Rodent Habenulo–Interpeduncular Pathway Expresses a Large Variety of Uncommon nAChR Subtypes, But Only the $\alpha 3\beta 4^*$ and $\alpha 3\beta 3\beta 4^*$ Subtypes Mediate Acetylcholine Release

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Recent studies suggest that the neuronal nicotinic receptors (nAChRs) present in the habenulo–interpeduncular (Hb–IPn) system can modulate the reinforcing effect of addictive drugs and the anxiolytic effect of nicotine. Hb and IPn neurons express mRNAs for most nAChR subunits, thus making it difficult to establish the subunit composition of functional receptors. We used immunoprecipitation and immunopurification studies performed in rat and wild-type (+/+) and $\beta 2$ knock-out (–/–) mice to establish that the Hb and IPn contain significant $\beta 2^*$ and $\beta 4^*$ populations of nAChR receptors (each of which is heterogeneous). The $\beta 4^*$ nAChR are more highly expressed in the IPn. We also identified novel native subtypes ($\alpha 2\beta 2^*$, $\alpha 4\beta 3\beta 2^*$, $\alpha 3\beta 3\beta 4^*$, $\alpha 6\beta 3\beta 4^*$). Our studies on IPn synaptosomes obtained from +/+ and $\alpha 2$, $\alpha 4$, $\alpha 5$, $\alpha 6$, $\alpha 7$, $\beta 2$, $\beta 3$, and $\beta 4^{-/-}$ mice show that only the $\alpha 3\beta 4$ and $\alpha 3\beta 3\beta 4$ subtypes facilitate acetylcholine (ACh) release. Ligand binding, immunoprecipitation, and Western blotting studies in $\beta 3^{-/-}$ mice showed that, in the IPn of these mice, there is a concomitant reduction of ACh release and $\alpha 3\beta 4^*$ receptors, whereas the receptor number remains the same in the Hb. We suggest that, in habenular cholinergic neurons, the $\beta 3$ subunit may be important for transporting the $\alpha 3\beta 4^*$ subtype from the medial habenula to the IPn. Overall, these studies highlight the presence of a wealth of uncommon nAChR subtypes in the Hb–IPn system and identify $\alpha 3\beta 4$ and $\alpha 3\beta 3\beta 4$, transported from the Hb and highly enriched in the IPn, as the subtypes modulating ACh release in the IPn.

Key words: habenula; nucleus interpeduncularis; nicotinic receptor subtypes; acetylcholine release; subunit composition; knock-out mice

Introduction

Evidence suggests the habenular complex (Hb) participates in various behaviors, including nociception, sleep–wake cycle, locomotion, anxiety-related responses, mood regulation, learning, and memory (De Biasi and Salas, 2008). Its role in reward phenomena has recently attracted much attention. The Hb and its inputs and outputs support self-stimulation (Morissette and Boye, 2008). Indeed, the Hb is activated by negative reward or absence of positive reward in humans (Shepard, 2006) and monkey (Matsumoto and Hikosaka, 2007). Stimulation of the lateral

Hb (LHb) suppresses dopamine neuron activity when an expected reward is withheld in rat (Ji and Shepard, 2007) and monkey (Matsumoto and Hikosaka, 2007). The medial Hb (MHb) projects almost exclusively to the interpeduncular nucleus (IPn) through the fasciculus retroflexus (fr) (Herkenham and Nauta, 1979; Klemm, 2004), whereas the LHb projects to a number of midbrain regions (Klemm, 2004; Lecourtier and Kelly, 2007; Geisler and Trimble, 2008).

The Hb and IPn are both highly enriched in cholinergic markers. The Hb receives cholinergic input from septal and basal forebrain nuclei through the stria terminalis (Contestabile and Fonnum 1983). Cholinergic cells, densely packed in the ventral portion of MHb, innervate rostral, central, and intermediate subnuclei of the IPn (Kimura et al., 1981; Houser et al., 1983; Contestabile et al., 1987; Eckenrode et al., 1987). The IPn also receives direct cholinergic input from forebrain nuclei through the fr bypassing the Hb (Contestabile and Fonnum, 1983; Fonnum and Contestabile, 1984; Albanese et al., 1985; Klemm, 2004):

The wealth of cholinergic neurons in the habenulo–interpeduncular pathway is paralleled by a high density of nicotinic acetylcholine receptors (nAChRs). The MHb and IPn express the highest density of nicotinic binding sites in mammalian brain

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and have the largest variety of nicotinic subunits (Clarke et al., 1985; Wada et al., 1989; Marks et al., 1992; Le Novère et al., 1996; Han et al., 2000, 2003; Whiteaker et al., 2000; Yeh et al., 2001; Cui et al., 2003). Electrophysiological and pharmacological experiments in rats or wild-type (+/+) and nicotinic subunit knock-out (–/–) mice have shown that nAChRs in MHb and IPn are heterogeneous, including both $\beta 2^*$ and $\beta 4^*$ receptors (Mulle et al., 1991; Connolly et al., 1995; Zoli et al., 1998; Quick et al., 1999; Whiteaker et al., 2002; Adams et al., 2004; Fonck et al., 2009). Although many nAChRs are presynaptic, some are postsynaptic in the IPn (Clarke et al., 1986; Mulle et al., 1991).

The habenulo–interpeduncular system may mediate some nicotine-induced behaviors. Effects of acute nicotine administration in rodent MHb and IPn include increases in Fos immunoreactivity (Seppa et al., 2001; Choi et al., 2006; Fonck et al., 2009) and cerebral blood volume (Choi et al., 2006). nAChRs in MHb and IPn may partially mediate locomotor effects of nicotine (Hentall and Gollapudi, 1995) and self-administration of addictive drugs (Glick et al., 2006; Taraschenko et al., 2007). However, functional roles of nAChRs in MHb and IPn remain essentially unknown. Aims of this study were to determine native nAChR subtypes in this system and define selected subtype function.

Materials and Methods

Animals. Adult male pathogen-free Sprague Dawley rats were obtained from Harlan-Nossan. Rat animal care and experimental procedures were approved by the University of Modena and Reggio Emilia and are in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC). Mice with nAChR subunit null mutations were bred and maintained at The Institute for Behavioral Genetics (Boulder, CO) in accordance with the guidelines and approval of the Animal Care and Utilization Committee of the University of Colorado, Boulder. All mice were generated from heterozygous matings, and genotype was determined by PCR analysis of tail DNA (Salminen et al., 2004). Original sources of the mutated mice were as follows: Dr. Jim Boulter (University of California, Los Angeles, Los Angeles, CA) ($\alpha 2$), Dr. John Drago (University of Melbourne, Victoria, Australia) ($\alpha 4$), Dr. Arthur Beaudet (Baylor College, Houston, TX) ($\alpha 5$, $\alpha 7$, $\beta 4$), Dr. Uwe Maskos (Pasteur Institute, Paris, France) ($\alpha 6$), Dr. Marina Picciotto (Yale University, New Haven, CT) ($\beta 2$), and Dr. Stephen Heinemann (Salk Institute, San Diego, CA) ($\beta 3$).

Materials. (+/–)[^3H]Epibatidine ([^3H]Epi) (specific activity, 70.6 Ci/mmol), [^{125}I] α Bungarotoxin ([^{125}I] α Bgtx) (specific activity, 200 Ci/mmol), [^3H]choline (methyl- ^3H) (60–90 Ci/mmol), and $^{86}\text{Rb}^+$ (initial specific activity, ~12 Ci/mg) were purchased from PerkinElmer Life and Analytical Sciences. The nonradioactive α Bgtx, epibatidine, acetylcholine iodide, diisopropylfluorophosphate, phenylmethylsulfonyl fluoride (PMSF), EDTA, EGTA, bovine serum albumin (BSA), atropine sulfate, acetylcholine iodide, tetrodotoxin, Tris, Triton X-100, Tween 20, glucose, nicotine sulfate, NaCl, KCl, MgSO_4 , CaCl_2 , and polyethylenimine were purchased from Sigma.

Antibody production and characterization. The subunit-specific polyclonal antibodies (Abs) used were produced in rabbit against peptides derived from the C-terminal (COOH) and/or intracytoplasmic loop (CYT) regions of rat, human, or mouse subunit sequences and affinity purified as described previously (Zoli et al., 2002). Most of the Abs have been described previously (Zoli et al., 2002; Champiaux et al., 2003; Gotti et al., 2005a,b, 2008) (supplemental Table 1A, available at www.jneurosci.org as supplemental material).

Antibody specificity was checked by means of quantitative immunoprecipitation or immunopurification experiments using nAChRs from different areas of the CNS of wild-type (+/+) and null mutant (–/–) mice, which allowed selection of Abs specific for the subunit of interest and established the immunoprecipitation capacity of each Ab (Zoli et al., 2002; Gotti et al., 2005a,b, 2007) (supplemental Table 1B, available at www.jneurosci.org as supplemental material).

Ab specificity was also tested by Western blotting (Gotti et al., 2008),

which indicated that some of the Abs were less specific for Western blotting than for immunoprecipitation.

Preparation of membranes and 2% Triton X-100 extracts from Hb and IPn. The tissues obtained from rats or mice were dissected, immediately frozen on dry ice or in liquid nitrogen, and stored at -80°C for later use. In every experiment, the tissues from Hb (0.05–0.20 g) or IPn (0.05–0.10 g) were homogenized in 5 ml of 50 mM Na phosphate, pH 7.4, 1 M NaCl, 2 mM EDTA, 2 mM EGTA, and 2 mM PMSF with a Potter homogenizer. The homogenates were then diluted and centrifuged for 1.5 h at $60,000 \times g$.

The procedures of homogenization, dilution, and centrifugation of the total membranes were performed twice, after which the pellets were collected, rapidly rinsed with 50 mM Tris HCl, pH 7, 120 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 2.5 mM CaCl_2 , and 2 mM PMSF, and then resuspended in the same buffer containing a mixture of 20 $\mu\text{g}/\text{ml}$ of each of the following protease inhibitors: leupeptin, bestatin, pepstatin A, and aprotinin. Triton X-100 at a final concentration of 2% was added to the washed membranes, which were extracted for 2 h at 4°C . The extracts were then centrifuged for 1.5 h at $60,000 \times g$, supernatants were recovered, and an aliquot of these resultant supernatants was collected for protein measurement using the BCA protein assay (Pierce) with bovine serum albumin as the standard.

Selective [^3H]epibatidine binding. To ensure that the $\alpha 7$ nAChRs did not contribute to [^3H]Epi binding, in both membrane or solubilized extracts, binding was performed in the presence of 2 μM α Bgtx, which specifically binds to $\alpha 7^*$ nAChR (and thus prevents [^3H]Epi binding to these sites).

Binding to the homogenates obtained from Hb or IPn membranes was performed overnight by incubating aliquots of the membrane with 2 nM [^3H]Epi at 4°C . Nonspecific binding (averaging 5–10% of total binding) was determined in parallel samples containing 100 nM unlabeled epibatidine. At the end of the incubation, the samples were filtered on a glass microfiber filter GF/C (Whatman) filter soaked in 0.5% polyethylenimine, washed with 15 ml of buffer (Na phosphate, 10 mM, pH 7.4, and 50 mM NaCl), and counted in a β counter.

The Triton X-100 extracts were labeled with 2 nM [^3H]epibatidine. Tissue extract binding was performed using DE52 ion-exchange resin (Whatman) as described previously (Champiaux et al., 2003).

[^{125}I] α Bungarotoxin binding. Binding experiments were performed by incubating Hb or IPn membranes overnight with 2–4 nM [^{125}I] α Bgtx at 20°C in the presence of 2 mg/ml BSA. Specific radioligand binding was defined as total binding minus nonspecific binding determined in the presence of 1 μM cold α Bgtx.

Immunoprecipitation by anti-subunit-specific antibodies of [^3H]Epi-labeled Hb or IPn receptors. Hb or IPn dissected from 30–40 rats or mice differing in $\beta 3$ or $\beta 2$ genotypes were immediately frozen at -70°C . The membranes (10–30 ml) were then extracted by addition of 2% Triton X-100 as described above and centrifuged. The extracts (100 μl) were labeled with 2 nM [^3H]Epi and incubated overnight with a saturating concentration of anti-subunit affinity-purified IgG (anti- $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, $\beta 2$, $\beta 3$, or $\beta 4$). Each immunoprecipitate was recovered by incubating the samples with beads containing bound anti-rabbit goat IgG (Technogenetics). The level of immunoprecipitation with each Ab was expressed as the percentage of [^3H]Epi-labeled receptors immunoprecipitated by the Abs (taking the amount present in the Triton X-100 extract solution before immunoprecipitation as 100%) or as femtomoles of immunoprecipitated receptors per milligram of protein.

To immunopurify the $\beta 2^*$ nAChRs, extracts (30 ml) were incubated three times with 5 ml of Sepharose-4B with bound anti- $\beta 2$ CYT Abs. The $\beta 2$ -depleted flow-through fraction was collected for additional processing. The bound $\beta 2^*$ population was eluted by incubation with 100 μM $\beta 2$ CYT peptide. The purified $\beta 2$ recovered populations (or the flow through devoid of $\beta 2$ -containing receptors) were analyzed by immunoprecipitation using subunit-specific Abs.

Immunoblotting and densitometric quantification of Western blot bands. The membranes obtained from mouse Hb and IPn were diluted 1:1 (v/v) with Laemli's buffer and then underwent SDS-PAGE using 9% acrylamide by loading 5 μg of membrane protein for both the Hb and IPn (except for the analysis of the $\beta 4$ subunit in which only 2.5 μg of IPn membrane protein was loaded). After SDS-PAGE, the proteins were elec-

trophoretically transferred to nitrocellulose membranes with 0.45 μ m pores (Schleicher and Schuell). The blots were blocked overnight in 5% nonfat milk in Tris-buffered saline (TBS), washed in a buffer containing 5% nonfat milk and 0.3% Tween 20 in TBS, incubated for 2 h with the primary antibody (1–2.5 μ g/ml), and then incubated with the appropriate peroxidase conjugated secondary Abs. After another series of washes, peroxidase was detected using a chemiluminescent substrate (Pierce).

The signal intensity of the Western blot bands was acquired using an Epson 4500 gel scanner. The developed films were scanned as a Tiff scale in eight-bit grayscale format at setting of 300 dpi. All of the films obtained from the separate experiments of different genotypes were acquired in the same way and scanned in parallel with the calibrated optical density step tablet from Stouffer. The images were analyzed using NIH ImageJ software (National Technical Information Service). The pixel values of the images were transformed to optical density values by the program using the calibration curve obtained by acquiring the calibrated tablet with the same parameters as those used for the images.

The immunoreactive bands were quantified in four separate experiments for both Hb and IPn. The optical density ratio was calculated by taking the optical density of the wild-type β 3 as 1. The values are the mean $\pm$ SEM of four separate experiments for each tissue for each genotype.

Release experiments. For both [3 H]ACh release and 86 Rb $^+$ efflux, crude synaptosomes were prepared from freshly dissected IPn and Hb. For [3 H]ACh release experiments, uptake of [3 H]choline into crude IPn synaptosomes as well as perfusion and release procedures of Grady et al. (2001) were used with the following modifications. Perfusion speed was 0.7 ml/min, with a 10 min wash period before stimulation with ACh or high potassium for 20 s. Fractions (10 s) were collected into 96-well plates using a Gilson FC204 fraction collector with multicolumn adapter, and radioactivity was determined after addition of 150 μ l of Optiphase Supermix mixture using a 1450 Microbeta scintillation counter (both from PerkinElmer Life and Analytical Sciences). Instrument efficiency was 20%. Baseline counts per minute were subtracted from peak samples, and then peak samples were summed and normalized to baseline to give units of release.

For 86 Rb $^+$ efflux experiments, the methods of Marks et al. (2002) were followed for uptake, superfusion, and detection of efflux. For these experiments, crude synaptosomes were prepared from IPn and Hb of β 2 and β 4 subunit null mutant genotypes (+/+ , +/- , -/-).

Results

Because rats have been consistently used for behavioral and lesioning studies of the Hb–IPn system and mice for the availability of genetically modified animals, we analyzed in detail the nAChR subtype content and composition of Hb and IPn in both species.

Subunit composition of nAChRs in rat Hb

We first determined the proportions of α Bgtx-sensitive ([125 I] α Bgtx binding) and α Bgtx-insensitive ([3 H]Epi binding) in the presence of 2 μ M α Bgtx) nAChRs in homogenates of rat Hb.

Figure 1. Subunit composition of rat Hb and IPn nAChRs before and after immunodepletion of β 2* receptors and mouse Hb and IPn nAChR from wild-type and β 2 subunit null mutant mice. Two percent Triton X-100 extracts from rat before (white) and after (gray) immunodepletion of β 2* receptors were preincubated with 2 nM [3 H]Epi and immunoprecipitated with the indicated subunit-specific Abs as described in Materials and Methods using saturating concentrations (20 μ g) of anti-subunit Abs. The amount immunoprecipitated by each antibody was subtracted from the value obtained in control samples containing an identical concentration of normal rabbit IgG, and the results are expressed as femtomoles of immunoprecipitated labeled [3 H]Epi nAChR per milligram of protein. Results are the mean $\pm$ SEM values of four to five experiments performed in triplicate per antibody. Rat extracts are shown in **A** and **B**. Extracts from solubilized mouse +/+ (white) and -/- (black) Hb and IPn are shown in **C** and **D**. Statistical analysis was performed by *t* test. Each Ab in control animals was compared with the same Ab tested in the β 2*-depleted extract by means of paired Student's *t* test, **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

[125 I] α Bgtx binding (33.0 ± 13.2 fmol/mg protein) was much less than [3 H]Epi binding (461.0 ± 26.2 fmol/mg protein).

To determine the subunit composition of the α Bgtx-insensitive nAChRs in the Hb, we immunoprecipitated [3 H]Epi-labeled receptors with subunit-specific Abs. Figure 1A and supplemental Table 2 (available at www.jneurosci.org as supplemental material) show the mean immunoprecipitation values obtained in four to five separate experiments for each subunit. By quantifying the number of receptors immunoprecipitated by the specific Ab as the percentage of the total number of [3 H]Epi receptors, we found that adult rat Hb contains detectable levels of almost all of the α Bgtx-insensitive nAChR subunits: high levels of α 3, α 4, β 2, β 3, and β 4, moderate levels of α 5, and no statistically significant levels of α 2 and α 6 subunits. The sum of the immunoprecipitated β 2* and β 4* (femtomoles per milligram of protein) [3 H]Epi-labeled receptors (414) was very close to the total number of [3 H]Epi labeled receptors (385) in the total extract, thus suggesting that β 2* and β 4* nAChRs are two primarily independent receptor populations in the Hb.

To examine the composition of the β 2* and β 4* receptor populations, we immunodepleted the habenular extract of β 2*

Figure 2. Immunoprecipitation analysis of the subunit content of purified $\beta 2^*$ nAChRs from Hb and IPn of rat. Extracts prepared from control rat Hb or IPn were incubated on an affinity column with bound anti- $\beta 2$ CYT Abs (see Materials and Methods) to bind the $\beta 2^*$ population, which was eluted from the column by means of incubation with the $\beta 2$ CYT peptide. The recovered $\beta 2^*$ nAChRs were labeled with 2 nM [3 H]Epi and then immunoprecipitated by the indicated subunit-specific Abs. The results are expressed as the percentage of the immunoprecipitation obtained with the $\beta 2$ Abs and are the mean $\pm$ SEM values of two to three immunoprecipitation experiments from each tissue.

receptors using an affinity column bearing an anti- $\beta 2$ antibody. Selective $\beta 2^*$ nAChR immunodepletion was confirmed by the fact that the number of immunoprecipitated $\beta 2$ -containing [3 H]Epi-labeled receptors decreased from $>60\%$ in the total habenular extract to $<1\%$ in the flow through of the anti- $\beta 2$ antibody column (Fig. 1A) (supplemental Table 2, available at www.jneurosci.org as supplemental material). To identify the subunit composition of the $\beta 4^*$ receptors, we immunoprecipitated [3 H]Epi-labeled receptors from the flow through of the anti- $\beta 2$ column by means of subunit-specific antisera. The flow through showed a considerable decrease in the number of the $\alpha 4^*$ and $\alpha 5^*$ receptors, a significant but more modest reduction in $\beta 3^*$ receptors, but no significant reduction in the number of $\alpha 3^*$ and $\beta 4^*$ receptors; the relative proportion of the sparsely expressed $\alpha 2^*$ and $\alpha 6^*$ receptors was only slightly modified (Fig. 1A) (supplemental Table 2, available at www.jneurosci.org as supplemental material) and did not differ significantly from zero.

Combining the results obtained from the total and $\beta 2$ -depleted rat Hb extracts, it can be concluded that the $\beta 2^*$ and $\beta 4^*$ nAChR populations are quantitatively similarly represented in Hb with (1) $\beta 2^*$ subunits contribute to 51% of the total habenular nAChRs, and 78% of these contain the $\alpha 4$ subunit (40% of the total), and 14% of the $\beta 2^*$ nAChRs were associated with the $\alpha 3$ subunits (7% of the total); and (2) $\beta 4^*$ subunits contribute to 49% of total habenular nAChRs, 88% of these contain the $\alpha 3$ subunit (43% of the total), and 24% of $\beta 4^*$ nAChRs were associated with the $\alpha 4$ subunits (12% of the total) (supplemental Table 2, available at www.jneurosci.org as supplemental material).

The $\beta 2^*$ and $\beta 4^*$ receptor populations of rat Hb both contain the accessory $\alpha 5$ and $\beta 3$ subunits but to different extents: higher levels of the $\alpha 5$ subunit are associated with the $\beta 2^*$ receptor (41% of $\beta 2^*$ vs 10% of $\beta 4^*$ nAChRs) and higher levels of the $\beta 3$ subunit with the $\beta 4^*$ receptors (45% of $\beta 4^*$ vs 26% of $\beta 2^*$ nAChRs).

Subunit composition of nAChRs in rat IPn

Binding studies revealed that the level of [3 H]Epi (974.1 ± 65.8 fmol/mg protein) was much higher than that of [125 I] α Bgtx binding (37.4 ± 5.2 fmol/mg protein) and also higher than the levels of [3 H]Epi binding in the habenular extract (see above).

Immunoprecipitation of [3 H]Epi-labeled receptors by means of subunit-specific antisera showed that adult rat IPn contains all of the nAChR subunits except $\alpha 6$: namely, high levels of $\alpha 3$, $\alpha 4$, $\beta 2$, $\beta 3$, and $\beta 4$, and moderate levels of $\alpha 2$ and $\alpha 5$ (Fig. 1B)

(supplemental Table 2, available at www.jneurosci.org as supplemental material). As in the Hb, the sum of the immunoprecipitated $\beta 2^*$ and $\beta 4^*$ [3 H]Epi-labeled receptors (742.8) was very close to the number of [3 H]Epi-labeled receptors in the total IPn extract (746.0), thus suggesting that $\beta 2^*$ and $\beta 4^*$ nAChRs are two primarily independent receptor populations in the IPn. However, unlike the Hb, the IPn has more $\beta 4^*$ than $\beta 2^*$ receptors (supplemental Table 2, available at www.jneurosci.org as supplemental material).

After immunodepleting the $\beta 2^*$ receptors, the immunoprecipitation of the flow through showed an almost total depletion of the $\alpha 2$ subunit, marked depletion of the $\alpha 4$ and $\alpha 5$ subunits, and a slight decrease in the number of receptors containing the $\alpha 3$, $\beta 3$, and $\beta 4$ subunits. There are, therefore, two main nAChR populations in the IPn: (1) $\beta 2$ subunits contribute to 38% of IPn nAChRs, and 35% of these are associated with the $\alpha 2$ subunit (13% of the total), 33% with the $\alpha 3$ subunit (13% of the total), and 54% with the $\alpha 4$ subunit (21% of the total); and (2) $\beta 4$ subunits contribute to 62% of IPn nAChRs, and of these 99% are associated with the $\alpha 3$ subunit (61% of the total) and 32% with the $\alpha 4$ subunit (20% of the total).

In addition, in the rat IPn, the $\beta 2^*$ and $\beta 4^*$ receptors are associated with $\alpha 5$ and $\beta 3$ accessory subunits: 46% of $\beta 2^*$ and 16% of $\beta 4^*$ nAChRs contain an $\alpha 5$ subunit, whereas 55% of the $\beta 4^*$ and 18% of $\beta 2^*$ nAChRs contain a $\beta 3$ subunit (supplemental Table 2, available at www.jneurosci.org as supplemental material) (see Fig. 6A).

Subunit composition of immunopurified rat $\beta 2^*$ nAChRs from Hb and IPn

To further characterize the possible $\beta 2^*$ receptor subtypes present in the Hb and IPn, we identified the subunits assembled with the $\beta 2$ subunit by immunoprecipitating the $\beta 2^*$ receptors bound by anti- $\beta 2$ antibodies. The captured $\beta 2^*$ nAChRs were eluted from the affinity column using an excess of the $\beta 2$ CYT peptide, labeled with [3 H]Epi, and then immunoprecipitated with subunit specific antisera. As shown in Figure 2, the subunit-specific Abs revealed that the mean $\pm$ SEM percentages ($n = 3$) of the purified [3 H]Epi-labeled $\beta 2^*$ nAChRs from rat Hb containing the different subunits were $1.6 \pm 1.1\%$ ($\alpha 2$), $26.6 \pm 11\%$ ($\alpha 3$), $85.4 \pm 3.2\%$ ($\alpha 4$), $26.6 \pm 14.7\%$ ($\alpha 5$), $4.0 \pm 4.0\%$ ($\alpha 6$), 100.0% ($\beta 2$), $23.5 \pm 5.8\%$ ($\beta 3$), and $13.5 \pm 2.4\%$ ($\beta 4$) (Fig. 2A).

This immunoprecipitation experiment qualitatively and quantitatively confirms that, in Hb, the $\beta 2$ subunit is principally associated with the $\alpha 4$ and to a much lower extent with the $\alpha 3$ subunit. Only a small fraction of the $\beta 2^*$ receptors have more than one principal ligand-binding subunit ($\alpha 2$, $\alpha 3$, $\alpha 4$, or $\alpha 6$). Levels of $\alpha 2$ and $\alpha 6$ subunits do not differ significantly from zero.

The same immunoprecipitation and immunoprecipitation experiments on the rat IPn showed that the mean $\pm$ SEM percentage ($n = 3$) of the purified [3 H]Epi-labeled $\beta 2^*$ nAChRs containing the different subunits were as follows: $26.9 \pm 3.8\%$ ($\alpha 2$), $38.7 \pm 16.0\%$ ($\alpha 3$), $60.7 \pm 7.4\%$ ($\alpha 4$), $35.9 \pm 5.6\%$ ($\alpha 5$), $0 \pm 0.0\%$ ($\alpha 6$), 100.0% ($\beta 2$), $20.9 \pm 6.3\%$ ($\beta 3$), and $36.8 \pm 8\%$ ($\beta 4$) (Fig. 2B). This immunoprecipitation experiment established that the recovery of the $\alpha 2$, $\alpha 3$, and $\alpha 4$ ligand-binding subunits was higher than that of the $\beta 2$ subunit alone, thus indicating the

presence of receptors with a complex subunit composition such as $\alpha 2\alpha 4\beta 2^*$, $\alpha 2\alpha 3\beta 2^*$, and/or $\alpha 3\alpha 4\beta 2^*$. However, the complexity of these receptors is even greater because some of them also contain the accessory subunits $\alpha 5$ and $\beta 3$, and, in addition, a fraction appears to contain the $\beta 4$ subunit.

Subunit composition of nAChRs in mouse Hb and IPn

When the number of receptors immunoprecipitated by the specific Abs was expressed as femtomoles per milligram of protein, we found that, similar to rat, Hb of adult wild-type mouse contains high levels of $\alpha 3$, $\alpha 4$, $\beta 2$, $\beta 3$, and $\beta 4$, and low levels of $\alpha 2$ and $\alpha 6$. A difference in expression was noted for $\alpha 5$, which was markedly less concentrated in mouse than in rat Hb (9% in mouse and 28% in rat) (Fig. 1A,C).

To distinguish the $\beta 2^*$ and $\beta 4^*$ receptors, we compared immunoprecipitation experiments using subunit-specific antibodies in both $\beta 2^{-/-}$ and $\beta 2^{+/+}$ mice (Fig. 1C) (supplemental Table 3, available at www.jneurosci.org as supplemental material). As in the case of $\beta 2$ -immunodepleted extracts of rat Hb, mouse $\beta 2^{-/-}$ Hb extracts were devoid of $\beta 2$ as expected, and $\alpha 4$ (–90%) and $\beta 3$ (–30%) subunits were significantly decreased. Similarly to rat, levels of $\alpha 2$, $\alpha 3$, $\alpha 6$, and $\beta 4$ were not significantly changed. Unlike the rat $\beta 2$ -immunodepleted extracts, in which significant decrease in the $\alpha 5$ subunits was observed, no statistically significant decrease in $\alpha 5$ was observed in $\beta 2^{-/-}$ mice.

We repeated the same approach using $\beta 2^{+/+}$ and $\beta 2^{-/-}$ IPn extracts, and we found that the $\beta 2^*$ and $\beta 4^*$ population were present at similar levels in the $+/+$, whereas, as expected, there was no $\beta 2^*$ population remaining in the $\beta 2^{-/-}$ extract. In addition, the $\beta 2^{-/-}$ extracts showed a selective loss of receptors containing the $\alpha 2$ (–100%), $\alpha 3$ (–47%), and $\alpha 4$ (–56%) subunits. This pattern differs from the rat in that $\alpha 5$ subunits are unchanged, whereas $\alpha 3$ subunits are decreased.

nAChR subtypes in rat and mouse Hb and IPn

Assuming the current stoichiometric rules on nAChR composition (Gotti et al., 2007) and the near 100% immunoprecipitating efficacy of our subunit-specific antibodies (for caveats, see Materials and Methods), some additional quantitative deductions can be made (supplemental Tables 2, 3, available at www.jneurosci.org as supplemental material) (see Fig. 6A,B); note that, when two percentages are in brackets, the first refers to rat and the second to mouse.

Habenula

In both rat and mouse Hb, $\beta 2^*$ nAChRs consist of a major $\alpha 4\beta 2^*$ (78 and 79%), a minor $\alpha 3\beta 2$ (14 and 13%), and two very small $\alpha 2\beta 2^*$ and $\alpha 6\beta 2^*$ (<5%) populations. More than half of the $\beta 2^*$ receptors in rat Hb contain either $\alpha 5$ (40%) or $\beta 3$ (26%) accessory subunits, in mouse Hb $\beta 3$ subunit is present in 12% of $\beta 2^*$ receptors, whereas only a few $\beta 2^*$ receptors contain the accessory $\alpha 5$ subunit (7%).

In both rat and mouse Hb, $\beta 4^*$ nAChRs consist of a major $\alpha 3\beta 4^*$ (89 and 100%) and a smaller $\alpha 4\beta 4^*$ (24 and 17%) population. Because the sum of $\alpha 3\beta 4$ and $\alpha 4\beta 4$ clearly exceeds 100%, we hypothesize the presence of a mixed $\alpha 3\alpha 4\beta 4$ receptor population in both rat (13%) and mouse (17%) Hb. This implies, indeed, that (non- $\alpha 3$) $\alpha 4\beta 4^*$ receptors may not exist in mouse. Many of these receptors also contain $\beta 3$ (45 and 56%) or $\alpha 5$ (11 and 15%) accessory subunits.

Interpeduncular nucleus

In both rat and mouse IPn, there are significant populations of $\beta 2^*$ nAChRs with $\alpha 2$ (35 and 33%), $\alpha 3$ (33 and 47%), and $\alpha 4$ (54

and 44%) subunits. Very few of the $\beta 2^*$ nAChRs contain $\alpha 6$ subunits. Both rat and mouse IPn have large populations of $\beta 4^*$ nAChRs combined with $\alpha 3$ (99 and 67%) or $\alpha 4$ (32 and 44%) subunits. In addition, the mouse shows a minor $\alpha 6\beta 4^*$ population (12%). None of the $\beta 4^*$ nAChRs in the IPn contain the $\alpha 2$ subunit. Given that the sum of the ligand-binding α subunits ($\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 6$) is higher than the immunoprecipitation value of the $\beta 2$ or $\beta 4$ subunits, we have to assume that some receptors contain two different ligand-binding interfaces and form complex subtypes. In addition, many $\beta 2^*$ receptors in rat IPn also contain $\alpha 5$ (47%), whereas none of the mouse $\beta 2^*$ nAChRs do. $\beta 3$ is at moderate to low level (18 and 8%) in $\beta 2^*$ nAChRs of both species. Many $\beta 4^*$ nAChRs of IPn also contain $\beta 3$ (55 and 83%) or $\alpha 5$ (16 and 35%) accessory subunits.

Effects of the genetic deletion of the $\beta 3$ subunit on nAChR subunit composition in the Hb and IPn

In situ hybridization studies have shown that the MHb expresses high levels of $\beta 3$ subunit mRNA (Deneris et al., 1989; Le Novère et al., 1996). The $\beta 3$ subunit is considered important not only for its role in determining the biophysical characteristics of the receptors containing this subunit (Broadbent et al., 2006; Kuryatov et al., 2008) but also for its role in determining assembly and/or transport of receptors (Gotti et al., 2005a; Drenan et al., 2008). Our immunoprecipitation and immunopurification studies showed that the Hb and IPn are enriched in $\beta 3^*$ nAChRs, particularly an $\alpha 3\beta 3\beta 4^*$ subtype that accounts for more than half of the $\beta 4^*$ nAChRs in the Hb and IPn of both rat and mouse. Therefore, in a series of experiments, we investigated the contribution of the $\beta 3$ subunit to the composition of nAChRs in the habenulo-interpeduncular system by means of immunoprecipitation and Western blotting studies of the Hb and IPn of $\beta 3^{+/+}$ and $\beta 3^{-/-}$ mice using subunit-specific antibodies.

Immunoprecipitation

The proportion of [3 H]Epi-labeled $\beta 3^*$ nAChR in the habenular extract was decreased from 26% of the total in the $\beta 3^{+/+}$ mice to only 1% in the $\beta 3^{-/-}$ mice; however, there was no significant difference in either the total [3 H]Epi-labeled extract or the relative amount of non- $\beta 3$ subunit-specific immunoprecipitates between $\beta 3^{+/+}$ and $\beta 3^{-/-}$ mice. However, although the proportion of immunoprecipitated [3 H]Epi-labeled $\beta 3^*$ nAChR in the IPn was 32% of the total in the $\beta 3^{+/+}$ mice and <1% in the $\beta 3^{-/-}$ mice, this decrease was paralleled by a decrease in [3 H]Epi labeling in total extract and in the relative content of several subunits: $\alpha 3$ (–39%), $\alpha 4$ (–26%), $\beta 2$ (–34%), and $\beta 4$ (–44%) subunits (Fig. 3A).

Western blotting

Samples obtained from the Hb and IPn of $\beta 3^{+/+}$ and $\beta 3^{-/-}$ mice were analyzed by loading the same amount of membrane protein (except for the $\beta 4$ sample in which the IPn protein loaded was half of Hb). Figure 3B shows the typical anti- $\alpha 3$, anti- $\alpha 4$, anti- $\beta 2$, anti- $\beta 3$, and anti- $\beta 4$ Ab labeling of samples.

To further define the difference in subunit composition between $\beta 3^{+/+}$ and $\beta 3^{-/-}$ mice and to compare the results obtained in immunoprecipitation and Western blotting, the $\alpha 3$, $\alpha 4$, $\beta 2$, $\beta 3$, and $\beta 4$ subunit signal intensity in the Hb and IPn of the two genotypes were expressed as the ratio of Ab labeling (taking the amount present in the samples from wild-type mice as 1.0) (Fig. 3C). The results are from four independent experiments using three separate membrane preparations. The immunoprecipitation and Western blotting data showed that, in the Hb,

Figure 3. Characterization of the nAChR subunit content of the Hb and IPn from $\beta 3^{+/+}$ and $\beta 3^{-/-}$ mice. **A**, Subunit composition of mouse habenula and nucleus interpeduncularis of $\beta 3^{+/+}$ and $\beta 3^{-/-}$ mice determined by immunoprecipitation. Results are the mean $\pm$ SEM values of three to four experiments performed in triplicate per antibody. Statistical analysis was performed by *t* test. Each Ab in $\beta 3^{+/+}$ mouse was compared with the same Ab tested in the $\beta 3^{-/-}$ extract by means of paired Student's *t* test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. **B**, The subunit content of habenula and nucleus interpeduncularis membranes from $\beta 3^{+/+}$ and $\beta 3^{-/-}$ mice was determined by Western blot analysis. The proteins (5 μ g) for all except the $\beta 4$ subunit (2.5 μ g) were separated on 9% acrylamide SDS gels, electrotransferred to nitrocellulose, probed with 1–2.5 μ g/ml of the indicated primary Abs, and then incubated with the secondary Ab (anti-rabbit conjugated to peroxidase; dilution, 1:40,000). The bound Abs were revealed by a chemiluminescent substrate (Pierce). **C**, Comparison of the quantitated results obtained in the habenula and nucleus interpeduncularis of $\beta 3^{+/+}$ and $\beta 3^{-/-}$ mice by immunoprecipitation (gray) and Western blotting (black). The ratios (mean values $\pm$ SEM) between $\beta 3^{+/+}$ (white) and $\beta 3^{-/-}$ measured by immunoprecipitation (IP; gray) and $\beta 3^{-/-}$ or Western blotting (WB; black) were calculated by taking the immunoprecipitation or optical density values of the wild type as 1 and are the results of three to four separate experiments for each tissue and genotype. Statistical analysis was performed by paired *t* test. Each Ab in $\beta 3^{+/+}$ was compared with the same Ab tested in the $\beta 3^{-/-}$ mice by means of paired Student's *t* test, * $p < 0.05$; ** $p < 0.01$. KO, Knock-out.

there were no significant changes in the levels of any of the subunits besides $\beta 3$: the mean $\pm$ SEM optical density ratios of the subunits were 1.23 ± 0.11 ($\alpha 3$ subunit), 1.11 ± 0.08 ($\alpha 4$ subunit), 1.12 ± 0.11 ($\beta 2$ subunit), and 1.18 ± 0.09 ($\beta 4$ subunit). However, in the case of the IPn, the ratios $\pm$ SEM were as follows: 0.68 ± 0.09 ($\alpha 3$ subunit, $p = 0.006$), 0.82 ± 0.05 ($\alpha 4$ subunit, $p = \text{ns}$), 0.71 ± 0.05 ($\beta 2$ subunit, $p = 0.03$), and 0.42 ± 0.07 ($\beta 4$ subunit, $p = 0.0013$), thus indicating a significant decrease in the amount of receptors containing these subunits. Immunoprecipitation

and Western blotting approaches are compared in Figure 3C and found to give highly concordant results.

In conclusion, our data showed that the lack of the $\beta 3$ subunit differentially affects the levels of the nAChR subtypes in the Hb and IPn: there is no change in the level of heteromeric receptors in the Hb, but a significant decrease is seen in the IPn, of mainly $\alpha 3\beta 4^*$ receptors with a lesser decrease in $\alpha 4\beta 2^*$.

Effects of the genetic deletion of $\alpha 2$, $\alpha 4$, $\alpha 5$, $\alpha 6$, $\alpha 7$, $\beta 2$, $\beta 3$, or $\beta 4$ subunits on acetylcholine release from IPn synaptosomes

Previously published data have shown that the nAChR mediating release of ACh from IPn synaptosomes is not affected by the $\beta 2$ null mutation (Grady et al., 2001) or by the $\alpha 3/\alpha 6\beta 2^*$ -selective antagonist α -conotoxin MII, but it is decreased by treatment with the $\alpha 3\beta 4^*$ -selective antagonist α -conotoxin Au1B (Luo et al., 1998; Grady et al., 2001). We extended this analysis to include the subunit null mutations for $\alpha 2$, $\alpha 4$, $\alpha 5$, $\alpha 6$, $\alpha 7$, $\beta 3$, and $\beta 4$ and repeated that of $\beta 2$ for purpose of comparison. Figure 4A shows the results for the $\beta 2$ and $\beta 4$ subunits, as well as for the accessory subunits $\alpha 5$ and $\beta 3$. As expected, the $\beta 2$ null mutation did not affect the release of [^3H]ACh from the IPn. Furthermore, the almost complete loss of release in the $\beta 4$ null mutants supported previous indications that the relevant receptor was likely of the $\alpha 3\beta 4^*$ subtype. Surprisingly, the $\alpha 5$ null mutation had no effect, whereas the $\beta 3$ homozygous as well as heterozygous null mutants at all concentrations showed significantly decreased [^3H]ACh release. The $\alpha 2$, $\alpha 4$, $\alpha 6$, and $\alpha 7$ subunit null mutations showed no differences among genotypes (Fig. 4B). These results indicate that ACh release in the IPn is mediated by the $\alpha 3\beta 3\beta 4$ and $\alpha 3\beta 4$ subtypes.

Effects of the genetic deletion of $\beta 2$ or $\beta 4$ subunits on $^{86}\text{Rb}^+$ efflux from Hb and IPn synaptosomes

To assess whether the two major subclasses $\beta 2^*$ and $\beta 4^*$ nAChRs are functional at pre-synaptic sites in Hb and IPn at terminals other than the cholinergic terminals described above, we made use of the $^{86}\text{Rb}^+$

efflux assay from synaptosomes prepared from these regions of mice with the $\beta 2$ or $\beta 4$ subunit null mutations. Although this assay does not determine which neurotransmitter is released, it does indicate which subtypes of nAChR are functional at terminals in the region assayed. Results (Fig. 5) indicate that, in the Hb, functional $\beta 2^*$ nAChRs are found, whereas there are few or no functional $\beta 4^*$ nAChRs. Evidence for function of both major subtypes is seen in the IPn synaptosomal preparation, in which significant decreases in $^{86}\text{Rb}^+$ efflux were found for both null mutations. A portion of the

functional $\beta 4^*$ nAChRs are likely those on cholinergic terminals measured with the [^3H]ACh release assay, although receptors of this subclass may be present on other terminals as well.

Discussion

Native subtypes in the habenulo–interpeduncular system of rats and mice

In this study, Hb and IPn were found to express two major and distinct nAChR populations, $\beta 2^*$ and $\beta 4^*$, with minor populations of $\alpha 7$. The diagrams in Figure 6 summarize the nAChR composition in Hb (A) and IPn (B) determined by quantitative immunoprecipitation.

The major $\beta 2^*$ nAChR expressed in Hb of both rats and mice is $\alpha 4\beta 2^*$ with minor amounts of $\alpha 3\beta 2^*$ nAChR (Fig. 6A). A significant fraction of $\beta 2^*$ receptors in rat contain accessory subunits, $\alpha 5$ or $\beta 3$. Because only one accessory position exists per receptor (Kuryatov et al., 2008), these two accessory subunits are mutually exclusive, and their presence indicates even greater nAChR heterogeneity. In a distinction between species, the mouse $\beta 2^*$ nAChRs in Hb include fewer accessory subunits.

In IPn of both species, $\beta 2^*$ nAChRs exist as three approximately equal populations of $\alpha 2\beta 2^*$, $\alpha 3\beta 2^*$, and $\alpha 4\beta 2^*$ (Fig. 6B), with a small fraction containing $\beta 4$ subunits. Restriction of $\alpha 2\beta 2^*$ to IPn agrees with previous *in situ* hybridization and reverse transcription-PCR studies showing localization of $\alpha 2$ mRNA in IPn and its absence in Hb (Wada et al., 1989; Marks et al., 1992; Sheffield et al., 2000). Detection of $\alpha 3\beta 2^*$ receptors in both regions agrees with decreases in α -conotoxin MII binding seen there in $\alpha 3^{-/-}$ mice (Whiteaker et al., 2002). Significant $\alpha 5$ was found in $\beta 2^*$ nAChRs in rat IPn, whereas $\alpha 5$ is absent in this population in mouse. For both species, low levels of $\beta 3$ were associated with $\beta 2^*$ nAChR in IPn.

The $\beta 4^*$ nAChR population, in both regions and species, is mainly associated with $\alpha 3$, in agreement with previously reported autoradiography studies in null mutant mice (Zoli et al., 1998; Marks et al., 2002; Whiteaker et al., 2002). Many of the $\beta 4^*$ nAChRs contain accessory subunits; more have $\beta 3$ than $\alpha 5$.

$\alpha 6$ subunit was detectable in mouse IPn but absent in rat. $\alpha 6^*$ nAChRs were not significantly decreased in $\beta 2^{-/-}$ mice but disappeared in IPn of $\beta 3^{-/-}$ mice, thus indicating a small population of $\alpha 6\beta 3\beta 4^*$ nAChRs exists in mouse IPn.

Although most $\beta 2$ and $\beta 4$ subunits form separate receptor populations, some $\beta 2\beta 4^*$ nAChRs exist in both regions (Fig. 2). Electrophysiological studies in rat Hb also presented evidence for

Figure 4. Effect of nAChR subunit null mutations on [^3H]acetylcholine release from IPn synaptosomes. [^3H]ACh release from IPn synaptosomes of $\beta 3^{+/+}$ (white bars), $\beta 3^{+/-}$ (gray bars), and $\beta 3^{-/-}$ (black bars) mice was stimulated by ACh. Release stimulated by 50 mM potassium did not differ by strain or genotype (mean $\pm$ SEM for K^+ -stimulated release, 31.4 ± 0.8). In addition, no significant differences were seen among $+/+$ mice for the different concentrations of ACh by strain. Data shown in A from $n = 6 \beta 2^{+/+}$, $n = 6 \beta 2^{+/-}$, $n = 6 \beta 2^{-/-}$, $n = 5 \beta 4^{+/+}$, $n = 7 \beta 4^{+/-}$, $n = 6 \beta 4^{-/-}$, $n = 11 \beta 3^{+/+}$, $n = 11 \beta 3^{+/-}$, and $n = 11 \beta 3^{-/-}$ mice, and in B from $n = 5 \alpha 2^{+/+}$, $n = 5 \alpha 2^{+/-}$, $n = 5 \alpha 2^{-/-}$, $n = 6 \alpha 4^{+/+}$, $n = 7 \alpha 4^{+/-}$, $n = 7 \alpha 4^{-/-}$, $n = 7 \alpha 6^{+/+}$, $n = 7 \alpha 6^{+/-}$, $n = 8 \alpha 6^{-/-}$, $n = 5 \alpha 7^{+/+}$, $n = 5 \alpha 7^{+/-}$, and $n = 5 \alpha 7^{-/-}$ mice. Significant differences by one-way ANOVA with Tukey's *post hoc* test are indicated by * for different from both $+/+$ and $+/-$ of same strain or + for different from $+/+$. No significant differences were found for the $\beta 2$, $\alpha 5$, $\alpha 2$, $\alpha 4$, $\alpha 6$, or $\alpha 7$ genotypes. For the $\beta 4$ null mutation, the $+/-$ mice did not differ from the $+/+$; however, the $-/-$ mice differed significantly from both the $+/+$ and $+/-$ mice ($F_{(2,15)} = 13.30$, $p < 0.05$; $F_{(2,15)} = 12.15$, $p < 0.05$; $F_{(2,15)} = 16.00$, $p < 0.05$ for 10, 30, and 100 μM ACh, respectively). Significant differences were also seen for the $\beta 3$ genotypes. The $-/-$ mice differed from both the $+/+$ and $+/-$ mice at the two lower ACh concentrations and from the $+/+$ at the highest concentration, whereas in addition, the $+/-$ mice differed from the $+/+$ at all concentrations ($F_{(2,30)} = 18.10$, $p < 0.05$; $F_{(2,30)} = 18.10$, $p < 0.05$; $F_{(2,30)} = 6.98$, $p < 0.05$ for 10, 30, and 100 μM ACh, respectively).

$\beta 2\beta 4^*$ nAChRs (Quick et al., 1999). Functional measures at individual cells may detect smaller populations of a subtype than methods applied here.

Location and function of subtypes in IPn

The numerous cholinergic terminals in the IPn are mainly projections from Hb through the fr (Clarke et al., 1986). Our

Figure 5. Effect of nAChR subunit null mutations on $^{86}\text{Rb}^+$ efflux from Hb and IPn synaptosomes. $^{86}\text{Rb}^+$ efflux from IPn synaptosomes of the $\beta 2$ and $\beta 4$ nAChR subunit +/+ (white bars), +/- (gray bars), and -/- (black bars) mice was stimulated by ACh. Data shown are from $n = 5$ $\beta 2$ +/+, $n = 5$ $\beta 2$ +/-, $n = 4$ $\beta 2$ -/-, $n = 5$ $\beta 4$ +/+, $n = 5$ –6 $\beta 4$ +/-, and $n = 4$ –5 $\beta 4$ -/- mice. Statistical analysis according to one-way ANOVA followed by Tukey's *post hoc* test; * $p < 0.05$ vs both +/+ and +/- mice of the same strain; + $p < 0.05$ vs +/+ mice. No significant differences in ACh-stimulated $^{86}\text{Rb}^+$ efflux were seen between $\beta 2$ +/+ and $\beta 4$ +/+ mice. For the $\beta 2$ null mutation with Hb synaptosomes, the $\beta 2$ -/- mice did not differ from the $\beta 2$ +/+ mice; however, the $\beta 2$ +/- mice differed significantly from both the $\beta 2$ +/+ and $\beta 2$ -/- mice ($F_{(2,11)} = 36.04$, $p < 0.001$; $F_{(2,11)} = 60.77$, $p < 0.001$; $F_{(2,11)} = 36.98$, $p < 0.001$ for 3, 30, and 300 μM ACh, respectively). Significant differences were also seen for the $\beta 2$ genotypes in IPn. The $\beta 2$ -/- mice differed from the $\beta 2$ +/+ mice at the two lower ACh concentrations ($F_{(2,11)} = 8.19$, $p < 0.01$; $F_{(2,11)} = 5.81$, $p < 0.05$; $F_{(2,11)} = 1.28$, NS, for 3, 30, and 300 μM ACh, respectively). No significant differences were found for the $\beta 4$ genotypes in Hb; however, $\beta 4$ gene deletion reduced $^{86}\text{Rb}^+$ efflux in IPn at the two higher ACh concentrations ($F_{(2,13)} = 0.79$, NS; $F_{(2,13)} = 5.70$, $p < 0.05$; $F_{(2,13)} = 6.27$, $p < 0.05$ for 3, 30, and 300 μM ACh, respectively).

[^3H]ACh release studies on these terminals clearly indicate that presynaptic $\beta 4^*$ nAChRs, but not $\beta 2^*$ nAChRs, modulate ACh release. Although deletion of $\alpha 2$, $\alpha 4$, $\alpha 5$, $\alpha 6$, or $\alpha 7$ subunits had no effect on nAChR-mediated ACh release, deletion of $\beta 3$ subunits reduced this response $\sim 50\%$. Deletion of $\beta 3$ also decreased expression of $\alpha 3$ and $\beta 4$ subunits by a similar amount (Fig. 3), establishing the presence of presynaptic $\alpha 3\beta 3\beta 4$ and $\alpha 3\beta 4$ controlling ACh release in the IPn.

Our immunochemical and functional experiments demonstrate that the IPn contains sizeable populations $\alpha 2\beta 2^*$, $\alpha 3\beta 2^*$, and $\alpha 4\beta 2^*$ nAChRs. $\alpha 2\beta 2^*$ nAChRs are expressed by intrinsic IPn neurons because, among the relevant nuclei, $\alpha 2$ mRNA is expressed only by the IPn (Wada et al., 1989). These are possibly the postsynaptic $\beta 2^*$ nAChRs identified previously using electrophysiological methods (Brown et al., 1983; Clarke et al., 1986; Mülle et al., 1991; Dineley-Miller and Patrick, 1992).

$\alpha 3\beta 2^*$ nAChRs must be located presynaptically in IPn because $\alpha 3$ mRNA is not expressed there. The afferents likely originate from MHb, highly enriched in $\alpha 3$ mRNA (Wada et al., 1989; Le Novère et al., 1996).

$\alpha 4\beta 2^*$ nAChRs could be expressed on intrinsic IPn neurons and/or on afferents to the IPn, and, therefore, could have both presynaptic and postsynaptic locations. Besides the massive input from the MHb, afferents project to the IPn from several other nuclei (Herkenham and Nauta, 1979; Groenewegen et al., 1986;

Shibata et al., 1986; Araki et al., 1988). These nuclei all express $\alpha 4$ and $\beta 2$ mRNAs (Wada et al., 1989) and may, in principle, be the origin of $\alpha 4\beta 2^*$ nAChRs. Nevertheless, MHb is likely the primary source of presynaptic $\alpha 4\beta 2^*$ nAChRs in IPn. Indeed, we measured decreases in levels of $\alpha 4$ and $\beta 2$ subunits in the IPn of $\beta 3$ -/- mice. These $\alpha 4\beta 2\beta 3$ nAChRs are likely synthesized in the MHb and transported to the IPn, because $\beta 3$ mRNA is not expressed in the IPn but is highly enriched in the MHb (Le Novère et al., 1996; Cui et al., 2003). This conclusion is consistent with the observation that bilateral habenular lesions decrease [^3H]nicotine binding sites (now known to be $\alpha 4\beta 2^*$) in the rat IPn by 35% (Clarke et al., 1986).

Our Rb^+ efflux results provide evidence that presynaptic $\beta 2^*$ nAChRs in IPn are functional, but $\beta 2^*$ nAChRs do not mediate ACh release. $\beta 2^*$ nAChRs may modulate release of other neurotransmitters because the IPn receives important noncholinergic inputs (Klemm, 2004; Lecourtier and Kelly, 2007). Although we have not yet identified which neurotransmitter(s) are released by $\beta 2^*$ nAChR, electrophysiological studies on IPn neurons suggest glutamate as a likely candidate (Girard et al., 2000).

In Hb synaptosomes, Rb^+ efflux indicates that $\beta 2^*$ nAChRs may be the only presynaptic functional subtype and may mediate release of several neurotransmitters here because dopaminergic (Phillipson and Pycock, 1982), GABA, glutamatergic (Meshul et al., 1998), and noradrenergic (Li et al., 1998) terminals have been identified in Hb.

Accessory subunits in $\alpha 3\beta 4^*$ nAChR populations

A significant fraction of the $\alpha 3\beta 4^*$ nAChR in the Hb and IPn of both rat and mouse contains the $\beta 3$ subunit. This is the first biochemical and functional characterization of this unique subtype from brain. This result was unexpected because peripheral $\alpha 3\beta 4^*$ nAChR, a well characterized subtype, contains $\alpha 5$ as accessory subunit (Conroy and Berg, 1995). $\beta 3$ subunits have been shown previously to be associated with the $\alpha 6\beta 2$ nAChR by immunopurification of mesostriatal dopaminergic and visual pathways (Zoli et al., 2002; Gotti et al., 2005b). The presence of $\beta 3$ not associated with $\alpha 6$ subunits in the Hb–IPn pathway agrees with *in situ* hybridization showing high levels of $\beta 3$ but not $\alpha 6$ subunit mRNA in Hb (Le Novère et al., 1996; Champtiaux et al., 2003; Cui et al., 2003). $\beta 3$ subunit mRNA is absent in IPn (Deneris et al., 1989; Cui et al., 2003); therefore, nAChRs containing $\beta 3$ are not synthesized in the IPn. The $\beta 3$ subunit may have a targeting role as suggested previously (Gotti et al., 2007; Kuryatov et al., 2008).

$\alpha 3\beta 3\beta 4$ nAChR has been expressed in oocytes (Groot-Kormelink et al., 1998; Boorman et al., 2000) in which functional studies indicate that incorporation of $\beta 3$ decreases mean channel open time and burst length with only minor changes in calcium permeability and pharmacological properties (Boorman et al.,

Figure 6. Diagrams reporting the content of nAChR subtypes in percentage of the total number of nAChRs in the rat and mouse Hb (**A**) and IPn (**B**). Note that, in the instances of $\beta 2^*$ nAChRs of rat and mouse interpeduncular nucleus and $\beta 4^*$ nAChRs of mouse interpeduncular nucleus, the sum of the α subunits is larger than the total number of receptors. In these cases, the existence of subtypes with different ligand-binding interfaces must be postulated, although present analysis does not allow their identification.

2003). Expression of human $\beta 3$ with $\alpha 3$ and $\beta 4$ subunits in oocytes resulted in only small changes in surface expression but suppressed function by $\sim 65\%$ (Broadbent et al., 2006). In our studies on $\alpha 3\beta 4$ and $\alpha 3\beta 3\beta 4$ nAChRs mediating [^3H]ACh release in mouse IPn, deletion of $\beta 3$ subunits decreased function by $\sim 50\%$, with no obvious change in EC_{50} . Absence of $\beta 3$ subunits also decreased the amount of $\alpha 3$ and $\beta 4$ subunits in IPn by $\sim 50\%$. It appears that absence of accessory subunit $\beta 3$ has not changed function as expected from oocyte studies using human sequences (Broadbent et al., 2006). Perhaps distribution of receptors normally localized at cholinergic terminals differs. Alternatively, the unchanged calcium permeability and pharmacology of these subtypes may be more relevant for this assay, or mouse may differ significantly from human.

Pathophysiology of $\alpha 3\beta 4^*$ nAChR subtypes

Nicotine can have both anxiolytic and anxiogenic effects. Behavioral studies indicate that subunit deletion of either $\beta 3$ or $\beta 4$ decreases anxiety-like behavior (Salas et al., 2003; Booker et al., 2007), suggesting that $\beta 3^*$ and $\beta 4^*$ nAChRs are involved in neuronal processes associated with anxiety. Our studies show that $\beta 4$ and $\beta 3$ subunits are not only colocalized but also coassembled in Hb and IPn and that this $\alpha 3\beta 3\beta 4$ nAChR modulates ACh release in the IPn. The IPn modulates impulses descending from the limbic forebrain to the source of serotonergic mesencephalic projections, a pathway implicated in anxiety and depression. Our data may link the $\alpha 3\beta 3\beta 4$ nAChR with the control of anxiety exerted by the IPn through the serotonergic pathways (Klemm, 2004).

Components of the Hb–IPn system are thought to play a major role in the physiology and pathophysiology of reward phenomena (see Introduction). In particular, a strong case has recently been made that $\alpha 3\beta 4^*$ nAChRs in the Hb–IPn system play

an important role in mediating self-administration of morphine and other addictive drugs (Glick et al., 2006; Taraschenko et al., 2007).

$\alpha 3\beta 4^*$ nAChRs have a restricted expression pattern in brain but are highly expressed in the Hb and IPn. Other uncommon nAChR subtypes ($\alpha 2\beta 2^*$, $\alpha 4\beta 3\beta 2^*$, $\alpha 3\beta 3\beta 4^*$, or $\alpha 6\beta 3\beta 4^*$) appear unique to this system. Although the role of $\alpha 3\beta 3\beta 4$ receptors in the Hb–IPn is partially defined, the anatomy and functions of the wide variety of subtypes described in this study remain almost unknown. Studies with cellular and subcellular anatomical approaches, as well as additional functional studies, will be necessary to clarify this complex issue. The characterization of nAChR subtypes present in the Hb–IPn system may be an important step in the development of new therapies for neuropsychiatric diseases, such as drug dependence, depression, and anxiety disorders.

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