QUANTITATIVE *IN VITRO* AUTORADIOGRAPHY OF
NEUROTRANSMITTER RECEPTORS
—APPLICATIONS TO DISEASED HUMAN BRAINS—

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The technique of *in vitro* autoradiography is widely utilized for observations of specific binding sites of neurotransmitters or drugs. The current trend of this research field is toward quantifying receptor densities in autoradiograms of brain sections. In this paper, the authors showed the method for quantification in our laboratory and confirmed that receptor densities obtained from *in vitro* autoradiography almost consisted with those from receptor binding assay. Since *in vitro* autoradiography can construct high-resolution maps of receptor distributions, it is the useful tool for studies of neural functions.

The authors performed the quantitative *in vitro* autoradiography in diseased human brains. Two cases of late cortical cerebellar atrophy (LCCA) were used in these experiments. Through morphometric analysis, it was observed that Purkinje cells in both case 1 and 2 were lost in half as many as those in control cases and the density of the granular cells was markedly reduced in the cerebellar cortex of case 2. In the dentate nucleus of case 1, the increase of GABA receptors as well as the decrease of GABA concentration was observed and this increase is considered to be receptor supersensitivity. In addition, the reduction of glutamate binding sites was observed in the dentate nucleus of both cases. Neurobiological basis of this receptor change is left for further investigations.

The technique of *in vitro* autoradiography is widely utilized for observations of specific binding sites of neurotransmitters (15) or drugs (7, 9). The current trend of this research field is toward quantifying receptor densities in autoradiograms of brain sections. This is especially significant for studies of the regulation of neural functions by drugs, lesions, diseases and so on, since the maintenance of receptors on membranes is one of the most important cellular functions.

In this paper, changes of neurotransmitter receptors in brains of patients with spinocerebellar degeneration (SCD) were observed autoradiographically, and the results were analysed quantitatively. Of the various types of SCD, olivopontocerebellar atrophy (OPCA) is the most common. As for OPCA, alterations of GABA and other neurotransmitters and these receptors have been reported (4-6, 11, 12). The present study was focused on brains of late cortical cerebellar atrophy

(LCCA), a rarer type of SCD.

MATERIALS AND METHODS

Quantitative receptor autoradiography

The authors studied to what extent receptor densities obtained from *in vitro* autoradiography on cryostat sections could be consistent with those calculated from receptor binding assay using brain homogenate. The brain of Japanese monkey was sagittally bisected, and one hemisphere was used for receptor binding assay and the other one for *in vitro* macroautoradiography. The amounts of specific ^3H -QNB binding sites were determined by these two techniques and the results were compared.

Human brains of LCCA

Two cases of LCCA and neurologically indifferent cases were used for the present study (3). Of LCCA two cases, case 1 was a 66-year-old man who died in 1983 after a progressive illness lasting 8 years. On neurological examinations, he showed ataxic gait, dysmetria, dysdiadochokinesis, explosive speech and saccadic eye movement. Pathological diagnosis was compatible to cortical cerebellar atrophy. Case 2 died at the age of 84 years in 1984 after a progressive cerebellar disorder lasting 28 years. Postural tremor on hands, ataxic gait and dysarthria were gradually developed and slowly progressed. There were familial involvements. Pathologically, Purkinje cell loss and proliferation of Bergmann's glia without apparent changes in the brainstem were observed. Autopsied human brains were sagittally bisected into hemispheres, and one hemisphere was used for histopathological observations and the other one for *in vitro* macroautoradiography of neurotransmitter receptors and biochemical quantitation of neurotransmitters, since it is very important to discuss densities of receptors in relation with cell numbers and neurotransmitters.

The *in vitro* autoradiographic technique was the one originally established by Young and Kuhar (20). GABAA-, glutamate- and aspartate binding sites were observed using ^3H -muscimol, ^3H -glutamate and ^3H -aspartate as radioactive ligands, respectively. We performed saturation experiments of these ligands on cryostat sections of the rat cerebellum and decided optimal experimental conditions for autoradiography (Table 1). In order to quantify autoradiograms, sections were co-exposed with autoradiographic [^3H] micro-scale (Amersham company) as standard. The standard contains various known amounts of radioactivity. Obtained autoradiograms were computer-analysed by IBAS II of Zeiss company.

RESULTS

Quantitative receptor autoradiography

Fig. 1 shows the relationship between optical density (OD), the absorption of light passed through the exposed film, and the level of radioactivity. The plastic containing eight different concentrations of tritium were apposed to a film (a Sakura macroautoradiograph film) for one month. The standard curve makes it possible

FIG. 1. Autoradiographic film response to increasing levels of radioactivity. The optical density was linearly related to the level of radioactivity.

to calculate the molar quantities of bound ligands according to the equation of Unnerstall *et al.* (14). Table 2 shows the comparison of specific ^3H -QNB binding sites obtained from both receptor binding assay and autoradiography. These two values almost consisted with each other.

Human brains of LCCA

The results of morphometric analysis are shown in Table 3. Purkinje cells in two LCCA cases were lost in half as many as those of control cases. The density of granule cells in case 1 was similar to that of normal control cases, while a marked reduction by more than 50% was observed in case 2. Cells in the inferior olive and pontine nucleus were preserved in two cases.

Glutamate, aspartate and GABA concentrations in the cerebellar areas and lower brainstem are shown in Table 4. Glutamate contents in LCCA cases, particularly in case 2, were lower than those of normal controls. The aspartate content in case 1 was within the normal range in the cerebellar areas and slightly increased in the inferior olive and pontine nucleus. In contrast, the aspartate content in case 2 was markedly reduced in all the regions. As for GABA concentration, the marked decrease in the dentate nucleus was observed in two cases.

As preliminary experiments of *in vitro* autoradiography, we performed saturation

TABLE 1. *Experimental conditions*

	Buffer	Ligand conc./ blank	Incubation (time/temp.)	Washing (time/temp.)
^3H -muscimol	Citrate buffer 0.31 M, pH 7.1	10 nM/0.2 mM GABA	30 min/25°C	1 min/25°C
^3H -aspartate	Tris HCl 50 mM, pH 7.4 +300 mM NaCl	600 nM/1 mM aspartate	20 min/4°C	5 min $\times$ 2/0°C
^3H -glutamate	Tris HCl 50 mM, pH 7.4 +2.5 mM CaCl_2	300 nM/1 mM glutamate	60 min/25°C	10 sec/0°C

TABLE 2. Specific binding of ^3H -QNB (fmol/mg protein)

	RRA	ARG
cerebral cortex	397.1	381.2
caudate n.	577.9	592.8
putamen	661.5	606.4
hippocampus	357.1	354.8

RRA: radioreceptor assay

ARG: autoradiography

TABLE 3. Neuronal cell density in the cerebellar cortex and the brainstem nuclei (Kanazawa et al.)

	Purkinje cells (cells/mm)	Granule cells ($\times 10^6$ cells/mm ³)	Inferior olivary nuclear cells (cells/mm ³)	Pontine nuclear cells (cells/mm ³)
controls	5,542 $\pm$ 1,075	2,254 $\pm$ 0.181	10754 $\pm$ 1675	18209 $\pm$ 2995
LCCA 1	2,440	2,002	11700	11020
LCCA 2	2,091	0,979	9450	18040

experiments on cryostat sections of the rat cerebellum. Fig. 2 shows the result of saturation experiments of ^3H -aspartate binding and Table 5 shows Kd and Bmax values of used ligands. Each of these three ligands had a saturable and single binding site.

Autoradiograms of ^3H -aspartate binding sites in the human cerebellum are shown in Fig. 3. In the cerebellum of case 2, ^3H -aspartate binding sites were markedly decreased. From these autoradiograms, receptor densities were calculated by above-mentioned method and the results are shown in Table 6. ^3H -aspartate binding sites of case 1 were almost within the normal range in all the brain regions. In contrast, those of case 2 were decreased in all the brain areas, in particular in the cerebral cortex, cerebellar cortex and dentate nucleus. Table 7 shows the regional

TABLE 4. Concentration of glutamate, aspartate and GABA in the cerebellum and the brainstem nuclei (Kanazawa et al.)

	glutamate			aspartate			GABA		
	controls	LCCA 1	LCCA 2	controls	LCCA 1	LCCA 2	controls	LCCA 1	LCCA 2
Anterior vermis	5.84 $\pm$ 0.43	3.98	1.07	1.70 $\pm$ 0.11	1.67	1.27	0.82 $\pm$ 0.14	0.91	0.94
Posterior vermis	6.21 $\pm$ 0.57	5.90	3.32	2.43 $\pm$ 0.21	2.29	0.98	1.10 $\pm$ 0.24	1.55	1.07
Cerebellar hemisphere	5.76 $\pm$ 0.32	4.27	3.77	2.03 $\pm$ 0.16	1.99	0.44	0.72 $\pm$ 0.13	0.87	0.97
Dentate nucleus	6.23 $\pm$ 0.84	6.42	3.60	2.05 $\pm$ 0.22	2.70	0.73	6.40 $\pm$ 0.77	0.99	0.74
Inferior olive	7.30 $\pm$ 1.35	5.76	2.82	1.45 $\pm$ 0.42	2.40	0.98	1.48 $\pm$ 0.19	1.38	1.57
Pontine nucleus	8.35 $\pm$ 0.78	7.80	3.63	1.70 $\pm$ 0.42	2.57	0.96	0.18 $\pm$ 0.06	0.47	0.16

Values are expressed as $\mu\text{mol/g}$ wet weight.

FIG. 2. The saturation curve and Scatchard plots of ^3H -aspartate binding sites. ^3H -aspartate binding revealed a single binding site whose K_d value was 670 nM.

FIG. 3. The autoradiographic distribution of ^3H -aspartate binding sites in the human cerebellum. ^3H -aspartate binding sites existed in the cerebellar cortex and dentate nucleus of normal control cases. In contrast, they were markedly decreased in the cerebellum of LCCA 2.

TABLE 5. *Kd and Bmax values of the used ^3H -ligands when applied to cryostat sections*

	Kd (nM)	Bmax (fmol/section)
^3H -muscimol	6.1	62.4
^3H -aspartate	670	710
^3H -glutamate	370	650

TABLE 6. *Regional distributions of ^3H -aspartate binding sites*

regions	specific binding (pmol/mg protein)			
	control 1	control 2	LCCA 1	LCCA 2
cerebral cortex	2.6	2.8	4.4	1.4
putamen	2.8	5.7	4.7	2.0
caudate n.	2.3		5.2	1.9
globus pallidus	1.1	1.4	1.6	0.8
hippocampus	3.9	4.3		2.9
cerebellar cortex	4.5	4.1	4.3	1.7
dentate n.	3.6		3.8	0.7

TABLE 7. *Regional distributions of ^3H -glutamate binding sites*

regions	specific binding (pmol/mg protein)			
	control 1	control 2	LCCA 1	LCCA 2
cerebral cortex	0.51	0.38	0.45	0.29
putamen	0.54	0.80	0.53	0.53
caudate n.	0.52		0.36	0.45
globus pallidus	0.53	0.38	0.35	0.37
cerebellar cortex	0.45	0.70	0.70	0.12
dentate n.	0.32		n. d.	n. d.

n.d.: not detectable

TABLE 8. *Regional distributions of ^3H -muscimol binding sites*

regions	specific binding (pmol/mg protein)			
	control 1	control 2	LCCA 1	LCCA 2
cerebellar cortex				
granular layer	0.77	0.80	0.83	0.56
molecular layer	0.40	0.35	0.39	0.37
dentate n.	n. d.	n. d.	0.18	n. d.

n.d.: not detectable

distribution of ^3H -glutamate binding sites. The way of changes of ^3H -glutamate binding sites in LCCA cases was almost the same as those of ^3H -aspartate binding sites. However, ^3H -glutamate binding sites were decreased to the white matter level in the dentate nucleus of case 1. Table 8 shows the regional distribution of

^3H -muscimol binding sites. ^3H -muscimol binding sites were increased in the dentate nucleus of case 1 and decreased in the cerebellar granular layer of case 2.

DISCUSSION

In vitro autoradiographic techniques have been only recently begun to be applied to the study of neurodegenerative disorders (17). Alterations of some neurotransmitter system play important roles in pathophysiology of these diseases. Although the neurobiological basis of diseases is complicated, it is significant to know specific patterns of neural dysfunctions in a given disease. Using quantitative autoradiography, one can map out neurotransmitter receptor distributions with a higher degree of anatomical resolution than radioreceptor assay. In this paper, the authors confirmed that quantification of *in vitro* autoradiograms was good for determining an absolute value of receptor numbers. Through this method, we will be able to find out abnormal cerebral functions hitherto undescribed in various diseases.

GABA is considered to be the inhibitory neurotransmitter of the cerebellar Purkinje cells as well as 3 interneurons, that is the basket, stellate and Golgi cells. Aspartate and glutamate have been implicated as excitatory neurotransmitters in the mammalian central nervous system. From many lesion studies, it is suggested that corticostriate (1, 8), corticothalamic, corticospinal (19), perforant (16) and hippocampal commissural (10) pathways and cerebellar granular cells (18) are probably glutaminergic. In addition, aspartate is likely to be the neurotransmitter of corticofugal fibers and climbing fibers from the inferior olive (13). It is reported that in the cerebral cortex both glutamate and aspartate are released by stimulation of the reticular formation (2). As for significance of the changes in glutamate receptors in the dentate nucleus, it is not easy to give an unequivocal answer, since there is no nerve terminals known to contain glutamate in this area. Such mismatches between neurotransmitters and receptors are known to exist for virtually all neurotransmitter-receptor systems. An interpretation may be possible that receptors are not necessarily associated with nerve terminals, including the view that some may represent the existence of transmitter-receptor interactions between non-contiguous cells.

In the dentate nucleus of case 1, the increase of GABAA receptors may be due to denervation supersensitivity of receptors resulting from the decreased GABA concentration. In contrast, the density of GABAA receptors in the dentate nucleus of case 2 was not increased, although GABA concentration was equally decreased. Differences of the duration of the illness, that is the passage of time, may cause the dissimilar behavior of GABAA receptors. The mechanisms of the decrease of both aspartate- and glutamate binding sites remain to be further investigated.

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