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Correlation between dopaminergic phenotype and expression of calretinin in the midbrain nuclei of the opossum (*Monodelphis domestica*): An immunohistological study

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We investigated distribution and morphology of neurons of the midbrain nuclei: the ventral tegmental area (VTA), substantia nigra (SN) and periaqueductal gray (PAG) of the adult grey short-tailed opossums that were double immunolabeled for the presence of calretinin (CR) and/or tyrosine hydroxylase (TH). The majority of TH-immunopositive neurons and fibers were located in the VTA, SN, and only scarce population of small neurons expressing TH was present in the PAG. In the SN 80% of TH-expressing neurons had large cell bodies, and only a small fraction had small perikarya. In the PAG populations of large and medium sized neurons were equal and 20% of neurons had small perikarya. Much scarcer population of TH-immunoreactive neurons in the PAG consisted of large or small neurons in its dorsal part (PAGd) and almost exclusively small neurons in the ventral part (PAGv). Distribution of neurons expressing TH and their types in the opossum are similar to those in rodents. The majority of CR-immunolabeled neurons were found in the VTA. In its subdivision, the parabrachal pigmented nucleus (PBP) cells expressing CR were approximately 28% more numerous than cells expressing TH. In spite of that, only 42% of TH-expressing neurons coexpressed CR. The high degree of colocalization TH and CR was observed in the SN. We propose that a higher percentage of TH/CR colocalization, which is observed in the opossums SN, may give them the ability to adapt to changes in their motor functions.

Key words: opossum, midbrain, calcium-binding proteins, calretinin, tyrosine hydroxylase, immunohistochemistry

INTRODUCTION

The results of several experiments show that behavior of rodents, particularly rats and mice and of the laboratory opossum (*Monodelphis domestica*) differs significantly (Wesierska et al. 2003, Blaszczyk and Turlejski 2005, Klejbor and Turlejski 2012). The opossums are more curious and active, showing lower level of anxiety-driven behavior than rats. They preferentially use the active exploration strategy, with a sacrifice of safety precautions, thus showing a natural tendency for risky strategies in behavior. This strategy may be advantageous for an animal feeding preferentially on small invertebrates that are moving fast and hiding.

Such distinct behavioral strategies may depend on differences in morphology and function of the brain systems that are involved in the integration of behavior. Both the motor system and the stress-related defensive behavior are influenced by the mesencephalic noradrenergic and dopaminergic neurons (Tzschentke 2001, Seamans and Yang 2004). Dopaminergic neurons are widely distributed in the several midbrain structures such as the ventral tegmental area (VTA), substantia nigra (SN) and periaqueductal gray (PAG).

Dopaminergic cells of these nuclei do not form a homogenous population, but belong to several neuronal types, form differing connections, have a variable content of neurotransmitter and also differ in expression of some

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calcium binding proteins (González-Hernández and Rodríguez 2000, Klejbor et al. 2006). Calcium binding proteins are thought to play multiple roles in regulation of neuronal functions (Li et al. 1995, Rintoul et al. 2001, Camp and Wijesinghe 2006, Burgoyne 2007, Todkar et al. 2012) and may be involved in synaptic plasticity, for instance in modulation of the long-term potentiation (DeFelipe 1997, Caillard et al. 2000, Edmonds et al. 2000, Nägerl et al. 2000, Schwaller et al. 2002). Moreover, disturbances in the intraneuronal Ca^{2+} levels are implicated

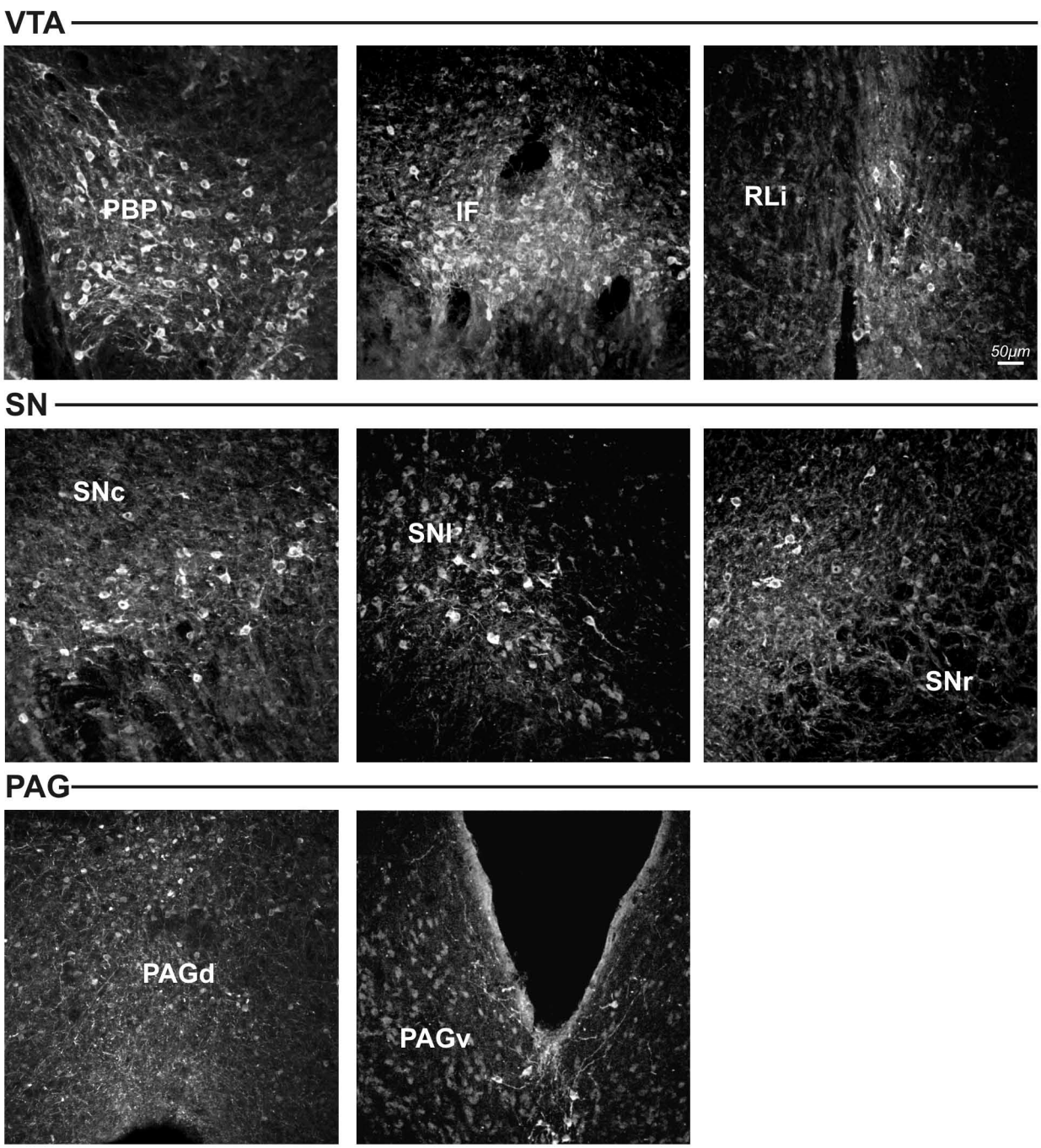

Fig. 1. Distribution of TH-immunoreactive cells and fibers within midbrain nuclei of the opossum. (VTA) ventral tegmental area; (PBP) parabrachal pigmented nucleus; (IF) interfascicular nucleus; (RLi) rostral linear nucleus; (SN) substantia nigra; (SNc) substantia nigra pars compacta; (SNl) substantia nigra pars lateralis; (SNr) substantia nigra pars reticulata (PAG) periaqueductal gray; (PAGd) dorsal periaqueductal gray; (PAGv) ventral periaqueductal gray. The scale bar in micrometers refers to all pictures.

in the pathogenesis of various neurodegenerative diseases like Parkinson, Alzheimer, amyotrophic lateral sclerosis, Huntington, epilepsy or brain ischemia (Heizmann and Braun 1992, Tsuboia 2000, Mattson 2007, Bezprozvanny 2009, Surmeier et al. 2010). Calcium binding proteins expression patterns have been widely used as neuronal markers to identify different cell types, especially interneurons (Celio and Heizmann 1981, Celio 1990). One of the calcium binding proteins, calretinin (CR), is distributed in various brain structures (Rogers 1992). In the VTA and SN of the midbrain structures CR is expressed (among others) in dopaminergic neurons.

In the present study we investigated distribution and morphology of tyrosine hydroxylase (TH) and CR expressing neurons in the midbrain nuclei (VTA, SN and PAG) of the adult, grey short-tailed opossums. We determined the correlations between expression of CR and TH in neurons of these nuclei.

METHODS

Animals

Nine adult, one year old grey short-tailed opossums were used. All animals were bred in the animal house of the Nencki Institute of Experimental Biology. The opossum is a solitary species, therefore the animals were kept in individual cages equipped with a small hiding place. Their environment was regulated as follows: light/dark cycle 14:10 (lights on at 08:00 AM), temperature 26°C and humidity 40–50%. They were fed dry food for kittens, canned meat for cats, fresh fruits and vitamins. The care and treatment of animals were in accordance with the guidelines for laboratory animals established by the National Institute of Health. The experiments were approved by the Local Ethics Committee for Animal Experimentation in Gdansk.

Experimental procedures

Animals were deeply anesthetized with lethal doses of Nembutal (80 mg/kg body weight) then perfused transcardially with a 0.9% solution of saline with heparin, followed by 4% paraformaldehyde solution in the 0.1M phosphate buffer (pH 7.4). The collected brains were post-fixed in 4% paraformaldehyde fixative for 3–4 hours. Then, they were placed in 15% sucrose solution (overnight at 4°C) followed by 30% sucrose solution until they sunk. After this, the brain coronal 40 µm-thick sections were cut on cryostat (Leica, Germany).

The series of sections were single or double-stained by standard immunohistochemical protocols using antibodies against calretinin and tyrosine hydroxylase.

The sections were washed three times in 0.01M phosphate buffered saline (PBS) followed by 2.5 h blocking in 10% normal goat serum and 0.3% Triton X-100 at room temperature. Then, the sections were incubated for 48 hours in 4°C with the primary antibodies: anti-calretinin (1:1 500, Millipore) or anti-tyrosine hydroxylase (1:1 000, Millipore). Afterward, sections were washed 3

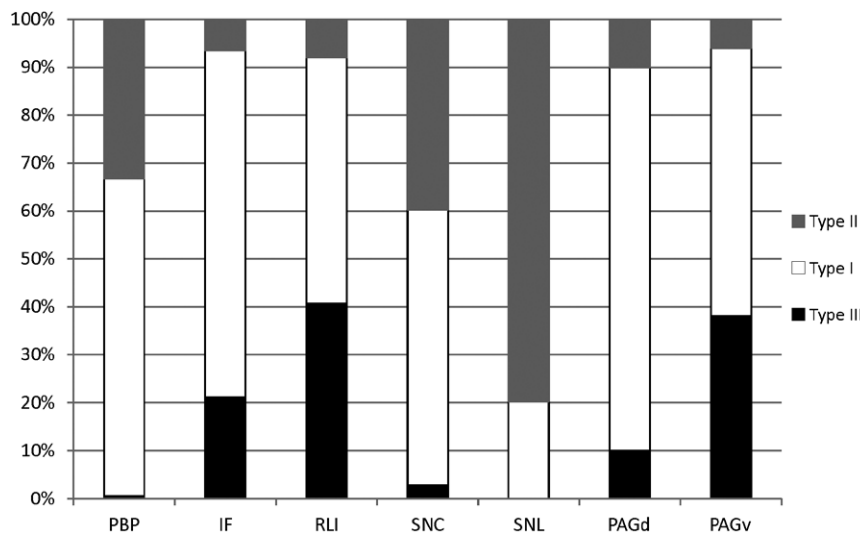

Fig. 2. Percentages of different types of TH-immunoreactive cells in the midbrain nuclei of the opossum

times in PBS and incubated for 2.5 h with the secondary antibody: 1:600 goat anti-rabbit antibody conjugated with Cy3 (111-165-144 Jackson ImmunoResearch Laboratories), for both calretinin and tyrosine hydroxylase. In case of double-staining the sections were incubated in a cocktail of primary antibodies: 1:1 500 rabbit anti-calretinin polyclonal antibody and 1:1 000 mouse anti-tyrosine hydroxylase monoclonal antibody. The sections were washed 3 times in PBS and incubated for 2.5

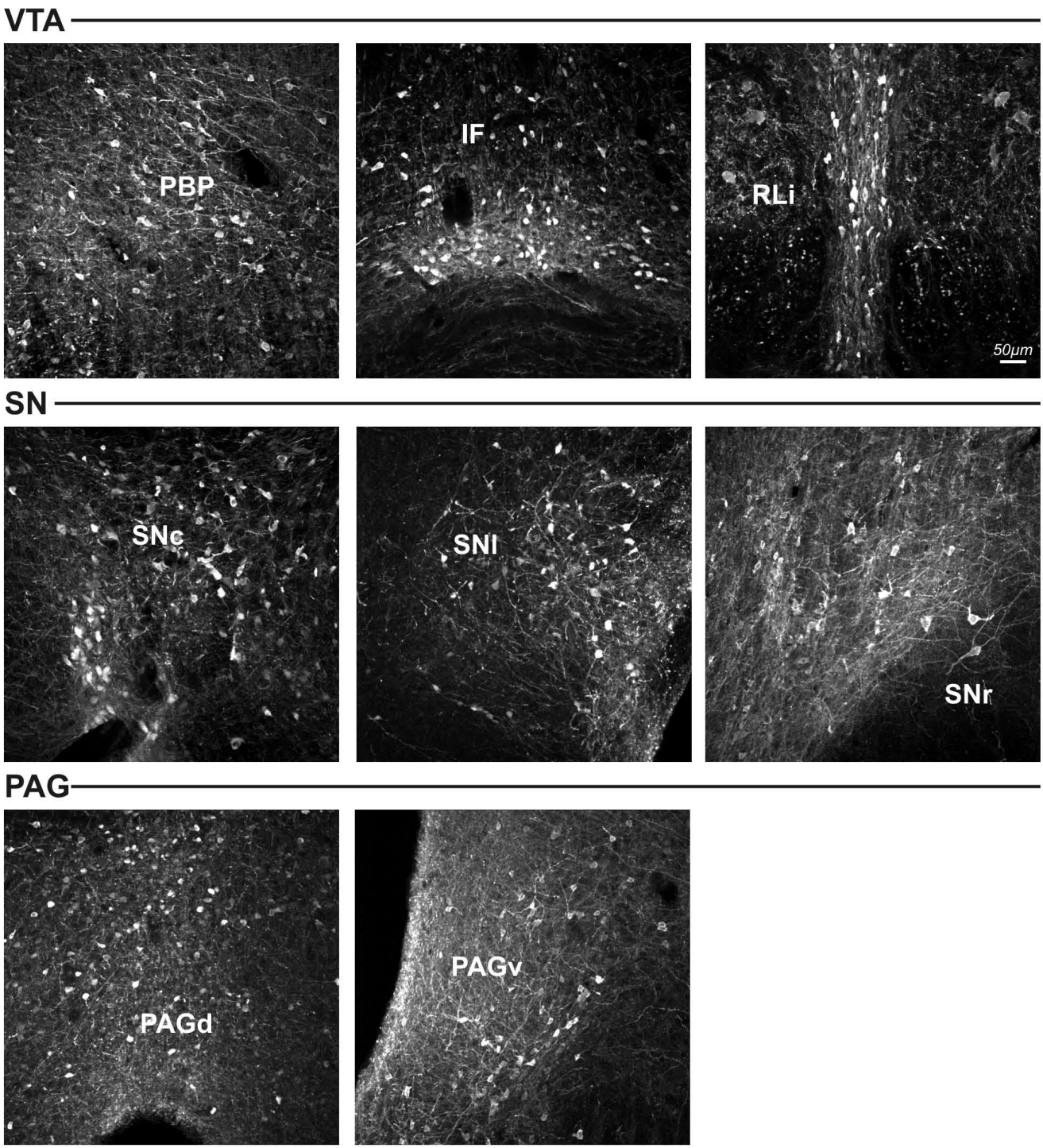

Fig. 3. Distribution of CR-immunoreactive cells and fibers within midbrain nuclei of the opossum. The scale bar in micrometers refers to all pictures.

Table I

Percentage of colocalization TH- and CR-immunoreactive neurons in the midbrain nuclei of the opossums

	CR-immunoreactive cells			TH-immunoreactive cells		
	CR+ [n]*	% of CR**	% of CR/TH**	TH+ [n]*	% of TH***	% of TH/CR***
IF	100	68	32	82	61	39
PBP	100	68	32	78	58	42
RLi	100	92	8	25	68	32
SNC	100	75	25	69	65	35
SNL	100	81	19	66	73	27
PAGd	100	99	1	6	91	9
PAGv	100	93	7	19	64	36

* Numbers of CR- or TH-immunoreactive cells; **Percent estimation of CR-only and CR/TH cells in CR-immunoreactive population of cells; ***Percent estimation of TH-only and TH/CR cells in TH-immunoreactive population of cells

h in room temperature in the mixture of secondary antibodies: 1:600 goat anti-mouse Cy3 (Jackson ImmunoResearch Laboratories), and goat anti-rabbit Alexa Fluor 488 (Molecular Probes). The sections were then washed, mounted on slides and cover-slipped with Kaiser's Glycerol gelatine for microscopy (Merck).

Quantitative analysis

Classification of cells morphological parameters containing TH and/or CR within the mesencephalic nuclei was analyzed with a LaserPix v.2.0 (Bio-Rad, UK) on CSLM images obtained with a 40× lens and zoom set to 1.9. The testing area size was 165×165 µm. At least five areas in every nucleus from each animal were evaluated. The first test area was chosen randomly and the remaining ones were selected by systematic random sampling. Neurons' profiles containing only TH, only CR and both TH and CR were counted and outlined. For each studied nucleus, the values of the polygonal area of cell profiles were obtained from the total of 25 areas in five opossums, which were then averaged, yielding the mean standard deviation (SD). The percentages of cells colocalizing and non-colocalizing TH and CR were estimated in reference to the total numbers of labeled cells counted in the areas described above.

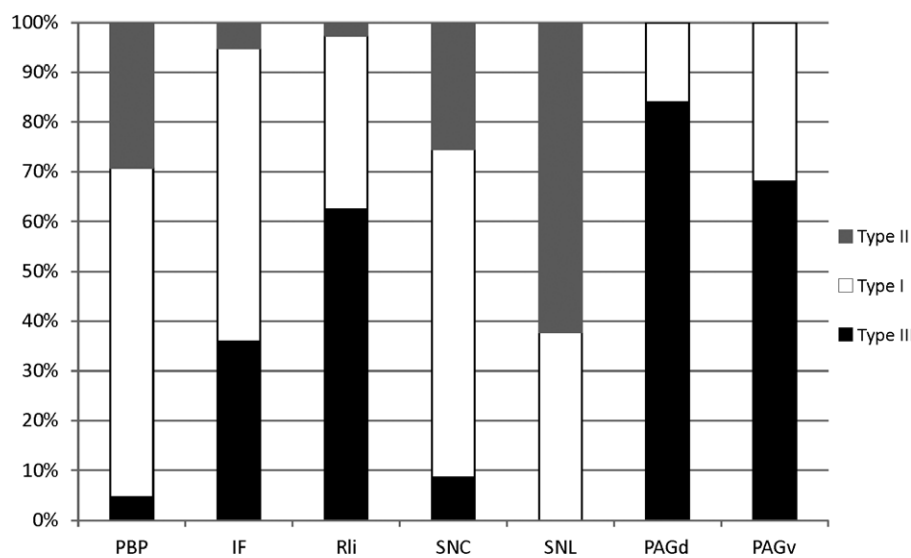

Fig. 4. Percentages of different types of CR-immunoreactive cells in the midbrain nuclei

RESULTS

The distribution and density of cells stained for TH and CR in the VTA, SN and PAG were examined using immunohistochemistry. We were able to distinguish three major morphologic types of labeled cells in the opossums' midbrain according to soma size and the shape of dendritic tree. Type I neurons were cells with medium size (square area between 100–200 μm²) fusiform or oval cell body, with dendrites emerging from the opposite poles. Type II neurons had a large soma (area over 200 μm²) and polygonally shaped cell bodies with a few thin and intensely stained dendrites. Type III neurons had small soma (area less than 100 μm²), ovoid or round cell bodies with a few thin and poorly stained dendrites.

Distribution of tyrosine hydroxylase in the opossum midbrain

The majority of TH-immunopositive neurons and fibers were located in the VTA and in the SN (Fig. 1). In the opossum we found that the VTA contained the medial nuclei: the rostral linear nucleus (RLi) and the interfascicular nucleus (IF) as well as the more laterally located parabrachial pigmented nucleus (PBP) and the SN subdivided into the pars compacta (SNC),

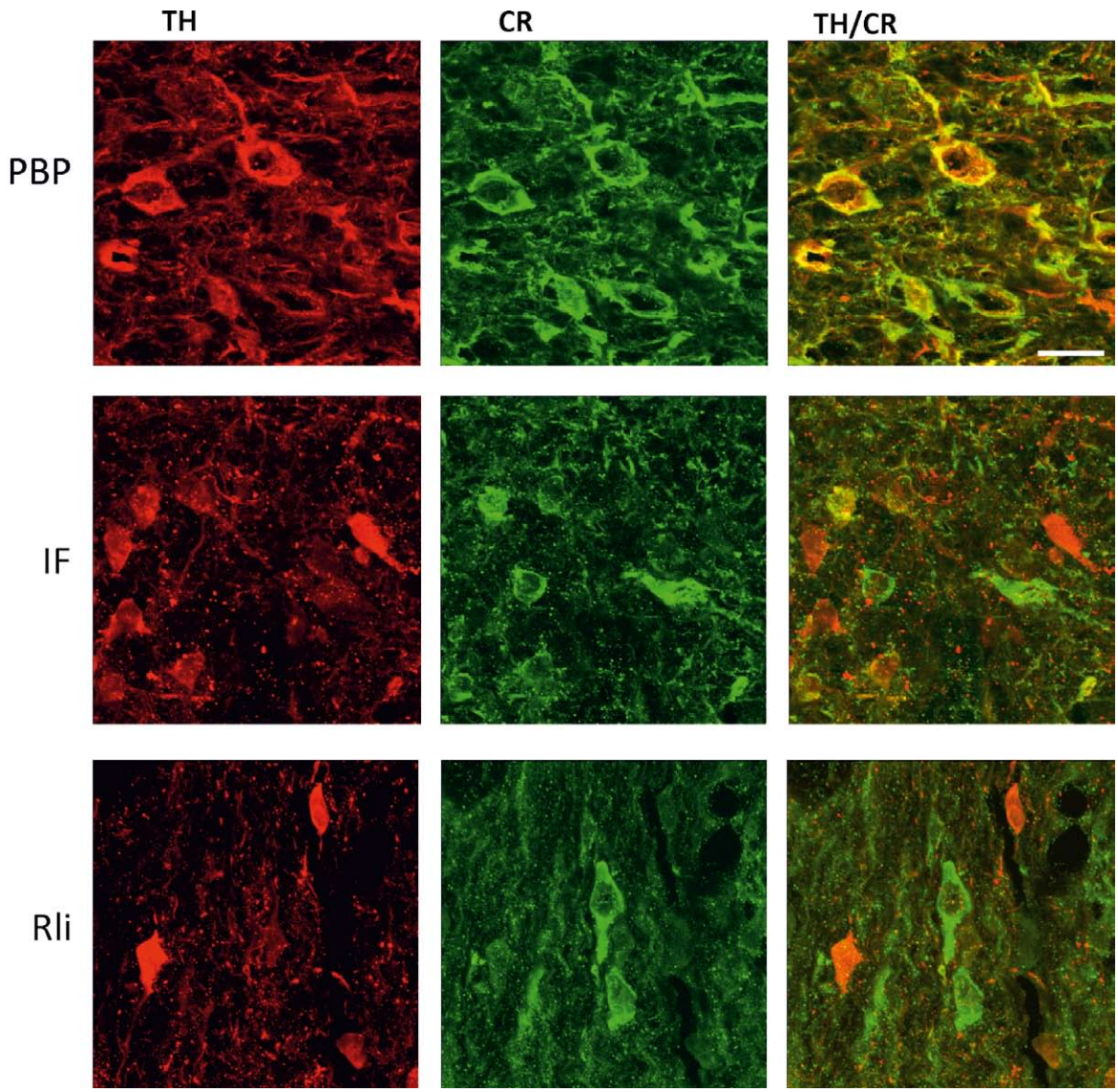

Fig. 5. Photomicrographs showing cells double immunolabeled for tyrosine hydroxylase (TH) and calretinin (CR) in the ventral tegmental area (PBP, IF and Rli). The scale bar in the upper right picture refers to all pictures and equals 20 μm.

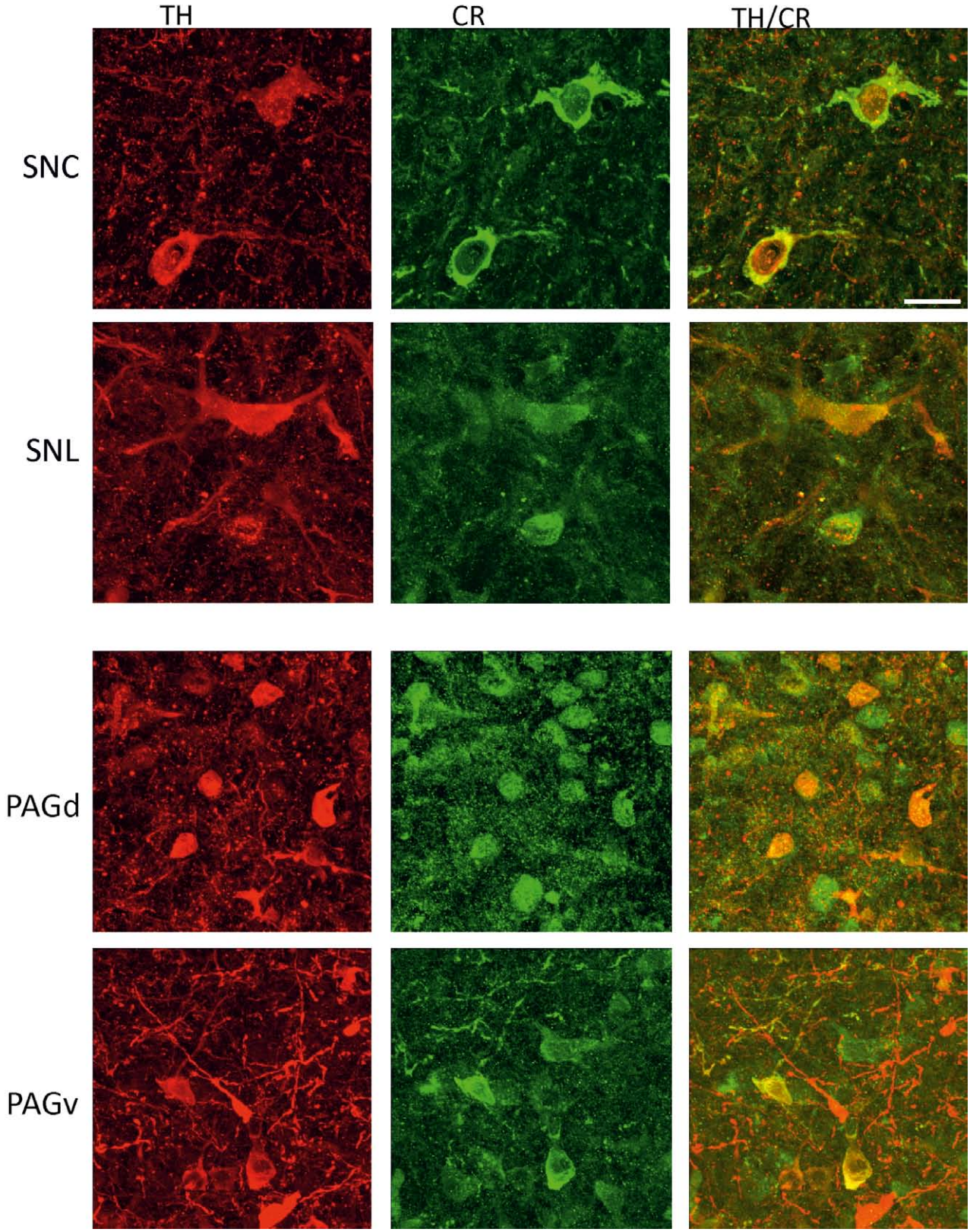

Fig. 6. Photomicrographs showing cells double immunolabeled for tyrosine hydroxylase (TH) and calretinin (CR) in the substantia nigra (SNC, SNL) and periaqueductal gray (PAGd and PAGv). The scale bar in the upper right picture refers to all pictures and equals 20 μm.

pars reticulata (SNR) and pars lateralis (SNL). In the PBP and IF nuclei, numerous and densely packed TH-labeled cells were observed, whereas in the RLi density of TH-labeled cells was lower (Fig. 1). In the PBP most of TH-stained cells belonged to type I (66%) and type II (33%) neurons. Only a minor fraction of TH-immunoreactive cells (1%) showed features characteristic for type III neurons (Fig. 2). The neuropil consisted of the dense meshwork of fibers, varying in diameter, sometimes with varicosities. Populations of TH-labeled neurons in the RLi showed a different pattern, as 41% of its neurons were of type III, 51% of type I, and remaining 8% belonged to type II (Fig. 2). Within this nucleus TH-labeled cells and fibers were arranged ventro-dorsally. In the IF the type I was the most abundant (72%), while type III constituted 21% and type II was rare (7%) (Fig. 2).

TH-immunoreactive cells in the SN were large and intensely stained (Fig. 1). Within SNC and SNL different types of TH-immunopositive neurons were observed. In the SNL neurons of type II predominated (80%) and the remaining 20% were of type I, while neurons of type III were not observed (Fig. 2). In the SNC the most common were neurons of type I (57%), type II neurons were less frequent (40%) and those of the type III were rarely observed (3%) (Fig. 2).

The low number of TH-immunoreactive cells was observed in the PAG, within both its ventral (PAGv) and dorsal (PAGd) parts. They constituted approximately one fourth of the brainstem dopaminergic neurons population. In both PAGd and PAGv subdivisions mainly small, round, sparsely distributed cells belonging to type I (80% and 56%, respectively) were observed (Fig. 2). In the PAGv the second largest population was type III neurons (38%), while only 6% belonged to the type II. In the PAGd type II and III neurons were present with equal frequency, each type constituting 10% of TH-labeled cells (Fig. 2).

Distribution of calretinin in the opossum midbrain

The majority of CR-labeled neurons and neuropil were located in the VTA, mainly in its PBP and IF parts (Fig. 3). A few CR-immunoreactive neurons were observed in the SNC, SNL, RLi, PAGv and PAGd. In the SNR the number of CR-stained cells was low, but a dense network of immunopositive dendrites and fibers was observed.

Neurons of type I (59%) predominated in the IF nucleus, while small, round cells of type III constituted 36% of the population. Cells with visible processes and medium soma size were scarce (Fig. 4). Various types of CR-immunoreactive fibers, short and smooth as well as long, thin and sometimes with varicosities were observed in the IF.

In the PBP cells expressing CR were approximately 28% more numerous than cells expressing TH. In this area the medium sized, round shaped type I neurons predominated (Fig. 4). However, the type II and III neurons were also observed (29% and 5%, respectively). Distribution of CR-immunoreactive neuropil in the PBP was similar to that in the IF nucleus.

In the RLi, we observed many fusiform and oval neurons with long dendrites (Fig. 3). These cells were the smallest CR-immunoreactive neurons among various nuclei of the VTA. Neurons of type III constituted 62 % of CR-immunoreactive cells, 35% of those neurons were of type I and neurons of the type II constituted the remaining 3% of the labeled cells (Fig. 4). Interestingly, in the RLi CR-labeled cells and fibers showed a vertical (ventro-dorsal) arrangement.

In the SNC the number of CR-immunoreactive cells was almost 45% higher than the number of TH expressing cells. The majority (66%) of CR expressing neurons in the SNC had a polygonal shape with clearly visible dendrites, therefore belonging to the type I (Fig. 4). The remaining CR-immunoreactive cells belonged to type II (25%) and type III (9%). The SNC neuropil contained densely packed CR-labeled fibers with varicosities. In the SNL two types of CR-immunoreactive cells, large, polygonal type II (62%) and medium sized type I (38%) were observed (Fig. 4). The dense network of long, thin CR-labeled fibers with numerous varicosities was also present. In the SN region the population of CR-labeled cells dominated over the population of TH-labeled cells by 52%. In the SNR the CR-immunopositive cells were sparsely distributed, while the CR-labeled neuropil was dense.

In both PAGd and PAGv, densely packed small, round CR-immunoreactive cell bodies were observed (Figs 3 and 4). They belonged predominantly to type III neurons (PAGd – 84%, PAGv – 68%). The remaining neurons were of the type I (PAGd – 16%, PAGv – 32%).

The number of TH-labeled cells in the opossum PAG was very low. Within PAGd CR-labeled cells were 16 times higher than numbers of TH-immunoreactive cells, while in the PAGv cells expressing CR were 5 times more numerous.

Colocalization of TH and CR immunoreactivity

The results of double-labeling immunohistochemistry that illustrate the relationships between TH- and CR-immunoreactive cells, were presented in Table I.

In the IF nucleus 39% of TH-immunoreactive cells expressed also CR (Fig. 5). The cells showing coexpression of both proteins belonged to type I neurons. Very similar results were obtained from PBP nucleus, where TH-immunoreactive cells revealed in 42% colocalization with CR. Within the RLi nucleus 32% of TH-immunoreactive cells showed colocalization with CR. Also in this nucleus the coexisting cells belonged to type I neurons (Fig. 5).

In the SNC 35% of the TH-labeled cells showed coexpression of CR (Fig. 6). Colocalizing cells belonged to type I (Fig. 6). Within the SNL cells expressing TH and CR simultaneously accounted for 73% of the population of cells expressing TH. Colocalizing cells were among the largest neurons studied, categorized as type II.

In the PAGd cells expressing TH and CR consisted of 9% of TH-labeled cells and belonged to the type III neurons, whereas 32% TH-labeled cells coexpressed CR (Fig. 6).

DISCUSSION

The present data show that dopaminergic cells expressing TH are located in the VTA, SN and PAG regions of the midbrain in the gray, short-tailed opossum (*Monodelphis domestica*). We found that in all three dopaminergic nuclei of the midbrain, neurons immunoreactive for either TH or CR belonged to one of three morphological types. The distribution and morphology of TH- and CR-immunoreactive cells in the mesencephalic dopaminergic nuclei of the opossum is generally similar to its distribution in rodents, such as the rat or mouse (Résibois and Rogers 1992, Rogers 1992, Isaacs and Jacobowitz 1994, Liang et al. 1996).

In the opossum we found that large TH-immunoreactive neurons were mainly located in the dorsal tier of the SNC, whereas smaller, densely packed TH-immunoreactive cells were placed in the IF. These findings are consistent with the distribution and types of dopaminergic neurons in the midbrain nuclei of the rat (Isaacs and Jacobowitz 1994). In contrast to this similarity of histological features of the dopaminergic nuclei in rodents and marsupials, dopaminergic cell groups in the human midbrain show somewhat different features. For example, humans have a more extensive ventral tier of the SN, smaller proportion of TH-immunoreactive neurons in the IF and larger proportion in the PBP than rats (McRitchie et al. 1996). Functional significance of these species-specific differences is yet unknown.

Our study revealed that dopaminergic midbrain structures are characterized by the high level of CR protein expression. We observed many CR-immunoreactive cells in the VTA, including PBP and IF and in the adjacent SNC brain region. CR immunolabeling of neuropil (dendrites, axons and endings) was intense in all of the midbrain nuclei. Interestingly, in the opossum CR-immunoreactive cells were found in both dorsal and ventral regions of the PAG (PAGd and PAGv, respectively). These round cells were among the smallest cells in these structures. The pattern of distribution of the CR perikarya and fibers in the SN/VTA complex observed in the opossum is similar to that present in rodents (Garcia-Segura et al. 1984, Hokfelt et al. 1984, Résibois and Rogers 1992, Rogers 1992), squirrel monkey (Fortin and Parent 1996, Parent et al. 1996) and humans (McRitchie et al. 1996). The morphology of CR-immunoreactive cells in all of the midbrain nuclei is similar to those described by Résibois and Rogers in the rat brain (Résibois and Rogers 1992). The degree of colocalization of TH and CR in the midbrain neurons did not differ between the opossum and rat. In the SN of the opossum, the percentage of CR/TH cells is slightly lower than in the rat but in other structures the percentage of double-labeled TH and CR cells was the same as in the rat (Garcia-Segura et al. 1984, Hokfelt et al. 1984, Résibois and Rogers 1992, Rogers 1992, Isaacs and Jacobowitz 1994).

Functional implications of expression of CR in midbrain neurons.

Management of intracellular Ca^{2+} signaling is essential for a cell's ability for continuous dynamic adaptation to changes of its activity evoked by external factors. Therefore, it is an important factor in reaction of animals to external stimuli and adaptation to its environment.

According to our observation in the opossum, as well as in the rat and mouse (Liang et al. 1996, Alfahel-Kakunda and Silverman 1997), CR is pres-

ent in both dopaminergic and non-dopaminergic neurons in the VTA and SN (probably GABA-ergic in the SN and GABA-ergic or glutaminergic in the VTA). Furthermore, a substantial proportion of dopaminergic neurons in the SN and VTA do not contain CR.

GABA-ergic and dopaminergic midbrain neurons have strikingly different firing properties. GABA-ergic neurons in the SN fire high-frequency, brief action potential bursts (Atherton and Bevan 2005, Zhou et al. 2006), whereas SN and VTA dopaminergic neurons fire low-frequency, long-duration bursts (Grace and Bunney 1984a, b, Hyland et al. 2002, Zhou et al. 2006). Firing properties of neurons are strongly influenced by intracellular calcium transients, whose time course and spatial spread are differentially affected by calcium-binding proteins. The absence of calcium binding proteins results in marked differences in cell firing properties (Bastianelli 2003). The functional benefits of CR differing distribution of CR in different cell populations are not fully understood, nor are the precise mechanisms through which CR (or indeed all calcium binding proteins), produce their diverse effects. Presumably, the presence, absence and colocalization of particular calcium binding and other proteins contributes to the firing specificity of cells. According to Bastianelli (2003), it is possible to hypothesize that the differential distribution of calcium binding proteins reflects different physiological requirements. For example, the stellate and basket cells in the cerebellum, that are GABA-ergic interneurons, fire in repetitive patches of spikes of very high frequency.

CR may function as a "fast" or "slow" buffer (Gall et al. 2003, Faas et al. 2007, Schwaller 2009). The presence of slow calcium buffers and absence of fast calcium buffers helps to sustain fast firing rates by keeping calcium levels at near resting levels. Interesting, it seems that CR might also act as a calcium sensor proteins (Billing-Marczak and Kuznicki 1999, Palczewska et al. 2001). It appears that this "multipotency" of CR may explain its presence in differently spiking midbrain neurons, providing them with the ability to adapt to periodically high loads of activity serving motor (SN) and motivation (VTA) functions that may highly differ in time.

Physiological function of CR has investigated in the cerebellar granule cells which, like SN neurons, are engaged in the control of movement (Schwaller et al. 2002, Schwaller 2009). Selective knockout of CR in mice produces disturbances of motor coordination and suggests a putative role for CR in the maintenance of calcium dynamics underlying motor adaptation (Schwaller et al. 2002, Schwaller 2009). In null-mutant CR−/− mice, action potentials and discharge properties of granule cells are altered (Bastianelli 2003, Gall et al. 2003, Camp and Wijesinghe 2006). CR is present in presynaptic terminals of granule cell axons (parallel fibers), which provide the input to the Purkinje cells. It suggests that CR could play a major role in Ca^{2+} dependent plasticity at these synapses (Schwaller et al. 2002). It seems likely that similar relationships can exist between neurons of the midbrain nuclei. Electrophysiological and morphological studies have shown that dopaminergic neurons of the SN receive a strong input from GABA-ergic neurons. Nigral GABA-ergic neurons expressing CR send local axon collaterals, which likely underlie the intranigral communication between GABA-ergic and dopaminergic neurons or among GABA-ergic neurons themselves (Carr and Sesack 2000, Lee and Tepper 2007, Nair-Roberts et al. 2008).

The present study shows that the midbrain CR-immunoreactive neurons in the opossum are heterogeneous in morphology and chemistry. Moreover, we found the high degree of CR and TH colocalization in the SN, which may give animals the ability to adapt to changes in their motor functions.

CONCLUSION

In spite of substantial differences in the balance of emotions and motivations between the opossum and rodents, dopaminergic structures of their brains, and in particular VTA and PAG seem to have a strikingly similar organization of the dopaminergic system. Significance of these findings remains to be explained.

ACKNOWLEDGEMENTS

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