FINAL REPORT

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1. Initial aims: The initial goal of our grant was to define mechanisms of neurodegeneration in the injured spinal cord and the regulation of such mechanisms by microglial signals. During the course of our studies, we obtained exciting results that lead us to focus on some aspects that we had not originally anticipated. Our research has taken us into a novel, exciting and rewarding direction. In particular, we investigated the role of plasma membrane calcium ATPase isoform 2 (PMCA2), an important calcium pump, in neuronal dysfunction and degeneration in the spinal cord and the effects of microglial signals on PMCA2 expression.

2. Project successes: Earlier studies in our laboratory indicated that neuronal PMCA2 levels are decreased in the spinal cord during inflammation. We had also reported that the decrease in PMCA2 might be one of the mechanisms downstream to kainate receptor-mediated glutamate excitotoxicity as exposure of spinal cord slice cultures to low but persistent levels of kainate causes a significant decrease in PMCA2 mRNA levels. Since extensive inflammation and excitotoxicity are also implicated in spinal cord injury, we assessed whether PMCA2 expression is altered after spinal cord contusion injury and whether microglial signals alter PMCA2 levels in neurons.We found that

- the expression of PMCA2, an important ion pump localized exclusively to neurons and playing critical roles in Ca^{2+} homeostasis is decreased 48 hours after spinal cord contusion injury in the rat. This happens, not only in the epicenter where there is extensive neuronal loss but also in the penumbra, due to regulation of expression rather than cell loss. In contrast, transcript levels of the other PMCA isoforms (1, 3 and 4) are not altered indicating that changes in PMCA2 are selective and specific to this isoform. (Elkabes S and Nicot A, 2003, Figures 1 and 2).
- inhibition of PMCA activity delays depolarization induced calcium transients, promotes axonal pathology and leads to neuronal loss in spinal cord neuronal cultures (Kurnellas et al, 2005).
- Microglial signals suppress neuronal PMCA2 levels in microglia-neuron co-cultures (Figure 3) leading to neuronal pathology (Figure 4). These effects are neither mediated by TNF-α nor by "direct actions" of kainic acid on neurons (Figure 5).
- Lack of PMCA2 in PMCA2-null mice causes a significant decrease in the number of motor neurons in the spinal cord (Kurnellas et al, 2005). Accordingly, these mice exhibit hindlimb weakness and loss of grip strength. In agreement with these results, Motor Unit Number Estimates (MUNE) are significantly decreased in PMCA2-null mice as compared to wild type controls (Figure 6).

Thus, we have defined some of the mechanisms that may be involved in secondary neuronal damage during SCI and determined microglial effects on such mechanisms.

Significance of the findings

Increasing evidence indicates that spinal cord contusion injury occurs in two phases. The first phase consists of the initial mechanical trauma, which is the cause of the primary injury. The second phase comprises several pathophysiological mechanisms including inflammation, glial activation and excitotoxicity, which are initiated by the first impact and are the primary

causes of the secondary injury. These changes exacerbate the functional deficits because they enhance neuronal death, axonal damage and loss of white matter.

Glutamate induced excitotoxicity plays a major role in secondary SCI. It is well known that glutamate causes neuronal dysfunction by increasing Ca^{2+} influx leading to intracellular Ca^{2+} overload, a trigger that promotes injury cascades. Abnormal elevations in intracellular Ca^{2+} levels may also be the consequence of defects in extrusion mechanisms. Our findings support this alternative possibility. Thus, restoration of normal calcium extrusion patterns may attenuate secondary neuronal injury and prevent exacerbation of neurological decline during SCI.

The hypothesis that emerged from these investigations is that glutamate and potentially microglial signals suppress PMCA2 expression, which in turn, promotes neuronal calcium overload leading to axonal and neuronal damage.

3. Project challenges

- To determine how blockade of PMCA2 activity affects neuronal integrity, we used pharmacological inhibitors. However, these pharmacological agents are not specific for PMCA isoform 2. They also block the activity of other PMCA isoforms. Therefore, we initiated studies to prevent transcription of PMCA2 by use of small interfering RNA in order to assess the specific involvement of this isoform in neuronal injury. The optimization of this approach has been challenging as efficacy of spinal cord neuronal transfection is low. We also have to use various siRNA sequences in combination in order to block transcription. We are still continuing these studies.

- As mentioned above, we are determining how suppression of PMCA2 activity leads to neuronal pathology. To address this issue in vivo, we used PMCA2 mutant mice with reduced or null pump activity. We first attempted to obtain the *deafwaddler* mouse, a spontaneous PMCA2 mutant with reduced PMCA2 activity, from commercial sources. However, the company was able to provide only a few animals very infrequently due to problems in their mice colonies. As these investigations were neither time nor cost effective, we contacted Dr. Gary Shull who has established a colony of PMCA2-null mice in his laboratory for the first time. Dr. Shull provided us with heterozygous breeding pairs. Establishment of our own colony was initially a challenge because of low breeding capacity of the pairs. However, we overcame this limitation and established our own colony. Subsequent studies with these mice were rewarding and indicated that lack of PMCA2 affects motor neurons.

- To determine whether microglial signals modulate PMCA2 levels, we established microglia-neuron co-cultures. The major challenge has been to obtain enough microglia from spinal cords in order to perform the studies as the yield is less than that obtained from brain regions such as the cerebral cortex. The second difficulty was to find the exact conditions to culture simultaneously neurons and microglia. After screening various compositions of defined media, we were successful to obtain healthy co-cultures.

4. Implications for future research

Our findings have led us to a new and previously unexplored research direction. We have provided insights into the potential role of aberrant calcium extrusion mechanisms in secondary neuronal damage after SCI with particular emphasis on PMCA2. The contribution of PMCAs to secondary neuronal injury in the spinal cord has not been explored. We have also shown that the reduction in PMCA2 expression may be downstream to glutamate excitotoxicity, although this effect appears to be mediated via glial cells rather than direct actions on neurons when the glutamate concentrations used are not lethal. Importantly, our studies indicate that microglial signals suppress PMCA2 levels leading to neuronal pathology. Elucidation of some principal mechanisms underlying neuronal/axonal damage during secondary spinal cord injury may identify therapeutic targets to prevent exacerbation of neurological deficits.

Our findings have implications for other diseases. We have reported that a decrease in PMCA2 expression also occurs during experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS; Nicot et al, 2003). Recent evidence indicates that axonal damage is an important correlate of permanent disability in these diseases, which causes paralysis in affected subjects.

Taken together, our investigations on SCI and EAE suggest that axonal damage in secondary SCI injury and during MS might develop via common molecular mechanisms. Although the events initiating SCI and MS are quite different, extensive inflammation of the spinal cord, microglia-mediated and excitotoxicity-induced neuronal damage appear to be shared features of these two pathological conditions. Thus, therapeutic strategies that attenuate secondary axonal injury during SCI may also be beneficial to prevent disease progression during MS.

5. Plan to continue this research

Our findings, which have relevance both to SCI and MS, have led to a NIH R01 grant. We are now especially focusing on regulation of neuronal damage by microglial signals with particular emphasis on calcium extrusion and influx mechanisms.

6. List of published manuscripts and abstracts

Kurnellas MP, Nicot A, Shull GE, **Elkabes S** (2004) Plasma membrane calcium ATPase deficiency causes neuronal pathology in the spinal cord: a potential mechanism for neurodegeneration in multiple sclerosis and spinal cord injury FASEB J (Epub Dec. 2, 2004), 19:298-300.

Elkabes S and Nicot A. (2003) Aberrant calcium extrusion mechanisms may contribute to secondary spinal cord injury. J. Neurol. 250 (2), 179.

Elkabes S, Nicot A, Kurnellas M (2003) Molecular mechanisms underlying neuronal/axonal pathology in spinal cord trauma and inflammation: the role of microglia. Second Symposium on NJCSCR, NJ, USA.

Elkabes S, Nicot A, Shull GE, Kurnellas MP (2004) Molecular mechanisms of neuronal pathology in the spinal cord: implications for multiple sclerosis and spinal cord injury. Abstracts Soc. Neurosci., 34th Annual Meeting.

Manuscript in preparation

Arguelles-Grande C, Donahue KC, Ehrlich D, **Elkabes S** (2006) Microglial signals modulate plasma membrane calcium ATPase 2 levels in the spinal cord leading to neuronal pathology.

7. SUMMARY OF MAJOR FINDINGS

Some of our findings have been published and can be found in attached manuscripts and abstracts. Some of the results presented below have not been published yet and will be presented in a manuscript which is in preparation.

PMCA2 expression is decreased in SCI

To analyze the expression of PMCA2 after spinal cord injury, we assessed transcript levels in spinal cord segments within 11 mm of the epicenter by real time PCR, 24 and 48 hours after contusion injury. We did not find any significant changes in PMCA2 transcript levels at 24 hours. In contrast, we observed about 80% decrease in PMCA2 mRNA at 48 hours post injury (Fig.1).

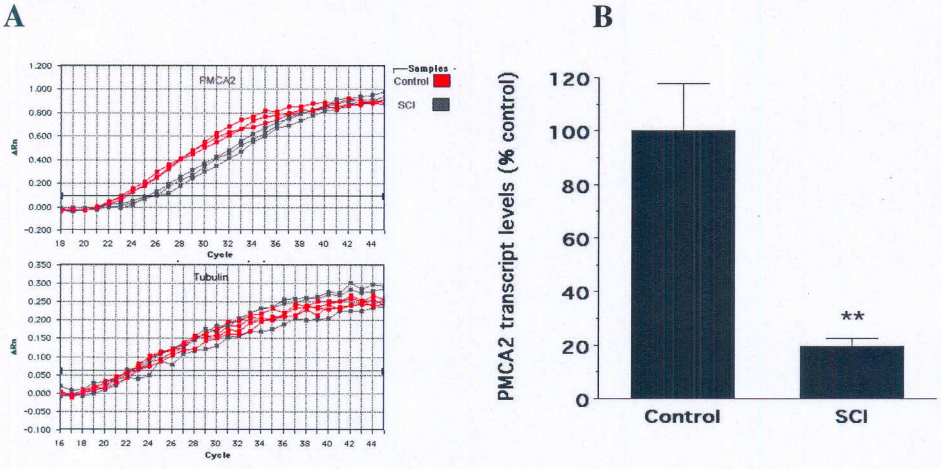

Figure 1. Expression of PMCA2 assessed by real time PCR. A) A representative experiment (n=3-4) B) Graphic representation of the data shown in A after normalization to α-tubulin, a housekeeping gene whose expression was not modified 48 hours after SCI. An 80% decrease in PMCA2 transcript levels occurred; ** $p<0.02$ by t-test.

Subsequently, we assessed whether the changes in PMCA2 are selective and specific to this isoform by quantifying transcript levels of all other members of the PMCA gene family. For these investigations, we employed the same RNA source as that used in the studies shown in Fig. 1. In contrast to PMCA2, the expression of PMCA1, 3 and 4 was not altered (Fig. 2). It is important to emphasize that like PMCA2, PMCA3 is also primarily expressed in neurons. These data, taken together suggest that the decrease in PMCA2 is not due to neuronal death but to regulation of PMCA2 gene expression.

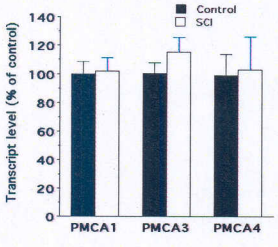

Figure 2. Expression of other PMCA isoforms in the spinal cord 48 hours after injury. A representative experiment (n=3) showing no significant changes in the expression of other PMCA isoforms. The source of RNA was the same as that used for the studies shown in figure 1. Values are normalized to α-tubulin, a housekeeping gene whose expression is not modified after injury.

Microglial signals decrease neuronal PMCA2 levels, in vitro

Activated microglia are abundant in the injury site and are thought to have both deleterious and beneficial effects. Therefore, we assessed whether microglial conditioned medium can suppress PMCA2 levels in microglia-neuron co-cultures. We cultured spinal cord neurons derived from 15 day old rat embryos in 6 well plates for one week, until differentiation and maturation. These cultures were 98% pure. We then exposed the neurons to microglia, which were obtained from mixed glial cultures derived from 3 day old rat pups. The microglia were plated at high density (7.5 X 10^4 cells/cm^2) in inserts, which were then placed on culture dishes containing neurons. This setting allows neurons to be exposed to soluble microglial substances without cell-cell contact. We chose to plate microglia at high density because, when isolated from their natural milieu and grown at high density, microglia are activated even in the absence of any exogenous stimulant. We found that microglial conditioned medium induces a significant decrease in the levels of PMCA2 which was assessed by western analysis. This was not due to neuronal death as quantification of Neu N positive neurons and MTT positive cells did not indicate any cell loss. We conclude that microglia secrete factors which can suppress the expression of PMCA2 in neurons.

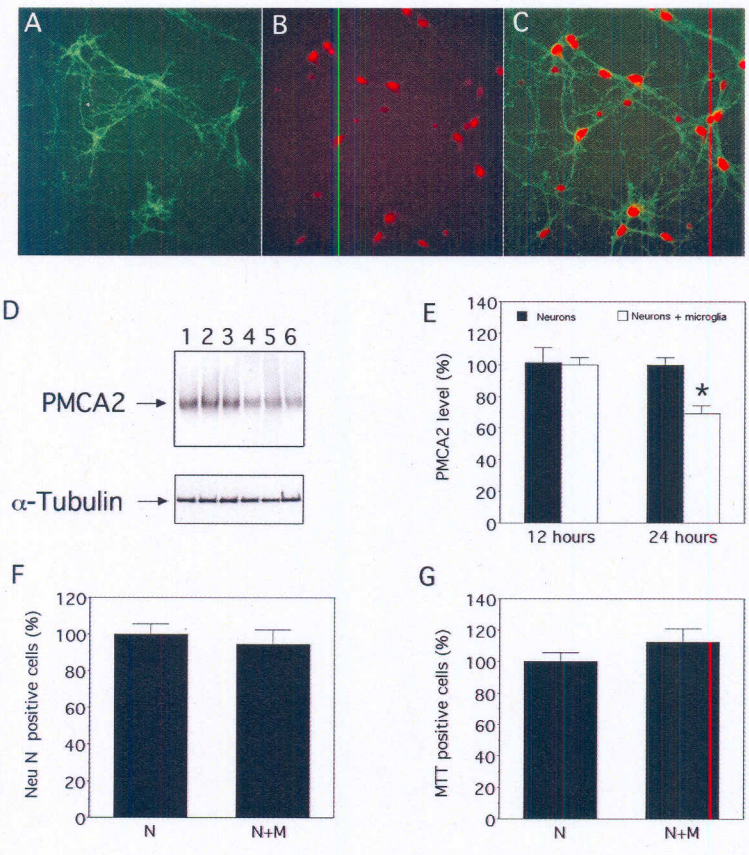

Figure 3. Modulation of neuronal PMCA2 levels by microglial signals. **A)** A neuronal culture labeled with PMCA2 antibody (FITC) showing immuno-reactivity around cell bodies and in processes. **B)** The same culture labeled with Neu N (Alexa Fluor), a marker of mature neurons. **C)** Merged picture indicating that PMCA2 is expressed in the majority of Neu N positive cells. **D)** Western blot of individual cultures treated with medium (1-3) or microglial conditioned medium (4-6) for 24 hours and probed with antibodies against PMCA2 (upper panel) or α-tubulin (lower panel), a control to correct for experimental variations. **E)** Quantification of PMCA2 levels in cultures treated with medium or microglial condition medium for 12 and 24 hours. The combined results obtained from three different experiments (n=8) is shown. * Significantly different from neurons kept in medium $p<0.0005$ by Student's t-test. Quantification of Neu N **(F)** and MTT **(G)** positive cells in neuronal cultures exposed only to medium (N) or microglial conditioned medium (N+M); (n=6).

To determine whether exposure of neurons to microglial cultures eventually leads to neuronal damage, we quantified the number of non phosphorylated neurofilament H (NF-H) positive cells in neuronal cultures exposed to medium alone or to microglial conditioned medium for 24 and 32 hours. We then labeled the cells with the SMI-32 antibody which recognizes a non-phosphorylated epitope of NF-H. The SMI-32 antibody has been used as a marker of neuronal injury in SC inflammation and other pathological conditions. Moreover, we have previously shown that inhibition of PMCA activity by pharmacological agents increases the number of SMI-32 positive spinal cord neurons, in vitro, which is then followed by neuronal death. Exposure of neurons to microglial conditioned medium for 24 and 32 hours significantly increased the number of SMI 32 positive neurons. These results together with our previous finding showing an increase of SMI32 positive cells in cultures treated with PMCA inhibitor which was then followed by neuronal loss, indicate that microglial signals may injure neurons by altering levels of PMCA2.

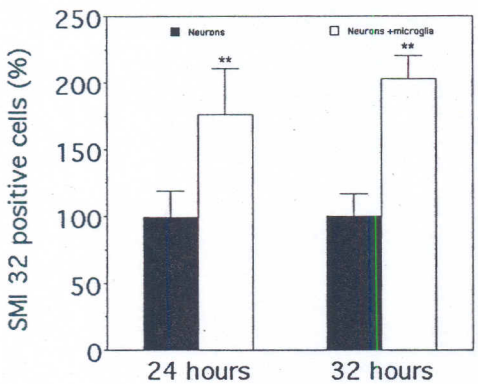

Figure 4. Microglia increase the number of SMI-32 immunoreactive cells in spinal cord neuronal cultures. Exposure of neurons to microglial cultures for 24 and 32 hours significantly increased the number of SMI-32 positive cells in the culture, suggesting neuronal pathology. ** Significantly different from controls (neurons maintained in medium alone), $p < 0.03$ by ANOVA, Scheffe's post-hoc test.

Microglial effects on PMCA2 expression are not mediated by glutamate or TNF-α

To identify the potential microglial agents, which suppress PMCA2 levels, we exposed spinal cord neuronal cultures to kainic acid and TNF-α. We used kainic acid because activated microglia are abundant source of glutamate and spinal cord neurons and especially motor neurons have AMPA/kainate receptors which mediate excitotoxicity. Moreover, our previous studies had indicated that exposure of spinal cord slices to low (4 μM) , non-lethal doses of kainate for 6 hours significantly decrease PMCA2 mRNA levels (Nicot et al, 2003). However, it was not clear whether this effect is direct on neurons or mediated by glia. To address this question, we exposed pure neuronal cultures to kainate (4 μM) for 6, 12 and 24 hours and quantified PMCA2 protein levels by western analysis. We did not find any significant effects (Figure 5). These results indicate that the reduction in PMCA2 levels in response to kainate in spinal cord slices is probably mediated via glia. Thus, it is possible that kainic acid act on glial cells which in turn release substances that act on neurons to modulate PMCA2 expression.

TNF-α receptors have been described in spinal cord neurons (Yan et al, 2003). Interestingly, low levels (25 ng/ml) of TNF-α induced a small but significant increase in PMCA2 whereas higher levels had no effect (Figure 5). Further investigations are necessary in order to identify the microglial signals which suppress PMCA2 expression.

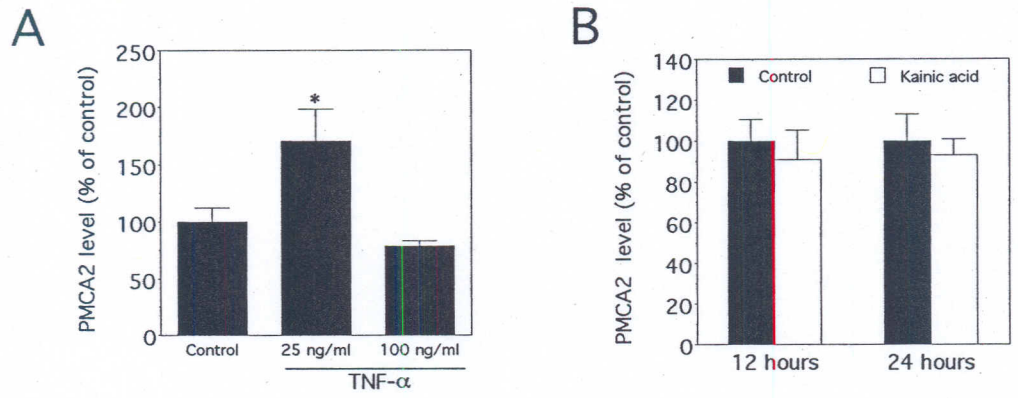

Figure 5. Effects of TNF-α and kainic acid on PMCA2 levels in spinal cord neuronal cultures. Low concentrations of TNF-a significantly increased PMCA2 levels whereas higher concentrations did not have any effect (**A**) * Significantly different from controls by ANOVA, Scheffe's post hoc test $p<0.03$; n=5-6. Four μM Kainic acid did not modulate PMCA2 levels in neurons (**B**).

Decreased motor unit number estimates (MUNE) in PMCA2-null mice

To determine how lack of PMCA2 affects innervation of muscles by spinal cord motor neurons, we assessed MUNE in PMCA2-null mice as compared to wild type controls. We found a significant decrease in MUNE in the knockout mice as compared to the wild type and heterozygous mice (Figure 6). These results further support our studies showing a loss of motor neurons in the spinal cord of PMCA2-null mice.

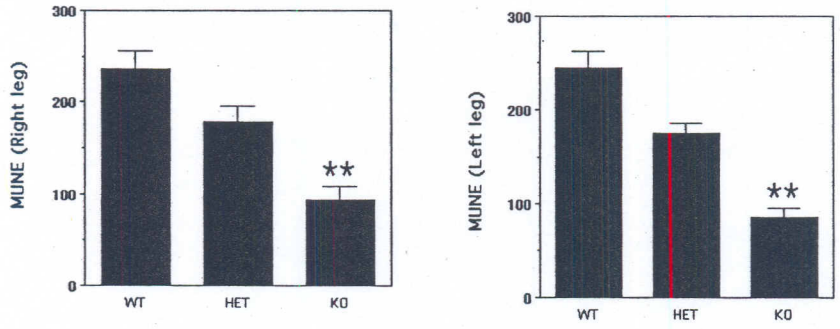

Figure 6. Motor number unit estimates suggest decreased innervation of muscles in the leg of the PMCA2 knockout mouse (KO) as compared to the heterozygous and wild type littermates. ** $p< 0.05$ by ANOVA, Scheffe's posthoc test (n=8).

Literature cited

Kurnellas MP, Nicot A, Shull GE, **Elkabes S** (2004) Plasma membrane calcium ATPase deficiency causes neuronal pathology in the spinal cord: a potential mechanism for neurodegeneration in multiple sclerosis and spinal cord injury FASEB J (Epub Dec. 2, 2004), 19:298-300.

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Yan P, Liu N, Kim GM, Xu J, Xu J, Li Q, Hsu CY, Xu XM. Expression of the type 1 and type 2 receptors for tumor necrosis factor after traumatic spinal cord injury in adult rats. Exp Neurol. 2003 Oct;183(2):286-97.