

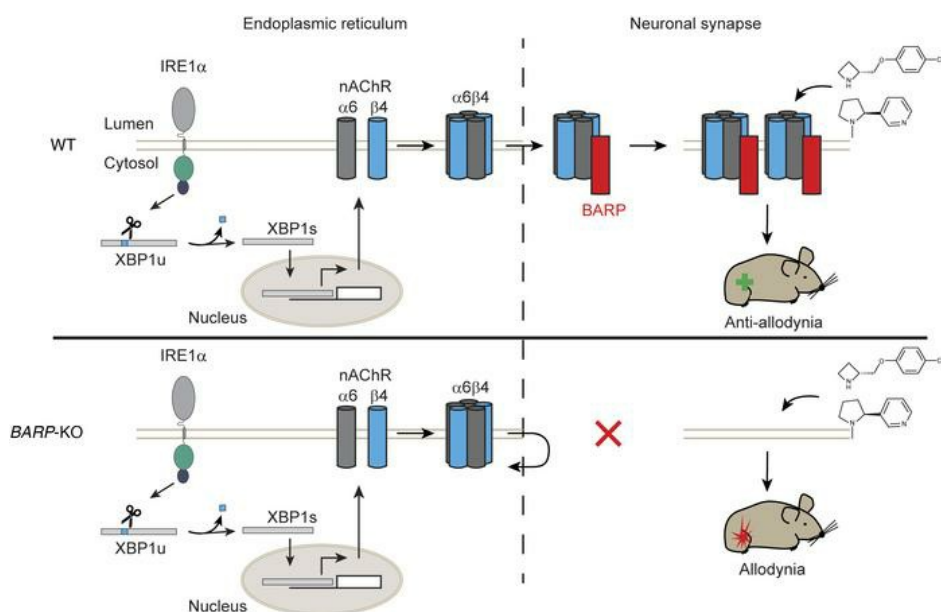
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J Clin Invest. 2020;130(11):6158-6170. <https://doi.org/10.1172/JCI140311>.

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Functional $\alpha 6\beta 4$ acetylcholine receptor expression enables pharmacological testing of nicotinic agonists with analgesic properties

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The $\alpha 6\beta 4$ nicotinic acetylcholine receptor (nAChR) is enriched in dorsal root ganglia neurons and is an attractive non-opioid therapeutic target for pain. However, difficulty expressing human $\alpha 6\beta 4$ receptors in recombinant systems has precluded drug discovery. Here, genome-wide screening identified accessory proteins that enable reconstitution of human $\alpha 6\beta 4$ nAChRs. BARP, an auxiliary subunit of voltage-dependent calcium channels, promoted $\alpha 6\beta 4$ surface expression while IRE1 α , an unfolded protein response sensor, enhanced $\alpha 6\beta 4$ receptor assembly. Effects on $\alpha 6\beta 4$ involve BARP's N-terminal region and IRE1 α 's splicing of *XBP1* mRNA. Furthermore, clinical efficacy of nicotinic agents in relieving neuropathic pain best correlated with their activity on $\alpha 6\beta 4$. Finally, BARP-knockout, but not NACHO-knockout mice lacked nicotine-induced antiallodynia, highlighting the functional importance of $\alpha 6\beta 4$ in pain. These results identify roles for IRE1 α and BARP in neurotransmitter receptor assembly and unlock drug discovery for the previously elusive $\alpha 6\beta 4$ receptor.

Introduction

Chronic pain is a serious unmet public health issue in need of non-opioid analgesics. Epibatidine has long been recognized as a potent analgesic through actions on nicotinic acetylcholine receptors (nAChRs), yet negative side effects preclude its clinical use. nAChRs transduce synaptic signals and mediate diverse physiological actions of ACh in the brain and periphery (1–6). nAChRs comprise a large family of homo- and heteropentamers assembled from 10 α ($\alpha 1$ – $\alpha 10$, $\alpha 8$ only found in chick) and 4 β ($\beta 1$ – $\beta 4$) subunits, each with discrete expression patterns. The $\alpha 4\beta 2$ receptor is thought to be the subtype mediating nAChR-induced analgesia; however, despite significant investment there are no approved nicotinic agents for analgesia, suggesting that a different subtype may be involved (7).

Recently, animal and human studies have implicated dorsal root ganglia-enriched (DRG-enriched) $\alpha 6\beta 4$ receptors in chronic pain states. Phenotypic analysis of inbred mice found that $\alpha 6$ levels in DRGs vary inversely with injury-induced allodynia, and loss-of-function variants in the $\alpha 6$ promoter region correlate with chronic postoperative pain (8). Furthermore, $\alpha 6$ -knockout ($\alpha 6$ -KO) mice suffer increased allodynia, whereas $\alpha 6$ gain-of-function models show attenuated pain levels (8). Collectively, $\alpha 6$ -containing recep-

tors in the DRG represent an attractive non-opioid approach for the treatment of pain.

Although nAChRs are appealing therapeutic targets, drug discovery and basic science efforts are hampered because most subtypes are not expressed as functional proteins in cell lines. Especially problematic are human $\alpha 6$ -containing receptors, which do not form functional channels in any recombinant expression system (9, 10). We previously identified NACHO, an endoplasmic reticulum (ER) protein, which serves as a client-specific chaperone that promotes function of many nAChRs and is essential for assembly of $\alpha 7$ homopentamers (11, 12). NACHO also mediates assembly of dopamine neuron-enriched $\alpha 6\beta 2\beta 3$ receptors, which also require additional protein accessories including β -anchoring and -regulatory protein (BARP), SULT2B1, and LAMP5 (13). Whether DRG-enriched $\alpha 6\beta 4$ shows similar dependency on NACHO and other accessory components for functional expression is unknown.

To assess this, we performed genome-wide cDNA screening for proteins that enable robust functional expression of human $\alpha 6\beta 4$ receptors. Our screen identified BARP and inositol-requiring enzyme-1 α (IRE1 α) as $\alpha 6\beta 4$ enhancers. BARP is a single-pass transmembrane protein that was first identified in association with voltage-gated calcium channel β subunits (14) and was later found as an accessory component of $\alpha 6\beta 2\beta 3$ nAChRs (13), while IRE1 α is a constituent of the unfolded protein response (UPR) (15). We found that the efficacy of nicotinic agonists to treat neuropathic pain best correlates with their activity on $\alpha 6\beta 4$ receptors. Furthermore, BARP-KO mice, which show diminished $\alpha 6\beta 4$ surface expression in DRG, have blunted antiallodynic responses to nicotine and ABT-594, a clinically effective nicotinic agent, suggesting that $\alpha 6\beta 4$ activity is critical for neuropathic pain relief.

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Authorship note: SG and WAE contributed equally to this work.

Conflict of interest: The authors are all full-time employees of Janssen Pharmaceutical Companies of Johnson and Johnson.

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Submitted: May 15, 2020; **Accepted:** August 6, 2020; **Published:** October 19, 2020.

Reference information: *J Clin Invest.* 2020;130(11):6158–6170.

<https://doi.org/10.1172/JCI140311>.

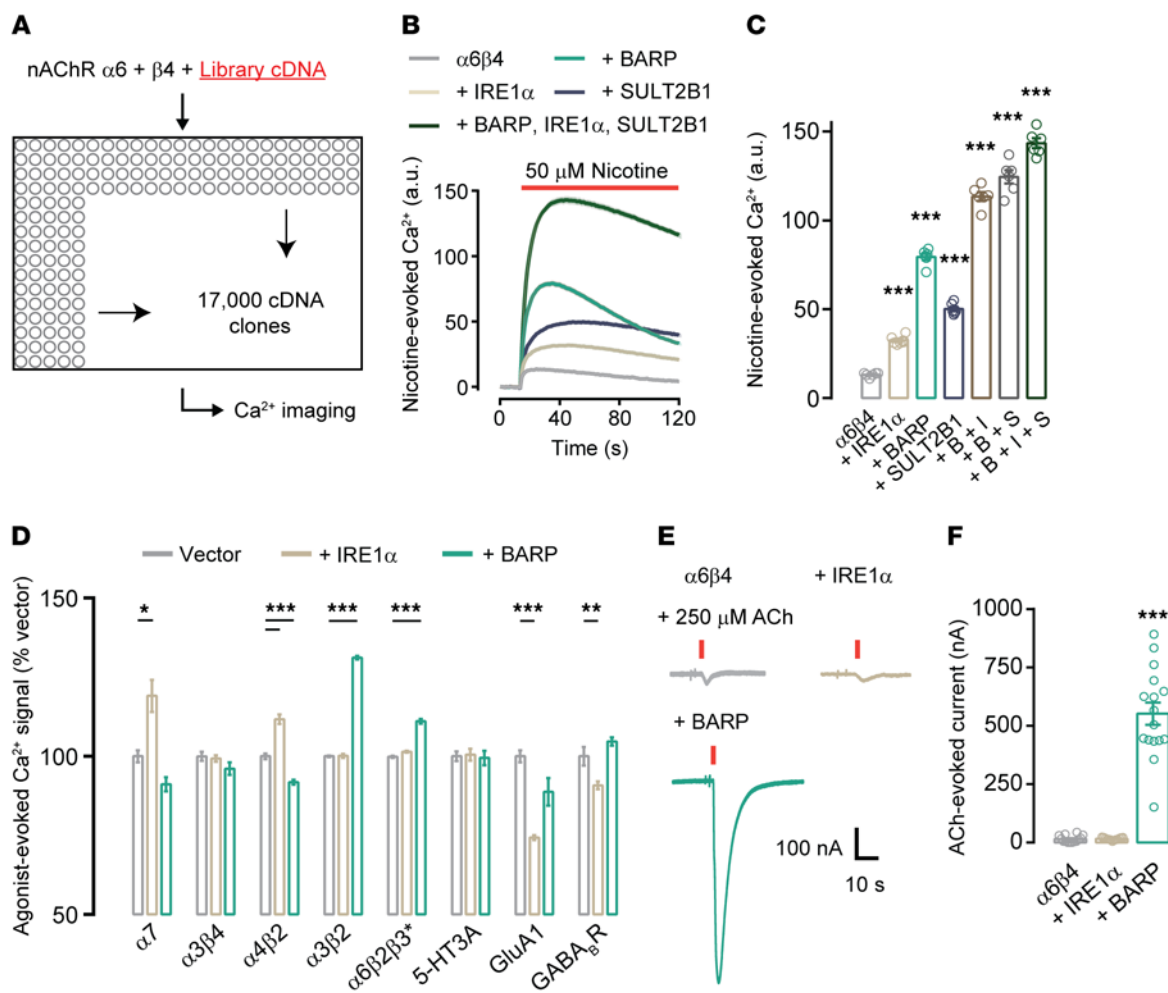


Figure 1. Genomic screening identifies chaperones for functional $\alpha 6\beta 4$ reconstitution. (A) Schematic of genomic screening. HEK293T cells were cotransfected with cDNAs encoding $\alpha 6$ and $\beta 4$ and individual plasmids from a genome-wide expression library. Ca^{2+} signals were measured in a fluorescence imaging plate reader (FLIPR). (B) Exemplary FLIPR traces showing nicotine-induced Ca^{2+} responses for indicated transfections. (C) Quantification of FLIPR signals. Activity of $\alpha 6\beta 4$ is enhanced by BARP, IRE1 α , and SULT2B1. $n = 6$ for each group. B, BARP; I, IRE1 α ; S, SULT2B1. (D) Quantification of FLIPR Ca^{2+} response from other nAChRs and other ion channels (5-HT3A and GluA1), and G protein-coupled receptor (GABA $_B$ R) cotransfected with either BARP or IRE1 α . nAChRs were stimulated with 50 μM nicotine, 5-HT3A with 100 μM serotonin, GluA1 with 100 μM glutamate plus 100 μM cyclothiazide, and GABA $_B$ R with 100 μM GABA. $n = 6$ each. Responses normalized to that of vector-transfected cells (100%). (E) Current traces from *Xenopus* oocytes injected with indicated cRNAs and stimulated with a 2-second pulse of 250 μM ACh. (F) Quantification of current amplitude (absolute value) responses in E: $n = 14, 13,$ and 16 oocytes for $\alpha 6\beta 4$, $\alpha 6\beta 4$ plus IRE1 α , and $\alpha 6\beta 4$ plus BARP, respectively. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by 1-way ANOVA with Dunnett's post hoc correction for multiple comparisons to vector (C, D, and F). C: $F_{6,35} = 511.4$. D: $\alpha 7$, $F_{2,15} = 18.08$; $\alpha 4\beta 2$, $F_{2,15} = 86.27$; $\alpha 3\beta 2$, $F_{2,15} = 1171$; $\alpha 6\beta 2\beta 3^*$, $F_{2,15} = 143.9$; GluA1, $F_{2,15} = 21.46$; GABA $_B$ R, $F_{2,15} = 12.85$. F: $F_{2,40} = 106.7$. Graphs are the mean \pm SEM and depict 1 experiment that was replicated with similar results.

Results

Genome-wide screening identifies BARP and IRE1 α as $\alpha 6\beta 4$ accessory proteins. To identify functional regulators of $\alpha 6\beta 4$, we cotransfected HEK293T cells with cDNAs encoding $\alpha 6$ and $\beta 4$ along with individual clones from a library of approximately 17,000 plasmids that express nearly all predicted human proteins. Using a fluorescence imaging plate reader (FLIPR) to measure nicotine-evoked Ca^{2+} responses, we identified 2 clones that augmented responses from $\alpha 6\beta 4$ (Figure 1, A-C). The clone that most robustly enhanced the FLIPR signal was BARP, which we previously identified as an auxiliary subunit of $\alpha 6\beta 2\beta 3$ receptors (11). In those previous studies, we found that SULT2B1, LAMP5, and NACHO are also accessories for $\alpha 6\beta 2\beta 3$ (13). Here, we found that SULT2B1, but not NACHO or LAMP5, also augmented nicotine-evoked responses

from $\alpha 6\beta 4$ (Figure 1, B and C; Supplemental Figure 1A; supplemental material available online with this article; <https://doi.org/10.1172/JCI140311DS1>).

The second-best enhancer of $\alpha 6\beta 4$ activity identified from the screen was IRE1 α of the UPR (15) (Figures 1, B and C). By contrast, 2 other UPR components, PERK and ATF6 (16), did not affect $\alpha 6\beta 4$ FLIPR responses (Supplemental Figure 1A). We confirmed that these nicotine-evoked Ca^{2+} signals were mediated by $\alpha 6$, as α -conotoxin MII, an $\alpha 6$ -selective antagonist (17), completely blocked responses from $\alpha 6\beta 4$, but not those from $\alpha 4\beta 2$ or $\alpha 3\beta 4$ (Supplemental Figure 1, B and C).

We next asked whether BARP and IRE1 α could also enhance responses from other nAChR subtypes. BARP promoted function of $\alpha 6\beta 2\beta 3^*$ (where * indicates the use of the $\beta 3$ mutant sub-

unit [V273S]) and $\alpha 3\beta 2$ but had no effects on the other nAChRs tested (Figure 1D). Interestingly, IRE1 α enhanced $\alpha 7$, $\alpha 4\beta 2$, and $\alpha 6\beta 2\beta 3^*$ to modest degrees (Figure 1D). Neither BARP nor IRE1 α increased responses from other ligand-gated ion channels including 5-HT $_3$ A or GluA1, or the class C GPCR GABA $_B$ R (Figure 1D).

We next explored the effects of BARP and IRE1 α using 2-electrode voltage clamp in *Xenopus* oocytes. Previous studies have been unable to produce large, reliable human $\alpha 6\beta 4$ currents in oocytes and instead have relied on chimeric or rodent receptors (9, 17–19). Similarly, we detected minimal to no ACh-evoked currents following oocyte injection with $\alpha 6$ and $\beta 4$ cRNAs (14.91 ± 4.016 nA). Strikingly, coinjection with BARP cRNA consistently enabled large ACh-evoked currents (551.9 ± 47.39 nA; Figure 1, E and F). By contrast, IRE1 α cRNA had no effect (15.78 ± 1.549 nA; Figure 1, E and F). In whole-cell patch clamp electrophysiology on HEK293T cells transfected with $\alpha 6\beta 4$ alone, small and inconsistent ACh-evoked (1 mM) currents were evoked (107.27 ± 44.96 pA). However, similarly to oocytes, HEK293T cells coexpressing BARP with $\alpha 6\beta 4$ produced larger, reliable currents (566.06 ± 226.02 pA), whereas those coexpressing IRE1 α did not (20.30 ± 10.43 pA; Supplemental Figure 1, D and E). In HEK293T cells, BARP enhanced the peak amplitude of ACh-evoked currents from $\alpha 6\beta 4$ but did not alter channel gating properties, as the steady-state-to-peak current ratio was the same in the absence or presence of BARP (Supplemental Figure 1F).

BARP and IRE1 α enable $\alpha 6\beta 4$ functional expression through different mechanisms. We next explored cellular mechanisms underlying the effects of BARP and IRE1 α on $\alpha 6\beta 4$. To assess $\alpha 6\beta 4$ surface expression, we used a $\beta 4$ subunit containing a C-terminal, extracellular HA tag that does not interfere with receptor function (12). Cotransfection with BARP in HEK293T cells robustly enhanced $\alpha 6\beta 4$ surface expression, whereas IRE1 α did not (Figure 2, A and B). This BARP effect was specific, as BARP did not enhance $\alpha 3\beta 4$ surface expression (Figure 2, A and B). We additionally tagged $\alpha 6$ with an extracellular V5 epitope and again found that BARP, but not IRE1 α , enhanced surface expression (Supplemental Figure 2, A and B). In alignment with functional data (Supplemental Figure 1A), we found that the $\alpha 6\beta 2\beta 3$ accessory protein SULT2B1 enhanced $\alpha 6\beta 4$ surface expression, while NACHO and LAMP5 did not (Supplemental Figure 2, A and B).

BARP is a single-pass transmembrane protein with cytosolic motifs that bind voltage-gated calcium-channel (VGCC) β subunits and negatively influences channel function (14). To determine whether BARP's effects on $\alpha 6\beta 4$ and VGCCs share a common essential domain, we created a series of BARP mutants. BARP202, a truncated version lacking its VGCC β subunit-binding domain, retained $\alpha 6\beta 4$ functionality for both FLIPR and surface expression (Supplemental Figure 3). Further truncation of BARP's intracellular region (BARP101) or deletion of BARP's transmembrane region (BARP Δ TM) disrupted effects on $\alpha 6\beta 4$ (Supplemental Figure 3). These experiments indicate that distinct domains are required for BARP's effects on $\alpha 6\beta 4$ and VGCCs.

As IRE1 α did not enhance $\alpha 6\beta 4$ surface expression, we assessed effects on receptor assembly. Orthosteric ligands such as [3 H]epibatidine interact at the interface of assembled nAChR subunits, and their binding quantifies receptor oligomerization (20). We

found that IRE1 α , but not BARP, increased [3 H]epibatidine binding to cotransfected $\alpha 6\beta 4$ (Figure 2C); however, neither BARP nor IRE1 α affected total $\alpha 6$ protein levels (Supplemental Figure 2C).

To assess protein interactions, we immunoprecipitated solubilized membranes from HEK293T cells cotransfected with $\alpha 6$ and $\beta 4$ along with accessory proteins. As expected, immunoprecipitation of V5-tagged $\alpha 6$ revealed association with $\beta 4$ (Figure 2D). We also observed an interaction with BARP and $\alpha 6\beta 4$, indicating that BARP directly binds to $\alpha 6\beta 4$. However, we observed no interaction with IRE1 α (Figure 2D). We confirmed the specificity of the immunoprecipitation, as BARP was absent from immunoprecipitation when cotransfected with only $\beta 4$ (Figure 2D, second lane). Taken together, our results demonstrate 2 separate roles for BARP and IRE1 α that work to augment $\alpha 6\beta 4$: IRE1 α enhances receptor assembly, while BARP promotes trafficking to the membrane by directly binding to $\alpha 6\beta 4$.

BARP acts on the transmembrane and cytosolic regions of $\alpha 6$. We next investigated which regions of $\alpha 6$ are required for BARP effects. Because BARP regulates $\alpha 6\beta 4$ but not $\alpha 4\beta 4$, we constructed $\alpha 6/\alpha 4$ receptor chimeras for cotransfection with $\beta 4$ (Figure 3A). BARP was inactive on a chimera containing the extracellular N-terminal region of $\alpha 6$ fused to the $\alpha 4$ transmembrane and intracellular regions ($\alpha 6$ N/4; Figure 3, B–D). Conversely, BARP robustly enhanced surface expression and FLIPR responses of the $\alpha 4$ N/6 chimera (Figure 3, B–D). We further confirmed functionality in oocytes and again found that BARP enhanced ACh-evoked currents from $\alpha 4$ N/6, but not from $\alpha 6$ N/4 chimeras (Supplemental Figure 4, A and B). Taken together, these results show that the N-terminal extracellular domain of $\alpha 6$ is expendable for BARP-mediated effects, and that BARP directly interacts with $\alpha 6\beta 4$ via its transmembrane regions.

RNase activity of IRE1 α is necessary and sufficient for $\alpha 6\beta 4$ assembly. Because IRE1 α does not physically associate with $\alpha 6\beta 4$, we wondered whether downstream signaling might be involved. Accumulation of unfolded proteins in the ER causes IRE1 α to dimerize and *trans*-autophosphorylate its kinase domain, which thereby activates its RNase domain to splice the transcription factor XBP1 to a unique protein, XBP1s (16). Accordingly, we generated IRE1 α mutants (Figure 4A) that abolish kinase (K599A, KINmut) or RNase (K907A, RNAmut) activities (21), as reflected by reduced XBP1s production (Figure 4B). Next, we cotransfected each mutant with BARP and measured FLIPR responses. Compared with WT IRE1 α , both mutants reduced upregulation of nicotine-evoked Ca $^{2+}$ responses from $\alpha 6\beta 4$ (Figure 4C). Additionally, STF-083010, an IRE1 α RNase inhibitor (22), blocked IRE1 α -mediated enhancement of $\alpha 6\beta 4$ function but had no effect on $\alpha 3\beta 4$ (Supplemental Figure 5, B–D). In line with IRE1 α 's role in $\alpha 6\beta 4$ assembly, IRE1 α KINmut and RNAmut reduced IRE1 α -mediated enhancement of [3 H]epibatidine binding (Figure 4D).

To isolate the contribution of IRE1 α 's RNase activity, we used a strategy developed by others (23) that combines an IRE1 α kinase-dead mutant (I642G, CONDmut) with a drug (1NM-PP1) that binds to the mutated kinase domain and initiates IRE1 α RNase activity independently of kinase activity (Figure 4A). Indeed, CONDmut showed reduced XBP1s, which was rescued with 1NM-PP1 (Figure 4B). In cells cotransfected with $\alpha 6\beta 4$ and BARP, CONDmut IRE1 α reduced the nicotine-evoked FLIPR

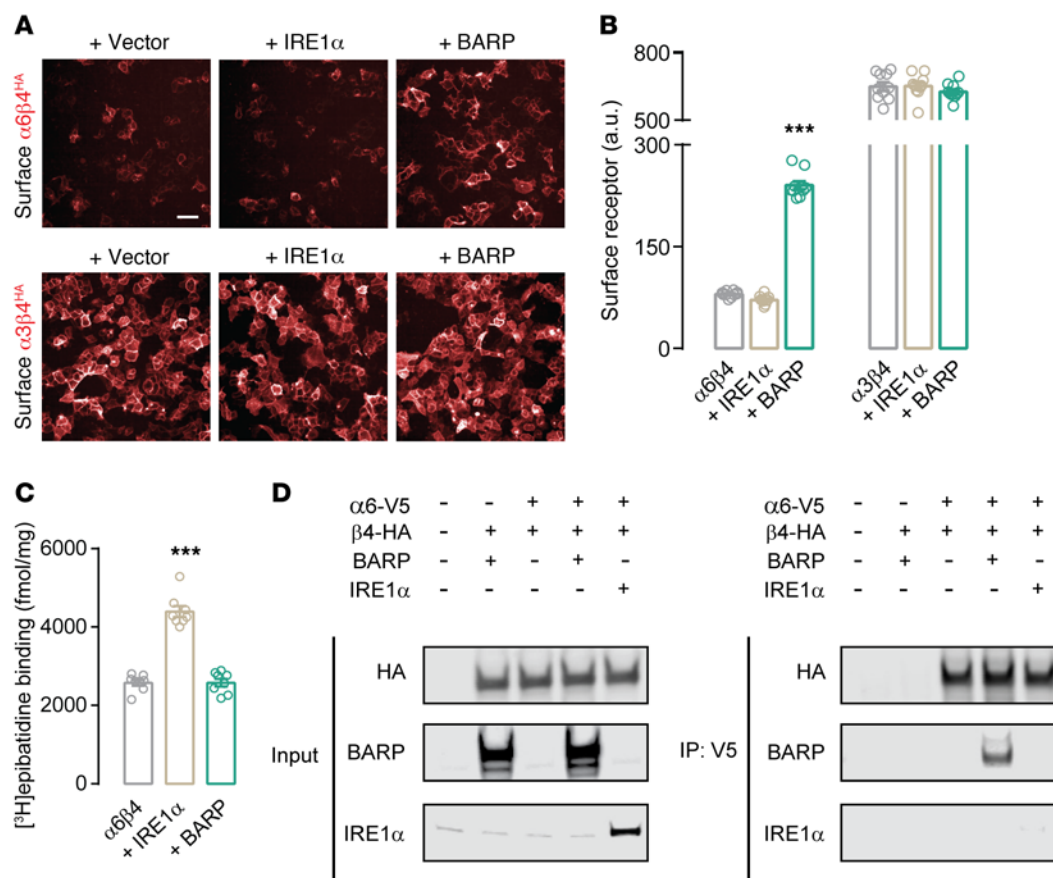


Figure 2. BARP and IRE1 α promote α 6 β 4 function through distinct mechanisms. (A) Confocal images of α 6 β 4 and α 3 β 4 surface staining in transfected HEK293T cells. The β 4 subunit C-terminus contained an HA tag, which was visualized with an anti-HA antibody. (B) Quantification shows that BARP, but not IRE1 α , enhances α 6 β 4 surface staining. Neither has effects on α 3 β 4. $n = 10$ for each group. Scale bar: 50 μ m. (C) Quantification of [3 H]epibatidine binding to HEK293T lysates transfected as indicated. $n = 8$ for each condition. (D) Immunoprecipitation with anti-V5 of solubilized HEK293T cells transfected as indicated. Immunoblotting shows that V5-tagged α 6 associates with β 4 and BARP, but not with IRE1 α . *** $P < 0.001$ by 1-way ANOVA with Dunnett's post hoc correction for multiple comparisons to vector (B and C). B: $F_{3,36} = 515.9$. C: $F_{3,28} = 106.7$. Graphs are the mean \pm SEM and depict 1 experiment that was replicated with similar results.

signal, which was restored with 1NM-PP1 (Figure 4E). Similarly, IRE1 α CONDmut reduced [3 H]epibatidine binding to HEK293T cells transfected with α 6 β 4 and this binding was also restored with 1NM-PP1 (Figure 4F). We next asked whether XBP1s itself can augment α 6 β 4 (Supplemental Figure 5A). Indeed, transfection of XBP1s enhanced α 6 β 4 assembly and function (Figure 4, G and H). Collectively, these experiments suggest that IRE1 α RNase activity targeting *XBP1* is critical for α 6 β 4 assembly.

Endogenous IRE1 α enhances α 6 β 4 assembly. To assess whether activation of endogenous IRE1 α promotes α 6 β 4 assembly, we used tunicamycin (Tm), which inhibits N-linked glycosylation, and thereby induces ER stress and stimulates the UPR (24, 25). Previous reports showed that Tm-induced IRE1 α activation peaks 4–8 hours after treatment and reduces back to baseline by 24 hours (25). Similarly, we found that 100 ng/mL Tm treatment for 4, but not 12 or 24 hours stimulated IRE1 α activity to increase XBP1s (Figure 5A). Furthermore, 100 ng/mL Tm treatment for 4, but not 12 or 24 hours enhanced α 6 β 4 FLIPR responses (Figure 5, B and C) and [3 H]epibatidine binding (Figure 5D) in α 6 β 4-plus-BARP cotransfections.

To explore effects of IRE1 α activation on nAChRs in neurons, we first transduced cerebrocortical cultures with lentivi-

ruses expressing α 6 and β 4 subunits. Similarly to HEK293T cells, 4-hour, but not 24-hour, Tm treatment enhanced [3 H]epibatidine binding (Figure 5E) and increased nicotine-evoked Ca^{2+} influx through α 6 β 4 in neurons (Figure 5F). Furthermore, the IRE1 α RNase inhibitor STF-083010 blocked the Tm-induced increase in [3 H]epibatidine binding (Figure 5E). Also, Tm significantly enhanced nicotine-induced Ca^{2+} responses in untransduced cortical neurons (Figure 5G), which express endogenous α 4 β 2 receptors that are also regulated by IRE1 α (Figure 1D).

To confirm that Tm effects on α 6 β 4 assembly specifically involve IRE1 α activation, we used CRISPR/Cas9 to disrupt IRE1 α in HEK293T cells. Immunoblotting demonstrated reduced or absent IRE1 α in IRE1 α -heterozygous (IRE1 α -HET) and IRE1 α -KO cell lines, respectively (Figure 5H). We also observed the expected reductions in Tm-induced XBP1s production in the IRE1 α -HET and IRE1 α -KO lines (Figure 5H).

Interestingly, IRE1 α -KO cells showed reduced nicotine-evoked α 6 β 4-mediated FLIPR responses (Figure 5I). By contrast, α 3 β 4 and 5HT3A responses increased in IRE1 α -KO cells (Supplemental Figure 6). In line with FLIPR data, IRE1 α -KO cells also exhibited reduced [3 H]epibatidine binding (Figure 5J). As expected, 4-hour

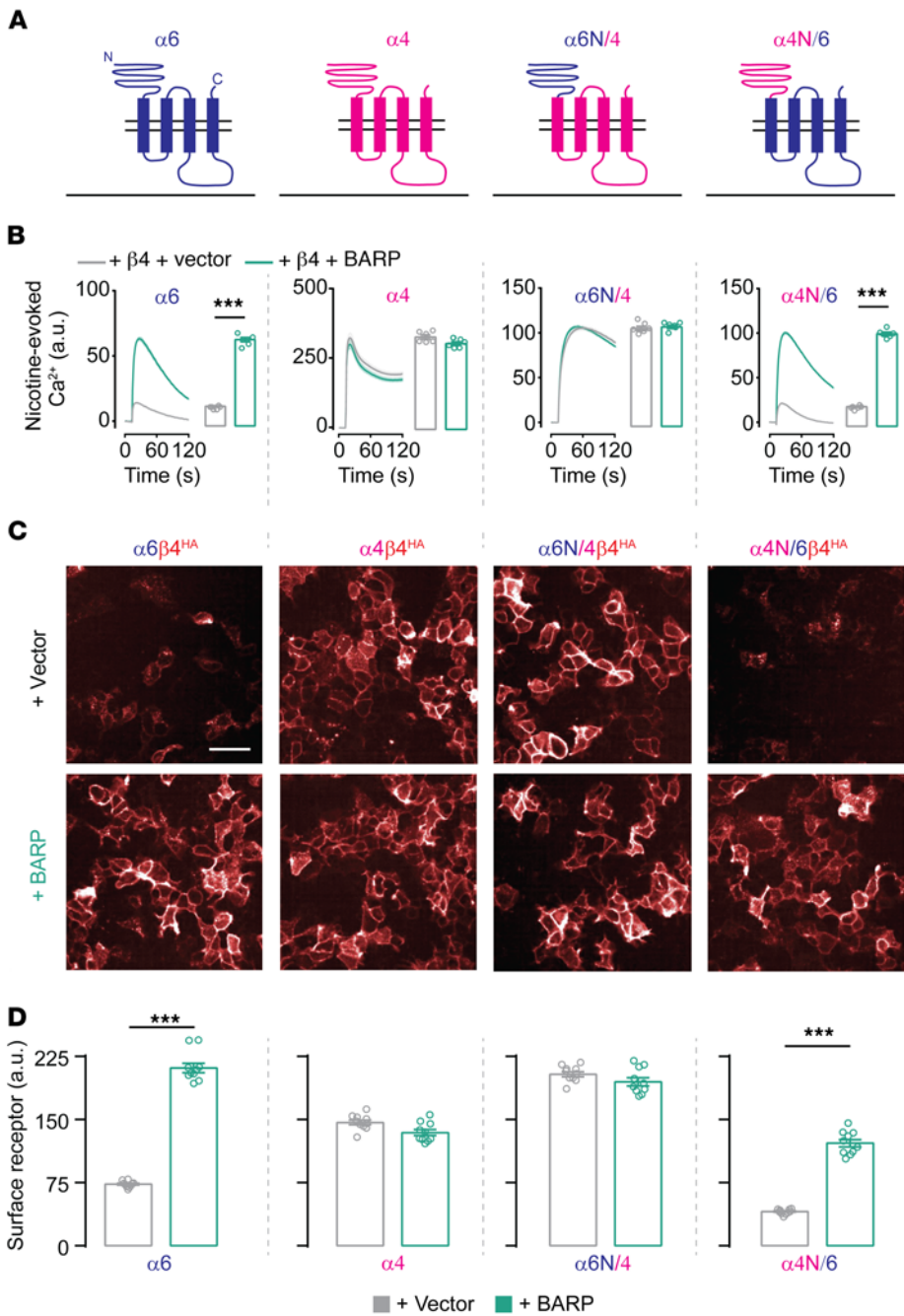


Figure 3. Transmembrane, but not N-terminal region, of α6 is critical for BARP effect. (A) Schematics of α6/α4 chimeras. (B) FLIPR traces and quantification of nicotine-evoked (50 μM) Ca²⁺ responses. *n* = 6 for each group. BARP enhances FLIPR responses only in α6- and α4N/6-containing receptors cotransfected with β4. (C) Representative confocal images for β4^{HA} surface staining in cotransfected HEK293T cells with (bottom) and without (top) BARP. Scale bar: 50 μm. (D) Quantification of surface receptor intensity in C. *n* = 10 for each group. All chimeric α6/α4 nAChRs were cotransfected with β4 into HEK293T cells. ****P* < 0.0001 by unpaired *t* test (B and D). B: α6β4, *t* = 29.95; α4N/6, *t* = 40.58. D: α6β4, *t* = 24.04; α4N/6, *t* = 18.19. Graphs are the mean ± SEM and depict 1 experiment that was replicated with similar results.

Tm application increased [³H]epibatidine binding to levels comparable to transfected IRE1α in WT cells (Figure 5K). In contrast, Tm failed to increase α6β4 [³H]epibatidine binding in IRE1α-KO cells but could be rescued when transfected with exogenous IRE1α (Figure 5K). These data demonstrate that endogenous IRE1α activity supports α6β4 assembly and function.

tested behaved as full agonists at α3β4. Together, these compounds' activities on α6β4 may provide an explanation for the clinical failure of ABT-894 in diabetic neuropathic pain.

To explore α6β4's role in neuropathic pain in vivo, we evaluated BARP-KO animals in the spared nerve injury (SNI) model of neuropathic allodynia (31). No difference in mechanical allodynia

BARP regulates sensory neuron α6β4 and nicotine-induced antiallodynia. α6 and β4 Subunit mRNAs are selectively coexpressed in DRG (Supplemental Figure 7A) (26). In addition, α6β4-mediated currents have been recorded in DRG neurons and α6β4 receptors there modulate pain responses (8, 27). Here, we detected a BARP-immunoreactive band in DRG and cerebral cortical protein extracts that comigrated with BARP from transfected HEK293T cells but was absent from BARP-KO tissues (Figure 6A). Because there are no suitable antibodies to detect endogenous α6 or β4 (28), we transduced isolated DRG neurons with V5-tagged α6 and β4 from WT and BARP-KO mice. Compared with WT neurons, BARP-KO neurons had reduced α6β4 surface expression but equivalent total α6 (Figure 6, B and C), showing that BARP promotes α6β4 surface expression in neurons. In contrast, BARP-KO mice showed no difference from WT in α4β2 or endogenous α7 surface expression (Supplemental Figure 7, B-E).

The nicotinic agonist ABT-594, which is closely related to epibatidine, effectively treated diabetic neuropathic pain in clinical trials, but its development was discontinued due to side effects (29). A follow-up compound, ABT-894, developed to circumvent the nicotinic side effects, was not efficacious (30). The lack of efficacy for ABT-894 was hypothesized to reflect its increased specificity for α4β2 receptor, suggesting that other nAChRs underlie ABT-594's efficacy in neuropathic pain (30).

We wondered whether differential effects of ABT-894 and ABT-594 on α6β4 receptors could explain their contrasting clinical efficacies. We found that ABT-594, nicotine, and epibatidine — all of which have analgesic effects — exhibited similar agonistic efficacies on α4β2 and α6β4 receptors (Figure 6, D and E; Supplemental Tables 1 and 2). In contrast, ABT-894 was only weakly efficacious at α6β4 as compared with α4β2 (Figure 6, D and E). All compounds

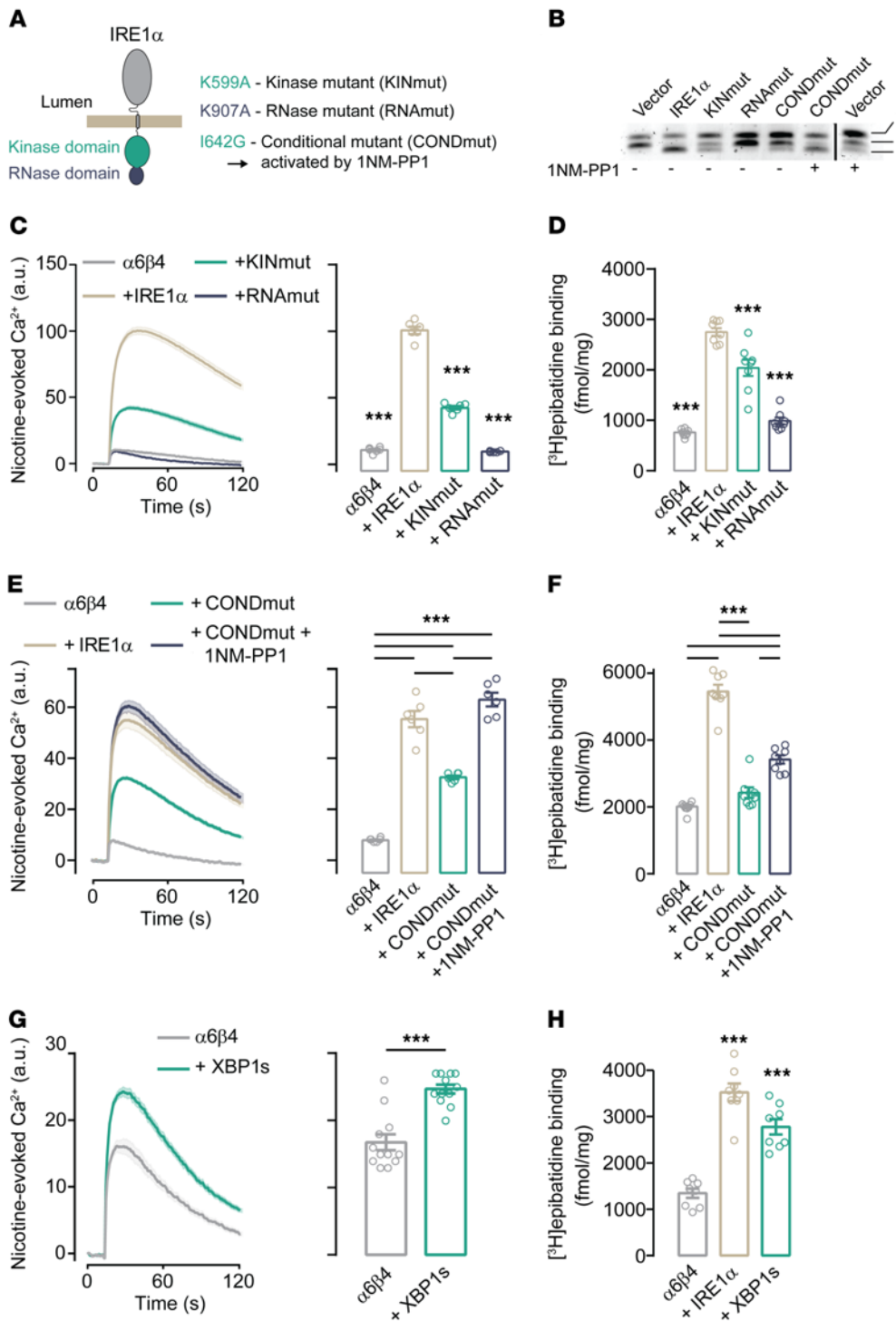


Figure 4. IRE1 α RNase activity and XBP1 splicing mediate assembly of α 6 β 4. (A) Schematics of IRE1 α kinase domain (K599A, KINmut), RNase domain (K907A, RNAmut), and conditional (I642G, CONDmut) mutants that were transfected into HEK293T cells. (B) RT-PCR shows that IRE1 α mutants decrease XBP1 splicing (lower band) as compared with WT IRE1 α . 1NM-PP1 rescues XBP1s in I642G CONDmut. u = full-length unspliced XBP1, s = spliced XBP1 (XBP1s), * = hybrid amplicon. (C) FLIPR traces (left) and quantification (right) of HEK293T cells transfected with WT IRE1 α , K599A mutant, or K907A mutant. IRE1 α and mutants were cotransfected with BARP. *n* = 6 for each group. (D) IRE1 α mutants reduce [³H]epibatidine binding in HEK293T cell lysates compared with WT IRE1 α . *n* = 8 for each condition. (E) FLIPR traces (left) and quantification (right) of I642G CONDmut without and with 5 μ M 1NM-PP1, which rescued the FLIPR response. (F) CONDmut reduces [³H]epibatidine binding. RNase activation of CONDmut with 5 μ M 1NM-PP1 increases α 6 β 4 assembly. *n* = 8 for each condition. (G and H) FLIPR traces (G, left), quantification (G, right), and [³H]epibatidine binding (H) from HEK293T cells cotransfected with α 6 β 4 and XBP1s as indicated. *n* = 12 for each condition in G, *n* = 8 for each group in H. ****P* < 0.001 by 1-way ANOVA with Dunnett’s post hoc test to correct for multiple comparisons to WT IRE1 α (C and D), 1-way ANOVA with Tukey’s multiple-comparisons post hoc test (E and F), unpaired *t* test (G), or 1-way ANOVA with Dunnett’s post hoc test to correct for multiple comparisons to vector (H). C: $F_{3,20} = 702.6$. D: $F_{2,28} = 92.92$. E: $F_{3,20} = 136$. F: $F_{3,28} = 111.7$. G: $t = 5.86$. H: $F_{2,21} = 49.87$. Graphs are the mean \pm SEM and depict 1 experiment that was replicated with similar results.

was measured before or up to 4 weeks after SNI surgery between WT and BARP-KOs (0.250 \pm 0.066 g, 0.166 \pm 0.043 g, respectively; Figure 6, F and G). Systemic nicotine administration exerts antiallodynia in rodent models of neuropathic pain (8, 32). Fitting with this, we found that intraperitoneal (i.p.) administration of nicotine (1.0 mg/kg) reduced mechanical allodynia following nerve injury in WT animals (Figure 6F). However, nicotine had no such relieving effect in BARP-KO mice (WT, 0.529 \pm 0.101 g; BARP-KO, 0.248 \pm 0.064 g; Figure 6F).

We next asked whether the antiallodynic deficits observed in BARP-KO mice were specific to α 6 β 4. NACHO is an essential chaperone for brain α 7 receptors and promotes assembly of other nAChRs such as α 4 β 2, α 3 β 2, and α 6 β 2 β 3 (11). Further, NACHO knockouts show dramatically reduced brain epibatidine binding (12) and striatal α -CTXMII binding (12). Importantly, we show here that α 6 β 4 occupies a privileged role compared with other nAChR subtypes, as NACHO has no effect on α 6 β 4 function (Supplemental Figure 1, A and E; Supplemental Figure 2, A and B).

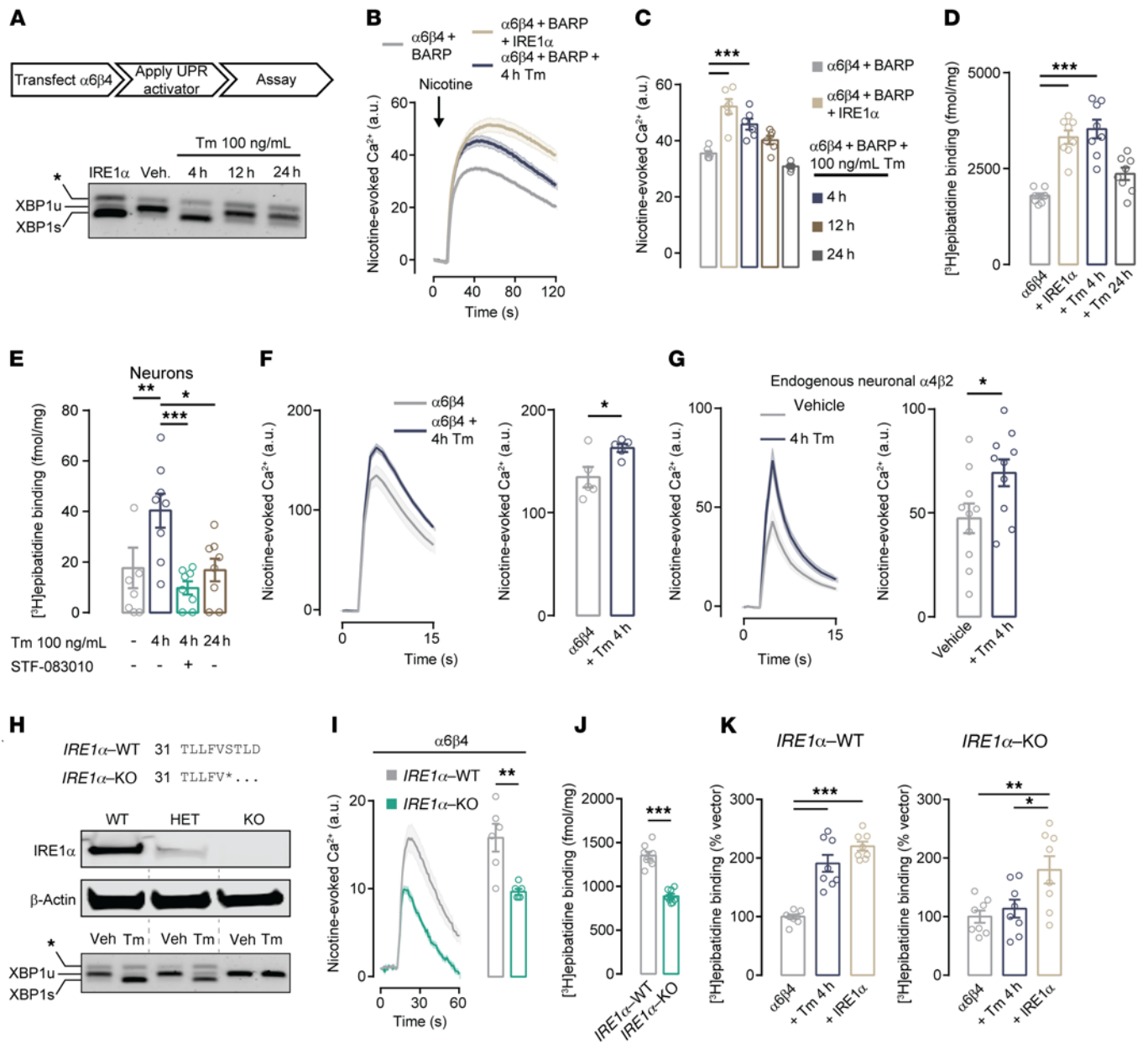


Figure 5. Endogenous IRE1α enhances α6β4 assembly. (A) HEK293T cells were transfected with α6β4 and treated with tunicamycin (Tm, 100 ng/mL) as indicated. Tm induced splicing of XBP1 (XBP1s) at 4 hours. XBP1u, unspliced XBP1. (B and C) Tm treatment for 4, but not 12 or 24 hours enhanced nicotine-induced (7.5 μM) α6β4 FLIPR responses. *n* = 6 for each condition except *n* = 5 for BARP + IRE1α. (D) [³H]epibatidine binding to α6β4 in HEK293T cells with indicated treatments. *n* = 8 each. (E) [³H]epibatidine binding to cortical neuron lysates transfected with α6β4. IRE1α inhibitor STF-083010 (20 μM) applied 30 minutes before Tm (100 ng/mL). *n* = 8 each. (F) Nicotine-evoked (50 μM) Ca²⁺ responses in cortical neurons transfected with α6β4 lentiviral particles. *n* = 5 for each condition. (G) Endogenous α4β2-mediated Ca²⁺ responses in cortical neurons. *n* = 10 for each condition. (H) Top: CRISPR/Cas9-mediated strategy for stop codon (*) insertion in IRE1α. Middle: Immunoblotting confirmed IRE1α protein knockout. Bottom: IRE1α activity in IRE1α-heterozygous (IRE1α-HET) and IRE1α-knockout (IRE1α-KO) lines. RT-PCR shows that the KO line lacks XBP1 splicing activity. (I) Nicotine-evoked (100 μM) FLIPR traces (left) and quantification (right) from IRE1α-WT and IRE1α-KO HEK293T cell lines transfected with α6β4 and BARP. *n* = 6 for each condition. (J) [³H]epibatidine binding in IRE1α-WT and IRE1α-KO cells transfected with α6β4. *n* = 8 each. (K) [³H]epibatidine binding to cells transfected with α6β4. Tm treatment increased binding in WT, but not IRE1α-KO cells, and cotransfection with IRE1α increased binding in both lines. *n* = 8 each. **P* < 0.05; ****P* < 0.001 by 1-way ANOVA with Dunnett's post hoc test compared with α6β4 alone (C and D), unpaired *t* test (F–J), or 1-way ANOVA with Tukey's post hoc test (E and K). C: *F*_{4,25} = 24.92. D: *F*_{3,28} = 22.29. E: *F*_{3,27} = 8.115. K: *F*_{2,21} = 41.93 for IRE1α-WT, *F*_{2,21} = 6.197 for IRE1α-KO. Graphs are the mean ± SEM and depict 1 experiment that was replicated with similar results.

To this end, we performed SNI surgery on *NACHO*-KO mice and tested mechanical allodynia. No difference in mechanical allodynia was observed after SNI surgery (Supplemental Figure 7F). Furthermore, *NACHO*-KO mice exhibited significant nicotine-

induced antiallodynia at levels comparable to WT (gram threshold levels WT, 0.854 ± 0.217 g; *NACHO*-KO, 0.566 ± 0.118 g), indicating that the deficits seen in *BARP*-KO mice were predominantly mediated via α6β4 (Supplemental Figure 7, F and G).

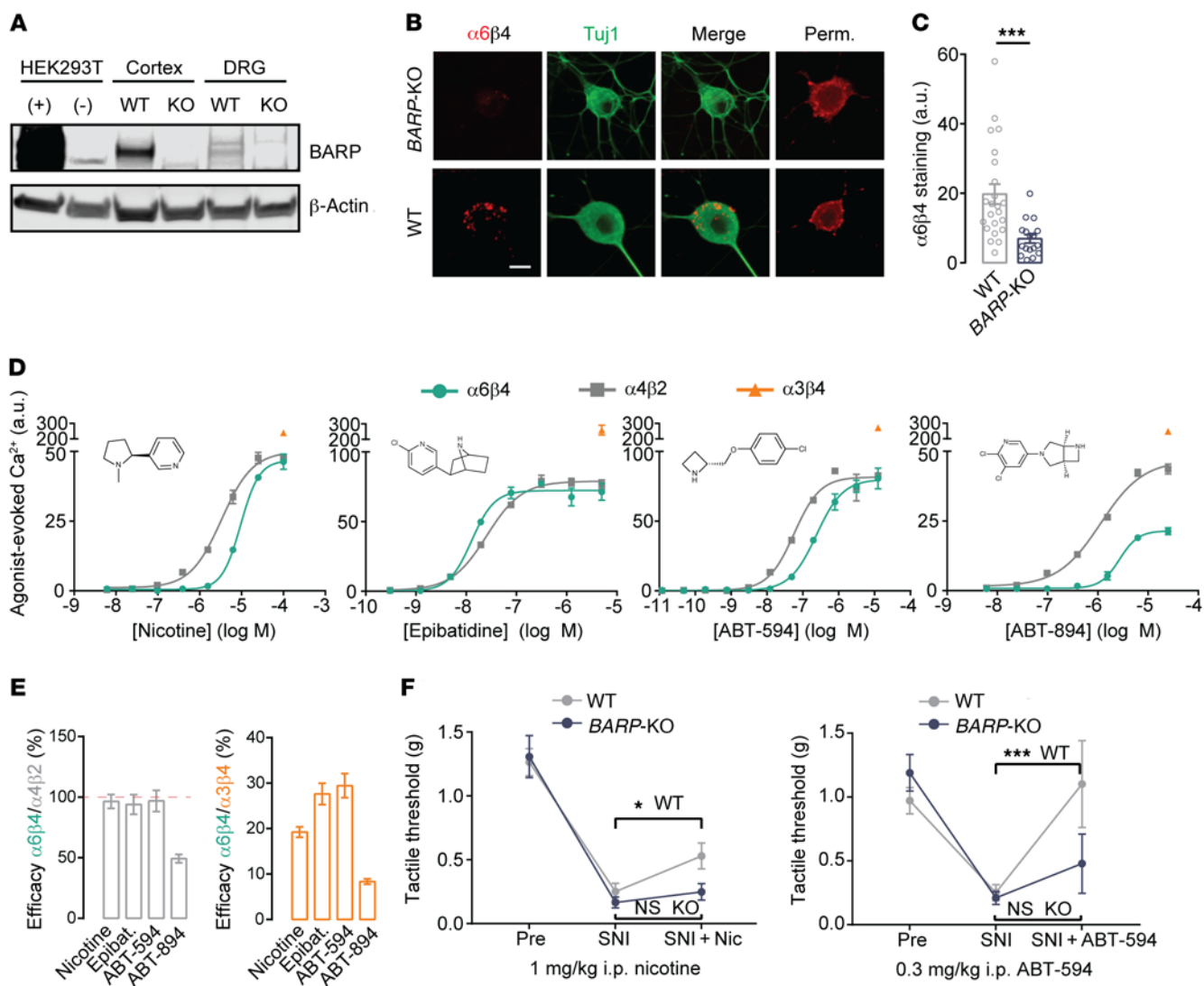


Figure 6. BARP promotes $\alpha 6\beta 4$ function in vivo and mediates antiallodynia. (A) Immunoblotting identified BARP protein in cerebral cortex and dorsal root ganglia (DRG) from WT but not *BARP-KO* mice. BARP-transfected HEK293T cells and β -actin served as controls. (B and C) Imaging (B) and quantification (C) of surface $\alpha 6$ in DRG neurons from WT and *BARP-KO* mice. Neurons were transfected with lentivirus expressing $\alpha 6$ -V5 and $\beta 4$ subunits and were stained with anti-V5 antibody. Surface staining for $\alpha 6$ is reduced in *BARP-KO* neurons, whereas permeabilized (Perm) neurons show similar total levels of receptor. Scale bar: 10 μ m. $n = 23$ and 17 neurons for WT and *BARP-KO*, respectively. (D) FLIPR responses in transfected HEK293T cells stimulated with indicated compounds. Cells transfected with $\alpha 6\beta 4$ were cotransfected with BARP, IRE1 α , and SULT2B1 (for values see Supplemental Tables 1 and 2). Shown are concentration-response curves for $\alpha 6\beta 4$ and $\alpha 4\beta 2$ and a single high concentration for $\alpha 3\beta 4$. (E) Quantification of maximal compound efficacy on $\alpha 6\beta 4$ vs. $\alpha 4\beta 2$ (left) or $\alpha 3\beta 4$ (right). (F and G) Mechanical allodynia was assessed in WT and *BARP-KO* mice before (Pre) and following spared nerve injury (SNI) surgery. WT, but not *BARP-KO* mice exhibited significant (F) nicotine-mediated and (G) ABT-594-mediated antiallodynia. $n = 10$ and 12 for WT and *BARP-KO*, respectively in F. $n = 11$ for each group for G. * $P < 0.05$; *** $P < 0.001$ by Mann-Whitney test (C) or linear mixed-effects model for repeated measures comparing SNI-baseline to SNI-treatment time point (F and G). C: $U = 57$. F: $P = 0.05$ for WT, SNI vs. SNI + nicotine; $P = 0.35$ for *BARP-KO*. G: $P < 0.001$ for WT, SNI vs. SNI + nicotine; $P = 0.4$ for *BARP-KO*. Graphs are the mean \pm SEM. Graphs in C–E are representative of 1 experiment and were replicated with similar results.

To provide further pharmacological evidence of non- $\alpha 4\beta 2$ -mediated antiallodynia in SNI, we pretreated WT animals with a potent $\alpha 4\beta 2$ antagonist, dihydro- β -erythroidine (DH β E), at a dose previously shown to block central effects of systemically administered nicotine (33, 34). After allodynia induction via SNI surgery, animals were injected subcutaneously with 1 mg/kg DH β E 20 minutes before nicotine administration and tested for paw withdrawal thresholds (35). Animals pretreated with DH β E and saline both exhibited robust nicotine-induced

antiallodynia (Supplemental Figure 7H; saline, 0.630 ± 0.171 g; DH β E, 0.702 ± 0.131 g).

Finally, to understand whether the clinical efficacy of ABT-594 may be mediated by $\alpha 6\beta 4$, we injected a separate cohort of *BARP-KO* mice with ABT-594 (0.3 mg/kg; i.p.). Importantly, ABT-594 alleviated mechanical allodynia in WT but not *BARP-KO* animals (Figure 6G), further supporting our model that BARP is crucial for $\alpha 6\beta 4$ function and nicotinic agent-induced antiallodynia (WT SNI, 0.252 ± 0.062 g; WT SNI + ABT-594, 1.102 ± 0.341 g; *BARP-KO*

SNI, 0.210 ± 0.051 g; *BARP-KO* SNI + ABT-594, 0.479 ± 0.232 g). Whereas previous studies found that the nonspecific nicotinic antagonist mecamylamine blocks ABT-594's antiallodynic effect (36), animals pretreated with DH β E retained significant ABT-594 antiallodynia (Supplemental Figure 7I; saline, 2.568 ± 0.422 g; DH β E, 2.059 ± 0.292 g), highlighting a non- $\alpha 4\beta 2$ mechanism.

Discussion

The cholinergic system participates in numerous neurodegenerative and neuropsychiatric disorders. However, many nAChRs cannot be functionally expressed, which has impeded drug discovery. Genome-wide cDNA screening here identified accessory proteins required for activity of the previously intractable $\alpha 6\beta 4$ receptor and unlock future high-throughput screening efforts. These findings expand the array of partner proteins that differentially control nAChRs. Whereas NACHO regulates most nAChRs, NACHO has no effect on $\alpha 6\beta 4$. The $\alpha 6$ -containing nAChRs themselves show selectivity, as NACHO, BARP, SULT2B1, and LAMP5 affect $\alpha 6\beta 2\beta 3$ (13), whereas $\alpha 6\beta 4$ is regulated by BARP, IRE1 α , and SULT2B1. These multiple layers of molecular control may allow precise regulation of nAChRs during specific developmental and physiological circumstances. Accordingly, $\alpha 6\beta 2\beta 3$ concentrates at presynaptic terminals of certain monoaminergic neurons, and this may be enabled by the lysosomal protein LAMP5, which displays a similar restricted distribution (37). On the other hand, LAMP5 is absent from DRG and does not regulate $\alpha 6\beta 4$.

IRE1 α promotes $\alpha 6\beta 4$ assembly via the UPR pathway. Previous studies in *C. elegans* identified a component of the ER membrane complex as critical for nAChR assembly and function (38). In mammals, nicotine-induced upregulation of nAChRs reduces the UPR including IRE1 α , suggesting an important interplay (39, 40). Furthermore, a recent report identified a direct chaperone role for IRE1 α on IP $_3$ receptors at mitochondria-associated membranes that did not require its kinase or RNase activities (41). In contrast, effects on $\alpha 6\beta 4$ require IRE1 α 's canonical kinase and RNase functions. Several observations suggest that this new IRE1 α role on nAChRs is physiologically relevant. First, IRE1 α enhanced responses from a subset of nAChR combinations, but IRE1 α did not affect other channels. Second, ATF6 and PERK, 2 other arms of the UPR, failed to enhance assembly of nAChRs, suggesting that IRE1 α 's effects are not a generalized consequence of UPR activation. Third, knockout of endogenous IRE1 α decreased $\alpha 6\beta 4$ binding and function. Finally, activating endogenous IRE1 α with Tm enhanced function of exogenous $\alpha 6\beta 4$ and endogenous $\alpha 4\beta 2$ in neurons.

Promotion of $\alpha 6\beta 4$ assembly by IRE1 α fits conceptually with the latter's role as a sensor of the UPR, which promotes protein folding in the ER during cell stress (16). Indeed, effects on $\alpha 6\beta 4$ involve the canonical IRE1 α pathway of autophosphorylation and splicing to form XBP1s. Once spliced, XBP1s translocates to the nucleus and upregulates chaperones such as Erdj4 and calnexin, which improves ER protein folding capacity (42–44). Accordingly, calnexin directly enhances assembly of muscle-type nAChRs (45). Precisely how XBP1s promotes $\alpha 6\beta 4$ assembly requires further study.

BARP promotes function of multiple nAChRs. BARP's robust enhancement of $\alpha 6\beta 4$ function in HEK cells, oocytes, and neurons complements previous studies showing that BARP promotes

function of $\alpha 6\beta 2\beta 3$ and $\alpha 3\beta 2$ receptors (13). Whereas BARP regulates gating of $\alpha 6\beta 2\beta 3$ and $\alpha 3\beta 2$ channels (13), it instead increases surface trafficking of $\alpha 6\beta 4$. Thus, BARP enhances multiple $\alpha 6$ -containing nAChRs via distinct mechanisms. Yeast 2-hybrid screening originally identified BARP as a negative regulator of VGCCs. These studies found that 2 intracellular domains toward the BARP C-terminus bind to calcium channel β subunits (14). However, the calcium channel-binding domains of BARP are not involved in regulation of $\alpha 6\beta 4$. Instead, we find that a more membrane-proximal region between BARP amino acids 101 and 202 regulates $\alpha 6\beta 4$. This suggests that BARP may function as a scaffold linking certain nAChRs with calcium channels. Such a model fits with experiments showing that cholinergic regulation of neurotransmitter release often involves calcium channel opening downstream of nAChRs (46, 47).

Targeting $\alpha 6\beta 4$ receptors to treat neuropathic pain. Functional reconstitution of $\alpha 6\beta 4$ receptors has important implications for neuropharmacology. Nicotine has long been recognized as an analgesic (48). Epibatidine is 200 times more potent than morphine in blocking nociception (49), but its side effects preclude clinical development (36). As several nAChR subtypes are expressed in DRG sensory neurons, and epibatidine is a pan-nicotinic agonist, the molecular target for its analgesia remains uncertain. $\alpha 4\beta 2$ nAChRs have been classically thought to mediate nAChR-induced analgesia, yet years of research have yielded no approved therapeutics, suggesting involvement of a different receptor subtype (7). Recently, a genomics screen of DRG tissue from 25 inbred mouse strains identified the $\alpha 6$ nAChR subunit as the major phenotypic contributor to allodynia (8). Furthermore, knockout mouse studies showed that $\alpha 6$ but not $\alpha 4$ is required for peripheral and spinal nicotine-induced analgesia (8). The precise mechanisms through which nicotinic agents mediate analgesia are uncertain. Previous work suggested that $\alpha 6\beta 4$ can produce analgesia via cross-inhibition of P2X2/3 receptors on DRG neurons (8, 50). Activation of nAChRs may also promote analgesia via blunting of TrpV1-mediated currents in DRG nociceptors (51).

Here, we find that *BARP-KO* mice, which have impaired $\alpha 6\beta 4$ surface expression in DRG, lack nicotine-mediated antiallodynia. Because BARP also promotes $\alpha 3\beta 2$ and $\alpha 6\beta 2\beta 3$ function, we leveraged the fact that NACHO has no effect on $\alpha 6\beta 4$ but promotes $\alpha 3\beta 2$ and $\alpha 6\beta 2\beta 3$ assembly. We observed normal nicotine-induced allodynia in *NACHO-KO* mice, suggesting that the deficits observed in *BARP-KO* mice are mediated predominantly via $\alpha 6\beta 4$ and not other nAChR subtypes. Supporting this, *BARP KO* did not alter surface expression of other nAChRs in DRG.

Phase II clinical studies found that ABT-594, a nicotinic agent related to epibatidine, was effective in treating diabetic neuropathic pain but had unacceptable side effects (29). The investigators hypothesized that activity at $\alpha 4\beta 2$ was mediating analgesia, while $\alpha 3\beta 4$ was predominantly responsible for the untoward side effects. As such, maximizing activity at $\alpha 4\beta 2$ while minimizing $\alpha 3\beta 4$ activity drove development of the follow-up compound, ABT-894. Unfortunately, ABT-894 was clinically ineffective (30). The study investigators hypothesized that "it is possible that ABT-594, which is a less selective $\alpha 4\beta 2$ [neuronal nicotinic receptor (NNR)] agonist than ABT-894, elicits some of its effects via activity at non- $\alpha 4\beta 2$ NNRs. If this is the case, the preferential affinity

of ABT-894 for $\alpha 4\beta 2$ NNRs may have been a disadvantage that resulted in a lack of analgesic efficacy” (30). Thus, the pharmacological difference between ABT-594 and -894 that accounts for their discrepant clinical efficacy is uncertain and likely involves non- $\alpha 4\beta 2$ nAChRs.

Our ability to express functional $\alpha 6\beta 4$ receptors showed that ABT-594 exhibits similar efficacy on $\alpha 4\beta 2$ and $\alpha 6\beta 4$. In contrast, ABT-894 exhibited only weak partial agonism on $\alpha 6\beta 4$ as compared with $\alpha 4\beta 2$ (Figure 6, D and E; Supplemental Table 1). We further show that the effective analgesics nicotine and epibatidine exhibit similar efficacies on $\alpha 4\beta 2$ and $\alpha 6\beta 4$. This unexpected pharmacology of $\alpha 6\beta 4$ provides a likely explanation for the differential clinical efficacy of ABT-594 versus ABT-894. That is, $\alpha 6\beta 4$ activity contributed to ABT-594’s clinical analgesia, yet ABT-894’s diminished $\alpha 6\beta 4$ efficacy may underpin the lack of its clinical efficacy. Supporting this model, ABT-594 exerts antiallodynic effects in WT mice but has no effect in *BARP*-KO mice, whose DRG have reduced $\alpha 6\beta 4$ surface expression. Furthermore, pretreatment with the potent $\alpha 4\beta 2$ antagonist DH β E failed to block ABT-594-induced antiallodynia, further supporting a non- $\alpha 4\beta 2$ mechanism for alleviating neuropathic pain in the SNI model. ABT-594 alleviates thermal and inflammatory pain, suggesting that $\alpha 6\beta 4$ agonists may be useful for a broad spectrum of pain conditions (36).

Methods

Cell culture and transfection. Expression plasmids for the following genes were used in this study (all human, GenBank accession numbers): *CHRNA6* (NM_004198), *CHRNA4* (NM_000744.6), *CHRNA3* (NM_000743.5), *CHRNA7* (NM_000746.5), *CHRNB2* (NM_000748.2), *CHRNB3* (NM_000749.5), *CHRNB4* (NM_000750), *BARP* (XM_017026555), *ERN1* (IRE1 α), *PERK* (NM_001313915.1), *ATF6* (NM_007348.4), *NACHO* (NM_021637.2), *SULT2B1* (NM_177973), *LAMP5* (NM_012261.4), *5HT3A* (NM_213621.4), *GLUA1* (NM_000827.4), *GABBR1* (NM_021903.2), and *GABBR2* (NM_005458.8).

HEK293T cells were initially purchased from ATCC and cultured in DMEM high-glucose medium supplemented with 10% FBS and 1 mM sodium pyruvate. Cells were transfected at 80%–90% confluence more than 4 hours after plating with FuGENE 6 or ViaFect Transfection Reagent (Promega) and incubated at 37°C. To promote surface expression, cells were switched to 30°C after 24 hours. All assays unless otherwise noted were performed 48–72 hours after transfection. For cDNA library screening, expression-ready cDNA plasmids from a Broad Institute library were cotransfected in 384-well plates with $\alpha 6\beta 4$ subunits in a 1:1:1 ratio.

For neuronal FLIPR and epibatidine experiments, rat cortical E18 neurons were purchased from BrainBits, dissociated with papain, and cultured in NbActiv media (BrainBits) at 37°C. Cultures were transduced with lentiviral particles expressing $\alpha 6$ and $\beta 4$ (Vigene Biosciences) at DIV 7 and assayed at DIV 13. Cultures were pretreated with compounds for indicated time periods before assay.

For DRG neuron staining, DRGs from adult WT or *BARP*^{-/-} mice were dissected in cold L-15 media supplemented with GlutaMAX (Thermo Fisher Scientific). DRGs were dissociated with collagenase/protease IV solution for 60 minutes. DRGs were triturated with a fire-polished glass pipette, resuspended in L-15 media, and filtered with a cell strainer. Cells were then pipetted on top of a 60%/30% Percoll

gradient and centrifuged at 800g for 20 minutes at 4°C. After centrifugation, the top layer was discarded, and neurons were collected and spun down at 200g for 10 minutes. The supernatant was aspirated, and cells were resuspended in Neurobasal A supplemented with B-27, GlutaMAX, and 25 ng/mL nerve growth factor. Neurons were then plated on glass coverslips coated with poly-D-lysine (PDL) and laminin. Cells were transduced 1 hour after plating and assayed 5 days later.

SNI and tactile threshold testing. For the SNI procedure, adult male mice (23–33 g) were deeply anesthetized with isoflurane and SNI surgery was performed as described previously (31). Briefly, the sural and common peroneal branches of the left sciatic nerve were ligated, leaving the tibial branch intact. Only mice that did not exhibit motor dysfunction (e.g., paw dragging) were used.

For tactile threshold testing, following a 30-minute acclimation on the von Frey stand, preinjury tactile thresholds were measured from the left hind paw and recorded before SNI surgery. Fifty-percent tactile paw withdrawal thresholds (PWTs) were measured from the left paw and recorded using calibrated von Frey filaments applied perpendicularly with sufficient force to bend slightly and held for 2–3 seconds against the plantar surface. Paw flinching during or immediately following removal of the stimulus was scored as positive. PWT was tested at preinjury levels and at 1, 3, 7, 14, and 21 days (21 days deemed SNI) following surgery. At 29 days following surgery, mice were i.p. injected with vehicle, 1.0 mg/kg (-)-nicotine (Millipore-Sigma), or 0.3 mg/kg ABT-594 (Tocris) and again tested at either 10 minutes (nicotine) or 1 hour (ABT-594) after injection. For DH β E pretreatment experiments, DH β E was dissolved in saline and administered subcutaneously at a final concentration of 1 mg/kg 20 minutes before nicotine or ABT-594 injection. Experimenters were blinded to genotype throughout the study.

Molecular biology. IRE1 α mutants (K907A, RNAmut; K599A, KINmut; I642G, CONDMut) were generated via site-directed mutagenesis and confirmed by sequencing. *BARP101* and *BAPR202* truncation mutants were generated by inserting a premature stop codon at amino acid 102 and 203, respectively.

The $\alpha 4N/6$ chimera consisted of the first 242 amino acids corresponding to the N-terminal extracellular region of $\alpha 4$ fused to the transmembrane and intracellular regions of $\alpha 6$ (amino acids 240–495). Conversely, the $\alpha 6N/4$ chimera consisted of the first 239 amino acids of $\alpha 6$ fused to the transmembrane and intracellular regions of $\alpha 4$ (amino acids 243–628). Loop chimeras substituted amino acids 331–600 for $\alpha 4$ and 328–465 for $\alpha 6$. All chimeras were confirmed by sequencing.

To generate IRE1 α -KO cell lines, a guide RNA targeting exon 2 of the IRE1 α gene was cotransfected into HEK293T cells with Cas9 enzyme (IDT Technologies) and single-stranded oligodeoxynucleotide (ssODN) for homology-directed repair using TransIT-X2 transfection reagent (Mirus, MIR6003). Serine at position 36 was mutated to a stop codon. Single clones were isolated, expanded, sequenced, and confirmed for hetero- or homozygosity with a Zero Blunt TOPO PCR Cloning Kit (Thermo Fisher Scientific, K280020SC). ssODN sequence: CAGATTTTGGAAAGTACCAGCACAGTGACGCTTCCT-GAAACCTGTGTTTGTGTAAACGCTGGATGGAAGTTTGCAT-GCTGTCAAGAGGACAGGCTCAATCAAATGG.

For XBP1s RT-PCR experiments, HEK293T cells were seeded in 6-well plates and treated with vehicle or Tm for the indicated time points. Cells were then collected and RNA isolated using an RNeasy Mini Kit (QIAGEN, 74104). cDNA was synthesized from total RNA

with a SuperScript IV First-Strand Synthesis System (Thermo Fisher Scientific, 18091050) according to the manufacturer's instructions. The following primers were used to amplify *XBP1* cDNA with PCR (25): *XBP1*sense, TTACGAGAGAAAACATGGC; antisense, GGGTCCAAGTTGTCCAGAATGC.

FLIPR assays. HEK293T cells were seeded at 15,000/well in 384-well glass-bottomed PDL-coated plates (Corning). At the time of assay, cells were washed once with FLIPR buffer (HyClone HEPES-buffered saline [GE Life Sciences] with [in mM] 149 NaCl, 4 KCl, 10 HEPES, 5 glucose, 2 CaCl₂, 1 MgCl₂; 300 mOsm and pH 7.4) and loaded for 1 hour at room temperature with Calcium5 dye (Molecular Devices). After dye loading, excess dye was removed, plates were washed once with FLIPR buffer, and then placed in a FLIPR Tetra (Molecular Devices) machine. Compound-induced Ca²⁺ responses were monitored and measured for 5 minutes with Screenworks software (Molecular Devices). For $\alpha 6\beta 2\beta 3^*$ responses, $\alpha 6/3$ chimera and $\beta 3$ mutant were used to enable larger responses (13). For $\alpha 7$ responses, cells were first incubated with the positive allosteric modulator PNU-120596 (5 μ M). Unless otherwise noted, all FLIPR transfections were carried out at a 3:1:5 ratio ($\alpha 6/\beta 4$ /chaperone). *XBP1*s transfections were carried out at a ratio of 3:1:0.1 ($\alpha 6/\beta 4$ /*XBP1*s). For neuronal assays, the FLIPR protocol was identical except that 3 mM CaCl₂ was used in FLIPR buffer. To isolate $\alpha 6\beta 4$ responses from endogenously expressed $\alpha 4\beta 2$, neurons were incubated for 5 minutes with 25 μ M DH β E before stimulation with nicotine.

Immunofluorescent surface staining. HEK293T cells were seeded at 10,000/well in 384-well glass-bottomed PDL-coated plates (Corning). All cell transfections included EGFP to label cytoplasm of transfected cells. For staining, cells were incubated for 1 hour with fluorescent-conjugated antibody against HA or V5 tag (Dylight HA-650 [26183-D650], Alexa Fluor V5-555 [37-7500-A555], Invitrogen) at 30°C. Cells were washed with PBS and then fixed for 45 minutes at room temperature with 4% paraformaldehyde. After washing, nuclei were stained with NucBlue solution (Invitrogen). Plates were imaged using the Opera Phenix confocal instrument (PerkinElmer) at $\times 20$ magnification. For image analysis, Columbus automated software was used (PerkinElmer). Briefly, EGFP labeling was used for automatic cell detection. Then, average fluorescence intensity was measured in a ring region surrounding the cell, but not including the cytoplasm, which represented surface staining.

For DRG neuron staining, cells were live stained with the same anti-V5 primary antibody as described above. Cells were additionally costained with β -3 tubulin (Tuj1) conjugated to Alexa Fluor 488 (eBioscience, 53-4510-82) to label cell bodies and processes. For cytoplasmic staining, cells were fixed then permeabilized for 20 minutes at room temperature with 0.3% Triton X-100 in PBS. Neurons were imaged at $\times 40$ magnification with a confocal microscope (Zeiss).

[³H]epibatidine binding. HEK293T cells or rat cortical E18 neurons were collected with PBS, spun down, and then resuspended in 50 mM ice-cold Trizma buffer (MilliporeSigma). Cells were homogenized for 30 seconds at max speed with a T-25 Ultra-Turrax homogenizer (Ika) and concentrations measured using the Pierce BCA assay (Thermo Fisher Scientific). Samples were run in 16 replicates and incubated with 10 nM [³H]epibatidine (PerkinElmer) for 2–3 hours at room temperature. In half of the samples, 10 μ M unlabeled epibatidine (Tocris) was coapplied to determine nonspecific binding. Samples were filtered and bound to polyethylenimine-treated 96-well GF/B filter

plates (PerkinElmer). Plates were washed with cold Trizma buffer and then desiccated for 20 minutes at 65°C. Each well received 60 μ L of MicroScient-O, and then plates were read with a TopCount NXT scintillation counter (PerkinElmer).

Protein biochemistry. For immunoblotting, cells were collected into RIPA buffer (Thermo Fisher Scientific) with Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). Cells were sonicated, spun down at 14,000g for 30 minutes at 4°C, and the supernatant was taken for further analysis. Protein concentrations were measured using the Pierce BCA assay. Protein lysates were denatured at 55°C for 30 minutes, proteins were resolved by SDS-PAGE, and blots were incubated overnight at 4°C with primary antibodies (anti- β -actin, Thermo Fisher Scientific [PA516914]; anti-IRE1 α , Cell Signaling Technology [3294S]; anti-HA, Thermo Fisher Scientific [26183]; anti-V5, Thermo Fisher Scientific [PA1993]; and anti-BARP [ref. 13]). Blots were then incubated for 1 hour at room temperature in the dark with infrared-conjugated secondary antibodies and imaged using the Odyssey system (Licor).

For coimmunoprecipitation, cells were solubilized 2 hours at 4°C on a shaker in buffer containing 100 mM NaCl, 1% *n*-dodecyl- β -D-maltoside, and 1 mM iodoacetamide. Samples were spun at 20,000g at 4°C for 45 minutes. These lysates were incubated with 3 μ g of immunoprecipitation antibody on a shaker overnight at 4°C. Protein-antibody complexes were captured with Protein A Dynabeads (Thermo Fisher Scientific). After washing, bound proteins were eluted by heating to 55°C for 30 minutes and were analyzed by immunoblotting.

Oocyte recordings. cRNAs were obtained via *in vitro* transcription with an mMessage mMACHINE T7 Transcription Kit (Thermo Fisher Scientific, AM1344). Stage 4/5 oocytes were purchased from Eco-Cyte Biosciences and injected with 60 ng total cRNA at a ratio of 1:1:2 $\alpha 6/\beta 4$ /chaperone. After injection, oocytes were placed in Modified Barth's Saline (MilliporeSigma) supplemented with gentamycin and incubated at 18°C. Recordings were made 3–5 days after cRNA injection and stimulated with 250 μ M ACh for 2 seconds. Oocytes were recorded using the 2-electrode voltage-clamp method and held at -60 mV. Currents were acquired using a HEKA amplifier with FitMaster software (HEKA Elektronik). Data were sampled at 5 kHz and filtered with a 4-pole Bessel filter at 1 kHz.

Electrophysiology. HEK293T cells in 6-well plates were cotransfected with $\alpha 6\beta 4$, chaperones, and EGFP, and 24 hours before recording were replated on 12-mm glass coverslips. For recordings, coverslips were placed in an extracellular bath composed of (in mM) 149 NaCl, 4 KCl, 10 HEPES, 5 glucose, 2 CaCl₂, and 1 MgCl₂. Borosilicate glass (World Precision Instruments) pipettes were pulled to obtain final resistances of 3–5 M Ω with a PC-10 micropipette puller (Narshige). Internal solution comprised (in mM) 145 CsCl, 2.5 NaCl, 10 HEPES, 4 MgATP, 1 EGTA; pH was adjusted to 7.4 with CsOH; osmolality was adjusted to 300 mOsm/kg with sucrose. After whole-cell recording from EGFP⁺ cells was achieved, cells were carefully lifted from the coverslip and moved toward a separate pipette with agonist. To record agonist-evoked responses, ACh was rapidly applied to cells held at -60 mV using a MXPZT piezoelectric actuator system (Siskiyou). Series resistances (3–15 M Ω) were compensated by at least 80%. All recordings were carried out with an Axopatch 200B amplifier (Molecular Devices). Signals were sampled at 25 kHz and low-pass filtered at 10 kHz. Data were acquired and analyzed

using pClamp10 software (Molecular Devices). Steady state/peak ratio was calculated as the amplitude of steady-state current during constant ACh application divided by the peak current.

Statistics. Results are represented as mean \pm SEM unless otherwise noted. For data sets with 2 groups, an unpaired, 2-tailed Student's *t* test was used to test for significance. For 3 or more groups, 1-way ANOVA was used. To adjust for multiple comparisons: when comparing multiple groups to a control group, Dunnett's post hoc test was used. If differences between all groups to each other were tested, then Tukey's post hoc test was used. Significance levels were set at **P* < 0.05, ***P* < 0.01, ****P* < 0.001 unless otherwise noted in figure legends. The D'Agostino and Pearson omnibus test with an α value of 0.05 was used to test for normality. EC₅₀ values and concentration-response curves were calculated using a variable slope, 4-parameter nonlinear regression model: $y = \text{bottom} + (x^{\text{Hill slope}}) \times (\text{top} - \text{bottom}) / (x^{\text{Hill slope}} + \text{EC}_{50}^{\text{Hill slope}})$. All statistics and graphing were carried out with Prism (GraphPad).

Study approval. BARP-KO and NACHO-KO mice were previously described (12, 13). All procedures were performed in accordance with Janssen (La Jolla) IACUC guidelines.

Author contributions

DK designed and conducted experiments, analyzed the data, and prepared the manuscript. SG helped conduct experiments. WAE performed mouse behavioral studies. GBD and JAM conducted HEK293T electrophysiology experiments. JL and ADW helped with pharmacological experiments. AB supervised behavioral studies. DSB designed experiments, supervised the project, and prepared the manuscript. All authors provided input for writing manuscript. Co-authorship was assigned by time of initial involvement.

Acknowledgments

The authors thank Ondrej Libiger for statistical advice, Bridget Kohlnofer for advice on CRISPR knockout generation, and Mike Morton for technical assistance with cell sorting.

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