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INTRODUCTION

Severe disruptions in ionic dynamics of the depolarizing neuron have long been recognized as possible mediators of excitotoxicity. In neurodegeneration leading to neuronal cell death, injury-induced imbalances in intracellular calcium ($[Ca^{++}]i$) have received considerable attention, leading many, including our own laboratory, to propose the "calcium mechanism" of neuronal injury and neurodegeneration. However, of possibly equal consequence to the responsiveness of an injured neuron is the state of the Na^{+}-Ca^{++} exchanger, and the influence of altered sodium dynamics to promote calcium overload, presynaptic membrane depolarization, and excitation. The pathophysiological importance of neuronal sodium channels to membrane stabilization has been recognized and studied in epilepsy, and more recently in peripheral neuropathy and other neurodegenerative conditions. The results of these studies have led to the cloning, sequencing and physiological characterization of at least four neuronal sodium channels. However, despite these discoveries and the exciting prospect of protecting neurons against excitotoxic insults with the development of novel sodium channel blocking drugs, the role of sodium channels in cell death mechanisms and neuroprotection has received relatively limited attention. It is becoming increasingly apparent that blockade of neuronal sodium channels may prevent, or at least attenuate, neurodegeneration and offer an exciting therapeutic approach for the treatment CNS injury. Therefore, the primary objective of this research proposal is to characterize the molecular expression and determine the functional significance of the respective neuronal sodium channel genes relative to the development and recovery mechanisms of the injury process. A second objective is to study the effects of sodium channel blockade on the molecular and cellular consequences of neuronal injury, and its influence to improve recovery and repair mechanisms. These molecular, cellular and pharmacological studies directly address the involvement of sodium channel mechanisms in neuronal injury and will provide significant insights to the role of brain sodium channels in injury-induced neurodegeneration, and the potential therapeutic consequences of sodium channel blockade (and/or decreased expression) on the neurodegeneration process.

BODY

I. Original "STATEMENT OF WORK"

Year 1:

Since we have successfully characterized and standardized the RT/PCR methods and determined that at least four of the neuronal sodium channels are expressed in normal rat brain, the focus of the first year will be to initiate experiments aimed at establishing the functional significance of each of the respective sodium channel genes to anoxia/ischemia-mediated neurodegeneration. This will be accomplished using antisense oligonucleotides (ASOs) and quantitative measures of neurodegeneration in both the *in vitro* neuronal culture model of hypoxia/hypoglycemia (H/H) injury (completed first) and the *in vivo* 72 hr recovery middle cerebral artery occlusion (MCAo) model. Also, *in vitro* pharmacological experiments (some of which are already underway) will be completed evaluating the neuroprotective properties of the sodium channel blocker mexiletine in three neuronal culture models of neurodegeneration (H/H, veratridine and glutamate). In parallel experiments, an *in vivo* dose-response experiment will be initiated in the rat MCAo 24 hr recovery model to determine the neuroprotective potency and efficacy of systemic mexiletine.

Year 2:

The functional studies using ASOs will be completed. Anticipating that these experiments will reveal which (if not all) of the sodium channels is functionally involved with developing neurodegenerative processes, we will begin comprehensive *in vitro* (H/H) and *in vivo* (72 hr MCAo) RT/PCR time-course experiments and examine the influence of the injury state on the expression of the sodium channel genes. We will also initiate *in situ* RT/PCR experiments to characterize the regional changes in the localization of the expressed sodium channels in normal and injured rat brain tissue.

Year 3:

The RT/PCR time-course and the *in situ* RT/PCR will be completed. Also, the experiments examining the effect of sodium channel blockade with mexiletine on injury related changes in sodium channel gene expression in the 72 hr MCAo model will be completed.

Justification for additional year (4th) funding:

As a result of studies conducted during the 2nd year of our 3 year research project an exciting and potentially critical discovery was made. Briefly, after first identifying and characterizing the change in sodium channel gene expression in normal and injured brain tissue using quantitative real-time RT/PCR, our SOW called for us to initiate *in situ* RT/PCR experiments with the purpose to anatomically localize the regional changes in the expression of the respective sodium ion channels during the injury process and recovery states. Our initial *in situ* experiments were designed to focus first on the rat brain sodium channel gene showing the most significant change during injury using the quantitative RT/PCR analysis, the rBI gene, which also happens to be one of the most prominent neuronal sodium channel genes in the rat brain (along with the rBII and rBIII genes). Our initial results have revealed the following: Not only is the rBI sodium channel gene down-regulated in the brain areas within the core injury site and in the proximal sites representing the penumbral zones of injury (as expected based upon our earlier RT/PCR expression studies in whole brain tissue), but we have discovered a dramatic down-regulation following injury (approaching 20-30% of normal) of the rBI channel in regions extending beyond the penumbral zone, including approximately 10% of the *contralateral hemisphere.* This was completely unexpected since A) there was no evidence from extremely sensitive TaqMan RT/PCR studies to indicate any dysfunction in gene expression from contralateral brain tissue and B) we are unaware of any observations to this effect reported in the literature with this or similar focal type brain injury models. Although the quantitative RT/PCR technique is highly sensitive and can measure as low as 10 copies of a given mRNA species in a sample, we may have failed to observes down-regulation of rBI mRNA in the contralateral hemisphere because tissue from the entire hemisphere was used. Keeping in mind the highly focal anatomical nature

of our injury model (MCAo), the contralateral hemisphere is uninjured and an underlying, developing injury process has never been described on the light microscopic (i.e. pathological) or neurologic (i.e. behavioral) level. This is extremely exciting and novel finding suggests that what has to this point been considered as uninjured, normal brain tissue instead exhibits significant changes at the molecular signaling level. The results of these findings were summarized in our annual report.

The ramifications of this discovery to our understanding of the injury process and developing neurodegeneration, especially as applied to neuroprotection mechanisms of delayed cell death and treatment strategies, are complex and far reaching. We have now confirmed this discovery in several animals for the rBI ion channel. As a totally unexpected finding, we are not funded under the current proposal to extend our research to more fully investigate this discovery, but it clearly represents a critical and exciting extension of our current studies that must be explored. It is essential that we not only evaluate the status of the rBI, rBII and rBIII sodium channel genes, but also the critical sodium-calcium exchanger proteins. Furthermore, it is important to determine whether this potentially damaging process is reversible, and more importantly whether it translates on a functional level and, therefore, represents a critical target for neuroprotection intervention.

Therefore, we are asking for supplemental extension of our existing contract to amend the time and budget in order to pursue this line of research. Specifically, we are proposing a two tier SOW where, during year 1 of the extension (STEP A) we will continue to us our *in situ* hybridzation technology to fully define and characterize the extent of the sodium

channel gene dysfunction to include the rBII, rBIII, and the sodium-calcium exhange genes NCX1, NCX2 and NCX3. If additional funding is available, during year 2 of the supplement (STEP B) this research will be extended electrophysiology patch-clamp studies from brain slices to determine the functional significance of this discovery to the recovery process and possible neuroprotection interventions.

Proposed Statement of Work for year 4:

Year 4: (Step A). Complete the comprehensive *in situ* hybridization analysis of the rBI, rBII, rB6 and the sodium-calcium exchanger (NCX1, NCX2, NCX3) gene expression profiles at early (3-24 hours) and late (72 h and 1 week) stages of the post injury process.

Year 4: (Step B). **THIS STEP WAS NOT FUNDED** and therefore the patch clamp studies were not performed. We propose to extend our results from *in situ* hybridization by directly assessing the functional significance of the altered gene expression. To do so, we will prepare slices from brain regions defined in injured animals as having altered levels of sodium channel mRNA (e.g. contralateral hemisphere and/or ipsilateral penumbral zone). Patch clamp recordings will be performed to assess changes in neuronal sodium currents and basic properties of electroresponsivity of these tissues compared to controls. Changes in electrophysiological properties could either increase or relieve a neuron's metabolic demands, and hence the degree of injury to which it is subject. After characterizing intrinsic changes, we will assess the efficacies/mechanisms of therapeutic agents (e.g. sodium channel blockers) and utilize this information to develop improved therapeutic strategies. Critically, we now have a fully functional electrophysiology lab set up in our laboratory, and the necessary research expertise in the person of CPT/Dr. Jed Hartings, to perform these experiments. The purchase of new equipment or addition of experienced personnel **will not** be required.

II. General Summary

Year 4 of our research project was extremely successful and we believe that we continue to meet, and in some instances exceed, our objective for the proposed research plan. There were no major administrative problems during Funding Year 04. Unexpected research problems encountered during the course of the year were dealt with aggressively and with complete success and are defined below in detail.

III. Administrative Problems/Accomplishments for Year'04

No significant administrative problems were encountered. All procurements proceeded on schedule and without delay.

IV. Research Problems/Accomplishments for Year'04

1. Description of Problems encountered:

During the fourth year funding period of the grant following technical problems were encountered:

1. As was the case described last year for the ASO experiments, we continued to experience cytotoxicity *in vitro* even with the newly synthesized ASOs. While the cytotoxicity is relatively mild, it greatly increases the variability of the experiment. This problem is unique only to the $Na_v1.1$ ASO (which is the most important channel to study) and only to the neuronal culture model. $Na_v1.1$ neuropathology is not evident in normal or injured rat brains following i.c.v. injections of the $Na_v1.1$ ASO; however, at two concentrations studied, it did not provide significant neuroprotection.
2. Due to budgetary restrictions we were unable to undertake electrophysiological studies proposed as part B of the SOW.

2. Description of Year'04 Accomplishments:

Review of Year'01 (Bullets):

1. Standardized the **non-quantitative** RT/PCR conditions and demonstrated expression of each of the four neuronal sodium channel genes in normal and injured rat brain.
2. Completed characterization of the conditions for real-time RT/PCR using TaqMan methodology and began our quantitative studies of differential expression of the four sodium channel genes in normal and injured rat brain tissue.
3. Initiated the *in situ* RT/PCR methods.
4. Initiated the *in vitro* antisense oligonucleotides (ASOs) studies.
5. Completed the i*n vitro* pharmacological experiments evaluating the neuroprotective properties of the sodium channel blockers mexiletine, QX-314, and the novel blocker RS100642 in three neuronal culture models of neurodegeneration (H/H, veratridine and glutamate). Manuscript was prepared for publication.
6. In parallel experiments, *in vivo* dose-response experiments were completed in the rat MCAo 24 hr recovery model demonstrating the excellent neuroprotection potency and efficacy of systemic post-treatment with RS100642, and of limited efficacy with mexiletine.

Review of Year'02 (Bullets):

1. Completed standardization the TaqMan quantitative RT/PCR (QRT/PCR) conditions during the 1st quarter of FY'02 and subsequently initiated and completed the quantitative characterization of the time-course of expression of each of the four neuronal sodium channel genes in normal and injured rat brain (out to 72 hours post-injury). (Manuscript submitted for Publication)
2. The *in vitro* functional studies using ASOs were begun. Preliminary results indicate that the blocking function expression of either the rBIII, PN1 or PN3 genes using ASOs

does not exhibit significant neuroprotection. However, consistent with our in vivo expression results, preliminary results suggest that rBI ASO is neuroprotective. Critically, we have determined that the rBI ASO is also producing increased protease activity and related cytotoxicity in normal cultured neurons, possibly interfering with the neuroprotection. This problem is currently being investigated (see above) and will need to be resolved prior to additional studies being initiated.

3. Initiated comprehensive *in vitro* RT/PCR time-course experiments on the expression of the sodium channel genes.
4. Initiated *in situ* RT/PCR experiments to characterize the regional changes in the localization of the expressed sodium channels in normal and injured rat brain tissue. Although several technical difficulties were encountered (see above) *successful in situ* hybridization studies have now been completed for the rBI sodium channel in the 24 hr injury model (Neuroscience Abstract submitted). Experiments are in progress to study the expression of other sodium channel genes (i.e. rBIII, PN1 and PN3).
5. As a logical extension of our *in vivo* gene expression studies, we have designed and synthesized the primers and probes for other sodium channels of possible functional importance, namely the rBII (rat brain II) sodium channel gene, and the sodium-calcium exchanger genes NCX1, NCX2 and NCX3. Preliminary studies using TaqMan QRT-PCR assay have been initiated.
6. We have extended *in vivo* MCAo our neuroprotection studies with RS100642 to now include 72 h recovery. In addition, **although not a part of our original research plan** using both pre-treatment and post-treatment protocols we have initiated experiments aimed at addressing the possible benefit of sodium channel blockade to treat the development of post-injury brain seizures and we have discovered potent anti-seizure actions of RS100642.
7. As part of the neuroprotection drug development studies described above in #6, we have also completed EEG neurotoxicity studies of RS100642 in normal, uninjured rats and have determined its safety at doses as high as 600 mg/kg. Again, this was not a part of our original research plan but it was necessary to include these studies as a results of 1) our discovery of brain neurotoxic EEG properties for mexiletine at doses very close to its neuroprotective dose and 2) our recent discovery that non-convulsant brain seizures, similar to what has been reported in human clinical studies, may be a serious consequence of experimental brain injury as well.

Review of Year'03 (Bullets):

1. The time-course characterization of the expression of sodium channel genes $Na_v1.1$, $Na_v1.3$, $Na_v1.7$ and $Na_v1.8$ (rBI, rBIII, PN1 and PN3) in the rat MCAo model by QRT-PCR has been completed and a **manuscript is published**.
2. Additional studies on the effects of ischemic insult on $Na_v1.2$ gene expression were undertaken and completed showing down-regulation of the same at 24 hr post-injury.
3. The *in vitro* functional studies using second set of NaCh ($Na_v1.1$, $Na_v1.2$ or $Na_v1.3$) ASOs were initiated. Preliminary results indicate that the blocking expression of either the $Na_v1.2$ or $Na_v1.3$ genes using ASOs does not exhibit significant neuroprotection. However, consistent with our *in vivo* expression results, preliminary results suggest that $Na_v1.1$ ASO is neuroprotective to a limited extent.

4. The *in situ* hybridization experiment to define the regional anatomical distribution of $Na_v1.1$ sodium channel gene expression in normal and at various time-post MCAo has been completed. Similar to QRT-PCR data, $Na_v1.1$ expression was down-regulated in subcortical and cortical regions of injured and, to some degree of contralateral hemispheres. Furthermore, in additional *in situ* hybridization studies expression of other NaCh gene was found to be unaffected following MCAo injury. **One abstract presented and one manuscript submitted and accepted with revision.**
5. The neuroprotective effects of sodium channel gene blocker, RS-1000642, on injury related changes in sodium channel gene expression by quantitative RT-PCR are currently underway. Preliminary results show that RS-1000642 treatment partially reversed the down-regulation of $Na_v1.1$ caused by MCAo injury. **One abstract submitted**
6. NaCh gene expression efficiency in normal fetal rat brain neurons in primary culture has been completed. The mRNA levels of $Na_v1.1$, $Na_v1.2$, $Na_v1.3$, $Na_v1.7$, $Na_v1.8$, and the house-keeping gene β-actin detected by quantitative RT-PCR demonstrate that $Na_v1.2$ is the most abundant of the five sodium channel genes expressed in normal fetal rat forebrain neurons (followed by $Na_v1.3$, $Na_v1.8$, $Na_v1.1$ and $Na_v1.7$). **One abstract submitted**
7. NaCh gene expression in primary forebrain neurons following veratridine toxicity has been completed. In this study, veratridine treatment of neurons produced no significant effects on the expression of all sodium channel genes studied, except $Na_v1.1$, which was significantly down-regulated. Treatment of neurons with neuroprotection doses of RS-100642 completely reversed this down-regulation by veratridine. **One abstract submitted and 1 manuscript submitted for publication.**
8. The expression of NCX1, NCX2 and NCX3 genes exhibited no significant changes in the same at any time points post-MCAo injury.
9. Using high–resolution (10 electrode placements) and computerized quantitative EEG analysis (qEEG), we have identified several non-convulsant (NCS) brain seizure events that are identical to the clinical brain injury state, namely spike-wave status activity, PLEDs, and IRDA, all in non-convulsant animals. The permanent MCAo injury best represents the clinical pathology. Unlike RS-100642, none of the 5 prototype AE drugs tested to date are significantly effective in stopping or attenuating the NCS. **One abstract presented, 1 abstract submitted, and 1 manuscript; a second manuscript has been accepted with revision.**

Review of Year'04 (Bullets):

1. The comprehensive *in situ* hybridization analysis of the rBI, rBII, rBIII is completed and similar to QRT/PCR studies only rBI and rBII exhibited down-regulation following MCAo injury.
2. The sodium-calcium exchanger (NCX1, NCX2, NCX3) gene expression profile at early (3-24 hours) and late (72 h and 1 week) stages of the post injury process is completed.
3. NaCh gene expression in primary forebrain neurons following veratridine toxicity has been completed. In this study, veratridine treatment of neurons produced no significant effects on the expression of all sodium channel genes studied, except $Na_v1.1$, which was significantly down-regulated. Treatment of neurons with

neuroprotection doses of RS-100642 completely reversed this down-regulation by veratridine. **1 manuscript has been published.**

4. The neuroprotective effects of sodium channel blocker, RS-1000642, on injury related changes in sodium channel gene expression by quantitative RT-PCR are completed. Our results show that RS-1000642 treatment partially reversed the down-regulation of $Na_v1.1$ caused by MCAo injury. **One abstract submitted and a manuscript has been accepted for publication in Neurotoxicity Research.**
5. In addition, **although not a part of our original research plan** we have undertaken immunocytochemical and Western blot analysis of rBI and rBII proteins. Preliminary results are comparable to those obtained using QRT/PCR method. **One abstract has been submitted.**
6. Also **not a part of our original research plan** we completed a comprehensive, time-course analysis of acute (hours) and delayed (days) neurobiology genomic profile following ischemic brain injury using Affymetric microarray technology. The results of these experiments, in addition to characterizing the genomic response to 12 functional neurobiology gene families out to 7 days post-injury, confirmed the selective down regulation in the rB1 and rBII sodium channel mRNA.

3. Details of accomplishments for Year'04:

1. The comprehensive *in situ* hybridization analysis of the rBI, rBII, rBIII is completed. RBI, rBII and rBIII mRNA were detected by *in situ* hybridization in the brains of sham-operated animals. In this study, the sodium channel oligonucleotide probes (synthesized by Genset Oligos Inc, La Jolla, CA) were complementary to rBI mRNA nucleotide 1113-1146, rBII 1472-1498, rBIII 2118-2143 and β-actin 37-64. A Blast search of the EMBL/Genbank databases indicated that there was no close homology between the probes directed against the sodium channel genes respectively and β-actin gene, or indeed any other sequences registered within these databases. The probes were 5' end-labeled with biotin. The biotinylated poly T Probe (Research Genetics, Huntsville, AL) was used as a positive control, and negative controls consisted of using a sense probe. In general, mRNA expression levels were low for rBI and rBII, with staining observed in neurons, axonal pathways and neuropil. The *in situ* hybridization signal for both rBI and rBII mRNA in sham animals was highest in layers 4 and 5 of isocortex. The hybridization signal in the piriform cortex of control animals was low compared with other cortical regions. Two hours post-MCAo injury little or no reduction was observed in the signal for rBI, rBII and rBIII mRNA. Adjacent sections stained with cresyl violet acetate did not reveal significant neuropathological changes at the two-hour time point. At six hours post-MCAo injury a small but notable down-regulation of rBI mRNA was detected in the ipsilateral infarct-damaged brain tissue. Injured areas that showed reduced rBI mRNA signals included the entire dorsal striatum, and primary motor, somatosensory and gustatory cortices. The peri-infarct region exhibited far less severe damage, and substantially less neuronal loss and was seen to extended into secondary motor cortex dorsally, and insular and piriform cortices ventrally. Major axonal pathways that exhibited neuropathological changes included the internal capsule, corpus callosum

and external capsule. At 24h and 48h post-MCAo injury, a significant reduction in signal was observed for rBI and rBII (no reduction in rBIII was noticed) throughout the same infarct-damaged areas. However, the down-regulation in rBII is not as dramatic as that demonstrated for the rBI gene (over 70% down-regulation for rBI v/s 35% for rBII gene expression). Severe neuropathological changes, including dramatic neuronal and axonal loss and leukocytic infiltration, were apparent in the cortex, striatum and associated fiber pathways. The loss in rBI and rBII mRNA signal correlated well with the loss of neurons and axons observed in cresyl violet stained sections. Most of the signal loss occurred in the ipsilateral hemisphere within the infarct-damaged areas. However, a significant loss of signal was also observed at 24h and 48h in the ipsilateral peri-infarct regions in secondary motor cortex, and in insular and piriform cortices.

2. The sodium-calcium exchanger (NCX1, NCX2, NCX3) gene expression profile at early (3-24 hours) and late (72 h and 1 week) stages of the post injury process is completed. We were unable to observe any significant changes in the expression profile of NCX1, NCX2 or NCX3 gene at 3, 15, 24 hr or 3 or 7 days post-MCAo.
3. NaCh gene expression in primary forebrain neurons following veratridine toxicity has been completed. Using quantitative RT-PCR we demonstrated the expression ratio of NaCh genes in normal fetal rat forebrain neurons to be $Na_v1.2 > Na_v1.3 > Na_v1.8 > Na_v1.1 > Na_v1.7$ (rBII > rBIII > PN3 > rBI > PN1). Veratridine treatment of neuronal cells produced neurotoxicity in a dose-dependent manner. The neuronal injury caused by veratridine (2.5 μM) resulted in a significant and exclusive down-regulation of the $Na_v1.1$ gene. Furthermore, treatment of veratridine-exposed neurons with neuroprotective concentration of RS100642 (200 μM) significantly reversed this selective down-regulation of $Na_v1.1$ gene expression. These findings document for the first time relative and quantitative changes in the expression profile of various NaCh genes in primary neuronal cultures following injury produced by activation of voltage-gated sodium channels, and suggest that the $Na_v1.1$ sodium channel gene may play a key role in neuronal injury/recovery. These results are published in Neurotoxicity Research, **5**:213-220, 2003.
4. The *in vivo* neuroprotective effects of sodium channel blocker, RS-1000642, on brain injury (MCAo) related changes in sodium channel gene expression by quantitative RT-PCR are completed. RS100642 (1.0 mg/kg), mexiletine (10 mg/kg) or vehicle was injected i.v. at 30 min, 2h, 4h and 6h post MCAo. Injured and contralateral hemispheres were dissected out 24 hours later. The expression of five NaCh genes, namely, $Na_v1.1$, $Na_v1.2$, $Na_v1.3$, $Na_v1.7$ and $Na_v1.8$ were evaluated by QRT-PCR. β-actin mRNA measurements in these samples provided sample amplification efficiency. Consistent with previous results, these results exhibited significant down-regulation of $Na_v1.1$ and $Na_v1.2$ genes following MCAo. Importantly, no significant differences in the expression of the other sodium channel genes were observed. RS100642 treatment significantly reversed the down-regulation of Na_v 1.1 (but not Na_v 1.2) at 24-48 h post-injury. Mexiletine treatment, on the other hand, had no significant effect on the down-regulation of either genes. These findings demonstrate that treatment with a neuroprotective dose of RS100642 significantly reverses the down-regulation of Na_v 1.1 caused by ischemic brain injury and suggests that RS100642 selectively targets the Na_v 1.1 α-subunit of the NaCh. Furthermore, our findings strengthen the hypothesis that ischemic injury may produce selective depletion of voltage-gated sodium channels, and suggest that the Na_v 1.1 NaCh

α-subunit may play a key role in the neuronal injury/recovery process. An abstract is published and a manuscript is in press. [Neurotoxicity Research, **5**, 2003 (In press)].

5. **Although not a part of our original research plan** we have undertaken immunocytochemical and Western blot analysis of rBI and rBII proteins. Our research till date collectively suggests that activity of $Na_v1.1$ NaCh α-subunit is detrimental to neuronal survival and that its down-regulation is an endogenous neuroprotective response to ischemic insult. Current study was undertaken to determine the effects of ischemic brain injury on the distribution of NaCh α-subunit $Na_v1.1$ protein by immunocytochemistry. Ischemic brain injury resulted in a severe decline in NaCh α-subunit $Na_v1.1$ protein in the ipsilateral hemisphere. The level of NaCh α-subunit $Na_v1.1$ protein progressively declined at each time point through 24 h post-injury, as found previously for mRNA levels with *in situ* hybridization. Further studies using Western blots are currently in progress to determine quantitatively the changes in NaCh α-subunit $Na_v1.1$ protein. Our studies collectively suggest that the regulation of $Na_v1.1$ sodium channel expression and activity is important in neuronal survival following focal ischemia. An abstract has been submitted.
6. In addition, **while also not a part of our original research plan** we completed a comprehensive, time-course analysis of acute (hours) and delayed (days) neurobiology genomic profile following ischemic brain injury using Affymetric microarray technology. Temporal changes in gene expression were measured using DNA microarrays following transient 2 h middle cerebral artery occlusion (MCAo) in rats. Total RNA was extracted from the injured hemisphere at 30 min, 4, 8, 24 h, 3 and 7 days post-MCAo for GeneChip® analysis using Affymetrix U34 Rat Neurobiology arrays (1322 functional genes). A total of 292 genes were differentially expressed: 181 genes were up-regulated, 104 genes were down-regulated, and 7 genes were bi-phasically up and down regulated. Among all differentially expressed genes, 90 were newly identified as associated with ischemic brain injury. Most of the affected genes were distributed among 12 functional categories. Immediate early genes, transcription factors, and heat shock proteins were up regulated as early as 30 min post-MCAo, followed by the up-regulation of inflammation, apoptosis, cytoskeletal, and metabolism genes, all of which peaked within 4-24 h of injury. Neurotrophic growth factors exhibited a sustained up-regulation beginning 24 h post-MCAo and persisting through 7 days post-injury. Three classes of genes, including ion channels, were conspicuously down-regulated between 8-24 h after injury. Specifically, we measured a selective down regulation in the rB1 and rBII sodium channel mRNA confirming our earlier results using the quantitative RT/PCR TaqMan approach.

KEY RESEARCH ACCOMPLISHMENTS for Year'04

- Manuscript entitled "Down-regulation of sodium channel Nav1.1 expression by veratridine and its reversal by a novel sodium channel blocker, RS100642, in primary neuronal cultures" was **published in Neurotoxicity Research 5: 213-220, 2003.**

- Manuscript entitled "The sodium channel blocker RS100642 reverses down-regulation of the sodium channel α-subunit Na_v 1.1 expression caused by transient ischemic brain injury in rats" **has been accepted for publication in Neurotoxicity Research 5: 2003.**

- An abstract entitled "Neuronal sodium channel gene expression and brain injury" was **presented** at the ATACCC2002 Conference, St. Petersburg, FL, 9-14 September, 2002.

- An abstract entitled "The sodium channel blocker RS100642 reverses the down-regulation of $Na_v1.1$ and $Na_v1.2$ sodium channel genes caused by ischemic brain injury in rats was **presented** at the **32nd Annual meeting of the Society for Neuroscience, 2002.**

- An abstract entitled "Down-regulation of sodium channel $Na_v1.1$ expression by veratridine and its reversal by the sodium channel blocker, RS100642, in primary neuronal cultures" was **presented at the 32nd Annual meeting of the Society for Neuroscience, 2002.**

- An abstract entitled "Down-regulation of sodium channel α-subunit $Na_v1.1$ mRNA and protein following ischemic brain injury" was **submitted for presentation at the 33rd Annual meeting of the Society for Neuroscience, 2003.**

- Using immunocytochemistry identified the regional distribution of $Na_v1.1$ protein in normal and injured brain and confirmed 1) the down-regulation was selective for this channel and not the rBIII and PN1 and PN3 channels.

- The effects of RS100642 to reduce brain injury correlate with a partial reversal of the $Na_v1.1$ (and not $Na_v1.2$) down-regulation caused by injury.

- The specific NaCh activator and neurotoxin veratridine selectively down-regulates only the $Na_v1.1$ gene in neuronal culture, and this effect is completely reversed by neuroprotective concentrations of RS100642.

REPORTABLE OUTCOMES for Year'04

Two manuscripts published
One manuscript In Press
One Manuscript accepted with revision
One Manuscript In Preparation
Three abstracts presented
One abstracts submitted

CONCLUSIONS: SEE ABOVE

REFERENCES: UNCHANGED FROM ORIGINAL PROPOSAL

REPORTABLE OUTCOMES FOR "ENTIRE" 4 YEAR PROJECT

Manuscripts (9)

Dave, J.R., Lin, Y., Ved, H.s., Koenig, M.L., Clapp, L. Hunter, J. and Tortella, F.C. RS-100642, a novel sodium channel blocker, provides differential neuroprotection against hypoxia/hypoglycemia, veratridine or glutamate mediated neurotoxicity in primary cultures of rat cerebellar neurons. Neurotoxicity Research 3(4): 381-395, 2001.

Yao, C., Williams, A.J., Cui, P,. Berti, R., Tortella, F.C. and Dave, J.R. Differential pattern of expression of voltage-gated sodium channel genes following ischemic brain injury in rats. Neurotoxicity Research 4(1): 67-75, 2002.

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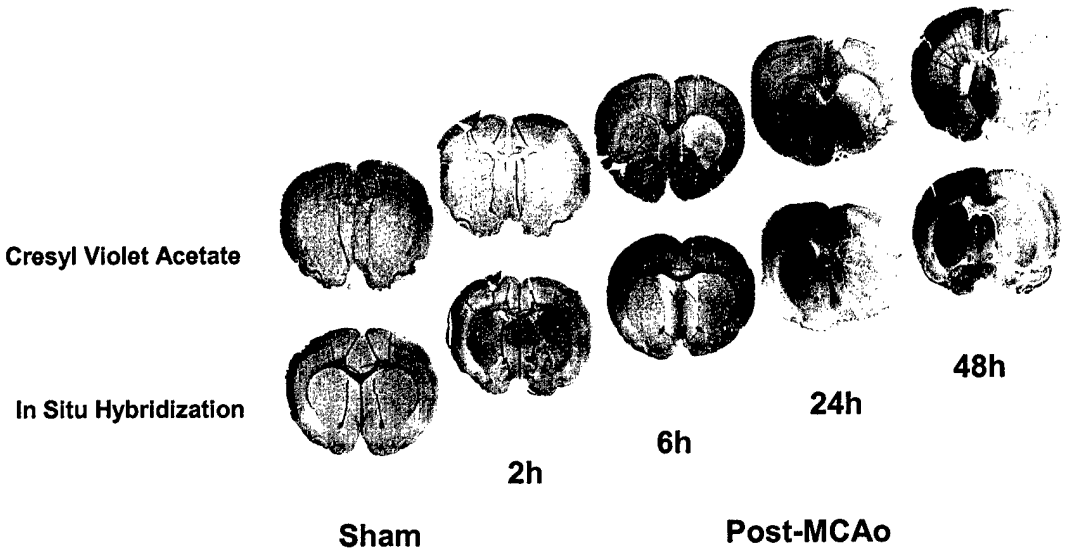
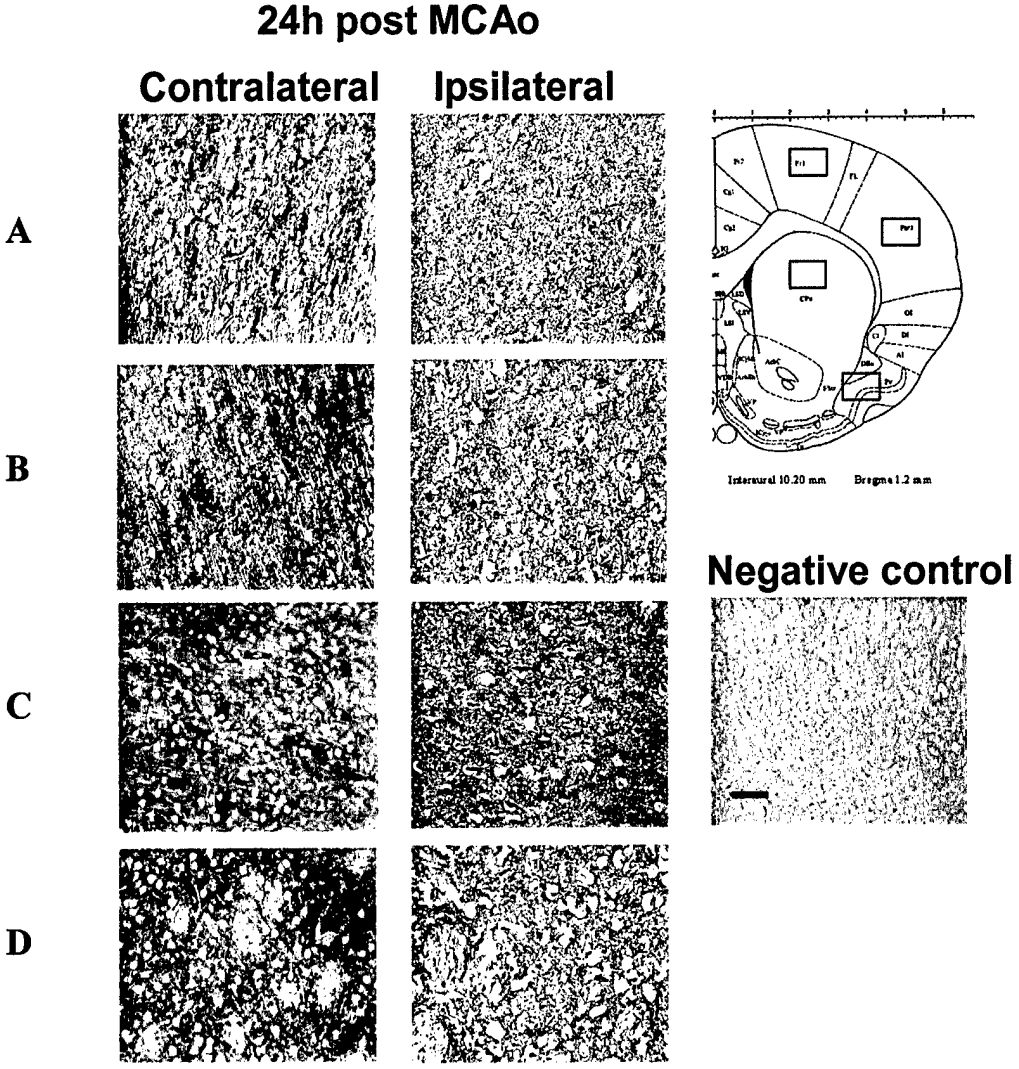
APPENDIX

Fig.1



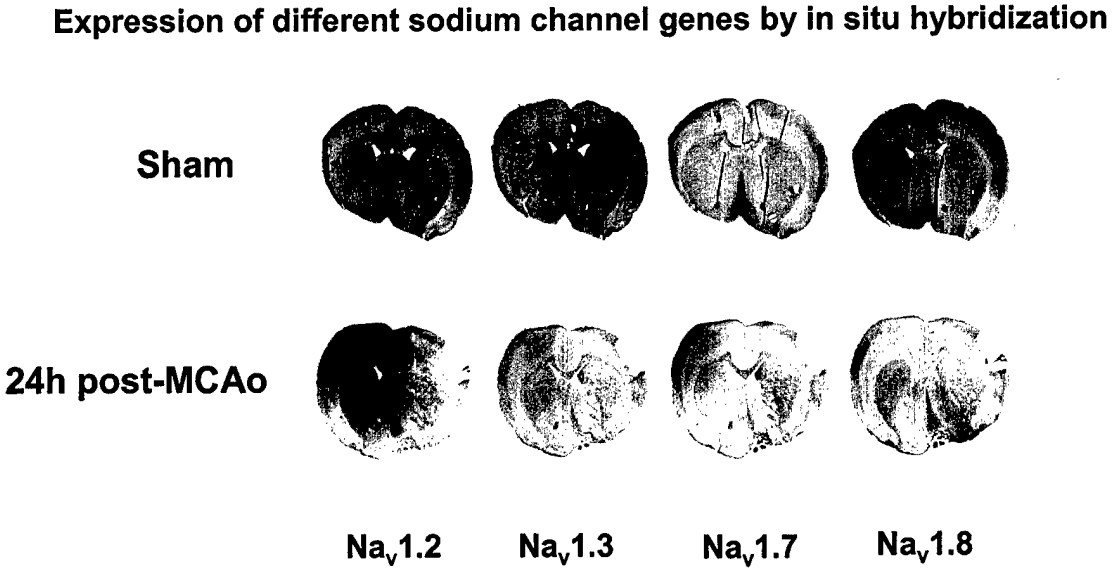
Sodium channel gene expression at different time points after MCAo injury . Corresponding sections stained with cresyl violet acetate are shown in the upper column. Notable loss of $Na_v1.1$ signal was observed in cortex and striatum at the 24h and 48h time points.

Fig 2



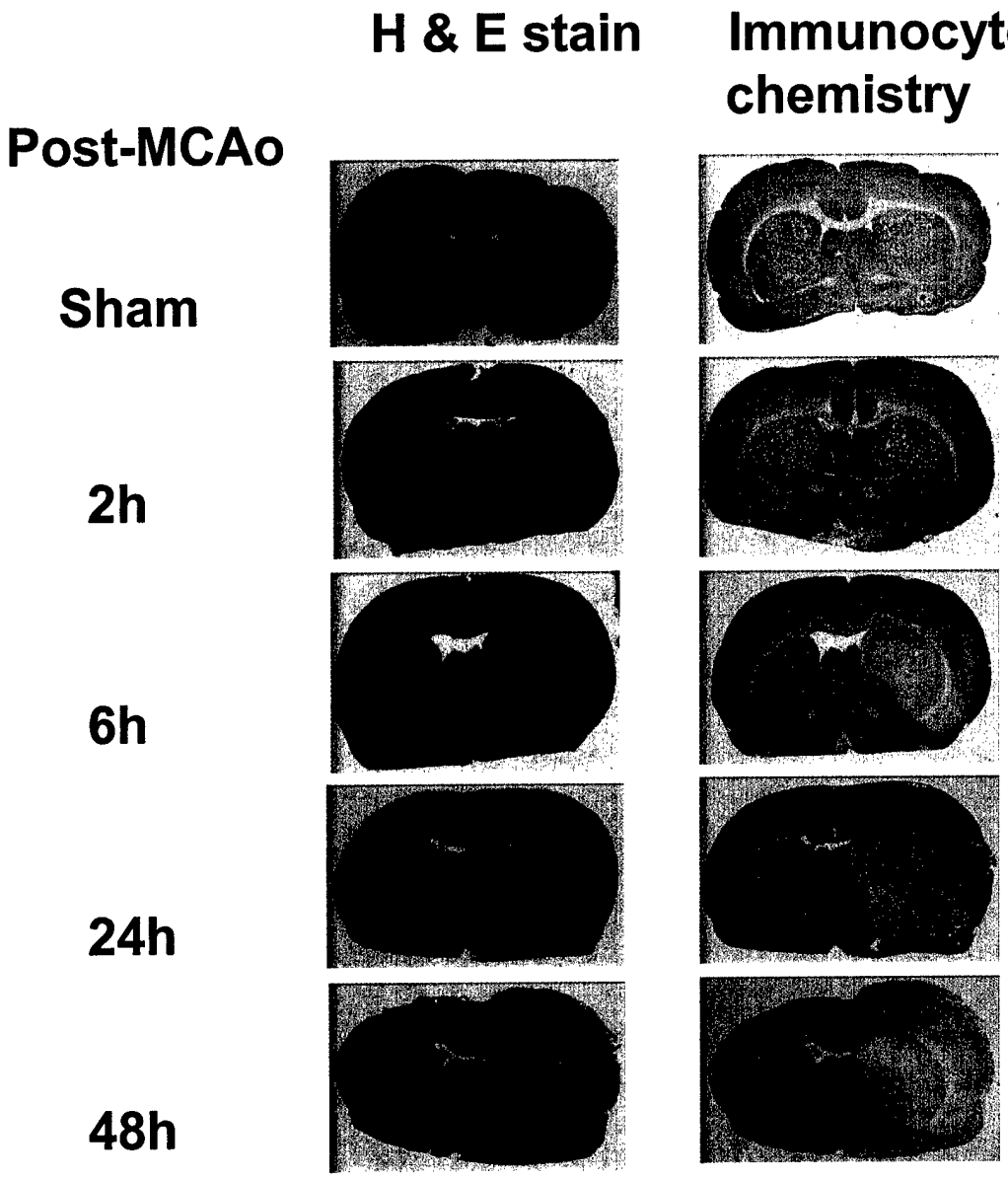
$Na_V1.1$ mRNA was expressed at significantly lower levels in the infarct at 24h post MCAo injury. Representative examples of the hybridization signal are shown for primary motor cortex (A), somatosensory cortex (B), piriform cortex (C), and dorsal striatum (D). Bar = 100μm.

Fig.3



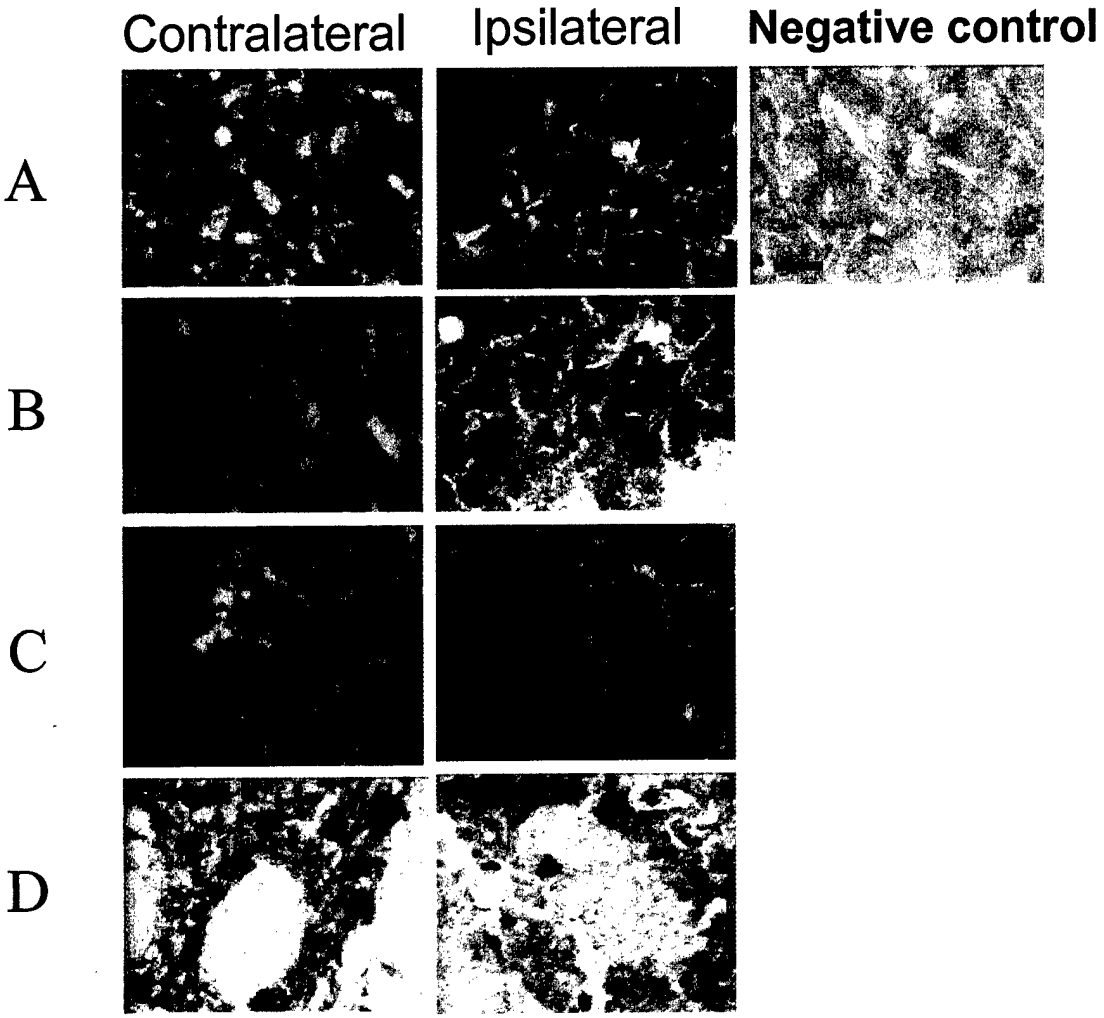
Comparision of other sodium channel genes $Na_V1.2$, $Na_V1.3$, $Na_V1.7$ and $Na_V1.8$ at 24h post-MCAo injury and in sham by *in situ* hybridization.

Fig. 4



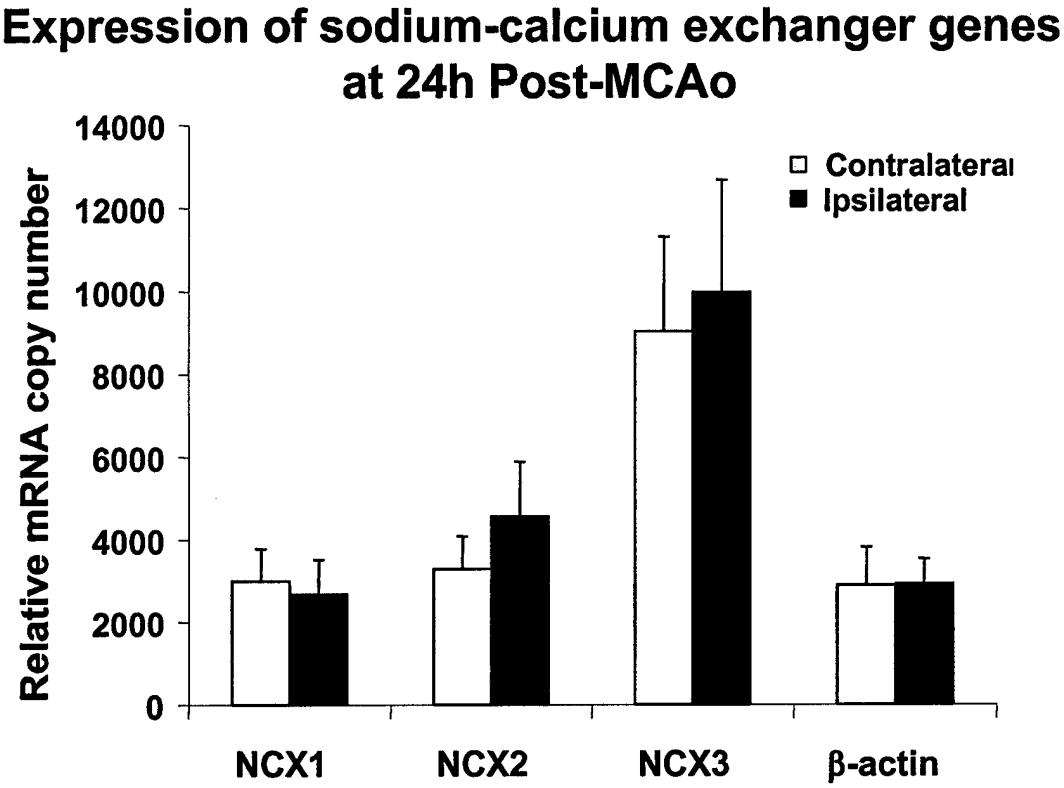
Sodium channel $Na_V1.1$ protein at different time points after MCAo injury as measured by immunocytochemistry. Corresponding sections stained with H & E are shown in the left column. Notable loss of $Na_V1.1$ signal was observed in cortex and striatum at the 6h, 24h and 48h time points.

Fig. 5



Sodium channel $Na_V1.1$ protein was expressed at significantly lower levels in the infarcted region at 24h post MCAo injury as measured by immunocytochemistry. Representative examples of the signal are shown for primary motor cortex (A), somatosensory cortex (B), piriform cortex (C), and dorsal striatum (D). Bar = 100 m.

Fig. 6



Expression of sodium-calcium exchangers NCX1, NCX2 and NCX3 genes were measured at 24h Post-MCAo by quantitative real time RT-PCR. Relative mRNA copy numbers on the contralateral and ipsilateral showed no significant differences.

Table 1: Ion channel genes expression at 30 minutes, 4h, 8h 24h, 3days and 7 days post MCAo injury by Microarray analysis. Four sodium channel genes, Na^+ channel I, Na^+ channel β-I, Na^+ channel II, Na^+ channel III were shown down-regulation at different time points after MCAo injury.

			Fold Change at time after injury					
			30min	4h	8h	24h	3D	7D
Ion Channel								
	Gene name	Gene Bank #						
1	K^+ inward rectifier (G protein-activated)	L77929	3	4	4			
2	K^+ channel r-ERG	Z96106	2					
3	K^+ channel	M81783		3				
4	K^+ channel K_v1	M27158			4			
5	K^+ channel (Ca^{2+} activated)	AF083341				3		
6	K^+ channel protein ERG	U75210				6		
7	K^+ channel 3 (ELK)	AJ007632						3
8	K^+ channel regulator 1	U78090	-3	-2		3		
9	K^+ channel $K_v4.3$ (Shal-related)	U42975	-3		-3			
10	K^+ channel $K_v4.3$	U92897		-4				
11	K^+ channel protein β	X70662			-4			
12	K^+ channel protein	M74898			-4			
13	K^+ channel 1	U27558			-2			
14	K^+ channel (inwardly rectifying)	X87635				-4		
15	K^+ channel $\alpha K_v9.1$	Y17606				-3		
16	K+ channel	Z34264					-3	-3
17	K+ channel protein (3145 bp)	X62840						-7
18	Ca^{2+} channel α-1 (pore-forming)	M99222	3			3		
19	Ca^{2+} channel (L-type voltage-dependent)	M89924				3		
20	Ca^{2+} channel β III	M88751		-2	-2	-2		
21	Ca^{2+} channel β 2 (L-type)	M80545			-3			
22	Ca^{2+} channel α-1	U14005				-2		
23	Ca^{2+} channel α-2 (L-type dihydropyridine-sensitive)	M86621					-3	
24	**Na^+ channel I**	M22253		-2	-2	-2		
25	**Na^+ channel β-1**	M91808					-3	*-2
26	**Na^+ channel II**	M22254					-3	*-3
27	**Na^+ channel III**	Y00766			-4			
28	Na^+, K^+-ATPase β 3	D84450						2
29	Na^+ K^+ -ATPase α-1	M28647						-2
30	Ca^{2+} ATPase-isoform 2	J03754						-3
31	Transferrin	D38380					4	
32	Transferrin receptor	M58040						3
33	Cyclic nucleotide-gated cation channel β	AJ000515			3			
34	Acid gated ion channel	AJ006519			-4			
35	Chloride channel-2	AF005720				-4		

Table 2. Comparison of the fold changes of selected genes analyzed by DNA microarray method and real-time QRT-PCR method.

Gene	Fold Change Value	
	Microarray	**QRT-PCR**
JAK 1	7.6	5.9
TNF-a	3.1	8.0
ELAM-1	6.3	20.0
ICAM-1	3.1	6.4
Ca2+/CaM-dependent protein kinase	-6.0	-2.9
mGluR1	-2.0	-2.0
C-myc	-7.7	-2.4
$Na_v1.1$	**-2.3**	**-4.1**

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Down-Regulation of Sodium Channel $Na_v1.1$ Expression by Veratridine and Its Reversal by a Novel Sodium Channel Blocker, RS100642, in Primary Neuronal Cultures†

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This study investigated the effects of veratridine-induced neuronal toxicity on sodium channel gene (NaCh) expression in primary forebrain cultures enriched in neurons, and its reversal by a novel sodium channel blocker, RS100642. Using quantitative RT-PCR, our findings demonstrated the expression ratio of NaCh genes in normal fetal rat forebrain neurons to be $Na_v1.2$ > $Na_v1.3$ > $Na_v1.8$ > $Na_v1.1$ > $Na_v1.7$ (rBII > rBIII > PN3 > rBI > PN1). Veratridine treatment of neuronal cells produced neurotoxicity in a dose-dependent manner (0.25-20 μM). Neuronal injury caused by a dose of veratridine producing 80% cell death (2.5 μM) significantly, and exclusively down-regulated the $Na_v1.1$ gene. However, treatment of neurons with RS100642 (200 μM) reversed the down-regulation of the $Na_v1.1$ gene expression caused by veratridine. Our findings document for the first time quantitative and relative changes in the expression of various NaCh genes in neurons following injury produced by selective activation of voltage-gated sodium channels, and suggest that the $Na_v1.1$ sodium channel gene may play a key role in the neuronal injury/recovery process.

Keywords: Veratridine, Primary neuronal cultures; Voltage-gated sodium channels; Gene expression; Quantitative RT-PCR

INTRODUCTION

Voltage-dependent sodium channels (NaChs) play a crucial role in neuronal excitability, and are considered to be one of several molecular targets for neuroprotection drug action. Under ischemic/excitotoxic conditions blockade of NaChs prevents excessive depolarization, limiting excitotoxic glutamate release. Further, NaCh blockade reverses the sodium dependent glutamate transporter and allows calcium extrusion, leading to reestablishment of ionic homeostasis (Lysko *et al.*, 1994). In fact, we recently reported in an *in vitro* study that a novel NaCh blocker, RS100641, provided neuroprotection against hypoxia/hypoglycemia and veratridine-mediated toxicity, and that it lowered the sustained rise in intracellular free calcium ions induced by veratridine or KCl (Dave *et al.*, 2001). Several *in vivo* studies using NaCh blockers such as tetrodotoxin and lamotrigine have described moderate neuroprotection in experimental models of brain ischemia (Lekieffre and Meldrum, 1993; Lysko *et al.*, 1994; Xie *et al.*, 1995; Carter, 1998: Kimura *et al.*, 1998), and in a recent study from our laboratory, RS100642 was described to significantly reduce cerebral infarction resulting from MCAo and attenuate injury-related brain seizures (Williams *et al.*, 2002). As such, changes in the transcription of NaCh genes could be involved in the neuronal injury associated with brain ischemia, as well as the neuronal plasticity associated with functional recovery.

The NaCh is composed of α and β-subunits, with the pore-forming α-subunit being both voltage-sensitive and ion specific. In different tissues and at different stages of development the α-subunit combines with a variable number of smaller β-subunits to form the bioactive channel (Isom *et al.*, 1992). The NaCh α-subunits belong to a multigene family, and cloning and electrophysiological characterization studies have documented the presence of ten different NaCh genes (e.g. rat brain I, II, III, rat SkM1, SkM2, rat NaCh6, PN1, PN3, NaN/SNS2, Na-G) in neuronal and muscle tissues in rodents (Noda *et al.*, 1986; Auld *et al.*, 1988; Kayano *et al.*, 1988; Gautron *et al.*, 1992; Waxman *et al.*, 1994; Schaller *et al.*, 1995; Akopian *et al.*, 1996; Sangameswaran *et al.*, 1996; 1997; Novakovic *et al.*, 1998). According to recently revised nomenclature, these voltage-gated NaCh genes are now referred to as $Na_v1.1$-$Na_v1.9$ and Na_x, respectively (Goldin *et al.*, 2000). However, the specific physiological

†The views of the authors do not purport to reflect the position of the Department of the Army or the Department of Defense, (para 4-3), AR 360-5.

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FIGURE 1 Representative bright-field micrographs (100x) of primary cultures of forebrain neurons stained with cresyl violet after either vehicle treatment (top-panel A), or veratridine (2.5 μM) treatment (middle-panel B), or veratridine plus RS100642 (200 μM) treatment (bottom-panel C).

roles distinguishing each of these isoforms remain unknown. One possible explanation for this is that the differences in the kinetic and pharmacological properties of these channels are subtle (Noda *et al.*, 1986; Auld *et al.*, 1998). Nevertheless, such subtle differences could be functionally important, because small changes in the voltage dependence of activation or inactivation could markedly affect excitability.

Using real-time quantitative RT-PCR, we recently reported on the relative expression of five neuronal NaCh genes ($Na_v1.1$, $Na_v1.2$, $Na_v1.3$, $Na_v1.7$, and $Na_v1.8$) in adult rat brain, and analyzed their time-related changes after ischemia/reperfusion brain injury. Our findings indicated that $Na_v1.1$ and $Na_v1.2$ NaCh genes were significantly down-regulated at 24 h post-injury (Yao *et al.*, 2002). The present studies were undertaken to determine the changes in expression of these five neuronal NaCh genes in primary cultures of fetal rat forebrain neurons following exposure to the neurotoxin and NaCh activator veratridine (Takahashi *et al.*, 1999; Dave *et al.*, 2001), with and without neuroprotection provided by the NaCh blocker; RS100642 (Dave *et al.*, 2001; Williams *et al.*, 2002).

METHODS

Cell Cultures

Research was conducted in compliance with the Animal Welfare Act, and other Federal statutes and regulation relating to animals and experiments involving animals and adheres to the principles stated in the *Guide for the Care and Use of Laboratory Animals*, NIH publication 85-23.

Cell cultures were prepared as described previously (Dave *et al.*, 2001). Briefly, adult timed-pregnant Sprague-Dawley female rats were purchased from Taconic Farms (Germantown, NY, USA) and enriched neuronal cultures were prepared from 15-day old rat embryos. Following euthanasia with carbon dioxide, the embryonic rats were removed from the uterus using aseptic techniques and placed in sterile neuronal culture media. Under a dissecting microscope, the brain tissue was removed from each embryo, taking care to discard meninges and blood vessels. The forebrain was separated by gross dissection and cells were dissociated by trituration of the tissue and plated at a density of 5×10^5 cells/well in 48 well culture plates pre-coated with poly-L-lysine. Cultures were maintained in a medium containing equal parts of Eagle's basal media (without glutamine) and Ham's F12 K media supplemented with 10% heat-inactivated horse serum, 10% fetal bovine serum, glucose (600 μg/ml), glutamine (100 μg/ml, penicillin (50 units/ml), and streptomycin (50 μg/ml). After 48 h, cytosine arabinoside (10 μM) was added to inhibit non-neuronal cell division. Our cultures typically yield 80-90% neurons and 10-20% glia and other cells (Ved *et al.*, 1991; DeCoster *et al.*, 1992).

Neurotoxicity Experiments

Cells were used in experiments after 6-8 days in culture. Cells were exposed to veratridine (0.25-20 μM) for 45 min in Locke's solution. At the end of treatment period the Locke's solution in each well was replaced with the original conditioned media. Morphological and cell viability assessments (MTT measurements) or total RNA extraction were done 24 h later. Cell damage was quantitatively assessed using a tetrazolium salt colorimetric assay with 3-[4,5-dimethylthylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma Chem. Co., Saint Louis, MO, USA). Briefly, this dye was added to each well (final

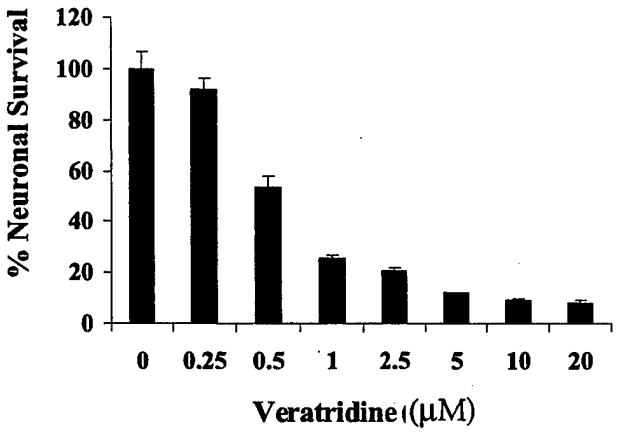

FIGURE 2 Demonstration that veratridine produced toxicity in primary cultures of fetal rat forebrain neurons in dose-related manner. Values are mean ± S.E.M. of 8-10 separate independent experiments.

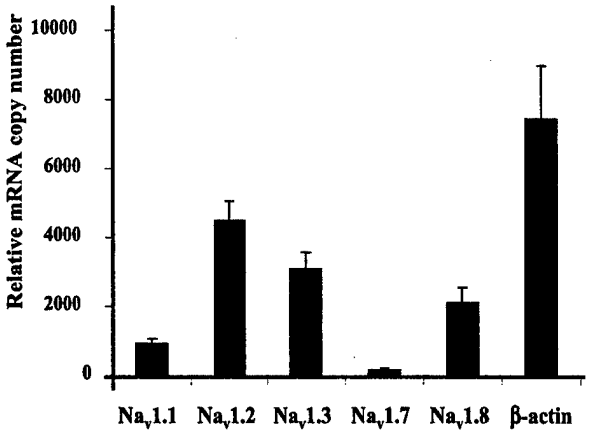

FIGURE 3 Relative expression efficiency of five sodium channel genes and β-actin gene in normal primary cultures of forebrain neurons. Values are mean ± S.E.M. of 8 separate culture plates.

concentration of 0.15 mg/ml) and cells were incubated with MTT for 1 h at 37°C. When the assay was terminated, the dye was solubilized by acidified isopropanol (0.1 N HCl in isopropanol) and the absorbance intensity of each sample measured at 540nm in a 96 well plate reader.

In separate experiments, neurotoxicity was produced by 2.5 μM veratridine. These neurons were then exposed to 200 μM RS100642, a maximal efficacious dose as described previously (Dave *et al.*, 2001). Values are expressed relative to vehicle-treated control cells that were maintained on each plate, and percentage changes in cell viability calculated. Differences in the cell viability among treatment groups were determined using one-way analysis of variance (ANOVA) and the Newman-Keuls Test.

Total RNA Isolation

The neuronal cells from 6-8 wells were pooled and after rinsing twice with phosphate-buffered saline were homogenized in TRIzol reagent (Life Technologies, Gaithersburg, MD, USA). Total RNA was extracted from the cells according to the manufacturer's suggested protocol. The total RNA concentration was determined by spectrophotometry at the absorbency 260 and 280 nm.

Primers and Probes

The primers and probes for NaCh genes $Na_v1.1$, $Na_v1.2$, $Na_v1.3$, $Na_v1.7$ and $Na_v1.8$, and house keeping gene β-actin were designed using the primer design software Primer Express™ and their sequences have been listed previously (Yao *et al.*, 2002) (The respective sequence positions of primers and the size of PCR amplifications are: 1363-1440, 78 bp; 7522-7600, 79 bp; 1488-1560, 73 bp; 2577-2648, 72 bp; 3424-3495, 72 bp and 42-108, 67 bp with accession nos. X03638, M22254, Y00766, U79568, U53833 and AA955429 respectively). Synthesis of these probes and primers was performed by Perkin-Elmer Applied Biosystems Foster City, CA. FAM (6-carboxyfluorescein) was used as the reporter and TAMRA (6-carboxy-tetramethyl-rhodamine) as the quencher dye. The housekeeping gene b-actin was used as an endogenous control in these samples to provide sample amplification efficiency.

Quantitative RT-PCR Reaction

RT and PCR were carried out using a GeneAmp RNA PCR Core Kit and TaqMan Universal PCR Master Mix kit (Perkin-Elmer) according to the manufacturer's specification. A two-step RT-PCR was performed. The RT reaction used 10 μg total RNA in a total volume of 100 μl containing 1x PCR Buffer II, 5 mmol/L $MgCl_2$, 1mmol/L of each dNTP, 2.5 μmol/L Random Hexamers, 1 U/μl RNase Inhibitor and MultiScribe Reverse Transcriptase. The RT reaction was carried out at 42°C for 15 min, 99°C for 5 min. The second cDNA synthesis and quantitative PCR were performed in the TaqMan Universal PCR Master Mix with 5-10 μl of each RT product ($Na_v1.1$ 5 μl, $Na_v1.2$ 5 μl, $Na_v1.3$ 8 μl, $Na_v1.7$ 10 μl and $Na_v1.8$ 8 μl; 1 μl of each RT product contains

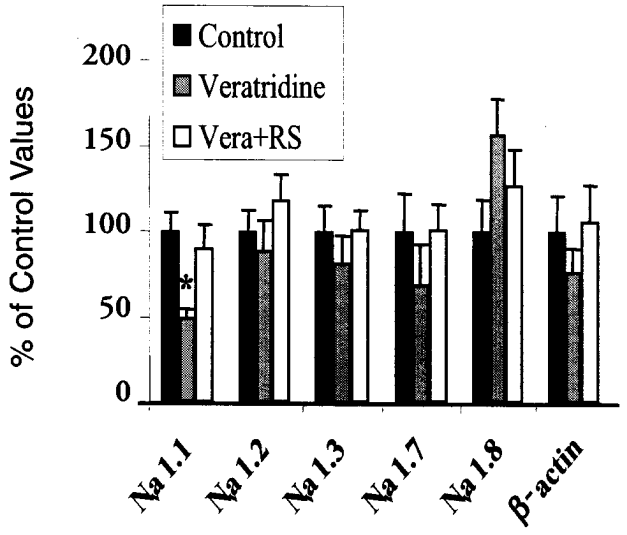

FIGURE 4 Demonstration that RS100642 (RS) reversed veratridine-mediated down regulation of $Na_v1.1$ gene expression in primary neuronal culture. Values are mean ± S.E.M. of 7-10 separate experiments. *, vs vehicle control values at $p < 0.05$.

0.1 μg total RNA), 100 nmol/L probe and 200 nmol/L primers in a total volume of 50 μl. PCR was performed at 50°C for 2 min, at 95°C for 10 min and then run for 40 cycles at 95°C for 15 seconds and again at 60°C for 1 min on the ABI PRISM 7700 Detection System. A single specific DNA band for $Na_v1.1$, $Na_v1.2$, $Na_v1.3$, $Na_v1.7$ and $Na_v1.8$ was observed on Southern gel electrophoresis analysis (data not shown). Using the formula provided by the manufacturer (Perkin-Elmer) and also described by Wang *et al.* (2000), the values were extrapolated to calculate the relative number of mRNA copies.

Statistical Analysis

Data are presented as mean ± S.E.M. Statistical comparisons (n = 8-14 culture plates from separate experiments carried out on separate days) were made by analysis of variance (ANOVA; Fisher's protected least squares difference) and values were considered to be significant when $p < 0.05$.

Chemicals

RS-100642 was synthesized at and obtained from the Department of Medicinal Chemistry, Roche Bioscience (Palo Alto, CA, USA). Veratridine and all other chemicals were of analytical grade and were purchased from the Sigma Chemical Co. (St. Louis, MO, USA).

RESULTS

Figure 1 demonstrates the morphological changes observed in primary neurons exposed to vehicle (A), veratridine (2.5 μM; B) or veratridine plus RS100642 (200 μM; C). Morphological changes in injured cells included a reduction in the number of neuronal processes or dendrites per neuron, and the cells often lost their round shape and appeared to be more fragmented (FIG. 1B). RS100642 treatment prevented most of these morphological changes (FIG. 1C).

As shown in figure 2, veratridine exposure was highly neurotoxic to forebrain neurons and produced a dose-related decrease in neuronal viability reaching a maximum of 80-90% cell death at concentrations of 2.5-20 μM. For subsequent experiments we used the 2.5 μM concentration of veratridine which consistently produced 80% neuronal injury.

Quantification of the relative level of mRNA for each sodium channel gene was first analyzed in normal fetal rat forebrain neurons in primary culture. The mRNA levels of $Na_v1.1$, $Na_v1.2$, $Na_v1.3$, $Na_v1.7$, $Na_v1.8$ and the house-keeping gene β-actin detected by quantitative RT-PCR demonstrate that $Na_v1.2$ is the most abundant of the five NaCh genes expressed in primary neuronal cultures (FIG. 3). Compared to β-actin gene expression, the levels of expression of each of the NaCh genes were $Na_v1.1$, 12.7%; $Na_v1.2$, 60.7%; $Na_v1.3$, 41.9%; $Na_v1.7$, 2.3% and $Na_v1.8$, 28.8%. Quantification of the level of relative mRNA for each NaCh gene was next analyzed in fetal rat forebrain neurons subjected to veratridine treatment alone, or veratridine plus RS100642 (200 μM) treatment (FIG. 4). As seen in figure 4, the mRNA levels of $Na_v1.2$, $Na_v1.3$, $Na_v1.7$, $Na_v1.8$ and the house-keeping gene β-actin were not significantly effected by veratridine exposure. However, the $Na_v1.1$ NaCh transcript was significantly down-regulated (greater than 50%) by veratridine. Furthermore, RS100642 treatment reversed the down-regulation caused by veratridine (FIG. 4). This reversal by RS100642 on veratridine-mediated down-regulation of $Na_v1.1$ correlates to its neuroprotective effect to completely prevent (i.e. 100% protection) against veratridine-mediated toxicity (FIG. 5).

DISCUSSION

The present study describes changes in brain type ($Na_v1.1$, $Na_v1.2$ and $Na_v1.3$) and peripheral nerve type ($Na_v1.7$ and $Na_v1.8$) NaCh gene expression using real-

time quantitative RT-PCR in primary cultures of fetal rat forebrain neurons. The significance of this study is the fact that of the five NaCh genes studied, veratridine treatment specifically down-regulated only one NaCh gene, i.e. $Na_v1.1$, while other NaCh genes were not significantly affected by direct activation of these voltage-gated NaChs. These data are consistent with our recent *in vivo* findings in which temporary middle cerebral artery occlusion (MCAo) produced a time-dependent down-regulation of $Na_v1.1$ gene in the injured hemisphere (Yao *et al.*, 2002). Furthermore, the present study is 1) the first to demonstrate a selective reversal of veratridine-mediated down-regulation of $Na_v1.1$ gene expression and 2) documents a direct correlation of RS100642 neuroprotection and reversal of NaCh gene expression.

In this study we demonstrated that among the five NaCh genes studied, the abundance of $Na_v1.2$ channel mRNA in fetal rat forebrain neurons is highest, followed by $Na_v1.3$, $Na_v1.8$, $Na_v1.1$ and $Na_v1.7$ mRNA. Although these results differ slightly from those reported earlier from our laboratory in adult rat brain (Yao *et al.*, 2002) where the abundance of $Na_v1.2$ channel mRNA is also greatest, but followed by $Na_v1.1$, $Na_v1.8$, $Na_v1.3$ and $Na_v1.7$ mRNA. It should be noted that in both cases $Na_v1.2$ gene expression is the most abundant, whereas the $Na_v1.7$ transcript is the least abundant. Our present results also differ slightly from those reported by Lara *et al.* (1996) in primary cultured neurons. However, their studies used a non-quantitative RT/PCR technique where the abundance of $Na_v1.3$ channel mRNA was reported to be maximal, followed by $Na_v1.2$ and $Na_v1.1$ ($Na_v1.7$ and $Na_v1.8$ genes were not studied). These differences may either be related our use of a quantitative RT/PCR technique (which has been reported to be more accurate, precise and reproducible than traditional RT/PCR) or differences in culture conditions (e.g. composition of culture media, enriched forebrain neuronal cultures in the present study vs whole brain neuron/glia mixed culture, etc.). Collectively, from the present study and our earlier *in vivo* study it appears that the $Na_v1.2$ NaCh may be one of the prominent ion channel genes expressed in both fetal and adult rat brain. However, translation of $Na_v1.2$ mRNA into an active channel remains to be established.

Furthermore, although expression of the $Na_v1.7$ gene in normal brain tissue has been previously demonstrated by us using QRT/PCR (Yao *et al.*, 2002) and by others using RT/PCR (Sangameswaran *et al.*, 1997), and in fetal brain neurons in the present study, the level of expression was the lowest of all NaCh genes expressed in adult or fetal neurons and likely functionally not important.

Our earlier *in vivo* report (Yao *et al.*, 2002) also demonstrated significant expression of the peripheral

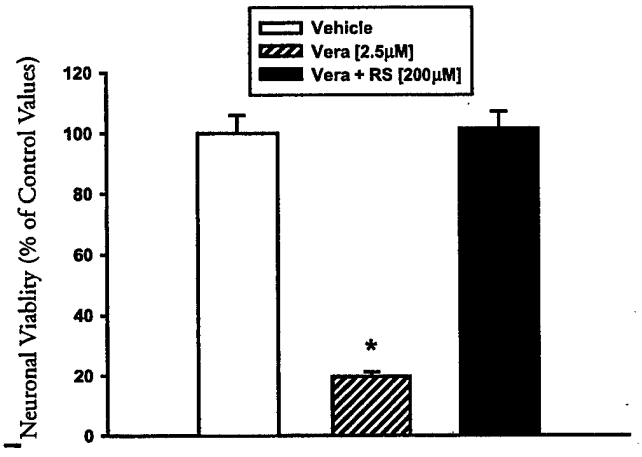

FIGURE 5 Demonstration that RS100642 (RS) treatment provided complete neuroprotection against veratridine-mediated neurotoxicity. Values are mean ± S.E.M. of 6 independent experiments. *, vs. vehicle control values at $p < 0.001$.

$Na_v1.8$ gene in normal and injured brain, which is consistent with our present results seen in fetal rat brain. This novel observation likely reflects the highly sensitive real-time QRT-PCR method used in the present study, as other investigators (Novakovic *et al.*, 1998) have failed to detect $Na_v1.8$ mRNA in rat brain using less sensitive *in situ* hybridization techniques.

The time-course of changes in $Na_v1.1$ gene expression in earlier *in vivo* studies revealed a significant down-regulation in the injured brain hemisphere from 6 h to 48 h post-MCAo, with a maximal decrease being observed at 24 h post-injury (Yao *et al.*, 2002). In the present study we selected the same optimal time-interval to demonstrate the effects of voltage-gated NaCh activation on regulation of these five NaCh genes in primary neuronal cultures. Similar to ischemic insult in adult rat brain, veratridine treatment of fetal rat brain neurons caused a significant down-regulation of $Na_v1.1$ NaCh gene. However, unlike the ischemic insult which also significantly down-regulated $Na_v1.2$ gene expression, veratridine treatment had no significant effects on regulation of this or any other NaCh gene *in vitro*.

Our findings that veratridine treatment down-regulates $Na_v1.1$ gene levels is in agreement with earlier report by Lara *et al.* (1996) in which treatment of whole brain neuronal cultures with scorpion neurotoxin for 60 h down-regulated $Na_v1.1$ expression. However, this study reported that similar treatments down-regulated $Na_v1.2$ and $Na_v1.3$ genes as well. As noted above, we failed to observe any significant down-regulation of these two NaCh genes in the present study. These differences may be related to either the difference in exposure time to

neurotoxin (60 h versus 45 min), the neurotoxin mechanism of insult, or to the use of RT-PCR techniques used by Lara and co-workers vs QRT-PCR technique used in the present study. Another earlier study showed that treatment of primary neuronal cultures with scorpion α toxin, batrachotoxin or veratridine caused a partial but rapid disappearance of surface NaCh density as measured by a decrease in the specific binding of saxitoxin and scorpion β toxin, and a decrease in specific ^{22}Na uptake (Dargent and Couraud, 1990). Though these binding or uptake studies do not provide any information as to which specific voltage-gated NaCh was down-regulated in response to sodium influx, it does support our present observations and strengthens the hypothesis that persistent activation may result in a molecular and functional down-regulation of these channels. On the other hand, other *in vivo* studies in rats with kainate-induced seizures have demonstrated the up-regulation of $Na_v1.2$ and $Na_v1.3$ NaCh gene expression in the hippocampus (Gastaldi *et al.*, 1997).

Though $Na_v1.2$ and $Na_v1.3$ expression was greater than $Na_v1.1$ expression in normal neurons, the exclusive down-regulation of the $Na_v1.1$ transcript by veratridine in the present study merits further attention. This finding suggests that although mRNA expression of the other two NaCh genes is greater, the $Na_v1.1$ gene may be functionally more important or more plastic in the fetal rat brain. The other possibility is that though the mRNA levels of $Na_v1.2$ and $Na_v1.3$ are greater than $Na_v1.1$, the levels of actual functional channel protein may not follow the same order of abundance. Further studies are currently underway in our laboratory to determine the respective protein levels of these NaChs in primary neuronal culture using immunohistochemistry and Western blot analysis. It is expected that these studies will provide added information about subcellular localization of various NaCh proteins. Although NaCh $Na_v1.3$ mRNA levels are not altered in the present study, it is worth mentioning that others have demonstrated generation of developmentally regulated isoforms of sodium channel $Na_v1.3$ mRNA α-subunit in rat brain, namely IIIN, an isoform that is predominant in neonatal brain vs IIIA, which is predominant in adult rat brain (Gustafson *et al.*, 1993).

The reversal of veratridine-mediated down-regulation of $Na_v1.1$ by the novel NaCh blocker RS100642 suggests that this blocker may have specificity towards $Na_v1.1$ channel. Alternatively, it is possible that RS100642 did not have any effect on the expression of those NaCh transcripts simply because veratridine itself had no significant effect on expression of the other NaCh genes. Additional immunocytochemical and electrophysiological experiments should provide added insight into this complex, yet interesting phenomenon.

In conclusion, the present study demonstrates expression of both brain type and peripheral type sodium channels in uninjured and injured fetal rat forebrain neurons. We have also shown significant and exclusive down-regulation in the expression of the $Na_v1.1$ gene following veratridine treatment. The reason for this decrease in $Na_v1.1$ gene expression following veratridine-mediated injury remains speculative. In view of the fact that NaCh blockers have been shown to be neuroprotective against various insults (Ashton *et al.*, 1997; Dave *et al.*, 2001) it is possible that vulnerable neurons may reduce the availability of $Na_v1.1$ sodium channel protein (by decreasing its expression) as a defense mechanism against further or delayed neurodegeneration. This hypothesis is indirectly supported by our present observation that the NaCh blocker RS100642 not only reverses veratridine-mediated down-regulation of $Na_v1.1$ gene expression, but does so at neuroprotection doses. Although the present study suggests a possible involvement of the $Na_v1.1$ sodium channel gene in the injury and/or recovery process, further studies are needed. For example, the use of antisense oligonucleotides to specifically evaluate the role of each NaCh subunit during the injury/recovery process may prove useful.

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The Sodium Channel Blocker RS100642 Reverses Down-Regulation of the Sodium Channel α-Subunit Na_v 1.1 Expression Caused by Transient Ischemic Brain Injury in Rats[1]

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In this study we evaluated the expression of five sodium channel (NaCh) α-subunit genes after transient middle cerebral artery occlusion (MCAo) in the rat and the effects of treatment with the NaCh blocker and experimental neuroprotective agent RS100642 as compared to the prototype NaCh blocker mexiletine. The expression of Na_v 1.1, Na_v 1.2, Na_v 1.3, Na_v 1.7, Na_v 1.8 and the housekeeping gene β-actin were studied in vehicle or drug-treated rats at 6, 24 and 48 h post-MCAo using real-time quantitative RT-PCR. RS100642 (1 mg/kg), mexiletine (10 mg/kg), or vehicle (1 ml/kg) was injected (i.v.) at 30 min, 2, 4, and 6 h post-injury. Following MCAo only the Na_v 1.1 and Na_v 1.2 genes were significantly down-regulated in the ipsilateral hemisphere of the injured brains. RS100642 treatment significantly reversed the down-regulation of Na_v 1.1 (but not Na_v1.2) at 24-48 h post-injury. Mexiletine treatment, on the other hand, had no significant effect on the down-regulation of either genes. These findings demonstrate that treatment with a neuroprotective dose of RS100642 significantly reverses the down-regulation of Na_v 1.1 caused by ischemic brain injury and suggests that RS100642 selectively targets the Na_v 1.1 α-subunit of the NaCh. Furthermore, our findings strengthen the hypothesis that ischemic injury may produce selective depletion of voltage-gated NaChs, and suggest that the Nav 1.1 NaCh α-subunit may play a key role in the neuronal injury/recovery process.

Keywords: Gene expression; Ischemia; Mexiletine; Middle cerebral artery occlusion; RS100642; Sodium channels; Sodium channel blockers

INTRODUCTION

Cerebral ischemia alters the expression of a variety of gene families including immediate early genes, cytokines, growth factors, neurotransmitter receptors, ion channels, apoptotic genes, etc. (Neumann-Haefelin *et al.*, 1994; Chen *et al.*, 1997; Gorter, *et al.*, 1997; Kawamata *et al.*, 1997; Carter, 1998; Berti *et al.*, 2002). Many of these gene-products have been implicated to ultimately play a role in neuronal survival or degeneration (Savitz *et al.*, 1999). Determining the molecular pathways and/or mechanisms responsible for the differential regulation of these genes is of paramount importance for understanding the molecular mechanisms underlying brain injury and recovery. Preventing alterations in the expression of key genes may provide a valuable neuroprotective strategy for treating cerebral ischemia.

Sodium channels, which are composed of α and β-subunits, play an important role in neuronal signaling by transmitting electrical impulses rapidly throughout cells and cell networks. Ten distinct α-subunits (Na_v 1.1 - Na_v 1.9, Na_x) and three β-subunits (β_1 – β_3) have so far been identified. Each α–subunit molecule consists of four homologous domains (I-IV) and each domain consists of six transmembrane segments (S1-S6) (Noda *et al.*, 1984). Cloning and electrophysiological characterization studies have documented the presence of ten different NaCh subtypes in brain and muscle tissue of rodents (Noda *et al.*, 1986; Auld *et al.*, 1988; Kayano *et al*, 1988; Gautron *et al.*, 1992; Waxman *et al.*, 1994; Schaller *et al.*, 1995; Akopian *et al.*, 1996; Sangameswaran *et al.*, 1996; 1997; Novadovic *et al.*, 1998). However, the specific physiological and pathophysiological roles distinguishing each of these isoforms remain unknown (Novakovic *et al.*, 2001). Neuronal NaChs are the molecular entities that generate and propagate action potentials in the nervous system, making them key suspects in excitotoxic damage after nervous system injury. It is well documented that excessive glutamate release, pathological Na^+

[1]*Research was conducted in compliance with the Animal Welfare Act, and other Federal statutes and regulation relating to animals and experiments involving animals and adheres to the principles stated in the Guide for the Care and Use of Laboratory Animals, NIH publication 85-23. The views of the authors do not purport to reflect the position of the Department of the Army or the Department of Defense, (para 4-3), AR 360-5.*

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influx, and the subsequent anoxic depolarization are considered to be involved in the pathogenesis of ischemic brain injury (Choi *et al.*, 1990, Stys *et al.*, 1998).

We recently reported in an *in vitro* study that a novel NaCh blocker RS100642 provided neuroprotection against hypoxia-hypoglycemia and veratridine-mediated toxicity, and that it lowered the sustained rise in intracellular free calcium ions induced by veratridine or KCl (Dave *et al*, 2001). In addition, RS100642 was recently shown to significantly reduce cerebral infarction resulting from MCAo and attenuated injury related brain seizures (Williams *et al*, 2002). Recently, using real-time quantitative RT-PCR, we reported on the relative expression of five NaCh α-subunit genes (Na_v 1.1, Na_v 1.2, Na_v 1.3, Na_v 1.7 and Na_v 1.8) in adult rat brain and analyzed their time-related changes. These findings indicated that Na_v1.1 and Na_v 1.2 were significantly down regulated at 24 h post-injury (Yao *et al*, 2002). To obtain further insight into the molecular mechanisms underlying neuroprotection with NaCh blockers, the present study investigated the effects of mexiletine and the mexiletine derivative RS100642 on NaCh gene expression following ischemic brain injury in the rat.

MATERIALS AND METHODS

1. Ischemic Brain Injury

Male Sprague-Dawley rats (270-330g; Charles River Labs, Raleigh, VA) were used in all of the following procedures as described previously (Tortella *et al.*, 1999). Anesthesia was induced by 5% halothane and maintained at 2% halothane delivered in oxygen. Indwelling intravenous (i.v.) cannulas (PE-50) were placed into the left jugular vein of all animals for drug delivery. Body temperature was maintained normothermic (37 ± 1°C) throughout all surgical procedures by means of a homeothermic heating system (Harvard Apparatus, South Natick, MA). Food and water were provided *ad libitum* pre- and post-surgery and the animals were individually housed under a 12h light/dark cycle. The facilities in which the animals were housed were maintained and fully accredited by the American Association of Laboratory Animal Care (AALAC).

Three to five days following the surgical procedures described above the rats were re-anesthetized and prepared for temporary focal ischemia using the endovascular suture filament method of middle cerebral arterial occlusion (MCAo) and reperfusion as described previously (Tortella et al., 1999). The endovascular suture remained in place for 2 h and then retracted to allow reperfusion of blood to the MCA. A sham group received surgery in which an identical procedure was followed, but without inserting the filament. Following MCAo surgery, animals were placed in recovery cages with ambient temperature maintained at 22°C.

Injections of vehicle (saline), RS100642 (1.0 mg/kg) or mexiletine (10.0 mg/kg) were given at 30 min, 2h, 4h and 6h post-MCAo. These are maximally effective neuroprotection doses following rat MCAo as previously reported (Williams and Tortella, 2002). Sham controls received saline injections. Rat brains were removed at 6, 24 and 48 h post-MCAo. From each injured brain a 2 mm thick coronal slice located 5 mm from the frontal pole was dissected, and both ipsilateral and contralateral hemispheres were separated for RNA extraction. The remaining brain tissue was stained with 2,3,5-triphenyltetrazolium chloride (TTC) (Tortella et al., 1999) to evaluate the presence of brain infarction. Computer-assisted image analysis was used to digitally image the posterior surface of each TTC-stained forebrain section (Loats Associates, Westminster, MD).

2. Total RNA Isolation

Tissue samples were homogenized in TRIzol Reagent (Life Technologies, Gaithersburg, MD, USA). Total RNA was extracted from the tissue according to the manufacturer's suggested protocol. The total RNA concentration was determined by spectrophotometry at the absorbency 260 and 280 nm.

3. Quantitative RT-PCR Reaction

Reverse transcription (RT) and PCR were carried out using a GeneAmp RNA PCR Core Kit and TaqMan Universal PCR Master Mix Kit (Perkin-Elmer) according to the manufacturer's specification. A two-step RT/PCR was performed. RT reaction used 14 µg total RNA in a total volume of 100 µl containing 1x PCR Buffer II, 5 mmol/L $MgCl_2$, 1mmol/L of each dNTP, 2.5 µmol/L Random Hexamers, 1 U/µl RNase Inhibitor and MultiScribe Reverse Transcriptase. RT reaction was carried out at 70°C for 5 min, 42°C for 15 min, 99°C for 5 min. Real-time PCR was performed using TaqMan Universal PCR Master Mix with different amounts of RT products (Na_v1.1 2.5 µl, Na_v1.2 0.5 µl, Na_v1.3 3 µl, Na_v1.7 12.5 µl, Na_v1.8 3 µl and β-actin 0.25 µl [1µl = 80 ng total RNA]), 100 nmol/L TaqMan probe and 200 nmol/L primers in a total volume of 50 µl. PCR was performed at 50°C for 2 min, at 95°C for 10 min and then run for 40 cycles at 95°C for 15 seconds and at 60°C for 1 min on the ABI PRISM 7700 Detection System.

4. Primer and Probes

The primers and probes for NaCh genes Na_v 1.1, Na_v1.2, Na_v 1.3, Na_v 1.7, Na_v 1.8 and house keeping gene β-actin were designed using the primer design software Primer Express™ and their sequences were the same as reported previously (Yao *et al*, 2002). Synthesis of probes and primers was performed by Applied Biosystems (Hayward, CA).

5. Compound

RS100642 (1-(2,6-dimethyl-phenoxy)-2-ethylaminopropane hydrochloride) and mexiletine (1-(2,6-dimethylphenoxy)-2-aminopropane hydrochloride) were obtained from Roche Biosciences (Palo Alto, CA). These drugs were dissolved in sterile saline solution immediately prior to testing and administered (i.v.) at doses of 1 mg/kg and 10 mg/kg of body weight, respectively.

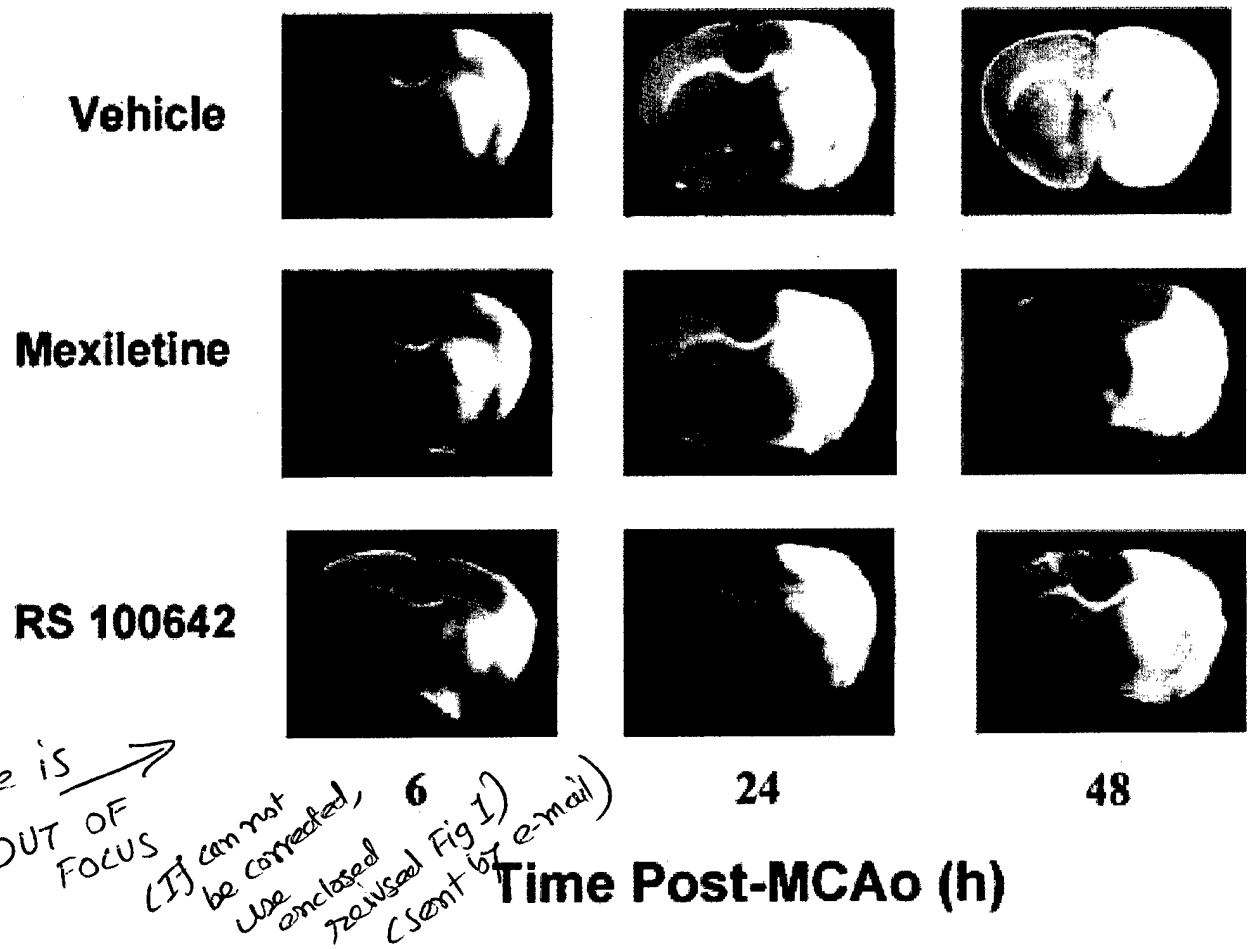

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FIGURE 1 Representative forebrain images (TTC-stained) from vehicle, mexiletine or RS100642 treated animals following 2 h MCAo and 4, 22 or 46 h reperfusion and recovery.

6. Data Analysis

Data are presented as the mean ± standard error of the mean. Statistical analysis was performed using two-tailed *t*-tests to evaluate the difference between contralateral and ipsilateral brain hemispheres. $P < 0.05$ was considered significant.

RESULTS

1. Brain Injury

All rats ($N = 10$/group) survived throughout the experiment. Figure 1 displays representative TTC-stained images of vehicle, RS100642, and mexiletine treated animals, indicating the progression of infarction from 6-48 h in each of the treatment groups. Notable injury in both cortical and subcortical regions was observed as early as 6 h post-MCAo, which increased in size out to 48 h post-MCAo. Treatment with RS100642 or mexiletine reduced the visible area of infarction as compared to vehicle treatment at each respective time point.

2. NaCh Gene Expression Following Treatment with Mexiletine or RS100642

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Figure 2 shows the effects of RS100642, mexiletine or vehicle treatment on expression of NaCh α-subunit genes Na_v 1.1, Na_v 1.2, Na_v 1.3, Na_v 1.7, Na_v 1.8 and β-actin at 24h post-MCAo. The expression of Na_v 1.1 and Na_v 1.2 genes was significantly down-regulated in the ipsilateral hemisphere of both the vehicle and mexiletine treated groups. However, following RS100642 treatment only Na_v 1.2 expression (ipsilateral vs contralateral) was significantly down-regulated. No significant changes in Na_v1.3, Na_v 1.7, or Na_v 1.8, or the house-keeping gene β-actin were observed in any treatment group studied. Figure 3 presents the time course change in Na_v 1.1 expression from 6-48 h following MCAo. No significant differences in mRNA levels were measured between the contralateral and ipsilateral hemispheres of sham injured animals (FIG. 3A). Na_v 1.1 was significantly down-regulated by 39-52% in all treatment groups at 6 h post-injury (FIG. 3B). However, at 24 and 48 h post-injury

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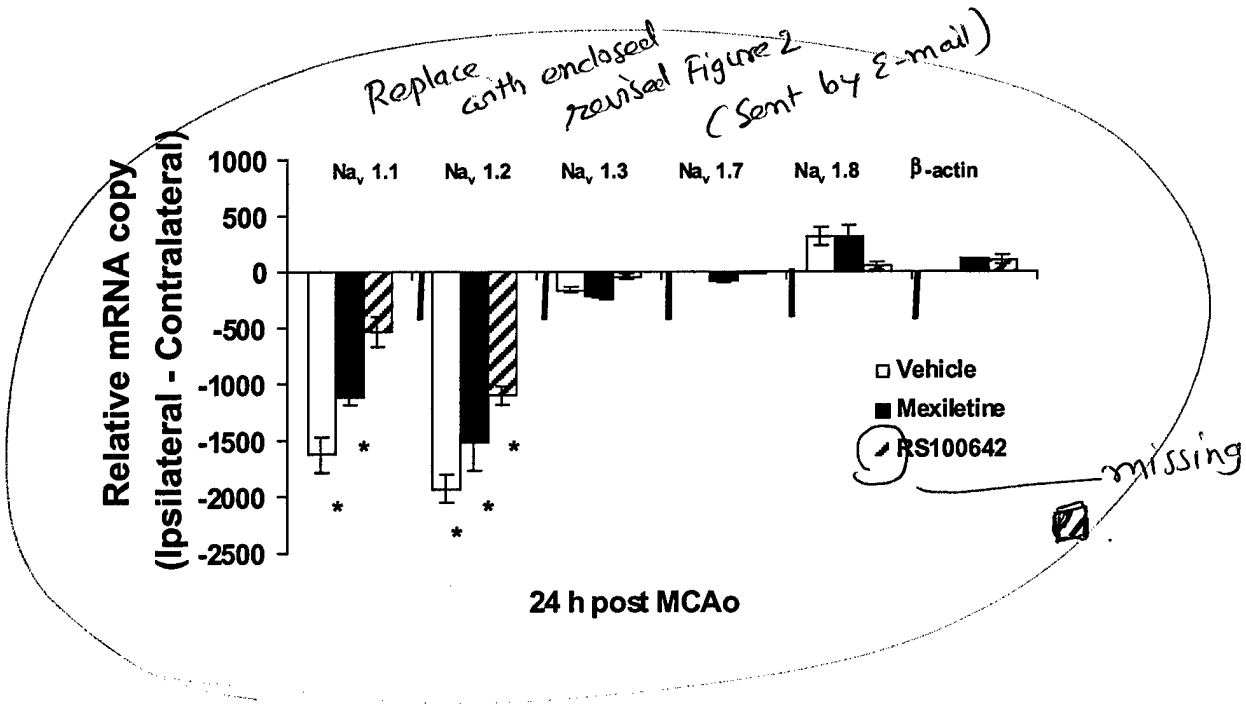

FIGURE 2 Relative expression of five NaCh α-subunits and β-actin (ipsilateral minus contralateral levels) at 24 h post-MCAo in vehicle (open bars), mexiletine (solid bars) or RS100642 (cross-hatched bars) treated animals. Values are mean ± SEM of 10 brain samples. Values marked with an asterisk (for Nav 1.1 and Nav 1.2) are significantly different between the contralateral and ipsilateral hemisphere at $P < 0.05$.

only the vehicle and mexiletine treated groups exhibited a significant down-regulation of $Na_v1.1$ between ipsilateral and contralateral hemispheres (FIG. 3C, 3D). Specifically, at 24 h post-MCAo Na_v 1.1 mRNA levels were significantly reduced by 73% and 61% in vehicle and mexiletine treated animals (FIG. 3C). RS100642 treated animals also exhibited a 30% decrease in mRNA expression at 24 h but there was not a significant difference between contralateral and ipsilateral values (FIG. 3C). Similarly, at 48 h post-MCAo Na_v 1.1 mRNA levels were significantly reduced by 76% and 62% in vehicle and mexiletine treated animals but RS100642 treated animals only exhibited a decrease of 38% which was not significantly different from the corresponding contralateral values (FIG. 3D).

DISCUSSION

The present study describes changes in brain type ($Na_v1.1$, Na_v 1.2 and Na_v 1.3) and peripheral nerve type (Na_v 1.7 and Na_v 1.8) NaCh α-subunit gene expression using real-time quantitative RT-PCR in rat brain following ischemia/reperfusion injury. We compared the treatment effects of two NaCh blockers (RS100642 and mexiletine) on the time course change in expression of these genes. The key finding of our investigations was that RS100642 selectively reversed the delayed down-regulation (24-48 h post-injury) of Na_v 1.1 with no significant effect on the expression of other α-subunit genes. These data are consistent with our recent *in vitro* findings, in which treatment of primary cultures of forebrain neurons with the NaCh activator veratridine selectively down-regulated Na_v 1.1 gene expression and was completely reversed by RS100642 treatment (Dave *et al*, 2003).

One of the primary factors detrimental to neurons following an ischemic insult is decreased energy supply in conjunction with an increased energy requirement due to excitotoxic over-activation of neurons. Considering the fact that increasing energy supply has been used as a strategy for protection against ischemic brain damage (Obrenovitch, 1995), down-regulation of NaChs may be another effective way to reduce energy demand. In fact, a large part of the energy consumed by the brain is used for maintenance of ionic gradients by Na^+/K^+ ATPase activity (Erecinska and Silver, 1989). Others have also suggested that decreasing neuronal membrane ion conductance may increase neuronal tolerance to ischemia (Jiang and Haddad, 1992; Cummins *et al.*, 1993). These studies reported that reduction in Na^+ conductance could reduce membrane excitability with preservation of membrane potential and input resistance in rat neurons. Further support of this relationship between down-regulation of NaChs and ischemic insult comes from the observation that diving turtles exhibit down-regulation of NaCh expression during hypoxic periods possibly as a survival/protective strategy (Perez-Pinzon *et al.*, 1992; Xia and Haddad, 1994). Reduction in the density of NaChs during oxygen deprivation may be primarily

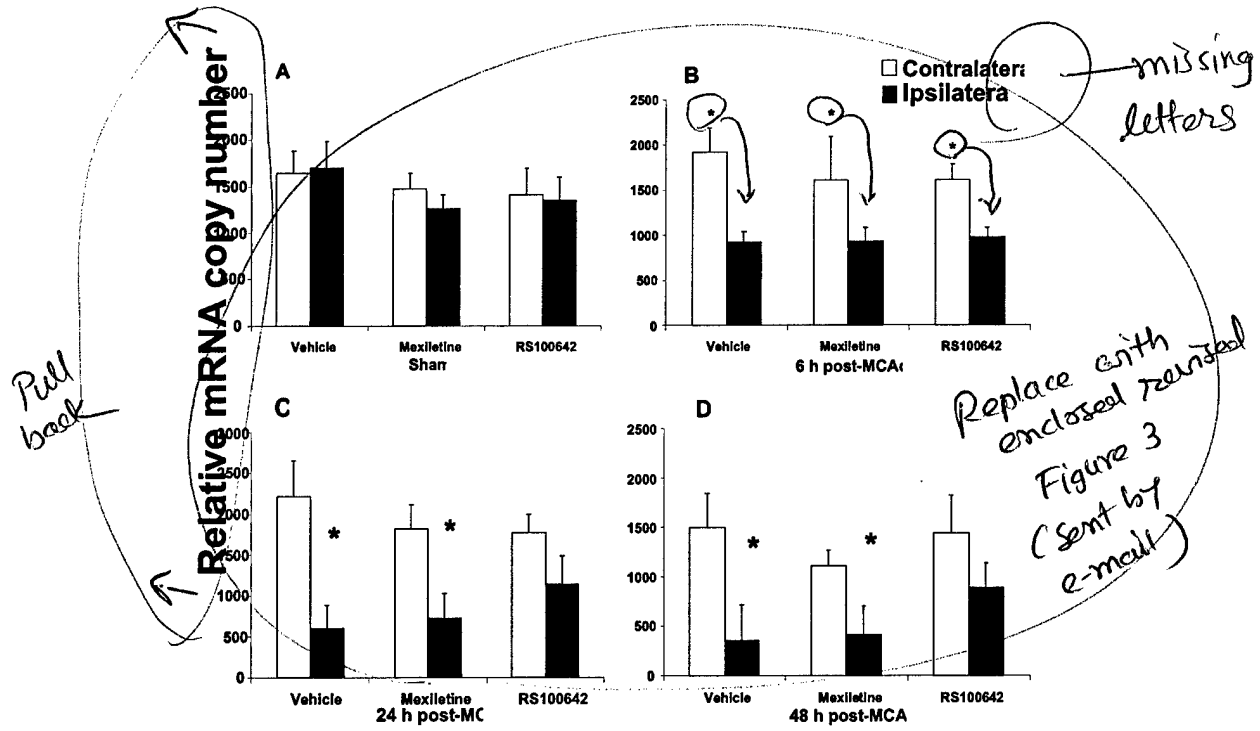

FIGURE 3 Relative expression of Na_v 1.1 in sham control (A) or in injured (MCAo) animals treated with vehicle, mexiletine or RS100642 at 6, 24 or 48 post-MCAo (B-D). Open bars represent values for contralateral hemispheres and filled bars represent values from injured (ipsilateral) hemispheres. Values are mean ± SEM of 10 brain samples. Values marked with an asterisk are significantly different from their respective contralateral values at $P < 0.05$.

responsible for decreased membrane excitability and energy demand. These changes have been demonstrated to contribute to the higher tolerance of the immature brain to hypoxia (Xia and Haddad, 1994). In the present study and in our earlier studies (Yao *et al.*, 2002) we have demonstrated that down-regulation of NaCh genes does in fact occur following ischemic brain injury in the rat. Several studies by other groups have also demonstrated a reduction in the density and functionality of NaCh proteins during hypoxic insults (Cherubini *et al.*, 1989; Dargent and Couraud, 1990; Cummins *et al.*, 1994; Xia and Haddad, 1994). Similarly, a decline in the number and conductance of NaChs (Dargent and Couraud, 1990) and down-regulation of NaCh gene expression (Na_v 1.1; Dave *et al.*, 2003) have been reported following increased sodium influx induced by NaCh activators such as veratridine.

RS100642 is a new analog of the use-dependent NaCh blocker mexiletine with voltage-gated NaCh blocking ability and neuroprotective properties (Dave *et al.*, 2001). In the earlier *in vitro* study we reported that in primary neuronal cultures RS100642 provided complete neuroprotection against injuries caused by hypoxia/hypoglycemia (i.e. ischemic injury) or the NaCh activator veratridine, but not glutamate (Dave *et al.*., 2001). Furthermore, in the same study RS100642 attenuated the rise in intracellular Ca^{2+} produced by veratridine or KCl, but not that by glutamate, indicating a selective modulation of NaChs by these insults. Additionally, although RS100642 was reported to possess NaCh binding properties similar to mexiletine (pK_i = 5.09 mM), it exhibited a more potent use-dependent reduction in compound action potentials as determined by rat vagus nerve preparations (Dave *et al.*, 2001).

In the present study significant down-regulation of the Na_v 1.1 gene started 6h post-MCAo in the ischemic hemisphere of the brain, and treatment with either NaCh blocker did not reverse the down-regulation of Na_v 1.1 at 6h post-injury. As noted above, this early down-regulation of NaChs may be part of the brain's immediate response to over-excitation induced by the excitotoxic state of an ischemic insult and during later stages of ischemia, constant down-regulation of NaCh genes may contribute substantially to a reduced neurophysiological recovery of the brain. As the reperfusion time was extended to 24 and 48h post-MCAo, we measured a significant recovery of Na_v 1.1 with RS100642 treatment but not mexiletine treatment. In fact, the delayed reversal of Na_v1.1 down-regulation (24-48 h post-MCAo) induced by treatment with RS100642 may indeed be associated with a return of neuronal function to pre-injury levels as these results correlate to the improved neurological and neurophysiological improvements we observed with this same dose of RS100642 for the treatment of MCAo injury as measured 24 and 72 h post-injury (Williams and Tortella, 2002). Importantly, RS100642 treatment alone did not alter normal Na_v 1.1 expression in sham-injured animals. In this context it is worth mentioning

that in our earlier study RS100642 was found to be more efficacious and 10-fold more potent for the treatment of MCAo injury than mexiletine (Williams and Tortella, 2002); and in the present study mexiletine failed to significantly reverse MCAo-mediated down-regulation of Na_v 1.1 or Na_v 1.2 genes. Studies are currently underway in our laboratory to determine the respective protein levels of these NaCh subunits using immunohistochemistry and Western blot analysis.

Pharmacological modulation of voltage-sensitive NaChs is considered to be a rational and effective therapeutic strategy against neuronal damage in cerebral ischemia (Rataud *et al.*, 1994; Carter 1998). The idea of a novel therapeutic compound with selective properties for blockade of one subtype of neuronal NaCh with little action on other NaCh subunits is attractive. However, the high degree of sequence homology between NaCh subunits makes it difficult to design selective NaCh blockers. Some studies with molecular biological approaches, particularly site-directed mutagenesis of NaCh proteins and functional expression of mutant channels have pinpointed regions that are involved in different functions of the channel (Catterall, 1992). Although at the present time the differences in interaction or binding affinity of RS100642 to Na_v 1.1 or Na_v 1.2 is not known, our data collectively suggest that RS100642 may be a potential selective NaCh blocker for the Na_v1.1 subunit. Future binding studies using recombinant subunit proteins may provide a clearer answer to channel selectivity of RS100642.

In conclusion, the present study supports the hypothesis that down-regulation of NaCh genes is an endogenous reaction of brain cells in the early stages of ischemic brain injury. Neuroprotective doses of the NaCh blocker RS100642, but not mexiletine, were able to reverse the down-regulation of Na_v 1.1 but only during the later stages of injury. Furthermore, effective treatment with RS100642 can be achieved without conspicuous undesirable changes in the expression of other NaCh subunits. These effects may suggest that RS100642 is selective for the Na_v 1.1 subunit, as the levels of Na_v 1.2 expression were still down-regulated following RS100642 treatment. Although the mechanism of selective reversal of Na_v 1.1 by RS100642 is still unclear, these effects may be related to the neuroprotective properties of this compound indicating that regulation of Na_v 1.1 expression may be an important strategy for improving outcome from ischemic brain injury.

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Neuroprotection assessment by topographic electroencephalographic analysis: effects of a sodium channel blocker to reduce polymorphic delta activity following ischemic brain injury in rats

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Abstract: The spatiotemporal electroencephalogram (EEG) pathology associated with brain injury was studied using high resolution, 10-electrode cortical EEG mapping in a rat model of middle cerebral artery occlusion (MCAo). Using this model we evaluated the ability of the novel sodium channel blocker and neuroprotective agent RS100642 to resolve injury-induced EEG abnormalities as a measure of neurophysiological recovery from brain injury. The middle cerebral artery (MCA) was occluded for 1 h during which a dramatic loss of EEG power was measured over the injured cortex with near complete recovery upon reperfusion of blood to the MCA region in all rats. The resultant progression of the MCAo/reperfusion injury (6-72 h) included the appearance of diffuse polymorphic delta activity (PDA) as visually indicated by the presence of high-amplitude slow-waves recorded from both brain hemispheres. PDA was associated with large increases in EEG power, particularly evident in outer "peri-infarct" regions of the ipsilateral parietal cortex as visualized using topographic EEG mapping. Post-injury treatment with RS100642 (1.0 mg/kg, i.v.) significantly reduced the PDA activity and attenuated the increase in EEG power throughout the course of the injury. These effects were associated with a reduction in brain infarct volume and improved neurological function. These methods of EEG analysis may be helpful tools to evaluate the physiological recovery of the brain from injury in humans following treatment with an experimental neuroprotective compound.

Key Words: sodium channels, EEG, MCAo, ischemia, RS100642, stroke

Introduction:

Normal brain function requires a complex integration of electrochemical signaling, which is altered in disease states or injuries such as epilepsy and traumatic or ischemic brain injury. Electrophysiological abnormalities that occur following recovery from brain injury include the induction of polymorphic delta activity (PDA), non-convulsive brain seizures (NCS), and periodic lateralized epileptic discharges (PLEDs) (1-4). Although the direct role of these electrophysiological phenomena as they pertain to the progression of brain injury has not been clearly defined they are certainly involved in the pathological outcome. Resolution of these electrophysiological disturbances may, in fact, correlate to a reduced cellular injury and improved functional recovery.

The use of clinical EEG is often supplanted by other neuroimaging techniques including magnetic resonance imaging (MRI), positron emission tomography (PET) and computerized tomography (CT). Quantitative and topographic EEG mapping may still be useful tools for evaluating the ischemic brain in clinical research, as well as providing an indirect measure of changes in cerebral metabolism (1, 5-9). EEG has been compared to cerebral blood flow in brain injury patients with a significant correlation measured between regional blood flow and changes in the delta EEG band (0-4 Hz) in human patients suffering unilateral cerebral infarction (10-13). Techniques such as topographic EEG mapping offer specific advantages as well as complements to other methods as a clinical assessment of brain injury including; 1) measurement of physiological changes (functional recovery) as opposed to anatomical changes (lesion size), 2) identification of specific electrocortical patterns associated with brain injury (i.e. NCS, PDA, and PLEDs),

3) temporal profile of changes occurring over time, and 4) a spatial relationship of electrical changes across the entire brain surface.

Loss of blood flow following brain ischemia restricts cerebral function by depleting available energy reserves and limiting oxidative metabolism, which are crucial for normal cellular function. Neurons rapidly lose the ability to maintain cellular membrane potentials due to the reduced ATP levels necessary for Na^+/K^+ ATPase activity. The resulting loss of ionic homeostasis leads to sustained increases in intracellular ions such as Na^+ and Ca^{++}. The consequence includes activation of cellular death cascades such as necrosis, apoptosis and inflammatory cell induced phagocytosis (14, 15). Pharmacological agents possessing voltage-gated sodium channel blocking activity are currently used clinically as anticonvulsants. Sodium channel blockers have also been shown to possess neuroprotective properties at least in experimental models of neuronal injury (16, 17). In particular, we have recently reported that the experimental sodium channel blocker RS100642 possesses both neuroprotective as well as anti-seizure effects in the MCAo/reperfusion model of ischemic brain injury in the rat without affecting body temperature(18). These studies indicated RS100642 to be an extremely well tolerated analog of mexiletine void of the cardiovascular and seizure-inducing side-effects associated with mexiletine (18, 19). The present data represents a comprehensive off-line EEG analysis of the same study where we utilize high resolution topographic EEG mapping in the rat MCAo model as a clinically relevant model of ischemic brain injury, which includes the sequela of EEG pathology observed in human brain injury (2, 3).

Materials and Methods:

Surgical Procedures and EEG Electrode Placement.

Male Sprague-Dawley rats (270-330g; Charles River Labs, Raleigh, VA) were used in all of the following procedures. Animals were housed in specially designed plexiglas cages (Dragonfly, Ridgeley, WV) equipped with free-swivel EEG commutators (Plastics One, Roanoke, VA) allowing EEG to be recorded continuously in awake, unrestrained animals. Anesthesia was induced by 5% halothane and maintained at 2% halothane delivered in oxygen. Indwelling intravenous (i.v.) cannulas (PE-50) were placed into the left jugular vein of all animals for drug delivery. Epidural electrodes (stainless steel screw electrodes, 0-80 x 1/8 in) were permanently implanted and fixed to the skull using dental acrylate cement (as described by Lu et al., 2001). Electrodes were stereotaxically placed as shown in figure 3, covering the bilateral frontal (F1-F2), parietal (P1-P4), and temporal (T1-T4) brain regions referenced to an electrode implanted over the occipital cortex (R). Odd numbers represent electrodes on the left hemisphere (contralateral to the injury site) and even numbers represent electrodes on the right hemisphere (ipsilateral to the injury site). Body temperature was maintained normothermic (37 ± 1°C) throughout all surgical procedures by means of a homeothermic heating system (Harvard Apparatus, South Natick, MA). Food and water were provided "ad libitum" pre- and post-surgery and the animals were individually housed under a 12 h light/dark cycle.

MCAo Procedure

Three to five days following the surgical procedures described above the rats were re-anesthetized with halothane and prepared for temporary focal ischemia using the filament method of MCAo and reperfusion (20) as described in detail elsewhere (21). Post-surgery, the animals were returned to their EEG recording cage and allowed to recover from anesthesia. The endovascular suture remained in place for 1 h and then the animal was reanesthetized under halothane and the filament retracted to allow reperfusion of blood to the MCA. Following the reperfusion surgery, animals were again placed in their EEG recording cage with ambient temperature maintained at 22°C.

Infarct analysis and neurological scoring

The methods and results from this study for measurement of both brain infarction and neurological scoring were reported in Williams et al. (2002). In brief, Triphenyltetrazolium chloride (TTC) was used to visualize brain infarction from 7 coronal brain slices, which were integrated to obtain a final core infarct volume (Inquiry Digital Analysis System, Loats Ass., Westminster, MD). Neurological scoring was based on a weighted 10-point scale, giving a positive score for each neurological deficit including forelimb flexion, shoulder adduction, reduced resistance to lateral push, and circling to the injured side of the body (modification of the method developed by Bederson et al., 1986 (22)).

EEG recording

EEG was recorded from Grass Model 7D (Grass Instruments, Natick, MA) polygraph and stored digitally using a Harmonie EEG software system (Astro-Med, West Warwick, RI) at a sample rate of 128 Hz. EEG signals were recorded from each of the 10 cortical electrodes on the morning of the experiment before induction of MCAo and continuously for 6 hours following the injury except during surgical procedures. Fifteen minute EEG samples were again recorded at 24, 48 and 72 h post-MCAo.

EEG analysis: visual, computational, and topographic mapping

The analog EEG signals were examined visually for signs of epileptic activity, sharp waves, spike/wave complexes, large amplitude slow waves, and/or depression of background activity. Behavioral changes were also noted including myoclonic or tonic convulsion, ataxia, head-weaving, sedation, and/or circling movement. Computer assisted spectral analysis was performed on a continuous 60 second sample of each of the 10-electrode EEG tracings pre-MCAo (baseline), 1, 6, 24, 48, and 72h post-MCAo. EEG frequencies were divided into 4 bands: delta (0-4 Hz), theta (4-8 Hz), alpha (8-12 Hz) and beta (12-30 Hz). The respective power scores from each of the frequency bands were graphed over the 72 h recovery period following MCAo. Power scores were used to generate a topographic map using the Harmonie EEG analysis software at each time point and again presented as the time course change during the progression of the injury over 72h.

RS100642 treatment.

RS100642 was received from Roche Pharmaceuticals (Palo Alto, CA). The compound was dissolved in a vehicle of distilled, deionized water immediately prior to testing and administered in a volume of 1ml/kg of body weight. Injections (1ml/kg, i.v.) of RS100642 (1.0mg/kg, n = 5) or vehicle (n = 5) were initiated 30 min post-occlusion with additional treatments given at 2, 4, 6, 24 and 48 h.

Data analysis.

Unless otherwise noted, statistical analyses on EEG parameters were performed by two-way ANOVA followed by Fisher's post-hoc analysis to evaluate both time and treatment effects following MCAo. Pearson correlation analysis was performed to evaluate the relationship between EEG power at each time point and neurological score or brain infarct volume.

Results:

Topographic EEG mapping of global power

Figure 1 displays the time course change in EEG power (0-30 Hz) from a representative vehicle (1A) and RS100642 (1B) treated animal, as seen across the entire cortical surface using topographic mapping of the EEG power values. The right column compares the histopathology at 72 h post-injury (TTC staining) of each animal to its corresponding topographic map throughout the course of the injury. Note the drop in EEG power ipsilateral to the injured hemisphere during the occlusion at 1 h, followed by recovery at 6 h, with dramatic increases from 24-48 h and gradual resolution beginning 72 h post-injury. Interestingly, we measured substantial increases in EEG power in the contralateral hemisphere throughout the course of the injury as well. Rats given RS100642 exhibited a similar drop in EEG power at 1 h over the ipsilateral cortex followed by recovery until marked resolution at 72 h. Importantly though, RS100642 treatment attenuated the increase in EEG power across the entire cortical brain surface.

Figure 2 presents topographic maps of the four EEG frequency bands at 24 h post-injury from a vehicle and RS100642 treated rat. The baseline (BL) maps of each animal are directly compared to the maps obtained at 24 h. The increases in power following MCAo shown to occur across the entire cortical surface are exclusively confined to the low frequency delta band as seen in the vehicle treated rat brain. RS100642 treatment is seen to attenuate the increased EEG delta power. All representative topographic maps were selected based on the average power scores for the entire group at each time point (see results of power analysis below).

Visual analysis of EEG waveform

Induction of MCAo caused a drop in EEG amplitude over the ipsilateral hemisphere in all animals, which continued for the 1 h duration of the occlusion and recovered following reperfusion of blood to the MCA (figs. 3&4). The appearance of PDA also occurred during reperfusion, predominantly in vehicle treated animals, as indicated by large slow-wave activity in the EEG signal (fig. 3, arrows). PDA primarily occurred in the ipsilateral parietal cortex (peri-infarct region) and continued at 24 and 48 h until eventually starting to dissipate 72 h post-MCAo. Interestingly, PDA was also evident in the uninjured, contralateral hemisphere. The RS100642 treatment group also exhibited the initial loss of EEG amplitude during the MCAo but had markedly reduced PDA activity following reperfusion. This reduction of PDA in the RS100642-treated animals was consistent throughout the entire course of the injury (fig. 4).

EEG Spectral analysis

Figure 5 demonstrates a representative spectral plot of baseline EEG as compared to EEG sampled at 1 h post-MCAo (immediately prior to reperfusion) and during a PDA episode as recorded 24 h post-MCAo. MCAo significantly reduced the power spectra across all frequencies. Post-reperfusion PDA activity, however, was associated with large increases in power in the low frequency delta band similar to the shift in EEG power across the brain surface to the low frequency range.

Figure 6 presents the time course change in EEG power from the T2 (ipsilateral temporal cortex), P2 (ipsilateral parietal cortex), and T1 (contralateral temporal cortex) electrodes. The spectral composition of cortical EEG waveforms was consistent

throughout the entire brain prior to injury (fig. 6). The average power distribution across the frequency spectrum (0-30 Hz) was divided into 25% delta, 40% theta, 15% alpha, and 20% beta (% of total power). Following MCAo injury these values were severely disturbed.

(i) ipsilateral hemisphere

Power values over the injured temporal cortex exhibited a drop at 1 h post-occlusion in all treatment frequency bands except for the delta band, which showed only a slight shift in power (fig. 3, T2 electrode). At 6 h post-injury, power values recovered to baseline although delta power was slightly increased in all groups. Significant changes were not observed until 24-48 h post-injury as compared to baseline. In vehicle treated animals delta power was significantly higher at 24 h in both the T2 ($p<0.05$) and T4 ($p<0.05$, not shown) regions, which gradually returned to baseline values by 72 h. In contrast, RS100642 treated animals exhibited no significant changes in EEG power values during the 24-72 h recovery period.

Frontal / Parietal power values over the injured cortex also showed a significant drop in all treatment groups at 1 h post-occlusion except for the delta band which showed only a slight shift in power (fig. 3, P2 electrode). At 6 h post-injury, power values recovered although delta power was increased. Again, significant changes were not observed until 24-48 h post-injury. In vehicle treated animals delta power was significantly higher (as compared to baseline) at 24 h in both the P2 ($p<0.05$) and P4 ($p<0.05$, not shown) regions, which gradually returned to baseline values by 72 h. RS100642 treated rats exhibited increases in delta power during the reperfusion period

but these increases were not significant ($p>0.05$). Additionally, all other band regions stabilized to near normal values during the reperfusion/recovery period following RS100642 treatment.

(ii) contralateral hemisphere

Power values over the uninjured temporal cortex in vehicle treated rats showed increased delta and theta activity at 1 h post-occlusion although not significantly ($p > 0.05$, fig. 6, T1 electrode). This trend of increased delta and theta power started to subside by 6 h but continued to be elevated throughout the later course of the injury (24-48 h) and eventually began to return to baseline values by 72 h. The increases in both delta and theta power were significant in the T1 region at 24 h post-injury ($p<0.05$). The alpha and beta bands in the contralateral hemisphere remained stable and seemed to be unaffected by MCAo and reperfusion in the ipsilateral hemisphere. RS100642 treated animals did not exhibit significantly altered power values in any of the band regions during the 24-72 h recovery period although a trend for increases in delta and theta power were observed as early as 6 h especially in the T1 brain region.

Frontal / Parietal power values over the contralateral hemisphere remained normal during the 1 h occlusion period but were significantly higher (as compared to baseline) during the reperfusion period (similar to the ipsilateral cortex) with increases predominately confined to the delta band region (data not shown). RS100642 treatment attenuated the increases in EEG delta power in this brain region as well.

Correlation between P2 and T2 delta power and neurological recovery

Figure 7 (upper panel) compares average EEG delta power to neurological score and infarct volume of vehicle treated rats. EEG power from both the T2 and P2 electrodes were significantly increased during the reperfusion period and began to subside by 72 h. We measured a moderate 0.468 correlation between the increase in P2 delta power and the increase in neurological deficit at 24 h post-MCAo. However, neurological score recovered dramatically at 48 and 72 h and revealed a low correlation (-0.090, -0.029, respectively) to P2 delta power. At 24 and 72 h post-MCAo the increase in T2 delta power also exhibited a moderate correlation (0.605 and 0.339, respectively) to the increase in neurological deficit. In contrast, at 48 h T2 delta power did not change significantly as compared to the dramatic recovery in neurological score with a low correlation (0.007) between the two parameters. EEG delta power and infarct volume were not significantly correlated at any time point.

Figure 7 (lower panel) indicates the effect of RS100642 treatment (as compared to vehicle, upper panel) on EEG delta power in the ipsilateral parietal and temporal cortices as related to the neuroprotective reduction in both neurological deficit and infarct volume. At 24 h post-MCAo RS100642 treatment reduced P2 and T2 delta power scores by 46% and 43%, respectively, which was associated with a 48% improvement in neurological score. Similarly, at 48 h RS100642 treatment reduced both P2 and T2 delta power scores by 56%, which was associated with a 63% improvement in neurological score. At 72 h no significant change in P2 delta power was measured but a 34% decrease in T2 power occurred, which was associated with a 77% improved neurological score.

This reduction of EEG delta power (34-63%, 24-72 h post-MCAo) following RS100642 treatment was also associated with a 65% reduction in core infarct volume.

Discussion:

Ischemic brain injury remains a major health concern with few experimental drugs available that provide significant relief of or recovery from the ensuing brain damage. In this study we explored the neurophysiological recovery of the rat brain following MCAo injury as assessed by quantitative EEG (QEEG) analysis and topographic mapping as compared to histological and neurological deficits. EEG monitoring allows researchers to assess the abnormal electrical activity induced by insults such as ischemia and thus provides a direct non-invasive real time assessment tool of brain infarction that may prove useful for the study of neuroprotection and functional recovery. Furthermore, EEG analysis provides a highly quantitative and descriptive neurophysiological link between neuronal pathology and the neurological deficits induced by altered brain function.

Sodium Channels (NaCHs) play an integral part in neuronal signaling involving the transduction of electrochemical signals between cells. NaCHs are predominately located along axons in the nodes of Ranvier with lower channel densities in the cell body and terminal synapses of neurons (23). Following injury however, it has been reported that the expression of NaCH genes are down-regulated (24). Sodium channels consist of the channel forming α-subunit and a channel modulating β-subunit. All known NaCH blockers bind to the α-subunit and modulate its function (25). Most of the therapeutic NaCH blockers, including RS100642, bind to the NaCH in a use-dependent manner (17). This property of use-dependence allows inhibition of repeated depolarizations, such as occurs during seizure or excitototoxicity, without altering normal neuronal firing. Many currently used NaCH blockers are not selective for NaCHs and may also bind other

closely related ion channels such as calcium channels (i.e. phenytoin, lifarizine). Recently, the development of specific NaCH blockers (i.e. BW619C89 (CeNeS Pharm.) and BIII-890-CL (Boehringer Ingelheim Pharm.)) has led to studies aimed at the treatment of Stroke (26, 27).

In this study we evaluated the post-injury EEG changes in a model of transient focal ischemia (i.e. MCAo) in rats, which we have previously reported as a clinically relevant experimental model of the EEG pathology associated with human brain injury (2, 3). Visual analysis of EEG changes following MCAo indicated that during occlusion of blood flow to the MCA a drop in amplitude was recorded in all rats regardless of treatment, which recovered following reperfusion. One of the key neurophysiological changes apparent following reperfusion was the appearance of PDA, predominately in the peri-infarct region of the injury but also present from nearly every electrode recorded across the entire brain surface. The prolonged increase in PDA common to all vehicle-treated rats appears to be a phenomenon inherent to reperfusion injury as previous studies of permanent MCAo have described either no PDA during the recovery phase, or a mix of PDA and intermittent rhythmic delta activity (2, 3). PDA has also been shown to be a common phenomenon of ischemic brain injury in humans and appears to indicate a greater degree of injury (5). In this study, treatment with RS100642 was highly effective at reducing the presence of PDA as well as EEG power throughout the course of the injury. Spectral analysis of the PDA waveforms revealed large increases in low frequency delta power, which corresponded the observed frequency of the PDA waveform (i.e. 1-4 Hz). Thus, the increases in delta power observed in the post-reperfusion EEG waveforms (24-72h) were predominately due to the underlying PDA.

The mechanisms underlying these changes in post-ischemic EEG patterns are currently unknown but may be related to the alteration in expression of NaCH genes. In particular, it has been reported that the down-regulation of NaCH genes following MCAo in rats is reversible with RS100642 treatment (28).

The use of topographic EEG mapping has been used in the past to detect subclinical stroke in asymptomatic patients as well as for predicting patients who will present with post cartotid endarterectomy induced neurological deficits (29). Furthermore, topographic EEG mapping has been reported as a useful, high-resolution tool for detecting and localizing subtle EEG changes following ischemic injury in humans (30, 31). In the present study, changes in EEG power across the brain surface were also visualized utilizing topographic EEG mapping of brain function, which allowed us to observe electrical changes across the entire brain surface during the course of the injury. For instance, topographic mapping demonstrated the unilateral drop in EEG power in the ischemic hemisphere during the occlusion of the MCA, which we have used in past studies as a correlate of a successful occlusion (18, 32, 33). Interestingly, early on following reperfusion EEG power recovered quite dramatically towards baseline values and by 24 h significant increases were observed across the entire brain surface of vehicle but not RS100642 treated rats. By dividing the power spectra into bands we verified that these increases were isolated to the delta band region. Once again, this increase in delta power over the injured region seems to be due to the appearance of PDA activity signifying an abnormal state of brain function.

One important aspect of our QEEG results suggests that there may be a correlation between EEG delta power during the early post-reperfusion period (24 h) and

functional outcome. Those animals that exhibited the largest increases in delta power at 24 h from the ipsilateral cortex suffered the most extreme neurological deficits during the reperfusion phase of the injury. Furthermore, the reduction of EEG delta power following treatment with RS100642 was associated with a similar degree of improvement in neurological score. Cohen et al. (1) reported a correlation between EEG slowing and infarct volume but not neurological function following MCAo, although they induced a permanent occlusion as opposed to the reperfusion model of MCAo that we have used. Currently, no clinical data has been reported evaluating QEEG as related to functional recovery in humans following ischemic brain injury (34). Assuming the relationship between PDA and neurological function also occurs in human head injury patients, QEEG analysis may provide an important measure of recovery following experimental treatment with a neuroprotective compound such as RS100642.

Another interesting aspect of the MCAo/reperfusion injury observed in this study was the EEG alteration in the "uninjured" contralateral hemisphere. This distant involvement or recruitment of brain activity peripheral to the site of injury has been termed diaschisis, which may contribute to neurological deficits observed post-injury (35). The effects of ameliorating EEG changes in the contralateral hemisphere are however unknown. These changes in the contralateral hemisphere do not correspond to brain regions that progress into pathologically necrotic tissue but these EEG alterations may be a consequence of the neuroregenerative adaptation of the brain to injury. Several studies have shown the ability of the brain to adapt to injury as related to CNS "rewiring" of neuronal networks (36, 37). In particular, this could explain the high EEG delta power activity observed from 24-72 h post-injury, a time period related to dramatic

improvements in neurological recovery even in vehicle-treated animals. Similarly, Carmichael and Chesselet (38) have recently reported a correlation between axonal sprouting and low frequency neuronal activity (0.1-4.0 Hz) 24-72 h following thermal-ischemic lesions to the sensorimotor cortex of the rat brain.

Conclusion

We have shown that post-injury treatment of ischemia/reperfusion brain injury in rats with RS100642 reduces the incidence PDA and attenuated the increases in EEG delta power across the entire brain surface as observed from spectral analysis of the EEG waveform and topographic EEG mapping. Importantly, the reduction in delta power in the ipsilateral brain hemisphere was correlated with improved neurological score as well as being associated with a reduction of infarct volume. In effect, these methods of QEEG analysis and topographic mapping may extend to clinical research for assessment of neurophysiological recovery induced by experimental neuroprotection compounds following ischemic brain injury in humans.

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Figure Titles:

Figure 1. Representative topographic EEG maps throughout the time course of the ischemia/reperfusion injury from vehicle and RS100642 treated rats. The right column shows the corresponding coronal brain slices of each rat stained with TTC to indicate infarct size.

Figure 2. Representative topographic EEG maps from each band region at 24 h post-injury as compared to baseline values from a representative vehicle and RS100642 treated rat. The increase in EEG power in vehicle treated rats is isolated to the delta band and is shown to occur across the entire cortical surface.

Figure 3. Representative EEG waveforms from a vehicle-treated rat following MCAo/reperfusion injury. The topographic location of each recording electrode is indicated on the rat skull to the right. Arrows indicate typical high amplitude, slow-wave polymorphic delta activity (PDA) waveforms.

Figure 4. Representative EEG waveforms following MCAo/reperfusion injury when rats were treated with RS100642 (1.0 mg/kg, i.v.) 30 min post-MCAo.

Figure 5. Comparison of spectral frequency plots from baseline (pre-MCAo), MCAo (during occlusion), and PDA (22 h post-reperfusion) waveforms. Note the dramatic loss of EEG power during occlusion of the MCA as opposed to the large increase associated with PDA.

Figure 6. Time course changes in EEG power are shown from each treatment group divided into four frequency bands; delta (0-4 Hz), theta (4-8 Hz), alpha (8-12 Hz), and beta (12-30 Hz). Samples shown are cortical EEG recordings from the T2 (core infarct region), P2 (peri-infarct region) and T1 (uninjured hemisphere) electrodes. Vehicle treatment is compared to treatment with RS1000642.

Figure 7. Comparison of EEG delta power (μV^2) scores from the P2 and T2 cortical electrodes to neurological score at 24, 48, and 72 h post-MCAo between vehicle (upper panel) and RS100642 (lower panel) treatment. Neurological scores are multiplied by 10 for comparison. Final core infarct volume (mm^3) is also shown as assessed 72 h post-MCAo. Values are given as mean ± S.E.M. * $P < 0.05$, independent t-test between each parameter of the vehicle (n = 5, upper panel) and corresponding RS100642 (n = 5, lower panel) treated group.

Footnotes:

*Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 edition. Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army.

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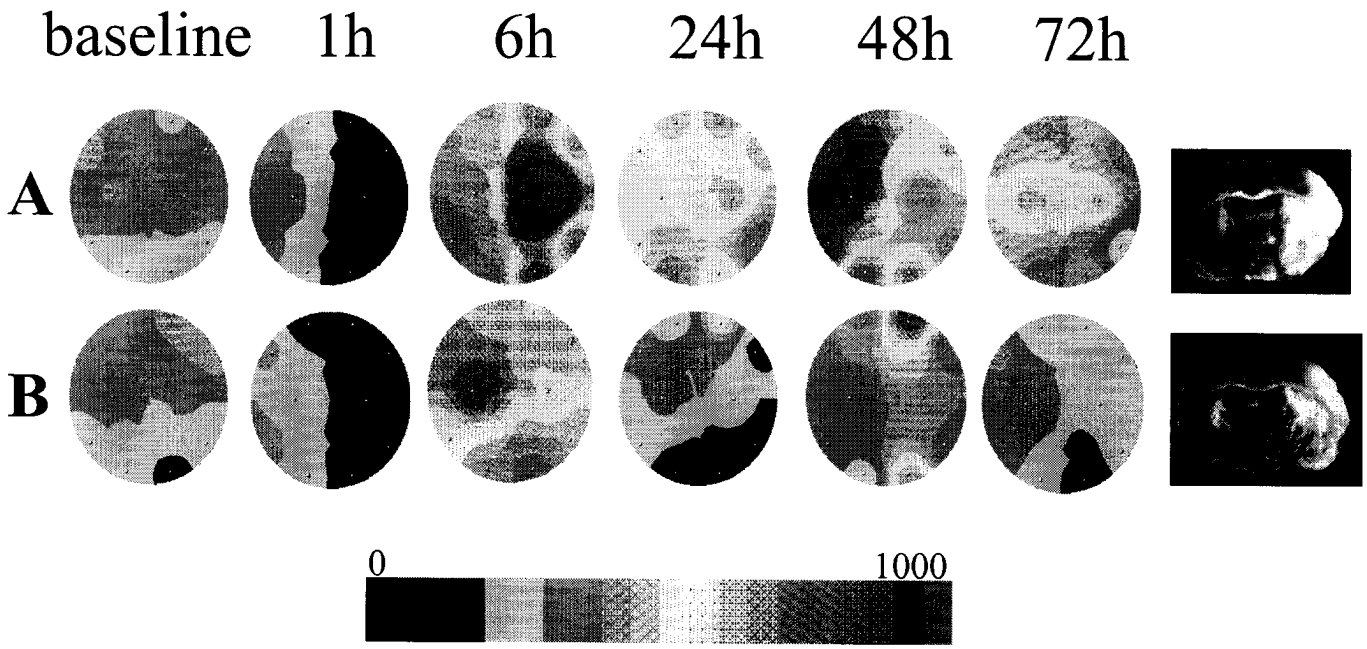
Figure 1



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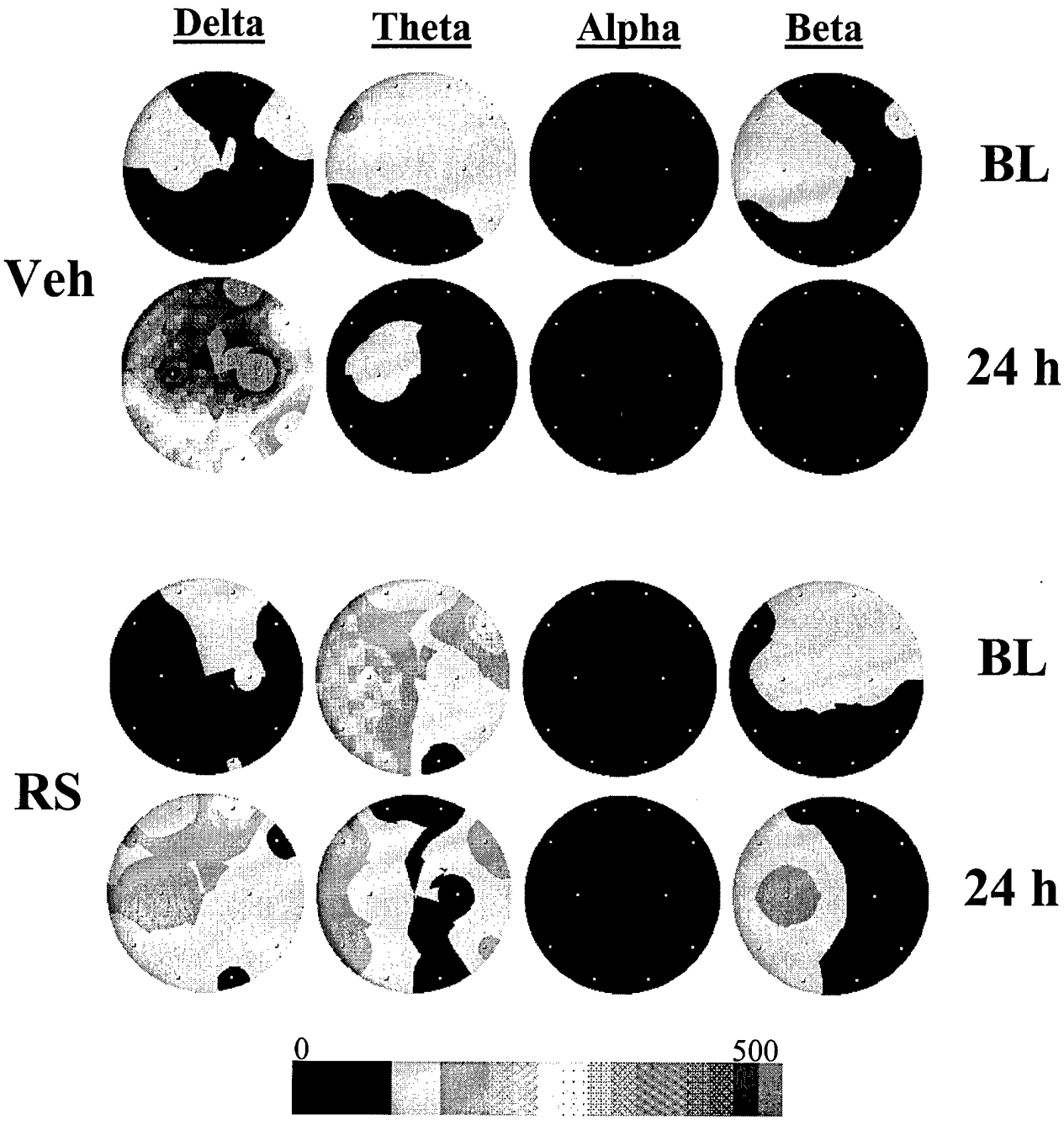


Figure 3

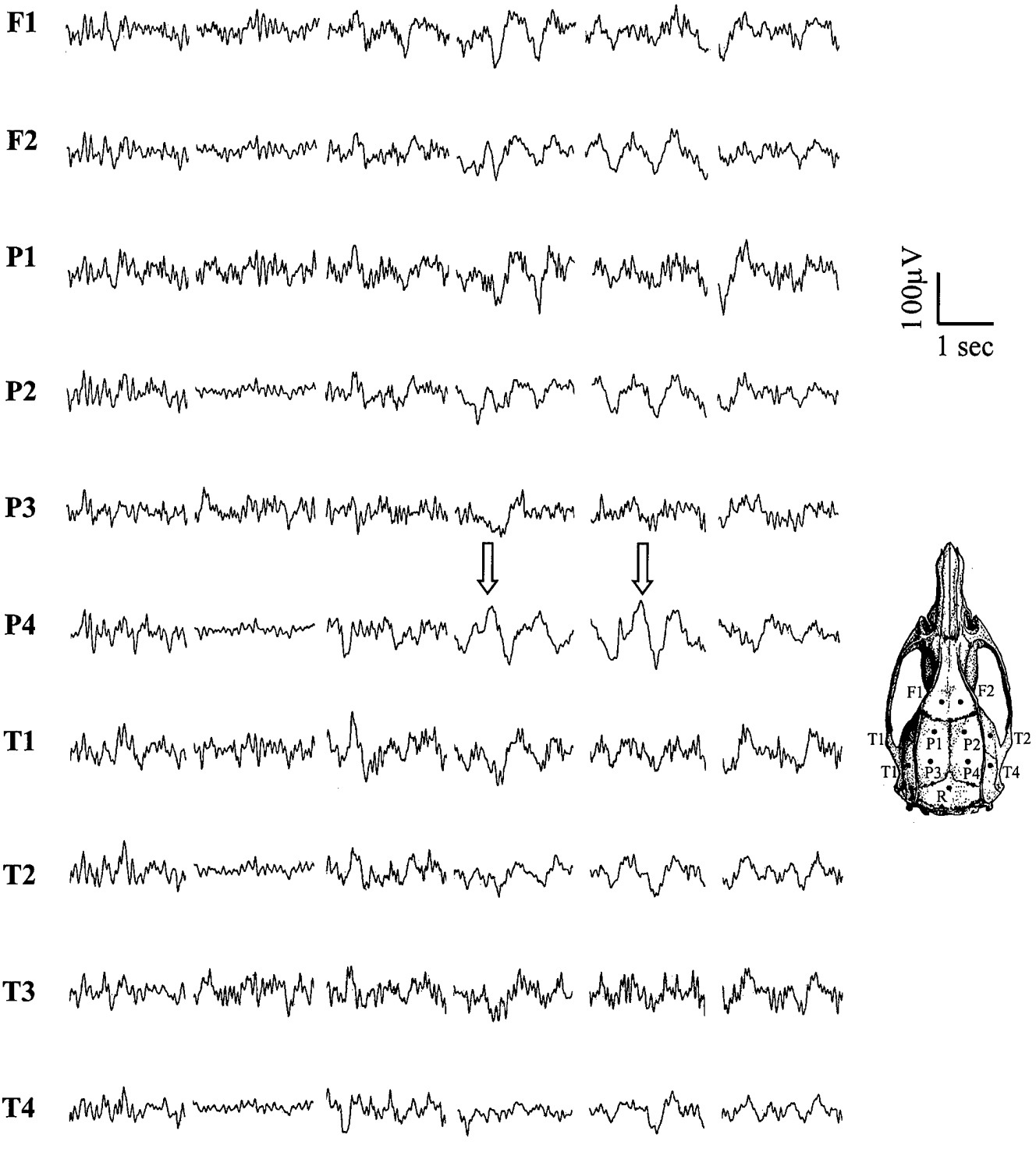

F1
F2
P1
P2
P3
P4
T1
T2
T3
T4
100µV
1 sec
F1
F2
T1
P1
P2
T2
T3
P3
P4
T4
R
Baseline
1
6
24
48
72
Timepoint post-MCAo (h)

Figure 4

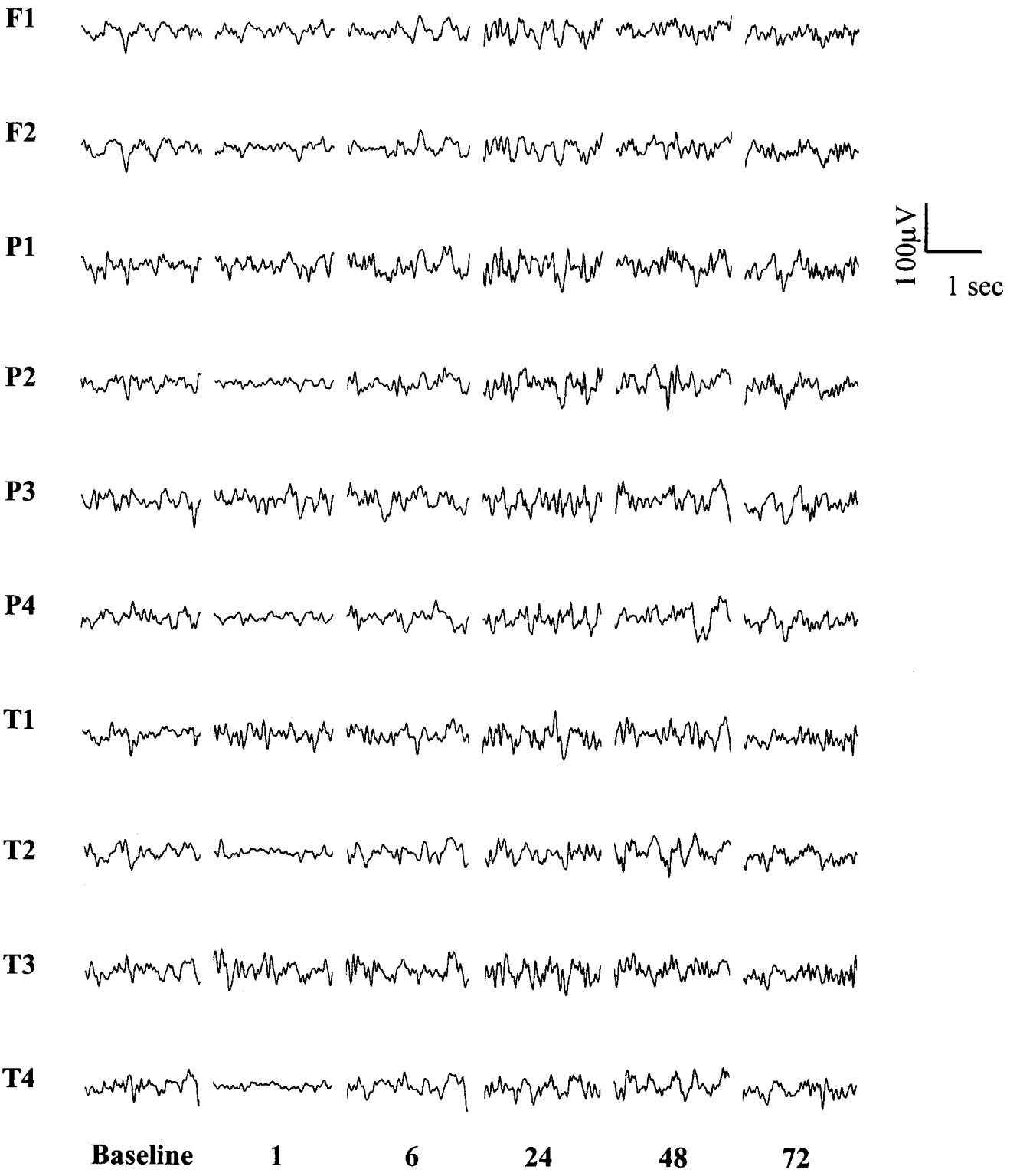

F1
F2
P1
P2
P3
P4
T1
T2
T3
T4
100μV
1 sec
Baseline
1
6
24
48
72
Timepoint post-MCAo (h)

Figure 5

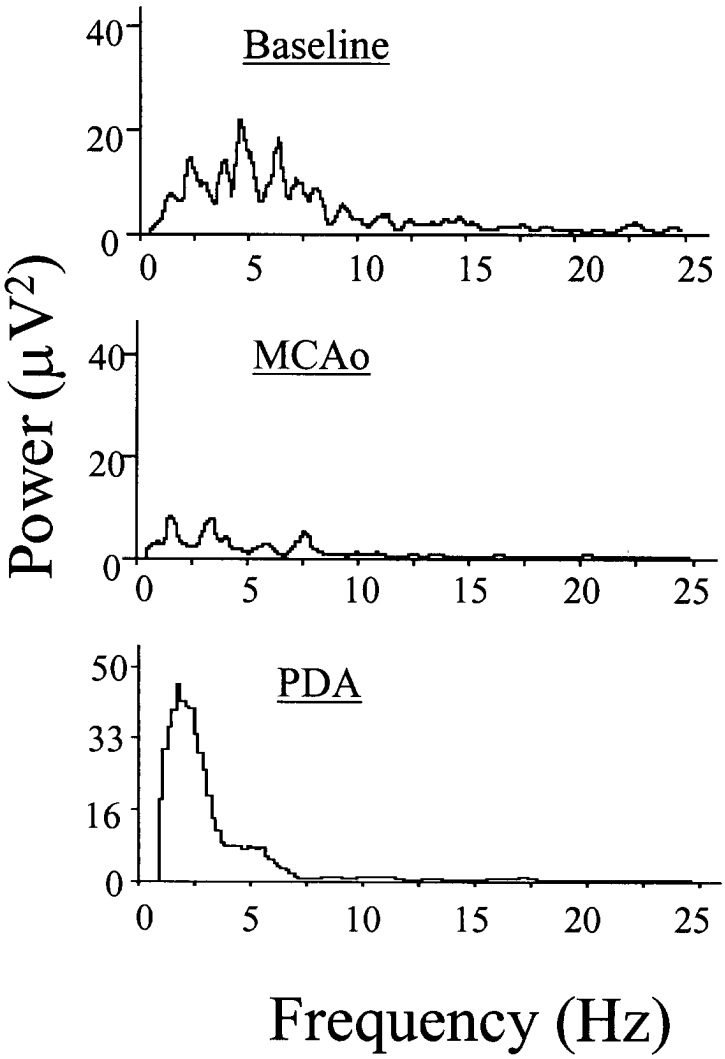

Baseline
MCAo
PDA
40
20
0
50
33
16
0
5
10
15
20
25
Power (μV²)
Frequency (Hz)

Figure 6

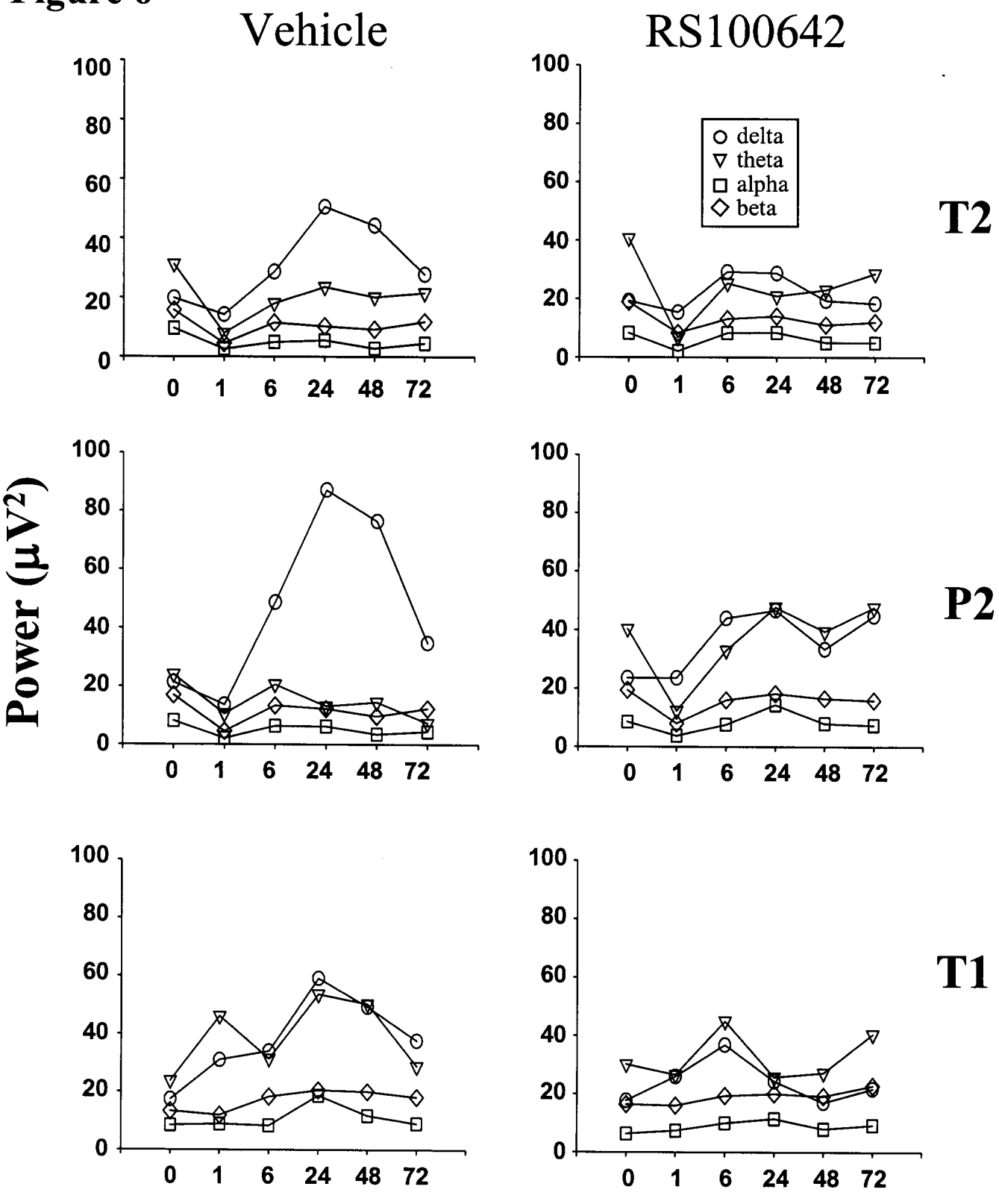
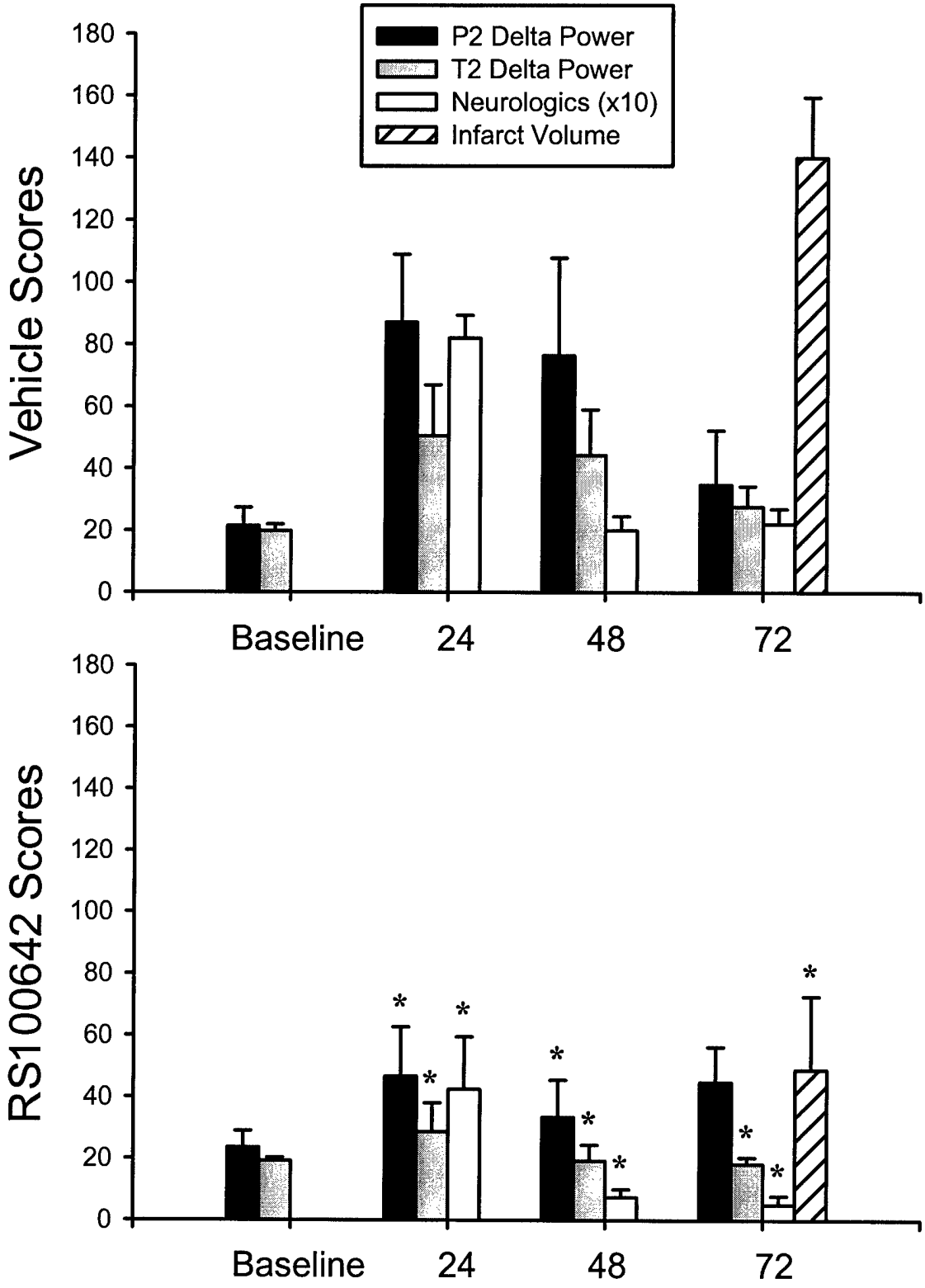

Vehicle
RS100642
T2
P2
T1
Power (μV²)
Time-point post-MCAo (h)
delta
theta
alpha
beta
0
20
40
60
80
100
0 1 6 24 48 72

Figure 7


P2 Delta Power
T2 Delta Power
Neurologics (x10)
Infarct Volume
Vehicle Scores
180
160
140
120
100
80
60
40
20
0
Baseline
24
48
72
RS100642 Scores
180
160
140
120
100
80
60
40
20
0
Baseline
24
48
72