Increased Monoamine Concentration in the Brain and Blood of Fetal Thalidomide- and Valproic Acid–Exposed Rat: Putative Animal Models for Autism

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ABSTRACT

Autism is defined as a congenital neurodevelopmental disorder in which serotonergic dysfunction may be involved in its pathogenesis. One of the characteristic laboratory findings in autistic patients is hyperserotonemia, although its mechanism has not been elucidated to date because of difficulties in studying human patients. Recent reports have demonstrated that thalidomide or valproic acid exposure during early embryonic days (first trimester) in humans causes higher incidence of autism. Morphologic abnormalities found in autism (*e.g.* cerebellar anomalies, reduced motor neuron numbers) have been reported in animals administered with these teratogens prenatally, suggesting the possibility of the use of these animals as an experimental autistic model. In this study, we evaluated monoamine levels in the brain and blood of rats exposed to teratogens prenatally. Of the groups exposed to thalidomide on embryonic day (E)2, E4, E7, E9, and E11, a significant increase of hippocampal serotonin was only observed in the group exposed on E9. Furthermore, E9

thalidomide and valproic acid exposure both resulted in an increase of hippocampal serotonin, frontal cortex dopamine, and hyperserotonemia. These results thus indicate that two potentially autism-inducing teratogens, thalidomide and valproic acid, have the same effect on early monoamine system development in the brain and the blood, which may explain the pathogenesis of autism. (*Pediatr Res* **52:** 576–579, 2002)

Abbreviations
5-HT, 5-hydroxytryptamine, serotonin
VPA, valproic acid
DA, dopamine
FGF, fibroblast growth factor
E, embryonic day
PN, postnatal day
PRP, platelet-rich plasma
5-HTT, 5-HT transporter

Autism is a congenital neurologic disorder characterized by impairment of socialization, abnormalities of communication, and limited activity and curiosity that usually develops in the first 3 y of life (1). The severity and specific nature of autism is heterogeneous, ranging from severe mental retardation to prodigious intelligence. The rate of autism among siblings and twins is higher than that in the general population (2), indicating a strong genetic component in the etiology of autism. Another observation of particular interest to autism is hyper-

serotonemia. Whole blood 5-HT levels have often been found to be elevated in autistic patients and relatives, suggesting involvement of 5-HT in the pathophysiology of autism [see Anderson *et al.* (3)].

Expression of the serotonergic phenotype in the rat brain begins on E12 in the cells of the rostral raphe nuclei [see Lauder (4)]. Preceding that, progenitors of 5-HT neurons as well as dopamine (DA) neurons are at the ventral isthmus in the neural tube, and early genes such as sonic hedgehog (*Shh*) and *FGF8* were thought to define the cell fate (5). Surprisingly, the 5-HT neuron is defined as early as the primitive streak period by the expression of the inducer gene *FGF4* (5). These findings support the notion that autism is a congenital defect of the normal monoamine neuron development *in utero*, especially early in embryogenesis.

Recent reports have demonstrated that thalidomide exposure during E20–E24 in humans, from the early somite stage to the

neural tube closure stage, caused higher incidence of autism, suggesting that drug toxicity through the placenta during this particular period induces autism (6).

On the basis of somite numbers in early embryos of rats and humans, E9–E11 in rats is considered to be from early somite stage to the beginning of neural tube closure, which corresponds to approximately E20–E24 in human embryos, that is, coincident with the period when the increase of autism complication is observed in the thalidomide-exposed infants (6).

Previous animal models exposed to thalidomide or other known teratogens linked to autism, VPA and ethanol (7, 8), *in utero*, showed a reduction of cell numbers in the cranial nerve motor nuclei (9), reductions in Purkinje cell number and cerebellar volume (10), or migration retardation of 5-HT neuron (11). These observations all parallel the reported human autistic pathologic findings; however, exposure to the teratogen in these experiments was performed at approximately the time of neural tube closure, at which time the spatial and temporal monoamine neuron cell fate had already been determined. We are, therefore, more interested in discovering whether teratogen administration in earlier developmental stages, such as the primitive streak stage and the early somite stage (E8–E10 in rats), is more detrimental to normal monoamine neuron differentiation or monoamine metabolism, which may lead to autism.

METHODS

Animals and teratogen exposure. Female Sprague-Dawley rats (SEASCO, Saitama, Japan, 10 wk old) were mated overnight. The day of insemination was designated E1.

We first selected E2, E4, E7, E9, and E11 for thalidomide administration. Control animals were administered mock solution on E9. On selected embryonic day at 1500 h, each group was administered 500 mg/kg thalidomide or mock solution orally using a feeding tube for infant (Atom Medical, Tokyo, Japan) attached to a 2.5-mL disposable syringe to the rats without sedation. Thalidomide (Wako, Osaka, Japan) was prepared by dissolving in 5% arabic gum (Wako) and distilled water; 5% arabic gum in distilled water was used as mock solution. The volume of each suspension and mock solution was adjusted not to exceed 2 mL per animal. Dams were housed individually and were allowed to raise their own litters. The pups were weaned on PN21 and were housed, same sex, two to three to a cage, until they were killed on PN35 for experiments.

We then administered the same amount of thalidomide or 800 mg/kg VPA, or mock solution to E9 female rats to see the monoamine concentration in the hippocampus, frontal cortex, medulla, cerebellum, and the blood in the offspring on PN50. VPA was prepared by dissolving 2-propylpentanoic acid (Sigma Chemical Co., St. Louis, MO, U.S.A.) in distilled water, and adjusted to pH 9.6 with sodium hydroxide.

Sample preparation and monoamine concentration measurement. Because blood and brain 5-HT levels are influenced by estrus cycle in females, we used the tissues only from male animals (12). Blood (approximately 2 mL) was collected after decapitation in a tube containing EDTA, then centrifuged at 1000 rpm for 10 min. Supernatant aliquots were stored at −20°C as PRP until assay. The hippocampus, frontal cortex, medulla, and cerebellum, all only from the left side, were immediately removed, frozen with liquid nitrogen, and stored at −80°C until assay. The concentrations of 5-HT and DA were determined by using HPLC with electrochemical detection as described elsewhere (13). Statistical evaluation was performed by grouped t test. These experiments were approved by the Community of Laboratory Animal Research Center in the University of Tsukuba.

RESULTS

The pregnancies of teratogen- or mock solution–administered females were otherwise healthy. No major anomalies, growth retardation, or reduced number of the delivered pups were observed in pups either in the teratogen-exposed groups or the control group. Mortality rate of these teratogen-treated rats is not different from the control group up to 20 wk of age.

Table 1 shows hippocampal 5-HT concentration measured on PN35 for each group. A significant increase of 5-HT was observed in the E9 thalidomide-exposed group compared with the control group ($t=3.074$, $p < 0.01$), which demonstrates that at approximately E9, the day of the first somite differentiation, embryos are the most susceptible to thalidomide.

To further investigate the consistency of the effect of E9 teratogen exposure, we then measured monoamine concentrations of various tissues in the brain and the blood of offspring from E9 thalidomide- and VPA-treated rats (Table 2). Significant increase in the 5-HT concentration in the hippocampus both in the thalidomide and the VPA groups ($t=4.319$, $p < 0.005$; $t=2.653$, $p < 0.05$, respectively) and in the cerebellum in the VPA group ($t=2.741$, $p < 0.05$) was observed compared with the control group, suggesting that VPA has a similar effect on the serotonergic system as thalidomide. 5-HT concentrations in other parts of the brain examined showed no significant difference between teratogen-treated groups and the control group. There was a significant increase in dopamine concentrations in the frontal cortex in both thalidomide and VPA groups compared with the control group ($t=4.080$, $p < 0.005$; $t=2.396$, $p < 0.05$, respectively; Table 2).

PRP 5-HT levels of E9 teratogen-exposed groups and the control group were also measured. As shown in Table 2, PRP 5-HT concentrations of thalidomide and VPA groups were approximately two to three times higher than the control group ($t=3.127$, $p < 0.01$; $t=3.558$, $p < 0.005$, respectively).

Table 1. *Effect of fetal thalidomide exposure on hippocampal 5-HT concentration*

Date administered	Mean 5-HT concentration (ng/g protein)
E9, mock solution ($n = 8$)	574.19 (27.29)
E2, thalidomide ($n = 5$)	666.05 (52.75)
E4, thalidomide ($n = 7$)	674.74 (45.63)
E7, thalidomide ($n = 6$)	516.33 (45.63)
E9, thalidomide ($n = 8$)	714.86 (20.03)*
E11, thalidomide ($n = 5$)	645.97 (70.56)

Values reported are mean (± SEM). Concentrations were measured on PN35.

* $p < 0.01$ *versus* control (E9, mock solution).

Table 2. *Increased monoamine levels in thalidomide- or VPA-exposed groups*

	Tissue	Mean monoamine level (ng/g protein)		Mean monoamine level (ng/g protein)		*p* value
5-HT	Hippocampus	Control	446.51 (28.19)	Thalidomide	579.90 (16.27)	<0.005
				VPA	536.12 (18.60)	<0.05
	Frontal cortex	Control	451.08 (19.60)	Thalidomide	484.77 (47.82)	NS
				VPA	540.04 (55.31)	NS
	Cerebellum	Control	74.45 (6.59)	Thalidomide	83.69 (7.71)	NS
				VPA	113.95 (14.04)	<0.05
	Medulla	Control	976.61 (67.26)	Thalidomide	937.63 (36.61)	NS
				VPA	1046.46 (84.22)	NS
DA	Frontal cortex	Control	671.03 (63.81)	Thalidomide	1063.96 (59.54)	<0.005
				VPA	1192.58 (169.37)	<0.05

	Tissue	Mean monoamine level (ng/ml PRP)		Mean monoamine level (ng/ml PRP)		*p* value
5-HT	Plasma (PRP)	Control	368.06 (72.05)	Thalidomide	1080.10 (228.33)	<0.01
				VPA	937.35 (157.15)	<0.005

Values reported are mean ($\pm$ SEM). Number of samples was 4–8 in each category. Comparison was made between E9 thalidomide- or VPA-exposed groups and control group on PN50.

DISCUSSION

In the present study, we demonstrated that thalidomide and VPA cause *1*) increased 5-HT in the hippocampus, *2*) increased DA in the frontal cortex, and *3*) hyperserotonemia when given on E9. These results indicate that thalidomide and VPA have a common pharmacologic effect(s), which can change the serotonergic system.

Functions of 5-HT in the brain include emotional and mood control, the circadian rhythm, respiration, sleep, food intake, and motor functions (11). Clusters of 5-HT neurons are located in the raphe nuclei of the brainstem. Rostral raphe nuclei start to express the 5-HT neuron phenotype on approximately E12 (14). Ascending 5-HT neurons are also first observed on the same day as rostral raphe nuclei, then promptly develop diverse axonal projection networks throughout the brain until birth (4).

Factors such as S-100β (15), brain-derived neurotrophic factor, neurotrophin-3 (16, 17), and bone morphogenic proteins (18) are thought to regulate 5-HT neuronal differentiation and maintenance on and after E11. Besides these, several early markers are also known to regulate 5-HT and DA neuron cell fates even before these neurons are differentiated. Ye *et al.* (5) showed that *Shh* and *FGF8* are necessary for induction of normal DA and 5-HT neurons on E9. The other molecule whose expression precedes 5-HT neuron differentiation is an E twenty-six (ETS) domain factor Pet-1 (19). Pet-1 RNA expression is observed in the developing hindbrain, which precedes the appearance of 5-HT neurons by 12–24 h. Conserved Pet-1 binding sites are found in promoter regions of several serotonergic genes, suggesting that Pet-1 may directly control 5-HT concentration and function by regulating expression of the serotonergic genes.

Among the genes that have Pet-1 binding sites, the *5-HTT* gene plays a key role as a 5-HT function modulator by controlling reuptake of 5-HT from extracellular space (20). We recently reported significant differences in genotype distribution and allele frequency of *5-HTT* promoter allelic polymorphism between sudden infant death syndrome victims and the

general Japanese population (21). Because it is generally agreed that *5HTT* promoter allelic polymorphism relates with *5-HTT* promoter activity (20), we hypothesized that low concentrations of 5-HT in the respiratory center owing to *5-HTT* overexpression may cause sudden infant death syndrome. *5-HTT* mRNA expression is observed in various tissues of developing embryos (*i.e.* placenta, yolk sac, heart tube, and neural crest-derived structures) as early as E8 in rats (22). Therefore, we assume that the teratogens, to which E9 developing embryos are exposed, may enter through the placenta and perturb the expression of monoamine-related transcription factor(s) and consequently alter the transcription efficiency of genes regulating monoamine concentrations (*i.e. 5-HTT*), resulting in an increase in the offsprings' monoamine concentration.

Recently, Betancur *et al.* (23) reported no significant relationship between 5-HT blood levels and *5-HTT* gene polymorphism in the autistic patients, which suggests that the hyperserotonemia in the autistic patients might be regulated by more than a single mechanism. Interestingly, thalidomide and VPA both are known to induce transient thrombocytopenia and exert a suppressive effect on tumor necrosis factor-α (24, 25), a proinflammatory cytokine that promotes inflammation and cellular apoptosis. These common pharmacologic effects of these teratogens might play roles in the mechanism of the hyperserotonemia in E9 teratogen-exposed rats as well as in autistic patients.

CONCLUSIONS

Our findings that thalidomide and VPA has the same effect on the brain and blood monoamine concentration when given on E9 shed light on the pathogenesis of autism from the point of view of monoamines. The rats studied after thalidomide or VPA administration can be at least useful biochemical models of autism especially to elucidate the precise effects of these teratogens on monoamine concentrations. Further studies including behavioral and learning ability tests are necessary to understand the precise phenotype of these rats.

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