

An Immunohistochemical Analysis on The Human Cerebellar Dopaminergic System

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Research

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Abstract

Background

The cerebellum now it has not considered a dopaminergic region. Despite, is traditionally reported only the presence of dopaminergic afferents to the cerebellum. Currently, studies attribute to the cerebellum a critical role in motor and cognitive functions and suggest a cerebellar involvement in dopamine related neurologic and psychiatric disorders. In several studies has been demonstrated mainly in the cerebellum of rodents a widely distribution of all dopaminergic receptor subtypes (DRD₁-DRD₅), a poor number of dopaminergic Purkinje neurons, and the presence of several dopaminergic neurons in the deep cerebellar nuclei. Data on an intrinsic dopaminergic neuronal system in the human cerebellum are lacking.

Methods

The aim of the present study of chemical neuroanatomy was to investigate in human cerebellum on the presence of a dopaminergic neuronal system, through an immunohistochemical approach based on the use of specific antibodies against the dopamine membrane transporter (DAT) and the dopamine receptor subtype 2 (DRD₂).

Results

The immunoreactions revealed the presence of DAT and DRD₂ positive neuronal cell bodies and processes of all the layers of the cerebellar cortex and in the dentate nucleus. These results are in agreement with previous studies, and suggest which the intrinsic cerebellar neuronal dopaminergic system may be involved in intrinsic and extrinsic (projective) cerebellar circuits.

Conclusions

This study open a new scenario on the interpretation of the cerebellar role in dopaminergic related brain disorders. Finally, this study may be an innovative critical element for the development of pharmacologic and non-pharmacologic therapeutic strategies for neurologic and psychiatric disorders related to dopamine.

Background

The cerebellum is traditionally not considered a dopaminergic area [1–3], in morphological studies has been detected only dopaminergic projection to the cerebellum, which presumably originating mainly by the ventral tegmental area (A₁₀) [4–7]. Although, the existence of an intrinsic cerebellar dopaminergic neuronal system in the human cerebellum is in part predictable. In the postmortem human cerebellum

has been detected significant levels of dopamine (DA), homovanillic acid (HVA), the most catecholamine metabolite [8–10], tyrosine hydroxylase (TH), the first enzyme involved in catecholamine biosynthesis, dopamine transporter (DAT), the membrane transporter involved in DA reuptake [11]. Positron emission tomography (PET) studies in monkey cerebellum and autoradiographic evaluations in human postmortem cerebellum has been detected the presence of selective dopamine transporter ligands (DAT-Ls) [12–14]. Moreover, in biochemical analysis has been revealed in rat and monkey cerebellum high levels of DA in the vermis lobules VII, VIII, IX, X, in the fastigial and dentate nuclei [11, 15–17].

In studies of chemical neuroanatomy on mammals cerebellar cortex, dopaminergic fibers has been observed in the granular layer in form of mossy fibers-like, fewer of them have been localized on the Purkinje neuron cell bodies and their dendritic arborizations, and only few fibers were detected in the molecular layer [18, 19]. In the adult rodents cerebellum by means of immunohistochemical, pharmacological, biochemical approaches has been demonstrated the presence of DAT immunoreactivity in Purkinje neurons and in several neurons of all deep cerebellar nuclei [20, 21] and into cerebellar synaptosomes has been evidenced an active [^3H]-DA transport and an endogenous release of DA [20, 22]. Furthermore, in adult rodents cerebellum in the lobules I, VII, VIII, IX, X has been observed a poor number of Purkinje neurons immunoreactive to the vesicular monoamines transporter 2 (VMAT₂), the protein transporter that carries DA from cytosol to synaptic vesicles [23], TH [24] and to DAT [19–21, 24–26]. Moreover, in clinical and experimental studies has been suggested a considerable role of the cerebellum in neurologic and psychiatric dopaminergic related disorders such as Parkinson's disease (PD) [27, 28], ataxias (ATX) [21, 30], schizophrenia (SCZ) [31–33], autism spectrum disorders (ASD) [33, 34], bipolar disorders (BD) [32, 35] and drug addiction (DGA) [36, 37].

Despite these studies, the data on an intrinsic neuronal cerebellar dopaminergic system in non-primate mammals are still incomplete, and detailed data on the existence of a human cerebellar neuronal dopaminergic system are often neglected or denied. Therefore, the aim of this study of chemical neuroanatomy by means of an immunohistochemical approach using specific polyclonal antibodies against DAT and dopamine receptor type 2 (DRD₂) is to evaluate the presence and the distribution of DAT and DRD₂ immunoreactive neuronal subpopulations in the human cerebellar cortex and in the dentate nucleus.

Methods

The study was carried out on human postmortem cerebellum samples taken in accordance with the guidelines of the 'Codice di Polizia Mortuaria' (art. 40 et seq., DPR 285, 10.09.1990) and 'Codice dei Comportamenti nella Università di Bari' (DR 2272, 02.07.2014).

The samples of human cerebellum were obtained from 4 healthy subjects aged between 20 and 63 years, 2 male and 2 females; (for each sex, one young and one senior) removed maximum of 36-40h after death. The standards for subject inclusion was the absence of neurological and psychiatric diseases in their medical history, and the absence of brain abnormalities at routine macroscopic examination.

From each cerebellum, were taken samples which corresponding to the left dentate nucleus and to the following cerebellar lobules: tonsilla (anterior lobe, left hemisphere), superior semilunar lobule (posterior lobe; left hemisphere) and inferior semilunar lobule (posterior lobe; left hemisphere).

Preparation of histological sections

From each lobules of cerebellar cortex, were obtained 3 fragments of 25-55 mm³, which included the entire cerebellar cortex and part of the underlying white matter; from each dentate nucleus were taken fragments that include its total extension surface considering the transverse axis of the nucleus. The fragments were fixed by immersion for 3h at 4°C in Zamboni's fluid [38]. We chose a fixative solution composed by 10% formaldehyde and a saturated concentration of picric acid, because in our previous experimental procedures the same fixative solution had shown to be optimal and useful for postmortem nervous tissue fixation, and for immunohistochemical visualization of different neuroactive molecules (i.e. enzymes, neuropeptides, calcium binding proteins) [For details see: 39-44].

The fragments after the fixation procedure, were washed and dehydrated in series of ethanol and incorporated in a semi-synthetic paraffin. The paraffin blocks were serially cut into 5µm sections, respectively oriented orthogonally to the long axis of the cerebellar cortex and to the major axis of the dentate nucleus. Before the immunostaining, randomly chosen sections from each fragment were stained with toluidine blue and submitted to light microscopic histopathological analysis to ascertain the normality of the nervous cerebellar tissue. In any case selected for the study, the microscopic structure of the cerebellum showed no pathological changes.

Immunohistochemistry for DAT and DRD₂

Briefly, from each sample series, 15 sections were selected at intervals of 150µm and subjected to immunohistochemistry for DAT or for DRD₂, consecutive sections were stained to toluidine blue. The sections were immunolabeled according to the following steps: rehydration in a descendent ethanol series; 2) immersion in 3% hydrogen peroxide solution, for 10 min at room temperature (RT) to inactive the endogenous peroxidase; 3) rinsing in PBS, pH 7.6, for 3×10 min at RT; 4) pre-incubation with donkey normal serum (Santa Cruz Biotechnology, CA, USA) diluted 1:10 in PBS for 1h at room temperature; 5) incubation with primary antibody anti-DAT diluted 1:200, a rabbit polyclonal antibody raised against a synthetic peptide from the 2nd extracellular loop (C-HLQQSHGIDDLGPPRW-OH) of the human DAT (for details see:45) (COVANCE – USA, Cat. Num. PRB-330P) or with primary antibody anti-DRD₂ diluted 1:50 a rabbit polyclonal antibody raised against KLH conjugated synthetic peptide derived from the amino acid sequence 214-260 of the human DRD₂ [for details see: 46, 47] (GENETEX – USA, Cat. Num. GTX17570), both dilutions were performed in a buffer solution (BS) containing 5% foetal calf serum in PBS; for 72 h at 4°C; 6) rinsing in PBS for 3×10 min at RT; 7) incubation with donkey anti-rabbit (Santa Cruz Biotechnology, CA, USA), diluted 1:100 in BS, for 1h at RT; 8) rinsing in PBS 3×10 min at RT; 9) incubation with the streptavidin-peroxidase complex solution (Vector Laboratories, CA, USA) for 40 min at RT; 10) rinsing in PBS 3×10 min at RT; 11) the sections tested with primary antibody anti-DAT were incubated

with the chromogen 3,3-diaminobenzidine tetrahydrochloride (DAB) for a brown reaction (Vector Laboratories, CA, USA) for 10 min at RT, the sections tested with the primary antibody anti-DRD₂ were incubated with 3,3-diaminobenzidine tetrahydrochloride (DAB) plus nickel solution for a gray-black staining (Vector Laboratories, CA, USA) for 20 min at RT.

Negative controls of immunohistochemistry for DAT and DRD₂

Negative controls of the immunoreactions were performed replacing the primary antibody with non-immune serum containing donkey normal serum (Santa Cruz Biotechnology, CA, USA) diluted 1:10 in BS. No specific immunostaining was observed in the negative controls, or with an excess of synthetic DAT or DRD₂ peptide

in place of the primary antibody.

Positive controls of immunohistochemistry for DAT and DRD₂

Positive controls of the DAT and DRD₂ immunoreactions were performed on paraffin sections of rat ileum by immersion in the above fixative solution and subjected to the same immunohistochemical procedure of the cerebellum sections (data not showed). (For details on the intestine distribution pattern of DAT and DRD₂ immunoreactivity see: 48, 49).

Qualitative analyses

The distribution pattern of DAT and DRD₂ immunoreactivity were analysed in all the immunostained section of cerebellar cortex and dentate nucleus; the qualitative morphological identification of the different immunoreactive cerebellar neuron types was carried out considering the following parameters: localization, cell body position, shape and cell body size, neuronal processes spatial arrangement, or through the comparison with the nearby stained toluidine blue sections [for further on the morphological parameters details see: 41-44, 50-54]. Microscopical analysis were performed with the light microscope Olympus Vanox-T and with the Spot Insight Color V3, Diagnostic Instruments Inc., USA.

Results

DAT immunoreactive neuronal bodies and processes in the cerebellar cortex

In the cerebellar cortex the DAT immunoreactivity were detected in neuronal cell bodies and processes distributed in the molecular layer (ML), in the Purkinje neuron layer (PL), in the granular layer (GL) and in the subjacent white matter (WM) (Fig. 1).

Molecular layer

In the ML were observed DAT immunonegative stellate neurons (Figs. 1, 2A-E), and, a diffuse DAT immunoreactivity in few perikarya of basket neurons localized in the deep zone of the layer or at the border

with the Purkinje neuron layer was occasionally detected (Figs. 2A, 2B). A strong DAT immunoreactivity in form of densely packed granular deposits, in the cytoplasm in variously oriented primary and secondary dendritic trunks of the Purkinje neurons was observed (Figs. 2C, 2D). Moreover, intensely DAT immunoreactive puncta (referable to axon terminals) were localized in the close relationship with the wall of the microvessels (Fig. 2E). In the ML fine and diffuse DAT immunoreactive puncta (referable to sectioned dendritic or axon processes, or axon terminals) were also widely distributed within the neuropil (Figs. 2C-E).

Purkinje neuron layer

A great number of the Purkinje neuron cell bodies presented a DAT immunoreactivity in form of grains dispersed within the perikarya (Figs. 1, 2A, 2C, 3A, 3B); in particular, the DAT immunoreactivity in form of grains within the primary dendritic trunks that extend into the ML were also detected (Figs. 1, 2A, 3A), and only occasionally DAT immunonegative Purkinje neurons were observed, and their cell bodies profile was surrounded by DAT immunoreactive puncta (Fig. 3B).

Granular layer

In the protoplasmic spaces of Held, where they are localized the synaptic glomeruli complex, DAT immunoreactive small and finely grains were localized among granules (Figs. 2A, 3B). Moreover, DAT immunoreactive clusters of puncta referred such as terminals of mossy fibers at cerebellar glomeruli complex were also observed (Figs. 4B). In addition, within the GL among the numerous immunonegative granules, few intensely DAT immunoreactive cell bodies of granules were also observed (Figs. 2A, 4B).

In addition, DAT immunoreactivity was also observed in a non-traditional large neuron type, it was identified on the basis of the localization, cell body position, cell body shape and size, spatial arrangement of neuronal processes as the synarmotic neuron [For further details: 41, 43, 44, 56, 57] (Fig. 4A).

Subcortical white matter

In the subcortical WM, numerous intensely DAT immunoreactive nerve fibers variously oriented, often in continuity with the positive processes located in the overlying GL were observed (Figs. 1, 2A, 4B).

DAT immunoreactive neuronal cell bodies and processes of the dentate nucleus

Numerous DAT immunoreactive neuronal cell bodies have been observed within the dentate nucleus (DN) and at the bordering to the nearby WM (Figs. 5, 6A, 6B); the DAT immunoreactive neurons on the basis of their morphological features were principally identified and subdivided in small, medium and large neuron types which respectively displayed a main diameter ranging of 6 to 9 μm , 18 to 25 μm and 18 to 40 μm [For further details see: 50-54].

The DAT immunoreactive small neuron types were scattered thorough within the DN (Figs. 6A, 6B, 7A, 7B), while, the DAT immunoreactive and immunonegative medium neuron types were preferentially localized in the intermediate zone of the DN (Figs. 5, 6A, 6B); in addition, both DAT immunoreactive neuron types showed in the cell bodies and processes an intense positivity in form of densely packed granular deposits.

In the DN, numerous DAT immunoreactive large neuron types characterized by a different distribution pattern, cell body shape and spatial organization of processes, were observed (Figs. 5, 6A, 6B, 7A, 7B). A first large neuron type showing a roundish cell body, was predominantly distributed in the internal zone of the DN (Figs. 5, 6A, 6B); a second large neuron type characterized by an ellipsoidal cell body was concentrated at the boundary of the DN (Figs. 6A, 6B); a third large neuron type presenting a large ellipsoidal cell body was localized in the external zone of the DN, characteristically from the internal pole of the cell body give rise a thickness dendritic trunk (Figs. 6A, 6B); a fourth large neuron type showed an elongated fusiform cell body were distributed within the DN (Figs. 6A, 6B). In addition, a large neuron type characterized by the cell body and neuronal processes in close relationship with the wall of microvessels was also observed (Figs. 7A, 7B) [For further details see: 41, 43, 44, 55, 57]. Moreover, in the neighboring white matter of the DN clusters of intensely DAT immunoreactive puncta was observed (referable to sectioned nerve fibers) (Figs. 5, 6A, 6B).

DRD₂ immunoreactive neuronal bodies and processes in the cerebellar cortex

In the cerebellar cortex the DRD₂ immunoreactivity was observed in neuronal cell bodies and processes distributed in all the three layers of the cerebellar cortex (Figs. 8A, 8B) and in the underlying WM (Fig. 11E).

Molecular layer

In the ML the DRD₂ immunoreactivity were detected in neuronal bodies and processes distributed throughout the layer (Fig. 8A). The DRD₂ immunoreactivity were observed in stellate neurons characterized by polygonal or spheroidal cell bodies distributed in the external zone of the layer (Figs. 9A, 9B), and in basket neurons with fusiform or roundish cell bodies localized in the internal zone of the layer (Figs. 8B, 9A). In stellate and basket neurons the DRD₂ immunoreactivity with a different degree of intensity, in form of densely packed granular deposits were observed in the perikaryon and in the cytoplasm of the proximal part of the processes (Figs. 8B, 9A, 9B). In addition, a strong DRD₂ immunoreactivity was also detected in the cytoplasm of the Purkinje neurons primary, secondary dendritic trunks and in their distal ramifications (Figs. 9A, 12A). In the neuropil of the layer a DRD₂ immunoreactivity in form of diffuse and fine puncta (referable to sectioned dendrites or axon processes, or axon terminals) were also observed (Figs. 9A, 9B, 12A).

Purkinje neuron layer

The DRD₂ immunoreactivity were detected in the cell bodies of numerous Purkinje neurons (Figs. 8B, 9A, 10, 11A, 12A). In the perikarya of the Purkinje neurons an intense and diffuse DRD₂ immunoreactivity in form of fine granular densely packed deposits was detected; the same DRD₂ immunoreactivity was also observed in the cytoplasm of the primary dendritic trunks which ascends into the ML (Figs. 9A, 10, 11A, 12A). In addition, immunonegative Purkinje neurons with a cell bodies profile surrounded by DRD₂ positive puncta were also observed (Fig. 12B).

Granular layer

In the GL the DRD₂ immunoreactivity was detected in cell bodies and processes of different neuron types, which include traditional neuron types of the layer, as the granules and the Golgi neuron (Figs. 10, 11A, 11B, 11E, 12A), and some non-traditional large neuron types of the GL (Figs. 10A, 11, 12A-D, 13A-C, 14A, 14B). The DRD₂ immunoreactive granules which present an intense and compact immunoreactivity, were detected in the external zone of the GL, beneath the cell bodies of the Purkinje neurons (Fig. 10) or in the internal zone of the layer at the boundary with the subjacent WM (Fig. 13E). The cell body of the Golgi neuron were localized in the external zone of the layer presented a strong and diffuse DRD₂ immunoreactivity in the perikaryon and in the axon-like process (Figs. 11A, 11B, 12A). The DRD₂ immunoreactivity were also detected in some non-traditional large neuron types, they were identified through accurate morphological analysis of their position in the zones of the GL, cell body shape and size, orientation, spatial arrangement of the processes [for details see: 41, 43, 44, 55-57]. The non-traditional large neuron types identified were the Lugano neuron (Figs. 12A, 13A), the candelabrum neuron (Fig. 13B), the triangular neuron (Fig. 14A), the ellipsoidal neuron (Fig. 13D), the globular neuron (Fig. 14B) and the perivascular neuron (Fig. 13C). In addition, within the GL were observed a DRD₂ immunoreactive neuron type characterized by spheroidal or ovoidal cell body, which displayed a main diameter ranging from 12 to 20 µm and presented a compact and heavy and DRD₂ immunoreactivity. The axon-like process originates from the cell body and is observable for part of its course (Figs. 11B, 11C). Fibers characterized by a DRD₂ moderate immunoreactivity variously oriented, throughout the layer, were observed (Fig. 11D). In addition, a DRD₂ immunoreactivity in form of small clusters and finely grains were localized within the space among granules in the protoplasmic spaces of Held, the sites in which are localized the synaptic glomeruli complexes (Figs. 8B, 12B). Here, were also detected DRD₂ immunoreactive clusters of puncta, corresponding to the axon terminals of mossy fibers at cerebellar glomeruli (Fig. 11D).

Subcortical white matter

In the underlying white matter, moderate DRD₂ immunoreactive nerve fibers were observed (Fig. 13E).

DRD₂ immunoreactive neuronal bodies and processes of the dentate nucleus

Numerous DRD₂ immunoreactive neuronal elements were observed within the DN and in the neighboring WM (Fig. 15); DRD₂ immunoreactive neurons on the basis of their morphological parameters were classified as small, medium and large neuron types that respectively displayed a main diameter ranging of 6 to 9 µm, 18 to 25 µm and 18-35 µm [For further details see: 50-54]. The DRD₂ immunoreactive small neuron types were scattered through the nucleus, they presented an intense immunoreactivity in form of densely packed granular deposits (Figs. 15, 16A). The DRD₂ immunoreactive medium neuron types were localized in the external and intermediate zones of the nucleus, their perikaryon presented a diffuse DRD₂ immunoreactivity in form of fine granules (Figs. 15, 16A, 16B).

Moreover, in the DN different DRD₂ immunoreactive large neurons types distinguished by distribution pattern, cell body shape and spatial organization of processes were observed (Figs. 16A, 16B). A large neuron type characterized by an ellipsoidal cell body was detected at the boundary of the DN (Figs. 15, 16A). A second large neuron type characterized by a large ellipsoidal cell body and a third large neuron type with an elongated fusiform cell body were both localized throughout within the DN (Figs. 15, 16A, 16B). Furthermore, in the neuropil of the DN and in the neighboring WM clusters of intensely DRD₂ immunoreactive puncta (referable to sectioned dendrites, axons, axon terminals, or nerve fibers) were observed (Figs. 15, 16A, 16B).

Discussion And Conclusion

The results of this study of light microscopic chemical neuroanatomy for DAT and DRD₂, provide the first morphological demonstration in the human cerebellum of an intrinsic neuronal dopaminergic system composed by neuronal cell bodies and processes of traditional neurons and non-traditional large neuron types distributed in the cerebellar cortex and in the DN.

The immunohistochemical detection, in the molecular layer of DAT and DRD₂ immunoreactive basket neurons and DRD₂ immunoreactive stellate neurons and the previously demonstration of all the dopamine receptor subtypes (DRD₁-DRD₅) in the neuropil and in the interneurons of the layer [58-62], allows us to suggest which DA may be involved in neurotransmission/neuromodulation mechanisms in the intrinsic circuits of the ML.

In addition, in the current study in the PN were observed DAT and DRD₂ immunoreactive cell bodies and processes of Purkinje neurons in the hemispheric lobules VII and IX, these findings are in accordance to immunohistochemical studies which reported in the cerebellar lobules VI-X an inconstant presence of dopaminergic Purkinje neurons [19-21, 26]. Furthermore, in the present study were also occasionally detected immunonegative Purkinje neurons, whose cell bodies profile were surrounded by DAT and DRD₂ immunoreactive ‘puncta’ (referable to axon terminals). Moreover, in previous studies were detected the presence of lobule IX of VMAT₂, TH and DAT immunoreactive axon terminals in close relationship to the cell bodies of the Purkinje neurons in the cerebellum of rodents and in non-human mammals primates cerebellum [19-21]. These last findings, were in some ways in accordance to the previous demonstrations

in the PN of extrinsic dopaminergic fibers, a poor number of dopaminergic cell bodies and processes of Purkinje neurons localized in several cerebellar lobules, and by the presence of all the dopamine receptor subtypes (DRD₁-DRD₅) mainly described in the layers of the cerebellar cortex [4, 5, 11, 19-21, 49, 60-62]. In addition, by means of a physiological and pharmacological combined approach has been demonstrated the presence of DAT immunoreactive Purkinje neurons and in which, DAT inhibitors, dopamine antagonists or DA exert a modulation of depolarization-induced slow currents (DISCs) [19]. Therefore, it is possible to hypothesize an involvement of the immunoreactive DAT and DRD₂ Purkinje neurons in dopaminergic signaling mechanisms in the cortico-nuclear projective circuits of the cerebellar cortex.

In the GL, were observed DRD₂ immunoreactive Golgi neurons and DAT and DRD₂ immunoreactive granules. Moreover, in the GL were also observed different DRD₂ immunoreactive non-traditional large neuron types [41, 44, 55], distributed in three zones of the GL; such as Lugano neurons, candelabrum neurons, perivascular neurons in the external zone, triangular neurons in the intermediate zone; ellipsoidal neurons and globular neurons in the internal zone [41, 43, 44, 55-57]. In the GL, were also detected the presence of DAT immunoreactive synarmotic neurons, the non-traditional large neuron type characteristically localized in the internal zone of the layer or in the subjacent matter of the folium, which could be involved in corticocerebellar and/or corticonuclear projective circuits [41, 43, 44, 55-56].

In addition, in the GL an intensely DRD₂ positivity were also observed in the cell body and processes of a neuron type has intermediate dimensions between the granules and the non-traditional large neuron types [41, 44, 55, 56, 64]. Although, this neuron type which we called 'big granule', was similar to the monodendritic neuron [65] and to the unipolar brush neuron [66, 67], it was mainly observed throughout within the GL of the cerebrocerebellum lobules, instead, studies on the distribution of the glutamatergic unipolar brush neuron demonstrated an it exclusively distribution in the vestibulocerebellum [67, 68]. Though, this initial morphological datum on the big granule constitutes an element of novelty, it needs further future morphofunctional insights.

Furthermore, in the DN has been found a widely DAT and DRD₂ imunoreactivity in cell bodies and processes of different neuron types. In many cases, it was possible to recognize them, through the evaluation of morphological parameters, as the small neuron type involved in intrinsic circuits, the medium neuron type mainly involved in extrinsic circuits of the DN [49, 50, 69-72], and at least 4 different large neuron types that include the central neuron, the border neuron, the intermediate asymmetrical neuron and the intermediate fusiform neuron, involved in projective circuits of the DN [47, 48, 52-54, 69, 72]. The findings of dopaminergic Purkinje neurons and synarmotic neurons in the cerebellar cortex and of dopaminergic projective neuron types in the DN, may suggest a relevant role of DA in cortico-nuclear interconnections in the human cerebellum.

In addition, the presence of DAT and DRD₂ immunoreactive fine puncta in the neuropil of the ML and in the space of Held of the GL, the sites of the cerebellar glomeruli complex and in the neuropil of the DN suggested a role of DA to the cerebellar mechanisms of synaptic plasticity [73, 74]. may be through the

dopamine and cAMP-regulated neuronal phosphoprotein (DARPP-32), widely expressed in the neurons of the cerebellum [75-77], that play a role in dopaminergic neuronal synaptic signaling [78].

In addition, studies attributed to the dorsal portion of DN a motor role and to the ventral portion an involvement in non-motor functions [79-82]. The findings of dopaminergic projective neuron types in the both portions of the DN, may suggest the existence of dopaminergic interconnections between the DN and the midbrain dopaminergic nuclei chiefly to the substantia nigra (A_9) and to the ventral tegmental area (A_{10}) and may also indicate the existence of direct and indirect dopaminergic interconnections to other basal ganglia nuclei, mainly to the subthalamic nucleus and the globus pallidus [83-87].

Moreover, the dopaminergic projective neuron types of the DN, may be involved in direct or indirect pathways to the regions of the limbic system such as the hypothalamus, amygdala and hippocampus [83, 85-88]. Furthermore, a recent study in mice suggested direct interconnections between the DN and the nucleus accumbens [89], this last data may evidenced a role of the dopaminergic projective neuron types of the DN in the modulation mechanisms of DA release in the mesolimbic system.

Moreover, the presence of dopaminergic perivascular neurons in the GL of the cerebellar cortex and in the DN, and presence of dopaminergic putative perivascular axon terminals, in close relationship to the wall of microvessels in the cerebellar cortex and in the DN of could represent the morphofunctional demonstration in the cerebellum of specific dopaminergic neuronal elements involved in regulatory mechanisms of the blood flow and of the blood brain barrier [90-94]. Furthermore, we can also suggest which dopaminergic cerebellar perivascular neurons may be involved in the amplification of the extra-synaptic dopaminergic signaling (volume transmission) [95-97].

In addition, the detection of cerebellar dopaminergic neurons suggest which DA may be act as a neurocotransmitter or neuromodulator with GABA or glutamate in traditional and non-traditional inhibitory and excitatory interneurons of the cerebellar cortex and of the DN [31, 41, 98-105] and with GABA/glycine or glutamate in traditional and non-traditional projective neuron types of the cerebellar cortex and of the DN [41, 106-112]. On this regard, previously, in other areas of the central nervous system has been demonstrated a cotransmitter role of DA with GABA or glutamate [113-117].

In conclusion, the finding of an intrinsic neuronal cerebellar dopaminergic system may provide the morphological basis of considerable interest for its applications in clinical neurology and psychiatry. In fact, we cannot exclude an involvement of the cerebellar dopaminergic neurons demonstrate in this study in DA related neurologic and psychiatric disorders such as PD, SCZ ASD, and DGA. Recently, studies demonstrate

a role of the cerebellum in the pathophysiology of motor and non-motor symptoms of PD [118-121], and as a target for the non-pharmacological treatment of the side effects caused by traditional dopaminergic therapies [122, 123]. Furthermore, studies suggest in SCZ and in ASD a role of the cerebellum, and in particular of the DN in the modulation of prefrontal circuitry involved in the dopaminergic release in the

prefrontal cortex and in cognitive abnormalities [126-128] and suggest a role of the cerebellum in innovative therapeutic non-invasive approaches for SCZ [129] and for ASD [130].

Abbreviations

Dopamine: DA; Homovanillic Acid: HVA; Tyrosine Hydroxylase: TH; Dopamine Transporter: DAT; Positron Emission Tomography: PET; Dopamine Transporters Ligands: DAT-Ls; Parkinson's Disease: PD; Ataxias: ATX; Schizophrenia: SCZ; Autism Spectrum Disorders: ASD; Bipolar Disorders: BD; Drug Addiction: DGA; Dopamine receptor type 2: DRD₂; Room Temperature: RT; Buffer Solution: BF; 3,3-diaminobenzidine tetrahydrochloride: DAB; Molecular Layer: ML; Purkinje Neuron Layer: PN; Granular Layer: GL; White Matter :WM; Monodendritic neuron: MN; Unipolar Brush Neuron: UBN.

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Authors' contributions

FP designed the study, performed the experiments and the analysis of the experimental data and participated in the writing of the manuscript. LP shared the study project and participated in the writing of the manuscript. GD shared the study project and participated in the writing of the manuscript. BA participated in the writing of the manuscript. BGA participated in the writing of the manuscript. BJJV participated in the writing of the manuscript. GM participated in the analysis of the experimental data and in the writing of the manuscript. CA participated in the writing of the manuscript. GG participated in the writing of the manuscript. MD participated in the writing of the manuscript. AG shared the study project and participated in the writing of the manuscript. BA shared the study project and participated in the writing of the manuscript.

Consent for publication

All authors provided critical feedback on the analysis and manuscript. The author(s) read and approved the final version of the manuscript.

Compliance with ethical standards

Competing interests

The authors declare that they have not conflict of interests

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Figures

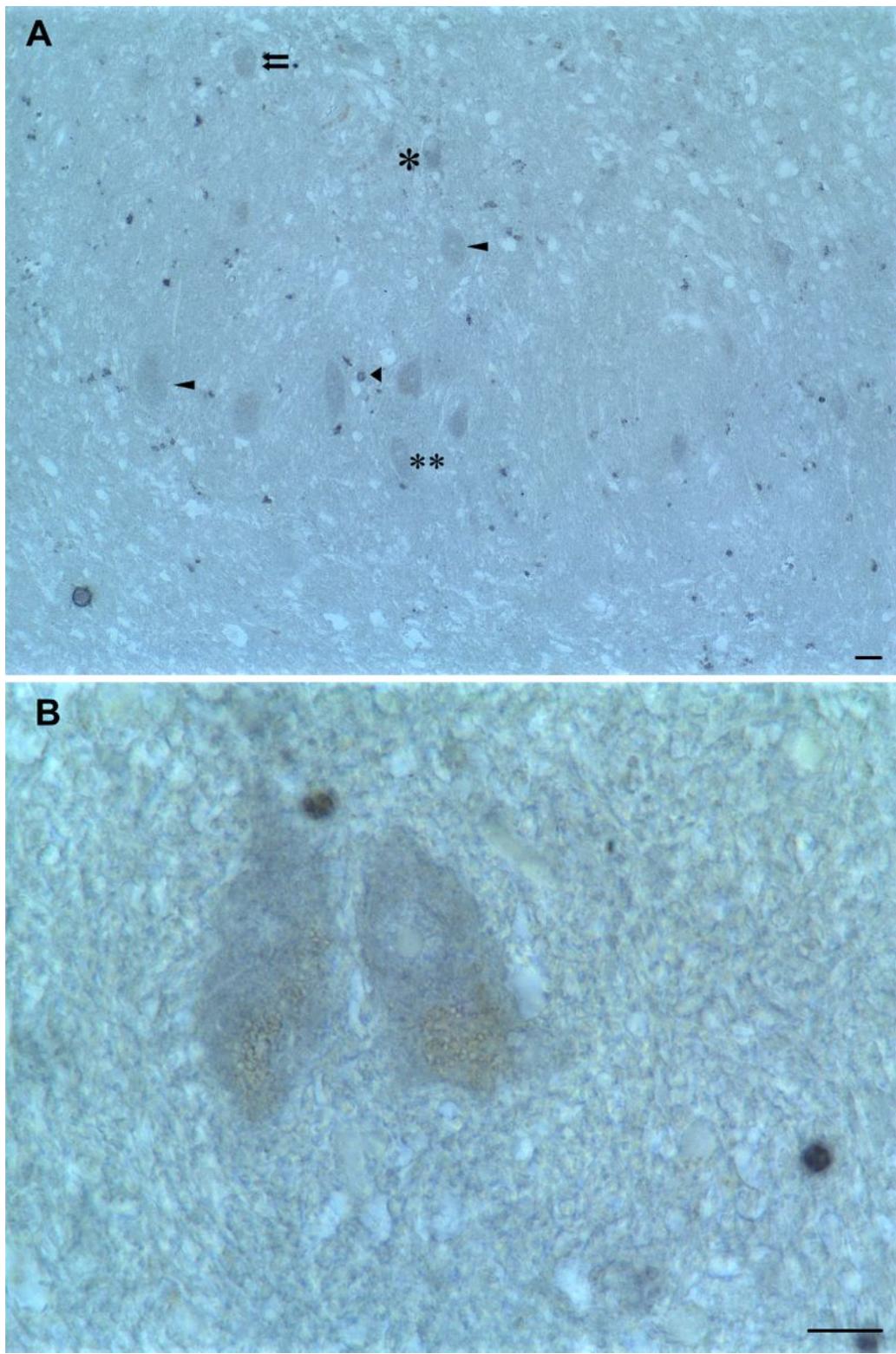


Figure 1

Dopamine transporter (DAT) immunoreactivity in the cerebellar cortex. The immunoreactivity is detectable in neuronal bodies and processes in the molecular layer (ML), in the Purkinje neuron layer (PL), in the granular layer (GL), in the white matter (WM). (Scale bar: 1A: 20 μ m).

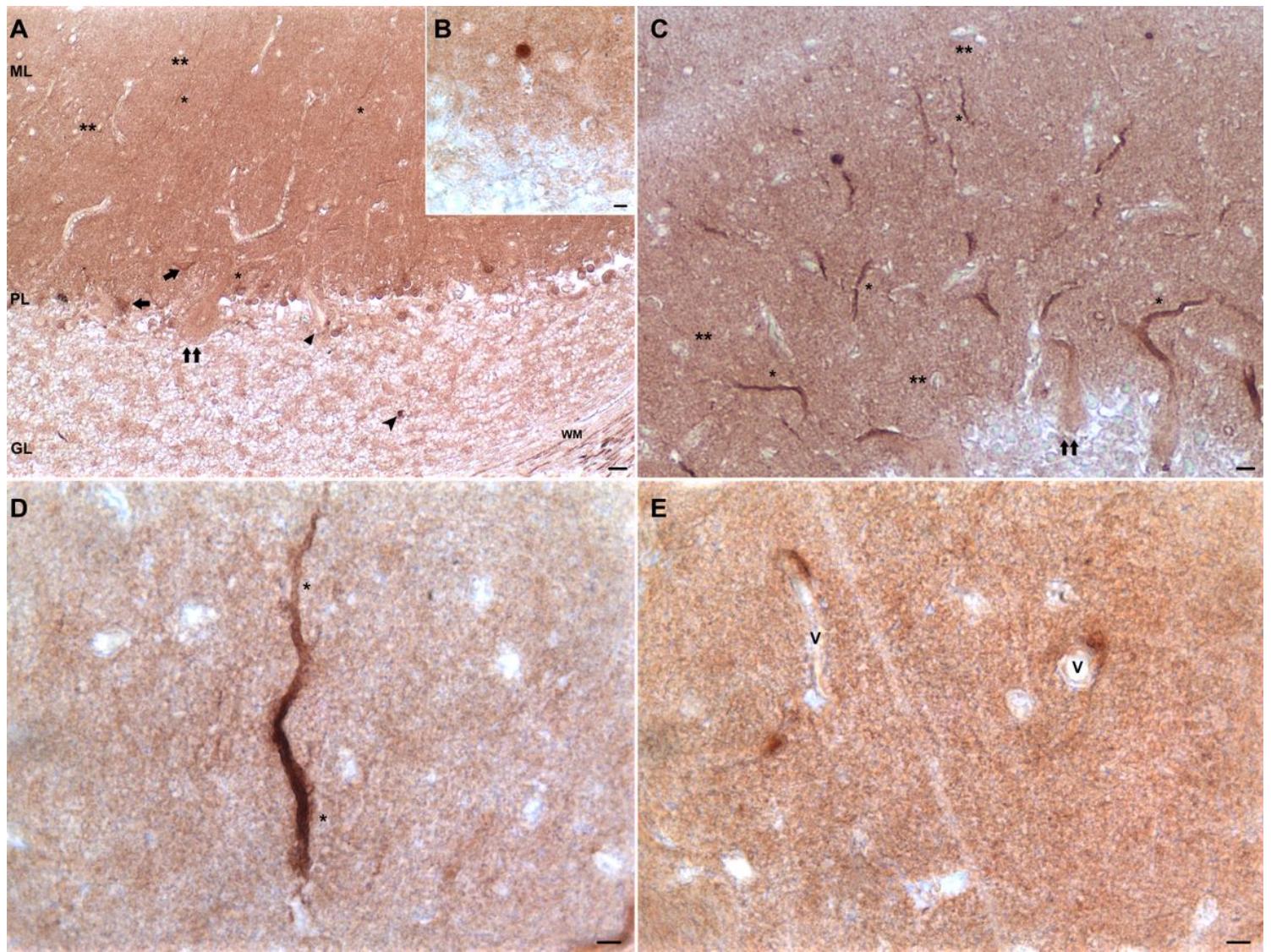


Figure 2

(A) Dopamine transporter (DAT) immunoreactivity in the molecular layer (ML), in the Purkinje neuron layer (PL), in the granular layer (GL) and in the subjacent white matter (WM). In the ML, DAT immunoreactivity in basket neurons (arrows); primary and secondary trunks dendritic and apical dendrites of Purkinje neurons (small single asterisk); negative stellate neurons (double asterisk). In the PL, DAT immunoreactive Purkinje neuron cell body (double arrows) and DAT immunoreactive primary trunk and apical dendritic (small asterisk), DAT immunonegative Purkinje neuron (big arrowheads); in the GL, immunoreactive granule cell body (small arrowheads); in the subjacent WM DAT immunoreactive fibers. (B) DAT immunoreactive basket neuron cell body; (C) ML, DAT immunoreactive secondary dendritic trunks and apical dendrites of Purkinje neurons (small asterisk); ML, DAT immunonegative stellate and basket neurons cell bodies (double asterisk). (D) ML, DAT immunoreactive dendritic trunk of Purkinje neuron; (E) ML, DAT immunoreactive axon terminals in close relationship to the wall of microvessels. (Scale bar: 2A: 20µm; 2B: 10µm; 2C: 20µm; 2D: 10µm; 2E: 10µm).

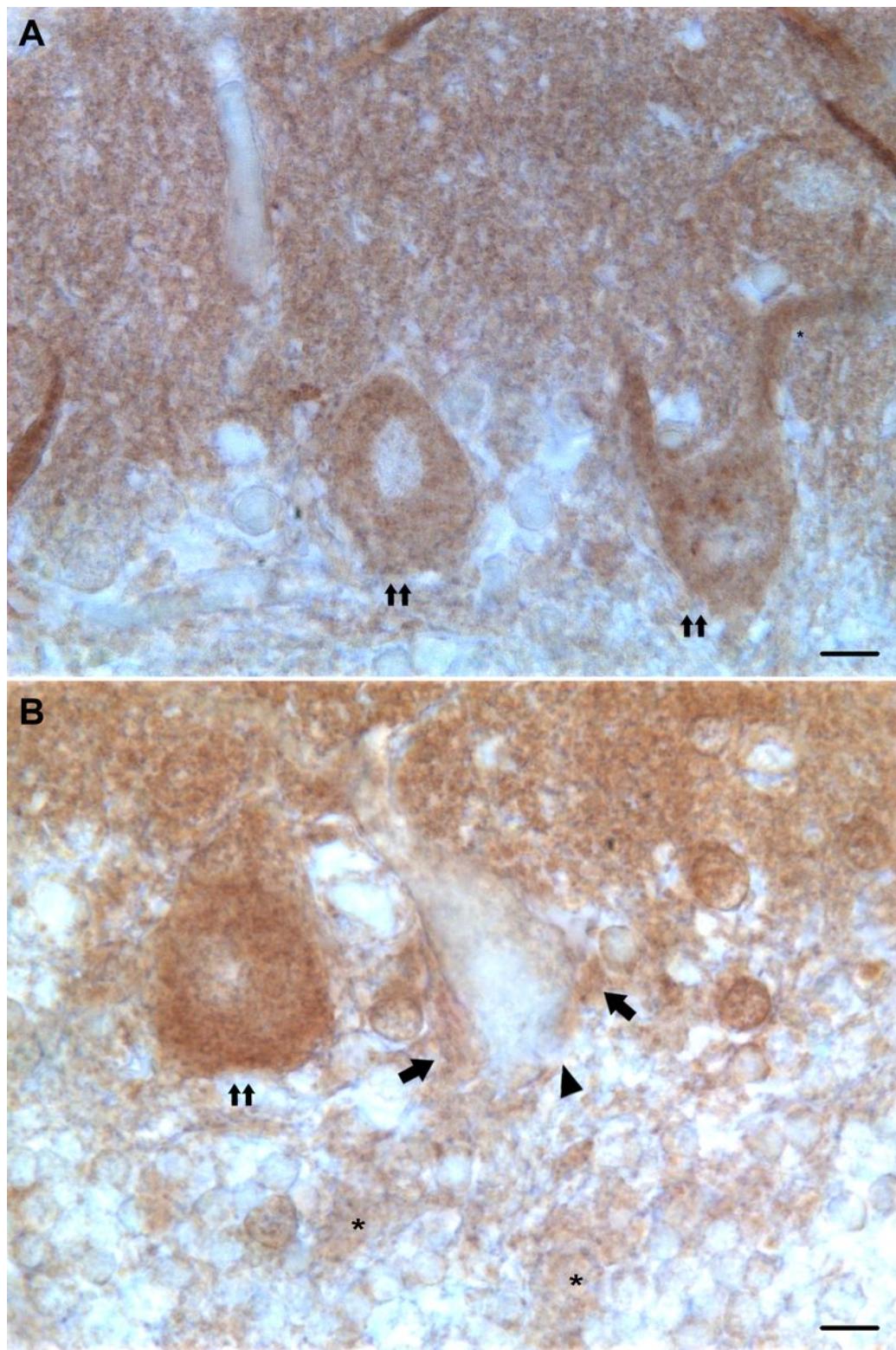


Figure 3

(A) PL, Dopamine transporter (DAT) immunoreactivity in the Purkinje neuron cell bodies (double arrows).
(B) PL, Dopamine transporter (DAT) immunoreactivity in Purkinje neuron cell body (double arrows), DAT immunonegative Purkinje neuron cell body (big arrowheads), DAT immunoreactivity in close relationship to the cell body of immunonegative Purkinje neuron (big arrows), DAT immunoreactivity in space of Held (big single asterisk) (Scale bar: 3A 10µm; 3B 10µm).

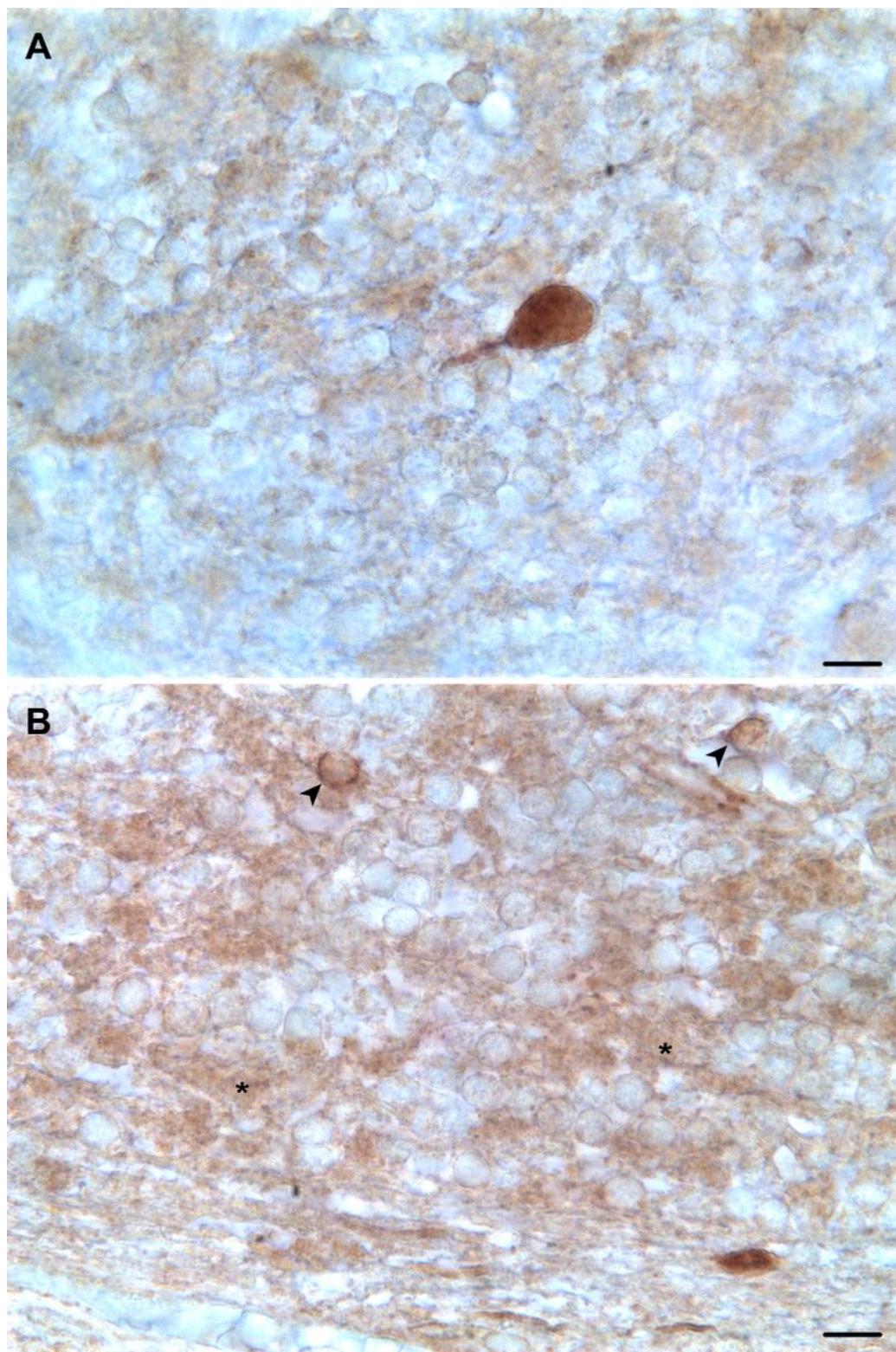


Figure 4

(A) GL, Dopamine transporter DAT immunoreactive perikarya and axon-like processes of the synarmotocneuron (B) DAT immunoreactive granules cell bodies (small arrowheads); clusters of DAT immunoreactivity in space of Held (big asterisk), DAT immunoreactive nerve fibers in the white matter; (Scale bar: 4A 60 μ m; 4B 10 μ m).

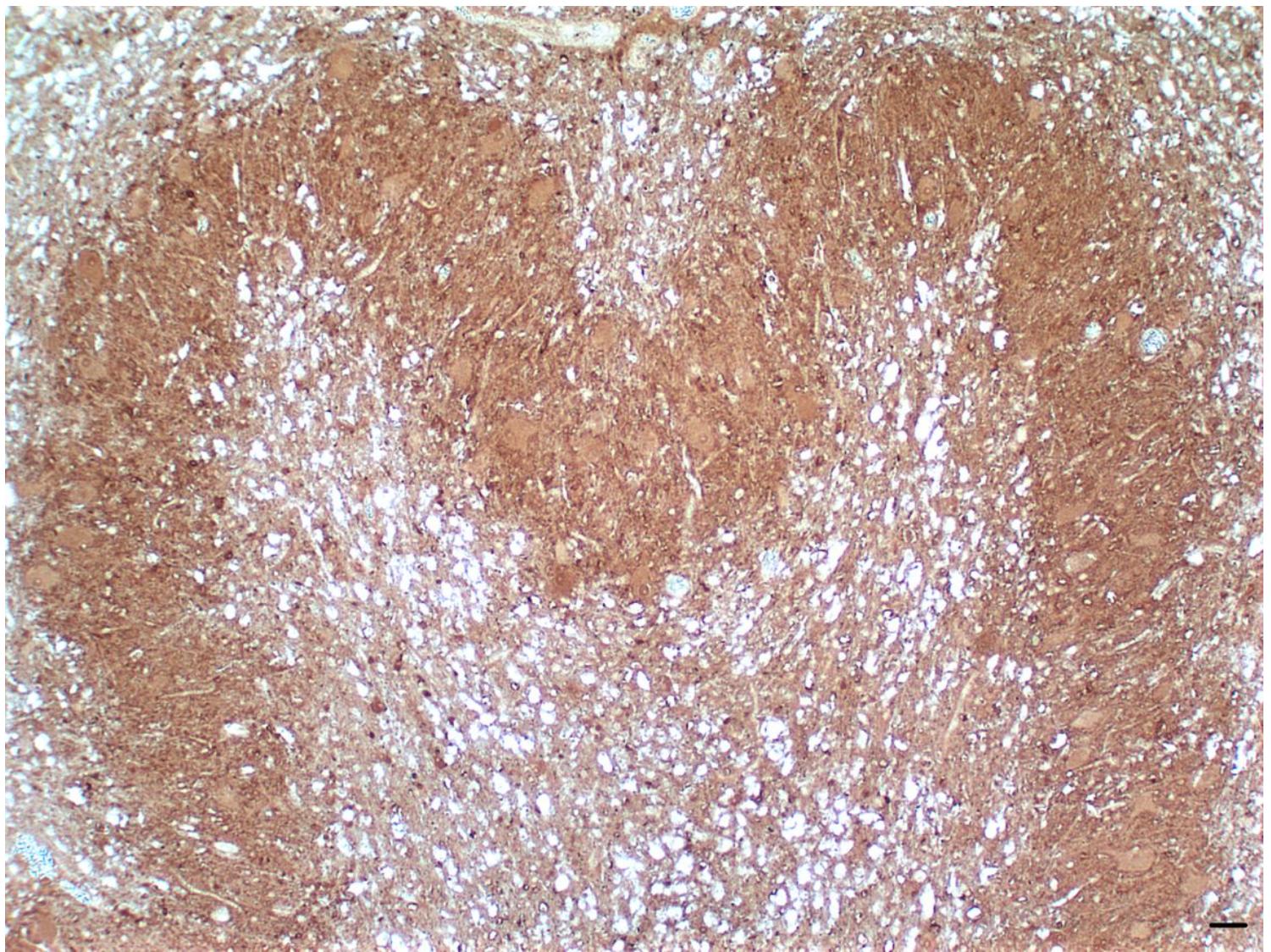


Figure 5

Distribution pattern of the dopamine transporter (DAT) immunoreactivity in the dentate nucleus gray matter and in the neighboring white matter. (Scale bar: 40 μ m).

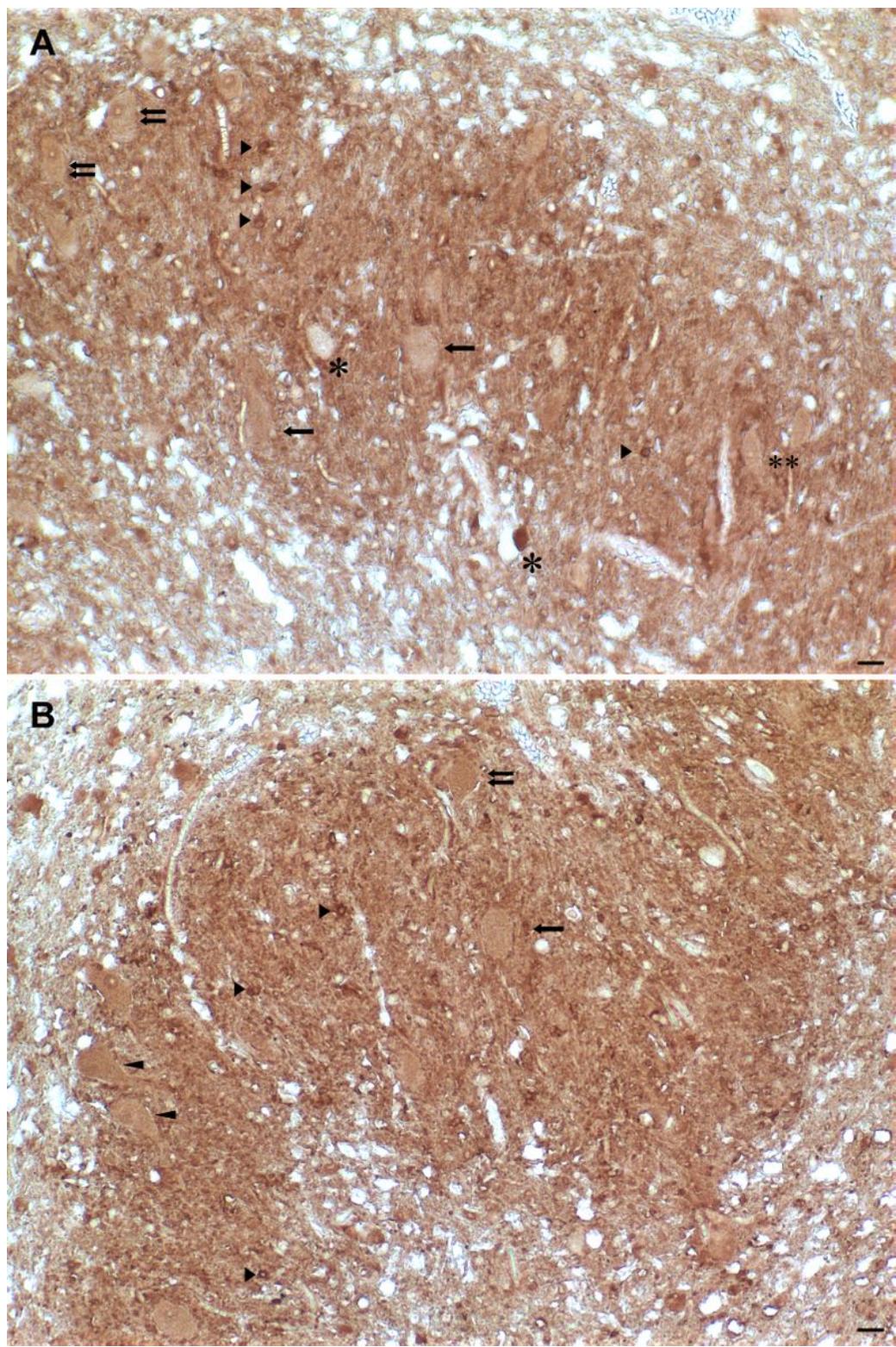


Figure 6

(A) DAT immunoreactive small neuron cell bodies (small triangular arrowheads); DAT immunoreactive and DAT immunonegative medium neuron cell bodies (medium asterisk); DAT immunoreactive border neuron cell bodies (small double arrows); DAT positive central neuron cell bodies (single small arrows); DAT immunoreactive intermediate fusiform neuron cell bodies (double asterisk); (B) DAT immunoreactive small neuron cell bodies (small triangular arrowheads), DAT immunoreactive intermediate asymmetrical

neurons (big triangular arrowheads), DAT immunoreactive central neuron cell body (single small arrows), DAT immunoreactive border neuron cell bodies (small double arrows), (Scale bar: 6A 20 μ m; 6B 20 μ m).

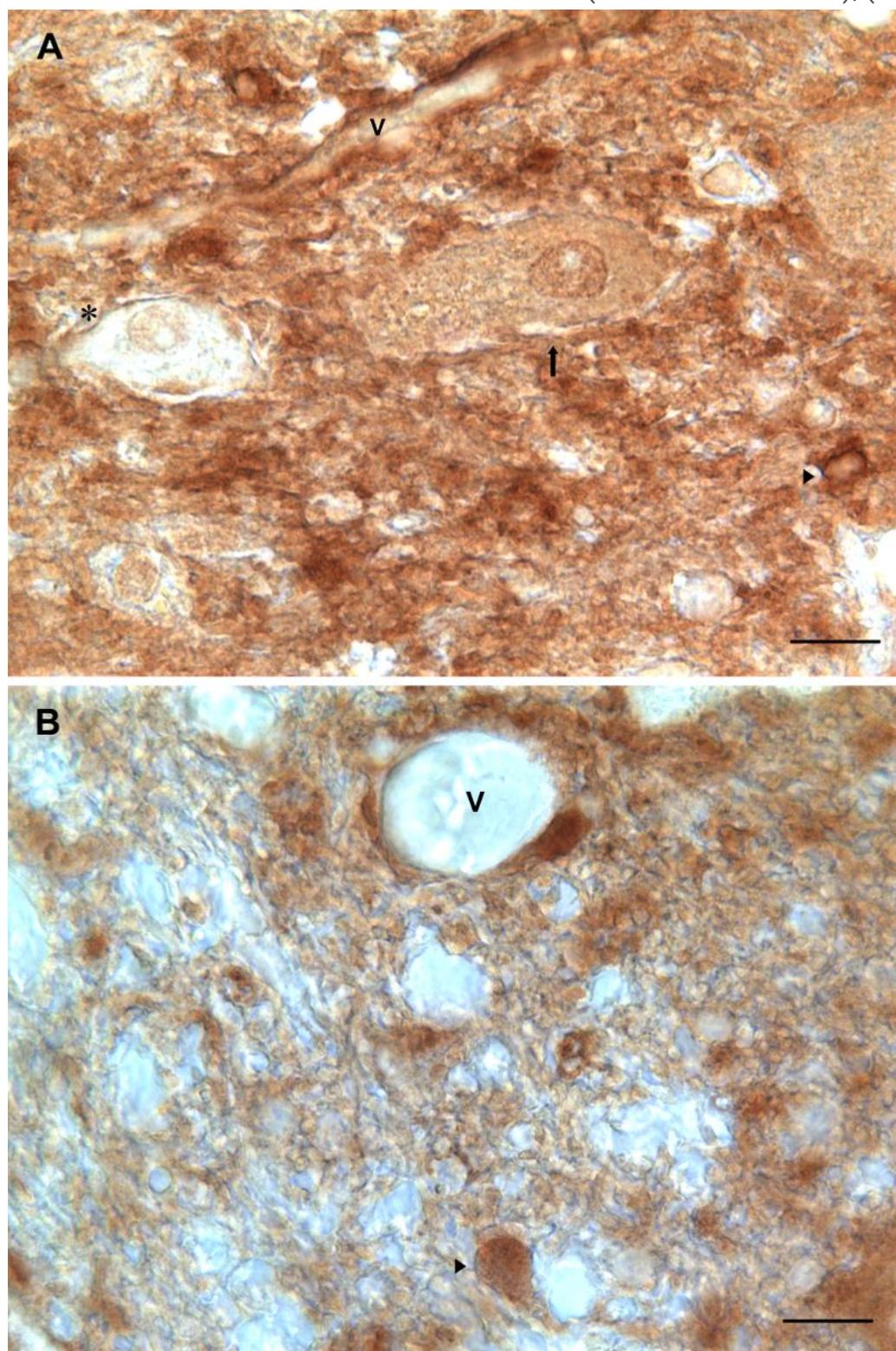


Figure 7

(7A) DAT immunoreactive small neuron cell body (small triangular arrowheads), small clusters of DAT immunoreactivity in close proximity of the wall of a microvessel (V), DAT immunoreactive cell body of a perivascular neuron type, DAT immunoreactive central neuron cell bodies (single small arrows). (7B) DAT

immunoreactive perivascular neuron cell body and processes in close relationship with a microvessel (V), DAT immunoreactive small neuron cell body (small triangular arrowheads), DAT immunoreactive small neuron cell body (small triangular arrowheads), (Scale bar: 7A 40 μ m; 7B 40 μ m).

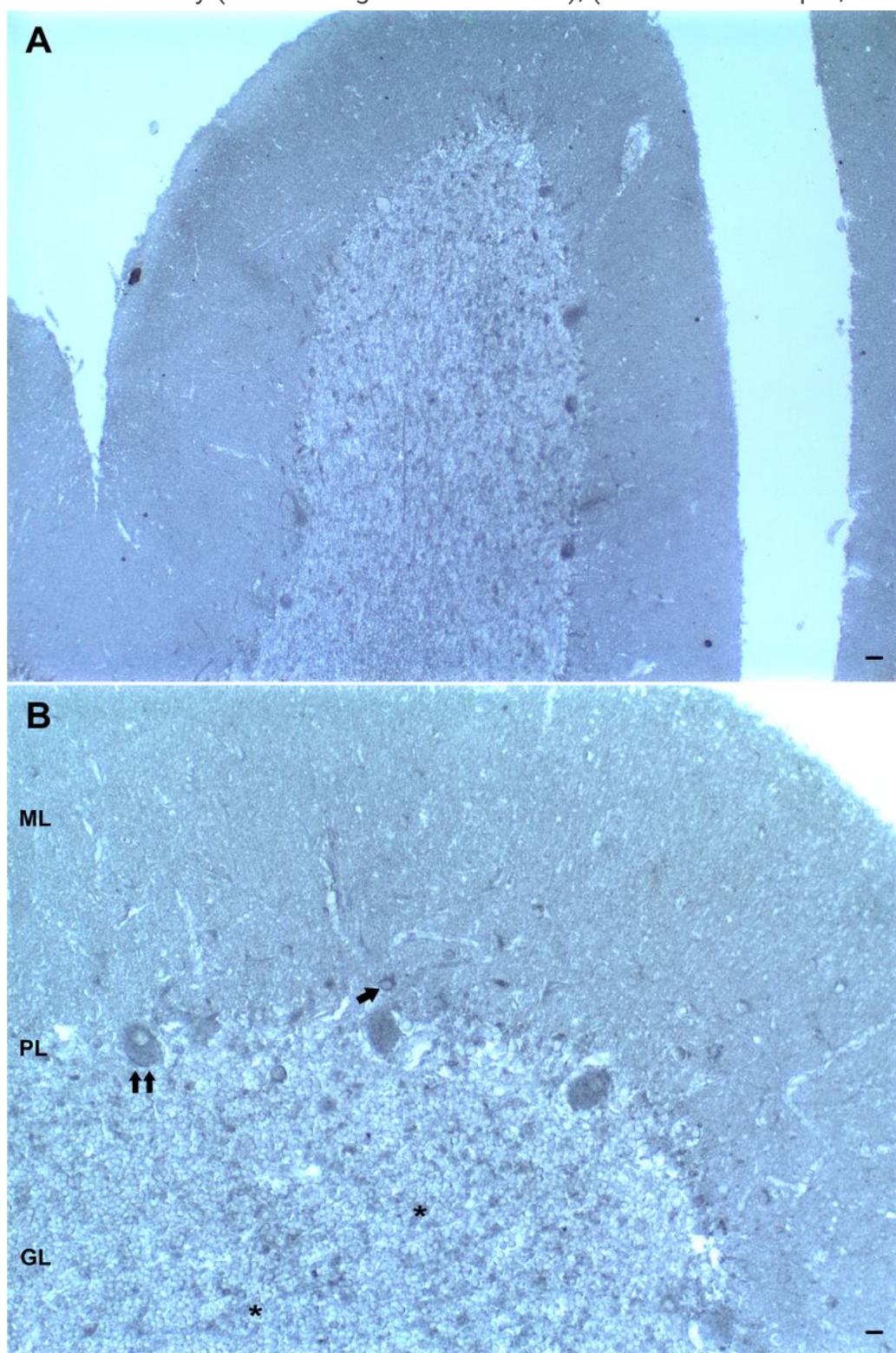


Figure 8

(8A) Distribution pattern of dopamine receptor type 2 (DRD2) immunoreactivity in the cerebellar cortex.
(8B) The DRD2 immunoreactivity is observable in neuronal bodies and processes in the layers of the

cerebellar cortex. In the ML, DRD2 immunoreactive basket neuron cell body (arrows), in the PL, DRD2 immunoreactive Purkinje neuron cell body (double arrows), in GL, DRD2 immunoreactive clusters in the space of Held (big asterisk), (Scale bar: 8A 40 μ m; 8B 20 μ m).

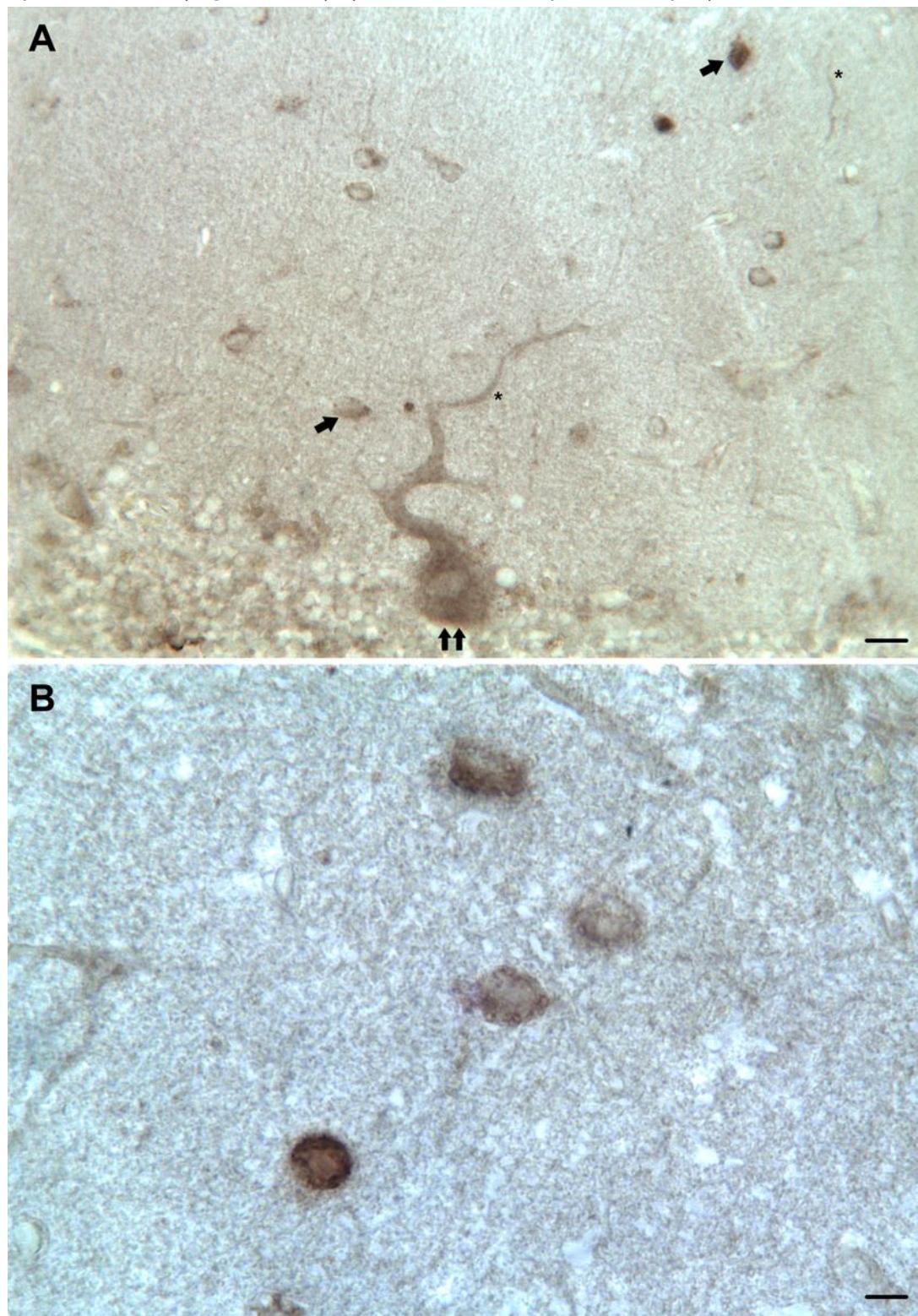


Figure 9

(9A) In the ML, DRD2 immunoreactive basket and stellate neuron cell bodies (arrows), DRD2 immunoreactive primary, secondary and apical dendrites of Purkinje neurons (small asterisk), DRD2

immunoreactive Purkinje neuron cell body (double arrows); (9B) cluster of DRD2 positive stellate cell bodies. (Scale bar: 9A: 20 μ m; 9B: 5 μ m).

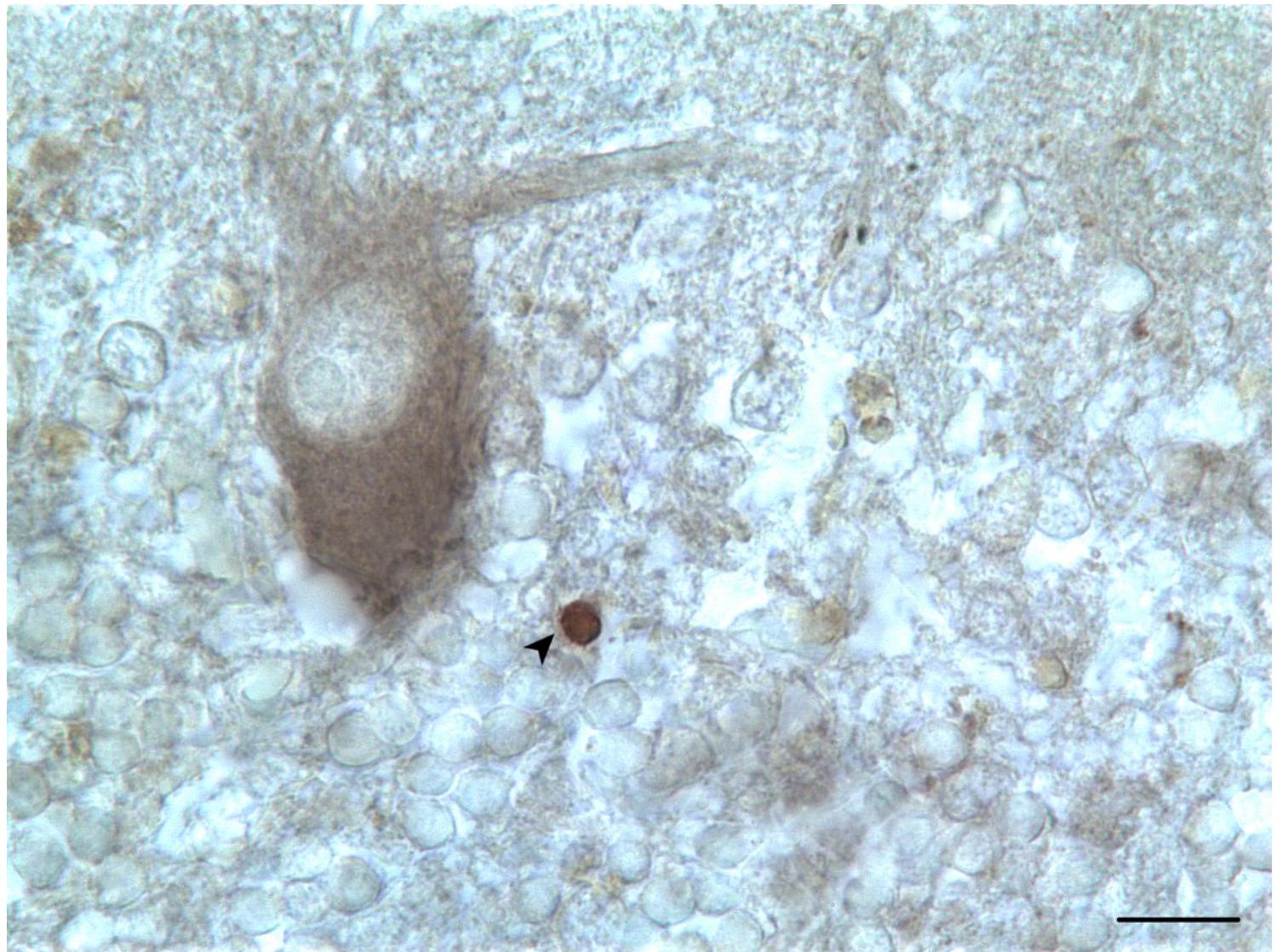


Figure 10

DRD2 immunoreactive Purkinje neuron cell body, DRD2 granule positive cell body (Scale bar: 40 μ m)

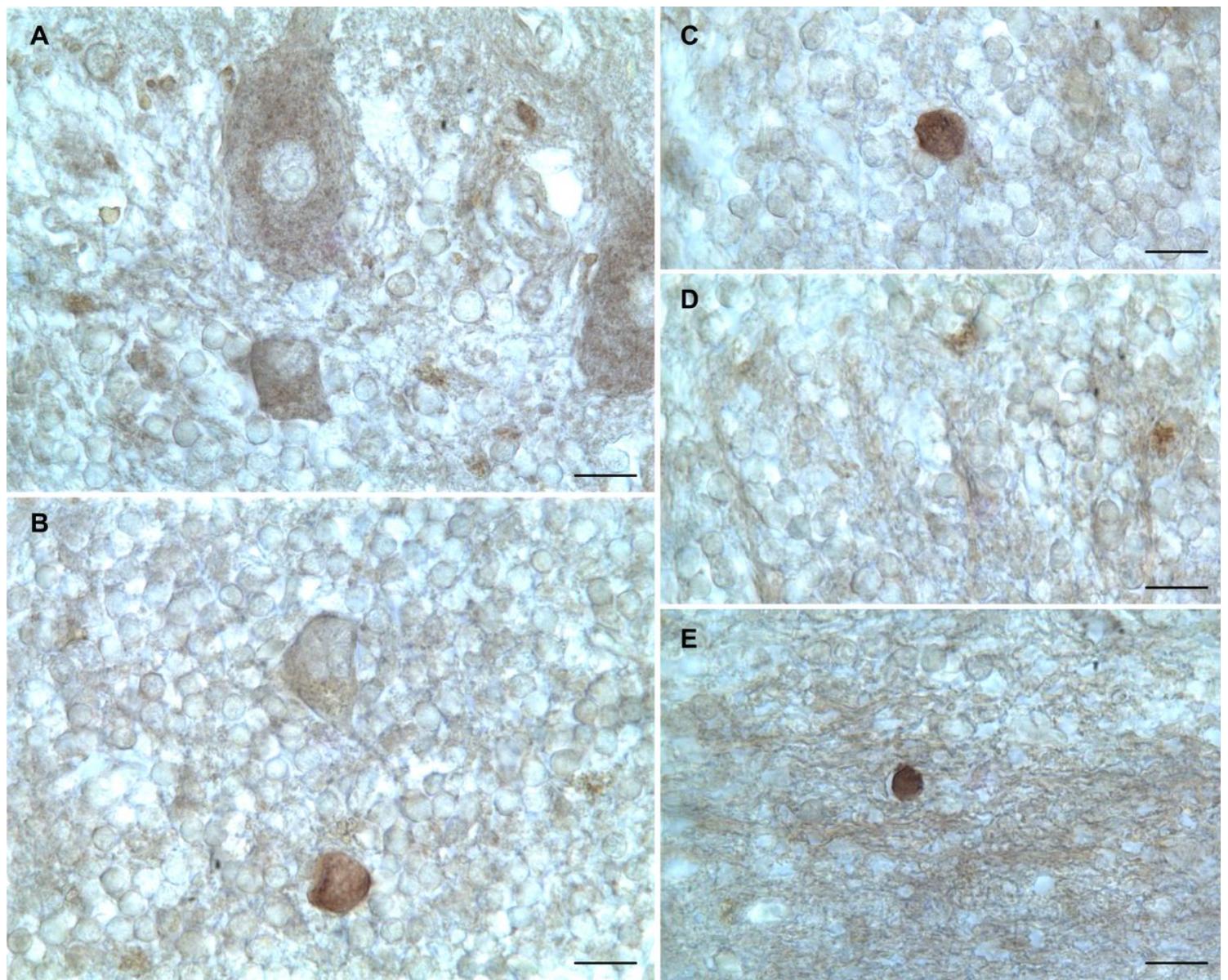


Figure 11

(13A) DRD2 immunoreactive Purkinje neuron and Golgi neuron cell bodies; (13B) DRD2 immunoreactive Golgi neuron cell body and axon-like process, DRD2 positive big granule cell body; (13C) DRD2 immunoreactive big granule cell body; (13D) DRD2 positive mossy-like fibers; (13E) DRD2 immunoreactive granule cell body and DRD2 immunoreactive fibers in the white matter. (Scale bar: 13A 20 μ m; 13B 20 μ m; 13C 20 μ m; 13D 20 μ m; 13E 20 μ m).

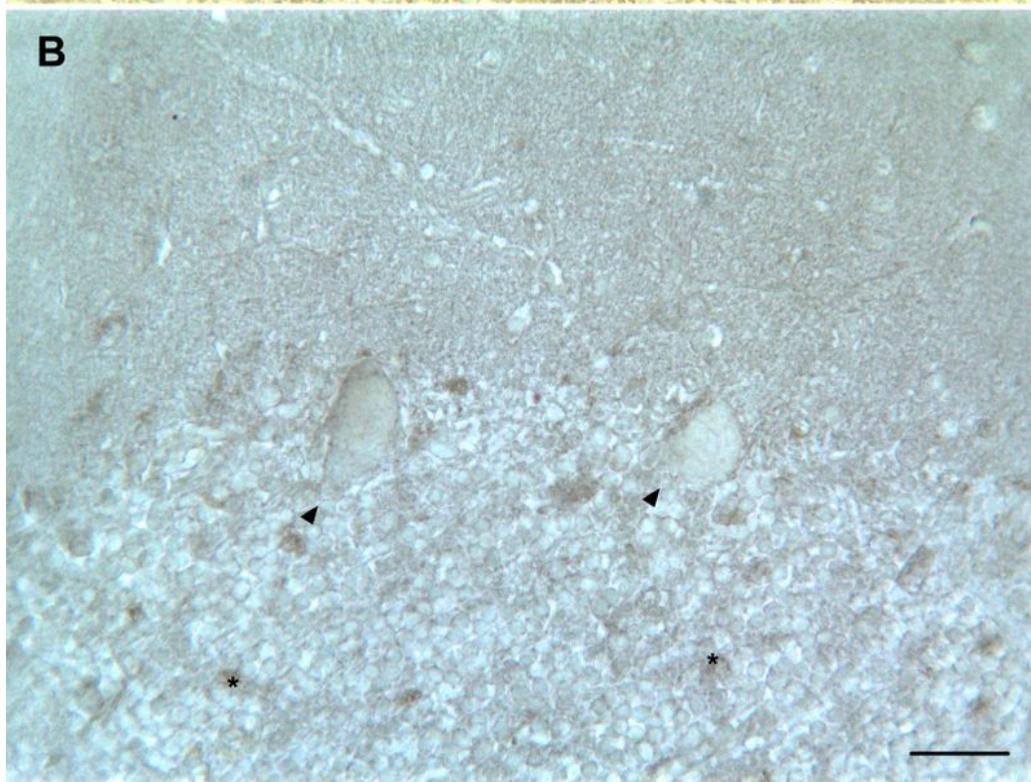
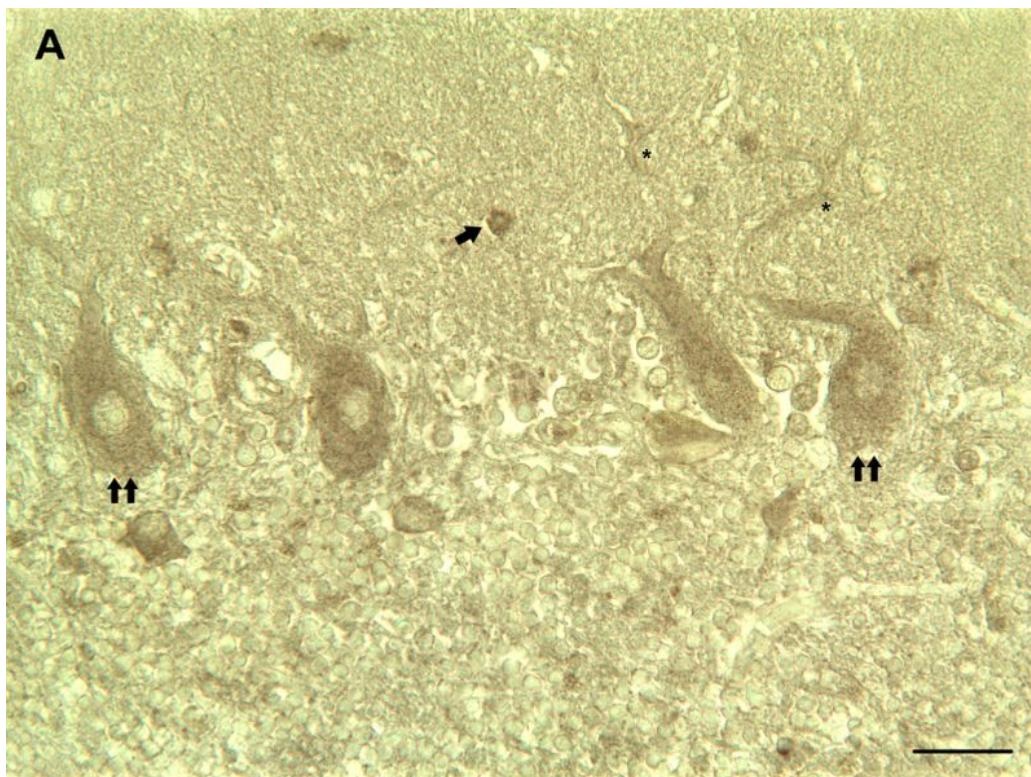


Figure 12

(12A) In the PL, group of DRD2 immunoreactive cell bodies of Purkinje neurons (double arrows), in the GL, immediately below the DRD2 immunoreactive cell bodies of the Purkinje neurons, from left to right DRD2 immunoreactive cell bodies of Golgi neuron, candelabrum neuron, Lugaro neuron; (12B) in the PL, group of DRD2 immunonegative cell bodies of Purkinje neurons (triangular arrowheads), DRD2 immunoreactive clusters in the space of Held (big asterisk). (Scale bar: 10A 40µm; 10B 40µm)

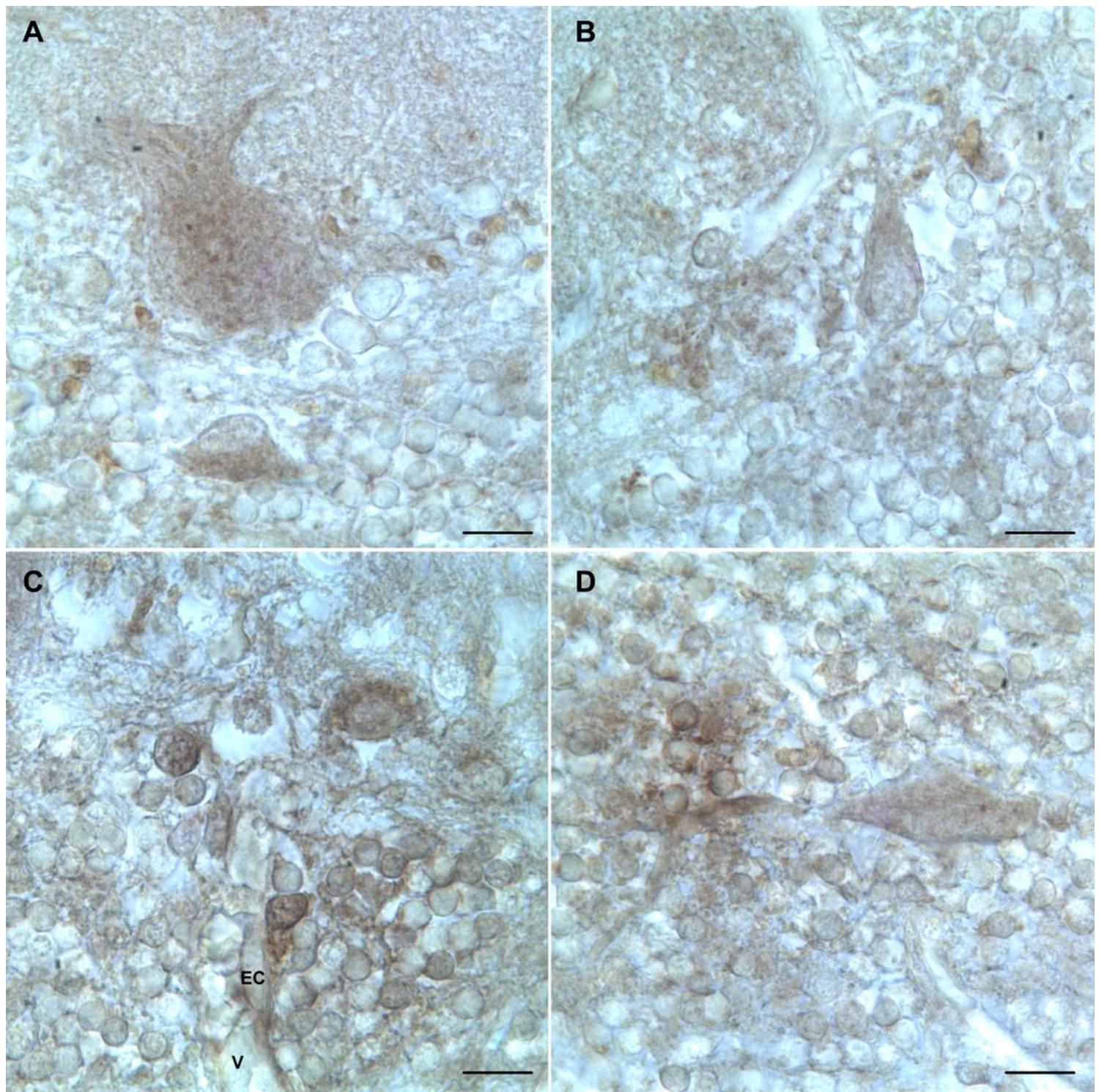


Figure 13

(13A) DRD2 immunoreactive Purkinje neuron and Lugano neuron cell bodies, (13B) DRD2 immunoreactive candelabrum neuron cell body, (13C) DRD2 positive perivascular neuron cell body, endothelial cell (EC), microvessel (V) (13D) DRD2 immunoreactive ellipsoidal cell body. (Scale bar: 12A 20 μ m; 12B 20 μ m; 12 C 20 μ m; 12D 20 μ m).

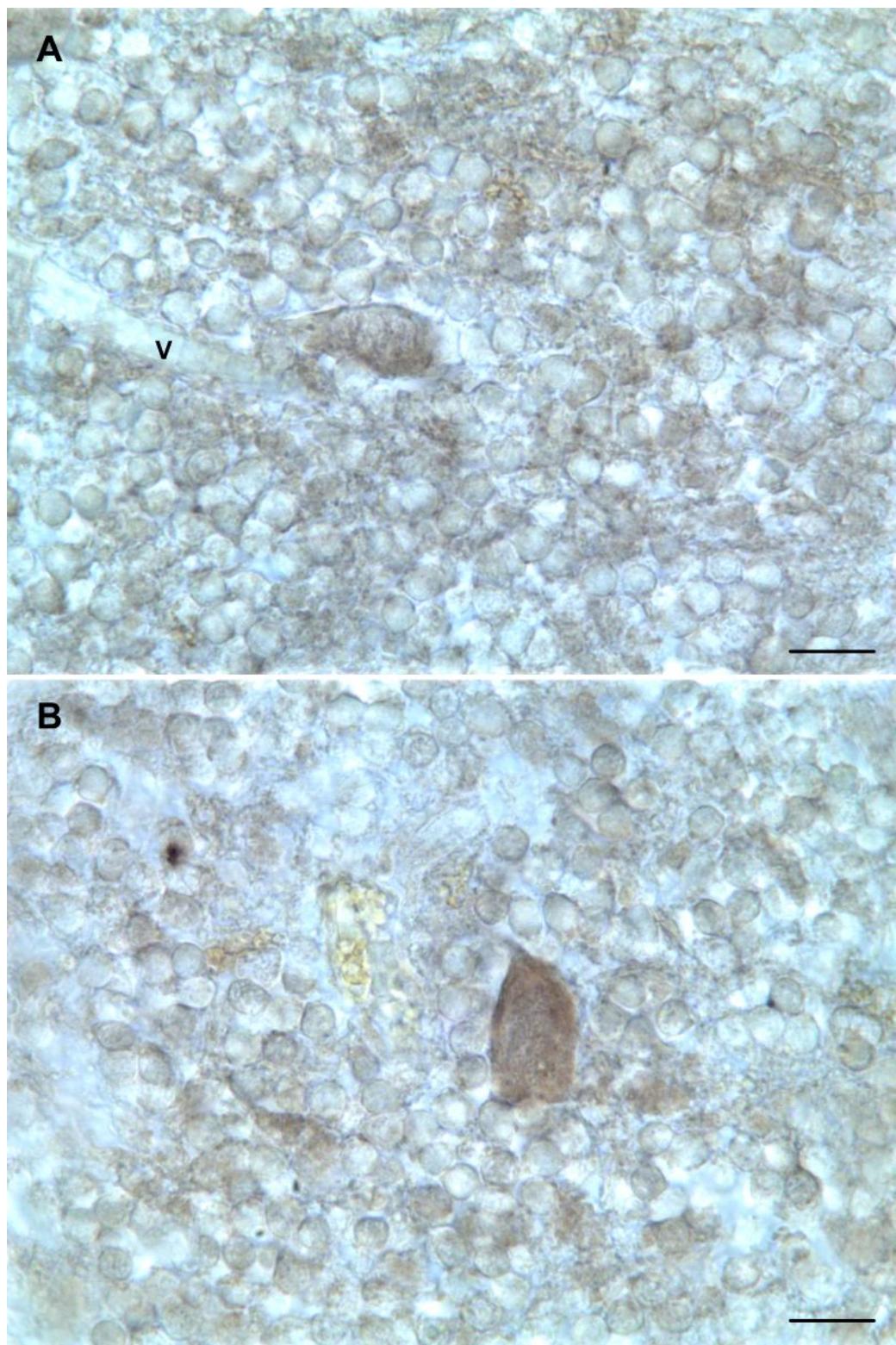


Figure 14

(14A) DRD2 immunoreactive triangular neuron cell body, (14B) DRD2 immunoreactive globular neuron cell body. (Scale bar: 14A 25 μ m; 14B 25 μ m).

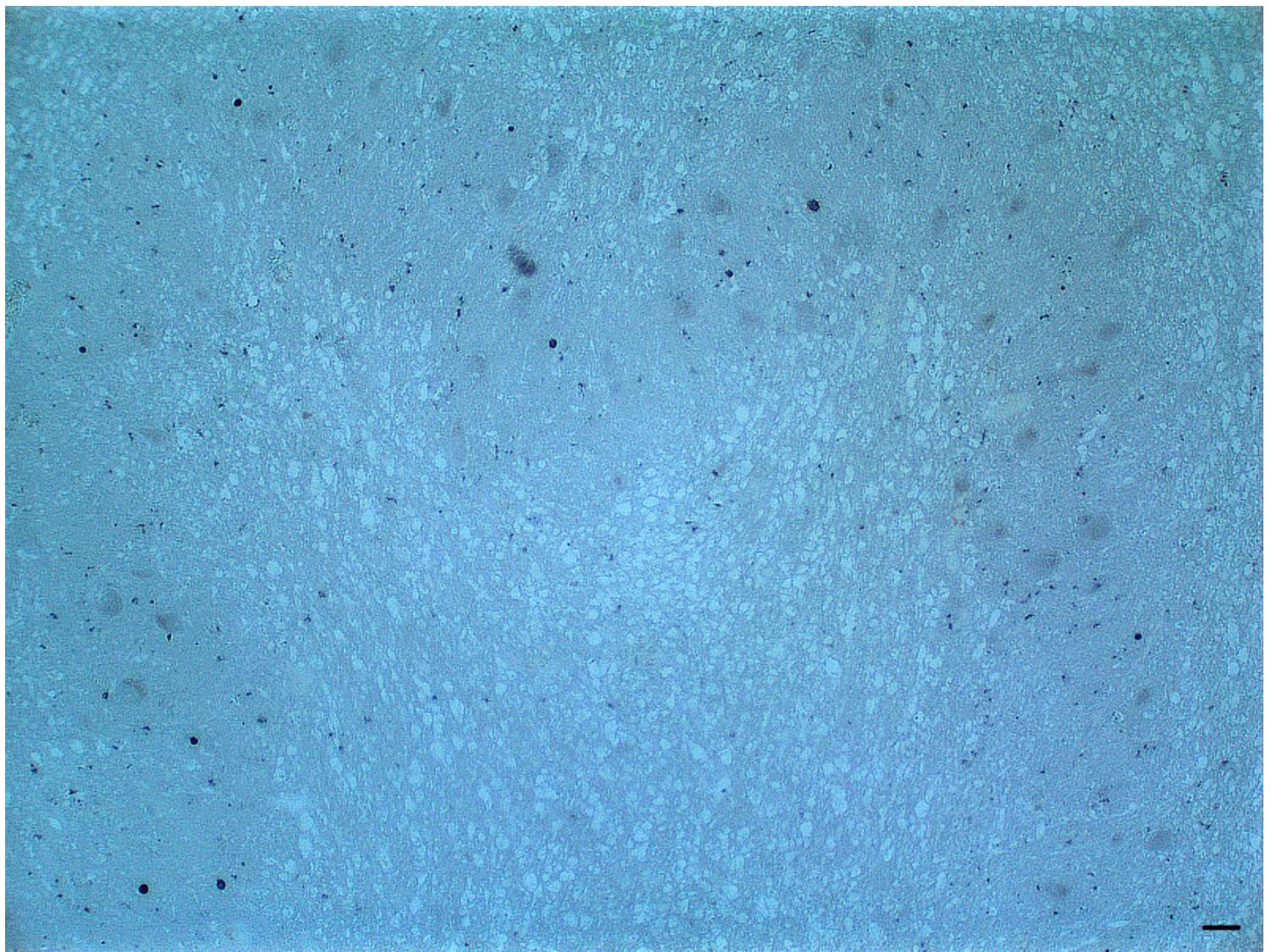


Figure 15

Distribution pattern of the dopamine receptor type 2 (DRD2) immunoreactivity in the dentate nucleus gray matter and in the neighboring white matter. (Scale bar: 40 μ m).

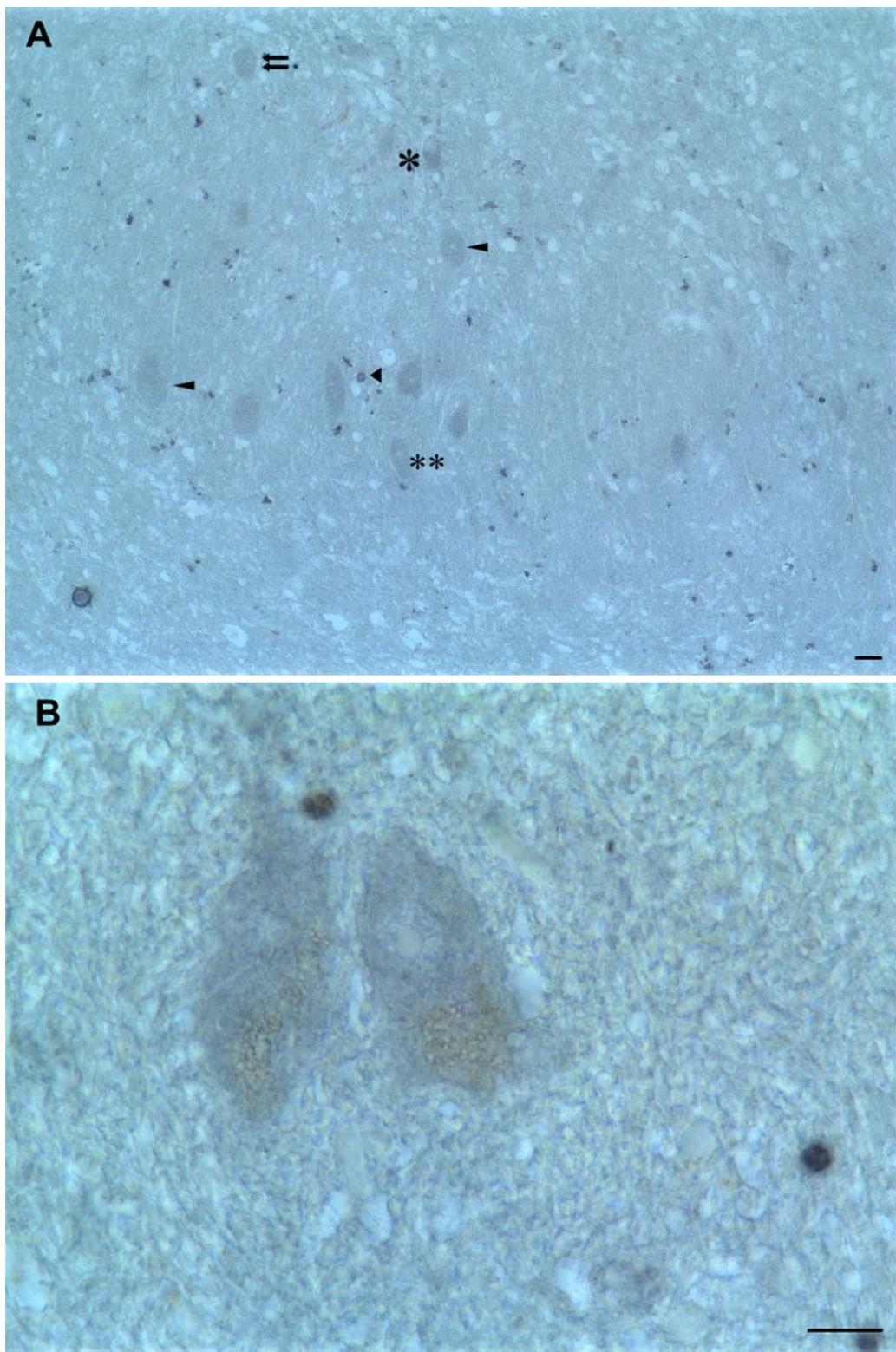


Figure 16

(16A) DRD2 immunoreactive small neuron cell body (small triangular arrowheads), DRD2 positive border neuron cell body (small double arrows), DRD2 positive intermediate asymmetrical neuron (big triangular arrowheads), DRD2 immunoreactive intermediate fusiform neuron cell body (double asterisk); (16B) DRD2 positive central neuron cell body. (Scale bar: 16A 20 μ m; 16B 10 μ m).

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