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The co-occurrence of substance P-like immunoreactivity and dynorphin-like immunoreactivity in striatopallidal and striatonigral projection neurons in birds and reptiles

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Using an immunofluorescence procedure that allows the simultaneous labeling of tissue for two different antigens, substance P-like immunoreactivity (SPLI) and dynorphin-like immunoreactivity (DLI) were observed to co-occur extensively in striatal neurons of the avian and reptilian basal ganglia and in fibers and terminals in the projection targets of the avian and reptilian striata. Thus, SPLI and DLI apparently co-occur extensively in striatopallidal and striatonigral projection neurons of the avian and reptilian basal ganglia. Since basal ganglia organization is fundamentally similar among amniotes, the present results suggest that SPLI and DLI may also co-occur extensively in striatal neurons in mammals.

In birds, reptiles and mammals, a neuropeptide highly similar or identical to substance P (SP) is present in striatopallidal and striatonigral projection neurons[4,14,18,22,24–26]. These SP-containing striatal neurons appear to represent a distinct subpopulation of the medium-sized neurons that make up the vast majority of the striatal neurons present in the basal ganglia[4,10,22,25]. Several recent studies have shown that members of the dynorphin family of peptides also appear to be present in striatopallidal and striatonigral projection neurons in the basal ganglia of birds, reptiles and mammals[20–23,27,28]. The present studies in White Carneaux pigeons and red-eared turtles were carried out to determine whether the dynorphinergic striatal neurons represent a neuronal population that is distinct from the population of SP-containing striatal neurons or whether dynorphin and SP are contained in the same striatal neurons.

An immunofluorescence double-labeling procedure was used that allows two different tissue antigens to be simultaneously and differentially labeled with two different fluorophores[8,19,24,30]. This double-labeling procedure, which has been referred to as the simultaneous immunofluorescence procedure, has been described previously in greater detail[8,24]. In the present study, SP-containing neurons and fibers were labeled using a rat monoclonal antibody (Accurate Chemical and Scientific Corporation) in conjunction with a tetramethylrhodamine isothiocyanate (TRITC)-conjugated secondary antiserum (goat anti-rat IgG), while dynorphinergic neurons and fibers were labeled using a rabbit antiserum against Dynorphin A_{1-17} (generously provided by L. Terenius of Uppsala University, Sweden) in conjunction with a fluorescein isothiocyanate (FITC)-conjugated secondary antiserum (goat anti-rabbit IgG). Animals received intraventricular injections of colchicine (100 μg in pigeons and 50 μg in turtles) to enhance perikaryal immunoreactivity. Following a postinjection survival time of 36–48 h (pigeons) or 96–120 (turtles), the animals were deeply anesthetized (ketamine for turtles and 35% chloral hydrate for pigeons) and their brains fixed by transcardial perfusion as described previously[24–26]. The brains were then sectioned frozen at 40 μm and processed according to the simultaneous immunofluorescence procedure[8,24]. Tissue incubations were carried out by first incubating tissue in a primary antisera cocktail containing the anti-SP antibody (at a 1:1000 dilution) and the anti-Dynorphin A_{1-17} antiserum (at a 1:250

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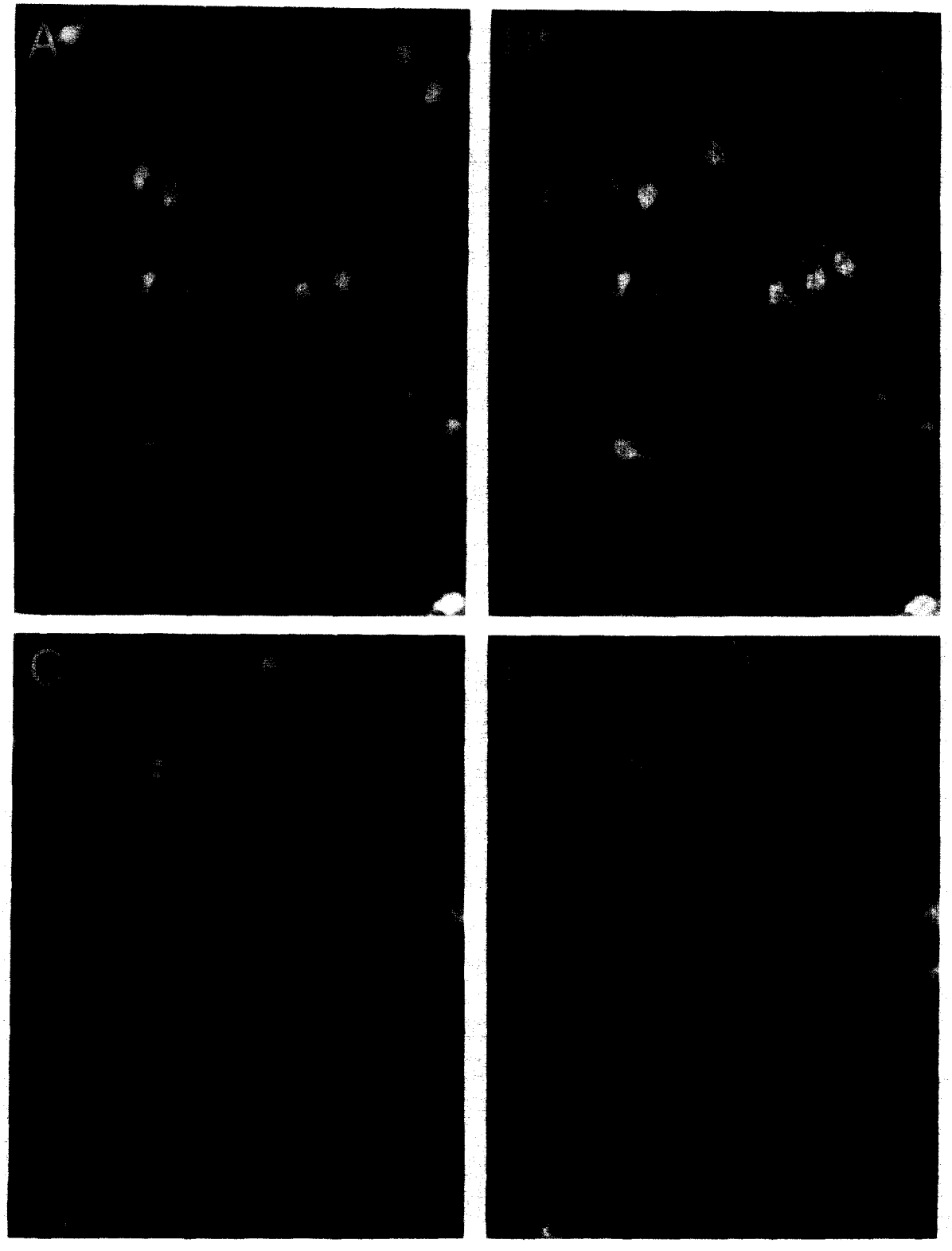

Fig. 1. Double-labeled neurons in tissue processed according to the simultaneous immunofluorescence procedure for the co-localization of substance P and Dynorphin A_{1-17}. The upper pair of photomicrographs, which present a single field of view of the avian medial striatum, show the SPLI-containing neurons (A) and the DLI-containing neurons (B) in this single field of view when viewed for TRITC-fluorescence and FITC-fluorescence, respectively. The lower pair of photomicrographs, which present a different field of view of the avian medial striatum than shown in the upper pair of photomicrographs, show the SPLI-containing (C) and the DLI-containing neurons (D) seen in this single field of view when viewed for TRITC-fluorescence and FITC-fluorescence, respectively. In general, SPLI-containing neurons were more intensely labeled than DLI-containing neurons. Consequently, DLI+ labeling in some of the more lightly labeled SPLI+ neurons is faint. Nonetheless, the vast majority of the SPLI+ neurons shown above (A and C) are also clearly labeled for DLI (B and D). The striatal neurons shown have a somal diameter of 10–15 μm.

dilution), followed by an incubation in a secondary antisera cocktail containing each of the secondary antisera at a 1:50 dilution. The primary antisera used here have been previously shown to be specific for the C-terminus of SP and the C-terminus of Dynorphin A_{1-17}, respectively[6,27,28]. To prevent any cross-reactive labeling of enkephalinergic neurons by the dynorphin antiserum, the primary antisera cocktail was blocked with 100 μmol leucine-enkephalin in all experiments.

Two types of controls ensured that the double-labeling observed in the present study was not the by-product of unintended antisera cross-reactivity. First, numerous neurons labeled only for substance P or only for Dynorphin A_{1-17} were observed outside the basal ganglia (particularly in the hypothalamus). Such single-labeled neurons would not have been observed if either of the secondary antisera cross-reacted with each other or with their non-targeted primary antiserum. Secondly, the specificity of the double-labeling was confirmed by a control study in which tissue was incubated in the primary antisera cocktail blocked with either 100 μmol SP or 100 μmol Dynorphin A_{1-17} (as well as with 100 μmol leucine-enkephalin), followed by normal incubation in the secondary antisera cocktail. In tissue processed in this fashion, only TRITC-substance P-labeling was observed when the primary antisera cocktail was blocked with Dynorphin A_{1-17} and only FITC-Dynorphin A_{1-17}-labeling was observed when the primary antisera cocktail was blocked with substance P. The intensity and distribution of the labeling observed for the unblocked antiserum was identical to that observed for that antiserum when the antisera cocktail was not blocked. This result indicates that: (1) the anti-substance P antibody does not cross-react with Dynorphin A_{1-17} and the anti-Dynorphin A_{1-17} antiserum does not cross-react with substance P, (2) neither of the secondary antisera bind to their non-targeted primary antiserum, and (3) the secondary antisera do not bind to each other.

The striatum in pigeons and turtles consists of two subdivisions: (A) a medial subdivision, termed the lobus parolfactorius (LPO) in pigeons and area d in turtles, and (B) a lateral subdivision, termed the paleostriatum augmentatum (PA) in both pigeons and turtles. In both species, the medial striatum projects to tegmental catecholaminergic cell groups corre-

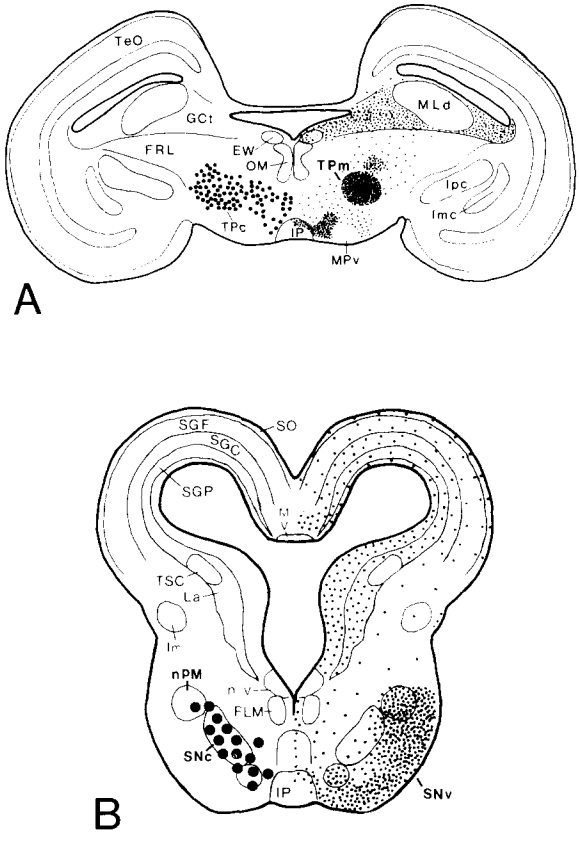

Fig. 2. Schematic line drawings of transverse sections through a midmesencephalic level illustrating the location and organization of the 'nigral' cell fields of the tegmentum in pigeons (A) and turtles (B). The right side of each schematic illustrates the location and density of the SP-containing fibers (SP-containing fibers in the pigeon tectum, although abundant, are not illustrated). The left side of each schematic shows the distribution of the dopaminergic neurons (filled circles) at this level of the midbrain. The TPc of pigeons and the SNc of turtles correspond to the substantia nigra, pars compacta of mammals, while the TPm of pigeons and the SNv of turtles correspond to the substantia nigra, pars reticulata of mammals. Abbreviations: EW, nucleus of Edinger–Westphal; FLM, medial longitudinal fasciculus; FRL, lateral reticular formation; GCt, central grey; IP, interpeduncular nucleus; Im, nucleus isthmi, pars rostralis; Imc, nucleus isthmi, pars magnocellularis; Ipc, nucleus isthmi, pars parvocellularis; La, nucleus laminaris of the torus semicircularis; MLd, nucleus mesencephali lateralis, pars dorsalis; MPv, nucleus profundus mesencephali, pars ventralis; MV, mesencephalic nucleus of the trigeminal nerve; nIV, trochlear nucleus; nPM, nucleus profundus mesencephali; OM, oculomotor nucleus; SGF, stratum griseum et fibrosum superficiale of the optic tectum; SGC, stratum griseum centrale of the optic tectum; SGP, stratum griseum profundum of the optic tectum; SNc, substantia nigra, pars compacta; SNv, substantia nigra pars ventralis; SO, stratum opticum of the optic tectum; TeO, optic tectum; TPc, nucleus tegmentipedunculopontinus, pars compacta; TPm, nucleus tegmentipedunculopontinus, pars medialis; TSC, torus semicircularis.

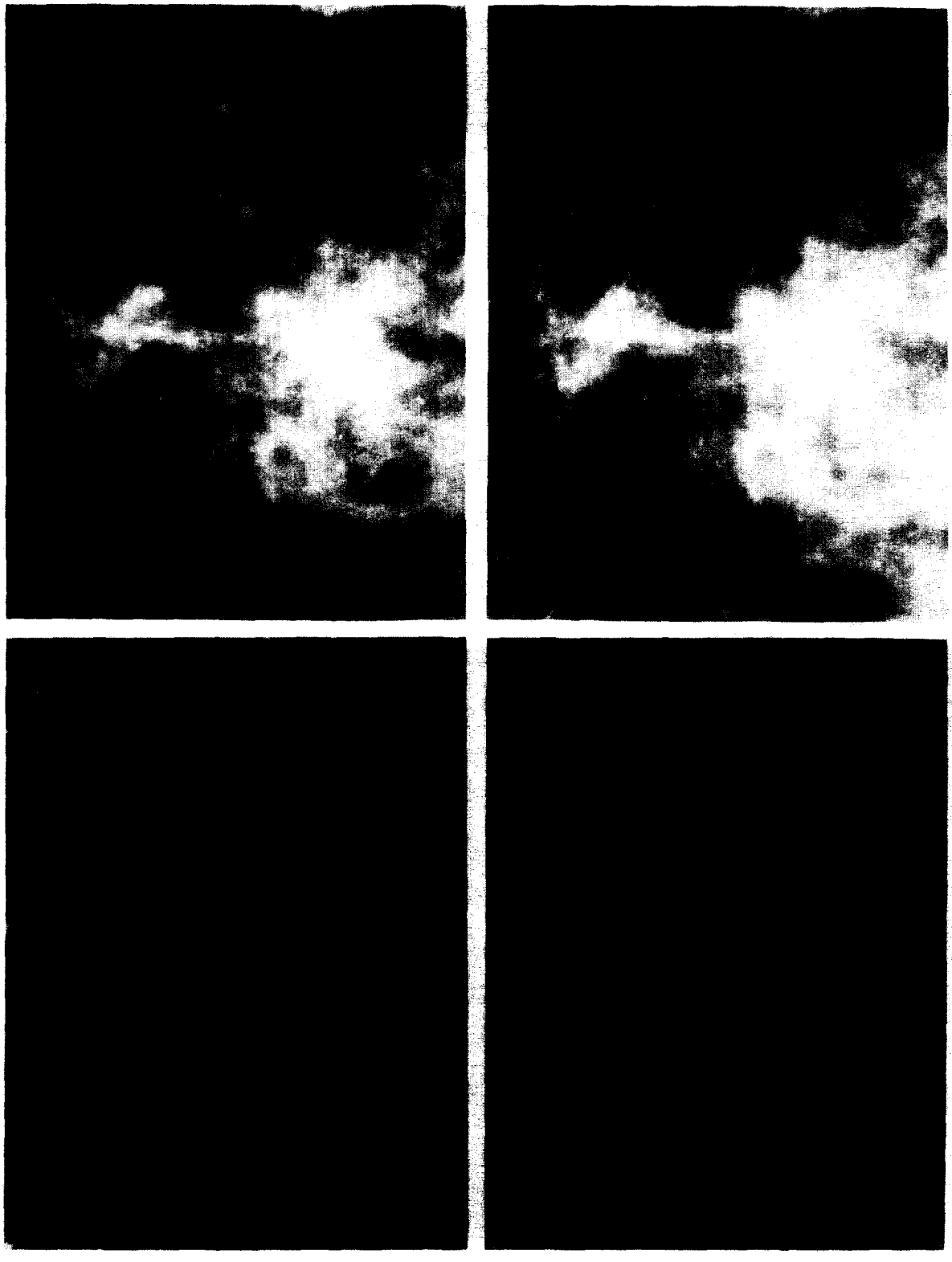

Fig. 3. Double-labeled fibers in tissue processed according to the simultaneous immunofluorescence procedure for the presence of substance P and Dynorphin A_{1-17}. The upper pair of photomicrographs were photographed using a 100× objective, while the lower pair were photographed using a 50× objective. The upper pair show TRITC-labeled SPLI-containing fibers and terminals in the dorsolateral edge of the substantia nigra of turtle (A) and the DLI-containing fibers in the same field of view (B). The overall labeling pattern for SPLI is largely identical to that for DLI and numerous individual boutons can be observed to be labeled in both photomicrographs. The lower pair of photomicrographs show TRITC-labeled SPLI-containing fibers and terminals in the turtle globus pallidus (C) and the DLI-containing fibers and terminals observed in the same field of view (D). As in the case of the upper pair of photomicrographs, the overall labeling patterns in the pair of photomicrographs are largely identical and numerous individual boutons can be observed to be labeled in both photomicrographs.

sponding to the ventral tegmental area (AVT), the substantia nigra (SN) and the nucleus tegmentipeduncularis pontinus (TP) of mammals[4,13,25]. The PA in both birds and reptiles projects to the portion of the basal ganglia corresponding to the mammalian globus pallidus[2,3]. In the present study, numerous SP-like immunoreactivity (SPLI)-containing neurons and numerous dynorphin-like immunoreactivity (DLI)-containing neurons were observed within the striata of colchicine-treated pigeons and turtles (Fig. 1). In both pigeons and turtles, these neurons possess medium-sized perikarya (10–15 μm). Within the medial striatum in both species, nearly all (95–99%) of the labeled cells examined for the co-occurrence of SPLI and DLI contained both SPLI and DLI (Fig. 1). The major tegmental projection of the medial striatum in birds and reptiles (as is also true in mammals) is to the nigral cell field of the tegmentum. The SPLI- and DLI-containing striatal projections to this nigral cell field (termed TP in birds and termed SN in turtles) terminate most densely in a portion of the nigral cell field that is relatively poorer in dopaminergic neurons than other portions of the nigral cell field[4,13,25]. In pigeons, this SPLI-rich and DLI-rich field is located within dorsomedial TP (TPm), while in turtles this field is located within the ventrolateral substantia nigra (SNv) (Fig. 2). The avian TPm and the turtle SNv thus appear comparable to the pars reticulata portion of the mammalian substantia nigra[30], based on the similarities in the densities of SPLI-containing fibers and DLI-containing fibers observed in these regions. In double-labeled avian and reptilian tissue examined at low power, the fiber labeling pattern for SPLI within the tegmental target areas (most strikingly the avian TPm and the reptilian SNv) of the medial striatum was nearly identical to that observed for DLI. High-power examination revealed that SPLI and DLI clearly co-occurred in the majority of the labeled fibers and boutons in the projection fields of the medial striatum (Fig. 3), although the large numbers and dense packing of the labeled fibers and terminals within these tegmental fields made it impractical to examine all fibers and terminals in single-fields of view for the co-occurrence of SPLI and DLI. The observation of such extensive co-occurrence of SPLI and DLI within medial striatal neurons and in fibers and boutons in medial striatal projection targets suggests that many (if not all) of the medial striatal neurons in which SPLI and DLI co-occur are striatonigral (or more broadly striatotegmental) projection neurons.

Within the lateral striatum in both pigeons and turtles, the co-occurrence of SPLI and DLI was extensive, but not as extensive as in the medial striatum. Although nearly all (95–99%) of the DLI-containing neurons observed in the lateral striatum contained SPLI, only 70–85% of the SPLI-containing neurons contained observable DLI. Both single-labeled neurons and double-labeled neurons possessed medium-sized perikarya. Within the projection target of the lateral striatum, termed the paleostriatum primitivum in pigeons and the globus pallidus in turtles[2–4,22,25,26], nearly identical labeling patterns for SPLI and DLI were observed in double-labeled material examined at low power. This was also observed to be the case in the ventral paleostriatum, which is rich in both SPLI-containing fibers and DLI-containing fibers and is comparable to the mammalian ventral pallidum[13,25]. When examined at high power, the vast majority of the labeled fibers and boutons in the paleostriatum primitivum and ventral paleostriatum of pigeons and in the globus pallidus and ventral paleostriatum of turtles were found to be clearly labeled for the presence of both SPLI and DLI (Fig. 3). Since lateral striatal neurons are reported to be the source of projections to the pallidal cell field of the avian and reptilian basal ganglia, the present results indicate that many (if not all) of the lateral striatal neurons in which SPLI and DLI co-occur are striatopallidal projection neurons.

Thus, the present results indicate that SPLI and DLI co-occur extensively in striatonigral and striatopallidal projection neurons in birds and reptiles. Whether the SPLI-only neurons and DLI-only neurons observed in the present study are neuronal populations that are distinct from those containing both SPLI and DLI or whether they simply contained one peptide in an amount that was subthreshold for immunohistochemical detection is at present uncertain.

Since the basal ganglia of all amniotes share a number of characteristics in common, particularly with respect to striatal organization[22], the extensive co-occurrence of SPLI and DLI in striatal neurons of birds and reptiles suggests that SPLI and DLI may also co-occur extensively in striatal neurons in mammals. Although the possibility that SPLI and DLI co-

occur extensively in striatal neurons in mammals has not been examined directly by double-label studies, the inference of such co-occurrence is consistent with the results of several single-label studies in mammals, which show that striatal neurons, pallidal fibers and nigral fibers containing SPLI have highly similar distributions to those containing DLI[9,16,18,28]. Further, since SP and the related tachykinin, substance K (SK), appear to be present in the same striatal neurons in mammals[17], the present results suggest that both SK and SP co-occur with dynorphin in mammalian striatal neurons. Separate SK-like and SP-like peptides have not, however, been identified in non-mammals; and thus, it is unknown if both an SK-like and an SP-like peptide co-occur with dynorphin in striatal neurons in birds and reptiles. Medium-sized striatal neurons in all amniotes are also known to contain GABA and enkephalin[1,9,10,22,23]. Several investigators have reported that the vast majority of the enkephalinergic striatal neurons (and their fibers) in mammals contain glutamic acid decarboxylase (GAD), the synthetic enzyme for γ-aminobutyric acid (GABA)[1,31] and that these enkephalin-containing/GABAergic neurons comprise over 40% of the medium-sized striatal neurons in mammals. These studies have further reported that many additional neurons (and their fibers) in the mammalian striatum contain GAD but not enkephalin. In birds also, enkephalinergic as well as non-enkephalinergic striatal neurons have been observed to contain GABA (using an anti-GABA antiserum courtesy of T.G. Kingan of Columbia University, NY) (A. Reiner, unpublished observation). Although it seems possible that at least some of the non-enkephalin-containing GABAergic striatal neurons contain SP/dynorphin, no published data are available on this point. The results of several previous studies do indicate, however, that it is unlikely that enkephalin co-occurs with SP/dynorphin to any great extent in neurons of the avian and mammalian striata[5,8,9]. Thus, enkephalin-containing/GABAergic neurons and SPLI-containing/DLI-containing neurons appear to make up two major distinct populations of striatal neurons in the amniote basal ganglia.

The functional significance of the co-occurrence of SPLI and DLI is uncertain. Opioid peptides, including dynorphin peptides, have been reported to inhibit substantia nigra neurons[11,29]. In contrast, several studies indicate that SP (as well as SK) excites nigral neurons[7,12,15]. These results suggest that SP and dynorphin may oppose one another in their actions in the mammalian substantia nigra. In this light, the co-occurrence of SP and dynorphin in striatonigral projection neurons appears paradoxical. Such co-occurrence would not, however, be paradoxical under either of two non-mutually exclusive circumstances: (1) if SP and dynorphin are released from nigral terminals under different conditions, or (2) if SP and dynorphin influence different nigral neurons. Whether or not the roles of SP and dynorphin within the globus pallidus are similar to those in the substantia nigra is at present uncertain. Although much further research is required to elucidate the functional significance of SP/dynorphin co-occurrence in striatal neurons, the presence of such co-occurrence clearly indicates that the neurotransmitter/neuromodulator interactions of individual striatal neurons with their projection targets is much more complex than previously realized.

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