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SUMMARY STATEMENT
(Privileged Communication)

Application Number: 1 R01 NS41635-01

ZNS1 SRB-R (1)
Review Group: NINDS SPECIAL EMPHASIS PANEL

Meeting Dates: SRG: OCT/NOV 2000 COUNCIL: JAN/FEB 2001 FD
Requested Start Date: 04/01/2001
NS01-004

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Project Title: Mechanisms and modulation of disease progression in ALS

SRG Action: Priority Score: 225
Human Subjects: 10-NO HUMAN SUBJECTS INVOLVED
Animal Subjects: 30-ANMLS INV.-VERIFIED, NO SRG CONCERNS OR COMMENT

PROJECT YEAR	DIRECT COSTS REQUESTED	ESTIMATED TOTAL COST
01	290,909	446,940
02	289,746	445,154
03	293,015	450,176
04	304,736	468,184
05	316,926	486,912
TOTAL	1,495,332	2,297,366

ADMINISTRATIVE BUDGET NOTE: The budget shown is the requested budget and has not been adjusted to reflect any recommendations made by reviewers. If an award is planned, the costs will be calculated by Institute grants management staff based on the recommendations outlined below in the COMMITTEE BUDGET RECOMMENDATIONS section.

NOTE TO APPLICANT FOLLOWS SUMMARY STATEMENT.

RESUME AND SUMMARY OF DISCUSSION:

The major strengths of the application include the experience and productivity of the investigative team, a well-written application with a straightforward, well-defined approach and, for the most part, reasonable and attainable end-point determinations. The application is, however, highly ambitious as it appears to cover several aspects of apoptosis and proposes the generation of at least two new transgenic mice models. Certain parts of the application are descriptive, and the investigation on the mechanisms of creatine and monocycline may not produce the desired mechanistic information. Overall, this application is likely to produce insights that will improve our understanding of some of the pathogenic mechanisms of ALS, as well as evaluate the effectiveness of neuroprotective agents with potentially significant therapeutic value for the treatment of this disease.

DESCRIPTION (provided by applicant):

The functional role of the caspase cell death family in neurodegeneration, in particular ALS, has been clearly demonstrated. We have shown that caspases- 1 and -3 are regulated at the transcription level in the mutant SOD1 G93A transgenic ALS mouse model. Caspases- 1 and -3 are specifically activated in ventral horn neurons in this mouse model. Adding relevancy to this finding, caspase- 1 and -3 activation have been demonstrated in the spinal cord of humans with ALS. Caspase inhibition, either by the caspase-1-dominant negative transgene, or by administration of the broad caspase inhibitor zVAD-fmk, slows disease progression and delays mortality in mutant SOD1 G93A mice. The broad goal of this study is to expand our understanding of the molecular and cellular pathways mediating neuronal apoptosis. This knowledge should contribute to the rational development of improved therapeutics for ALS. With this goal in mind, we wish to evaluate the cell autonomous and non-cell autonomous signals modulating disease progression in ALS. The aims of this study include: 1) Evaluate the non-cell autonomous functional interaction between caspase- 1 and iNOS in ALS mice. A detrimental feedback loop appears to play a role between caspase-1-generated mature IL-1B and iNOS-generated NO. 2) Caspase-1 and caspase-3 are regulated at the expression and activation levels. The regulation of additional caspases will be evaluated. 3) Investigate the mechanisms of creatine-mediated neuroprotection. 4) Evaluate a potential therapeutic role for minocycline in ALS. 5) Since neuroprotection conferred by caspase inhibition and Bcl-2 overexpression occurs by acting at different stages of the cell death pathway, we hypothesize that the combination of caspase inhibition and Bcl-2 overexpression will provide greater neuroprotection than either alone. A proper knowledge of the caspase-mediated pathways will aid in designing rational pharmacotherapy. Since the mechanisms of cell death in these devastating diseases appear to be shared, furthering the understanding of the mechanisms of neurodegeneration in ALS will likely result in benefits to other neurodegenerative diseases, such as Huntington’s, Parkinson’s, and Alzheimer’s disease.

CRITIQUE 1:

SIGNIFICANCE: ALS is one of the neurodegenerative diseases that have a genetic anomaly (SOD1 mutations) that has been reasonably well-described. However, the molecular basis for the expression of phenotype for the familial, as well as the sporadic ALS, remains elusive. The neuroanatomical features of the disease share similarities with other neurodegenerative disorders and include premature motor neuron loss with features of apoptosis, protein aggregation and elements of oxidative stress. Several transgenic mice have been developed with hopes to elucidate the molecular mechanism(s) that lead to the development of a pathogenic phenotype and screening of potential therapies. This study proposes to extend previous observations on the role of caspases and nitric oxide in the execution of apoptosis in the ALS-transgenic mice. The application will also examine the potential protective effects of creatine and minocycline in ameliorating symptoms, or expression, of phenotype in ALS mice. The proposed studies are important and significant because they are investigating potential mechanisms of neuronal death, and potential interventions that hold the promise towards development of clinical treatments for the disease.

Approach: The majority of the application is designed to investigate the role of apoptosis in ALS with the primary focus on caspase 1, other caspases and the potential role of nitric oxide in the propagation of apoptosis in ALS mice. Three potential neuroprotective molecules, creatine, minocycline and inhibitors of apoptosis will be evaluated in the same model. The rationale for the proposed experiments is reasonable and, for the most part, is derived from the previous publications, and the expertise of the PI and the investigative team. Overall, the approach is sound and well-organized, but in parts diffuse and descriptive.

The first specific aim is a logical extension of the previous observations implicating caspase-1 as a critical component of cell death in models of neuronal injury. The major goal of this aim is to generate new transgenic mice by crossbreeding G93A mSOD1 mice with caspase-1 KO mice. A recognizable problem is the difference in genetic background, and it is advised that the effect of the genetic background be tested to be resolved before embarking onto the crossbreeding experiments. A number of interesting observations will be made in these mice, mostly down stream effects of caspase-1 activity. It is unclear as to how the PI and collaborators will distinguish the activity of iNOS versus nNOS, as the products of NOS are the same for both proteins, and most antibodies and inhibitors are not able to differentiate the different NOS isoforms.

The second specific aim proposes to generate another transgenic mouse. Mice lacking iNOS will be crossed with the mSOD1 ALS mice. The background is also a problem in this aim and should be considered up front, not after the breeding experiments.

The third aim investigates the expression of different caspases in the mSOD1 ALS mice. It is unclear why this effort is put forward, in view of the published data that caspase 1, 3 and 9 appear to be a part of the execution of neuronal death. Additional effort to measure caspase 6 and 8 appears to dilute the efforts of the PI, since considerable obstacles regarding antibodies and specific inhibitors are also apparent.

The fourth aim evaluates the protective mechanisms of creatine. The published and preliminary data indicate that creatine is capable of protecting both apoptotic and necrotic death. Whereas a hypothesis and a research plan are proposed to investigate the release of cytochrome-c, and activation of caspase 9 and 3, there is no plan to examine the protection against necrotic or other forms of neuronal death. Published data has suggested that cell death induced from metabolic alterations has features of apoptosis, but it may also represent other forms of cell death, such as delayed necrosis. Moreover, this aim, as well as the fifth aim, appears to be an entirely separate investigation that will require a significant amount of effort and time to accomplish. Testing the efficacy of creatine and minocycline to delay, and/or rescue mice with FALS SOD1 mutations, is straightforward and does not require much effort. However, the desire of the PI to investigate, in depth, the potential molecular mechanism for the protective effect(s) implies that substantial effort is required which will certainly dilute the efforts of the investigative team. Essentially, this proposal is two separate investigations, and despite the excellent publication record of the PI, may be physically impossible to complete in the period of a single application.

The fifth aim evaluates the protective effects of minocycline in the ALS-transgenic mice. From the published preliminary data and discussions, it is unlikely that a singular molecular mechanism will be responsible for neuroprotection. From this perspective, the gene array maybe informative in identifying clusters of genes which expression may be influenced by minocycline. However, the gene array approach is not coupled to protein expression and it will remain an interesting observation without significant impact on the mechanism of minocyclin neuroprotection.

The sixth aim is an extension of the previous work, which demonstrated that overexpression of Bcl-2 extends the life span of mSOD1 ALS mice. The proposed studies will advance the previous findings by investigating the effects of Bcl-2 on caspase expression and function. This aim relates to the first three aims, and it could have been productive to determine the expression of Bcl-2 in the two new proposed mice models (aims 1 and 2).

Innovation: The approach is state of the art, but not innovative. It builds on the hypotheses and transgenic mice developed previously. However, it fully utilizes the experience and tools developed in the past by the investigative team, and aims to provide mechanistic information.

Investigators: Dr. Friedlander recently has made significant contributions in Huntington’s disease and recently in ALS. He has published a number of excellent articles regarding the role of caspase-1 and apoptosis in these neurodegenerative disorders. He has also collaborated with a number of laboratories, and an impressive list of collaborators and consultants is provided in this application. Overall, he is highly-qualified to perform the proposed work in this application.

Environment: Resources and personnel available in the laboratories of Dr. Friedlander provide confidence for successful completion of the work.

CRITIQUE 2:

Significance: This proposal focuses on further understanding the mechanism and modulation of motor neuron degeneration in a mouse model of ALS. This is significant because the mechanisms for selective motor neuron degeneration in ALS are unknown. There are currently no effective treatments for ALS. This proposal addresses questions that are mechanistically and therapeutically directed.

Approach: A strength of this proposal is that an animal model of ALS will be used to understand mechanisms of motor neuron degeneration. This model is widely used in this field. Another strength is that outcome measures will be behavioral and neuropathological. Furthermore, some of the proposed experiments can be perceived as a logical extension of earlier work. This proposal addresses questions that are mechanistically and therapeutically directed.

Some major weaknesses in this application are manifested in the preliminary data, experimental design and methods, and the interpretations and expectations of the results. There are several concerns regarding the preliminary studies that have bearing on the proposed experiments.

An underlying theme in this proposal is that motor neuron degeneration in mSOD1 is apoptosis. It is still not certain whether apoptosis contributes to the neurodegeneration in the mSOD1 model of ALS. The applicant has ignored reports demonstrating a lack of apoptosis in mice with ALS (Migheli et al., 1999, Nature Med). However, the applicant's data indicate that caspase-1 and caspase-3 modulate the disease progression (Friedlander et al., 1997; Li et al., 2000). Bcl-2 overexpression also modulates the disease progression (Kostic et al., 1997). There is still no structural evidence for apoptosis in mSOD1 mice, although biochemical evidence is available (Vukosavic et al., 1999).

The applicants imply that motor neurons in mSOD1 mice undergo apoptosis and that caspase-1 has a role in this process through the production of IL1B. These conclusions cannot be made with certainty based on their data. Caspase inhibition with zVAD-fmk delays the onset of the disease, extends survival, and, as determined at 110 days, protects some motor neurons and decreases IL1B levels. The finding that inhibition of caspases only delays the disease might suggest that apoptosis does not have a definitive role in motor neurodegeneration in these mice, and that cytokine-mediated inflammation and necrosis is more critical. Alternatively, the mode of death is shifted with caspase inhibition from apoptosis to necrosis.

Elevated levels of IL1 may be secondary to the motor neuron degeneration. Measurements of IL1 should be made over time during the disease progression and correlated with motor neuron disease. Interestingly, caspase-1 and IL1 production may favor motor neuron survival in adult animals (de Bilbao et al., 2000 Neuroscience). Thus, an upregulation of caspase-1 in mSOD1 mice might be a compensatory survival mechanism.

It would be beneficial if their recent work (Li et al., 2000) were expanded and confirmed before other experiments are performed that are based on this earlier work. For instance, the conclusion that zVAD-fmk protects motor neurons is based on small numbers of animals and, perhaps, not particularly rigorous statistical analyses. The protection was seen only at cervical levels of spinal cord, but not at lumbar levels. Furthermore, a critical question is whether the neuroprotection is sustained. The neuropathological measurements were made only at 110 days. It is reasonable to assume that because the onset of disease was delayed, and survival is extended, that the neuropathology is delayed. The animals die from the disease, nevertheless, so the neuropathology is likely to be only delayed. Moreover, a structural analysis should be done on the neurons in the caspase inhibitor-treated animals.

The lack of protection of lumbar neurons has been explained, hypothetically, as a concentration-dependent effect. This idea should be tested.

The proposed crossbreeding experiments have not been done, so these experiments could potentially provide original information. However, the results of the crossbreeding experiments might not be straightforward, and it is difficult to be enthusiastic about these matting experiments without preliminary data. Apoptosis can be caspase-independent. In addition, studies have shown that if caspases are inhibited in cells stimulated to undergo apoptosis, they die anyway by necrosis. A central question in Aim 1 is that caspase-1 deletion will block motor neuron degeneration in mSOD1 mice. However, it is already known

that production of 1L-1 has no autonomous function in apoptosis.

Caspase-1-/- mice are also deficient in the processing of IL1, IL6, IL18, TNF, and IFN (in addition to IL1B). Thus, these cytokines must be considered, as well.

A photograph of activated caspase-1 in the spinal cord of a 90 day old mSOD1 is shown. It is hard imagine that this truly represents activated caspase-1. It seems too abundant. Furthermore, glial cells in white matter are labeled, in contrast to what is stated. The antibody used needs to be characterized better. Full-length blots with appropriate exposures might show that this antibody detects the pro-enzyme and possibly other proteins. Based on the western blots shown in their published work (Li et al., 2000), this antibody reacts with procaspase-1. The applicants should be more critical of their reagents.

In Specific Aim 2, the paracrine idea involving non-autonomous cell death mechanisms is interesting. However, crossbreeding experiments with iNOS-deficient mice might not be fruitful. iNOS up-regulation in mSOD1 mice might very well be a consequence of the disease, rather than a mechanism for motor neuron degeneration. In support of a possible role, iNOS activity and protein levels are induced at early symptomatic stages. But the animals are already symptomatic and are likely to show pathology, thus induction of iNOS is possibly a response to injury. The broad spectrum inhibitor of NOS, LNAME, has no effect in mSOD1 mice (Facchinetti et al., 1999). Therefore, it is very possible that a negative result will be the outcome of this experiment.

In Specific Aim 3, the time-course analysis of the levels of different caspases will be important. However, some questions need to be addressed to ensure the maximal amount of useful information. What parts of spinal cord will be analyzed (entire cord or ventral horns)? Localization studies need to be done. Biochemical measurements of activity need to be done.

The proposed experiments in aim 4 are interesting and creative. It would be nice to see some dose-response effects with different concentrations of creatine phosphate. It will also be important to demonstrate that the antibodies that will be used for the Apaf1 and caspase-9 immunoprecipitations are highly specific. Furthermore, some experiments should be done in the presence of specific kinase and phosphatase inhibitors, and caspase inhibitors.

Specific aim 5: The experiments to evaluate the effect of minocycline in mSOD1 mice might provide novel information. This drug crosses the blood brain barrier and can be given systemically. They have experience working with this drug in HD mice. However, there is still no direct evidence that minocycline prevents neurodegeneration. Minocycline has multiple actions, so the precise mechanisms of the actions of this drug will be difficult to know.

The gene array experiments proposed in Aim 5 appear to be an add-on. They are not well-developed with regard to possible mechanisms and results. Furthermore, it is not indicated how they will confirm the results. There is much concern about the interpretation of results based on spinal cord tissue extracts for the gene expression profiles. The applicants must consider the following possibility. Many different types of cells will be activating, or suppressing, cell death or survival signals and degenerative, regenerative, or reactive

signals. Degenerating, and regenerating, neuronal and glial cells (astroglia, oligodendroglia, and microglia), as well as non-CNS inflammatory cells derived from the vasculature (the applicants have shown leukocyte infiltration), will be represented in the extracts of RNA. The contributions of these differential cellular responses to the gene expression results, particularly data based on tissue homogenates, will have to be deciphered appropriately. Thus, gene expression assays could be suspect with regard to interpretation. It will be critical to determine whether the applicants are studying apoptosis of motor neurons, neuroglia, or inflammatory responses. In a similar vein, the applicants could be studying reactive or compensatory changes.

The in vitro experiments, designed to understand mechanisms of minocycline neuroprotection, are premature. There is no direct in vivo evidence that minocycline prevents cell death. It may protect against neurologic deficits through enhanced plasticity.

Specific Aim 6: This would be a worthwhile experiment, providing that the earlier work on caspase inhibition (Li et al, 2000) was more convincing.

Innovation: The proposed experiments are designed around a transgenic mouse model of ALS. The overall plan is to use this model to understand the modulation of motor neuron degeneration in these mice. The crossbreeding experiments proposed in Aims 1 and 2 are not innovative or creative. Modest effects (if any) are predicted.

Investigator: Dr. Friedlander (the PI) has strong credentials in working with transgenic mouse systems as models of human neurodegenerative disease. He can oversee the proposed experiments.

Environment: The resources appear to be appropriate for the proposed studies

HUMAN SUBJECTS/VERTEBRATE ANIMALS:

No concerns.

GENDER, MINORITY AND CHILDREN REPRESENTATION:

N/A.

BUDGET:

The experimental design does not justify 5 years of funding. The major experiments in this proposal are the crossbreeding experiments and the monocycline therapy experiment. This work can be done in 4 years. The costs for supplies should be cut by ~60% for each category listed under supplies. Direct costs could be cut to 225,000/year.

NOTICE: The NIH has modified its policy regarding the receipt of amended applications. Detailed information can be found by accessing the following URL address: http://grants.nih.gov/grants/policy/amendedapps.htm

NIH announced implementation of Modular Research Grants in the December 18, 1998 issue of the NIH Guide to Grants and Contracts. The main feature of this concept is that grant applications (R01, R03, R21, R15) will request direct costs in $25,000 modules, without budget detail for individual categories.

Further information can be obtained from the Modular Grants Web site at

http://grants.nih.gov/grants/funding/modular/modular.htm

MEETING ROSTER

ATIONAL INSTITUTE OF NEUROLOGICAL DISORDERS AND STROKE SPECIAL EMPHASIS PANEL
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ZNS1 SRB-R (01)
December 13, 2000 - December 14, 2000

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Consultants are required to absent themselves from the room during the review of any application if their presence would constitute or appear to constitute a conflict of interest.