

## **Blessing, Chapter 2: Methodological Issues**

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*First identify your neuron.* R. McAllen (personal communication)

Our understanding of the manner in which the CNS controls a particular function often reflects characteristics of the methods used to investigate the function. The choice of methods may be unduly influenced by the subject matter. Thus, the characteristic respiratory rhythms of many lower brainstem neurons ensured that electrophysiology dominated early studies of central respiratory control. Electrical stimulation of the brain and spinal cord was used less commonly, probably because this procedure does not affect respiratory function in a quantitative, easily controlled manner. In contrast, electrical stimulation was extensively used in early studies of central cardiovascular control, presumably because the procedure elicited dramatic changes in arterial pressure and heart rate. Less emphasis was placed on extracellular recording techniques, possibly because it was difficult to find neurons with a clear cardiac cycle rhythm.

As a second example, one might consider the history of the study of the mechanisms controlling cerebral blood flow. Great emphasis is placed on the regulation of brain blood flow by metabolic factors. Evidence for the physiological importance of the nerves surrounding the larger cerebral vessels is considered "vague and equivocal" (Kety, 1950). However, most methods currently used to measure cerebral blood flow have a time resolution of several minutes, hardly sufficient to detect changes occurring over seconds, the time course likely to reflect neurally mediated changes in cerebral blood flow (see Chapter 5).

Different investigators may compare and contrast results obtained with quite different measures of particular functions, assuming that they reflect a common underlying event. One investigator may measure arterial pressure change, or blood flow to an organ, and another investigator may measure sympathetic nerve activity to the same organ in response to, for example, electrical stimulation of some brainstem center. The nerve recording technique detects responses occurring milliseconds after the stimulation, whereas the pressure or flow techniques measure responses occurring within seconds. Studies of "stress" may apply nociceptive stimuli for seconds, minutes, or hours. Apparently incompatible conclusions may reflect the failure of the investigators to appreciate the different time scales.

Comprehensive understanding of the way particular functions are controlled by the nervous system is more likely to be gained when different experimental techniques are applied to the same problem. Each method has strengths and weaknesses. The following discussion emphasizes potential problems with each method. How different authors have dealt with these problems has influenced the papers chosen as sources of evidence for the regulation of the various homeostatic functions reviewed in this book.

## **Lesions: Electrolytic, Thermal, and Mechanical**

Electrolytic, thermal, and mechanical lesions affect all the elements of the neuropil, including cell bodies, fibers, glia, and blood vessels. A lesion in one area might well affect a neighboring region by interfering with its blood supply. Damage to axons of passage is particularly important in interpretation of brainstem lesion studies. Acute lesions may have excitatory effects, sometimes related to iron deposition when anodal currents are used.

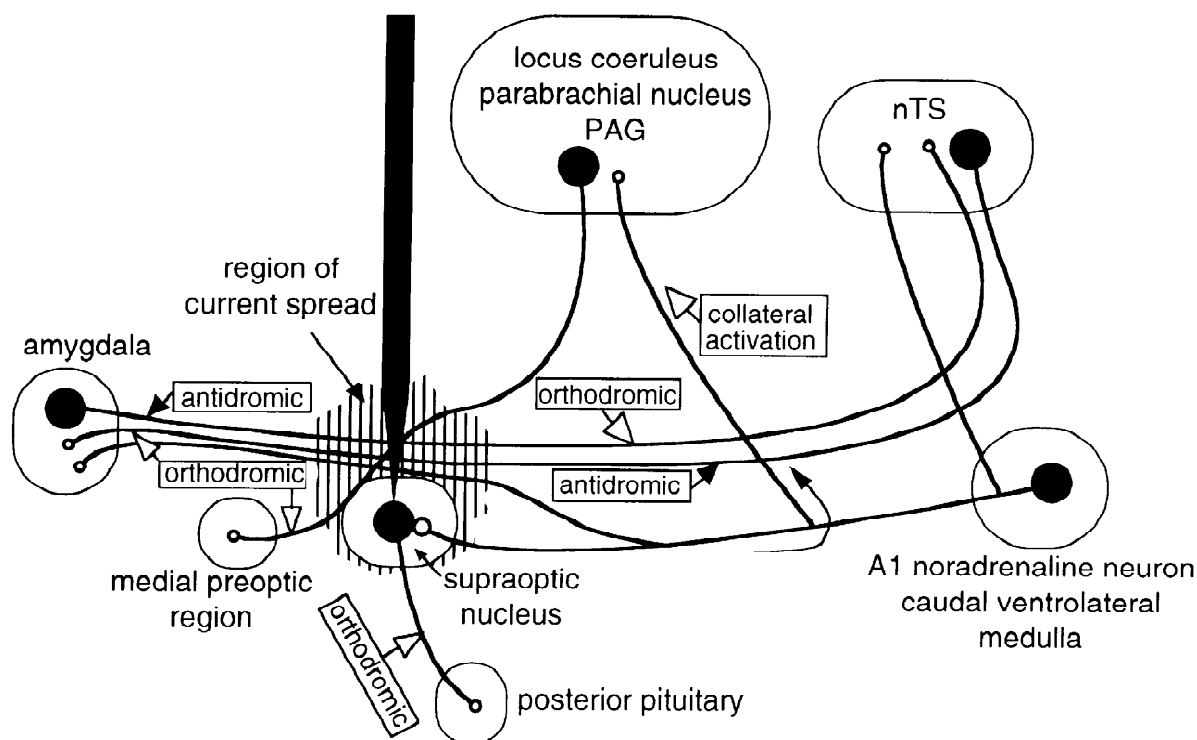
Preservation of function after a CNS lesion indicates that the lesioned structure is not necessary for the function at the level of sophistication at which the function is measured. The intact nervous system achieves finely tuned, complex control of particular activities. Apparent preservation of function after a lesion may reflect the simplified manner in which the function is assessed. The locus coeruleus is not necessary for survival in the animal house, but survival in the wild minus the locus coeruleus may be another question.

Even when the measure of function is appropriate, it is dangerous to assume that preservation of function indicates that a lesioned structure is not relevant to the function. As well as the hierarchical multiple representations described by Hughlings Jackson (see Chapter 1), there may be parallel circuits at a given level. In addition, interpretation of the outcome of chronic lesions may be confounded by recovery of function due to axonal regrowth or by recovery via presently unknown mechanisms.

## **Electrical Stimulation of Neural Structures**

The advantages of electrical stimulation as a tool to study the nervous system are obvious, particularly with respect to the precise control of the timing of the stimulus. The limitations are equally obvious, but they are often ignored. Whitteridge (1952:137) said that electrical stimulation of the central end of the cut vagal trunk "should be a punishable offence," emphasizing the difficulty of knowing which afferents are responsible for any effects observed. Results depend on the stimulus parameters, as noted at the beginning of this century for blood pressure changes following peripheral nerve stimulation (Gruber, 1917).

There are additional problems of interpretation when electrical stimulation is applied within the CNS, where neuroanatomical arrangements are more complex. Electrical stimulation aimed at a particular neuronal group may activate fibers passing through the region. This problem is particularly acute when the technique is used in the brainstem where passing fibers are abundant (see brainstem neuroanatomical sections in Figs. 3.1 and 8.2). No doubt many investigators have shared the experience of Amoroso et al. (1954), who noted that "a great variety of vasomotor responses were obtained from various areas of the medulla." More complex confounding responses may also occur. Fibers activated by electrical stimulation will conduct action potentials antidromically just as readily as they conduct them orthodromically. Not a great deal of attention has been paid to the functional effects of antidromic activation on collaterals of the activated cell. Figure 2.1 indicates a sample of the neural pathways that may be activated with relatively minor current spread during electrical stimulation of such an apparently homogeneous structure as the supraoptic nucleus. It is no wonder that electrical stimulation has produced some apparently paradoxical effects.



**Figure 2.1** Complex effects elicited by electrical stimulation aimed at the supraoptic nucleus. Many nerve fibers are stimulated orthodromically and/or antidromically. Collaterals of stimulated fibers may also be activated.

### Application of Neurochemical Agents to the Cerebrospinal Fluid

If a drug freely crosses the blood-brain barrier, then application of the drug to the cerebrospinal fluid (CSF) is soon equivalent to systemic application. If a drug crosses poorly, then appropriately dose-related peripheral responses after application of the agent to the CSF indicate that the brain or spinal cord contains receptors for the drug. Unless the CSF pathways are strategically isolated (e.g., by occluding the aqueduct), it is difficult to know the anatomical location of these receptors. Intrathecal administration into the spinal CSF is probably an exception, but even with this procedure it may be necessary to keep the spinal CSF separate from the brain CSF by the use of wax or a similar agent. Since there are virtually always multiple CNS pathways containing receptors for a particular drug, interpretation of studies using CSF application is difficult.

### Application of Neurochemical Agents to Focal Brain Regions

When investigators with a pharmacological background measured changes in physiological parameters (blood pressure, respiration, etc.) in response to the application of drugs to specific brainstem regions, rapid advances were made in our understanding of the neuronal circuitry underlying particular functions. Pharmacologists commonly used agents affecting dendritic and perikaryal function rather than axonal processes so that the problem of fibers of passage was elegantly bypassed. Loeschcke, and then Feldberg, were originators and champions of this procedure, with Feldberg investigating a myriad of homeostatic functions, initially using regional CSF blocks and then continuing with his ingenious focal applications of agents to the ventral surface

of the medulla oblongata. The major limitation of these pharmacological studies was the investigators' failure to look sufficiently carefully beneath the ventral surface, that is, their neglect of neuroanatomy. In Feldberg's fascinating book (1982) and in major reviews by Feldberg (1976) and Loeschcke (1982) there is not one diagram of a transverse section of the medulla oblongata; thus it has always been difficult to correlate the ventral surface work with the results of others who investigated functions of nerve cells in specific medullary regions.

Appreciation of the widespread distribution of perikaryal receptors for L-glutamate (nearly always excitatory) and GABA (nearly always inhibitory) led to the introduction of focal intracerebral microinjections of these agents to excite or inhibit neuronal cell bodies in specific brain regions. Application of these microinjections to CNS sites was especially illuminating in studies of central cardiovascular control. Advantages and possible disadvantages of the method have been reviewed (Goodchild et al., 1982; Lipski et al., 1988). The best studies use reasonably small intracerebral injection volumes, (preferably less than 250 nl, since 1  $\mu$ l is a cubic millimeter), demonstrate a dose-response effect, show that the effect is regionally specific, and, where appropriate, show that usually inhibitory agents (such as GABA) have effects opposite to usually excitatory agents (such as L-glutamate). The availability of specific receptor agonists and antagonists has helped validate the intracerebral injection procedure.

### **Extracellular Electrophysiological Recordings from Single Neurons**

A major limitation in the application of this obviously vital technique has been the failure of many investigators to select their neurons according to preestablished criteria. This is especially important when recordings are made from the dispersed and intermixed neuronal populations that occur in many brainstem regions. Failure to record from identified subclasses of neurons often results in the commonly reported conclusion that, in response to a particular experimental procedure, "one third of the sampled neurons increased their discharge rate, one third decreased their rate, and one third exhibited no change." Unplanned selection also biases the sample so that, for example, recordings are more likely to be from larger spontaneously discharging neurons.

Antidromic activation is a most useful tool for specifying the sub-populations from which electrophysiological recordings are made (Lipski, 1981), especially now that the projection patterns of many brainstem neurons have been established by neuroanatomical means. Some "antidromically proven" electrophysiologically demonstrated neural pathways disappeared with the introduction of retrograde intraaxonal transport of histologically demonstrable markers.

Putative neurotransmitter-related agents can be applied to single neurons during extracellular recordings. There have been discrepancies between studies using iontophoretic application and studies using micropressure injections. The latter procedure has the advantage that a dose-response relationship can be established. The possible spread of the injected agent when the pressure technique is used may not always be disadvantageous, given the increasing appreciation of the extent of the dendritic trees possessed by many brainstem neurons, including cells in apparently tightly packed regions such as the dorsal motor nucleus of the vagus.

Extracellular recording from the lower brainstem in the unanesthetized or the decerebrate animal has proven a difficult task, reflecting the mobility of this part of the brain in relation to the skull.

### **Intracellular Recordings**

Technical considerations have meant that most in vivo recordings of brainstem neurons have been made from large neurons, such as those in neuronal circuits for respiratory control. Smaller cells have usually been studied in in vitro preparations, using tissue blocks or slices. The importance of neuronal selection according to preestablished criteria applies to intracellular as well as extracellular recordings. Antidromic activation may not be possible in slices, but identification of neurons by prior retrograde tracing procedures has been most helpful, as has identification by intracellular fill with histologically identifiable agents at the time of the recording.

An obvious limitation of the in vitro technique is that it is usually impossible to alter physiological inputs to the cells. The presence, within the slice, of axonal connections of the impaled neuron has allowed some electrophysiological manipulation of these connections, as in studies utilizing axons in the tractus solitarius as inputs to intracellularly identified neurons in medullary slices. However, the situation is rarely as advantageous as that which occurs in brain regions such as the hippocampus.

### **Spike-Triggered Averaging**

Because synaptic delay time is often short compared with axonal conduction time, calculation of latency is not always sufficient to establish monosynaptic inputs in orthodromic stimulation studies. The most reliable electrophysiological technique of establishing monosynaptic inputs is that of spike-triggered averaging. Intracellular recordings of the transmembrane potential are made from the neuron hypothesized to receive the monosynaptic input. The oscilloscope or other averaging device is then repeatedly triggered from the spontaneous extracellularly recorded spike of the neuron hypothesized to provide the monosynaptic input. The synaptic noise is averaged over many sweeps to detect those inhibitory or excitatory postsynaptic potentials occurring in fixed temporal relationship to the discharge of the triggering neuron. The technique is especially suited to situations in which the triggering neuron has a strong input to the target neuron. The technique is less sensitive when the target neuron is driven by weak inputs from any particular neuron. The technique can falsely suggest a monosynaptic input if the discharge of the triggering neuron is driven by an unidentified third cell that also has a monosynaptic input to the target neuron. The amplitude and the rate of change of the potentials depend on how close the contacts are to the cell some; inputs to dendrites may be difficult to detect.

### **Biochemical Assays of Neurotransmitter-Related Agents in CSF and Brain Regions**

Because of the complexity and intermingling of brainstem neuronal pathways for different homeostatic functions, and the involvement of the same neurotransmitters in pathways mediating these different functions, measuring levels of neurotransmitter-related agents in the CSF has not often proven a productive strategy. Unfortunately, the same is true concerning postmortem biochemical

studies of dissected or micropunched brainstem regions. A major problem is that of ascertaining the contribution of axons, nerve terminals, perikarya, and even blood vessels as sources of the neurotransmitter detected.

In vivo studies using strategically positioned push-pull cannulae, dialysis probes, and electrochemical detectors have made a much more significant contribution. It is possible to relate the intensity of the signal to perturbations in physiological inputs, and this, together with documentation of regional specificity of transmitter release, has ensured the value of this experimental approach.

### **Receptor Binding Studies**

Specific binding of a pharmacologically characterized agent to appropriately homogenized brain material, together with appropriate displacement of binding by specific antagonists, suggests the presence of particular receptors. The data need to be interpreted with caution since the inference from binding site to receptor is sometimes invalid. When homogenized brain material is used it is usually impossible to specify the location of the receptors or even to determine whether they are on neuronal, glial, or vascular structures. Binding and displacement studies can also be carried out on histological sections, as in receptor autoradiography. When this is done, the histological pattern of the binding provides valuable information concerning the location of the receptors. Neural pathways involved with the receptors can be manipulated by, for example, prior unilateral lesioning of relevant pathways. Such combination studies have increased the value of receptor autoradiography.

### **Histological Demonstration of Neuronal Constituents**

The fluorescence histochemical detection of catecholamines in neurons was one of the first tools known to label subpopulations of neurons selectively according to the specific transmitter-related substances they contained. The brainstem catecholamine-containing cell groups immediately seemed fascinating; in Floyd Bloom's happy description, they glowed in the dark. Later, immunohistochemical techniques made myriad neuronal antigens glow in the dark, and then Sternberger's peroxidase-DAB procedure made it possible to see them in the light.

### **Histological Demonstration of Activity-Dependent Neuronal Markers**

#### **[<sup>3</sup>H]2-deoxyglucose**

[<sup>3</sup>H]2-deoxyglucose is taken up and phosphorylated by neuronal and glial elements according to their rate of glucose utilization. The phosphorylated molecule cannot be further metabolized. If [<sup>3</sup>H]2-deoxyglucose is used, subsequent tissue autoradiograms provide information concerning the metabolic rate of the elements taking up the tracer. Theoretically, brain regions whose metabolism is changed by maneuvers such as altering peripheral baroreceptor or chemoreceptor inputs can be identified by such autoradiographs. In practice, interpretation of the autoradiographs is complex, because the resolution of the technique is insufficient to discriminate between labeled perikarya and labeled nerve terminals. Both neuronal elements, and possibly glia, contribute to the labeling. If the nerve terminals are inhibitory, then increased [<sup>3</sup>H]2-deoxyglucose uptake in the region may well

reflect reduced activity of perikarya in the region. The technique has not made a major contribution to the definition of brainstem pathways mediating homeostatic functions.

### **Fos (early gene-product) immunohistochemistry**

Introduction of fos immunohistochemistry has provided a powerful tool for classifying nerve cells according to whether they are activated by particular stimuli. It is possible to study whole populations of cells, not just the small sample available to the electrophysiologist. Absence of labeling can be as informative as the presence of labeling, and multiple brain areas can be examined in each study. The functional stimuli can be applied to either anesthetized or unanesthetized animals. The activation must be over an hour or so, but this is well suited to many studies of brainstem function. The activated cells can be additionally characterized in terms of their neuronal connections and transmitter content. Not all types of neurons express the fos protein so that absence of the antigen must be interpreted with caution. Glial cells may also be labeled. The procedure obviously does not reveal cells that are inhibited by a particular experimental manipulation, nor can it reveal monosynaptic connections. Nevertheless, the availability of an activity-dependent histological marker in the neuronal cell body is a major advance. Results from these studies are proving a rich source of hypotheses to direct conventional studies such as extra- and intracellular recordings. Introduction of other early gene-related antigens is extending the technique.

### **Establishment of Neuronal Connectivity with Intra-axonally Transported Agents, Including Transneuronal Transport of Viruses**

Introduction of intraaxonal transport of horseradish peroxidase (HRP) revolutionized neuroanatomy (LaVail and LaVail, 1972); everyone became a latter-day Cajal! HRP and the various lectin tracers, including those labeled with gold particles, as well as the various fluorescent dye tracers, remain excellent tools for determining neuronal projections by retrograde labeling. Transneuronal tracing with live viruses (Ugolini et al., 1989) has greatly extended the possibilities of the retrograde tracing procedures. When Gerfen and Sawchenko (1984) described the use of *Phaseolus vulgaris* leucoagglutinin as an anterograde tracing agent, they provided a means of labeling the axonal terminal boutons of particular neurons so clearly that neuronal connectivity can frequently be inferred at the light microscopic level.

### **New Imaging Techniques**

Advances in imaging techniques, especially position emission tomography (PET) and magnetic resonance imaging (MRI), have renewed interest in the physiological investigation of the human brain. PET techniques focus on the link between cerebral blood flow and metabolism. Use of the technique remains confined to a few major institutions, reflecting the complexity of the technology and equipment associated with synthesis of compounds with short half-life radioactive labels. The resolution of MRI is continually improving so that regional images of changes in cerebral blood flow, occurring over short time periods (e.g., less than 1 minute), may soon be feasible. This will prove

most important, since MRI scanners are available at most major medical centers, and MRI imaging is done routinely by technical staff, with no necessity for intravenous injections.

As a general rule, functional imaging studies require conscious subjects. The necessity for keeping the head still while the images are obtained means that experimental animals are less suitable as subjects. The larger size of the human brain is also an advantage given the current spatial resolution of the various procedures.

### **A Note on Sympathetic and Parasympathetic Final Motoneurons**

The term postganglionic neuron is misleading. Postganglionic axon is a reasonable term, but the perikaryon is ganglionic rather than postganglionic. The term final motoneuron (corresponding to lower motoneuron in the somatic system) is useful for referring to sympathetic and parasympathetic motoneurons.

In neuroanatomical studies of sympathetic and parasympathetic innervation of particular organs we must live with certain ambiguities. Retrograde tracing studies, including transneuronal viral studies (see Chapter 3), do not distinguish between innervation of specialized nonvascular structures (e.g., sinoatrial node, sweat gland, iris muscle) within a peripheral organ, and innervation of the blood vessels within the organ. For some organs, such as the skin, the kidney, and the penis, the vasculature is a principal determinant of the specific function of the organ. For other organs, the role of the blood vessels is predominantly nutritive (e.g., skeletal muscle). If a viral tracer is transneuronally transported to the intermediolateral column of the spinal cord after injection into skeletal muscle, then the labeled sympathetic preganglionic cells are presumably vasomotor in function. The same tracer injected into the iris muscle may be transneuronally transported to sympathetic preganglionic cells concerned with either vasomotor or pupillary function.

### **More Terminology**

Neurons can be classified according to the chemicals released from the axon terminals that alter the discharge tendency of the postsynaptic neuron. The suffix '-ergic' is often used, as in cholinergic, glutamatergic, and enkephalinergic. As the distinction between neurotransmitter and neuromodulator becomes more blurred, and as we learn more concerning the number of potential neurotransmitter candidates in a single neuron, it becomes less and less appropriate to refer to neurons as something-ergic. If one abandons -ergic, then substitution of -containing, although grammatically correct, is cumbersome. The suffix has usually been omitted in this book. The term for the designated transmitter/ neuromodulator is sometimes used as an adjective, as in noradrenaline neuron.

### **Anesthesia and the Study of Physiology**

Our human experience of pain and distress encouraged the discovery of anesthetic agents, and we accept that these should be used to prevent similar experiences in animals used for physiological experiments. The site and mechanism of action of most general anesthetics is still a mystery. Virtually by definition, they diminish forebrain function, together with many other integrated CNS inputs to the neural circuitry under investigation. In anesthetized animals the range of stimuli useful for eliciting

responses is limited. It is no use showing a snake to an anesthetized subject. On the other hand, treatments that elicit marked responses in anesthetized animals may have little or no apparent effect in conscious animals. In anesthetized animals, for example, systemic administration of  $\alpha$ -adrenergic receptor-blocking agents generally causes a major fall in arterial pressure. The same treatment in unanesthetized animals may have little or no effect on arterial pressure. Presumably conscious animals compensate via vasoconstricting mechanisms that bypass the receptor blockade, but the exact mechanism is not understood at present.

How the individual actually works is both more and less than the sum of the component parts demonstrated in anesthetized or in vitro preparations. We need to remember the context in which particular information was obtained. A proper understanding of how the individual actually works requires a synthesis of information obtained by different techniques—from in vitro preparations and from both anesthetized and unanesthetized subjects.