

## Somatostatin and neuropeptide Y are almost exclusively found in the same neurons in the telencephalon of turtles

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(Accepted 14 July 1987)

**Key words:** Co-occurrence; Cortex; Dorsal ventricular ridge; Basal ganglion; Somatostatin; Neuropeptide Y; Evolution; Turtle

In mammals, somatostatin and neuropeptide Y (NPY) are largely found in the same neurons of the telencephalon. To determine if this is a phylogenetically ancient feature of telencephalic organization, the brain of red-eared turtles was examined using immunofluorescence double-labeling procedures. The results showed that somatostatin and NPY are found almost exclusively in the same neurons in the telencephalon of turtles, but these neuropeptides rarely co-occur in neurons outside the telencephalon. Thus, the extensive co-occurrence of NPY and somatostatin appears to be a feature of telencephalic organization that was present in the reptilian common ancestors of mammals and modern reptiles.

The neuropeptides somatostatin (SS) and neuropeptide Y (NPY) have both been shown to be present in many neurons and fibers throughout the central nervous system in a variety of mammalian species<sup>1, 2, 7, 8, 10, 12, 19, 25, 40</sup>. Although these two neuropeptides are typically found in different neurons throughout much of the mammalian nervous system, within the telencephalon SS and NPY are largely found in the same neurons<sup>5, 34, 37, 38, 39, 41</sup>. Neurons containing both SS and NPY are particularly abundant in the striatal portion of the basal ganglia and in the cortex. Within the basal ganglia, essentially all of the SS+ neurons contain NPY and almost all of the NPY+ neurons (90% or more) contain SS<sup>34, 37</sup>. The neurons containing SS and NPY in the striatum appear to be medium-sized aspiny neurons that have local circuit connections within the striatum, but do not project outside of the striatum<sup>34, 37</sup>. Within the cortex, nearly all NPY+ neurons also contain SS<sup>5, 13, 37–39</sup>, although many SS+ cortical neurons (20% in primates) have been observed that do not contain NPY. Several investigators have concluded that the cortical neurons containing SS and/or NPY are non-pyramidal local circuit neurons of the cortex<sup>13, 24</sup>.

The high degree of co-occurrence of SS and NPY at telencephalic levels and the rare co-occurrence of these peptides outside the telencephalon is a striking feature of the distribution of these neuropeptides in the nervous system of mammals. It would be of interest to know if this feature of brain organization is unique to mammals. SS has been shown to be a neuropeptide whose structure is evolutionarily well-conserved<sup>16</sup>. Further, somatostatin is present in abundance and widely distributed in the central nervous system of a number of different non-mammalian species<sup>3, 9, 14, 35, 36, 42</sup>. Immunohistochemical studies have specifically shown that SS+ neurons are abundant in the telencephalon of amphibians, reptiles and birds<sup>3, 14, 36, 42</sup>. Little is known, however, about NPY or its distribution in the brains of non-mammals. Nonetheless, there are some immunohistochemical data suggesting that an NPY-like peptide is present and widely distributed in the brains of non-mammals<sup>15, 20, 29, 33</sup>. Further, since other members of the family of peptides to which NPY belongs (i.e. the pancreatic polypeptide family) appear to be evolutionarily conservative<sup>21</sup>, it is likely that the NPY-like substances in the brains of these non-mammals are

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similar in structure to NPY in mammals. The possible colocalization of NPY with SS in neurons of the telencephalon has not, however, been studied in any non-mammalian group. Since mammals are descended from reptiles, it seemed reasonable to study the phylogenetic antiquity of SS/NPY co-occurrence in telencephalic neurons in a living reptilian species. Among living reptiles, turtles appear to have the branch point closest to the mammal–reptile divergence; and thus, red-eared turtles were examined in the present study. It was thought that if SS and NPY are typically found in the same neurons in the telencephalon of turtles, then this feature must also have been present in the stem reptiles that were the common ancestors to both turtles (as well as the other living reptiles) and mammals.

Male and female adult red-eared turtles (*Pseudemys scripta*) were used in the present study. Turtles were deeply anesthetized with ketamine (1 ml/kg) and perfused transcardially with 50–100 ml of 6% dextran in 0.1 M phosphate (pH 7.2) buffer followed by 300–400 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). The brains were post-fixed overnight in the same fixative in 0.1 M phosphate buffer (pH 10.5). Although some turtles received intraventricular injections of colchicine (100–200 mg in 3 ml) 5 days prior to being sacrificed, it was found that it was not necessary to use colchicine to visualize the neurons containing SS and NPY. Single-label immunohistochemical techniques were used in the present study, as described previously<sup>27,30,32</sup>, to separately study the distributions of SS and NPY in the brain of red-eared turtles. A simultaneous immunofluorescence double-labeling procedure was used to examine the co-localization of NPY and SS. The details of this double-labeling procedure have been described previously<sup>4,11,26,28,31,43</sup>. To label SS+ perikarya, a mouse monoclonal antibody that is highly specific for SS was used<sup>40</sup>. This antibody was

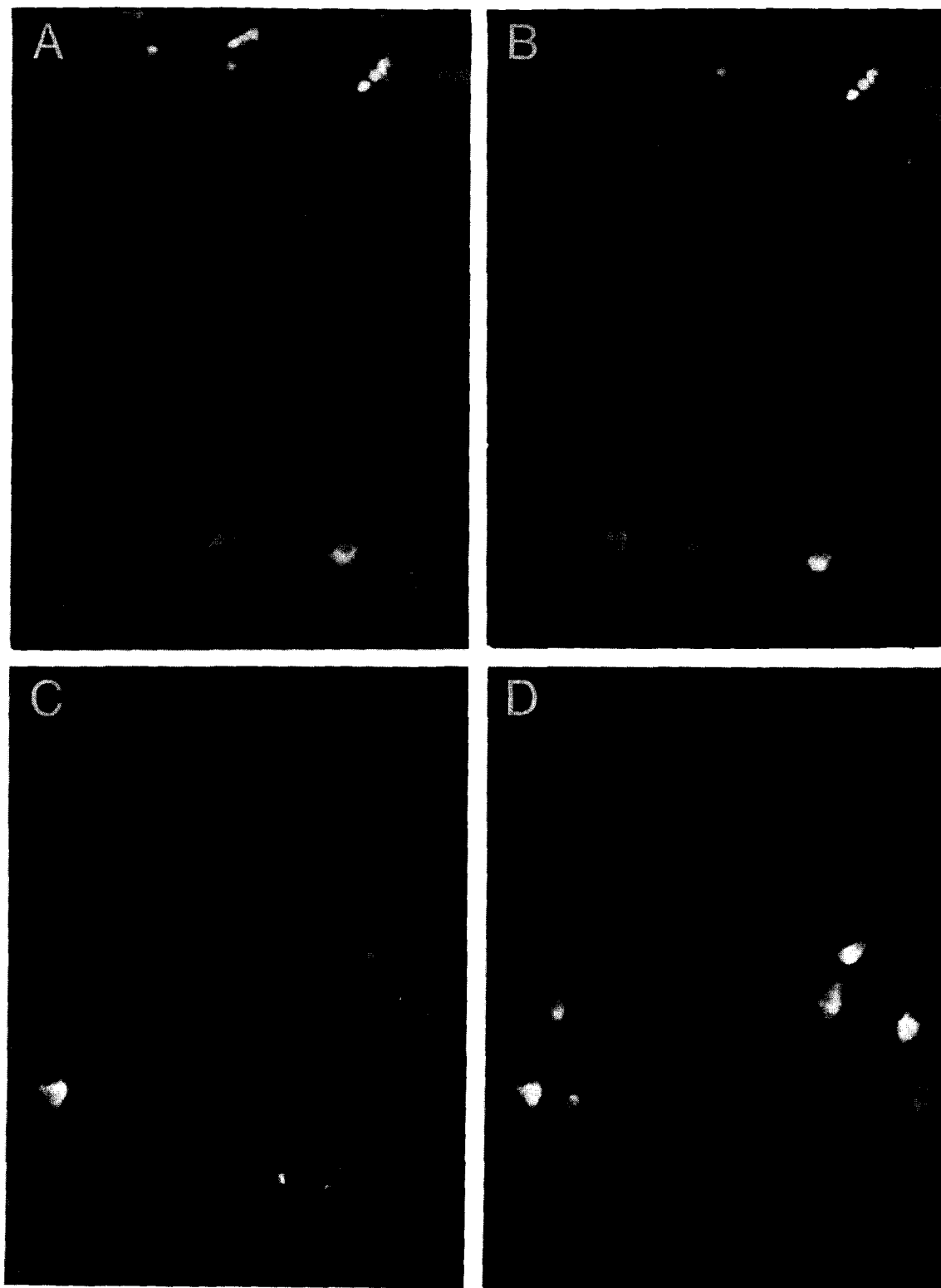
used at a 1:500 dilution and its production and characteristics have been described previously<sup>40</sup>. An antiserum raised in sheep and directed against NPY was used to localize NPY+ perikarya. This antiserum, which was used at a 1:500 dilution, appears to be specific for NPY<sup>4</sup>. The production and characteristics of this antiserum have been described previously<sup>4</sup>.

Single-labeling studies showed that the distribution of SS observed with the monoclonal antibody was indistinguishable, both at telencephalic levels and at subtelencephalic levels, from that previously described for the turtle brain by Bear and Ebner<sup>3</sup> and Weindl et al.<sup>42</sup>, each of whom used a different polyclonal anti-SS antiserum. The anti-NPY antiserum also yielded labeling of numerous perikarya throughout the brain, but the distribution of NPY+ neurons in the midbrain and hindbrain showed little overlap with the distribution of SS+ perikarya. At the levels of the hypothalamus and in the telencephalon, the distributions of NPY and SS showed considerable overlap. In the case of specific portions of the telencephalon (such as the basal ganglia, dorsal ventricular ridge or cortex), the neurons singly labeled with either the anti-SS antibody or the anti-NPY were indistinguishable from one another in their morphology. Since these results implied the possible colocalization of SS and NPY, at least at telencephalic levels, this possibility was examined directly using the simultaneous immunofluorescence procedure.

To carry out this double-labeling procedure, sections were simultaneously incubated at 4 °C for 48–72 h in a primary antisera cocktail containing each primary antiserum at a 1:500 dilution. The sections were subsequently washed 3 times in 0.1 M phosphate buffer and then incubated at room temperature for 1 h in a secondary antisera cocktail containing rabbit anti-sheep IgG conjugated to fluorescein isothiocyanate (at a 1:50 dilution) and rabbit

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Fig. 1. The upper pair of photomicrographs (A and B) shows a single field of view of turtle dorsal cortex from a section that had been simultaneously fluorescein-labeled for NPY (A) and rhodamine-labeled for SS (B). Superficial is toward the top in both photomicrographs. A was taken with the filters for viewing fluorescein in place, while B was taken shortly thereafter with the rhodamine filters in place. As can be seen, all 4 of the NPY+ neurons shown in A were also labeled for SS, as shown in B. The lower pair of photomicrographs (C and D) shows a single field of view of turtle medial cortex from a section that had been simultaneously fluorescein-labeled for NPY (C) and rhodamine-labeled for SS (D). Superficial is toward the top in both photomicrographs. C was taken with the filters for viewing fluorescein in place, while D was taken shortly thereafter with the rhodamine filters in place. As can be seen, all of the NPY+ neurons shown in C were also labeled for SS, as shown in D.



anti-mouse IgG conjugated to tetramethylrhodamine isothiocyanate (at a 1:50 dilution). All antisera were diluted with a solution of 0.1 M phosphate buffer (pH 7.2), containing 0.3% Triton X-100 and 0.02% sodium azide, as described previously<sup>26–28,30–32</sup>. The sections were then washed 3 times in phosphate buffer and mounted on gelatin-coated slides. After air-drying, the sections were cover-slipped with a solution of 9:1 glycerin–0.05 M carbonate buffer (pH 9.0) and examined with a Leitz epi-illumination fluorescence microscopy system. It was then possible to identify the NPY+ neurons in a single field of view using the filters for visualizing fluorescein and determine if any of those neurons were labeled for SS by switching to the rhodamine filters (without changing the field of view). Control procedures were carried out as described previously to ensure that the double-labeling observed was not the spurious consequence of unintended antisera cross-reactivity or of fluorophore cross-emission. The efficacy of this double-labeling procedure has been clearly demonstrated in several previous studies<sup>4,11,26–28,31,43</sup>.

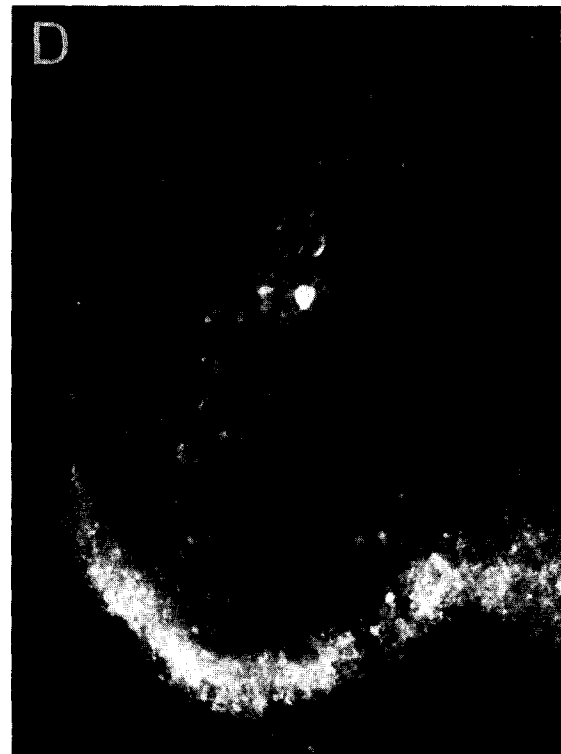
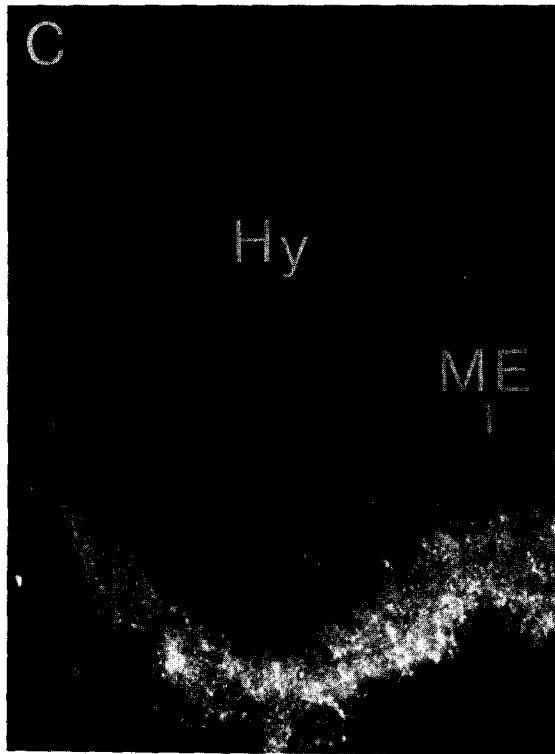
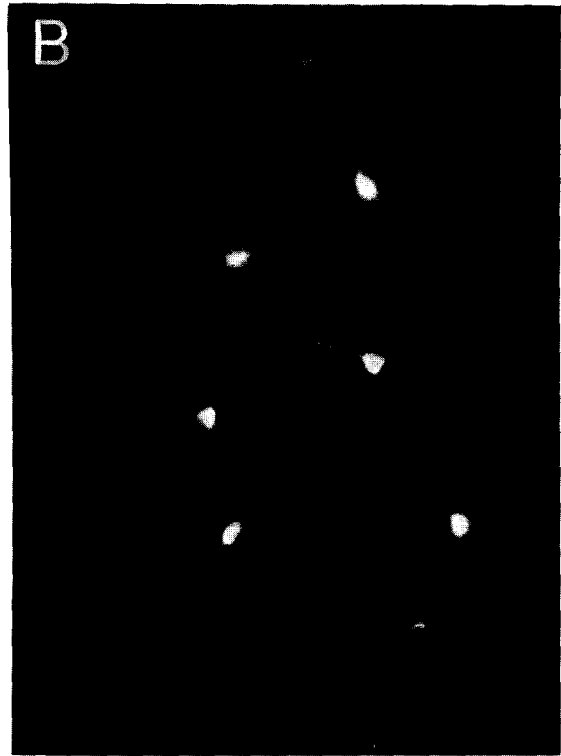
The double-labeling studies revealed that nearly all of the neurons within the telencephalon that contained either NPY or SS contained both. Less than 5% of the labeled neurons labeled for either did not contain both. Regions where NPY+/SS+ neurons were particularly abundant included the 3 cortical fields (the medial cortex, the dorsal cortex and the olfactory cortex) (see Fig. 1), the dorsal ventricular ridge (which embryologically and hodologically resembles the neocortex in mammals<sup>22,29</sup>) and the striatum (Fig. 2). The neurons in the cortex tended to be large stellate neurons (20–25  $\mu\text{m}$  in diameter) resembling mammalian non-pyramidal cortical neurons, while the striatal neurons tended to be medium-sized (12–18  $\mu\text{m}$  in diameter) and more fusiform in shape,

as has been described in previous studies on the distribution of SS in the turtle telencephalon<sup>3,42</sup>. Previous studies on turtles have also reported that some SS+ perikarya are present within the boundaries of the pallidal portion of the turtle basal ganglia<sup>3</sup> and we found that these neurons also were typically double-labeled for both SS and NPY. Neurons that were single-labeled for SS or NPY did not appear to be concentrated in any one portion of the telencephalon. There were some differences, however, between the basal ganglia and other portions of the telencephalon in terms of the types of single-labeled cells present. Specifically, in the cortex and dorsal ventricular ridge, the neurons that did not contain both NPY and SS tended to be labeled for SS, while in the basal ganglia the neurons that did not label for both NPY and SS tended to be labeled for NPY. In contrast to the extensive co-occurrence of SS and NPY in telencephalic neurons, within the diencephalon SS and NPY were not observed to be co-localized in the same neurons. This was true even in the hypothalamus (Fig. 2), in which SS+ perikarya and NPY+ perikarya are present and show some overlap in their distributions. The observation that diencephalic neurons were not double-labeled further reinforces the conclusion that the extensive double-labeling observed in the case of telencephalic neurons was not due to an artifact of the double-labeling procedure employed.

The present results thus indicate that, as in mammals, NPY and SS are largely found in the same neurons at telencephalic levels. In mammals, these neurons are abundant in the striatum and cortex. Similarly, in turtles, these neurons are abundant in both the striatum and in the cortical or cortex-like regions (i.e. the dorsal ventricular ridge) overlying the basal ganglia. Further, the morphology of the cortical SS+/NPY+ neurons appears to be similar in turtles

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Fig. 2. The upper pair of photomicrographs (A and B) shows a single field of view of turtle striatum from a section that had been simultaneously fluorescein-labeled for NPY (A) and rhodamine-labeled for SS (B). The lateral ventricle is evident toward the upper right in both photomicrographs. A was taken with the filters for viewing fluorescein in place, while B was taken shortly thereafter with the rhodamine filters in place. As can be seen, all of the NPY+ neurons shown in A were also labeled for SS, as shown in B. C and D show a single field of view of turtle basal hypothalamus (Hy) from a section that had been simultaneously fluorescein-labeled for NPY (C) and rhodamine-labeled for SS (D). The median eminence (ME) is evident along the lower edge of both photomicrographs. C was taken with the filters for viewing fluorescein in place, while D was taken shortly thereafter with the rhodamine filters in place. As can be seen, the labeling pattern for NPY in C is clearly different than that for SS, as shown in D. Neurons labeled for NPY in C are not labeled for SS in D and vice versa. Further, the distributions of labeled terminals in the median eminence, although showing some overlap for NPY and SS, are also clearly different, with the NPY+ terminals being more broadly distributed and the SS+ fibers being more concentrated in a narrow band.



and mammals, as is also true of the striatal SS+/NPY+ neurons in both groups. These considerations suggest that the cortical and striatal SS+/NPY+ neurons in mammals are homologous as populations to the cortical and striatal SS+/NPY+ neuronal populations, respectively, in turtles. Based on the definition of the term homology, another way of stating this conclusion is that the present results support the inference that cortical and striatal SS+/NPY+ neurons were present in the telencephalon of the reptilian common ancestor of mammals and turtles, and both mammals and turtles have inherited their populations from this ancestor. Further evidence favoring this conclusion would be provided if it were shown that SS and NPY also co-occur extensively in telencephalic neurons in the members of the other reptilian groups (lizards, snakes and alligators) and in birds.

Assuming that there has been some degree of conservatism also in the role that the SS+/NPY+ neurons play in telencephalic function, one implication of the present results is that it may be possible to learn about the overall functional significance of these telencephalic neuronal populations by studying them in turtles. Recent studies by Kriegstein et al. have shown that the turtle telencephalon is a useful model system for physiological and pharmacological approaches for studying neurotransmitter- and neuropeptide-mediated interactions among different neuronal populations<sup>6,17,18</sup>. Since little is known of the functional significance of the co-occurrence of SS and NPY or of the possible synergistic postsynaptic actions these neuropeptides have in the telencephalon, turtles might provide a good model system in which to explore the role of the neurons containing these substances in cortical and striatal functions.

Although the present results suggest that SS+/NPY+ neurons were present in the telencephalon of stem reptiles, this neuronal population may have, in fact, arisen even before the evolutionary appearance of reptiles. Several published studies indicate that SS is present in telencephalic neurons in amphibians and fish<sup>9,14,36</sup>, and there is evidence to indicate that NPY

is also found in telencephalic neurons in fish and amphibians (ref. 33 and A. Reiner, unpublished observations). Although the co-occurrence of SS and NPY in telencephalic neurons in amphibians and fish has not yet been explored, the data from these single-labeling studies raise the possibility that these telencephalic neurons arose very early in vertebrate evolution.

The fact that many SS+/NPY+ neurons are present in both the basal ganglia and cortex (two disparate telencephalic regions), but essentially nowhere else in the brain in turtles and mammals, however, is puzzling. One hypothesis to account for the common presence of SS+/NPY+ neurons in both striatum and cortex is that the SS+/NPY+ neurons may have arisen before the pallial portion of the telencephalon (which corresponds to the cortical portion in land vertebrates) and the basal ganglia were distinct telencephalic regions. A pallium and basal ganglia, however, have been identified in all jawed vertebrates<sup>23,29,33</sup>. Thus, if the basal ganglia and pallium did differentiate from a common telencephalic region during vertebrate evolution, such a differentiation must have occurred before the evolutionary appearance of jawed vertebrates. If, in fact, SS+/NPY+ neurons appeared within the telencephalon of primitive vertebrates before the telencephalon had become differentiated into a pallium and basal ganglia, then SS+/NPY+ neurons should be present in the pallium and basal ganglia of all jawed vertebrates. Further studies will be required to see if this is the case and to see if such neurons are also present in the brains of living agnathans.

Mr. Simon Maddocks of the Waite Agricultural Research Institute, University of Adelaide, helped prepare the antiserum to NPY. This research was supported by NS-19620 (A.R.) and by the National Health and Medical Research Council, the National Heart Foundation of Australia, and the Neurosurgical Research Foundation of South Australia (J.R.O.).

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