[11] Using Osmotic Minipumps for Intracranial Delivery of Amino Acids and Peptides

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Concomitant with the revolution in molecular biological techniques applied to neurobiology has come an ever-increasing appreciation of the roles that peptides and amino acids play in the development, maintenance, and function of the nervous system. For example, the nerve growth factor family of neurotrophins, insulin and the insulin-like growth factors, fibroblast growth factor, and transforming growth factors are all known to play an integral role in both the developing nervous system and in adults. Amino acids, or their derivatives, are implicated as the "fast" or "classic" transmitter in most central nervous system (CNS) synapses and disturbances of transmission in these systems are associated with diseases ranging from excitotoxicity to depression. Similarly, it is now understood that colocalization of one or more neuropeptides with a classic transmitter is the norm rather than the exception in CNS neurons. Because virtually all neuropeptide receptors (apart from growth factor receptors) belong to the G protein-linked superfamily of receptors, neuropeptides can modulate intracellular protein kinase signaling cascades as well as influence neuronal excitability by altering conductance properties of ion channels. Thus, from this brief introduction it should be apparent that any understanding of the functions of and interactions between cells in the central nervous system must involve studies of peptides and amino acids.

For the investigator interested in peptide or amino acid neurobiology *in vivo*, it is important to optimize the approach to studying and/or perturbing these systems. How does one introduce peptides, amino acids, or pharmacological agents for their receptors into the CNS? One option is the peripheral administration of a particular compound. The existence of saturable blood–brain barrier (BBB) transport systems for amino acids is well documented and it appears that there are also saturable transport systems for some peptides [see Section III of this volume, and Schwartz *et al.* (1)]; however, some compounds do not pass and the extent to which these systems are effective for most peptides is unclear. Moreover, it is not always experimentally desirable or economically feasible to administer peptides peripherally in doses that are sufficient to achieve pharmacological activity centrally. Therefore, it is necessary to devise a method for introducing these compounds directly into the brain. To this end, osmotic minipump delivery

systems have been used successfully for over 15 years for direct administration of peptides and amino acids into the brain. In this chapter we summarize the experiences from our laboratories and review the work of others in which osmotic minipumps have been used to infuse peptides, amino acids, and drugs through indwelling cannulas into the cerebral ventricles or into specific CNS tissue sites. In the course of these discussions we emphasize technical considerations for using minipump delivery systems and highlight potential limitations, pitfalls, and controls to consider when using these systems.

Intracerebroventricular Injection versus Microinjection into Brain

General Considerations

Brain Interstitial Fluid and Cerebrospinal Fluid Formation and Movement

Early investigators believed the blood–brain barrier to be impermeable to virtually all solutes, and the cerebrospinal fluid (CSF) to be the principal vehicle for nutrient delivery to the brain (2). A common misconception stemming from this belief was that brain interstitial fluid (ISF) is derived from CSF, and that intracerebroventricular (icv) administration results in effective distribution of a drug throughout the brain. It is now evident, however, that circulating nutrients are provided to the CNS principally via transport across the BBB endothelium, and that CSF, in fact, is a relatively ineffective vehicle for solute delivery to many brain areas. Moreover, brain ISF and CSF differ in their origin, nutrient and electrolyte content, pattern of flow, and clearance mechanisms. These considerations emphasize the important differences in distribution of agents following icv administration versus direct infusion into brain tissue.

The microvasculature of the CNS is characterized by an endothelium with many unique features. First, the barrier function of brain vasculature is attributable to the extensive network of tight junctions (zonula occludens) that characterize this endothelium (3). Second, the many nutrient transport systems (e.g., glucose and amino acid transporters) expressed by brain microvascular endothelium are vital to the normal functioning of neural and glial elements within the CNS (4, 5). The formation of brain ISF represents a third specialized feature of the BBB endothelium (6). Once formed, brain ISF flows along perivascular interstitial channels, ultimately draining into cervical lymphatics, with a small fraction entering the CSF (7, 8).

This pattern of brain ISF production, flow, and removal is distinctive, having little in common with the movement of CSF. In contrast to brain ISF, CSF formation occurs largely within the choroid plexus (9, 10). This specialized organ, composed of a vascular tuft invested by a tightly opposed

epithelium (the "blood–CSF barrier"), is present in lateral, third, and fourth cerebral ventricles. The CSF formed at the choroid plexus is the product of an energy-dependent secretory process (11). A smaller contribution to CSF formation is provided by bulk movement of ISF out of the brain and into subarachnoid and ventricular CSF (12). It is noteworthy that solute concentrations are not in equilibrium between brain ISF and CSF; rather, there is a net movement of most solutes from brain ISF to CSF down a concentration gradient, and few solutes are delivered to the brain via the CSF under physiological circumstances. Once formed, CSF flows about the subarachnoid and ventricular surfaces of the brain in a rostral-to-caudal direction (13), and is removed principally across arachnoid villi that project into venous sinuses draining the brain.

Solute Removal from Cerebrospinal Fluid

That CSF volume and pressure remain constant over time reflects the equal rates at which CSF formation and removal occur. In the rat, this rate represents a complete turnover of the CSF volume (0.15 ml) every 1–2 hr, such that the half-life of most CSF solutes is ~0.75 hr (9). Cerebrospinal fluid removal occurs via bulk flow, and solutes are generally cleared from CSF with the same rate constant regardless of size. Exceptions to this rule include certain neuropeptides and neurotransmitters, for which high-affinity receptors or enzymatic clearance mechanisms are present on the surface of the brain. In such cases, clearance from CSF may occur much more rapidly. Angiotensin II, for example, has a CSF half-life of <1 min, owing to enzymatic degradation (14).

Sink Action of Cerebrospinal Fluid and Solute Diffusion into Brain Tissue

What, then, is the fate of intraventricularly administered peptides and amino acids? The rapid rate of bulk flow CSF turnover relative to the rate of diffusion of solutes from CSF into the brain creates a "sink effect" (9), which has important consequences for intraventricularly administered agents. Because CSF-borne solutes penetrate brain tissue by diffusion, the efficacy of the intraventricular route of administration is critically dependent on the distance of the target site within the brain from the ventricular or subarachnoid surface (6). Because the CSF-to-brain concentration ratio falls exponentially with increasing distance from the ependymal surface of the brain, it is now clear that many brain areas are exposed to trivial fractions of CSF solute concentrations. In addition to distance from the CSF, other factors that limit solute penetration from CSF to brain include high molecular size, sequestration and/or inactivation by brain cells, and high permeability across cell membranes, which result in more rapid solute clearance from

CSF into the circulation (6). In the case of biologically active peptides, it is estimated that structures located >1 mm from CSF are exposed to levels that may be <0.1% of the CSF concentration (6). Even if this estimate is inaccurate by an order of magnitude, it is clear that during icv administration, pharmacological levels of peptides must be present in CSF to provide physiological concentrations in many brain regions.

The above considerations influence not only the level of a CSF-borne peptide or amino acid to which a given brain area is exposed, but also the time necessary for this exposure to occur. The time interval required for a peptide to travel from CSF to a given brain area is determined by the same factors that influence its concentration in brain tissue; for example, the distance from the ependymal surface and the diffusability of the agent. Whereas sodium diffuses 3 mm in <1.5 hr, for example, albumin requires more than a day to travel the same distance (6). It may be anticipated, therefore, that if the efficacy of a large peptide hinges on delivery to a brain area situated at a considerable distance from the CSF (e.g., deep within the cerebral cortex or caudate nucleus), several hours may pass following icv administration before any exposure to the peptide occurs. Conversely, agents that elicit a rapid response on icv injection (e.g., angiotensin II-induced drinking behavior) may be safely assumed to be acting at a site near the ependymal surface of the brain.

Implications for Efficacy of Intracerebroventricular Delivery versus Microinjection

The preceding discussion of fluids and their movements in the CNS emphasizes both the limited distribution of peptides and amino acids following icv administration and the nonphysiological nature of this approach. As highlighted by Pardridge (6), in order to achieve significant peptide concentrations in brain tissue 2 mm removed from CSF, the required CSF concentration could result in blood levels that paradoxically exceed those achieved in brain.

Despite these limitations, the icv route has proved useful in the hands of many. Third ventricular infusion of insulin in the rat, for example, results in reduced food intake (15) and altered neuropeptide gene expression within the hypothalamic arcuate nucleus (1, 16) at doses that do not significantly affect circulating insulin or glucose levels (<3 mU/24 hr). This outcome may reflect the close proximity of the arcuate nucleus to the ependyma of the third ventricle, which facilitates exposure to insulin during intraventricular infusion. Nonetheless, the observation that direct intrahypothalamic insulin infusion yields comparable effects on food intake at doses two to three orders of magnitude lower than those required during icv infusion reiterates the

nonphysiological nature of the icv route for administering peptides to the brain.

In contrast to the icv method, administration of peptides or amino acids directly into brain by either microinjection or continuous infusion reliably yields high local concentrations in the area surrounding the injection site. The injection volume impacts significantly on the radius of drug distribution about the injection site. For studies involving brain areas where many sensitive structures reside in close proximity to one another, small injection volumes are critical for mapping responses to peptides. For example, Stanley has reported orexigenic effects of neuropeptide Y to be highly localized within the rat hypothalamus (17) by using microinjection volumes of 10 nl, rather than the larger volumes (500 nl) traditionally used. Microinjection of this smaller volume limited distribution of the peptide to within 0.8 mm of the injection site. Moreover, although more technically demanding, this approach evidently facilitated identification of discretely responsive hypothalamic areas undetected in previous studies using larger injection volumes.

Minipump Delivery Systems

Advantages and Disadvantages

As with any experimental system, there are benefits and limitations to the use of osmotic minipump delivery paradigms. Foremost among the advantages is that a substance can be administered with no intervention from the investigator or disturbance to the animal once the pump has been implanted. This implies that all treatments can be performed on conscious, freely moving animals. Obviously, this is a requirement for any behavioral study (e.g., feeding, reproductive, or learning paradigms), but is also advantageous for *in vivo* studies in general because stress of handling is minimized. In addition, long-term studies are facilitated by minipump systems and help eliminate variables such as different injection times, depending on the investigator's schedule and different skills in administering compounds by different laboratory workers. Furthermore, continuous infusion avoids peaks and troughs of concentrations of administered agents, so that a steady state concentration can be achieved. Last, minipump infusions can generally be accomplished with a minimum of tissue damage at the site of cannula placement.

There are also several limitations to minipump infusions that must be considered carefully. In prolonged infusion paradigms it must be determined that the peptide, amino acid, or pharmacological agent to be infused is stable at 37°C for the period of the experiment. Thus, preliminary experiments must be conducted in which the compound to be infused is incubated at 37°C

for an appropriate period, after which its chemical identity or biological activity verified. Similarly, a common problem for peptides is precipitation at concentrations used for infusion when stored in saline or artificial CSF. Therefore, at the end of the infusion period it is important to verify the concentration and biological activity of the peptide in the infusate, e.g., by radioimmunoassay (RIA) or bioassay.

The minipump paradigm is not totally intervention free because minor surgery is required at the time of pump implantation. Thus, although usually not a problem in rodents, investigators must monitor animals for signs of infection and stress as the result of surgery and must allow sufficient time for the animal to recover from effects of anesthesia before beginning an experiment. It is important to carry out parallel experiments with animals receiving infusion only of vehicle to control for effects that are due solely to pump or cannula implantation or vehicle infusion. When infusions are performed directly into brain tissue, it is also important to verify the extent of any tissue damage due to the infusion. Thus, in behavioral paradigms, microscopic analysis of the infusion site should be performed at the end of the experiment. In experiments in which such post hoc analysis is not possible, for example, when the end point is the assay of brain tissue extracts, preliminary or parallel experiments should be performed in which microscopic analysis is performed on similarly cannulated and infused tissue. Some authors have found that in prolonged perfusion paradigms, pump-derived cytotoxins reach concentrations sufficient to ablate a area of tissue 1–3 mm in diameter (18). This difficulty does not appear to represent a significant problem in shorter term experiments and may be obviated in long-term experiments by modifications to the minipumps to reduce flow rates [see Hagg, this volume, and Hagg *et al.* (19)]. Yet another concern is that infusion paradigms are rarely ideal in that icv peptide infusions may have limited delivery to the brain area of interest (see above) or, conversely, may exert nonspecific or confounding effects on brain structures unrelated to the hypothesis being tested. On the other hand, direct tissue infusion is limited in its effectiveness by the degree of diffusion from the tissue site, so that the concentration of infused agent will fall off as an exponential function of the distance from the infusion site. For small brain areas such as hypothalamic nuclei, this latter concern is usually not a problem but in larger brain areas such as cortex or hippocampus, experiments must be confined to the region of diffusion. For example, Kasamatsu *et al.* (20) estimated a 500-fold dilution of infused agent at a 3-mm distance from the infusion cannula. For a discussion of methods to determine tissue concentrations of infused agent, the reader is referred to the section Practical Considerations, below.

The continuous nature of minipump infusion can also be problematic because a variety of neuronal and neuroendocrine systems are believed to be

regulated in a pulsatile fashion. Therefore, continuous infusion would not mimic the true *in vivo* nature of peptide or amino acid interaction in that system. This difficulty has been addressed by some authors by filling tubing alternately with a dose of drug to be infused separated by a small volume of air. This paradigm is applicable when pulses occur over 1- to 2-hr time spans but would be unsuitable for circadian-type experiments. These latter experiments would require that infusion in the intervening time be with vehicle that could be separated from agent by a small air pocket. Again, in using any type of pulsatile infusion paradigm it would be necessary to verify that air infusion did not lead to significant tissue damage.

Last, investigators must be cognizant of the potential for infusion failure either due to an occluded cannula (see below) or minipump failure. In long-term studies, it is simple to remove any remaining fluid from the pump chamber and determine that the volume remaining corre- nds to the initial volume added minus the volume calculated to be delivered. In short-term experiments in which infusion is performed for only a few hours, it is often desirable to load the agent to be infused into polyethylene (PE) tubing (rather than directly into the minipump), then load the minipump with saline containing a dye such as bromophenol blue. Successful infusion is then verified when the blue dye is pumped into the PE tubing to displace the infusate. Knowing the distance the dye is pumped into the tubing and the volume of the tubing, one can easily determine if the desired volume of infusate was delivered.

Examples

Examples of studies in which osmotic minipumps have been used to deliver peptides, amino acids, or associated drugs are given in Table I. Even in this abbreviated list, it can be seen that minipump delivery systems have been used successfully to address numerous neurobiological problems with a variety of agents.

Practical Considerations for Cannulation and Minipump Use

Procedure for Third Ventricular Cannulation and Minipump Placement in Rat

We prefer to induce and maintain general anesthesia via inhalation of halothane vaporized with 100% oxygen, as it affords minute-to-minute control

TABLE I Peptides, Amino Acids, and Drugs Infused Using Osmotic Minipumps

Compound infused[a]	Site	Experimental paradigm	References[b]
Insulin	icv	Feeding	1, 2
	icv	Feeding, gene expression	3
Nerve growth factor	Hippocampus	Reinnervation	4
	icv	Memory	5
Fibroblast growth factor		Cell death	6
Horseradish peroxidase	Lateral geniculate	Cell labeling	7
Neuropeptide Y	icv	Feeding	8
Corticotropin-releasing factor	icv	Feeding, obesity	9, 10
Cholecystokinin	icv	Feeding	11
Leupeptin	icv	Long-term potentiation	12
Angiotensin II, III	icv	Blood pressure	13
Neurotensin, substance P, TRH	icv	Dopamine receptor regulation	14
Enkephalin, β-endorphin, morphine	icv	Dependence	15
Interleukin 3	icv	Trophic factor	16
Glucose	icv	Feeding	17, 18
Glutamate, aspartate, GABA	Striatum, hippocampus		19
GABA	Visual cortex		20
APV	Visual cortex	Cortical plasticity	21
Ibotenic acid	Visual cortex	Lesion mapping	22
Dopamine, dopamine agonist	icv	Experimental parkinsonism	23
MPP$^+$	Substantia nigra	Experimental parkinsonism	24
Norepinephrine	Visual cortex	Cortical plasticity	25, 26
	Hypothalamus	Feeding	27

Histamine	Suprachiasmatic nucleus	Feeding	28
[^{3}H]Pro, -Leu	Hypothalamus, striatum	Peptide biosynthesis	29, 30
[^{35}S]Met, -Cys	Hypothalamus, hippocampus, striatum	Peptide biosynthesis	30–32
Morphine	icv	Pulsed delivery	33

[a] TRH, Thyrotropin-releasing hormone; GABA, γ-aminobutyric acid; APV, 2-amino-5-phosphonovaleric acid; MPP^+, N methyl-4 phenylpyridinium cation.

[b] Key to references: (1) H. Ikeda, D. B. West, J. J. Pustek, D. P. Figlewicz, M. R. Greenwood, D. Porte, Jr., and S. C. Woods, *Appetite* **7,** 381 (1986); (2) K. Nagai, T. Mori, T. Nishio, and H. Nakagawa, *Biomed. Res.* **3,** 175 (1982); (3) M. W. Schwartz, A. J. Sipols, J. L. Marks, G. Sanacora, J. D. White, A. Scheurink, S. E. Kahn, D. G. Baskin, S. C. Woods, D. P. Figlewicz, and D. Porte, Jr., *Endocrinology* (*Baltimore*) **130,** 3608 (1992); (4) T. Hagg, H. L. Vahlsing, M. Manthorpe, and S. Varon, *J. Neurosci.* **10,** 3087 (1990); (5) W. Fisher, A. Bjorklund, and F. H. Gage, *J. Neurosci.* **11,** 1889 (1991); (6) K. J. Anderson, D. Dam, S. Lee, and C. W. Cotman, *Nature* (*London*) **332,** 360 (1988); (7) A. J. Weber and R. E. Kalil, *J. Comp. Neurol.* **220,** 336 (1983); (8) B. Beck, A. Stricker-Krongrad, J.-P. Nicolas, and C. Burlet, *Int. J. Obesity* **16,** 295 (1992); (9) F. Rohner-Jeanrenaud, C.-D. Walker, R. Greeco-Perotto, and B. Jeanrenaud, *Endocrinology* (*Baltimore*) **124,** 733 (1989); (10) K. Arase, N. S. Shargill, and G. A. Bray, *Am. J. Physiol.* **256,** R751 (1989); (11) R. R. Schick, C. W. Stevens, T. L. Yaksh, and V. L. W. Go, *Brain Res.* **448,** 294 (1988); (12) U. Staubli, J. Larson, O. Thibault, M. Baudry, and G. Lynch, *Brain Res.* **444,** 153 (1988); (13) K. Katahira, H. Mikami, T. Ogihara, K. Kohara, A. Otsuka, Y. Kumahara, and M. C. Khosla, *Am. J. Physiol.* **256,** H1 (1989); (14) S. M. Simasko and G. A. Weiland, *Eur. J. Pharmacol.* **106,** 653 (1984); (15) E. Wei and H. Loh, *Science* **193,** 1262 (1976); (16) M. Kamegai, K. Niijima, T. Kunishita, M. Nishizawa, M. Ogawa, M. Araki, A. Ueki, Y. Konishi, and T. Tabira, *Neuron* **2,** 429 (1990); (17) J. D. Davis, D. Wirtshafter, K. E. Asin, and D. Brief, *Science* **212,** 81 (1981); (18) J. Panksepp and J. Rossi, III, *Behav. Brain Res.* **3,** 381 (1981); (19) R. M. Mangano and R. Schwarcz, *Brain Res. Bull.* **10,** 47 (1983); (20) S. Brailowsky, C. Silva-Barrat, C. Menini, D. Riche, and R. Naquet, *Electroencephalogr. Clin. Neurophysiol.* **72,** 147 (1989); (21) M. F. Bear, A. Kleinschmidt, Q. Gu, and W. Singer, *J. Neurosci.* **10,** 909 (1990); (22) M. R. Dursteler, R. H. Wurtz, and W. T. Newsome, *J. Neurophysiol.* **57,** 1262 (1987); (23) J. G. de Yebenes, S. Fahn, V. Jackson-Lewis, P. Jorge, M. A. Mena, and J. Reiriz, *J. Neural Transm.* **27,** suppl., 141 (1988). (24) D. J. S. Sirimathsinghji, R. P. Heavens, S. J. Richards, I. J. M. Beresford, and M. D. Hall, *Neuroscience* **27,** 117 (1988); (25) J. D. Pettigrew and T. Kasamatsu, *Nature* (*London*) **271,** 761 (1978); (26) T. Kasamatsu, T. Itakura, and G. Jonsson, *J. Pharmacol. Exp. Ther.* **217,** 841 (1981); (27) T. Shimazu, M. Noma, and M. Saito, *Brain Res.* **369,** 215 (1986); (28) N. Itowi, K. Nagai, H. Nakagawa, T. Watanabe, and H. Wada, *Physiol. Behav.* **44,** 221 (1988); (29) J. E. Krause, J. P. Advis, and J. F. McKelvy, *Endocrinology* (*Baltimore*) **111,** 344 (1982); (30) J. E. Krause, A. J. Reiner, J. P. Advis, and J. F. McKelvy, *J. Neurosci.* **4,** 775 (1984); (31) J. D. White, J. E. Krause, and J. F. McKelvy, *J. Neurosci.* **4,** 1262 (1984); (32) J. D. White, C. M. Gall, and J. F. McKelvy, *Proc. Natl. Acad. Sci. U.S.A.* **83,** 7099 (1986); (33) V. C. Raynet, I. C. A. F. Robinson, and J. A. Russell, *J. Physiol.* (*London*) **396,** 319 (1988).

over the anesthesia level and facilitates rapid postoperative recovery, although other methods may be used effectively.

Cannulation of the third ventricle is described here in detail. The first step is to insert a guide sleeve; the cannula itself is inserted through the guide sleeve at the time of pump implantation. Our method calls for stereotaxic insertion of a 22-gauge stainless steel guide sleeve (model C313G; Plastics One, Roanoke, VA) through the midline of the brain into the third ventricle. The coordinates are calculated as follows: the anterior–posterior (A–P) coordinate is calculated as 0.20 times the difference between the bregma A–P and the interaural (IA) line A–P coordinates. The dorsal–ventral (D–V) coordinate is calculated as 0.68 times the difference between the superior sagittal sinus D–V and the IA line D–V coordinates. Following the removal of a small (0.625 cm^2) piece of overlying skull, the cannula guide sleeve is inserted through the sagittal midline of the brain after visualizing and gently retracting the superior sagittal sinus. The guide sleeve is then fixed into place with dental acrylic and the overlying skin sutured to enclose all but the proximal 1 cm of the guide sleeve. An obturator is inserted at the conclusion of the surgical procedure and maintained in position during the subsequent recovery period.

After at least 1 week of postoperative recovery, the animals should be ready for entry into a study protocol. Animals are lightly reanesthetized, using halothane prior to surgical implantation of the osmotic minipump into the interscapular subcutaneous space. Prior to implantation, the osmotic minipump (model 2001; Alzet, Palo Alto, CA) and connecting PE tubing are primed by submersion in isotonic saline for 12 hr at room temperature. It is important to realize that icv infusion (and hence the study protocol) begins immediately on connection of the PE tubing from the primed pump to the infusion cannula (model C313, 26 gauge; Plastics One), which is inserted into the guide sleeve. To minimize variability in measured parameters on initiation of the study protocol, it is essential to minimize surgical and anesthetic stress at the time of pump implantation.

Dosing calculations are based on the desired infusion rate, which has been standardized at 1 μl/hr at 37°C for most products. Therefore, if one desires a continuous infusion of a peptide at a rate of 1 μg/hr, the pump should be filled (usual pump volume is 200 μl) with a solution containing 1 $\mu g/\mu l$ of the peptide of interest.

Verifying Cannula Placement and Patency

Correct placement of the cannula can be assessed in two ways prior to pump implantation. The first is simply to look for reflux of CSF following insertion of the infusion cannula, which is evident in the majority of successful cannu-

lations. The second approach is to inject angiotensin II through the infusion cannula (30 ng in 3 μl of saline) in the unanesthetized rat and monitor for a drinking response (>5 ml in 30 min) (21). This drinking response is transient, as angiotensin II has an extremely short half-life, and adverse effects have not been observed in our hands with this approach. In general, we find this test to be both specific and sensitive for establishing intraventricular location of the cannula prior to study, although quantitative data are not available.

At the time of sacrifice, patency of the infusion system and verification of cannula placement may be confirmed by injection of methylene blue dye (5 μl) through the PE tubing and internal cannula, and into the third ventricle. Resistance to flow indicates obstruction of the infusion system; if there is no resistance to flow, but blue staining does not appear in the third ventricle (which can be determined by visual examination of the ventral surface of the brain), cannulation of the third ventricle was likely unsuccessful. This staining procedure does not interfere with histochemical methods such as *in situ* hybridization. However, such a lack of interference should be confirmed for more specialized anatomical analyses. Finally, assay of CSF sampled from the cisterna magna at the time of sacrifice for determination of the level of infused peptide or amino acid may help to verify successful infusion. However, cisternal levels of biologically active peptides may not be in equilibrium with levels present in the third ventricle, if CSF clearance occurs independent of bulk flow. Nonetheless, cisternal levels should be higher in experimental animals receiving icv peptide infusion than in vehicle-treated controls.

Estimates of Tissue Concentrations Using Radiolabeled Compounds

For some studies, in which compounds are infused directly, it may be critically important to derive an estimate of the tissue concentration of the infused amino acid, peptide, or homologous drug, for example, to demonstrate that a peptide produces physiological effects in the concentration range that is optimal for specific receptor interaction. Bear *et al.* (18) have effectively approached this problem by coinfusion of tracer amounts of labeled APV (an *N*-methyl-D-aspartate [NMDA] receptor antagonist) and experimentally relevant amounts of unlabeled APV. At the end of the infusion period, the animal is deeply anesthetized, and the brain is then exposed and frozen *in situ*, using liquid nitrogen. The brain is then removed as rapidly as possible and, with as little thawing as practical, 500-μm tissue sections are prepared from the region surrounding the infusion site, using a McIlwain tissue chopper. Each slice is placed into 1 ml of water and sonicated to disrupt the tissue. One-half of the sonicate is removed for protein content determination and from the protein content an estimate of tissue wet weight is derived.

(Preliminary control experiments using noninfused tissue permit a wet weight determination to be made prior to sonication, which is then correlated with protein content to allow derivation of wet weight per milligram protein from infused tissue.) The remaining sonicate is then used for determination of total radioactivity and, knowing the specific activity of the total labeled and unlabeled compound infused, a value for nanomoles of compound per milligram tissue (wet weight) is derived.

The value derived for tissue radioactivity (or nanomoles of compound) per milligram tissue (wet weight) represents the combination of free extracellular compound, receptor-bound compound, and, perhaps, internalized compound or radioactivity. It therefore becomes necessary to construct a standard curve in which tissue radioactivity can be related to the extracellular level of compound. This standard curve can be constructed by incubating tissue slices in known concentrations of labeled compound. After determining the length of time for tissue radioactivity levels to stabilize, which may be as long as 12–18 hr, it is then known that the free extracellular concentration of labeled compound is equal to the bath concentration. The slices are then removed, filtered rapidly with nylon mesh to remove the bath radioactivity and tissue protein, and radioactivity levels determined as above. By incubating tissue slices in a range of radiolabeled compound concentrations, it is possible to construct a standard curve for nanomoles of compound per milligram tissue protein (or wet weight) vs extracellular compound concentration. From this standard curve, the value derived for nanomoles of infused compound per milligram tissue (wet weight) is used to calculate free extracellular concentrations of infused compound.

This method can be used to derive an estimation for extracellular tissue concentrations of infused drugs as a function of distance from the cannulation site or in a given brain region following icv infusion. For either icv or direct infusion, this determination relies on the infusion time being of sufficient duration for the tissue concentration of the infused compound to reach equilibrium. For shorter duration infusions, the derived tissue concentration will not be absolutely accurate but nevertheless will yield a rough estimation of concentrations for the investigator. A second, and significant, caveat to this method is that it is optimally suited for drugs that are not significantly metabolized by brain tissue. If this method is to be used for determining local peptide concentrations, the initial experiments using brain slices must also include a method such as high-performance liquid chromatography purification of radiolabeled peptide at the end of the incubation period to determine what fraction of radioactivity represents intact peptide. Naturally, with a steady state bath incubation method, the peptide will achieve an equilibrium in which the total tissue radioactivity represents the balance between diffusion of peptide into the tissue from the bath and the sum of extracellular

intact peptide, extracellular degraded peptide, receptor-bound peptide, and internalized peptide. When the amount of intact peptide radioactivity is determined and compared to total tissue radioactivity, this same ratio can then be applied to values derived from infusion experiments (provided they are of sufficient duration to estimate a steady state reasonably). This method should not be used for radiolabeled amino acids, the incorporation of which into protein yields an additional difficulty in interpretation.

Summary

Osmotic minipump delivery systems can be designed to deliver virtually any compound into the CNS via indwelling cannulas for administration via the CSF or into specific tissue sites. When used with appropriate care and recognizing the limitations to conclusions derived from the data obtained, these systems can be used to approach a wide variety of behavioral, physiological, and biochemical questions.

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