

The co-expressing D1–D2 neurons in the striatum of the common tree shrew (*Tupaia glis*)

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Abstract The striatum contains D1 and D2 neurons within the striosome and matrix regions. The objective of this study was to identify the distribution of D1 and D2 striatal neurons in tree shrews by fluorescent immunohistochemistry using D1, D2 and substance P antibodies. Animal brain sections were immunolabeled with antibodies against the D1 receptor and substance P and then counterstained for nuclei with DAPI. It was revealed that 91% of D1 labeled neurons in the striatum were in close contact with substance P labeled terminals. Thus, substance P antibody was used as a marker for D1 neurons. Brain sections were incubated with antibodies against substance P and D2 receptors and then counterstained for nuclei with DAPI. The results showed that 88% of total neurons were in close contact with substance P labeled terminals in the striosome and 30% were D2 labeled neurons that were in contact with substance P labeled terminals. The matrix contained 98% of D2 labeled neurons, and 29% of D2 labeled neurons that were in close contact with substance P labeled terminals. In conclusion, approximately 30 % of striatal neurons in either the striosome or matrix expressed both D1 and D2 receptors. These co-expressing D1-D2 neurons probably send different outputs from those of D1 or D2 neurons. **Chiang Mai Medical Journal 2017;56(3):115-23.**

Keywords: striatum, D1 neuron, substance P, D2 neuron, tree shrew

Introduction

The striatum is the main structure found in the basal ganglion, and is associated with body movement planning and behavioral learning (1). It consists of the putamen and a dorsal striatum called the caudate nucleus. The ventral striatum contains the nucleus accumbens. The striatum receives glutamatergic excitatory

input from the cortex and dopaminergic inhibitory input from the substantia nigra pars compacta (SNc). There are two types of neurons within the striatum. The first is called striatal projection neurons (96% of medium spiny neurons). The others are referred to as interneurons (4% of aspiny neurons). The medium

spiny projection neurons can be divided into type I (D1) and type II (D2) dopaminergic receptor expressing neurons. Both D1 and D2 receptors are associated primarily with the spines, dendrites and perikarya of the medium spiny neurons in both the patch/striosome and matrix compartments of the striatum. The change in dopamine receptors was found to relate to symptoms found in Schizophrenia patients (2,3). Several studies have found that D1 and D2 receptors segregate to the dopaminergic pathway. The D1 receptors are involved in direct pathway neurons, which are the substance P containing neurons or striato-GPi or striatonigral neurons. These neurons promote desired movements. However, D2 receptors are involved in indirect pathway neurons that are enkephaline containing neurons or striato-GPe neurons (4). These neurons suppress unwanted movements. The D1 neurons are stimulated by the dopamine neurotransmitter that consequently increases striatal output, while D2 neurons, which are inhibited by the dopamine neurotransmitter, decrease striatal output. The striatal activities of these two pathways are normally in balance. Therefore, D1 and D2 receptors mediate the response of striatal neurons to the dopaminergic input from SNc.

The striatum is a heterogeneous structure containing patch/striosome and matrix regions (5), which differ according to their neuronal chemical contents, development, and neuronal connections. The striosome and matrix were stained densely with substance P antibody and parvalbumin, respectively (6,7). Both the striosome and matrix contain D1 and D2 neurons. Substance P labeled terminals in the striatum were found to be in contact with D1 neurons that are projected to the substantia nigra par reticulata (SNr) in a direct pathway (8).

Although the D1 and D2 receptors are found mostly in various striatal neurons, their co-localization has been reported in several species (9). The localization of dopamine receptors has led to a better understanding of the effects that dopamine has on regulating striatal neurons and its role in direct and indirect dopaminergic pathways. However, the localization of D1 and D2 neurons in the tree shrew striatum has not been studied. Therefore, the

aim of this study was to localize the D1 and D2 neurons in the patch/striosome and matrix of the tree shrew striatum in order to understand the dopaminergic pathway better in humans.

Methods

Two male tree shrews, with an average weight of 120 gm., were used in this study. They were sacrificed by ether until they showed no evidence of reflex. Their thoraxes were opened for perfusion. Phosphate buffer saline (PBS) was perfused through the left ventricle in order to flush the blood out of the body, and 500 mL³ of 4% paraformaldehyde solution was administered. The brains were extracted from the skulls and sectioned in the coronal plane to a 50 µm thickness by vibratome (Leica s1200). Every fifth section was chosen and placed in one row.

In order to reveal the distribution of substance P labeled terminals and D2 labeled receptors, two rows of brain sections were processed for single immunolabeling, with primary antibodies used against substance P made in a rat (MediMABS) at a dilution of 1:1500, while those against D2 receptors were made in a rabbit (Millipore) at a dilution of 1:1K. These brain sections were then reacted with Alexa Fluor 488 anti-rat (Invitrogen) for substance P labeling, and Alexa Fluor 488 anti-rabbit (Invitrogen) for D2 receptor labeling as secondary antibodies. The two rows of the tree shrew brain sections were then counter stained with 4', 6-diamidino-2-phenylindole (DAPI) for DNA fluorescent or nuclear staining, in order to identify the target neuronal nuclear of the substance P-labeled terminals and D2 neuronal nuclear. In order to examine whether or not the substance P-labeled terminals contact the D1 expressing cells, one row of the tree shrew brain sections was double immunolabeled with the anti-substance P made in a rat (MediMABS) at a dilution of 1:1500, and the anti-D1 antibodies were made in a rabbit (Millipore) at a dilution of 1:1K. These were then reacted with the Alexa Fluor 488 anti-rat (Invitrogen) and Alexa Fluor 555 anti-rabbit (Invitrogen) for substance P and D1 receptor labeling, respectively. These sections were then counter stained with DAPI. It was assumed that the neurons contacted by substance P labeled terminals were D1, as suggested in a previous study (8). In order to localize the D1 or target neurons of the substance P labeled terminals and D2 neurons, one row of the brain sections was double immunolabeled with anti-substance P and anti-D2 antibodies, as described for double immunolabeling for substance P terminals and D1 receptors.

Data analysis

The striatal sections containing the DAPI labeled nuclei, double immunolabeling with D1 receptors and substance P labeled terminals were examined under a fluorescent microscope. The striatal neurons were characterized by a DAPI labeled nuclei with a D1 labeled receptor or surrounding substance P labeled terminals. The number of the D1 expressing neurons and that of surrounding substance P labeled terminals were counted. The neurons expressing D1 receptors and those surrounded by substance P labeled terminals also were examined and analyzed.

The sections containing the DAPI labeled nuclei and double immunolabeling with D2 receptor and substance P labeled terminals were examined. The D2 neurons characterized by DAPI labeled nuclei and surrounded by D2 labeled receptors were calculated. The DAPI labeled nuclei surrounded by substance P labeled terminals were identified as possible D1 neurons and counted. Those expressing D2 receptors and those contacted by substance P labeled terminals were characterized as co-expressing D1–D2 receptor neurons and calculated as well.

Results

D1 neurons contacted mostly by substance P labeled terminals

Figure 1 illustrates the substance P labeled terminals, D1 labeled receptors and DAPI labeled nuclei in the striosome/patch region of the tree shrew striatum. The merged image illustrates that all neurons, which express the D1 receptors, were contacted by the substance P labeled terminals. From a total of 68 D1 expressing neurons, most of them were surrounded by the substance P labeled terminals (62/68 or 91.87 % in Table 1). Sequentially, the substance P labeled terminals in the striosome/patch specifically contact D1 expressing neurons. These images suggest that the neurons surrounded by substance P terminals are probably D1, as suggested in previous studies (8). This study insists that the neurons contacted by substance P labeled terminals are D1 expressing neurons. Therefore, substance P was used as the marker for identifying D1 neurons in this study.

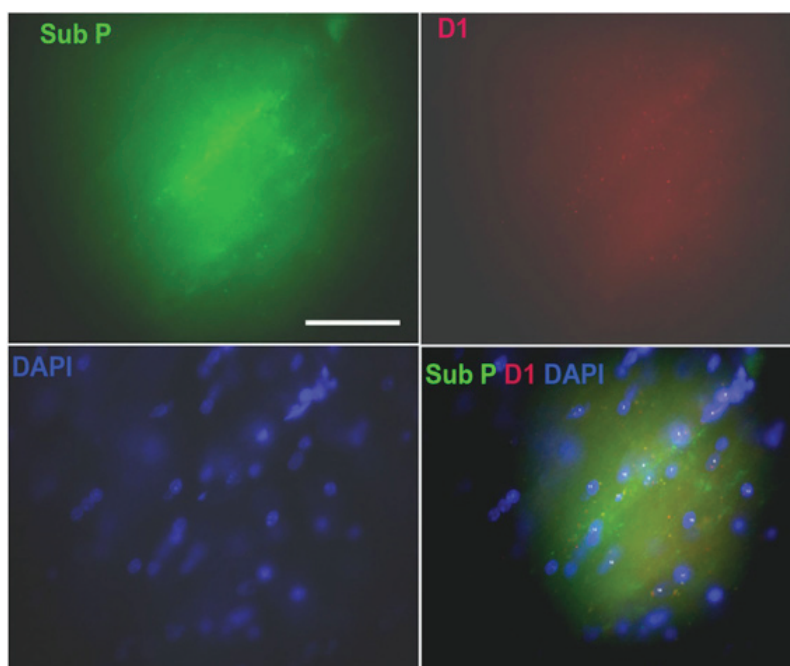


Figure 1. The immunofluorescence images of D1 expressing neurons and substance P terminals in the patch region of the tree shrew striatum. The substance P labeled terminals are in the upper left image, the D1 labeled receptors are recognizable in the upper right image, and the DAPI stained nuclei are seen in the lower left image. The merged image (the lower right one) shows that the DAPI labeled nuclei are surrounded by substance P terminals and D1 receptors. The neurons containing DAPI labeled nuclei are numbered and were observed. The scale bar in the upper left image is equal to 50 μ m, which applies to all of the images in this figure.

Table 1. The number of the D1 expressing neurons and that of neurons which that are contacted by the substance P labeled terminals in the striosome/patch

Image no.	D1 expressing neurons	The D1 neurons contacted by substance P terminals
01	18	18
04	7	6
05	11	11
06	3	3
07	7	5
08	9	7
09	13	12
total	68 (100%)	62 (91.18 %)

Possible D1 neurons and D2 expressing neurons found in the striosome/patch and matrix, respectively

Figure 2A shows that most of the striatal neurons containing the DAPI stained nuclei have made contact with substance P labeled terminals. The substance P labeled terminals were found to surround the DAPI labeled nuclei in the patch region, and they probably make contact on the dendrites or dendritic spines of the medium spiny neurons of the striatum. Figure 2B shows that the D2 labeled receptors are peripheral to and overlying the DAPI stained nuclei of the striatal neurons in

the matrix region. These D2 labeled receptors are probably on the soma and proximal dendrites of the striatal neurons.

Figure 3A illustrates the striosome/patch and matrix of the tree shrew striatum. The striosome/patch contains a dense collection of substance P labeled terminals, while the matrix contains mainly D2 labeled receptors. As described above, most D1 expressing neurons are contacted by the substance P labeled terminal. Thus, the striosome/patch that contains substance P labeled terminals surrounding the DAPI labeled nuclei likely contains D1 neurons. Possible D1 neurons in the strio-

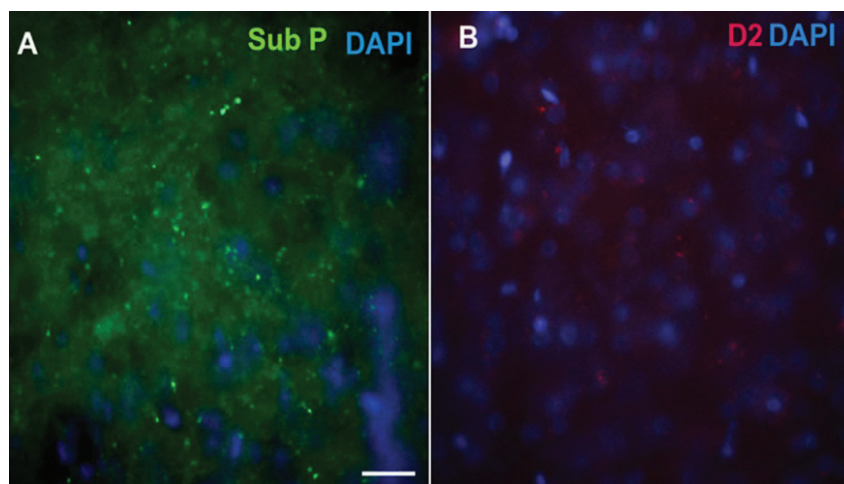


Figure 2. The distribution of substance P labeled terminals and that of D2 labeled receptors on the striatal neurons. The substance P labeled terminals (green) are distributed densely in the patch region of the tree shrew striatum. These terminals are close to the DAPI stained nuclear (blue) containing neurons (A). The D2 labeled receptors (red) are found mostly in the matrix region. The D2 receptors are on the DAPI stained nuclear (blue) containing neurons (B). The scale used in A and B is 50 μ m.

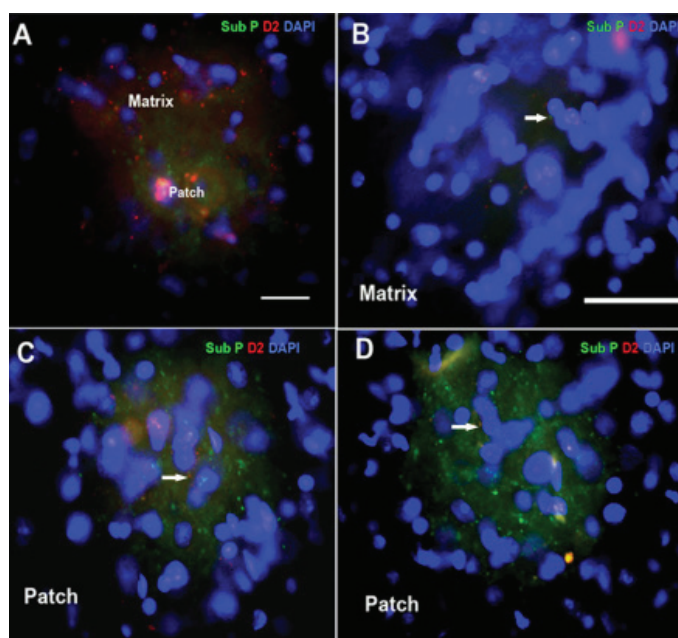


Figure 3. The immunofluorescence images of D2 expressing neurons and substance P terminals in the tree shrew striatum. The matrix region has a high density of D2 neurons (A) and some of them also are in contact with substance P labeled terminals (arrows). The patch region has a dense distribution of substance P labeled terminals (C and D), of which some of them also express D2 receptors (arrows in C and D). The scale is 100 μ m in A and 50 μ m in B, C and D.

some/patch are approximately 57.76% (93 of 161 in Table 2), and neurons expressing only D2 receptors are 11.80% (19 of 161 in Table 2). The D2 expressing neurons in the matrix are approximate 68.89% (31 of 45 in Table 3), and those contacted only by substance P labeled terminals, or possible D1 neurons, are 2.22 % (1 of 45 in Table 3).

However, neurons were contacted by substance P labeled terminals, with expressed D2 receptors found in the striosome/patch (Figure 3C and 3D) and matrix (Figure 3B). Presumably, co-expressing D1-D2 neurons are found in both the striosome/patch and matrix. The co-expressing D1- D2 neurons in the striosome/patch were slightly higher (30.43%, 49 of 161 in Table 2) than those in the matrix (28.83%, 13 of 45 in Table 3).

Discussion

The reciprocal connections between the striosome/ patch and substantia nigra

This study found that the striosome/patch

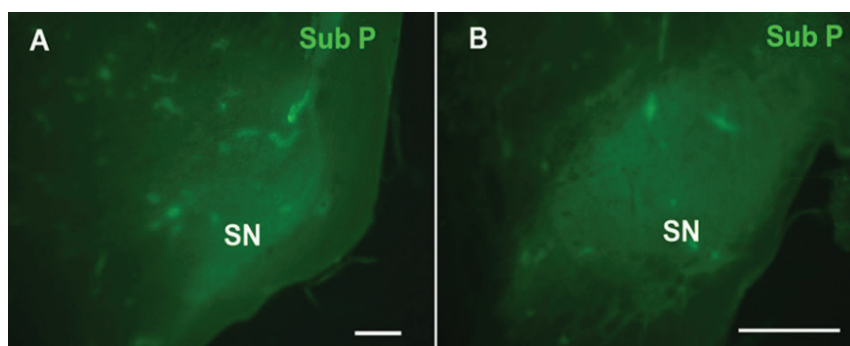
of the tree shrew striatum contains substance P labeled terminals, as reported in Day-Brown et al., 2010. The substance P terminals form on the outlines of the neurons. The substantia nigra in the brain sections is revealed as a rich substance P region (Figure 4). Thus, the SP terminals in TS striatum might originate from the SN. The striatal neurons of the tree shrew are typically medium spiny neurons (MSN) (10). The D1 expressing neurons, which were contacted by the SP in this study, are possibly MSN. A previous study in rats reported that substance P labeled terminals synapsed on the striatonigral spiny neurons that were in the direct pathway of the basal ganglia circuit (8). These striatonigral neurons project into the substantia nigra pars reticularis (SNr) (11) and/or substantia nigra pars compacta (SNc) (12). The D1 expressing neurons in the patch of the tree shrew striatum are probably in the direct pathway of the basal ganglia, and send their axon to the substantia nigra. However, projection of the striatal neurons in the patch to the SN in tree shrews will need to be confirmed by the anatomical tract tracing technique.

Table 2. The number of possible D1, D2 expressing and co-expressing D1 and D2 neurons in the striosome/patch regions of the striatum

Image no.	Possible D1	D2 expressing	co-expressing D1- D2	Striatal neurons
3	12	0	4	16
4	4	2	5	11
5	15	0	2	17
6	8	0	6	14
7	7	1	6	14
8	7	5	7	19
9	4	2	10	16
10	9	0	5	14
11	3	0	0	3
13	2	1	3	6
14	5	3	0	8
15	2	0	1	3
16	15	5	0	20
Total (%)	93 (57.76)	19 (11.80)	49 (30.43)	161 (100)

Table 3. The number of D1 (contacted by substance P labeled terminals), D2 and co-expressing D1 and D2 neurons in the matrix regions

Image no.	D1	Expressing D2	co-expressing D1-D2	Striatal neurons
1	1	17	4	4
2	0	5	6	6
12	0	9	3	3
Total (%)	1 (2.22)	31 (68.89)	13 (28.89)	13 (28.89)

**Figure 4.** The dense staining of substance P in the substantia nigra (SN) of the tree shrew. The scale in A and B is 100 μ m and 50 μ m, respectively.

The D1 and D2 expressing neurons as postsynaptic targets of the dopaminergic terminals from the substantia nigra

The D2 and D1 receptors of the tree shrew striatum can be identified by immunolabelling them against their antibodies. The D1 and D2 labeled receptors should be located on the membrane of the medium spiny neurons. The D1 and D2 receptors are the targets of the do-

paminergic neurotransmitter that is produced by the dopaminergic neurons in substantia nigra compacta (SNc). The postsynaptic targets of the dopaminergic terminals are dendritic spines, dendritic shafts, and soma (10) and they contain these dopaminergic receptors. The D1 and D2 receptors are expressed by neurons in the direct and indirect pathways, respectively (13). Further study will be needed to examine

whether the dopaminergic terminals prefer contacting either D1 or D2 expressing neurons.

D1 and D2 neurons that receive excitatory input from the cortex and thalamus and inhibitory input from the substantia nigra

The striatum is the major input region of the basal ganglion. It receives the glutamatergic excitatory inputs from the cortex and thalamus. The tree shrew striatum receives thalamic inputs from the pulvinar nucleus (6). The pulvinostriatal terminals make up the asymmetrical or excitatory synapses on the dendritic spines of the striatal neurons. The D1 and D2 neurons in the striatum receive the dopaminergic inhibitory inputs from the nigrostriatal neurons in the SNc. The dopaminergic terminals of the nigrostriatal neurons make up the symmetrical or inhibitory synapse on the dendritic spines, dendritic shafts, and cell bodies (10). Normally, the excitatory synapses are found on the dendritic spines, while the inhibitory synapses are found on the soma. However, the D1 and D2 receptors on the postsynaptic target have a different effect on the responses of the MSN after glutamatergic input (14). Further study will be needed to determine the locations of D1 and D2 receptors on the MSN of the tree shrew striatum.

Double labeling for type 1 (D1) and type 2 (D2) dopaminergic receptors

This study could not double label D1 and D2 receptors in the same brain section because the antibodies against them were made in the same host species. Substance P immunolabelling terminals contacting the neuron were used as indirect detection of D1 neurons (8). This study indicated that some of the D2 expressing neurons also were contacted by the substance P labeled terminals in the tree shrew striatum. These neurons are presumably co-expressing D1 and D2 neurons. In order to examine co-localization of the D1 and D2 expressing neuron specifically, the host of D1 and D2 antibodies for immunolabeling should differ, which requires the use of NeuN staining for neuron staining rather than DAPI staining.

Conclusion

The tree shrew striatum is a heterogeneous structure composed of the patch/striosome and matrix regions. Each region is histochemically different. The patch/striosome contains a high percentage of substance P terminals, while the matrix contains mainly D2 receptors. Approximately 57.76% of the total neurons in the patch are contacted by substance P terminals and thought to be D1 neurons, as suggested by Bolam and Izzo in 1988; and about 68.89% of the neurons in the matrix are D2 neurons. These D1 and D2 neurons are medium sized, spiny and projecting. The D1 neurons project into the substantia nigra pars reticularis (SNr) and globus pallidus internus (GPi), while the D2 neurons project into the globus pallidus externus (GPe). However, the co-expressing D1 and D2 neurons are found to make up 30.43% of total neurons in the patch/striosome, while being slightly less present in the matrix. These co-expressing neurons in both patch and matrix regions of the tree shrew striatum probably send their terminals to the substantia nigra, and are involved in both direct and indirect pathways of the basal ganglia.

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References

1. Graybiel AM. Building action repertoires: memory and learning functions of the basal ganglia. *Curr Opin Neurobiol.* 1995;5:733-41.
2. Seema MV. Forty-five years of schizophrenia: personal reflection. *Hist Psychiatry.* 2006;67:363-73.
3. Toda M, Abi-Dargham A. Dopamine hypothesis of schizophrenia: making sense of it all. *Curr Psychiatry Rep.* 2007;9:329-36.
4. Gerfen C. Synaptic organization of the striatum. *J Electron Microscop Tech.* 1988;10:265-81.

5. Goldman-Rakic PS. Cytoarchitectonic heterogeneity of the primate neostriatum: Subdivision into island and matrix cellular compartments. *J Comp Neurol.* 1982;205:398-413.
6. Day-Brown J, Wei H, Chomsung R, Petry H, Bickford ME. 2010. Pulvinar projections to the striatum and amygdala in the tree shrew. *Front Neuroanat.* 2010;15:143. doi: 10.3389/fnana.2010.00143.
7. Rice MW, Roberts RC, Melendez-Ferro M, Perez-Costas E. Neurochemical characterization of the tree shrew dorsal striatum. *Front Neuroanat.* 2011; 5:1-17.
8. Bolam J, Izzo P. The postsynaptic targets of substance P-immunoreactive terminals in the rat neostriatum with particular reference to identified spiny striatonigral neurons. *Exp Brain Res.* 1988; 70:361-77.
9. Reinius B, Blunder M, Brett FM, Eriksson A, Patra K, Jonsson J, et al. Conditional targeting of medium spiny neurons in the striatal matrix. *Front Behav Neurosci.* 2015;9:71. doi: 10.3389/fnbeh.2015.00071.
10. Seeharach K, Days-Brown J, Tocharus C, Bickford ME, Quiggins R. Synaptic organization of dopamine input in striatum of tree shrew, *Tupaia glis* Proceedings of the anatomy association Thailand 35th AAT Annual Conference. 2012; 98.
11. Fejer J, Crossman A. Identification of different subpopulations of neostriatal neurons projecting to globus pallidus or substantia nigra in the monkey: a retrograde fluorescence double-labeling study. *Neurosci Lett.* 1984;49:7-12.
12. Wang H, Deng YP, Reiner A. In situ hybridization histochemical and immunohistochemical evidence that striatal projection neurons co-containing substance P and enkephalin are overrepresented in the striosomal compartment of striatum in rats. *Neurosci Lett.* 2007;425:195-9.
13. Deng, YP, Lei WL, Reiner A. Differential perikaryal localization in rats of D1 and D2 dopamine receptors on striatal projection neuron types identified by retrograde labeling. *J Chem Neuroanat.* 2006; 32:101-16.
14. Surmeier DJ, Ding J, Day M, Wang Z, Shen W. D1 and D2 dopamine-receptor modulation of striatal glutamatergic signaling in striatal medium spiny neurons. *Trends Neurosci* 2007;30:228-35.

การแสดงออกร่วมกันของเซลล์ประสาท D1 และ D2 ในสไตรเอตัมของกระแต (*Tupaia glis*)

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สไตรเอตัมมีเซลล์ประสาท 2 ชนิด ได้แก่ เซลล์ประสาท D1 และเซลล์ประสาท D2 อยู่ในบริเวณสไตรโอโซมและแมทริกซ์ ตามลำดับ วัตถุประสงค์ในศึกษานี้เพื่อแสดงการกระจายตัวของเซลล์ประสาทสไตรเอตัม D1 และ D2 ในกระแตโดยวิธีฟลูออเรสเซนซ์ อิมมูโนฮิสโตเคมีสตรี้ โดยใช้แอนติบอดีต่อรีเซพเตอร์ D1, รีเซพเตอร์ D2 และซัสแตนท์ P แผ่นสมองถูกนำมาติดฉลากอิมมูโนด้วยแอนติบอดีต่อรีเซพเตอร์ D1 และซัสแตนท์ P จากนั้นนำมาย้อมสีทับเพื่อหานิวเคลียสด้วย DAPI พบว่าร้อยละ 91 ของเซลล์ประสาทติดฉลาก D1 ในสไตรเอตัมถูกใกล้ชิดด้วยปลายประสาทที่ติดฉลากด้วยซัสแตนท์ P ดังนั้น ซัสแตนท์ P ถูกใช้เป็นตัวบ่งชี้สำหรับเซลล์ประสาท D1 แผ่นสมองถูกนำมาแช่ในแอนติบอดีต่อซัสแตนท์ P และรีเซพเตอร์ D2 และจากนั้นย้อมสีทับสำหรับนิวเคลียสด้วย DAPI ผลการศึกษาพบว่ามีร้อยละ 88 ของจำนวนเซลล์ประสาททั้งหมดในบริเวณสไตรโอโซมถูกใกล้ชิดด้วยปลายประสาทติดฉลากด้วยซัสแตนท์ P และร้อยละ 30 เป็นเซลล์ประสาทติดฉลาก D2 ที่ถูกใกล้ชิดด้วยปลายประสาทติดฉลากซัสแตนท์ P ส่วนบริเวณแมทริกซ์มีร้อยละ 98 เป็นเซลล์ประสาทติดฉลากด้วย D2 และร้อยละ 29 เป็นเซลล์ประสาทติดฉลากด้วย D2 ที่ถูกใกล้ชิดด้วยปลายประสาทติดฉลากด้วยซัสแตนท์ P สรุปได้ว่า ประมาณร้อยละ 30 ของเซลล์ประสาทสไตรเอตัมทั้งบริเวณสไตรโอโซมและ/หรือแมทริกซ์แสดงออกทั้งรีเซพเตอร์ D1 และ D2 เซลล์ประสาทที่แสดงออกทั้งรีเซพเตอร์ D1 และ D2 ร่วมกันเหล่านี้น่าจะส่งเอาท์พุทแตกต่างไปจากเอาท์พุทของเซลล์ประสาท D1 หรือเซลล์ประสาท D2 **เชียงใหม่ เวชสาร 2560;56(3):115-23.**

คำสำคัญ: สไตรเอตัม เซลล์ประสาท D1 ซัสแตนท์ P เซลล์ประสาท D2 กระแต