improved seizure control and resulted in no motor deficit after operation [9,10].

Although the number of inhibitory interneurons was decreased in the neocortex of FCD, very few studies of basal ganglia of FCD have been reported [11,12]. In the present study, we demonstrated the correlation of each interneuron subtype distribution in the neocortex and basal ganglia, using interneuron markers of GAD, CR, CB, PV and NPY. Our data may well provide a basic understanding of FCD epileptogenesis.

2. Materials and methods

2.1. Sample preparation

All cerebral tissues used in the present study were approved for research usage by the parents and ethical committees of the hospital and institute. Four cases of intractable epilepsy were selected for this study, with FCD and abnormal signals in the deep white matter on the imaging, and surgical resection of the lesions containing neocortex and deep cerebral structures. Their clinical features are summarized in Table 1. Average age of onset was 8.3 months of age, and age at surgical resection ranged from 3 months to 13 years. Studied samples were part of the tissues removed for therapeutic reasons after careful assessment of the epileptogenic areas, determined by analysis of seizure semiology, ictal and interictal electroencephalography, magnetoencephalography, electrocorticogram, MRI, fluorodeoxyglucose positron emission tomography and interictal single photon emission computed tomography. We then performed resection of the lesions including neocortex, white matter and deep brain structure. We pathologically evaluated the serial section including malformed cortex and the underlying caudate nucleus. The age-matched controls used were summarized in Table 1. After resection, all brains were fixed in 10% buffered formalin or 4% paraformaldehyde and embedded in paraffin. The serial sections were cut 4–6 μm thickness for histological and immunohistological examination. For pathological diagnosis, the sectioning tissues were stained by hematoxylin and eosin (HE) and Klüver–Barrera (KB) methods. The tissues diagnosed as FCD by two individual neuropathologists were classified by ILAE classification [7]. Two parts of the resection tissue, neocortex and basal ganglia, were analyzed on immunohistochemistry.

2.2. Immunohistochemistry

Our immunohistochemistry technique was previously described in detail [13]. The primary antibodies were incubated at 4 °C for 16–72 h. To investigate the presence of interneuron in the neocortex and caudate nucleus of the basal ganglia, we used the mouse monoclonal antibodies against Calretinin (CR, clone5A5, dilution of 1:100, Thermo Fisher Scientific Anatomical Pathology, Fremont, CA) and Calbindin D-28K (CB, clone CB955, 1:300, Sigma, St. Louis, MO), glutamic acid decarboxylase (GAD, 1:100, Enzo life Sciences, Plymouth Meeting, PA), parvalbumin (PV, 1:500, Sigma), and the polyclonal rabbit antibody against Neuropeptide tyrosine (NPY, 1:100, Phoenix Pharmaceuticals, Burlingame, CA).

2.3. Comparison of immunopositive cell counts between cortex and basal ganglia

Various immunopositivities of cells comprising the neocortex and caudate nucleus were observed in all cases. The number of immunopositive cells, excluding glial cells and endothelial cells, was counted in 5 fields of each region (neocortex and caudate nucleus) at a magnification of 200 times. The number was corrected per 100 nuclei in each region for each case as the immunopositive cell density. For statistical analysis, Student-*t* test was used for comparison between two parts. Student-*t* test was applied using statistical software (SPSS; SPSS Inc., Illinois, USA) at a significance level of $P<0.05$.

3. Results

All FCD cases were suspected by MRI and the other examinations before surgery and resected lesions (Fig. 1). We examined neurological and mental status before and after operation. As a result, no changes were found in all patients. The neuropathology of our cases showed abnormal lamination of the cortices, dysmorphic neurons and/or balloon cells, some heterotopic neurons in the white matter, and some normal-looking neurons, and was diagnosed as FCD IIa or IIb (Table 1, Fig. 2).

The resected caudate nucleus histologically identified the small number of dysmorphic neurons or balloon cells (Fig. 2C and F). Some neurons in the neocortex and caudate nucleus displayed GAD+, CR+, CB+, PV+ and NPY+ in each case (Fig. 3). In the neocortex, the concentration of each cell type was less in FCD than in controls (Fig. 4). The concentrations of GAD+, CR+ and CB+ cells showed significant differences between FCD and controls. That of GAD+ cells was $4.8\pm0.8\%$ (average ± standard deviation) in controls and $1.8\pm0.6\%$ in FCD ($P=0.0194$). That of CR+ cells was $10.6\pm4.3\%$ in controls and $2.8\pm2.2\%$ in FCD ($P=0.0472$). That of CB+ cells was $6.0\pm2.2\%$ in controls and $2.8\pm2.1\%$ in FCD ($P=0.0385$). That of PV+ cells was $7.6\pm6.5\%$ in controls and $3.6\pm2.7\%$ in FCD ($P=0.6311$), while that of NPY+ cells was $0.7\pm0.6\%$ in controls and $0.2\pm0.1\%$ in FCD ($P=0.1802$).

Table 1
Clinicopathological profiles of FCD patients and age-matched control.

Case		Sex	Age		Lesion	Pathology	Seizure	Intelligence (DQ or IQ)	Cause of death
			Onset	Surgery					
FCD	1	f	11M	13Y	F	FCD IIa	GTC	43	
	2	f	10M	5Y9M	F	FCD IIa	GTC	59	
	3	m	0M	3M	F	FCD IIa	GTC	ND	
	4	m	1Y	1Y1M	F	FCD IIb	CPS	91	
CTL	1	m	0M			BE, SAH		ND	Neonatal asphyxia
	2	f	2M			SAH		ND	CHD
	3	m	2M			HIE, PE, PVL		ND	BPD, pneumonia
	4	f	6M			PVL, HM		ND	SIDS
	5	m	1Y			BE, HE		ND	DIC, hyperlactemia
	6	f	1Y4M			SAH, SDH		ND	Dehydration, diarrhea
	8	f	2Y			BE, HE		ND	HUS
	7	f	8Y			Cbl		ND	ALL

m; male, f; female, Y; year(s), M; month(s), GTC; generalized tonic–clonic seizure, CPS; complex partial seizure, IQ; intelligent quotient, DQ; developmental quotient, F; frontal lobe, FCD; focal cortical dysplasia, CTL; control, ND; not done, Pathological diagnosis was due to the recent international classification (see in text). BE; brain edema, SAH; subarachnoid hemorrhage, HIE; hypoxic–ischemic encephalopathy, PE; porencephaly, PVL; periventricular leukomalacia, HM; hypomyelination, HE; hemorrhage, SDH; subdural hemorrhage, Cbl; cerebellar infarction, *CHD*; congenital heart disease, *BPD*; bronchopulmonary dysplasia, *SIDS*; sudden infant death syndrome, *HUS*; hemolytic uremic syndrome, *ALL*; acute lymphoblastic leukemia.

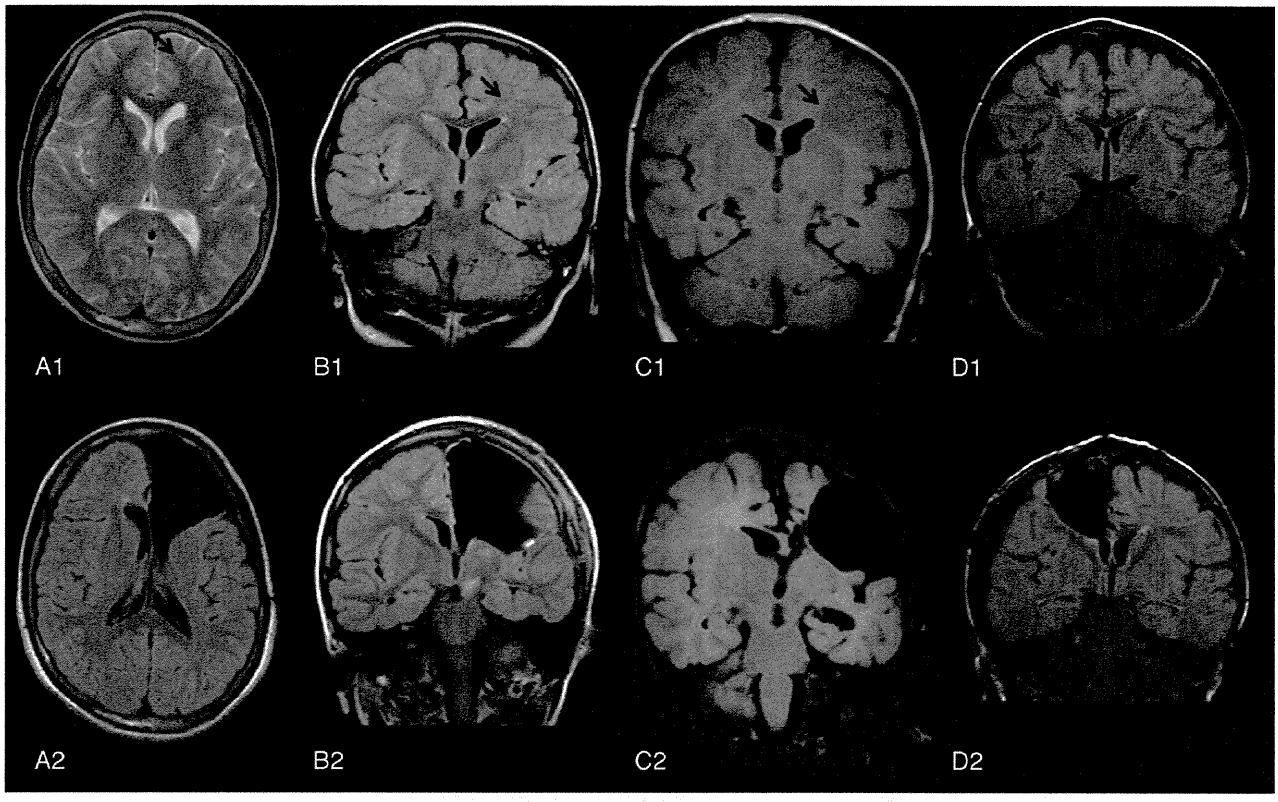

Fig. 1. MRI of pre-operation and post-operation of subjects. A1 is pre-operation MRI, axial view/T2-weighted image, and A2 is post-operation, axial view/FLAIR image of case 1 in Table 1. B1 is pre-operation MRI, coronal view/FLAIR image, and B2 is post-operation, coronal view/FLAIR image of case 2 in Table 1. C1 is pre-operation MRI, coronal view/FLAIR image, and C2 is post-operation, coronal view/FLAIR image of case 3 in Table 1. D1 is pre-operation MRI, coronal view/FLAIR image, and D2 is post-operation, coronal view/FLAIR image of case 4 in Table 1. Arrows indicate abnormal signals in the white matter and/or neocortex. In B1, there is definitive laterality of the caudate nuclei.

On the contrary, in the caudate nucleus the concentration of each cell type was higher in FCD than in controls (Fig. 4). The concentrations of CR+ and CB+ cells showed significant differences between FCD and controls. The GAD+ cell level was $3.0 \pm 2.1\%$ in controls and $4.2 \pm 2.8\%$ in FCD ($P = 0.5409$). The CR+ cell level was $1.2 \pm 0.4\%$ in controls and $4.2 \pm 2.6\%$ in FCD ($P = 0.0475$). The CB+ cell level was $0.1 \pm 0.2\%$ in controls and $7.6 \pm 4.1\%$ in FCD ($P = 0.0017$). The PV+ cell level was $1.8 \pm 2.0\%$ in controls and $4.1 \pm 3.2\%$ in FCD ($P = 0.2583$). The NPY+ cell level was $0.6 \pm 0.3\%$ in controls and $0.7 \pm 0.4\%$ in FCD ($P = 0.2386$).

4. Discussion

Neocortical interneurons play an important role in modulating neocortical plasticity and output [14]. GABAergic interneurons appear to perform important regulatory functions on developmental processes of neuronal migration, proliferation, and the postnatal development of cortical circuitry [15]. GABAergic inhibitory interneurons are morphologically distinct from excitatory pyramidal cells and account for 20–25% of all neocortical neurons. In the cortical dysplasia of epilepsy patients, there were reduced numbers of CR+ and PV+ interneurons [12,16,17]. Recent studies reported that several genes were involved in the reduction of the interneurons in the cortex [13]. Mice lacking the transcription factors responsible for regulation of differentiation (*Dlx1*, *Dlx2* or *Mash1*) and regionalization (*Nkx2.1*) in the basal telencephalon have reduced numbers of cortical GABAergic interneurons at the time of birth [18]. It is known that mice with targeted mutation of the gene encoding urokinase plasminogen activator receptor have a 50% reduction in neocortical GABAergic interneurons at embryonic and perinatal ages. This strain exhibits spontaneous seizure activity and higher susceptibility to pharmacologically induced convulsions [19]. *Dlx1*-lacking mutant mice showed CR- and somatostatin (SST)-positive interneuron loss in neocortex and hippocampus, cortical dysrhythmia, and generalized electrographic seizures [20]. Reduction of GABAergic interneurons may directly result in epileptogenesis. GABA is also co-localized with CB, CR and NPY. CB and CR belong to the large family of calcium-binding proteins (CBPs), which are characterized by the presence of a variable number of helix–loop–helix motives binding Ca^{2+} ions with high affinity. These proteins are involved in regulating calcium pools important for synaptic plasticity [21]. Neuropeptides differ from classical neurotransmitters in size, synthesis and mechanism of action. GABAergic interneurons co-localized with one or more neuropeptides are specifically targeted by serotoninergic and catecholaminergic afferents. NPY+ neurons in the neocortex are GABAergic, medium-sized, spiny and exhibit ultrastructural characteristics typical for neurons producing and releasing peptides.

In previous studies, the number of inhibitory neurons in patients with intractable epilepsy and mammalian models shows a variable aberrant pattern, such as a decreased or increased number of GABAergic interneurons [3,22]. Interestingly, approximately 35% of neocortical interneurons in the human brain originate in the subventricular zone [1]. Moreover, mutation of ARX, an initiation factor of interneuron migration, demonstrated many residual GABAergic interneurons in the white matter and striatum of the human brain [13]. Development of interneurons occurs from deep structures. Many cortical GABA-containing interneurons originate in the subpallial telencephalon and migrate tangentially to reach their final destination [23]. CR-containing

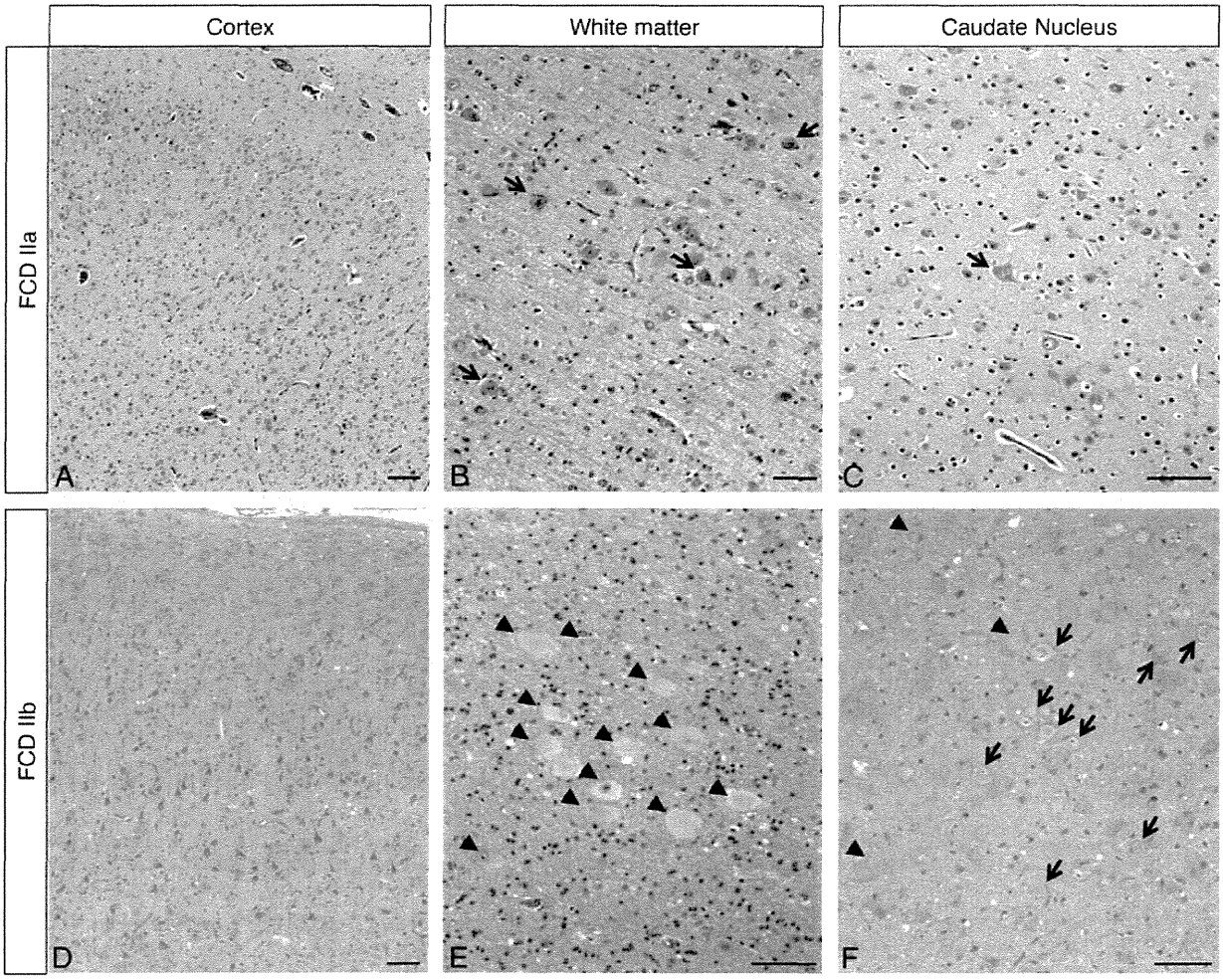

Fig. 2. Characteristic pathology. A shows unlayer neocortex and some large dysmorphic neurons (arrows) (case 2 in Table 1). B exhibits many heterotopic neurons in the white matter, which are variously sized and disorientated (the same case of A). C shows some dysmorphic neurons in the caudate nucleus (the same case of A). D, E and F are the neocortex, white matter and caudate nucleus of case 4 in Table 1, respectively. D shows unlayer neocortex and numerous disoriented neurons. E reveals many balloon cells (arrowheads). F demonstrates not only balloon cells (arrowheads) but also many dysmorphic neurons (arrows). Each scale bar indicates 100 μm.

interneurons primarily arise from the dorsal caudal ganglionic eminence. SST- or PV-containing interneuron progenitors primarily migrate from the Nkx2.1-expressing domain of the medial ganglionic eminence [24].

A factor which directs interneurons to the cortex or the striatum on migration, was discovered recently. Migrating interneurons expressing neuropilins, receptors for semaphorins, are directed to the cortex; those lacking them go to the striatum. Loss of neuropilin function increases the number of interneurons that migrate into the striatum [25]. Our results demonstrated that interneurons in patients were fewer in the cortex and more numerous in the striatum compared with controls. Interestingly, even our small case number indicated a significantly different distribution of interneurons between the neocortex and deep gray matter. The results suggest the dysfunction of interneuron migration factors in our cases. The abnormalities in the numbers of interneurons in the striatum would also serve to explain the pathophysiology in terms of striatal disorders. Besides a decreased number of interneurons in the cortex, an abnormally increased number of interneurons in the striatum may be related to the epileptogenesis of cortical dysplasia. Normal-looking neurons of our cases may have abnormalities of neuronal maturation and differentiation [17]. The recent experimental study in rat hippocampal CA1 slices demonstrated that the prototypic form of seizure activity was driven by fast-spiking interneurons [26]. The previous study suggested that fast-spiking network alone could drive the prototypic form of electrically-induced seizure-like oscillations through their excitatory GABAergic transmissions through gap junction-mediated communications [26]. Meanwhile, partial removal of the striatum with the cortical margin and the insular cortex in the present cases, especially at the reoperations, resulted in seizure freedom. Therefore, the imbalance of striatal and neocortical interneurons might be one of the causes of epileptogenesis.

In this study, the density of CR+ and CB+ interneurons had a significant increase in the caudate nucleus, but decreased in the neocortex. Surprisingly, the rate of GAD+ and PV+ interneurons also evidenced the same pattern of those of CR+ interneurons unless there was a significant difference. On the other hand, the rate of NPY+ interneurons exhibited no change between cerebral cortex and basal ganglia. We could not elucidate the reasons why the density of CB+ interneurons is higher in the FCD than in controls. From our data, the abnormal CBPs+ interneurons might influence not only the neocortex but also the basal ganglia. Recently, dysfunction and decreased number of interneurons are known to play an important role in epileptogenesis [27,28]. We speculate that the imbalance of

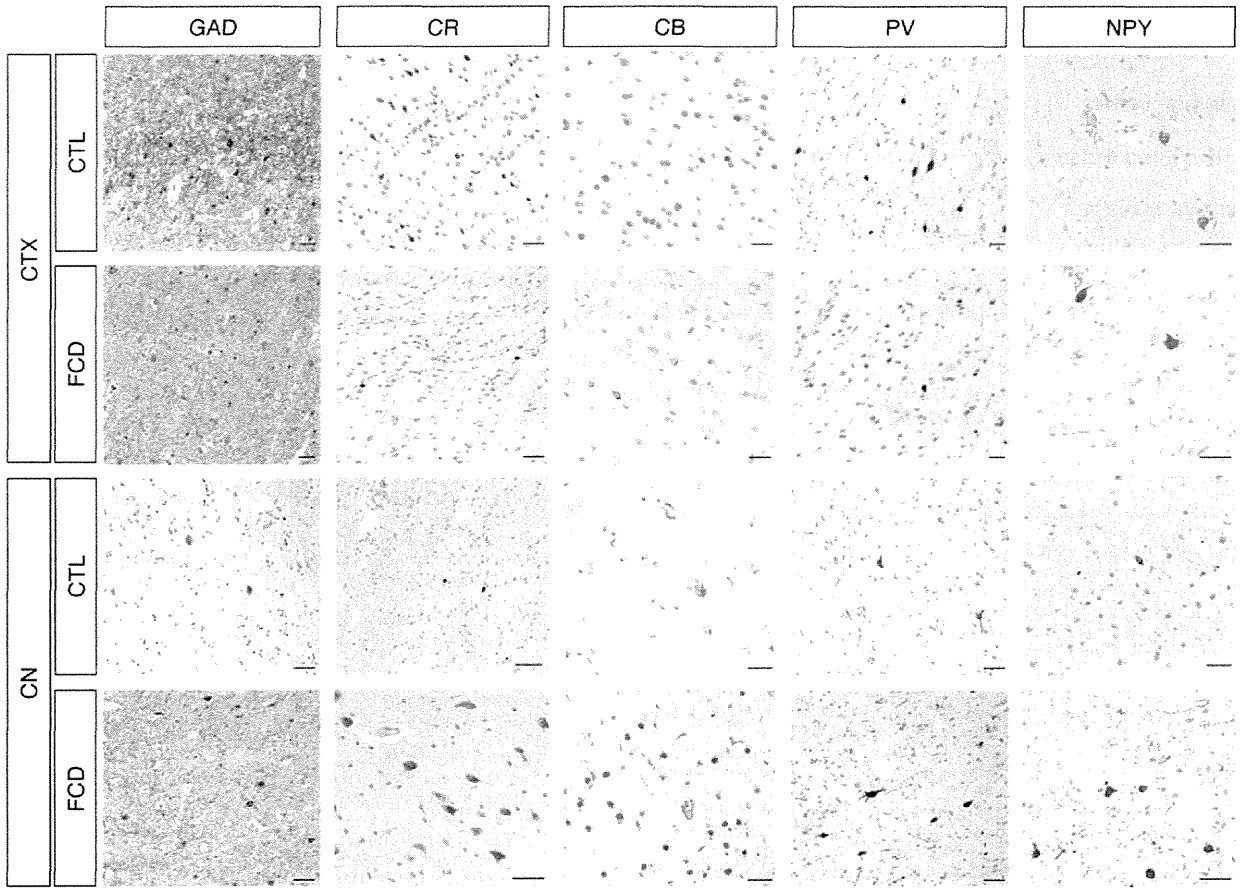

Fig. 3. Immunoreactivities for interneuron subtype markers. In neocortex, the numbers of immunoreactive cells of controls are relatively more than those of FCD. On the contrary, in caudate nucleus, the numbers of immunoreactive cells of controls are relatively less than those of FCD. Moreover, we noticed that immunoreactive cells of FCD are generally small in size. Each scale bar indicates 100 μm.

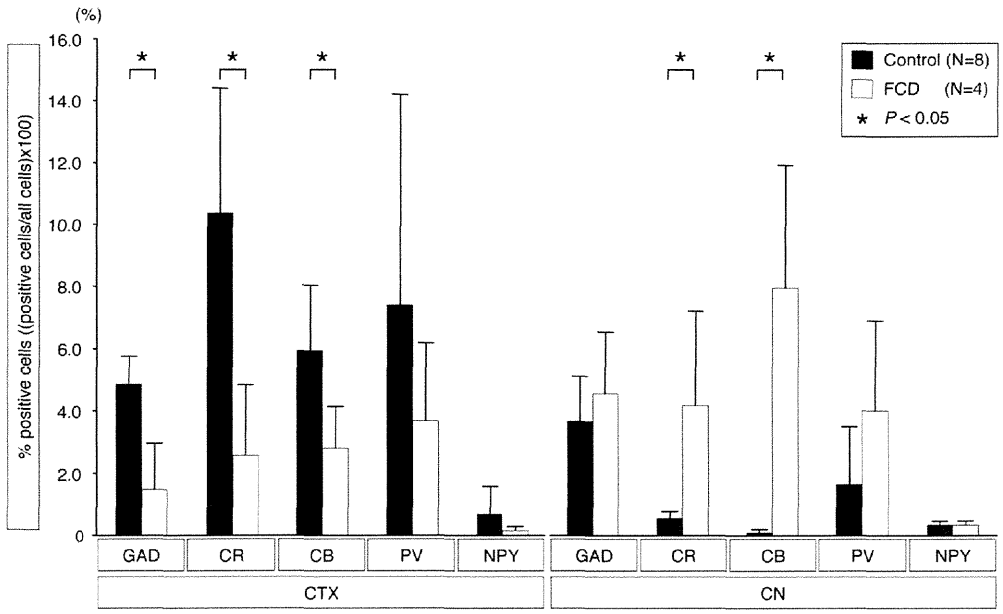

Fig. 4. Correlation between controls and FCD cases of interneuron subtype marker expression. In neocortex, there are significant differences in GAD+, CR+ and CB+ cell concentrations between controls and FCD. On the contrary, CR+ and CB+ cell concentrations in the caudate nucleus show a reverse significant difference.

interneurons, especially in the CBP-positive interneurons, may affect the epileptogenesis of FCD.

Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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