



Requirements for Hazard Assessment of Chemical Substances and the Nervous System

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I believe that it is a national mission to provide a safe environment for children to grow up and to protect the nervous system from various hazardous substances during the early development period. It is critical to establish proper appraisal methods for assessing which of the many chemicals that surround us are genuinely hazardous to the nervous system. In order to correctly understand how chemical substances affect the nervous system, it is imperative to have an accurate knowledge of the uniqueness of the nervous system as an organ.

1. Basic brain cell components and functions

The nervous system has a variety of functions, such as learning, memory, motor, sensation, thinking and language. All these functions are handled by information processing performed by the neuron network created by the neurons, one of the cellular components of the brain.

The brain generally consists of several billion neurons. Numerous neural fibers (axons) and projections (dendrites) extend from each neuron and communicate electrically with one another across a small gap called a synapse. (Figure 1)

At the synapse, chemicals called neurotransmitters, such as acetylcholine, glutamic acid and GABA), are released from the end of the axons to bind with receptor proteins in the neighboring neuron. This will then open up ion channels of elements such as sodium, potassium, chloride and calcium to excite or inhibit the cells

electrically. The electric charge is then carried by the axons from one neuron to another so that the entire neuron network comes to be engaged in this process of information processing. Considering that approximately 1,000 synapses exist at each neuron, the complexity and enormous scale of the neuron network engaged in the processing of the electric signals is easily imagined.

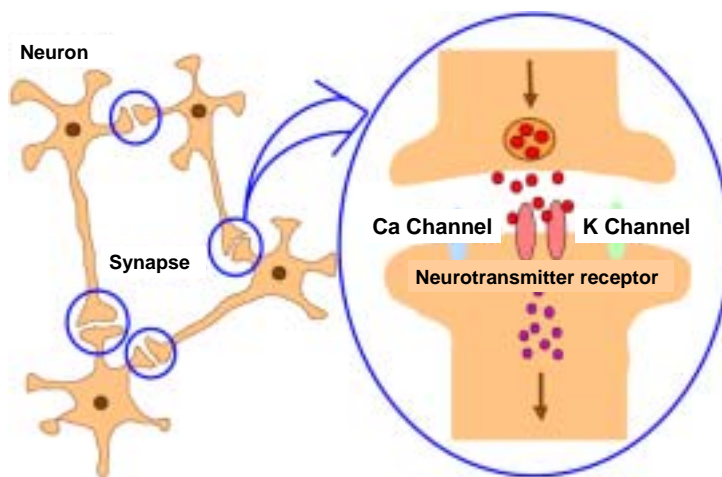


Figure 1. Neuron network and synapse created by the neurons

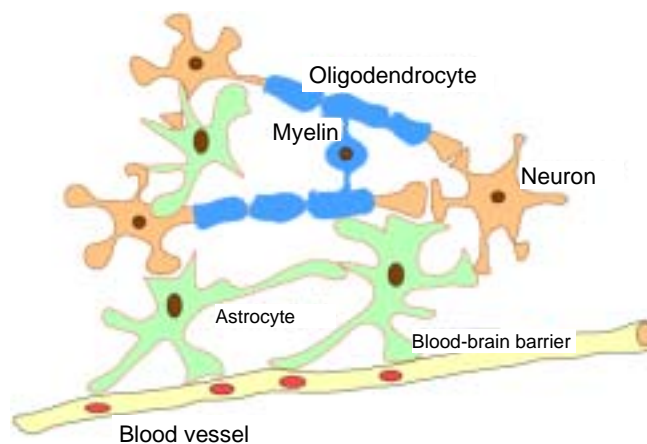


Figure 2. Cells forming the brain



Glial cells called astrocytes and oligodendrocytes also exist in the brain besides the neurons (Figure 2). Astrocytes outnumber neurons by approximately ten to one and have functions in supporting the survival of neurons and preventing hazardous substances from entering the brain by forming blood-brain barriers. Oligodendrocytes have extensions that wrap around the nerve tissues in many layers and form myelin -- a structure that serves in some way as an insulator, ensuring that the electric signals produced by the neurons are transmitted effectively. So, in essence, the glial cells such as astrocytes and oligodendrocytes actively support the functions of the neuron, while the neurons carry out various functions that the brain is in charge.

2. Development of the nervous system

The nervous system is created at an early stage of the development of an embryo, from tissue known as the neural plate. The edges of the neural plate rise until they meet and then merge with each other at the back to form a structure called a neural tube. This is the initial form of the nervous system. In the center of this structure there is a tube that will ultimately develop into the cerebral ventricle. In the deep part of the tissue facing the cerebral ventricle are a group of cells called neural stem cells, each of which divides vigorously in order to proliferate. At a certain stage in this process of repeated duplication, some of the cells are gradually directed to develop into neurons and, after some time, these cease to divide. Beyond this point, no further division takes place in these cells. Not long after the neurons have formed, the glial cells are also formed from the still-dividing neural stem cells. Both the newly formed neurons and glial cells migrate from the wall of the cerebral ventricle to a special part of the brain tissue. By

the time this migration is complete, a number of projections have grown out from the neurons. At the point where their migration has ceased, the neurons link to one another through the synapse to form a neuron network (Figure 3). The gap between the neurons is then filled, and oligodendrocytes will wrap around the nerve tissue and form myelin. This is the process by which the structure of the nervous system is completed.

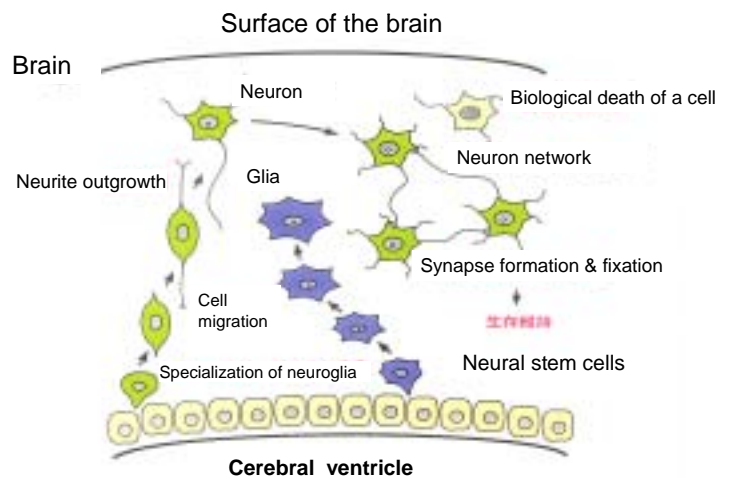


Figure 3. Development Process of the brain

In the case of the human brain, the birth of neurons and formation of the neural network occur at a very early stage of embryonic development. Although there is not yet sufficient data to determine the precise timing, it is generally considered that the network is almost completely formed by the 16th week of development (Figure 4). Sufficient amount of data has been gathered about the development of the nervous system for rodents, such as rats and mice, which are often used in research. The development process of the nervous system between rodents and humans is almost identical. The only significant difference is the duration and the timing until the formation of the nervous system is completed. For humans, the formation of the structure of the nervous system and the maturation of the glial cells is completed over the 10-month



period in a uterus. Whereas in rodents, although the development of the neurons and cell migration are finished by the time of birth, it will take another four weeks after the birth for the

formation of the neuron network through the synapses and the proliferation and migration of the glial cells.

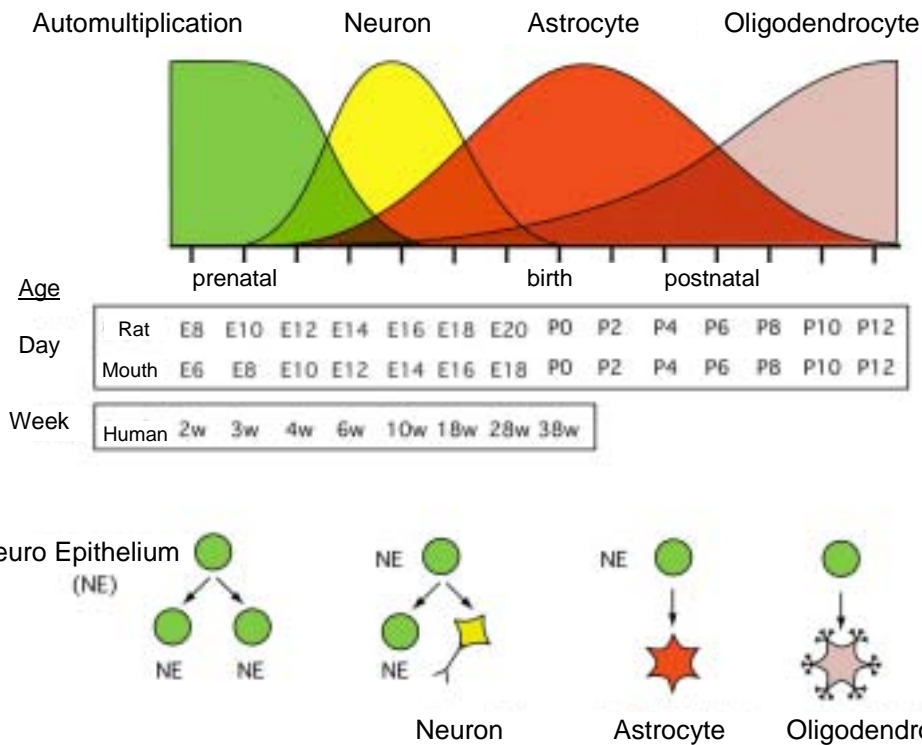


Figure 4. Development of Brains: Human and Rodent

* Courtesy of Noriko Okuma, Tohoku University Graduate School of Medicine

The structure of the human nervous system is mostly complete prior to birth, but from the point of view of a function, it will continue to develop over a long period of time after birth – in a way, throughout a person’s entire life. This is because a neuron network has a flexibility to adapt to the constant functional and physical changes although it may look rigidly completed on the appearance. The neuron network has a characteristic to change the number of the active synapses or the shape of axons depending on the various types of stimulation from the environment, including learning and sensational stimulation. This characteristic is called the plasticity of the nervous system. A typical

example of the plasticity of the nervous system can be seen in the case of the development of monozygotic twins, where the structure of the nervous systems is expected to be almost identical. According to environmental factors encountered during postnatal development, the personality of each twin can be different from one another.

3. Currently available hazard assessments for chemical substances and the nervous system

It is not an easy task to assess the effect of chemicals on the nervous system, especially the neuron network, which is formed by the delicate and sophisticated process and is very sensitive to



react to a wide range of stimulants. For most organs, an assessment method need only determine whether the cells are alive or not, or to be able to accurately indicate that the function of a certain group of cells has been disturbed. However, this is obviously not sufficient with the nervous system. It is important that any method attempting to assess effects on the nervous system needs to be able to measure a variety of effects on a wide range of groups of cells involved at different development stages. Not only that, the concept of plasticity must be introduced.

Guidelines on assessment methods for determining chemical effects on nervous systems have been established in many countries. One such example, shown here, is a guideline on tests for neurotoxicity of pesticides (Table 1). As seen in this example, the method employed is in vivo testing using mostly rodents and birds. Pathological tests, as employed in this example,

make it possible to detect whether the cells in the nervous system are alive or whether any deformity has occurred, while function tests will enable us to make some estimation on the degree of effect on the information processing function in the nervous circuits, and these results are actually used for the purpose of hazard assessment.

Assessment methods based on in vivo tests do have their own value. Yet, there are some with problematic aspects. One issue is that the current method of testing for effects on the nervous system takes too long, restricting our capability to respond to the tens of thousands of chemicals thought to be of concern. Also, many of the tests are aimed at gaining a general view of the function of the neuron network after the nervous system has been more or less completed. Therefore, it is difficult to apply the results of these tests to examining the effects on the neuron network at the developing stages or the effects on each synapse.

Table 1. Tests on neurotoxicity caused by chemicals and pesticides

	Guidelines	Animal	Test items	Purpose
Acute neurotoxicity	GLP No. 6283* OECD 424 US OPPTS 870.6200	Rat	General symptoms, function test, pathological test	Acute toxic risk assessment (Accidental ingestion, exposure, etc.)
Neurotoxicity by oral administration	GLP No. 6283* OECD 424 US OPPTS 870.6200	Rat	General symptoms, function test, ophthalmological test, pathological test	Risk assessment on long-term exposure through food and/or drinking water
Developmental Neurotoxicity	US OPPTS 870.6300	Rat	General symptoms, function test, pathological test	Risk assessment on prenatal/postnatal exposure during in utero and breast-feeding periods
Acute delayed neuro-toxicity	GLP No. 6283* OECD 418 US OPPTS 870.6100	Chicken	General symptoms, biochemical test, pathological test	Acute toxic risk assessment (Accidental ingestion, exposure, etc.)
Delayed neuro-toxicity by 28-day repeated oral administration	GLP No. 6283* OECD 419 US OPPTS 870.6100	Chicken	General symptoms, function test, pathological test	Risk assessment on long-term exposure through food and/or drinking water

* Ministry of Agriculture, Forestry and Fisheries Notice 1999 No.6283 Guidelines "On Good Laboratory Practice Standards for Toxicological Studies on Agricultural Chemicals"



4. Requirements for future hazard assessment of chemical substances and the nervous system

How then, can we establish a more accurate and effective system of assessment? I would like to propose one way of hazard assessment method, employing cultivated cells and tissue slices taken from the nervous system of rodents for the following reasons:

- 1) As mentioned above, the stages of development of the nervous system, including genetic programs, are similar between rodent and human beings.
- 2) There is established technology available for cultivating neurons and individual glial cells.
- 3) Using cultivated cells or slices will enable observation of the development of the human prenatal nervous system not only in its early developmental stage but also during postnatal developmental stages, including plasticity.
- 4) Not only whether the cells are alive or not, but also the function of neurons and the situations of information processing taking place at the neuron network can be examined relatively swiftly and conveniently.
- 5) Because the time required for assessment can be shortened, while a relatively large number

of examples are examined.

The following methods are available today for determining whether cultivated neurons are alive or dead (Table 2).

1. PI method
2. Hoechst staining
3. MTT Assay
4. Annexin-V staining
5. LDH release

For examining functions of neurons and neurocircuit, the following highly technical methods have been established:

- 1) Observation of the division and migration of the neural stem cells
- 2) Examination of the maturation level of the neuron and glial cells
- 3) Measurement of the quantity of released neurotransmitters
- 4) Measurement of neurotransmitter receptors
- 5) Measurement of synapse-related proteins
- 6) Imaging of the neuron's electric firing
- 7) Imaging of neuron network development
- 8) Observation of the plasticity of the nerves (long-term potentiation and depression)

Table 2: Methods for measuring cultured nerve cell death

Measurement Method	Principles
PI method	Colorimetric method for detecting cell death Detects PI bound to DNA after passing through non-functioning cell membranes
Hoechst Staining	Colorimetric method for detecting cell death Detects nucleus fragmentation characteristic of apoptosis
MTT Assay	Detection method for live cells Uses succinic acid dehydrogenase in mitochondria of live cells for cell vitality index
Annexin-V Staining	Colorimetric method for detecting cell death Detects Annexin-V bound to phosphatidyl serine exposed by changes in cell membrane structure
LDH release	Detection method for dead cells Uses lactate dehydrogenase (LDH) released from dead cells as index of cell death



5. Conclusion

It is impossible to examine all chemical substances with the currently available assessment methods for assessing hazards to the nervous system. It is, therefore, more effective to employ a method using cultivated nerve cells as the first phase screening assessment.