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13. ABSTRACT *(Maximum 200 Words)*

The purpose of this research project is to study the role of neuronal sodium channels in mechanisms of neuronal injury, neurodegeneration, and neuroprotection. The primary objective of this research project is to characterize the expression, and study the functional significance, of neuronal sodium channels during the process of injury and recovery. Also, the effects of sodium channel blockade (using antisense and channel blockers) on gene expression and neurodegeneration will be studies.

The progress during our 3rd year of this project wss excellent. Several important and critical discoveries providing a greater understanding of the injury/neurodegeneration process and the role of brain NAChs in this process were elucidated. Our key observations were 1) using ASO for the Na1.1 NaCh it was demonstrated that functional blockade of this channel results in brain neuroprotection following injury; 2) our in situ hybridization experiments defined the regional anatomical distribution of Nav1.1 sodium channel gene expression in normal and at various time-post MCAo and confirmed our QRT-PCR data showing the Na1.1 expression to be down-regulated in subcortical and cortical regions of injured and, to some degree of contralateral hemispheres. In addition, these in situ hybridization studies revealed that the expression of other NaCh gene appears not to be of significance to the brain injury process; 3) our preliminary results show that treatment of neurons with RS-1000642 at least partially reverses the down-regulation of Nav1.1 caused by MCAo injury. Importantly, evidendc eindicates that this is an effect selective for the Na1.1 NaCh and not other NAChs; 4) Based upon our QRT-PCR resutls, it does not appear that sodium-calcium exhanger genes, namely NCX1, NCX2 and NCX3, re inolved in the NaCh mechanisms of ischemia brain injury, at least not at early time-points post-MCAo injury; 4) Finally, our comprehensive EEG studies have identified several non-convulsant (NCS) brain seizure events identical to the clinical brain injury and that of all the AEDs tested to date, only the sodium channel blocker RS-100642 significantly stops or attenuates the NCS neuropathology.

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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.

II. General Summary

Year three 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 FY'03. Unexpected research problems encountered during the course of the year were dealt with aggressively and with complete success and are defined below in detail. We do not believe at this time that these setbacks will effect our meeting our stated objectives for the third and final year of the project.

III. Administrative Problems/Accomplishments for Year'03

No significant administrative problems were encountered. All procurements proceeded on schedule and without delay. Since we were unable to find a replacement for our part-time technician slot, we requested and have received approval for re-budgeting the excess salary funding into the supplies/materials funding for our supplemental FY'04 year.

IV. Research Problems/Accomplishments for Year'03

1. Description of Problems encountered:

During the third 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 problems 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.

2. Description of Year'03 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.**

3. Details of accomplishments for Year'03:

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. For the most part, the results of this study were described in detail in our year 02 report. During the first 2 months of year 03 it was necessary to increase the group size for a few of the time points. Once completed the manuscript was submitted and has now been published.
2. Additional studies on the effects of brain injury on $Na_v1.2$ gene expression was undertaken and completed showing that there is also a down-regulation in the $Na_v1.2$ gene at 24 hr post-injury. However, the down-regulation in $Na_v1.2$ is not as dramatic as that demonstrated for the $Na_v1.1$ gene (over 70% down-regulation for $Na_v1.1$ v/s 35% for $Na_v1.2$ gene expression).
3. Because of problems encountered and described earlier with the NaCh ASOs, the ***in vitro*** functional studies in primary cultures were extended using second set of NaCh ($Na_v1.1$, $Na_v1.2$ or $Na_v1.3$) ASOs. These set of ASOs were generated from a site that is at least 500 bp away from the original set of ASOs. However, preliminary ***in vitro*** results indicate that the blocking expression of either the $Na_v1.2$ or $Na_v1.3$ genes using these new ASOs does not exhibit significant neuroprotection. Importantly and consistent with our ***in vivo*** NaCh expression results, data obtained from recent experiments in the MCAo injured brain suggest that **$Na_v1.1$ ASO is neuroprotective** to a limited extent when animals are treated 24 hr before the insult with ASO concentrations ranging from at 1-5 μM. Studies are currently in progress to determine the neuroprotective effects of longer pretreatment period (36 or 48 hr) using even lower ASO concentrations (0.1-1 μM). Once the optimal treatment period is established, additional studies will be undertaken to determine if combination of $Na_v1.1$ ASO with other sodium channel gene ASOs could improve the degree of neuroprotective. However, as mentioned in last year's annual report $Na_v1.1$ ASO produces mild cytotoxicity and we have seen a similar effect with the new $Na_v1.1$ ASO, as well as a similar increase in protease activity that results in cell detachment from the culture wells. Once again, this situation was unique to only the $Na_v1.1$ ASO, as the other three sodium channel ASOs did not produce any toxicity and had no effect on protease activity. Also, this cytotoxicity has only been observed in the in vitro primary neuronal cultures and no in the in vivo brain injured tissue as examined by TTC and H&E staining and light microscopy. At this point we have decided to continue with the *in vivo* studies.
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. $Na_v1.1$ mRNA was 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 $Na_v1.1$ mRNA nucleotide 1113-1146, rBIII 2118-2143, PN1 1472-1498, PN3 1565-1588 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 $Na_v1.1$, with staining observed in neurons, axonal pathways and neuropil. The *in situ* hybridization signal for $Na_v1.1$ 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 $Na_v1.1$ 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 $Na_v1.1$ mRNA was detected in the ipsilateral infarct-damaged brain tissue. Injured areas that showed reduced $Na_v1.1$ 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 throughout the same infarct-damaged areas. 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 $Na_v1.1$ 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. Additionally, a small but noticeable reduction in $Na_v1.1$ mRNA signal was also observed in the contralateral piriform cortex at the 24h and 48h post-injury time points. No noticeable enhancement of signal was observed in any cell population in the ipsilateral or contralateral hemispheres at any time point studied. The piriform cortex is connected to its contralateral counterpart via the anterior commissure. It is possible that the drop in $Na_v1.1$ signal contralateral to the injury was due to a transynaptically-mediated down-regulation of sodium channel gene transcription as a response to a loss of activity coming from the injured hemisphere. Since more evidences to support these changes are required by the publishers, protein analysis such as histochemistry and Western blotting are under progress now.

5. The neuroprotective effects of sodium channel gene blocker, RS-100642, on injury related changes in sodium channel gene expression by quantitative RT-PCR are currently underway. RS--100642 ~~treatment (i. v.~~ 1.0 mg/kg, iv) or vehicle ~~performed~~ were injected 30 min, 2h, 4h and 6h post-MCAo ~~in rats~~. (We reported in our FY02 annual report that this is most effective neuroprotective dose of RS-100642 and provided over 65% neuroprotection in core infarcted brain region following MCAo). 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 quantitative RT-PCR. β-actin mRNA measurements in these samples provided sample amplification efficiency. Our results demonstrate that $Na_v1.1$ and $Na_v1.2$ genes were significantly down-regulated following ischemic insult. No significant differences in the expression of other sodium channel genes were observed following MCAo. RS-100642 treatment partially reversed down-regulation of $Na_v1.1$ and $Na_v1.2$ genes. These findings suggest that the $Na_v1.1$ and $Na_v1.2$ sodium channel genes may play a key role in neuronal injury/recovery and further suggest that the neuroprotective effects of RS-100642 may be mediated in part by restoration of the sodium channel expression.;

6. NaCh gene expression efficiency in normal fetal rat brain neurons in primary culture was initiated. Quantification of the level of relative 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 and shown in 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 $Na_v1.7$). Compared to β-actin gene expression, the relative level 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%.
7. NaCh gene expression in primary forebrain neurons following veratridine (a specific sodium channel activator and neurotoxin) was studied. Exposure of veratridine 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. Therefore, for subsequent experiments we are using the 2.5 μM concentration of veratridine that consistently produces 80% neuronal injury. Quantification of the level of relative mRNA for each sodium channel gene was analyzed in fetal rat forebrain neurons subjected to veratridine treatment alone, or veratridine plus RS-100642 (200 μM) treatment. 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, RS-100642 treatment reversed the down-regulation caused by veratridine. This reversal by RS-100642 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.
8. During last reporting period we indicated that we have designed and synthesized probes and primers for three sodium calcium exchanger genes (NCX1, NCX2 and NCX3) and proposed to initiate studies investigating the effect of injury on the expression of three sodium-calcium exchanger genes. To our surprise, the expression of NCX1, NCX2 and NCX3 genes showed no significant changes from basal levels at any time point studied up to 24h post-MCAo injury. It is possible that the turn-over rate of these exchanger proteins is delayed and that we may have to study longer time-intervals following injury. However, since these studies on the regulation of these genes was not part of the original proposal, and since we can not detect any significant changes in the same following MCAo insult, we have decided to not to continue these experiments during our supplemental year 04.
9. As a follow-up to our discovery that MCAo brain injuries are associated with the emergence of non-convulsant seizure activity (NCS) which is significantly attenuated by

RS100642, we initiated new experiments aimed at: 1) a more comprehensive quantitative description of this NCS pathology and a comparison of the efficacy of RS100642 to other "prototype" anti-epileptic drugs. Using high–resolution (10 electrode placements) and computerized quantitative EEG analysis (qEEG), we have identified several NCS 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 was found to better represent the clinical pathology. Of the 5 prototype drugs tested to date which are know to be relatively refractory in the treatment of this type of clinical brain seizure condition (namely, phenobarbital, ethosuximide, fos-phenytoin, diazepam and midazolam), none were significantly effective in stopping or attenuating the NCS, whereas RS100642 is highly effective. One **manuscript has been published** and a **second manuscript has been accepted with revision**.

KEY RESEARCH ACCOMPLISHMENTS for Year'03

- Manuscript entitled "RS-100642-198, a novel sodium channel blocker, provides differential neuroprotection against hypoxia/hypoglycemia, veratridine or glutamate mediated neurotoxicity in primary cultures of rat cerebellar neurons" was **published in Neurotoxicity Research 3(4): 381-395, 2001.**

- Manuscript entitled "Differential pattern of expression of voltage-gated sodium channel genes following ischemic brain injury in rats" **was published in Neurotoxicity Research 4(1): 67-75, 2002.**

- Manuscript entitled "Neuroprotective effects of the sodium channel blocker RS100642 and attenuation of ischemia-induced brain seizures in the rat" **was published in Brain Research 932:45-55, 2002.**

- Manuscript entitled "Sodium channel $Na_v1.1$ gene expression after ischemic injury: an in situ hybridization study **was submitted NeuroReport and accepted with revision.**

- Manuscript entitled "Down-regulation of sodium channel Nav1.1 expression by veratridine and its reversal by a novel channel blocker, RS-100642, in primary neuronal cultures" has been **submitted for Publication, June 2002**.

- An abstract entitled "Naeuronal sodium channels and brain injury: Molecualr expression and neuroprotection studies" was **presented** at the ATACCC2001 Conference, Fort Walton Beach, FL, 9-14 September, 2001.

- Abstracts entitled "Expression of sodium channel genes following ischemic brain injury: an in situ hybridization study" was **presented** at the **31st Annual meeting of the Society for Neuroscience.**

- An abstract entitled "Molecular expression of neuronal sodium channels in brain injury" was **presented** at the International Symposium on Molecular Medicine, Vadodara, India, 20-24 January, 2002.

- Abstracts 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" and "The sodium channel blocker RS-100642 reverses the down-regulation of $Na_v1.1$ and $Na_v1.2$ sodium channel genes caused by ischemic brain injury in rats" were **submitted for presentation to the 32st Annual meeting of the Society for Neuroscience.**

- Demonstrated using ASO technology that at least in vivo, blocking expression of only $Na_v1.1$, but not $Na_v1.2$ or $Na_v1.3$, offers significant, albeit limited, neuroprotection form the injury.

- Using in situ hybridization identified the regional distribution of $Na_v1.1$ 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 and 2) that it extends into the contralateral, uninjured hemisphere

- The effects of RS100642 to reduce brain injury correlate with a partial reversal of the $Na_v1.1$ down-regulation caused by injury.

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

- Using high-resolution EEG analysis, identified and characterized brain seizure events identical to relevant clinical pathology described for NCS in brain injured patients and demonstrated that this pathology, which is sensitive to treatment with the sodium channel blocker RS100642, was refractory to a number of prototype AE drugs.

REPORTABLE OUTCOMES for Year'03

Three manuscripts published (see appendices)
Two manuscripts accepted with revision (see appendices)
One manuscript submitted for publication (see appendices)
Three abstracts presented (see appendices)
Two abstracts submitted (see appendices)

CONCLUSIONS: SEE ABOVE

REFERENCES: UNCHANGED FROM ORIGINAL PROPOSAL

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Research report

Neuroprotective effects of the sodium channel blocker RS100642 and attenuation of ischemia-induced brain seizures in the rat

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Abstract

Seizurogenic activity develops in many patients following brain injury and may be involved in the pathophysiological effects of brain trauma and stroke. We have evaluated the effects of the use-dependent sodium channel blocker RS100642, an analog of mexiletine, as a neuroprotectant and anti-seizure agent in a rat model of transient middle cerebral artery occlusion (MCAo). Post-injury treatment with RS100642 (0.01–5.0 mg/kg) dose-dependently reduced brain infarction, improved functional recovery of electroencephalographic (EEG) power, and improved neurological outcome following 2 h of MCAo and 24 h recovery. This effect was more potent and offered a larger reduction of brain infarct volume than a maximal neuroprotective dose of mexiletine (10.0 mg/kg). Furthermore, brain seizure activity recorded following 1 h MCAo and 72 h of recovery in injured rats was either completely blocked (30 min pre-MCAo treatment) or significantly reduced (30 min post-MCAo treatment) with RS100642 (1.0 mg/kg) treatment resulting in greater than 60% reduction of core brain infarct. These results indicate that brain seizure activity during MCAo likely contributes to the pathophysiology of brain injury and that RS100642 may be an effective neuroprotective treatment not only to decrease brain injury but also to reduce the pathological EEG associated with focal ischemia. Published by Elsevier Science B.V.

Theme: Disorders of the nervous system

Topic: Ischemia

Keywords: Brain injury; MCAo; Sodium channels; Seizures; Neuroprotection

1. Introduction

Thromboembolic stroke is the third leading cause of death in the United States and Europe and may result in severe functional disability among survivors [5]. Furthermore, seizures have been reported to occur in up to 50% of brain injured patients depending on the type of injury induced, including traumatic brain injury, hemorrhage, or stroke [15,29,37]. Anticonvulsant therapy with agents possessing sodium channel blocking properties is currently available for the treatment of seizures (i.e., phenytoin and carbamezapine) but to date the prophylactic use of anticonvulsants following brain ischemia is not widely practiced except in those patients who develop overt convulsant seizure activity [2]. Recently electroencephalographic (EEG) studies of brain-injured patients have indicated that the presence of seizure activity is actually higher than previously estimated due to the 'nonconvulsant' nature of post-traumatic brain seizures [37].

The normally functioning brain is solely dependent upon blood-delivered glucose as an energy source for the production of adenosine-triphosphate (ATP). Positron emission tomography (PET) studies in humans during epileptic seizures have indicated an acute rise in glucose utilization occurring during ictal activity [22]. Consequently, following a cerebral ischemic episode, seizure-induced increases in glucose metabolism could rapidly deplete cellular energy stores in the ischemic regions. Upon depletion of energy reserves the ischemic brain critically compromises its ability to maintain ATP-dependent cellular membrane potentials, possibly resulting in an influx of sodium and calcium ions through voltage-gated ion

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channels leading to the demise of the cell through a cascade of excitotoxic cellular events [21]. Effectively, the reduction of seizure activity following brain ischemia may be a crucial target for neuroprotection, and a rational complement to any neuroprotection strategy.

Sodium channel blockers have been studied as therapies to relieve the excitotoxic effects of cerebral ischemia due to the membrane stabilizing properties of these compounds [36]. Most therapeutic sodium channel blocking compounds are use-dependent and only significantly block channels in the inactivated state. The result is that low frequency or basal levels of sodium channel function is left relatively unaffected, reducing toxicity, while high frequency stimulation (i.e., seizure activity) is attenuated [32]. In the current study, we have evaluated the effects of the novel use-dependent sodium channel blocker RS100642 to not only reduce brain injury following focal ischemia, but additionally to block ischemia-induced brain seizures.

2. Materials and methods

2.1. Surgical procedures including middle cerebral artery occlusion (MCAo)

Male Sprague–Dawley rats (270–330 g; Charles River Labs, Raleigh, VA, USA) were used in all of the following procedures. 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. Depending on the experiment (see below), either 2 or 10 epidural electrodes (stainless steel screw electrodes, 0–80×1/8 in) were permanently implanted and fixed to the skull using dental acrylate cement [35]. Body temperature was maintained normothermic (37±1 °C) throughout all surgical procedures by means of a homeothermic heating system (Harvard Apparatus, South Natick, MA, USA). Food and water were provided 'ad libitum' pre- and post-surgery and the animals were individually housed under a 12 h light—dark cycle. The facilities in which the animals were housed were maintained and fully accredited by the American Association of Laboratory Animal Care. In conducting the research described in this report, the investigators adhered to the *Guide for the Care and Use of Laboratory Animals*, as promulgated by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council.

3–5 days following the surgical procedures described above the rats were re-anesthetized and prepared for temporary focal ischemia using the filament method of MCAo and reperfusion as described elsewhere [33]. Briefly, the right external carotid artery was isolated and its branches coagulated. A 3-0 uncoated monofilament nylon suture with rounded tip was introduced into the internal carotid artery via the external carotid artery and advanced (approximately 20 mm from the carotid bifurcation) until a slight resistance was observed, thus occluding the origin of the middle cerebral artery (MCA). The endovascular suture remained in place for 1 or 2 h and then retracted to allow reperfusion of blood to the MCA. Following MCAo surgery, animals were placed in recovery cages with ambient temperature maintained at 22 °C. Sham-treated animals received all surgical procedures but the filament was not inserted into the MCA.

2.2. Neuroprotection dose response (2 h MCAo/22 h recovery)

Two cortical electrodes were placed over the right (injured) parietal cortex (4 mm right, 4 mm right and 4 mm posterior to bregma). Rectal temperatures were monitored pre-MCAo and 0.5, 2, 4, 6, 24 h post-MCAo. EEG samples were recorded in the anesthetized animal pre-MCAo, during reperfusion and again at 24 h. Intravenous injections of either vehicle or RS100642 (0.01–5.0 mg/kg) were given at 0.5, 2, 4, and 6 h post-MCAo. EEG power was calculated from 2 min EEG samples using QND EEG analysis software (Neurodata, Pasadena, CA, USA). Power values at 2 h were compared to baseline, pre-MCAo values to obtain the percent drop in power. Percent recovery of EEG power was calculated as the increase at 24 h compared to the drop at 2 h. If the animals did not show at least an 80% drop in EEG amplitude immediately prior to reperfusion they were excluded from the study. Neurological evaluations (see below) were scored pre-MCAo, immediately before reperfusion at 2 h, and again at 24 h. At 24 h post-MCAo final EEG and neurologic measures were taken, following which animals were euthanized and brains collected for infarct analysis.

2.3. Brain seizures/long-term recovery (1 h MCAo/70 h recovery)

Ten cortical electrodes were permanently fixed to the rat skull, located as shown in Fig. 1. Animals were housed in custom designed plexiglas recording chambers (Dragonfly, Ridgeley, WV, USA) equipped with multi-channel swivel commutators (Plastics One, Roanoke, VA, USA). On the morning of the experiment, the rats were connected to the swivel system by flexible shielded cables providing a noise-free connection from the unrestrained rat to a Grass Model 7D polygraph and digital analysis system using Harmonie software (Astro-Med, West Warwick, RI, USA) while permitting freedom of movement by the animals during all phases of the experiment. EEG samples were recorded in awake, unanesthetized animals pre-MCAo, during the 1 h MCA occlusion, and continuously for 5 h following reperfusion. Fifteen-minute EEG samples were again recorded at 24, 48, and 72 h post-MCAo. Ictal seizure events were defined as generalized epileptiform

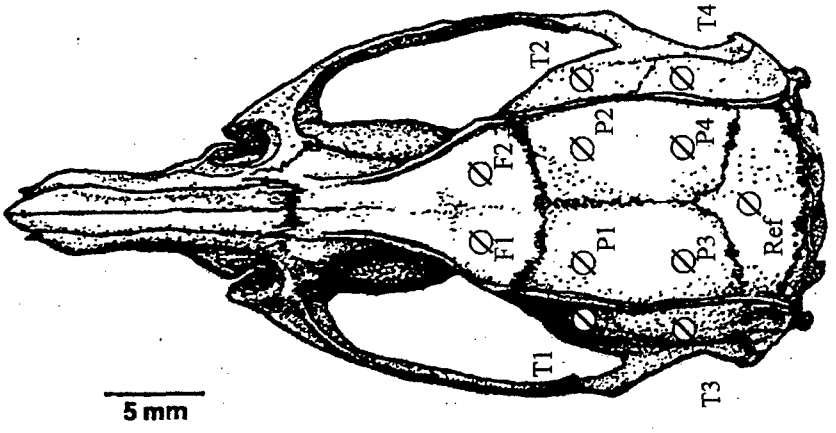

Fig. 1. Location of cortical EEG electrodes on the rat skull.

spike/slow-wave activity. Rectal temperatures were monitored pre-MCAo and 0.5, 2, 4, 6, 24, 48, and 72 h post-MCAo. Intravenous injections of vehicle or RS100642 (1.0 mg/kg) were administered either at 0.5, 2, 4, and 6 h post-MCAo or 0.5 h pre-MCAo and 2, 4, and 6 h post-MCAo. Neurological evaluations (see below) were scored pre-MCAo, immediately before reperfusion at 1 h, and again at 24, 48, and 72 h. At 72 h post-MCAo the animals were euthanized and brains collected for infarct analysis.

2.4. Neurological testing

Neurologic scores (NS) were derived using a 10-point sliding scale. Each animal was examined for reduced resistance to lateral push (score=4), open field circling (score=3), and shoulder adduction (score=2) or contralateral forelimb flexion (score=1) when held by the tail (modified from Ref. [4]). Rats extending both forelimbs toward the floor and not showing any other signs of neurological impairment were scored 0. Using this procedure, maximal neurological severity was measured as an NS=10. In the present study, all rats subjected to MCAo either exhibited a neurological score of 10 when examined 1 or 2 h post-occlusion (i.e., immediately prior to reperfusion) or they were dropped from the study. Sham animals were also observed for neurological deficits.

2.5. Infarct analysis

From each rat brain, analysis of ischemic cerebral damage included total and core infarct volumes and hemispheric infarct size (calculated as percentage of total hemispheric volume, to exclude the possible contributing effect of hemispheric edema to infarct size). Infarcted regions were evaluated using TTC (2,3,5-triphenyl tetrazolium chloride) staining from seven coronal sections (2 mm thick) taken from the region beginning 1 mm from the frontal pole and ending just rostral to the cortico–cerebellar junction. Core injury was defined as brain tissue completely lacking TTC-staining while total injury was specified as all ipsilateral tissue showing a loss of stain as compared to the contralateral, uninjured hemisphere. Computer-assisted image analysis was used to calculate infarct volumes and has been described in detail elsewhere [33]. Briefly, the posterior surface of each TTC stained forebrain section was digitally imaged (Loats Associates, Westminster, MD, USA) and quantified for areas (mm^2) of ischemic damage. Sequential integration of the respective areas yielded total and core infarct volumes (mm^3). Similarly, ipsilateral and contralateral hemispheric volumes were measured where hemispheric swelling (edema) was expressed as the percent increase in size of the ipsilateral (occluded) hemisphere over the contralateral (uninjured) hemisphere.

2.6. Data analysis

Data are presented as the mean±standard error of the mean. Unless otherwise noted, statistical analysis of neuroprotective recovery was done by analysis of variance (ANOVA) followed by Dunnet's post-hoc test to compare individual treatment doses to the vehicle, control group. Statistical analyses were calculated using Minitab Statistical Analysis software program. For the neuroprotection ED_{50} the criteria for identifying a positive responder was defined as previously described [34]. Potency and ED_{50} calculations were performed using the Pharmacological Calculations Computer Programs [31].

2.7. Compound

RS100642 was received from Roche Biosciences (Palo Alto, CA, USA). The compound was dissolved in a vehicle of distilled, deionized water immediately prior to testing and administered in a volume of 1 ml/kg of body weight.

3. Results

3.1. MCAo injury

Control vehicle-treated rats subjected to MCAo/reperfusion injury and either 24 or 72 h of recovery exhibited striatal and cortical brain infarction in the right hemisphere from approximately 3 to 11 mm from the frontal pole (Fig. 2). Two hours of MCAo followed by 24 h of recovery resulted in total infarct volume in control rats of 378±12 mm^3 and core infarct volume of 220±16 mm^3 with the total infarct representing approximately 36%, and the core infarct approximately 21%, hemispheric infarction. One hour of MCAo followed by 72 h of recovery resulted in a total infarct volume in control rats of 284±12 mm^3 and core infarct volume was 140±19 mm^3 with the total representing 32%, and the core 16%, hemispheric infarction. MCAo resulted in significant hemispheric edema representing approximately an 8 and 6% increase in cerebral volume compared to the contralateral, uninjured hemisphere for the 24 and 72 h endpoints, respectively. Neurologic function, which was severely impaired at 1 and 2 h post-MCAo (NS=10±0), exhibited a significant degree of spontaneous recovery (NS=7.8±1.1 at 24 h and NS=2.2±0.5 at 72 h). However, none of the injured vehicle-treated animals completely recovered neurologic function (NS=0), with at least contralateral forelimb flexion and shoulder adduction (NS=3) still evident in all rats examined at 24 h, and at least contralateral forelimb flexion (NS=1) evident in all rats at 72 h post-injury. Sham-treated animals exhibited normal TTC staining and no neurological deficits.

All injured animals lost approximately 13–18% body weight over the 24 h recovery period and a 16–23% loss at 72 h, regardless of treatment group, with no significant differences in body weight loss between groups. In vehicle-treated control animals, MCAo caused a transient, mild hyperthermia that began to return to normal values by 6–24 h post-occlusion (Table 1). At 48–72 h animals exhibited a mild, albeit non-significant, drop in body

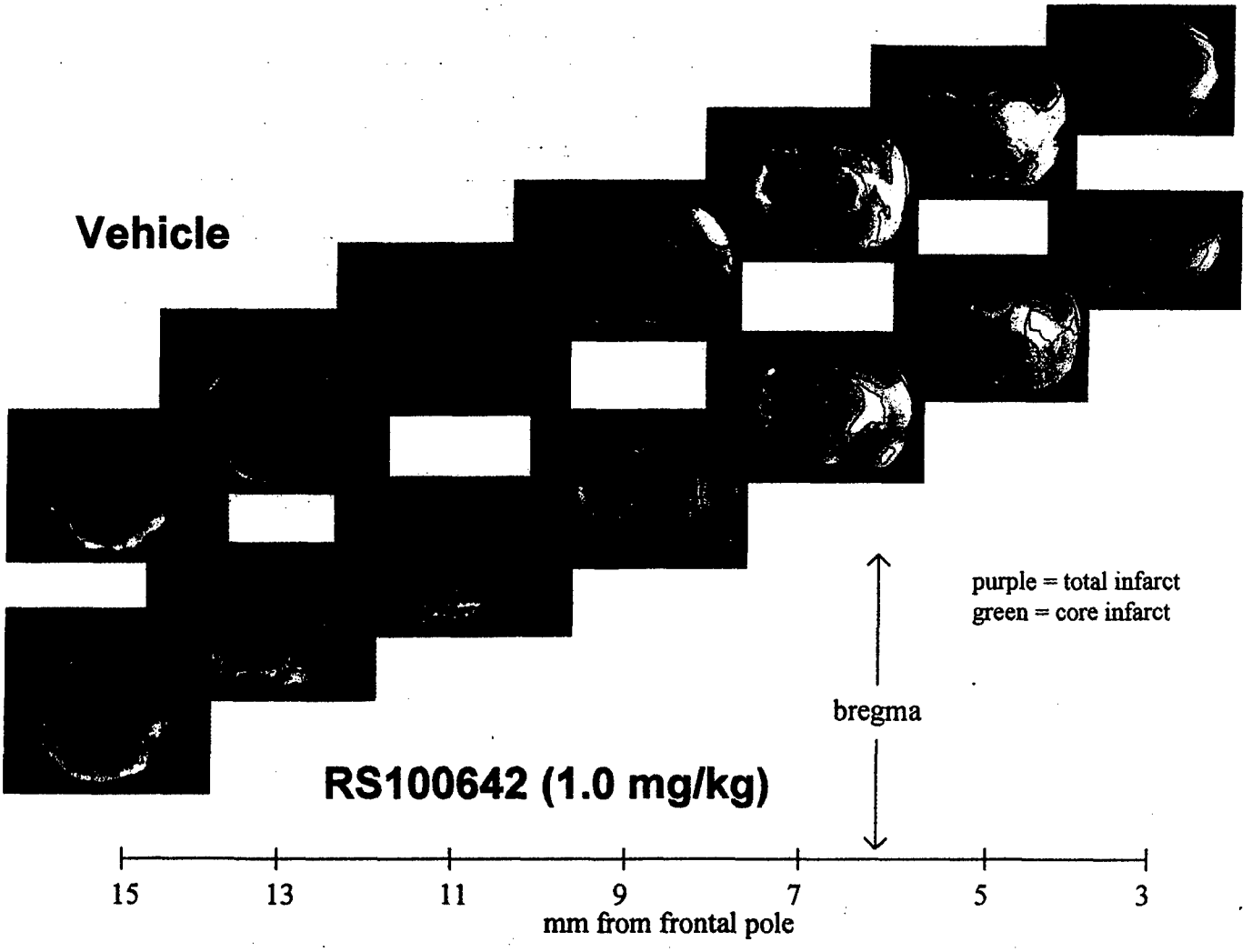

Fig. 2. Representative coronal brain sections stained with TTC from vehicle and RS100642-treated animals following 2 h MCAo and 22 h reperfusion and recovery.

Table 1
Body temperature changes following 2 h MCAo/reperfusion injury

Group[a]	Baseline[b]	2 h	4 h	6 h	24 h
Vehicle	37.0±0.7	38.3±0.7	37.1±0.8	37.5±0.7	36.6±0.8
RS (0.01 mg/kg)	36.4±0.3	38.0±0.6	37.2±0.8	36.4±0.9	36.6±0.7
RS (0.03 mg/kg)	36.7±0.3	37.7±0.5	36.7±0.9	36.7±0.7	36.5±0.4
RS (0.1 mg/kg)	36.6±0.2	38.2±0.5	37.1±0.7	36.8±0.2	36.0±0.3
RS (1.0 mg/kg)	36.6±0.4	37.4±0.4	36.8±0.4	36.0±0.5	36.2±0.6
RS (5.0 mg/kg)	36.4±0.5	38.2±0.6	37.1±0.9	36.8±0.8	36.0±0.7
Mex (10 mg/kg)	36.3±0.7	37.3±1.4	37.4±0.6	37.2±0.5	37.0±0.7

[a] Treatments with vehicle, RS100642 (RS), or mexiletine (Mex) were administered starting 30 min post-MCAo.
[b] Timepoints shown are post-MCAo.

temperature (36.7±0.7 °C, 35.6±0.4 °C and 35.7±0.6 °C, respectively), which was similar to our earlier findings [33,38].

3.2. Neuroprotection dose response (2 h MCAo/24 h recovery)

Post-treatment with RS100642 (administered i.v. starting a 0.5 h post-occlusion) significantly reduced ischemic infarction throughout the brain measured at 24 h post-occlusion (Fig. 2). A dose-dependent decrease in infarct volume was seen in both total and core infarction (Fig. 3) corresponding to a neuroprotection in these regions of 57±10 and 64±12%, respectively, at the maximal efficacious dose (1.0 mg/kg), which corresponds to 16 and 8% hemispheric infarction in total and core regions, respectively. The neuroprotection ED_{50} (and 95% confidence limits) based on reduction of core infarction was 0.026 (0.021–0.31) mg/kg. The effects of RS100642 to alter hemispheric swelling due to edema following MCAo were not statistically significant. At all doses and time-points, temperature measurements from RS100642 treated animals (Table 1) were not significantly different from the corresponding control, vehicle-treated animals ($P>0.05$, ANOVA).

A progressive improvement in both EEG power and neurologic score was seen with RS100642 treatment at 24 h post-occlusion as compared to the vehicle-treated group (Fig. 4A and B). At the 1.0 mg/kg dose this recovery corresponded to a 24% increase in EEG power over vehicle-treated animals and an improved neurological score of 2.1±0.8 as compared to 7.8±1.1 for the vehicle-treated group. One RS100642 animal showed complete recovery (NS=0) and 4 animals only showed signs of contralateral forelimb flexion (NS=1).

Post-injury treatment with mexiletine (Fig. 5) resulted in a 37±14% reduction of total, and 53±16% reduction of core, infarction which corresponds to 24 and 10% hemispheric infarction in total and core regions, respectively. This recovery also corresponded to a 28% increase in EEG power over vehicle-treated animals and an improved neurological score of 3.3±1.2 as compared to 7.8±1.1 of

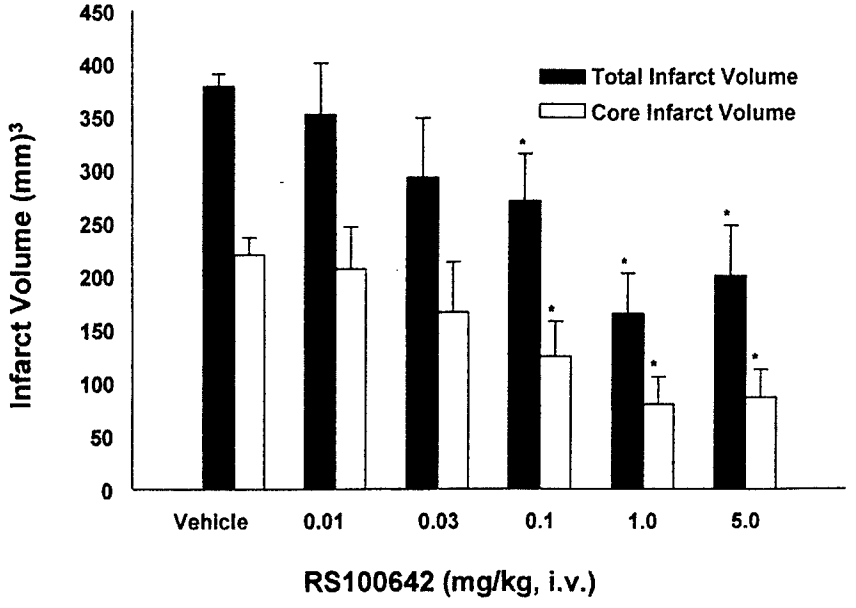

Fig. 3. Dose response effect of RS100642 to decrease brain infarct volume following 2 h MCAo and 22 h reperfusion and recovery ($n=6–8$ per group). *$P<0.05$, **$P<0.01$, ANOVA followed by Dunnet's post hoc analysis.

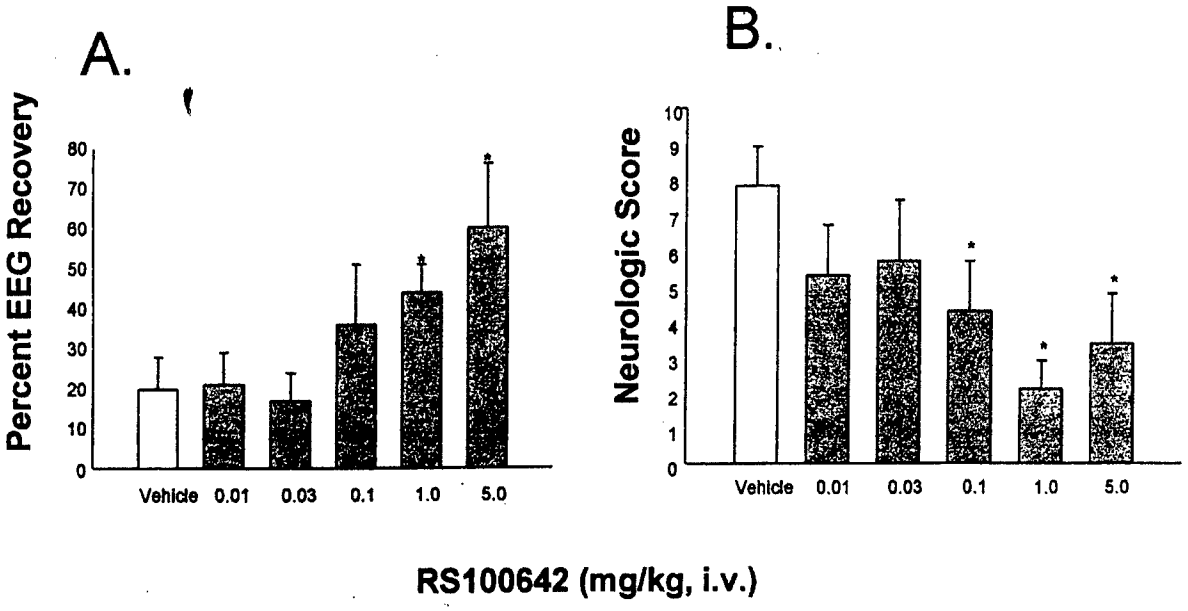

Fig. 4. Effect of RS100642-treatment following 2 h of MCAo and 22 h recovery and reperfusion to (A) increase recovery of EEG power *$P<0.05$, ANOVA followed by Dunnet's post hoc analysis, (B) improve neurological outcome *$P<0.05$, **$P<0.01$, Mann–Whitney U-test.

the vehicle-treated group. No significant effect on cerebral edema was measured as compared to the vehicle group. Critically, testing higher doses of mexiletine (i.e., 20 mg/kg) resulted in convulsant seizures in two animals and therefore further studies with mexiletine were halted.

3.3. Long term recovery (1 h MCAo/72 h recovery)

Both pre- and post-MCAo treatment with RS100642 (1.0 mg/kg) resulted in a significant decrease in cerebral infarction when evaluated after 72 h of MCAo/reperfusion (Fig. 6A). The reduction of total and core infarct volumes corresponded to 51±14 and 60±18% (pre-treatment group) and 46±12 and 65±14% (post-treatment group), respectively. Similarly, the overall percent hemispheric infarction was significantly reduced (total=16%, core=6%, pre-treatment) and (total=17%, core=5%, post-treatment). No significant effect on cerebral edema was measured as compared to the vehicle group. For the pre- and post-treated groups there was a significant improvement in

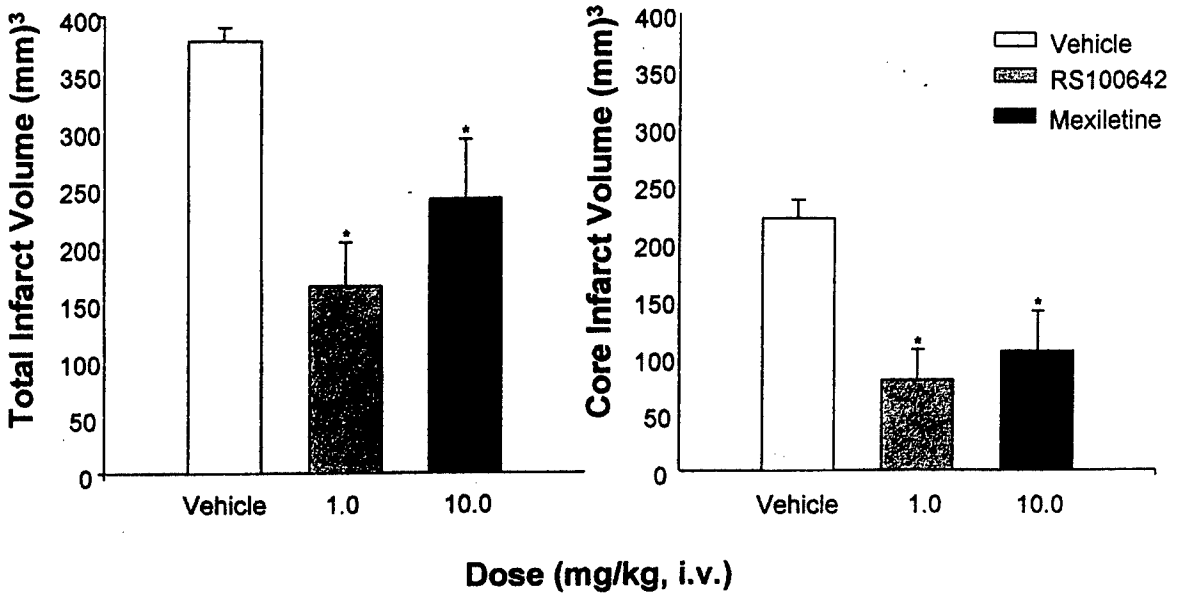

Fig. 5. Effects of maximally efficacious doses of RS100642 and mexiletine to reduce total (A) and core (B) brain infarct volume following 2 h MCAo and 22 h reperfusion and recovery. *$P<0.05$, **$P<0.01$, ANOVA followed by Dunnet's post hoc analysis.

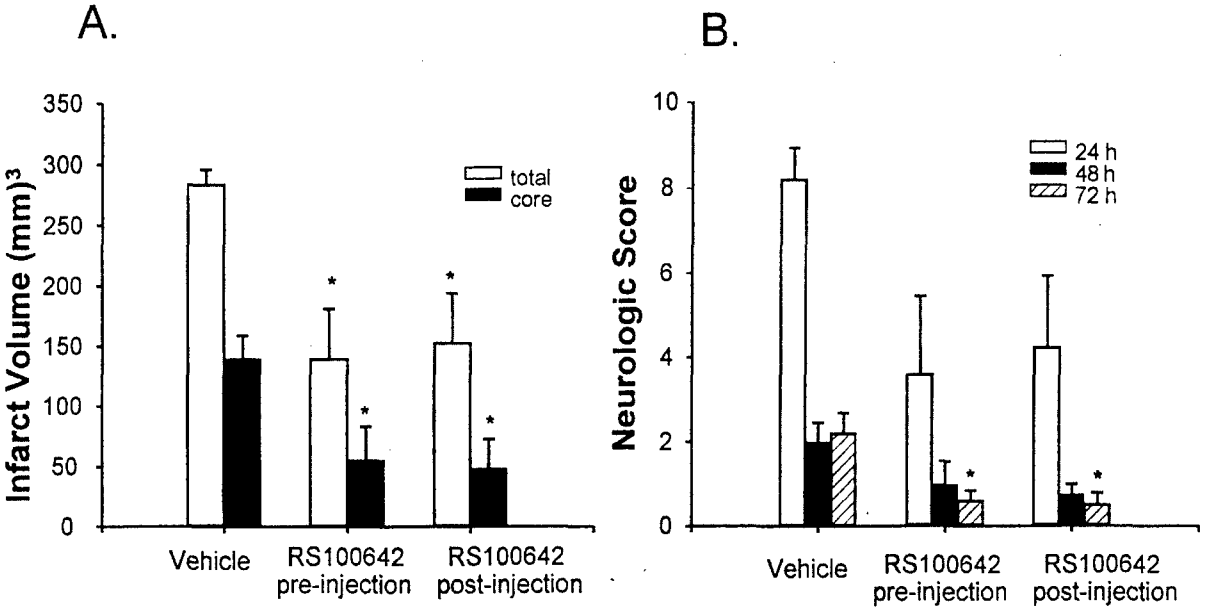

Fig. 6. Effect of 30 min pre- or 30 min post-MCAo treatment with RS100642 (n=4–5 per group) to (A) reduce brain infarct volume *P<0.05, ANOVA followed by Dunnet's post hoc analysis, and (B) improve neurological outcome *P<0.05, Mann–Whitney U-test, following 1 h MCAo and 70 h reperfusion and recovery.

neurological recovery at 72 h post-MCAo, NS=0.6±0.2 and 0.5±0.3, respectively (Fig. 6B) where two animals in each group exhibited complete recovery of neurological function (NS=0). Temperature measurements from RS100642 treated animals (data not shown) were not significantly different from the corresponding control, vehicle-treated animals (P>0.05, ANOVA).

3.4. Ischemia-induced brain seizure activity

MCAo-injured animals exhibited non-convulsant seizures (NCS), predominately during the first hour of injury, which were attenuated following reperfusion of blood to the brain (as observed from EEG recordings). However, no behavioral convulsions were observed during brain seizure activity and in general animals were conscious and ambulatory, although occasionally wet dog shake (WDS) behavior was observed during NCS. The NCS were generalized spike/slow-wave complexes occurring at a frequency of 1–2 per second and lasting 134±33 s with 4.2±1.2 ictal events occurring in four out of five vehicle-treated animals (Fig. 7). On average, NCS began 26±6 min following occlusion of the MCA in vehicle-treated animals. Furthermore, NCS were generalized to the entire brain and were recorded from all 10 cortical electrodes. Each consecutive seizure spike gradually increased in amplitude (Fig. 7) and following resolution of seizure activity a period of post-ictal EEG depression occurred. Pre-treatment with RS100642 (1.0 mg/kg) completely blocked all NCS activity. With 30 min post-MCAo treatment of RS1000642 (1.0 mg/kg), three of four animals experienced NCS but only 1 event occurred per animal and was shorter in duration (39±12 s, P<0.05, independent t-test as compared to vehicle group).

4. Discussion

RS100642, a new sodium channel blocker and neuroprotective agent, is an analog of the use dependent sodium channel blocker mexiletine [10]. Previous in vitro studies with RS100642 have reported complete neuroprotection against injuries caused by hypoxia/hypoglycemia (i.e., ischemic injury) or the Na^+ channel activator veratridine, but not glutamate, in primary neuronal cultures. Furthermore, in these same cultures there was an attenuation of the rise in intracellular Ca^{2+} due to veratradine or KCl but not glutamate [10], indicating a selective modulation of sodium channels. Additionally, RS100642 was also reported to possess sodium channel binding properties similar to mexiletine (pK_i=5.09 μM) but exhibited a more potent use-dependent reduction in compound action potentials as determined by rat vagus nerve preparations [10]. At concentrations up to 10 μM RS100642 binding affinity is selective for sodium channels, with no significant affinity for other neuronal receptor systems including opiate, glutamate, GABA, or other voltage-gated ion channels (John Hunter, Roche Biosciences, personal communication).

In rodent models of focal ischemia sodium channel modulation with various sodium channel blockers, including mexiletine, lamotrigine, and BW619C89, has been described as neuroprotective [13,16,30] indicating the potential for targeting neuronal sodium channels as a neuroprotection mechanism to treat ischemic brain injury.

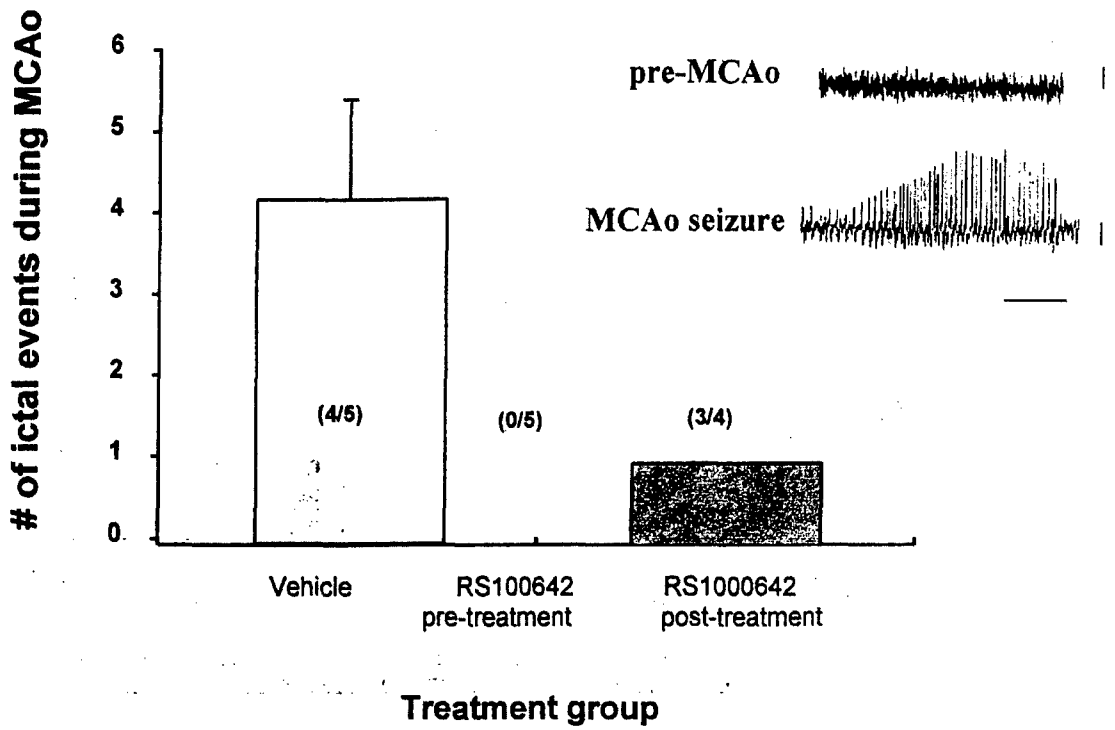

Fig. 7. Effect of 30 min pre- or 30 min post-MCAo treatment with RS100642 to reduce the number of ictal seizure events. The number of animals exhibiting seizure activity are given in parentheses. Baseline and ictal seizure activity are shown with vertical bars representing 100 μV and the horizontal bar representing 1 min of EEG recording.

This study describes the in vivo dose-dependent neuroprotection profile of post-injury treatment with RS100642 to reduce brain injury and improve functional recovery from experimental transient focal ischemia and reperfusion in the rat, as well as block ischemia-induced brain seizure activity.

The pharmacological basis for achieving neuroprotection against ischemic injury with sodium channel blockers is unresolved but changes in ionic sodium dynamics influencing ischemic injury have been clearly established [36]. Although the brain only comprises 2% of the total human body weight it consumes 25% of the available glucose and 20% of systemic oxygen. Nearly half of the central nervous systems energy supplies are utilized to drive Na^+–K^+-ATPase ion pumps, which repolarize neurons following an action potential as well as maintain ionic balance in glial cells [19]. A complex series of events occurs when cells lose their source of glucose and oxygen following ischemia. One major consequence is that mitochondria are unable to produce the ATP needed by the ionic pumps [36]. Glial cells then become unable to absorb extracellular glutamate, a process dependent on the Na^+ gradient created by Na^+–K^+-ATPase pumps. Additionally, reverse operation of the glutamate transporter also increases extracellular glutamate levels, leading to excitotoxicity or overstimulation of the post-synaptic neuron. To further exacerbate the problem of increased excitation, the post-synaptic neurons become unable to pump out the accumulated intracellular Na^+ and cannot reestablish ionic gradients. The result is the necrotic degradation of cells due to osmotic swelling [9]. Even for surviving cells an increase in intracellular Ca^{2+} levels, due to opening of Ca^{2+} permeable ion channels and reverse operation of the Na^+/Ca^{2+} exchanger, leads to a cascade of cellular death mechanisms involving delayed Ca^{2+}-mediated apoptotic processes of cellular degradation [21].

In this study we have used a model of temporary MCAo and reperfusion to produce brain injury in the rat closely resembling clinical stroke pathology [28]. Histological evaluation of injured tissues following MCAo using TTC staining revealed brain regions to be either completely infarcted (those areas completely lacking TTC stain) or pathologically compromised (regions of light pink staining). TTC is reduced to a red-formazon product in normal brain tissue due to the presence of active mitochondrial enzymes, and has been shown to correlate to other common histological markers of brain injury [3,24]. This model of in vivo transient MCAo has been widely used as an experimental model of ischemic brain injury and has proven to be highly sensitive to various neuroprotective drug interventions [8,14,20,26,33,38,42]. In this study, post-injury treatment with RS100642 dose-dependently reduced brain infarct volume, increased recovery in EEG power over the injured cortex and showed improvement of neurological recovery as evaluated 24 h post-injury. The correlation of increased EEG recovery with a reduction of

brain infarction is similar to the effects seen with other neuroprotective compounds in this model, including the high affinity *N*-methyl-D-aspartate antagonist conantokin-G and an experimental anti-inflammatory agent PS519 [26,38].

Although the temporal pathobiology of brain injury may continue for several weeks, by 72 h the injury has reached maximum hemispheric infarction volume [17]. As such, the neuroprotective effects of RS100642 were also confirmed following longer recovery periods of 72 h. Importantly, the neuroprotective effects of RS100642 were not likely due to drug-induced brain hypothermia since rectal temperatures, which have been shown to correlate to brain temperatures [40,43], never fell below normal levels following MCAo and treatment with RS100642.

Coincident with the neuroprotective alleviation of brain injury following RS100642 treatment we measured a significant and profound reduction of brain seizure activity. Initiation of NCS following MCAo injury generally began within 30 min of MCAo. Although pre-ictal treatment with RS100642 completely blocked this activity, post-ictal treatment was also able to reduce seizure activity and still provide a significant and comparable neuroprotective effect. Previously, we have described the spatio-temporal pathophysiology of brain ischemia in the MCAo model using high-resolution 10-electrode topographic mapping in the rat [18,39]. These studies revealed the presence of ischemia-induced brain seizures as well as significant disruption of the EEG activity throughout the injured brain. Although the direct relation between these types of seizures and their possible involvement in promoting the pathology of the brain injury has not been firmly established, this seizure activity is undoubtedly an inherent part of the overall pathophysiology of ischemic brain injury and likely detrimental to recovery. Sustained or repeated Na^+ channel openings has been shown to induce persistent Na^+ conductance across cellular membranes likely due to a shift in Na^+ channel gating properties, reducing the likelihood of Na^+ channel inactivation [1,6]. Persistent Na^+ conductance can induce long-lasting depolarizing plateau potentials and has been proposed to underlie the presence of repeated depolarizing waves associated with seizures, spreading depression, as well as the sustained depolarizations of ischemic brain tissues [7,32]. Following brain injury, the modulation of sodium channels to reduce cellular depolarizations not only ameliorates the mechanisms of glutamate-induced excitotoxicity but can also maintain cellular membrane potentials by reducing activity of Na^+/K^+ pumps (the major source of neuronal energy use) and in effect reduce the load on the already compromised energy reserves. Clinically, the therapeutic use of anticonvulsants following brain injury in not widely practiced except in those patients who develop convulsive seizure activity [2].

Sodium channels exhibit different electrophysiological properties including the classical fast inactivating, tetrodotoxin (TTX)-sensitive channels of α-subunits I, II, IIA, III, and PN1, slow inactivating, TTX-resistant α-subunits PN3 and NaN, and others including Na6 and NaG [11,12,23]. Each of these subunits also exhibits different patterns of expression throughout the central and peripheral nervous system [12]. Furthermore, it has been implicated that down-regulation of sodium channels during hypoxia may be a survival strategy of diving turtles, whose brains are deprived of oxygen for extended periods of time [25]. Our laboratory is currently evaluating the temporal profile of sodium channel expression in the MCAo model to determine the optimal time course of intervention as well as specific sodium channel subunits to target in neuroprotective drug development [41].

Treatment with RS100642 also provided an improved safety profile (i.e., neuroprotective to neurotoxic index) as compared to mexiletine. Mexiletine evaluation in this study was limited by its effect to induce seizure toxicity at a dose (20 mg/kg) only twice that of its determined neuroprotective dose (10 mg/kg). Not only was RS100642 more efficacious and more potent than mexiletine in reducing brain infarction and improving functional recovery, it produced no signs of neurotoxicity in the neuroprotection dose range. Importantly, separate experiments in normal rats given acute subcutaneous injections of RS100642 up to 600 mg/kg also provided no evidence of brain seizure activity (unpublished observations). Smith and Meldrum [30] have reported a 31% reduction of total infarct volume following treatment with lamotrigine (20 mg/kg, i.v.) when given immediately after MCAo as well as a significant reduction when delayed 1 h post-MCAo (although only cortical infarction was reduced) but its effect was limited at higher doses (50 mg/kg, i.v.) by toxicity. Neuroprotection with the lamotrigine analog BW619C89 has also been reported in the MCAo model to provide a 20% reduction of total infarction (30 mg/kg, i.v.) when delivered 45 min post-MCAo [13]. Other compounds with sodium channel blocking properties including carbamezapine and phenytoin have also shown efficacy as neuroprotectants in the MCAo model when delivered 30 min post-occlusion offering a 25 and 39% reduction of cortical infarction, respectively [27]. In comparison, RS100642 provided a 64% reduction in core infarction when delivered 30 min post-MCAo, with no overt behavioral or EEG signs of toxicity.

In conclusion, a comprehensive dose–response study has established the neuroprotective efficacy of RS100642 to effectively reduce brain infarct volume and improve functional recovery following post-injury treatment in a focal cerebral brain injury model of stroke. Furthermore, this neuroprotective effect was associated with an attenuation of ischemia-induced seizure activity. Collectively, these results support the possible clinical efficacy of RS100642 as a post-injury treatment of ischemic type brain injury. Further studies with this compound will be aimed at evaluating long term functional recovery using

high resolution EEG mapping and defining the 'therapeutic window' of treatment by delaying time of initial treatment post-injury.

References

[1] C. Alzheimer, P. Schwindt, W. Crill, Postnatal development of a persistent Na+ current in pyramidal neurons from rat sensorimotor cortex, J. Neurophysiol. 69 (1993) 290–292.

[2] A. Arboix, L. Garcia-Eroles, J. Massons, M. Oliveres, E. Comes, Predictive factors of early seizures after acute cerebrovascular disease, Stroke 28 (1997) 1590–1594.

[3] J. Bederson, L. Pitts, S. Germano, M. Nishimura, R. Davis, H. Bartkowski, Evaluation of 2,3,5-triphenyltetrazolium chloride as a stain for detection and quantification of experimental cerebral infarction in rats, Stroke 17 (1986) 1304–1308.

[4] J. Bederson, L. Pitts, M. Tsuji, M. Nishimura, R. Davis, H. Bartkowski, Rat middle cerebral artery occlusion: evaluation of the model and development of a neurologic examination, Stroke 17 (1986) 472–476.

[5] R. Bonita, Epidemiology of stroke, Lancet 339 (1992) 342–344.

[6] A. Brown, P. Schwindt, W. Crill, Different voltage dependence of transient and persistent Na+ currents is compatible with modal-gating hypothesis for sodium channels, J. Neurophysiol. 71 (1994) 2562–2565.

[7] E. Busch, M. Gyngell, M. Eis, M. Hoehn-Berlage, K. Hossmann, Potassium-induced cortical spreading depressions during focal cerebral ischemia in rats: contribution to lesion growth assessed by diffusion-weighted NMR and biochemical imaging, J. Cereb. Blood Flow Metab. 16 (1996) 1090–1099.

[8] J. Callaway, M. Knight, D. Watkins, P. Beart, B. Jarrott, Delayed treatment with AM-36, a novel neuroprotective agent, reduces neuronal damage after endothelin-1-induced middle cerebral artery occlusion in conscious rats, Stroke 30 (1999) 2704–2712, Discussion 2712.

[9] A. Carter, The importance of voltage-dependent sodium channels in cerebral ischaemia, Amino Acids 14 (1998) 159–169.

[10] J. Dave, Y. Lin, H. Ved, M. Koenig, L. Clapp, J. Hunter, F. Tortella, RS-100642-198, a novel sodium channel blocker, provides differential neuroprotection against hypoxia/hypoglycemia, veratridine or glutamate-mediated neurotoxicity in primary cultures of rat cerebellar neurons, Neurotoxicity Res. 3 (2001) 381–395.

[11] S. Dib-Hajj, L. Tyrrell, J. Black, S. Waxman, NaN, a novel voltage-gated Na channel, is expressed preferentially in peripheral sensory neurons and down-regulated after axotomy, Proc. Natl. Acad. Sci. USA 95 (1998) 8963–8968.

[12] P. Felts, S. Yokoyama, S. Dib-Hajj, J. Black, S. Waxman, Sodium channel alpha-subunit mRNAs I, II, III, NaG, Na6 and hNE (PN1): different expression patterns in developing rat nervous system, Brain Res. Mol. Brain Res. 45 (1997) 71–82.

[13] S. Graham, J. Chen, J. Lan, M. Leach, R. Simon, Neuroprotective effects of a use-dependent blocker of voltage-dependent sodium channels, BW619C89, in rat middle cerebral artery occlusion, J. Pharmacol. Exp. Ther. 269 (1994) 854–859.

[14] S. Kawasaki-Yatsugi, C. Ichiki, S. Yatsugi, M. Shimizu-Sasamata, T. Yamaguchi, YM90K, an AMPA receptor antagonist, protects against ischemic damage caused by permanent and transient middle cerebral artery occlusion in rats, Naunyn Schmiedebergs Arch. Pharmacol. 358 (1998) 586–591.

[15] M. Kotila, M. Moskowitz, Epilepsy after stroke, Epilepsia 33 (1992) 495–498.

[16] E. Lee, I. Ayoub, F. Harris, M. Hassan, C. Ogilvy, K. Maynard, Mexiletine and magnesium independently, but not combined, protect against permanent focal cerebral ischemia in Wistar rats, J. Neurosci. Res. 58 (1999) 442–448.

[17] P. Lipton, Ischemic cell death in brain neurons, Physiol. Rev. 79 (1999) 1431–1568.

[18] X.C. Lu, A. Williams, F.C. Tortella, Quantitative electroencephalography spectral analysis and topographic mapping in a rat model of middle cerebral artery occlusion, Neuropathol. Appl. Neurobiol., in press.

[19] J. Magistretti, D. Ragsdale, A. Alonso, Direct demonstration of persistent Na+ channel activity in dendritic processes of mammalian cortical neurones, J. Physiol. 521 (Pt. 3) (1999) 629–636.

[20] I. Margaill, S. Parmentier, J. Callebert, M. Allix, R. Boulu, M. Plotkine, Short therapeutic window for MK-801 in transient focal cerebral ischemia in normotensive rats, J. Cereb. Blood Flow Metab. 16 (1996) 107–113.

[21] L. Martin, N. Al-Abdulla, A. Brambrink, J. Kirsch, F. Sieber, C. Portera-Cailliau, Neurodegeneration in excitotoxicity, global cerebral ischemia, and target deprivation: a perspective on the contributions of apoptosis and necrosis, Brain Res. Bull. 46 (1998) 281–309.

[22] C. Meltzer, P. Adelson, R. Brenner, P. Crumrine, C.A. Van, D. Schiff, D. Townsend, M. Scheuer, Planned ictal FDG PET imaging for localization of extratemporal epileptic foci, Epilepsia 41 (2000) 193–200.

[23] S. Novakovic, E. Tzoumaka, J. McGivern, M. Haraguchi, L. Sangameswaran, K. Gogas, R. Eglen, J. Hunter, Distribution of the tetrodotoxin-resistant sodium channel PN3 in rat sensory neurons in normal and neuropathic conditions, J. Neurosci. 18 (1998) 2174–2187.

[24] C. Park, A. Mendelow, D. Graham, J. McCulloch, G. Teasdale, Correlation of triphenyltetrazolium chloride perfusion staining with conventional neurohistology in the detection of early brain ischaemia, Neuropathol. Appl. Neurobiol. 14 (1988) 289–298.

[25] M. Perez-Pinzon, M. Rosenthal, T. Sick, P. Lutz, J. Pablo, D. Mash, Downregulation of sodium channels during anoxia: a putative survival strategy of turtle brain, Am. J. Physiol 262 (1992) 712–715.

[26] J. Phillips, A. Williams, J. Adams, P. Elliott, F. Tortella, Proteasome inhibitor PS519 reduces infarction and attenuates leukocyte infiltration in a rat model of focal cerebral ischemia, Stroke 31 (2000) 1686–1693.

[27] J. Rataud, F. Debarnot, V. Mary, J. Pratt, J. Stutzmann, Comparative study of voltage-sensitive sodium channel blockers in focal ischaemia and electric convulsions in rodents, Neurosci. Lett. 172 (1994) 19–23.

[28] E. Ringelstein, R. Biniek, C. Weiller, B. Ammeling, P. Nolte, A. Thron, Type and extent of hemispheric brain infarctions and clinical outcome in early and delayed middle cerebral artery recanalization, Neurology 42 (1992) 289–298.

[29] A. Salazar, B. Jabbari, S. Vance, J. Grafman, D. Amin, J. Dillon, Epilepsy after penetrating head injury. I. Clinical correlates: a report of the Vietnam Head Injury Study, Neurology 35 (1985) 1406–1414.

[30] S. Smith, B. Meldrum, Cerebroprotective effect of lamotrigine after focal ischemia in rats, Stroke 26 (1995) 117–121, Discussion 121-2.

[31] R. Tallrida, R. Murray, Manual of Pharmacological Calculations With Computer Programs, Springer-Verlag, New York, 1987.

[32] C. Taylor, L. Narasimhan, Sodium channels and therapy of central nervous system diseases, Adv. Pharmacol. 39 (1997) 47–98.

[33] F. Tortella, P. Britton, A. Williams, X. Lu, A. Newman, Neuroprotection (focal ischemia) and neurotoxicity (electroencephalographic) studies in rats with AHN649, a 3-amino analog of dextromethorphan and low affinity NMDA antagonist, J. Pharmacol. Exp. Ther. 291 (1999) 399–408.

[34] F. Tortella, P. Britton, A. Williams, X. Lu, A. Newman, Neuroprotection (focal ischemia) and neurotoxicity (electroencephalographic) studies in rats with AHN649, a 3-amino analog of dextromethorphan and low-affinity *N*-methyl-D-aspartate antagonist, J. Pharmacol. Exp. Ther. 291 (1999) 399–408.

[35] F. Tortella, J. Rose, J. Moreton, J. Hughes, J. Hunter, EEG spectral

analysis of the neuroprotective kappa opiods enadoline and PD117302, J. Pharmacol. Exp. Ther. 282 (1997) 286–293.

[36] J. Urenjak, T. Obrenovitch, Neuroprotection—rationale for pharmacological modulation of Na(+)-channels, Amino Acids 14 (1998) 151–158.

[37] P. Vespa, M. Nuwer, V. Nenov, E. Ronne-Engstrom, D. Hovda, M. Bergsneider, D. Kelly, N. Martin, D. Becker, Increased incidence and impact of nonconvulsive and convulsive seizures after traumatic brain injury as detected by continuous electroencephalographic monitoring, J. Neurosurg. 91 (1999) 750–760.

[38] A. Williams, J. Dave, J. Phillips, Y. Lin, R. McCabe, F. Tortella, Neuroprotective efficacy and therapeutic window of the high-affinity *N*-methyl-D-aspartate antagonist conantokin-G: in vitro (primary cerebellar neurons) and in vivo (rat model of transient focal brain ischemia) studies, J. Pharmacol. Exp. Ther. 294 (2000) 378–386.

[39] A. Williams, F. Tortella, Topographic EEG mapping following experimental stroke in rats and treatment with the neuroprotective sodium-channel blocker RS100642, Soc. Neurosci. Abstr. 26 (2000).

[40] D. Xue, Z. Huang, K. Smith, A. Buchan, Immediate or delayed mild hypothermia prevents focal cerebral infarction, Brain Res. 587 (1992) 66–72.

[41] C. Yao, F. Tortella, A. Williams, J. Dave, Real-time quantitative RT-PCR assay for voltage-gated sodium channel genes in rat brain: effects of focal ischemia, Soc. Neurosci. Abstr. 26 (2000).

[42] S. Zausinger, E. Hungerhuber, A. Baethmann, H. Reulen, R. Schmid-Elsaesser, Neurological impairment in rats after transient middle cerebral artery occlusion: a comparative study under various treatment paradigms, Brain Res. 863 (2000) 94–105.

[43] Y. Zhang, P. Lipton, Cytosolic Ca2+ changes during in vitro ischemia in rat hippocampal slices: major roles for glutamate and Na+-dependent Ca2+ release from mitochondria, J. Neurosci. 19 (1999) 3307–3315.

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Differential Pattern of Expression of Voltage-gated Sodium Channel Genes Following Ischemic Brain Injury in Rats†

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This study investigated the effects of brain ischemia on sodium channel gene (NaCh) expression in rats. Using quantitative RT–PCR, our findings demonstrated the expression ratio of NaCh genes in normal rat brain to be $Na_v1.1 > Na_v1.8 > Na_v1.3 > Na_v1.7$ (rBI > PN3 > rBIII > PN1). In contrast, brain injury caused by middle cerebral artery occlusion (MCAo) for 2 h followed by reperfusion significantly down-regulated $Na_v1.3$ and $Na_v1.7$ genes in both injured and contralateral hemispheres; whereas the $Na_v1.8$ gene was down regulated in only the injured hemisphere (though only acutely at 2 or 2–6 h post-MCAo). However, the time-course of NaCh gene expression revealed a significant down-regulation of $Na_v1.1$ only in the ischemic hemisphere beginning 6 h post-MCAo and measured out to 48 h post-MCAo. In a separate preliminary study $Na_v1.2$ (rBII) gene was found be expressed at levels greater than that of $Na_v1.1$ in normal rats and was significantly down regulated at 24 h post-MCAo. Our findings document, for the first time, quantitative and relative changes in the expression of various NaCh genes following ischemic brain injury and suggest that the $Na_v1.1$ sodium channel gene may play a key role in ischemic injury/recovery.

Keywords: Ischemia; Stroke; Voltage-gated sodium channels; Gene expression; Quantitative RT–PCR

INTRODUCTION

Cerebrovascular disease is a major cause of death and disability (Bonita, 1992) caused by the metabolic failure of neurons and glia due to deprivation of continuous supply of oxygenated blood. Insults such as stroke or ischemia are known to trigger a vicious cycle of electrical and chemical events (Lee *et al.*, 1999; Carter, 1998). It is now widely accepted that neuronal cell death caused by ischemia results from a cascade of events, ranging from excessive presynaptic release of excitatory amino acids (EAA), alterations in cellular ionic dynamics, toxic postsynaptic accumulation of intracellular calcium $[Ca^{2+}]_i$, and ultimately the activation of secondary signaling mechanisms leading to acute or delayed injury processes (Choi, 1987; DeCoster *et al.*, 1992; Mattson *et al.*, 1992). This process of excitotoxicity is also believed to include a series of genomic events from the expression of immediate early genes to the synthesis of proteins, which in turn regulate the expression of other genes (Evan *et al.*, 1992; Smeyne *et al.*, 1993; Dave and Tortella, 1994; Lu *et al.*, 1997).

Voltage-dependent sodium channels (NaChs) may play a crucial role in neuron excitability and are considered one of the several cellular targets for neuroprotective mechanisms of action. It has been suggested that under ischemic/excitotoxic conditions blockade of sodium channels not only prevents the excessive depolarization and limits excitotoxic glutamate release (through reversal of the sodium-dependent glutamate transporter), but also allows calcium extrusion leading to reestablishment of the ionic homeostasis (Lysko *et al.*, 1994). In fact, several *in vivo* studies using sodium channel blockers such as tetrodotoxin and lamotrigine have

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described moderate neuroprotection in experimental models of brain ischemia (Lekieffre and Meldrum, 1993; Lysko *et al.*, 1994; Xie *et al.*, 1995; Kimura *et al.*, 1998), and in a recent preliminary study from our laboratory, a novel NaCh blocker RS-100642-198 was described to significantly reduce cerebral infarction resulting from MCAo (Williams *et al.*, 1999). As such, changes in the transcription of NaChs genes could be involved in the neuronal injury associated with brain ischemia, as well as the neuronal plasticity associated with functional recovery.

The sodium channel is comprised of α and β-subunits, with the pore forming α-subunit 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 sodium channel α-subunits belong to a multigene family, and cloning and electrophysiological characterization studies have documented the presence of ten different sodium channel genes (e.g. rat brain I, II, III, rat SkM1, SkM2, rat NaCh6, PN1, PN3, NaN/SNS2, Na-G) in the central and peripheral nervous systems and other excitable tissues (heart and muscle) 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 recent nomenclature, these voltage-gated sodium channel genes are now referred to as $Na_v1.1$–$Na_v1.9$ and Na_x, respectively (Goldin *et al.*, 2000). However, the specific physiological roles distinguishing each of these isoforms remain unknown. One possible explanation for this is that the differences in the functional properties of these channels are subtle (Noda *et al.*, 1986; Auld *et al.*, 1988). Nevertheless, such subtle differences could be functionally important, because small changes in voltage dependence of activation or inactivation could markedly affect excitability.

In order to assess the relationship between differential gene expression and function, it is essential to accurately and quantitatively measure mRNA levels. While a number of techniques, such as Northern blotting and semi-quantitative reverse transcription-polymerase chain reaction (RT–PCR) are available to measure levels of mRNA expression, certain limitations exist, including the insensitive and potentially inaccurate quantitation of mRNA that may be normally expressed in low abundance. Indeed, using these non-quantitative techniques our initial pilot studies revealed highly variable results in NaCh gene expression in injured rat brain (Cui *et al.*, 1999). Recently described methods for quantitative RT–PCR are more sensitive, more accurate and able to detect and amplify low abundance mRNA (Livak *et al.*, 1995; Gibson *et al.*, 1996). Using quantitative RT–PCR, the present studies were therefore undertaken to determine the time-related changes in expression of four neuronal sodium channel genes, namely $Na_v1.1$ and $Na_v1.3$ (rBI and rBIII) and peripheral nerve type $Na_v1.7$ and $Na_v1.8$ (PN1 and PN3) in rat brain following focal brain ischemia produced by MCAo. An additional preliminary study was also begun to determine changes in $Na_v1.2$ gene expression post-MCAo.

MATERIALS AND METHODS

Focal Ischemic Surgery

Middle cerebral artery occlusion was carried out according to our previously described method (Phillips *et al.*, 2000; Williams *et al.*, 2000). Briefly, male Sprague-Dawley rats weighing 270–330 g (Charles River Labs, Raleigh, VA) were anesthetized by 5% halothane and maintained at 2% halothane. An intraluminal filament occluded the right middle cerebral artery for a period of 2 h followed by reperfusion for 0, 4, 22 and 46 h. Control groups of rats received sham surgery, in which an identical procedure was followed but without inserting the filament (no occlusion). From each injured brain a 2 mm thick coronal slice located 5 mm from the frontal pole was dissected for RNA extraction. From this slice injured and contralateral hemispheres were dissected out. The remaining brain tissue was stained with 2,3,5-triphenyltetrazolium chloride (TTC; see Williams *et al.*, 2000) in order to ascertain that the MCAo was successful.

Total RNA Isolation

The 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.

Primers and Probes

The primers and probes for sodium channel 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 are presented in Table I. Synthesis of these probes and primers was performed by Perkin-Elmer Applied Biosystems Foster City, CA. FAM (6-carboxy-fluorescein) was used as the reporter and TAMRA (6-carboxy-tetramethyl-rhodamine) as the quencher dye. The housekeeping gene β-actin was used as an endogenous control in these samples to provide sample amplification efficiency.

TABLE I The sequences of forward and reverse primers and probes used for quantitative RT–PCR of five sodium channel genes and β-actin gene expression. The probes consisted of a reporter dye (6-carboxyfluorescein; FAM) at 5′ position and a quencher dye (6-carboxy-tetramethyl-rhodamine; TAMRA) at 3′ position

Gene	Primer/probe	Sequence (5′-3′)	Base pairs
$Na_v1.1$	Forward primer	CAT TTC TGT CCC TGT TTC GAC TG	23
	Reverse primer	TCC CGG CAG CAC GC	14
	Probe	TGA CTC AGG ACT TCT GGG AAA ATC TTT ACC AAC	33
$Na_v1.2$	Forward primer	GAA GCT GTC ACC GCT GCA T	19
	Reverse primer	TCC CTG TTA GAC TTC TGG ACA GCA	24
	Probe	CTC TCT TGC TGA TGC CGC TGT ATC TTA ACC	30
$Na_v1.3$	Forward primer	CCC AAC TACGGC TAC ACA AGC	21
	Reverse primer	CCT GAG TCA TGA GTC GAA ACA GG	23
	Probe	TTG ACA CCT TCA GCT GGG CCT TCT TGT	27
$Na_v1.7$	Forward primer	CAA CTC GCT GGG CGC A	16
	Reverse primer	CCG ACC ACG GCA AAA ATG	18
	Probe	CTG ACC CTG GTG CTG GCC ATC A	22
$Na_v1.8$	Forward primer	TCC TTG GGC CAC AGG CT	17
	Reverse primer	AAA CTC TCA AAC CAG CTG TGC TC	23
	Probe	AGG TGC GCA AGA CCT GCT ACC GC	23
β-actin	Forward primer	GTC TCA CGT CAG TGT ACA GGC C	22
	Reverse primer	TTG GTC TCC CTG GGA GTG G	19
	Probe	CCC TGG CTG CCT CAA CAC CTC AA	23

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 1 × PCR Buffer II, 5 mmol/l $MgCl_2$, 1 mmol/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 RT products contains 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 s 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/group) were made by analysis of variance (ANOVA; Fisher's protected least squares difference) and values were considered to be significant when $P < 0.05$.

RESULTS

Infarct Analysis

All rats survived throughout the experiment. Computer-assisted image analysis was used to digitally image the posterior surface of each TTC-stained forebrain section (Loats Associates, Westminster, MD). Figure 1 show the representative images of coronal sections demonstrating ipsilateral infarcts in the ischemic hemisphere, and the

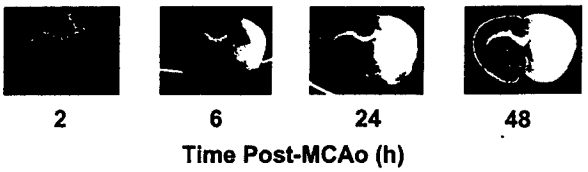

FIGURE 1 Representative forebrain images showing injury at different time after MCAo. 2 mm thick brain sections were stained with TTC as described earlier.

corresponding non-infarcted contralateral hemisphere, taken from animals at 2–48 h post-MCAo. At 2 h post-MCAo (no reperfusion) there was no obvious ischemic damage to the cerebral tissue. The severity of the ischemic injury was progressive as the reperfusion time extended from 6 to 48 h post-MCAo.

Standard Curves

Calibration curves for quantification of sodium channel gene expression were constructed on a 1:2 serial dilution of DNA Template Reagents (Perkin Elmer) (Fig. 2; top). These standard curves demonstrate that quantitation of each target gene was linear on a scale of at least seven orders of magnitude with excellent correlation factors of 0.99 and slope values ranged between 3.6 and 4.2 (Fig. 2; bottom).

NaCh Gene Expression Efficiency in Normal Rat Brain

Quantification of the level of relative mRNA for each sodium channel gene was first analyzed in normal rat brain. The mRNA levels of $Na_v1.1$, $Na_v1.3$, $Na_v1.7$, $Na_v1.8$ and the house-keeping gene β-actin detected by quantitative RT–PCR and shown in Fig. 3 demonstrate that $Na_v1.1$ is the most abundant of the four sodium channel genes expressed in normal rat brain. Compared to β-actin gene expression, the relative level of expression of each of the NaCh genes were $Na_v1.1$, 18%; $Na_v1.3$, 8.3%; $Na_v1.7$, 7.3% and $Na_v1.8$, 9.6%. The $Na_v1.1$ gene expression was significantly greater than the other NaCh genes, whereas the differences between these channels (i.e. $Na_v1.3$, $Na_v1.7$ and $Na_v1.8$) were very small and not significant.

NaCh Gene Expression During MCAo Injury

NaCh gene expression was quantified at various time points following MCAo and the results are shown in Fig. 4(A–D). There was no significant difference in $Na_v1.1$ gene expression in the contralateral (i.e. uninjured) hemisphere (compared to the same hemisphere from sham controls) at any time point studied. However, a significant down-regulation of $Na_v1.1$ gene expression was detected in the ischemic hemisphere from 6 h post-MCAo to 48 h post-MCAo with a maximal decrease being observed at 24 h post-injury (Fig. 4A).

Compared to sham controls, $Na_v1.3$ and $Na_v1.7$ genes were significantly down regulated in both injured and contralateral hemispheres (though only acutely at 2 or 2–6 h post-MCAo). The $Na_v1.8$ gene was significantly down regulated in only the injured hemisphere at 2–6 h post-MCAo (Fig. 4B–D). However, there was no significant difference in the expression levels of these three NaCh genes at any time in the injured hemisphere when compared with their contralateral hemispheres.

In separate preliminary experiments we have initiated expression studies of the $Na_v1.2$ sodium channel gene and to date have obtained data for the 24 h post-MCAo time-point. In normal uninjured rat brain the expression of $Na_v1.2$ was found to be higher than that of $Na_v1.1$ (or $Na_v1.3$, $Na_v1.7$, $Na_v1.8$) expression. Compared to β-actin gene expression, the relative level of $Na_v1.1$ expression in normal rat brain was approximately 35%. In injured rats, $Na_v1.2$ gene expression was also significantly down-regulated, though not to the same degree as $Na_v1.1$ (30% $Na_v1.2$ vs. 75% $Na_v1.1$), in brains 24 h post-MCAo (Table II).

DISCUSSION

The present study describes changes in brain type (rBI, rBII and rBIII or $Na_v1.1$, $Na_v1.2$ and $Na_v1.3$) and peripheral nerve type (PN1 and PN3 or $Na_v1.7$ and $Na_v1.8$) sodium channel gene expression using quantitative RT–PCR in the MCAo model of rat brain injury. Although expression of peripheral nerve type sodium channel $Na_v1.7$ has been documented in normal rat brain (Sangameswaran *et al.*, 1997), to our knowledge, this is the first time that $Na_v1.8$ expression has been demonstrated in normal or injured rat brain. Furthermore, the significance of this study is the fact that this is the first and only demonstration of changes in expression of NaCh genes following ischemic brain injury, in particular the regionally selective down-regulation measured for the $Na_v1.1$ gene.

The application of quantitative RT–PCR was critical to our success in studying NaCh gene expression in rat brain since unlike other gene families such as early immediate genes, the abundance of normal endogenous NaCh mRNA is low (Dib-Hajj et al., 1996; Sangameswaran *et al.*, 1997; Waxman *et al.*, 2000) and the turnover of functional channels is relatively very slow (Waechter *et al.*, 1983; Schmidt and Catterall, 1986). In fact, as noted earlier, our pilot studies using non-quantitative RT–PCR or Northern hybridization exhibited results of NaCh gene expression in injured rat brain which while

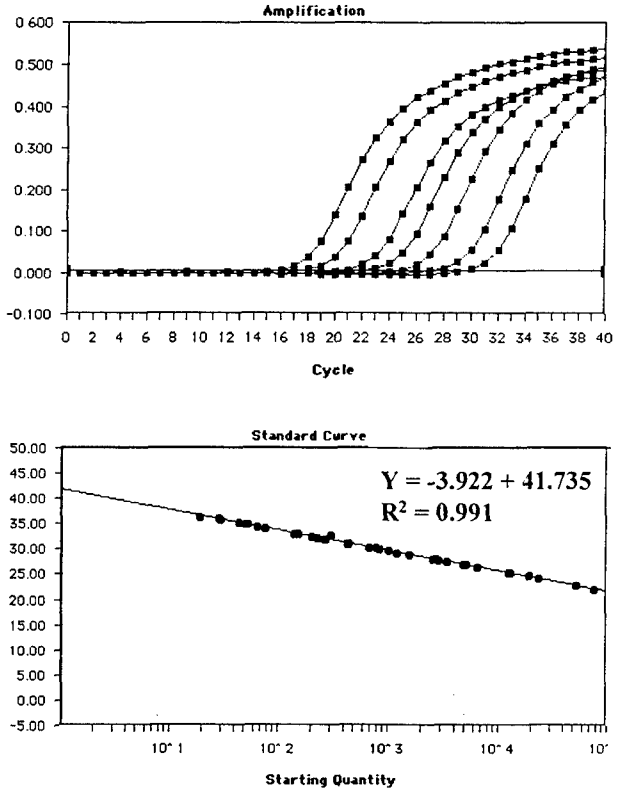

FIGURE 2 Upper panel: A representative standard curve showing real-time amplification plots of the standard DNA. Lower panel: A representative standard curve showing linear correlation between threshold cycle and the initial amount of copy numbers.

demonstrating similar trends to those reported here, were highly variable and statistically non-significant (Cui *et al.*, 1999).

In the present study, we demonstrated that among the five sodium channel genes studied, the abundance of $Na_v1.2$ channel mRNA in normal rat brain is maximum, followed by $Na_v1.1$, $Na_v1.8$, $Na_v1.3$ and $Na_v1.7$ mRNA. These results differ slightly from those reported earlier in rat dorsal root ganglia (DRG) by Sangameswaran *et al.* (1997) where $Na_v1.1$ expression was maximum, followed by $Na_v1.7$, $Na_v1.8$ and only a minimum of $Na_v1.3$ expression ($Na_v1.2$ gene was not included in this study). Although it is not surprising that the order of NaCh genes expression in the present study (i.e. brain neurons) and that reported by Sangameswaran and co-workers (i.e. peripheral DRG neurons) appears different, it is surprising that in both studies it has now been shown that the $Na_v1.1$ gene was one of the prominent NaCh gene expressed. From these collective results it appears that the $Na_v1.1$ NaCh may be one of the prominent ion channels expressed in both central and peripheral nervous systems. Of course, translation of $Na_v1.1$ 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 the RT–PCR method (Sangameswaran *et al.*, 1997), our report is the only study describing significant

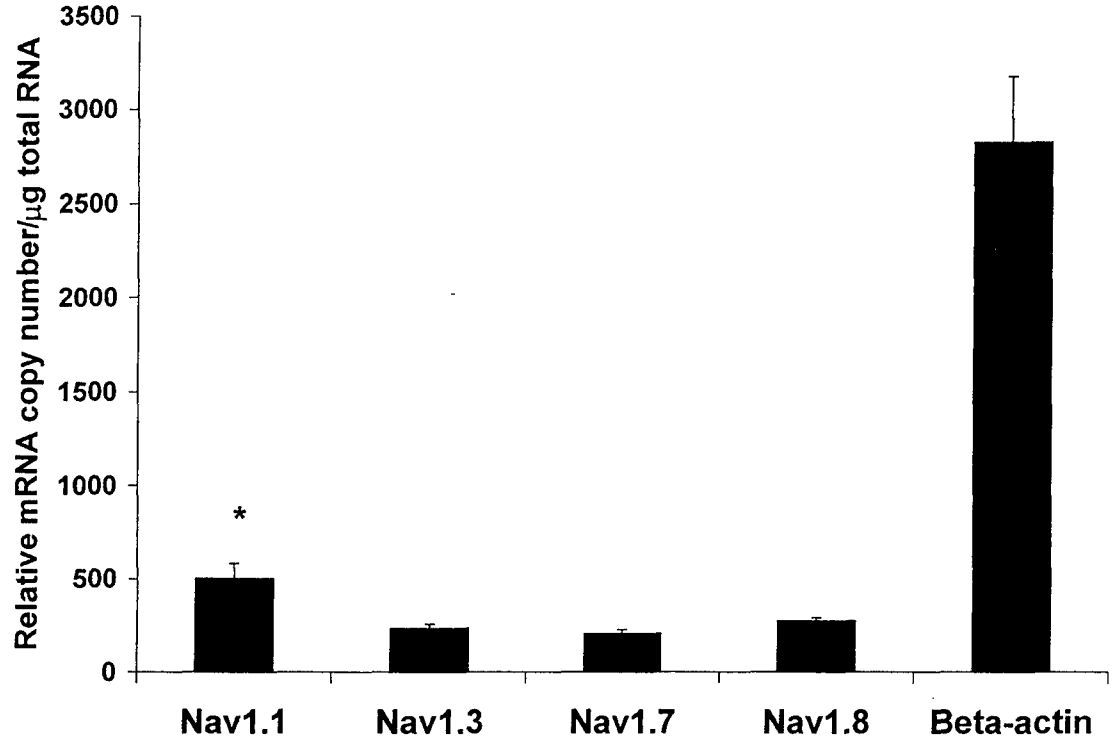

FIGURE 3 Relative expression efficiency of four sodium channel genes and (-actin gene. Values are mean ± S.E. of eight separate brain samples. Values marked with an asterisk (for $Na_v1.1$) are significantly different from $Na_v1.3$, $Na_v1.7$ and $Na_v1.8$ values.

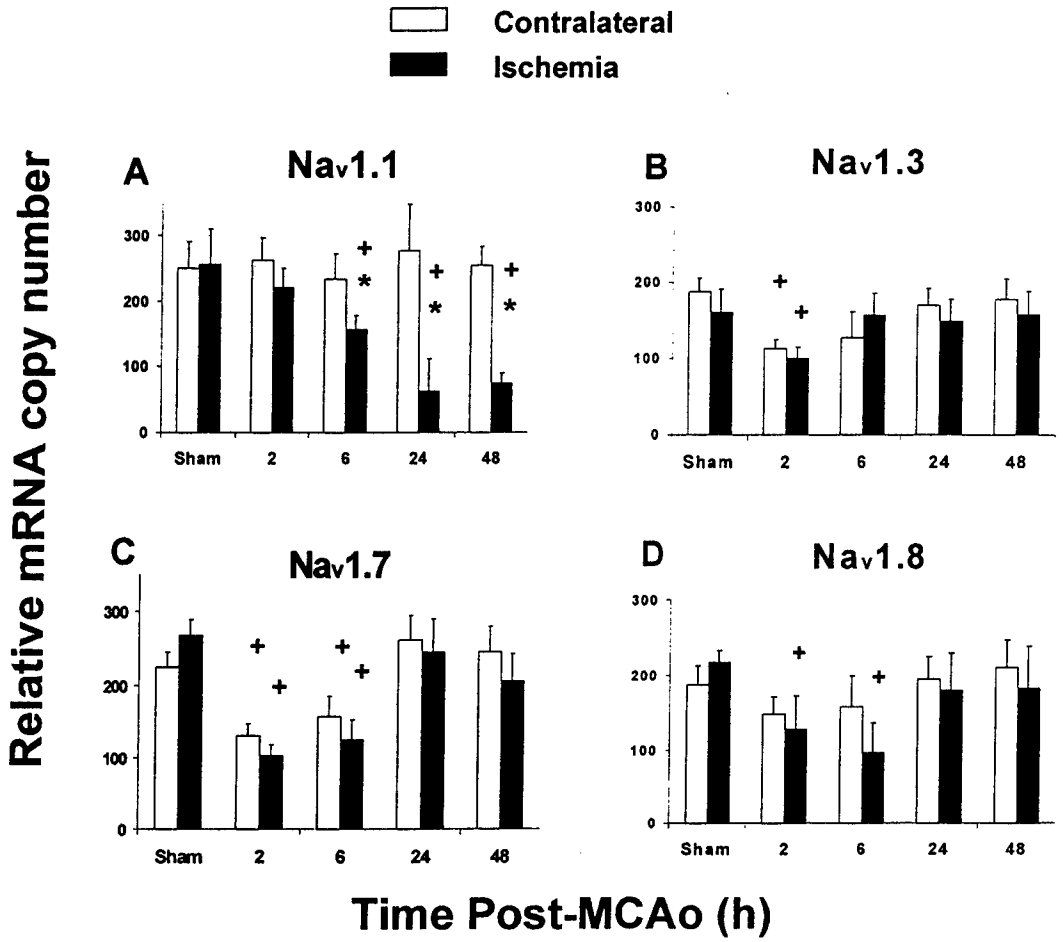

FIGURE 4 Time course of sodium channel $Na_v1.1$ (Panel A), $Na_v1.3$ (Panel B), $Na_v1.7$ (Panel C) and $Na_v1.8$ (Panel D) genes expression following MCAo injury. Open bars represent values for contralateral hemispheres and filled bars represent values from injured (ipsilateral) hemispheres. Values are mean ± S.E. of eight separate brain samples. RT products used for PCR were $Na_v1.1$ 5 μl, $Na_v1.3$ 8 μl, $Na_v1.7$ 10 μl and $Na_v1.8$ 8 μl (1 μl of RT products contains 0.1 μl total RNA. Values marked with a plus (+) sign are significantly different from the sham control values and those marked with an asterisk (*) are significantly different from the values for their respective contralateral hemispheres at $p < 0.05$.

levels of $Na_v1.8$ gene expression in both normal and injured rat brain. This discovery could simply reflect the highly sensitive quantitative RT–PCR method used in the present study, as other investigators (Novakovic *et al.*, 1998) have also failed to detect $Na_v1.8$ mRNA in rat brain using *in situ* hybridization technique. In this context it is worth mentioning that using *in situ* hybridization technique (Felts *et al.*, 1997) also failed to observe the presence of $Na_v1.7$ mRNA in either hippocampus, cerebellum and spinal cord during development (E17–P30) in rats.

The time-course of changes in $Na_v1.1$ gene expression in our study revealed a significant down-regulation in the ischemic hemisphere from 6 to 48 h post-MCAo with a maximal decrease being observed at 24 h post-injury. Similarly $Na_v1.8$ NaCh gene was also down regulated in injured hemisphere, though only acutely. Compared to sham controls, $Na_v1.3$ and $Na_v1.7$ genes were also significantly down regulated, but this was seen in both injured and contralateral hemispheres (though acutely at 2 or 2–6 h post-MCAo). However, when compared with their contralateral hemispheres, there was no significant difference in the expression levels of these genes (i.e. $Na_v1.3$, $Na_v1.7$ and $Na_v1.8$) in the injured hemisphere at any time interval studied. In view of the fact that there is a significant neuroanatomical and functional link between the brain hemispheres, one might expect profound cellular changes in the contralateral hemisphere of the brain following ischemic insult. However our results show that $Na_v1.3$, $Na_v1.7$ and $Na_v1.8$ sodium channel genes did not exhibit significant difference between the hemispheres, but rather only an acute reduction in the expression of these NaCh genes in both the hemispheres. Though speculative, it is possible that the contralateral hemisphere may have enough uncompromised sodium channel on the neuronal plasma membrane that the signaling mechanism to activate or inhibit NaCh gene expression may not have triggered. In fact in a recent EEG topography study in a similar MCAo injury model, we observed that EEG activity in the contralateral hemisphere was also altered as inter-

TABLE II Preliminary data showing expression of $Na_v1.1$, $Na_v1.2$ and β-actin genes in injured and uninjured hemispheres of rat brain 24 h post-MCAo (values are mean ± S.E.M of nine individual brain samples. Values marked with asterisks are significantly different from those obtained from uninjured hemisphere. $^*p < 0.025$; $^{**}p < 0.0001$ (Paired *T*-test))

	Relative mRNA copy number		
	$Na_v1.1$	$Na_v1.2$	β-actin
Uninjured hemisphere	508 ± 51.6	1082 ± 154.6	2935 ± 623.1
Injured hemisphere	126 ± 30.5**	753 ± 94.3*	2986 ± 455.1

mittent rhythmic activities (4–6 Hz) were observed in the frontal and central parietal regions, and increased beta activity in the temporal cortical regions, of the contralateral hemisphere (Lu *et al.*, unpublished observations). These results indicate that the ischemic insult also affects neuronal activity (and likely membrane conductance) in the contralateral hemisphere. In this context, using *in situ* hybridization for the $Na_v1.1$ gene we have discovered that the expression of this gene is in fact decreased on the contralateral hemisphere at 24 h post-MCAo (Williams *et al.*, 2001). Additional studies are currently in progress evaluating more time points and the other NaCh genes.

In separate preliminary experiments, we have begun to study the expression of the $Na_v1.2$ sodium channel gene and to date have obtained data for the 24 h post-MCAo time-point. Consistent with other reports (Lara *et al.*, 1996; Felts *et al.*, 1997; Gastaldi *et al.*, 1997), in normal uninjured rat brain the expression of $Na_v1.2$ was found to be higher than that of $Na_v1.1$ (or $Na_v1.3$, $Na_v1.7$, $Na_v1.8$) expression. In injured rats, $Na_v1.2$ gene expression was also significantly down-regulated, albeit not to the same degree as $Na_v1.1$ (30% $Na_v1.2$ vs. 75% $Na_v1.1$). However, $Na_v1.2$ gene expression has only been studied at the single time-point of 24 h post-MCAo injury. Therefore, it is possible that $Na_v1.2$ sodium channel gene may be modulated differently at other time intervals post injury and studies are currently in progress to establish the entire time course of not only $Na_v1.2$ expression, but also the $Na_v1.6$ ion channel gene.

Gene expression following cerebral ischemia involves the up-regulation of several gene families which are involved in the progression of brain injury. These genes include immediate early genes, heat shock proteins, cytokines, adhesion molecules, and apoptotic genes. Glutamate-induced calcium influx due to cerebral ischemia is considered to be one of the prominent mediators of this genomic response (Savitz and Rosenbaun, 1999). However, in some cases down-regulation of possible signaling mechanisms, such as excitatory amino acid (EAA) receptors and voltage-gated ion channels, has also been shown following brain injury and it is possible that this post-ischemic target receptor down-regulation may represent an endogenous protection mechanism of cells to reduce EAA excitotoxicity. Examples include the reduction of NR2A, NR2B, GLUR2 and GLUR3 in the hippocampal CA1 region of the rat following global ischemia (Zhang *et al.*, 1997; Pellegrini-Giampietro *et al.*, 1994). Also, in primary neuronal cultures from rats scorpion neurotoxin (a sodium channel agonist) decreased the expression of $Na_v1.1$, $Na_v1.2$, and $Na_v1.3$ (Lara *et al.*, 1996) and in rats with kainite-induced seizures, the down-regulation of $Na_v1.2$ and $Na_v1.3$ (adult subtype) NaCh gene expression in the hippocampus has also been documented (Gastaldi *et al.*, 1997), indicating that persistent activation may relate to a molecular and functional down-regulation of these channels. Importantly, this down-regulation has also shown to be sensitive to the calcium-dependent CaM kinase II activation (Carlier *et al.*, 2000). Thus, the increases in intracellular calcium following brain ischemia may induce signaling cascades involved in altering the expression of NaChs. Finally, as an interesting footnote it has also been reported that diving turtles exhibit reduced NaCh expression during hypoxic periods, possibly as a protective adaptation strategy (Perez-Pinzon *et al.*, 1992).

Chronic receptor stimulation during brain injury may be due to increased glutamate levels, which is involved in excitotoxic injury (Lipton and Rosenberg, 1994). Our data have confirmed that the down regulation of several NaCh genes does in fact occur following focal cerebral ischemia. Importantly, the down regulation observed in glutamate receptors (Lipton and Rosenberg, 1994) as well as that measured for voltage-gated NaChs in the present study, was delayed post-injury (i.e. >2–6 h post-injury). This becomes important in future drug development studies, which may target specific receptor subunits. If the target receptor is being down-regulated, efficacy of drug treatment targeting these receptors may be lost. Thus, studying the time course of gene expression of excitatory amino acid and voltage-gated ion channels is a crucial direction for future research consistent with advanced drug development targets.

In conclusion, the present study reports expression of both brain type and peripheral type sodium channels in uninjured and injured rat brain. We have also demonstrated time-dependent changes in the expression of these four sodium channel genes

following MCAo injury. Although, $Na_v1.3$, $Na_v1.7$, $Na_v1.8$ gene expression did not significantly differ (compared to contralateral hemisphere) following MCAo injury, $Na_v1.1$ gene expression showed a clear and dramatic time-related decrease and preliminary experiment suggest that $Na_v1.2$ expression may be similarly affected. The reason for this decrease in $Na_v1.1$ gene expression following MCAo injury remains speculative. In view of the fact that sodium channel blockers have been shown to be neuroprotective against ischemic insult (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. 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, possibly using antisense oligonucleotides, to specifically evaluate the role of each NaCh subunit during the injury/recovery process.

References

Akopian, A.N., Siviltti, L. and Wood, J.N. (1996) "A tetrodotoxin-resistant voltage-gated sodium channel expressed by sensory neurons", *Nature* **379**, 257–262.

Ashton, D., Willems, R., Wynants, J., Van Reempts, J., Marrannes, R. and Clincke, G. (1997) "Altered Na^+-channel function as an *in vitro* model of the ischemic penumbra: Action of lubeluzole and other neuroprotective drugs", *Brain Res.* **745**, 210–221.

Auld, V.J., Goldin, A.L., Kraft, D.S., Marshall, J., Dunn, J.M., Catterall, W.A., Lester, H.A., Davidson, N. and Dunn, R.J. (1988) "A rat brain Na^+ channel alpha subunit with novel gating properties", *Neuron* **1**, 449–461.

Bonita, R. (1992) "Epidemiology of stroke", *Lancet* **339**, 342–344.

Carlier, E., Mabrouk, K., Moulard, M., Fajloun, Z., Rochat, H., De Waard, M. and Sabatier, J.M. (2000) "Ion channel activation by SPC3, a peptide derived from the HIV-1 gp120 V3 loop", *J. Pept. Res.* **56**, 427–437.

Carter, A.J. (1998) "The importance of voltage-dependent sodium channels in cerebral ischaemia", *Amino Acids* **14**, 159–169.

Choi, D.W. (1987) "Dextrorphan and dextromethorphan attenuate glutamate neurotoxicity", *Brain Res.* **403**, 333–336.

Cui, P., Tortella, F.C., Williams, A.J., Hunter, J.C., Sangmeswaran, L. and Dave, J.R. (1999) "Expression of tetrodotoxin-sensitive and tetrodotoxin-resistant sodium channel genes in rat brain following ischemic injury", *Advances in Ion Channel Research, (Abstract)*, 12.

Dave, J.R., Lin, Y., Ved, H.S., Koenig, M.L., Clapp, L., Hunter, J. and Tortella, F.C. (2001) RS-100642-198, a novel sodium channel blocker, provides differential neuroprotection against hypoxia/hypoglycemia, veratridine or glutamate-mediated neurotoxicity in primary cultures of tat cerebellar neurons. *Neurotoxicity Res.* **3**, 381–395.

Dave, J.R. and Tortella, F.C. (1994) "Regional changes in c-fos mRNA in rat brain after i.v. or i.c.v. NMDA injections", *Neuroreport* **5**, 1645–1648.

DeCoster, M.A., Koenig, M.L., Hunter, J.C. and Tortella, F.C. (1992) "Calcium dynamics in neurons treated with toxic and non-toxic concentrations of glutamate", *Neuroreport* **3**, 773–776.

Dib-Hajj, S., Black, J.A., Felts, P. and Waxman, S.G. (1996) "Down-regulation of transcripts for Na channel alpha-SNS in spinal sensory neurons following axotomy", *Proc. Natl. Acad. Sci. USA* **93**, 14950–14954.

Evan, G.I., Wyllie, A.H., Gilbert, C.S., Littlewood, T.D., Land, H., Brooks, M., Waters, C.M., Penn, L.Z. and Hancock, D.C. (1992) "Induction of apoptosis in fibroblasts by c-myc protein", *Cell* **69**, 119–128.

Felts, P.A., Yokoyama, S., Dib-Hajj, S., Black, J.A. and Waxman, S.G. (1997) "Sodium channel alpha-subunit mRNAs I. II, III, NaG, Na6 and hNE (PN1): different expression patterns in developing rat nervous system", *Brain Res. Mol. Brain Res.* **45**, 71–82.

Gastaldi, M., Bartolomei, F., Massacrie, R.A., Planells, R., Robaglia-Schlupp, A. and Cau, P. (1997) "Increase in mRNAs encoding neonatal II and III sodium channel alpha-isoforms during kainate-induced seizures in adult rat hippocampus", *Brain Res. Mol. Brain Res.* **44**, 179–190.

Gautron, S., Dos Santos, G., Pinto-Henrique, D., Koulakoff, A., Gros, F. and Berwald-Netter, Y. (1992) "The glial voltage-gated sodium channel: cell- and tissue-specific mRNA expression", *Proc. Natl. Acad. Sci. USA* **89**, 7272–7276.

Gibson, U.E., Heid, C.A. and Williams, P.M. (1996) "A novel method for real time quantitative RT–PCR", *Genome Res.* **6**, 995–1001.

Goldin, A.L., Barchi, R.L., Caldwell, J.H., Hofmann, F., Howe, J.R., Hunter, J.C., Kallen, R.G., Mandel, G., Meisler, M.H., Netter, Y.B., Noda, M., Tamkun, M.M., Waxman, S.G., Wood, J.N. and Catterall, W.A. (2000) "Nomenclature of voltage-gated sodium channels", *Neuron* **28**, 365–368.

Isom, L.L., De Jongh, K.S., Patton, D.E., Reber, B.F., Offord, J., Charbonneau, H., Walsh, K., Goldin, A.L. and Catterall, W.A. (1992) "Primary structure and functional expression of the beta 1 subunit of the rat brain sodium channel", *Science* **256**, 839–842.

Kayano, T., Noda, M., Flockerzi, V., Takahashi, H. and Numa, S. (1988) "Primary structure of rat brain sodium channel III deduced from the cDNA sequence", *FEBS Lett.* **228**, 187–194.

Kimura, M., Sawada, K., Miyagawa, T., Kuwada, M., Katayama, K. and Nishizawa, Y. (1998) "Role of glutamate receptors and voltage-dependent calcium and sodium channels in the extracellular glutamate/aspartate accumulation and subsequent neuronal injury induced by oxygen/glucose deprivation in cultured hippocampal neurons", *J. Pharmacol. Exp. Ther.* **285**, 178–185.

Lara, A., Dargent, B., Julien, F., Alcaraz, G., Tricaud, N., Couraud, F. and Jover, E. (1996) "Channel activators reduce the expression of sodium channel alpha-subunit mRNA in developing neurons", *Brain Res. Mol. Brain Res.* **37**, 116–124.

Lee, J.M., Zipfel, G.J. and Choi, D.W. (1999) "The changing landscape of ischemic brain injury mechanisms", *Nature* **399**, A7–14.

Lekieffre, D. and Meldrum, B.S. (1993) "The pyrimidine-derivative, BW1003C87, protects CA1 and striatal neurons following transient severe forebrain ischemia in rats", *Neurosci.* **56**, 93–99.

Lipton, S.A. and Rosenberg, P.A. (1994) "Excitatory amino acids as a final common pathway for neurologic disorders", *N. Engl. J. Med.* **330**, 613–622.

Livak, K.J., Flood, S.J., Marmaro, J., Giusti, W. and Deetz, K. (1995) "Oligonucleotides with fluorescent dyes at opposite ends provide a quenched prove system useful for detecting PCR product and nucleic acid hybridization", *PCR Methods Appl.* **4**, 357–362.

Lu, X.C., Tortella, F.C., Ved, H.S., Garcia, G.E. and Dave, J.R. (1997) "Neuroprotective role of c-fos antisense oligonucleotide: *in vitro* and *in vivo* studies", *Neuroreport* **8**, 2925–2929.

Lysko, P.G., Webb, C.L., Yue, T.L., Gu, J.L. and Feuerstein, G. (1994) "Neuroprotective effects of tetrodotoxin as a Na^+ channel modulator and glutamate release inhibitor in cultured rat cerebellar neurons and in gerbil global brain ischemia", *Stroke* **25**, 2476–2482.

Mattson, M.P., Cheng, B., Davis, D., Bryant, K., Lieberburg, I. and Rydel, R.E. (1992) "beta-Amyloid peptides destabilize calcium homeostasis and render human cortical neurons vulnerable to excitotoxicity", *J. Neurosci.* **12**, 376–389.

Noda, M., Ikeda, T., Kayano, T., Suzuki, H., Takeshima, H., Kurasaki, M., Takahashi, H. and Numa, S. (1986) "Existence of distinct sodium channel messenger RNAs in rat brain", *Nature* **320**, 188–192.

Novakovic, S.D., Tzoumaka, E., McGivern, J.G., Haraguchi, M., Sangameswaran, L., Gogas, K.R., Eglen, R.M. and Hunter, J.C. (1998) "Distribution of the tetrodotoxin-resistant sodium

channel PN3 in rat sensory neurons in normal and neuropathic conditions", *J. Neurosci.* **18**, 2174–2187.

Pellegrini-Giampietro, D.E., Cozzi, A. and Moroni, F. (1994) "The glycine antagonist and free radical scavenger 7-Cl-thio-kynurenate reduces CA1 ischemic damage in the gerbil", *Neuroscience* **63**, 701–709.

Perez-Pinzon, M.A., Rosenthal, M., Sick, T.J., Lutz, P.L., Pablo, J. and Mash, D. (1992) "Downregulation of sodium channels during anoxia: a putative survival strategy of turtle brain", *Am. J. Physiol.* **262**, R712–R715.

Phillips, J.B., Williams, A.J., Adams, J., Elliott, P.J. and Tortella, F.C. (2000) "Proteasome inhibitor PS519 reduces infarction and attenuates leukocyte infiltration in a rat model of focal cerebral ischemia", *Stroke* **31**, 1686–1693.

Sangameswaran, L., Delgado, S.G., Fish, L.M. and Koch, B.D. (1996) "Structure and function of a novel voltage-gated, tetrodotoxin-resistant sodium channel specific to sensory neurons", *J. Bio. Chem.* **271**, 5953–5956.

Sangameswaran, L., Fish, L.M., Koch, B.D., Rabert, D.K., Delgado, S.G., Ilnicke, M., Jakeman, L.B., Novakovic, S., Wong, K., Sze, P., Tzoumaka, E., Stewart, G.R., Herman, R.C., Chan, H., Eglen, R.M. and Hunter, J.C. (1997) "A novel tetrodotoxin-sensitive, voltage-gated sodium channel expressed in rat and human dorsal root ganglia", *J. Biol. Chem.* **272**, 14805–14809.

Savitz, S.I. and Rosenbaun, D.M. (1999) "Gene expression after cerebral ischemia", *The Neuroscientist*, 238–253.

Schaller, K.L., Krzemien, D.M., Yarowsky, P.J., Krueger, B.K. and Caldwell, J.H. (1995) "A novel, abundant sodium channel expressed in neurons and glia", *J. Neurosci.* **15**, 3231–3242.

Schmidt, J.W. and Catterall, W.A. (1986) "Biosynthesis and processing of the alpha subunit of the voltage-sensitive sodium channel in rat brain neurons", *Cell* **46**, 437–444.

Smeyne, R.J., Vendrell, M., Hayward, M., Baker, S.J., Miao, G.G., Schilling, K., Robertson, L.M., Curran, T. and Morgan, J.I. (1993) "Continuous c-fos expression precedes programmed cell death *in vivo*", *Nature* **363**, 166–169.

Waechter, C.J., Schmidt, J.W. and Catterall, W.A. (1983) "Glycosylation is required for maintenance of functional sodium channels in neuroblastoma cells", *J. Biol. Chem.* **258**, 5117–5123.

Wang, Y., Chen, X. and Colvin, R.A. (2000) "Expression of the Na^+/Ca^{2+} exchanger ameliorates ionomycin-induced cell death", *Biochem. Biophys. Res. Commun.* **276**, 93–96.

Waxman, S.G., Kocsis, J.D., Black, J.A. and Type, III, . (1994) "sodium channel mRNA is expressed in embryonic but not adult spinal sensory neurons, and is re-expressed following axotomy", *J. Neurophysiol.* **72**, 466–470.

Waxman, S.G., Cummins, T.R., Dib-Hajj, S.D. and Black, J.A. (2000) "Voltage-gated sodium channels and the molecular pathogenesis of pain: a review", *J. Rehabil. Res. Dev.* **37**, 517–528.

Williams, A.J., Phillips, J., Hunter, J.C. and Tortella, F.C. (1999) RS100642, A novel sodium channel blocker, reduces infarct volume and improves functional recovery following MCAo and reperfusion in rats. 29th Neurosci. Annual Meeting (Abstract) **25**.

Williams, A.J., Dave, J.R., Phillips, J.B., Lin, Y., McCabe, R.T. and Tortella, F.C. (2000) "Neuroprotective efficacy and therapeutic window of the high-affinity N-methyl-D-aspartate antagonist conantokin-G: *in vitro* (primary cerebellar neurons) and *in vivo* (rat model of transient focal brain ischemia) studies", *J. Pharmacol. Exp. Ther.* **294**, 378–386.

Williams, A.J., Tortella, F.C., Yao, C., Yu, Z.Y., Hale, S.L., Berti, R. and Dave, J.R. (2001) Expression of sodium channel genes following ischemic injury: An *in situ* hybridization study. 31st Neurosci. Annual Meeting (Abstract) **27**.

Xie, Z., Yip, S., Morishita, W. and Sastry, B.R. (1995) "Tetanus-induced potentiation of inhibitory postsynaptic potentials in hippocampal CA1 neurons", *Can. J. Physiol. Pharmacol.* **73**, 1706–1713.

Zhang, L., Hsu, J.C., Takagi, N., Gurd, J.W., Wallace, M.C. and Eubanks, J.H. (1997) "Transient global ischemia alters NMDA receptor expression in rat hippocampus: correlation with decreased immunoreactive protein levels of the NR2A/2B subunits, and an altered NMDA receptor functionality", *J. Neurochem.* **69**, 1983–1994.

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RS-100642–198, a Novel Sodium Channel Blocker, Provides Differential Neuroprotection Against Hypoxia/Hypoglycemia, Veratridine or Glutamate-Mediated Neurotoxicity in Primary Cultures of Rat Cerebellar Neurons*

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The present study investigated the effects of RS-100642–198 (a novel sodium channel blocker), and two related compounds (mexiletine and QX-314), in *in vitro* models of neurotoxicity. Neurotoxicity was produced in primary cerebellar cultures using hypoxia/hypoglycemia (H/H), veratridine or glutamate where, in vehicle-treated neurons, 65%, 60% and 75% neuronal injury was measured, respectively. Dose-response neuroprotection experiments were carried out using concentrations ranging from 0.1–500 μM. All the sodium channel blockers were neuroprotective against H/H-induced injury, with each exhibiting similar potency and efficacy. However, against veratridine-induced neuronal injury only RS-100642–198 and mexiletine were 100% protective, whereas QX-314 neuroprotection was limited (i.e. only 54%). In contrast, RS 100642–198 and mexiletine had no effect against glutamate-induced injury, whereas QX-314 produced a consistent, but very limited (i.e. 25%), neuroprotection. Measurements of intraneuronal calcium ($[Ca^{2+}]_i$) mobilization revealed that glutamate caused immediate and sustained increases in $[Ca^{2+}]_i$ which were not affected by RS-100642–198 or mexiletine. However, both drugs decreased the initial amplitude and attenuated the sustained rise in $[Ca^{2+}]_i$ mobilization produced by veratridine or KCl depolarization. QX-314 produced similar effects on glutamate-, veratridine- or KCl-induced $[Ca^{2+}]_i$ dynamics, effectively decreasing the amplitude and delaying the initial spike in $[Ca^{2+}]_i$, and attenuating the sustained increase in $[Ca^{2+}]_i$ mobilization. By using different *in vitro* models of excitotoxicity, a heterogeneous profile of neuroprotective effects resulting from sodium channel blockade has been described for RS-100642–198 and related drugs, suggesting that selective blockade of neuronal sodium channels in pathological conditions may provide therapeutic neuroprotection against depolarization/excitotoxicity via inhibition of voltage-dependent Na^+ channels.

Keywords: Excitotoxicity; Ischemia; Neuronal cultures; Neuroprotection; Neurotoxicity; Sodium channel blockers

* 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 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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INTRODUCTION

It is widely accepted that neuronal cell death results from a cascade of events, ranging from excessive presynaptic release of excitatory amino acids (EAA), alterations in cellular ionic dynamics, toxic postsynaptic accumulation of intracellular calcium $[Ca^{2+}]_i$, and ultimately the activation of secondary signaling mechanisms leading to acute or delayed injury processes (Choi, 1987; DeCoster *et al.*, 1992; Mattson *et al*, 1992). This process of excitotoxicity is now believed to include a series of genomic reactions from the expression of immediate early genes to the synthesis of proteins which in turn regulate the expression of other genes (Evan *et al.*, 1992; Smeyne *et al.*, 1993; Dave and Tortella, 1994; Lu *et al.*, 1997). In many cases the primary pathophysiological event associated with the initiation of neuronal injury is ischemia, or oxygen/energy deprivation leading to metabolic failure of the neuron and triggering EAA/glutamate release. Consequently, many reported studies have utilized various *in vitro* and *in vivo* models of pre- and post-synaptic hyperglutamatergic activity to study the molecular mechanism of neuronal injury.

Earlier studies from our laboratory and by others have implicated rapid changes in $[Ca^{2+}]_i$ dynamics as a seminal cause of EAA mediated neuronal death (Choi *et al.*, 1987; DeCoster *et al.*, 1994; Dave *et al.*, 1997). However, a precise balance of intracellular ions is crucial for the survival and recovery of an injured neuron, and hence, intracellular sodium dynamics and the state of Na^+-Ca^{2+} exchanger may be of equal importance. The Na^+-Ca^{2+} exchanger is considered one of the principal mechanisms by which neuronal $[Ca^{2+}]_i$ homeostasis is maintained (Sanchez-Armass and Blaustein, 1987; Blaustein, 1988), and several studies have proposed that the increase in intracellular calcium ($[Ca^{2+}]_i$) during hypoxia and ischemia is caused in large part by an increase in intracellular sodium $[Na^+]_i$ mediated by the reverse mode of Na^+-Ca^{2+} exchangers (Lysko *et al*, 1994; Calabresi *et al*, 1999). *In vitro*, anoxia has been reported to induce an increase in intracellular sodium levels leading to neurotoxicity, and the removal of extracellular Na^+ prevented anoxia-mediated morphological changes in hippocampal neurons (Friedman and Haddad, 1994). Furthermore, several *in vitro* and *in vivo* studies have demonstrated neuroprotective effects of use-dependent sodium channel blockers against ischemic/hypoxic injury (Lynch *et al*, 1995; Sun and Faden, 1995; Stys and Lesiuk, 1996; Campbell *et al*, 2000). Collectively, these studies suggest an important role of sodium channel mechanisms in neurodegeneration resulting from cellular ischemia/hypoxia.

The present studies were undertaken to determine the relative neuroprotective effects of RS-100642–198, a novel sodium-channel blocker that is structurally related to mexiletine, and two known, use-dependent sodium channel blockers (i.e. mexiletine and QX-314). The effects of these drugs were studied in three distinct *in vitro* neuronal injury models, namely, neurotoxicity induced by pre-synaptic glutamate release (i.e. hypoxia/hypoglycemia), post-synaptic glutamatergic (i.e. ligand gated) activity (i.e. glutamate), or the voltage-gated sodium channel activator veratridine. To understand, in a limited manner, the mechanism of action of these drugs, the excitotoxic dynamics of $[Ca^{2+}]_i$ was also studied using real-time laser cytometry in primary neurons depolarized by glutamate or KCl.

METHODS

Cell cultures

Adult time-pregnant Sprague-Dawley female rats were purchased from Taconic Farms (Germantown, NY) and enriched neuronal cultures were prepared from the brains of 17- day old rat embryos. Following euthanasia with carbon

dioxide, 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 cerebellum 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 medium (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 (DeCoster *et al*, 1992; Ved *et al*, 1991).

Neurotoxicity experiments

Cells were used in experiments after 6–8 days in culture. Cells were exposed to glutamate (80 μM) or veratridine (20 μM) for 45 min in Locke's solution. H/H was induced by incubating the cells in a humidified airtight chamber saturated with 95% nitrogen:5% CO_2 gas for 2 hr in Locke's solution without added glucose. At the end of the treatment period (45 min for glutamate or veratridine and 2 hr for H/H), the Locke's solutions in each well were replaced with conditioned media (original) and morphological and cell viability (MTT measurements) assessments were made 24 hr 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). Briefly, this dye was added to each well (final 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 (540 nM) of each sample was measured in a 96 well plate reader. In each toxicity model RS-100642–198 (0.1–500 μM), mexiletine (0.1–500 μM) or QX-314 (0.1–500 μM) were tested for neuroprotective potencies.

Values are expressed relative to vehicle-treated control cells (for glutamate or veratridine toxicity) that were maintained on each plate or relative to normoxic control cells maintained on a different plate, and percentage changes in cell viability calculated. Percent neuroprotection was calculated using the following formula in which "insult" refers to H/H, veratridine or glutamate treatments and "SCB" refers to a particular sodium channel blocker (either RS 100642, mexiletine or QX-314). Normoxic control values were used as "$\text{Survival}_{(\text{vehicle})}$" for the H/H model in the following formula. Differences in the cell viability and % neuroprotection among treatment groups were determined using one-way analysis of variance and the Newman-Keuls Test.

$$\% \text{ Neuroprotection} = \frac{\text{Survival}_{(\text{insult+SCB})} - \text{Survival}_{(\text{insult})}}{\text{Survival}_{(\text{vehicle})} - \text{Survival}_{(\text{insult})}} \times 100$$

Measurement of intracellular free Ca^{2+} ($[Ca^{2+}]_i$)

Changes in $[Ca^{2+}]_i$ were determined using the fluorescent Ca^{2+}-sensitive dye fluo-3. Neurons were loaded with the membrane-permeable acetoxymethyl ester form of the dye by exposure to a reduced Ca^{2+} (0.2 mM) medium containing fluo-3-AM (5 μM) for 1 hr (37°C), then washed and maintained in Locke's solution at 37°C. Fluorescence changes in individual neurons were monitored using the InSight Plus confocal scanning laser microscope system (Meridian Instruments, Okemos, MI). Sequential image scans of fields containing 10–50 neurons (250×250 μm^2) were collected every 10 s to construct kinetic profiles of the effects of glutamate, KCl or veratridine on $[Ca^{2+}]_i$ in the presence and absence

of RS-100642–198, mexiletine or QX-314. A typical experiment lasts 500 sec and measurements were made every 10-sec. Each experiment was begun with a 50–60 sec period of pre-experimental basal Ca^{2+} measurements followed by the addition of either a sodium channel blocker or vehicle (for another 40–50 sec), followed by the addition of glutamate, veratridine or KCl and termination at 500 sec. To verify adequacy of dye loading and neuronal viability, the Ca^{2+} ionophore ionomycin (2 μM) was added 1–2 min before the end of each experiment. Neurons not responding or responding only very weakly to the treatment with ionomycin were not counted in these studies.

[^{3}H]-Batrachotoxin binding studies

Frozen brains from male Sprague-Dawley rats (200–400 g; Charles River) were obtained from Pel-Freez Biologicals (Rogers, AR). The cerebral cortices were removed and homogenized in 10 volumes of Tris-sucrose buffer (10 mM Tris-HCl, 250 mM sucrose, pH 7.4 at 4°C) using 10 strokes with a glass-glass, hand-held homogenizer. The homogenates were filtered through nylon mesh and then centrifuged at 1000-x g for 15 min. The pellets were resuspended in a Hepes buffer (50 mM Hepes, 130 mM choline chloride, 5 mM glucose, 5.4 mM KCl, pH 7.4 at 25°C) and frozen at –70°C until use.

Rat cerebral cortical membranes were incubated with 16 nM [^{3}H]-batrachotoxin ([^{3}H]-BTX; Dupont-NEN, Boston, MA) in 250 μl of Hepes buffer (50 mM Hepes, 130 mM choline chloride, 5 mM glucose, 5.4 mM KCl, pH 7.4 at 25°C). Non-specific binding was defined in the presence of 1 mM veratridine. Membranes were incubated for 60 min at 25°C and then filtered using GF/B glass fiber filters that had been pre-treated with 0.3% polyethyleneimine (PEI). Filters were rinsed with 3 × 1 ml of ice-cold 50 mM Tris-HCl (pH 7.4 at 4°C) and bound radioactivity was determined by scintillation counting (Packard Top-count). For data analysis, [^{3}H]-BTX inhibition curves were generated with 10 concentrations of inhibitor and analyzed by fitting data to a four-parameter logistic equation. IC_{50} values were then converted to K_I values using the Cheng-Prusoff equation.

Rat vagus nerve in vitro

Extracellular recordings were made of supramaximally stimulated C-fiber compound action potentials (CAPs) in rat vagus nerves in vitro, according to standard "grease-gap" techniques (Marsh *et al.*, 1987). Briefly, 2–3 cm lengths of vagus nerve were removed from male Sprague-Dawley rats (200–400 g; Charles River, MA) desheathed and placed in Marsh ganglion baths (Hugo Sachs Electronics, type 858). Nerves were bathed in oxygenated Krebs solution (following in mM: NaCl 119, KCl 2.5, $CaCl_2$ 2.0, $MgSO_4$ 1.3, $NaHCO_3$, 26.2, NaH_2PO_4 1.0 and glucose 11.0). The portion of the nerve in the center section of each bath was continuously superfused with control or drug-containing solution, heated to 34°C, at a flow rate of ~5 ml/min. A dedicated data acquisition system, using an analog-digital converter (Axon Instruments, Foster City, CA) displayed, stored and analyzed the AC voltage records. The acquisition program also directed a motorized valve (Hamilton Co., Reno, NV) to switch between solutions at set intervals. During each experiment, simultaneous recordings were made from up to four nerves. Cumulative concentration-inhibition curves for C-spikes were constructed using 10 min exposures to 1, 10, 100 and 1000 μM RS-100642–198 and mexiletine. Tonic block was measured with a slow rate of nerve stimulation (0.03 Hz) and phasic (or use-dependent block) with a 30-sec burst of 30 Hz stimulation at the end of each 10 min superfusion period. IC_{50} values for tonic and phasic inhibition were determined by SUNR techniques.

TABLE I *In vitro* Sodium Channel (NaCH) Properties of RS 100642–198

Assay	*RS 100642–198*	*Mexiletine*
[^{3}H]-Batrachotoxin[a]		
pK_i	5.09 [5.09–5.09]	4.94 [4.80–5.04]
Rat Vagus Nerve[b]		
Tonic IC_{50} (μM)	140 [120–160]	210 [160–280]
Phasic IC_{50} (μM)	130 [110–140]	200 [160–250]

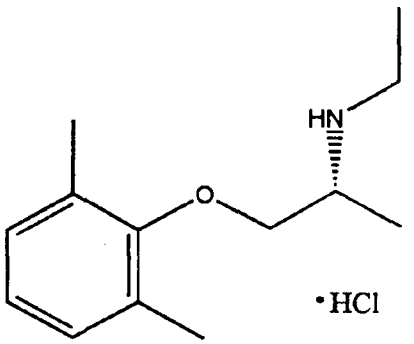
a. [^{3}H]-batrachotoxin labeled sodium channels in rat cerebral cortex.
b. Use-dependent reduction in compound action potentials recorded from the vagus nerve (Tonic, 0.3 Hz; Phasic, 30 Hz).

Chemicals

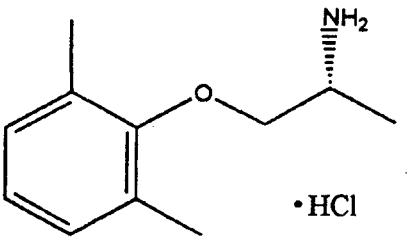
RS-100642–198 was synthesized at and obtained (along with mexiletine) from the Department of Medicinal Chemistry, Roche Bioscience (Palo Alto, CA). QX-314 was purchased from Alomone Labs (Jerusalem, Israel). As illustrated in Figure 1, both RS-100642–198 (1-(2′,6′-dimethyl-phenoxy)-2-ethylaminopropane hydrochloride) and mexiletine (1-(2′,6′-dimethylphenoxy)-2-aminopropane hydrochloride) are phenoxyaminopropanes and QX-314 (1-(2′,6′-dimethyl-phenylcarbamoylmethyl) triethylammonium bromide) is a phenylcarbamoyl compound. Veratridine and all other chemicals were of analytical grade and were purchased from the Sigma Chemical Co. (St. Louis, MO).

RESULTS

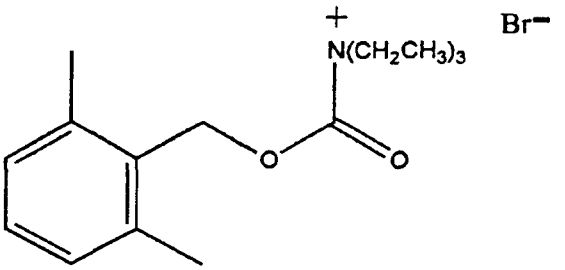
RS-100642–198 and mexiletine both produced a concentration-dependent inhibition of [^{3}H]-batrachotoxin labeled sodium channels present in rat cerebral cortical membranes (Table I). In addition, each compound, as expected, produced both a tonic and a small additional component of use-dependent inhibition of vagus nerve C-spikes *in vitro*. The K_i (radioligand binding) and IC_{50} values for the tonic:phasic components of the CAP (electrophysiology) are also displayed in Table I.



RS 100642-198



Mexiletine



QX-314

FIGURE 1 Chemical structures of two known, use-dependent sodium channel blockers (mexiletine and QX-314), and a novel sodium channel blocker, RS 100642–198

Figure 2 demonstrates the morphological changes observed in primary neurons exposed to vehicle/normoxic conditions (A), H/H (B), veratridine (20 μM; C) or glutamate (80 μM; D).

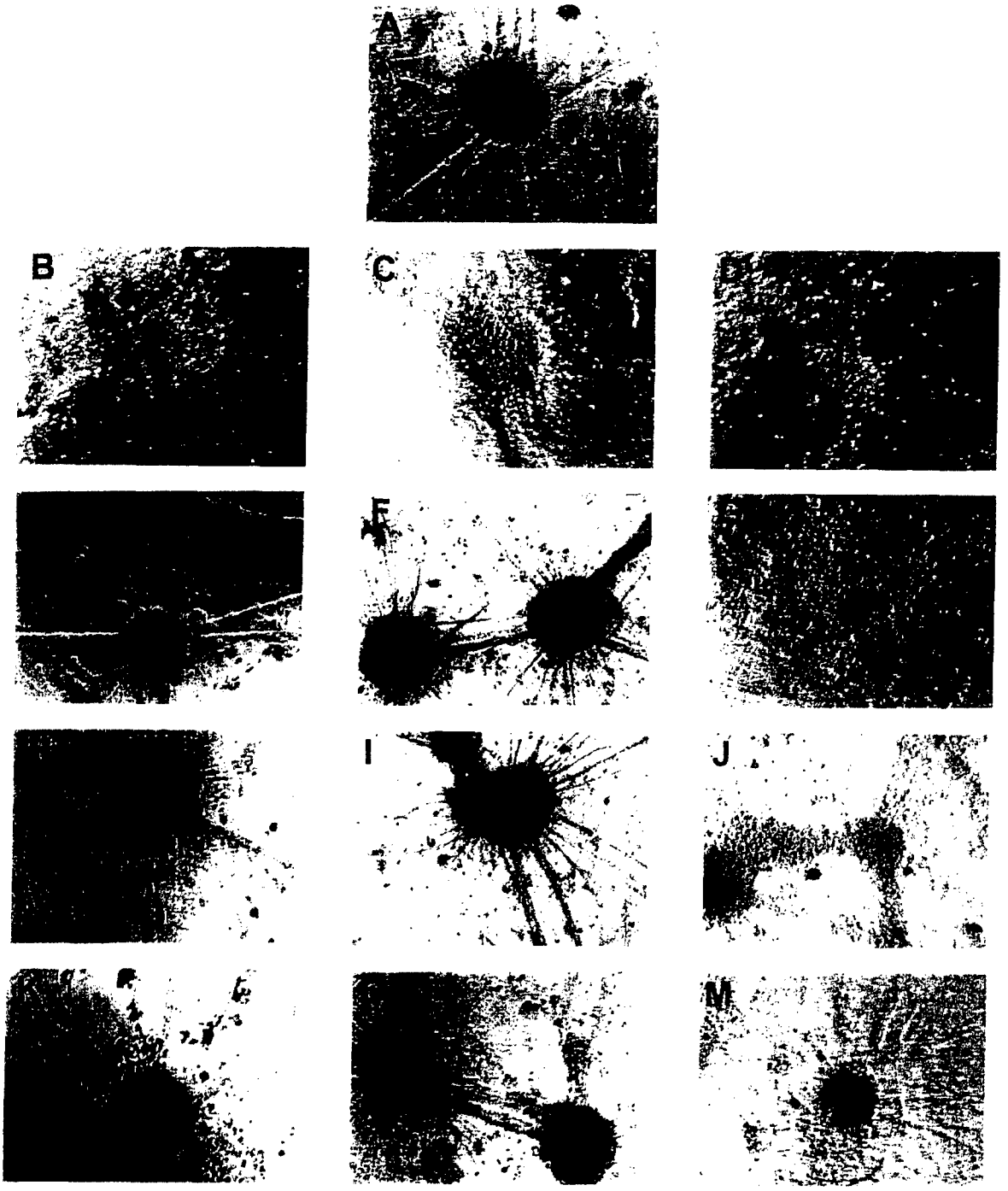


FIGURE 2 Representative bright-field micrographs (80x) of primary cultures of cerebellar neurons stained with MTT stain after either vehicle treatment (panel A) or H/H, veratridine (20 μM) or glutamate (80 μM) treated neurons (Panels B, C and D, respectively). Panels E, F and G: Neurons treated with 200 μM RS-100642–198 and subjected to H/H, veratridine or glutamate treatment, respectively. Panels H, I and J: Neurons treated with 200 μM mexiletine and subjected to H/H, veratridine or glutamate, respectively. Panels K, L and M: Neurons treated with 100 μM QX-314 and subjected to H/H, veratridine or glutamate treatment, respectively

In all the three models of neurotoxicity, cells lost their neuronal processes and shrank (Fig 2, B-D). Cell shrinkage was most pronounced following glutamate or veratridine treatment, otherwise the morphology of the respective injuries appeared similar. In general, in injured cells the number of neuronal processes or dendrites per neuron was also reduced compared to control/normoxic cells. In the veratridine-neurotoxicity model, the cells appeared to be more fragmented and often lost their round shape (Fig 2, C). Figure 2 (E-M) also shows the effect of RS-100642–198 (200 μM), mexiletine (200 μM) or QX-314 (100 μM) in neurons exposed to H/H, veratridine or glutamate. RS-100642–198 (Fig 2, E-G) or mexiletine (Fig 2, H-I) treatment prevented most of these morphological changes in H/H and veratridine-induced toxicity, although neither drug had a significant effect against glutamate-induced toxicity (Fig 2, G and J). QX-314, on the other hand, had a partial effect against H/H (Fig 2, K) and veratridine (Fig 2, L) neurotoxicity, but an even smaller effect on glutamate (Fig 2, M) mediated morphological changes. Cells treated with QX-314 appeared to have fewer but healthier neuronal processes, and cells bodies were less fragmented.

As shown in Figure 3, H/H, veratridine or glutamate exposures were highly neurotoxic to cerebellar neurons. In three separate experiments these treatments produced approximately 65%, 60% and 75% neuronal injury, respectively.

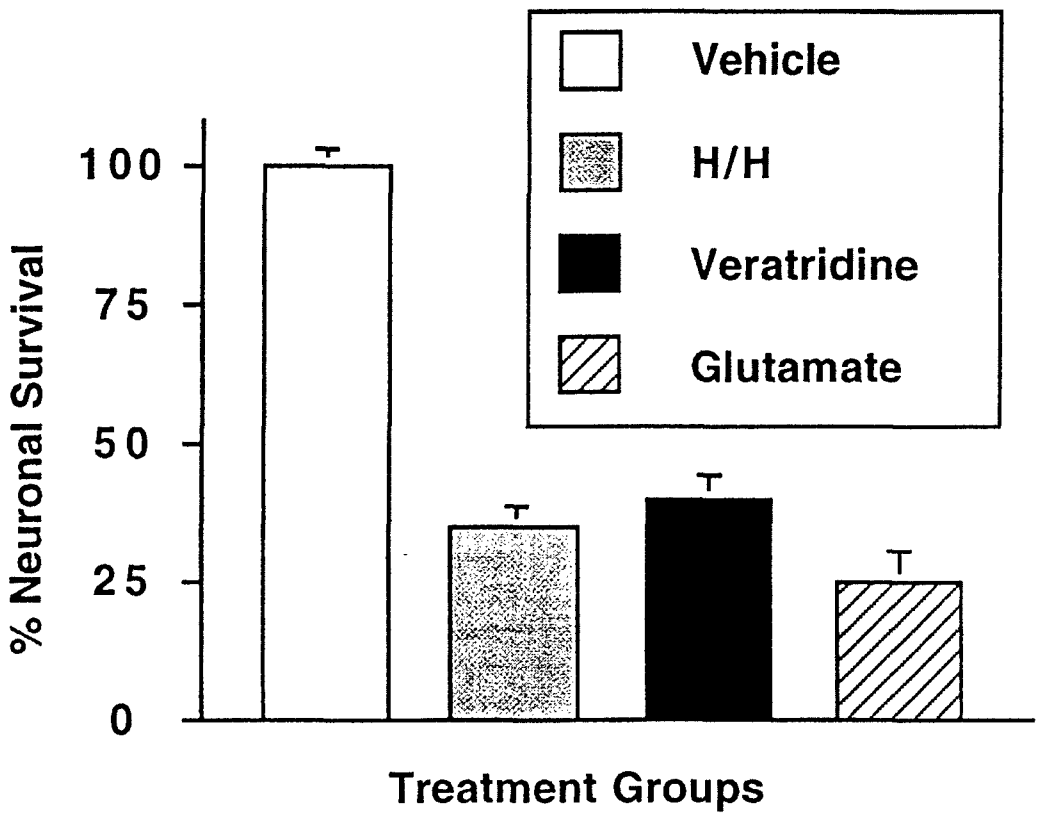


FIGURE 3 Demonstration that 2 hr of hypoxia/hypoglycemia (H/H), or exposure to veratridine (20 μM) or glutamate (80 μM) for 45 min produced significant neurotoxicity in primary cultures of cerebellar neurons. Values are mean ± SE of 4–6 determinations. All the three treatments groups are significantly different from vehicle control group at $p < 0.05$ (student's t-test). Similar results were obtained in at least 6 separate experiments

TABLE II Neuroprotective EC_{50}s (μM) and [95% CLs] of various sodium channel blockers against hypoxic/hypoglycemic (H/H), veratridine (20 μM) or glutamate (80 μM) insults

	H/H	*Veratridine*	*Glutamate*
RS-100642–198	60.8 [42.8–86.3] (76%)	6.9 [2.1–229] (100%)	No effect
Mexiletine	76.4 [58–101] (87%)	6.1 [2.7–13.9] (100%)	No effect
QX-314	144 [79.7–257.1] (66%)	12.1 [1.5–96.6] (50%)	11.5 [0.6–228] (25%)

Values in parenthesis are maximum neuroprotection observed.
Values are calculated from 4 separate experiments (n=12 wells/concentration).

RS-100642–198, mexiletine and QX-314 all produced a dose-dependent neuroprotection against H/H injury (Figure 4), with EC_{50} values of 61, 76 and 144 μM, and maximal efficacies of 76%, 87% and 66% neuroprotection, respectively (Table II).

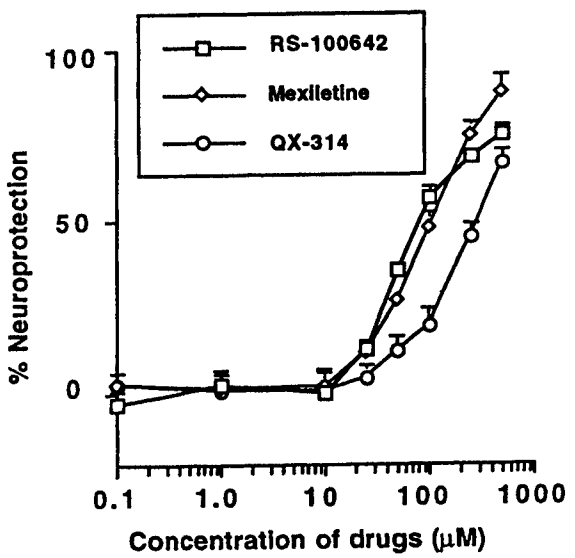


FIGURE 4 Representative dose-responses curve of neuroprotection by mexiletine, QX-314 and RS 100642–198 against hypoxia/hypoglycemia. Values are mean ± SE of 4 separate determinations. Similar results were obtained in at least two separate experiments

Against veratridine-induced injury RS-100642–198 and mexiletine were 100% efficacious while QX-314 neuroprotection was limited (only 54%; Fig 5). Importantly, RS-100642–198 and mexiletine exhibited significant neuroprotection at concentrations as low as 1 μM, whereas QX-314 neuroprotection was not significant until concentrations of 10–50 μM were used. The respective neuroprotection EC_{505} for RS-100642–198, mexiletine and QX-314 were 7, 6 and 12 μM, respectively. These results are also summarized in Table II. Although these drugs by themselves did not produce cytotoxicity at concentrations up to 2 mM (data not presented), in the presence of veratridine both RS-100642–198 and mexiletine, at concentrations of 500 μM (Fig 5) and higher, were neurotoxic (data not presented).

In contrast to its neuroprotective effect against H/H and veratridine induced injury, RS-100642–198 was not neuroprotective against glutamate-induced injury at concentrations up to 500 μM. Similar results were seen with mexiletine. QX-314 produced a consistent, yet very limited (25%; range 19–28%), neuroprotection over a narrow dose-range of 50–200 μM. Interestingly, similar to veratridine induced neurotoxicity, RS-100642–198 and mexiletine were only cytotoxic when higher concentrations (> 100 μM) were tested with glutamate. These results are shown in Figure 6.

Neuronal $[Ca^{2+}]_i$ mobilization was measured in individual neurons and, consistent with prior observations (DeCoster et al., 1994), glutamate (80 μM) or KCl (25 mM) caused immediate and sustained increases in $[Ca^{2+}]_i$ which remained elevated throughout the 500 sec observation period (Table III). A similar increase in $[Ca^{2+}]_i$ was also observed with veratridine (20 μM) treatment. In these experiments, the basal/resting levels of $[Ca^{2+}]_i$ ranged from 70–355 nM. Additions of either glutamate, veratridine or KCl caused a peak increase in $[Ca^{2+}]_i$ to 1600–2800 nM (Table III). Post-peak levels of $[Ca^{2+}]_i$ ranged from 500–2400 nM. Glutamate caused the great-

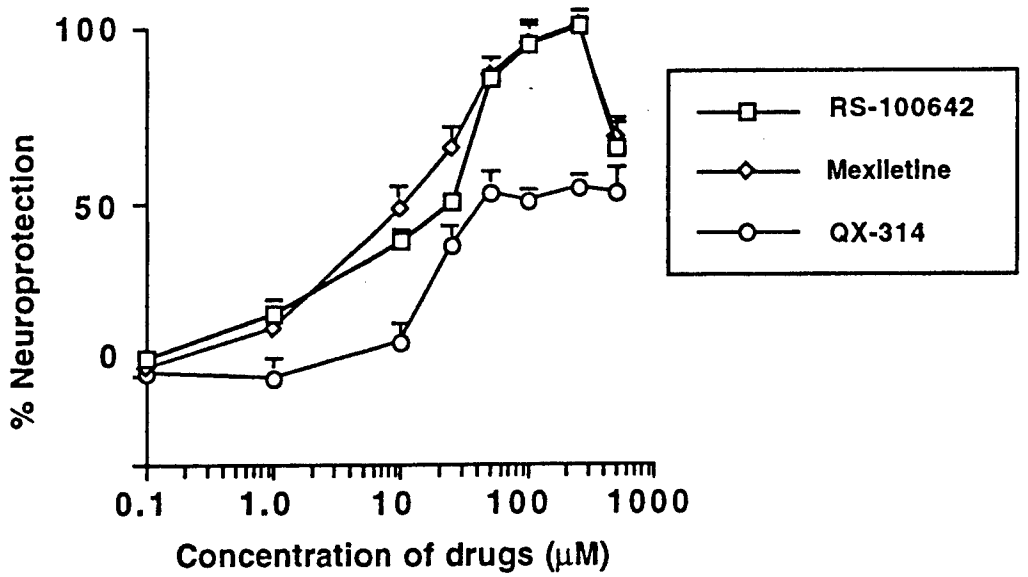


FIGURE 5 Representative dose-responses curve of neuroprotection by mexiletine, QX-314 and RS 100642–198 against veratridine. Values are mean ± SE of 4 separate determinations. Similar results were obtained in at least two separate experiments

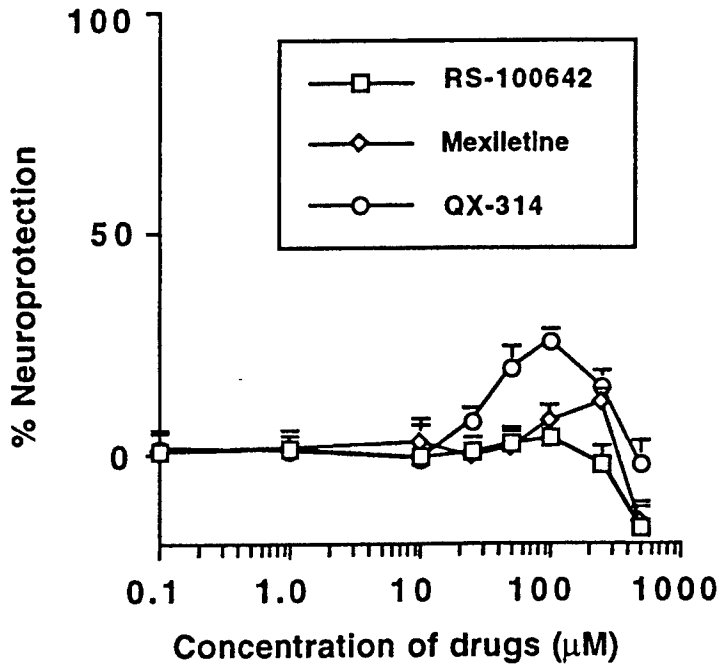


FIGURE 6 Representative dose-responses curve of neuroprotection by mexiletine, QX-314 and RS 100642–198 against glutamate. Values are mean ± SE of 4 separate determinations. Similar results were obtained in at least two separate experiments

est increase in $[Ca^{2+}]_i$ (Peak = 2500–2800 nM; post-peak = 1800–2400 nM). Veratridine and KCl induced increases in $[Ca^{2+}]_i$ reached only to 1600–2000 nM (not as high as glutamate) at peak response, and decreased to a post-peak level of 500–900 nM. RS-100642–198 (or mexiletine) had no effect on glutamate-induced increase in $[Ca^{2+}]_i$. However, both drugs decreased the initial amplitude and attenuated the sustained rise caused by veratridine- or KCl-induced depolarization (Table III). In contrast, QX-314 produced similar effects on glutamate-, veratridine- or KCl-induced responses, effectively decreasing the amplitude and delaying the initial Ca^{2+} spike and attenuating the sustained increase in Ca^{2+} mobilization (Table III).

DISCUSSION

This study describes the neurotoxicity produced by H/H, veratridine and glutamate and demonstrates that the novel sodium channel blocker RS-100642–198 provides almost complete neuroprotection against H/H injury, or neuronal injury directly produced by sodium channel activation (e.g. by veratridine). However, RS-100642–198 failed to provide any significant neuroprotection against glutamate-mediated neurotoxicity. Similar results were obtained with mexiletine. By contrast, QX-314 was the only compound which provided limited (20–25%), but consistent, protection against neurotoxicity caused by glutamate.

As indicated earlier, a primary pathophysiological event associated with several types of neuronal injury is ischemia or tissue anoxia possibly leading to excitotoxic glutamate release. In this regard many studies have utilized various *in vitro* models of ischemia, e.g. hypoxia, hypoglycemia and cyanide exposure to study the molecular or cellular mechanisms of ischemia/EAA-mediated neuronal injury (Buisson and Choi, 1995; Lockhart *et al*, 1995; Lynch *et al*, 1995; Sun *et al*, 1997; Moro *et al*, 1998). Consistent with these models, we previously demonstrated that a combination of hypoxia and hypoglycemia for a period of 2 hr resulted in reproducible neuronal injury of approximately 60–70% (Lin *et al*, 1997), whereas hypoxia alone required a longer time period to induce a neuronal injury which was inconsistent and variable. Therefore, in the present study we utilized the combination of hypoxia and hypoglycemia *in vitro* to produce neuronal injury. Furthermore, along with the H/H model, we also used two additional excitotoxicity models to study the neuroprotective properties of RS-100642–198: glutamate-induced injury, a widely utilized neurotoxicity model and considered a "gold standard" in this research area, and veratridine-induced injury, a neurotoxicity model based on sodium channel opening mediated depolarization.

TABLE III Effects of sodium channel blockers on mobilization of intracellular $[Ca^{2+}]_i$ following glutamate, KCl or veratridine treatment

Treatment		*Peak Ca^{2+}*	*Post-peak Ca^{2+}*
Glutamate	+ Vehicle	2718 ± 117	2213 ± 135
	+ RS 100642–198	2632 ± 105	2155 ± 186
	+ Mexiletine	2598 ± 143	2250 ± 166
	+ QX-314	1485 ± 133*	985 ± 125*
KCl	+ Vehicle	1816 ± 101	710 ± 76
	+ RS 100642–198	1292 ± 95*	221 ± 67*
	+ Mexiletine	1156 ± 76*	245 ± 38*
	+ QX-314	1386 ± 87*	341 ± 23*
Veratridine	+ Vehicle	1958 ± 132	896 ± 95
	+ RS 100642–198	1135 ± 105*	258 ± 89*
	+ Mexiletine	1048 ± 135*	189 ± 82*
	+ QX-314	1143 ± 137*	219 ± 44*

Values are intracellular calcium in nM and are mean ± SEM of 25–45 individual neurons. Values marked with an asterisk (*) are significantly different from vehicle control groups at $p < 0.05$ (Student's *t*-test). Similar results were obtained in two additional experiments. Concentrations of various compounds used were: 80 μM glutamate, 25 mM KCl, 20 μM veratridine, 200 μM RS 100642–198, 200 μM mexiletine and 200 μM QX-314.

Although the primary mechanism of action for H/H-mediated neuronal injury involves energy deprivation resulting in metabolic failure of the neuron, several studies have implicated activation of the glutamate/NMDA receptor complex as the imminent cause of neuronal death (Lockhart *et al* 1995; Sun *et al*, 1997). However, a recent study has demonstrated that activation of the glutamate/NMDA receptor complex occurs secondary in H/H injury, and injury is due to a primary activation of calcium and sodium channels leading to enhanced glutamate efflux into the extracellular space (Kimura *et al.*, 1998). Our present data also support such a hypothesis. Here both RS 100642–198 and mexiletine were equipotent, and exhibited comparable neuroprotection efficacies against H/H mediated neurotoxicity. However, both failed to provide any neuroprotection against glutamate mediated neurotoxicity, indicating that H/H-mediated neuronal injury involves upstream mechanisms of excitotoxic neuronal injury, or modulation of sites on the post-synaptic membrane complex sensitive to sodium channel induced depolarization.

The cytotoxic effects of glutamate have been demonstrated to be primarily as a result of an increase in intracellular calcium ion concentrations (Collingridge and Lester, 1989). Our earlier (Dave *et al*, 1997; De Coster *et al* 1994) and present findings support the above hypothesis and suggest a close correlation between neurotoxic and calcium-mobilizing effects of glutamate. Although the precise mechanism by which glutamate mediates neurotoxicity still remains to be fully defined, the initial blocking of neuronal calcium influx certainly provides significant neuroprotection (Choi *et al.*, 1987; DeCoster *et al.*, 1994; Dave *et al.*, 1997), suggesting a crucial role for Ca^{2+}-dependent cellular events. The present study demonstrates that the novel sodium channel blocker, RS-100642–198, failed to provide neuroprotection against glutamate toxicity and had no significant effect on glutamate-mediated increases in $[Ca^{2+}]_i$. However, it clearly attenuated both the veratridine and KCl mediated increases in $[Ca^{2+}]_i$. In this context, it has been suggested that under ischemic conditions blockade of sodium channels not only prevents the excessive depolarization and limits excitotoxic glutamate release (through reversal of the sodium-dependent glutamate transporter), but also allows calcium extrusion leading to reestablishment of the ionic homeostasis (Lasko *et al.*, 1994). Alternatively, under conditions of sustained Ca^{2+} influx, the Na^+-Ca^{2+} exchange system in primary neuronal cultures has been shown to manifest a Ca^{2+} extrusion system that attenuates delayed glutamate excitotoxicity (Andreeva *et al*, 1991). These data collectively support a key role for Na^+-Ca^{2+} exchanger system in the ischemic injury.

Veratridine induced neurotoxicity primarily involves activation of voltage-dependent sodium channels leading to intracellular sodium ion overload within the neurons. One of the ways by which appropriate sodium and calcium ion concentrations are maintained within the cell is by the Na^+/Ca^+ exchanger. Any significant alterations in the concentrations of either calcium (e.g. by glutamate or hypoxia/hypoglycemia) or sodium (e.g. by veratridine or KCl) will result in an ionic imbalance within the neuron, and, if uncorrected, can result in cell death. Several *in vivo* studies have demonstrated rapid alterations in the distribution of ions across the cell membrane following CNS trauma. For example, in spinal cord injury extracellular levels of both Na^+ and Ca^{2+} at the site of impact decrease initially within the first few minutes only to be followed by a significant increase in these elements over a period of hours to days (Kwo *et al.*, 1989; Moriya *et al.*, 1994). Furthermore, exposure of cultured hippocampal neurons to glutamate has been reported to produce a significant increase in the cytosolic sodium concentration (Pinelis *et al.*, 1994). It is widely accepted that these ionic shifts initiate the progressive neuronal degeneration and loss of function that occur after CNS injury. It has been

reported that high concentrations of veratridine or KCl increase intracellular Na^+ in primary cultures of neurons, respectively, by activation of voltage-sensitive Na^+ channels or by directly opening these channels and by depolarization of the cell membrane leading to Na^+ influx, resulting in neuronal death (Takahashi *et al.*, 1999). Consistent with this are the neuroprotective effects of tetrodotoxin against veratridine-induced toxicity which have also been described in cultured rat cerebellar neurons (Lysko *et al.*, 1994). The present study demonstrates that the novel sodium channel blocker, RS-100642–198, and mexiletine provided almost complete neuroprotection against veratridine toxicity and suppressed both the KCl and veratridine mediated increases in $[Ca^{2+}]_i$. These results demonstrate the importance of intracellular Ca^{2+} dynamics in neuronal survival and further strengthen the hypothesis that voltage-sensitive Na^+ channels may play a key role in neurotoxicity and that blocking of these channels by appropriate Na^+ channel blockers, which also attenuate excitotoxic increases in $[Ca^{2+}]_i$, could provide complete neuroprotection.

Of three different sodium channel blockers studied only QX-314 provided limited, but consistent, neuroprotection against a glutamate insult. Unlike RS-100642–198, QX-314 also produced an effect on glutamate-mediated Ca^{2+} mobilization, suppressing the immediate rises in $[Ca^{2+}]_i$ within 20–50 sec of the glutamate insult (albeit only at high [100–200 μM] concentrations). This partial suppression of glutamate-mediated increases in $[Ca^{2+}]_i$ by QX-314 compares favorably to its limited neuroprotection measured against glutamate injuries and was also seen following KCl or veratridine exposures. In contrast, in earlier experiments we have measured nearly complete suppression of glutamate-mediated increases in $[Ca^{2+}]_i$ by the non-competitive NMDA antagonist MK-801, which in turn also produced complete neuroprotection against glutamate toxicity (DeCoster *et al.*, 1994). The similar effects observed with QX-314 on KCl and veratridine suggest a non-selective blocking action of QX-314 on excitotoxic depolarizing responses. Alternatively, these limited actions of QX-314 on glutamate-mediated increases in $[Ca^{2+}]_i$ and neurotoxicity may be due to a sodium channel blocking action from the "extracellular" membrane surface, an action not previously reported for this drug.

We have shown in this study differential effects of various sodium channel blockers on either hypoxia/hypoglycemia, glutamate or veratridine-mediated neurotoxicity. RS-100642–198 (and mexiletine) provided nearly complete neuroprotection against H/H or veratridine injury yet failed to protect against glutamate excitotoxicity, suggesting the possibility that in these injury models different types of sodium channels are being activated. Alternatively, intracellular Ca^{2+} mobilization may be the primary event driving glutamate neurotoxicity while activation of neuronal Na^+ channel occurs secondary. This hypothesis is supported by Ca^{2+} mobilization data reported herein demonstrating that RS-100642–198 (and mexiletine) had no effect on glutamate-mediated increases in $[Ca^{2+}]_i$, but indeed suppressed KCl- or veratridine-mediated increases in $[Ca^{2+}]_i$. Consistent with this are the results of other studies describing similar actions where, for example, flunarizine was shown to provide complete neuroprotection against veratridine toxicity, but was ineffective against glutamate mediated neurotoxicity (Pauwels *et al.*, 1989). Furthermore, in cerebellar granule cells, the rate of $[Ca^{2+}]_i$ extrusion has been reported to be much slower following glutamate pulse than that after a comparable elevation in $[Ca^{2+}]_i$ induced by K^+ depolarization (Kiedrowski *et al.*, 1994). In this study, K^+ depolarization-mediated transient increase in $[Na^+]_i$ was much lower (from 4 mM to 13 mM) than that from glutamate (4 mM to 60 mM), further supporting the hypothesis that in these injury models probably different types of sodium channels may be activated.

Sodium channel blockers including lidocaine, QX-314, quinidine or lorcainide have also been shown to provide significant neuroprotection against H/H injury in cortical neurons but only when combined with other glutamate receptor antagonists (Lynch *et al.*, 1995). Other *in vivo* studies using sodium channel blockers such as tetrodotoxin and lamotrigine have described moderate neuroprotection in experimental models of brain ischemia (Lekieffre and Meldrum, 1993; Xie *et al.*, 1995; Kimura *et al*, 1998). Although lidocaine was reported to be ineffective in a cat model of focal cerebral ischemia (Shokunbi *et al.*, 1986), the sodium channel blocker and glutamate release inhibitor BW1003C87 has been shown to attenuate cerebral edema following experimental brain injury in rats (Okiyama *et al.*, 1995). The neuroprotective effects of tetrodotoxin in gerbil global brain ischemia has been documented by Lysko *et al* (1994), and in a recent preliminary study from our laboratory, RS-100642–198 treatment significantly reduced cerebral infarction resulting from middle cerebral artery occlusion (MCAo) (Williams *et al*, 1999).

Collectively, these studies support the theory that sodium channel blockers provide neuroprotection in both *in vitro* and *in vivo* experimental brain injury models and suggest a possible key role for selective types of sodium channels in peripheral and central nervous system related neuropathologies. The voltage-gated sodium channel comprises of α and β-subunits, with the pore forming α-subunit 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 sodium channel α-subunits belong to a multigene family and cloning and electrophysiological characterization studies have documented presence of at least eleven different sodium channel genes (e.g. rat brain I, II, IIA, III, rat Na6, PN1, PN3, NaG, rat SkM1, rat heart 1, NaN/SNS2) in the central and peripheral nervous systems and other excitable tissues (heart and muscle) in rodents (Gautron *et al.*, 1992; Schaller *et al.*, 1995; Waxman *et al.*, 1994; Akopian *et al.*, 1996; Sangmeswaran *et al.*, 1996; 1997; Novakovic *et al.*, 1998). In a recent preliminary study from our laboratory, MCAo for 2 hr followed by reperfusion in rats exhibited a time-related decrease in rat brain type I (rBI) sodium channel expression (Yao *et al.*, 2000) suggesting the possible involvement of rBI neuronal sodium channel gene expression in CNS injury.

Development of selective blockers to target individual sodium channels and the role of sodium channels in neurodegeneration and repair has received limited attention. Although, the sodium channel blockers used in the present study are non-selective and probably do not target any single neuronal sodium channel, they have provided crucial information regarding efficacy and potency in different neurotoxicity models. In conclusion, our results demonstrate that the novel sodium channel blocker RS-100642–198 provided almost complete neuroprotection against neuronal injuries caused by cellular depolarization and sodium channel activation, rather than cellular consequences of post-synaptic glutamate hyperexcitability.

References

Akopian, A.N., Sivilotti, L. and Wood, J.N. (1996) A tetrodotoxin-resistant voltage-gated sodium channel expressed by sensory neurons. *Nature* **379**:258–262.

Andreeva, N., Khodorov, B., Stelmashook, E., Cragoe, E. and Victorov, I. (1991) Inhibition of Na^+/Ca^{2+} exchange enhances delayed neuronal death elicited by glutamate in cerebellar granule cell cultures. *Brain Res* **548**:322–325.

Blaustein, M.P. (1988) Calcium transport and buffering in neurons. *Trends Neurosci* **11**:438–443.

Buisson, A. and Choi, D.W. (1995) The inhibitory mGluR agonist, S-4-carboxy-3-hydroxyphenylglycine selectively attenuates NMDA neurotoxicity and oxygen-glucose deprivation-induced neuronal death. *Neuropharm* **34**:1081–1087.

Calabresi, P., Marfia, G.A., Centonze, D., Pisani, A. and Bernardi, G. (1999) Sodium influx plays a major role in the membrane depolarization induced by oxygen and glucose deprivation in rat striatal spiny neurons. *Stroke* **30**:171–179.

Campbell, C.A., Barone, F.C., Benham, C.D., Hadingham, S.J., Harries, M.H., Harling, J.D., Hills, J.M., Lewis, V.A., Mackay, K.B., Orlek, B.S., White, R.F., Parsons, A.A. and

Hunter, A.J. (2000) Characterization of SB-221420-A-a neuronal Ca^{2+} and Na^{+} channel antagonist in experimental models of stroke. *Euro. J. Pharmacol* **401**:419–428.

Choi, D.W. (1987) Dextrorphan and dextromethorphan attenuate glutamate neurotoxicity. *Brain Res* **403**:333–336.

Choi, D.W., Peters, S. and Visekul, V. (1987) Dextrorphan and levorphanol selectively block N-methyl-D-aspartate receptor-mediated neurotoxicity on cortical neurons. *J Pharmacol Exp Ther* **242**:713–720.

Collingridge, G.L. and Lester, R.A.J. (1989) Excitatory amino acid receptors in the vertebrate central nervous system. *Pharmac Rev* **40**:143–210.

Dave, J.R. and Tortella, F.C. (1994) Regional changes in *c-fos* mRNA in rat brain after i.v. or i.c.v. NMDA injections. *NeuroReport* **5**:1645–1648.

Dave, J.R., Koenig, M.L., Tortella, F.C., Pieringer, R.A., Doctor, B.P. and Ved, H.S. (1997) Dodecylglycerol provides partial protection against glutamate toxicity in neuronal cultures derived from different regions of embryonic rat brain. *Mol Chem Neuropathology* **30**:1–13.

DeCoster, M.A., Koenig, M.A., Hunter, J.C. and Tortella, F.C. (1992) Calcium dynamics in neurons treated with toxic and non-toxic concentrations of glutamate. *NeuroReport* **9**:773–776.

DeCoster, M.A., Conover, J.R., Hunter, J.C. and Tortella, F.C. (1994) The neuroprotective k opioid CI-977 alters glutamate-induced neurotoxic calcium signaling. *NeuroReport* **5**:2305–2310.

Evan, G.I., Wyllie, A.H., Gilbert, C.S., Littlewood, T.D., Land, H., Brooks, M., Waters, C.M., Penn, L.Z. and Hancock, D.C. (1992) Induction of apoptosis in fibroblasts by c-myc protein. *Cell* **69**:119–128.

Gautron, S., Dos Santos G., Pinto-Henrique, D., Koulakoff, A., Gros, F. and Berwald-Netter, Y. (1992) The glial voltage-gated sodium channel: cell- and tissue-specific mRNA expression. *Proc Natl Acad Sci USA* **89**:7272–7276.

Isom, L.L., Dejongh, K.S. and Catterall, W.A. (1994) Auxiliary subunits of voltage-gated ion channels. *Neuron* **12**:1183–1194.

Kiedrowski, L., Brooker, G., Costa, E. and Wroblewski, J.T. (1994) Glutamate impairs neuronal calcium extrusion while reducing sodium gradient. *Neuron* **12**:295–300.

Kimura, M., Sawada, K., Miyagawa, T., Kuwada, M., Katayama, K. and Nishizawa, Y. (1998) Role of glutamate receptors and voltage-dependent calcium and sodium channels in the extracellular glutamate/aspartate accumulation and subsequent neuronal injury induced by oxygen/glucose deprivation in cultured hippocampal neurons. *J Pharmacol Exp Ther* **285**:178–185.

Kwo, S., Young, W. and Decrescito, V. (1989) Spinal cord sodium, potassium, calcium, and water concentration changes in rats after graded contusion injury. *J Neurotrauma* **6**:13–24.

Lekieffre, D. and Meldrum, B.S. (1993) The pyrimidine-derivative, BW1003C87, protects CA1 and striatal neurons following transient severe forebrain ischemia in rats. *Neurosci* **56**:93–99.

Lin, Y., Dave, J.R., Clapp, L., Koenig, M., Ved, H., Hunter, J.C. and Tortella, F.C. (1997) Differential effects of the sodium channel blockers mexiletine and QX-314 in primary rat cerebellar neurons: I. Protection against glutamate or hypoxic injury. *Soc Neurosci Abst* **23**:1475.

Lockhart, B.P., Soulard, P., Benicourt, C., Privat A. and Junien, J.L. (1995) Distinct neuroprotective profiles for sigma ligands against N-methyl-D-aspartate (NMDA), and hypoxia-mediated neurotoxicity in neuronal culture toxicity studies. *Brain Res* **675**:110–120.

Lu, X-C. M., Tortella, F.C., Ved, H.S., Garcia, G.E. and Dave, J.R. (1997) Neuroprotective role of *c-fos* antisense oligonucleotide: *in vitro* and *in vivo* studies. *NeuroReport* **8**:2925–2929.

Lynch, J.J. iii, Yu, S.P., Canzoniero, L.M., Sensi, S.L. and Choi, D.W. (1995) Sodium channel blockers reduce oxygen-glucose deprivation-induced cortical neuronal injury when combined with glutamate receptor antagonists. *J Pharmacol Exp Ther* **273**:554–560.

Lysko, P.G., Webb, C.L., Yue, T-L., Gu, J-L. and Feuerstein, G. (1994) Neuroprotective effects of tetrodotoxin as a Na^{+} channel modulator and glutamate release inhibitor in cultured rat cerebellar neurons and in gerbal brain ischemia. *Stroke* **25**:2476–2482.

Marsh, S.J., Stanfeld, C.E., Brown, D.A., Davey, E. and McCarthy, D. (1987) The mechanism of action of capsaicin on sensory C-type neurons and their actions *in vitro*. *Neuroscience* **23**:275–289.

Mattson, M.P., Cheng, B., Davis, D., Bryant, K., Lieberburg, I, and Rydel, R.E. (1992) β-Amyloid peptides destabilize calcium homeostasis and render human cortical neurons vulnerable to excitotoxicity. *J Neurosci* **12**:376–389.

Moriya, T., Hassan, A.Z., Young, W. and Chesler, M. (1994) Dynamics of extracellular calcium activity following contusion of the rat spinal cord. *J Neurotrauma* **11**:255–263.

Moro, M.A., DeAlba, J., Leza, J.C., Lorenzo, P., Fernandez, A.P., Bentura, M.L., Bosca, L., Rodrigo, J. and Lizasoain, I. (1998) Neuronal expression of inducible nitric oxide synthase after oxygen and glucose deprivation in rat forebrain slices. *Eur J Neurosci* **10**:445–456.

Novakovic, S.D., Tzoumaka, E., Mcgivern, J.G., Haraguchi, M., Sangameswaran, L., Gogas, K.R., Eglen, R.M. and Hunter, J.C. (1998) Distribution of the tetrodotoxin-resistant sodium channel PN3 in rat sensory neurons in normal and neuropathic conditions. *J Neurosci* **18**:2174–2187.

Okiyama, K., Smith, D.H., Gennareli, T.A., Simon, R.P., Leach, M. and McIntosh, T.K. (1995) The sodium channel blocker and glutamate release inhibitor BW1003C87 and magnesium attenuate regional cerebral edema following experimental brain injury in the rat. *J Neurochem* **64**:802–809.

Pauwels, P.J., Van Assouw, H.P., Leysen, J.E. and Janssen, P.A. (1989) Ca^{2+}-mediated neuronal death in rat brain neuronal cultures by veratridine: protection by flunarizine. *Mol Pharmacol* **36**:525–531.

Pinelis, V.G., Segal, M., Greenberger, V. and Khodorov, B.I. (1994) Changes in cytosolic sodium caused by a toxic glutamate treatment of cultured hippocampal neurons. *Biochem Mol Biol Internatl* **32**:475–482.

Rose, C.R. (1997) Intracellular Na^{+} regulation in neurons and glia: Functional implications. *The Neuroscientist* **3**:85–88.

Sanchez-Armass, S. and Blaustein, M.P. (1987) Role of sodium-calcium exchange in regulation of intracellular calcium in nerve terminals. *Am J Physiol* **252**:C595–603.

Sangameswaran, L., Delgado, S.G., Fish, L.M., Koch, B.D., Jakeman, L.B., Stewart, G.R., Sze, P., Hunter, J.C., Eglen, R.M. and Herman, R.C. (1996) Structure and function of a novel voltage-gated, tetrodotoxin-resistant sodium

channel specific to sensory neurons. *J Biol Chem* **271**:5953–5956.

Sangameswaran, L., Fish, L.M., Koch, B.D., Rabert, D.K., Delgado, S.G., Ilnicka, M., Jakeman, L.B., Novakovic, S., Wong, K., Sze, P., Tzoumaka, E., Stewart, G.R., Herman, R.C., Chan, H., Eglen, R.M. and Hunter, J.C. (1997) A novel tetrodotoxin-sensitive sodium channel expressed in rat and human dorsal root ganglia. *J Biol Chem* **272**:14805–14809.

Schaller, K.L., Krzemien, D.M., Yarowsky, P.J., Krueger, B.K. and Caldwell, J.H. (1995) A novel, abundant sodium channel expressed in neurons and glia. *J Neurosci* **15**:3231–3242.

Shokunbi, M.T., Gelb, A.W., Peerless, S.J., Mervart, M. and Floyd, P. (1986) An evaluation of the effect of lidocaine in experimental focal cerebral ischemia. *Stroke* **17**:962–966.

Smeyne, R.J., Vendrell, M.V., Hayward, M., Baker, S.J., Milao, G.G., Schilling, K., Robertson, L.M., Curan, T. and Morgan, J.I. (1993) Continuous *c-fos* expression precedes programmed cell death *in vivo*. *Nature* **363**:166–169.

Stys, P.K. and Lesiuk, H. (1996) Correlation between electrophysiological effects of mexiletine and ischemic protection in central nervous system white matter. *Neurosci* **71**:27–36.

Sun, F-Y. and Faden, A.I. (1995) Neuroprotective effects of 619C89, a use-dependent sodium channel blockers, in rat traumatic brain injury. *Brain Res* **673**:133–140.

Sun, X., Shin, C. and Winderbank, A.J. (1997) Calmodulin in ischemic neurotoxicity of rat hippocampus *in vitro*. *NeuroReport* **8**:415–418.

Takahashi, S., Shibata, M. and Fukuuchi, Y. (1999) Role of sodium ion influx in depolarization-induced neuronal cell death by high KCl or veratridine. *Eur J Pharmacol* **372**:297–304.

Ved, H.S., Gustow, E. and Pieringer, R.A. (1991) Regulation of neuronal differentiation in enriched primary cultures from embryonic rat cerebra by platelet activating factor and the structurally related glycerol ether lipid, dodecylglycerol. *J Neurosci Res* **30**:353–358.

Waxman, S.G., Kocsis, J.D. and Black, J.A. (1994) Type III sodium channel mRNA is expressed in embryonic but not adult spinal sensory neurons and is re-expressed following axotomy. *J Neurophysiol* **72**:466–470.

Xie, Z., Yip, S., Morishita, W. and Sastry, B.R. (1995) Tetanus-induced potentiation of inhibitory postsynaptic potentials in hippocampal CA1 neurons. *Can J Physiol Pharmacol* **73**:1706–1713.

Yao, C., Tortella, F.C., Williams, A.J. and Dave, J.R. (2000) Real-time quantitative RT-PCR assay for voltage-gated sodium channel genes in rat brain: Effects of focal ischemia. *Neurosci. Abstr.* **26**: 1109 (Abstract).

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Neuronal sodium channels and brain injury: Molecular expression and neuroprotection studies. J.R. Dave, C. Yao, A.J. Williams, R. Berti, and F.C. Tortella. Division of Neuroscience, Walter Reed Army Institute of Research, Silver Spring, MD 20910.

Voltage-dependent sodium channels (NaChs) play a crucial role in neuron excitability and are considered a primary target of neuroprotection drug development. In a recent study, we have demonstrated that a novel sodium channel blocker, RS-100642 provided dose-dependent neuroprotection against ischemic injury *in vivo* and *in vitro*. The objective of ongoing studies is to determine if NaCh genes are expressed differentially following ischemic brain injury. Using the ABI Prism 7700 Sequence Detection System a real-time RT-PCR assay has been developed. Our findings demonstrate that the expression ratio of NaCh genes in normal rat brain to be rBI>PN3>rBIII>PN1. Brain injury in adult male rats was produced by middle cerebral artery occlusion (MCAo) for two hours followed by reperfusion. Injured or contralateral brain hemispheres removed 24 hours later. This injury significantly down-regulated rBIII and PN1 genes in both injured and contralateral hemispheres, whereas the PN3 gene was down regulated in only the injured hemisphere (though only acutely at 2 or 2-6 h post-MCAo). However, the time-course of NaCh gene expression revealed a significant down-regulation of rBI only in the ischemic hemisphere beginning 6 h post-MCAo and measured out to 48 h post-MCAo. In a separate preliminary study the rBII gene (the most prominent NaCh gene in adult rat brain) was found be expressed at levels greater than that of rBI in normal rats, and was also significantly down regulated at 24 hr post-MCAo. These findings suggest a possible key role for rBI and rBII sodium channel genes in ischemia-mediated neuroinjury and recovery. In view of the fact that sodium channel blockers are known to provide significant neuroprotection against ischemic injury, it is suggested that neurons may down-regulate expression of selective brain NaCh genes as a defense mechanism against delayed neuronal injury.

46.21

GDNF AND NGF REVERSE CHANGES IN REPRIMING KINETICS OF TTX-S SODIUM CURRENTS FOLLOWING PERIPHERAL AXOTOMY OF DRG NEURONS. T.R. Cummins[1]*, A. Lefler[1], S.D. Dib-Hajj[1], W.H. Hormuzdiar[1], J.A. Black[1] and S.G. Waxman[1]. *1. Dept Neurology, Yale University VAMC, West Haven, CT, USA.*

Control C-type rat DRG neurons predominantly express slowly-inactivating TTX-resistant (TTX-R) and slowly-repriming TTX-sensitive (TTX-S) Na+ currents. Following axotomy TTX-R current densities are reduced and rapidly repriming TTX-S currents predominate. In parallel with these changes, Nav1.8 and Nav1.9 mRNA levels are decreased and Nav1.3 expression is increased. The dramatically different Na+ current kinetics after axotomy may alter DRG excitability and could contribute to chronic pain associated with injury of sensory neurons. In earlier studies we demonstrated that neurotrophic factors, GDNF (glial derived growth factor) and NGF (nerve growth factor), can modulate TTX-R Na+ channel expression in DRG neurons. We now report on the role of GDNF and NGF in modulating the changes of TTX-S Na+ current repriming (recovery from inactivation) after nerve injury (sciatic n. ligation). Using osmotic pumps, GDNF, NGF, GDNF+NGF or Ringer solution was delivered intrathecally. Ten days following axotomy the TTX-S Na+ current reprimed almost threefold faster in axotomized neurons treated with Ringer solution (τ=57 ms at -80 mV) than in uninjured neurons (τ=146 ms). This dramatic change in repriming kinetics was partially rescued by GDNF and NGF, and fully rescued by treatment with GDNF+NGF (τ=164 ms). Both GDNF and NGF were also able to reverse the increase in Nav1.3 mRNA that occurs following axotomy, with GDNF+NGF producing the largest effect. Our data indicate that both GDNF and NGF can partially reverse an important effect of axotomy on the electrogenic properties of sensory neurons. *Supported by VA, NMSS, PVA and EPVA.*

46.22

MODULATION OF VOLTAGE-GATED NA+ CHANNEL EXPRESSION BY ITS C IN RAT AND HUMAN PROSTATE CANCER CELLS. D. Stewart[1]* and M.B.A *Biology Dept., Imperial College, London, United Kingdom.*

Previous experiments revealed that blocking VGSC activity by tetrodotoxin (TTX) r sive capacity of the highly metastatic cell lines MAT-LyLu and PC-3 and had no eff LNCaP [1,2]. In the present study, we have investigated whether modulation of VGS affect its own expression. AT-2, MAT-LyLu, PC3 and LNCaP cells were grown as 2], in the presence and absence of 1 μM TTX or 20 μM Veratridine (VRD) for 48 primary antibody against α subunit of VGSCs (TCS) was utilised for immunocytoche Western blotting (WB). Pre-absorption of the antibody with the antigen and skipp antibody were used as controls. Plasma membrane (PM) and internal organelle memt tions were prepared and WB was performed as before (3). Immunoblotting results ind ence of the expected band above the 205 kD marker in PM of highly metastatic M PC-3 cells. In addition, a low level of VGSC expression was found in the lowly meta LNCaP cells, mainly in IM. On the whole, TTX treatment up-regulated VGSC expr lines studied, whilst VRD treatment had the opposite effect, i.e. down- regulated. Th VGSC up-regulation was greater especially in PM of AT-2 and LNCaP cells. After V VGSC expression in PM and IM of all the cell lines were decreased. The IC experi same results. These observations suggest that VGSC activity can modulate its own e and human prostate cancer cells. 1- Grimes, JA *et al FEBS Lett* 369, 290-4 (1995) 2- *et al Am J Path* 150 1213-21 (1997) 3- Stewart, D *et al. J. Phys* 525P, 96-7P.

46.23

EXPRESSION OF SODIUM CHANNEL GENES FOLLOWING ISCHEMIC INJURY: AN *IN SITU* HYBRIDIZATION STUDY. A.J. Williams[1], F.C. Tortella[1]*, C. Yao[1], Z.Y. Yu[3], S.L. Hale[2], R. Berti[1] and J.R. Dave[1]. *1. Div. of Neuroscience, Walter Reed Army Institute of Research, Silver Spring, MD, USA, 2. Div. of Pathology, Walter Reed Army Institue of Research, Silver Spring, MD, USA, 3. Resuscit. Med., Naval Medical Research Center, Silver Spring, MD, USA.*

Voltage-dependent sodium channels (NaChs) can modulate neuronal excitability and are considered primary targets of several neuroprotective drugs. We have previously reported (Yao *et al.*, Neurosci. Abstr. 26, 2000) the expression of mRNA for the NaCh genes rBI, rBIII, PN1 and PN3 in normal and ischemic rat brain by quantitative RT-PCR where the relative abundance of NaCh mRNAs in normal rat brain was rBI>PN3>rBIII>PN1, and the rBI gene exhibited a significant down-regulation in the ischemic hemisphere measured form 6-48 h post-injury. The objective of this study was to determine the differential and anatomical distribution of these NaCh mRNAs following brain injury produced by middle cerebral artery occlusion (MCAo) using *in situ* hybridization techniques. Preliminary results have revealed a low hybridization signal of rBI mRNA not only in the ischemic region but also in the area of the contralateral hemisphere proximal to the ischemic region. This observation was unexpected since that part of the contralateral hemisphere exhibiting lower rBI mRNA hybridization appeared normal when stained with 2,3,5-triphenyltetrazolium chloride (TTC). Additional *in situ* studies are currently in progress to determine the expression profile of other NaCh (rBIII, PN1 and PN3) genes following MCAo. These findings begin to define the anatomical distribution of NaCh gene expression in rat brain, and provide further information on their possible role in the cellular mechanisms of CNS injury. *Supported by DoD Funding.*

46.24

NA+ AND K+ CHANNEL ISOFORM EXPRESSION DURING DEVELOPMENTAL ELECTRICAL ACTIVITY: A PATCH CLAMP AND SINGLE CELL RT-PCR ANALYS INJE CELLS IN CEREBELLAR SLICES. M.K. Jarvinen[1]*, D.M. Porter[2] and R.A. M *chemistry, 2. Physiology, Dartmouth Coll Med Sch, Hanover, NH, USA.*

The role(s) of specific voltage-dependent sodium channel (Na_v) isoforms in shaping activity of neurons in the brain remain elusive. Therefore, patch clamp recording and s tive, single-cell RT-PCR was used to analyze electrical activity and Na_v gene expressic Purkinje cells. Recordings of the spontaneous and evoked activity of Purkinje cells in a slices from PN 4-5, PN 8-10, PN 17-20 mice revealed developmental changes in the ra of electrical activity, firing threshold, and action potential duration. Cell contents were t and used for cDNA synthesis. RT-PCR analyses included nested sets of primers spec ($Na_v1.1$ – $Na_v1.6$) and β ($Na_vβ1.1$, $Na_vβ1.1A$, $Na_vβ2.1$, $Na_vβ3.1$) subunits, potassium sequences ($K_v3.1$, $K_v3.2$, $K_v3.4$), calbindin D28K (as a Purkinje cell marker), and the expressed gene for GAPDH (used as an internal standard for semi-quantitative analysis) terms of subunit gene expression were detected, with some isoforms never detected (N: $Na_vβ3.1$), others detected at all ages examined ($Na_v1.6$, $Na_vβ1.1$, $Na_vβ2.1$, $K_v3.2$, K others exhibiting varied patterns of expression ($Na_v1.1$, $Na_v1.2$, $Na_vβ1.1A$, and $K_v3.$ identify changes in voltage-dependent ion channel expression in Purkinje cells during and provide a basis for analysis of Purkinje cells in which the pattern of Na_v gene expre changed through genetic alteration or transient expression. *Supported by NIH (NS2 Hitchcock Foundation.*

46.25

DOPAMINERGIC INHIBITION OF SODIUM CHANNEL GENE EXPRESSION IN RAT MELANOTROPES. L.F. Lopez-Santiago[1] and G. Cota[1]*. *1. Dept. of Physiology, Biophysics and Neuroscience, Cinvestav-IPN, Mexico City, Mexico.*

We have previously reported that melanotropes isolated from the neurointermediate lobe (NIL) of the rat pituitary gland exhibit a 40-50% decrease in sodium current density from postnatal day 3 (P3) to P12. The decrease coincides with the innervation of melanotropes by hypothalamic dopaminergic neurons and can be completely reversed by chronic *in vivo* treatment with sulpiride, a D2 dopamine receptor antagonist. Since the onset of innervation persistently reduces melanotrope sodium channel activity without altering channel gating properties or affinity for tetrodotoxin, a decrease in the rate of channel production may underlie the suppression of sodium current density. To test this hypothesis, total RNA was isolated from NILs at P3 and P12. RNA samples were then analyzed by semi-quantitative RT-PCR using sequence-specific primers for mRNAs encoding different sodium channel subunits. Channel transcript levels were normalized to cyclophilin mRNA. At P3, the intensity of the mRNA signals for alpha subunits followed the order of Nav1.2 > Nav1.3 > Nav1.6 > Nav1.1 > Nav1.5; the mRNAs for Nav1.4, Nav1.7 and Nav1.8 were no detectable. In addition, the beta-2 mRNA was expressed at a higher level than the beta-1 mRNA. Finally, we found that the transition from P3 to P12 was accompanied by a 30-45% reduction in Nav1.2, Nav1.3 and Nav1.6 mRNAs, with no significant changes in transcript levels for Nav1.1, Nav1.5, beta-1 or beta-2 subunits. These results indicate that a differential downregulation of mRNAs encoding sodium channel alpha subunits contributes to the suppression of melanotrope sodium current density by the onset of dopaminergic innervation. *Supported by Conacyt grant 26391N to G.C.*

46.26

MODULATION OF SODIUM CHANNEL ß1 SUBUNIT-MEDIATED ANKYRIN RE BY AN INTRACELLULAR TYROSINE RESIDUE. J.D. Malhotra[1]*, M.C. Koop Kazen-Gillespie[1], M. Hortsch[2] and L.L. Isom[1]. *1. Pharmacol, 2. Cell and Developm Univ Michigan, Ann Arbor, MI, USA.*

Sodium channel ß subunits function as cell adhesion molecules and participate in hom sion resulting in ankyrin recruitment to cell contact sites. The cytoplasmic domain of single tyr residue that may be critical for ankyrin recruitment. We tested our hypoth mutants replacing this residue with ala (ß1Y181A), phe (ß1Y181F), or glu (ß1Y181E cated construct that deleted all residues beyond the tyr (ß1L182STOP). Ankyrin rec observed in ß1L182STOP, suggesting that residues I166-Y181 contain the major ankyr activity. Ankyrin recruitment was preserved in ß1Y181F but abolished in ß1Y181E, su phosphorylation of Y181, mimicked in the mutant by introducing a negative charge, m ankyrin interactions. ß1 subunits are recognized by anti-phosphotyr antibodies, suggesti may be phosphorylated in vivo. Preliminary data show that fyn kinase may be responsi phorylation. Stimulation of transfected cells with FGF increased the level of ß1 subur precipitated by anti-phosphotyr. AnkyrinG and ß1 are coimmunoprecipitated from brai and transfected cells expressing ß1 and ankyrinG in the absence of sodium channe ß1Y181E and ankyrinG did not associate in transfected cell lines, indicating that phosp ß1 critical is for ankyrin association. Homophilic cell adhesion through ß1 subunits m to axonal fasciculation or channel placement at nodes of Ranvier. Phosphorylation of ß1 may be important for regulation of ankyrin recruitment. *Supported by 1R01MH59980*

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Neuronal sodium channels and brain injury: Molecular expression and neuroprotection studies.

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Voltage-dependent sodium channels (NaChs) play a key role in neuron excitability and are considered a primary target of neuroprotection drug development. Recently we have demonstrated that a novel sodium channel blocker, RS-100642 provide a dose-dependent neuroprotection against ischemic injury in both *in vivo* and *in vitro*. The objective of present studies is to determine if NaCh genes are expressed differentially following ischemic brain injury. Using a real-time quantitative RT-PCR assay our findings demonstrate that the expression ratio of NaCh genes in normal rat brain to be $Na_v1.1$> $Na_v1.1$ $Na_v1.8$> $Na_v1.3$> $Na_v1.7$. In this brain injury model adult male rats were subjected to ischemic insult by middle cerebral artery occlusion (MCAo) for two hours followed by reperfusion. Injured or contralateral brain hemispheres removed 24 hours later. This injury significantly down-regulated $Na_v1.3$ and $Na_v1.7$ genes in both injured and contralateral hemispheres, whereas the $Na_v1.8$ gene was down regulated in only the injured hemisphere (though only acutely at 2 or 2-6 h post-MCAo). However, the time-course of NaCh gene expression revealed a significant down-regulation of $Na_v1.1$ only in the ischemic hemisphere beginning 6 h post-MCAo and measured out to 48 h post-MCAo. In a separate preliminary study the $Na_v1.2$ gene (the most prominent NaCh gene in adult rat brain) was found be expressed at levels greater than that of $Na_v1.1$ in normal rats, and was also significantly down regulated at 24 hr post-MCAo. These findings suggest a possible key role for $Na_v1.1$ and $Na_v1.2$ sodium channel genes in ischemia-mediated neuroinjury and recovery. In view of the fact that sodium channel blockers are known to provide significant neuroprotection against ischemic injury, it is suggested that neurons may down-regulate expression of selective brain NaCh genes as a defense mechanism against delayed neuronal injury.

DOWN-REGULATION OF SODIUM CHANNEL $Na_v1.1$ EXPRESSION BY VERATRIDINE AND ITS REVERSAL BY THE SODIUM CHANNEL BLOCKER, RS100642, IN PRIMARY NEURONAL CULTURES. J.R. Dave*, C. Yao, J.R. Moffett, R. Berti, M.L. Koenig and F.C. Tortella. Div. Neurosciences, Walter Reed Army Inst. Res., Silver Spring, MD 20910.

We recently reported that ischemic brain injury produced down-regulation of $Na_v1.1$ and $Na_v1.2$ voltage gated sodium channel genes (Neurotox. Res. 4, 2002). The objectives of the present study were 1) to determine if veratridine-induced toxicity would down-regulate sodium channel gene (NaCh) expression in primary forebrain cultures enriched in neurons and 2) study the effect of the sodium channel blocker RS 100642 on veratridine and NaCh gene responses. Primary cultures of rat fetal (E = 15) forebrain neurons were used after 7 days *in vitro*. 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 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. Government Support

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. C. Yao*, F. C. Tortella, X. Lu, J. R. Moffett, A. J. Williams, R. Berti and J. R. Dave. Walter Reed Army Inst. Res. Silver Spring, MD 20910.

Voltage-dependent sodium channels (NaChs) play a crucial role in neuronal excitability and are considered as one of the several cellular targets for neuroprotection. In a recent *in vivo* study we reported that ischemic brain injury in rats produced by middle cerebral artery occlusion (MCAo) caused significant down-regulation in expression of the voltage-gated sodium channel genes $Na_v1.1$ and $Na_v1.2$ (Neurotoxicity Res. 4, 2002). Furthermore, we have also reported that a novel sodium channel blocker, RS100642, provides significant neuroprotection (approximately 65% reduction in total infarcted tissue) against MCAo-mediated brain injury (Brain Res. 923, 2002). The objective of the present study was to determine if RS100642 neuroprotection involves modulation of these these down-regulated sodium channel genes . Adult male SD rats were subjected to (MCAo) for two hours followed by reperfusion. RS100642 (1.0 mg/kg, iv) or vehicle was injected 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 quantitative RT-PCR. β-actin mRNA measurements in these samples provided sample amplification efficiency. Consistent with previous results, the present data signifcnat downregulation 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 partially reversed the down-regulation of $Na_v1.1$ and $Na_v1.2$ further suggesting that the $Na_v1.1$ and $Na_v1.2$ sodium channel genes play a key role in neuronal injury/recovery and that that the neuroprotective effects of RS100642 may be mediated, in part, by restoration of the sodium channel expression. Government Support

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Down-regulation of sodium channel $Na_v1.1$ expression by veratridine and its reversal by a novel sodium channel blocker, RS-100642, in primary neuronal cultures*

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ABSTRACT

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, RS-100642. 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 RS-100642 (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 sodium channels prevents excessive depolarization, limiting excitotoxic glutamate release. Further, sodium channel 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 sodium channel blocker, RS-100641, 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 sodium channel 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; Kimura *et al*, 1998), and in a recent study from our laboratory, RS-100642 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 NaChs genes could be involved in the neuronal injury associated with brain ischemia, as well as the neuronal plasticity associated with functional recovery.

The sodium channel 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 sodium channel α-

subunits belong to a multigene family, and cloning and electrophysiological characterization studies have documented the presence of ten different sodium channel 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 sodium channel genes are now referred to as $Na_v1.1$-$Na_v1.9$ and Na_x, respectively (Goldin *et al*, 2000). However, the specific physiological 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 hr post-injury (Yao *et al*, 2002). The present studies were undertaken to determine the changes in expression of these five neuronal sodium channel genes in primary cultures of fetal rat forebrain neurons following exposure to the neurotoxin and sodium channel activator veratridine (Takahashi *et al*, 1999; Dave *et al*, 2001), with and without neuroprotection provided by the NaCh blocker; RS-100642 (Dave *et al*, 2001; Williams *et al*, 2002).

METHODS

CELL CULTURES

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) 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 solutions in each well was replaced with the original conditioned media. Morphological and cell viability assessments (MTT measurements) or total RNA extraction were done 24 hr 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). Briefly, this dye was added to each well (final 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 RS-100642, 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 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 sodium channel 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). 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 β-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 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). Veratridine and all other chemicals were of analytical grade and were purchased from the Sigma Chemical Co. (St. Louis, MO).

RESULTS:

Figure 1 demonstrates the morphological changes observed in primary neurons exposed to vehicle (A), veratridine (2.5 μM; B) or veratridine plus RS-100642 (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 1, B). RS-100642 treatment prevented most of these morphological changes (Fig 1, C).

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 sodium channel genes expressed in primary neuronal cultures (Fig 3). Compared to β-actin gene expression, the level 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%. Clearly, the expression levels for the $Na_v1.2$ gene were significantly greater than that for the other NaCh genes.

Quantification of the level of relative mRNA for each sodium channel gene was next analyzed in fetal rat forebrain neurons subjected to veratridine treatment alone, or veratridine plus RS-100642 (200 μM) treatment (Figure 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, RS-100642 treatment reversed the down-regulation caused by veratridine (Fig 4). This reversal by RS-100642 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 (Figure 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 $Na_v1.8$) sodium channel 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 sodium channels. 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 RS-100642 neuroprotection and reversal of sodium channel gene expression.

In this study we demonstrated that among the five sodium channel 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 that 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 versus 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 by Yao *et al* (2002) also demonstrated significant expression of the peripheral $Na_v1.8$ 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 sodium channel 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 hr versus 45 min), the neurotoxin mechanism of insult, or to the use of RT-PCR techniques used by Lara and co-workers versus 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 sodium channel was down-regulated in response to sodium influx, it does support our present observations and strengthen the hypothesis that persistent activation may result in a

molecular and functional down-regulation of these channels. Other *in vivo* studies in rats with kainite-induced seizures have demonstrated the down-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 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.

The reversal of veratridine-mediated down-regulation of $Na_v1.1$ by the novel sodium channel blocker RS-100642 suggests that this blocker may have specificity towards $Na_v1.1$ channel. Alternatively, it is possible that RS-100642 did not have any effect on the expression of those sodium channel 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 sodium channel 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 sodium channel blocker RS-100642 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.

REFERENCES

Akopian, A. N., Siviltti, L., and Wood, J. N. (1996). A tetrodotoxin-resistant voltage-gated sodium channel expressed by sensory neurons. *Nature* **379**: 257-262.

Ashton, D., Willems, R., Wynants, J., Van Reempts, J., Marrannes, R., and Clincke, G. (1997). Altered Na^+-channel function as an in vitro model of the ischemic penumbra: Action of lubeluzole and other neuroprotective drugs. *Brain Res.* **745**: 210-221.

Auld, V. J., Goldin, A. L., Kraft. D. S., Marshall, J., Dunn, J. M., Catterall, W. A., Lester, H. A., Davidson, N., and Dunn, R. J. (1988). A rat brain Na^+ channel alpha subunit with novel gating properties. *Neuron* **1**: 449-461.

Carter, A. J. (1998). The importance of voltage-dependent sodium channels in cerebral ischemia. *Amino Acids* **14**: 159-169.

Dargent, B., and Couraud, F. (1990). Down-regulation of voltage-dependent sodium channels initiated by sodium influx in developing neurons. *Proc. Natl. Acad. Sci. USA* **87**: 5907-5911.

Dave, J. R., Lin, Y., Ved, H. S., Koenig, M. L., Clapp, L., Hunter, J., and Tortella, F. C. (2001). RS-100642-198, a novel sodium channel blocker, provides differential neuroprotection against hypoxia/hypoglycemia, veratridine or glutamate-mediated neurotoxicity in primary cultures of tat cerebellar neurons. *Neurotoxicity Res.* **3**: 381-395.

DeCoster, M.A., Koenig, M.L., Hunter, J.C. and Tortella, F.C. (1992). Calcium dynamics in neurons treated with toxic and non-toxic concentrations of glutamate. *NeuroReport* **9**:773-776.

Gastaldi, M., Bartolomei, F., Massacrier A., Planells, R., Robaglia-Schlupp, A., and Cau, P. (1997). Increase in mRNAs encoding neonatal II and III sodium channel alpha-isoforms during kainate-induced seizures in adult rat hippocampus. *Brain Res. Mol. Brain Res.* **44**: 179-190.

Gautron, S., Dos Santos, G., Pinto-Henrique, D., Koulakoff, A., Gros, F., and Berwald-Netter, Y. (1992). The glial voltage-gated sodium channel: cell- and tissue-specific mRNA expression. *Proc. Natl. Acad. Sci. USA* **89**: 7272-7276.

Goldin, A.L., Barchi, R.L., Caldwell, J.H., Hofmann, F., Howe, J.R., Hunter, J.C., Kallen, R.G., Mandel, G., Meisler, M.H., Netter, Y.B., Noda, M., Tamkun, M.M., Waxman, S.G., Wood, J.N. and Catterall, W.A.(2000). Nomenclature of voltage-gated sodium channels. *Neuron* **28**: 365-368.

Isom, L. L., De Jongh, K. S., Patton, D. E., Reber, B. F., Offord, J., Charbonneau, H., Walsh, K., Goldin, A. L., and Catterall, W.A. (1992). Primary structure and functional expression of the beta 1 subunit of the rat brain sodium channel. *Science* **256**: 839-842.

Kayano, T., Noda, M., Flockerzi, V., Takahashi, H., and Numa, S. (1988). Primary structure of rat brain sodium channel III deduced from the cDNA sequence. *FEBS Lett.* **228**: 187-194.

Kimura, M., Sawada, K., Miyagawa, T., Kuwada, M., Katayama, K., and Nishizawa, Y., (1998). Role of glutamate receptors and voltage-dependent calcium and sodium channels in the extracellular glutamate/aspartate accumulation and subsequent neuronal injury induced by oxygen/glucose deprivation in cultured hippocampal neurons. *J. Pharmacol. Exp. Ther.* **285**: 178-185.

Lara, A., Dargent, B., Julien, F., Alcaraz, G., Tricaud, N., Couraud, F., and Jover, E. (1996). Channel activators reduce the expression of sodium channel alpha-subunit mRNA in developing neurons. *Brain Res. Mol. Brain Res.* **37**: 116-124.

Lekieffre, D., and Meldrum, B. S. (1993). The pyrimidine-derivative, BW1003C87, protects CA1 and striatal neurons following transient severe forebrain ischemia in rats. *Neurosci.* **56**: 93-99.

Lysko, P. G., Webb, C. L., Yue, T. L., Gu, J. L., and Feuerstein, G. (1994) Neuroprotective effects of tetrodotoxin as a Na^+ channel modulator and glutamate release inhibitor in cultured rat cerebellar neurons and in gerbil global brain ischemia. *Stroke* **25**: 2476-2482.

Noda, M., Ikeda, T., Kayano, T., Suzuki, H., Takeshima, H., Kurasaki, M., Takahashi, H., and Numa, S., (1986). Existence of distinct sodium channel messenger RNAs in rat brain. *Nature* **320**: 188-192.

Novakovic, S. D., Tzoumaka, E., McGivern, J. G., Haraguchi, M., Sangameswaran, L., Gogas, K. R., Eglen, R. M., Hunter, J. C. (1998). Distribution of the tetrodotoxin-resistant sodium channel PN3 in rat sensory neurons in normal and neuropathic conditions. *J. Neurosci.* **18**: 2174-2187.

Sangameswaran, L., Delgado, S.G., Fish, L.M., Koch, B.D., (1996). Structure and function of a novel voltage-gated, tetrodotoxin-resistant sodium channel specific to sensory neurons. *J. Bio. Chem.* **271**: 5953-5956.

Sangameswaran, L., Fish, L. M., Koch, B. D., Rabert. D. K., Delgado, S. G., Ilnicke, M., Jakeman, L. B., Novakovic, S., Wong, K., Sze, P., Tzoumaka, E., Stewart, G. R., Herman, R.C., Chan, H. Eglen, R.M., and Hunter, J.C. (1997). A novel tetrodotoxin-sensitive, voltage-gated sodium channel expressed in rat and human dorsal root ganglia. *J. Biol. Chem.* **272**: 14805-14809.

Schaller, K. L., Krzemien, D. M., Yarowsky, P. J., Krueger, B. K., Caldwell, J. H. (*1995*). A novel, abundant sodium channel expressed in neurons and glia. *J. Neurosci.* **15**: 3231-3242.

Takahashi, S., Shibata, M. and Fukuuchi, Y. (1999) Role of sodium ion influx in depolarization-induced neuronal cell death by high KCl or veratridine. *Eur. J. Pharmacol.* **372**:297-304.

Ved, H.S., Gustow, E. and Pieringer, R.A. (1991) Regulation of neuronal differentiation in enriched primary cultures from embryonic rat cerebra by platelet activating factor and the structurally related glycerol ether lipid, Dodecylglycerol. *J. Neurosci. Res.* **30**:353-358.

Wang, Y., Chen, X. and Colvin, R.A. (2000). Expression of the Na^+/Ca^{2+} exchanger ameliorates ionomycin-induced cell death. *Biochem. Biophys. Res. Commun.* **276**: 93-96.

Waxman, S. G., Kocsis, J. D., and Black, J. A. (1994). Type III sodium channel mRNA is expressed in embryonic but not adult spinal sensory neurons, and is re-expressed following axotomy. *J. Neurophysiol.* **72**: 466-470.

Williams, A.J. and Tortella, F.C. (2002). Neuroprotective effects of the sodium channel blocker RS100642 and attenuation of ischemia-induced brain seizures in the rat. Brain Research (In Press).

Xie, Z., Yip, S., Morishita, W., and Sastry, B. R. (1995). Tetanus-induced potentiation of inhibitory postsynaptic potentials in hippocampal CA1 neurons. *Can. J. Physiol. Pharmacol.* **73**: 1706-1713.

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

Figure legends

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).

Figure 2: Demonstration that veratridine produced toxicity in primary cultures of fetal rat forebrain neurons in dose-related manner. Values are mean ± S.E. 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. of 8 separate culture plates.

Figure 4: Demonstration that RS-100642 reversed veratridine-mediated down regulation of $Na_v1.1$ gene expression in primary neuronal culture. Values are mean ± S.E. of 7-10 separate experiments. Value marked with an asterisk is significantly different from the vehicle control values at $p < 0.05$.

Figure 5: Demonstration that RS-100642 treatment provided complete neuroprotection against veratridine-mediated neurotoxicity. Values are mean ± S.E. of 6

independent experiments. Value marked with an asterisk is significantly different from the vehicle control values at $p < 0.001$.

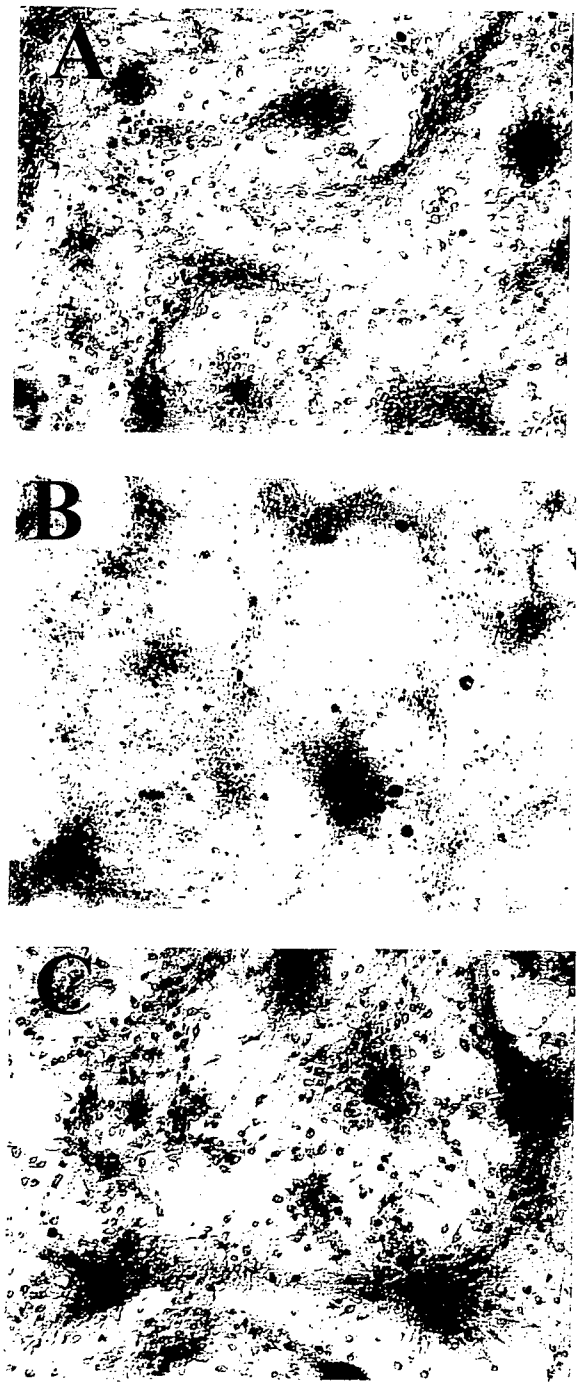

Figure 1

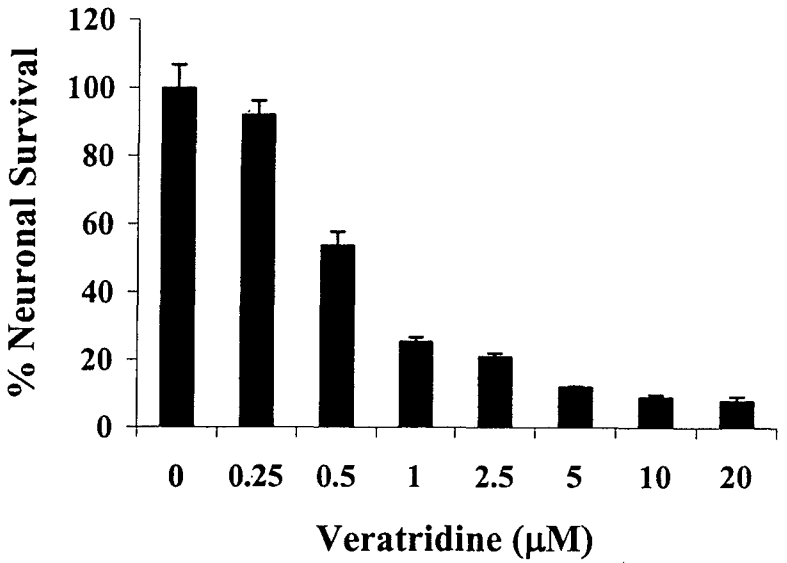
% Neuronal Survival
120
100
80
60
40
20
0
0
0.25
0.5
1
2.5
5
10
20
Veratridine (μM)

Figure 2

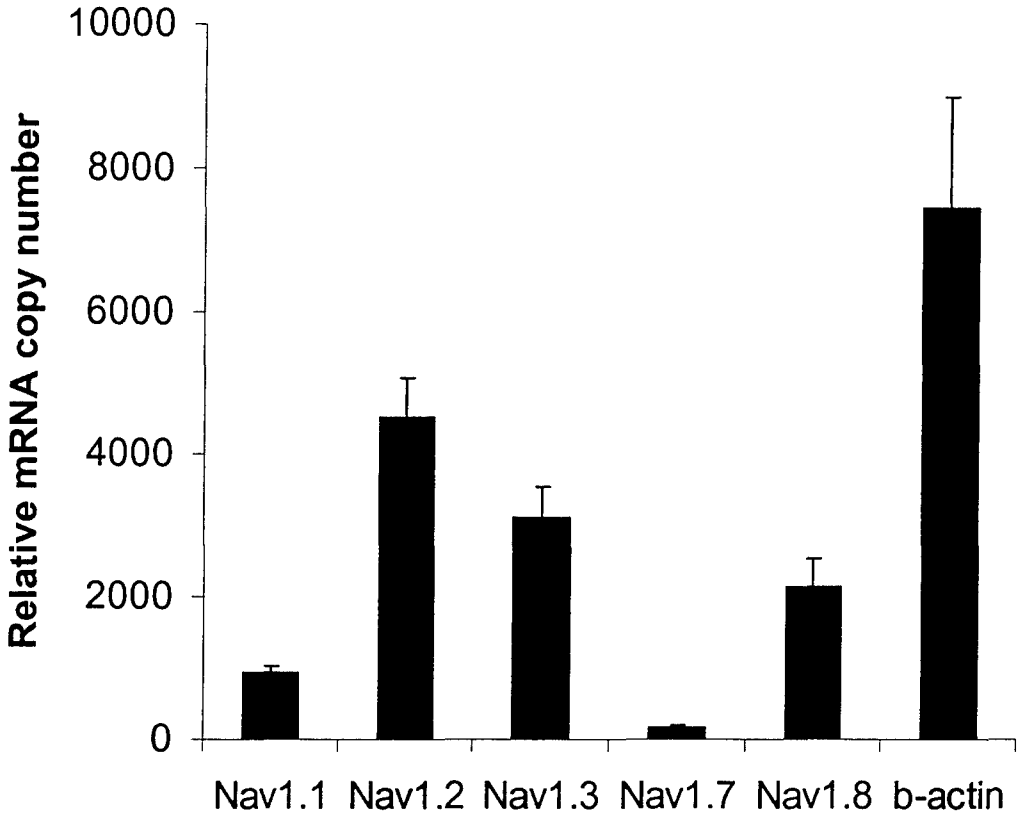
10000
8000
6000
4000
2000
0
Relative mRNA copy number
Nav1.1
Nav1.2
Nav1.3
Nav1.7
Nav1.8
b-actin

Figure 3

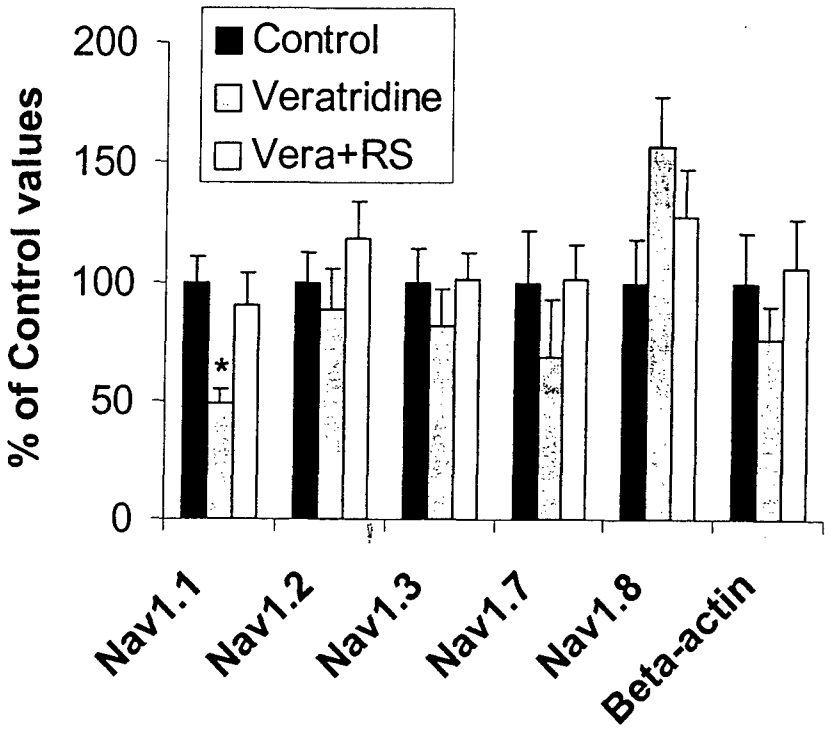
Control
Veratridine
Vera+RS
% of Control values
200
150
100
50
0
*
Nav1.1
Nav1.2
Nav1.3
Nav1.7
Nav1.8
Beta-actin

Figure 4

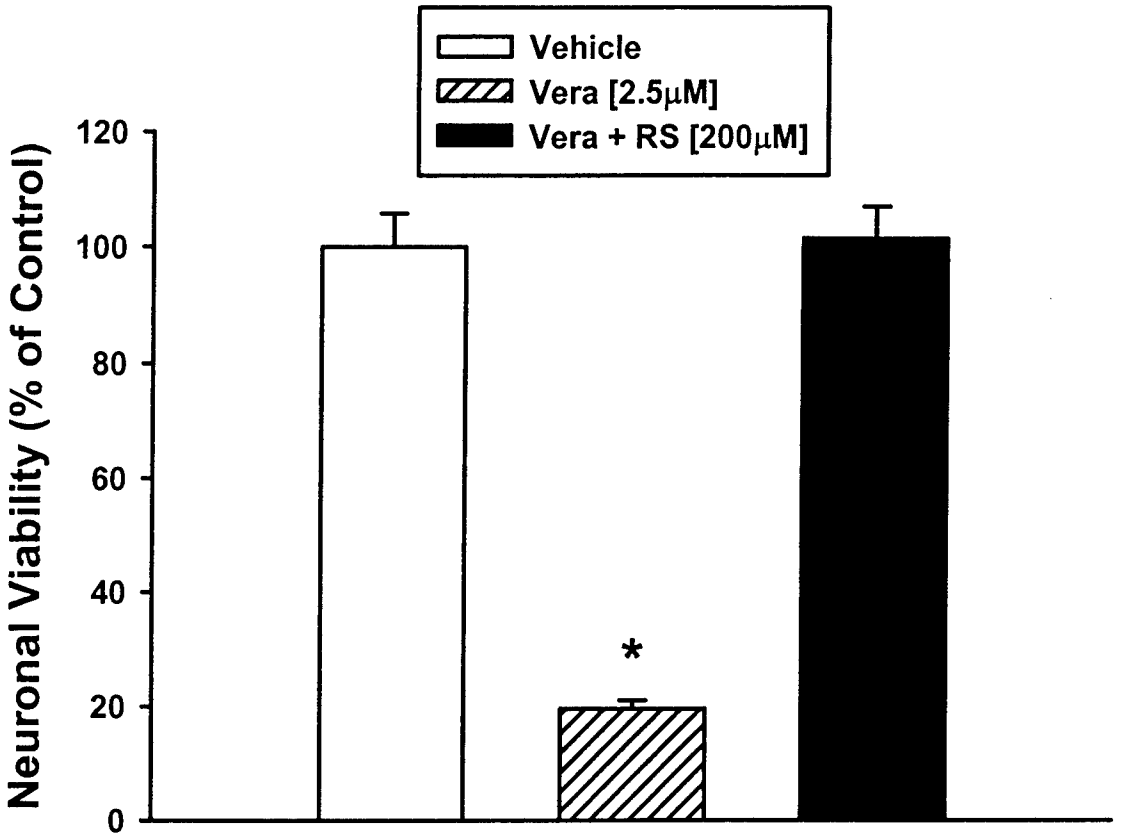
Vehicle
Vera [2.5μM]
Vera + RS [200μM]
Neuronal Viability (% of Control)
120
100
80
60
40
20
0
*

Figure 5

Sodium channel $Na_v1.1$ gene expression after ischemic injury: an *in situ* hybridization study*

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14 pages, 2 figures

Running Title: $Na_v1.1$ *In situ* hybridization after MCAo injury

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*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, (paragraph 4-3), AR 360-5.

Abstract

Neuronal voltage-gated sodium channels (NaChs) generate and propagate action potentials in the nervous system, making them key suspects in excitotoxic damage after CNS injury. Previously we reported on the expression of NaCh genes in normal and ischemic rat brain by quantitative RT-PCR, revealing a significant reduction of $Na_v1.1$ gene expression in the ischemic hemisphere, suggesting that $Na_v1.1$ may play an essential role in ischemic injury. The present study used *in situ* hybridization to examine alterations in the distribution of Nav1.1 gene expression after MCAo injury. Our results confirm the loss of $Na_v1.1$ mRNA within the injured ipsilateral structures, and also suggest that the expression of $Na_v1.1$ may be down-regulated transynaptically in the cerebral hemisphere contralateral to the ischemic injury.

Keywords: Voltage-gated sodium channel, ischemia, middle cerebral artery occlusion, MCAo, reperfusion

Introduction

Sodium channels represent a diverse family of transmembrane protein complexes, encoded by different genes, and with different physiological properties and functions. Voltage-gated sodium channels (NaChs) are those whose gating properties depend on the electrical potential of the cellular membrane, as opposed to those that respond to neurotransmitters, pH, or other ligands. Originally thought to be nonplastic, it is now known that NaCh activity can be modulated, primarily by way of subunit phosphorylation [1]. Recent research has also shown that the expression of sodium channels within neurons is not a static process [2]. Indeed, plasticity in the expression of genes encoding voltage-gated sodium channels can alter the deployment of sodium channel proteins, thereby changing the electrical properties of neurons [3]. These changes may be important in disease states and trauma characterized by hypo- or hyper-excitability, or where altered impulse trafficking contributes to neuronal pathophysiology.

Neuronal NaChs are comprised of alpha and beta subunits. Nine distinct alpha subunits ($Na_v1.1$ - $Na_v1.9$) and three beta subunits ($\beta1 - \beta3$) have so far been identified [4]. The genes encoding alpha subunits $Na_v1.1$, $Na_v1.2$ and $Na_v1.3$ are primarily expressed in the CNS, while those for $Na_v1.8$ and $Na_v1.9$ are expressed predominantly in the peripheral nervous system (e.g., dorsal root ganglion neurons). Genes encoding $Na_v1.6$ and $Na_v1.7$ are distributed more evenly in both the peripheral and central nervous systems, while those encoding $Na_v1.4$ and $Na_v1.5$ are restricted to skeletal and cardiac muscle respectively. The distribution of different sodium channel isotypes in the CNS is regulated temporally and spatially [5], and can be altered significantly by neuronal injury, such as that associated with epilepsy [6]. Our lab has recently shown that in the MCAo model of ischemic brain injury, a subpopulation of injured animals experiences non-convulsive epileptic seizures, and that the seizure activity may contribute to some of the post-MCAo neuropathology [7]. As such, the study of regional changes in mRNA expression for voltage-gated sodium channels after MCAo injury may provide

information useful in devising treatments to reduce post-ischemic neuropathological consequences.

We have recently reported on the expression of mRNA for the NaCh genes $Na_v1.1$, $Na_v1.2$, $Na_v1.3$, $Na_v1.7$ and $Na_v1.8$ in normal and ischemic rat brain by quantitative RT-PCR [8]. The relative abundance of NaCh mRNAs in normal rat brain was $Na_v1.2$ > $Na_v1.1$ > $Na_v1.8$ > $Na_v1.3$ > $Na_v1.7$. Among the mRNA levels examined, the $Na_v1.1$ gene exhibited the most significant down-regulation in the ischemic hemisphere measured from 6-48h post-injury.

Quantitative RT-PCR performed on tissue samples cannot identify the neuronal populations that express the genes, or which neurons are involved in the observed changes in mRNA levels following ischemic injury. Therefore, the current study was aimed at defining the regional distribution of $Na_v1.1$ mRNA in the same focal ischemia model using *in situ* hybridization techniques.

Methods

A. Ischemic brain injury

The middle cerebral arterial occlusion (MCAo) model of focal cerebral ischemia in the rat has been described previously [9]. Briefly, MCAo was carried out in four adult male Sprague-Dawley rats at each time point (n=16 total), with one sham operated animal for each group (n=4 total). The right middle cerebral artery was occluded by the intraluminal filament method for 2h followed by reperfusion for 0, 4, 22 and 46 hours to produce focal ischemic brain injury. Control groups of rats received sham surgery in which an identical procedure was followed, but without inserting the filament.

B. *In situ* hybridization

Brains were embedded in paraffin and cut at thickness of 10μm. The *in situ* hybridization technique used was a modification from Emson and Gait [10] and Rollwagen, et al. [11]. Sections were deparaffinized with xylene, and permeabilized with proteinase K for 3 to 5 minutes at 37°C, followed by acetylation with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 minutes at room temp. Hybridization was performed overnight at 37°C with the $Na_v1.1$-biotinylated DNA probe at a concentration of 20-80 fm/μl in hybridization buffer. Posthybridization washes (1 X Stringent Wash Solution) were undertaken for 30 min at 45°C. After blocking nonspecific binding with Dako Biotin Blocking System (Dako Corp. Carpinteria, CA), signals were detected by incubation with streptoavidin-alkaline phosphatase (AP) conjugate (DAKO Corp.). The reaction was developed in bromochloroindolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) substrate.

In this study, the sodium channel oligonucleotide probe (synthesized by Genset Oligos Inc, La Jolla, CA) was complementary to $Na_v1.1$ mRNA nucleotide sequence 1113-1146. A Blast search of the EMBL/Genbank databases indicated that there was no close homology with any other registered sequences. The probe was 5' end-labeled with biotin. The biotinylated poly T Probe (Research Genetics, Huntsville, AL) was used as a positive control, and the negative control consisted of using a sense probe. Representative

tissue sections from each injured brain were also stained with cresyl violet acetate to assess both the success and the extent of MCAo injuries.

Results

Two hours after MCAo and subsequent reperfusion the animals were sacrificed at 2h, 6h, 24h or 48h post-injury. Tissue blocks were taken from the region perfused by the middle cerebral artery and processed for *in situ* hybridization. A sense-probe for $Na_v1.1$ mRNA was used as a negative control, and the hybridization signal observed showed only light, background staining (data not shown).

$Na_v1.1$ mRNA was detected by *in situ* hybridization in the brains of sham-operated animals (Fig. 1). In general, mRNA expression levels were low for $Na_v1.1$, with staining observed in neurons, axonal pathways and neuropil. The *in situ* hybridization signal for $Na_v1.1$ mRNA in sham animals was highest in layers 3, 4 and 5 of isocortex. The hybridization signal in the endopiriform area and insular cortex of control animals was low compared with other brain regions.

As seen in Figure 1, no reduction was observed in the signal for $Na_v1.1$ mRNA at two hours post-MCAo injury. Adjacent sections stained with cresyl violet did not reveal significant neuropathological changes at the 2h time point. By six hours post-MCAo injury a loss of cresyl violet staining was observed primarily in the striatum. At this time point, a slight down-regulation of $Na_v1.1$ mRNA was detected in the ipsilateral hemisphere (Fig. 1). Injured areas that showed noticeably reduced $Na_v1.1$ mRNA signals at the 6h time point included the primary motor and somatosensory cortices.

At 24h and 48h post-MCAo injury, a significant reduction in $Na_v1.1$ mRNA signal was observed throughout the dorsal striatum, and the primary motor, somatosensory and gustatory cortices (Figs. 1 and 2). Severe neuropathological changes, including neuronal and axonal loss and leukocytic infiltration, were apparent in the cortex, striatum and associated fiber pathways at these two later time points. The loss in $Na_v1.1$ mRNA signal correlated well with the loss of neurons and axons observed in cresyl violet stained sections (Fig. 1). In addition, a noticeable reduction in $Na_v1.1$ mRNA signal was observed in the contralateral piriform cortex at the 24h and 48h post-injury time points (Fig 1). Peri-infarct regions exhibited less severe damage, and substantially less neuronal

loss, and these included secondary motor cortex dorsally, and insular and piriform cortices ventrally.

Discussion

Preliminary results obtained in our lab have shown that $Na_v1.2$ mRNA is the most abundant sodium channel transcript in the uninjured rat brain [8] $Na_v1.2$ mRNA was also found to be down-regulated after MCAo injury (~30% reduction), but to a significantly lesser degree than the $Na_v1.1$ transcript (~75% reduction). For this reason, the current study concentrated on the changes in $Na_v1.1$ mRNA.

The normal abundance of endogenous voltage-gated sodium channel mRNA is relatively low when compared with many "housekeeping genes" [2]. This may be related to the fact that turnover of functional NaChs in the adult central nervous system is relatively slow [12]. The low level of transcript translates to a relatively low *in situ* hybridization signal. Despite this fact, the $Na_v1.1$ mRNA signal was observable, with enhanced staining in layers 3, 4 and 5 of isocortex, and reduced staining in the endopiriform area and insular cortex. The *in situ* hybridization signal observed in control brains clearly indicated regional differences in the expression of $Na_v1.1$ mRNA in the normal rat brain.

Previous quantitative RT/PCR studies from our laboratory have demonstrated that $Na_v1.1$ mRNA expression is down-regulated or lost in the injured hemisphere after MCAo injury [8]. In agreement with those quantitative RT/PCR data, the $Na_v1.1$ mRNA hybridization signal was slightly down-regulated in the cortex at 6hr. The loss in $Na_v1.1$ mRNA signal was greatest at 24 hours and 48 hours post-MCAo injury. Less dramatic but noticeable decreases in mRNA signals were observed in ipsilateral structures immediately adjacent to the core-infarct region.

The most unexpected finding was a small but apparent loss of $Na_v1.1$ signal in the contralateral piriform cortex, suggesting an indirect response to ischemia in the non-injured hemisphere. The piriform cortex is connected to its contralateral counterpart via the anterior commissure. It is possible that the drop in $Na_v1.1$ signal contralateral to the injury was due to a transynaptically-mediated down-regulation of sodium channel gene transcription as a response to a loss of neuronal input from the injured hemisphere. For example, it has been reported that deafferentation in the olfactory bulb results in a

reduction in the expression of sodium channel genes in the postsynaptic neurons [13]. Conversely, it is also possible that neurons in the contralateral piriform cortex down-regulate NaCh gene expression in response to excessive activity in the anterior commissure due to epileptiform activity in the injured, ipsilateral piriform cortex. In fact, in recent EEG topography studies of MCAo injury, we observed generalized post-injury seizure activity in some injured animals, and found that EEG activity was altered in both the ipsilateral and contralateral hemispheres [7, 14]. These results indicate that the ischemic insult also affects neuronal activity (and likely membrane electrogenic properties) in the contralateral, presumably uninjured, hemisphere. In this regard, it is worth noting that a down-regulation of sodium channel genes has been reported in the hippocampus of human epilepsy patients [6].

A question that arises in the present investigation is whether the loss of $Na_v1.1$ mRNA signal is due to post-ischemia neuronal attrition, or due to down-regulation of mRNA production in surviving neurons. At the 6-hour time point, many neurons were still visible within the core-infarct region, suggesting that the loss of signal was due to a down-regulation of $Na_v1.1$ transcript. Based on the extent of the neuropathological injury observed in cresyl violet stained sections at 24h and 48h post-MCAo, and the lack of histochemically labeled neurons within the infarct area, it is likely that much of the reduction of $Na_v1.1$ mRNA signal was directly attributable to neuronal death. Down-regulation of signal in surviving neurons outside the core infarct region was apparent in secondary motor cortex, and in the insular and piriform cortices. Down-regulation of $Na_v1.1$ mRNA was also seen in the contralateral piriform cortex as well, where overt neuropathological changes were not observed.

Conclusion:

The present findings begin to define the anatomical distribution of $Na_v1.1$ mRNA expression in rat brain, and the changes associated with forebrain ischemia. Such data may provide information on the possible roles these cation channels play in the cellular mechanisms of CNS injury in general, and ischemic brain injury in particular.

References

1. Cantrell A.R. and Catterall W.A. (2001) Neuromodulation of Na+ channels: an unexpected form of cellular plasticity. *Nat. Rev. Neurosci.* **2**, 397-407.

2. Waxman, S.G. (2000). The neuron as a dynamic electrogenic machine: modulation of sodium- channel expression as a basis for functional plasticity in neurons. *Philos Trans R Soc Lond B Biol Sci.* **355**:199-213.

3. Waxman SG. (2001). Acquired channelopathies in nerve injury and MS. *Neurology* **56**:1621-1627.

4. Novakovic, S. D., Eglen, R. M., and Hunter J. C. (2001). Regulation of Na+ channel distribution in the nervous system. *Trends Neurosci.* **24** (8): 473-478.

5. Hill, B. (1992) Ionic channels of excitable membranes. Sunderland, MA, Sinauer.

6. Whitaker, W. R., Faull, R. L., Dragunow, et al., (2001). Changes in the mRNAs encoding voltage-gated sodium channel types II and III in human epileptic hippocampus. *Neurosci.* **106** (2): 275-285.

7. Hartings J.A., Williams A.J., and Tortella F.C. (2001). EEG characterization of non-convulsive seizures following focal cerebral ischemia in the rat. *Neurosci. Abstr. 27 (1); 1463.*

8. Yao C., Williams A.J., Cui P., Berti R., Tortella F.C. and Dave J.R. (2001). Differential pattern of expression of voltage-gated sodium channel genes following ischemic brain injury in rats. *Neurotoxicity Research* (in press).

9. Tortella, F.C., Britton, P., Williams, A., et al., (1999). Neuroprotection (focal ischemia) and neurotoxicity (electroencephalographic) studies in rats with AHN649, a

3-amino analog of dextromethorphan and low-affinity N-methyl-D-aspartate antagonist. *J.Pharmacol.Exp.Ther.* **291** (1): 399-408.

10. Emson, P. C. and Gait, M.C. (1992). In situ hybridization with biotinylated probes. In "In Situ Hybridization, A Practical Approach" (D. G. Wilkinson, Ed.), pp. 45-59, Oxford Univ. Press. Oxford.

11. Rollwagen, F.M., Yu Z.Y., Li Y.Y., et al., (1998). IL-6 rescues enterocytes from hemorrhage induced apoptosis in vivo and in vitro by a bcl-2 mediated mechanism. *Clin. Immunol. Immunopathol.* **89**(3): 205-13.

12. Waxman, S. G., Dib-Hajj, S., Cummins, T. R., et al., (2000). Sodium channels and their genes: dynamic expression in the normal nervous system, dysregulation in disease states. *Brain Res.* **886**: 5-14.

13. Schmidt, J. W., and Catterall, W. A. (1986). Biosynthesis and processing of the alpha subunit of the voltage-sensitive sodium channel in rat brain neurons. *Cell* **46**: 437-444.

14. Sashihara, S., Waxman, S. G., and Greer, C. A. (1997). Down-regulation of Na+ channel mRNA in olfactory bulb tufted cells following deafferentiation. *Neuroreport* **8**: 1289-1293.

15. Lu, X.-C. M., Williams, A. J., and Tortella, F. C., (2001). Quantitative electroencephalography spectral analysis and topographic mapping in a rat model of middle cerebral artery occlusion. *Neuropath. Appl. Neurobiol.* **27**:1-18.

Figure 1

Sodium channel mRNA expression at different time points after MCAo injury as shown by *in situ* hybridization. Corresponding sections stained with cresyl violet acetate are shown in the left column. Notable loss of $Na_v1.1$ signal was observed in cortex and striatum at the 24h and 48h time points. Note the loss of $Na_v1.1$ mRNA in the contralateral piriform cortex at the 24h and 48h time points (arrows).

Figure 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.

Figure 1

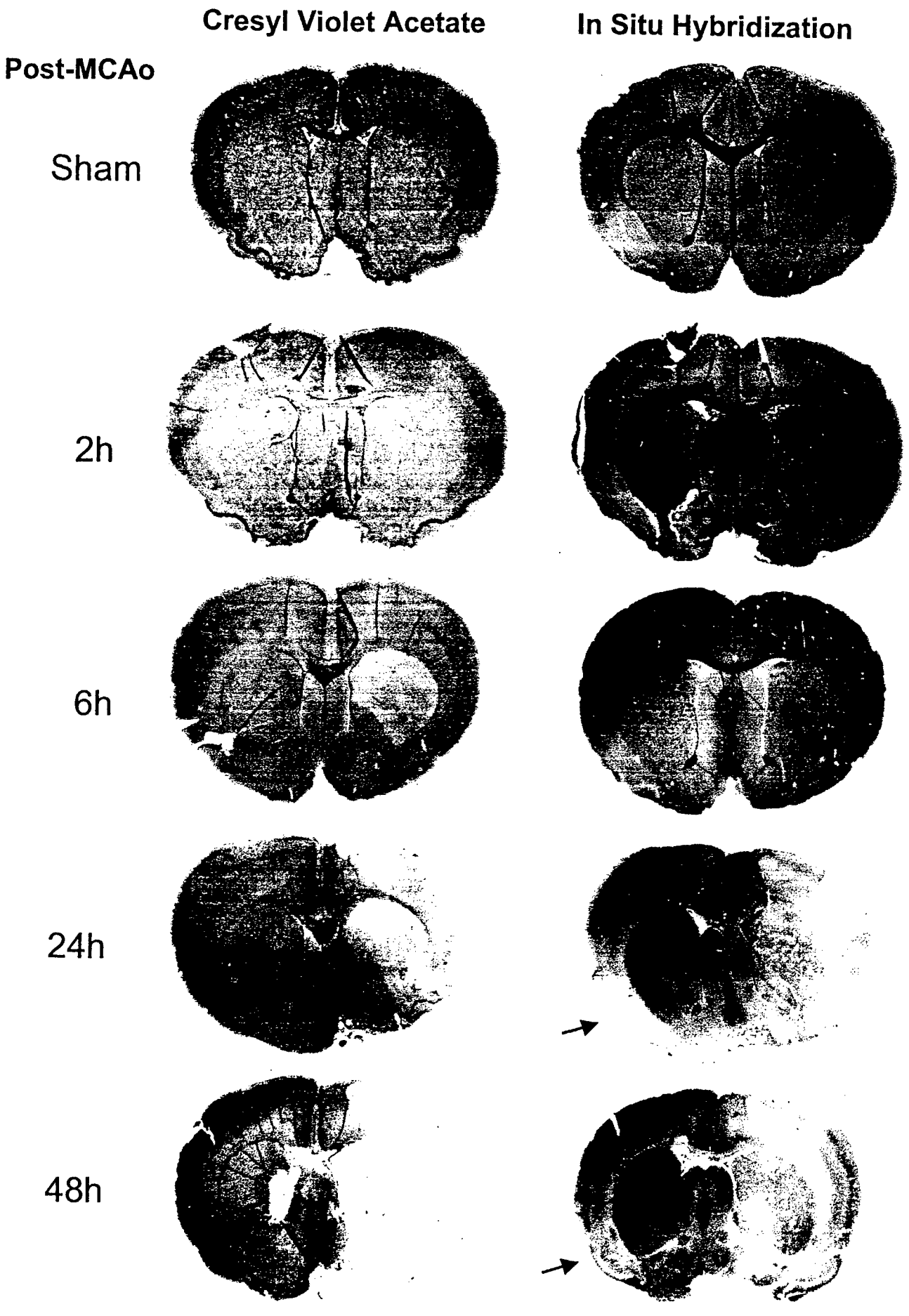
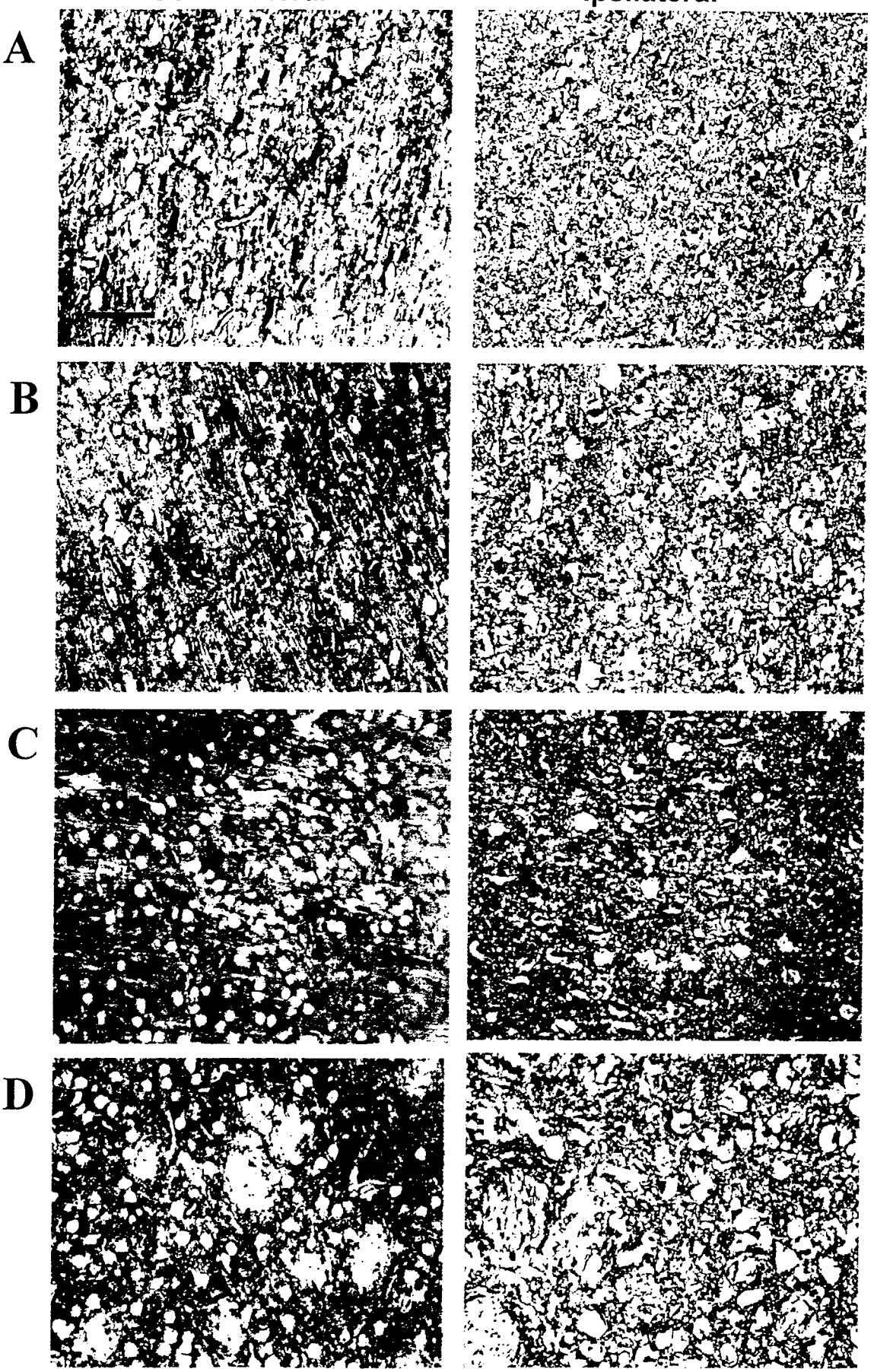

Cresyl Violet Acetate
In Situ Hybridization
Post-MCAo
Sham
2h
6h
24h
48h

Figure 2



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Occurrence of Non-Convulsive Seizures, Periodic Epileptiform Discharges, and Intermittent Rhythmic Delta Activity in Rat Focal Ischemia

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ABSTRACT

A significant proportion of neurologic patients suffer electroencephalographic (EEG) seizures in the acute phase following traumatic or ischemic brain injury, including many without overt behavioral manifestations. Although such non-convulsive seizures may exacerbate neuropathological processes, they have received limited attention clinically and experimentally. Here we characterize seizure episodes following focal cerebral ischemia in the rat as a model for brain injury-induced seizures. Cortical EEG activity was recorded continuously from both hemispheres up to 72h following middle cerebral artery occlusion (MCAo). Seizure discharges appeared in EEG recordings within 1h of MCAo in 13/16 (81%) animals and consisted predominantly of generalized 1-3 Hz rhythmic spiking. During seizures animals engaged in quiet awake or normal motor behaviors, but exhibited no motor convulsant activity. Animals had a mean of 10.6 seizure episodes within 2h, with a mean duration of 60 sec per episode. On average, seizures ceased at 1h 59min post-MCAo in permanently occluded animals, and did not occur following reperfusion at 2h in transiently occluded animals. In addition to seizures, periodic lateralized epileptiform discharges (PLEDs) appeared over penumbral regions in the injured hemisphere while intermittent rhythmic delta activity (IRDA) recurred in the contralateral hemisphere with fronto-parietal dominance. PLEDs and IRDA persisted up to 72h in permanent MCAo animals, and early onset of the former was predictive of prolonged seizure activity. The presentation of these EEG waveforms, each with characteristic features replicating those in clinical neurologic populations, validate rat MCAo for study of acutely induced brain seizures and other neurophysiological aspects of brain injury.

Keywords: ischemia, electroencephalography, epilepsy, non-convulsive seizure, PLED, IRDA, MCAo, *in vivo*, paroxysm, rat

INTRODUCTION

Electrophysiological events play a key role in the progression of brain injury from regions of primary ischemic or traumatic insult to adjacent regions of secondary injury (28,29). High extracellular potassium and excess glutamate release from the injury core lead to depolarization and increased metabolic demand in neighboring neuronal tissue (3,26). When metabolic failure ensues, as especially occurs under hypoxic/hypoglycemic conditions, neuronal membranes depolarize, leading to further glutamate release, spreading ionic imbalances, and eventual cell death (22,26). Epileptiform activity is a pathological sequela of brain injury reflecting this state of hyperexcitability in brain circuits and abnormally prolonged depolarization of individual neurons. Such activity may be epiphenomenal, merely a consequence of electrochemical imbalances that have already developed, or rather may itself contribute to metabolic stress and exacerbate injury in participating neurons.

In patients suffering ischemic and traumatic brain injury, as well as other cerebral insults, electroencephalographic (EEG) seizure activity can occur in the absence of overt clinical manifestations (13,19,40,51). Jordan (17,18) and others (51) pioneered the use of continuous electroencephalographic (EEG) monitoring throughout the acute phase following injury and found that 34% of patients admitted to the Neuroscience Intensive Care Unit suffered non-convulsive seizures (NCS), of which 76% met the criteria for non-convulsive status epilepticus (NCSE; continuous seizures for >30min.). Similar studies have yielded

varying estimates of NCS incidence depending on subject criteria (17% incidence (51); 26% (18); 38% (3); 55% (13)).

Despite the prevalence of NCS, Treiman et al. (47) found that 85% of NCSE patients were refractory to anti-convulsant therapy, compared to 45% of patients with generalized convulsive status epilepticus (GCSE; see also (9,40,51)). Similarly, Jordan (18) reported that NCSE was controlled within 3h of treatment in only 25% of treated patients. The refractoriness of NCSE to treatment is particularly challenging given that the presence of NCSE is associated with higher mortality in brain trauma patients (58,59), more so even than when compared with GCSE (47). Together these clinical data suggest distinct neurophysiological and pharmacological substrates for NCS and emphasize the need to develop experimental models for its further study.

Here we sought to determine the potential of rat focal cerebral ischemia to serve as an *in vivo* model of brain injury-induced seizures by fully describing the incidence and character of seizure discharges we have observed previously (24,54) following middle cerebral artery occlusion (MCAo). Results show that distinct episodes of generalized seizure discharges occur in a high proportion of MCAo-injured animals, but without overt manifestations or disturbance of normal motor behavior. We have also identified, and herein describe, the occurrence of periodic lateralized epileptiform discharges (PLEDs) and intermittent rhythmic delta activity (IRDA) in rat MCAo. Together, the concurrence of these pathological and ictal activities and the congruity of their characteristics with homologous phenomena in clinical populations establish rat MCAo as an attractive model of electrophysiological aspects of brain injury pathology.

MATERIALS AND METHODS

Surgical Procedures

Male Sprague-Dawley rats (Charles River Labs, Raleigh, VA) weighing 275-350g were used in these experiments. All surgical procedures were approved by the Walter Reed Army Institute of Research Animal Care and Use Committee and conform to policies outlined in the *Guide for the Care and Use of Laboratory Animals.*

Electrode Implantation. Animals were anesthetized with ketamine and xylazine (70 mg/kg and 6 mg/kg i.m., respectively). Epidural EEG electrodes, consisting of stainless steel screws (0-80 x 1/8in. for bipolar montage; 0-50 x 1/8in. for 10-electrode montage) soldered to insulated Ni chrome wire (0.2mm diameter), were implanted and fixed to the skull with dental acrylic (45). The free end of the wire was soldered to a multi-pin connector (March Electronics, West Hempstead, NY), also secured by dental acrylic.

EEG Recording Montages. Two EEG montages were used: a 10-electrode referential recording montage and a 2 bipolar electrode montage. For the 10-electrode montage, electrodes were implanted bilaterally in the skull over the frontal, parietal, occipital, anterior temporal, and posterior temporal regions of the brain. In the second configuration, a bipolar recording was made from each hemisphere through electrodes placed over the parietal and occipital cortices. In both montages, a reference electrode was placed posterior to lambda over the transverse sinus. Three animals subjected to permanent MCAo received the 10-electrode montage. Because it was evident from these recordings that all NCS activity could be detected by monitoring parietal cortex bilaterally (see Results), recordings from subsequent animals were made with the two bipolar electrode configuration.

Middle Cerebral Artery Occlusion. Animals were subjected to MCAo by the intraluminal filament method 2-3 days following electrode implantation. Anesthesia was induced with 5% halothane delivered in oxygen and was maintained with 1.5% halothane throughout surgery. Briefly, the right external carotid artery was ligated, cauterized, and cut, and its branches were coagulated. A 35mm length of 3-0 nylon monofilament (Ethicon, Somerville, NJ) with a rounded tip was then inserted into the internal carotid artery via the proximal end of the external carotid artery stump. The filament was advanced approximately 20 mm beyond the bifurcation of the internal and external carotid arteries, when slight resistance was encountered. Animals subjected to transient MCAo were briefly re-anesthetized at 2h and reperfused by retraction of the filament. For sham MCAo surgeries, the same procedures were followed but the filament was advanced only 10mm beyond the internal-external carotid bifurcation and was left in place until sacrifice at 24h. During all surgical procedures animals were maintained at 37.0°C by a homeothermic heating blanket (Harvard Apparatus, Holliston, MA).

Experimental Procedures

Animals were provided food and water ad libitum throughout the study and were individually housed under a 12h light/dark cycle. On the day of MCAo surgery, animals were transferred from their cages to custom-designed plexiglass EEG recording chambers (Dragonfly Inc., Ridgeley, WV) equipped with multi-channel swivel commutators (Plastics One, Roanoke, VA). The male end of the multi-pin connector on the rat skull was connected to the swivel system via a flexible shielded cable, allowing free movement of the animals during recordings. The swivel commutator was interfaced with a model 7D Grass polygraph

(Natick, MA) and a computerized EEG recording system (Stellate Harmonie software, Astro-Med, West Warwick, RI).

Prior to MCAo surgery, rectal temperatures and neurologic function were assessed and baseline EEG recordings were made continuously for at least 1h. Animals were then removed from their custom recording chambers for MCAo surgery. Immediately after surgery, animals were returned to recording chambers and continuous EEG recordings were again initiated. For the following 6h post-MCAo, continuous digital and polygraph EEG records were obtained and animals were observed carefully for signs of tonic/clonic motor convulsions and other abnormal behaviors. All animal behaviors, as well as movement artifacts, were annotated manually on the polygraph EEG records to document coincidence with seizure discharges and other electrographic events. Continuous digital EEG records were also obtained for the full duration of the study (24h or 72h) and stored on compact discs for subsequent quantitative and spectral analysis. Rectal temperatures were taken at 0.5h, 1h, 2h, 4h, 6h, and 24h and neurologic assessments were made at 1h post-MCAo and prior to sacrifice at either 24h or 72h.

Neurologic Assessment. Neurologic function was assessed by testing for four behaviors: (1) contralateral shoulder adduction (curling) and (2) unequal forelimb flexion when held by the tail and suspended above the floor, (3) counterclockwise circling during free ambulation, and (4) unequal resistance to left lateral displacement compared to right displacement. Any animals which did not exhibit all four of these behaviors were excluded from further study.

Infarct Assessment. Animals subjected to permanent MCAo were allowed to recover 72h, and those subjected to transient MCAo recovered 24h. At this time animals were deeply

anesthetized with 5% halothane and euthanized by decapitation. Forebrains were then harvested and cut in seven 2mm coronal sections beginning 1mm from the rostral pole. Sections were stained with 2,3,5-triphenyltetrazolium chloride (TTC) and digitally imaged using a computerized analysis system (LOATS Inc., Westminster, MD). Areas of infarction were defined as those completely lacking red TTC stain, and total infarct volumes were computed by integrating the infarct areas of sequential brain sections (53).

Data Analysis

In addition to neurologic assessment, total power of the EEG waveform was used as a criterion for adequate MCAo. When MCA blood flow is properly occluded, the amplitude of potentials recorded from the injured hemisphere is considerably reduced within 0.5h. We computed the percentage drop in EEG power in the global frequency band (0-35 Hz) in the injured hemisphere (parietal electrode in 10-e˙ montage) at 0.5h and at 2h post-MCAo compared to baseline recordings taken prior to MCAo surgery (e.g. % drop = $100\times(Power_{base}-Power_{2h})/Power_{base}$). Artifact-free, one minute EEG records during quiet, awake behavior (i.e. during low amplitude, high frequency EEG in contralateral hemisphere) were selected from digitally stored ipsilateral records to obtain power estimates. Only animals that showed both >60% drop in total power at 0.5h and a net decrease in power at 2h relative to pre-injury baseline were included in the study. These criteria are based on a larger, unpublished data set showing that all animals not satisfying these criteria have exceptionally small core infarct volumes.

Continuous 24 or 72h digitally stored EEG records were scanned in entirety at a display resolution of 9mm/sec for detection and scoring of NCS episodes. An NCS was defined in this study as either generalized or focal epileptiform activity occurring

continuously for at least 10sec with an amplitude unambiguously greater than background (19). If sequential NCS events were separated by <10sec, they were scored as a single episode; otherwise they were considered distinct. Rhythmic spikes sometimes occurred at amplitudes within the physiologic range either independently or antecedent to readily detectable, large amplitude ictal discharges. Such low amplitude activity was not scored as NCS due to its difficulty of detection and ambiguity of onset/offset.

Intermittent rhythmic delta activity (IRDA) and periodic lateralized epileptiform discharges (PLEDs) were also identified by scanning digital EEG records at a display of 9 mm/sec, and are addressed separately from NCS. IRDA consisted of readily identifiable, brief (<10 sec) bursts of rhythmic, large amplitude waves in the delta-theta (3-8 Hz) frequency range. PLEDs were identified as interictal spikes, sharp, or slow waves recurring with a variable period of 1-8 sec (mean ~2 sec; see Results). For quantitation of PLEDs, inter-discharge intervals were measured in digital records as the time between peaks of sequential discharges, and durations of individual discharges were measured as the time of excursion from baseline of the wave's most prominent phase. For each animal, the inter-discharge intervals and waveform durations were measured for the first 50 discharges appearing in the record. All data are reported as means ± S.D.

RESULTS

A total of 42 animals were subjected to MCAo in this study, of which 14 were excluded due to inadequate occlusion as assessed by EEG criteria (drop in total EEG power post-MCAo; see Methods), 6 based on neurologic examination at 1h post-MCAo, 3 due to sub-arachnoid hemorrhage, and 3 due to death prior to 24h. Post-ischemic ictal activity was

thus examined and quantified in 7 permanent and 9 transient MCAo animals. As reported previously (53), animals experienced a slight hyperthermia following MCAo (37.4 ± 0.7°C at 1h), but temperatures returned to baseline levels (36.5 ± 0.4°C) within 6h (36.7 ± 0.8°C). Animals also experienced a mean loss of 15.1 ± 2.6% body weight in the 24h period following injury. Of animals subjected to permanent MCAo, 3 died prior to their 72h recovery and the remaining 4 had a mean core infarct volume of 241 ± 65 mm^3. Of transient MCAo animals, 7/9 had a mean core volume of 214 ± 50 mm^3; two others had small core infarcts but showed significant regions of penumbral injury. Three additional animals were subjected to sham MCAo and exhibited no sign of neurologic impairment and neither seizure discharges nor other EEG abnormalities.

Non-Convulsive Seizure Discharges

In three animals, continuous 72h EEG recordings, initiated immediately following MCAo surgery, were made with 10-electrode montages covering frontal, parietal, occipital and temporal regions bilaterally in order to determine the spatiotemporal extent of seizure activity throughout the cerebral cortex following permanent MCAo. Figure 1 shows bilateral recordings from parietal and occipital cortices of a typical post-MCAo ictal event. The seizure initiates in the ischemic hemisphere and then generalizes bilaterally to all cortical regions. As the seizure develops the amplitude of rhythmic spikes first augments and then diminishes before termination.

The generalized seizure illustrated in Figure 1 is representative of the ictal activity most commonly observed after MCAo. Variations on this basic pattern included rhythmic sharp waves and occasional rhythmic spike-and-wave and polyspike discharges (Figure 2). Continuous polyspiking was often recorded in the initial and terminal phases of seizure

episodes. Typically rhythmic patterns occurred in the 1-4 Hz range and were observed in all cortical regions. In three animals focal seizures appeared only in the injured hemisphere (Fig. 2). These consisted of rhythmic 1-4 Hz spikes and high frequency, polyspike activity in nearly equal abundance. Like generalized events, they followed an evolving sequence of augmenting amplitude and changing frequency.

Importantly, no motor tonicity or behavioral convulsions were observed during either focal or generalized electrographic seizures. Animals exhibited restful, awake behavior with occasional wet dog shakes, or engaged in motor behaviors indistinguishable from those observed in the absence of ictal EEG patterns. These included circular ambulation, stereotypic grooming, and occasional pawing/scratching on the sides of the cage.

Seizure Incidence and Duration

'Trend' graphs (48,50) showing changes in the total power of the EEG record over ~3h post-MCAo illustrate the time course and incidence of seizure activity in two representative animals (Fig. 3). The large peaks correspond to seizure events such as that shown in Fig. 1. Several brief and distinct episodes can be observed in the few hours following injury. The majority of seizure events could be easily identified through trends monitoring, although some low amplitude rhythmic ictal activity could only be identified by inspection of the EEG record at slow speed.

Seizure incidence was quantified by defining specific criteria for individual episodes and identifying times of seizure onset and offset (see Methods). Of animals subjected to permanent MCAo, 6/7 (85%) had their first seizure episode within 1h post-MCAo, at a mean time of 35 ± 20 min. Similarly, 7/9 (78%) transient MCAo animals exhibited seizures within 1h, with a mean latency of 28 ± 13 min. The mean latency for the

two groups combined was 31 ± 16 min. In permanently occluded animals, the final seizure episode ended 119 ± 57 min post-MCAo, and 96 ± 103 min after initiation of the first seizure. In transiently occluded animals, seizures never occurred following reperfusion at 2h.

Animals subjected to permanent MCAo had a mean of 11.4 ± 5.1 seizure events within the first 2h of occlusion, which is nearly identical to the mean number of seizure events in transient MCAo animals prior to reperfusion (10.0 ± 6.2). The two groups combined had a mean of 10.6 ± 5.6 events within 2h post-MCAo. The average total number of seizure episodes over the full 72h recovery period following permanent MCAo was only slightly higher (12.5 ± 5.2) than that for the first 2h period. One permanently occluded animal exhibited no seizures within the first several hours of injury, but later experienced 8 seizure events, beginning 8h 45min post-MCAo and terminating 63min after inception.

As suggested by Figure 3, in most animals seizures were not continuous throughout the period they were observed, but rather were episodic in nature. Mean durations for seizure episodes were computed for each animal. Excluding one outlier, the mean duration of individual seizure events for 12/13 animals was 60.0 ± 32.7sec, with events being separated by an average inter-seizure interval of 6min 44sec (± 4min 2sec). The mean total time for which the EEG recordings were occupied with seizure activity was 11min 47sec (± 10min 38sec). The outlying animal had a mean seizure duration of 4min 39sec, a mean interval of 90sec, and a total seizure time of 1h 23min 40sec. There were no significant correlations between seizure incidence and either infarct volumes or neurological outcomes at 24h post-injury.

Periodic Lateralized Epileptiform Discharges

Another type of epileptiform activity appearing prominently in MCAo-injured animals was PLEDs. The characteristics and distribution of PLEDs throughout the cortex were rather homogenous across different animals when they appeared, and these are illustrated in Figure 4. The six channels shown in Fig. 4A, taken from a 10-electrode recording of one animal, illustrate that PLEDs occurred mainly in the ischemic hemisphere over regions of penumbral injury. These include ipsilateral occipital, parietal, and frontal cortices, although PLEDs tended to distribute bilaterally in frontal regions. PLEDs did not occur in temporal regions, which TTC staining revealed as areas of core infarct.

Measures of individual PLED waveform durations were unimodal and normally distributed, with a mean of 230 ± 41ms (range: 130-310ms; see Fig. 4B). The mean durations computed for different animals varied with a S.D. of 13ms. Though sometimes triphasic, individual PLED discharges were most frequently biphasic with an initial negative phase (Fig. 4B). PLEDs did not occur with a metronomic periodicity as was the case for rhythmic spike seizures, but rather had a stereotyped pseudoperiodic rhythmicity (Fig. 4A). Combining data from all animals, sharp/slow waves recurred at a mean interval (period) of 2.18 ± 0.66sec (0.46 Hz), with a coefficient of variation (CV) for the inter-discharge interval of 0.30 (Fig. 4C). The range of inter-discharge intervals sampled was 1.4-4.0sec, with 98% being <3.8sec. The mean intervals computed for different animals ranged from 1.82 to 2.55sec, and varied with a S.D. of 0.30sec. CVs for individual animals averaged 0.27 and ranged from 0.19 to 0.34.

PLEDs occurred in 6/7 animals subjected to permanent MCAo. All animals first showed >60% drop in total power in the injured hemisphere within 0.5h of occlusion. In some animals PLEDs emerged on this depressed background *prior* to the appearance of

seizure discharges; in other animals PLEDs began only after several seizure episodes or following the cessation of ictal episodes. In the former instance, PLEDs recurred as an inter-ictal pattern and in all instances continued after cessation of seizure episodes. Following seizure cessation, PLEDs ranged in incidence from nearly continuous to occasional/episodic, alternating most frequently with arrhythmic polymorphic delta activity. PLEDs occurred frequently through 24h post-injury, and although their incidence tapered with time, they continued to appear through the 72h survival period. In animals subjected to transient MCAo, PLEDs occurred with a slightly lower incidence (4/9 animals; $p=0.09$, χ^2) since they were rarely observed following reperfusion at 2h and sometimes had a late (>2h) onset in permanently occluded animals.

Considering both transient and permanent groups together, in 6/16 animals PLEDs emerged within 1h post-MCAo, *prior* to the first seizure episodes. These animals had a significantly greater number of seizure events within 2h (14.8 ± 4.0) than all other MCAo-injured animals (4.8 ± 3.7; $p<0.01$, two-tailed *t*-test), and even all other animals exhibiting seizures, with or without subsequent PLEDs (6.1 ± 2.9; $p<0.01$, two-tailed *t*-test; Fig. 4D). One animal had PLEDs but no seizures, while 4/6 animals with no PLEDs did exhibit seizures. Animals with and without PLEDs did not significantly differ in their core infarct volumes (two-tailed *t*-test).

Intermittent Rhythmic Delta Activity

While PLEDs dominated the injured hemisphere, the *contralateral* hemisphere exhibited a recurrent pattern similar to that described as IRDA. IRDA consisted of 2-6 sec bursts of large amplitude activity in the 4-7 Hz range, was strongly lateralized, and predominated in frontal and parietal electrodes (Fig. 5A). Concomitant bursts of activity

could be observed in occipital and temporal electrodes during frontal/parietal IRDA, but the activity was less rhythmic, more broadly distributed in its frequency composition, and smaller in amplitude (Fig. 5A).

IRDA bursts most commonly occurred in isolation, but in some cases recurred continually. Figure 5C illustrates such an episode in which several 3-4 sec IRDA bursts occur in sequence. In this instance bursts are easily identified by abrupt amplitude shifts during onset and offset, as well as by their distinctive rhythmicity (Fig. 5B). Often, however, large amplitude activity, identical to IRDA in duration and dominant frequency, would also appear with less rhythmic structure (Fig. 5D,E). These IRDA bursts frequently occurred in sequence with longer epochs of high amplitude, paroxysmal patterns on a background of low amplitude, desynchronized EEG (Fig. 5D,F). Spectral analysis revealed consistently that such periods of activity were also dominated by spectral power in the 4-7 Hz range (Fig. 5E,F).

The onset of contralateral IRDA typically occurred within 1h of MCAo, either prior to or amongst seizure episodes. Its appearance was most frequent and sometimes continual (e.g. Fig. 5C-F) for several hours following termination of seizures, and persisted as an intermittent pattern through 72h in permanent MCAo animals, albeit with decreasing frequency. Because IRDA was frontally dominant, with less rhythmicity and smaller amplitude in parietal and occipital cortices, this pattern of activity was difficult to positively identify in animals recorded with bipolar electrode montages. Consequently we were not able to determine what effect, if any, reperfusion of the injured hemisphere had on the presentation of contralateral IRDA.

DISCUSSION

Here we have presented a comprehensive description of EEG seizure activity accompanying rat focal ischemia. We found that 81% of animals exhibited seizures within the first hour post-MCAo and experienced an average of 10.6 seizures within 2h. Seizures averaged 60sec in duration and ceased following reperfusion. In addition, we have described the appearance of two neuropathologic EEG activities, PLEDs and IRDA. These results complement our previous report mapping the spatiotemporal extent of voltage attenuation and altered EEG spectral content (e.g. enhanced delta activity) in rat MCAo (24). Together these data validate rat MCAo as a pre-clinical model of ischemic neurophysiology, as well as a novel and robust model for the study of post-ischemic ictal activity.

This model offers several advantages over existing animal models for the study of acutely induced seizures. Because seizures occur in rat MCAo as distinct, identifiable events, they are amenable to quantitation and allow for statistical analysis of the effects of pharmacological intervention (54). A brain injury model for pharmacological studies also circumvents problems of drug interaction inherent to seizure models in which epileptic conditions are chemically induced (e.g. 21). Unlike weight drop and fluid percussion models of traumatic brain injury, however, the intraluminal filament method of MCAo does not require opening of the cranial cavity, thus allowing unrestricted access to the brain for monitoring of experimental parameters. Finally, an *in vivo* model of thrombotic/embolic ischemia producing EEG seizures is more relevant and interpretable in pathophysiologic terms than chemical and electroshock models of epilepsy.

Although the mechanisms of post-ischemic seizures are incompletely understood, depolarizations induced by high extracellular potassium ($[K^+]_o$) are likely to mediate the

acutely induced ictal events described here. In rat MCAo, neuronal depolarization in the ischemic core is accompanied by a rise in $[K^+]_o$ to approximately 60 mM, and frequent waves of spreading depression can propagate these ionic imbalances throughout the ischemic hemisphere (29). In rat hippocampal slices, $[K^+]_o$ levels of only 8.5 mM are sufficient to elicit spontaneous 20-90 sec seizures (46,4). These seizures are mediated by somal currents (42), including low voltage-gated persistent Na^+ currents (2), and are not dependent on chemical synaptic transmission (16). Glutatmate-mediated currents may provoke the enhanced excitability underlying recurrent epileptiform activity in the sub-acute phase of injury (44).

Non-Convulsive Seizures

A critical aspect of the seizures identified in this study is their non-convulsive nature. Careful monitoring of ongoing behavior and concurrent EEG recordings revealed that seizure discharges occurred during normal wakeful activity without motor convulsions or behavioral arrest. In addition, seizures meet both primary (repetitive generalized or focal spiking at ~3 Hz) and secondary (onset with incrementing voltage and slowing frequency, and offset of decreasing voltage; Figs. 1&2) criteria for clinical NCS as described by Jordan (19).

Classically, NCSE has referred to absence status and complex partial status epilepticus (CPSE). Increasingly, however, the term 'NCSE' is used to describe a distinct form, also termed 'subtle' generalized status epilepticus (47; see 31), which is commonly associated with severe medical illness in which patients are obtunded or comatose (9,23,40,47). Although both CPSE and 'NCSE' occur following structural brain lesions (CPSE, see 36), recent studies have focused on the occurrence of the latter after traumatic

head injury, stroke, or following the control of convulsive status epilepticus (17,18,23,47,51). The importance of treatment has been a matter of controversy in CPSE (8,20), but mortality is high and success of treatment low in cases of 'NCSE' (9,7,23,40,47).

The seizure events described here are distinguished from 'NCSE' by the animals' awake and behaving state and by their limited duration. The generalized nature of the seizures, however, would also preclude their characterization as 'complex partial'. Given these considerations, the most appropriate term for these seizures, used in a purely descriptive manner, is simply 'non-convulsive seizures'. The responsiveness of these NCS events to anti-convulsant compounds, which is presently being studied, will better determine the appropriateness of these seizures as a model of the refractory NCSE condition.

Periodic Lateralized Epileptiform Discharges

In addition to NCS, we found that a prominent neurophysiological sequela of MCAo injury in the rat is the appearance of PLEDs. The high incidence of PLEDs in MCAo injury is not surprising given clinical findings that PLEDs are most commonly associated with structural lesions (5,13,38), and stroke in particular (see also 53,57).

The characteristics of PLEDs described in this model replicate well those described in clinical populations (for review see 32). In an inventory of patients with PLEDs (14), in all cases but one PLEDs were found to emerge from simplified background activity, such as increased relative delta power and voltage attenuation (see also 38,57). Following rat MCAo injury, simplification also consisted of diffuse slowing/polymorphic delta activity and voltage attenuation (24). In this context, or in the period of suppression following an NCS episode, PLEDs emerged. During permanent occlusion EEG power in the injured hemisphere remains attenuated >2h post-injury and only gradually recovers over subsequent

days. In this context of prolonged simplification, we found that PLEDs persist intermittently for the full duration of the 72h recovery period. On the other hand, PLEDs cease when animals are reperfused after 2h and EEG power recovers (see also 24).

PLEDs as occur in rat MCAo exhibit waveforms and periodicity that are nearly identical to those described in clinical populations (38,57). Individual waveform durations in rat MCAo ranged over values that straddled classification as sharp (<200ms) and slow (>200ms) waves. Gross et al. (14) found that PLEDs in 63% of their subjects consisted of sharp/slow waves, and that an additional 37% consisted of sharp waves (see also 11,32,57). We did not observe PLEDs consisting of spikes, as occur in some clinical instances of PLEDs (41,57).

While the classical definition describes PLEDs as recurring with metronomic periodicity, often the interval between sequential discharges fluctuates in a characteristic 'pseudoperiodic' manner (25). For instance, Gross et al. (14) measured the periodicity of PLEDs in 57 patients and found C.V. values that ranged from 0.14 to 1.16, with a mean of 0.36. The MCAo model replicates this idiosyncratic feature of human PLEDs, as the mean C.V. for inter-discharge periods was 0.29. This pseudoperiodicity was stereotyped, as all animals had similar C.V. values. The range of inter-discharge periods described here (Fig. 4C) also replicates that described in patient populations, where periods range from 0.5 to 8 sec, with 1-3 sec periods occurring most frequently (14,32,57).

Finally, the rat MCAo model captures several salient aspects of the relationship between PLEDs, NCS, and brain injury. Most surprisingly, we found a highly significant association between the occurrence of PLEDs before NCS and prolonged NCS series. This is in agreement with previous findings of an association between severe seizure activity and

PLEDs (34,37,38,53,57). For instance, Snodgrass et al. (41) found that over 50% of all patients they catalogued with PLEDs exhibited electrographic status epilepticus. Furthermore, Garzon et al. (11) found that *mortality was significantly higher in status epilepticus when PLED occurs as the first ictal pattern.* Importantly, studies have shown that PLEDs lead to local increases in metabolism (15) and cerebral blood flow (33) for several days following termination of SE, suggesting that they may contribute to the spread of secondary injury and play a causal role in associated high mortality.

Intermittent Rhythmic Delta Activity

In addition to NCS and PLEDs, the rat MCAo model replicates characteristics of a third EEG abnormality, IRDA, which is associated clinically with neurologic insult. Similar to the data reported here for rat MCAo, bursts of IRDA in human patients last 2-6 seconds and occur mainly in the delta frequency band (0-4 Hz), but also in the lower theta range (4-8 Hz) (30). The occurrence of higher (6-8 Hz) frequencies was more common in our experiments.

In clinical populations IRDA usually appears predominantly in frontal regions and is sometimes referred to as frontally predominant intermittent rhythmic delta activity (FIRDA) or monorhythmic frontal delta (35,10). Remarkably, in the MCAo-injured rat brain IRDA also presented as a frontally dominant pattern. The rat IRDA pattern, however, differed from those observed clinically in that its appearance was restricted to the hemisphere *contralateral* to MCAo injury (Fig. 5); in clinical populations it most commonly appears bilaterally (30,35). The lateralized appearance of IRDA is noteworthy, given the minimal EEG changes in background activity observed contralaterally following MCAo (24). Cresyl violet and hematoxylin and eosin staining show no evidence of pathology in regions

generating IRDA, although it is possible that contralateral circuits have enhanced excitability (55,56).

Schaul et al. (35) reported patient sub-populations with IRDA that exhibited normal background activity. Together with present results, this suggests that IRDA can be evoked by mild dysfunction such as, for instance, subtle disruptions of subcortical structures and/or fiber tracts innervating the cortex due to increased transventricular pressure (12). In patient populations IRDA is associated with a wide variety of neurologic disorders (10), with a predominance of causes related to structural pathology, as in the case of MCAo (35).

Conclusions

The similarities between abnormal EEG waveforms in the rat MCAo model and in human populations suggest that they reflect the same phenomena, with common underlying mechanisms. Heretofore, the circuit and pathological bases of PLEDs and IRDA have mainly been the object of speculation based on clinical data, and an *in vivo* model of NCS has been lacking. The rat MCAo, as presented here, is the first animal model of brain injury which exhibits these neurologically important yet poorly understood phenomena and holds promise for their future pathological and electrophysiological investigations.

REFERENCES

1. Aminoff, M.J. 1998. Do nonconvulsive seizures damage the brain?-No. *Arch. Neurol.* 55:119-120.
2. Azouz, R., Jensen, M.S., and Yaari, Y. 1996. Ionic basis of spike after-depolarization and burst generation in adult rat hippocampal CA1 pyramidal cells. *J. Physiol.* 492:211-223.

3. Bergsneider, M., Hovda, D.A., Shalmon, E., Kelly, D.F., Vespa, P.M., Martin, N.A., Phelps, M.E., McArthur, D.L., Caron, M.J., Kraus, J.F., and Becker, D.P. 1997. Cerebral hyperglycolysis following severe traumatic brain injury in humans: a positron emission tomography study. *J. Neurosurg.* 87:803-805.

4. Borck, C. and Jefferys, J.G.R. 1999. Seizure-like events in disinhibited ventral slices of adult rat hippocampus. *J. Neurophysiol.* 82:2130-2142.

5. Chatrain, G.M., Shaw, C.-M., and Leffman, H. 1964. The significance of periodic lateralized epileptiform discharges in EEG: an electrographic, clinical, and pathological study. *Electroenceph. Clin. Neurophysiol.* 17:177-193.

6. Cox, S.B., Woolsey, T.A., and Rovainen, C.M. 1993. Localized dynamic changes in cortical blood flow with whisker stimulation corresponds to matched vascular and neuronal architecture of rat barrels. *J. Cereb. Blood Flow Metab.* 13:899-913.

7. DeLorenzo, R.J., Waterhouse, E.J., Towne, A.R., Boggs, J.G., Ko, D., DeLorenzo, G.A., Brown, A., and Garnett, L. 1998. Persistent Nonconvulsive Status Epilepticus After the Control of Convulsive Status Epilepticus. *Epilepsia* 39:833-840.

8. Drislane, F.W. 1999. Evidence against permanent neurologic damage from nonconvulsive status epilepticus. *J. Clin. Neurophysiol.* 16:323-31.

9. Drislane, F.W. and Schomer, D.L. 1994. Clinical implications of generalized electrographic status epilepticus. *Epilepsy Res.* 19:111-21.

10. Fariello, R.F., Orrison, W., Blanco, G., and Reyes, P.F. 1982. Neuroradiological correlates of frontally predominant intermittent rhythmic delta activity (FIRDA). *Electroenceph. Clin. Neurophysiol.* 54:194-202.

11. Garzon, E., Fernandes, R.M.F., and Sakamoto, A.C. 2001. Serial EEG during human status epilepticus: evidence for PLED as an ictal pattern. *Neurology* 57:1175-1183.

12. Gloor, P., Ball, G., and Schaul, N. 1977. Brain lesions that produce delta waves in the EEG. *Neurology* 27:326-33.

13. Grand'Maison, F., Reiher, J., and Leduc, C.P. 1991. Retrospective EEG abnormalities in partial status epilepticus. *Electroencephal. Clin. Neurophysiol.* 79:264-270.

14. Gross, D.W., Wiebe, S., and Blume, W.T. 1999. The periodicity of lateralized epileptiform discharges. *Clin. Neurophysiol.* 110:1516-1520.

15. Handforth, A., Cheng, J.T., Mandelkern, M.A., and Treiman, D.M. 1994. Markedly increased mesiotemporal lobe metabolism in a case with PLEDs: further evidence that PLEDs are a manifestation of partial status epilepticus. *Epilepsia* 35:876-881.

16. Jensen, M.S. and Yaari, Y. 1988. The relationship between interictal and ictal paroxysms in an in vitro model of focal hippocampal epilepsy. *Ann Neurol.* 24:591-598.

17. Jordan, K.G. 1993. Continuous EEG and evoked potential monitoring in the neuroscience intensive care unit. *J. Clin. Neurophysiol.* 10:445-475.

18. Jordan, K.G. 1995. Neurophysiologic monitoring in the neuroscience intensive care unit. *Neurol. Clin.* 13:579-626.

19. Jordan, K.G. 1999. Continuous EEG Monitoring in the Neurscience Intensive Care Unit and Emergency Department. *J. Clin. Neurophysiol.* 6:14-39.

20. Kaplan, P.W. 2000. No, some types of nonconvulsive status epilepticus cause little permanent neurologic sequelae (or: "The cure may be worse than the disease"). *Neurophysiol. Clin.* 30:377-82.

21. Krsek, P., Mikulecka, A., Rastislav, D., Zdenek, H., Kubova, H., and Mares, P. 2001. An animal model of nonconvulsive status epilepticus: a contribution to clinical controversies. *Epilepsia* 42:171-180.

22. Lipton, P. 1999. Ischemic cell death in brain neurons. *Physiol. Rev.* 79:1431-1568.

23. Litt, B., Wityk, R.J., Hertz, S.H., Mullen, P.D., Weiss, H., Ryan, D.D., and Henry, T.R. 1998. Nonconvulsive status epilepticus in the critically ill elderly. *Epilepsia* 39:1194-1202.

24. Lu, X.-C.M., Williams, A., and Tortella, F. 2001. Quantitative EEG spectral analysis and topographic EEG mapping applied in a rat model of middle cerebral artery occlusion. *Neuropathol. Appl. Neurobiol.* 27:481-95.

25. Markand, O.N. and Daly, D.D. 1971. Pseudoperiodic lateralized paroxysmal discharges in electroencephalogram. *Neurology* 21:975-81.

26. McIntosh, T.K., Smith, D.H., Meaney, D.F., Kotapka, M.J., Gennarelli, T.A., and Graham, D.I. 1996. Neuropathological sequelae of traumatic brain injury: relationship to neurochemical and biomechanical mechanisms. *Lab. Invest.* 74:315-342.

27. Meltzer, C., Adelson, P., Brenner, R., Crumrine, P., Van, C.A., Schiff, D., Townsend, D., and Scheuer, M. 2000. Planned ictal FDG PET imaging for localization of extratemporal epileptic foci. *Epilepsia* 41:193-200.

28. Mies, G., Iijimi, T., and Hossman, K.-A. 1993. Correlation of peri-infarct DC shifts and ischaemic neuronal damage in rat. *Neuroreport* 4:709-711.

29. Nedergaard, M. and Hansen, A.J. 1993. Characterization of cortical depolarizations evoked in focal cerebral ischemia. *J. Cereb. Blood Flow Metab.* 13:568-574.

30. Neufeld, M.Y., Chistik, V., Chapman, J. and Korczyn, A.D. 1999. Intermittent rhythmic delta activity (IRDA) morphology cannot distinguish between focal and diffuse brain disturbances. *J. Neurol. Sci.* 164:56-59.

31. Niedermeyer, E. and Ribeiro, M. 2000. Considerations of nonconvulsive status epilepticus. *Clin. Electroencephal.* 13:192-195.

32. Pohlmann-Eden, B., Hoch, D.B., Cochius, J.I., and Chiappa, K.H. 1996. Periodic lateralized epileptiform discharges - a critical review. *J. Clin. Neurophysiol.* 13:519-530.

33. Raroque, H.G., Devoux, M.D. Sr., Gonzalez, P.C.W., and Leroy, R.F. 1991. Regional cerebral blood flow with single photon emission computed tomography in PLEDs and BIPLEDs. *Epilepsia* 32(suppl):77.

34. Reiher, J., Rivest, J., Grand'Maison, F., and Leduc, C. 1991. Periodic lateralized epileptiform discharges with transitional rhythmic discharges: association with seizures. *Electroenceph. Clin. Neurophysiol.* 78:12-17.

35. Schaul, N., Lueders, H., and Sachdev, K. 1981. Generalized, bilaterally synchronous bursts of slow waves in the EEG. *Arch. Neurol.* 38:690-692.

36. Scholtes, F.B., Renier, W.O., and Meinardi, H. 1996. Non-convulsive status epilepticus: causes, treatment, and outcome in 65 patients. *J. Neurol. Neurosur. Ps.* 61:93-95.

37. Schraeder, P.L. and Singh, N. 1980. Seizure disorders following periodic lateralized epileptiform discharges. *Epilepsia* 21:647-53.

38. Schwartz, M.S., Prior, P.F., Scott, D.F. 1973. The occurrence and evolution in the EEG of a lateralized periodic phenomenon. *Brain* 96:613-22.

39. Silbert, P.L., Radhakrishnan, K., Sharbrough, F.W., and Klass, D.W. 1996. Ipsilateral independent periodic lateralized epileptiform discharges. *Electroenceph. Clin. Neurophysiol.* 98:223-226.

40. Simon, R.P. and Aminoff, M.J. 1986. Electrographic status epilepticus in fatal anoxic coma. *Ann. Neurol.* 20:351-355.

41. Snodgrass, S.M., Tsuburaya, K., and Ajmone-Marsan, C. 1989. Clinical significance of periodic lateralized epileptiform discharges: relationship with status epilepticus. *J. Clin. Neurophysiol.* 6:159-172.

42. Somjen, G.G., Aitken, P.G., Giacchino, J.L., and McNamara, J.O. 1985. Sustained potential shifts and paroxysmal discharges in hippocampal formation. *J. Neurophysiol.* 53:1079-1097.

43. Striano, S., DeFalco, F.A., Zaccaria, F., Fels, A., Natale, S., and Vacca, G. 1986. Paroxysmal lateralized epileptiform discharges (PLEDS). Clinical-EEG correlations in twenty cases. *Acta Neurol. (Napoli)* 8:1-12.

44. Sun, D.A., Sombati, S., and DeLorenzo, R.J. 2001. Glutamate injury-induced epileptogenesis in hippocampal neurons: an in vitro model of stroke-induced "epilepsy". *Stroke* 32:2344-50.

45. Tortella, F., Rose, J., Moreton, J., Hughes, J., and Hunter, J. 1997. EEG spectral analysis of the neuroprotective kappa opiods enadoline and PD117302. *J. Pharmacol. Exp. Ther.* 282:286-293.

46. Traynelis, S.F. and Dingledine, R. 1988. Potassium-induced spontaneous electrographic seizures in the rat hippocampal slice. *J. Neurophysiol.* 59:259-276.

47. Treiman, D.M., Meyers, P.D., Walton, N.Y., Collins, J.F., Colling, C., Rowan, A.J., Handforth, A., Faught, E., Calabrese, V.P., Uthman, B.M., Ramsay, R.E., and Mamdani, M.B. 1998. A comparison of four treatments for generalized convulsive status epilepticus. *N. Engl. J. Med.* 339:792-798.

48. Treiman, D.M., Walton, N.Y., and Kendrick, C. 1990. A progressive sequence of electroencephalographic changes during generalized convulsive status epilepticus. *Epilepsy Res.* 5:49-60.

49. Vespa, P.M., Nenov, V., and Nuwer, M.R. 1999. Continuous EEG monitoring in the intensive care unit: early findings and clinical efficacy. *J. Clin. Neurophysiol.* 16:1-13.

50. Vespa, P., Nuwer, M.R., Nenov, V. et al. 1997. Incidence of nonconvulsive and convulsive seizures in the ICU following traumatic brain injury: increased incidence detected by continuous EEG monitoring. *Crit. Care Med.* 25:A120.

51. Vespa, P.M., Nuwer, M.R., Nenov, V., Ronne-Engstron, E., Hovda, D.A., Bergsneider, M., Kelly, D.F., Martin, N.A., and Becker, D.P. 1999. Increased Incidence and Impact of Nonconvulsive and Convulsive Seizures After Traumatic Brain Injury as Detected by Continuous Electroencephalographic Monitoring. *J. Neurosurg.* 91:750-760.

52. Wadman, W.J., Juta, A.J.A., Kamphuis, W., and Somjen, G.G. 1992. Current source density of sustained potential shifts associated with electrographic seizures and with spreading depression in rat hippocampus. *Brain Research* 570:85-91:

53. Walsh, J.M. and Brenner, R.P. 1987. Periodic lateralized epileptiform discharges – Long-term outcome in adults. *Epilepsia* 28:533-536.

54. Williams, A.J. and Tortella, F.C. 2002. Neuroprotective effects of the sodium channel blocker RS100642 and attenuation of ischemia-induced brain seizures in the rat. *Brain Res.* 932:45-55.

55. Williams, A.J., Tortella, F.C., Yao, C., Yu, Z.Y., Hale, S.L., Berti, R., and Dave, J.R. 2001. Expression of sodium channel genes following ischemic injury: an in situ hybridization study. *Soc. Neurosci. Abstr.* Vol 27, Program No. 46.23.

56. Woods, L., Rema, V., and Ebner, F.F. 2001. Onset of interhemispheric effects following stroke-like lesions to adult rat S1 cortex. *Soc. Neurosci. Abstr.* Vol. 27, Program No. 393.2.

57. Young, G.B., Goodenough, P., Jacono, V., and Schieven, J.R. 1988. Periodic lateralized epileptiform discharges (PLEDs): electrographic and clinical features. *Am. J. EEG Technol.* 28:1-13.

58. Young, G.B. and Jordan, K.G. 1998. Do nonconvulsive seizures damage the brain? Yes. *Arch. Neurol.* 55:117-9.

59. Young, G.B., Jordan, K.G., and Doig, G.S. 1996. An assessment of nonconvulsive seizures in the intensive care unit using continuous EEG monitoring: an investigation of variables associated with mortality. *Neurology* 47:83-9.

FIGURE LEGENDS

Figure 1. Seizure discharges in an MCAo-injured rat. **(A)** Recordings from 4 of 10 electrodes show representative generalized seizures occurring in injured **(I)** and contralateral **(C)** hemispheres. Decreased power in the injured hemisphere during interictal periods is

evident. **(B)** Seizure initiation shown on an expanded time scale. Time lines delineate 3 sec segments.

Figure 2. Waveforms of seizure discharges following MCAo. Time scale bar applies to top three traces. Time lines for focal seizure mark 3 sec segments.

Figure 3. Continuous long-term monitoring of seizure activity. 'Trends' graphs show estimates of total EEG power based on consecutive 2.5 sec segments and are plotted in 10 sec bins. **(A)** and **(B)** illustrate EEG changes for two rats subjected to permanent and transient MCAo, respectively. Power fluctuations prior to MCAo reflect animals' natural shifts in levels of activity/vigilance. Following MCAo, seizure events are evident as large, transient increases in total power. Power recovers and seizure episodes cease upon reperfusion **(B)**. Asterick (*) denotes the two seizure events illustrated in Figure 1.

Figure 4. PLEDs recur in injured hemisphere. **(A)** and **(B)** Recordings illustrate the spatial distribution, pseudoperiodicity, and waveforms of PLEDs. Time lines in **(A)** and **(B)** mark 3 sec segments. **(C)** Distribution of inter-discharge intervals of PLEDS measured in all animals (see Methods). **(D)** graphs the mean number of seizure events (± S.D.) within 2 h for animals in which PLEDs emerged **before** the first seizure episode, and for those in which PLEDs occurred in the wake of seizures or did not appear at all (**after**).

Figure 5. IRDA appears in contralateral hemisphere. **(A)** 10-electrode recording of an IRDA burst showing fronto-parietal dominance. Time lines delineate 3 sec segments. Subsequent

panels show recordings from the contralateral frontal electrode. **(B)** IRDA illustrated with an expanded time scale. **(C)** Series of consecutive IRDA bursts. **(D)** Examples of less rhythmic IRDA and associated high amplitude activity. **(E)** and **(F)** Power spectra of selected segments of the EEG record marked by dashed lines in **(D)**.

Figure 1.

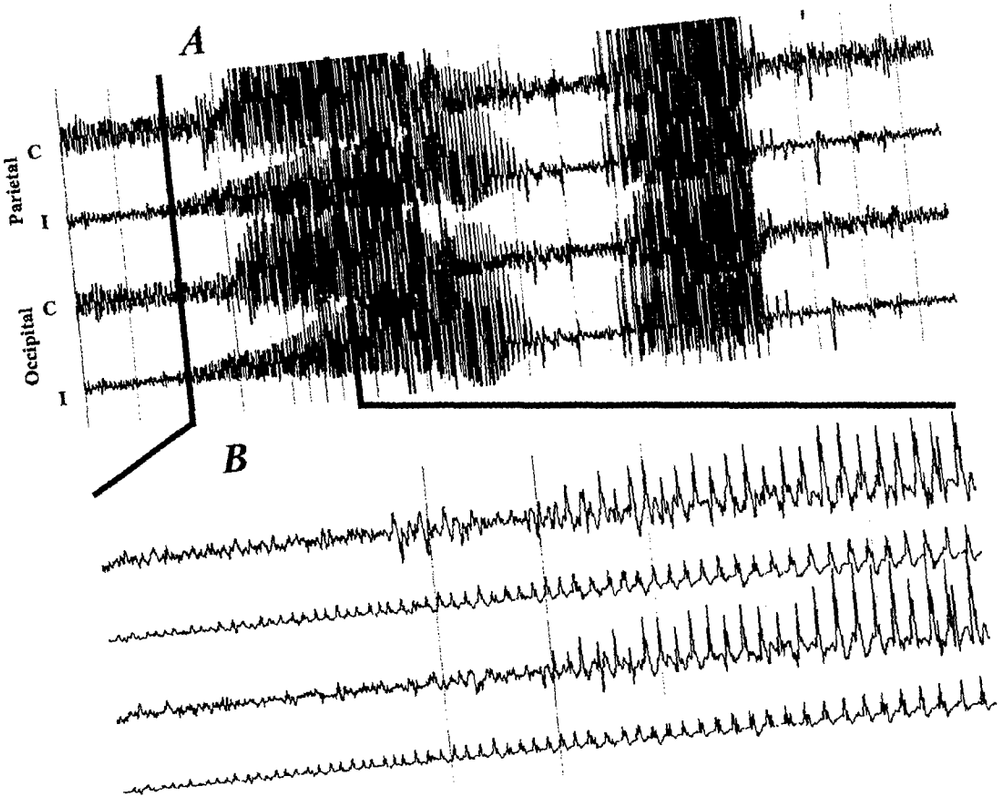

Figure 2.

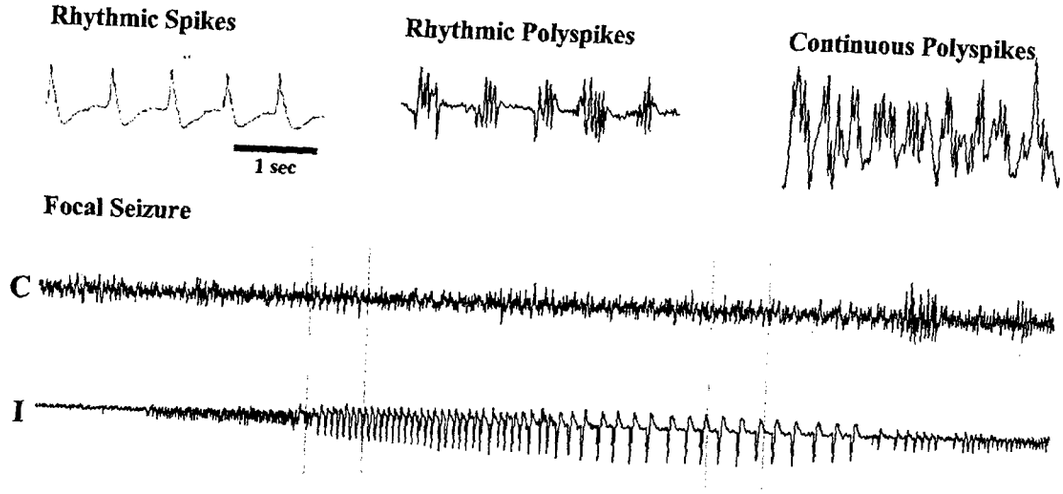
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Figure 3.

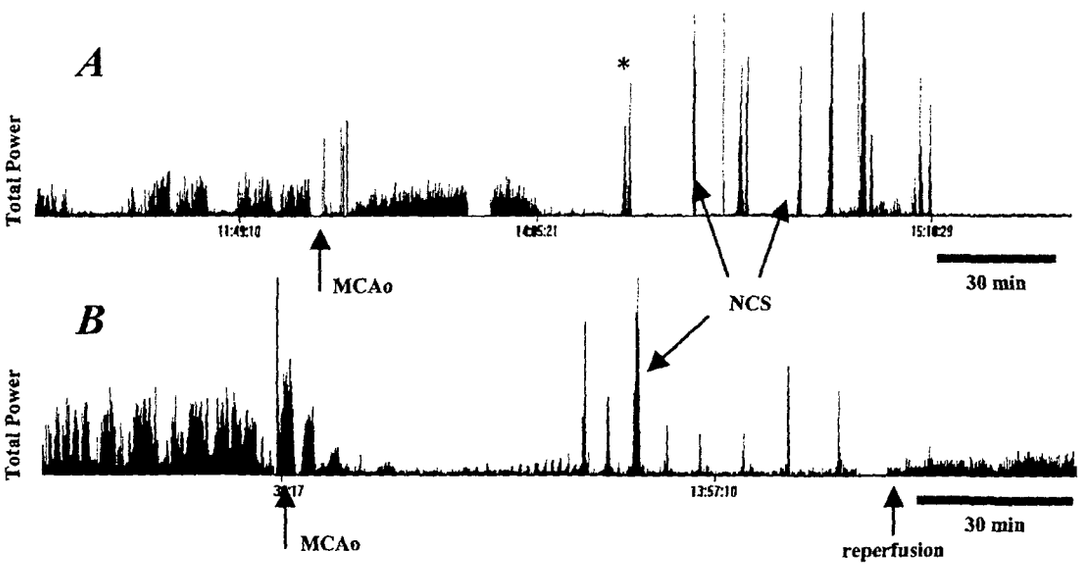
A
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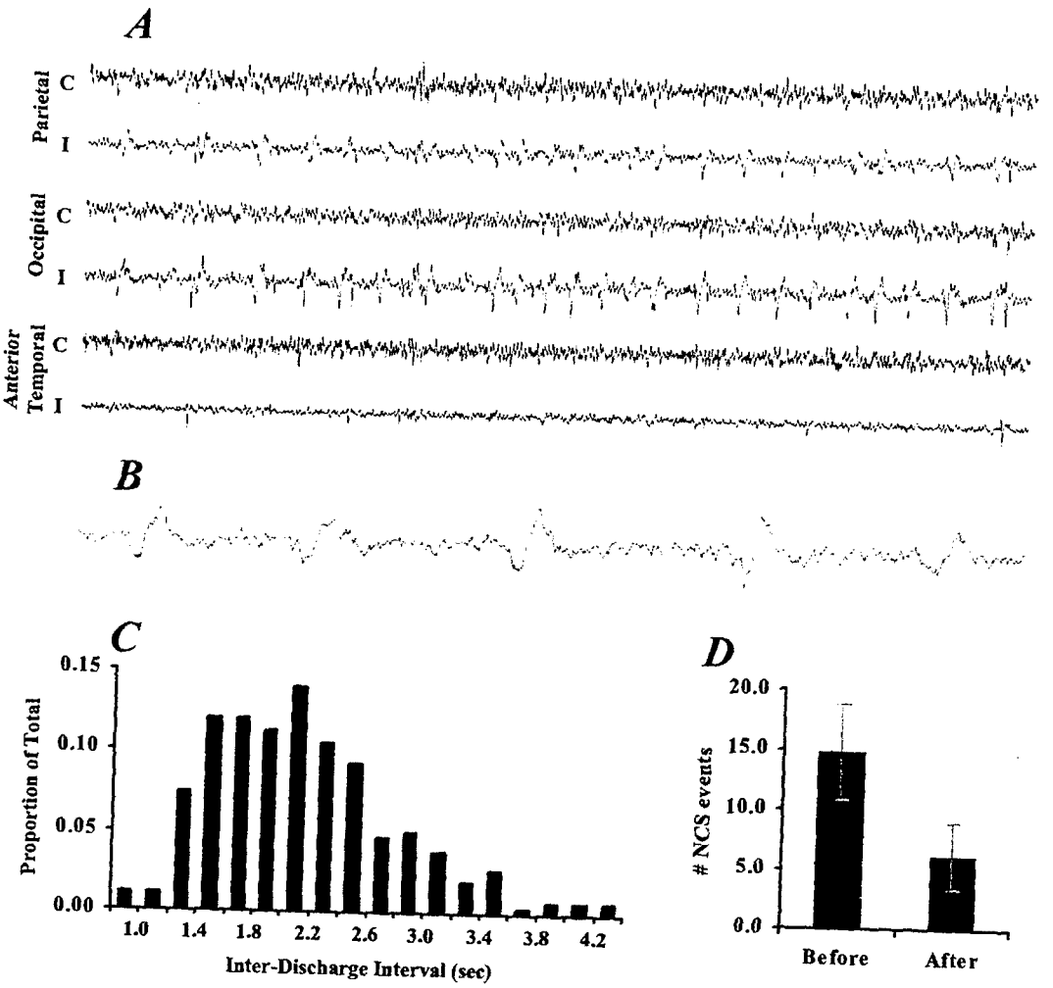
Figure 4.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Anterior Temporal
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B
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Proportion of Total
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0.05
0.00
1.0
1.4
1.8
2.2
2.6
3.0
3.4
3.8
4.2
Inter-Discharge Interval (sec)
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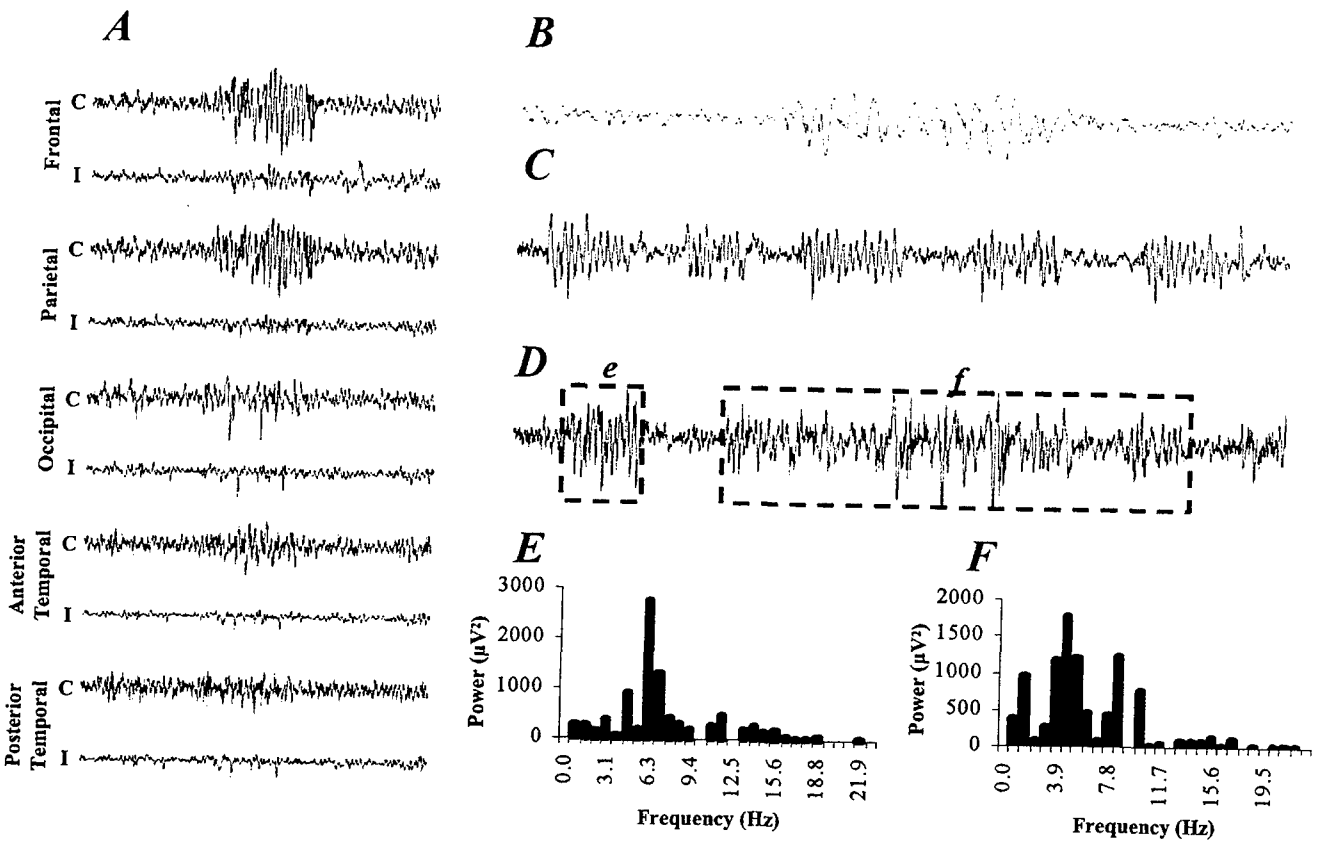
NCS events
20.0
15.0
10.0
5.0
0.0
Before
After

Figure 5.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Power (μV²)
3000
2000
1000
0
0.0
3.1
6.3
9.4
12.5
15.6
18.8
21.9
Frequency (Hz)
F
Power (μV²)
2000
1500
1000
500
0
0.0
3.9
7.8
11.7
15.6
19.5
Frequency (Hz)