THE JOURNAL OF COMPARATIVE NEUROLOGY 439:235–247 (2001)

Presynaptic Localisation of the Nicotinic Acetylcholine Receptor β2 Subunit Immunoreactivity in Rat Nigrostriatal Dopaminergic Neurones

IAN W. JONES,[1] J. PAUL. BOLAM,[2] AND SUSAN WONNACOTT[1]*
[1]Department of Biology and Biochemistry, University of Bath, Claverton Down, Bath BA2 7AY, United Kingdom
[2]MRC Anatomical Neuropharmacology Unit, University of Oxford, Oxford OX1 3TH, United Kingdom

ABSTRACT

Nicotinic acetylcholine receptors (nAChR) are widely distributed in the central nervous system, where they exert a modulatory influence on synaptic transmission. For the striatum, pharmacological evidence supports the presence of presynaptic α3β2* and α4β2* nAChR that modulate dopamine release from nigrostriatal terminals. The objective of this study was to examine the precise subcellular distribution of the nAChR β2 subunit in these neurones and its localisation at presynaptic sites. Double immunolabelling with tyrosine hydroxylase (TH) at the confocal level revealed that the cell bodies and axon terminals (synaptosomes) of nigrostriatal neurones were also immunoreactive for the nAChR β2 subunit. Double-preembedding electron microscopy confirmed that β2-immunogold labelling was enriched in TH-positive terminals in the dorsal striatum. Quantitative analysis of doubly immunogold-labelled sections in postembedding electron microscopy showed that 86% of TH-positive axonal boutons are also labelled for the nAChR β2 subunit, whereas 45% of β2 subunit-immunolabelled boutons do not contain TH. Thus the β2 subunit is localised within at least two populations of axon terminals in the dorsal striatum. In these structures, 15% of β2 subunit immunoreactvity was at the plasma membrane but was rarely associated with synapses. These findings are compatible with functional presynaptic β2-containing nAChR that may be stimulated physiologically by acetylcholine that diffuses from synaptic or nonsynaptic sites of acetylcholine release. These results demonstrate the presynaptic localisation of an nAChR subunit in nigrostriatal dopaminergic neurones, providing morphological evidence for the presynaptic nicotinic modulation of dopamine release. J. Comp. Neurol. 439:235–247, 2001.

Indexing terms: electron microscopy; immunocytochemistry; nigrostriatal dopamine pathway; synaptosomes

Neuronal nAChR are pentameric ligand-gated cation channels that are widely distributed in the mammalian brain, albeit at relatively low densities. Nine nAChR subunits (α3–α7; β2–β4) have been identified in the mammalian central nervous system (CNS), giving the potential for numerous nAChR subtypes with differing pharmacological and biophysical characteristics (Role and Berg, 1996). Identification of the subunit composition and precise subcellular location of nAChR subtypes is critical to understanding the function of these receptors in the CNS and for the design of pharmacological agents to treat conditions in which nAChR are implicated. Therapeutic targets for nicotinic ligands include smoking cessation and Parkinson's disease (Decker and Arneric, 1999). Dopamine

Contract grant sponsor: Biological and Biotechnological Sciences Research Council, United Kingdom; Contract grant number: 86/C09501; Contract grant sponsor: Royal Society, United Kingdom; Contract grant number: 21565; Contract grant sponsor: Wellcome Trust; Contract grant number: 049452.

*Correspondence to: S. Wonnacott, Department of Biology and Biochemistry, University of Bath, Claverton Down, Bath BA2 7AY, United Kingdom. E-mail: bsssw@bath.ac.uk

Received 21 February 2001; Revised 30 May 2001; Accepted 25 July 2001

neurotransmission in the "reward" and motor pathways is a key element in these conditions, and nicotine and nicotinic agonists increase dopamine release from mesolimbic and nigrostriatal neurones in vitro and in vivo (Wonnacott, 1997; Di Chiara, 2000). There are rather few cases in which nAChR have been shown to mediate synaptic transmission in the brain (Jones et al., 1999); the current consensus is that nAChR may exert a predominantly modulatory influence on neuronal activity and transmitter release (Role and Berg, 1996; Wonnacott, 1997). Nigrostriatal dopaminergic neurones have received considerable attention in this context: The nigrostriatal pathway comprises neurones whose axons project from cell bodies in the substantia nigra pars compacta (SNc) to the dorsal striatum. There is ample evidence from in vitro experiments on synaptosome preparations for the facilitation of basal dopamine release by presynaptic nAChR on striatal dopaminergic terminals (see, e.g., Rapier et al., 1990; Grady et al., 1992; el-Bizri and Clarke, 1994; Soliakov and Wonnacott, 1996, 2001). Somatodendritic nAChR can excite dopaminergic SNc neurones (Clarke et al., 1985; Picciotto et al., 1998; Klink et al., 2001) and may also exert a modulatory influence, provoking sustained increases in intracellular Ca^{2+} (Tsuneki et al., 2000).

In the rat, most nAChR subunits (α3–α7 and β2–β4) have been reported to be expressed in the SNc by using in situ hybridisation (Wada et al., 1989, 1990; Le Novere et al., 1996) or RT-PCR (Charpantier et al., 1998). Recently, single-cell RT-PCR analysis of SN and ventral tegmentum neurons led to the conclusion that all dopaminergic neurons express mRNA for the α4 and β2 subunits, whereas varying proportions of these neurons express α3, α5, α6, α7, and β3 mRNA (Klink et al., 2001). Immunocytochemical studies corroborate the localisation of several nAChR subunit proteins in the rat SNc (Swanson et al., 1987; Hill et al., 1993; Dominguez et al., 1994), with labelling of the cell bodies and dendrites of dopaminergic neurones demonstrated at the light/confocal (Goldner et al., 1997; Arroyo-Jimenez et al., 1999) and electron microscopic levels (Sorenson et al., 1998). Additionally, there is in vitro pharmacological evidence for functional nAChR on cell bodies of rat nigrostriatal neurones (Klink et al., 2001): Two β2 subunit-containing subtypes were inferred from their differential sensitivity to α-conotoxin-MII, in addition to a smaller population of α7 subunit-containing nAChR (designated *β2** and *α7**, respectively). This interpretation is corroborated in mouse SN by the absence of responses in transgenic animals lacking expression of the β2, α4, or α7 subunit (Tsuneki et al., 2000; Klink et al., 2001).

The presence of at least two subtypes of nAChR on nigrostriatal terminals has been inferred from the pharmacological characterisation of the presynaptic nicotinic modulation of dopamine release in vitro. One subtype, which is sensitive to α-conotoxin-MII, is proposed to contain α3 and β2 subunits (Kulak et al., 1997; Kaiser et al., 1998), whereas the putative composition of the other subtype includes α4 and β2 subunits (Sharples et al., 2000). Recent immunoconfocal studies support the presence of the nAChR α4 and α3/α5 subunits in rat striatal synaptosomes (Nayak et al., 2000) and suggest that the two subtypes are segregated to different axon terminals. However, the neurochemical identity of the labelled synaptosomes was not established, and the low resolution of this technique does not permit more subtle questions of localisation to be addressed.

We have chosen to focus initially on the localisation of the β2 nAChR subunit, because this is the most abundant subunit and is common to both of the putative nAChR subtypes associated with dopamine terminals. The latter view is consistent with the absence of nicotine-evoked dopamine release in β2-null mutant mice (Picciotto et al., 1998; Grady et al., 2001). Our preliminary immunoelectron microscopy studies showed immunogold labelling of synaptosomes, consistent with the nAChR β2 subunit on the surface of a subpopulation of rat striatal axon terminals (Wonnacott et al., 2000). As with the confocal studies by Nayak and colleagues (2000), the neurochemical identity of these β2 subunit-containing axon terminals could not be assessed easily in this protocol, and neither of these approaches allows quantitative analysis. The aim of the present study, therefore, was to determine the precise subcellular localisation of the nAChR β2 subunit in relation to the dopaminergic axons and axon terminals in the dorsal striatum, to provide ultrastructural evidence for presynaptic β2 subunit-containing nAChR that can modulate dopamine release. To address this, double-labelling experiments with antibodies against tyrosine hydroxylase (TH) and β2 nAChR subunit were carried out at the confocal and electron microscopic levels.

MATERIALS AND METHODS

Animals

Twelve- to fifteen-week-old adult male Sprague Dawley rats (250–350 g) from the in-house breeding colony at the University of Bath were used in this study. All experiments were performed within the guidelines given by The Animals (Scientific Procedures) Act, 1986. For gel electrophoresis and synaptosome experiments, the rats were killed by cervical dislocation, followed by decapitation, and the brains quickly removed and dissected accordingly. For tissue slices to be processed for microscopy, the rats were deeply anaesthetised with chloral hydrate (350 mg/kg i.p.), perfused transcardially with 50 ml 0.1 M phosphate buffer (PB; pH 7.4) followed by 250 ml fixative, and decapitated and the brains removed for processing.

Antibodies

Three antibodies raised against the nAChR β2 subunit were used in this study; sc1449 (goat polyclonal; Santa Cruz Inc., Santa Cruz, CA), mAb270 (rat monoclonal, gift from Prof. J. Lindstrom, University of Pennsylvania), and NARB2 (mouse monoclonal; Novocastra Laboratories Ltd., Newcastle Upon Tyne, United Kingdom). Mouse monoclonal anti-TH (mAb318) was purchased from Chemicon International Inc. (Temecula, CA), and rabbit polyclonal anti-TH (TZ1010) was from Affiniti Research Products Ltd. (Exeter, United Kingdom). Biotin-, peroxidase-, and fluorochrome-conjugated antibodies were obtained from Vector Laboratories Inc. (Burlingame, CA). Gold-conjugated antibodies were purchased from British Biocell International Ltd. (Cardiff, United Kingdom). All other chemicals were purchased from Sigma-Aldrich Plc. (Poole, United Kingdom). Experiments were conducted at room temperature unless stated otherwise.

Peptide synthesis and antibody preabsorbtion

The commercial goat polyclonal antibody sc1449 was raised against a peptide corresponding to the C-terminus of the human nAChR β2 subunit (LFQNYTTTTFLHSDHSAPSS) and affinity purified by the manufacturer. To confirm the specificity of this antibody in studies on rat tissues, preabsorption controls were performed using the corresponding rat C-terminal peptide (LFQNYTATTFLHPDHSAPSS), which shares 90% identity with the human peptide immunogen. The rat peptide was commercially synthesised at the University of Bristol. For preabsorption studies, sc1449 was incubated with a molar excess of the rat peptide in phosphate-buffered saline (PBS), pH 7.4, for 1 hour on a rotator. The preabsorbed antibody was then centrifuged at 10,000 rpm in a microfuge (Heraeus Instruments, Osterode, Germany) for 5 minutes to remove any particulate matter prior to use.

Immunoprecipitation

A freshly dissected rat brain was homogenised in 5 volumes of ice-cold lysis buffer (PBS), pH 7.4, containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 100 μg/ml phenylmethylsulphonyl fluoride, 33 μg/ml aprotinin, and 1 mM sodium orthovanadate and incubated at 4°C for 30 minutes. Following centrifugation (15,000*g* for 20 minutes at 4°C), the supernatant fraction (lysate) was diluted twofold in PBS containing 2% bovine serum albumin and incubated at 4°C for 10 minutes. The sample was centrifuged as described above and the supernatant incubated with sc1449 (1 μg/ml final concentration) at 4°C for 30 minutes. Next, 3 μg/ml rabbit anti-goat IgG was added to the sample and incubated for a further 30 minutes. The immune complex was "precipitated" by incubating the sample with 100 μg/ml protein A-sepharose at 4°C for 1 hour. The sample was centrifuged as described above and the pellet washed three times in PBS prior to boiling in 200 μl electrophoresis sample buffer (0.2 M Tris buffer, pH 6.7, containing 10% glycerol, 5% β-mercaptoethanol, 5% SDS, 200 μg/ml bromophenol blue). Following centrifugation, the supernatant was loaded onto a 10% SDS-polyacrylamide gel, which was run at 100 V, and then stained for protein content using the standard Coomassie method. The gel was subsequently recorded digitally using an Arcus II scanner (Agfa, Brentford, United Kingdom) and labelled in Photoshop 6.0 (Adobe, Uxbridge, United Kingdom) running on a standard personal computer.

SDS-PAGE and Western blotting

The SN and ventral tegmental area (VTA) were rapidly dissected from the brains of three rats and homogenised in lysis buffer as described above. Following centrifugation (15,000*g* for 20 minutes at 4°C), the protein content of the lysate was determined by the Bradford assay (Bio-Rad Laboratories Inc., Hercules, CA) and diluted in lysis buffer to give a protein concentration of 1 μg/ml. The lysate was mixed with an equal volume of electrophoresis sample buffer, boiled for 5 minutes, and loaded onto a 10% SDS polyacrylamide gel, which was then run at 100 V. The gel was transferred to a nitrocellulose membrane (Schleicher and Shuell, Dassel, Germany) by Western blotting according to standard techniques. The nitrocellulose membrane was blocked in 4% nonfat milk powder (Bio-Rad Laboratories Inc.) in PBS for 1 hour, followed by incubation in primary antibody (sc1449, 1 μg/ml) in PBS containing 0.2% Tween-20 (PBSTw) overnight at 4°C. After PBS washes (3 × 10 min), the membrane was incubated in secondary antibody (horseradish peroxidase-conjugated anti-goat IgG; 1μg/ml) in PBSTw for 2 hours. The membrane was washed as described above, and immunoreactive protein bands were detected using the chemiluminescence technique (Amersham Pharmacia Biotech AB, Uppsala, Sweden). The autoradiographic film was digitally recorded and labelled as described above. Negative controls were performed by incubating the membrane in sc1449 preabsorbed with the rat C-terminal peptide or omitting the primary antibody from the procedure.

Confocal microscopy (tissue)

Immunoconfocal microscopy was performed according to the method of Arroyo-Jimenez et al. (1999). Anaesthetised rats (n = 3) were perfused with 250 ml 4% paraformaldehyde in PB, the brains removed, and 50 μm sections through the SN and dorsal striatum collected using a vibrating microtome. The sections were blocked for 1 hour in 2% foetal calf serum in PBS containing 0.2% Triton X-100 (PBST), followed by incubation in primary antibodies (sc1449 at 5 μg/ml and mAb318 at 1/1,000 dilution) in PBST overnight at 4°C. After PBS washes (3 × 10 minutes), the sections were incubated in fluorochrome-conjugated secondary antibodies (anti-goat FITC and anti-mouse Texas red, both at 1 μg/ml) in PBST for 2 hours. The sections were washed as described above and mounted on glass slides in VectorShield mountant (Vector Laboratories Inc.) prior to viewing in a Zeiss LSM 510 confocal microscope. Images were labelled using the confocal software and recorded digitally. Controls were performed by preabsorbing sc1449 with the rat C-terminal peptide or by omitting one or both primary antibodies from the procedure.

Confocal microscopy (synaptosomes)

Synaptosomes were prepared from freshly dissected striata of three rats and isolated on Percoll gradients according to the method of Soliakov and Wonnacott (1996). The purified synaptosomes (fraction 4) were resuspended in gradient buffer (5 mM HEPES buffer, pH 7.4, containing 320 mM sucrose), fixed in 4% paraformaldehyde containing 0.1% saponin in gradient buffer, washed in PBS (3 × 10 minutes), and then blocked in 2% foetal calf serum in PBS for 1 hour. The synaptosomes were washed in PBS as described above and then incubated in primary antibodies (sc1449 at 5 μg/ml and mAb318 at 1/1,000 dilution) in PBS overnight at 4°C. After PBS washes, the synaptosomes were incubated in fluorochrome-conjugated secondary antibodies (anti-goat FITC and anti-mouse Texas red, both at 1 μg/ml) in PBS for 2 hours. The synaptosomes were washed in PBS and suspended in a drop of VectorShield mountant on glass slides prior to viewing in a Zeiss LSM 510 confocal microscope. Images were recorded as described above. Controls were performed by omitting one or both primary antibodies from the procedure.

Preembedding immunoelectron microscopy

Single-preembedding immunoelectron microscopy was performed according to standard techniques (see Bernard et al., 1997). Anaesthetised rats (n = 5) were perfused with 250 ml 3% paraformaldehyde plus 0.1% glutaralde-

hyde in PB, the brains removed, and 70 μm sections through the SN and dorsal striatum collected using a vibrating microtome. The sections were cryoprotected in PB containing 25% sucrose and 10% glycerol and then subjected to three cycles of freeze–thaw in liquid nitrogen to aid the penetration of immunoreagents into the tissue. The sections were then blocked for 1 hour in 2% normal rabbit serum in PBS prior to incubation in primary antibody (sc1449 at 5 μg/ml or mAb318 at 1/1,000 dilution) in PBS overnight at 4°C. The sections were washed in PBS (3 × 10 minutes) and incubated in secondary antibody (biotin-conjugated anti-goat for sc1449 or mouse IgG at 1 μg/ml for mAb318) for 2 hours. After PBS washes, the sections were incubated in an avidin-biotin-peroxidase complex (ABC reagent; Vector Laboratories Inc.) for 1 hour and washed in PBS and immunoreactive sites revealed by incubation with 0.005% hydrogen peroxide in the presence of 660 μM diaminobenzidine in 0.05 M Tris buffer, pH 7.4. The sections were washed in PB (3 × 10 minutes), postfixed in 1% osmium tetroxide in PB for 35 minutes, dehydrated through an ethanol series, and flat embedded on microscope slides in Durcupan resin (Fluka U.K.). The resin was cured at 60°C. Areas of interest were cut from the sections and 60 nm ultrathin sections collected onto nickel slot grids using an Ultracut E microtome (Leica Microsystems Inc., Bannockburn, IL). The ultrathin sections were counterstained in 2% uranyl acetate and lead citrate according to standard techniques and viewed in a Jeol 1200EX transmission electron microscope operating at 80 kV. Areas of interest were photographed and micrographs prepared on glossy paper (MGIV; Ilford, Knutsford, United Kingdom) in a dark room. Controls were performed by preabsorbing sc1449 with the rat C-terminal peptide or by omitting the primary antibody from the procedure.

Double-preembedding immunoelectron microscopy was performed according to the method of Bernard et al. (1999). Tissue sections from three rats were prepared as for single labelling prior to incubation in both primary antibodies (sc1449 at 5 μg/ml and mAb318 at 1/1,000 dilution) in PBS overnight at 4°C. The sections were washed in PBS (3 × 10 minutes) and incubated in secondary antibodies (1 nm gold-conjugated anti-goat IgG at 1/200 dilution and biotin-conjugated anti-mouse IgG at 1 μg/ml) in PBS overnight at 4°C. The sections were washed in PBS and postfixed for 10 minutes in 1% glutaraldehyde in PBS and the gold particles enhanced by incubation in silver reagent (British Biocell) for 5 minutes. After PBS washes, the sections were incubated in ABC reagent, and immunoreactive sites were revealed by incubation with diaminobenzidine and processed for electron microscopy as described above. Controls were performed by omitting one or both of the primary antibodies from the procedure.

Postembedding immunoelectron microscopy

Postembedding immunoelectron microscopy was performed according to the methods of Baude et al. (1993) and Fujiyama et al. (2000). Anaesthetised rats (n = 3) were perfused with 250 ml 3% paraformaldehyde plus 0.1% glutaraldehyde in PB, the brains removed, and 0.5–1-mm-thick slices of striatum collected using a vibrating microtome. The sections were slam-frozen on a copper plate cooled in liquid nitrogen (MM80E; Leica Microsystems Inc.) to preserve ultrastructure, freeze-substituted in methanol, and embedded in resin (Lowicryl HM20; Agar Scientific Ltd., Stanstead, United Kingdom) according to the manufacturer's instructions. The resin blocks were polymerised under UV light, and ultrathin sections (60 nm) were cut using an Ultracut E microtome and collected on gold mesh or nickel slot grids. The sections were etched briefly (3 seconds) in a saturated solution of sodium ethanolate in absolute ethanol, washed in distilled water, and blocked in 2% normal human serum in PBST for 1 hour. The sections were then incubated in primary antibodies (sc1449 at 10 μg/ml and mAb318 at 1/1,000 dilution) in blocking solution overnight, washed in PBS (3 × 10 min), and incubated in secondary antibodies (10 nm gold-conjugated anti-goat IgG at 1/50 dilution and 18 nm gold-conjugated anti-mouse IgG at 1/50 dilution) in blocking solution containing 0.5% polyethylene glycol for 2 hours. The sections were washed as described above, postfixed in 1% glutaraldehyde, counterstained in 2% uranyl acetate and lead citrate according to standard techniques, and examined in a Jeol 1200EX transmission electron microscope operating at 80 kV. Photomicrographs of immunolabelled structures were obtained as described above. Controls were performed by preabsorbing sc1449 with the rat C-terminal peptide or by omitting one or both primary antibodies from the procedure. In addition, TH double labelling was carried out by coincubation of the sections with two anti-TH antibodies (mAb318 at 1/1,000 dilution and TZ1010 at 1/500 dilution) using 10 nm anti-rabbit IgG and 18 nm anti-mouse IgG secondary antibodies (both at 1/50 dilution).

Analysis of immunogold labelling

Sections of double-preembedded dorsal striatum from three rats were analysed independently by two investigators. Because immunogold labelling does not penetrate tissue as far as diaminobenzidine labelling, all observations were made within 3 μm of the section surface to avoid false-negative results. In this region, the proportions of silver-enhanced immunogold particles within TH-positive and TH-negative structures were recorded for each rat using a random search pattern. In addition, the mean percentage area occupied by TH-positive structures in the same sections of the dorsal striatum was calculated from electron micrographs (5 or 10 per rat at 21,000×). The area occupied by the immunoreactive structures was estimated using a digitising pad and NIH Image software (NIH, Bethesda, MD). The proportion of β2 subunit immunogold particles associated with TH-positive structures was compared to the percentage area that these TH-positive structures occupy in the striatum.

Doubly postembedded sections of dorsal striatum from two rats were analysed from micrographs (at 20,000×) taken at random over the tissue. Immunolabelled axons and axonal boutons in 10 × 1 μm^2 random grids per rat were characterised according to whether they were immunopositive for the nAChR β2 subunit only, TH only, or both proteins. Axonal boutons were identified on the basis of their morphological characteristics, including size and the presence of synaptic vesicles and mitochondria. A structure was deemed immunoloabelled for either protein if it contained a minimum of two immunogold particles for the respective protein. In addition, the cytoplasm or plasma membrane association of each β2 subunit immunogold particle was recorded. Immunogold particles were deemed "membrane-associated" if they touched or were

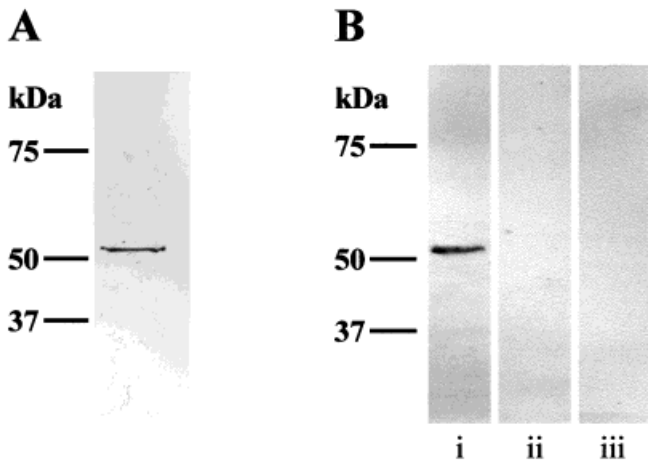

Fig. 1. Specificity of the anti-nAChR β2 subunit antibody sc1449 was confirmed by immunoprecipitation (A) and Western blot analysis (B). **A:** Coommassie-stained gel showing sc1449 immunoprecipitation of a 54 kDa protein, consistent with the β2 subunit (Deneris et al., 1988), from whole rat brain lysate. **B:** Western blot detection of a 54 kDa protein (lane i) from rat SN/VTA lysate. No band was detected following preabsorbtion of the primary antibody with the rat C-terminal peptide (lane ii) or omission of the primary antibody (lane iii).

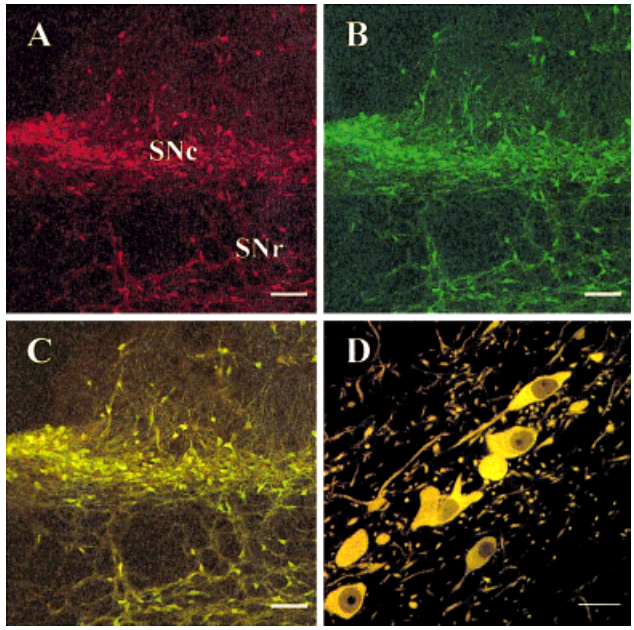

Fig. 2. Immunofluorescence confocal microscopy reveals the colocalisation of nAChR β2 subunit and TH in rat SNc. TH immunoreactivity (**A**) and nAChR β2 immunoreactivity (**B**) in the same section. **C** and **D** show overlay images of A and B. The nAChR β2 subunit and TH are colocalised in the cytoplasm of cell bodies and processes within the SNc. Staining is also seen in displaced dopaminergic neurones within the substantia nigra pars reticulata (SNr). In the absence of the primary antibodies, no immunofluorescence was detected. Scale bars = 200 μm in A–C, 20 μm in D.

within one immunogold particle diameter (10 nm) of the plasma membrane.

RESULTS

Antibody characterisation

Three anti-nAChR β2 subunit antibodies (sc1449, mAb270, and NARB2) were examined in this study. All three antibodies showed comparable immunolabelling in confocal experiments when the tissue was fixed in paraformaldehyde alone. However, only the polyclonal antibody, sc1449, worked effectively in pre- and postembedding electron microscopy, when glutaraldehyde, a stronger fixative than paraformaldehyde, was used (albeit at a low concentration of 0.1%). The inability of two of the antibodies to bind to proteins in tissues fixed for electron microscopy probably reflects the masking by glutaraldehyde of relevant antigenic epitopes on the nAChR β2 subunit. Therefore, only the results obtained with sc1449 are presented.

Sc1449 was raised against a 20-amino-acid peptide from the extracellular C-terminal domain of the human nAChR β2 subunit. The corresponding rat β2 subunit C-terminal sequence (see Materials and Methods) shares 90% identity with the peptide immunogen (18 of 20 amino acids), indicating that there is a high probability that the antibody will recognise the rat β2 subunit. There is not more than 5% identity between the peptide immunogen and any other rat nAChR subunit (for example, 1 of 20 amino acids with the α3 subunit). To confirm that sc1449 recognises the rat β2 subunit, immunoprecipitation and Western blot analyses of rat brain extracts were performed (Fig. 1). The antibody immunoprecipitated a single protein band of 54 kDa (Fig. 1A), consistent with the predicted molecular weight for the rat nAChR β2 subunit (Deneris et al., 1988). A band of similar size was recognised on Western blotting (Fig. 1B, lane i). No protein bands were detected when sc1449 was preabsorbed with a molar excess of the rat C-terminal peptide sequence or when the antibody was omitted from the protocol (Fig. 1B, lanes ii, iii).

Immunoconfocal microscopy

The distributions of TH and nAChR β2 immunoreactivity were compared by using confocal microscopy in 50 μm sections of rat SN and dorsal striatum. Within the SNc (Fig. 2), all cell bodies and dendrites immunopositive for TH (Fig. 2A) were also labelled by the anti-β2 subunit antibody (Fig. 2B), as shown in the overlay (Fig. 2C, and at higher magnification in Fig. 2D). A few displaced dopaminergic neurones were also immunopositive for both TH and nAChR β2 subunit within the SNr. Preabsorbtion of sc1449 with the rat C-terminal peptide corresponding to the immunogen abolished β2 subunit immunoreactivity in the SNc (not shown). Additional control experiments in which sections were incubated in a single primary antibody followed by both secondary antibodies demonstrated that there was no cross-reactivity between the secondary antibodies and that the colocalisation observed was not due to spectral crossover. No neurones immunopositive for only TH or nAChR β2 subunit were observed in either the SNc or the SNr (n = 3 rats).

When the same technique was applied to the striatum, no cell bodies were labelled by either the anti-TH or the anti-nAChR β2 subunit antibodies (not shown). There was, however, strong labelling of TH-containing axons throughout the dorsal striatum by mAb318. In contrast

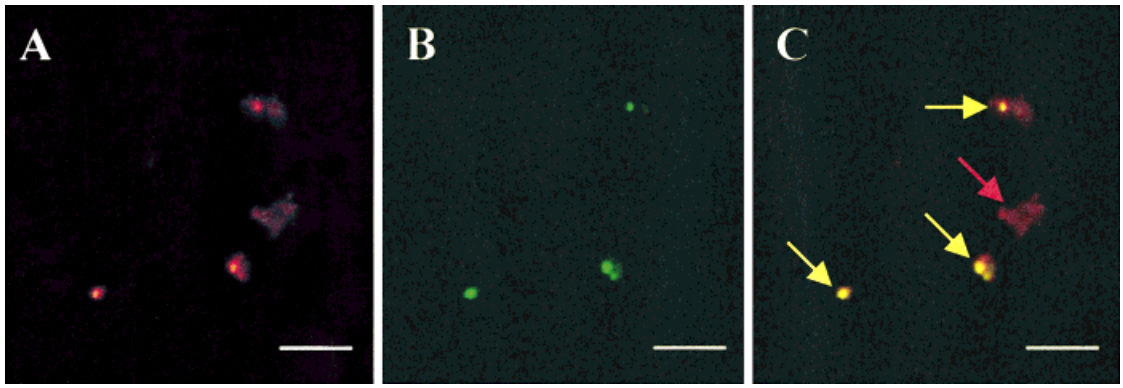

Fig. 3. Immunoconfocal microscopy reveals the presence of TH (**A**) and nAChR β2 subunit (**B**) immunoreactivity within striatal synaptosomes. By overlaying the two images (**C**), it can be seen that a proportion of the synaptosomes is labelled for both nAChR β2 subunit and TH (yellow arrows), whereas others are labelled for TH only (red arrow). Scale bars = 10 μm.

there was a weaker, diffuse labelling with the anti-β2 subunit antibody sc1449, which could not be unequivocally attributed to axons or axon terminals at the resolution of the confocal microscope.

To address more directly the presence in axon terminals of presumptive nAChR containing the β2 subunit, immunoconfocal analysis was applied to striatal synaptosomes (Fig. 3). A small proportion of synaptosomes was immunopositive for TH (Fig. 3A) and for the nAChR β2 subunit (Fig. 3B). Superimposition revealed that many of the TH-positive terminals also contained the β2 subunit (Fig. 3C, yellow arrows). Some weakly labelled TH-containing structures that were not positive for the β2 subunit were also observed (Fig. 3C, red arrow), as well as a population of β2-positive, TH-negative structures (not shown). Because of the aggregation of synaptosomes during processing, it was not possible to perform accurate quantitative analysis of the distribution of TH or β2 immunoreactivity, but these experiments provide qualitative evidence for the presence of the nAChR β2 subunit in dopaminergic terminals within the rat striatum. For a more detailed analysis of the precise subcellular localisation of nAChR β2 subunit immunoreactivity, immunoelectron microscopy was performed on sections of rat dorsal striatum and SNc.

Immunoelectron microscopy

Single-labelling studies, employing standard immunoperoxidase methods (Bernard et al., 1997), revealed β2 subunit immunoreactivity in cell bodies and dendrites within the SNc (Fig. 4A,B) and axons and axon terminals within the dorsal striatum (Fig. 4C–E). Although the neurochemical identity of these neurones could not be determined, many of the labelled axon terminals in the dorsal striatum formed symmetric synapses with dendritic spines (illustrated in Fig. 4D), similar to those formed by dopaminergic neurones (Fig. 4F). In addition, β2 subunit immunoreactivity was also seen in a population of axon terminals forming asymmetric synapses with dendrites (Fig. 4E). No β2 subunit immunolabelling was seen when sc1449 was preabsorbed with a molar excess of the rat C-terminal peptide sequence or when the antibody was omitted from the protocol (not shown).

To elucidate whether the TH and β2 subunit immunoreactivities are colocalised in the same structures, SN and dorsal striatum sections were subjected to double preembedding immunoelectron microscopy (Bernard et al., 1999) using silver-enhanced gold particles to localise the nAChR β2 subunit and diaminobenzidine to identify TH immunoreactive neurones (Fig. 5). In agreement with the confocal observations (Fig. 2), the results clearly indicate that TH and nAChR β2 subunit are colocalised in the same neuronal cell bodies and dendrites within the SNc (Fig. 5A,B). Control experiments (Fig. 5E–H), in which one of the primary antibodies was omitted from the staining procedure, reveal patterns of immunoreactivity comparable to those in single-labelling experiments, demonstrating that there is no cross-reactivity with either secondary antibody. When both primary antibodies were omitted from the protocol, no immunolabelling was observed (not shown). In the dorsal striatum, nAChR immunoreactivity was localised within both TH-positive (Fig. 5C,D) and TH-negative (not shown) axons and axon terminals. Quantitative analysis of dorsal striatum sections from three rats by two independent investigators revealed that a mean of 17.5% of silver-enhanced gold particles was associated with TH-positive structures (range 7–22%; four observations per rat; $n > 300$ immunogold particles per observation). The TH-positive structures occupied a mean of 2.6% of the area of the sections analysed (range 1.3–4.5%), indicating an average 6.7-fold enrichment of labelling by the silver-enhanced gold particles of TH-positive structures compared with TH-negative structures (range 4.2–8.8-fold).

A major limitation of the preembedding technique is the poor penetration of the immunoreagents through the section. This is a particular problem for the gold-conjugated antibodies, which penetrate only 3–4 μm into the section. Consequently, all observations were made within 3 μm of the section surface to avoid false-negative results. This immunoreagent penetration problem and the possibility of the silver reagent fusing several gold particles together and masking the precise particle location make quantitative analysis unreliable. To overcome this, we performed postembedding immunoelectron microscopy on Lowicryl-embedded dorsal striatum (Fig. 6). The advantage of this technique is that immunolabelling is performed on the surface of ultrathin sections, so antibody penetration is not a limitation, and the precise subcellular location of

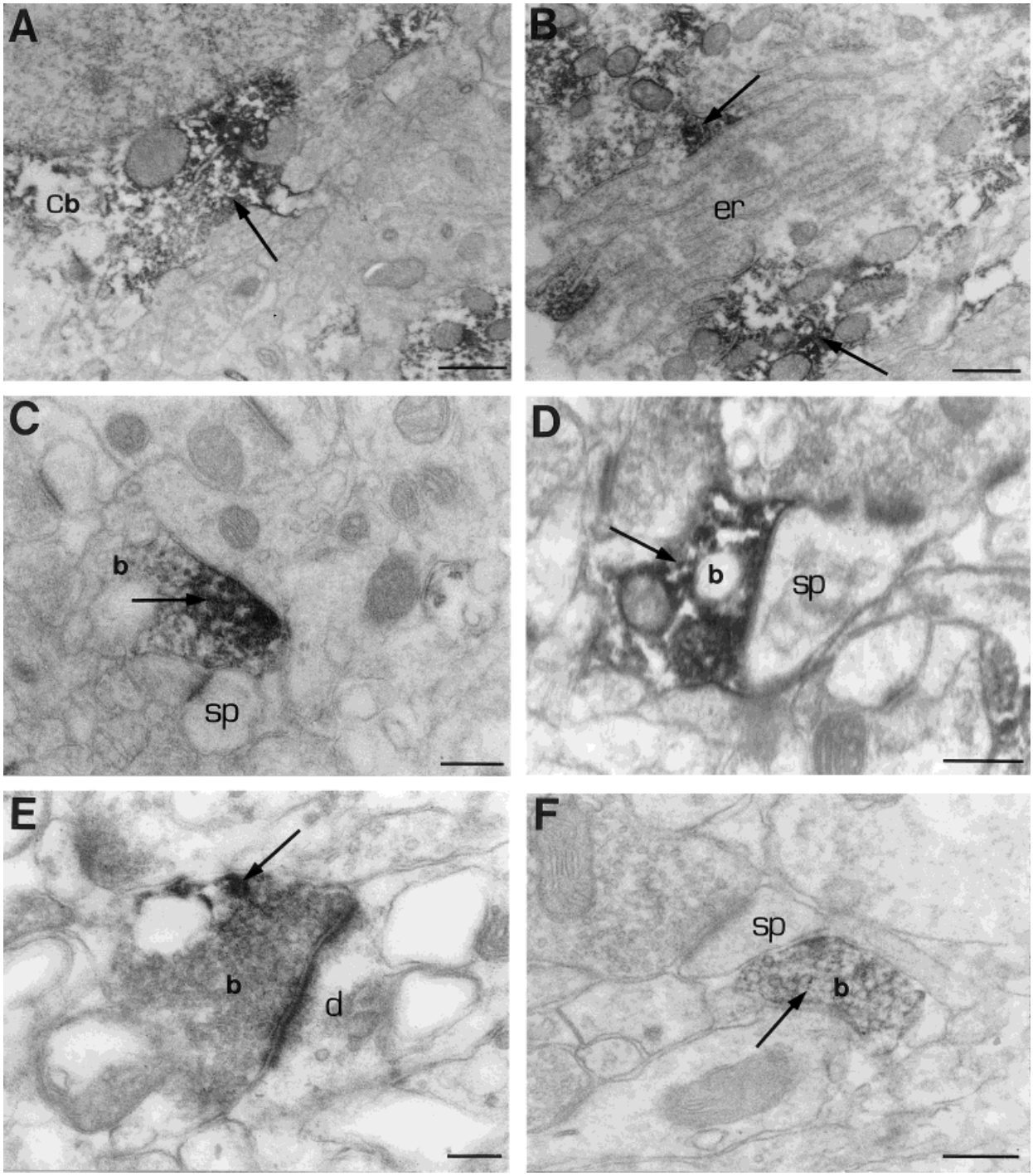

Fig. 4. Single preembedding immunoelectron microscopy reveals the presence of TH and nAChR β2, indicated by deposition of electron-dense diaminobenzidine reaction product (arrows) in the SNc and dorsal striatum. **A,B:** nAChR β2 immunoreactivity in the cytoplasm of a cell body (Cb) in A and peripheral to the endoplasmic reticulum (er) in a proximal dendrite in B within the SNc. **C–E:** nAChR β2 immunoreactivity in axonal boutons (b) within the dorsal striatum. In C and D, immunolabelled boutons are observed apposed to dendritic spines (sp). In E, an immunolabelled bouton forms an asymmetric synapse with a dendritic shaft (d). F: TH immunoreactivity in an axonal bouton (b) apposed to a dendritic spine (sp) within the dorsal striatum. Scale bars = 500 nm in A,B, 200 nm in C,D,F, 100 nm in E.

immunogold particles can be established. In addition, several different-sized immunogold particles can be used for colocalisation studies. The major disadvantage is the poor retention of morphology in freeze-substituted tissue, which can hamper the identification of some cellular components.

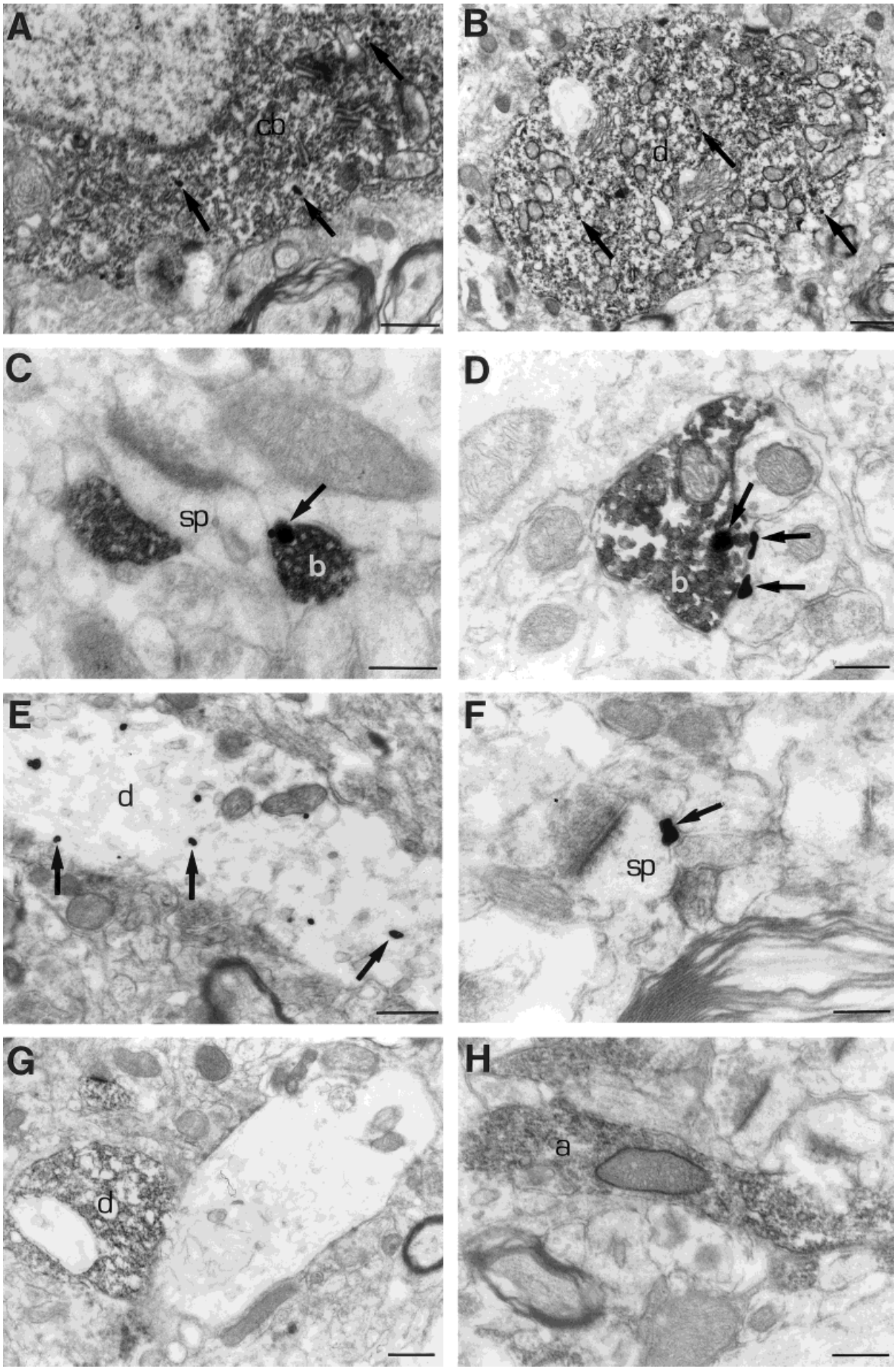

Figure 5

In agreement with the preembedding results, the postembedding double-labelling experiments, employing different sizes of immunogold particles, demonstrated colocalisation of the nAChR β2 subunit and TH within axons and axon terminals within the dorsal striatum (Fig. 6A–D). In addition, as revealed by double-preembedding experiments, β2 subunit immunoreactivity was observed in a population of TH-negative axons and axonal boutons that typically formed asymmetric synapses with dendrites (Fig. 6E). No β2 subunit immunolabelling was seen when sc1449 was preabsorbed with a molar excess of the rat C-terminal peptide sequence (not shown). To confirm that the double labelling observed is not due to cross-reactivity between the secondary antibodies, control experiments were performed in which tissue sections were incubated with only one primary antibody, either anti-nAChR β2 subunit or anti-TH antibody, followed by incubation in both secondary antibodies. The results (Fig. 6F,G) show that only one size of immunogold particle is present on the section in each case, demonstrating that that there is no cross-reactivity between the antibodies. As an additional control, to confirm the specificity of the anti-TH antibody, double labelling for TH was undertaken using two different anti-TH antibodies. The immunogold particles associated with each antibody were found to colocalise within the same axons (Fig. 6H) and axon terminals. The nonreactivity of the other anti-β2 subunit antibodies in the presence of glutaraldehyde precluded a similar analysis for the nAChR β2 subunit.

Quantitative analysis of the nAChR β2 subunit and TH double labelling in sections from two animals shows that 66.6% of labelled axons were immunopositive for both proteins, 8.8% for TH only, and 24.6% for β2 subunit only (n = 228 axons). Among the labelled axon terminals, 49.2% were immunopositive for both TH and β2 subunit, 7.7% for TH only, and 43.1% for β2 subunit only (n = 130 axon terminals). This equates to 86% of TH-positive axonal boutons containing the nAChR β2 subunit, whereas 45% of β2 subunit labelled boutons do not contain TH. The majority of the β2 subunit immunogold particles was cytoplasmic (84.7%), with a smaller proportion (15.3%) associated with the cell membrane (i.e., touching the membrane or within one gold particle diameter of it; n = 1,209). This contrasts with immunolabelling for TH, in which only 3.9% (n = 1,282) of immunogold particles was associated with the cell membrane.

Fig. 5. Double-preembedding immunoelectron microscopy reveals the subcellular localisation of the nAChR β2 subunit within dopaminergic nigrostriatal neurones. nAChR β2 subunit immunoreactivity is identified by silver-enhanced gold immunoparticles (some indicated by arrows); TH immunoreactivity is identified by diaminobenzidine reaction product. **A,B:** nAChR β2 subunit and TH colocalisation within the cytoplasm of a cell body (cb; A) and dendrite (d; B) in the SNc. **C,D:** nAChR β2 subunit and TH colocalisation within axonal boutons (b) in the dorsal striatum. In C, the axonal bouton is closely apposed to a dendritic spine (sp). **E,F:** Control experiments in which the anti-TH antibody was omitted from the staining procedure. Note that β2 subunit immunolabelling alone persists in a dendrite (d) within the SNc (E) and an axon terminal apposed to a dendritic spine (sp) within the dorsal striatum (F). **G,H:** Control experiments in which the anti-nAChR β2 subunit antibody was omitted from the staining procedure. Note that TH immunolabelling alone persists in a dendrite (d) within the SNc (G) and an axon (a) within the dorsal striatum (H). Scale bars = 1 μm in B, 500 nm in A,E,G, 200 nm in C,D,F,H.

DISCUSSION

The objective of the present study was to define the precise cellular and subcellular localisation of the nAChR β2 subunit in the adult rat nigrostriatal pathway. To this end, the nAChR β2 subunit was identified within the cytoplasm and on membranes of dopaminergic cell bodies and dendrites within the SNc and axon terminals within the dorsal striatum. These data thus provide the first ultrastructural corroboration for the presence of presynaptic β2-containing nAChR on dopaminergic axonal boutons in the striatum, previously inferred from pharmacological observations (Kulak et al., 1997; Kaiser et al., 1998; Sharples et al., 2000; Grady et al., 2001).

Technical considerations

Both immunoconfocal and immunoelectron microscopy were employed to address the question of nAChR β2 subunit localisation in the nigrostriatal pathway. In using the relatively mild fixation regime (paraformaldehyde alone) required for confocal microscopy, three anti-nAChR β2 subunit antibodies (sc1449, mAb270, and NARB2) were found to immunolabel neurones in confocal experiments, compared to just one (sc1449) in electron microscopy experiments, in which only relatively low levels of glutaraldehyde (0.1%) could be used. Higher glutaraldehyde concentrations, in which tissue is better preserved, were found to be deleterious to immunoreactivity of the β2 subunit detected by sc1449 and therefore could not be used in this study. The success of sc1449 in glutaraldehyde-fixed tissue may be due to its polyclonal nature, insofar as it has a better chance of recognising unmasked epitopes on the β2 subunit than monoclonal antibodies. However, the monoclonal anti-TH antibody mAb318 is clearly effective in both pre- and postembedding studies, as illustrated by double labelling with the polyclonal anti-TH antibody TZ1010 (Fig. 6H). To confirm the specificity of sc1449, which was raised to the C-terminus of the human β2 subunit, in studies on rat tissues, preabsorption experiments were performed using a peptide corresponding to the C-terminus of the rat β2 subunit. Under all the fixation and incubation strategies employed in this study, preabsorption abolished sc1449 immunoreactivity.

Two immunolabelling strategies were applied at the electron microscopic level, where subcellular resolution is achieved: single labelling with diaminobenzidine and double labelling with diaminobenzidine and/or immunogold particles. The localisation of proteins by the deposition of diaminobenzidine reaction product is an extremely sensitive technique as a result of the amplification of the signal by the avidin-biotin-peroxidase complex. Single labelling clearly showed β2 immunoreactivity in neuronal structures within the SNc and dorsal striatum (Fig. 4). However, in that the reaction product is diffusable within these structures and has a particularly high affinity for membranes, the precise subcellular distribution of proteins cannot be deduced. In contrast, the use of immunogold particles in postembedding studies allows the precise subcellular distribution of proteins to be identified, because the gold particles do not diffuse within labelled structures and remain in close association with the target protein. Thus, a gold particle that is physically touching a plasma membrane or is within one particle diameter of it has been taken to represent membrane localisation.

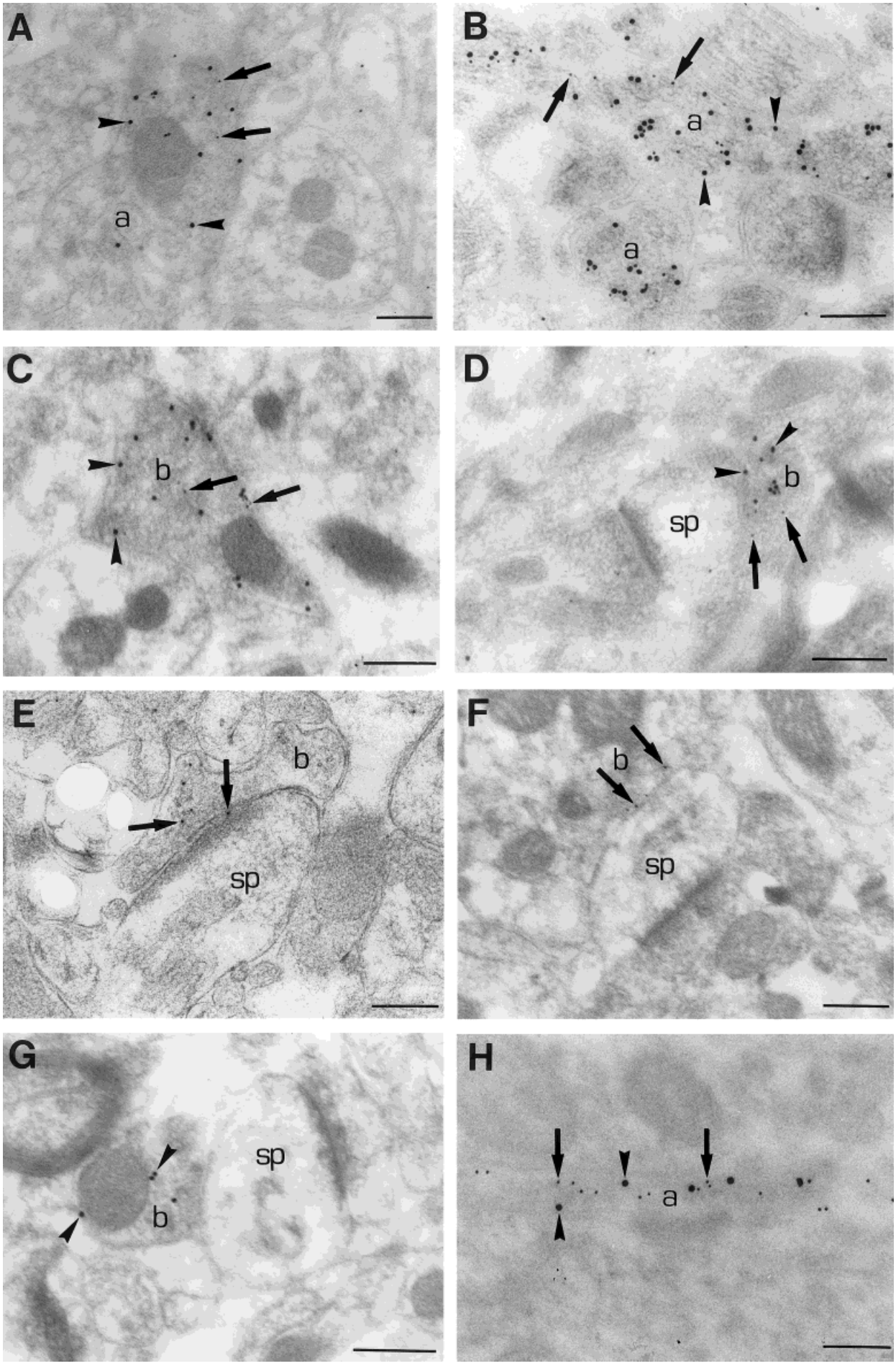

Figure 6

Controls conducted in parallel to the double-labelling experiments included the omission of one or both of the primary antibodies from the staining protocols. By leaving out one of the primary antibodies, but incubating the samples in both secondary antibodies, an estimation of the cross-reactivity between these secondary antibodies could be ascertained. Careful selection of secondary antibodies, especially the host species, and adjustments to the blocking conditions were conducted during the development of the experiments to ensure that secondary antibody cross-reactivity was avoided (Figs. 5E–H, 6F,G). Omission of both primary antibodies from the staining protocols revealed negligible background labelling imparted by secondary and tertiary reagents. Again, this was achieved by careful optimisation of reagent concentrations and blocking conditions.

Colocalisation of nAChR β2 subunit and TH immunoreactivity in the SNc

The immunoconfocal (Fig. 2) and immunoelectron (Figs. 4, 5) microscopy studies, showing that the cell bodies and dendrites of dopaminergic neurones within the SNc express the nAChR β2 subunit, are in agreement with data from Sorenson et al. (1998). No neurones or processes immunoreactive for the β2 subunit or TH alone were observed, implying, first, that there are no nondopaminergic neurones or afferents in the SNc expressing the β2 subunit and, second, that all the nigrostriatal dopaminergic neurones contain this nAChR subunit. Arroyo-Jimenez and colleagues (1999) identified two populations of nAChR α4 subunit-containing neurones within the SNc, one that was TH-positive and a minor population that was TH-negative. Our results suggest that the latter neuronal population does not contain the β2 subunit. Klink et al. (2001) recently presented biochemical and pharmacological evidence for the presence of β2* nAChR on GABAergic neurones in the rat SN. However, in that study, the proportion of GABAergic neurones was found to peak at 16 days of age, and the prevalence of this phenotype was suggested to be regulated developmentally. The present examination was performed on tissue from 12–15-week-old rats, in which β2-expressing GABAergic neurons may have declined below detectable levels.

At the subcellular level, the nAChR β2 subunit was found predominantly in the cytoplasm of TH-positive cell bodies and dendrites within the SNc (Fig. 5). This labelling presumably represents the expression, assembly, and trafficking of the β2 subunit to membrane destinations in the SNc and striatum. Occasionally β2 subunit immunolabelling was observed to be associated with somatodendritic membranes (seen in Fig. 5A,B,E), suggesting that β2-containing nAChR may be present at these sites. This is consistent with evidence for functional β2-containing nAChR on rat and mouse SNc neurones (Picciotto et al., 1998; Tsuneki et al., 2000; Klink et al., 2001). The nAChR α4 subunit has previously been localised to the soma and dendrite surface of nigrostriatal neurones (Arroyo-Jimenez et al., 1999). That observation, together with the present data, implies that some of the nAChR expressed on the plasma membrane of dopaminergic neurones may be of the α4β2* subtype, as proposed by Klink et al. (2001).

Localisation of nAChR β2 subunit immunoreactivity in striatal axons and axon terminals

The presence of the nAChR β2 subunit within the rat dorsal striatum was suggested by a diffuse staining observed in immunoconfocal analysis of labelled sections (this study; Hill et al., 1993). In that no intrinsic striatal neurones were β2 immunoreactive, this diffuse staining is likely to represent the presence of the β2 subunit in afferents projecting into, or through, the dorsal striatum. This interpretation was also proposed by Swanson and colleagues (1987) to account for the moderate labelling of the rat striatum by [^{125}I]mAb270, now recognised to be a β2 subunit-specific antibody. The presence of the β2 subunit in the striatum was confirmed by immunoconfocal microscopy of isolated axon terminals (synaptosomes; Fig. 3). The results show that the β2 subunit is expressed in a proportion of striatal synaptosomes, consistently with our previous immunogold labelling of striatal synaptosomes for the β2 subunit (Wonnacott et al., 2000). Colocalisation with TH shows that many of these synaptosomes are dopaminergic and therefore of nigrostriatal origin.

Immunoelectron microscopy permitted ultrastructural verification of the presence of the β2 subunit in nigrostriatal axons and axonal boutons. Double-preembedding experiments on sections of dorsal striatum demonstrated that there is an average 6.7-fold enrichment of nAChR β2 immunogold particles in TH-positive axons and axon terminals (data from three rats). This association was further quantified by analysis of doubly postembedded samples, revealing that 86% of TH-positive axonal boutons in the dorsal striatum contained the nAChR β2 subunit, with a small proportion only singly labelled for TH. In that all dopaminergic cell bodies within the SNc are immunoreactive for the nAChR β2 subunit (Fig. 2), the absence of the β2 subunit from a proportion of TH-positive axon terminals may be a technical artefact, simply reflecting the low density of nAChR in these structures. Alternatively, the β2 subunit may be targeted to a subpopulation of dopaminergic terminals.

In addition to the double-labelled structures seen in confocal and immunoelectron microscopy experiments, single-labelled structures immunopositive for the β2 subunit alone were also detected in the dorsal striatum (illustrated in Fig. 6E). In postembedded samples, a substantial proportion (45%) of β2 subunit-labelled boutons was TH-negative. These frequently differed morphologically from

Fig. 6. Subcellular localisation of nAChR β2 subunit (10 nm gold particles, some indicated by arrows) and TH (18 nm gold particles, some indicated by arrowheads) in axons (a) and axonal boutons (b) in the dorsal striatum, revealed by postembedding immunoelectron microscopy. **A–E:** nAChR β2 and TH double labelling. **F,G:** Single labelling. **H:** TH double labelling with 10 nm (TZ1010) and 18 nm gold particles (mAb318). A,B: Colocalisation of nAChR β2 subunit and TH within axons (a). C,D: Colocalisation of nAChR β2 subunit and TH within axonal boutons (b), one shown closely apposed to a dendritic spine (sp) in D. E: β2 Subunit immunoreactvity within a TH-negative axonal bouton (b) in asymmetric synaptic contact with a dendritic spine (sp). F,G: Control experiments in which sections were incubated in one of the primary antibodies (nAChR β2 subunit in F and TH in G) but both of the secondary antibodies. Note that immunolabelling for only one size of gold particle was present in the immunolabelled axonal boutons (b) in each case, indicating that the secondary antibodies do not cross-react. H: Double labelling of TH in an axon using two different anti-TH primary antibodies. Scale bars = 200 nm in A–D,F–H, 100 nm in E.

TH-positive axon terminals by forming asymmetric synapses onto dendrites (see Fig. 6E; see also Fig. 4E). Although the neurochemical identity of these axon terminals could not be defined in these experiments, several neuronal populations projecting into the striatum have been shown to form this type of synapse, including corticostriatal and thalamostriatal glutamatergic afferents and serotonergic afferents from the dorsal raphe nucleus (Bolam and Bennett, 1995). The latter population is a good candidate for the β2-positive, TH-negative boutons; [^{3}H]nicotine binding (which predominantly labels α4β2 nAChR) in the rat striatum is decreased by serotonergic lesions (Schwartz et al., 1984). Moreover, nicotine stimulates 5-hydroxytryptamine release from rat striatal synaptosomes, consistent with the presence of nAChR on serotonergic terminals (Reuben and Clarke, 2000). The unique pharmacological profile of this response did not unequivocally define the subunit composition of the presynaptic nAChR responsible; however, the α4β2 nAChR is one candidate.

Axonal boutons immunoreactive for both TH and the β2 subunit with the double-preembedding and -postembedding techniques typically formed symmetric synapses with dendritic spines in the dorsal striatum. In these axonal boutons, 15.3% of β2 subunit immunoreactivity was associated with the plasma membrane, in contrast to TH immunoreactivity, where only 3.9% of immunogold particles were membrane associated. Insofar as TH is a cytoplasmic enzyme, the value of 3.9% is likely to be disproportionately high because larger gold particles were used. The findings thus strongly indicate that a proportion of the nicotinic receptor is associated with the plasma membrane and could reflect surface expression of functional β2-containing nAChR on axon terminals within the dorsal striatum.

Immunolabelling for the β2 subunit with pre- and postembedding techniques was rarely associated with the synaptic specialisations of TH-positive terminals (Figs. 5C,D,F), suggesting a nonsynaptic role for β2-containing nAChR. Quantitative analysis of this association was hampered by the difficulty of identifying symmetric synapses in postembedded material, resulting from the poor retention of morphology and the relatively small size of dopaminergic synapses in the dorsal striatum. In this area of the brain, the source of the endogenous ligand, acetylcholine, is local interneurones. Analysis of choline acetyltransferase (ChAT)-immunostained terminals in the rat striatum has revealed that these form synapses with GABAergic medium spiny neurones (Wainer et al., 1984; Izzo and Bolam, 1988), but axoaxonic synapses were rarely observed (Izzo and Bolam, 1988). Other immunoelectron microscopy studies have addressed the number of synaptic contacts made by cholinergic varicosities. Such analysis indicates that fewer than 10% of axon terminals and varicosities have synaptic membrane differentiation. This suggests a low frequency of synaptic contacts such that, in addition to synaptic transmission, diffuse (paracrine) transmission by acetylcholine is also likely to occur (Descarries et al., 1997). Thus, stimulation of nAChR on dopaminergic terminals may be by acetylcholine that has diffused from sites of release. Future experiments with nAChR subunits and cholinergic neurone markers, such as ChAT, will be aimed to address this relationship directly.

The observation that essentially all dopaminergic terminals are β2 immunoreactive, indicating that all terminals bear nAChR, and the apparent extrasynaptic membrane localisation of the β2 subunit, allow us to speculate on the significance of presynaptic nAChR. Nigrostriatal dopaminergic neurones possess highly branched innervation of the striatum, and it has been estimated that each neurone forms between 2.5×10^5 and 5×10^6 synaptic boutons (J.P. Bolam, unpublished observations). The presence of β2* nAChR on every bouton, coupled with the proposed paracrine delivery of acetylcholine, would facilitate the co-ordination of nerve terminal activity in response to cholinergic tone. Hence, a function of presynaptic nAChR might be to coordinate the diffuse regulation of terminal arborisations. This would be achieved through local depolarisation and through increases in intracellular Ca^{2+} (Soliakov et al., 1995; Soliakov and Wonnacott, 1996) that, in addition to facilitating transmitter release, may also interface with intracellular signalling cascades (see, e.g., Soliakov and Wonnacott, 2001) to regulate diverse aspects of neuronal activity. Similar dual modes of activation, through depolarisation and Ca^{2+} signalling, are also mediated by β2* nAChR on the soma of these neurones (Picciotto et al., 1998; Tsuneki et al., 2000). Hence, nAChR may serve comparable modulatory roles at the somatodendritic and axon terminal levels.

In summary, the demonstration of nAChR β2 subunit immunoreactivity on nigrostriatal dopaminergic terminals provides an anatomical substrate for the pharmacological evidence for presynaptic nAChR that modulate dopamine release in the striatum. The β2 subunit is localised predominantly at nonsynaptic sites on dopaminergic axon membranes in the striatum, where it may participate in functional nAChR responding to acetylcholine released in a paracrine manner. The ability to localise precisely, in a single section, an nAChR subunit and a neurochemical marker of the cellular element in which they are located represents an important development in the elucidation of the precise composition and distribution of nAChR subtypes in the circuitry of the basal ganglia.

ACKNOWLEDGMENTS

We thank Prof. J. Lindstrom for the generous gift of mAb270 and Caroline Francis and Ursula Potter for technical assistance.

LITERATURE CITED

Arroyo-Jimenez MM, Bourgeois JP, Marubio LM, Le Sourd AM, Ottersen OP, Rinvik E, Changeux JP. 1999. Ultrastructural localization of the alpha4-subunit of the neuronal acetylcholine nicotinic receptor in the rat substantia nigra. J Neurosci 19:6475–6487.

Baude A, Nusser Z, Roberts JD, Mulvihill E, McIlhinney RA, Somogyi P. 1993. The metabotropic glutamate receptor (mGluR1 alpha) is concentrated at perisynaptic membrane of neuronal subpopulations as detected by immunogold reaction. Neuron 11:771–787.

Bernard V, Somogyi P, Bolam JP. 1997. Cellular, subcellular, and subsynaptic distribution of AMPA-type glutamate receptor subunits in the neostriatum of the rat. J Neurosci 17:819–833.

Bernard V, Levey AI, Bloch B. 1999. Regulation of the subcellular distribution of m4 muscarinic acetylcholine receptors in striatal neurons in vivo by the cholinergic environment: evidence for regulation of cell surface receptors by endogenous and exogenous stimulation. J Neurosci 19:10237–10249.

Bolam JP, Bennett BD. 1995. Microcircuity of the neostriatum. In: Ariano MA, Surmeier DJ, editors. Molecular and cellular mechanisms of neostriatal function. Austin, TX: R.G. Landes Company. p 1–19.

Charpantier E, Barneoud P, Moser P, Besnard F, Sgard F. 1998. Nicotinic

acetylcholine subunit mRNA expression in dopaminergic neurons of the rat substantia nigra and ventral tegmental area. Neuroreport 9:3097–3101.

Clarke PB, Hommer DW, Pert A, Skirboll LR. 1985. Electrophysiological actions of nicotine on substantia nigra single units. Br J Pharmacol 85:827–835.

Decker MW, Arneric SP. 1999. Nicotinic acetylcholine receptor-targeted compounds: a summary of the development pipeline and therapeutic potential. In: Arneric SP, Brioni JD, editors. Neuronal nicotinic receptors: pharmacology and therapeutic opportunities. New York: John Wiley & Sons. p 395–411.

Deneris ES, Connolly J, Boulter J, Wada E, Wada K, Swanson LW, Patrick J, Heinemann S. 1988. Primary structure and expression of beta 2: a novel subunit of neuronal nicotinic acetylcholine receptors. Neuron 1:45–54.

Descarries L, Gisiger V, Steriade M. 1997. Diffuse transmission by acetylcholine in the CNS. Progr Neurobiol 53:603–625.

Di Chiara G. 2000. Behavioural pharmacology and neurobiology of nicotine reward and dependence. In: Clementi F, Fornasari D, Gotti C, editors. Handbook of experimental pharmacology 144: neuronal nicotinic receptors. Berlin: Springer. p 603–750.

Dominguez DT, Juiz JM, Peng X, Lindstrom J, Criado M. 1994. Immunocytochemical localization of the alpha 7 subunit of the nicotinic acetylcholine receptor in the rat central nervous system. J Comp Neurol 349:325–342.

el-Bizri H, Clarke PB. 1994. Blockade of nicotinic receptor-mediated release of dopamine from striatal synaptosomes by chlorisondamine administered in vivo. Br J Pharmacol 111:414–418.

Fujiyama F, Fritschy JM, Stephenson FA, Bolam JP. 2000. Synaptic localization of GABA(A) receptor subunits in the striatum of the rat. J Comp Neurol 416:158–172.

Goldner FM, Dineley KT, Patrick JW. 1997. Immunohistochemical localization of the nicotinic acetylcholine receptor subunit alpha6 to dopaminergic neurons in the substantia nigra and ventral tegmental area. Neuroreport 8:2739–2742.

Grady S, Marks MJ, Wonnacott S, Collins AC. 1992. Characterization of nicotinic receptor-mediated [^{3}H]dopamine release from synaptosomes prepared from mouse striatum. J Neurochem 59:848–856.

Grady SR, Meinerz NM, Cao J, Reynolds AM, Picciotto MR, Changeux JP, McIntosh JM, Marks MJ, Collins AC. 2001. Nicotinic agonists stimulate acetylcholine release from mouse interpeduncular nucleus: a function mediated by a different nAChR than dopamine release from striatum. J Neurochem 76:258–268.

Hill JAJ, Zoli M, Bourgeois JP, Changeux JP. 1993. Immunocytochemical localization of a neuronal nicotinic receptor: the beta 2-subunit. J Neurosci 13:1551–1568.

Izzo PN, Bolam JP. 1988. Cholinergic synaptic input to different parts of spiny striatonigral neurons in the rat. J Comp Neurol 269:219–234.

Jones S, Sudweeks S, Yakel JL. 1999. Nicotinic receptors in the brain: correlating physiology with function. Trends Neurosci 22:555–561.

Kaiser SA, Soliakov L, Harvey SC, Luetje CW, Wonnacott S. 1998. Differential inhibition by alpha-conotoxin-MII of the nicotinic stimulation of [^{3}H]dopamine release from rat striatal synaptosomes and slices. J Neurochem 70:1069–1076.

Klink R, de Kerchove d'Exaerde A, Zoli M, Changeux JP. 2001. Molecular and physiological diversity of nicotinic acetylcholine receptors in the midbrain dopaminergic nuclei. J Neurosci 21:1452–1463.

Kulak JM, Nguyen TA, Olivera BM, McIntosh JM. 1997. Alpha-conotoxin MII blocks nicotine-stimulated dopamine release in rat striatal synaptosomes. J Neurosci 17:5263–5270.

Le Novere N, Zoli M, Changeux JP. 1996. Neuronal nicotinic receptor alpha 6 subunit mRNA is selectively concentrated in catecholaminergic nuclei of the rat brain. Eur J Neurosci 8:2428–2439.

Nayak SV, Ronde P, Spier AD, Lummis SC, Nichols RA. 2000. Nicotinic receptors co-localize with 5-HT(3) serotonin receptors on striatal nerve terminals. Neuropharmacology 39:2681–2690.

Picciotto MR, Zoli M, Rimondini R, Lena C, Marubio LM, Pich EM, Fuxe K, Changeux JP. 1998. Acetylcholine receptors containing the beta2 subunit are involved in the reinforcing properties of nicotine. Nature 391:173–177.

Rapier C, Lunt GG, Wonnacott S. 1990. Nicotinic modulation of [^{3}H]dopamine release from striatal synaptosomes: pharmacological characterisation. J Neurochem 54:937–945.

Reuben M, Clarke PB. 2000. Nicotine-evoked [^{3}H]5-hydroxytryptamine release from rat striatal synaptosomes. Neuropharmacology 39:290–299.

Role LW, Berg DK. 1996. Nicotinic receptors in the development and modulation of CNS synapses. Neuron 16:1077–1085.

Schwartz RD, Lehmann J, Kellar KJ. 1984. Presynaptic nicotinic cholinergic receptors labeled by [^{3}H]acetylcholine on catecholamine and serotonin axons in brain. J Neurochem 42:1495–1498.

Sharples CG, Kaiser S, Soliakov L, Marks MJ, Collins AC, Washburn M, Wright E, Spencer JA, Gallagher T, Whiteaker P, Wonnacott S. 2000. UB-165: a novel nicotinic agonist with subtype selectivity implicates the alpha4beta2* subtype in the modulation of dopamine release from rat striatal synaptosomes. J Neurosci 20:2783–2791.

Soliakov L, Wonnacott S. 1996. Voltage-sensitive Ca^{2+} channels involved in nicotinic receptor-mediated [^{3}H]dopamine release from rat striatal synaptosomes. J Neurochem 67:163–170.

Soliakov L, Wonnacott S. 2001. Involvement of protein kinase C in the presynaptic nicotinic modulation of [^{3}H]-dopamine release from rat striatal synaptosomes. Br J Pharmacol 132:785–791.

Soliakov L, Gallagher T, Wonnacott S. 1995. Anatoxin-a-evoked [^{3}H]dopamine release from rat striatal synaptosomes. Neuropharmacology 34:1535–1541.

Sorenson EM, Shiroyama T, Kitai ST. 1998. Postsynaptic nicotinic receptors on dopaminergic neurons in the substantia nigra pars compacta of the rat. Neuroscience 87:659–673.

Swanson LW, Simmons DM, Whiting PJ, Lindstrom J. 1987. Immunohistochemical localization of neuronal nicotinic receptors in the rodent central nervous system. J Neurosci 7:3334–3342.

Tsuneki H, Klink R, Lena C, Korn H, Changeux JP. 2000. Calcium mobilization elicited by two types of nicotinic acetylcholine receptors in mouse substantia nigra pars compacta. Eur J Neurosci 12:2475–2485.

Wada E, Wada K, Boulter J, Deneris E, Heinemann S, Patrick J, Swanson LW. 1989. Distribution of alpha 2, alpha 3, alpha 4, and beta 2 neuronal nicotinic receptor subunit mRNAs in the central nervous system: a hybridization histochemical study in the rat. J Comp Neurol 284:314–335.

Wada E, McKinnon D, Heinemann S, Patrick J, Swanson LW. 1990. The distribution of mRNA encoded by a new member of the neuronal nicotinic acetylcholine receptor gene family (alpha 5) in the rat central nervous system. Brain Res 526:45–53.

Wainer BH., Bolam JP, Freund TF, Henderson Z, Totterdell S, Smith AD. 1984. Cholinergic synapses in the rat brain: a correlated light and electron microscopic immunohistochemical study employing a monoclonal antibody against choline acetyltransferase. Brain Res 308:69–76.

Wonnacott S. 1997. Presynaptic nicotinic ACh receptors. Trends Neurosci 20:92–98.

Wonnacott S, Kaiser S, Mogg A, Soliakov L, Jones IW. 2000. Presynaptic nicotinic receptors modulating dopamine release in the rat striatum. Eur J Pharmacol 393:51–58.