Brain Pathology 8: 795-807(1998)

SYMPOSIUM: Transgenic Models of Neurodegeneration

The Lurcher Mutation and Ionotropic Glutamate Receptors: Contributions to Programmed Neuronal Death *in vivo*

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The recent positional cloning and physiological characterization of the lurcher mutation resulted in the identification of a novel stimulus that results in neurodegeneration. The catastrophic loss of cerebellar Purkinje cells in lurcher heterozygotes has now been strongly associated with a large constitutive inward current which ultimately activates a programmed form of neuronal death. The completely penetrant and focal nature of the lurcher phenotype gives us an opportunity to investigate the manner in which neurons respond to an aberrant signal in the context of the brain parenchyma. Although there is no human genetic disease that is equivalent to the lurcher mutation at this time, its triggering of programmed neuronal death enables us to pose and address questions that are relevant to a large number of human neurological diseases. The advantage of working in a genetically manipulable *in vivo* mammalian system is evident: we can address questions relating to gene function in the nervous system in a context that is physiological. Classical genetic analyses looking for molecules that suppress or modify the lurcher phenotype are under way and have now been supplemented with two novel techniques developed in our laboratory: biolistic transfection of cerebellar slices and Bacterial Artificial Chromosome modification. The integration of these novel and classical approaches will facilitate the testing of hypotheses, developed during the course of our study of the lurcher mutation, which explore the propagation of abnormal signals and the initiation of programmed neuronal death in neurons.

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Introduction

Neurodegeneration can be defined as the abnormal loss of neurons in a developing or mature animal. While neurodegeneration has been recognized as the cause of abnormal central nervous system (CNS) function in humans and experimental animals for many decades, the wide variety of physiological insults resulting in death of differentiated neurons obscured possible similarities in effector mechanisms among these myriad disorders. During the past several years, an appreciation that some aspects of the underlying pathogenic mechanisms operating in broad categories of these diseases may be similar has arisen from experimental results in three areas. First, genetic studies have led to the identification of a large number of mutations in familial neurodegenerative diseases, revealing at least three broad etiologic categories: the triplet repeat diseases, the ion channelopathies, and the protein aggregation disorders (27). Second, it has become apparent that the loss of cells from the CNS in response to these genetic insults can occur by apoptosis, an orderly program of death similar to the one occurring in all neuronal populations during development. Third, components of the response pathways that operate in neurons when homeostasis is altered are beginning to be identified. These developments have led to a working model of neuronal loss for at least some forms of neurodegenerative disease; the model postulates that an initial insult issues from the mutant molecule and is propagated throughout the affected neuron, that a “metabolic integrator” akin to those controlling the cell cycle in dividing cell populations senses this aberrant signal, and that effector pathways that are activated by the integrating device either attempt to restore homeostatic control or initiate the orderly removal of the damaged neurons from the nervous system. While this framework for experimentation has stimulated basic research, in no case has a complete understanding of the pathogenic mechanism underlying a specific neurodegenerative disorder been developed. In this review, we report progress toward this goal in the

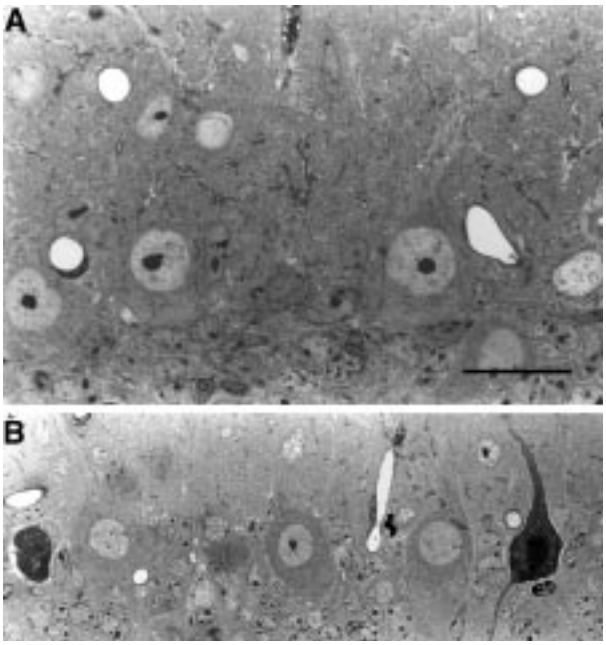

Figure 1. P12 cerebella from *Lc*/+ mice and their wild-type littermates, immunostained against calbindin, a Purkinje cell-specific marker. **A.** Parasagittal section of a wild-type cerebellum. In this field, the monolayer of Purkinje cell somata can be readily appreciated. At P12, the Purkinje cell dendritic arbor is already quite extensive, forming a delicate mesh of tertiary dendrites in the molecular layer. **B.** Parasagittal section of a *Lc*/+ cerebellum. The monolayer of Purkinje cell somata is disrupted as multiple Purkinje cells are missing. Some of the Purkinje cells appear to be relatively normal (arrowhead), but most present some sign of degeneration such as an irregularly condensed soma (arrow A) or axonal varicosities (arrow B)

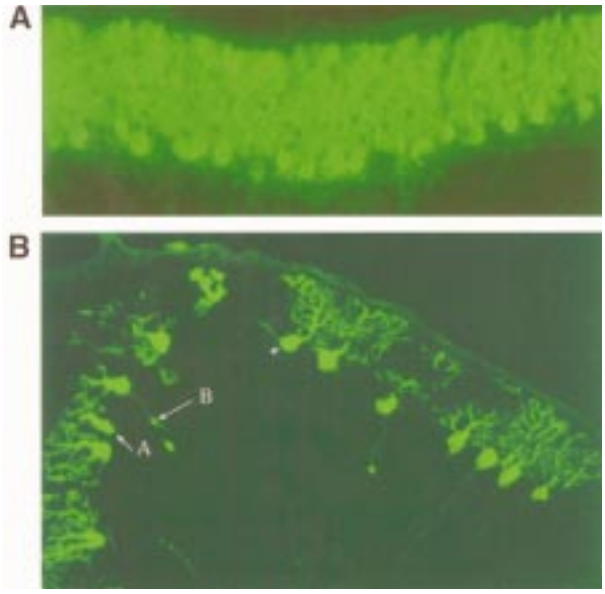

Figure 2. Methylene-blue-stained light micrographs of postnatal day 12 (P12) cerebella from *Lc*/+ mice and their wild-type littermates. **A.** In this section of wild type cerebellum, the typical morphology of normal Purkinje cells with lightly stained and round nuclei can be readily appreciated. **B.** In a section of *Lc*/+ cerebellum, some Purkinje cells still appear normal at P12, but two others display characteristics of apoptotic cells such as a condensed cytoplasm and nucleus as well as irregular cytoplasmic and nuclear membranes. Occasionally, a pyknotic cell is seen being engulfed by a cell (arrow) with glial characteristics such as a lightly staining and convoluted nucleus.
(A,B) Scale bar represents 20uM.

lurcher (*Lc*) mutant mouse and discuss the implications of our current knowledge of this disease for a general understanding of mammalian neurodegeneration.

The lurcher phenotype

Lc is a semidominant mouse mutation first described by Philips as an ataxic mouse strain with gross cerebellar abnormalities (49). Heterozygous *Lc* mice develop ataxia during the second postnatal week due to the loss of cerebellar Purkinje cells (Figure 1)(9). Homozygous lurcher animals are much more severely affected, dying at birth due to massive loss of neurons in the hindbrain and brainstem (10, 50). Analysis of mouse chimeras created by fusion of wild type and heterozygous *Lc* (*Lc*/+) embryos established that the *Lc* gene acts cell autonomously in cerebellar Purkinje cells *in vivo* (58). These studies also established that the death of other cerebellar cell types in *Lc*/+ animals is a consequence of Purkinje cell loss and thus not a direct effect of the *Lc* mutation (58, 59). *Lc*/+ Purkinje cells do not die when crossed onto a genetic background (staggerer / staggerer mice - *sg/sg*) that prevents their terminal differentiation, demonstrating a requirement for maturation of Purkinje cells prior to their degeneration as a consequence of lurcher gene action (41). The mode of cell death in *Lc*/+ Purkinje cells has been extensively characterized by two laboratories (45, 61). Both studies report features characteristic of apoptosis (Figure 2), although a definitive genetic demonstration that Purkinje cell death in these animals is programmed has not yet been reported.

While the precise phenotype of *Lc* mice does not correspond to any known human disease, several features of *Lc* animals are reminiscent of human neurodegenerative disorders: the phenotypic sensitivity to gene dosage, the focal death of specific neuronal populations followed by much more widespread neuronal loss, and the requirement of neuronal maturation for the onset of cell death have all been observed in human disease. Neither the cell autonomous nature of a mutation nor the mode

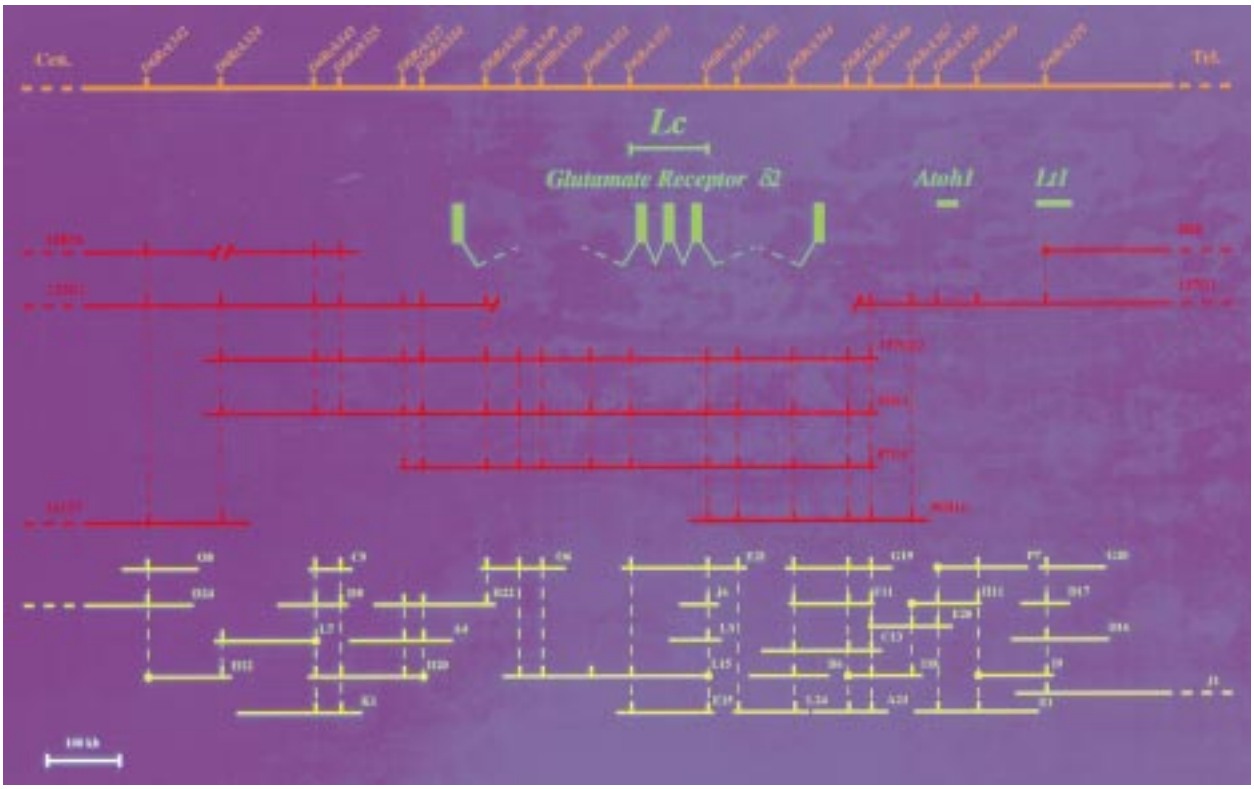

Figure 3. A transcript map of the *Lc* region. This map demonstrates the position of the critical *Lc* region and of the three transcripts that have been found within the YAC contig. Hybridization to a panel of YAC and BAC clones revealed that Lt1 colocalizes with marker *D6Rck329* and *Atoh1* with marker *D6Rck368.* Three exons of Grid2 were found to map within the minimal *Lc* region (exons A, B, and C); they are represented by three rectangles over the YAC contig. The two additional rectangles mark the approximate 5' and 3' ends of *Grid2* which were determined by using the entire *Grid2* cDNA as a probe to screen the complete panel of BAC clones.

of cell death can be definitively established in cases of human neurodegeneration, but suggestive evidence supporting each of these points has been obtained. Given these considerations and the obvious experimental advantages to studying a spontaneous neurologic mutant mouse strain, it seemed evident that careful studies of the pathogenic process in *Lc*/+ mice could contribute to our knowledge of human neurodegeneration.

Positional cloning of the *Lc* mutation

To begin a molecular dissection of the *Lc* phenotype, we first sought to identify the *Lc* mutation by traditional positional cloning. Thus, a 504-animal intersubspecific backcross using the original *Lc* mutation was generated by Norman and colleagues (44) and used to map the *Lc* mutation to a very small segment of mouse Chromosome 6. Further refinement of this map demonstrated that the critical genetic interval carrying the *Lc* mutation was a 110 kb chromosomal segment flanked by polymorphic marker *D6Rck353* on the centromeric side and polymorphic marker *D6Rck357* on the telomeric side (15). A search for genes within this critical 110 kb genomic DNA segment revealed the presence of three exons of the ionotropic glutamate receptor delta-2 (*Grid2*) gene within this critical interval. Furthermore, we demonstrated that the *Grid2* gene extends over approximately 800 kb of genomic DNA, completely encompassing the 110 kb D6Rck353–D6Rck357 segment (Figure 3). Analysis of *Grid2* exons from the critical genetic interval revealed a single missense mutation which results in the substitution of a threonine residue for an alanine residue in the third transmembrane domain of *Grid2* (Figure 4a and b) (67). This point mutation was present in both the original *Lc* allele and in a recently isolated allele (Lc^{i}) that arose on an inbred genetic background (16), providing conclusive evidence that the mutation is responsible for the lurcher phenotype. The position of the mutation in the third transmembrane span of *Grid2* is intriguing, since it highlights a nine amino acid domain that is highly conserved among all ionotropic glutamate receptors, including those present in *Caenorhabtidis elegans* and *Drosophila*

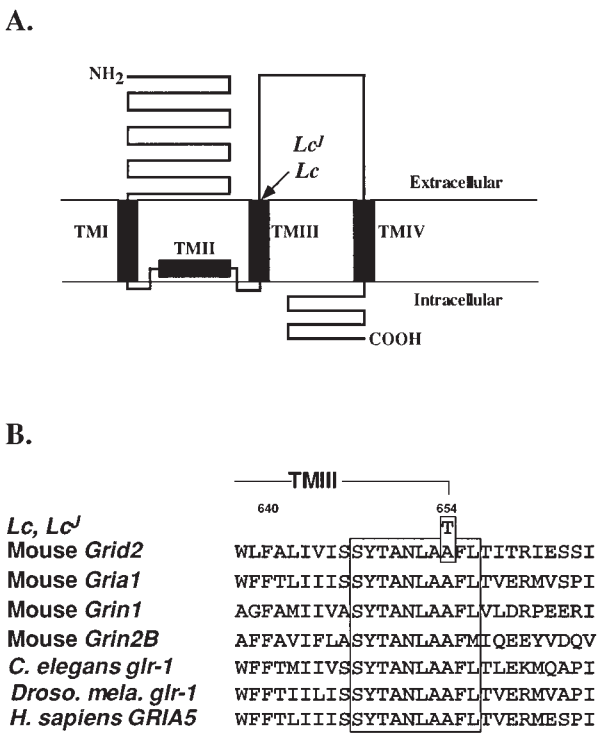

Figure 4. The *Lc* mutation occurs in an evolutionarily conserved domain of Grid2. **A.** A diagram of the topology of ionotropic glutamate receptors. The amino acid change caused by the *Lc* point mutation takes place in the third transmembrane domain (TMIII), near the extracellular surface. **B.** Conservation of the affected Ala residue in members of the ionotropic glutamate receptor family from different species. The *Lc* and *Lc*^{J} mutations result in an Ala-to-Thr substitution at a.a. position 654 (small box) of mouse *Grid2* in a highly conserved domain of nine amino acids (large box) in TMIII. Six representative members of the ionotropic glutamate receptor family are used to demonstrate the conservation of this domain through evolution: mouse *Gria1* (genbank accession no. X57497), mouse *Grin1* (D10028), mouse *Grin2B* (D10651), *C. elegans glr-1* (U34661); *D. melanogaster GluR-1* (M97192), and human *Gria5* (L19058). The significance of this extensive conservation throughout the evolution of ionotropic glutamate receptors is unknown.

melanogaster (Figure 4b). While the function of this domain in receptor activity has not been investigated, its evolutionary conservation and the dramatic phenotypic effect of the *Lc* missense mutation suggest that it plays an important role in this class of receptors.

Lc is a gain-of-function mutation

The identification of the *Lc* mutation as a missense mutation in the *Grid2* gene (the *Grid2*^{Lc} allele), taken together with the semidominant nature of the lurcher phenotype, immediately suggested that it might be a gain of function mutation. Definitive proof that this hypothesis is correct came from phenotypic comparison of *Grid2*^{Lc} with a null mutation (the *Grid2*$^{-/-}$ allele) in the *Grid2* gene generated by gene targeting (30). While both *Lc* alleles of *Grid2* result in ataxia and loss of motor learning, the *Grid2*$^{-/-}$ allele is recessive and does not result in either Purkinje cell degeneration in the cerebellum or perinatal death when carried in a homozygous state. These phenotypic differences are important for two reasons. First, when considering the mechanisms resulting in Purkinje cell death in lurcher animals, it is important to realize that the properties of the *Grid2*^{Lc} receptor may not accurately reflect the role of the wild type *Grid2* molecule *in vivo*. Second, the dose dependent effect of the *Grid2*^{Lc} allele is readily explained because functional ionotropic glutamate receptors are thought to be either tetramers or pentamers (6, 8, 33, 57, 60). Thus, if the presence of wild type subunits in the mixed channels of *Lc*/+ animals can mitigate the effects of the mutant subunits, then the phenotypic severity of this gain-of-function allele will be lessened in heterozygous animals.

Grid2 is an "orphan" ionotropic glutamate receptor

The delta family (*Grid1* and *Grid2*) of ionotropic glutamate receptors was isolated using low stringency hybridization and degenerate reverse transcriptase - PCR to identify additional members of the ionotropic glutamate receptor superfamily (2, 38, 63). Sequence similarity comparisons clearly indicates that these two genes are members of the ionotropic glutamate receptor family, since they share approximately 20-30% identity with both NMDA and AMPA/kainate receptors; in addition, the predicted membrane topology of the delta receptors is the same as that proposed for other family members (2, 38, 63). However, sequence comparisons also indicate that the *Grid1* and *Grid2* genes are much more highly related to one another than they are to either the NMDA or AMPA/kainate subfamilies (Figure 2), suggesting that they might comprise a separate functional subclass of this superfamily of receptors. This suggestion is supported by functional analysis of the delta family of receptors.

Ionotropic glutamate receptors are ligand-gated ion channels that can function either as homopentamers or heteropentamers. The different family members have been extensively investigated after expression in cultured cells or *Xenopus laevis* oocytes, revealing significant differences in their responses to agonist binding and in their physiological properties (29). In contrast to

other members of this gene family, neither *Grid1* nor *Grid2* have been shown to bind glutamate or to display ion channel activity alone or in combination with other members of the family (2, 38). Furthermore, immunoprecipitation studies of *Grid2* from extracts of cerebellum failed to reveal interacting proteins with the correct stochiometry to be considered as candidates for additional subunits of this receptor (40). The delta receptors, therefore, remain "orphan" receptors because there is no data that identifies them as being responsive to glutamate or other known agonists of ionotropic glutamate receptors. As a result, the ion channel activities of the wild type *Grid1* and *Grid2* receptors remain unknown.

In spite of the lack of functional data concerning the properties of *Grid2*, a great deal is known concerning its pattern of expression *in vivo*. *Grid2* is expressed at high levels in cerebellar Purkinje cells, and at lower levels in some brainstem neurons (2, 38). In general, the cells affected by the lurcher mutation correspond to those known to express the receptor, as expected from the cell autonomous action of the *Lc* mutation (58). Detailed *in situ* hybridization and immunocytochemical localization studies have indicated that *Grid2* is expressed as early as embryonic day 15 in cerebellar Purkinje cells, and that its level of expression in this cell type increases significantly after birth (55). One of the most interesting properties of *Grid2* is its specific subcellular localization; it is found only in the postsynaptic density of those Purkinje cell dendritic spines that make contact with the parallel fibers of granule cells in the molecular layer of the cerebellar cortex (Figure 3) (32, 54, 64). This contrasts with the localization of other ionotropic glutamate receptors in Purkinje cells, which can be found both at the parallel fiber synapse and at the climbing fiber synapse, the other major excitatory input to Purkinje cells (32, 65).

A large, constitutive inward conductance is observed in *Lc*/+ Purkinje cells

To investigate the physiological effects of the *Lc* mutation, and to gain insight into its mode of action, Purkinje cells in thin slices of cerebellar vermis from postnatal day 10 and 11 mutant and wild type animals were analyzed electrophysiologically (67). *Lc*/+ Purkinje cells exhibited a dramatic and severe physiological phenotype: when compared to wild type Purkinje cells, *Lc*/+ Purkinje cells required a holding current of larger magnitude to clamp the neuron at -70 mV. In addition, measurements of the initial currents and membrane conductances in the affected cells were much greater, and their resting potential was elevated

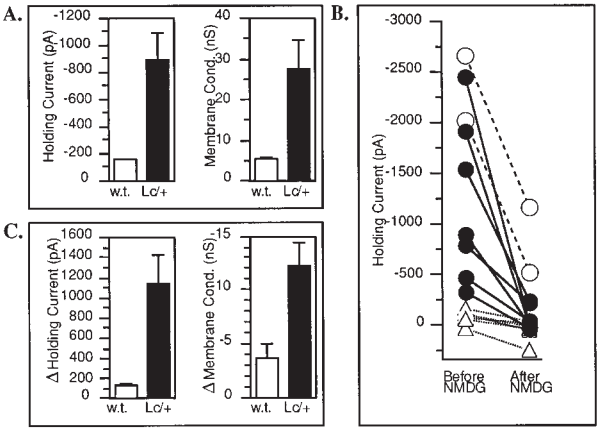

Figure 5. Physiological characterization of *Lc*/+ Purkinje cells: *Lc*/+ Purkinje cells express a large, constitutively active and selective resting conductance. **A.** Average values of the holding current and membrane conductance in Purkinje cells from wild-type (wt) and *Lc*/+ mice were determined on first entering whole-cell mode. **B.** Holding current before and after exchange of normal extracellular saline (containing 154 mM Na+) with NMDG-substituted saline (containing 27 mM Na^+ and 138 mM NMDG) are plotted for individual *Lc*/+ and wild-type recordings. Filled circles, "healthy" *Lc*/+ recordings (n=7). Open circles, "compromised" *Lc*/+ recordings (n=2). Open triangles, "healthy" wild-type recordings (n=6). **C.** Average changes in holding current and membrane conductance induced by NMDG substitution. The large magnitude of the changes is specific to *Lc*/+ Purkinje cells.
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(Figure 5). In addition, these abnormal currents could be largely eliminated by the substitution of N-methyl-D-glucamine (NMDG), a relatively large organic cation, for most of the Na^+ in the external saline, demonstrating that the *Lc*-specific conductance is selective and not the result of poor *Lc*/+ membrane integrity or leakage at the pipette/membrane interface (Figure 5). Furthermore, the large reduction in holding current magnitude and decrease in membrane conductance caused by reducing the external Na^+ concentration strongly suggested that Na^+ is a major current carrier of the *Lc*-specific, constitutive, inward current. All of these results were obtained in the presence of tetrodotoxin, a potent voltage-gated Na^+ channel blocker that should eliminate the release of glutamate evoked by any action potentials arising spontaneously within the slice preparation; this suggests that the constitutively active current in *Lc*/+ Purkinje cells does not depend on activation by ambient neurotransmitters.

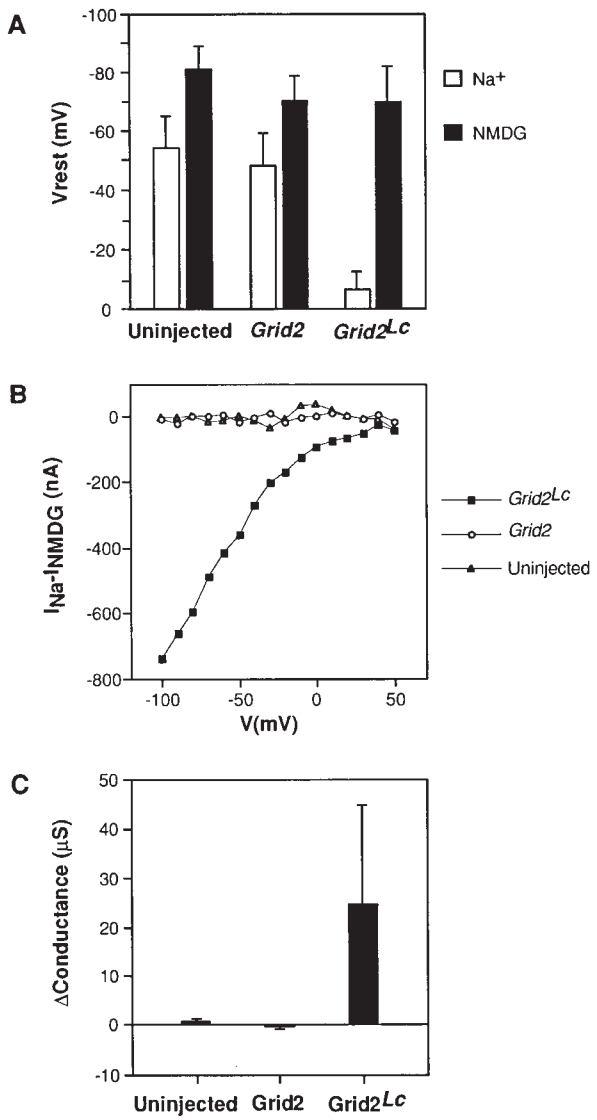

Figure 6. Physiological characterization of Grid2^{Lc} in oocytes. A large, constitutive conductance is observed in *X. laevis* oocytes injected with *Grid2*^{Lc}. Error bars in (**A**) and (**C**) represent s.e.m. of two electrode voltage measurements from 4 uninjected oocytes, 5 oocytes injected with wild-type *Grid2* cRNA, and 15 oocytes injected with *Grid2*^{Lc} cRNA. **A.** Average resting membrane potentials (Vrest) of uninjected oocytes and oocytes injected with *Grid2* and *Grid2*^{Lc} cRNAsin external Na^+ bath (open bars) and in external NMDG bath (filled bars). **B.** Current-voltage relationships of two representative oocytes before and after NMDG substitution. Open squares (Na^+) and triangles (NMDG) are from the oocyte injected with Grid2^{Lc} cRNA.**C.** Average changes in whole-cell conductance at -60 mV membrane potential before and after NMDG substitution.
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Grid2^{Lc} encodes a constitutively active homomeric channel

To examine the properties of the *Grid2*^{Lc} channel, the cRNAs coding for the wild type and mutant alleles of *Grid2* were assayed for electrophysiological activity in *Xenopus laevis* oocytes. Consistent with previous reports (2, 38), oocytes expressing wild type *Grid2* were not significantly different from their uninjected counterparts in resting potential observed either in the absence or presence of NMDG. In contrast, injection of *Grid2*^{Lc} cRNA, which was prepared by changing a single nucleotide from G to A at position 1960 in the full-length cDNA sequence to recreate the *Grid2*^{Lc} allele, produced a dramatic depolarization in the resting potential. This depolarization could be completely reversed by replacement of external Na^+ with NMDG (Figure 6) (67). Since these changes in resting potential and whole cell conductance were observed in the absence of any ligand, these measurements demonstrated that cells injected with the mutant *Grid2*^{Lc} expressed a large, constitutive conductance under physiological conditions.

The discovery that the expression of *Grid2*^{Lc} in Xenopus oocytes results in the formation of a large, constitutively active conductance is important for two reasons. First, and most important, the fact that the currents observed in *Lc*/+ Purkinje cells and *Grid2*^{Lc} expressing *Xenopus laevis* oocytes display the same basic properties strongly suggests that the major electrophysiological phenotype of *Lc*/+ Purkinje cells result from the direct action of the mutant allele. Second, the formation of a constitutively active channel by expression of the *Grid2Lc* allele in oocytes provides the first evidence that wild type *Grid2* subunits are competent to form homomeric channels. It seems unlikely that this single amino acid change could confer the ability to form a homomeric channel upon a wild type protein that normally requires a different subunit to assemble into an active channel. This result thus suggests that the failure to observe channel activity of wild type *Grid2* in injected oocytes or transfected mammalian cells may reflect the inability to properly activate these channels rather than an inherent lack of channel forming properties in the wild type protein. The inability to properly inactivate these channels could result from our ignorance concerning the relevant ligand, from a requirement for an additional subunit to participate in gating, or a combination of the latter two possibilities.

Neurodegeneration in *Lc* mice is similar to delayed cell death following ischemia

Since the original demonstration that brain lesions

can occur in animals exposed to glutamate (46), excitotoxic cell death due to prolonged exposure to this neurotransmitter has been thought to play a role in neurodegeneration *in vivo* (11, 51). Recent evidence implicating glutamate toxicity in delayed neuronal death following ischemia (12) and aberrant processing of astrocytic glutamate transporter mRNAs in sporadic cases of amyotrophic lateral sclerosis (ALS) (35) has supported this general hypothesis. The demonstration that neuronal death in lurcher mice results from the constitutive activation of an ionotropic glutamate receptor provided the first genetic proof that this type of pathway can be the primary cause of neurodegeneration *in vivo* (67). Obviously, this raises important issues concerning the mechanisms of neuronal cell death in these disorders, and suggests that *Lc* mice can provide an important experimental system in which to address them.

The prevailing model for glutamate toxicity has been developed through *in vitro* studies using glutamate to elicit excitotoxic cell death in cultured neurons; in this system, it has been shown that Ca^{2+} influx into cells through the NMDA and/or AMPA receptors is a critical step in initiating neuronal death and that the mechanism of death is necrosis (12, 48, 53). *In vivo*, the situation appears to be more complex. Although our knowledge of the precise mechanisms involved in widespread neuronal death following ischemia is still quite primitive, a synthesis of the available data suggests that the immediate necrotic death of neurons at the site of an ischemic lesion may be very closely related to excitotoxic cell death in culture (12). On the other hand, the delayed neuronal death that is found in hypoperfused areas near the site of ischemia appears to occur through the activation of ionotropic glutamate receptors and of an apoptotic pathway (12, 19, 36, 56). This is consistent with genetic results demonstrating a role for the apoptotic machinery in ischemic cell death *in vivo* (13, 39, 47). Given the mechanistic distinctions between these two forms of cell death, it is of obvious importance to study the cell death pathway in any case of neurodegeneration where activation of glutamate receptors is thought to play an important role.

Studies of Purkinje cell loss in *Lc*/+ animals has provided strong evidence that these cells are dying *in vivo* through an apoptotic mechanism. Thus, at both the light and electron microscopic levels, the morphology of dying *Lc*/+ Purkinje cells is typical of neurons undergoing an apoptotic death (Figures 1 and 2). Furthermore, several genes that are known to be expressed during the programmed death of neurons are expressed in *Lc*/+ Purkinje cells prior to their death *in vivo*, and the nuclei of *Lc*/+ Purkinje cells contain nicked DNA ends during the death process (45). These results are certainly consistent with the activation of an apoptotic process in response to the *Grid2*^{Lc} stimulus, suggesting that neurodegeneration in this case is very closely related to the delayed death of neurons following ischemia, and that it does not resemble the necrotic process observed in studies of cultured neurons exposed to excess glutamate.

The specificity and timing of Purkinje cell death in *Lc*/+ animals

The discovery of the *Grid2*^{Lc} allele provided an opportunity to understand the lurcher phenotype in the context of the very detailed knowledge of the expression and location of the *Grid2* protein *in vivo*. The fact that those cell populations first dying in the cerebellum of *Lc*/+ animals, or in the brainstem of *Lc*/*Lc* embryos, are those known to express the *Grid2* gene (2, 38) might have been predicted from the genetic demonstration that the lurcher phenotype is cell autonomous (58). However, the timing of cell death in lurcher animals is not so readily explained. The *Grid2* gene is first expressed at embryonic day 15 in cerebellar Purkinje cells, and the protein accumulates to quite high levels in individual Purkinje cells by birth (55). Yet, there is no evidence of apoptosis in this cell population until approximately postnatal day 8, when degenerating Purkinje cells are first observed. Neuronal loss occurs quickly thereafter, and 95% of Purkinje cells are dead by postnatal day 25 (9). On the other hand, in (*Lc*, *sg* / +, *sg*) double mutants, Purkinje cells do not die postnatally (41). While neither the requirement for Purkinje cell maturation nor the timing of Purkinje cell death in *Lc*/+ mice are yet understood, a possible explanation for these results has been provided by the demonstration that the *Grid2* protein is redistributed within Purkinje cells from a homogeneous distribution throughout the dendritic arbor to the postsynaptic density of the parallel fiber synapse at approximately the same time that Purkinje cell death commences (9, 54). These results suggest that localization of the *Grid2*^{Lc} channels to the synapse might be important for the activation of apoptotic death in *Lc*/+ Purkinje cells and raise the general issue of the importance of subcellular localization to the generation of aberrant signals that initiate neurodegeneration *in vivo*.

Why do neurons die in response to constitutive activation of *Grid2*^{Lc}?

In trying to formulate a working hypothesis for the mechanisms involved in Purkinje cell death in *Lc*/+ mice, we have considered two very different models of

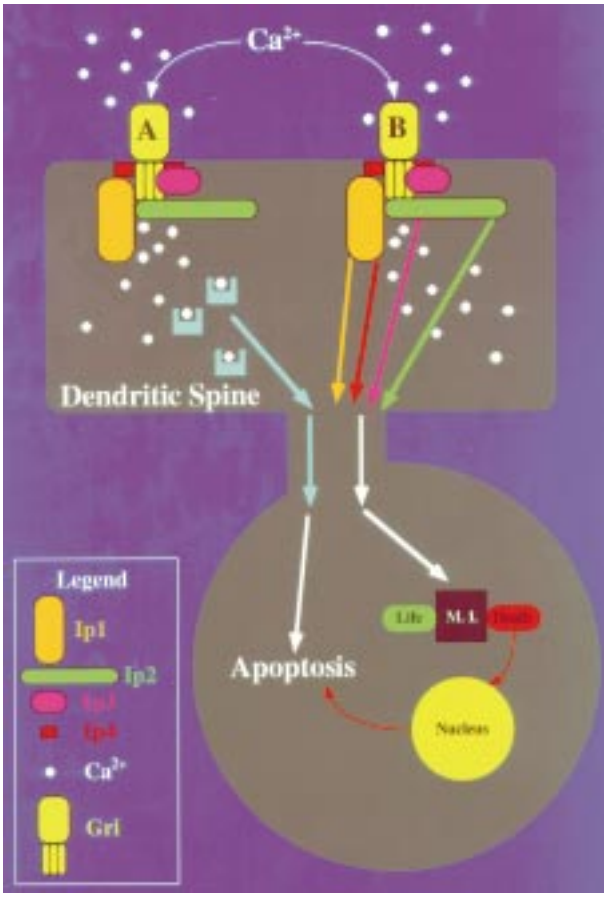

Figure 7. Two models of the initiation of apoptosis by ionotropic glutamate receptors. We propose two models to explain the manner in which an apoptotic process is initiated in the context of delayed cell death following an ischemic insult. In model A (presented to the left), the abnormal Ca^{2+} influx plays a major role by interacting with and activating signaling molecules that ultimately initiate the neuronal death program. In model B, Ca^{2+} may play a role in activating the various signaling modalities of a particular ionotropic glutamate receptor complex; it is the persistent signaling from this receptor complex that is ultimately interpreted by the metabolic integrator. The integrator then makes a decision whether to initiate apoptosis. {Gri - Glutamate receptor, ionotropic; Ip - Gri Interacting protein}{N.B. The number of Ip proteins is completely arbitrary; none of the Gri complexes have been completely characterized at this time.}

neurodegeneration. The first is based on the demonstrated involvement of Ca^{2+} in excitotoxic cell death in cultured neurons. *In vitro* results suggest that excitotoxic cell death in culture, even when induced slowly by low concentrations of the excitotoxic agent, occurs through necrosis (12, 53). These data suggest that the excitotoxic pathway may be quite distinct from the delayed apoptotic death of neurons in ischemia or death of *Lc*/+ Purkinje cells. However, it seems quite possible that necrosis and apoptosis simply represent different cellular responses to the same primary metabolic event. In this case, it is not the nature of the signal emanating from the constitutively activated receptor that dictates the choice of pathway, but the quantitative impact it has upon the cell. Were this to be the case, the primary signal in both forms of cell death would be elevated intracellular Ca^{2+} levels (Figure 7). While a mechanism by which elevated intracellular Ca^{2+} levels would activate apoptosis has not been established, the robust signaling between the synapse and cell nucleus through CREB activation (14) provides a precedent for the type of mechanism that may be relevant in this form of neurodegeneration. A key prediction of this model is that it is the ion flux through the activated channel per se that is the initiating event in neurodegeneration.

The second model we are considering derives from our previous attempts to organize facts concerning the general properties of neurons and the characteristics of neurodegeneration into a framework that is analogous to the one developed for the action of oncogenes in cell transformation (25, 26, 27). Incorporated into this idea are three key elements: the involvement of neurodegenerative disease genes in the activation of inappropriate signal transduction events, the integration of these aberrant metabolic signals by intracellular mechanisms akin to cell cycle checkpoints, and the activation of programmed cell death in response to these signals as the sole effector pathway downstream from these metabolic integrators (Figure 7). The central tenet of this hypothesis is that all cells contain a mechanism for integrating signal transduction events with internal metabolic information and that programmed cell death is the dominant effector pathway that is activated by this mechanism in postmitotic neurons in response to aberrant stimuli. Strong evidence that activation of programmed neuronal death plays a role in both delayed cell loss following ischemia (12) and in Purkinje cell death in *Lc*/+ mice (45) has been obtained. Furthermore, the existence of cell death "checkpoints" as natural regulatory mechanisms for the initiation of apoptotic death is now established. The critical issue, therefore, is the nature of the stimulus that results in activation of the cell death pathway in response to ionotropic glutamate receptor activation. According to this hypothesis, one might predict that the signal transducing capability of ionotropic glutamate receptors, as well as their role as ligand gated ion channels, may be crucial for activation of the cell death pathway. It is becoming increasingly apparent that large complexes of proteins involved in various modalities of signaling –such as α-actinin (62), calmodulin (20, 62),

Glutamate Interacting Protein (GRIP) (17), PSD-93 (7), PSD-95 (31, 43), and SAP102 (42)- are assembled onto the C-terminal cytoplasmic domain of ionotropic glutamate receptors, and evidence is now accumulating that these complexes are critical for the *in vivo* functions of these molecules (52). Furthermore, the neuronal isoform of nitric oxide synthase, which interacts with the C-terminal domain of the NR1 receptor, has been implicated in the initiation of programmed cell death following activation of NR1-containing ionotropic glutamate receptors (5, 34). While the coincident ion flux through the channel may be important in signal generation, its proposed role would be to act through the C-terminal signaling components associated with the receptor. Thus, a critical role for the C-terminus that is independent from its possible involvement in gating the channel is proposed.

To distinguish between these possibilities, or to determine their relative contribution in a specific neurodegenerative disorder, will require an understanding of the precise signals emanating from the mutant receptor, their integration by the cell, and the molecular mechanism responsible for activation of the programmed death pathway. While *in vitro* systems will be very useful in illustrating the menu of possible pathways that should be considered in trying to understand neurodegeneration, a clear definition of the molecular mechanisms participating *in vivo* will require genetic analysis of these events in the context of the intact CNS. It is toward this end that we have directed our research into Purkinje cell death in lurcher mutant mice.

New methods for the analysis of neurodegeneration in vertebrates

The most powerful studies of programmed cell death have been conducted by Horvitz and colleagues in the nematode *Caenorhabtidis elegans* (18, 28). These studies have been conducted using a classical genetic approach, and have resulted in the definition of both a conceptual framework for understanding normal developmental cell death, and the identification of molecules that are fundamental to programmed cell death in all organisms. While this sort of approach has been attempted in both zebra fish (1, 22) and mice (23, 24), in neither case has a complex pathway emerged using a strict genetic approach. In spite of this, the very powerful array of biochemical and molecular techniques that have been developed over the past decade and the robust application of human genetics to this problem, ensure that a large number of candidate molecules relevant to the process of neurodegeneration will be identified. To begin to examine the functions of molecules identified using these methodologies, we have developed two techniques that are of general utility.

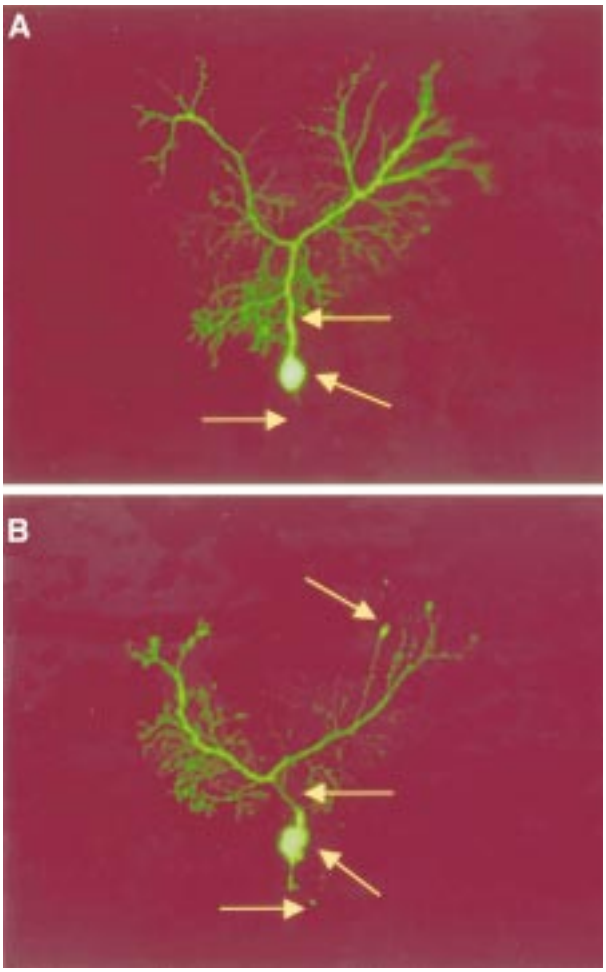

Figure 8. Biolistic transfection of Purkinje cells in slices of cerebellum. **A.** P10 Purkinje cell transfected with *Grid2* and eGFP and cultured for 48 hours. The extensive dendritic arbor of this Purkinje cell is outlined by eGFP. Of note are the rounded soma, thick stem dendrite, and smooth axon that are characteristic of Purkinje cells. **B.** P10 Purkinje cell transfected with *Grid2*^{Lc} and eGFP and cultured for 48 hours. This neuron, its morphology outlined by the expression of eGFP, is undergoing apoptotic degeneration. Its soma is elongated and irregularly condensed and gives rise to a stunted dendritic arbor which contains abnormal varicosities in its distal branches. In addition, there is a dramatic decrease in the caliber of the stem. Finally, the axon displays several varicosities or "torpedoes" that are found in degenerating neurons.

Cells transfected with *Grid2*^{Lc} thus recapitulate events that occur in *Lc*/+ Purkinjecell death in vivo, providing an in vitro system within which to test molecules which may block apoptosis.

The first method we have developed is directed toward rapid analysis of protein function in slice preparations from mammalian brain. This is based on the use

of biolistic transfection to introduce foreign DNA into cells in slices of the developing or mature brain (3, 37). The critical feature of this methodology that can be exploited for analysis of gene function is the fact that cotransfection of multiple DNAs using the biolistic methodology is nearly 100% efficient, presumably due to the fact that the DNA is actually physically introduced into the nucleus (3, 4). This property of biolistic methodology is critical for functional analysis, since it is possible to both mark the transfected cell of interest and assay gene function within that cell. Given this fact, one can design properly controlled experiments in which the inherent variability in both the efficiency of transfection by the biolistic method and the health of the individual slice preparations can be eliminated from consideration. An illustration of this type of experiment is presented in Figure 8. The ability to design this type of experiment can allow us to rapidly dissect proteins or domains involved in the lurcher pathway so that only those experiments that precisely test an hypothesis are carried forward into transgenic mice.

The second method we have developed is designed to correct the very frequent problem of position effects when trying to analyze gene function in transgenic mice. In the majority of instances, gain of function analysis in mice has been extremely difficult due to the inability to achieve proper expression with conventional transgenic constructs, particularly if the dosage of the gene product is important for understanding its action. In the case of neurodegeneration, gene dosage *in vivo* often has a dramatic effect on the resulting phenotype. Thus, to pursue a genetic strategy using transgenic mice, it is critical that reproducible temporal and spatial patterns of transcription, and copy number dependent expression can be obtained. This is particularly important for analysis of lurcher mutant mice, since the phenotype results from a gain of function mutation that should produce the lurcher phenotypes even on a wild type background if the correct levels of expression are achieved. To overcome these difficulties, we have developed an efficient method for modification of Bacterial Artificial Chromosomes (BACs) that allows the use of genomic fragments between 100 and 500 kb for transgenic analysis (64). Using this methodology, we have been able to construct modified BACs for marker insertion, structure-function analysis, and overexpression studies; in each case, the gene of interest is expressed accurately when the modified BAC is reintroduced into the mouse genome (64). The use of this methodology can now allow gain of function genetic analysis that reflects the *in vivo* role of the wild type or mutant protein. Thus, BAC constructs expressing the *Grid2*^{Lc} allele or any desired derivative of this gene can be assayed *in vivo* in a reliable manner. In this way, one can test models for lurcher gene action at the correct gene dosage *in vivo*. Taken together with the biolistic methodology described above, these two tools significantly enhance the arsenal with which we can attack the problem of neurodegeneration *in vivo*.

Future Directions

The *Lc* mutation is thus emerging as an *in vivo* system with which to study the response of neurons to an aberrant stimulus. The advantage of *Lc* is that the initial physiological insult is well characterized both at the genetic and at the physiological level. We can now begin to dissect the manner in which an abnormal stimulus is propagated through the dendritic and somatic cytoplasm and ultimately forces the "metabolic integrator" to initiate a program of cell death (Figure 7). This analysis must begin with the characterization of the *Grid2* signaling complex but can take advantage of classical genetic studies as well. We can readily cross the *Lc* mutation onto a variety of other backgrounds containing either spontaneous or targeted mutations whose phenotype appears relevant to our analysis. Any insight gained from the latter genetic studies or the biochemical approach at the level of the receptor can be quickly tested in our *in vitro* system using biolistic transformation of cerebellar slices. Finally, *in vitro* results can be challenged with our *in vivo* system by introducing subtle mutations into genes of interest and expressing them in a controlled manner using BAC modification and transgenic technology. This integrated system of analysis should prove fruitful because any hypothesis can be quickly and convincingly tested in several complimentary systems.

Acknowledgements

We would like to thank Drs. J. Zuo for his contributions to the cloning and characterization of the *Lc* mutation; Drs. L. Feng, D. J. Norman, and W. Jiang for their contribution to the cloning and histopathological analysis of the *Lc* mutation; as well as K. Takahashi and Dr. D. Linden for their physiological characterization of *Lc* Purkinje cells in slices of cerebellum. P.L.D. is supported by the National Institutes of Health / National Institute of General Medical Sciences (NIH/NIGMS) grant GMO7739(P.L.D.), and N.H. is supported by the Howard Hughes Medical Institute.

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