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Topographic distribution and quantitative analysis of retrograde-labelled cells in the substantia nigra following administration of fluoro gold in the caudate putamen nucleus

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Summary. The organization of the efferent projections of the substantia nigra following the injection of 0.4 µl of 2% Fluoro Gold into the caudate-putamen nucleus of rats of both sexes was studied. After a survival period of 72 h, the greatest number of labelled cells was visualized in the substantia nigra, with lower numbers in adjacent structures (ventral tegmental area, zona incerta and medial lemniscus).

The cells featured an intensely fluorescent gold-coloured soma and were varied in shape (round or oval); short sinuous projections arose out of them, corresponding to the dendrites and axons of the neurons.

In the statistical study, no significant differences were observed between males and females.

A similar pattern of distribution was found ranging from the rostral to the caudal regions of the substantia nigra, occupying the whole of the extent of the substantia nigra; this distribution was most predominant in the rostro-caudal transition of the pars compacta of the substantia nigra.

Key words: Caudate-putamen, Fluoro Gold, Retrograde axonal transport, Substantia nigra, Rat

Introduction

Cell organelles and proteins that are synthesized in the soma of nerve cells need to be transported along the axon or the dendrites towards the synaptic regions. There is also a return flux towards the neuronal body which can be visualized by retrograde axonal transport techniques.

Since the first fluorescent neuronal tracers Evans blue and DAPI (4-6 diamino-2 phenylindole) were introduced, recently a large number of new fluorescent tracers for retrograde neuronal labelling have been identified. Among them, fluoro gold (FG) introduced by Schmued and Fallon in 1986 has received considerable attention (Reep et al., 1988; Schmued et al., 1989; Chang et al., 1990; Chen and Su, 1990; Dado et al., 1990; Du and Qiao, 1990; Garrett et al., 1991; Soriano et al., 1991; Sugitani et al., 1991; Van Asselt et al., 1991; among others) and has shown great advantages over other retrograde tracers; an intense fluorescence, great resistance to decolouring, great freedom in the survival period of the animals (from 2 days to 2 months) and its compatibility with neurohistochemical techniques.

The substantia nigra (SN), located between the brain stem and the tegument of the midbrain, is undoubtedly an important centre of the extrapyramidal motor pathway; it is mainly influenced by the striatus and is related to neighbouring structures (scheme 1).

The efferent projections of the SN from the striatum have been studied in different species of animals, using different tracers to do so (Anden et al., 1964; Moore et al., 1971; Ungerstedt, 1971; Carpenter and Peter, 1972; Maler et al., 1973; Kuypers et al., 1974; Nauta et al., 1974; Beckstead et al., 1979; Van Kooy, 1979; Van Kooy and Wise, 1980). However, few studies have analyzed retrograde axonal transport using FG as tracer. In view of this, we were prompted to evaluate the topographic distribution, cellular morphology and number of FG-positive cells at the level fo the SN of adult rats, from the rostral portions to the caudal ones, following administration of this tracer in the caudate-putamen nucleus.

Materials and methods

Twenty albino Wistar rats (10 males and 10 females), with weights ranging between 250 and 300 g were used. The animals were deeply anaesthetized i.p. with Ketamine (10 mg/kg b.w.) and placed in a stereotaxic

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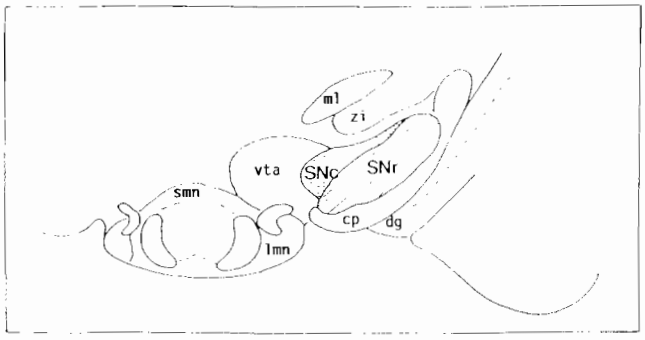


Scheme 1. Relationships of the substantia nigra with neighbouring structures. SNc: Substantia nigra, compacta. SNr: substantia nigra, reticular. cp: cerebral peduncle. vta: ventral tegumental area. zi: zona incerta. ml: medial lemniscus. smn: suprammamillary nucleus. lmn: lateral mammillary nucleus. dg: dentate gyrus.

apparatus for the administration of fluoro gold (FG, Fluorocrome Inc) dissolved in distilled water at a concentration of 2% into the caudate putamen nucleus according to the coordinates of the atlas of Paxinos and Watson (1986), using a Hamilton mycrosyringe to inject 0.4 µl of FG.

After a survival period of 3 days, under deep sodium pentobarbital anaesthesia the animals were perfused via the ascending aorta with 4% paraformaldehyde in phosphate buffer.

After removing the animals' brains these were postfixed with 30% sucrose in PBS for 12 h. Brain sections were taken on a freezing microtome, taking serial frontal sections 40 µm in thickness in the rostro-caudal sense. These were mounted on slides with gelatin, air-dried and then covered with one drop of D.P.X.

In order to clearly differentiate the different zones of the SN and overlap them onto the sections taken for fluorescence, two consecutive sections were taken, using one series for Nissl staining and the consecutive section for visualization under a fluorescence microscope. In this way it was possible to locate the topographic distribution of the retrograde FG-labelled neurons with accuracy at the level of the SN.

To analyze the cells topographically, an X-Y plotter connected to the microscope was employed, then drawing the distribution maps corresponding to the labelled cells, based on the graphic design obtained with the computer, and overlain over the preparations stained with the Nissl technique.

The intense infill by labelled granules of the cell body made it feasible to perform a quantitative analysis of the cells labelled with FG. To do this, the serial frontal sections containing the whole of the SN from the rostral to caudal sections, at an interval of 240 µm, were visualized, obtaining the statistical values of the different zones of the SN and adjacent structures examined.

Cell counting was carried out on a computer. In the statistical analysis the Kolmogorov-Smirnov test was used to evaluate the normality of the distribution of the variables used; the Mann-Whitney «U» test to check the quantitative and qualitative variables of two categories; Student's «t» test for paired data, in quantitative variables with normal distribution, and the Friedman test to compare paired quantitative variables of more than two categories. The Pearson correlation was also used to evaluate the relationship among quantitative variables. All data were analyzed with the SPSS/Pc + statistical package (Statistical Package for the Social Sciences) for compatible personal computers and the CIA commercial program to evaluate the confidence levels.

Results

We report the results obtained using conventional microscopy for the sections stained by the Nissl method, later analysing the findings obtained under fluorescent light to observe the FG-positive cells, together with the topographic distribution and quantitative analysis of the fluorescent cells.

There were no morphological, quantitative or distributive differences between the male and female animals, as shown in Table 3 and graph 1. Accordingly, the results are analysed jointly for both sexes.

1.- Conventional light microscopy. Nissl staining

At low power magnification, the SN appeared as a lamina in the shape of a postero-medially concave half-moon, occupying, at the frontal peak, the whole of the cerebral peduncle (Fig. 1). The numerous neurons were pigmented, as evidenced under the microscope.

The convex portion, ventral, limited by the brain stem, is the pars reticular (SNr) and was formed of large cells. The concave part, which is dorsal, corresponds to the pars compacta (SNc) and had a high density of medium-sized neurons.

Compared with the high cellular density of the pars compacta, the cells of the pars reticular were diffusely distributed (Fig. 1).

The clear morphological distinction between both cell types is shown in figures 2 and 3 at greater magnification. Thus, together with strongly stained cells, which prevail in the pars compacta (Fig. 2) there were others that were weakly stained and mainly belonged to the pars reticular (Fig. 3), although, in lesser numbers, they could also be visualized in the pars compacta.

2.- Fluorescence microscopy

Observation under the fluorescence microscope of the injection of FG at the level of the caudate-putamen nucleus revealed 3 zones that were relatively concentric (Fig. 4): a central zone, corresponding to the needle, surrounded by a small degree of necrosis; another intermediate zone, peripheral to the previous one and strongly fluorescent, corresponding to the region of maximum activity in cellular uptake, and a third zone, the peripheralmost of all, displaying the greatest degradation of the tracer, with a weaker degree of cell

staining.

After studying the sections under fluorescent light, the retrograde FG-labelled cells were found to feature a cytoplasm occupied by strongly fluorescent gold-coloured granules, characteristic of the tracer, together with different sinuous prolongations, less strongly labelled, existing from the cell body (Figs. 5, 6, 6a, 6b).

Together with these strongly fluorescent cells, other less strongly labelled could also be visualized (Fig. 6).

The shape of these cells varied; thus, as well as cells with an oval body (Fig. 5), out of which arose prolongations in different directions, there were other cells that were rounder in shape and were occupied by fluorescent granules (Fig. 5), with some dendritic

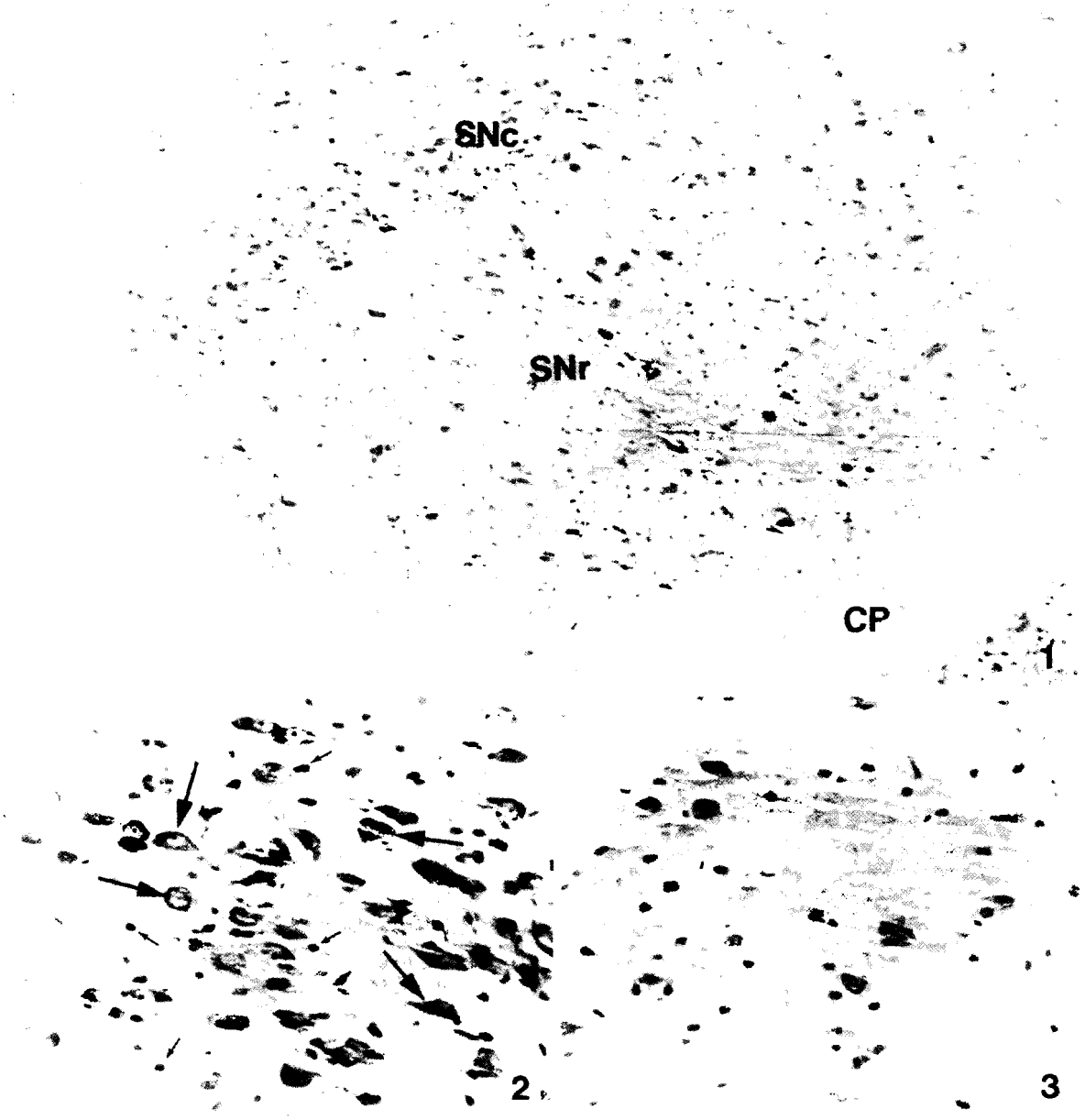


Fig. 1. Low-power view of a frontal section of the substantia nigra of the rat appearing as a half-moon-like formation, appended to the cerebral peduncle (cp). Two portions can be differentiated: pars compacta (SNc) and pars reticular (SNr) owing to their different cell density. Nissl Stain. x 50

Fig. 2. Substantia nigra, pars compacta. Note the high cell density and the strong Nissl stain. Two cell types can be differentiated: some that are round or oval (thick arrow); and others, smaller, dispersely distributed throughtout the SNc (thin arrow). x 150

Fig. 3. Substantia nigra, pars reticular. Note the more diffuse distribution of the Nissl-stained cells with respect to the SNc and their lower number. x 150

processes leaving the soma. Also, apart from these dendrite-like prolongations, a long, thicker, prolongation corresponding to the axon of the neuron was also seen (Figs. 6, 6a, 6b).

3.- Location of FG positive cells

The topographic distribution of the neurons labelled with FG in the SN was predominant in the whole of the pars compacta in the rostro-caudal sense, as reflected in scheme 2. Some disperse FG-labelled cells were also visualized in the area immediately dorsal to the SNc, corresponding to the ventral tegmental area (VTA), zone incerta (ZI) and medial lemniscus (ML).

At caudal levels, where the SN changes laterally in a progressive fashion and decreases in size, the limit between the SNc and the SNr was hard to delimit, the labelled cells appearing as a continuous whole between the group of the SNc and the dorso-lateral part of the SN (Scheme 2).

4.- Quantitative results

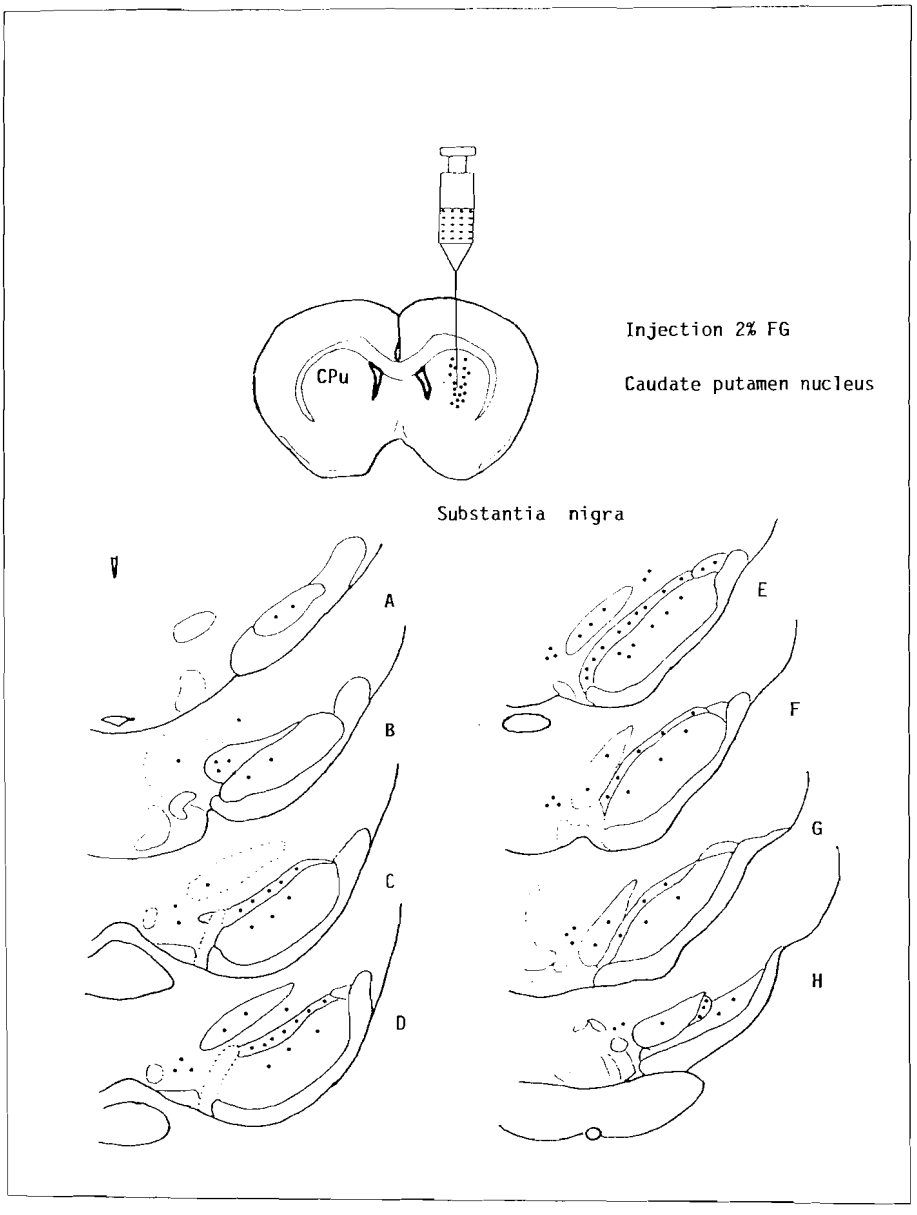
The assumption of normality can be verified with the Kolmorogrov-Smirnov test and also with the indices of Kurtosis and Skewness; however, the assumption of normality is not often compared with small samples because tests for normality with few cases are not very potent.

Using this test it was possible to verify that the distribution of cells in the rostral and caudal regions of the SNc was normal ($p > 0.05$) (Table 1). In other areas adjacent to the SNc, such as in the medial lemniscus, at rostral levels, distribution was not normal ($p = 0.04$); accordingly, the non-parametric test was used for these cases (Table 1).

There were no significant differences ($p > 0.05$) between the male and female animals in any of the areas studied according to the Mann-Whitney «U» test; a non-parametric test for quantitative variables (number of cells) and qualitative variables of two categories (male and female animals) (Table 2).

The arithmetic means obtained for each of the areas considered are shown in Table 3 and graph 1. The SNc zone was striking as the area where the greatest numbers of FG-labelled neurons were observed ($\bar{X} \pm SD$) 15.20 ± 2.07 for the rostral levels and 24.05 ± 1.67 for the caudal levels.

Statistically significant differences were found between the number of cells in the rostral regions with respect to the caudal ones in the SNc ($p < 0.001$), the number of caudal cells being



Scheme 2. Schematic representation of the topographic distribution of retrograde-labelled neurons in the substantia nigra after the injection of fluoro gold into the caudate-putamen nucleus.

Zone of injection of FG into the caudate-putamen nucleus (CPu) and frontal sections of the left half of the substantia nigra, from rostral to caudal (A-H) (240 μm separation).

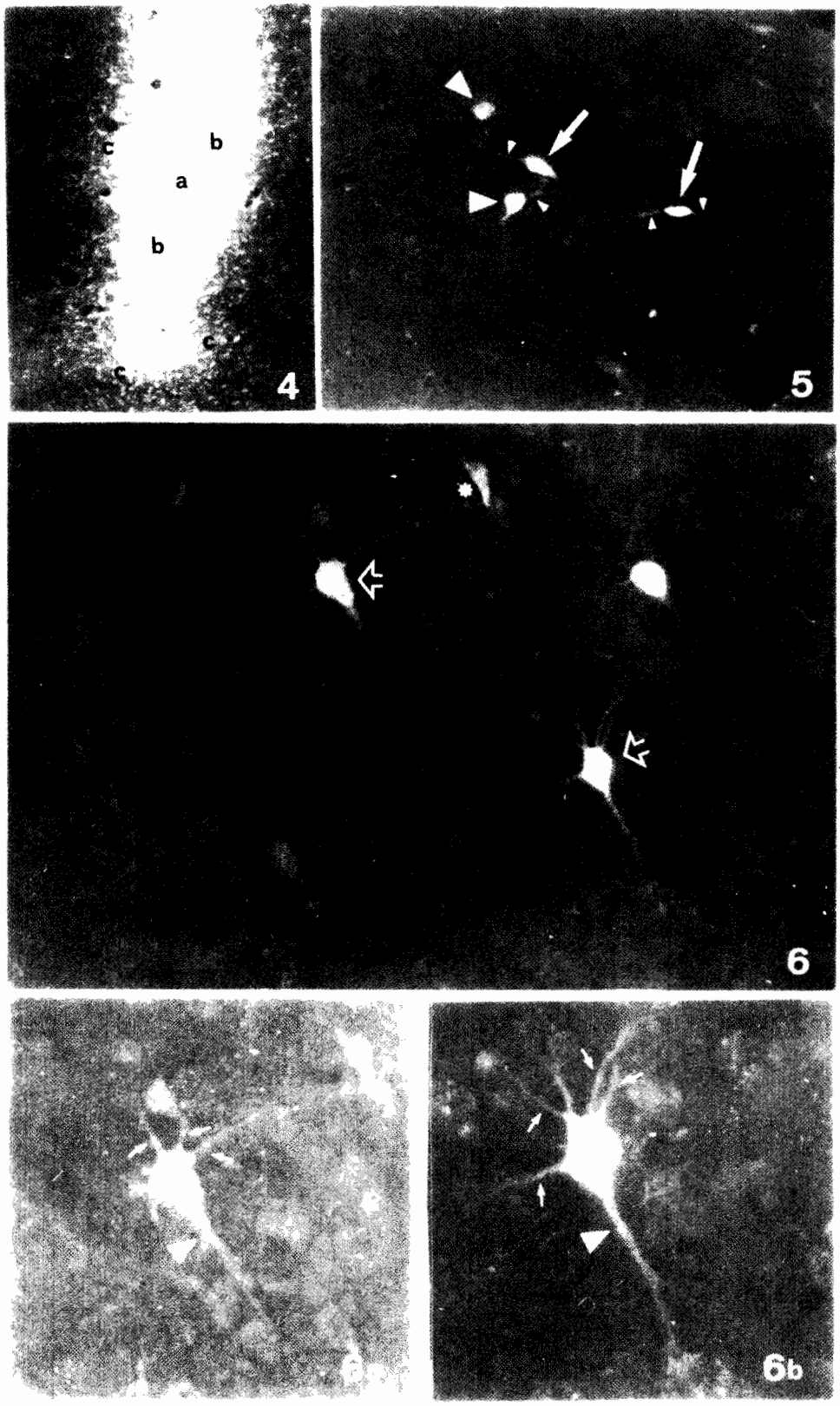


Fig. 4. Fluoro gold injection at the level of the caudate-putamen nucleus. Three concentric zones can be observed. **a:** Central zone, corresponding to the needle and surrounding necrosis; **b:** Intermediate zone, of maximum fluorescence; and **c:** peripheralmost zone of dispersion of the tracer. x 35

Fig. 5. FG-positive cells in the rostral portion of the SNc. Note differentiation between some cells with an oval soma (thick arrows) and small prolongations exiting the cell body (thin arrows) and others, whose soma is rounder (arrowheads), out of which stem small short prolongations (thin arrows). x 90

Fig. 6. FG-fluorescent cells of the substantia nigra (pars compacta) at caudal level. Strongly-stained cells (arrows) can be seen along with other less intensely stained ones (asterisk). x 110

Figs. 6a and 6b. Detail of cells labelled in retrograde with FG in which the pronounced infilling with fluorescent granules of the cell soma can be appreciated. Out of the soma arise small short and sinuous prolongations (arrows), corresponding to the dendrites together with a thicker and longer prolongation (arrowhead) corresponding to the axon of the neuron. x 150

Table 1. Kolmogorov-Smirnov index.

AREA	ROSTRAL LEVEL	CAUDAL LEVELS
SNc	0.700	0.645
SNr	0.227	0.551
VTA	0.186	0.493
ZI	0.262	0.339
ML	0.044*	0.086

The distribution of cells labelled with FG in the rostral and caudal regions, in both sexes, is normal ($p > 0.05$), except in the medial lemniscus (*) of the rostral levels where this distribution pattern was not observed.

Table 2. Mann-Whitney "U" test.

AREA	ROSTRAL LEVEL	CAUDAL LEVELS
SNc	0.5157	0.4194
SNr	0.9051	0.7240
VTA	0.3176	0.2750
ZI	0.5733	0.6076
ML	0.8048	0.5645

There were no statistically significant differences between the male and female animals ($p > 0.05$) for any of the areas considered.

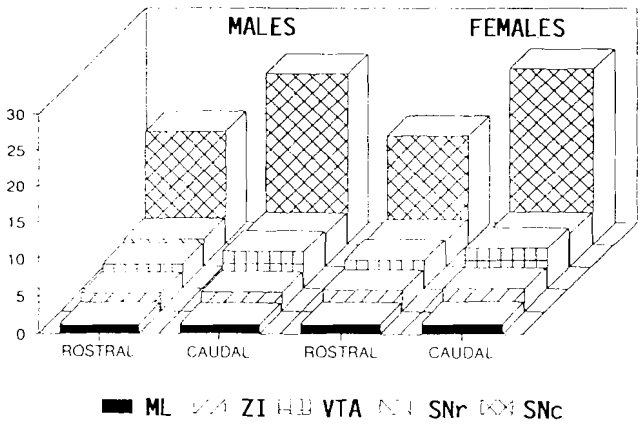
Table 3. Arithmetic means.

AREA	ROSTRAL LEVEL	CAUDAL LEVELS
SNc	15.20±2.07	24.05±1.67
SNr	3.55±0.94	4.45±1.67
VTA	3.50±1.36	5.30±1.38
ZI	2.50±1.10	2.85±0.99
ML	1.30±0.80	1.30±0.73

greater than rostral cells in the population between 7.78 and 9.95 (confidence interval, 95%).

According to the Friedman test, there were also statistically significant differences between the SNc of the rostral levels and the SNc of the caudal levels, and the rest of the rostral levels considered.

There was no correlation (Pearson correlation) between the number of cells in either level (rostral and caudal), and the number of rostral and caudal cells therefore seems to behave independently in each animal. However, a positive correlation ($p < 0.01$) was observed in the SNc at rostral level with the SNr at rostral level (0.64), VTA (0.64) and ZI (0.60). There was also positive correlation ($p < 0.01$) in the SNc at caudal levels with the ML area of the caudal levels (0.55).



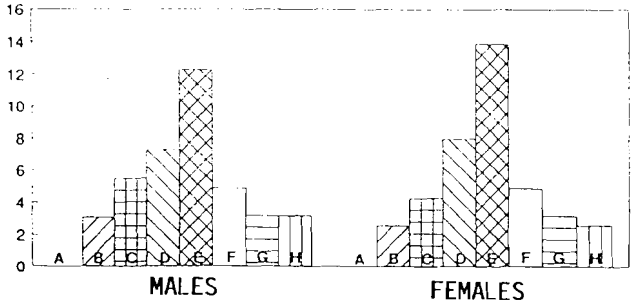
Graph 1. Comparison between male and female animals and the number of FG-positive neurons found at the rostral and caudal levels for each of the areas considered. The SNc is striking as the area in which the highest proportion of cells labelled with FG was observed.

The quantitative study of the different serial sections showed that the highest proportion of FG-positive neurons was present at the rostro-caudal limit of the SNc (Graphic 2), zones D with 7.65±1.35 (SD) and E with 13.10±2.25 (SD) being those with the greatest numbers of FG-positive neurons. There was a correlation ($p < 0.001$) of 0.75 between zone D and E.

Discussion

Knowledge of the morphology of the neuronal elements of a given pathway of the CNS is perhaps the last step in unravelling the morphology of the whole of the system. However, in view of the complexity of the CNS, insight into the fine architecture of any given zone is very difficult. It is therefore necessary to use tracers that, in a retrograde fashion, will permit selective labelling of the neurons for later morphological description. With this in mind, we attempted to evaluate the efferent projection of the SN using FG after administration of this substance into the caudate-putamen nucleus to gain further understanding of this pathway.

Many studies have analyzed the morphology of the SN, using different techniques and animal species (Bak, 1967; Hanaway et al., 1970; Rinvik and Grofova, 1970; Gulley and Wood, 1971; Parizek et al., 1971; Agid et al., 1973; Sotelo et al., 1973; Schwyn and Fox, 1974; Butcher and Marchand, 1978; Butcher and Talbot, 1978; Domesick, 1979). However, these authors made no



Graph 2. Representation of the number of FG-positive cells at the level of the SNc, dividing this latter into eight zones in the rostro-caudal sense. Note the high number of cells in zone E with respect to the rest of the zones considered. No significant differences were found between the male and female animals.

distinction between male and female animals, using only one sex. In our study, we did not observe significant differences as regards sex in any of the parameters examined (morphology, topographic distribution and quantitative analysis of the cells).

As initially demonstrated by Dahlstrom and Fuxe (1964), using immunofluorescent methods, and later immunohistochemical techniques (Hökfelt et al., 1976; Pickel et al., 1977), most dopaminergic cells of the midbrain are located in the pars compacta of the SN, whereas the non-dopaminergic cells of the nigral complex are found in the pars reticular of the SN. These data have been confirmed by different authors (Hanaway et al., 1970; Rinvik and Grofova, 1970; Gulley and Wood, 1971; Parizek et al., 1971; Pickel et al., 1977; Domesick, 1979).

The basophile nature observed in this work of the strongly stained cells, which were predominant in the pars compacta of the SN, is due to the massive accumulation of Nissl substance, filling almost the whole of the cytoplasm. Such cells would correspond to the dopaminergic cells previously reported by other authors. By contrast, the cytoplasm of the weakly-stained cells is translucent because the Nissl substance is diffusely distributed, leaving large unstained areas.

The observation in the present study of some neurons labelled with FG at the level of the VTA could be accounted for in terms of the idea that the projection from the VTA to the accumbens nucleus courses all the way along the ventral striatum and the medial half of the caudate putamen, as reported by Gonzalez et al. (1990), thus partially coinciding with the projection of compact substantia nigra. Additionally, the cells of the SN observed by us with the Nissl stain were similar to those found in the VTA.

Injections with high concentrations of tracer (5-10%) at an injection volume between 0.2-0.5 µl afford an intense retrograde label. However, they also produce an undesirable degree of necrosis in the zone. Accordingly, most authors, among which we include ourselves, consider that a good retrograde labelling with an insignificant lesion can be achieved with low concentrations (2-3%). Using a 2% concentration, we obtained only a small zone of necrosis around the needle, an area concentric to the necrosis of brilliant fluorescence remaining; this would correspond to the zone of maximum activity.

Although FG permits considerable variability in the survival time of the animals, this period does strongly affect the later labelling of the cells, as reported earlier by Schmued and Fallon (1986). Thus, short survival times (1-2 days) give an accumultion of vesicles in the cytoplasm, whereas with periods of 4 days to 4 weeks vesicles accumulate in the soma, extending from there to the dendritic prolongations.

In the present work, with a survival time of 72 hours, it was possible to visualize a high concentration of tracer in the soma, dendrites and axon; accordingly, for us this survival period is optimum to evaluate the whole cell.

Despite this, there is no absolute consensus among the different authors as regards the optimum survival time; in this sense, there are other factors that affect good visualization of the cell labelling, although they remain to be discovered owing to the strong variability in the survival times used by the different authors.

Once the tracer has occupied the inside of the cells, its intensity is not lost even though it remains there for some time. We observed this on visualizing the preparations after a lapse of two months. This confirms the strong resistance to decolouring described by Schmued and Fallon in 1986.

Although few works have addressed the topographic distribution of FG-labelled cells at the level of the SN after administration of the tracer to the caudate-putamen nucleus, there are studies that have analyzed this pathway using other tracers (Kuypers et al., 1974; Beckstead et al., 1979; Bentivoglio et al., 1979; Van Kooy, 1979), the organization of the efferent projections coinciding with the same topographic sites determined by us with FG, although most of the above-mentioned authors did not perform an exhaustive study of the different zones of the SN from the rostral to the caudal levels, as was performed in this study.

We observed that the greatest predominance of FG-positive cells occurred at the level of the SNc throughout its rostro-caudal extension. This confirms the results obtained by other authors using other tracers.

Although it is fairly well known, currently the attention of many studies is being focused on the morphological, neurochemical and neuropharmacol-ogical analysis of this extrapyramidal pathway.

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