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## ESPECIAL CONSIDERATIONS FOR NEUROTOXICOLOGICAL RESEARCH

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## I. CONTRASTS BETWEEN HUMAN AND ANIMAL EXPOSURE

In the case of reports on human exposure, behavioral changes can only be related to pathological studies of postmortem tissue or to physiological studies on the exposed subject. While blood and urine samples have been used to detect abnormal levels of toxicants (such as lead), these samples give little indication of the detailed nature of damage to the nervous system. However, neurotransmitter-related characteristics of serum platelets may be of use in diagnosis of a variety of psychiatric and neurological disorders.<sup>94,97,131</sup> Alterations of platelet  $\alpha_2$  adrenergic receptors under some conditions can be taken as representative of changes of the corresponding receptor species with central nervous tissue.<sup>32</sup> This approach has been criticized as inappropriate in several circumstances.<sup>40</sup> However, some blood-based assays have been demonstrated to have clinical value in assessment of neurotoxic damage. For example, erythrocyte levels of sodium-potassium-dependent ATPase are depressed in miners receiving an occupational exposure to mercury.<sup>151</sup> Erythrocyte fragility has been proposed as a sensitive test for neurotoxic organic solvents and a correlation between hemolysis and neurotoxicity has been made.<sup>10</sup> Such an approach could be of value in detection of neurotoxic damage in humans.

Another limitation of data derived from humans is that subtle behavioral changes are often not recognized. However, undramatic sensory, motor, or psychic deficits may critically affect a person's judgment or ability to optimally carry out tasks. Unfortunately, all too often the first indication that a compound is harmful comes from rather undetailed reports of rather major damage to humans.

One of the ultimate goals of the toxicological study of animals is to understand and predict parallel events that might occur in humans. This is not an easy extrapolation since changes in rats tend to be most predictive of changes in other rats. Not only do species vary considerably in their metabolic treatment of toxic agents, but even different strains within a species can have very distinct responses.<sup>19</sup> Such biological features are relatively minor in comparison to the enormous behavioral diversity among species. Another limitation is that human exposure to toxicants tends to involve many variables, as opposed to the defined experimental situation in the laboratory. These unknown variables can seriously modulate the harmfulness of a toxicant. For example, heavy metals in combination can either antagonize or exacerbate each other's toxicity. A mixture of mercury and lead salts can act in a very strongly synergistic manner<sup>144</sup> as can concurrent administration of manganese and lead.<sup>36</sup>

Antagonistic effects between various toxic agents have also been described. Methyl mercury toxicity is alleviated by cadmium, another toxic metal, so that a mixture of both compounds is less toxic than either alone.<sup>59</sup> Such interactions may be very critical in environmental situations and the search for synergism and antagonism is an important area.<sup>116</sup> Because of the large number of potential interactions that are not merely additive, laboratory studies may throw light on "why a compound is harmful," but cannot easily approach the question of "whether the compound is harmless under a variety of conditions."

The choice of an experimental species involves several factors. Some of these are scientific such as metabolic and anatomical characteristics, while some are not, such as the availability and cost of a species. The limited availability of data relating cell biology to behavior in normal untreated animals may also limit an unambiguous correlation of toxic events and behavioral change.

## II. PROBLEMS OF EXPERIMENTAL DESIGN AND INTERPRETATION OF DATA

### A. Experimental Design

There are two conflicting concepts concerning the design of studies intended to increase understanding of the mechanisms of toxicity. One of these approaches stresses the importance of a sound hypothesis preceding experimentation. Studies are then carried out, that relate to this hypothesis so that the extent to which it is pertinent can be evaluated. It is desirable to use a variety of unrelated methods which can test a given theory by relatively independent means. This approach has an inherent bias in that it is focused so as to largely eliminate the consideration of alternatives.

The second means of throwing light on toxicity is to carry out a broad series of measurements that are not directed towards a specific hypothesis. This may be a relatively objective procedure but tends to be diffuse and time consuming. The inherent weakness of each of the two designs can be reconciled by initially using a broad conceptual framework as a means of pinpointing those parameters that are vulnerable to toxic attack. The data from this approach can then form the basis of a more detailed hypothesis which can then be tested by more targeted techniques.

An example of this "research by ever increasing resolution" is our work on acrylamide. The initial hypothesis was that certain neuronal circuits were more vulnerable than others to this neurotoxic agent. A series of receptor binding assays suggested that dopaminergic circuitry was especially affected by acrylamide.<sup>1</sup> This prompted the development of a more focused approach including regional analysis of levels of dopamine and its metabolites and demonstration that treated animals were unusually sensitive to drugs believed to act on the dopamine system.<sup>25</sup> The resolution increased as other questions were asked:

1. Is the affected dopamine receptor pre- or postsynaptic?<sup>80</sup>
2. Is the active agent acrylamide or a metabolite thereof?<sup>2</sup>
3. Are such changes mediated by the endocrine system?<sup>168</sup>

However, the primary mechanisms of action of acrylamide on the central nervous system remain elusive.

Several other factors impinge on experimental design. For example, the availability of space or funds may dictate the animal species and methodology used. The generation of the most meaningful data for the least cost is usually a major issue. Another consideration is what constitutes the final goal of an investigation. Increased knowledge about the primary mechanism of action of a neurotoxic agent may require relatively high doses of that agent to be administered for a short period. However, attempts to develop an animal model exhibiting changes similar to those seen in exposed humans may involve long times of

exposure to relatively low doses of toxicants. The latter situation may involve many complex indirect effects that are difficult to understand but highly relevant to the real life hazards presented by a chemical. The biochemical study of neurotoxicity can have several goals, and each objective requires a differing research strategy. Thus, it is important to clearly define the goal one is pursuing. Two examples of these follow.

#### *1. The Detection of Initial Sites of Impact of a Neurotoxic Agent*

This goal is of value in furthering our understanding of the direct mechanisms involved in the initiation of toxicity. Such work may be oriented toward the more ubiquitous types of cell component. General metabolic events such as rates of oxidative phosphorylation and macromolecule synthesis may find neurological expression, when altered throughout an animal. One feature of the approach of identifying the initial sites of action of xenobiotics is that the opportunity exists of reproducing such effects *in vitro*. This allows interactions to be studied under more defined conditions and strengthens the argument that a primary event is involved. Thus, lead will inhibit  $\delta$ -aminolevulinic acid-dehydratase *in vivo* and *in vitro*,<sup>118</sup> suggesting a direct action on the heme biosynthetic pathway.

Direct sites of action of neurotoxic chemicals may not always be within the nervous system. Many chemicals are metabolized by enzymes to form toxic materials. The liver is especially capable of both detoxification and "toxification" reactions. Tetraethyl- and tetramethyl-lead compounds appear to be hepatically metabolized to more neurotoxic materials than they themselves are.<sup>70</sup> Damage to peripheral glands such as testis or adrenals can upset the normal hypothalamohypophyseal feedback mechanisms by which their secretion is regulated. For example, while manganese is generally thought to directly damage certain nerve pathways, it also has a damaging effect on the testis.<sup>82</sup> The resulting reduction in testosterone levels<sup>81,95</sup> may also modify an animal's behavioral characteristics.

For these reasons, the distinction between a neurotoxic chemical and a generally toxic chemical is by no means a clear one. In addition, the ultimate expression of all forms of toxicity has a behavioral component.

#### *2. The Correlation of Behavioral Changes Caused by Toxic Agents, with Alterations of Nerve Transmission*

This type of study attempts to account for the altered behavior of animals treated with a toxic agent, in terms of deranged underlying neuronal circuitry. This involves the measurement of chemicals or phenomena that are confined to distinct physiological and morphological components of nerve tissue. Such changes may be indirect and at the terminus of a series of secondary effects of the toxic agent.

This approach does not attempt to directly identify primary molecular mechanisms. Delineation of damaged neuronal species can, however, give clues to the series of steps that lead to altered behavior. This goal generally involves assay of neurotransmitter-related parameters since it is here that changes immediately relevant to behavior take place. Neuropharmacological agents generally exert their effects on a relatively limited cell population, often confined to a single neuronal class. On the other hand, many environmentally prevalent neurotoxic agents such as lead probably act at several sites common to most tissues (Figure 1). This tends to broaden the number of potential goals that neurotoxicity studies may have in comparison to pharmacological research.

### **B. Variables Which May Interact with a Neurotoxic Response**

Several features of experimental design may modify the effect of a toxic agent upon the chemical and behavioral features of an organism. It is important to delineate such influences. At worst they may cause erroneous interpretation of data, while at best they may throw light upon mechanisms of action of neurotoxicants.

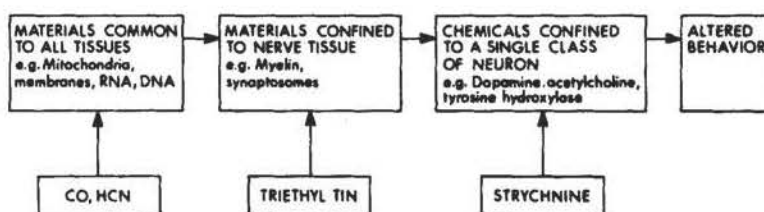


FIGURE 1. Possible sites of action of neurotoxic agents.

### 1. *Animal Species and Strain*

The most important features of neurotoxic action are likely to be generalizable. If two strains from within the same species show major differences in response to exposure to a neurotoxic agent, it is unlikely that results can be applicable to humans. However, such data may contribute to knowledge concerning different metabolic pathways and adaptive strategies adopted within a single species. Albino rats and mice have widespread laboratory use, but it should be borne in mind that such strains may exhibit relatively distinctive or abnormal responses to pharmacological agents.<sup>47</sup> When rats and mice are compared, they may exhibit very different responses to neurotoxicants although they are somewhat related species.<sup>39</sup> Inbred animal strains, while providing rather homogeneous experimental material, may exhibit genetic depression and significantly inferior responses to stimuli.<sup>77</sup> The combination of biochemical and behavioral approaches to the study of neurotoxicants often involves *in vitro* assays from animals that have experienced behavioral testing. This can have a profound effect on results obtained. For example, glutamic acid dehydrogenase levels are depressed in the hippocampus after a kainic acid-induced lesion. The rate of behavioral recovery and of restoration of normal enzyme levels is markedly accelerated by daily experience with a maze task<sup>71</sup> and behavioral testing can have long-term effects on the serum endocrine profile.<sup>169</sup> The rat is widely used and this facilitates comparisons between laboratories. It is relatively insensitive, however, to certain neurotoxicants including manganese and teratogens such as thalidomide,<sup>149</sup> and is behaviorally governed to a major extent by its olfactory sense. The extended gestational period of the guinea pig maximizes the maternal influences on the fetus, and this may present a specific advantage. Conversely, direct effects on embryos in the absence of maternal influences can best be studied in an egg, usually from a quail or chick. The chick also has fewer nonspecific and sudden lethal reactions to toxins.<sup>138</sup> A fundamental question in toxicological research is the extent to which data derived from various species can be extrapolated to humans. In view of the incomparable sophistication of human mentation, this question is especially difficult to answer in neurotoxicological studies. The use of primates is expensive and potentially liable to adverse societal judgment. However, it is obvious that such species are more likely to exhibit responses to toxic agents that closely resemble the corresponding effects on humans. Such research has great potential value and can be carried out humanely and with appropriate safeguards. It is essential that this type of work not be totally abandoned in the face of social pressures.

### 2. *Animal History*

Many environmental factors can influence the effect of a toxic agent on animal behavior or neurochemistry. Thus, dopamine metabolism is altered by caging density<sup>103</sup> and basal neurotransmitter receptor binding is affected by whether an animal has previously been subjected to a behavioral test battery.<sup>167</sup> An animal that has been repeatedly handled also has a differing endocrine response than its unhandled counterpart.<sup>168</sup> Neurochemical and neuroendocrine changes caused by exposure to a toxicant may only be apparent in handled



animals or only in naive animals. Therefore, there is no general optimal procedure; rather there is a need to be aware of such factors. Dietary factors may also be important and can affect rates of absorption and metabolism of a toxicant.<sup>139</sup> The case for the use of purified, defined diets in toxicological research has recently been clearly presented.<sup>176</sup>

Developmental studies involving maternal interactions with offspring can pose particular problems. It is known that lead treatment of lactating mothers can cause developmental delay in pups. This may be due to a direct effect of lead, altered food consumption of mother or pup, or a change in maternal behavior.<sup>14</sup> Although these factors are difficult to dissect apart, the overall situation has important implications for the human population.

### 3. Toxicant Administration

The means of dosing experimental animals can be by several routes. Injection can be intravenous, subcutaneous, intraperitoneal, or directly into a specific cerebral site. In addition, the diet could contain the agent to be studied, or it could be administered by gavage. Each route has distinctive features. Dietary ingestion of an agent does not subject the animal to any potentially stressful manipulation, but the delivered dose is hard to control since the absorption rate of the agent and the appetite of the animal may be variable factors. Repeated intraperitoneal injections of saline have been shown to alter the development of rat pups, perhaps by modifying the maternal response<sup>109</sup> and to alter several endocrine and neuropeptide-related parameters.<sup>81</sup> Intracranial injection is rather invasive but largely eliminates the need to consider hepatic metabolism of the toxic agent. The stress of such injections is known to inhibit cerebral protein synthesis,<sup>55</sup> and thus the overall activity level of the brain is likely to be altered by this procedure. The liver has capacities to detoxify or otherwise modify many administered circulating chemicals. Such metabolic changes may also produce the actively neurotoxic agents.<sup>72</sup> The vehicle in which the toxic agent is delivered may itself have the capacity to alter animal behavior or biology. Complex interactions have been found to occur between chlordecone and the commonly used vehicle dimethylsulfoxide. This solvent may derange the hypothalamo-pituitary-adrenal axis.<sup>9</sup> Even the acetate component of lead acetate may have an effect on weight and activity levels of treated rats.<sup>13</sup>

The possibility that an agent may cause altered body temperature should be kept in mind. Brain polysomes are especially sensitive to hyperthermia,<sup>76</sup> and hypothermia also adversely affects cerebral macromolecule synthesis.<sup>33</sup> Thus, many biochemical or behavioral consequences of a toxicant may be mediated by altered temperature. Certain environmentally prevalent neurotoxic agents such as DDT are known to cause damage to thermoregulatory mechanisms.<sup>170</sup>

### 4. Time Relations of Biochemical and Behavioral Events

Since behavioral effects may be only indirectly related to the initial biochemical effects of the toxic agent, a clear relation of behavioral changes and the concentration of the administered agent cannot be expected.<sup>101</sup>

Tricyclic antidepressants rapidly block catecholamine reuptake systems but, when administered to humans, psychic changes are not evident until these drugs have been administered for about 3 weeks.<sup>90</sup> Thus, a major biochemical effect is not temporally related to mood changes. This does not mean that the two events are not connected. The process of adaptation to a drug or toxic agent may be that feature which results in behavioral change. The correlation of behavioral change and known major biochemical changes is also not clear in the case of several organophosphates. These compounds cause a severe but transient depression of a specific enzyme (neurotoxic esterase) while paralysis ensues at a considerably later time. There is a clear linear relation between the ability of compounds to block this esterase and their ability to cause paralysis, but no cause-effect pathway has yet been uncovered.<sup>85</sup>

### III. SPECIALIZED FEATURES OF THE NERVOUS SYSTEM RELEVANT TO ITS VULNERABILITY TO TOXIC AGENTS

#### A. Biochemical Features

The existence of a considerable population of chemicals specific to the nervous system creates a range of potential targets for specific neurotoxic events. Potential targets include a distinctive proteolipid complex-myelin, which ensheathes most neurons. The hydrophobic nature of myelin allows a preferential accumulation of lipophilic materials, such as organic solvents. Such a partition can result in a considerable concentration of certain toxicants, facilitating injury of the laminated myelin sheath. Demyelination is observed in a variety of toxically induced neuropathies.<sup>155</sup> Such damage can impair normal neuronal function and, because of the relatively low rate of replacement of some myelin components, can be relatively persistent. Peripheral nerves and certain central tracts such as the optic nerve appear the most sensitive areas.<sup>142</sup>

The process of myelination is susceptible to a variety of toxic agents. These may act by directly damaging myelin or may exert an adverse effect on general development and thus indirectly retard myelin deposition. Inorganic lead may partially act in this latter manner.<sup>114</sup> Another way in which myelin development can be indirectly affected is by way of neuronal damage, because axonal diameter plays a role in determining the extent of oligodendroglial production of myelin.<sup>64</sup> Myelination may even be affected by the extent of electrical activity in the ensheathed nerve.<sup>91</sup>

Another area of nervous system distinctiveness is in the complex chemicals and processes involved in the process of neurotransmission. The transfer of information across the synapse involves a variety of unique events. These include the release and reuptake of neurotransmitters and the specific high affinity binding of transmitters to extracellular sites. Such superficial binding can elicit a series of major intracellular responses ranging from alterations in ion flux to activation of enzymes.<sup>152</sup>

Interference with any of these interrelated processes can disrupt nerve function. In addition, the equilibrium between synthesis and catabolism of neurotransmitter species is a critical element in nerve function. Thus, the inhibition of acetylcholinesterases by some organophosphate insecticides can have a lethal effect by allowing acetylcholine levels to build up and thus cause cholinergic hyperactivity.<sup>106</sup> Some neurotransmitter species such as dopamine are especially sensitive to oxidation and this process may be catalyzed by certain metal ions such as manganese.<sup>37</sup> The possibility exists that such oxidative products are sometimes harmful due to their close but incomplete resemblance to the parent molecule. For example, 6-hydroxy-dopamine is selectively toxic to adrenergic neurons.<sup>108</sup>

#### B. Anatomical and Morphological Features

The mature nervous system possesses almost no capacity to replace lost neurons. This lack of proliferative and regenerative capacity, however, is tempered by a great adaptive capacity that persists throughout adulthood. The ability of an organism to adapt to nerve damage if given sufficient time makes the behavioral detection of such damage a challenging task. In many cases, abnormal behavior can be elicited only by subjecting the animal to a stressor. Such stressors can unmask deficits by creating a situation to which the animal cannot adapt. Techniques used involve dietary restriction,<sup>156</sup> physical stress,<sup>136</sup> and pharmacological agents.<sup>35,163</sup> The latter approach has the advantage that a specific biochemical lesion can often be inferred (see later section). The low rate of cell division of the mature brain makes it more resistant to agents blocking such division, such as antimetabolic agents and X-irradiation, both of which are used in treatment of cancer. However, the developing brain undergoes rapid cell proliferation, generally during fetal life in most mammals. The range of susceptibilities of the fetal brain therefore can differ very significantly from that

of the adult brain.<sup>53,111</sup> Thus, the effects of gestational exposure to toxic agents can only be determined by direct experimentation and generally not by analogy with the adult situation. Another feature that makes consideration of the immature brain very unlike the mature brain is the incomplete development of the blood-brain barrier. This barrier prevents the ready access to the brain of many blood-borne materials.<sup>122</sup> Some areas of the brain such as the neurohypophysis are not protected by this membrane barrier<sup>20</sup> even in the adult and may thus become unusually sensitive to circulating toxic substances. The blood-brain barrier also can be a handicap to the access of desirable therapeutic agents to the brain. Some neurotoxic agents are thought to act by damaging this barrier<sup>126</sup> or by altering general vascular permeability.<sup>83</sup> The blood-brain barrier may be transiently opened, e.g., with mannitol, and this may facilitate the study of the neurotoxic component of a generally toxic material such as vincristine.<sup>164</sup>

Axoplasmic transport is a process that represents an exaggeration of a property found in all cells. This is due to the highly asymmetric nature of neurons. It has been stated that if the neuronal soma were the size of a tennis ball, some axons would have a length corresponding to 3 miles.<sup>68</sup> This results in an enhanced emphasis on the movement of proteins and other materials along the axon, and is associated with an unusually high content of microtubule protein in nerve tissue, in the form of neurotubules. This makes the nervous system sensitive to certain kinds of antimitotic agents such as colchicine and vinblastine although the rate of mitosis in adult nerve tissue is very low. Thus, these agents can have behavioral effects that are not related to their potent antimitotic activity.<sup>41</sup> Other agents such as *n*-hexane, methylbutylketone, and aluminum interfere with axoplasmic transport but have no antimitotic properties.<sup>113</sup> The agents probably damage the neurofilaments.<sup>148</sup>

### C. Physiological Features

An outstanding characteristic of the mature brain is its requirement for a high and continuous energy supply. A large proportion (20 to 25%) of the organisms glucose and oxygen need is required for this organ which constitutes under 5% of body weight.<sup>153</sup> Not only is this need large, but it is also continuous. While a temporary shutdown of the circulation can be withstood by most organs, the brain will develop irreversible damage in a few minutes.<sup>153</sup> Consciousness can only be sustained for 2 sec by humans whose cerebral circulation is withdrawn.<sup>48</sup> This acute dependence on an energy source is not found in the immature brain and is not characteristic of the peripheral nervous system. A toxicant blocking energy production in a relatively general way, such as cyanide or carbon monoxide, can have specifically neurotoxic sequelae that persist.<sup>18,107</sup>

The brain also has distinctive nutritional requirements. For example, because phenylalanine hydroxylase is not present in the brain, tyrosine becomes an essential amino acid for this organ, unlike liver. The inability to convert phenylalanine to tyrosine can under some circumstances (phenylketonuria) make phenylalanine a neurotoxic agent.<sup>115</sup>

### D. The Relationship between the Body and the Brain

The brain and body reciprocally regulate and influence each other. In addition to its neurotransmitter role, the nervous system influences the rest of the body by neurotrophic and neuroendocrine means. The state of differentiation of most body tissues is controlled by one of these latter means. Directly innervated tissues such as muscle depend on neurotrophic factors to maintain their structure, while noninnervated tissues often depend on hormones, ultimately under hypothalamic control two major consequences emerge from this.

1. The hypothalamus is the area where around 40,000 nerve fibers mediate between the central nervous system and the rest of the body, by way of release factors, trophic hormones, and endocrine glands (Figure 2). Thus, damage in this rather small area



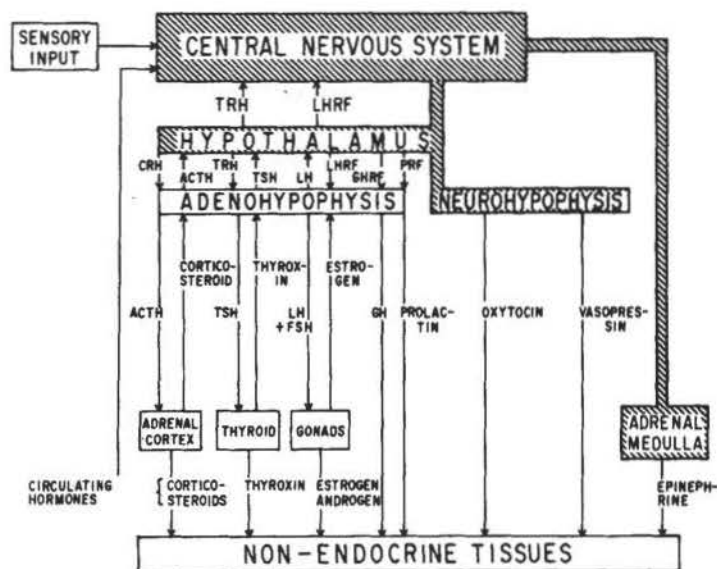


FIGURE 2. The hypothalamic pituitary linkage of nervous and nonnervous tissues.

can have major reverberations. In view of the incompleteness of the blood-brain barrier (previously discussed), the hypothalamus is also more exposed to systemically circulating materials.

- Major toxic damage can appear to be remote from a primary hypothalamic lesion. By this means, a neurotoxic agent (such as monosodium glutamate) can cause major changes in sexual function and body weight while CNS changes are confined to a very small area — the arcuate nucleus.<sup>124</sup> Sometimes, general systemic damage may be underlaid by specific damage to nerve tissue. For example, an important role of the sympathetic nervous system may be the prevention of carcinogenesis,<sup>159</sup> probably by way of the immune system.<sup>158</sup> Thus, sympathetic nerve growth promoting materials such as nerve growth factor can protect against carcinogenesis<sup>171</sup> and many toxic agents interfering with the sympathetic nervous system could result in reduced immunological surveillance.<sup>159</sup>

#### IV. BIOCHEMICAL AND PHARMACOLOGICAL APPROACHES TO NEUROTOXICOLOGICAL STUDY

Since a large proportion of vertebrate biochemical processes are represented within the brain, neurochemistry encompasses a wide range of study. This area is further expanded since there is no clear dividing line between brain chemistry, neuroendocrinology, neurophysiology, molecular biology, and cell biology. In the search for understanding of nervous function, one must frequently work at the interface between two more disciplines and from this the new concept of neurobiology has arisen.

While one cannot say that any of the myriad possibilities of neurochemistry should *not* be studied in neurotoxicology, there are certain areas which undoubtedly are relevant. In general, the more specialized features of brain chemistry are likely to be the targets of agents that are specifically neurotoxic, and each of the following sections will be addressed primarily to the aspects of cerebral metabolism that are pronouncedly distinctive. These properties may be quantitatively distinct (e.g., the high energy dependence of central nervous tissue and axoplasmic transport processes) or qualitatively unique to nerve tissue (e.g., myelin- or neurotransmitter-related processes).

Candidates for use in neurotoxicological study can be broadly classified: enzymes; chemical composition of the brain; metabolic activity of the brain; myelin- and membrane-related phenomena; axoplasmic transport; neurotransmitter-related parameters; neuroendocrine factors; and tissue culture.

### A. Enzymes

Of the several hundred enzyme systems that can be assayed, a few can be considered especially important for toxicity studies. Such importance can be for one of two reasons.

1. The enzyme may be the rate-limiting step in a metabolic path and thus any change in its level could alter the rate of synthesis or breakdown of a chemical and thus the concentration of that chemical's concentration within the brain. An example of such an enzyme is tyrosine hydroxylase which appears to be rate limiting to catecholamine synthesis.<sup>119</sup> In many cases, inhibition of an enzyme can cause it to become rate limiting. Thus, fluoracetic acid owes its toxicity to its conversion to fluorocitrate. The consequent inhibition of citrate synthetase blocks the Krebs cycle and causes cessation of oxidative metabolism.<sup>98</sup> A host of neurological disorders exist where the underlying cause is the genetic absence of an enzyme.<sup>27</sup> In the majority of such mutations, it is the catabolism of brain chemicals that is blocked. The subsequent accumulation of abnormal amounts of metabolic intermediary products is generally very damaging. However, genetic diseases where an anabolic enzyme is missing (e.g., phenylketonuria) are also known.<sup>45</sup> The wide variety of genetic neuropathological diseases that are known illustrates the many points at which nervous function is vulnerable.
2. A more limited selection of enzymes can be considered as biochemical markers. These enzymes are known to be associated with a particular cell type or subcellular fraction.<sup>11</sup> Alternatively, these enzymes when present in abnormal amounts can be indicators of a certain type of pathological change.<sup>63,86,141</sup> Thus, these enzymes can be very useful in determination of the locus of action of neurotoxicants.

Neurotoxic effects may be detectable in humans by means of analyses carried out on biological fluids such as blood-urine and this could be utilizable as a screening procedure on high risk populations such as those working in certain industrial processes. Erythrocyte  $\text{Na}^+$ ,  $\text{K}^+$ , ATPase levels have been found to vary with different clinical phases of manic and depressive disorders.<sup>4,78,120</sup> These levels have also been shown to be depressed in miners exposed to mercury.<sup>151</sup> Damage to the blood-brain barrier may be detected by the presence of a specific creatine kinase in serum.<sup>29,128</sup> Another test might involve measurements of serum transketolase, a thiamine-dependent enzyme which is reported to play an important role in the maintenance of the myelin sheath.<sup>54,179</sup> An attractive aspect of measurement of serum transketolase levels is that variations may correlate with recovery as well as the onset of toxic responsiveness.<sup>112</sup> Similarly, serum  $\beta$ -hydroxylase has been suggested as an indicator of toxically induced excessive sympathetic nervous activity.<sup>89</sup> Platelets have been used by several workers to study the role of biogenic amines in psychiatric and neurological disorders. Biogenic amine pharmacodynamics of platelets have been correlated with Downs syndrome, mental retardation, infantile autism, hyperactivity, schizophrenia, Parkinsonism, Huntington's disease, and headaches.<sup>157</sup>

Several enzymes exist where an alteration of the activity level has implications as to the status of nerve tissue. One short-term biochemical test which is of interest in connection with its possible utility for predicting long-term neurotoxic effects is the "neurotoxic esterase" test described by Johnson.<sup>86</sup> This test, which was developed in chickens, may be of some use in predicting toxicity of organophosphates. The lysosomal  $\beta$ -glucuronidase and  $\beta$ -galactosidase levels have been found to be greatly elevated in a variety of central and

**Table 1**  
**DIAGNOSTIC ENZYMES**

Enzyme	Marker for
Cholesterol esterase	Myelin content
2'3'-Cyclic nucleotide phosphodiesterase	Myelin content (protein component)
Galactose-UDP ceramide transferase	Rate of myelination, oligodendroglia
Na <sup>+</sup> K <sup>+</sup> ATPase	Cell surface membranes
	Ion transport phenomena
Mg <sup>2+</sup> ATPase	Oxidative phosphorylation failure
RNA polymerase	Nuclei
Succinic dehydrogenase	Mitochondria
	Oxidative metabolism
NADPH-cytochrome reductase	Intracellular membranes
Lactic dehydrogenase	Cytosol
β-Glucuronidase	Lysosomes
	Astroglial, microglial hypertrophy
	Wallerian degeneration
Superoxide dismutase	Aging, membrane protection
Choline acetylase	Cholinergic neurons
Dopamine β-hydroxylase (DBH)	Noradrenergic neurons
Neurotoxic esterase	Organophosphate toxicity
Ornithine decarboxylase (ODC)	Cell proliferation, activation, hypertrophy

peripheral neuropathological conditions.<sup>50,88</sup> It would appear that acid phosphatase may also provide an indication of lysosomal damage or macrophage abundance.<sup>50</sup> Ornithine decarboxylase levels are elevated in tissues undergoing proliferation, hypertrophy, regeneration, or activation<sup>138</sup> and may provide clues as to the initial site of neurotoxic damage.<sup>16,51</sup> The onset of, or extent of, myelination may be reflected by tissue levels of cholesterol,<sup>49</sup> cholesterol esterase,<sup>60</sup> or 2', 3'-cyclic nucleotide 3'-phosphohydrolase.<sup>12,93</sup> The numbers of synapses may be reflected by tissue levels of neurotransmitter compounds, of transmitter-metabolizing enzymes (e.g., tyrosine hydroxylase, dopamine β-hydroxylase, glutamate decarboxylase, etc.<sup>26,30,46</sup> or by gangliosides.<sup>49</sup> Functional integrity of mitochondria may be reflected by tissue levels of citrate synthetase, Mg<sup>2+</sup> ATPase, or succinate dehydrogenase.<sup>162</sup> Plasma membrane functional ability may be reflected by levels of Na<sup>+</sup> + K<sup>+</sup> ATPase<sup>5</sup> or by levels of transmitter- or modulator-sensitive adenylate cyclase.<sup>73</sup> Table 1 lists a selection of enzymes and the parameters for which they may be considered as indicators.

### **B. Chemical Composition of the Brain**

The chemical composition of the brain is altered in many disease states and the assay of certain materials may provide clues as to sites of action of toxic agents. As with enzymes, certain chemicals can be considered as markers for specific cell types or subcellular fractions. Excessively low amounts of substances or abnormally high concentrations may be equally damaging.<sup>132</sup> This kind of survey can be especially well applied to the developing brain where the accretion of many brain-specific chemicals in a certain sequence is essential for normal maturation.<sup>57</sup> Table 2 shows some of the substances that may be indices for various neurobiological processes. Again, many examples of neurological disease are known where a change in concentration of a material is a diagnostic concomitant. These neurological disorders frequently present a distinct neuropathological profile.

### **C. Metabolic Activity of the Brain**

Because the brain is dependent on a high and unceasing nutrient supply, any derangement of this supply is likely to result in serious neurological sequelae. Conversely, reduced

**Table 2**  
**DIAGNOSTIC CHEMICALS**

Substance	Marker for
Cerebral GD <sub>1</sub> gangliosides Spingomyelin DNA	Neurons Schwann cells, oligodendroglia Alterations in cell number (Gliosis, regional degeneration, aging)
14-3-2 Protein S100 Protein Proteolipid protein Cerebroside Acetylcholine Norepinephrine	Neurons Glia Myelin protein Myelin lipid Cholinergic neurons Noradrenergic neurons
Urinary or cerebrospinal fluid Homovanillic acid 5-Hydroxyindolacetic acid	Catecholamine turnover Indoleamine turnover

metabolic supply to a specific brain area has been shown in some cases to reflect functional inactivity of that area, while hyperactivity of a brain region can be detected by excessive vascular supply.<sup>96</sup> In this manner epileptic foci have been located within the brain.<sup>166</sup> The introduction of deoxyglucose as a means of measuring glucose supply to discrete brain regions has made possible accurate determination of relative rates of blood flow through brain areas.<sup>66,154</sup> This could be a very useful approach in neurotoxicological studies especially when the agent in question is suspected of causing localized rather than general brain damage. Good methods now exist that enable rapid evaluation of the integrity of the blood-brain barrier.<sup>22</sup> These involve double labeling techniques and combining a rapid diffusible substance with a relatively impermeable chemical.<sup>122</sup> Even small alterations of the ability of the brain to exclude certain materials can be measured by this means. Some toxic agents have been shown damaging to this barrier.<sup>15,38,105,160</sup> In addition to chemical insults,<sup>126</sup> microwave irradiation has also been reported to damage the blood-brain barrier.<sup>125</sup>

Brain metabolic study also includes assay of rates of macromolecule synthesis. The intensity of cerebral protein synthesis has been suggested as a useful index of general neurotoxicity.<sup>56</sup> However, nonspecific phenomena such as body temperature of treated animals must always be taken into consideration.<sup>33</sup> Since cell proliferation rates are very low in mature nerve tissue, the rate of DNA synthesis may be a useful clue in detecting abnormal cell proliferation such as gliosis. The precise dating of the birth of neurons by <sup>3</sup>H-thymidine administration during gestation may also allow the detection of delayed or abnormal neurogenesis in animals prenatally exposed to neurotoxicants.<sup>127</sup>

The effect of toxic agents upon cerebral metabolism may be mediated by interference with critical cofactors such as pyridoxal phosphate which, if deficient, results in seizures. This effect is probably due to the vitamin B<sub>6</sub> requirement of glutamic acid decarboxylase, a key enzyme in determining the ratio of the excitatory transmitter, glutamate to the inhibitory  $\gamma$ -aminobutyric acid.<sup>134</sup> Hyperbaric oxygen can dramatically alter metabolic rates by causing convulsions. This has been attributed to the unusual sensitivity of glutamic acid decarboxylase to oxidative destruction. Damage to this enzyme leads to reduced rates of formation of the inhibitory neurotransmitter, GABA.<sup>177</sup> These oxidative processes may be mitigated by superoxide dismutase.<sup>79</sup>

#### **D. Myelin- and Membrane-Related Phenomena**

Since many neurotoxic agents are known to cause neuropathy, the study of myelin com-

ponents could be of use in neurotoxicological evaluation. Such studies can be divided into three areas, namely measurement of the concentrations of the lipid and protein components of myelin, measurement of their rates of synthesis and decay, and assay of myelin-related enzymes. Neurological abnormalities of myelin include total or partial failure of myelination. Since the accretion of lipid and protein constituents are closely linked, it is sometimes hard to know where the primary point of failure is located.<sup>64,91,110,165</sup> Using mouse mutants,<sup>150</sup> it has been shown that partial failure of myelin formation is not incompatible with life. However, total lack of myelin is lethal at the time when myelination should be proceeding rapidly. Why the 10-day-old rat is not dependent upon myelin while the 30-day-old rat cannot survive without it, is not clear. The demyelinating disease, multiple sclerosis, generally has a variable course with periodic remissions. This illustrates the ability of remyelination to occur in the adult central nervous system. The majority of toxicological studies to date have examined peripheral rather than central nervous system myelin. Damage to the blood-brain barrier may result upon an autoimmune attack upon myelin by circulating lymphocytes so that toxic agents may affect myelin in this indirect manner. Thus, the role of viral agents in facilitating myelin-related disease may be relevant to toxicological studies.<sup>65</sup> Malnutrition has been shown to have a specific deleterious effect on myelination so the laying down of this nervous system-specific material may be unusually vulnerable to environmental factors.<sup>174</sup>

Many new ways of examining membrane structure now exist. These include electron spin resonance, fluorescence spectrometry, circular dichroism, and optical rotary dispersion analyses. Abnormal membrane fluidity and changed solvent penetrability may occur in response to relatively nonspecific organic toxic agents such as chloroform or ethanol.<sup>74</sup>

#### **E. Axoplasmic Transport**

This energy- and calcium-dependent process is disturbed in toxically induced peripheral neuropathies and in muscular dystrophy. Axoplasmic migration of materials occurs bidirectionally and seems to have two major functions.

1. The supply of macromolecules and organelles from the cell body where the bulk of anabolic processes are located, to the energy-utilizing presynaptic terminus which has limited synthetic capacities. These materials are essential for maintenance of basal biochemical metabolism and for neurotransmission, neurosecretion, and neurotrophic functions.
2. The exchange of information between the cell body and the nerve endings. Retrograde transport processes appear to inform the soma about the nature and status of the innervated larger cell. This has been best studied in the cases of neuromuscular innervation and the dependence of sympathetic neurons on target cell-generated nerve growth factor.<sup>145,161</sup> Axoplasmic transport is related to the axonal neurotubular and neurofilamentous fibers which run longitudinally within the axon. Neurofilaments are known to be deranged in Alzheimer's disease (premature senility) and aluminum has been implicated as a toxic agent that may influence the formation of pathological neurofibrillary tangles.<sup>148</sup> While several reports on axoplasmic transport in peripheral neuropathies exist,<sup>31,32,129,140</sup> little work has been carried out on disturbances of axoplasmic flow within the central nervous system. The avian visual system offers the possibility of quantitation of this process, by virtue of a large retina whose ganglion cells innervate a prominent and discrete target organ — the optic tectum. Toxic agents or isotopes can be injected into a single eye and the partner uninjected eye can often be treated as a control.<sup>17,21,133,172</sup>

#### **F. Neurotransmitter-Related Parameters**

Many ways exist of biochemically evaluating transmitter function within the brain. Levels



of transmitters have been measured frequently in toxicological studies and appear to be sensitive to a variety of compounds. It is possible to determine turnover rates of compounds by following their decline after administration of agents that block their synthesis. A more satisfactory way of assaying transmitter half-lives is by use of labeled precursors followed by liquid chromatographic separation of radioactivity incorporated into a specific compound.

The determination by high performance liquid chromatography of metabolic breakdown products from catecholamines and indolamines in urine, serum, or cerebrospinal fluid is possible. Increases in levels of such metabolites can be considered indices of metabolic activity of neuronal pathways related to specific transmitters. This approach is useful because it can be performed repeatedly in the same animal. In addition, these methods may have applicability to testing in humans who have been inadvertently exposed to toxic agents. Homovanillic and 5-hydroxyindolacetic acid levels in cerebrospinal fluid or urine are used as indicators of catecholamine and serotonin metabolism, respectively, while vanillmandelic acid levels may be used to estimate norepinephrine catabolism. Changed levels of these catabolites have also been reported in neurotoxicological studies.<sup>7</sup>

Dynamic events at the synaptic region may be studied by measurement of transmitter movements between cell fractions, rather than by their synthesis or degradation. These "translocations" can be used to study pre- or postsynaptic changes. They include the study of calcium-dependent presynaptic release of neurotransmitters, or determination of the activity of presynaptic high affinity, sodium dependent reuptake mechanisms. Neurotransmitter receptor sites within brain areas can also be measured and in a few instances, a biological consequence of such transmitter-receptor interaction can be determined (e.g., the various catecholamine-stimulated adenylyl cyclases).<sup>175</sup> All these translocation phenomena are modulated by pharmacological agents and they also appear to be responsive to relatively low levels of toxic agents.<sup>23,24</sup>

Radioactive drugs with a narrow range of targets have been used as tools with which to analyze neurotransmitter receptor sites.<sup>178</sup> Where phylogenetic studies have been reported, the characteristics of receptors across vertebrate species are remarkably similar.<sup>104</sup> Since the preponderance of this site is often regulated by the intensity of neuronal activity, deranged neuronal circuits can be detected using labeled ligands.<sup>58</sup> A wide variety of diseases have been shown to be correlated with unusual levels of these receptors. Such changes can often aid in understanding the neuronal derangements caused by the disease. Clinical syndromes which may have characteristic receptor changes include Parkinsonism, Huntington's disease, asthma, schizophrenia, pheochromocytoma, chronic heart disease, myasthenia gravis, and epilepsy.<sup>152</sup> The basis of receptor studies is the frequently reported inverse relation between the activity level of a nerve pathway and the neurotransmitter binding capacity of the postsynaptic receptors. Such a generalization has exceptions and is further complicated by the existence of presynaptic receptors which may not exhibit such response. However, the concept has been successfully applied in delineating vulnerable neuronal circuits after exposure to neurotoxic agents. These include acrylamide,<sup>1,25</sup> manganese,<sup>146</sup> organophosphates,<sup>6,67,100,143</sup> organochlorines,<sup>3,147</sup> and various heavy metals.<sup>8,44,61,75</sup> It should be borne in mind that this type of study is useful in accounting for altered behavior following exposure to neurotoxicants. However, such events may be at the terminus of a long sequence, and thus receptor study is unlikely to throw light on the initial molecular impact sites of a neurotoxic agent.

The development of new neurotransmitter-related chemicals whose major site of action in biological systems is well delineated has several applications to neurotoxicology. Some of these agents have been of great value in adding to our understanding of a variety of neurological and psychiatric disorders.<sup>152</sup> Similarly, the exacerbation or alleviation of signs of neurotoxicity by chemicals of known specificity can give clues as to sites of action. Pharmacological intervention by inhibition of a circuit that is already impaired can enhance

behavioral abnormality by stressing a neuronal system beyond the capacity of the organism to generate an adaptive response.<sup>2,35,62,99</sup> This "double-hazard" means of unmasking occult damage to the nervous system is termed a pharmacological challenge. In this manner, the availability of drugs of increasing selectivity can be beneficially exploited by toxicologists.<sup>17,3</sup>

An increasing number of compounds are known that are closely related to neurotransmission but have not been clearly established as neurotransmitters. These compounds appear to influence neurotransmission by altering the rates of the release and binding of neurotransmitters and are termed neuromodulators. Such chemicals may include peptides such as enkephalins, endorphins, substance P, and other compounds such as prostaglandins. The mode of action of such regulatory materials is the subject of much current research.<sup>92</sup> Very low levels of neural peptides can be assayed with radioimmune antibody techniques, and one neuron can be shown to possess several types of peptides.<sup>84</sup> A limitation of this method is the potential for cross-reaction with related compounds that antibodies raised to a single chemical may possess. The use of monoclonal antibodies is likely to increase the resolution capacity of this procedure.<sup>135</sup>

### **G. Neuroendocrine Factors**

The nervous system, by way of the hypothalamic-hypophyseal axis, regulates the major part of systemic hormone production. Conversely, several circulating endocrine materials are known to exert direct chemical and behavioral effects on the brain. The distinction between neurotransmitter and neuroendocrine function rests largely on whether the target tissue is adjacent to or distant from the nerve ending. But the two functions possess several common features. Neurosecreted materials, like transmitters, migrate distally along the axon and are secreted at the terminus by a calcium-dependent, exocytotic process. Target cells of neural and endocrine systems are often activated by release molecules impinging on specific receptor sites on the external cell surface. This event can pass a transmembrane signal to the interior of the cell, frequently resulting in modulation of cyclic nucleotide parameters. Interference with neurosecretory processes can have profound consequences for somatic metabolism. Because the infundibulum of the hypothalamus contains only a few thousand key neurons, this area might be especially vulnerable to toxic attack. As for the peptide neuromodulators, it is now possible to measure minute quantities of endocrine-related materials by radioimmune assay. Partially because of their special role in maintaining the homeostasis of many physiological functions, neuroendocrine systems are extremely sensitive in responding to external stimuli or to internal change of body systems. For example, mild stress causes an increase in plasma level of corticosterone and prolactin.<sup>81</sup> Therefore, it appears that neuroendocrine parameters can serve as a sensitive index to monitor how the animals respond to various external stimulus such as environmental neurotoxicants. However, endocrine changes may not always give a clue to the specific mechanism of action of a toxic agent. Certain hormonal parameters seem vulnerable to a wide variety of differing compounds. For example, serum testosterone levels are depressed by a wide variety of compounds including manganese,<sup>81</sup> acrylamide,<sup>7</sup> chlordecone and triethyl lead,<sup>81a</sup> inorganic lead,<sup>28</sup> and monosodium glutamate.<sup>121</sup> Although this may represent a nonspecific stress response, testosterone levels cannot be readily depressed by a simple stress which profoundly alters corticosterone and prolactin levels.<sup>81</sup> The idea has been proposed that the effects of toxic agents invariably involve nonspecific stress in addition to the distinctive toxicity and that such stress modulates the neuroendocrine axis.<sup>136</sup> This would account for the wide range of chemicals that can depress circulating testosterone levels.

### **H. Tissue Culture**

Tissue culture offers a rapid, inexpensive way of testing a variety of toxic agents. This system has been used with great success in the development of mutagenicity testing of large

numbers of compounds. While the neurobiological equivalent of the Ames test is still a long way off, such a simple system utilizing a controlled environment with few variables offers great promise in the neurotoxicological testing of compounds in the future.<sup>52,117</sup> Monoclonal cell lines of both neuronal and glial origin are available, and these have been shown to possess many of the biochemical, morphological, and physiological characteristics of neurons and glia, although they are derived from malignant variants. Many features of cerebral maturation can be reliably reproduced in cell culture including the cessation of proliferation, the onset of neurotransmitter- and myelin-related metabolism, and the formation of extensive cell processes (neurites). Several enzymes and specific proteins could be assayed with ease and little expense. In addition, this system allows the possibility of rapid morphological assessment of toxicity. Neuroblastoma cells have been used in the study of organophosphate-induced neurotoxicity<sup>69</sup> and mercury toxicity.<sup>130</sup>

The development of cell culture expertise will also allow the production of antibodies from monoclonal cell lines. These are derived from lymphocytes of immunized animals that have been transformed by fusion with neoplastic variants using Sendai virus. A homogeneous cell line, producing a single molecular species of antibody, can be produced by this means. Immunocytochemistry with such defined antibodies can give exact information as to the anatomical localization of specific proteins and other chemicals.

## V. CONCLUSIONS AND DIRECTIONS FOR THE FUTURE

Neurotoxic damage can be caused by many pharmacological agents, biological neurotoxins, and insecticides. These types of compounds have an inherent manmade or evolutionary design and are generally harmful in rather selective ways. Neurotoxic injury can also result from nonspecific, environmentally prevalent chemicals. These may be of natural origin (e.g., various metals or minerals) or may be products of industrial processes. Unlike pharmacological agents and natural toxins such as snake venoms, nonspecific toxic agents are not designed to affect biological function. They are, therefore, likely to exert their effects by interacting with more than a single biological site. This potential multiplicity makes it difficult to identify the precise mechanisms by which damage to the organism is effected. For this reason, sites of action of toxic agents are generally less well understood than those of pharmaceutical agents.

Altered behavior is an end point of the effects of all toxic agents, but not all such materials are classified as neurotoxicants. Neurotoxicity is generally understood to imply a direct effect on nerve tissue. However, most environmental agents that appear to primarily cause damage to the nervous system are also harmful to other tissues and organs.

Toxic agents are often on the continuum between random denaturation events, such as those caused by altered pH or heat, and the specific limited interactions that a pharmacological agent may have with a few protein species. Our understanding of the series of events caused by a toxicant is partly determined by progress in the understanding of neuroscience. Compounds that help in delineation of neurotoxic mechanisms are not necessarily the environmentally prevalent species. "Model" compounds with a more limited spectrum of action can be very useful in determining which cerebral parameters are unusually critical or sensitive.

One goal of the toxicologist is to design therapeutic approaches. Cholestyramine has been used to treat chlordecone toxicity, and the use of chelation therapy in heavy metal toxicity is well known. There are presently few such beneficial therapies, but the use of specific antisera to toxicants presents a potentially valuable means of accelerating elimination of toxic agents.<sup>43</sup> Also, substance P counteracts neurotoxic damage to developing adrenergic neurons and this peptide has been proposed as a potential therapeutic agent,<sup>87</sup> and vitamin B<sub>6</sub> may protect against acrylamide neurotoxicity.<sup>102</sup> When the details of sites of action of toxicants become known, the possibility arises of designing selective antagonists. For ex-

ample, *N*-methylaspartate neurotoxicity can be blocked by compounds binding at the same synaptic site.<sup>123</sup> The initial phase of development of a discipline often involves the tedious accumulation of a data base upon which hypotheses can be formed. The current state of neurotoxicology involves the gathering of much basic data and is reminiscent of the state of pharmacology around 30 years ago. We can expect the gradual emergence of patterns which will increasingly allow the formulation of those generalizations which form the core of a discipline. It is clear that this can only be brought about by simultaneous utilization skills from several established areas of neuroscience.

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