Editorial Commentary

Neurotransmission in Central Cardiovascular Control
10 Suggestions for Microinjections

Paul M. Pilowsky

In the article by Lin et al, new information is provided concerning the central actions of the acetylated growth hormone secretagogue peptide, ghrelin. In this study the authors show that microinjection of ghrelin into the nucleus tractus solitarius caused small but significant changes in blood pressure and sympathetic nerve activity. To a certain extent, this study is a useful addition to our knowledge about central cardiovascular regulation. Many brain nuclei are now established to be important in the control of the circulation and the reflexes that maintain cardiovascular stability. However, the neurotransmitters that the neurons at these sites use, the receptors that they interact with, and the second messenger systems involved in transducing the effects of neurotransmitter release into intracellular events that range from channel opening to gene transcription are incredibly poorly understood. Each additional piece of information, such as that provided by Lin et al, is valuable. To their credit, they provide raw data showing the response and they used localized microinjection techniques to restrict the response to one brain nucleus. Most importantly, they used sympathetic nerve recording to prove that the effects observed are due, at least in part, to the effects of changes in sympathetic activity. Is this adequate? In examining the article by Lin et al, a number of areas for further investigation are presented. Lin et al raise some of these questions, such as a possible role for tonically released ghrelin. In this brief commentary I would like to suggest a list of necessary experiments and methodological approaches that should be used in modern experiments of this type. It may be that for various reasons it is not possible to achieve all of these, but it should be a goal.

1. As with all in vivo experiments, the animal species and strain, and drugs used (anesthesia, atropine, curare-form agents, heparin etc) should all be listed. The number of situations in which very different responses can be obtained under different anesthetics is now well established. Broadly speaking, anesthetics fall into 3 main categories: brain stem anesthetics (barbiturates, halothane, etc), all of which depress sympathetic activity and reflexes; so-called supramedullary anesthetics such as ketamine, urethane, chloralose, xylazine, alfadalone-alfaxalone etc, that tend to elevate sympathetic activity; and, of course, awake, freely moving animals that have recently had surgery to implant an invasive catheter to measure blood pressure or other variables either directly or via telemetry. Each of these approaches can be valid in different circumstances, but the details must be clearly laid out.

2. The wide availability of easy-to-use reasonably priced multibarrel micropipette pullers means that all experiments of this type can usually be done with unilateral or bilateral injections of, for example, vehicle, glutamate to localize sites, agonist, and antagonist. In our experience, 4 barrels are the maximum that can easily be used. This is because each barrel must be observed during pressure microinjection to ensure that the meniscus in the tubing falls. This is the only reliable method to conduct pressure injection. With 5 barrels, 1 is hidden in the middle of the other 4. A marker dye can be put into the vehicle or glutamate barrel. Refilling single barrel electrodes and expecting to return to the same site is risky.

3. Injection sites must be marked with dye and retrieved histologically. Many techniques are available; recently we have found that the wide availability of colloidal gold is excellent for this type of work. Many other approaches are described by ourselves, and others, and are equally useful. Moreover, it is imperative that injection sites be shown in histological sections with standard nuclei marked so that others may compare the results with their own and others’ data. The nucleus tractus solitarius, for example, is a tremendously complicated nucleus with many functions all packed into a tube with a diameter of only a few hundred microns extending over many millimeters rostrocaudally. If possible, sections should also be processed for immunohistochemistry, which is a powerful and easily acquired technique. Naturally, a relevant antigen should be chosen for examination. Alternatively, in situ hybridization can be used although this is a less straightforward technique.

4. The choice of dose should be explained rationally. This is as much to prevent missing a real effect as it is to prevent causing spurious effects that should be detected by vehicle injection anyway. Normally, it is reasonable to start with a dose that is 10 times larger than that used in steady-state bath pharmacological studies with the same agent. This accounts for the many differences between in vivo and in vitro pharmacology that are not elaborated on here.

5. If possible, a dose-response curve should be prepared. Even if from only 3 doses and conducted as a cumulative dose-response curve, this provides valuable
confirmation of specificity, as well as being crucial in antagonist and other studies.

6. An explanation of the likely region affected should be provided. Agents such as glutamate and GABA have very high affinity uptake mechanisms, and their influence does not stray far from their site of injection. In fact, the region affected will largely depend on the volume of the injectate and the speed of injection. Peptides, on the other hand, and many other drugs may have much wider spheres of influence.

7. It is therefore, important to make injections around the region of interest and to make an effect-response map. If an effect of an agonist is found, then antagonist experiments should be conducted if possible. If more than one class of antagonist is available then multiple types should be tested to ameliorate the possibility that the antagonist itself has effects. The presence of an effect from the antagonist alone is often taken as evidence for a tonic effect. This may or may not be so depending on the nature of the antagonist-receptor interaction, and caution must be observed.

8. More than just one parameter should be measured. This is especially important in close-packed nuclei with many functions that may influence each other. Blood pressure and heart rate are simply the minimum. ECG (allowing measurement of chronotropic and dromotropic effects), phrenic nerve discharge, regional sympathetic nerve activity, regional blood flow, and plasma hormone levels are among the many other variables that should be considered.

9. Statistical evaluation should be appropriate. Randomization does not mean that tests can be randomly chosen from the drop-down menu on the computer. I personally recommend Sokal and Rohlf. It is a superb text with straightforward explanations and easy to follow examples of quite technical material.

I propose these 10 as a starting point in any investigation that involves microinjection into the brain. Although not commandments, they are almost all essential, and in the modern age easily achievable; the greater the number successfully addressed, the more powerful the study. The study by Lin et al. covers many, albeit not all, of these and is therefore deserving of our interest.

Acknowledgments

Work in the author’s laboratory is supported by grants from the National Health and Medical Research Council of Australia (211023, 211196); the National Heart Foundation of Australia (G00S0716); the Garnett Passe and Rodney Williams Memorial Foundation; the North Shore Heart Research Foundation (14–00/01, 17–00/01); and Northern Sydney Health.

References

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_Hypertension_. 2004;43:945-946; originally published online March 1, 2004;
doi: 10.1161/01.HYP.0000122807.18408.77
_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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World Wide Web at:
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