Tuberoinfundibular peptid 39 and its receptor in the central nervous system of rodent, macaque and human

Ph.D. thesis

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1.   Introduction

Parathyroid hormone receptor 2 (PTH2 receptor), which shows 70% sequence homology to the parathyroid hormone (PTJ) receptor is expressed in the central nervous system especially in diencephalon and the brain stem. Previous studies confirmed the existence of the endogenous ligand of the PTH2 receptor, which is the recently identified tuberoinfundibular peptid of 39 residues (TIP39). Based on rodent studies the PTH2 receptor is widely expressed in the brain, while TIP39 expressing neurons are located only in two areas, in the thalamus and the rostral pons.

In the thalamus, TIP39 expressing neurons are present in the subparafascicular area of the thalamus (SPF) divided into two cell groups. One group of thalamic TP39 neurons is located in the periventricular gray of thalamus (PVG) along the 3rd ventricle, the other more caudally and laterally in the parvicellular SPF and the intralaminar complex of the thalamus (PIL). Thalamic TIP39 neurons receive neuronal input from the hypothalamus and limbic structures. The main efferentation from the SPF-PVG group targets the limbic system, while the SPFp-PIL neurons´ main projections are towards the hypothalamus.

The other TIP39 cell group is in the ponto-mesencephalic junction, medial to the lateral lemniscus where cells form a well circumscribed nucleus which is called the medial paralemniscal nucleus (MPL). The afferentation of MPL comes from the auditory system and the hypothalamus, the efferentation targets the auditory system and the spinal cord. There are interconnecting neuronal pathways between the thalamic and pontine TIP39 cells.

In contrast to the TIP39 cells, PTH2 receptor expressing cells are widely distributed in the subcortical areas based on rodent studies using reverse transcription PCR, in situ hybridization, immunohistochemistry and transgenic mice. Dense PTH2 receptor expression was found in limbic structures, the hypothalamus, certain areas of the thalamus, brainstem and even in the spinal cord. In rats, the PTH2 receptor expressing cell bodies are detectable using immunohistochemistry and the topography is consistent with the in situ hybridization results. In contrast, in mice, only the PTH2 receptor immunopositive fibers can be visualized using immunostaining, although the PTH2 receptor expressing cell bodies were identified by

in situ hybridization histochemistry. We found similar findings in our primate studies suggesting the existence of a fast axonal transport of the PTH2 receptor peptide from the perikaryon to the axon terminals. Previous rodent studies revealed the glutamatergic nature of the PTH2 receptor positive terminals.

The PTH2 receptor shows a constant expression in the brain throughout the lifespan while TIP39 is abundantly expressed only in the early postnatal life, and rapidly decreases after puberty and reactivates only under special physiological conditions.

The physiological role of the TIP39-PTH2 receptor system is not well understood yet. In TIP39 knockout mice, increased anxiety and depression-like behavior patterns were observed. Intracerebroventricular administration of TIP39 leads to increased tolerance of nociceptive stimuli, increase of corticotropin releasing hormone (CRH) and inhibition of growth hormone (GH) release. Rat studies suggest that the stress response to auditory stimuli including CRH release is mediated by TIP39 cells. Recent investigations in dam rats revealed that the TIP39 cells in the SPFp-PIL and MPL are induced during lactation and participate in the maternal adaptation.

2.   Objectives

1.1.   TIP39 expressing neurons during embryonic and early postnatal life in rats

The ontogenesis of TIP39 neurons during postnatal life has been described in a detailed rodent study. TIP39 cells were found in newborn rats in both known locations, the subparafascicularis area of the thalamus (SPF) and the medial paralemniscal nucleus (MPL) The TIP39 expression showed a marked increase until the 14th postnatal day, and started to decline after the 33rd postnatal day, and became barely detectable by the 125th postnatal day. The decrease during the period of puberty is more pronounced in male animals. The transient expression pattern of TIP39 suggests that the peptide may have a role in the ontogenesis and reproduction. To better understand the ontogenesis of the TIP39 cell groups we investigated the expression pattern of TIP39 during embryonic and early postnatal period.
In our study we addressed the further questions:

1) When does TIP39 appear during the embryonic life?

2) Is there any difference in the expression pattern of the thalamic (SPF) and pontine (MPL) TIP39 neuron groups?

3) Can we observe the compartmentalization of the SPF TIP39 cells during embryonic life into the PVG and PIL subdivisions?

4) Is there any area of TIP39 expression, which is not known in adult rats?

1.2.   TIP39-PTH2 system in the primate brain

In contrast to rodents, where the TIP39-PTH2 receptor system has been described, no data in human or primates has been published. The only available information was that the genes of TIP39 and PTH2 receptor were present in the human genome. To better understand the potential physiological or pathophysiological role of TIP39 in human we investigated the TIP39-PTH2 receptor system in the primate brain (macaque and human). Since the TIP39-PTH2 receptor system harbors a pronounced hypothalamic representation, we addressed its anatomical connections to the hypothalamo-hypophyseal axis, and its role in the neuroendocrine regulation. According to published data, TIP39 increases the secretion of ACTH via CRH release, and decreases the secretion GH via somatostatin. We wanted to find the morphological background of these effects.

We addressed the following questions in primate studies:

1) Is TIP39 expressed in the primate brain? What is the topographical distribution of TIP39 cells?

2) Is the PTH2 receptor expressed in the primate brain? What is the topographical distribution of PTH2 receptor expressing structures?

3) Is there any difference regarding the expression pattern of the TIP39-PTH2 receptor system between rodents, macaque and human?

4) Are we able to confirm the glutamatergic nature of PTH2 receptor immunopositive fibers?

5) Is there any direct anatomical connection between the TIP39-PTH2 receptor system and the hypothalamo-hypohyseal axis, especially regarding the regulation of CRH and somatostatin release?

6) What is the potential physiological role of the TIP39-PTH2 receptor system according to our and previously published data?

3.   Materials and methods

All procedures involving rats were carried out according to experimental protocols approved by the Animal Examination Ethical Council of the Animal Protection Advisory Board at the Semmelweis University, Budapest and were conducted in accordance with international standards on animal welfare as defined by the European Communities Council Directive of 24 November 1986 (86/609/EE) and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Macaque tissue collection and all procedures were performed according to protocols approved by the Animal Care and Use Committee of the National Institute of Mental Health following ethical review, and in accordance with the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and conformed to international guidelines on the ethical use of animals.

Human brain samples were collected in accordance with the Ethical Rules for Using Human Tissues for Medical Research in Hungary (HM 34/1999) and the Code of Ethics of the World Medical Association (Declaration of Helsinki). Tissue samples were taken during brain autopsy at the Department of Forensic Medicine of Semmelweis University in the framework of the Human Brain Tissue Bank, Budapest, or at the Department of Pathology of the University of Pécs, as approved by institutional ethics committees of the Semmelweis University or the University of Pécs. The medical history of the subjects was obtained from medical or clinical records, interviews with family members and relatives, as well as from pathological and neuropathological reports. All personal identifiers had been removed and samples coded before the analyses of tissue.

Animal experiments and histochemical studies were performed in the Institute of Anatomy, Histology and Embryology of the Semmelweis University Medical School in collaboration with Dr. Ted B. Usdin (NIH) regarding macaque studies.

We used standardized, previously published methods to demonstrate TIP39 expressing cells in the rat embryo and for the mapping of TIP39 and PTH2 receptor expression and immunoreactivity in human and macaque brain. Some of the methods (e.g. TIP39 immunostaining in human brain) did not work properly. The methods providing reproducible and demonstrative results in our studies are listed in the table below.

rat	TIP39	immunocytochemistry
	TIP39	double immunostaining (CGRP)
	TIP39	in situ hybridization
macaque	TIP39	in situ hybridization
	PTH2R	immunocytochemistry
	PTH2R	double immunostaining (VGLUT)
	PTH2R	in situ hybridization
human	PTH2R	RT-PCR
	PTH2R	immunocytochemistry
	PTH2R	double immunostaining (CRH, SS)

Similar to mice, no PTH2 receptor immunopositive cell bodies, but only fibers were detectable using immunohistochemistry in the human brain. Because of this similarity, we used mice data instead of rats for comparison in the primate PTH2 receptor mapping studies. The fibers were demonstrated using immunolabeling while the perikarya were localized by detecting PTH2 receptor mRNA. The results obtained using identical methods were compared in mice and primates.

Two different methods were used to detect PTH2 receptor mRNA in primates: RT-PCR in humans and in situ hybridization in macaque. In situ hybridization did not work in human samples, most likely due to the degradation of mRNA. The RT-PCR samples were obtained from a 56 years old, and a 89 years old subject, while the macaque tissue was collected from a 3 days old animal. Since the expression of the PTH2 receptor was reported to be independent from age and gender in rodent, we compared the human and monkey data to each other.

Prior to the RT-PCR assays, the mRNA quality was checked for each human sample to eliminate the possibility that mRNA degradation in the postmortem period leads to a negative signal. In addition, the presence of the housekeeping gene GAPDH was shown in each sample using a lower cycle number to demonstrate that mRNA was efficiently transcribed into cDNA. In contrast, a high cycle number was used for the PTH2 receptor so that even a small amount of mRNA would be detected. A negative control sample without RNA was used to exclude the possibility of contamination at either the RNA or the cDNA level. The reproducibility of our data was also confirmed when nine samples of the same brain regions were analyzed from two different human brains. Indistinguishable results were obtained for six samples while three samples produced clear bands from both brains that differed in their intensity.

Regarding the PTH2 receptor expressing cells, the comparison between human and macaque was done by two different methods (RT-PCR and in situ hybridization histochemistry). These results were then compared to previously published mice data.

The expression of TIP39 is the most intense in the thalamic SPFp-PIL region immediately after the birth, TIP39 mRNA in situ hybridization experiments were carried out in the brain of a 3 day old macaque – there were no human samples that young available. The same brain was used for PTH2 receptor mRNA in situ hybridization studies. The brain of a 9 year old monkey, sacrificed for other research purposes was used in PTH2 receptor immunolabeling studies. Based or rat studies we know that the expression of the PTH2 receptor is independent on gender and age, this made possible to compare the data from two monkeys of different age, and so we were able to minimize the use of macaques in the study. Three separately developed different anti-TIP39 antibodies were tested in human, but immunolabeling did not work for either one in human samples. In contrast, PTH2 receptor immunostaining resulted in demonstrative visualization of the PTH2 receptor positive fiber network. Therefore, it was possible to compare the results to previous mice PTH2 receptor immunohistochemistry data.

4.   Results

4.1. A TIP39 expression in embryonic and early postnatal age in rats

Our results showed marked differences between the thalamic and pontine TIP39 areas as to their expression of TIP39 during embryonic age. The medial paralemniscal nucleus (MPL) showed TIP39 expression already at embryonic day 14.5 (ED-14.5), but in the medial part of the SPF (SPF-PVG) no TIP39 appeared until ED-20.5.

Even the SPF itself, which is divided into a medial periventricular (SPF-PVG) and a caudal lateral, parvicelullar part in the posterior intralaminar complex of the thalamus (SPFp-PIL), showed inhomogeneous expression pattern during the embryonic life. We demonstrated the TIP39 cells appeared in the PIL from ED-14.5, the most intensive expression was found on ED-16.5, and after that, it decreased gradually until postnatal day 5 when it reached the very low level of adult animals. In contrast, in the medial SPF_PVG area, TIP39 immunoreactivity appeared only on ED-20.5, and the immunolabeling was getting more pronounced until postnatal day 5. All these differences between the time course of TIP39 expression between the PVG and PIL TIP39 cell groups are consistent with the theory that these neurons form two distinct subdivisions, with different neuronal connections, regulation and development.

We discovered a new TIP39 positive cell group in the amygdalo-hippocampal transitional zone at ED-16.5. These neurons disappeared during early postnatal life, and we could not detect them in adult animals.

An important finding of our developmental study was the transient appearance of TIP39 expression, which was observed in two cases: 1. There is an early appearing, marked expression in the pSPF-PIL, which significantly decreases during early postnatal life. 2. Transient expression of TIP39 takes place during embryonic life in the amygdalo-hippocampal transitional zone, which disappears after the birth. The transient appearance of TIP39 immunolabeling in both cell bodies and fiber network suggest that TIP39 may have a role in the ontogenesis. There is no evidence available at present, whether there is a degeneration process which affects TIP39 neurons, or the neurons migrate from PIL to PVG, or only the expression of TIP39 ceases. The topography of TIP39 neurons, their

intense hypothalamic connections and the transient activity suggest some role of these neurons in the processes of reproduction.

4.2. **The expression of TIP39 and PTH2 receptor in the primate brain**

4.2.1. TIP39 expressing neurons in the brain of macaque

We demonstrated TIP39 expressing neurons in a 3 day old macaque brain using in situ hybridization. TIP39 cells were found in two locations, identical as described in rodents: the rostral medial (SPF-PVG) and the caudal lateral (SPFp-PIL) division of the subparafascicular area of the thalamus and the medial paralemniscal nucleus in the pons (MPL). We were not able to detect TIP39 mRNA in human samples, which can be explained in two ways: 1. According to published data, the expression of TIP39 markedly decreases during postnatal life, and reactivates only under special physiological circumstances (e.g. lactation). The human mRNA was isolated from the brains of aging subjects. 2. There were no specific TIP39 containing areas among the samples obtained using micropunch technique. Considering that the PTH2 receptor expressing cell bodies, and the PTH2 receptor immunolabeled fibers show extreme homology between rodents, human and non-human primates, we suppose, that the distribution of TIP39 cells is similar to that found in rodents and macaque.

4.2.2. PTH2 receptor expressing neurons in the brain of human and macaque

The expression of PTH2 receptor was shown using RT-PCR in human, and in situ hybridization in macaque. We confirmed wide distribution of PTH2 receptor mRNA in the central nervous system. PTH2 receptor expression was found in the cortex and especially in the septum pellucidum in human. While the human amygdala showed only moderate expression using RT-PCR, in situ hybridization studies in macaque showed high expression level in the medial and central amygdaloid nuclei. The difference can be explained with the larger size of the human tissue sample obtained using micropunch technique, which might have contained the other, PTH2 receptor non-expressing lateral and basal amygdaloid nuclei as well.

Both methods resulted in high expression levels in the diencephalon and the brainstem: in the medial hypothalamus, the medial geniculate body and the pontine tegmentum. In

contrast, no evidence of PTH2 receptor expression was shown in the ventral and mediodorsal thalamic nuclei, and in the pulvinar. A low expression level of the PTH2 receptor was found in the lateral geniculate body, subthalamic nucleus, ventral tegmental area, and the pontine formatio reticularis. No expression was found in the mesencephalon: in the substantia nigra and the red nucleus. In summary, the human RT-PCR and the monkey in situ hybridization date are very similar. There is only one single location where we found marked interspecies difference. This is the praetectal area, which showed high expression in human using RT-PCR, but no expression in macaque by in situ hybridization histochemistry. The different age of subjects or real interspecies differences may explain this finding.

4.2.3. Comparison of PTH2 receptor mRNA expression in the brain of rodents and primates
PTH2 receptor expression, demonstrated using RT-PCR in human is very similar to that found in mice. High expression levels were found in the septum, caudate nucleus, medial geniculate body, hypothalamus, pontine tegmentum and the cerebellum, while a low level of expression was found in the cortex, hippocampus, amygdala, lateral geniculate body, ventral tegmental area, the pontine formatio reticularis, the dorsal vagal complex and spinal trigeminal nucleus. No expression of the PTH2 receptor was found in most of the thalamic nuclei, the suprachiasmatic nucleus, the mamillary body, the substantia nigra, the red nucleus and the motor nuclei of the cranial nerves. All these brain regions show very precise correlation of our results with mice PTH2 receptor mRNA data. We were able to perform more detailed comparison using in situ hybridization histochemistry. We found remarkable correlation between the distributions of the PTH2 receptor in mouse and macaque brain even at the subregional level (e.g. within the hypothalamus and the medial geniculate body). Differences were shown only in two areas, the ventral part of the lateral hypothalamic area and the parabigeminal nucleus, where we found well defined PTH2 receptor expressing cells in macaque, but only scattered cells in mouse. This may represent an interspecies expressional difference.

4.2.4 PTH2 receptor immunopositivity – fibers and axon terminals in primates
According to our results, human PTH2 receptor expressing cell bodies do not show immunolabeling – due to the supposed rapid axonal transport – so PTH2 receptor immunostaining outlines only the fiber networks of these neurons. Dense network of PTH2

receptor positive fibers was found in the endocrine hypothalamus, the medial preoptic area, the paraventricular and arcuate nuclei, and in the median eminence. Pronounced immunostaining was shown in the septum, the paraventricular nucleus of the thalamus and the periaqueductal gray. PTH2 receptor positive fibers were visible in somatosensory and viscerosensory areas, the trigeminal sensory nuclei, the nucleus tractus solitarii, and the lateral parabrachial nucleus. Only hypothalamic regions were investigated in macaque, but the results were identical to human data. Human studies were performed on samples collected from diencephalic and brain stem areas. These data were almost homologous to the mice data based on PTH2 receptor immunolabeling studies.

4.2.5. The supposed machanism of action of TIP39 on the hypothalamo-hypophyseal axis

The glutamergic nature of PTH2 receptor immunoreactive fibers was described previously in rats. Double immunolabeling studies done in macaque brain showed dense VGLUT-2 immunoreactive network in the septum and the hypothalamus. Some of these fibers showed also PTH2 receptor immunolabeling, but all of the PTH2 receptor positive terminals were stained with anti-VGLUT-2 antibody. This colocalization suggests that the PTH2 receptor terminals in the septum, hypothalamus and supposedly in other brain areas harbor glutamatergic nature – similar to rodents.

Double immunolabeling was performed in human hypothalamus using anti-PTH2 receptor and anti-somatostatin or anti-corticotropin releasing hormone (CRH) antibodies. Somatostatin secreting cells are located in the periventricular nucleus projecting to the median eminence where somatostatin is released, reaches the pituitary via portal vessels and inhibits growth hormone (GH) secreting cells. Some of the somatostatin containing terminals in the median eminence showed co-localization with the PTH2 receptor suggesting that TIP39 acts to facilitate somatostatin release via the PTH2 receptor and to inhibit growth hormone secretion from the pituitary.

Our data suggest another mechanism in the regulation of CRH secretion. The CRH positive cells in the paraventricular hypothalamic nucleus did not co-localize with PTH2 receptor, but the PTH2 receptor immunoreactive terminals apposed closely the CRH immunoreactive perikarya. CRH cell bodies did not contain PTH2 receptor according to our results, but they are innervated by excitatory glutamatergic synapses, and some of these synapses showed

PTH2 receptor immunoreactivity. We suppose that the TIP39 modulates CRH secretion via these glutamatergic terminals as it was shown previously in hypothalamic tissue.

Our results can provide the anatomical background for the previously described actions of the TIP39-PTH2 receptor neuromodulator system on the hypothalamo-hypophyseal axis. Both ways of action of TIP39 on the hypothalamus suggests presynaptic modulation by directly facilitating somatostatin release in the median eminence, and indirectly, via excitatory glutamatergic synapses increasing CRH secretion of hypothalamic paraventricular neurons. Our hypothesis of axono-axonal presynaptic modulation is also supported by our findings that PTH2 receptor protein is rapidly transported towards the axon terminals, and that the subregional distribution of TIP39 and PTH2 receptor positive axons is very similar.

4.3. Summary

We first demonstrated the transient expression of the TIP39 in rat embryos suggesting a role of TIP39 in the ontogenesis and reproductive processes. We demonstrated that TIP39 cells in thalamic SPF are divided into two subdivisions, which suggest separate functions of the 2 groups of TIP39 neurons.

In primate studies we first demonstrated the presence of the TIP39-PTH2 receptor neuromodulator system, showing the expression of the PTH2 receptor and TIP39 mRNAs and the mapping of the PTH2 receptor immunoreactivity. The topography found in primates is very similar to that in rodents suggesting that the TIP39-PTH2 receptor system has similar role in the regulation of endocrine, reproductive and viscerosensory functions in humans as well. Based on our results in the human hypothalamus we suppose that the TIP39-PTH2 receptor neuropeptide system acts on the hypothalamo-hypophyseal system via presynaptic modulation.

5. Conclusions

a. The expression of TIP39 in rats starts on embryonic day 14, first in the pontine medial paralemniscal nucleus (MPL), the caudal lateral, parvicellular part (SPFp-PIL) of the SPF, and in the amygdalo-hippocampal transition zone. We first

described the presence of TIP39 expression in the amygdala, which is detectable only during the embryonic life.

b. The early appearance of TIP39 expression in the subparafascicular area is localized in the caudal lateral, parvicellular part (SPFp-PIL) of the SPF. Neurons in the medial part of the PSF (SPF-PVG) start to express TIP39 only later, at the end of the embryonic life. TIP39 expression in the SPF-PVG persists during early postnatal life, but in the SPFp-PIL TIP39 the expression significantly decreases immediately after birth, and reactivates only under special circumstances (e.g. lactation) according to published data. The developmental difference between the two TIP39 expressing compartments of SPF argues for the discrimination of the SPFp-PIL and SPF-PVG cell groups, which is also supported by previous connectional and functional studies.

c. We first confirmed the presence of TIP39 and PTH2 receptor in the central nervous system of human and macaque. TIP39 neurons are located in two areas of the primate brain: in the thalamic subparafascicular area, where the cells form two compartments, the SPFp-PIL and SPF-PVG cell groups, and in the pontine MPL. Pronounced PTH2 receptor expression is present in the septum, caudate nucleus, medial geniculate body, hypothalamus, pontine tegmentum and in the cerebellum.

d. Detailed mapping revealed strong homology in the expression pattern of PTH receptor mRNA and immunoreactivity between primate (human and macaque) and rodent brain, even in subregional level.

e. We confirmed the glutamatergic nature of PTH2 receptor immunoreactive fibers in the primate hypothalamus.

f. Our studies on the CRH and somatostatin secreting cells of human hypothalamus suggest that the TIP39 neuropeptide acting on the PTH2 receptor influences the CRH and somatostatin production via presynaptic modulation. Our results provide the anatomical background of this theory and explain the hormonal responses to TIP39 administration published previously.

6. List of publications

6.1. Publications related to the theses

1. Varga T, Mogyoródi B, **Bagó AG**, Cservenák M, Domokos D, Renner É, Gallatz K, Usdin TB, Palkovits M, Dobolyi A: Paralemniscal TIP39 is induced in rat dams and may participate in maternal functions. Brain Struct Funct 217(2):323-325, 2012, IF: 4,982

1. **Bagó AG**, Dimitrov E, Saunders R, Seress L, Palkovits M, Usdin TB, Dobolyi A.: Parathyroid hormone 2 receptor and its endogenous ligand tuberoinfundibular peptide of 39 residues are concentrated in endocrine, viscerosensory and auditory brain regions in macaque and human. Neuroscience 162(1):128-47, 2009, IF: 3,292

2. **Brenner D***, **Bagó AG***, Gallatz K, Palkovits M, Usdin TB, Dobolyi A: Tuberoinfundibular peptide of 39 residues in the embryonic and early postnatal rat brain, J. Chem. Neuroanat. 36(1):59-68, 2008, IF: 2,12, <u>*The first two authors contributed to the manuscript equally.</u>

3. **Bagó AG**, Palkovits M, Usdin TB, Seress L, Dobolyi A: Evidence for the expression of parathyroid hormone 2 receptor in the human brainstem. Ideggyogy. Sz./Clin. Neurosci. 61(3-4):123-6, 2008

6.2. Abstracts related to the theses

1. **Bagó A**, Dobolyi Á**,** Dimitrov E, Saunders R, Seress L, Palkovits M, Usdin TB: A parathormon-2-receptor (PTH2R) és ligandjának (TIP39) kimutatása emberi és majom agyban.A Magyar Anatómus Társaság 15. Kongresszusa, Parathyroid hormone 2 receptor and its endogenous ligand TIP39 in the brain of macaque and human. 15th Conference of Hungarian Society of Anatomists, Budapest, Hungary, 2009

2. Dobolyi Á, **Bagó AG**, Seress L, Usdin TB, Palkovits M: Parathyroid hormone 2 receptor in the human brain − a combined RT-PCR and fluorescent amplification immunocytochemistry study. BrainNet Europe 2nd International Conference on Human Brain Tissue Research, J. Neural Transm. 115(12), 1730., Munich, Germany, 2008

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2. Palkovits M, Harvey-White J, Liu J, Kovacs ZS, Bobest M, Lovas G, **Bagó AG,** Kunos G: Regional distribution and effects of postmortal delay on endocannabinoid content of the human brain, Neuroscience 152(4):1032-9, 2008, IF:3.35

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