Basal Forebrain Neuronal Loss in Mice Lacking Neurotrophin Receptor p75

There is substantial evidence from in vitro studies that nerve growth factor (NGF) exerts an important influence on the development and survival of central neurons (1). The effects of NGF are thought to be mediated through the high-affinity tyrosine receptor kinase trkA and the low-affinity p75 receptor (2). Several studies suggest that the p75 receptor initiates an apoptotic signal that leads to neuronal death (3).

The role of NGF in the development and survival of central neurons in intact systems is less clear. The use of null mutant mice provides a useful strategy for delineating the role of specific receptors and factors during in vivo development. Mice that are deficient in NGF and trkA have been produced, and the effect of the mutation on central neurons was investigated (4). Despite the observation of impairment of peripheral neuronal development, neither the lack of NGF nor the absence of trkA receptors prevented the development and survival of septal cholinergic neurons, cells that normally express trkA and p75 receptors and that can be rescued by the administration of NGF after traumatic lesion. These two studies, however, did not provide quantitative data (4).

We previously generated mice that carry a targeted mutation for p75 and found significant impairment of the peripheral nervous system in those animals (5). Here, we describe the effect of lack of p75 receptor on central neurons in the basal forebrain. We used unbiased quantitative stereology to avoid quantitative artifacts arising from reference volume distortion between experimental and control conditions (6). While the number of cells per histological section is the apparent value of interest, the section is only a sample of the entire structure being examined. Expressing the quantitative values relative to the histological section can result in two quantitative artifacts. The first, known as overprojection and truncation, results from incorrectly identifying cells within the sample (focus) plane. For many years, this was corrected by the use of mathematical models, such as the Abercrombie method, but is now better addressed by the use of the disector principle (6).

The second quantitative artifact results from not accounting for changes in the reference volume. To express the quantitative value relative to histological sections is to express it as a density ratio. Values expressed as number of cells per unit area, or as number per section, could be affected by changes in either the numerator or denominator of the expression. Because the total number of the cells in the tissue is the true value of interest, the appropriate approach is to estimate the total number using an unbiased stereological approach in which three-dimensional samples are taken with equal probability throughout the entire reference volume, such as the optical disector (7).

As part of a wider characterization of the developmental role of p75 on central neurons, we quantified the septal region of adult p75 null mutant mice and their wild-type littermates with the use of the optical disector procedure on a confocal microscope (8). The volume of the septal region was significantly reduced in p75 null mutant mice (Table 1A). Determination of the absolute number of cholinergic neuronal subpopulations revealed a significant loss in ChAT-positive, but not trkA-positive, neurons in p75 null mutant mice (Table 1A). Furthermore, there was a significant loss in the total number of septal neurons in p75-null mutant mice (Table 1A). Because of the significant loss of total septal neurons, the relative proportion of ChAT-positive and trkA-positive neurons showed an apparent, but nonsignificant, increase in p75 null mutant mice (Table 1B). This last result emphasizes the danger of relying on ratios to determine cell numbers in the absence of compelling evidence that one of the values in the ratio is unchanged between conditions.

Our results, which demonstrate cholinergic neuronal loss within the septum of mice carrying a targeted mutation of p75, are at variance with the report by Van der Zee et al. (9). They present quantitative results as number of cells per section, which would make their data subject to the limitation of the Abercrombie method and reference volume distortion artifacts. Because the volume of adult p75 null mutant mice septum was reduced (Table 1A), the greater number of cholinergic cells found by Van der Zee et al. (9) was likely an apparent increase resulting from distortion. Furthermore, the changes in reference volume over the developmental time frame they examined (9) make comparison of number per section over this time period irrelevant.

The difference between our results and those of Van der Zee et al. represents not just technical issues, but also conceptual differences. They conclude from their data that a greater number of cholinergic neurons in p75 null mutant mice and wild-type mice administered with a factor to interfere with p75 binding provides in vivo evidence for p75-mediated apoptosis. Although our data do not disprove this hypothesized role for p75 in vivo, they do render the report by Van der Zee et al. unreliable as evidence in support of this hypothesis. Rather, the loss of total septal neurons in the p75 null mutant mice raises new issues about p75-mediated effects on central neurons.

The reference volume of the tissue examined is often determined by the Cavalieri method. Choline acetyltransferase-positive (ChAT+) trkA-positive (trkA+), and total neuronal absolute number (Nabs) within the septum were determined independently with the use of unbiased stereology. There was a significant loss of ChAT+ neurons and trkA+ neurons in p75 null mutant mice and in the total number of septal neurons in p75 null mutant mice. Because of this latter loss, the expression of results as a ratio of either ChAT+ or trkA+ to total neuronal number gives an apparent increase in these populations in the p75 null mutant mice, when in fact there are fewer of these cells present (Table 1A).

Table 1. Quantitation of the septum of wild-type (WT) and p75 null mutant (p75 null) mice with the use of unbiased stereology. (A) Septal volume in p75 null mutant mice was significantly decreased as determined by the Cavalieri method. Choline acetyltransferase-positive (ChAT+) trkA-positive (trkA+), and total neuronal absolute number (Nabs) within the septum were determined independently with the use of the optical disector method. There was a significant loss of ChAT+ neurons and the total number of septal neurons in p75 null mutant mice. (B) Because of this latter loss, the expression of results as a ratio of either ChAT+ or trkA+ to total neuronal number gives an apparent increase in these populations in the p75 null mutant mice, when in fact there are fewer of these cells present (Table 1A). SEM, standard error of the mean; P values from Student’s t test, NS, not significant.

<table>
<thead>
<tr>
<th>Volume (mm³)</th>
<th>ChAT (Nabs)</th>
<th>trkA (Nabs)</th>
<th>Total neurons (Nabs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>p75 NM</td>
<td>WT</td>
<td>p75 NM</td>
</tr>
<tr>
<td>Mean</td>
<td>4.22E+09</td>
<td>16165</td>
<td>11839</td>
</tr>
<tr>
<td>± SEM</td>
<td>2.04E+08</td>
<td>1013</td>
<td>1318</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.0119</td>
<td>&lt;0.0328</td>
<td>(NS)</td>
</tr>
<tr>
<td>Change</td>
<td>−25%</td>
<td>−27%</td>
<td>−17%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&lt;−37%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ChAT/Total</th>
<th>trkA/Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>p75 NM</td>
</tr>
<tr>
<td>Mean</td>
<td>1.83%</td>
</tr>
<tr>
<td>± SEM</td>
<td>0.17%</td>
</tr>
<tr>
<td>Change</td>
<td>+20%</td>
</tr>
</tbody>
</table>
amine in a study can vary significantly between control and experimental conditions. Changes in reference volume are at issue whether the experiment involves physical damage to a tissue; administration or withholding of a factor; comparison between developing, adult, or aged tissue; or (as we demonstrated) a change in the geometry whether the experiment involves biological tissues underscores the importance of using unbiased stereology to provide estimates of the absolute quantitative parameters being investigated.

Daniel A. Peterson
John T. Leppert
Kuo-Fen Lee
Fred H. Gage
Salk Institute
10010 North Torrey Pines Road
La Jolla, CA 92037, USA
E-mail: dpeterson@salk.edu

REFERENCES AND NOTES
1. Y. A. Barde, Neuron 2, 1525 (1989).
8. p75-deficient mice and age-matched control mice used in this study were maintained on Balb/C and 129 outbred genetical background, and all procedures were performed in accordance with the Salk Institute guidelines. Adult p75 null mutant (N = 6) and wild-type littermate (N = 6) mice were processed for immunofluorescence as previously described (6, 1998). A one-in-six series of coronal sections was selected and stained with polyclonal antibodies to ChAT (goat, 1:500, Chemicon, Temecula, CA) and trkA (RTA, 1:2000; gift of L. Reichardt, Univ. of California, San Francisco) and labeled with FITC- or Cy5-conjugated secondary antibodies (Jackson Immuno-Research, West Grove, PA) and counterstained with propidium iodide (1:1000; Molecular Probes, Eugene, OR). Sections were imaged on a Bio-Rad MRC1024UV confocal microscope running CoMOS v7.0A and using a custom macro program (stefan@lab available by anonymous FTP at 198.202.67.48) to implement the optical dissector sampling procedure. This program controlled the X-Y-Z motorized stage to move the sample in a defined sampling grid array and then imaged a three-dimensional image stack of 17-mm depth at 1-mm intervals at each sample site. The section top surface was determined and then the focus was moved through a 2-mm guard region to establish the top exclusion plane according to the optical dissector procedure (see references 6 and 7). Identification of propidium iodide–stained neurons required a 10-μm diameter minimum soma with a euchromatic nucleus; the plane of image passing through the nucleus was used to define the selection of the cell for counting. Septum volume was calculated by the Cavalieri procedure from the following anatomical boundaries: dorsal, corpus callosum; ventral, ventral brain surface; lateral, lateral ventricle walls and linear extension of these to the ventral surface; rostral, rostral genu of the corpus callosum; and caudal, last section with a complete anterior commissure. The numerical density (N) obtained from the optical dissector was multiplied by the reference volume (Vref) to give absolute numbers (N

10. We thank L. Kitabayashi for technical assistance and P. Yates for assistance with the macro development. This work was supported by NINDS P01 NS29121, NIA P01 AG006088, NIA P01 AG10435-04, and the American Paralysis Association. K.F.L. is a recipient of a Basil O’Connor Award of the March of Dimes Foundation.

Response: After performing additional detailed quantitative analysis of the mouse tissues using the optical dissector method, we confirm our earlier conclusion (1) that mice with a null-mutation of the p75 NGF receptor have more cholinergic basal forebrain neurons than do wild-type controls.

The main criticisms of Peterson et al. are (i) that we presented our data as a neuron per section and (ii) that we did not use the optical dissector method in our studies. First, our numbers were derived from eight standardized sections throughout the rostrocaudal extent of the septal region of each mouse. Second, we have now recounted cholinergic neurons in the medial septum according to the optical dissector method in every third section throughout the rostrocaudal extent of the septal nucleus of six DNA control (3 Balb/c and 3 129/Sv mice) and six p75-deficient (Balb/c × 129/Sv) mice. Counts were made in the entire coronal plane of the nucleus and not just in points selected according to a grid. The total number was calculated by multiplying the counts by a factor of three. Counting in the entire coronal plane in one-third of the tissue (3 dimensions) should give an accurate total number. The rostrocaudal distance of the septal nucleus was measured from the anterior commissure, and the total number of cholinergic neurons in the medial septum area was 53% higher in the p75-deficient mice, confirming our previous result (1). The number of cholinergic neurons in the vertical limb of the diagonal band was also higher. Our previous numbers, derived through application of the Abercrombie correction formula to numbers derived from every third brain section, were the same as that obtained by use of the dissector method (Fig. 1).

We have tried to determine what could be the cause of the apparent discrepancy between our study and that of Peterson et al. With the use of their described boundaries, we find that the “septal” volume is the same in p75-deficient and control mice. Moreover, the volume occupied by the cholinergic neurons is the same in p75-deficient and control mice. Peterson et al., however, find that p75-deficient mice have a smaller septum/diagonal band area than their controls (70% of control). This result may be indicative of a general developmental problem of their p75-deficient mice, a possibility that could be analyzed by measuring brain and body weight. It is conceivable that there are differences in the genetic background of the p75-deficient mice used in these two studies that are not related to p75 itself, but affect general development. These mice are both on a Balb/c × 129/Sv background and ultimately derive from the same founder mice produced in R. Jaenisch’s laboratory (Massachusetts Institute of Technology, Boston). However, the first breeders provided to our source (Jackson Laboratories, Bar Harbor, Maine) may have represented an insufficient number of generations of inbreeding. As we understand the situation, the mice in the study by Peterson et al. were inbred separately over the past several years, and the two colonies may therefore have undergone genetic segregation.

Unexpectedly, the actual volume of the total region in their control mice was about twice that in ours. Moreover, their total cholinergic neuron number was about four times greater than ours (about 11,000 and 16,000 as opposed to about 4000 and 3000 in our p75-deficient...
mice and controls, respectively). Unbiased stereologic analysis (3) and our own previous studies show that this region contains 6000 to 8000 cholinergic neurons in adult rats. The septal/diagonal band complex of mice is smaller than that in rats, and it is clear from brain sections that mice have about half the cholinergic neurons per section of equal thickness as do rats. Our numbers and those reported by others (4) for mice (3000 to 4000 total) are consistent with this observation.

The greater volume and total cholinergic neuron number in the Peterson et al. study may be indicative of some systematic error in their calculations. One factor that could have contributed to the discrepancy would be the possibility that Peterson et al. used tissue sections that were not representative of the medial septal/diagonal band or used a sampling grid that did not give neurons an equal likelihood of being counted. However, it is unclear from their comment how many sections were analyzed, what internal reference point was used to ensure that similar sections were sampled between animal groups, from which rostro-caudal level the sections were taken, and how the sampling grid was applied (interpoint distance and so forth). Cholinergic neurons are not distributed in a homogeneous fashion, and small differences in the sampling area in any of the three dimensions would result in substantial numerical differences. If these sampling errors exist, they could be exaggerated if a brain region that includes large areas that are not occupied by cholinergic neurons is used as the reference volume. In addition, some mouse strains are known to lack (to various degrees between animals) fusion of the corpus callosum, which can prevent accurate determination of the genu of the corpus callosum (one of the boundaries used by Peterson et al.), which would make accurate volume determination difficult.

In conclusion, we have confirmed our previous published results by using the optical disector method for counting neurons in the entire coronal plane of the medial septum in every third section through the septal nucleus (which represents the entire 3-dimensional extent of the nucleus). Therefore, the apparent discrepancy between our results and those of Peterson et al. is most likely not due to differences in quantification methods, but may reflect a genetic difference between the two mouse colonies that is not related to p75. Our results (1), that p75-deficient mice have more basal forebrain cholinergic neurons than control mice and that they do not show the apoptosis observed in control mice, probably more closely reflect the (death-mediating) role of p75. This was further strengthened by our finding (1) that a p75-interfering peptide mimicked the phenotype of the p75-deficient mice, that is, prevented the postnatal death of the cholinergic neurons in control mice. Our results also are consistent with the observations by others that p75 can mediate cell death (5). To resolve the issue of which results (which mice) most closely represent the role of p75 in the basal forebrain, it will be of great interest to analyze the recently created p75-deficient mice, which are on a different genetic background (C57BL/6J; Jackson Laboratories).

Note added in proof: A study (6) from the laboratory of Frank Longo (University of California, San Francisco) replicates our results that adult p75-deficient mice have 50% more cholinergic neurons in the basal forebrain. They obtained p75-deficient mice from Jackson Laboratories and, through backcrossing, produced deficient and wild-type control litters. With the use of highly controlled stereological methods, the numbers of neurons were blindly assessed by people from two laboratories (F. M. Longo and W. C. Mobley, also at University of California, San Francisco).

K. Hsiao et al. (1) describe evidence of a memory deficit that is correlated with an elevation in amyloid-containing plaques in a transgenic mouse that overexpresses the so-called Swedish mutation of amyloid precursor protein. This comment questions whether the evidence actually shows such a memory deficit.

First, although the performance of the transgenic mice was impaired, they did show signs of learning. Figure 2E in the report demonstrates that the 9- to 10-month-old transgenic mice performed more poorly than did wild-type mice in the visible platform subtest of the Morris water maze, a result which is usually taken to indicate a sensory-motor impairment, not a memory deficit. On two of the four individual days of the subtest, such differences were statistically significant; but it is not shown whether an overall analysis would also produce a significant result. In the absence of basic measures of locomotor activity, vegetative functions, or sensory capacity, perhaps these 9- to 10-month-old animals are sluggish. By the fourth block of trials, the transgenic mice improved, although they remained significantly slower than controls. Thus, the transgenic animals were learning something: This conclusion is confirmed in figure 2A in the report, in which improvement in latency for learning the task of finding the hidden platform is similar on a percentage basis for old transgenic mice (40 to 20 s) and young nontransgenic mice (20 to 10 s). Hsiao et al. emphasize the absolute latency difference, suggesting that this represents a memory deficit. But the “AD mice” actually improve more than nontransgenics. From the visible and hidden platform test data, one can conclude that the transgenic animals required more time to perform, yet learned over days (decreasing their mean latency) at a rate similar to that of controls.

Second, there appears to be no relation between the appearance of plaques in aging and the decline in performance. In figure 2B in the report, which shows time in each quadrant with the platform missing, there is essentially no change shown with aging in the target quadrant for the transgenic animals (39% at 2 months, 35% at 6 months, and 34% at 10 months), although table 1 in the report indicates a significant increase in the number of plaques with aging.
Basal Forebrain Neuronal Loss in Mice Lacking Neurotrophin Receptor p75

Daniel A. Peterson, John T. Leppert, Kuo-Fen Lee and Fred H. Gage

Science 277 (5327), 837-839.
DOI: 10.1126/science.277.5327.837