Final Report-TBI-John Pintar, PI; Steve Levison, Teresa Wood, Co-PI

Introduction

During this project period of support from the New Jersey Commission on Traumatic Brain Injury, we made substantial progress toward the original goals of our project, which was to investigate the role of IGF binding proteins following traumatic brain injury. Initial reviews of the project were very positive about the hypothesis and significance of our proposal, which had two aims. The first proposed aim was to further characterize outcomes in wild-type and IGFBP mutant mice following puncture wound. We had previously documented upregulation of several of the IGFBPs and proposed to determine whether loss of any upregulated IGFBP affected any of several parameters histologically. The second goal was to introduce a compression model for TBI (controlled cortical impact) and determine whether IGFBP expression was altered following this type of injury.

The major, overarching comment of the reviewers was that we should focus on introducing the compression injury model since it is currently thought to be a far better model to provide information related to the goals of the NJ Commission on TBI. As a consequence, we have placed our efforts in the first project period on establishing this compression model and, importantly, were successful in that regard as documented below. We also began substantial histological work on this model, which we propose to extend in the second funded project period, which will focus on analysis of one IGFBP which is up-regulated following CCI TBI. While there is clearly much histologic work to be done on this newly-introduced model (which is ongoing), we concentrated initial efforts with this model on this project period to determine whether any of the IGFBPs that we had previously shown to be upregulated following the puncture wound model might also be upregulated in wild-type mice following compression injury. If that were the case, we could then determine the consequences of mutation using the general overall strategy we had proposed for analyzing the puncture wound model. In one major finding from this grant, we have found that IGFBP-2 is dramatically up-regulated on the ipsalateral side of lesion following TBI as documented below.

TBI experiments were performed using the e-Controlled Cortical Impactor (CCI) manufactured by Custom-Design in Virginia. Following approved protocols, TBI was produced using this impactor with the injury depth of deformation 1 mm, velocity at 4 m/sec and duration of 150 msec. Ten-twelve mice are now routinely and reproducibly put through this procedure in one day, so that we have been able to begin examine time-course outcomes following injury of a single cohort of mice. An initial low power histologic assessment indicated that the cavity formed by the wound is clear at day 3 and maintained at day 7. This pattern of lesion is now generally reliable and is similar to that shown in analysis of mice that recieved a lateral compression injury of similar severity (J. Neurotrauma 23:124).

Once the model was established, we have determined in several experiments whether there were any changes in IGFBP expression following CCI. Following lateral

CCI using the conditions above, cohorts of mice were maintained for 0, 1, 3, and 7 days. Brains were then isolated, fresh-frozen, and processed for in situ hybridization with several 35S-labelled IGFBP probes. Sections from four-six fresh-frozen brains have been examined at each age so far and have led to findings that, following one more confirmation, will be prepared for publication.

One specific IGFBP, IGFBP-2, was reproducibly found to be upregulated in the parenchyma adjacent to the injury site, with the results for the expression pattern similar at each time point following CCI. Low IGFBP-2 expression is un-injured mice is normally seen at the X-ray level only in the meninges and choroids plexus. However, even only one day following CCI, however, low levels IGFBP-2 expression are detected in near the lesion site. IGFBP-2 while prominent up-regulation is apparent in the nearby meninges. The upregulation of IGFBP-2 remains clear and more dramatic at both day 3 and 7 following CCI. The higher levels of expression at day 3 are readily apparent with expression relatively evenly distributed through the brain parenchyma adjacent to the lesion site. By day 7 following lesion, expression is focused around the lesion site though expression appears to be increased from that of day 4. These results clearly demonstrated that IGFBP-2 expression is upregulated near the impact site for at least 7 days and suggest that perhaps the increased expression maintained over an ever longer period. This long-lasting expression is in contrast to our earlier work with IGFBP expression in the puncture wound model, where IGFBP-2 was upregulated 2-4 days post-lesion, but had resolved to normal levels by day 7 following injury (see initial grant for discussion of that earlier data). Emulsion autoradiography is continuing to give at least an idea of whether the even distribution in fact represents a more discrete cellular expression near the wound site though the fresh-frozen sections will not allow more discrete histologic analysis. The overall pattern of IGFBP-2 expression seen following CCI is generally similar to that of reactive gliosis described for the mouse following lateral CCI in the above referenced paper, which we are now confirming with GFAP.

Since fresh-frozen sections give poor microscopic detail, we have began to use perfusion fixed brains to provide cellular resolution following autoradiography. In initial attempts, X-ray film signals were very weak even following several days of exposure, probably because of over-fixation (initially 4% paraformaldehyde, O/N post-fix), so we are currently reducing the formaldehyde concentration to 2% with a reduced (30') post-fixation, in an attempt to increase RNA accessibility so that we can obtain more information about the cellular sites of IGFBP-2 expression via dual in situ/immunocytochemical protocols with which we have had prior experience.

This exciting result clearly makes the study with IGFBP-2 KO mice an important and well-justified one that we have begun. We re-expanded the IGFBP-2 line, which was backcrossed onto C57Bl6/J background, and now have initial litters of IGFBP-2 KO mice that we can use for experimental analysis. We will focus on BuDR incorporation in SVZ precursors and quantitate apoptotic and GFAP and neural cells as proposed for the puncture wound model though some initial experiments had technical problems with trypsin treatment, which we have now resolved. Since one reviewer of the initial indicated that more development of the counting protocol would be helpful, we have

continued stereological assessment with other project in the lab as well. For example, we have now extenisve series of cell counts following BuDR in WT and mu opioid receptor (MOR-1) knock-out mice, which is being prsented at the upcoming Soc. Neuroscience meeting in Chicago. In addition, some additional specific end-points, such as integrity of the blood-brain barrier using IgG, for histological analysis of CCI have recently been established in the C57Bl6/J mouse (Saatman et al, 2006, ref above) and these will also be used as we compare the impact of CCI on the IGFBP-2 KO and WT mice. The proposed analysis of activated caspase is ongoing and is concentrated at the earlier ages (<8 days post CCI), as suggested by one reviewer, while longer outcome times will still be performed to determine whether there, for example, any genotype dependent changes in cortical area, again using protocols we have already established to determine hippocampal area in opioid KO mice.

Finally, we continued to increase our behavioral expetise since we propose to initiate at least some behavioral analysis of CCI WT and IGFBP-2 mice. We have established several standard behavioral tests (locomotory, anxiety, Morris water maze) that we have primarily used for analysis of opioid mutant mice. It is clear from the above work of Saatman et al. that basal locomotory function and Morris water maze testing are sensitive to different levels of CCI. If an altered CCI response is mediated by the increased IGFBP-2 expression, it is possible that behavioral as well as morphological effects will be seen. While the anatomic comparison of proliferation, apoptosis, and the reactive gliotic response between WT and IGFBP-2 mice will remain our highest priority, we have previously brought to our facility and maintained mice with spinal cord contusion injuries (which require careful care to ensure that the bladder empties) without lethality and measured changes in locomotory recovery as we detailed in the original application. Thus, maintaining CCI mice for behavioral testing should not be a problem. In any case, we will grossly monitor the IGFBP-2 mice for obvious behavioral differences from WT following CCI prior to sacrifice for morphology as we continue these studies.

Additional relevant findings

Since we anticipate that other IGFBPs will also be up-regulated, we have begun the process of producing combinatorial IGFBP mutants on the C57BL6/J background, so that any compensatory/redundant functions can be assessed. IGFBP-2/3 and IGFBP-2/4 strains are being produced (IGFBP-2/5 cannot be produced because genes are adjacent and will not recombine). Although technical problems with the in situs for those probes have prevented us from having definitive evidence on this issue at this time, we expect clear data on their expression shortly, as well as on expression of IGF-1, IGF-II and IGF-1R, as suggested by one reviewer. In addition, we are crossing the IGFBP-2 mice with C57 mice with a nestin/GFP, which will allow us to visually observe any SVZ progenitor changes as a consequence of IGFBP-2 deletion.

We also developed qPCR analytic protocols for all IGFBP expressed in the brain. Primers sets were designed, showed only one peak in dissociation curves, and detected signals in brain cDNA with expected primer sizes verified by agarose electrophoresis.

Thus we have established qPCR, which can be used to quantitate expression following CCI. Thus we developed the capability to quantitate the extent of any altered upregulation of any other IGFBPs in response to IGFBP-2 mutation biochemically as well as histologically. Using these protocols we have also specifically shown that IGFBP-2 is expressed at reasonably high levels in SVZ neurospheres while IGFBP-4 is also expressed. While we agree that detailed extensions of the project to this area in beyond the scope of this proposal, we do believe that this initial result shows feasibility in future funding efforts for this overall project.