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Abnormal Expression of Neuropeptide Y in the Cerebellar Purkinje Cells of the Ataxic Mutant Mice, *Pogo*

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The *pogo* mouse is an autosomal recessive ataxic mutant that arose spontaneously in the inbred *KJR/MsKist* strain derived originally from Korean wild mice. The ataxic phenotype is characterized by difficulty in maintaining posture and the consequent inability to walk straight. In our previous study about pogo mice cerebellum, we reported the Purkinje cell abnormalities and ectopic expression of tyrosine hydroxylase (TH) in Purkinje cell.

In this study, we have provided an abnormal expression of NPY in ataxic mutant pogo mice for the first time. There was increased immunoreactivity for NPY in Purkinje cell of ataxic pogo (*pogo/pogo*) mice compared to those of heterozygote non-ataxic pogo mice (*pogo/+*, control group). In our previous study, TH is also expressed abnormally in Purkinje cells of ataxic mutant pogo (*pogo/pogo*) mouse cerebellum. To compare the expression patterns of TH and NPY within some Purkinje cell using double immunofluorescence, most of NPY-immunoreactive Purkinje cells in the ataxic pogo mice are TH-immunoreactive Purkinje cells. However, all of TH-immunoreactive Purkinje cells are not express the NPY. These data reveal that abnormal NPY-immunoreactivity in the ataxic pogo (*pogo/pogo*) cerebellum is restricted to a subset of cells within the ectopic TH-immunoreactive Purkinje cell subset. These results further suggest that Purkinje cell abnormalities contribute to motor ataxia in the ataxic pogo mouse.

Key words : *Pogo* mouse, Cerebellum, NPY, Tyrosine Hydroxylase, Purkinje cell

Introduction

Neuropeptide Y (NPY) is a 36 amino-acid neurotransmitter/neuromodulator abundantly expressed in many areas of the mammalian brain (Allen et al. 1983, De-Quidt et al. 1986). NPY exerts several biological functions in the central and peripheral nervous system. These functions include the activation of feeding behavior (Clark et al. 1984), regulation of circadian rhythm (Albers and Ferris, 1984), modulation of memory (Flood et al. 1987), inhibition of anxiogenic activity (Heilig et al. 1994). Resently, NPY coexists with GABA in neurons of dentate hilus and it inhibits the excitatory neural transmission in the hippocampus. Moreover, NPY is supposed as one of the endogenous anticonvulsive substance (Colmers et al. 1988).

Although much is known concerning the distributions of NPY in hypothalamus, amygdaloid complex, olfactory bulb, striatum, and neocortex, but little is

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known about NPY expression in cerebellum. The presence of NPY-immunoreactivity has been described in the adult cerebellum, in specific olivocerebellar compartments and in a small number of mossy fibers (Ueyama et al. 1994), and its expression in the olivocerebellar system is up-regulated during the second postnatal week (Morara et al. 1997). For this reason, NPY and its receptor were participated in the postnatal development of cerebellum (Neveu et al. 2002).

The *pogo* mouse is an autosomal recessive ataxic mutant that arose spontaneously in the inbred *KJR/MsKist* strain derived originally from Korean wild mice (Hyun et al. 2001). The *pogo* mutation is inherited as a trait on chromosome 8 (Jeong et al. 2000, Hyun et al. 2001). The ataxic phenotype is characterized by difficulty in maintaining posture and side to side stability, faulty coordination between limbs and trunk, and the consequent inability to walk straight (Hyun et al. 2001). In our previous study about pogo mice cerebellum, we reported the Purkinje cell abnormalities (Jeong et al. 2000) and ectopic expression of tyrosine hydroxylase (TH) in Purkinje cell (Jeong et al. 2001).

This study was undertaken to clarify whether NPY had any relations to expression of ataxia. We examined the expression of NPY in pogo mice cerebellum and topological relationship compared to the ectopic TH expression by immunohistochemical and double immunofluorescence technique.

Material and Methods

1. Animals

Mice used in this study came from a breeding colony maintained at the KRIBB (Korea Research Institute of Bioscience and Biotechnology, Daejun) and housed in a constant room temperature (23˚C), constant humidity (45～50%), with a 12 h light : dark cycle and access to food and water *ad libitum*. Adult (all 6 month-old) homozygote pogo (*pogo/pogo*, n = 10) and non-ataxic littermates (heterozygote, *pogo*/+, n = 10) mice were used in this experiment. Non-ataxic littermates were used as controls. All experimental procedures were carried out in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals.

2. Tissue preparations

All animals were deeply anaesthetized with sodium pentobarbital (60 mg/kg body weight) and transcardinally perfused with 0.9% NaCl in 0.1M phosphate buffer (PB, pH 7.4) followed by 100 mL of Bouin's solution without acetic acid. Cerebella were removed and immersed in the same fixative for 4 hours. Postfixed cerebella were dehydrated, clearing (with xylene), embedded in paraffin and sectioned serially in the coronal plane at 5 μm. Sections were deparaffinized with xylene and dehydrated with alcohol series. Dehydrated sections were irradiated with microwave for 5 min. in 10 mM citrate buffer (pH 6.0) and processed for immunohistochemistry.

3. Immunohistochemistry

The tissue sections of each series were then incubated in 0.1 M PBS containing 10% normal goat serum (Jackson ImmunoRes., West Grove, PA) for 60 min. and then left in 0.1 M PBS containing the anti-NPY (1 : 1,000, Incstar, Stillwater, USA) for 16～18 hours at 4˚C. Sections were then washed three times for 15 minutes each in 0.1 M PBS and incubated in 0.1 M PBS containing biotinylated anti-mouse and anti-rabbit IgG (1 : 200, Vector, Burlingame, CA) for 1 hour at 4˚C. After incubation in secondary antibody, the section were washed 3 times for 15 minutes in 0.1 M PBS and the reacted with the Vectastain ABC elite kit (1 : 200, Vector, Burlingame, CA) for 1 hour. This was followed by 3 × 10 min. washed in 0.1 M PBS. These immunoreactive products were visualized with

0.01% 3, 3′−diaminobenzidine tetrachloride (DAB, Sigma, USA) in 0.03% H_2O_2 as a chromogen.

To confirm the specificity of the various primary antiserum, tissue sections were incubated in various primary antiserum combined with various blocking concentrations of the primary antibody at 4℃ for 18 hours and then processed as described above.

4. Double immunofluorescence

Cerebellar sections for fluorescence immunohistochemistry were washed for 15 minutes each in 0.1 M PBS (pH 7.4). The tissue was then incubated in PBS containing 10% normal goat serum (Jackson ImmunoRes., West Grove, PA) for 60 minutes and incubated in PBS containing a combination of the primary antibodies [anti−NPY (1 : 1,000) and anti−TH (1 : 10,000)] for 16～18 hours at 4℃. Following incubation in primary antibodies, sections were washed for 15 minutes in 0.1 M PBS buffer and then left in PBS containing Cy3−conjuagted goat anti−rabbit secondary antibody and Cy2−conjugated donkey anti−mouse secondary antibody (both at 1 : 1,000) for 24～48 hours at 4℃. After incubation in secondary antibody, the sections were washed for 15 minutes in 0.1 M PBS, mounted onto poly−L−lysine coated slides, air−dried overnight, cleared in 0.1 M PBS, and coverslipped with nonfluorescing mounting medium (Fluorsave Reagent, Calbiochem, La Jolla, CA).

5. Image capture and Photography

Immunohistochemistry and double immunofluorescence was quantified using a computer−based image analysis system, which included a Leica digital camera (DC−100), connected to an IBM computer, mounted on a Leica DMR light microscope. Images of the immunostained cerebellar sections were captured using a Leica capturing program. The digital signals from the camera were converted into grayscale digital images of 741 × 540 pixels. Selected sections were assembled in Adobe Photoshop 6.0. Images were cropped and adjusted for brightness and contrast but otherwise unmanipulated.

Result

1. Anti−NPY immunohistochemistry in pogo mice cerebellum

In this study, we observed NPY−immunoreactivity in the ataxic homozygous pogo (*pogo*/*pogo*) mice cerebellum (Fig. 1A, 1C, 1E, 1G, 1I, 2A) but in the heterozygous pogo (*pogo*/＋) mice (Fig. 1B, 1D, 1F, 1H, 1J, 2B). NPY−immunoreactivity was conspicuous in a significant subpopulation of cerebellar Purkinje cells in *pogo*/*pogo* mice (Fig. 1A, 1C, 1E, 1G, 1I). These NPY−immunoreactive Purkinje cells were present in all lobules of the cerebellar vermis and hemispheres in both anterior (Fig. 1A, 1C) and posterior lobes (Fig. 1E, 1G, 1I), and dispersly distributed in coronal sections. In section through the cerebellum of the ataxic pogo (*pogo*/*pogo*) mice immunoperoxidase stained for NPY, reaction product is deposited in perikarya of Purkinje cells, but not in the dendrites (Fig. 2A). In sections through the cerebellum of the littermate control of *pogo*/＋, a few faintly stained NPY−immunoreactive Purkinje cells were observed in the posterior vermal lobules of the cerebellum (Fig. 1D, 2B).

2. Anti−TH and anti−NPY double immunofluorescence in pogo mice cerebellum

Double immunofluorescence demonstrated excellent correspondence between the patterns of distribution of Purkinje cell immunoreactivity for the two antigens. The boundaries between immunoreactive and non−immunoreactive Purkinje cells coincided in both anterior and posterior lobes of the vermis and hemispheres. Double immunofluorescence [TH (Fig. 3A, 3D, 3G, 3J)

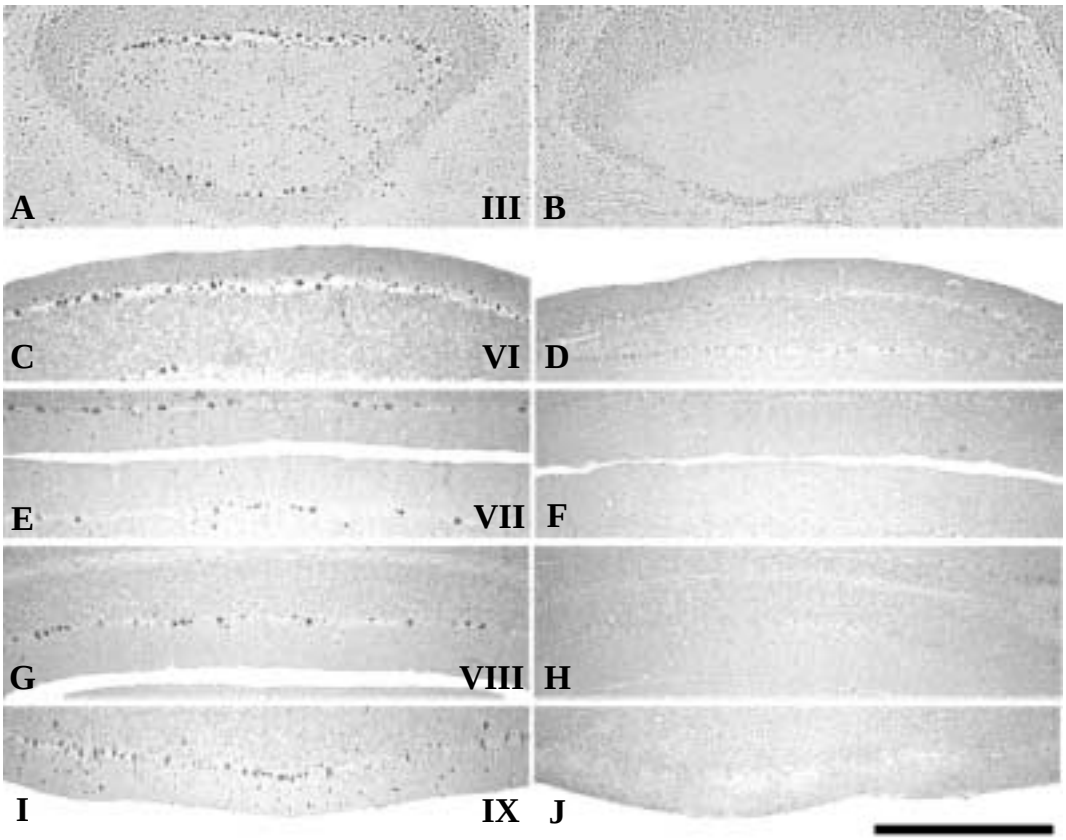

Fig. 1. Distribution of NPY-immunoreactive Purkinje cells in the cerebellum of ataxic homozygote pogo (*pogo*/*pogo*) mice [A, C, E, G, I] and littermate control of *pogo*/+ mice [B, D, F, H, J]. NPY-immunoreactivity was observed in the Purkinje cells of ataxic pogo mice. Scale bar = 250 μm.

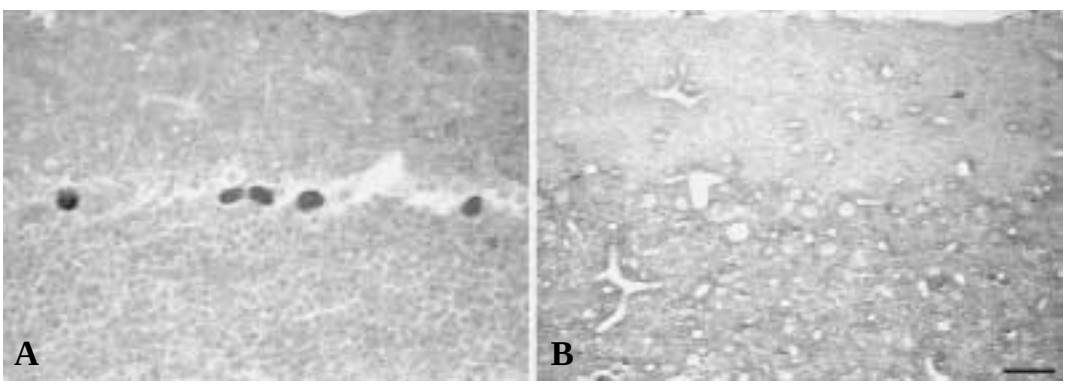

Fig. 2. High magnification of NPY-immunoreactive Purkinje cells in the cerebellum of ataxic homozygote pogo (*pogo*/*pogo*) mice (A) and littermate control of *pogo*/+ mice (B). NPY-immunoreactivity was observed in the perikarya of Pukinje cells of ataxic pogo mice. Scale bar = 50 μm.

and NPY (Fig. 3C, 3F, 3I, 3L)] in *pogo*/*pogo* mouse cerebellar sections revealed the characteristic pattern of TH-immunoreactive Purkinje cells, but no clear banding pattern could be observed for TH fluorescence and NPY fluorescence in the same sections. TH-immunoreactive Purkinje cells were observed and appeared to be somewhat more numerous than NPY-immunoreactive Purkinje cells in same sections (Fig. 3).

Within the TH-immunopositive and NPY-immunoreactive Purkinje cells in *pogo*/*pogo* cerebellum, double fluorescence revealed that most of NPY-immunoreactive Purkinje cells are TH-immunoreactive Purkinje cells (Fig. 3B, 3E, 3H, 3K). However, all of TH-immunoreactive Purkinje cells are not expressed the NPY (Fig. 3). Based on the facts that have been investigated so far, NPY-immunoreactive Purkinje cells are also TH-immunoreactive but represent only a subset of the TH-immunoreactive Purkinje cells.

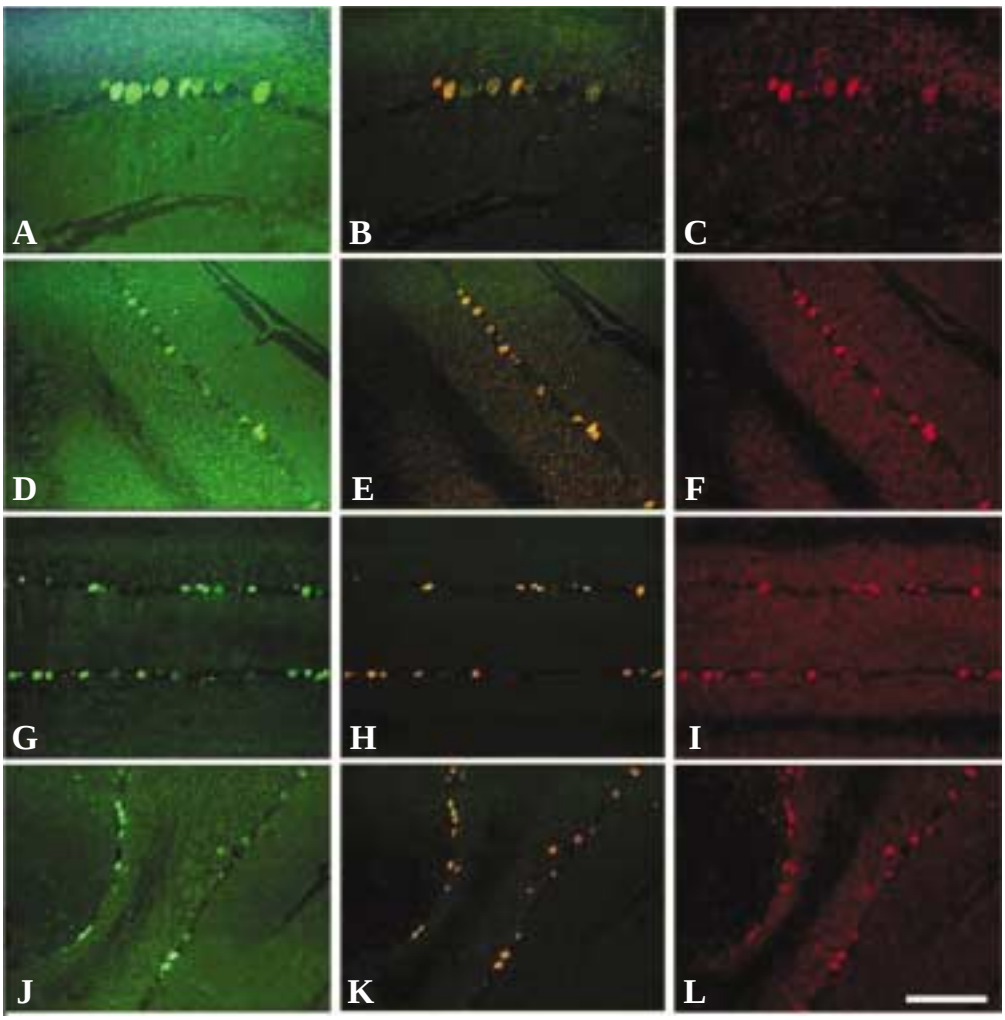

Fig. 3. Coronal sections of double-immunofluorescence stained for TH (green color, A, D, G, J) and NPY (red color, C, F, I, J) in ataxic pogo mice cerebellum. All TH-immunoreactive Purkinje cells are showed a NPY-immunoreactivity but not all NPY-immunoreactive Purkinje cells express TH (orange color, B, E, H, K). Scale bar = 200 μm.

Discussion

NPY is a member of the pancreatic polypeptide (PP) family and shows homology to both PP and peptide YY (Tatemoto et al. 1982). NPY has a widespread distribution in the mammalian central nervous system (Adrian et al. 1983, Allen et al. 1983, Hu et al. 1987, Busch-Sorenson et al. 1989). In the rodent, the highest concentrations of NPY are found in the hypothalamus, pituitary and pineal gland. Moderate concentrations are found in the olfactory bulb, septum, striatum, thalamus and pons (Chronwall et al. 1985). Negligible amounts were detected in the cerebellum (De Quidt and Emson, 1986). The presence of NPY-immunoreactivity has been described in the adult cerebellum, in specific olivocerebellar compartments and in a small number of mossy fibers (Ueyama et al. 1994).

In this study, we have provided a ectopic expression of NPY in ataxic mutant pogo mice for the first time. There was increased immunoreactivity for NPY in Purkinje cell of ataxic pogo (*pogo*/*pogo*) mice compared to those of heterozygote non-ataxic pogo mice (*pogo*/+, control group). In our previous study, TH is also expressed abnormally in Purkinje cells of ataxic mutant pogo (*pogo*/*pogo*) mouse cerebellum. We have reported this observation to show a selective, highly reproducible pattern of ectopic TH expression in parasagittal bands of Purkinje cells of the ataxic mutant pogo (*pogo*/*pogo*) mouse (Jeong et al. 2002). However in this study, NPY-immunoreactive Purkinje cell was not forming the parasagittal bands. To compare the expression patterns of TH and NPY within some Purkinje cell, sections through the ataxic pogo vermis were double immunofluorescence labeled by using anti-TH and anti-NPY. In same regions, TH-immunoreactive and NPY-immunoreactive Purkine cells are very similar. Most of NPY-immunoreactive Purkinje cells in the ataxic pogo mice are TH-immunoreactive Purkinje cells. However, all of TH-immunoreactive Purkinje cells are not expressed the NPY. Based on the facts that have been investigated so far, NPY-immunoreactive Purkinje cells are also TH-immunoreactive but represent only a subset of the TH-immunoreactive Purkinje cells.

Abnormal TH expression in Purkinje cells has appeared in cerebellum of several ataxic mutant mice (Sawada et al. 1990, Austin et al. 1992, Abbott et al. 1996), including pogo (Jeong et al. 2002), and is implicated in the onset and/or development of ataxia (Sawada et al. 1999). Since the transcription of the TH gene is facilitated by Ca^{2+}, TH expression in the mutant Purkinje cells indicates functional abnormality by alterations in intracellular Ca^{2+} concentration (Kilbourne et al. 1992, Nagamoto-Combs et al. 1997).

Recently, there are a number of reports previously studied NPY in epilepsy mouse (Takahashi et al. 1997, Reibel et al. 2001, Vezzani et al. 2002). In the Noda epileptic rat (NER), increased contents of NPY-immunoreactivity were found in the striatum and amygdale of 8-week NERs with partial seizure (Takahashi et al. 1997). Also they reported an elevated Corticotropin-releasing factor (CRF)-immunoreactivity in NER. The mechanism why they showed an increased expression for NPY and CRF is unclear. In ataxic *pogo* mice cerebellum, we observed an elevated expression of CRF (in press) and NPY. These data are consistent with previous study with NER (Takahashi et al. 1997). NPY treatment increased the CRF mRNA and the CRF peptide level (Sheriff et al. 1998). These studies suggest that NPY upregulated the CRF gene. However, the molecular basis of NPY induced upregulation of the CRF gene is not known. It is possible that NPY mediated increase in CRF mRNA may also be through the CREB pathway (Sheriff et al. 1998).

In this study, we have now extended our new finding of abnormal expression of NPY in Purkinje cell of *pogo* mice cerebellum. These data reveal that abnormal NPY-immunoreactivity in the ataxic pogo (*pogo*/*pogo*) cerebellum is restricted to a subset of cells with in the ectopic TH-immunoreactive Purkinje cell sub-

set. These results further suggest that Purkinje cell abnormalities contribute to motor ataxia in the ataxic pogo mouse.

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선천성 운동실조 Pogo 마우스 소뇌 조롱박세포의 비정상적 NPY 발현

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간추림 : 본 연구는 한국산 야생마우스에서 유래된 선천성 운동실조 마우스인 *pogo* 마우스 소뇌에서 비정상적으로 발현되는 neuropeptide Y (NPY)를 면역조직화학염색법으로 확인하고, *pogo* 마우스 소뇌를 포함한 여러 운동실조 마우스 조롱박세포에서 비정상적으로 발현되는 tyrosine hydroxylase (TH)과의 발현차이를 이중형광면역염색으로 비교 확인하기 위하여 시행하였으며 다음과 같은 결과를 얻었다.

대조군으로 사용한 heterozygote pogo (*pogo*/+) 마우스 소뇌에서는 소수의 NPY-면역반응 조롱박세포들이 소뇌후엽에서 관찰되었다. 이에 반하여, 운동실조 pogo (*pogo*/*pogo*) 마우스 소뇌에서는 NPY-면역반응 조롱박세포들이 소뇌반구와 소뇌벌레의 모든 소엽에서 관찰되었다. NPY-면역반응 조롱박세포에서 NPY-면역반응은 조롱박세포의 세포체에서만 관찰되었으며 세포돌기인 가지돌기와 축삭에서는 관찰되지 않았다. 이전에 본 연구자들이 보고한 바 있는 운동실조 pogo 마우스 소뇌의 비정상적인 TH발현과의 차이를 확인하기 위하여 동일 절편에서 TH와 NPY 이중면역형광염색을 실시하여 그 차이를 확인하였다. 대부분의 NPY-면역반응 조롱박세포는 TH-면역반응성을 보였지만, 모든 TH-면역반응 조롱박세포는 NPY-면역반응을 보이지 않기에 NPY-면역반응 조롱박세포들은 TH-면역반응 조롱박세포들의 부분집합인 것으로 보인다. 이상의 결과들은 선천성 운동실조 마우스 소뇌 조롱박세포의 형태적이상과 운동실조와의 상관관계를 밝히는 기초자료로 유용할 것으로 생각된다.

찾아보기 낱말 : *Pogo* 마우스, 소뇌, NPY, tyrosine hydroxylase, 조롱박세포

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