Functional Development of Neuronal Networks in Culture -An *in vitro* Assay System of Developing Brain for Endocrine Disruptors

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Here, I would like to discuss about our work entitled "Functional Development of Neuronal Networks in Culture." As previous speakers have discussed, environmental chemicals influence not only the reproductive system. Receptors of sex hormones are abundantly present in the central nervous system, and hormones play important roles in the development of brain. Thus, the brain may become another major target of endocrine disrupters. In fact, recent studies have revealed that various environmental chemicals influence the brain functions. Children exposed to PCBs in the Great Lakes exhibit impairment of intellectual development. In Yusho-disease, the cognitive impairment in infants is observed. The relationship between herbicide or pesticide and Parkinson's disease has been reported. There are an increasing number of studies about the protection from Alzheimer's disease by estradiol (female hormone) or phytoestrogen. Furthermore, metals including lead, aluminum or tin have various adverse effects on neural systems.

During the developmental stages of the brain, from pregnancy to infancy, neurons begin to differentiate and begin to form complex networks which were connected with synapses. It is widely accepted that the formation of synapses and their plasticity are based on the molecular mechanism of learning and memory. Here, I demonstrate the developmental changes of brain weights and the synaptic densities. These changes occur before nearly three years of age. Environmental chemicals can enter into the infant's brain through mother's body or mother's milk, or from other environmental sources. Unfortunately, the development of the blood brain barrier is not sufficient during these periods. Moreover, most of endocrine disrupting chemicals are lipophilic and easily pass through the blood brain barrier. The functions of the liver and the kidney of infants are not complete, and therefore, these chemicals cannot metabolize and cannot be excreted. Considering these results together, it is highly possible that environmental chemicals can enter into the infant's brain and influence the development of functional network at this significant period.

Our aim is to investigate which environmental chemicals effect the functional development of brain. For this purpose, it is critical to establish the *in vitro* screening system for synapse formation. We employed several methods including the calcium imaging system, the immunohistochemical observation, and the expression of synapse-specific genes to evaluate the synapse formation between the primary cultured neurons of rat cerebral cortex, cerebellum, and hypothalamus. Then, we added thyroid hormones, which are essential for the neuronal development, to a culture of neurons and investigated their effects on synapse formation. Next, we plan to search for compounds, which disrupt neuronal development, using thyroid hormone enhanced synapse formation as an indicator.

We have already developed the long-term culture system of rat cerebral cortical neurons. From the brains of 18-days embryonic rats, the cerebral cortex was dissected out, dissociated, and plated on culture dishes. After 3-7 days in culture, neurons begin to extend neurites and to form synaptic contacts. The expression of receptors of neurotransmitter, or neuron-marker proteins, occurs in the same period. Cultured neurons after 170 days *in vitro* (approximately 5 months) were stained by antibody to MAP2 (microtubule associated protein 2; a neuron marker protein) and anti-synaptophysin (a synapse marker protein which localizes in the presynaptic terminal). The laser confocal microscope analysis reveals the

existence of matured synapses (dot-like structures) on neuronal processes and cell bodies of cultured neurons. Our system of primary cultured neurons has these benefits such as holding neuron-specific characteristics and could be routinely maintained more than one month. In our brains, there are various types of synapses: excitatory, inhibitory, *etc.*, and numerous synapses form the complex neuron-networks. Here is an image observed by laser confocal microscope of three weeks cultured neurons immunostained by antibody to MAP2 and antibody to NMDA receptor (a receptor of excitatory neurotransmitter and localized on the postsynaptic area of excitatory synapses). Other neurons were stained by anti MAP2 and anti GAD (glutamic acid decarboxylase, which recognizes the enzyme of inhibitory neurotransmitter, GABA). Therefore, the cultured neurons of rat cerebral cortex also exhibit various types of synapses as well as *in vivo* brains. We can analyze the type, the number and the distribution of these synapses.

We have also developed the functional assay system for evaluating synapse numbers using calcium-imaging system. We expose calcium-sensitive fluorescent dye, fura-2, to cultured rat cerebral cortical neurons and observed by a fluorescent microscope equipped with a high-sensitive video camera. The slide shows the pseudo color image of intracellular calcium levels of cultured neurons. Using this system, we could monitor the spatio-temporal changes of intracellular calcium levels of numerous neurons at the same time. After seven days *in vitro*, cultured neurons exhibit periodical changes of intracellular calcium levels spontaneously. Here, neurons labeled 1 to 7 showed the periodic changes of intracellular calcium levels without any stimulation. These spontaneous oscillatory changes of intracellular calcium levels were synchronized among most neurons. Neuron (V) in the slide represents the observation of the membrane potential at the same time. These periodic changes of intercellular calcium levels were due to the synaptic firings, maybe due to the conversion of miniature EPSPs. These spontaneous and synchronized changes of intracellular calcium levels appeared during the development of cultured neurons. After seven days in culture, almost all neurons show the synchronized calcium oscillation. Usually, the frequency is three to four spikes per minites. We have also observed the synapses between cultured neurons by electron microscopy and counted the number of synapses at the same time. Interestingly, the frequency of the calcium oscillation and the number of synapses counted by electron microscopy was strongly correlated. Therefore, we can conveniently evaluate the synapse formation and can estimate their numbers by observing this frequency.

Slide shows a summary of the strategy of our research. In cultured neurons, it is widely accepted that the extension of neuritis firstly occurs, and the formation of excitatory synapses, and then inhibitory synapses are formed. The balance between the formation of excitatory and inhibitory synapses is crucial for the development of functional neuron networks. We tried to apply various chemicals on cultured neurons and observe the development of functional and morphological synapses using calcium-imaging and immunohistochemistry. Using this system, at first we investigated the effects of thyroid hormones on synapse formation. Next, after we had confirmed that thyroid hormones enhanced synapse formation, we searched for compounds, which interact with thyroid hormones and disrupt the thyroid-related synapse formation.

As Dr. Zoeller and Dr. Koibuchi have demonstrated, thyroid hormones play many important roles in the development of the brain. There is mounting evidence that thyroid hormones like T3 or T4 cause cell proliferation and cell survival, neurite outgrowth, synaptogenesis, regeneration, and the effect of cytoskeleton in the central nervous system. Furthermore, severe thyroid deficiency such as cretinism is characterized by mental retardation. Hyperthyroidism in both mother and infants also results in permanent deficiency in neurological and intellectual development. Recent studies have suggested a relationship between thyroid hormone and ADHD, attention-deficit hyperactivity disorder.

We applied thyroid hormones T3 and T4 on cultured cortical neurons at three days *in vitro* under the serum-free condition. After eight days in culture, we observed the changes of intercellular calcium

levels. This is the typical pattern of intercellular calcium levels of randomly chosen four neurons. In these experimental conditions, cultured neurons exhibit synchronized and spontaneous calcium changes at two to three spikes per minute. However, the addition of T3 or T4 significantly increased this frequency and showed more frequent synchronized spikes. While, the short-term application of thyroid hormones during the observations did not exhibit any changes, thus, thyroid hormones influence not synaptic transmission but influenced synapse formation. The increase of calcium oscillation's frequency was in a dose dependent manner of T3 or T4. Furthermore, to confirm that thyroid hormones really enhance the synapse formation, we observed the synapse morphologically using the immunohistochemical technique. Synapsin 1 is a presynaptic protein and is widely used as a good synapse marker protein. We double-immunostained cultured neurons by anti-MAP2, red, and anti-synapsin 1 and observed them by laser confocal microscope. These green dot-like immunoreactive changes, these are represented by synapses, and these synapses are significantly increased in thyroid hormone treated neurons. The increase of synapsin 1 was also confirmed by western blotting. These results suggested that thyroid hormone really enhance synapse formation between cultured neurons and that the enhancement can be quantitatively evaluated by the frequency of calcium oscillation.

Now, we have established the assay system for evaluating synapse formation, and the screening became possible. So we applied various chemicals and thyroid hormones to cultured neurons at the same time. After one week, we observed the calcium oscillation and evaluated whether these chemicals influence thyroid-hormone induced synapse formation or not. This screening assay is now ongoing. However, during this process, several compounds were revealed to inhibit thyroid-hormone induced synapse formation. For example, amiodarone, a widely known drug which has anti-thyroidal functions, inhibits the T4 induced increase of frequency significantly. Furthermore, amitrol, a herbicide, also inhibited the thyroid hormone-induced increase of calcium oscillation. However, in this experimental condition, the application of amitrol or amiodarone itself did not influence the calcium oscillation.

The molecular mechanism underlying how thyroid hormones enhance the synapse formation is also our interest. Thyroid hormones may influence numerous pathways. However, we are now focusing on MAP1B (microtubule associated protein 1B). We have already revealed MAP1B, and its phosphorylation is crucial for synapse formation between cultured cortical neurons. The inhibitor of protein kinase, k-252b, which inhibits the phosphorylation of extracellular domain of proteins, was revealed to block the synapse formation. MAP1B is involved in the phosphorylation process of the ecto-protein kinase. MAP1B is originally a cytoskeleton protein, and therefore, many people had believed that MAP1B was localized in the intracellular area. However, we found this protein has a transmembrane domain and is localized in post synaptic areas. Thus, we investigated the effect of thyroid hormones on MAP1B expression. The mRNA level of MAP1B was analyzed by northern blotting method. After 24 hours exposure to T3 or T4 caused the induction of MAP1B while in the same experimental condition, estradiol did not influence the expression of MAP1B. Furthermore, our collaborators, Dr. Tsuda's group at Toyama Medical and Pharmaceutical University, are now investigating various gene expressions including synaptophysin induced by thyroid hormones.

The effect of thyroid hormones on synapse formation is not restricted to the cerebral cortex. This is the result of our collaborator Dr. Nagata and Dr. Kimura-Kuroda. They applied thyroid hormones to Purkinje cells in cerebellum and observed their morphological changes by laser confocal microscopy. Dendrites of Purkinje cells were stained by antibody to calbindin D-28 and by antibody to synapsin 1. Both stainings were enhanced by thyroid hormones. They also compared the dendritic areas of Purkinje cells stained by anti-calbindin antibody using the quantitative image analysis, and found that thyroid hormones enhance the extension of dendrites in dose dependent manner. Furthermore, bisphenol A, a

widely known endocrine disrupting chemical, inhibits the thyroid hormone-induced extension of dendrite of Purkinje cells significantly.

Finally, let me talk about our approach for developing another *in vitro* neuronal model. We have now investigated the effects of cerebral cortex and cerebellum. However, considering that the hypothalamus is largely implicated in the emotion or behavior, hypothalamic neurons may also become a target of endocrine-disrupting environmental chemicals.

Immortalized hypothalamic neurons (GT 1-7 cells) are derived from mouse hypothalamic neurons. The characteristics of GT1-7 cells which mimic hypothalamic neurons *in vivo* have made GT 1-7 cells as a good tool for the study of neuroendocrine system. Therefore, we applied bisphenol A and other several endocrine disrupting chemicals to GT 1-7 cells and observed the cell viability and morphological changes now.

In conclusion, we have developed the *in vitro* screening system for evaluating the synapse formation between cultured neurons using calcium-imaging and immunohistochemistry. Using this system, thyroid hormones were revealed to enhance the synapse formation in cerebral cortical neurons, and the existence and the extension of dendrites of cultured cerebellum Purkinje cells.

Using the thyroid hormone-induced enhancement of synapse formation as an indicator, we screened several compounds, which influenced the neuronal development. Our results indicate that cultured neurons of the central nervous system will become a useful tool for the practical screening and the study of molecular mechanism of neurotoxicity induced by these environmental chemicals. This work was supported by CREST of JST.

Q&A

Koibuchi : Thank you very much. Now his paper is open for discussion. Any questions? Yes, please.

Q : Here we are talking about calcium imaging, but has this been cultured using neurons alone?

Kawahara : In the beginning, there were initially glia cells in the cerebral cortex, but we later used serum-free conditions. I think they almost completely disappeared under the observation conditions. In the FRA2 image, we are at least unable to clearly see glia cells; what we see under these conditions are neurons only.

Q: What I am interested in is the synchronicity of neurons – the synchronicity of neurons with oscillation of calcium. I think you might have something to say about synchronization in the primary culture of GnRH of the hypothalamus even though there might not be synaptic conduction. What are your thought concerning this?

Kawahara : In the case of JT mansem cells of the hypothalamus, this is referred to as a "gap junction," and I think this has probably been confirmed conforms for the most part. Finally, I brought up JT mansem cells, because I think we should watch for the effect on gap junction as well as synapse formation. At least, concerning cerebrocortical neurons and hippocampus neurons, so far we have not observed gap junction using electron microscopes and so on. Siapusy has been confirmed using electron microscopes and so on, but what has been observed in the cerbrocortex and hippocampus has been mainly synaptogenesis.

Koibuchi : Any more questions? Yes, please.

Q : I have an interest in the phenomenon that synaptogenesis is driven or stimulated by thyroid hormone. But have you ever checked the effect of other steroid hormones, like estradiol, on synaptogenesis? Is this phenomenon specific for thyroid hormone or not?

Kawahara : Thank you for the question. That is interesting. I have also applied estradiol or other estrogen related hormones to the cerebrocortical neurons. But we have not observed significant effects.

We have also acquired tamoxifen, an anti-estrogen drug. Tamoxifen caused apoptotic neural death in hypothalamus and hippocampus, but not the cerebral cortex, so maybe the effects of thyroid hormone and estrogen related hormones are different in at least the cerebral cortical neurons, I think.

Q : Thank you very much.

Kawahara : Thank you very much.

Koibuchi : Regarding the BPA action of Purkinje cell arborization: since Purkinje cells also produce neurosteroids, the action of BPA could be exerted through the inhibition or change in neurosteroidal level produced in Purkinje cells rather than inhibition of thyroid hormone action.

Kawahara : That mechanism is very interesting, but at least at present, we have no idea for answering this question. So we must take many possibilities I think as you suggested. We would like to try it.

Koibuchi : Thank you. Any more questions? We are running behind time. Thank you very much.