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TEMPORALLY- AND SPATIALLY-REGULATED TRANSCRIPTIONAL ACTIVITY OF THE NICOTINIC ACETYLCHOLINE RECEPTOR β4 SUBUNIT GENE PROMOTER

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Abstract—Signaling through nicotinic acetylcholine (nACh) receptors underlies a diverse array of behaviors. In order for appropriate signaling to occur via nACh receptors, it is necessary for the genes encoding the receptor subunits to be expressed in a highly regulated temporal and spatial manner. Here we report a transgenic mouse approach to characterize the transcriptional regulation of the gene encoding the nACh receptor β4 subunit. nACh receptors containing this subunit play critical roles in both the central and peripheral nervous systems. We demonstrate that a 2.3-kilobase pair fragment of the β4 5′-flanking region is capable of directing reporter gene expression in transgenic animals. Importantly, the transcriptional activity of the promoter region is cell-type-specific and developmentally regulated and overlaps to a great extent with endogenous β4 mRNA expression. These data indicate that the 2.3-kilobase pair fragment contains transcriptional regulatory elements critical for appropriate β4 subunit gene expression.

Key words: nicotinic receptor, gene expression, transcription.

Signaling through neuronal nicotinic acetylcholine (nACh) receptors underlies several fundamental biological processes both during development and in the adult (Albuquerque et al., 2009). In the CNS, presynaptic nACh receptors modulate release of most classical neurotransmitters including norepinephrine, ACh, glutamate and GABA (Albuquerque et al., 2009; Dani and Bertrand, 2007; Engelman and MacDermott, 2004). Postsynaptic nACh receptors are intimately involved in fast ACh-mediated synaptic transmission in addition to activity-dependent gene expression, which is critical for synaptic plasticity (Albuquerque et al., 2009; Dani and Bertrand, 2007; Hu et al., 2002; Ji et al., 2001). Within the peripheral nervous system, nACh receptors mediate fast excitatory transmission in most, if not all, autonomic ganglia and are involved in modulating visceral and somatic sensory transmission (Genzen et al., 2001; Hu and Li, 1997; Steen and Reeh, 1993; Sucher et al., 1990; Wang et al., 2002). More recently, numerous studies have revealed the expression of nACh receptors on non-neuronal cells and evidence is accumulating indicating that the receptors play crucial roles in signal transduction underlying many physiological processes outside the nervous system (Gahring and Rogers, 2006; Spindel, 2003; Wessler and Kirkpatrick, 2008). The importance of nACh receptor-mediated signaling is reflected in the many pathologies in which cholinergic signal transduction is compromised. For example, significant alterations in nACh receptor expression and function have been documented in several diseases such as Alzheimer's disease, autosomal dominant nocturnal frontal lobe epilepsy, schizophrenia, Parkinson's disease, Tourette's disease and megacystis-microcolon-intestinal hypoperistalsis syndrome (De Fusco et al., 2000; Isacson et al., 2002; Lena and Changeux, 1997; Perl et al., 2003; Perry et al., 2001; Richardson et al., 2001; Silver et al., 2001; Steinlein et al., 1995; Teaktong et al., 2003; Whitehouse et al., 1988). In addition, nACh receptors are key players in the initial steps and subsequent downstream health consequences of nicotine addiction (Kedmi et al., 2004; Laviolette and van der Kooy, 2004). Significantly, there is a growing awareness that nACh receptors may directly contribute to the pathogenesis of lung cancer (Catassi et al., 2008; Egleton et al., 2008; Schuller, 2008, 2009; Song et al., 2008).

Neuronal nACh receptors are pentameric ligand-gated ion channels assembled from a family of subunits that include α2–α10 and β2–β4 (Albuquerque et al., 2009). The α2–α6 subunits can form functional receptors in combination with the β2–β4 subunits while the α7–α10 subunits are

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Abbreviations: β-Gal, β-galactosidase; CDRG, cervical dorsal root ganglion; Cg/Rs, cingulate/retrosplenial cortex; CNR4, conserved noncoding region 4; Cpu, caudate putamen; CRG, cervical root ganglion; DG, dentate gyrus; DRG, dorsal root ganglion; ED, embryonic day; FrA, frontal association cortex; GFAP, glial fibrillary acid protein; IZ, intermediate zone; LHb, lateral habenula; LRG, lumber root ganglion; M, medulla oblongata; MAR, matrix attachment region; MHb, medial habenula; miRNAs, microRNAs; nACh, nicotinic acetylcholine; NeuN, neuron-specific nuclear marker; NGF, nerve growth factor; NLS, nuclear localization signal; P, pons; PBS, phosphate-buffered saline; PD, postnatal day; Pir, piriform cortex; PMCo, posteromedial cortical amygdaloid nucleus; PV, paraventricular thalamic nucleus; VA, ventral anterior thalamic nucleus; VZ, ventricular zone.

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capable of forming homomeric nACh receptors. In addition, the α9 and α10 subunits form unique heteromeric receptors (Elgoyhen et al., 2001; Lustig et al., 2001; Taranda et al., 2009) as do the α7 and β2 subunits (Liu et al., 2009). Importantly, each nACh receptor subtype exhibits distinct electrophysiological and pharmacological properties, reflecting the broad array of signaling pathways in which nACh receptors are involved (Albuquerque et al., 2009). The functional diversity exhibited by the neuronal nACh receptor family is a consequence, in part, of the differential expression of the various subunit genes leading to the incorporation of distinct subunits into mature receptors. A major goal in the field is to uncover the molecular mechanisms underlying the temporal and spatial expression of the nACh receptor subunit genes.

Several laboratories, including our own, have focused on elucidating the transcriptional mechanisms regulating expression of the α3, α5 and β4 subunit genes. These genes are particularly interesting from a regulatory point-of-view as they are tightly clustered in the genome (Boulter et al., 1990), perhaps reflecting their coordinate regulation. Several lines of evidence support this hypothesis. First, the three subunits have extensively overlapping expression patterns (Azam et al., 2002; Dineley-Miller and Patrick, 1992; Gahring et al., 2004; Genzen et al., 2001; Hellström-Lindahl et al., 1998; Keiger et al., 2003; Liu et al., 1998; Rust et al., 1994; Vincler and Eisenach, 2003; Winzer-Serhan and Leslie, 1997; Zoli et al., 1995). Second, the promoter regions of the α3, α5 and β4 subunit genes can all directly interact with and be trans-activated by the widely expressed transcription factors Sp1 and Sp3 (Bigger et al., 1996, 1997; Boyd, 1996; Campos-Caro et al., 1999; Valor et al., 2002; Yang et al., 1995) and the more spatially-restricted regulatory factors Sox10 and SCIP/Tst-1/Oct-6 (Fyodorov and Deneris, 1996; Liu et al., 1999; Yang et al., 1994). Third, α3, α5 and β4 mRNA levels are coordinately up-regulated during neural development (Corriveau and Berg, 1993; Levey et al., 1995; Levey and Jacob, 1996) and coordinately down-regulated following denervation (Zhou et al., 1998). Finally, two transcriptional regulatory elements, β43′ and conserved noncoding region 4 (CNR4), have been shown to play key roles in directing expression of the clustered nACh receptor genes in a tissue-specific manner with β43′ being important for expression in the adrenal gland and CNR4 being critical for expression in the pineal gland and superior cervical ganglion (Xu et al., 2006). CNR4 also appears to play a role in directing nACh receptor expression in the brain (Xu et al., 2006).

While these advances have shed considerable light on the molecular details of nACh receptor expression, it is likely that *cis* elements in addition to β43′ and CNR4 are involved in regulating clustered gene expression in the nervous system given that neither of these elements completely recapitulated receptor gene expression in vivo (Xu et al., 2006). Here we describe a transgenic mouse approach in which a 2.3-kilobase pair region of the nACh receptor β4 subunit 5′-flanking DNA was used to drive expression of β-galactosidase (β-gal). This region of the β4 subunit gene contains several regulatory elements we previously showed to direct cell-type-specific expression in vitro (Liu et al., 1999). The present work demonstrates that this region also directs spatially restricted expression in vivo and further, that this regulatory function is developmentally regulated.

EXPERIMENTAL PROCEDURES

Construction of the β4 promoter/β-galactosidase transgene

A 2346-base pair SstI/HindIII fragment of the rat β4 subunit gene was treated with Klenow fragment of DNA polymerase I to generate blunt ends. This fragment spans nucleotides −2208 to +137 relative to the β4 transcription initiation site (Hu et al., 1994). The blunt-ended SstI/HindIII fragment was subcloned into the β-gal expression vector, pSG-MAR, which had been digested with SmaI, to generate the construct pSGB4SH (Fig. 1B). pSG-MAR contains the β-gal coding sequence, a nuclear localization signal (NLS) as well as the 5′ matrix attachment region (MAR) of the chick lysozyme gene (Fuchs et al., 2002). The NLS was included to facilitate comparison between β-gal activity with β4 RNA expression and to reduce background staining (Mercer et al., 1991). The MAR was included to insulate the transgene from insertion positional effects (Phi-Van and Stratling, 1996). The construct was verified by nucleotide sequencing (Nucleic Acid Facility, University of Massachusetts Medical School).

Cell culture and transfection

The rat pheochromocytoma cell line, PC12 (Greene and Tischler, 1976), was cultured and transfected as previously described (Liu et al., 1999). Briefly, the transfections were done using a liposome-mediated approach (Lipofectamine, Invitrogen, California, USA). The cells were transfected with pSGB4SH and a luciferase expression construct, pGL-Promoter (Promega Corporation, Wisconsin, USA). The cells were differentiated with 100 ng/ml nerve growth factor (NGF; Upstate Biotechnologies, Inc., New York, USA) for 2 days following transfection and then harvested and assayed for β-gal (Galacto-Star, Applied Biosystems, California, USA) and luciferase (Luciferase Assay System, Promega) activities in a Lumimark microplate luminometer (Bio-Rad, California, USA). To correct for differences in transfection efficiencies between dishes, the β-gal activity in each sample was normalized to the luciferase activity in that same sample.

Generation of transgenic mice

pSGB4SH was digested with NotI to release the β4/β-gal transgene (Fig. 1B). Following agarose gel electrophoresis, the transgene fragment was excised and the DNA was extracted from the gel using a QIAquick Gel Extraction Kit (QIAGEN, California, USA). The purified DNA was injected into pronuclei followed by implantation into pseudopregnant females. The C57BL/6 x SJL F2 hybrid mouse strain was used for all transgenic experiments. Injection of DNA and all subsequent steps up to and including the generation of founder animals were performed by the University of Massachusetts Medical School Transgenic Animal Modeling Core. Transgenic founders were identified by polymerase chain reaction. Founders were mated with C57BL/6 x SJL F2 hybrid mice to establish transgenic lines. Adequate measures were taken to minimize pain and discomfort to the animals. All procedures were conducted in accordance with the rules of the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

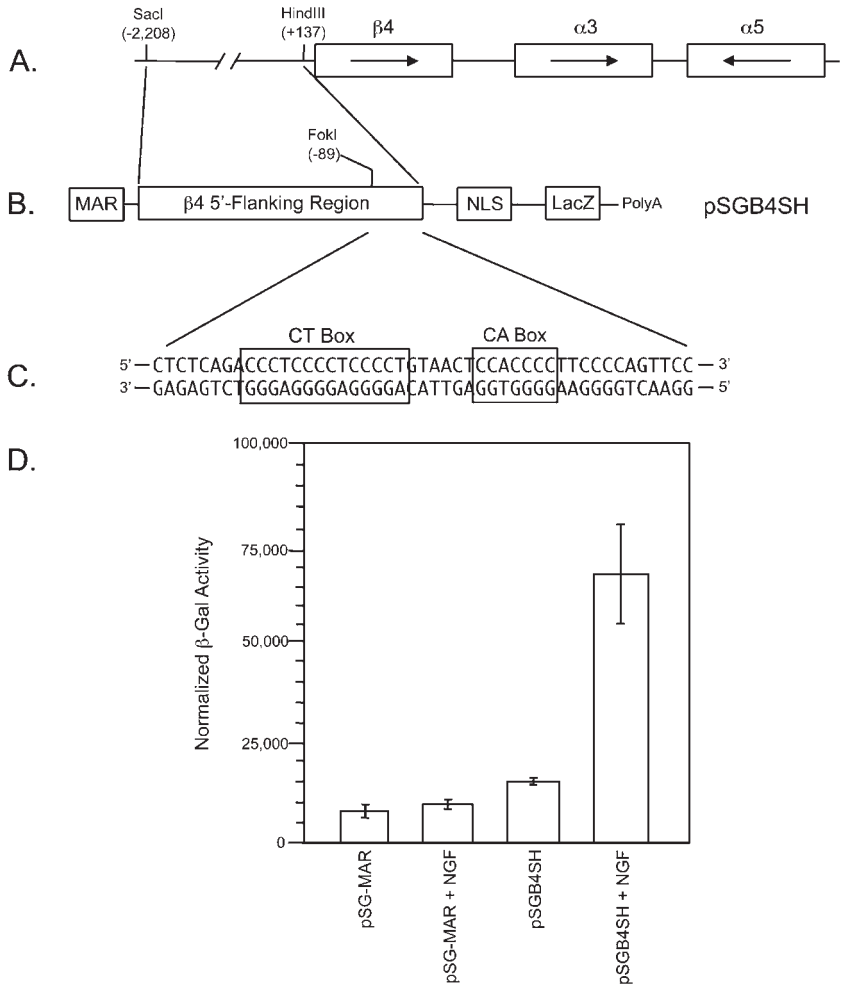

Fig. 1. The nACh receptor β4 subunit promoter region used for transgenesis. (A) The β4/α3/α5 gene cluster. The 2.3-kb SacI/HindIII fragment of the β4 promoter region is indicated on the left. Arrows indicate direction of transcription. The nucleotide positions of the SacI and HindIII sites are relative to the major transcription initiation site. (B) Structure of the linearized construct, pSGB4SH, used to generate transgenic animals. MAR, matrix attachment region; NLS, nuclear localization sequence. (C) Nucleotide sequences of the CT and CA boxes of the β4 promoter region. (D) The vector, pSG-MAR, and pSGB4SH were transfected into PC12 cells along with a luciferase construct in which the SV40 promoter drives expression of the firefly luciferase gene. Cells were treated with NGF (100 ng/ml for 2 days) as indicated. β-Gal activity was normalized to luciferase activity to correct for differences in transfection efficiencies. Error bars represent standard deviations of the means.

Determination of transgene copy number

Transgene copy number of the four transgenic lines was determined using absolute quantification-based real-time PCR (Yuan et al., 2007). PCR reactions were performed with primers designed to amplify a fragment of the β-gal coding sequence present in the β4 promoter/β-gal transgene. The sequence of the upper strand primer was 5′-GATTTCCATGTTGCCACTCGCTTTA-3′ while that of the lower strand primer was 5′-TTCAGCAGCAGCAGACCATTTTCAA-3′. All PCR reactions were set up in triplicate and included 100 nanograms of genomic DNA as template. Two positive control samples of known copy number (a generous gift from Ricardo Medina) were used in this analysis. These control samples were isolated from transgenic animals that contain a targeted β-gal coding sequence. One of these animals has one copy of the β-gal transgene and the other has two copies. The value obtained for the single copy positive control sample was set as 1 in each experiment. This value was used to estimate the copy number for all other samples including the control sample with two copies. In quadruplicate assays run with the two-copy control, transgene copy numbers ranged from 2.005 to 2.274 with an average value of 2.07. In order to determine copy number for our four β4/β-gal transgenic lines, three DNA samples from each line were run in qPCR experiments. The quantities derived from the standard curve for each set of three animals were averaged and then divided by the value for the 1-copy positive control.

Histochemical analysis of transgenic mice

Two ages of transgenic mice were studied: embryonic day (ED) 18.5 and postnatal day (PD) 30. Mice were anesthetized with pentobarbital and perfused transcardially with cold 0.1 M sodium phosphate buffer/2 mM $MgCl_2$ followed by fixative (cold 4% paraformaldehyde). Tissues were then dissected and post-fixed for 5–6 h (ED18.5) or 4 h (PD30). Fixed tissues were transferred to 30% sucrose/2 mM $MgCl_2$, in 1× phosphate-buffered saline (PBS) and incubated at 4 °C overnight. Tissues were embedded in Tissue-Tek (Miles, Indiana, USA) and quick frozen on dry ice. If not used immediately, the samples were stored at −70 °C. Sectioning was done on a Leica CM3050S cryostat at −30 °C generating either 14 μm (ED18.5) or 25 μm (PD30) thick sections that were transferred directly onto Superfrost glass slides (Fisher,

Pennsylvania, USA). Slides were air-dried at room temperature, washed with sodium phosphate buffer and then incubated overnight at 37 °C with β-gal staining solution (0.1 M $NaHPO_4$, 0.1 M NaH_2PO_4, 2 mM $MgCl_2$, 0.1% sodium deoxycholate, 0.02% NP-40, 10 mM $K_3(Fe)CN_6$, 10 mM $K_4(Fe)CN_6$, 1 mg/ml X-gal). The slides were subsequently washed with 1× PBS and incubated with distilled water either for 1 h (ED18.5) or overnight (PD30). Slides were then counter-stained with Neutral Red (1% w/v in 37 mM sodium acetate), dehydrated through a graded series of ethanol (50%, 70%, 90% and 100%) and cleared with xylene. The slides were air-dried overnight at room temperature in a fume hood followed by the application of cover slips. Microscopy was done using a Zeiss Axiovert 200M microscope with a high resolution Retiga 1300R CCD camera and Slidebook image analysis software. Anatomical analysis was done with the aid of Kaufman (Kaufman, 1995) and Paxinos and Franklin (Paxinos and Franklin, 2001).

Triple immunofluorescence

Slides were prepared as described above for the PD30 animals (25-μm sections). The samples were washed twice for 10 min in 1× PBS then permeablized for 20 min in 0.1% Triton X-100 in PBS. Following three washes in PBS, the sections were incubated with 1% BSA in PBS for 3 h. The BSA solution was removed and the sections were incubated overnight with primary antibodies. The antibodies and their dilutions were as follows: rabbit anti-β-gal (1:100; MP Biomedicals, Ohio, USA), mouse anti-neuron-specific nuclear marker (NeuN; 1:100; Millipore, Massachusetts, USA) and chicken anti-glial fibrillary acid protein (GFAP; 1:500; Millipore). The dilutions were made with 1% BSA in PBS. After antibody incubation, the slides were washed five times for 6 min with 1× PBS followed by a 2-hour incubation with a 1:100 dilution of each secondary antibody. The following antibodies were used (all from Invitrogen, California, USA): Alexa 488 (goat anti-rabbit), Alexa 350 (goat anti-mouse IgG1) and Alexa 594 (goat anti-chicken). The sections were washed five times for 10 min with 1× PBS and mounted with Vectashield (Vector Laboratories, California, USA). Microscopy was done as described above.

RESULTS AND DISCUSSION

In vitro functional characterization of the β4/β-galactosidase transgenic construct

An approximately 2.3-kilobase pair fragment of the nACh receptor β4 subunit 5′-flanking DNA (Fig. 1A) was subcloned into the β-gal expression vector, pSG-MAR (Fuchs et al., 2002) generating the construct pSGB4SH (Fig. 1B). This region of the β4 promoter contains two transcriptional regulatory elements we previously characterized in vitro in the context of a luciferase-based reporter vector. These elements are referred to as a CT box and a CA box based on their nucleotide compositions (Fig. 1C). The 19-base pair CT box contains three repeats of the sequence 5′-CCCT-3′ (Hu et al., 1995) and directly interacts with the regulatory factors heterogeneous nuclear ribonucleoprotein K and Purα (Du et al., 1997, 1998). The CA box (5′-CCACCCC-3′) directly interacts with the general transcription factors Sp1 and Sp3 (Bigger et al., 1996, 1997) and the more spatially-restricted factor Sox10 (Liu et al., 1999). To confirm that the transcriptional activity of pSGB4SH, a β-gal-based system, is similar to that of the previous β4/luciferase constructs, transient transfections were carried out using the rat pheochromocytoma cell line, PC12 (Greene and Tischler, 1976). pSGB4SH was trans-

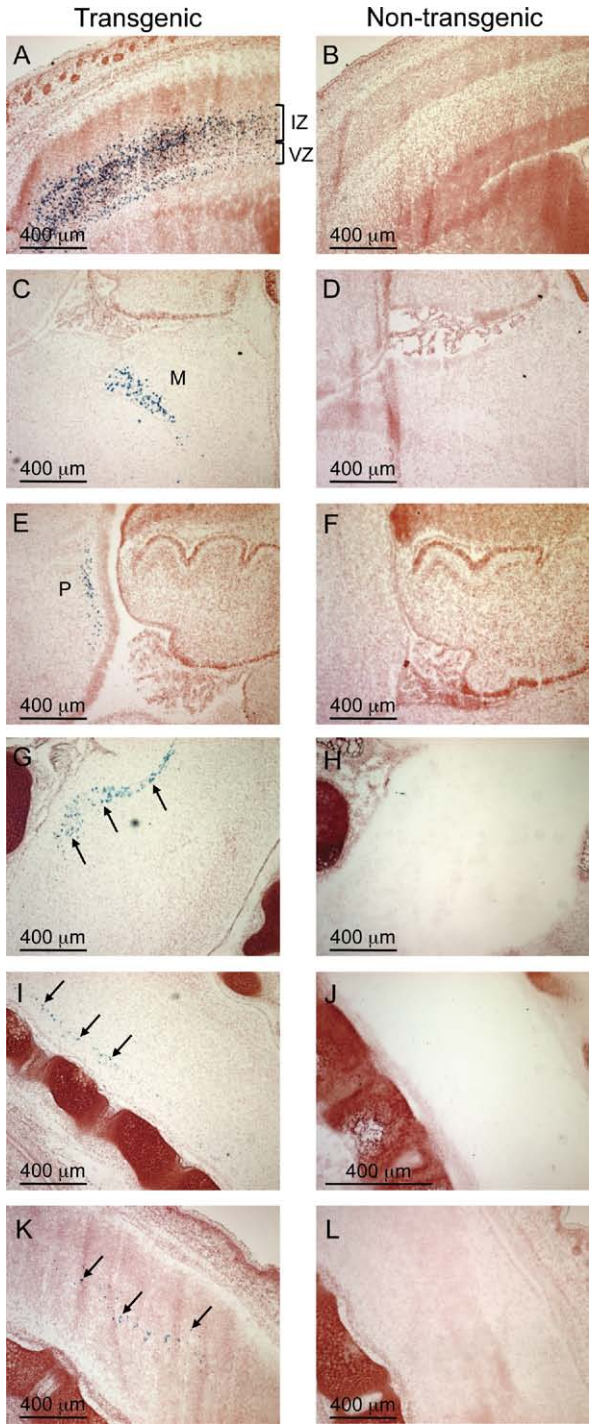

Fig. 2. nACh receptor β4 subunit promoter activity in the CNS of ED18.5 transgenic animals. Sagittal sections of transgenic (A, C, E, G, I and K) and non-transgenic (B, D, F, H, J and L) ED18.5 mouse brain and spinal cord are shown. The sections were stained for β-gal activity and counter-stained with Neutral Red. Note that β-gal staining is restricted to nuclei as the β4/lacZ transgene contains a nuclear localization signal (see Fig. 1B). (A and B), cortex (IZ, intermediate zone; VZ, ventricular zone); (C and D), medulla oblongata (M); (E and F), pons (P); (G and H), upper cervical region of the spinal cord; (I and J), mid-thoracic region of the spinal cord; (K and L), mid-lumbar region of the spinal cord. Arrows in panels (G, I and K) indicate β-gal-expressing cells.

fected into parallel cultures of PC12 cells with one set of cells being treated with NGF for 2 days following transfection and the other set being used as untreated controls. As shown in Fig. 1D, there was a significant increase in β-gal activity following NGF treatment reflecting an increase in β4 promoter activity. These data are consistent with our previous work using the luciferase-based reporter vector (Hu et al., 1994; Liu et al., 1999) and indicated that the new construct was suitable for transgenic work.

β4 promoter activity in ED18.5 transgenic mouse embryos

To determine whether the 2.3-kilobase pair β4 promoter region functions *in vivo* and if so, in a cell-type-specific manner reflecting endogenous β4 mRNA expression, the β4/β-gal transgene was excised from the pSGB4SH backbone and used to generate several founder lines of mice (see *Experimental Procedures* for details). Nine founder lines were obtained. Of these, one died unexpectedly, one showed no β-gal staining, two did not breed well and one showed extremely intense β-gal staining throughout the body and was not pursued. The remaining four lines (lines 30, 39, 54 and 447) were analyzed for β-gal staining at ED18.5 and PD30 as described below. Using a quantitative PCR approach (Yuan et al., 2007), transgene copy number was determined for each line and revealed that line 30 has approximately seven copies, line 39 has 26 copies, line 54 has 12 copies and line 447 has 10 copies (see *Experimental Procedures* for details).

In general, β-gal staining was substantially less intense in embryos as compared to adults for all four founder lines indicating a developmental effect on β4 promoter activity. In fact, founder lines 30 and 39 showed almost no detectable staining in the CNS at ED18.5 but showed extensive staining at 1 month of age (see below). The two other lines (lines 54 and 447) however, did exhibit significant β-gal staining early in development, but again, the staining was more intense in older animals (see below). As shown in Figs. 2–4, the β4 promoter region directs rather striking cell-type-specific expression of the β-gal gene in ED18.5 transgenic embryos of founder lines 54 and 447. In the CNS, there is very strong β-gal staining in the cortex (intermediate and ventricular zones, Fig. 2A), the medulla oblongata (Fig. 2C), the pons (Fig. 2E) and the spinal cord (Fig. 2G, I, K). Less intense staining is seen in other regions of the CNS including the piriform cortex, subiculum, lateral and medial habenular nuclei and the pontine area (Table 1). These results are consistent with *in situ* work done by others characterizing expression of endogenous β4 mRNA (Winzer-Serhan and Leslie, 1997; Zoli et al., 1995). In addition to these regions, endogenous β4 mRNA has been detected in the pineal gland. Our transgene construct did not yield β-gal expression in this region. This is most likely due to the absence of CNR4, a transcriptional regulatory region shown by the Deneris group to be important for pineal gland expression of the clustered nACh receptor genes (Xu et al., 2006). CNR4 is located several kilobase pairs upstream of the β4 promoter region being tested in this work (Xu et al., 2006).

Outside of the CNS, there was remarkable β-gal staining in the spinal ganglia (Fig. 3), the intestine (Fig. 4A) and the tongue (Fig. 4C) of ED18.5 transgenic animals. There

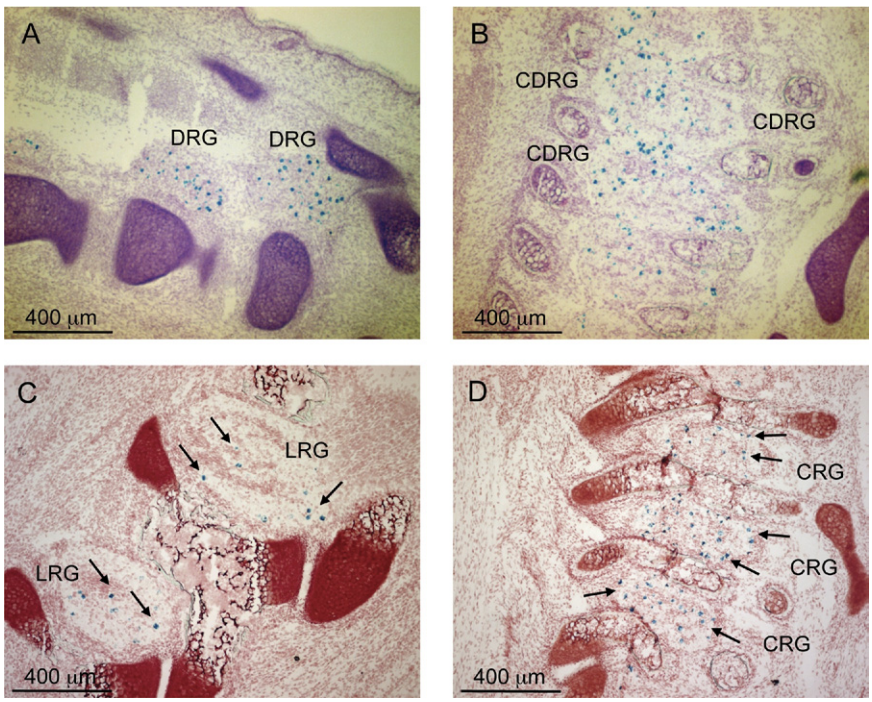

Fig. 3. nACh receptor β4 subunit promoter activity in spinal ganglia of ED18.5 transgenic animals. Sagittal sections of transgenic ED18.5 animals were stained for β-gal activity and counter-stained with Neutral Red. (A) dorsal root ganglion (DRG); (B) cervical dorsal root ganglion (CDRG); (C) lumbar root ganglion (LRG); (D) cervical root ganglion (CRG). Arrows in panels (C and D) indicate β-gal-expressing cells.

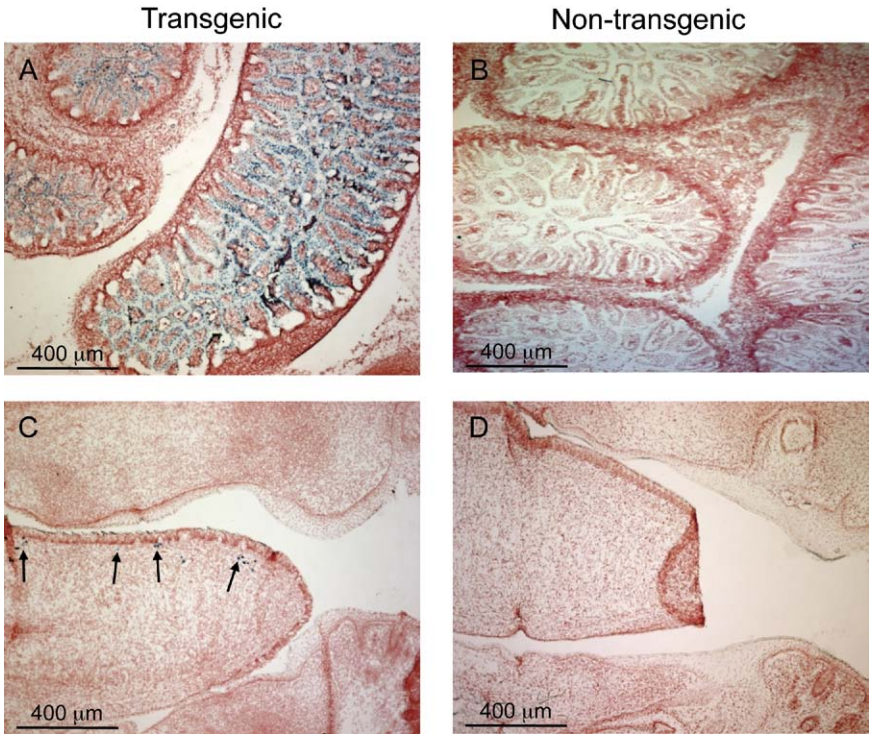

Fig. 4. nACh receptor β4 subunit promoter activity in the intestine and tongue of ED18.5 transgenic animals. Sagittal sections of transgenic (A and C) and non-transgenic (B and D) ED18.5 animals were stained for β-gal activity and counter-stained with Neutral Red. (A and B) intestine; (C and D) tongue. β-gal staining of the tongue appears to be restricted to or near the longitudinal intrinsic muscle (arrows indicate β-gal-expressing cells).

were lower levels of β-gal staining in the retina, trigeminal ganglia and lips (Table 2). Again, for the most part, these results are consistent with β4 mRNA localization studies (Winzer-Serhan and Leslie, 1997; Zoli et al., 1995). Two notable exceptions are the intestine and the lips, for which we are not aware of any reports describing the presence of β4 mRNA in ED18.5 animals. The presence of β-gal staining in these areas may be due to positional effects on transgene expression or it could be due to the lack of a cell-type-specific repressor element we previously identified in an intron of the nACh receptor α3 subunit gene, which we showed represses β4 promoter activity in cells that do not express the β4 subunit gene (Fuentes Medel and Gardner, 2007).

Table 1. β4/lacZ transgene expression in the central nervous system of ED18.5 embryos

Central nervous system region	30	39	54	447
Cerebral cortex	−	−	++++	++
Piriform cortex	−	−	+	+
Subiculum	−	−	++	+
Lateral habenula	−	−	+	+
Medial habenula	−	−	+	+
Medulla oblongata	−	−	++	+
Anterior pontine area	−	−	++	+
Posterior pontine area	−	−	++	+
Pons	−	−	++	++
Pineal gland	−	−	−	−
Thalamus	−	−	−	+
Ventral spinal cord	−	−	+	++
Dorsal spinal cord	−	−	+	++

Expression levels were scored as follows: −, no expression; +, low level; ++, intermediate level; ++++, very high level.

β4 promoter activity in the brains of transgenic PD30 mice

As mentioned above, in contrast to embryonic animals, all four founder lines exhibited significant β-gal staining at 1

Table 2. β4/lacZ transgene expression outside the central nervous system of ED18.5 embryos

Tissue	30	39	54	447
Retina	+	−	+	+
Cervical root ganglia	−	−		
Ventral	−	−	+	++
Dorsal	−	−	+	++
Thoracic root ganglia	−	−		
Ventral	−	−	+	+
Dorsal	−	−	+	+
Lumbar root ganglia	−	−		
Ventral	−	−	+	+
Dorsal	−	−	+	+
Trigeminal ganglia	−	−	+	−
Intestine	−	++++	+++	−
Tongue	++	−	+	−
Lips	+	−	+	+

Expression levels were scored as follows: −, no expression; +, low level; ++, intermediate level; +++, high level; ++++, very high level.

Table 3. β4/lacZ transgene expression in the central nervous system of PD30 animals

Brain region	30	39	54	447
Forebrain/cortex				
Layer 1	−	+	+	+
Layer 2	−	+	+	−
Layer 3	−	−	+	+
Layer 5	+	++	++++	++
Anterior olfactory nucleus	−	++	+	+
Basal nucleus (Meynert)	+	+	−	+
Caudate putamen (striatum)	+	+	++	++
Lateral globus pallidus	−	+	+	+
Olfactory bulb	+	+	++	+
Olfactory tubercle	+	−	+	++
Agranular insular cortex	+	+	+++	+
Auditory cortex	−	+	++	+
Cingulate cortex, area 1	−	+	++	++
Cingulate/retrosplenial cortex	+	+	+	−
Cortex-amygdala transition zone	−	+	+	+
Dorsal endopiriform nucleus	+	+	++++	++
Entorhinal cortex	+	+	+++	+
Frontal association cortex	+	+	++	++
Granular insular cortex	−	+	+	+
Orbital cortex	+	+	+++	++
Primary motor cortex	−	+	++	++
Secondary motor cortex	+	+	++	++
Piriform cortex	++	+++	++++	+++
Perirhinal cortex	+	+	+++	+
Prelimbic cortex	+	−	+++	++
Retrosplenial agranular cortex	+	+	+	+
Primary somatosensory cortex	+	+	++	++
Secondary somatosensory cortex	+	+	++	+
Temporal association cortex	−	+	+++	+
Primary visual cortex	+	+	+	++
Secondary visual cortex	−	+	+	+
Hippocampus/corpus callosum				
Parasubiculum	−	+	+	+
Presubiculum	−	+	+	+
Subiculum	−	++	++++	+
Molecular layer of the dentate gyrus	−	++++	++	++
Oriens layer of the hippocampus	−	++	+	+
Habenular nuclei				
Lateral habenular nucleus	+	+	+	+
Medial habenular nucleus	−	+++	++	+
Preoptic area				
Lateral preoptic area	+	+	+	+
Medial preoptic area	+	+	+	+
Median preoptic nucleus	+	+	−	+
Medial preoptic nucleus, medial part	−	+	+	+
Thalamus				
Anteromedial thalamic nucleus	−	++	+	+
Basomedial amygdaloid nucleus, anterior part	+	+	++	+
Caudal interstitial nucleus of the medial, longitudinal fasciculus	−	+	++	+
Central medial thalamic nucleus	−	+	+	+
Dysgranular insular cortex	−	+	++	+
Dorsolateral orbital cortex	+	+	++	+
Dorsal penduncular cortex	+	+	+	++
Gustatory thalamic nucleus	+	+	+	−
Laterodorsal thalamic nucleus, dorsomedial part	+	+	+	−
Lateral posterior thalamic nucleus, mediorostral part	−	+	+	+
Mediodorsal thalamic nucleus	+	+	+	+
Medial geniculate nucleus	−	++	++	+
Posterior thalamic nuclear group	−	+	++++	+
Paratenial thalamic nucleus	−	+	+	+
Paraventricular thalamic nucleus	+	+	++	+
Precommissural nucleus	+	+	+	+
Reuniens thalamic nucleus	−	+	+	+
Stria medullaris of the thalamus	−	+	+	+
Ventral anterior thalamic nucleus	+	+	+	+
Ventrolateral thalamic nucleus	−	+	+	+++
Ventromedial thalamic nucleus	−	+	+	+
Ventral posteromedial thalamic nucleus	−	+	+	+
Hypothalamus				
Dorsomedial hypothalamic nucleus	++	+	+	−
Lateral hypothalamic area	+++	+	+	+
Medial forebrain bundle	+	+	+	−
Perifornical nucleus	+	+	+	−
Posterior hypothalamic area	+	+	+	−
Ventromedial hypothalamic nucleus	+	+	+	+
Amygdala				
Anterior amygdaloid area	−	+	+	+
Anterior cortical amygaloid nucleus	−	+	+	+
Medial amygdaloid nucleus	+	+	+	+
Posterolateral cortical amygdaloid nucleus	−	++	+++	++
Posteromedial cortical amygdaloid nucleus	+	++	++	+
Other midbrain structures				
Dorsolateral periaqueductal gray	+	+	−	+
Deep mesencephalic nucleus	+	+	+	−
Inferior colliculus	+	+	++	+
Retroparafascicular nucleus	+	+	+	−
Subbrachial nucleus	+	+	+	+
Superior colliculus	+	+	−	+
Pons				
Laterodorsal tegmental nucleus	+	+	+	−
Lateral mammillary nucleus	+	+	+	−
Lateral parabrachial nucleus, ventral part	+	+	−	+
Medial lemniscus	+	++	−	+

Expression levels were scored as follows: −, no expression; +, low level; ++, intermediate level; +++, high level; ++++, very high level.

month of age. Analysis of β-gal staining revealed broad and substantial β4 promoter activity in the brains of transgenic animals. In particular, there was significant staining in the cortex, hippocampus, thalamus, amygdala, medial habenula and the colliculus. For the most part, the broad β-gal staining (Table 3) is consistent with previous studies that determined β4 subunit mRNA expression using *in situ* hybridization (Azam et al., 2002; Dineley-Miller and Patrick, 1992; Winzer-Serhan and Leslie, 1997). In addition, the present results are consistent with immunostaining work that indicated widely dispersed expression of the

nACh receptor β4 subunit protein in the adult mouse nervous system (Gahring et al., 2004). As discussed by Gahring et al., the regulated widespread expression of the β4 subunit gene likely reflects its role in a variety of diverse functions in the nervous system.

Forebrain/cortex. As with nACh receptor β4 mRNA (Dineley-Miller and Patrick, 1992; Winzer-Serhan and Leslie, 1997) and protein (Gahring et al., 2004), significant β4 promoter activity was detected in layer 5 of the cortex (Fig. 5A) and the piriform cortex (Fig. 5C). Slightly lower promoter activity was observed in the frontal association cortex and caudate putamen (Fig. 5E, G, respectively) with there being lower levels in the cingulate/retrosplenial cortex (Fig. 5I). There was also substantial β4 promoter activity in other regions of the forebrain/cortex (Table 3) including the olfactory bulb, agranular insular cortex, auditory cortex, cingulated/retrosplenial cortex, piriform cortex and the visual cortex, amongst others. In addition, there were regions where β4 promoter activity was seen for which we know of no reports of β4 mRNA expression, such as the entorhinal, orbital and prelimbic cortices (Table 3). Again, this may be due to the lack of other transcriptional regulatory elements in the β4/β-gal transgene, such as the cell-type-specific repressor element discussed above. Alternatively, it could be due to positional effects on transgene expression.

Hippocampus/subiculum. The hippocampus is an interesting region in which to study nACh receptor β4 expression, as it is known that β4 expression in this structure significantly varies across mouse strains (Gahring et al., 2004). However, it is clear that across strains, there is β4 expression, both mRNA and protein, in the dentate gyrus, CA1 field, CA3 field and the subiculum (Dineley-Miller and Patrick, 1992; Gahring et al., 2004; Winzer-Serhan and Leslie, 1997). Consistent with these observations, we see strong β4 promoter activity in these same regions (Fig. 6A, Table 3).

Habenular nuclei. The habenula is the region of highest nACh receptor β4 subunit expression in the brain (Dineley-Miller and Patrick, 1992; Duvoisin et al., 1989; Gahring et al., 2004; Winzer-Serhan and Leslie, 1997). As expected, β4 promoter activity was quite strong in the medial habenula, but was lower in the lateral habenula (Fig. 6C). Interestingly, β4 promoter activity was much higher in PD30 animals versus ED18.5 animals. This is in contrast to β4 mRNA levels—which are high throughout development (Winzer-Serhan and Leslie, 1997; Zoli et al., 1995)—and may reflect the absence of other regulatory elements in the β4/β-gal transgene, for example, CNR4, that may be active early in development (Xu et al., 2006).

Thalamus/hypothalamus. As discussed above, significant β4 promoter activity was observed in the medial habenula, a region that expresses high levels of β4 mRNA. In addition, the β4 subunit is also expressed in the paraventricular thalamic nucleus, medial geniculate nucleus, the posterior thalamic nuclear group, the retroparafascicular nucleus, the inferior colliculus and the ventral anterior and ventral posteromedial thalamic nuclei (Gahring et al., 2004). We detected β4 promoter activity in these same regions (Fig. 6C, E, Table 3). With the exception of these structures and the interpeduncular nucleus (see below), nACh receptor β4 expression is either very low or undetectable in the thalamus and hypothalamus (Dineley-Miller and Patrick, 1992; Duvoisin et al., 1989; Gahring et al., 2004; Winzer-Serhan and Leslie, 1997). Thus, it was surprising to observe significant β4 promoter activity in both of these regions (Table 3). Again, this could be due to the absence of the cell-type-specific repressor element mentioned earlier. Interestingly, while β4 mRNA and protein expression is high in the interpeduncular nucleus (Dineley-Miller and Patrick, 1992; Gahring et al., 2004; Winzer-Serhan and Leslie, 1997), we detected no β4 promoter activity in this region (not shown). However, Deneris and colleagues reported that CNR4 drives high transgene expression in the interpeduncular nucleus (Xu et al., 2006). The absence of CNR4 in the β4/β-gal transgene used in the current study may explain why no β4 promoter activity was seen in this nucleus.

Amygdala and pons. Two other brain regions of the PD30 mouse where significant β4 promoter activity was detected are the amygdala and the pons. In particular, strong β4 promoter activity was seen in the posteromedial cortical amygdaloid nucleus (Fig. 6G) and the posterolateral cortical amygdaloid nucleus (Table 3). Lower, yet significant, β4 promoter activity was observed in the pons, most notably in the laterodorsal tegmental nucleus, the lateral mammillary nucleus and the lateral parabrachial nucleus (ventral part) as well as in the medial lemniscus (Table 3). To our knowledge, the amygdala and pons have not been reported to express the nACh receptor β4 subunit, once more raising the issue as to why β4 promoter activity is seen in these two brain regions in multiple founder lines. As before, one possibility is the lack of a cell-type-specific repressor element in the β4/β-gal transgene (Fuentes Medel and Gardner, 2007), although it is possible that the endogenous promoter is subject to regulation, negative in this case, to which the transgene promoter is immune.

The β4 promoter is active in neurons

Expression of nACh receptor β4 subunit protein has been detected in non-neuronal cells within the brain (Gahring et al., 2004). In order to determine if the β4 promoter activity we observed throughout the brain is or is not neuron-specific, triple immunofluorescence was carried out using anti-GFAP to label glial cells, anti-NeuN to label neurons and anti-β-gal to label β-gal-expressing cells. As shown in Fig. 7, in each region tested, β-gal labeling over-lapped that of NeuN but not GFAP, indicating that the transgene β4 promoter activity is neuron-specific.This suggests that other regulatory elements are involved in the non-neuronal expression of the β4 subunit described by Gahring et al. (2004). Alternatively, post-transcriptional or epigenetic regulatory mechanisms may be at work in non-neuronal cells.

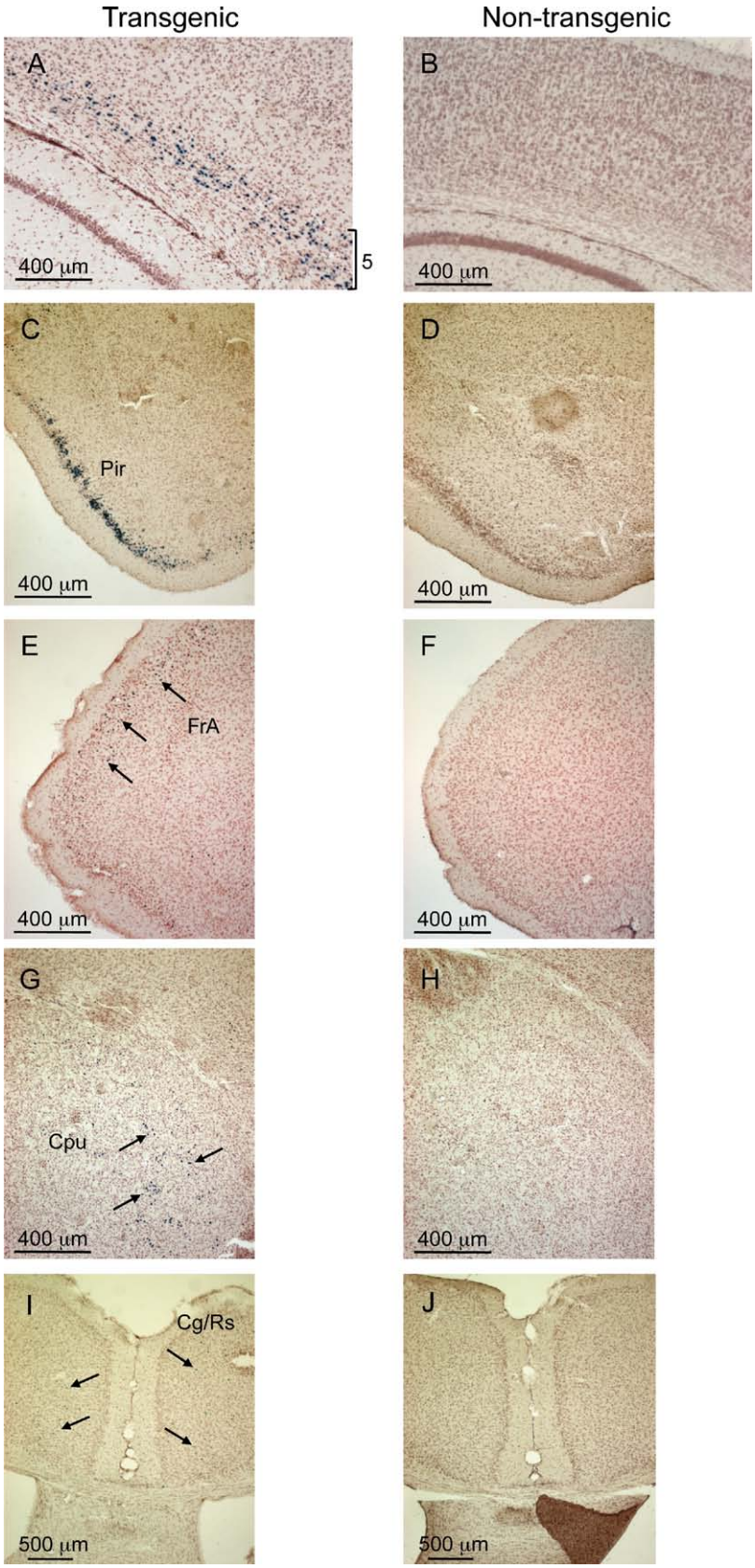

Fig. 5. nACh receptor β4 subunit promoter activity in the forebrain and cortical regions of PD30 transgenic animals. Coronal sections of transgenic (A, C, E, G and I) and non-transgenic (B, D, F, H and J) PD30 mouse brains are shown. The sections were stained for β-gal activity and counter-stained with Neutral Red. (A and B) layer 5 of the cortex ("5"); (C and D) piriform cortex (Pir); (E and F) frontal association cortex (FrA); (G and H) caudate putamen (Cpu); (I and J) cingulate/retrosplenial cortex (Cg/Rs). Arrows in panels (E, G and I) indicate β-gal-expressing cells.

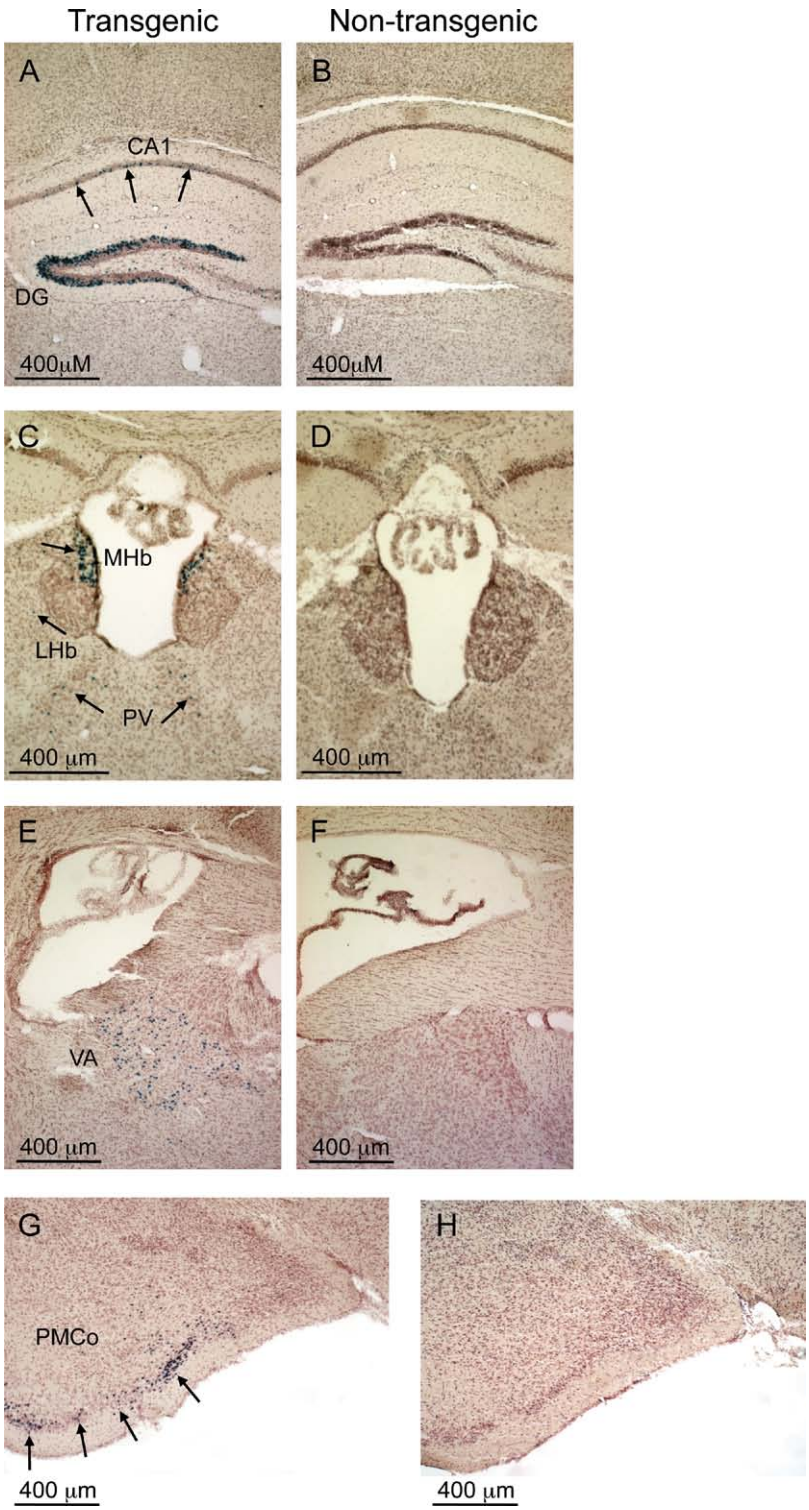

Fig. 6. nACh receptor β4 subunit promoter activity in the CNS of PD30 transgenic animals. Coronal sections of transgenic (A, C, E and G) and non-transgenic (B, D, F and H) PD30 mouse brains are shown. The sections were stained for β-gal activity and counter-stained with Neutral Red. (A and B) hippocampus (CA1) and dentate gyrus (DG); (C and D) medial (MHb) and lateral (LHb) habenula and paraventricular thalamic nucleus (PV); (E and F), ventral anterior thalamic nucleus (VA); (G and H), posteromedial cortical amygdaloid nucleus (PMCo). Arrows in panels (A, C and G) indicate β-gal-expressing cells.

In summary, we have used a transgenic mouse model to study the transcriptional activity of the nACh receptor β4 subunit gene promoter. As described above, the activity of the β4/β-gal transgene used in this study paralleled, for the

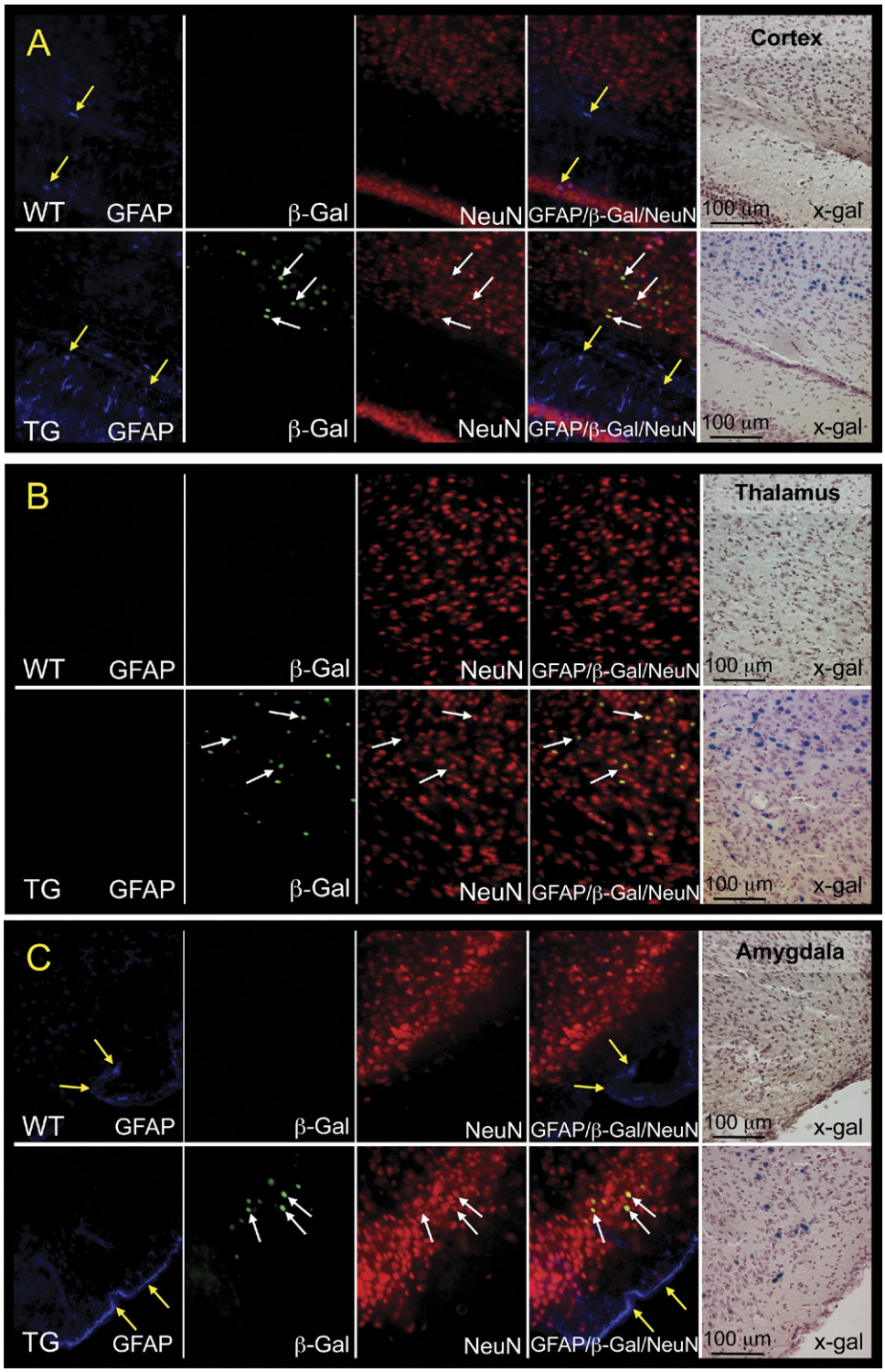

Fig. 7. The nACh receptor β4 subunit promoter directs neuron-specific gene expression. Triple immunofluorescence was done on coronal sections of transgenic (TG) and non-transgenic (WT) PD30 mouse brains. (A) cortex; (B) thalamus; (C) amygdala. GFAP, glial fibrillary acid protein (yellow arrows); NeuN, neuron-specific nuclear marker; β-gal, β-galactosidase. Overlap of the three immunostained sections indicates specific β-gal staining in neuronal nuclei (white arrows). β-gal histochemistry (right panels) was done to confirm β-gal activity in the transgenic animals. Note: There was no GFAP staining in the thalamus (panel B).

most part, endogenous β4 subunit mRNA expression both spatially and temporally. These results indicate that the 2.3-kilobase pair fragment of the nACh receptor β4 subunit 5′-flanking DNA contains transcriptional regulatory ele-

ments critical for appropriate cell-type-specific and developmentally regulated expression of the β4 subunit gene. Although the precise regulatory elements within the 2.3-kilobase pair region responsible for the *in vivo* activity of the β4 promoter have not been determined, strong candidates are the CT and CA boxes that we previously characterized in cell culture systems. The significance of these elements in vivo is currently being pursued. However, it is clear that the 2.3-kilobase pair fragment does not contain all of the required positive regulatory elements that govern expression of the β4 gene, as there are regions of the brain that express relatively high levels of the β4 gene but in which no transgene activity was detected. In some cases, as described above, this may be due to the absence of specific regulatory elements in the transgene used in the present study. For example, the CNR4 element identified by the Deneris group and shown to drive transgene expression in the pineal gland, adrenal gland and specific regions of the brain, was not part of the transgene construct. It is also possible that other, yet to be identified positive regulatory elements are also required for appropriate β4 gene expression. In addition to the β4 promoter activity that corresponds to endogenous β4 mRNA expression, we also detected promoter activity in brain regions that have not been reported to express the β4 subunit. These observations likely underscore the importance of negative regulatory elements in β4 gene expression and may be a consequence of the absence of a cell-type-specific repressor in the transgene used in this study. We previously demonstrated in vitro that this repressor, referred to as "α3 intron 5", exerts its transcriptional effects in non-β4-expressing cells but is relatively inactive in β4-expressing cells (Fuentes Medel and Gardner, 2007).

Finally, it is important to note that at least two other modes of regulation are likely to play key roles in expression of the nACh receptor β4 subunit gene (as well as other nACh receptor subunit genes). First, in addition to the cis-acting elements described thus far, chromatin remodeling most probably is critical for appropriate β4 gene expression. This is a relatively understudied area in terms of nACh receptor gene expression and is a focus of current research efforts. Second, post-transcriptional mechanisms are another level of control of nACh receptor expression, particularly in response to extracellular stimuli. One such mechanism may involve small 21–24 nucleotide long regulatory molecules, referred to as microRNAs (miRNAs) (Ambros, 2004). miRNAs are predicted to regulate the majority of mammalian protein-coding genes, typically by binding to complementary sites in the 3′-untranslated regions of target mRNAs and guiding them to an RNA-induced silencing complex (Kosik, 2006). Interestingly, a miRNA, miR-1, has been reported to target nACh receptors in *C. elegans*, leading to changes in nACh receptor properties (Simon et al., 2008). We are investigating whether miRNAs play a role in the expression of mammalian nACh receptor genes.

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