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Pronounced Cellular Diversity and Extrasynaptic Location of Nicotinic Acetylcholine Receptor Subunit Immunoreactivities in the Chicken Pretectum

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The diversity of nicotinic ACh receptor (AChR) expression in the chick lateral spiriform nucleus (SpL) was assessed using subunit-specific monoclonal antibodies (mAbs) and laser scanning confocal microscopy. The late embryonic SpL was immunoreactive for mAbs against the α2, α5, α7, α8, and β2 AChR subunits. Distinct neuronal cell classes were determined using pair-wise staining of mAbs. Approximately 90% of the neurons in the SpL contained both α5-like immunoreactivity (LI) and β2-LI, with no neurons having only one of these subunit-LIs. Approximately 70% of the neurons contained α2-LI. All α2-LI neurons contained α5/β2-LI; thus, neurons having α2-LI are a subset of those having α5- and β2-LI. Fewer neurons, approximately 20%, contained α7-LI. A subset of α7-positive neurons were immunoreactive for other subunits; for example, some α7-positive neurons also contained α2-LI. Fewer than 15% of the neurons contained α8-LI. Some of the α8-LI-containing neurons contained α7-LI. The 14 week post-hatch SpL resembles the late embryonic nucleus in the percentage of neurons immunoreactive for α2, α5, α7, α8, and β2 AChR subunits, and in the presence of multiple classes based on AChR subunit immunoreactivity. In addition, α4-LI was found in about 20% of the 14 week SpL neurons. Double-label immunofluorescence experiments with mAbs to AChRs and to synaptic vesicle antigens showed that most clusters of α5-LI and β2-LI are extrasynaptic. The pronounced diversity of AChR subunit expression and the extrasynaptic location of AChR-LI suggest that AChR-like molecules in the SpL do not function solely to respond to transmitter focally released from presynaptic terminals.

[Key words: nicotinic ACh receptors, extrasynaptic, diversity, optic tectum, nucleus spiriformis lateralis, chicken brain]

Nicotinic ACh receptors (AChRs) are well-characterized members of a supergene family of ligand-gated channels that include glycine receptors, $GABA_A$ receptors, and $5\text{-}HT_3$ receptors. Muscle AChRs are pentamers containing four subunits in the stoichiometry $\alpha_2\beta\gamma$(or ϵ)δ. Muscle cells synthesize either $\alpha1_2\beta1\gamma\delta$ or $\alpha1_2\beta1\epsilon\delta$ receptors, depending upon their state of innervation (reviewed in Hall and Sanes, 1993). In contrast, neuronal AChRs are more diverse and are composed of several subunits derived from a family of homologous genes named α2-α9 and β2-β4 (Sargent, 1993; Elgoyhen et al., 1994). In the nervous system, each of the AChR subunit mRNAs is expressed in a unique subset of neurons, and some neurons express as many as six different transcripts and, presumably, proteins (reviewed in Role, 1992; Sargent, 1993). These diverse expression patterns imply that AChRs have diverse subunit compositions and functional roles.

Although the presence of AChR subunits has been demonstrated in neurons in the CNS, little is known about how these subunits are distributed within individual nuclei or whether all neurons in a nucleus express the same subunits. Characterizing the cellular distribution of AChR subunits within a nucleus containing cholinoceptive neurons would provide useful information about the functional complexity of AChRs in the CNS. Furthermore, despite the experiments cited above, there is little evidence for nicotinic AChR-mediated synaptic responses or localization of AChR subunits at synaptic sites in the CNS (Sargent, 1993). Therefore, finding AChR subunit-LI at synaptic sites would be important evidence for a classical receptor role for nicotinic AChRs in the CNS.

To study the basis and consequences of neuronal AChR diversity it is important to focus on a well-defined system where the subunits can be readily detected. The nucleus spiriformis lateralis (SpL) in the chicken is such a system. The SpL is a thalamic, pretectal nucleus that receives cholinergic innervation from the semiluminaris nucleus (SLu) and projects to the optic tectum (Reiner et al., 1982; Sorenson et al., 1989). The SpL neuronal cell bodies are cholinoceptive (Sorenson and Chiappinelli, 1990) and are recognized by mAbs specific for the α5, α7, and β2 AChR subunits (Swanson et al., 1983; Britto et al., 1992a). In addition, neurons containing both β2- and α7-LI have been observed in the SpL (Britto et al., 1992b) indicating that the pattern of AChR subunit expression may be complex. We have examined the extent of this complexity by using subunit-specific anti-AChR mAbs and double-label immunofluorescence techniques. We found an extensive and unexpected diversity in AChR subunit immunoreactivities within the chick SpL nucleus. Moreover, many clusters of AChR immunoreactivity are extrasynaptic. Together, these findings raise questions about the role played by AChRs in the SpL.

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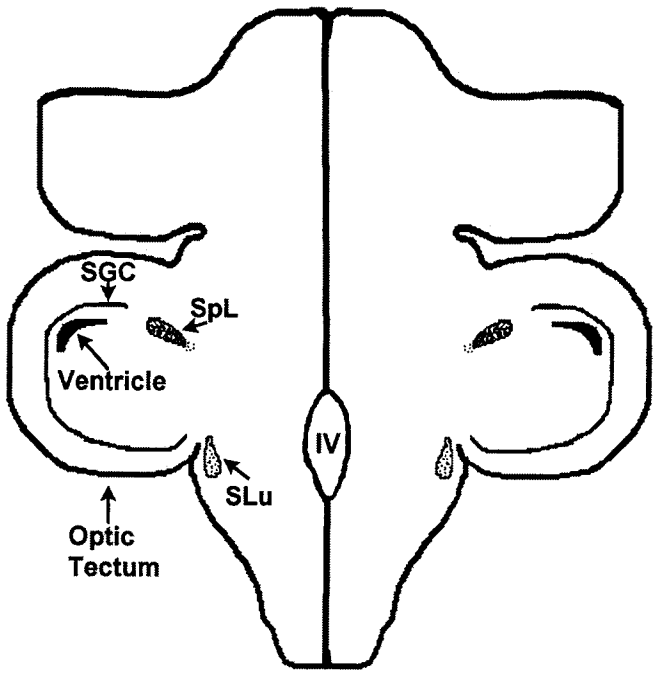

Figure 1. This schematic of a horizontal section of a chicken brain shows the location of the SpL, the SLu (which supplies the SpL with cholinergic innervation), and the SGC layer of the optic tectum (which receives innervation from the SpL).

These results have been presented in abstract form (Ullian and Sargent, 1994).

Materials and Methods

Embryonic day 20–21 chicks and 12–14 week pullets (*Gallus domesticus*), obtained locally, were decapitated and their brains were removed and immersion-fixed in 1% formaldehyde in sodium phosphate buffer (0.11 M, pH 7.3) for 1–2 hr (embryos) or 3–5 hr (pullets) at 4°C. In a few instances, pullets were anesthetized and perfused with 1% formaldehyde in sodium phosphate buffer and subsequently immersion-fixed, as before. Frozen sections were prepared from brains cryoprotected in 30% sucrose in PBS (0.015 M NaCl, 0.28 M Na phosphate, pH 7.3) and sectioned (20 μm) in either coronal or horizontal planes. The sections were then mounted on gelatin-coated slides. The SpL nucleus is a pretectal nucleus located near the junction between the mesencephalon and diencephalon (Fig. 1). The nucleus, whose borders are visible in cryostat sections when viewed with dark-field optics, was identified using a stereotaxic atlas of the chick brain (Keunzal and Mason, 1988), previously published SpL immunohistochemistry (Britto et al., 1992a,b), and with the help of Dr. Harvey Karten (UC San Diego).

Rat anti-AChR mAbs 35, 268, 270, 289, 305, 318, 319, and 323 and mouse anti-AChR mAb 306 were kindly provided by Dr. Jon M. Lindstrom (University of Pennsylvania). Mouse anti-AChR mAb A3-15 was kindly provided by Dr. Darwin K. Berg (UC San Diego). Mouse antisynaptic vesicle-associated glycoprotein (SV2) mAb 10h (Buckley and Kelly, 1985) was kindly provided by Dr. Steven S. Carlson (University of Washington). Cyanine 3.18 or cyanine 5.18 conjugated goat anti-rat and goat anti-mouse IgGs (hereafter abbreviated Cy3- or Cy5-GAR and Cy3- or Cy5-GAM) were purchased from Jackson Immunoresearch (West Grove, PA).

The percentage of neurons stained with each mAb was determined in double-label experiments in which all neurons were labeled with ethidium bromide. Ethidium bromide also stains non-neuronal cells, but these are many times smaller than neurons and readily distinguishable from them. Frozen sections were incubated in 10–30 nM anti-AChR mAb in PBS containing 5% normal goat serum and 0.3% Triton X-100 (hereafter abbreviated SGT) for 1 hr, washed in PBS for 10 min, incubated in 10–30 nM Cy5 conjugated secondary antibody and 12 nM ethidium bromide in SGT for 1 hr, washed in PBS for 10 min, and mounted in 4% *n*-propyl gallate in 90% glycerol, 10% PBS to retard photobleaching (Giloh and Sedat, 1982). To control for mAb specificity, we used mAbs made in the same species and having the same isotype that did not produce a signal in the nucleus. All large cells, assumed to be neurons on the basis of their size and the presence of neurites, were scored "by eye" as being either positive or negative for mAb staining. This approach was made possible by the high signal-to-noise ratio for staining in our experiments: neurons within the SpL were either well stained or unstained by any particular antibody. We verified the validity of this approach by performing a few experiments using image analysis to score neurons as positive or negative (see Results).

Double label experiments with rat and with mouse primary antibodies were done by incubating slide-mounted frozen sections with the following solutions (1) SGT for 10–20 min; (2) both primary mAbs, applied simultaneously at 10–30 nM, for 30–60 min; (3) PBS for 10 min; (4) Cy3-GAR or GAM and Cy5-GAM or GAR in SGT for 30–60 min; and (5) PBS for 10 min. Sections were mounted as before. Secondary antibodies were immunoadsorbed against the conspecific IgG to prevent cross-reactivity.

Double labeling with two primary antibodies from the same species (rat) was done with at least one primary Ab directly conjugated to a cyanine fluorochrome. Monoclonal antibodies 35 and 270 were reacted with cyanine 3.18 and cyanine 5.18, respectively, according to instructions supplied by the vendor (Biological Detection Systems, Pittsburgh, PA), and were applied at 30–50 nM in SGT. The sequence of incubation steps was (1) SGT for 10–20 min; (2) unconjugated primary rat mAb (e.g., 323) for 30 min; (3) PBS for 10 min; (4) Cy3- or Cy5-GAR for 30 min; (5) PBS for 10 min; (6) nonstaining mAb, which should be bound by unoccupied antigen binding sites on the Cy3- or Cy5-labeled GAR, thus preventing directly labeled rat mAb from subsequently binding there, for 30 min; (7) PBS for 10 min; (8) Cy3-mAb 35 or Cy5-mAb 270 for 30 min; and (9) PBS for 10 min. All incubations were done at room temperature.

The relationship between clusters of AChR-LI and synaptic sites was analyzed in 200 μm vibratome sections of brains fixed by immersion in 1% formaldehyde in PBS. Sections were incubated in directly-labeled 30–50 nM Cy3-mAb 35 or Cy5-mAb 270 in PBS containing 5% normal goat serum overnight at 4°C, washed three times for 15 min each in PBS at room temperature, and then incubated with anti-SV2 mAb 10h in SGT for 6 hr at 4°C. Following three 15 min washes in PBS at room temperature, the sections were incubated overnight with 10–30 nM Cy3-GAM or Cy5-GAM in SGT at 4°C. After the three 15 min washes in PBS, the sections were mounted as before.

The specificity of most mAbs used in these experiments has been shown by immunoblotting. Blotting of gels show mAb A3-15 is specific for α3 (Vernallis et al., 1993); mAb 289 is specific for α4 (Whiting et al., 1987); mAb 35 is specific for α5 and α1 (Tzartos et al., 1981, Conroy et al., 1992); mAb 306, 318, and 319 are specific for α7 (Schoepfer et al., 1990, Conroy et al., 1992); mAb 305 is specific for α8 (Schoepfer et al., 1990); and mAb 270 is specific for β2 (Whiting and Lindstrom, 1986a,b, Conroy et al., 1992). However, mAb 35 is not specific only for native AChRs containing the α5 subunit: it appears to recognize AChRs containing the α3 subunit (Haselbeck et al., 1994) and it recognizes an AChR of unknown subunit composition that also binds α-Bgt (Pugh et al., 1995). However, the SpL is not immunoreactive for the α3 subunit (see Results), and the newly identified AChR (Pugh et al., 1995) is not detected in chicken brain. The anti-α2 mAb 323 was made against an α2 specific peptide sequence, but has not yet been shown to be specific for α2 in immunoblots (Lindstrom, Schoepfer, Whiting, and Gore, unpublished results).

We used a Bio-Rad MRC-600 laser scanning confocal imaging system with a krypton-argon ion laser having excitation lines at 568 nm and 647 nm to excite the cyanine fluorophores and collect images. The Cy3 emission was collected between 578 and 618 nm (orange-red) and Cy5 emission was collected between 664 and 696 nm (far red, Sargent, 1994). Ethidium bromide emission was collected between 578 and 618 nm (orange-red). The laser was attenuated with a 3% transmission neutral density filter to reduce photobleaching. Images were collected with a Nikon 60× planapo oil objective with a numerical aperture of 1.4 and with the confocal detector aperture set at 3 mm, yielding an optical section thickness of ca. 1.2 μm in the orange-red to far-red (half-height). Most images were obtained by signal averaging and are printed without alteration of look up tables.

The percentage of AChR clusters at synaptic sites was determined by imaging boutons stained with anti-SV2 mAb 10h and AChR clusters stained with Cy3-conjugated mAb 35 in vibratome sections. AChR clus-

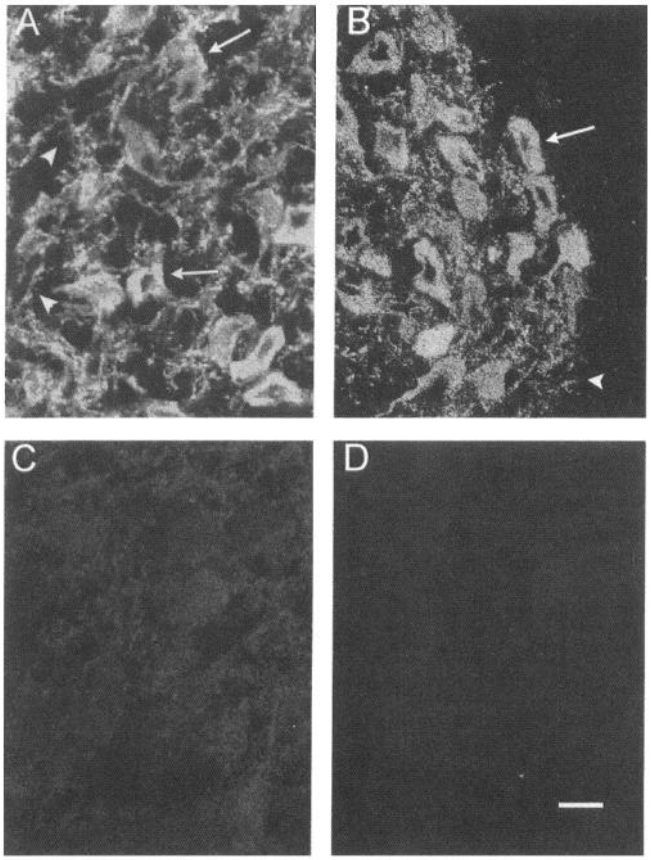

Figure 2. mAb 270 binds specifically to many neurons within the embryonic SpL. *A* and *B* show examples of SpL staining with mAb 270 (β2), while *C* and *D* show examples of staining with negative control mAb 319. Both mAbs are of the same subtype (IgG1), and were applied simultaneously. Note the clear difference in staining between *A* and *C*, and *B* and *D*. *Arrows* in *A* and *B* point to stained neuronal cell bodies, while *arrowheads* point to stained neuropil. Scale bar in *D*, 20 μm.

ters and synaptic boutons were identified with an image analysis program (OPTIMAS) by setting thresholds so that the program marked all objects previously considered by eye to represent AChR clusters or boutons. The degree of overlap between AChR clusters and boutons was determined using a digitizing pad.

Results

Detection of AChR subunit immunoreactivity in chick SpL

An example of specific neuronal AChR subunit immunoreactivity is seen in Figure 2, *A* and *B*, with mAb 270. Both cell bodies (arrows) and neuropil (arrowheads) are stained in this example. The stained cell bodies presumably belong to neurons, given their size (diameter: 20–40 μm) and morphology. A control mAb (Fig. 2*C,D*) of the same subtype as mAb 270 (IgG1) and used in the same experiment on different sections did not produce a detectable signal, indicating that nonspecific association of either primary or secondary IgGs with chick tissue cannot account for the signal.

The fraction of neurons labeled with each mAb was assessed in double label experiments by dividing the number of mAb-stained neurons by the total number of neurons, determined using ethidium bromide, which stains neurons quantitatively (Table 1). The percentage of neurons immunoreactive for each subunit varied from 90% (α5/β2) to less than 15% (α8, Table 1). The

Table 1. Percentages of SpL neurons immunoreactive for AChR subunits

mAb	Specificity	Embryonic	Adult	*p*
35	α5	93 ± 6%	89 ± 17%	0.8
270	β2	90 ± 5%	90 ± 12%	0.8
323	α2	72 ± 13%	59 ± 38%	0.9
306	α7	21 ± 14%	27 ± 14%	0.6
305	α8	13 ± 12%	11 ± 19%	0.9
289	α4	0%	18 ± 12%	0.009
A3-15	α3	0%	0%	N.A.

Data show the percentage of neurons containing each AChR subunit-LI for both embryonic and adult SpL. Embryonic data were taken from embryos at 20–21 d of development. Adult data were taken from 14 week hens. Values represent the mean and SD taken from 5–8 fields of neurons. The number of neurons analyzed for each sample ranged from 60 to 100. None of the adult means were significantly different from the embryonic means for the same mAb, except mAb 289.

fact that the immunoglobulins (IgGs) mAb 323 (α2), mAb 306 (α7), and mAb 305 (α8) did not stain all the neurons in the SpL is unlikely to be due to incomplete penetration of the antibodies into the 20 μm thick sections during the 30–60 min incubation periods, since the IgGs mAbs 35 and 270 presumably did completely penetrate the sections, inasmuch as they stained nearly all neurons in the nucleus. Another possible explanation for our finding that only some neurons are stained with mAbs 323 (α2), 306 (α7), and 305 (α8) is that these antibodies stain neurons only weakly, such that some cells fall below our threshold for immunoreactivity, which is determined by eye. This possibility would be evident if the frequency distribution of gray values (pixel intensities) for neurons stained with these mAbs overlapped with that for neurons stained with a control mAb. Figure 3 shows that this is not the case for mAb 323 (similar results were obtained with mAbs 306 and 305). The frequency distribution of neurons stained with mAb 323 is bimodal and contains two populations of gray values: a large population that does not overlap with gray values measured in a control incubation and a smaller population that overlaps almost completely with control gray values (Fig. 3). This result confirms the validity of classifying cells as immunopositive or immunonegative "by eye," especially since the fraction of neurons that stain with mAb 323 is nearly identical when assessed quantitatively and by eye. Thus, the mAb 323-negative neurons are not simply the most lightly stained of a unimodal population but represent a distinct class of cells with no detectable immunoreactivity. The widely varying percentages of neurons immunoreactive for each of several AChR subunits suggests the existence of diverse cell types in the SpL nucleus. We have confirmed this by simultaneously staining tissue for immunoreactivity using pairs of subunit-specific mAbs.

Neuron classes in the SpL

Many neurons within the SpL are stained with either mAb 35 (α5) or mAb 270 (β2) (Table 1), and we therefore expected many neurons to contain both α5-LI and β2-LI in double label experiments. Indeed, a majority of neurons in the nucleus are immunoreactive for both mAb 35 (α5, Fig. 4*A*, left panel) and mAb 270 (β2, Fig. 4*A*, right panel). Double staining of a majority of SpL neurons with both mAb 35 (α5) and mAb 270 (β2) could be artifactual. One potential problem is that light

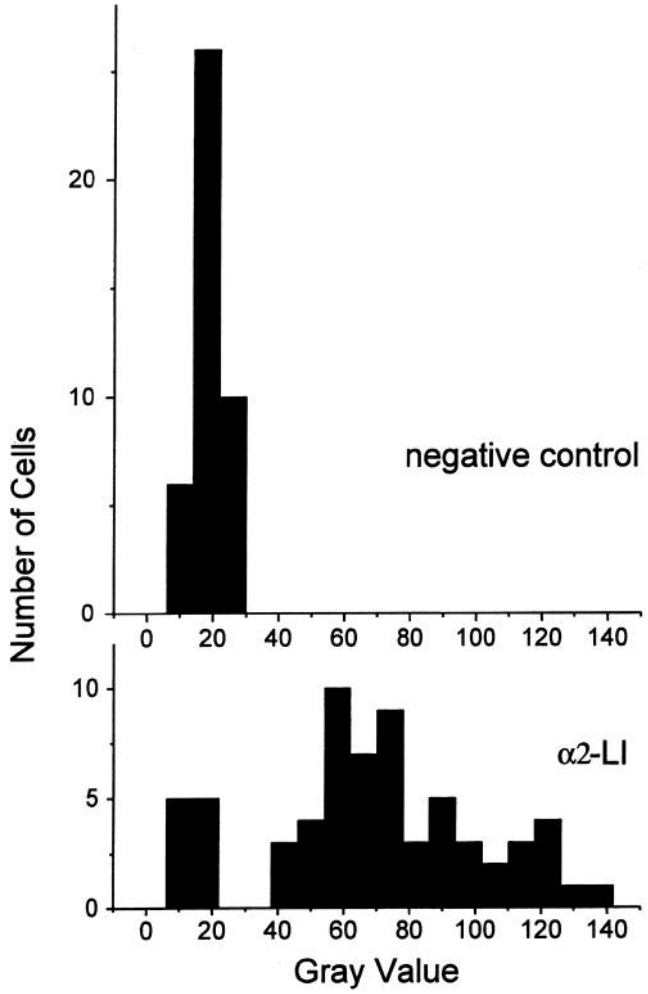

Figure 3. The frequency distribution of neurons stained with anti-α2 mAb 323 is bimodal and contains two populations of gray values: a large population that does not overlap with gray values measured in a control incubation and a smaller population that overlaps completely with control gray values.

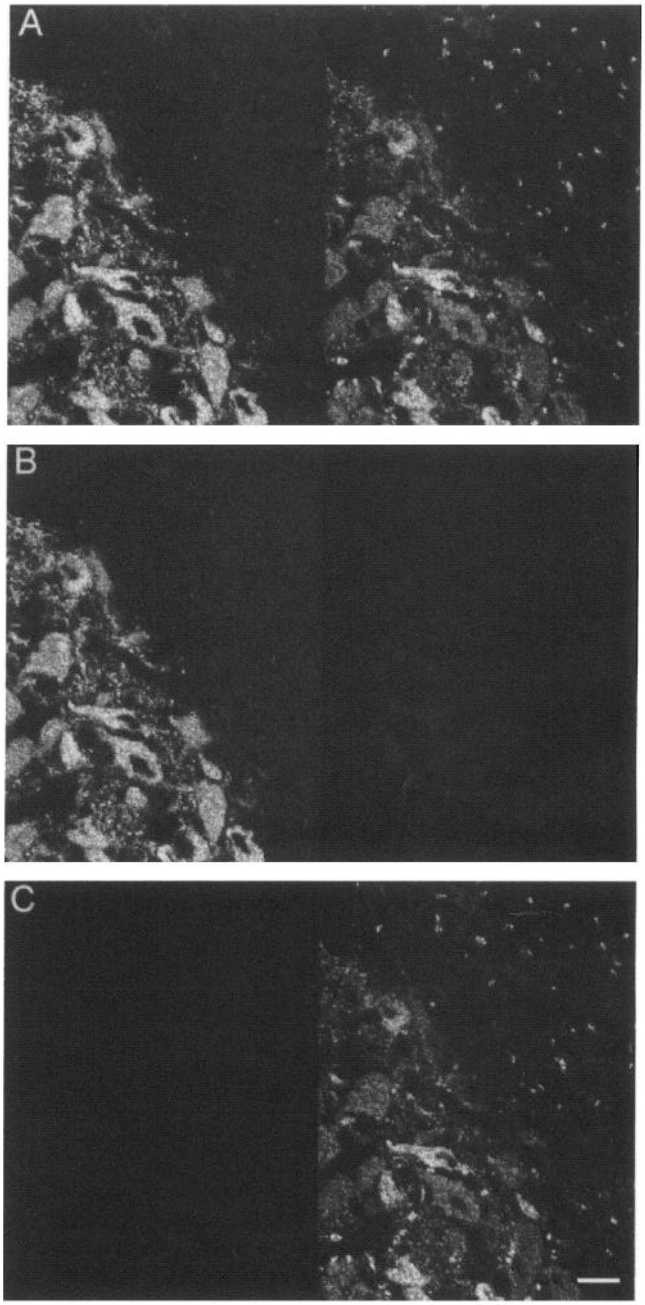

Figure 4. Dual staining of SpL neurons with both mAb 35 (Cy3) and mAb 270 (Cy5) cannot be explained by spectral crossover between Cy3 and Cy5 fluorochromes. *A* shows images from a field of SpL neurons stained with both mAb 35 (Cy3) and mAb 270 (Cy5) excited with both the 568 nm excitation line and the 647 nm excitation line. The signal in the left channel (*orange-red*) arises principally from Cy3, since it is present when only the 568 nm line is used (*B*) and absent when only the 647 nm line is used (*C*). Likewise, the signal in the right channel (*far-red*) arises principally from Cy5, since it is present when only the 647 nm line is used (*C*) and absent when only the 568 nm line is used (*B*). Scale bar, 20 μm.

emitted by one fluorophore may be collected by the channel designed for the other fluorophore (spectral crossover). Except under extreme conditions, Cy5 will not emit detectably in the Cy3 channel (578–618 nm). However, Cy3 does emit slightly in the Cy5 channel (664–696 nm), but as illustrated in Figure 4, *B* and *C*, the extent of crossover is insignificant. A second source of artifact that could explain why nearly all SpL neurons stain for both mAb 35 (α5) and mAb 270 (β2) arises because both mAbs were made in a common species (rat). We have eliminated the obvious problem of indirectly visualizing the binding of each mAb by labeling one of them directly with fluorochrome. Our experiments are typically done by incubating the tissue first with the unconjugated primary mAb (e.g., 35), then with a conjugated secondary Ab, and finally with the directly labeled primary mAb (e.g., 270). It is still possible, however, that the mAb colocalization results because the directly conjugated mAb is recognized by the bound secondary Ab, one of whose antigen binding sites is free. To eliminate this possibility we have preceded the final step with an additional one, in which the tissue is incubated with an unconjugated rat mAb (see Materials and Methods). The colocalization of α5-LI and β2-LI is thus unlikely to be an artifact; moreover, we note that other experiments utilizing two mAbs from the same species do not result in complete colocalization (see below). Thus, our results are best explained by assuming that nearly all neurons are immunoreactive for both α5 and β2 AChR subunits.

Approximately 70% of the neurons in the SpL contained α2-LI (Table 1). Figure 5*C* illustrates the type of staining seen in

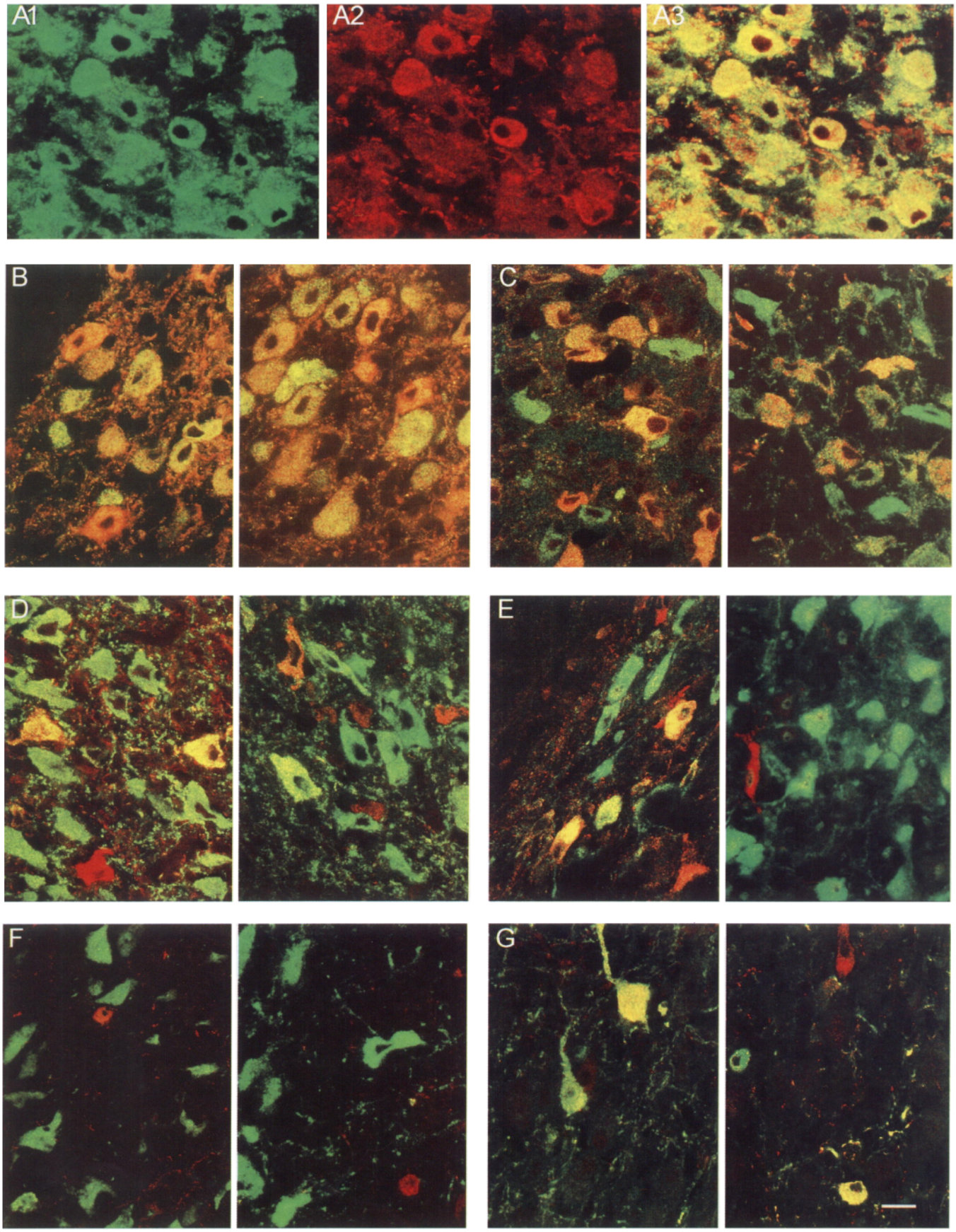

Figure 5. Double labeling reveals multiple classes of neurons in the SpL, based on their expression of AChR subunit-LI. *A* shows a field of SpL neurons stained with both mAb 35 (*A1*, Cy3 channel, rendered *green*) and with mAb 270 (*A2*, Cy5 channel, rendered *red*), and a color merge of

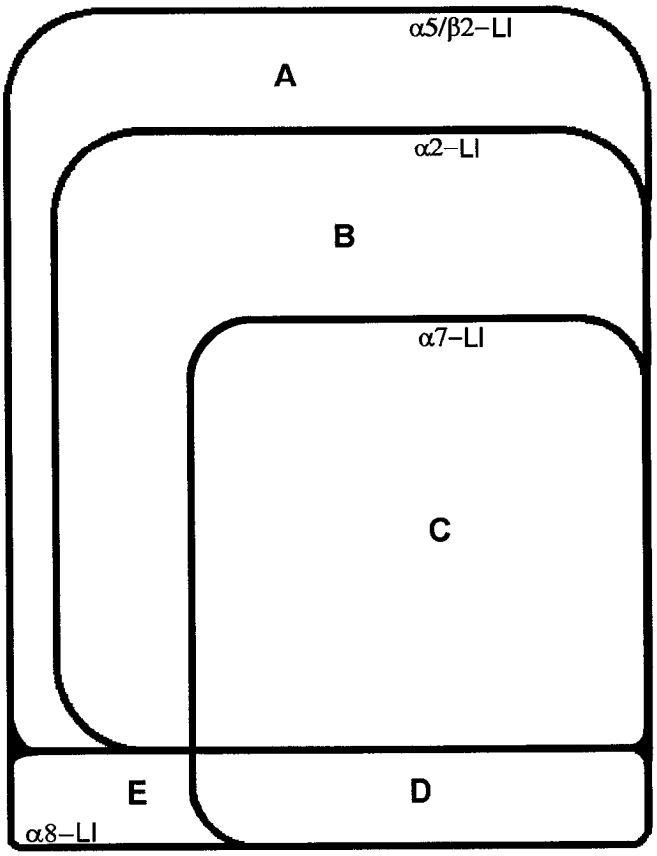

Figure 6. The Venn diagram illustrates the neuronal classes in the SpL, based on the presence of AChR-LI. The *outer rectangle* represents all neurons in the SpL, while the set sizes are proportional to the fraction of cells containing each subunit-LI. Note that the majority of neurons contain both α5- and β2-LI. Cells expressing other subunit-LIs bear distinct relationships with this set of neurons: cells containing α2-LI are a subset of α5/β2-positive neurons, cells containing α7-LI comprise a set that intersects with α5/β2-positive neurons, and cells containing α8-LI comprise a set that does not intersect with α5/β2-positive neurons. The Venn diagram illustrates that at least five classes of neurons exist within the SpL (classes *A–E*).

the SpL after double labeling with mAb 323, which recognizes α2 (Fig. 5*C*, red color), and mAb 35, which recognizes α5 (Fig. 5*C*, green color). Note that neurons containing both subunit-LIs (yellow neurons in Fig. 5*C*) are prevalent. Neurons containing only α5-LI are also present (Fig. 5*C*, green neurons); however, there are no neurons containing only α2-LI (Fig. 5*C*, red). Similar results were obtained in double label experiments using mAb 323 (α2) and mAb 270 (β2, not shown). Consequently, α2-LI is present in a subset of the neurons that also contain α5-LI/β2-LI. Note that our interpretation would require modification if mAb 35 were not specific for α5 in these experiments. If, for example, mAb 35 recognized either α5 or α2, then neurons having α2-LI and not α5-LI (which would be mAb 35 positive and mAb 323 positive) would be misinterpreted as having both α2-LI and α5-LI. We can argue against the possibility that mAb 35 recognizes a set of SpL neurons having α2-LI and not α5-LI, since we find that mAb 35 recognizes precisely the same set of neurons recognized by mAb 268 (not shown), which recognizes an epitope on the α5 subunit distinct from that recognized by mAb 35 (Whiting et al., 1987; Conroy et al., 1992). It is very unlikely that both mAbs 35 and 268 crossreact with α2 subunits, given their different epitope specificities. These results suggest that there are two classes of α5/β2-immunoreactive cells: those that contain α2-LI and those that do not. Approximately 20% of the SpL neurons were stained with mAb 306 (α7, Table 1). Double label experiments with mAb 35 (α5) and mAb 306 (α7) show neurons that contain α5-LI alone (Fig. 5*D*, green neurons), neurons that contain α7-LI alone (Fig. 5*D*, red neurons), and neurons that contain both (Fig. 5*D*, yellow neurons). Similarly, double label experiments with mAb 323 (α2) and mAb 306 (α7) show neurons that contain α2-LI alone (Fig. 5*E*, green neurons), neurons that contain α7-LI alone (Fig. 5*E*, red neurons), and neurons that contain both (Fig. 5*E*, yellow neurons). Thus, α7-LI is present in at least two populations of neurons: (1) those that lack α5-LI (and therefore lack α2-LI), and (2) those that contain α2-LI (and therefore contain β2/α5-LI).

Fewer than 15% of neurons in the SpL contained α8-LI (Table 1). Double label experiments with mAb 35 (α5) and mAb 305 (α8) revealed neurons that contain α5-LI only, neurons that contain α8-LI only, but no neurons containing both α5 and α8-LI (Fig. 5*F*). Double label experiments with mAb 306 (α7) and mAb 305 (α8) showed neurons that contain α7-LI, neurons that contain α8-LI, and neurons that contain both α7 and α8-LIs (Fig. 5*G*). This experiment helps to define at least three classes of neurons having α7-LI. Previously, we showed that some α7-positive neurons contained α2-LI, while others did not. Of those that did not, we can now say that some are immunoreactive for α8, while others are not. Thus, among the neurons containing α7-LI, there are those that contain α2-, α5-, and β2-LI; those that contain α8-LI but not α2-, α5-, or β2-LI; and those that lack α8-, α2-, α5-, and β2-LI. Neurons containing α8-LI in double label experiments show two different patterns: neurons containing α8-LI only and neurons containing α7- and α8-LI. None of the neurons in the embryonic SpL stained for mAb 289 (α4), although this mAb did stain neurons in other nuclei and SpL neurons at later stages of development (see below).

We found no neurons within the SpL that were immunoreactive for anti-α3 mAb A3-15, although this mAb did stain virtually all neurons in the chicken ciliary ganglion.

These data demonstrate that several classes of neurons exist

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these two panels (*A3*, *yellow*). All neurons in the field stained with both mAbs (*A3*). *B* shows two different merged images of SpL neurons stained with mAb 35 (*green*) and mAb 270 (*red*); again note that all neurons contain both immunoreactivities. *C* shows color merges of SpL neurons stained with mAb 35 (α5, *green*) and mAb 323 (α2, *red*). The merged images show neurons that contain both α2-LI and α5-LI (*yellow-orange*), and neurons that contain α5-LI but not α2-LI (*green*), but no neurons that contain α2-LI but not α5-LI (no red). *D* shows SpL neurons stained with mAb 35 (α5, *green*) and with mAb 306 (α7, *red*). The merged images show that there are three classes of neurons: α5-LI only (*green*), α7-LI only (*red*), and both α5-LI and α7-LI (*yellow*). *E* shows SpL neurons stained with mAb 323 (α2, *green*) and mAb 306 (α7, *red*). The merged images show three classes of neurons: those containing α2-LI only (*green*), those containing α7-LI only (*red*), and those containing both LIs (*yellow*). *F* shows neurons stained with mAb 35 (α5, *green*) and mAb 305 (α8, *red*). The merged images show two classes of neurons: those containing α5-LI only (*green*) and those containing α8-LI only (*red*). No neurons were found with both α5-LI and α8-LI (*yellow*). *G* shows double labels with mAb 305 (α8, *green*) and mAb 306 (α7, *red*). The merged images show three classes of neurons; those containing α8-LI (*green*), neurons containing α7-LI (*red*), and neurons containing both LIs (*yellow*). Scale bar in *G*, 20 μm.

within the SpL, based on the presence of AChR-LI. The Venn diagram shown in Figure 6 illustrates the neuronal classes and their relation to each other. The outer rectangle represents all neurons in the SpL, while the set sizes are proportional to the fraction of cells containing each subunit-LI. Since α5/β2-positive neurons and α8-positive neurons are non-intersecting sets, and since together these sets account for all neurons (Table 1), we suspect that all neurons in the SpL are immunoreactive for at least one AChR subunit. Note that the majority of neurons contain both α5- and β2-LI. Neurons expressing other subunit-LIs bear distinct relationships with this set of neurons: neurons containing α2-LI are a subset of α5/β2-positive neurons, neurons containing α7-LI comprise a set that intersects with α5/β2-positive (and α2-positive) neurons, and neurons containing α8-LI comprise a set that does not intersect with α5/β2-positive neurons. The existence of at least five unique classes of neurons (Fig. 6, classes A–E) is demonstrated by the results of double label experiments illustrated in Figure 5. For example, double label experiments with mAbs 323 (α2) and 306 (α7) reveal that some neurons have α2-LI, but not α7-LI. Since these neurons contain α2-LI, they must also have α5-LI and β2-LI, because neurons with α2-LI are a subset of the neurons with α5/β2-LI. Analogously, since these neurons contain α5/β2-LI, they cannot also contain α8-LI, because neurons having α5/β2-LI do not overlap with the neurons having α8-LI. These neurons are therefore placed in category B (α2+, α5+, α7−, α8−, β2+). Four of the five cell classes illustrated in the Venn diagram are evident from directly analyzing the outcome of the double label experiments as we have just detailed for category B. However, the cells in category A (α2−, α5+, α7−, α8−, β2+) are assumed to exist by inference. Neurons that are α2-negative and α5/β2-positive must be α8-negative, but we don't know if they are also α7-negative (this could be established with triple label experiments). Nonetheless, we can argue that at least some of these neurons are α7-negative by examining the percentage of cells immunoreactive for each subunit (Table 1); about 20% of neurons in the SpL are positive for α5/β2 and negative for α2 (the 90% α5/β2-positive minus the 70% α2-positive), and an equal proportion are positive for α7. Double label experiments show that a portion of these α7-positive neurons are both α5/β2-negative and α2-negative, and another portion are both α5/β2-positive and α2-positive. This does not leave enough α7-positive neurons available to fill the 20% gap left by the α5/β2-positive, α2-negative neurons. Therefore, at least some of these α5/β2-positive, α2-negative neurons must also be α7-negative (category A). It is possible that other α5/β2-positive, α2-negative neurons are α7-*positive*; if so, these neurons represent a sixth class: one not illustrated in the Venn diagram (α2−, α5+, α7+, α8−, β2+). Clearly, more experiments are needed to complete the remainder of the Venn diagram. However, it is at least as important to understand the basis and the consequences of this diversity as to document all of its details.

We repeated these immunofluorescence experiments on 12–14 week chickens to determine if the degree of diversity in embryonic expression of AChR-LIs is sustained. The percentages of cells containing α2-LI, α5-LI, α7-LI, α8-LI, and β2-LI did not change significantly in the 12–14 week SpL (Table 1; $0.6 < P < 0.9$). Several cell classes present in embryonic chicks (Fig. 6) are present as well. For instance, α2-LI is still present in a subset of the cells containing α5-LI (not shown). However, an additional AChR-LI, α4-LI, is present in the 14 week old SpL, but absent in the embryonic SpL (Fig. 7). We calculate that 18% of all neurons in the SpL contain α4-LI (Table 1). All cells containing α4-LI also contain α5- and β2-LI in the 14 week old SpL (Fig. 7*A*). Therefore, neurons containing α4-LI are a subset of the neurons containing α5/β2-LI.

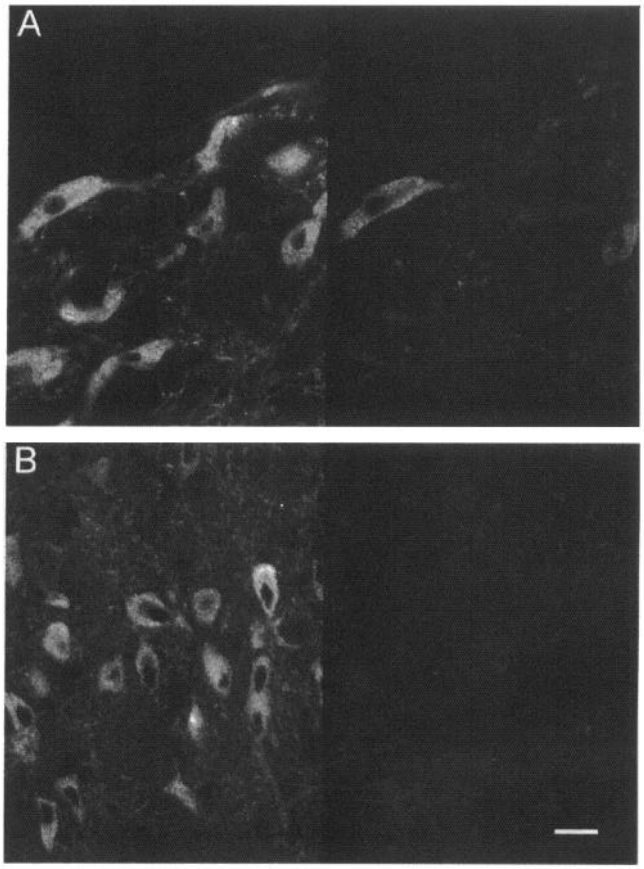

Figure 7. Neurons in adult, but not embryonic SpL, have α4-LI. *A* shows a field of 14 week SpL neurons double labeled with mAb 35 (α5, Cy3, *left panel*) and mAb 289 (α4, Cy5, *right panel*). *B* shows a field of embryonic SpL neurons similarly stained. Note that α4-LI is only present in a subset of adult SpL neurons (*A, right panel*) and that all neurons containing α4-LI also contain α5-LI (*A, left and right panels*). Scale bar in *B*, 20 μm.

Detection of AChR subunit immunoreactivity in the chick optic tectum

The SpL neurons project to a layer of the optic tectum called the stratum griseum centrale (SGC, Reiner et al., 1982). Swanson et al. (1983) showed that the SGC of posthatch 2 d chicks contains α5-LI, and since this immunoreactivity declines after ipsilateral SpL lesioning, they suggested that SpL neurons transport AChRs to the SGC (Swanson et al., 1983). To identify candidate AChR subunits in addition to α5 that might be transported to the optic tectum from the SpL, we examined the optic tectum for immunoreactivity corresponding to other AChR subunits found in the SpL. Britto et al. (1992a) found mAb 270 β2-LI in the SGC, and we here report similar results (Fig. 8). The staining is characterized by bright, neuropil-like immunoreactivity with few stained neuronal cell bodies (Fig. 8*A*). The stained neuropil is presumed to represent the SGC of the optic tectum, based on the description of cellular lamination according to Kuenzal and Mason (1988). The optic tectum also stained both with mAb 306 (α7) and mAb 305 (α8, Fig. 8*B,C*). However,

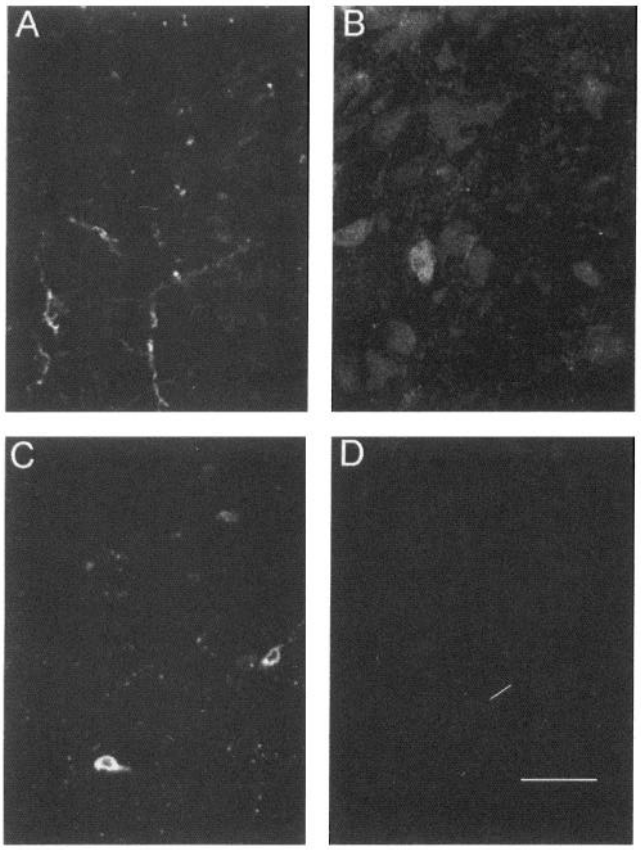

Figure 8. The SGC layer of the embryonic optic tectum is immunoreactive for AChR subunits. *A* shows mAb 270 (β2) staining in the SGC. Note the neuropil-like staining, with no cell bodies visible in the field. *B* shows SGC staining with mAb 306 (α7). Both punctate neuropil-like staining and cell body staining are evident. *C* shows an examples of mAb 305 (α8) staining to SGC. There is both cell body and punctate, neuropil-like staining. In contrast, *D* shows that mAb 323 (α2) does not stain the SGC. Scale bar, 50 μm.

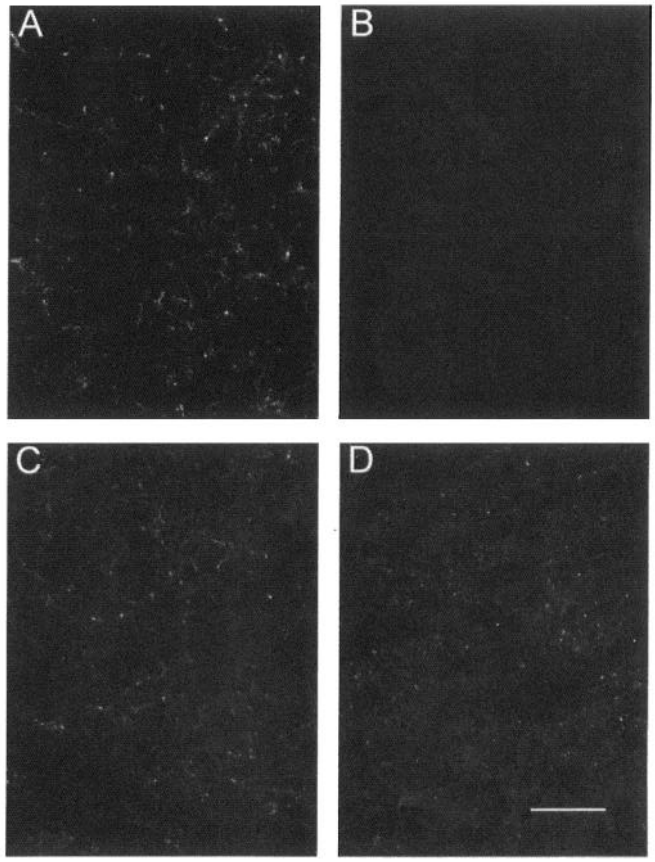

Figure 9. The adult optic tectum, but not the embryonic optic tectum, contains α5-LI. *A* shows punctate α5-LI in the SGC of the adult optic tectum. However, *B* shows that α5-LI is not present in the embryonic SGC layer of the optic tectum. *C* and *D* show that β2-LI is present in the adult (*C*) and embryonic (*D*) optic tectum SGC. Scale bar, 50 μm.

unlike mAb 270, both mAb 306 and mAb 305 also stained cell bodies within or near the SGC. Figure 8*B* shows an example produced with anti-α7 mAb 306; there is both punctate neuropil staining and light immunoreactivity present in many neuronal cell bodies, which lacked extensive stained processes. Figure 8*C* (α8-LI) also shows punctate neuropil staining; here, however, neuronal cell bodies are intensely stained and have short immunoreactive processes. In contrast, the optic tectum SGC showed no immunoreactivity for mAbs 323 (α2, Fig. 8*D*) or 35 (α5, not shown). Thus, the SGC of the late embryonic optic tectum is immunoreactive for β2 (mAb 270), α8 (mAb 305), and α7 (mAb 306) but not for α5 (mAb 35), α2 (mAb 323), or α4 (mAb 289) AChR subunits.

We repeated the staining of the SGC in 14 week old chickens to determine whether novel AChR-LIs appear after hatching. Overall, the 14 week SGC resembles the late embryonic SGC in containing β2-LI (Fig. 9*C,D*) and α7- and α8-LI (not shown), while lacking α2- and α4-LI (not shown). The 14 week optic tectum differs from its embryonic counterpart in displaying α5-LI (Fig. 9*A,B*). Swanson et al. (1983) showed that α5-LI is present in 2 d posthatch chickens, and we find that its expression is sustained during the subsequent several months.

Synaptic localization of AChRs

The occurrence of AChR clusters at synaptic sites was assessed in vibratome sections of the SpL of both late embryonic and 14 week animals. Figure 10*A1* shows an optical section of an embryonic neuron stained for mAb 35 (α5, green), while Figure 10*A2* shows the same neuron stained for synaptic sites (SV2, red). Figure 10*A3* shows a color merge of Figure 10, *A1* and *A2*, and illustrates that some clusters of AChR-LI are synaptic. However, Figure 10*B–D* shows that many AChR clusters are extrasynaptic. In Figure 10*D* examples of both synaptic and extrasynaptic clusters of AChR-LI are indicated by arrows and arrowheads, respectively. Many AChR clusters do not appear simply to be slightly displaced from synaptic sites, but exist in isolation from detectable SV2-LI. We calculated, using image analysis software, that only 25 ± 20% of the AChR clusters are located at synaptic sites in the embryonic SpL (n = 20 neurons). The 14 week old SpL also has a preponderance of extrasynaptic AChR clusters (Fig. 10*E–G*). At this age only 11 ± 10% of the AChR clusters are located at synaptic sites as determined by SV2 staining (n = 20 neurons, significantly different from embryonic mean, p = 0.009).

Discussion

Despite the wide distribution of AChR subunit mRNAs in the CNS (e.g., Wada et al., 1989), there are few central synapses where AChRs are thought to mediate fast synaptic transmission

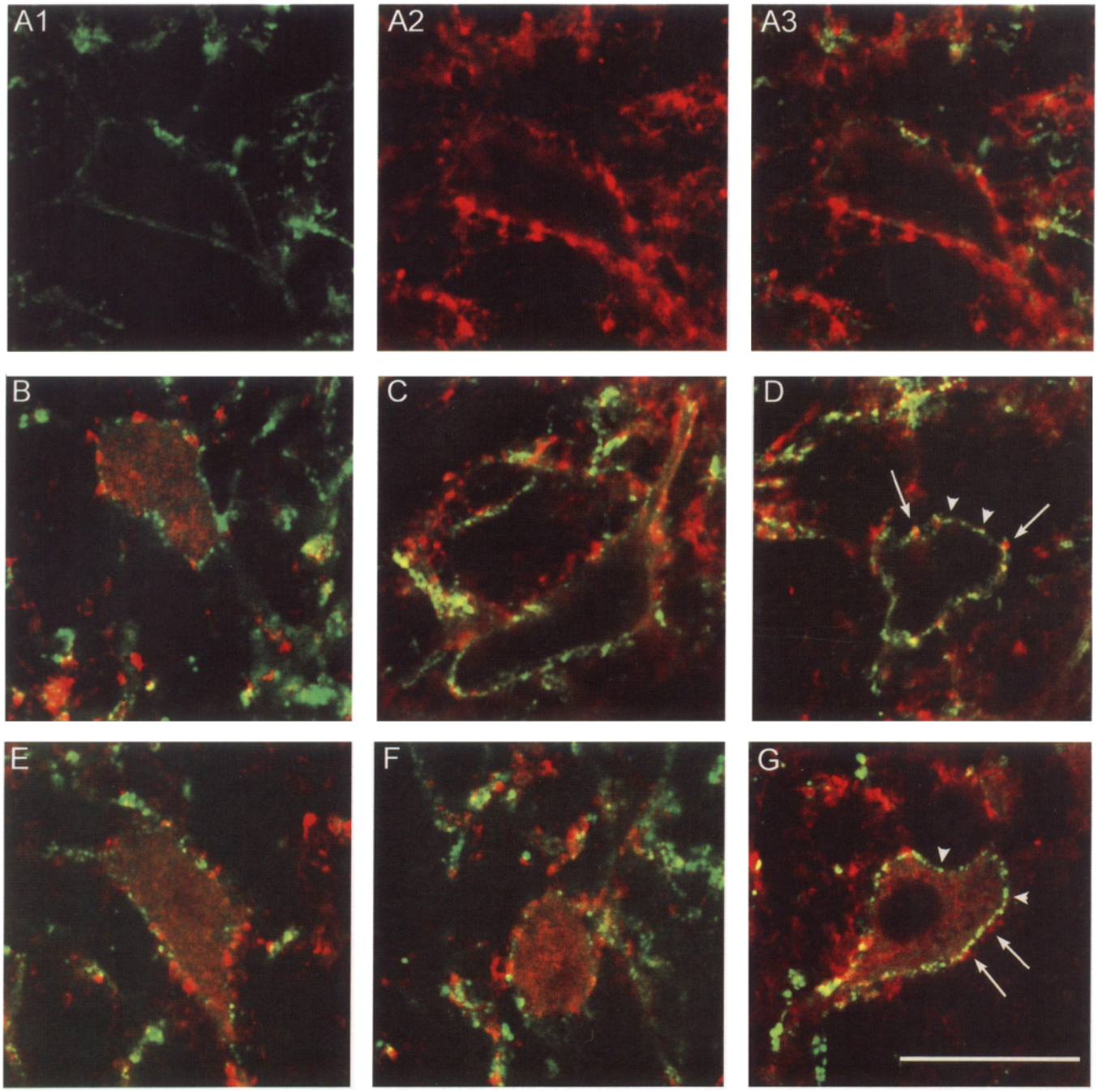

Figure 10. Synaptic and extrasynaptic AChR surface clusters are found in both embryonic and adult SpL. *A* shows an optical section of a single SpL neuron. *A1* shows surface AChR clusters stained with mAb 35 (*green*) while *A2* shows synaptic boutons stained with mAb 10h (*red*). *A3* is a color merge of *A1* and *A2*; this merged image allows comparison of the relative location of the AChR surface clusters and synaptic boutons. *B–D* show additional merged images of AChR clusters (*green*) and synaptic boutons (*red*) from other embryonic SpL neurons. These figures illustrate that some AChR clusters are at synaptic sites (*D, arrows*) while many AChR clusters are extrasynaptic (*D, arrowheads*). *E–G* show merged images of AChR clusters and synaptic boutons from adult SpL. Again, some AChR clusters are synaptic (*G, arrows*) and many clusters are extrasynaptic (*G* arrowheads). Scale in *G*, 20 μm.

(Nicoll et al., 1990); exceptions include the spinal cord motor neuron–Renshaw cell synapse (Eccles et al., 1954) and retinal amacrine cell–ganglion cell synapses (Masland et al., 1984). Similarly, there have been few instances where AChRs have been shown to be located at postsynaptic sites within the CNS (Schröder et al., 1989). The AChR subunit-LIs expressed in the SpL can only mediate synaptic transmission if they are clustered at cholinergic synapses. In our experiments on embryos only 25% of the clusters of α5 and β2 AChR-LI were associated with immunocytochemically visualized synaptic boutons. The numerous extrasynaptic AChR clusters are unlikely to be an artifact due to incomplete staining of vesicle antigen, because many SV2 sites were strongly labeled (Fig. 10). Therefore, these clusters of AChR subunit-LI truly may be extrasynaptic. Extrasynaptic AChR clusters have been found elsewhere (Jacob and Berg, 1983; Jacob et al., 1984; Sargent and Pang, 1989; Sargent

et al., 1989; see also Smiley and Yazulla, 1990; Baude et al., 1993; Liu et al., 1994; Smiley et al., 1994). The further reduction in the fraction of clusters of AChR-LI at synaptic sites in the adult implies that the preponderance of extrasynaptic AChR clusters in the embryo is not a developmental artifact, stemming from the fact that the proper connections between the SLu and SpL are not yet established.

The structural diversity of neuronal AChRs has been studied in the CNS, where various techniques have detected α2, α3, α4, α5, α7, α8, β2, and β4 subunits (reviewed in Deneris et al., 1991; Lukas and Bencherif, 1992; Role, 1992; Patrick et al., 1993; Sargent, 1993). The α7 subunit appears in at least two classes of AChRs in chicken brain; those that contain the α8 subunit and those that lack it (Schoepfer et al., 1990). In chick retina, there appear to be AChRs that contain the α8 subunit but not the α7 subunit (Keyser et al., 1993). The α7 and α8 subunits, which account for virtually all high-affinity α-Bgt binding in chicken brain, apparently do not associate with any of the other known α or β subunits (Schoepfer et al., 1990, Conroy et al., 1992). In chicken brain, the α5 subunit associates with the α4 subunit and separately with the α3 subunit; some AChRs appear to contain α3, α4, and α5 subunits (Conroy et al., 1992). Some of these AChR classes may also contain the β2 subunit (Whiting et al., 1987; Conroy et al., 1992; see also Flores et al., 1992). Thus, some neuronal AChRs contain at least four unique subunits and may therefore be as complex as muscle AChRs (Conroy et al., 1992).

Our experiments on the SpL extend the analysis of AChR structural diversity to the level of a single nucleus and suggest that different neurons within the same nucleus may assemble AChRs having different subunits. In the SpL, α5-LI and β2-LI are found in most neurons and could be components of the same AChR (Conroy et al., 1992). In contrast, neurons containing α7-LI as well as both α5-LI and β2-LI (Fig. 6) probably do not assemble these three subunits into a single AChR type, since α7 subunits do not coassemble detectably with α5 or β2 subunits in chicken brain (Schoepfer et al., 1990; Conroy et al., 1992). SpL neurons expressing α7-LI and α8-LI may form AChRs containing both subunits (Schoepfer et al., 1990). No α3-LI is found in the SpL, so we assume that α3-containing AChRs (Conroy et al., 1992) are accounted for by other brain regions.

The diverse neuronal types based on AChR subunit-LI are consistent with the diverse pharmacology reported in the SpL by Weaver et al. (1994), who found that nicotinic responses of some SpL neurons were blocked potently by the AChR antagonist trimethaphan, while responses of other neurons were not affected. Thus, both immunocytochemical and pharmacological studies suggest that AChR subunit composition within the SpL may differ on a neuron-to-neuron basis. In contrast, AChR subunit expression in the chicken ciliary ganglion is apparently uniform: virtually all neurons express each of the subunits found in the ganglion as a whole (Boyd et al., 1988; Corriveau and Berg, 1993; Sargent and Wilson, 1994). Ciliary ganglion neurons may serve fewer functions than SpL neurons and may not require such a diverse group of nicotinic AChRs.

It is notable that we obtained different results using three mAbs specific for the α7 subunit (Schoepfer et al., 1990); mAbs 306 and 318 produced staining of a small population of SpL neurons, but mAb 319 did not. This discrepancy may be related to differences in specificity, since the eptitope for mAbs 306 and 318 is largely nonoverlapping with that for mAb 319 (McLane et al., 1992). In contrast to the results in the SpL, all three anti-α7 mAbs (306, 318, 319) stain neurons in the ciliary ganglion (not shown). The tissue differences in the binding of 319 may be explained if the epitope for 319 is exposed in fixed ciliary ganglia, but buried in the SpL. Perhaps a cytoplasmic membrane protein in the SpL (similar to the 43K protein of muscle) is crosslinked to α7 by the fixative, resulting in occlusion of this epitope.

The appearance of α4-LI in the 14 week SpL implies that developmental regulation of this subunit is different from other AChR-LIs. The α4 mRNA is abundant in 1 d posthatch chicks (Morris et al., 1990). It is possible that the α4 subunit is suddenly expressed after hatching, as the visuomotor system of the chick is first used. Since the SpL is part of the avian visuomotor system (Reiner et al., 1982), it is possible that these changes are visually driven. It is notable that Keyser et al. (1993) have described changes in the relative amounts of α7- and α8-LI in the chicken retina at the time of hatching.

A presynaptic function for AChRs in the optic tectum would be suggested if AChR subunits were transported to the tectum from the SpL. There is pharmacological evidence for presynaptic nicotinic AChRs in the CNS, although in very few instances do we have a solid understanding of either their functional role or the source of ACh (see Sargent, 1993). The β2 subunit is a candidate for transport from the SpL to the optic tectum, since it is present in most neurons in the SpL and in a layer of the optic tectum that receives an SpL projection, the SGC. It seems unlikely that the β2-LI within the SGC arises intrinsically, since few β2-immunoreactive neuronal cell bodies are present there. To confirm that the β2-LI in the SGC of the optic tectum is transported from the SpL, additional experiments would be needed, such as metabolic labeling and immunoprecipitation (Henley et al., 1986).

The α5 subunit is another candidate for transport from the SpL to the optic tectum (Swanson et al., 1983). However, while α5-LI is present within the optic tectum in posthatch chicks as well as 14 week chickens (Swanson et al., 1983; Fig. 9*A*), we failed to find α5-LI in the optic tectum at late embryonic stages, as previously observed by Swanson et al. (1983). These results suggest that α5 subunit expression in the optic tectum is developmentally regulated. If the α5 subunit in the SGC originates from SpL projections, then these projections may not form until after hatching. Alternatively, the projections may preexist, and the axonal transport of the α5 subunit may be developmentally regulated.

The argument that the α7 and α8 subunits may be transported from the SpL to the optic tectum is less compelling than for the β2 and α5 subunits, since neuronal cell bodies immunoreactive for the α7 and α8 subunits are present in the optic tectum and may account for the punctate staining in the SGC layer (Fig. 8*B,C*).

Another source of AChR subunit-LI could be the retinal ganglion cells that project to the optic tectum. Although retinal ganglion cells are immunoreactive for β2, α3, α5, α7, and α8 subunits (Whiting et al., 1991; Britto et al., 1992b; Anand et al., 1993; Keyser et al., 1993) and may transport some or all of these subunits to the optic tectum (Britto et al., 1992a), the vast majority of retinal ganglion cells do not project to the SGC, but rather to more superficial layers (Crossland et al., 1973, 1974).

In conclusion, our experiments reveal a surprising diversity of neuron types based on expression of AChR subunit-LIs in the SpL, and they identify subunits that might be targeted to the optic tectum, where they could act as presynaptic AChRs. The

observed degree of diversity exceeds what would be expected if AChRs in the SpL serve solely a classical receptor role by responding to ACh focally released from presynaptic terminals. Our experiments also demonstrate that most AChR clusters on SpL neurons are extrasynaptic. This constitutes further evidence that AChRs do not serve a classical role in the SpL.

References

Anand R, Conroy WG, Schoepfer R, Whiting P, Lindstrom J (1991) Neuronal nicotinic acetylcholine receptors expressed in *Xenopus* oocytes have a pentameric quaternary structure. J Biol Chem 266: 11192–11198.

Anand R, Peng X, Ballesta JJ, Lindstrom J (1993) Pharmacological characterization of α-bungarotoxin-sensitive acetylcholine receptors immunoisolated from chick retina: contrasting properties of α7 and α8 subunit-containing subtypes. Mol Pharmacol 44:1046–1050.

Baude A, Nusser, Z, Roberts JDB, Mulvihill E, McIlhinney J, Somogyi P (1993) The metabotropic glutamate receptor (mGluR1α) is concentrated at presynaptic membrane of neuronal subpopulations as detected by immunogold reaction. Neuron 11:771–787.

Boyd RT, Jacob MH, Couturier S, Ballivet M, Berg DK (1988) Expression and regulation of neuronal acetylcholine receptor mRNA in chick ciliary ganglia. Neuron 1:495–502.

Britto LR, Keyser KT, Lindstrom JM, Karten HJ (1992a) Immunohistochemical localization of nicotinic acetylcholine receptor subunits in the mesencephalon and diencephalon of the chick (*Gallus gallus*). J Comp Neurol 317:325–340.

Britto LRG, Hamassaki-Britto DE, Ferro ES, Keyser KT, Karten HJ, Lindstrom JM (1992b) Neurons of the chick brain and retina expressing both α-bungarotoxin-sensitive and α-bungarotoxin-insensitive nicotinic acetylcholine receptors: an immunohistochemical analysis. Brain Res 590:193–200.

Buckley K, Kelly RB (1985) Identification of a transmembrane glycoprotein specific for secretory vesicles of neural and endocrine cells. J Cell Biol 100:1284–1294.

Conroy WG, Vernallis AB, Berg DK (1992) The α5 gene product assembles with multiple acetylcholine receptor subunits to form distinctive receptor subtypes in brain. Neuron 9:679–691.

Corriveau RA, Berg DK (1993) Coexpression of multiple acetylcholine receptor genes in neurons: quantitation of transcripts during development. J Neurosci 13:2662–2671.

Crossland WJ, Cowan WM, Kelly JP (1973) Observation on the transport of radioactively labeled proteins in the visual system of the chick. Brain Res 56:77–105.

Crossland WJ, Cowan WM, Rogers LA, Kelly JP (1974) The specification of the retino-tectal projection in the chick. J Comp Neurol 155:127–164.

Deneris ES, Connolly J, Rogers SW, Duvoisin R (1991) Pharmacological and functional diversity of neuronal nicotinic acetylcholine receptors. Trends Pharmacol Sci 12:34–40.

Eccles JM, Fatt P, Koketsu K (1954) Cholinergic and inhibitory synapses in a pathway from motor-axon collaterals to motoneurons. J Physiol (Lond) 126:524–562.

Elgoyhen AB, Johnson DS, Boulter J, Vetter DE, Heinemann S (1994) α9: an acetylcholine receptor with novel pharmacological properties expressed in rat cochlear hair cells. Cell 79:705–715.

Flores CM, Rogers SW, Prabreza LA, Wolfe BB, Keller KJ (1992) A subtype of nicotinic cholinergic receptor in rat brain is comprised of α-4 and β-2 subunits and is up-regulated by chronic nicotine treatment. Mol Pharmacol 41:31–37.

Giloh H Sedat JW (1982) Fluorescence microscopy: reduced photobleaching of rhodamine and fluorescein protein conjugates by n-propyl gallate. Science 217:1252–1255.

Hall ZW, Sanes JR (1993) Synaptic structure and development: the neuromuscular junction. Neuron 10:99–121.

Haselbeck RC, Conroy WG, Romano SJ, Berg DK (1994) Expression of neuronal ACh receptor genes in transfected HEK-293 cells. Soc Neurosci Abstr 20:466.11.

Henley JM, Lindstrom JM, Oswald RE (1986) Acetylcholine receptor synthesis in retina and transport to optic tectum in goldfish. Science 232:1627–1629.

Jacob MH, Berg DK (1983) The ultrastructural localization of alpha-bungarotoxin binding sites in relation to synapses on chick ciliary ganglion neurons. J Neurosci 3:260–271.

Jacob MH, Berg DK, Lindstrom JM (1984) Shared antigenic determinants between *Electrophorus* acetylcholine receptor and a synaptic component on chicken ciliary ganglion neurons. Proc Natl Acad Sci USA 81:3223–3227.

Keyser KT, Britto, LRG, Schoepfer R, Whiting P, Cooper J, Conroy W, Brzozowska-Prechtl A, Karten HJ, Lindstrom J (1993) Three subtypes of α-bungarotoxin-sensitive nicotinic acetylcholine receptors are expressed in chick retina.. J Neurosci 13:442–454.

Kuenzel WJ, Mason M (1988) A stereotaxic atlas of the brain of the chick (*Gallus domesticus*). Baltimore: Johns Hopkins UP.

Lindstrom J, Schoepfer R, Whiting P (1987) Molecular studies of the neuronal acetylcholine receptor family. Mol Neurobiol 1:281–337.

Liu H, Brown JL, Jasmin L, Maggio JE, Vigna SR, Mantyh PW, Basbaum AI (1994) Synaptic relationship between substance P and the substance P receptor: light and electron microscopic characterization of the mismatch between neuropeptides and their receptors. Proc Natl Acad Sci USA 91:1009–1013.

Lukas RJ, Bencherif M (1992) Heterogeneity and regulation of nicotinic acetylcholine receptors. Int Rev Neurobiol 34:25–131.

Masland RH, Mills JW, Cassidy C (1984) The function of acetylcholine in the rabbit retina. Proc R Soc Lond [Biol] 223:121–139.

Matter J-M, Matter-Sadzinski L, Ballivet M (1990) Expression of neuronal nicotinic acetylcholine receptor genes in the developing chick visual system. EMBO J 9:1021–1026.

McLane KE, Wu X, Lindstrom JM, Conti-Tronconi BM (1992) Epitope mapping of polyclonal and monoclonal antibodies against two α-bungarotoxin a subunits from neuronal nicotinic receptors. J Neuroimmunol 38:115–128.

Morris BJ, Hicks AA, Wisden W, Darlison MG, Hunt SP, Barnard EA (1990) Distinct regional expression of nicotinic acetylcholine receptor genes in chick brain. Mol Brain Res 7:305–315.

Mulle C, Vidal C, Benoit P, Changeux J-P (1991) Existence of different subtypes of nicotinic acetylcholine receptors in the rat habenulo-interpeduncular system. J Neurosci 11:2588–2597.

Nicoll RA, Malenka RC, Kauer JA (1990) Functional comparison of neurotransmitter receptor subtypes in mammalian central nervous system. Physiol Rev 70:513-565.

Patrick J, Séguéla P, Vernino A, Amador M, Luetje C, Dani JA (1993) Functional diversity of neuronal nicotinic acetylcholine receptors. Prog Brain Res 98:113-120.

Pugh PC, Corriveau RA, Conroy, WG, Berg, DK (1995) Novel subpopulation of neuronal acetylcholine receptors among those binding alpha-bungarotoxin. Mol Pharm 47:717–725.

Reiner A, Brecha NC, Karten HJ (1982) Basal ganglia pathways to the tectum: The afferent and efferent connections of the lateral spiriform nucleus of pigeon. J Comp Neurol 208:16–36.

Role LW (1992) Diversity in primary structure and function of neuronal nicotinic acetylcholine receptor channels. Curr Opin Neurosci 2:254–262.

Sargent PB (1993) The diversity of neuronal nicotinic acetylcholine receptors. Annu Rev Neurosci 16:403–443.

Sargent PB (1994) Double-label immunofluorescence with the laser scanning confocal microscope using cyanine dyes. Neuroimage 1:288–295.

Sargent PB, Pang DZ (1989) Acetylcholine receptor-like molecules are found in both synaptic and extrasynaptic clusters on the surface of neurons in the frog cardiac ganglion. J Neurosci 9:1062–1072.

Sargent PB, Pike SH, Nadel DB, Lindstrom JM (1989) Nicotinic acetylcholine receptor-like molecules in the retina, retino-tectal pathway, and optic tectum of the frog. J Neurosci 9:565–573.

Sargent PB, Wilson HL (1994) Distribution of nicotinic acetylcholine receptor subunit immunoreactivities on the surface of chick ciliary ganglion neurons. In: International symposium on nicotine: the effects of nicotine on biological systems II (Clarke PBS, Quik M, Thurau K, Adlkofer F, eds), abstract P1. Boston: Birkhauser.

Schoepfer R, Conroy WG, Whiting P, Gore M, Lindstrom J (1990) Brain α-bungarotoxin binding protein cDNAs and mAbs reveal subtypes of this branch of the ligand-gated ion channel gene superfamily. Neuron 5:35–48.

Schröder H, Zilles K, Maelicke A, Hajós F (1989) Immunohisto- and cytochemical localization of cortical nicotinic cholinoceptors in rat and man. Brain Res 502:287–295.

Sorenson EM, Parkinson D, Dahl JL, Chiappinelli VA (1989) Immunohistochemical localization of choline acetyltransferase in the chicken mesencephalon. J Comp Neurol 281:641–657.

Sorenson EM, Chiappinelli VA (1990) Intracellular recording in avian brain of a nicotinic response that is insensitive to K-bungarotoxin. Neuron 5:307–315.

Smiley JF, Yazulla S (1990) Glycinergic contacts in the outer plexiform layer of the *Xenopus laevis* retina characterized by antibodies to glycine, GABA, and glycine receptors. J Comp Neurol 299:375–388.

Smiley JF, Levey AI, Ciliax BJ, Goldman-Rakic PS (1994) D_1 dopamine receptor immunoreactivity in human and monkey cerebral cortex: predominant and extrasynaptic localization in dendritic spines. Proc Natl Acad Sci USA 91:5720–5724.

Swanson LW, Lindstrom J, Tzartos S, Schmued L, O'Leary D, Cowan WM (1983) Immunohistochemical localization of monoclonal antibodies to the nicotinic acetylcholine receptor in chick midbrain. Proc Natl Acad Sci USA 80:4532–4536.

Tzartos SJ, Rand DE, Einarson BL, Lindstrom JM (1981) Mapping of surface structures of electrophorus acetylcholine receptor using monoclonal antibodies. J Biol Chem 256:8635–8645.

Ullian EM, Sargent PB (1994) Cellular diversity in the expression of nicotinic acetylcholine receptor subunits in the chick central nervous system. In: International symposium on nicotine: the effects of nicotine on biological systems II (Clarke PBS, Quik M, Thurau K, Adlkofer F, eds), abstract P1. Boston: Birkhauser.

Vernallis AB, Conroy WG, Berg DK (1993) Neurons assemble acetylcholine receptors with as many as three kinds of subunits while maintaining subunit segregation among receptor subtypes. Neuron 10:451–464.

Wada E, Wada K, Boulter J, Deneris E, Heinemann S, Patrick J, Swanson LW (1989) Distribution of alpha2, alpha3, alpha4, and beta2 neuronal nicotinic receptor subunit mRNAs in the central nervous system: a hybridization histochemical study in the rat. J Comp Neurol 284:314–335.

Weaver WR, Wolf KM, Chiappinelli VA (1994) Functional heterogeneity of nicotinic receptors in the avian lateral spiriform nucleus detected with trimethaphan. Mol Pharmacol 46:993–1001.

Whiting PJ, Lindstrom J (1986a) Purification and characterization of a nicotinic acetylcholine receptor from chick brain. Biochemistry 25: 2082–2093.

Whiting PJ, Lindstrom J (1986b) Pharmacological properties of immuno-isolated neuronal nicotinic receptors. J Neurosci 6:3061–3069.

Whiting PJ, Liu R, Morley BJ, Lindstrom JM (1987) Structurally different neuronal nicotinic acetylcholine receptor subtypes purified and characterized using monoclonal antibodies. J Neurosci 7:4005–4016.

Whiting PJ, Schoepfer R, Conroy WG, Gore MJ, Keyser KT, Shimasaki S, Esch F, Lindstrom JM (1991) Expression of nicotinic acetylcholine subtypes in brain and retina. Mol Brain Res 10:61–70.