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Immunohistochemical Study of Neuronal Changes in the Hippocampus and Cerebellum of Intoxication and Drug-related cases

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Abstract

Background: Drug abuse is spreading to controlled substances and also to pharmaceuticals. There are many drug problems in society today. In forensic autopsy cases, drugs such as methamphetamine, amphetamine, illegal herbal products, and psychotropic drugs are occasionally detected. Many of these drugs are drugs that act on the central nervous system (CNS).

Aims: To clarify drug-induced neuronal injury histologically is important for forensic autopsy as well as for clinical medicine. This study examines neuronal changes in the hippocampus and cerebellum immunohistochemically in intoxication and drug-related cases.

Materials and Methods: Fourteen drug-related cases were selected from forensic autopsy cases within 48 hours of the postmortem interval. The hippocampus and cerebellum were observed with Hematoxylin-eosin and Luxol fast blue. Immunohistochemical staining was performed using antibodies against Microtubule-associated protein 2 (MAP2), Glucose transporter 5 (GLUT5), Neuronal nuclei (NeuN), Heat shock protein 70 kDa (Hsp70), and Glial fibrillary acidic protein (GFAP). In the hippocampus, neurons were observed at each sector, including the hilus, CA3, CA2, CA1, and the subiculum (SUB). The granule cell layer and molecular layer were also observed. In the cerebellum, the

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Purkinje cell layer, granule layer, and molecular layer were observed. Neurons of the dentate nucleus were also examined. All cases had their blood alcohol concentration measured. A drug screening test was performed and the detected drugs were analyzed quantitatively.

Results: In the hippocampus, immunoreactivities to MAP2, GLUT5, NeuN, Hsp70 and GFAP decreased sequentially in CA2 and CA1 pyramidal cells than in CA3. In the cerebellum, the positive rate of Purkinje cells with MAP2 and GLUT5 were 28.6% and 64.3%, respectively, and that of Hsp 70 was 42.9%.

Conclusion: Drug-induced neuronal injury was examined immunohistochemically. It was considered that CA2 and CA1 pyramidal cells in the hippocampus and Purkinje cells in the cerebellum might be damaged by drug ingestion. This tendency was thought to be particularly pronounced for neuroexcitatory/stimulant drugs. Further clarification of the actual state of drug-induced neuronal injury by the accumulation of target drugs and cases is required.

Keywords: neuronal changes, drug related forensic autopsy cases, immunohistochemistry, hippocampus, cerebellum

INTRODUCTION

Drug abuse is spreading to controlled substances and also to pharmaceuticals. There are many drug problems in society today. In forensic autopsy cases, drugs such as methamphetamine, amphetamine, illegal herbal products, and psychotropic drugs are occasionally detected. Many of these drugs are drugs that act on the central nervous system (CNS). To clarify drug-induced neuronal injury histologically is important not only for forensic autopsy, but also for clinical medicine.

This study examines neuronal changes in the hippocampus and cerebellum immunohistochemically in drug-related cases.

MATERIAL AND METHODS

Cases / Samples

Fourteen drug-related cases were selected from cases autopsied at Fukuoka University and the Tokyo Medical Examiner's Office within 48 hours of the postmortem interval. All cases were drug-related intoxication deaths or suspected as such (Table 1).

Two cardiovascular deaths were selected as controls and compared with the drug-related cases. The hippocampus and cerebellum were collected from those forensic autopsy cases.

Table 1. Summary of the examined cases

*M: male; F: female

Case No.	Age	Sex*	Causative substances
1	55	M	Methamphetamine
2	75	M	Methamphetamine
3	31	M	Methamphetamine/illegal herbal products
4	32	M	Illegal herbal products
5	31	M	Cocaine
6	27	M	Cocaine
7	40	M	Caffeine
8	21	M	Caffeine
9	19	F	Methanol
10	19	M	Ethanol
11	38	F	Toluene
12	45	F	Amlodipine, intoxicated with ethanol
13	90	F	Bleach (sodium hypochlorite)
14	37	M	Sevoflurane

Immunohistochemical examination

The hippocampus and cerebellum were fixed with 10% formalin neutral buffer solution for 3 weeks, then paraffin-embedded, and sliced into 3.5μm sections. Conventional histochemical staining was observed with Hematoxylin-eosin (**HE**) and Luxol fast blue (**LFB**).

Immunohistochemical staining was performed using antibodies against Microtubule-associated

protein 2 (**MAP2**) (1: 200, Abcam plc, UK), Glucose transporter 5 (**GLUT5**) (1: 200, Abcam plc, UK), Neuronal nuclei (**NeuN**) (1: 1000, Abcam plc, UK), Glial fibrillary acidic protein (**GFAP**) (1: 1000, Abcam plc, UK), and Heat shock protein 70 (**Hsp70**) (1: 100, Abcam plc, UK) with the EnVision Detection SystemsPeroxidase/DAB, Rabbit/Mouse (Dako, Japan), according to the manufacturer's instructions. MAP2, GLUT5, NeuN and Hsp70 were for neurons; GFAP was for astrocytes.

In the hippocampus, neurons were observed at each sector, including the hilus, CA3, CA2, CA1, and the subiculum (SUB). The granule cell layer and molecular layer were also observed. In the cerebellum, the Purkinje cell layer, granule layer, and molecular layer were observed. Neurons of the dentate nucleus were also examined. Immunoreactivities of each antibody were elucidated as positive or negative. "Positive" cases were designated as those in which several immune-positive neurons were observed. "Positive-minus" cases were those in which only a few neurons were positive, and "negative" had no stained immunoreactivity. "GFAP-positive" cases were those in which astrocytes were observed. Morphologically, neuronal degeneration, such as cell shrinkage, the reduction of cytoplasm with pyknosis, swelling, and loss, were observed. For astrocytes, the swelling of cytoplasm and fibrillary astrocytes were examined morphologically.

Alcohol and volatiles analysis

Screening and quantitative analyses for ethanol, other alcohols (methanol), and other volatiles (toluene) were performed using headspace gas chromatography with flame ionization detection (HS-GC-FID) on a QP-2010 Plus GC (Shimadzu, Kyoto, Japan). Separation was achieved on a Rtx-1 analytical column (60 m × 0.53 mm i.d., film thickness of 7.0 μm) (Restek, Bellefonte, PA, USA) (1). In our department, ethanol can be measured at a low concentration, with a limit of quantitation of 0.04 mg/mL.

Screening for alcohol and volatiles identified ethanol, methanol, and toluene from some of the cases.

Toxicological screening analysis

The screening of blood and urine samples was performed using gas chromatography-mass spectrometry (GC-MS) on a QP-2010 Ultra GC-MS (Shimadzu, Kyoto, Japan). Separation was achieved by fast GC-MS using tandem columns connected by a SilTite μ-Union (SGE Analytical Science Pty Ltd., Ringwood, Victoria, Australia). The tandem columns were a Rtx-200MS (2 m × 0.18 mm i.d., film thickness of 0.4 μm) (Restek, Bellefonte, PA, USA) followed by BPX-5 (3.9 m × 0.15 mm i.d., film thickness of 0.25 μm) (SGE Analytical Science Pty Ltd., Ringwood, Victoria, Australia). The injection

was performed in the splitless mode with a purge period of 0.4 min. The initial oven temperature was 80°C, held for 0.5 min, followed by a 70°C/min ramp to 200°C, and a 50°C/min ramp to 320°C that was held for 2 min. The helium carrier gas flow rate was 5.1 mL/min (390 kPa at 80°C) and the injection was performed at a high pressure of 570 kPa (13.9 mL/min, 0.4 min). The injector was set to 300°C, the interface to 320°C, and the ion source to 230°C (2,3).

Toxicological screening analyses on blood and urine sample identified methamphetamine, cocaine, illegal herbal products such as α-PHP, caffeine, amlodipine, bleach (sodium hypochlorite), and sevoflurane from some of the cases.

Quantitative analysis of drugs detected by screening

Quantitation was performed using either GC, GC-MS, or liquid chromatography – tandem mass spectrometry (LC-MS/MS) depending on the drug. For alcohols/volatiles, the method described in the "*Alcohol and volatiles analysis*" section was also used for quantitation. For bleach (sodium hypochlorite) and sevoflurane, the method described in section "*Toxicological screening analysis*" was used. For the remaining compounds (methamphetamine, illegal herbal products (α-PHP, etc.), cocaine, caffeine, amlodipine), quantitation was performed on a LCMS-8045 triple quadrupole LC-MS/MS (Shimadzu) using a Kinetex XB-C18 (100 mm x 2.1 mm, 2.6 μm, Phenomenex) column for separation. Two microliters of extract were injected into the LC system via autosampler. The mobile phase consisted of a gradient between 0.1% formic acid in water containing 10 mM ammonium formate (mobile phase A), and 100% methanol (mobile phase B). The gradient was 10% B, increased to 80% B at 6 min, held at 80% B for 4 min, and returned to 10% B for 5 min. The total run time was 15 min. The sample was introduced to the MS/MS by positive ESI. Detection of the target analytes was in the multiple reaction monitoring (MRM) mode. The MRM transitions and optimal collision energies for the target analytes were determined by direct injection of each methanolic standard at a concentration of 10 ng/mL. A deuterium compound or a compound with a similar chemical structure was used as internal standards (4-9).

RESULTS AND DISCUSSION

In the hippocampus, the granule cell layer, molecular layer, hilus, CA3, CA2, CA1, and SUB were observed. In the cerebellum, the Purkinje cell layer, granule layer, and molecular layer were also examined. An example of the immunohistochemical findings is shown in Fig. 1.

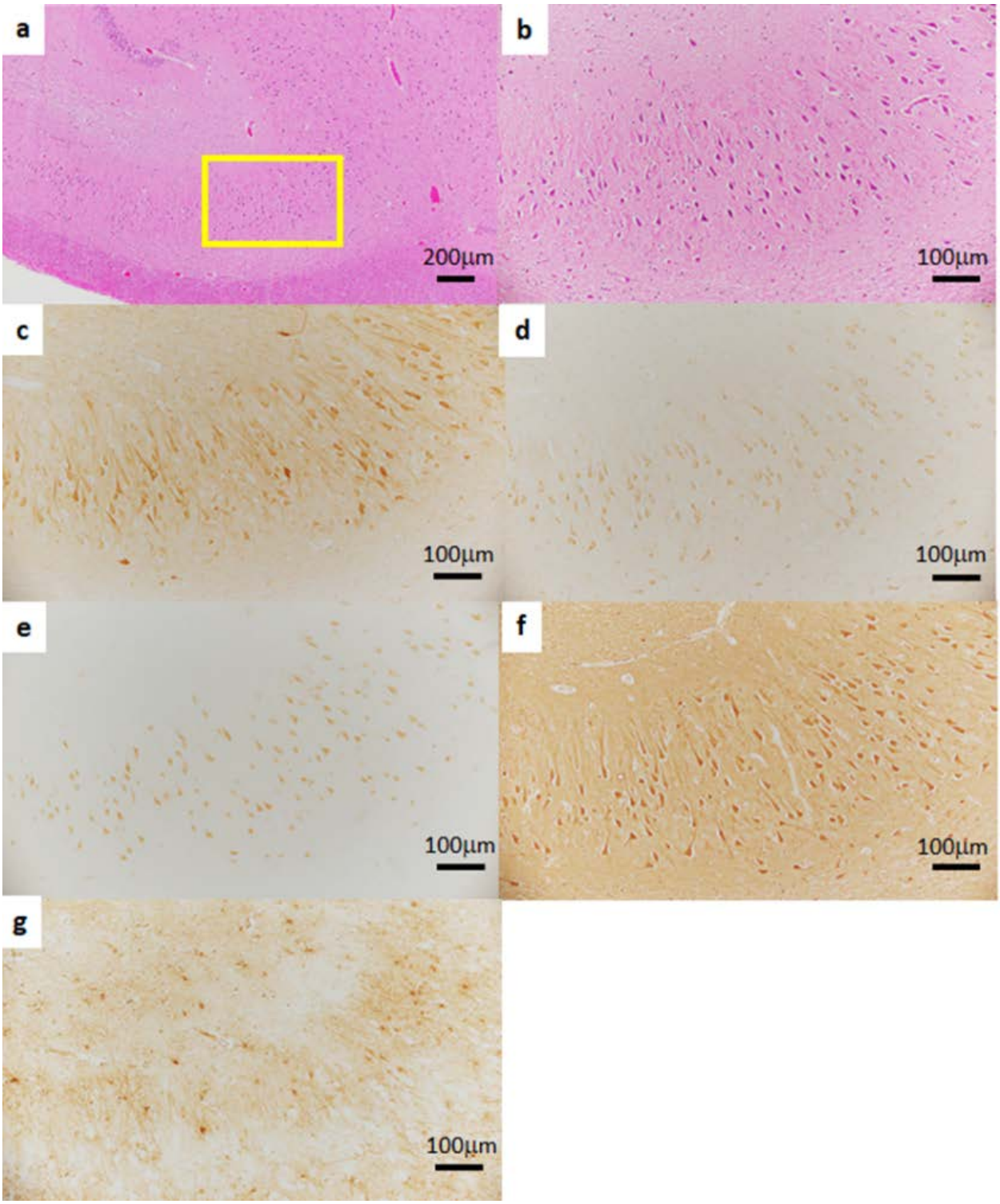

Figure 1. Immunohistochemical findings in the hippocampus: Case 5

(a) A part of the hippocampus
(b) - (g) An enlarged figure (marked yellow square of Fig.1a)
(a),(b): HE; (c): MAP2; (d): GLUT5; (e): NeuN; (f): Hsp70; (g): GFAP

Table 2. Immunopositive rate (%) of each marker in the hippocampus and cerebellum

Markers	Hippocampus							Cerebellum			
MAP2	Granule	Molecular	DG	CA3	CA2	CA1	SUB	Purkinje	Granule	Molecular	Dentate
Total	7.1	0.0	85.7	71.4	53.8	21.4	28.6	28.6	0.0	28.6	57.1
Group A	25.0	0.0	80.0	60.0	50.0	0.0	50.0	50.0	0.0	0.0	75.0
Group B	0.0	0.0	75.0	75.0	50.0	25.0	25.0	25.0	0.0	50.0	75.0
Group C	0.0	0.0	20.0	60.0	60.0	40.0	20.0	0.0	40.0	60.0	60.0
GLT5	Granule	Molecular	DG	CA3	CA2	CA1	SUB	Purkinje	Granule	Molecular	Dentate
Total	50.0	7.1	64.3	85.7	78.6	78.6	71.4	64.3	0.0	28.6	78.6
Group A	60.0	0.0	100.0	100.0	80.0	80.0	80.0	60.0	0.0	40.0	100.0
Group B	50.0	0.0	50.0	75.0	75.0	75.0	50.0	100.0	0.0	25.0	50.0
Group C	40.0	20.0	40.0	80.0	80.0	80.0	80.0	40.0	0.0	20.0	80.0
NeuN	Granule	Molecular	DG	CA3	CA2	CA1	SUB	Purkinje	Granule	Molecular	Dentate
Total	64.3	0.0	42.9	57.1	35.7	28.6	7.1	0.0	64.3	0.0	14.3
Group A	60.0	0.0	40.0	60.0	40.0	40.0	0.0	0.0	80.0	0.0	0.0
Group B	50.0	0.0	50.0	50.0	25.0	25.0	25.0	0.0	75.0	0.0	25.0
Group C	80.0	0.0	40.0	60.0	40.0	20.0	0.0	0.0	40.0	0.0	20.0
HSP70	Granule	Molecular	DG	CA3	CA2	CA1	SUB	Purkinje	Granule	Molecular	Dentate
Total	57.1	0.0	92.9	92.9	78.6	64.3	42.9	42.9	28.6	21.4	100.0
Group A	80.0	0.0	100.0	100.0	80.0	80.0	60.0	40.0	20.0	20.0	100.0
Group B	50.0	0.0	75.0	100.0	100.0	75.0	50.0	50.0	50.0	25.0	100.0
Group C	40.0	0.0	100.0	80.0	60.0	40.0	20.0	40.0	20.0	20.0	100.0
GFAP	Granule	Molecular	DG	CA3	CA2	CA1	SUB	Purkinje	Granule	Molecular	Dentate
Total	7.1	7.1	50.0	71.4	50.0	28.6	14.3	7.1	57.1	57.1	28.6
Group A	20.0	0.0	60.0	80.0	40.0	0.0	20.0	20.0	100.0	60.0	20.0
Group B	0.0	25.0	75.0	75.0	50.0	50.0	0.0	0.0	25.0	50.0	0.0
Group C	0.0	0.0	20.0	60.0	60.0	40.0	20.0	0.0	40.0	60.0	60.0

The immunoreactivity was calculated as the percentage of positive cases among all the drug-related cases.

Immunoreactivity of each antibody in the hippocampus

The immunoreactivities in granule cells with each antibody were positive in only a few neurons or negative. So, neuronal changes of granule cell layers in the hippocampus and cerebellum were

not suitable for observing immunohistochemically in drug-related cases. Granule cells are excitatory neurons responsible for output to the CA3 region (10)。 In the hippocampus, the positive rate of granule cells with GLUT5, NeuN, and Hsp 70 were 50.0%, 64.3%, and 57.1%, respectively. MAP2 and GLUT5 were both 7.1%. Immunoreactity of GLUT5 and NeuN showed that about 60% of granule cells were damaged, so it was considered Hsp 70 was expressed in a neuroprotective reaction. MAP2 are proteins of neuronal structure, but the immunoreactivities were low. So it was suspected that degeneration of the neuronal structure had occurred. The immunoreactivities with MAP2 were lower in CA3, CA2, and SUB. MAP2 is a microtubule-associated protein with almost specific localization in dendrites and cell bodies, and is often used as a cytoplasmic and dendrite marker (11); normal neurons are MAP2-positive. So, it was considered that neurons of CA3, CA2, and SUB were damaged in intoxication and drug-related cases.

GLUT5 is a fructose transporter and is also present in neurons (12). GLUT5 expression levels and fructose uptake rates are also significantly affected by diabetes, hypertension, obesity, and inflammation (12). The immunoreactivites of neurons were lowest in the hilus and the rate was 64.3%. In contrast, CA3, CA2,CA1, and SUB were all above 70%. Therefore, it was considered that damage by drugs was not remarkable with GLUT5.

NeuN is a marker found in the nucleus and cytoplasm of neurons (13), and is a tissue-specific splicing regulator RNA-binding Fox3 (Rbfox3) (14). The immunoreactivites to neurons in the hilus and CA3 were 42.9% and 57.1%, respectively, and that of CA2 and CA1 were both about 30%. The least reactive area was SUB and the rate was 7.1%. It has been shown that NeuN expression in neurons is decreased in cerebral ischemia (15). So, it might be that the mechanism of damage to neurons by drugs is similar to ischemia.

Hsp70 is a molecular chaperone and a family of heat shock proteins. Hsp70 helps protect cells from stress (16). The Hsp70-immunoreactivity to neurons in the hilus and CA3 were both above 90%, and that of CA2 and CA1 were 78.6% and 64.3%, respectively. The least reactive area was SUB and that immunoreactivity was 42.9%. So, it was considered that neurons in the hippocampus were damaged and Hsp70 was expressed.

GFAP is a type III intermediate filament protein that is expressed in astrocytes (17), and often used as an astrocyte marker. GFAP is proposed to play a role in astrocyte-neuron interactions as well as cell-cell communication (18). The rate of immunoreactivity with GFAP were decreasing in the order of CA3, CA2, CA1 and SUB, and that was similar to Hsp70. So, it was thought that GFAP was compatible with the damage of neurons.

Immunoreactivity of each antibody in the cerebellum

Purkinje cells form inhibitory synapses with GABA as a neurotransmitter to neurons in the cerebellar nucleus (dentate nucleus, etc.) on the white matter side. On the cortical side, Purkinje cells extend molecular layer dendrites and form inhibitory synaptic connections with astrocytes. Granule cells make excitatory synaptic connections with dendrite spinous processes of Purkinje cells (19).

NeuN is not naturally expressed in Purkinje cells (13). In this study, there were no NeuN-positive cases. So, we considered about the immunoreactivity to Purkinje cells among other antibodies. The positive rate of Purkinje cells with MAP2 and Hsp 70 were 28.6% and 42.9%, respectively. It was considered that from the result of MAP2, the Purkinje cells were damaged and Hsp 70 was expressed in a neuroprotective reaction. There were no immunoreactivities to granule cells with MAP2 and GLUT5. However, the positive rate of NeuN was 64.3% and that of Hsp 70 was 28.6%. These results were in contradiction with MAP2 and GLUT5, so the damage to the granule cells was difficult to explain. The positive rate of GFAP was 57.1%, so it might be considered that astrocyte-neuron interactions had occurred and there was damage to the neurons. In the molecular layer, the positive rates of MAP2, GLUT5 and Hsp70 were above 20%, and that of GFAP was 57.1%. So it might be considered that there was damage of neurons and the astrocytes reacted to them. It might also be considered that there was damage to the synaptic connections from the Purkinje cells.

In the dentate nucleus, the positive rate of Hsp70 was 100% with neurons. Therefore, it was considered that neuroprotective reaction was caused. In contrast with Hsp70, the positive rate of MAP2, GLUT5 and NeuN were 57.1%, 78.6%, and 14.3%, respectively. Except for NeuN, the result might mean neurons were protected.

Changes in drug-induced immunoreactivity in the hippocampus and the cerebellum

We considered about the relationship between the actions of drugs and neuronal changes. The 14 cases were divided into 3 groups based on the drug's effects on the nervous system. Group A is neuroexcitatory/stimulant drugs (n= 5); Group B is neuroinhibitor/depressors (n= 4); Group C is neuroexcitatory/stimulant drugs mixed with neuroinhibitor/depressors, or other drugs and toxic substances (n= 5). We compared the positive rates of the 14 cases and each group, and considered about the differences of neuronal changes between stimulant drugs and depressors (Table2).

Group A: In the hippocampus, MAP2 and GFAP were 0% in CA1 pyramidal cells of Group A, but of the 14 cases, these were 21.4% and 28.6%, respectively. It is well known that the pyramidal cells and Purkinje cells in CA1 are more vulnerable to ischemia than other pyramidal cells in the hippocampus and cerebellar neurons (20). The positive rate of Purkinje cells in the

cerebellum was 50% with MAP2, and that was higher than CA1 in the hippocampus. Moreover, the positive rate of granule cells in the cerebellum was 100% with GFAP. So, it might be thought that a neuroprotective reaction occurred to the Purkinje cells, and the cause of damage to the pyramidal cells in CA1 were ischemia and also the effect of stimulant drugs.

Group B: In the cerebellum of Group B, the positive rate of Purkinje cells was 100% with GLUT5, and that of granule cells was 50% with Hsp70. These rates were higher than those of the 14 cases in total. It might be considered that Purkinje cells are inhibitor neurons and there were neuroprotective reaction in granule cells, so the damage by the depressive drugs was lower.

CONCLUSION

Drug-induced neuronal injuries were examined immunohistochemically. It was considered that the CA2 and CA1 pyramidal cells in the hippocampus and Purkinje cells in the cerebellum might be damaged by drug intoxication. This tendency was thought to be particularly pronounced for neuroexcitatory/stimulant drugs. Continued research with further accumulation of target drugs and cases is required to clarify the actual state of drug-induced neuronal injury.

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Conflict of Interest Statement: The authors declare that they have no conflict of interest.

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