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14. ABSTRACT Our major focus of year 1 of this grant were Aims 1 and 4. In Aim 1, we proposed to identify a maximally effective P2X7 receptor antagonist, as well as to optimize its dose in a weight drop model of experimental spinal cord injury. Solid progress has been made. The neuroprotective effect of the highest tolerated dose of four P2X7 receptor antagonists has been tested in rats and 3 P2X7 receptor antagonists have been tested in mice exposed to spinal cord injury. To identify actions of the potential new medication not related to P2X7 receptor inhibition, we obtaining permission from DOD to use mice instead of rats as our experimental model. Of 3 agents tested so far, only the P2X7 receptor antagonists BBG provided a significant neuroprotective effects in both rats and mice. The data collected shows: 1) BBG effectively reduces the severity of spinal cord injury in rats, and 2) also protects in mice exposed to similar injury; 3) P2X7 receptor KO mice exhibit significantly less injury than wild-type littermates; 4) BBG has no neuroprotective effect in P2X7 receptor KO mice; 5) combined these observations provide direct evidence suggesting that BBG educe the severity of SCI by antagonizing P2X7 receptors. The major goal of Aim 4, an additional focus of year 1, was to define the transcriptional events associated with spinal cord injury in spinal astrocytes and microglia, with a specific focus on those associated with and regulated by P2X7R activity-dependent transcription. We have made significant progress in this technologically challenging Aim, by establishing the injury-associated expression profiles of sorted adult spinal microglia, which we are now analyzing to define optimal microglial targets for intervention. Indeed, our analysis has already strongly validated the innate immune system as perhaps the critical initiator of spinal inflammatory injury, and has suggested a number of previously unconsidered targets for therapeutic intervention in SCI; these data have also clarified the role of P2X7R-associated transcripts in this process. In addition, we have developed and validated new sort protocols by which to isolate spinal astrocytes, which we are now employing for a parallel analysis of the response of spinal astroglia to injury; this is a necessary prerequisite to our planned analysis of paracrine interactions between spinal astroglia and microglia in response to SCI, and the role of P2X7R activation in that process.					
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Development of a Small Molecule P2X7R Antagonist as a Treatment for Acute Spinal Cord Injury

Introduction

The proposed studies were based on the observation that ATP release and activation of P2X7 receptors drives the innate inflammatory response initiated by spinal cord injury. P2X7 receptor activation activates and coordinates a number of downstream signaling events, including the release of pro-inflammatory cytokines (Di Virgilio et al., 2009). We proposed that suppressing activation of P2X7 receptors - the very initial steps in the innate inflammatory response to spinal cord injury is more efficient than previous attempts to target complex downstream pathways involved in secondary injury. The objectives of the proposal were to (1) screen P2X7 receptor antagonists for their neuroprotective activities in a weight drop model of spinal cord injury in rodents; (2) define the clinical indications for administering the P2X7 receptor antagonist in various models of SCI in rats; (3) use a FDA accredited commercial laboratory, to obtain GLP rat and rabbit safety and toxicity data to support an IND application necessary to conduct a collapse phase 1 + 2 clinical trial after completing of the proposed studies, and (4) define the cellular target for P2X7 receptor blockade.

The development of a systemic treatment that reduces, or even prevents the secondary damage associated with SCI and which could be given to soldiers and others following injury on the battlefield has the potential to dramatically improve the outcomes of these injuries, improving the quality of life and reducing the health care needs for thousands of individuals each year. The prospect of administering a small molecule agent with no known adverse effects is particularly attractive in the setting of acute traumatic injury.

Body (Progress report)

In Year 1 of this grant we focused on 2 of its 4 Aims, specifically Aims 1 and 4.

Aim 1

We have over the past 12 months systematically tested the toxicity of the P2X7 receptor antagonists, MRS2159, KN-62, and A-740003 in non-injured control rats to establish the maximal dose tolerated. We found that the maximal dose tolerated of MRS2159 (30-50 mg/kg), KN-62 (20-40 mg/kg), a-740003 (10-40 mg/kg). The neuroprotective effects of these agents in the setting of acute spinal cord injury were next compared to control vehicle, as well as to rats treated with brilliant blue G (BBG, 10 mg/kg). Adult female Sprague-Dawley rats (220-250 g) were anesthetized with i.p. injections of a mixture containing 8 mg/kg ketamine and 10 mg/kg xylazine. For surgery, a midline incision was made on the back region and a laminectomy was performed aseptically at the T11- T12 level. Before SCI, a catheter (PE-10 tubing) was placed in the left femoral vein after carefully separating nerves and blood vessels. Immediately afterward, the exposed dorsal surface of the cord was subjected to a 10 g weight-drop impact from a height of 12.5 mm. Vehicle (0.9% NaCl) or MRS2159, KN-62, A-740003, or BBG were given intravenously 10-15 min after the weight drop. BBG served as our positive control, because this P2X7 receptor antagonist previously consistently has improved functional recovery and reduced lesion volume in rats exposed to traumatic weight drop injury of the spinal cord (Peng et al., 2009). We found that MRS2159 (50 mg/kg) and KN-62 (40 mg/kg) did not provide a significant functional improvement in the maximal doses tolerated by the rats,

whereas A-740003 (40 mg/kg) significantly improved locomotor function in rats exposed to traumatic spinal cord injury compared to control vehicle-treated littermates.

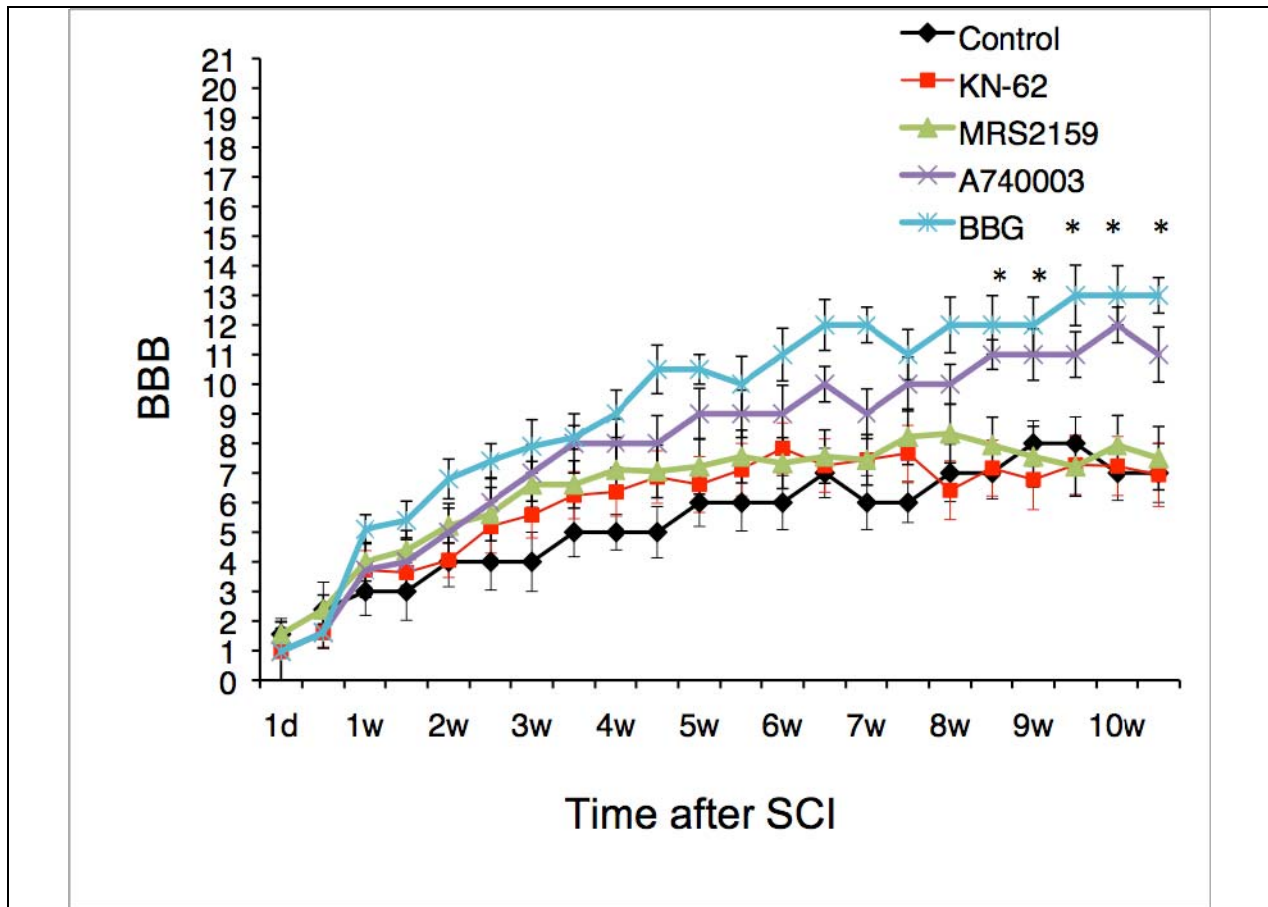


Fig. 1. BBB score of functional recovery for 10.5 weeks after weight drop injury of spinal cord in rats. The P2X7 receptor antagonists MRS2159 (50 mg/kg) and KN-62 (40 mg/kg) failed to improve functional recovery when the rats received the highest dose tolerated without side-effects. In contrast, A 740003 (40 mg/kg) and BBG (10 mg/kg) provided significant protection since the locomotor functions of rats that received either A730004 or BBG were significantly better starting at 8.5 weeks compared to control vehicle animals exposed to similar injury (n = 16 in all groups, *, p < 0.05, ANOVA).

The rats were perfusion-fixed with 4% paraformaldehyde 10.5 weeks after being exposed to spinal cord injury. For quantification of spinal cord damage, 20-µm-thick cyosections were stained with the combined Klüver-Barrera (Luxol Fast blue and Cresyl Violet) standard procedure to detect myelin. For morphometry, every tenth section was selected, and its image was captured by using a digital camera. Severe disruption of tissue organization and/or the loss of staining were identified as lesion areas. The identified areas in individual sections were measured by using imageJ software. The lesion volume was obtained by the sum of total lesion area multiplied by distance between the sections (200 µm). As expected based on the functional analysis, MRS2159 and KN-62 did not reduce tissue loss after spinal cord injury

(Figure 2). However, surprisingly a significant decrease in lesion volume was not noted in rats receiving A730004 despite that this group exhibited significantly better locomotor function than control vehicle treated rats exposed to the same injury. However, lesion volume was significantly reduced in rats receiving BBG (positive controls) in accordance with previous observations (Figure 2). Based on the observation that A730004 improved functional recovery, but did not reduce the traumatic lesion, we felt it was critically important to test whether the effects of the P2X7 receptor antagonists are mediated by inhibiting P2X7 receptors, or through other non-specific effects.

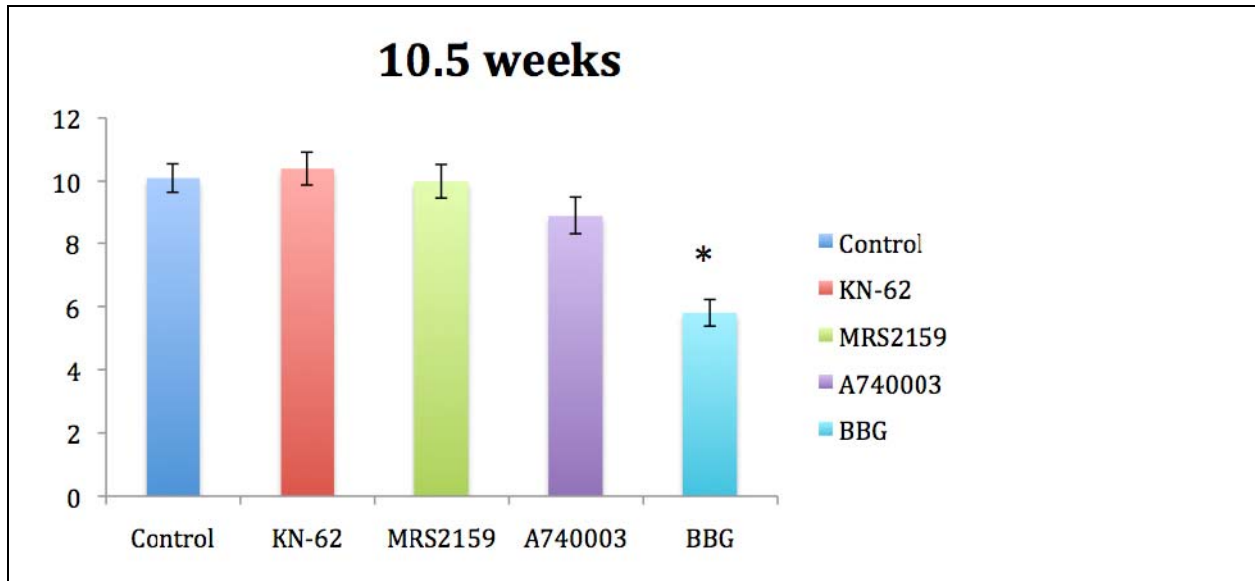


Fig. 2. Traumatic lesion in rats exposed to weight drop spinal injury 10.5 weeks earlier

The different groups (n = 16) were compared by using ANOVA with Tukey-Kramer posthoc test. *, p < 0.05 compared to control vehicle.

In work funded by NY State Spinal Cord Injury Board, we have for several years worked at optimizing a murine model of weight drop injury in mice. The murine model of spinal cord injury is technically more difficult and often associated with a higher degree of variability than the rat model. However, we have been able to reduce the variability of the mice model so that it is directly comparable to the rat model (Figure 3). We obtained permission from DOD to conduct the remaining experiments in mice. The major advantage of using mice is that the P2X7 receptor antagonists can be tested in P2X7 receptor knockout mice and that effect of the drugs not related to P2X7 receptor inhibition can be identified. We have directly compared the effects of spinal cord injury in P2X7 receptor KO mice and wildtype littermates. Adult female mice (8-10 weeks old) were anesthetized with a mixture of ketamine (60mg/kg, i.p.) and xylazine (10mg/kg, i.p.). A laminectomy over the dorsal portion of T11 was performed and the vertebral column was held with fine clamps at the T10 and T12 level. The exposed dorsal surface of the spinal cord was subjected to a 3g weight-drop with tip diameter of 0.5 mm flat surface, modified NYU impactor (Peng et al., 2009), from a height of 6.75mm using a modified NYU impactor.

We have so far tested the neuroprotective effect of MRS2159, KN-62, and BBG in the mice model of spinal cord injury. The observation in mice so far can be summarized as follows: MRS2159 and KN-62 did not affect the severity of spinal cord injury, whereas BBG potentially improve recovery of locomotor functions. We are in the middle of completing the analysis of A730004. The consistency of the neuroprotective effects of BBG without any toxicity and the lack of is very promising. BBG also potentially reduced inflammatory reaction to traumatic injury evaluated 4 days after spinal cord injury (Fig. 2). The chemical structure of BBG is almost identical to blue food color that since 1928 has been wide used as a food color with essentially no report on side-effects except for 2 cases in which very high amount of BBG were added to feeding tubes in comatose patients. However, in neither case, it was possible to positively link deterioration of the patients' conditions to BBG intake.

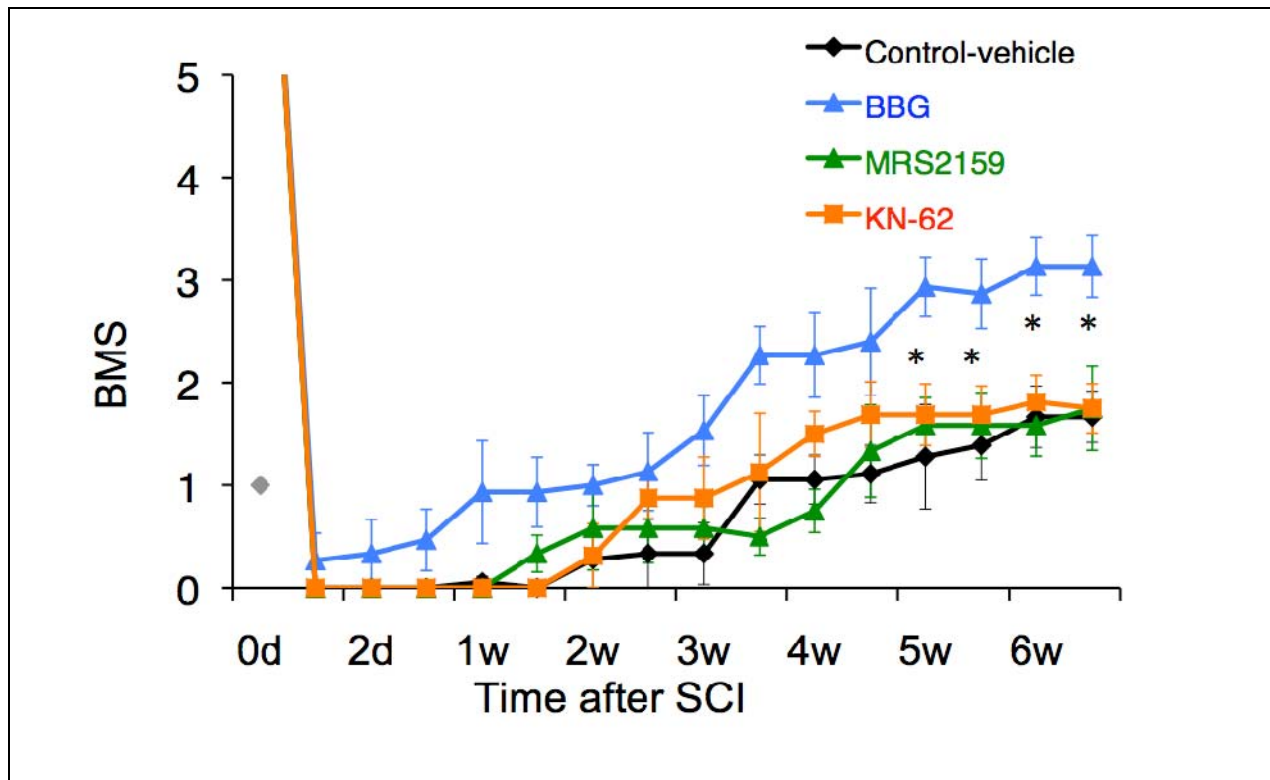
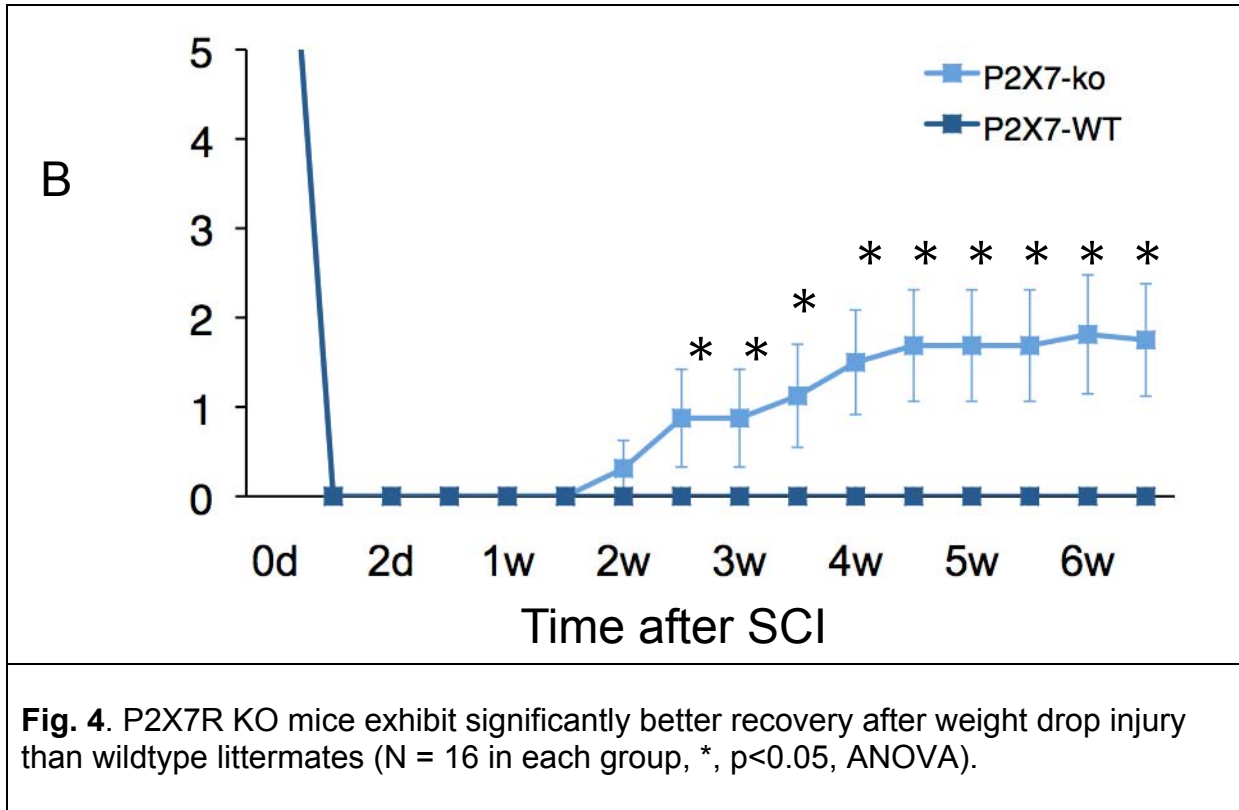


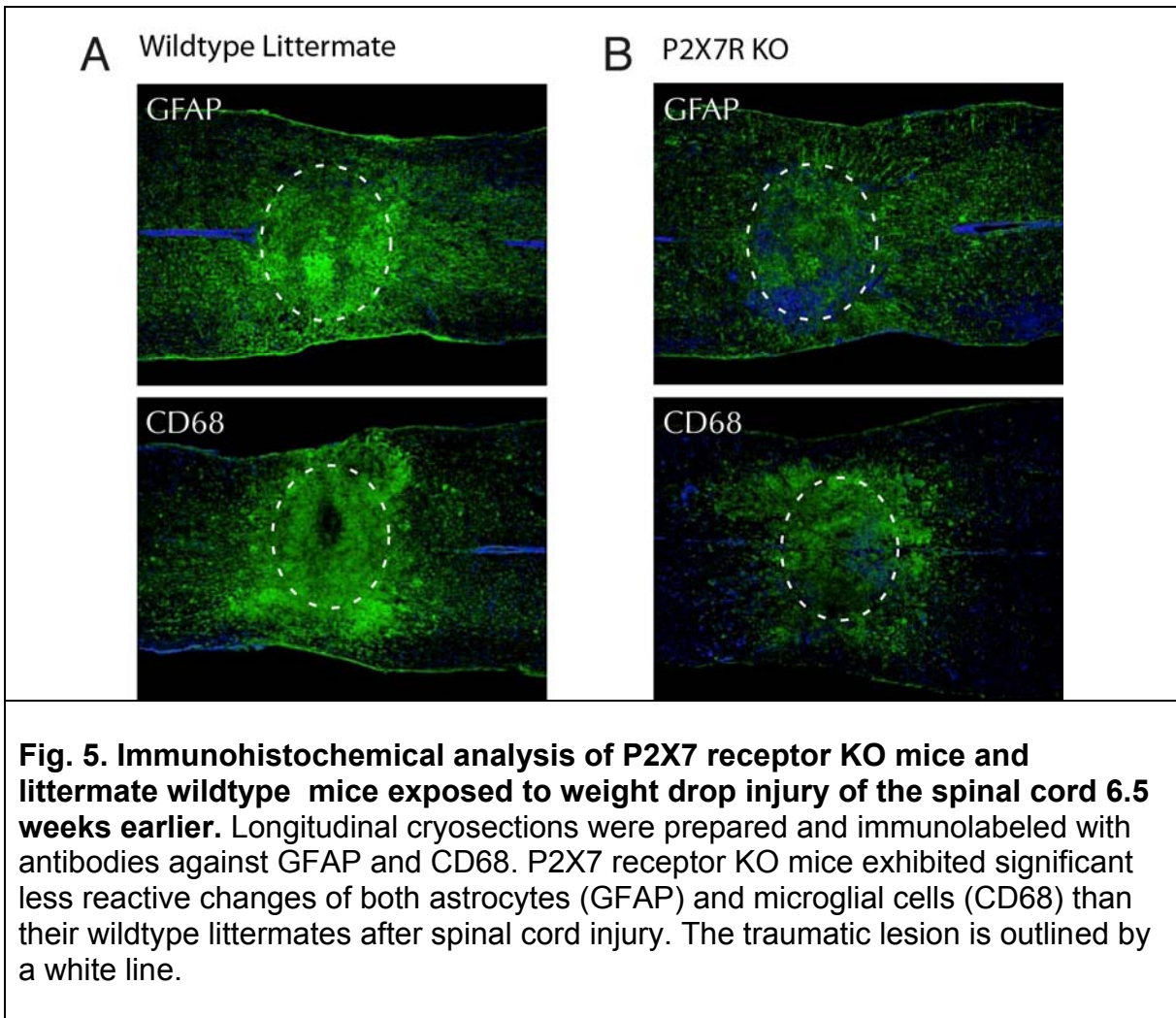
Fig. 3. Mice were exposed to weight drop injury and functional recovery evaluated using the BMS scoring system. BBG (10 mg/kg) significantly improved locomotor function after week 5, whereas MRS2159 and KN-62 had no effect compared to vehicle controls exposed to the same injury (N = 16 in each group, *, $p < 0.05$, ANOVA).

As the negative control, all P2X7 receptor antagonists that reduce the severity of spinal cord injury, whether it is improvement of locomotor function or a reduction in the traumatic lesion, will be tested in P2X7 receptor knockout mice. Only antagonists that fail to improve functional recovery and/or reduce tissue injury in P2X7 receptor KO mice act through antagonizing P2X7 receptors. As shown in Figure 4, deletion of P2X7 receptors provides a significant improvement of locomotor function starting 2.5 weeks after spinal cord injury. Figure 4 also

shows that recovery of wildtype littermates to P2X7 receptor KO mice recovered poorly compared to C57 controls shown in Figure 3. The P2X7 receptor mice are on FVB background. We are presently backcrossing this line to generate P2X7 receptor KO mice on C57 background.



The P2X7 receptor Ko mice and their littermate wiltypes were 6.5 weeks after spinal cord injury anesthetized with ketamine (80 mg/kg) and xy lazine (10 mg/kg) and perfused transcardially with 4% paraformaldehyde in PBS. The spinal cords were dissected, postfixed overnight at 4 °C, and then transferred to 30% sucrose for processing into 20 µm longitudinal cryosections. These sections were blocked for 30 min at room temperature in a solution containing 10% normal donkey serum and 0.5% Triton X-100, and incubated overnight at 4 °C with primary antibodies against GFAP (1:500, Sigma), Cx43 (1:500, Sigma), and CD68 (1:50, Serotec all antibodies need numbers so people can order them selves). All fluorescent- conjugated secondary antibodies (Jackson Immunoresearch) were used at 1: 250. After immunolabeling, the sections were counterstained with DAPI (1:5000, Invitrogen) for 10 min at room temperature and a coverslip mounted. Images were collected with a confocal microscope (FV500, Olympus) with FluoView (Olympus) software by using a 20x oil objective lens (NA 1.3). Nonbiased stereological approaches were used to evaluate the lesion volume and fluorescence intensity. Images were subsequently analyzed with custom-made Matlab software (Wang et al., 2004). Figure 5 shows that reactive gliosis detected as increased GFAP (astrocytes) and CD68 (microglial cells) were significantly reduced in P2X7 receptor KO mice compared to their wildtype littermates exposed to the same weightdrop injury 6.5 weeks earlier.



Aim 4

Another focus of effort in year 1 of this proposal has been Aim 4, the intent of which is to identify and systematize the transcriptional events accompanying injury in each P2X7R-expressing spinal cord cellular phenotype. When we investigated the patterns of GFP expression in *bac* transgenic P2X7R-GFP mice, we found that P2X7R expression was more widespread than expected among spinal cord glia, among which astrocytic and microglial GFP expression proved ubiquitous. As a result, sorting on the basis of GFP expression became unnecessary, as GFP expression was already high and essentially uniform among each target phenotype. Rather, we reasoned that sorting on the basis of markers for phenotype alone would be sufficient for the purposes of our study, and would allow us to better focus on the downstream transcriptional events associated with P2X7R receptor activation in each cellular phenotype, as a function of injury. On that basis, in the past year we concentrated on developing fluorescence activated cell sorting (FACS) protocols by which to isolate spinal cord microglia and astrocytes, and on optimizing our cellular yields to permit maximal RNA extraction from areas of injury. Such genomic analysis of isolated adult spinal cord phenotypes has never been accomplished, yet we have already made rapid progress in doing so. We initiated this effort by targeting microglia and astrocytes, as our transgenic analysis of the

P2X7R-GFP transgenics revealed essentially ubiquitous expression of P2X7R by both of these phenotypes.

Whereas we had been aware that P2X7R protein expression is high by spinal microglia, our previous observations had suggested relatively low expression by astroglia. We thus focused our initial efforts in transcriptonal analysis upon microglia. We have now sorted microglia from both normal and injured spinal cords, using a CD11b-directed strategy that we developed for this purpose, and which we were able to further optimize. We have profiled the expressed RNAs of these isolated microglia, and are in the process of analyzing the resultant genomic data sets.

We also attempted to sort astroglial from these cords, but achieved only low yields, as our previously published glut1-directed FACS strategy proved much less efficient in injured adult mice than in uninjured normals, and also proved inefficient for human cells. In pilot studies in which we attempted to sort astroglia from human spinal cord samples, we were disappointed in the net efficiency of glut1-targeted extraction, which we believe is dependent on the maturation stage of the targeted astroglia, and itself dynamically regulated by injury. These difficulties may have been a function of our available antisera, which are directed at a specific epitope of the glut1 ectodomain whose presentation may vary with both maturation and injury, as well as by species. To address this issue, we are designing new glut1 ectodomain targets that may yet prove useful; nonetheless, we are proceeding under the assumption that glut1-based selection will not be optimal for FACS isolation of astrocytes, since the validity of our genomic comparisons between normal and injured tissues ultimately requires the injury state-independence of the antigens used to isolate our cells for study.

Because of this unanticipated difficulty in astrocytic selection, we screened other potential astroglial markers, and identified CD44 as a far superior target for ectodomain-based isolation. CD44 is a membrane-bound heparin receptor uniformly expressed by astroglia, and it has been used as a target for FACS isolation of a variety of systemic phenotypes, especially in malignancies. In the brain and spinal cord, however, it is expressed only by astrocytes and glial progenitors, the latter of which are easily separated out by FACS using other markers (NG2, A2B5, CD140a); we have found CD44 to yield reliable and reproducible FACS isolations, and our preliminary studies thus far have revealed little acute injury-dependent variation in its level of expression. On that basis, we have begun to accumulate CD44-sorted isolates for genomics comparison from normal murine spinal cord, from injured cord, and from normal human spinal cord as well. Our assessment of the resultant profiles, and their clustering on P2X7R-regulated transcripts, will be a major focus of the next half-year, after which we would anticipate being able to assess the effects of P2X7R receptor antagonism on the expression of those same downstream P2X7R-regulated genes and pathways.

It is also worth noting that the high transcription of the P2X7R mRNA, as suggested by its high reporter expression in the *bac* transgenic, suggested to us that P2X7R protein expression and membrane insertion might be dynamically regulated in astrocytes. This presents the possibility of an active, environmentally-dependent regulation of P2X7R translational efficiency in astrocytes; such a mechanism could permit the highly dynamic modulation of P2X7R in response to injury-induced reactive activation. Such a mechanism would comprise a highly adaptive strategy for acute astrocytic reaction to injury, and hence an attractive potential target for intervention. Our experiments of the next year will thus also include analytic arms dedicated to determining whether P2X7R translation might be tonically suppressed via cognate miRNAs, and if so, whether P2X7R-induced inflammation and spinal neuronal death might be moderated through strategies intended to increase the degradation of P2X7R mRNA prior to its injury-associated translation.

Key Research Accomplishments

- 1) Established that MRC2159 and KN-62 do not reduce the severity of spinal cord injury in the highest doses tolerated by either rats or mice
- 2) Established that BBG consistently reduces the severity of spinal cord injury in rats
- 3) Shown for the first time that BBG also improve functional recovery of locomotor functions in mice
- 4) Shown that P2X7 receptor deletion (P2X7R KO mice) reduces reactive changes in astrocytes (GFAP) and microglial cells (CD68)
- 5) We established the injury-associated expression profiles of sorted adult spinal microglia, which we are now analyzing to define optimal microglial targets for intervention. This analysis has strongly validated the innate immune system as perhaps the critical initiator of spinal inflammatory injury
- 6) We developed and validated a new CD44-based spinal dissociation and FACS sort protocol by which to isolate spinal astrocytes, which we are now employing for a parallel analysis of the transcriptional responses of spinal astroglia to injury

Reportable outcomes

Two manuscripts are under review:

Critical role of connexin 43 in secondary expansion of traumatic spinal cord injury

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Abstract: Spinal cord injury (SCI) is often complicated by secondary ischemic injury as a result of the innate inflammatory response to traumatic injury and tissue swelling. Prior studies have shown that excessive ATP release from peri-traumatic regions contributes to the inflammatory response to SCI by activation of low affinity P2X7 receptors. Since connexin hemichannels constitute an important route for astrocytic ATP release, we here evaluated the impact of deletion of connexins (Cx30/Cx43) in astrocytes on post-traumatic ATP release. *In vivo* bioluminescence imaging showed a significant reduction in ATP release after weight-drop injury in mice with deletion of Cx43 compared with Cx43-expressing littermates. Moreover, astrogliosis and microglia activation were reduced in peri-traumatic areas of mice lacking Cx43. Motor recovery was also significantly improved and the traumatic lesion smaller in mice with deletion of Cx43. Combined, these observations demonstrate that astrocytic hemichannels contributes to post-traumatic ATP release, which in turn aggravates secondary injury and restrains functional recovery following experimental spinal cord injury. Cx43 hemichannels may thereby constitute a new therapeutic target in spinal cord injury.

and

Astrocytic Cx43 hemichannels and gap junctions play a crucial role in development of chronic pain following spinal cord injury

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Background: Chronic neuropathic pain is a frequent consequence of spinal cord injury (SCI), yet despite recent advances, up-stream releasing mechanisms and effective therapeutic options remain elusive. Previous studies have demonstrated that SCI results in excessive ATP release to the peritraumatic regions and that purinergic signaling, among glia cells, likely plays an essential role in facilitating inflammatory responses and nociceptive sensitization. We here sought to assess the role of Connexin 43 (Cx43) as a mediator of CNS inflammation and chronic pain.

Results: To determine the extent of Cx43 involvement in chronic pain, a weight-drop SCI was performed on transgenic mice with Cx43/Cx30 deletions. SCI induced robust and persistent neuropathic pain including thermal hyperalgesia and mechanical allodynia in wild-type control mice, which developed after 4 weeks and maintained after 8 weeks. Notably, SCI-induced thermal hyperalgesia and mechanical allodynia were prevented in transgenic mice with Cx43/Cx30 deletions. SCI-induced gliosis, detected as upregulation of glial-fibrillary-acidic-protein (GFAP) in the spinal cord astrocytes, was also reduced in the knockout mice, when compared to littermate controls. In comparison, a standard regimen of post-SCI treatment of minocycline attenuated neuropathic pain to a significantly lesser degree than Cx43 deletion.

Conclusions: Taken together these findings suggest that Cx43 is critically linked to the development of central neuropathic pain following acute SCI. Since Cx43/Cx30 is expressed by astrocytes, our data also support an important role of astrocytes in the development of chronic pain.

Conclusions

The analysis of functional recovery and lesion volume has required considerable more effort than anticipated. Several reasons for not accomplishing all goals exist. First, we were concerned that the P2X7 receptor antagonists included in the study would have effects other than just inhibiting P2X7 receptors. For example, BBG is a blue food color and may target multiple other signaling pathways. A logical approach to define the actions of the agents is to evaluate their effects in P2X7 receptor Ko mice. However, traditionally. However, the mice models of weight drop spinal injury are traditionally more variable than similar models in rats. We have over the past couple of years refined our model of weight drop injury in mice in work sponsored by NY State Spinal Cord Injury Board. The consistency of injury and functional recovery in the murine spinal cord injury model is now very similar or even better than the rat model (compare figures 1 and 3). After obtaining permission from DOD to conduct the remaining experiments in mice, we have switched to conduct experiments proposed in Aims 1 and 2 in mice rather than rats. This change in strategy necessitated a repeat of control vehicle injury as well as an evaluation neuroprotective action of the P2X7 receptor antagonist indeed is P2X7 receptors (Please see Fig. 1). We believe that this change in plan was advantageous

for future studies and that the change of species in itself added valuable information, since testing in multiple species is one of the requirements for an IND application. The major accomplishments of this first year of findings are still substantial as BBG has been shown to consistently and potently reduce the severity of spinal cord injury in both rats and mice. Moreover, experiments including a large number of animals (rats = 16, mice = 16) have shown that KN62 and MRS2159 are not neuroprotective in the setting of spinal cord injury. The most significant observation that has come out of the studies is that spinal cord injury is associated with significantly less injury and reactive changes in astrocytes and microglial cells in P2X7 receptor KO mice (Figure 5) and is linked to faster regaining of locomotor functions (Figure 4) than in littermate wildtype exposed to the same injury. This observation is fundamental and important, because it provides data supporting a role in P2X7 receptor activation in aggravating spinal cord injury that does not depend on pharmacology.

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Appendices

N/A