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REVIEW

Regulation of Brain Development by Thyroid Hormone and its Modulation by Environmental Chemicals

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THYROID hormone (TH; T3, 3,5,3'-triiodothyronine; T4, 3,5,3',5'-tetraiodothyronine, thyroxine) plays an important role in development and functional maintenance of the central nervous system [1]. TH deficiency during pre- and early postnatal period results in abnormal brain development known as cretinism in humans. However, the molecular mechanism of TH action in brain is not fully understood. In particular, brain development is severely affected by TH deficiency during a limited period, called the "critical period" of TH action, but the mechanism generating such a period is not known. Although TH treatment immediately after birth is sufficient to prevent brain damage induced by neonatal hypothyroidism (*i.e.*, by thyroid dysgenesis), such treatment cannot fully rescue the abnormal brain development induced by hypothyroidism *in utero* (*i.e.*, by maternal iodine deficiency). Thus, each brain region has a distinct critical period, during which neurons are particularly sensitive to TH.

The effect of TH is mainly exerted through nuclear TH receptor (TR), a ligand-dependent transcription factor [2], although TH action at non-genomic site has also been proposed [3]. Thus, TH action in brain may also be exerted mainly through TH-TR interaction.

To clarify the role of the TH-TR system in brain, one approach is to identify the target genes that are regulated by TH, while another is to understand the mechanism of brain-specific TR action, such as to identify the factors responsible for the generation of the critical period. For such purposes, we have been using the developing rodent cerebellum as a model system [4]. There are several advantages of using this model. First, although rodents are born much earlier than humans, the developmental pattern is not greatly different between the two species. Second, the rodent cerebellum largely develops postnatally, and TH deficiency during such a period markedly affects its development. Thus, postnatal rodent cerebellum is a good model to study TH action in developing brain, since TH status is easier to manipulate in postnatal animal than in fetus. In the present article, the molecular mechanism of TH action in developing brain is reviewed mainly by introducing our previous studies using rodent cerebellum model. Fig. 1 shows typical examples of the effect of perinatal hypothyroidism on brain development.

This article also summarizes our recent studies on the effect of environmental pollutants on brain development. Exposure to certain pollutants may cause abnormal brain development similar to that seen in perinatal hypothyroidism [5]. In particular, brain damage by perinatal exposure of polychlorinated biphenyls (PCBs) has been considered to be induced in part through disruption of the TH system [6]. However, the mechanism of PCB action has not been well characterized. Recent findings have provided several clues to our further understanding of the possible molecular mechanism of PCB action.

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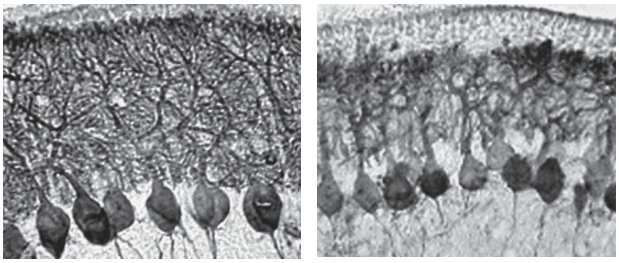

Fig. 1. Abnormal Purkinje cell dendrite arborization in perinatal hypothyroid rat cerebellum. Purkinje cell is immunohistochemically stained using a calbindin antibody.

Physiological action and metabolism of TH in developing brain

T3, an active form of TH, is produced locally in the brain by the 5'-deodination from T4, which preferentially enters the developing brain than T3 [7]. Such preference is probably because brain-specific organic anion transporter (Oatp14) at blood-brain barrier (BBB), a major TH transporter at BBB, preferentially binds to T4 [8]. After crossing the BBB, T4 is taken up by astrocytes and deiodinated by type 2 iodothyronine deiodinase [9]. T3 is then transferred to neurons or oligodendrocytes possibly through monocarboxylate transporter 8 (MCT8) [10], where it bind to the TR expressed in these cell types to regulate transcription of target genes. TRs are widely distributed in the developing brain including cerebellum [11, 12].

In the developing rodent cerebellum, perinatal hypothyroidism induces various morphological abnormalities. Such abnormalities include: a) delayed proliferation and migration of granule cells from the external granule cell layer (EGL) to the internal granule cell layer (IGL); b) decreased arborization of Purkinje cell dendrites; c) decreased synaptogenesis between parallel fibers and Purkinje cell dendrite; d) delayed disappearance of synapses between climbing fibers and Purkinje cell bodies, and decreased synaptogenesis between climbing fibers and Purkinje cell dendrites; and e) decreased synaptogenesis between mossy fibers and granule cell dendrites in the IGL [1, 4]. These abnormalities cannot be fully rescued unless TH is replaced within 2 weeks after birth.

Possible molecular mechanisms of thyroid hormone action on cerebellar development

Regulation of gene expression by thyroid hormone

As discussed above, TH action in brain is mainly exerted through binding to nuclear TRs [2]. TR binds to specific nucleotide sequences termed TH-responsive element (TRE), as a homodimer or heterodimer with retinoid X receptor (RXR) [13]. Upon binding to TRE, it recruits a series of proteins termed coactivator or corepressor in a ligand-dependent manner, regulating the transcription of target genes [14].

In the developing cerebellum, TH positively or negatively regulates the expression of many genes. To identify TH-responsive genes in the developing cerebellum, we have applied cDNA microarray technique, to detect a large number of transcripts, whose expression is altered by perinatal hypothyroidism [15]. Although TH-responsive gene can be extensively and exhaustively screened with this approach, such technique may not always be useful to identify “key” genes, which play a primary role in hypothyroidism-induced abnormal brain development. Thus, simultaneously with microarray studies, we took another approach. We have studied mutant animals showing abnormal cerebellar phenotype similar to that seen in perinatal hypothyroid animals. Phenotypic homology between hypothyroid animals and such mutant animals may indicate that there is a cross-talk between TH system and mutated genes within such model animals. Through investigation of the mutant animals, we have identified several candidate genes that may play a key role in TH-regulated brain development.

Among candidate genes, we hypothesized that neurotrophic factors including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin (NT)-3 and NT-4/5, play critical roles in TH-regulated brain development. These factors regulate processes of neuronal development similar to those regulated by TH, such as dendrite arborization, neurite extension and synaptogenesis [16]. In the developing cerebellum, BDNF and NT-3 play particularly important roles. Mutation of BDNF induces an abnormal cerebellar development similar to that in hypothyroid animal [17]. Indeed, previous studies have shown that the expression of BDNF and NT-3 are under control of TH in the developing cerebellum [18–20]. As shown in Fig. 2, NT-3 expression is down-regulated by hypo-

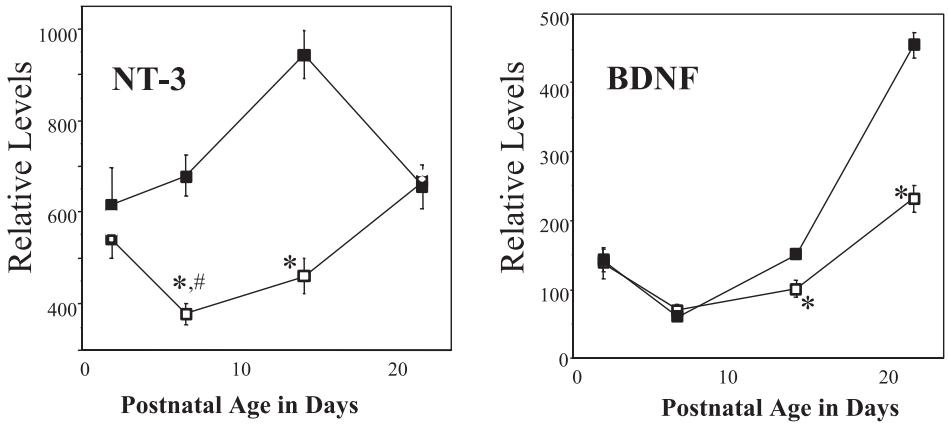

Fig. 2. The effects of perinatal hypothyroidism on levels of NT-3 and BDNF mRNA in the developing rat cerebellum. Rats were rendered hypothyroid by administering 0.05% propylthiouracil as a drinking water to their dams. ■: euthyroid animal, □: hypothyroid animal. *: $p<0.01$ vs euthyroid animals of the same age. #: $p<0.01$ vs postnatal age 2 days.

thyroidism in rat cerebellum only during the first two weeks after birth, whereas BDNF expression is decreased at later stage. Replacing NT-3 or BDNF can partly rescue the abnormal phenotype seen in perinatal hypothyroidism [18], indicating that abnormal phenotype seen in hypothyroidism may be due to the decreased expression of these genes. BDNF contains multiple transcription start sites, which are connected to different promoters. Each promoter activity is regulated in a brain region- and developmental stage-specific manner. The expression of BDNF gene is regulated by TH in a promoter-specific manner [20]. Although the TRE has not yet been identified in the promoter region of these genes, we concluded that these genes may play critical roles in abnormal brain development induced by perinatal hypothyroidism.

Another gene that may play a critical role in TH-regulated cerebellar development is retinoid receptor-related orphan receptor α (RORα), an orphan receptor that belongs to the steroid/thyroid hormone receptor superfamily. RORα is strongly expressed in the cerebellar Purkinje cell, and plays an essential role in its development [21]. A natural mutant mouse, called *staggerer*, in which RORα gene is mutated, shows abnormal cerebellar development similar to that in perinatal hypothyroid mouse, particularly within Purkinje cells. The growth of dendrite and synaptic connection with parallel fibers are greatly disrupted, which are also observed in hypothyroid animals, although *staggerer* mouse has severer phenotype. As shown in Fig. 3, we have identified a cross-talk between TR and RORα. RORα expression is in part regulated by TH only during postnatal cerebellar development in rat and mouse [19, 22]. We have also identified that the expression pattern of BDNF, NT-3 and their receptors in developing *sg* mouse cerebellum is very much similar to that in perinatal hypothyroid mouse (manuscript in preparation). Furthermore, in the transient transfection-based reporter gene assay, the RORα augments TR-mediated transcription [23]. These results indicate that RORα is required for full function of TR in the developing cerebellum, and thus closely related to TH-mediated brain development.

In addition to these genes, many other TH-regulated genes may play key roles in TH-mediated brain development. Through the screening process of such genes, however, we came to consider it impossible to list up such genes completely. Instead, we decided to examine the brain-specific action of the TR-TH system, which may be another potent approach to understand further the role of TH in brain development. For example, the critical period of TH action, during which TH action is much greater than at any other period, is not so clear in other organs. Thus, the clarification of the molecular mechanisms generating such a critical period of TH action may contribute greatly to improve our understanding of the role of TH in brain, as discussed in the following section.

Possible mechanisms generating critical period of TH action in brain

A striking feature of TH action in the brain is the existence of a critical period of its action. Interestingly,

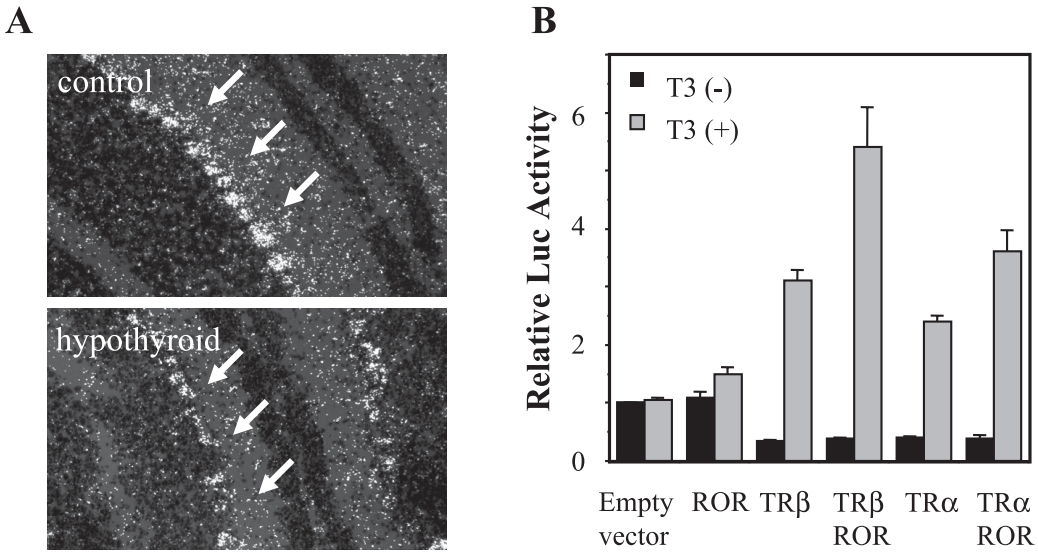

Fig. 3. A: *In situ* hybridization histochemistry for RORα mRNA in postnatal day 15 rat cerebellum. Arrows indicate hybridization signal in the Purkinje cell. B: The effect of RORα on TR-mediated transcription, studied using a transient transfection-based reporter gene assay. Equal amount of expression vectors containing TR or ROR was transfected into CV-1 cells. One hundred nM of T3 was added at 24 hrs before harvesting.

T3 binding capacity [24] and TR mRNA levels [25] are greater in adult brain than during the critical period. Hence, TH sensitivity is likely to be controlled by other unknown mechanisms. As discussed above, TH regulates the expression of many target genes mainly during the perinatal critical period. For example, TH regulates the expression of myelin basic protein (MBP) [26] and Purkinje cell-specific protein (PCP)-2 [27] gene only during the first 2 postnatal weeks in the rodent cerebellum [2].

One possible factor responsible for the altered TH sensitivity is nuclear protein that competitively binds to TRE to inhibit TR action. One example is chicken ovalbumin upstream promoter-transcription factor (COUP-TF). It is strongly expressed in the fetal cerebellum when TH is not effective in its development, and inhibits TR-mediated transcription of PCP-2 gene, probably by competing with TR on TRE [28]. However, COUP-TF is not ubiquitously expressed in the whole subset of neurons. Furthermore, the nucleotide sequence of TRE and its flanking region could greatly affect such binding. Thus, a more generalized mechanism may be involved in generating the critical period.

We hypothesized that the differential expression of cofactors during development may alter the TR-mediated transcription in the brain. SRC-1 is the most abundant coactivator in the brain [29]. It is expressed in many regions as early as embryonic 11 days in mouse [30]. Mutation of SRC-1 gene induces an abnormal brain development similar to that seen in perinatal hypothyroid animal [31]. Initially, we examined the expression of p160 coactivators and several representative corepressors such as N-CoR and SMRT at mRNA level [32]. There was no marked change in their expression before or after the critical period. Then, the change in SRC-1 expression at protein level in the developing rat cerebellum was further examined using immunohistochemistry and Western blot analyses [33]. Western blot analysis revealed that the protein level is greatest at postnatal day 15, when the expression of many TH-responsive genes is greatly affected by altered thyroid status. Furthermore, as shown in Fig. 4, while TRs are almost ubiquitously expressed in the neuronal cells even during development, a regional difference in SRC-1 immunoreactivity was seen. For example, SRC-1 was not expressed in the proliferative zone of the EGL. This finding is consistent with previous reports showing that TH is not effective in promoting proliferation or morphological alteration of external granule cells, although TRs are expressed in this cell type [34, 35]. These results indicate that differential expression of cofactors for TR may at least in part be responsible for the change in TH sensitivity in the developing brain. However, the change in cofactor expression cannot fully account for the change in TH sensitivity in brain. Trials to identify

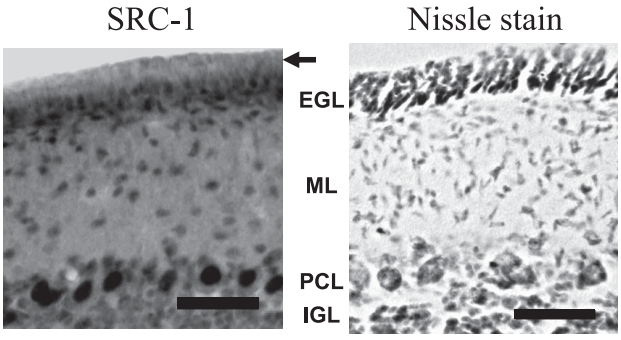

Fig. 4. Immunohistochemical staining of SRC-1 (left panel) and Nissle staining (cresyl violet, right panel) of adjacent section from postnatal days 15 rat cerebellum. Note that SRC-1 immunostaining (nucleus) is not observed in the proliferative zone of the EGL (arrow). Bars = 100 mm
Abbreviations: EGL, external granule cell layer; ML, molecular layer; PCL Purkinje cell layer; IGL, internal granule cell layer

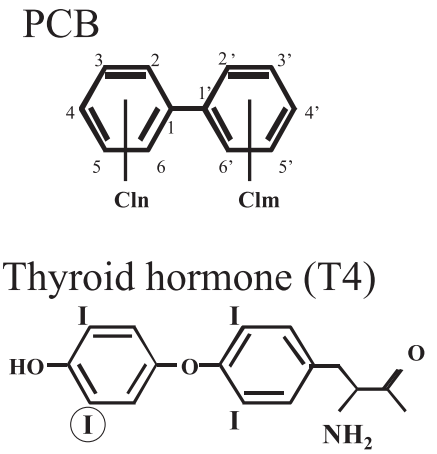

Fig. 5. Chemical structure of PCB and thyroid hormone. Cln and Clm denote that Cl can bind to 2–6 or 2'–6' site of each benzene ring. Removal of iodine in the circle produces T3.

additional factors responsible for alteration of TH sensitivity are currently underway.

Modulation of thyroid hormone action by environmental chemicals

Environmental chemicals are a group of chemical compounds or chemical elements present in the air, food, soil, dust, or other environmental media (*e.g.,* consumer products), some of which are synthetic chemicals, and others are natural compound (*i.e.,* plant products). Some such environmental chemicals seem to affect the mammalian endocrine system, hence they are termed "endocrine disrupting chemicals" or "environmental hormones". Exposure to such substances have been associated with developmental, reproductive and other health disorders in wildlife and laboratory animals. These endocrine-disrupting chemicals may also affect to humans. However, unlike endogenous hormones, the magnitude of action of such chemicals varies greatly among species. Thus, limited information is available regarding the effect of such chemicals on human health.

Many of the endocrine disrupting chemicals are chlorinated compounds, including dioxins, polychlorinated biphenyls (PCBs) and organochlorine pesticides such as DDT. Among these compounds, PCB is one of most well-known substance that may affect the developing brain. Because its chemical nature is similar to that of oil (lubricative, low electric conductivity), but inflammable, PCB was widely used in industrial and household appliances, such as electrical fluid in transformers and capacitors, hydraulic lubricants, paints, and copy paper. Due to its toxicity, however, its production was banned in 1972. Most PCBs still exist globally in environment because of its chemical stability, and use of PCB-containing appliances is still allowed. Probably because of such reasons, the levels of PCB in various human tissues have not greatly altered until recently [36], and nano-pico molar order of PCB is still contained in blood, breast milk and almost all natural products [37]. As shown in Fig. 5, its chemical structure is similar to that of TH, since both contain two benzene rings, which are halogenated at various degrees. PCBs contain 206 congeners due to differences in chlorination. Perinatal exposure to PCB results in abnormal brain development similar to that seen in perinatal hypothyroidism [6]. The intellectual development of child is partly retarded with increased PCB concentration in maternal milk, which reflects the perinatal exposure level [38]. Initially, it was thought that the effect of PCB was exerted through inhibition of TH secretion from thyroid gland, or competitive inhibition of TH binding to TH-binding protein such as transthyretin in blood [39], both of which may induce hypothyroxinemia in plasma. However, although PCB treatment induces decrease in plasma TH in experimental animals, the PCB exposure level and the changes in plasma TH level are not correlated

with one another in humans [40]. Even in the rat, although perinatal PCB exposure induces a decrease in the total T4 levels in plasma, the growth rate of PCB-treated animal is identical to that of control animal, indicating that the animal is generally euthyroid [41]. Thus, the major action of PCB to induce abnormal brain development may not be through the decrease in plasma TH, but rather, PCB may act directly on TR to modulate its action.

To examine the direct action of PCB on TR-mediated transcription, we performed a transient transfection-based reporter gene assay [42]. One example of our result is shown in Fig. 6. A hydroxylated PCB (4-OH-2',3,3',4',5'-penta-chlorobiphenyl) suppressed TR-mediated transcription at a much lower dose (100 pM) than that previously reported. Interestingly, the dose-response relationship was not so evident, and the magnitude of suppression at 100 pM and 5 μm was not so greatly different, indicating that the suppression may not be due to competitive inhibition of T3 binding to TR. We have examined several congeners including a PCB mixture commercially used for various purposes (Aroclor 1254), and obtained similar results [43]. Furthermore, the effect of PCB was greater in neuron-derived clonal cells than other clonal cells of different tissue origin [42]. These results indicate that PCB may affect TR-mediated transcription in a tissue specific manner. Taken together with previous results showing that PCB exposure may induce abnormal development particularly in the neuronal function, the developing brain may be more vulnerable to PCB exposure than other organs.

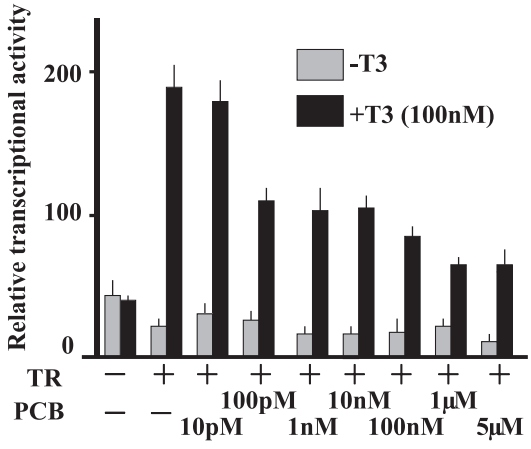

Fig. 6. The effect of polychlorinated biphenyl on TR-mediated transcription. Expression vector containing human TRβ1, and reporter vector containing a TRE at upstream region of luciferase gene was transfected into CV-1 cell. Indicated amount of PCB (4-OH-2',3,3',4',5'-pentachlorobiphenyl) was added simultaneously with T3. Cells were harvested at 24 h after the treatment.

We further examined the mechanism of suppression of TR-mediated transcription by PCB. PCB did not dissociate coactivators from TR in the presence of T3 *in vitro* and *in vivo* nor did it recruit corepressors to TR. Instead, it dissociated TR from TRE [43]. These results indicate that the site of action of PCB in TR molecule may not be ligand-binding domain, but may be another domain such as a DNA-binding domain. Trials to identify the site of action of PCB are currently underway.

Future perspectives

Although the importance of TH in brain development is well recognized, the mechanism of TH action in brain has yet to be clarified. By using the rodent cerebellum as a model system, we have been studying the mechanisms of TH action in the developing brain. We hope that our findings will contributed to further understanding of such mechanisms. However, there are still a number of questions to be addressed. For example, brain phenotype of perinatal hypothyroid animal and TR-knockout animal are quite different except for hearing impairment [44]. Furthermore, TRα deletion partly prevents abnormal cerebellar development of perinatal hypothyroid mouse induced by anti-thyroid drug treatment [45]. On the other hand, transgenic animals expressing a mutant TR that does not bind to TH show abnormal cerebellar development similar to that in perinatal hypothyroid animals [46]. These results indicate that abnormal brain phenotype by perinatal hypothyroidism could be due to dominant-negative action of unliganded TR that suppresses transcription of TH-regulated genes. To further test this hypothesis, brain-specific dominant-negative inhibition of TH using a genetic approach is necessary, since general hypothyroidism alters expression of many peripheral substances, which then indirectly affect brain development.

On the other hand, there are still many uncertainties in the effect of endocrine disrupting chemicals on TH system. In the case of PCB, for example, although PCB suppressed TR-mediated transcription, previous studies have indicated that it may not bind directly to

TR [39, 47]. Thus, PCB may bind to an unknown TR-binding protein, which may then induce conformational changes in TR DNA binding domain to dissociate TR from TRE. Since the action of PCB is greater in neuron-derived cells than that in cells derived from other organs, such protein may be strongly expressed in neurons. Trials to identify PCB-inducible TR binding protein are currently underway.

In addition to suppression of TR action, PCB may act at multiple sites in the central nervous system. Previous studies have shown that it might also directly bind to neuronal membrane to alter excitability. For example, acute treatment of PCB 77, a coplanar-type PCB, reduces the magnitude of long-term potentiation in the hippocampus [48]. We also identified that PCB treatment induces an increase in resting membrane potential of cultured brainstem neuron with the decrease in magnitude of depolarization by low pH [49]. We have further identified that the increase in resting membrane potential induced an increase in intracellular calcium, which then induces the expression of calcium-inducible genes such as c-Jun [50]. Taken together with the suppressive action of PCB on TR-mediated transcription, PCB may act at multiple sites in the neuron to alter their development. Until recently, the main site of action of endocrine disrupting chemicals was considered to be the reproductive organs, but more attention should be paid to their effect on the developing brain.

To determine the toxicity of synthetic chemicals, it is very important to construct a sensitive and reliable assay system. Our recent findings have shown that environmental chemicals may not only act as an agonist or antagonist of hormones, but also act at allosteric sites of receptors. Further, it may simultaneously act on the cellular membrane to alter its excitability. Thus, screening systems to examine toxicity should be constructed by considering such multiple pathways. We hope that our data will provide important clues to generate reliable screening systems.

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