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Memorandum to Jean Harry from Bob Garman Re: the morphometry component of the perchlorate effects protocol:

NOTE: Comments from Jean Harry are in quotes. Responses from Bob Garman are bolded.

"I have reviewed the protocol and have a number of concerns."

1) "How is the tissue fixed?"

Jean, we had intended to use 10 percent neutral buffered formalin for the PND 10 and 22 day animals, because this is comparable to what we have been doing in the past with Day 11 and adult rats on developmental studies. This approach would also allow us to compare data from the PND 10 rats with those from the PND 11 rats from the prior study. However, we realize that formalin is not likely to be the optimal fixative for the Gestational Day 21 rats and PND 5 rats. We expect to receive specimens from rats of this age from Argus in the very near future, and sets of these brains will be fixed either in formalin or in Bouin's solution. I imagine that we will be using the latter fixative for these early time points, because this fixative would "firm up" the brains, somewhat. What we have to work out is the time in fixative prior to transferring to alcohol or formalin, as well as modifications in our processing times for optimal staining and sectioning. This will all be spelled out prior to conduct of this component of the study, but we will need some time to validate the procedures.

2) "For the blocking of the brain, a matrix guide should be used and these sections not cut freehand. Both the anatomical location identifiers should be given as well as section location on the matrix - this will allow for consistency in plane, consistency hopefully between hemispheres, and also by location # in the matrix - give a measurement of any increase or decrease size of the brain. I think that these are commercially available through Ted Pella co."

Jean, we have had quite a bit of experience with both "free sectioning" and sectioning using brain molds/matrix guides and feel that we get much more consistent sections with free sectioning. This has also been my experience in examining sections obtained using brain molds that have been sent to made by other laboratories. The use of these molds is particularly problematic when dealing with young-aged rats. Even at PND 11, there is significant variation in brain size. One cannot, therefore, get a mold that is of the appropriate size for every brain. Although matrix molds may be helpful in obtaining vertical sections, trying to cut a brain in a mold when the brain is smaller than the mold actually makes the procedure more difficult. The brains tend to move around within these molds during sectioning and are harder to hold in position than when placed directly on a bench top. Furthermore, the younger-aged brains are sometimes traumatized by these molds. To section brains from rats of Gestational Day 21, PND Day 5, and PND Date 10 would probably require somewhere between 4 and 6 different molds of slightly different size for each time point. Even if we did have such molds available to us, the individual knife grooves in the molds might not line up with the specific anatomic area through which we would like to cut. Other morphologists with whom I have spoken have voiced similar opinions.

Jean, we have modified our approach to trimming these brains since performing the last perchlorate study. Previously, we embedded multiple coronal sections of each brain in two paraffin blocks. Now, we singly embed the four coronal sections on which we anticipate taking measurements and, in addition, take multiple step sections. These step sections are approximately 60 micrometers apart. On the PND 11 rats, we generally take three such step sections (but take more if necessary). The extra slices of brain tissue are multiply embedded in the fifth paraffin block (*i.e.*, a total of five blocks of brain tissue).

3) "In addition, each section should be identified by an atlas picture and Anterior/Posterior coordinates."

Jean, our current approach on standard developmental neurotoxicity studies is to scan each image that is measured using a film scanner and to archive this image on a CD-R disc. In the raw data, I also put in sheets of "thumbnail photos" of each measured section, along with a figure legend indicating the individual animal numbers. By so doing, it is easy to scan the sheet of images and determine the consistency of each level. I believe that this is more appropriate than merely stating the page number of a reference atlas or the Bregma coordinates. It is not possible to state a specific bregma location in the protocol for each of the measured sections, because there is going to be some slight variation unless serial sections are taken (which is cost and time prohibitive on this study). However, by having a scan image available, one can easily establish section level consistency, as well as print out scans for additional measuring. It is also possible to print these scans onto acetate sheets and then lay these on top of each other to compare section levels and sizes of neuroanatomic areas.

3) "In the young animals, I question how well the two sections through the cerebellum will be able to be accomplished consistently."

I would like to continue taking coronal sections on the cerebella of the PND 10 rats both in order to have these comparable to the sections examined previously from PND 11 rats and to also allow me to examine certain prominent areas of apoptosis present along the floor of the fourth ventricles. However, I believe that the cerebella from the Gestational Day 21 and PND 5 rats should be sagittal in orientation. I would propose performing a slightly parasagittal slice on these cerebella and then having step sections cut into the block until the midpoint is reached. Then, we would measure the diagonal AP length and diagonal height (at a perpendicular intersection) in a fashion similar to that reported by Pat Rodier in a number of her papers. I heartily agree with you that achieving replicable coronal sections on such young-aged brains would be problematic.

5) "It should be noted in the protocol that the brain section should not be trimmed to full face (as is often done with other organs) but that the block should be aligned for a full face cut initially.

Jean, this has always been our approach on all studies involving morphometry, but this can be stated in the protocol if you wish. We never change the block angle or knife angle, and we take our first section as soon as possible (even if not a full-face section). In studies performed in-house, we also always use the same microtome. Because of the large numbers of brains and very tight time schedule for this particular study, multiple histotechnicians will need to be involved. This will undoubtedly result in some slight variation, but sectioning will be standardized to as great an extent as possible.

6) Having examined such slides and conducted morphometrics I would strongly suggest the following. Rather than a written description of measurement sites - or in addition to the contractor needs to provide you with a stereotaxic atlas figure giving the appropriate anterior/posterior plane of cut - and on that figure identify the region for measurement. Each section should be related back to the reference A/P site in atlas. This will provide a systematic evaluation of plane of cut.

As mentioned, above, each measured section will be scanned and the individual images available on a CD-R disc. In addition, representative sections from one or more annals will have the positions and angles of measurement depicted with lines. (I had not planned, however, to have these lines on every scanned image because of time constraints.) If, in addition, a reference to a brain atlas is desired, this can be provided. However, the exact level at which a measurement is taken is, in my mind, less important than the inter-animal consistency for that particular level.

7) "Measurements should be taken from both hemispheres but not averaged for a mean measurement per animal - each should be analyzed with hemisphere as an identifying factor. In this way we can keep the data as collected and still take into account the hemisphere/plane of cut difference. If the variance between hemispheres in a group of animals is greater than/or close to a treatment effect we will have some idea as to the validity of the data."

Jean, the measurement from each side will be recorded separately, and these measurements will be available for examination. However, I would suggest that mean values (of the two sides) be used for statistical analysis. In my experience, side differences are generally the result of oblique sections. However, if a statistical analysis shows a slight difference in one side and not in the other, this is, in my experience, likely to be interpreted as a neurotoxic end point by the regulatory agencies.

8) "Measurement of the cc should not be conducted where the written description seems to identify. Along the midline there is a high incidence of edema artifact due to the ventricle location and has been shown to not be reliable. This would be especially critical in the young animals. I would recommend three areas for measurement. one along the midline (that will allow the contractor to compare data to any other control data that is on file, one at the most consistently narrow/compact point of the cc. and the other at the widest peak. >

Jean, I agree with your comments regarding the midpoint of the corpus callosum. Because of this problem, we no longer measure the corpus callosum at the midpoint. Instead, I take bilateral measurements at the level of the external granular layer of the overlying cingulate cortex. These, again, are usually averaged for statistical analysis (but are generally within a few micrometers of each other). If you feel that the midpoint, as well as the more lateral region (widest dimension, which also includes the overlying cingulum) should also be measured, I can easily do this. However, our original estimate was for only 8 or 9 measurements to be taken.

8) Hippocampus - width of the Ammon's horn, dentate blade, (thus dentate granule cell layer thickness) - Width of the CA1 and CA3 pyramidal cell layers (width of cell band at each site) - again identified by atlas reference and illustration. Similar approaches should be made with cerebellar measurements and layers. This will be difficult in the day 11 pups as there is a significant amount of migration happening and the internal - external granule cell layers are maturing.

Jean, I am uncertain whether the above paragraph represents your recommendation or your interpretation of the SOP. Although I have measured the thickness (or number of cells) in the pyramidal layer of the hippocampus, before, my usual approach is to measure the full-thickness of the dentate gyrus, extending from the alveus to the external capsule. The location and angle of measurement would, of course, be documented on one of the coronal sections that are archived with the other scanned images. I can also measure the thickness of the pyramidal cell layer in the CA1 and CA3 regions. However, once again, such measurements were not included in the original estimate.

10) All morphometrics should be conducted with digital images of each section and use of a quantitative software package such as NIH Image. Each image and site of measurement - as well as actual measurement length needs to be documented. If this is not done in the initial study it will only have to be conducted later.

Jean, as mentioned, above, we will have digital images of each measured section available for review. However, the logistics of this particular study (especially the timetable) are problematic. It will be much faster to measure the specified neuroanatomic areas using an ocular micrometer (this to be done by the pathologist evaluating the tissues both for lesions and consistency of cut - *i.e.* not by a technician). It would take considerably more time to scan each image and analyze the same with the NIH Image program. If additional measurements are to be performed at a later point in time, the

individual scanned coronal sections can easily be magnified to full-screen size or printed out at a standard size (such as 8 x 10 inches) and then re-measured. Jean, this study is calling for 640 brains to be processed and embedded and for at least 320 of these (high dose and control) to be analyzed morphometrically. The expected turnaround time was initially set at approximately two weeks final tissue collections in mid-March and Report due April 1. Now, the committee would like to know how many animals can be completed by April 1. My initial hope was that we would have 5 to 6 months to do this study. I am sure that you would agree that the most important aspect of the study is that the histologic sections be achieved in a standardized artifact-free fashion. This dictates that only a small number of technicians should be involved in the trimming and sectioning of these brains. Although subsequent analyses may need to be performed on these sections, it will be a physical impossibility to conduct the morphometric aspects of this study in the way that you suggest and in the time frame that has been dictated.

11) These are the major comments for now. If the plane of cut is not consistent - and this will be difficult in the younger animals, this study will not offer any better data than the previous study.

Jean, I heartily agree with your summary statement. Unfortunately, the main emphasis on this study appears to be turnaround time. We are planning to do the very best that we can with it under the imposed time constraints. My suggestion would be to take our time and to perform the study in step-wise fashion, starting first with the PND 10 rats, having only the data from these animals available by April 1. We could examine the data from the PND 10 rats, first, and then decide how to proceed with the other age groups. By examining the PND 10 rats, first, we would have some comparison with the prior perchlorate study. Depending upon the findings for the PND 10 rats, this might even modify our approach to examining the younger-aged animals. By the way, we have made the decision to dissect (slice), process, and block all of the brains from the rats at any particular age time point so that there will be no differences that might be attributable to shrinkage or that might develop as a result of different fixation times or other unforseen factors. However, we would initially prepare slides and microscopically examine only the rats from the high dose and control groups.

I very much appreciate your input, Jean, and I would like to discuss further with you any differences in opinion that we may still have *re:* the most appropriate way to conduct the morphometric aspects of this study (*i.e.* under the imposed time constraints). However, I thought it would be best to first put some of my comments into print form for your review. I will plan to call you during the coming week to that we can discuss these points further.

Bob Garman
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