 (TM2) corresponding to Glu247 in α4. It was previously shown that mutating this acidic residue to alanine (α4E247A) relieves polyamine block of α4β2 channels in Xenopus laevis oocytes. Loss of negative charge in the α4E247A also reduces calcium permeability through the mutant receptor. Accordingly, we found that a E247A mutant (Fig. 3a) co-transfected with β2 evinced minimal nicotine-evoked Ca2+ influx in HEK293T cells (Fig. 3b), and this was unaffected by preincubation with DFMO or co-transfection with SAT1. By contrast, DFMO or SAT1 enhanced surface expression of α4E247A/β2 similar to wildtype α4β2 (Fig. 3c, d). These data establish distinct mechanisms for polyamine regulation of nAChR gating and trafficking.

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Similarly, exchanging the TM3-TM4 loop of homomeric α7 receptors on the intracellular loop that can mediate polyamine regulation of nAChR assembly with that of α6 (Supplementary Fig. 4d) abolished SAT1-mediated upregulation of alpha-bungarotoxin (α-Bgt) binding sites on α7 (Supplementary Fig. 4e, f), which demonstrates that the intracellular loop determines polyamine-regulation of both α4 and α7 nAChR assembly.

Previous studies noted that, within the T3-TM4 loop, a subdomain proximal to TM4 contains conserved positively and negatively charged residues. Interestingly, we observed certain negatively charged residues (Glu and Asp) in this subdomain that are conserved between α7 and α4 but diverge in α6 to uncharged Asn (Supplementary Fig. 5a, b). Mutating those Glu and Asp to Ala in α7 (E438A or D451A) and α4 (E569A or D582A) blocked SAT1-mediated upregulation of α7 assembly (Supplementary Fig. 5c, d) and α4β2 surface expression (Supplementary Fig. 5e, f). By contrast, these mutations did not blunt effects of Ric-3 on α7 or NACHO on α4β2. Replacing the corresponding Asn residues (N134 or N447) to Glu within the α6 cytosolic loop conferred moderate but significant SAT1 and DFMO sensitivity to α6β4 receptors (Supplementary Fig. 5g, h). These experiments identify negatively charged residues within the TM3-TM4 cytosolic loop that can mediate polyamine regulation of nAChR assembly and surface expression. Future studies are needed to fully elucidate these mechanisms.

Polyamines regulate nAChR gating and assembly independently. To further distinguish mechanisms for polyamine control of nAChR assembly and gating, we compared effects of the membrane-permeant polyamine analog BenSpm with philanthotoxin-343 (PhTx-343), which cannot enter cells and engages the channel pore via a long polyamine tail. Whereas BenSpm abrogated SAT1-mediated enhancement of basal surface levels of α4β2, whereas BenSpm abolished SAT1-mediated enhancement of surface α4β2, PhTx-343 did not (Fig. 5a, b). By contrast, both BenSpm and PhTx-343 pretreatment abolished nicotine-evoked Ca\(^{2+}\)\(^{+}\) with or without SAT1 co-transfection (Fig. 5c). These data demonstrate that only cell-permeant polyamines can control of nAChR surface trafficking.

To more completely elucidate these mechanisms, we compared effects of BenSpm and PhTx-343 on α4-containing and α6-containing receptors, as SAT1 regulates trafficking of α4β4 but not α6β4 (Fig. 4b). As expected, pre-treatment with BenSpm, but not PhTx-343, reversed SAT1-mediated enhancement of α4β4 surface expression and neither had effect on α6β4 surface expression (Fig. 5d, e). By contrast, both BenSpm and PhTx-343
application abolished nicotine-evoked Ca^{2+} signaling from either α4β4 or α6β4 (Fig. 5f).

We next evaluated effects of BenSpm and PhTx-343 on the α6/α4 chimeric constructs, which swap the extracellular N-terminal or TM3-TM4 cytosolic loop domains. As predicted, the α4 cytosolic loop was necessary and sufficient for BenSpm pre-treatment to reverse SAT1-mediated enhancement of receptor surface expression, and PhTx-343 did not alter surface expression for any chimera (Fig. 5e and Supplementary Fig. 6, d, e). By contrast, acute application of either BenSpm or PhTx-343 blocked nicotine-evoked Ca^{2+}-signaling from all receptor chimeras in a concentration-dependent manner (Fig. 5f, Supplementary Fig. 6a-c). These molecular biological and pharmacological studies establish distinct mechanisms and protein domains for polyamine control of nAChR trafficking and gating.

Polyamines regulate assembly of neuronal nAChRs. We next explored whether polyamines upregulate endogenous nAChRs in neurons. Accordingly, we treated cultured rat cortical neurons with DFMO and quantified α7 nAChR surface expression with fluorescent α-Bgt. As a positive control, we incubated neurons with 100 μM nicotine and found a significant increase in surface α-Bgt binding sites (Fig. 6a, b). By contrast, neither DFMO nor nicotine altered surface expression of the AMPA-type glutamate receptor subunit GluA1 (Fig. 6a, b). Preincubating neurons with DFMO also increased the nicotine-evoked Ca^{2+} signal (Fig. 6c), an effect that was comparable to the enhancement seen following nicotine pretreatment. These nicotine-evoked Ca^{2+} responses reflect α7 activity, as they require the α7-specific positive allosteric modulator (PAM) PNU-120596/1534 and were abolished by the α7-specific inhibitor α-Bgt (Fig. 6c).

[^3H]Epibatidine binding in brain largely reflects α4β2 receptors and was absent in cerebral cortex of α4 knockout mice. Importantly, incubating cortical neurons with DFMO or transducing them with a lentivirus expressing SAT1 enhanced levels of [^3H]epibatidine binding (Fig. 6d), indicating an increased α4β2 receptor assembly. To confirm polyamine regulation of neuronal α4β2, we transduced cortical neurons with α4-expressing and β2-expressing lentiviruses. Levels of [^3H]
epibatidine binding to the transduced neuronal membranes were ~20 times higher than untransduced neurons, and this was further increased with DFMO pretreatment (Fig. 6d). Importantly, neither DFMO preincubation nor SAT1 transduction altered binding sites for the GABA receptor ligand [3H]flunitrazepam (Fig. 6d). Taken together, these data show that polyamines regulate assembly of neuronal nAChR but not AMPA or GABA receptors.

Polyamines modulate nicotine-mediated neuroprotection. Nicotine has well-established neuroprotective properties and can mitigate excitotoxicity in vivo and in vitro. This neuroprotection is mediated both by α7 and α4β2 nAChRs. To assess the functional consequence of polyamine upregulation of receptor assembly, we asked whether this nicotine-mediated neuroprotection could be enhanced by reducing cellular polyamine levels. We induced excitotoxicity by challenging cultured neurons for one hour with glutamate (30 µM) and quantified cell survival and cytosolic cytchrome C (CytC), which is a trigger for apoptosis. We also surface-labeled α7 receptors with fluorescent α-Bgt. In line with previous reports, glutamate challenge induced excitotoxicity and increased cytosolic CytC. Co-application of 100 µM nicotine reduced excitotoxicity, but glutamate did not alter surface expression of α7. On the other hand, neurons pretreated with either DFMO or nicotine showed a three-fold increase in surface α-Bgt-binding sites (Supplementary Fig. 7a, d) and a concordant reduction of glutamate-induced CytC release (Fig. 6e; Supplementary Fig. 7c). Nicotine or DFMO preincubation also augmented nicotine-mediated protection from glutamate-mediated excitotoxic cell death (Fig. 6e and Supplementary Fig. 7b).

To more directly link the neuroprotective effects of DFMO with nAChRs, we studied NACHO KO mice, which have dramatically reduced nAChR function in brain. We found that nicotine blunted glutamate-induced cell death in neurons from wild-type but not NACHO KO mice (Fig. 7a–c). Fitting with results from our rat cortical neuron experiments, we found that pre-treating wild-type mouse neurons with DFMO increased surface α-Bgt staining (Fig. 7e) and mitigated glutamate-induced increases in cytosolic CytC (Fig. 7d). By contrast, DFMO pre-treatment did not augment α-Bgt binding levels in NACHO KO neurons. Accordingly, DFMO preincubation did not confer subsequent nicotine-induced protection from excitotoxic neuronal death in NACHO KO neurons (Fig. 7c). Collectively, these experiments show that polyamines regulate functional assembly of nAChRs, which promotes neuroprotection in wild-type, but not NACHO KO neurons.

Discussion

This study identifies an unexpected role for polyamines in controlling assembly of neuronal α4β2 and α7 receptors. Our
genome-wide analysis found that SAT1 increases assembly of α4β2 receptors to a much greater extent than any other protein screened, including the nicotinic receptor chaperone NACHO. Polyamine-regulated assembly is specific for certain nAChRs, is distinct from other nAChR chaperone mechanisms, and requires negatively charged amino acids within the α4 or α7 cytosolic loop. Blocking polyamine synthesis with DFMO upregulates neuronal α4β2 and α7 surface levels and promotes nicotinemediated neuroprotection, which provides a new angle for drug discovery.

The biogenesis of pentameric nAChRs is a complex and tightly-regulated process. Seminal studies in the 1980s found that nicotine and other orthosteric ligands upregulate brain nACh protein levels, and this likely participates in nicotine dependence. In C. elegans, Ric-3 is required for efficient assembly of worm nAChRs; in mammalian brain, NACHO is required for α7 and many other nAChRs. Additional proteins synergize with NACHO for assembly of specific mammalian nAChRs. Ric-3 and certain Bcl-2 family proteins work with NACHO to promote function of α7, whereas BARP, LAMP5, and

Fig. 5 Polyamine analogs regulate nAChR gating and assembly by distinct mechanisms. a Fluorescent anti-HA labeling of non-permeabilized HEK293T cells transfected with cDNAs encoding extracellular HA-tagged β2 and α4 with or without SAT1. Scale bar = 50 μm. b Quantification of surface staining for α4β2 receptors with or without SAT1 and BenSpm or PhTx343 pre-treatment (n = 6). BenSpm occluded the SAT1-enhanced receptor surface expression (p < 1e−4) but PhTx343 had no effect (p = 0.86). c Nicotine-evoked Ca2+ signal was fully blocked by both BenSpm or PhTx343 (p < 1e−4) (n = 6). d Schematics of chimeric constructs of α4-α6. e Normalized anti-HA surface expression from cells transfected and treated with BenSpm or PhTx343 as indicated (n = 5). Only BenSpm occluded SAT1-mediated enhancement of surface receptor expression of α4β2 (p < 1e−4), α6NT/α4β2 (p < 1e−4) and α6/α4-loopβ2 (p < 1e−4). Both SAT1 and BenSpm effects on receptor surface expression required the α4 cytosolic loop. f Both BenSpm and PhTx343 fully inhibited nicotine-evoked Ca2+ in all receptor combinations. All cDNA combinations included BARP and SULT2B1. Quantifications are displayed as mean ± SD. ***p < 0.001, One-way ANOVA between the groups was used for panels b, c and e. Comprehensive dose-response studies are in Supplementary Fig. 5. Source data for panel b, c and e are provided as a Source Data file.
SULT2B1 conspire with NACHO to enhance function of α6-containing receptors. Our discovery of SAT1 regulation identifies the polyamine pathway as an additional controlling mechanism for α4β2 and α7 receptors.

These multiple layers of nAChR regulation utilize distinct mechanisms. In the case of α7, NACHO mediates subunit oligomerization. Subsequent regulation by nicotine, Ric-3, and Bcl-2 proteins synergizes with NACHO to promote protein folding, receptor surface expression, and channel function. Nicotine and other orthosteric ligands promote assembly through α7’s ACh binding domain whereas Bcl-2 proteins and polyamines engage the receptor cytosolic loop. However, polyamine regulation involves a distinct mechanism, as we find SAT1 augments receptor assembly atop NACHO, nicotinic ligand, Ric-3, or Bcl-2 family proteins.

It is intriguing to ask why nAChRs—but not other receptors in the Cys-loop superfamily including 5-HT3 and GABA<sub>A</sub> receptors—require accessories for functional expression. One possibility is that multiple assembly mechanisms provide regulatory nodes for controlling nAChR function. NACHO transcription is upregulated by physiological stimuli, and Bcl-2 family protein levels are dynamically induced during developmental and pathological processes. Furthermore, protein accessories may also determine nAChR cellular localization. The α6-containing nAChRs specifically concentrate at presynaptic terminals of specific monoaminergic neurons, and this may be enabled by the lysosomal protein, LAMP5, which displays a similar restricted distribution.

Polyamines are ubiquitous in biology and play multiple roles in cell growth, survival, and differentiation. In neurons, polyamines control gating of several important ion channels. Cytosolic polyamines confer inward rectification to certain potassium channels, AMPA receptors, and nAChRs. Elegant biophysical studies showed that neuronal depolarization draws cytosolic polyamines into the channel pore, which precludes ion flow. This polyamine site is accessible to extracellular...
philanthotoxins\textsuperscript{50}, which have long polyamine tails that can engage the channel.

Our studies decisively establish that regulation of α\textsubscript{7} and α\textsubscript{4}β\textsubscript{2} assembly by polyamines is distinct from their classical role in controlling ion channel gating. First, we find that SAT1 increases α\textsubscript{4}β\textsubscript{2} and α\textsubscript{7} function even when recorded at hyperpolarized potentials that preclude nAChR gating control by cellular polyamines\textsuperscript{22} (Fig. 1c, d). Second, SAT1 promotes assembly of non-functional α\textsubscript{4}β\textsubscript{2} receptors that cannot bind ACh (Fig. 3e–i). Third, extracellular philanthotoxin acutely blocks α\textsubscript{4}β\textsubscript{2} receptor function but philanthotoxin does not affect receptor assembly (Fig. 5a–c). Fourth, chronic but not acute application of a cell-permeable polyamine analog, BenSpm, reverses the effects of SAT1 on α\textsubscript{7} and α\textsubscript{4}β\textsubscript{2} receptor assembly and function (Fig. 2d).

Interestingly, we find that SAT1 does not augment function of α\textsubscript{6}-containing nAChRs. Taking advantage of this α\textsubscript{6}-subunit specificity, our α\textsubscript{6}/α\textsubscript{4} chimeras determine that polyamine regulation of channel assembly involves the α\textsubscript{4} cytosolic loop. Again, this is

Fig. 7 DFMO promotes nicotine-mediated neuroprotection in a NACHO-dependent fashion. a, b Images of cortical neurons (DIV 20) from wild-type (a) or NACHO KO (b) mice. As indicated, neurons were pretreated with DFMO (5 mM, 4th row) or and challenged with 30 μM glutamate (Glu, 2nd–4th row) in the absence (2nd row) or presence (3rd–4th row) of 100 μM nicotine. The cells were stained for MAP2 (left panel) and cytochrome-C (CytC middle panel) and surface α-Bgt647 (right panel). White squares indicate regions that are magnified. c–e Graphs quantify neuronal survival (c), CytC (d) and surface α-Bgt647 (e) (n = 6). Acute nicotine increases cell survival (p < 10\textsuperscript{-4}) and reduces CytC mobilization (p = 0.0002) during glutamate toxicity in wild-type neurons, but not in NACHO KO neurons (p = 0.9). DFMO pre-treatment further promotes nicotine-mediated cell survival (p < 10\textsuperscript{-4}) and reduces CytC mobilization (p = 0.0002) in wildtype neurons. DFMO significantly enhances surface α-Bgt647 labeling of wild-type (p < 10\textsuperscript{-4}) but not of NACHO KO neurons (p = 0.9). **p < 0.01, ***p < 0.001, n.s = not significant, one-way ANOVA between the groups. Data displayed as mean ± SD. Source data for panel c–e are provided as a Source Data file.
distinct from the pore region that determines polyamine control of rectification.

As α7 and α4β2 are the most abundant nAChRs in human brain and control diverse aspects synaptic signaling and plasticity, polyamine regulation of their assembly has important physiolog-
ical and pathophysiological implications. Polyamine levels in neurons are dynamically regulated over both short-time and long-time scales. Acutely, synaptic transmission increases synthesis of polyamines, which modulate integrative neuronal properties by reducing AMPA receptor currents[51] and provide an excitability buffer by negatively regulating Na+ channels[52]. Long-lasting changes in synaptic transmission associated with patho-
logical processes such as epilepsy dramatically upregulate ODC1[53], which our study predicts would downregulate nAChR assembly. Indeed, α4-containing nAChRs levels are decreased in pirmiform cortex of kindled mice[54].

Polyamines and nAChRs share compelling links to neu-
ropsychiatric disorders. Numerous genetic, genomic, and bio-
chemical studies identify alterations in the polyamine pathway in major affective disorders and suicide[55]. An SNP in the promoter region of SAT1 that reduces expression is associated with suicide in a French-Canadian founder population[55]. Postmortem studies find reduced SAT1 protein and mRNA levels in precen
gyrus and cortical frontal lobe[56]. Also, polyamine levels increase during anxiety episodes, and this polyamine stress response pathway[19] overexpression[60] and nicotine[36] protect animals from kainate-

Methods

Genes and molecular biology and cell culture. The following genes are studied here: (Human forms) CHRNA4 (NM_000744.6), CHRNA2 (NM_000748.2), CHRNA7 (NM_000746.5), CHRNA6 (NM_004198), CHRNA4 (NM_005705), TMEM35A or NACHO (NM_021367.2), Ric-3 (NM_024357.5), Bcl-2 (NM_138578.2), Mcl-1 (NM_021960.4), SAT1 (NM_002970.3), ODC1 (NM_002539); (mouse forms) 5HT-3A (NM_013561), AMD1 (NM_138578.2), Mcl-1 (NM_021960.4), SAT1 (NM_002970.3), ODC1 (NM_002539); (mouse forms) SHT-A (NM_013561), AMD1 (NM_009665).

Chimeric constructs used in this study, with residues in the parenthesis are: a6N7α1 (6β2-369), a6N7α6 (6α4-124/6β2-405), a6β4/6α6 loop (6β2-405

exchanged to α4,311-400), a6β4/6α6 loop (α4,311-400 exchanged to α6β2-405), a6β4/6α6 loop (α6β2-405 exchanged to α6β4-311). In brief, for α6NT/α4 chimera, β4, α6–259 Region and α6–259 was linearized and amplified with PCR. The primers for α6–259 contained complementary overhangs immediately upstream and downstream of amplified region. PCR products were ligated using In-Fusion HD Cloning Plus kit (Takara, 683910) in accordance with manufacturer’s protocol. All other chimeras were generated following similar strategy and were confirmed by sequencing. Site-directed mutagenesis was done with two complementary primer reactions (primer details provided in Supplementary Table 1), and all mutations were confirmed by sequencing. The α7-HA, β2-HA, and HA-4 constructs contained a PSGA linker and HA tag immediately following the C-terminal residue.

Radioligand binding assays. HEK293T cells or cortical neurons were harvested in 50 mM ice-cold TrisHCl buffer (pH 7.4). Cells were homogenized for 30 s using the T-25 Ultra-Turrax homogenizer (Ika) and total protein concentration of the homogenate was determined by the Pierce™ BCA Protein Assay (Thermo Scientific). Cell homogenates were incubated with 10 nM [3H]epibatidine (for nAChR) or with 30 nM [3H]flunitrazepam (for GABA) in 96-well plates for 3 h at room temperature. Non-specific binding was determined by co-incubation of the cell samples with 10 μM unlabeled epibatidine or 100 μM unlabeled flunitrazepam. Assays were terminated by filtration through polyethyleneimine-treated 96-well Unifilter GF/B plates (PerkinElmer). Filter plates were washed with 500 μL TrisHCl buffer and then desiccated at 65 °C for 30 min. MicroScint-0 scintillant

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cocktail (50 µL, PerkinElmer) was added to each well and plates read with a TopCount NXT scintillation counter (PerkinElmer).

**Electrophysiology.** HEK293T cells were seeded (1 million/well) on uncoated six-well plates and transfected with cDNA combinations (total 2 µg/well) using FuGENE®6 transfection reagent (Promega Corporation). EGFP plasmid (10% of total cDNA) identified transfected cells. After 24 h, cells were dissociated using CellStripper® dissociation reagent (Corning) and re-seeded on 12 mm glass coverslips (40,000/well). Electrophysiological recordings were done 48 h after transfection using external solution composed of Hyclone® HEPES-buffered saline (149 mM NaCl, 4 mM KCl, 10 mM HEPES, 2 mM CaCl₂, 1 mM MgCl₂, and 5 mM Glucose at pH 7.4; 300 mmOsm osmolality). Intracellular solution contained (mM): 140 potassium gluconate, 10 HEPES, 4 Mg-ATP, 0.4 Na-GTP, and 0.6 EGTA (pH 7.3). To study most receptors, fast perfusion of compounds was achieved with the Perfusion Fast-Step system (Warner Instruments). To study α7 nACHRs, ultrafast perfusion of compounds was achieved with a piezo-driven perfusion system and theta glass (Siskovo) on to eGFP-expressing cells. The membrane holding potential was ~70 mV. All recordings were performed at room temperature using an Axopatch 200B amplifier (Axon Instruments) and signals were filtered at 2 kHz and digitized at 10 kHz. For α7 nACHRs, signals were filtered at 10 kHz with a digitization rate of 50 kHz. Data acquisition and subsequent analysis were done with pClamp9 software (Axon Instruments).

**Statistics.** Results are represented as mean ± SD unless stated otherwise. All F1P1R assay, immunostaining experiments and radioligand binding assay in HEK293T cells and rat neurons were replicated thrice. Significance analyses between two datasets were performed with nonparametric Mann–Whitney U test, while statistical analyses between three or more datasets used one-way ANOVA (GraphPad Prism, Carlsbad, CA). Significance level α = 0.05 was set.

**Reference**

**References**

1. Role, L. W. & Berg, D. K. Nicotinic receptors in the development and modulation of CNS synapses. *Neuron* 16, 1077–1085 (1996).
2. Gotti, C. & Clementi, F. Neuronal nicotinic receptors: from structure to pathology. *Prog. Neurobiol.* 74, 363–396 (2004).
3. Lindstrom, J. Nicotinic acetylcholine receptors in health and disease. *Mol. Neurobiol.* 15, 193–222 (1997).
4. Hogg, R. C., Raggenbass, M. & Bertrand, D. Nicotinic acetylcholine receptors: in vitro and in vivo characterization. *J. Biol. Chem.* 265, 18788–18793 (1990).
5. Prince, R. J. & Sine, S. M. Epibatidine binds with unique site and state selectivity to muscle nicotinic acetylcholine receptors. *J. Biol. Chem.* 273, 10337–10343 (1998).
6. Yang, X. et al. A public genome-scale lentiviral expression library of human ORFs. *Nat. methods* 6, 659–661 (2011).
7. Kessel, H. S., Patel, R. N., Franzyk, H. & Mellor, I. R. Block of nicotinic acetylcholine receptors by philanthotoxins is strongly dependent on their subunit composition. *Sci. Rep.* 6, 38116 (2016).
8. Bewley, M. et al. Structures of wild-type and mutant human spermide/ spermine N1-acetyltransferase, a potential therapeutic drug target. *Proc. Natl. Acad. Sci. USA* 103, 2063–2068 (2006).
9. Gerner, E. W. & Myskens, F. L. Jr. Polynucleotides: an overview of their structure and function. *Chem. Rev.* 97, 1025–1069 (1997).
10. Morales-Perez, C. L., Noviello, C. M. & Hibbs, R. E. X-ray structure of the human α6β2 nicotinic receptor. *Nature* 538, 411–415 (2016).
11. Stokes, C., Treinin, M. & Papke, R. L. Looking below the surface of nicotinic acetylcholine receptors. *Trends Pharmacol. Sci.* 36, 514–523 (2015).
12. Tchkonov, D. B., Mellor, I. R. & Usherwood, P. N. Modeling noncompetitive antagonism of a nicotinic α7 acetylcholine receptor. *Biophys. J.* 87, 159–170 (2004).
13. Kawai, H. & Berg, D. K. Nicotinic acetylcholine receptors containing α7 subunits on rat cortical neurons do not undergo long-lasting inactivation even when up-regulated by chronic nicotine exposure. *J. Neurochem.* 78, 1306–1313 (2001).
14. Hurst, R. S. et al. A novel positive allosteric modulator of the α7 neuronal nicotinic acetylcholine receptor: in vitro and in vivo characterization. *J. Neurosci.* 25, 4396–4405 (2005).
15. Marabito, L. M. et al. Reduced antinociception in mice lacking neuronal nicotinic receptor subunits. *Nature* 398, 805–810 (1999).
16. Borlongan, C. V. et al. [3H]-nicotine protects against systemic kainic acid-induced excitotoxic effects. *Exp. Neurol.* 136, 261–265 (1995).
17. Akaike, A., Tamura, Y., Yokota, T., Shimosaka, S. & Kimura, J. Nicotine-induced protection of cultured cortical neurons against N-methyl-D-aspartate receptor-mediated glutamate cytotoxicity. *Brain Res.* 664, 181–187 (1994).
18. Martín, P., Mauz, M., Desagher, S., Glowinski, J. & Preumont, J. Nicotine protects cultured striatal neurons against N-methyl-D-aspartate receptor-mediated neurotoxicity. *Neuroreport* 9, 1977–1980 (1994).
19. Dajas-Bailador, F. A., Lima, P. A. & Wonnacott, S. The α7 nicotinic acetylcholine receptor subtype mediates nicotine protection against NMDA excitotoxicity in primary hippocampal cultures through a Ca2⁺-dependent mechanism. *Neuropharmacology* 39, 2799–2807 (2000).
20. Hejmadi, M. V., Dajas-Bailador, F., Barns, S. M., Jones, B. & Wonnacott, S. Neuroprotection by nicotine against hyposia-induced apoptosis in cortical cultures involves activation of multiple nicotinic acetylcholine receptor subtypes. *Mol. Cell. Neurosci.* 24, 779–786 (2003).
21. Kharbanda, S. et al. Role for Bcl-xL as an inhibitor of cytosolic cytochrome C accumulation in DNA damage-induced apoptosis. *Proc. Natl. Acad. Sci. USA* 94, 6939–6942 (1997).
42. Orrenius, S. & Zhivotovsky, B. Cardiolipin oxidation sets cytochrome c free. *Nat. Chem. Biol.* **1**, 188–189 (2005).
43. Green, W. N. & Millar, N. S. Ion-channel assembly. *Trends Neurosci.* **18**, 280–287 (1995).
44. Schwartz, R. D. & Kellar, K. J. Nicotinic cholinergic receptor binding sites in the brain: regulation in vivo. *Science* **220**, 214–216 (1983).
45. Lester, H. A. et al. Nicotine is a selective pharmacological chaperone of acetylcholine receptor number and stoichiometry. *Implic. Drug Discov. AAPS J.* **11**, 167–177 (2009).
46. Halevi, S. et al. The C. elegans ric gene is required for maturation of nicotinic acetylcholine receptors. *EMBO J.* **21**, 1012–1020 (2002).
47. Tran, P. V., Georgieff, M. K. & Engeland, W. C. Sodium depletion increases sympathetic neurite outgrowth and expression of a novel TMEM35 gene-derived protein (TUF1) in the rat adrenal zona glomerulosa. *Endocrinology* **151**, 4852–4860 (2010).
48. Youle, R. J. & Strasser, A. The BCL-2 protein family: opposing activities that mediate cell death. *Nat. Rev. Mol. Cell Biol.* **9**, 47–59 (2008).
49. Tiveron, M. C. et al. LAMP5 fine-tunes GABAergic synaptic transmission in defined circuits of the mouse brain. *PLoS ONE* **11**, e0157052 (2016).
50. Stromgaard, K. et al. Solid-phase synthesis and biological evaluation of a derivatized protein (TUF1) in the rat adrenal zona glomerulosa. *Endocrinology* **151**, 4852–4860 (2010).
51. Aizenman, C. D., Munoz-Elias, G. & Cline, H. T. Visually driven modulation of glutamatergic synaptic transmission is mediated by the regulation of intracellular polyamines. *Neuron* **34**, 623–634 (2002).
52. Fleidervish, I. A., Libman, L., Katz, E. & Gutnick, M. J. Endogenous polyamines regulate cortical neuronal excitability by blocking voltage-gated Na+ channels. *Proc. Natl Acad. Sci. USA* **105**, 18994–18999 (2008).
53. Herberg, L. J., Rose, I. C., de Belleruche, J. S. & Mintz, M. Ornithine decarboxylase induction and polyamine synthesis in the kindling of seizures: the effect of alpha-difluoromethylornithine. *Epilepsy Res.* **11**, 3–7 (1992).
54. Takechi, K., Suemaru, K., Kiyoi, T., Tanaka, A. & Araki, H. The alpha2beta2 nicotinic acetylcholine receptor modulates autism-like behavioral and motor abnormalities in pentyleneetetrazol-kindled mice. *Eur. J. Pharmacol.* **775**, 57–66 (2016).
55. Limon, A., Mamdani, F., Hjelm, B. E., Vawter, M. P. & Sequeira, A. Targets of polyamine dysregulation in major depression and suicide: Activity-dependent feedback, excitability, and neurotransmission. *Neurosci. Biobehav. Rev.* **66**, 80–91 (2016).
56. Sequeira, A. et al. Implication of SSAT by gene expression and genetic variation in suicide and major depression. *Arch. Gen. Psychiatry* **63**, 35–48 (2006).
57. Gandelman, J. A., Newhouse, P. & Taylor, W. D. Nicotine and networks: Potential for enhancement of mood and cognition in late-life depression. *Neurosci. Biobehav. Rev.* **84**, 289–298 (2018).
58. Peron, A. et al. Snyder-Robinson syndrome: a novel nonsense mutation in spermine synthase and expansion of the phenotype. *Am. J. Med. Genet. A* **161A**, 2316–2320 (2013).
59. Ghasemi, M. & Hadipour-Nikitarash, A. Pathologic role of neuronal nicotinic acetylcholine receptors in epileptic disorders: implication for pharmacological interventions. *Rev. Neurosci.* **26**, 199–223 (2015).
60. Kaasinen, K., Koistinaho, J., Alhonen, L. & Janne, J. Overexpression of spermidine/spermine N-acetyltransferase in transgenic mice protects the animals from kainate-induced toxicity. *Eur. J. Neurosci.* **12**, 540–548 (2000).
61. Nordberg, A. Nicotinic receptor abnormalities of Alzheimer’s disease: therapeutic implications. *Biol. Psychiatry* **49**, 200–210 (2001).

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**Author contributions**

M.D. and M.L. performed cDNA library screening. J.A.M. conducted electrophysiology experiments. M.D. and H.Y. performed immunocytochemistry assays. M.D. conducted radioligand binding assays. D.K. and S.G. provided chimeric cDNA constructs. M.D. and D.S.B. wrote the manuscript. All authors contributed to the discussion and editing of the paper. D.S.B. supervised the project.

**Competing interests**

All contributing authors are full-time employees in Johnson and Johnson.

**Additional information**

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