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Using temporal genetic switches to synchronize the unfolded protein response in cell populations in vivo

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Abstract

In recent years, recognition of the importance of protein aggregation in human diseases has increasingly come to the fore and it is clear that many degenerative disorders involve activation of a metabolic signaling cascade known as the unfolded protein response (UPR). The UPR encompasses conserved mechanisms in cells to monitor and react to changes in metabolic flux through the secretory pathway. Such changes reflect an imbalance in cell homeostasis and the UPR integrates several signaling cascades to restore homeostasis. As such, the UPR is simply interpreted as a protection mechanism for cells as they perform their normal functions. A number of groups have suggested that the UPR also can eliminate cells in which homeostasis is lost, for example during disease. This notion has kindled the rather paradoxical concept that inhibiting the UPR will ameliorate degenerative disease. However, several in vivo studies in the nervous system indicate that curtailing UPR function either exacerbates disease or may reduce severity through unexpected or unidentified pathways. Perhaps the notion that the UPR protects cells or eliminates them stems from widespread use of suboptimal paradigms to characterize the UPR; thus, too little is currently known about this homeostatic pathway. Herein, I describe the development of genetic switch technology (GST) to generate a novel model for studying UPR diseases. The model is geared toward obtaining high resolution in vivo detail for oligodendrocytes of the central nervous system, but it can be adapted to study other cell types and other UPR diseases.

Keywords

Pelizaeus-Merzbacher disease; spastic paraplegia; leukodystrophy; UPR disease; misfolding; protein aggregation; myelin; neurodegeneration; CHOP; GAD153; Ddit3

1. INTRODUCTION

The unfolded protein response (UPR) is a near-ubiquitous metabolic stress signaling cascade emanating in higher animals from the endoplasmic reticulum (ER) to the nucleus and the cytoplasm that has been studied extensively over the last two decades in a multitude of experimental paradigms and cell types in vitro and in vivo. A common dictum that has emerged from the plethora of reviews that summarize the essential features of this work is that the UPR protects cells from the toxic effects of unfolded/misfolded proteins in the secretory pathway but, perhaps paradoxically, triggers apoptosis. Several models have been proposed to define circumstances under which positive or negative outcomes (i.e. cell survival versus cell death) arise during metabolic stress so as to account for this apparent

dichotomy in UPR function (Gow and Wrabetz, 2009; Lin et al., 2009; Rutkowski and Kaufman, 2007).

Whichever the model, the dichotomy has galvanized perceptions of the UPR and popularized the search for components that switch the cascade between protective and destructive outputs, particularly for signaling that involves the PRKR-like endoplasmic reticulum kinase (PERK/eIF2 α). This PERK pathway is comprised of a negative-feedback, temporal delay circuit (Fig. 1) that inhibits ribosome assembly through a phosphorylation-dephosphorylation cycle of the alpha subunit of eukaryotic initiation factor 2 (eIF2 α) and promotes expression of several transcription factors and a regulatory subunit of protein phosphatase 1 (reviewed in Wek and Cavener, 2007).

Herein, I briefly comment on some of the key variable findings reported for the function of the PERK pathway, which have revolved around the role played by the transcription factor, C/EBP-homologous protein (CHOP/GADD153/Ddit3). CHOP was the first protein reported to trigger the switch from cell survival to cell death in the PERK pathway (Zinszner et al., 1998). However, subsequent studies do not show a strong relationship between CHOP expression and cell death, at least in the nervous system (Lin et al., 2005; Pennuto et al., 2008; Southwood et al., 2002). The ambiguities in CHOP function that are revealed by these studies highlight the need for new ideas and new tools to characterize the function of the PERK pathway. Accordingly, I describe a novel set of genetic tools that may help to achieve this goal and increase the effectiveness with which in vivo studies can be used to examine the pathophysiology of UPR diseases. To illustrate the application of these tools, I focus on animal models for a well-characterized neurodegenerative disease, Pelizaeus-Merzbacher disease (PMD), that is caused by mutations in the *PROTEOLIPID PROTEIN 1 (PLP1)* gene and serves as a model UPR disease (Southwood et al., 2002). Importantly, the genetic tools detailed below are generalizable to other UPR diseases and may benefit the investigation of these diseases in animal models in vivo.

2. VARIABLE FUNCTIONS OF CHOP IN THE PERK SIGNALING PATHWAY

In early studies, Ron and colleagues (Zinszner et al., 1998) proposed CHOP as a proapoptotic transcription factor and reported that ablation of the *Chop* gene abolishes UPR-induced cell death in kidney. More recently, ablation of CHOP expression in *Myelin P₀* (*Mpz*) gene mutant mice was shown to attenuate demyelination in the peripheral nervous system, although not as a consequence of reducing Schwann cell apoptosis but rather through an unknown mechanism (Pennuto et al., 2008).

In contrast, studies from my group have indicated that CHOP is a pro-survival protein in the central nervous system (CNS). We have demonstrated that ablation of the *Chop* gene in *Plp1* mutant mice significantly increases apoptosis in oligodendrocytes undergoing a UPR (Southwood et al., 2002). Importantly, these data are consistent with findings from other studies that have used gene ablation to inactivate components at several different levels of the PERK pathway (Lin et al., 2007; Lin et al., 2005; Lin et al., 2008). Two examples illustrate this point.

The first example involves data from *Perk*-null heterozygous and homozygous mice. If CHOP is a proapoptotic protein, then ablating the *Perk* gene should suppress *Chop* gene expression and reduce cell death under UPR conditions; however, the reverse appears to be the case. Ablating *Perk* in embryonic stem cells increases their susceptibility to UPR-induced cell death in vitro (Harding et al., 2000). Inactivating *Perk* in mice causes pancreatic β -cell death and diabetes, presumably because the PERK pathway (and CHOP expression) is critical for normal development and the regulation of metabolic activity in the secretory pathway (Harding et al., 2001). Furthermore, immune-mediated demyelination and

oligodendrocyte apoptosis are increased during allergic experimental encephalomyelitis in mice that are hypomorphic for the *Perk* gene (Lin et al., 2007) most likely because the PERK pathway serves to protect oligodendrocytes.

In the second example, *Gadd34*-null homozygotes have been examined. If CHOP represents a proapoptotic signal, then inactivation of the *Gadd34* gene to suppress the dephosphorylation of phospho-eIF2 α should sustain UPR signaling, prolong CHOP expression and exacerbate cell death. Again, the reverse is the case. Oligodendrocyte apoptosis is reduced in response to IFN γ -mediated induction of the UPR in *Gadd34*-null mice (Lin et al., 2008). Together, these examples indicate that inactivation of the *Perk*, *Chop* or *Gadd34* genes in oligodendrocytes during a UPR yields the consistent result that PERK pathway signaling, which invariably involves CHOP expression and its nuclear localization, promotes oligodendrocyte survival and diminishes the negative impact of UPR disease.

The inconsistencies that these and other studies reveal for the function of PERK signaling, and the role played by CHOP, necessitate a re-evaluation and an expansion of experimental methods used to define this pathway. Because of the difficulty of teasing out mechanistic details in vivo, most studies have been performed in vitro. However, such experiments appear prone to cell culture artifacts (Sharma and Gow, 2007; Sharma et al., 2007); thus, the development of novel paradigms should be focused on tools to characterize the UPR at high resolution in vivo.

3. MUTATIONS IN THE *PLP1* GENE - A MODEL UPR DISEASE

Pelizaeus-Merzbacher disease is a rare, allelic X-linked recessive leukodystrophy caused by deletion, duplication or mutation of the *PROTEOLIPID PROTEIN 1 (PLP1)* gene (Gencic et al., 1989; Hudson et al., 1989; Raskind et al., 1991). By and large, males are affected (but see Hurst et al., 2006; Nance et al., 1993) with pleiotropic clinical symptoms manifested in the first year of life and perinatally in the most severely affected individuals (reviewed in Garbern, 2007; Inoue, 2005). The primary defect originates in oligodendrocytes, which express the *PLP1* gene and synthesize myelin principally within the white matter of the CNS.

Proteins encoded by the *PLP1* gene (PLP1 and its smaller splice-isoform DM20) are the major polytopic transmembrane proteins targeted to CNS myelin sheaths through the secretory pathway (Fig. 2a). These sheaths are large lipid-rich membrane sheets that are synthesized as lamellipodial expansions of oligodendrocyte processes and spirally-wrap and electrically-insulate axons to enable saltatory conduction (recently reviewed by Baron and Hoekstra, 2010). During myelinogenesis, as much as 10% of the mRNA in oligodendrocytes encodes *PLP1* gene products, rendering it one of the most highly expressed genes known (Milner et al., 1985). Accordingly, genetic lesions that significantly increase expression level or alter splicing of the primary transcript, or mutations in the coding region that destabilize the three-dimensional structure of the proteins, might be expected to perturb the secretory pathway and induce metabolic stress leading to activation of the UPR.

To date, missense and nonsense mutations in *PLP1* are the only genetic lesions in PMD that have been found to induce the UPR (Gow, 2004; Gow et al., 1994; Gow et al., 1998; Southwood et al., 2002). Thus, mutant PLP1, and in some cases DM20 (Gow and Lazzarini, 1996), accumulate in the ER causing metabolic stress and activation of the UPR (Fig. 2b). Resolution of the stress, by degrading the accumulated nascent protein chains negatively regulates the UPR and allows resumption of global protein synthesis. However, resumption of the expression of mutant *Plp1* gene products again causes accumulation in the secretory pathway and triggers another cycle of metabolic stress. Such iterative cycles of UPR activation increase the risk of apoptosis for individual cells (reviewed in Gow and Wrabetz,

2009). Overexpression of PLP1, which accounts for approximately 50% of *PLP1* mutations, is caused by duplication or triplication of the gene. The pathophysiology appears to involve cholesterol accumulation in lysosomes or altered lipid metabolism (Karim et al., 2010; Simons et al., 2002). Alternatively, the absence of *PLP1* gene expression leads to a subtle late-onset phenotype characterized by length-dependent axonal degeneration (Garbern et al., 2002; Griffiths et al., 1998; Rosenbluth et al., 2006).

Excellent naturally-occurring rodent models of PMD have been available for decades (Billings-Gagliardi et al., 1995; Dautigny et al., 1986; Gencic and Hudson, 1990; Nadon and Duncan, 1995; Schneider et al., 1992) and have been characterized in great detail (reviewed in Werner et al., 1998). Moreover, the specific missense mutations found in two of the mouse mutants, *msd* (A243V) and *rsh* (I187T), are also found in patients (Kobayashi et al., 1994; Yamamoto et al., 1998) and confer analogous symptoms in terms of relative disease severity, degree of pathology, extent of oligodendrocyte apoptosis and relative decreases in life span.

4. PROBLEMS WITH CURRENT PARADIGMS USED TO CHARACTERIZE THE UPR

In diseases for which the UPR is thought to be the primary trigger of pathology, the target cells are often post-mitotic, differentiated and fully functional prior to the metabolic stressor. In contrast, the majority of experiments focused on defining UPR pathways and the functions of its signaling cascades are performed *in vitro* with undifferentiated cells, such as transformed cell lines or mouse embryonic fibroblasts that are perpetually proliferative or merely contact inhibited at high density. Accordingly, it may be of little surprise that components of the UPR found to be critical for function *in vitro*, turn out to be irrelevant *in vivo* (e.g. Di Sano et al., 2006; Lamkanfi et al., 2004; Obeng and Boise, 2005; Sharma and Gow, 2007; Sharma et al., 2007). Perhaps this disconnect between *in vitro* and *in vivo* studies arises from the paradigms used, as well as nuances in UPR-activated pathways in different cell types and/or under conditions of proliferation versus differentiation.

Prime advantages of using proliferative cells in culture include their cellular homogeneity and the ease with which they can be expanded, treated and analyzed to generate timecourse data of UPR-related changes. Thus, tunicamycin or a comparable UPR-inducing agent is added to the medium, *which defines time zero for UPR induction*, and essentially all of the cells progress in synchrony down a common pathway culminating in metabolic stress. In contrast, activation of the UPR *in vivo* is typically a dyssynchronous event that plays out stochastically at the level of individual cells. *In other words, there is no time zero in tissue samples.* Furthermore, tissues are typically heterogeneous and the UPR may be induced in only one cell type. In such instances, all phases of UPR pathogenesis are simultaneously represented in the target cell population. In addition, any changes that can be measured in population-based assays from the harvested tissue, such as altered gene expression by northern blotting or qPCR, appear muted by the background of cells in which the UPR is not active. These problems severely constrain the sensitivity and types of experiments that can be performed *in vivo* and often increase variability in the results.

5. A NOVEL IN VIVO GENETIC SWITCH SOLVES MANY OF THESE PROBLEMS

To overcome many disadvantages of *in vivo* paradigms in which to characterize the UPR, my laboratory is developing novel genetic switch technology (GST) in transgenic mice. UPR induction in these animals will be cell type specific, synchronous and will activate a fluorescent tag to enable the identification, characterization and harvesting of stressed cells

at a known time interval after induction of the UPR. The description of components of GST is divided into two parts.

Part 1 describes the generation of a knockin mutant *msd* allele of the *Plp1* gene that is constitutively inactivated (i.e. transcriptionally silent) during mouse development. This allele is activated by inducing loxP-Cre mediated recombination in oligodendrocyte nuclei, which induces mutant *Plp1* gene expression and the UPR. Subsequently, oligodendrocytes undergo apoptosis *en masse*.

Part 2 describes a transgene for oligodendrocyte-specific expression of the Cre recombinase, CreER^{T2}. This recombinase is a fusion protein with a variant of the human estrogen receptor (Leone et al., 2003) and is expressed by mature myelinating oligodendrocytes as an inactive protein bound to hsp90 in the cytoplasm. Upon treatment of mice with tamoxifen, CreER^{T2} translocates to the nucleus and recombines genomic DNA at loxP sites, thereby engaging the genetic switch described in Part 1.

5.1 Part 1 - Knockin construct to enable temporal activation of the mutant *Plp1* gene and UPR induction in oligodendrocytes

To induce the UPR in oligodendrocytes, a targeting vector (designated *Neo^{msd}*) was generated for homologous recombination in embryonic stem cells. This genomic construct introduces into the *Plp1* gene the *msd* mutant allele and several other elements (Fig. 3A).

5.1.1 Required materials

Sources of DNA for the targeting constructs

- Source of genomic DNA for the gene of interest from the library of choice. This library should be derived from the embryonic stem cells that will be used for gene targeting. Alternatively, long-range PCR using TaKaRa DNA polymerase (Clontech, Mountain View, CA) can be used to clone the desired region from the gene of interest.
- Plasmid vectors for sub-cloning genomic fragments and the selectable neomycin resistance marker gene (e.g. pKJ-1, Rudnicki et al., 1992), designated the *PGKneo* cassette. These reagents can usually be obtained from a transgenic Core facility or a company that will perform the gene targeting work in the embryonic stem cells.
- The *PGKneo* cassette needs to be flanked by loxP sites (i.e. a floxed *PGKneo* cassette) to facilitate its removal when activating expression of the mutant *Plp1* gene. In the event that a floxed cassette is not available from the transgenic core, it can be generated by cloning two loxP sites into a bacterial plasmid and inserting the *PGKneo* cassette between them. The DNA sequence of a loxP site is 34 bases long: 5' – ATA ACT TCG TAT AGC ATA CAT TAT ACG AAG TTA T – 3'.
- The CreER^{T2} coding region can be obtained through a materials transfer agreement with Dr P. Chambon at the Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC) in France.

Kits

- Quickchange kit (Agilent Technologies, Santa Clara, CA) for mutagenesis of genomic DNA to introduce coding region mutations or restriction endonuclease sites as well as to insert the floxed *PGKneo* cassette.

Other reagents

- DNA restriction endonucleases for cloning (New England Biolabs, Ipswich, MA).

- Corn oil (Cat. # C8267, Sigma-Aldrich, St Louis, MO).
- Tamoxifen (Cat. # T5648, Sigma-Aldrich) for intraperitoneal injection or gavaging of mice. Heat 5 ml of corn oil in an 18 ml glass vial at 42°C for 30 min. Add 100 mg of tamoxifen and vortex intermittently over several hours until dissolved. Store up to 1 month at 4°C protected from light. For single doses, up to 0.15 mg/g body weight can be used. For multiple doses use no more than 0.075 mg/g/day.

Genetically engineered mice: The *ROSA26* allele is a defined locus on mouse chromosome 6 that has been used extensively for the targeted insertion of a wide variety of transgenes (Nyabi et al., 2009) including fluorescent protein reporters under transcriptional control of ubiquitously expressed house-keeping genes. We use a *ROSA26-EGFP* strain designated *Tomato* mice (Jackson Laboratories, *Gt(ROSA)26Sor^{tm4}(ACTB-tdTomato,-EGFP)Luo/J*, Stock # 007576) in which EGFP is tethered to the plasma membrane of expressing cells (Muzumdar et al., 2007).

5.1.2 Construct design—Thus, an *Eco RI* genomic fragment containing exons 2 – 7 of the *Plp1* gene was cloned from a *129SvEv* genomic library (Stecca et al., 2000). The *msd* mutation, which substitutes valine for alanine in PLP1 (Gencic and Hudson, 1990), and an additional neutral polymorphism to create an *Eco RI* restriction endonuclease site, were inserted into exon 6 of the construct using site-directed mutagenesis (Quickchange kit, Agilent Technologies, Santa Clara, CA). A second round of mutagenesis was used to create an *Avr II* restriction site located 70 bp downstream of the 5' splice site in intron 6. A floxed *PGKneo* cassette was subcloned into the *Avr II* site in the antisense orientation with respect to transcription of the *Plp1* gene. The *PGKneo* positive selectable marker has two functions. Initially it is used for selection of ES cell clones in which the targeting vector has undergone homologous or non-homologous integration into the genome. Subsequently, the cassette is used to regulate expression of the mutant *Plp1* allele.

In knockin mice, the targeted insertion of the *Neo^{msd}* allele into the *Plp1* gene constitutively silences the locus during development, presumably by antisense-mediated degradation. Thus, strong transcription from the ubiquitously-expressed *PGKneo* cassette yields antisense *Plp1* transcripts that hybridize to sense *Plp1* transcripts, suppress their splicing and reduce their stability (Boison and Stoffel, 1994; Stecca et al., 2000). Male *Neo^{msd}* mice, which harbor a single X-chromosome, are functionally null for *Plp1* expression and females with one *Neo^{msd}* allele are carriers.

Targeting vectors frequently include negative selectable markers, such as thymidine kinase or diphtheria toxin under transcriptional control of a ubiquitous promoter. We have found in previous studies that negative selection reduces the number of ES cell colonies obtained after electroporation, but they do not significantly increase the likelihood of obtaining homologous recombinants (Stecca et al., 2000). Accordingly, we do not use negative selection.

5.2 Part 2 - A transgene construct to induce Cre recombinase expression in oligodendrocytes and trigger the genetic switch

Previous studies in transgenic mice have demonstrated that the proximal 1.9kb of the canonical mouse myelin basic protein (MBP) promoter/enhancer region drives high level oligodendrocyte-specific expression for the entire life of the mouse, with no expression outside of the CNS (Gow et al., 1992). This contrasts with a number of other MBP promoter cassettes that are leaky in other cell types or are suppressed within a few months after birth (Foran and Peterson, 1992; Turnley et al., 1991). As illustrated in Fig. 3B, 1.9kb of the MBP promoter is used to express the CreER^{T2} fusion protein and a 1.6kb genomic *Bam HI – Pst I*

fragment from the human β -globin gene comprising part of exon 2 through the end of exon 3 is used for splicing and polyadenylation signals.

The net effect of *MCreER^{T2}G* transgene expression is accumulation of the recombinase in the cytoplasm of mature, myelinating oligodendrocytes. Immature pre-myelinating cells do not express the transgene. A single-dose of tamoxifen ($\leq 0.15\text{mg/g}$) dissociates CreER^{T2} from the hsp90 complex in a proportion of oligodendrocytes, depending on the dose, and enables nuclear translocation. The dose-response for nuclear translocation does not appear to be linear; more than a 30-fold decrease in the number of CreER^{T2}-positive oligodendrocyte nuclei is observed for a 16-fold decrease in tamoxifen (unpublished data). Multiple tamoxifen doses on successive days substantially increase oligodendrocyte labeling.

Recombination of the *Neo^{msd}* allele at loxP sites (Fig. 3A), which constitutes the genetic switch, removes the *PGKneo* cassette and spliced mutant *Plp1* mRNA accumulates in the cytoplasm. Translation of this mRNA results in accumulation of mutant *Plp1* gene products in the ER, which activates the UPR. Because *Plp1* gene expression is triggered by tamoxifen treatment, a large number of oligodendrocytes throughout the CNS will undergo synchronized induction of the UPR and will subsequently undergo apoptosis. The half-life of tamoxifen in the mice is less than eight hours (Borgna and Rochefort, 1981) so the wave of *Plp1* expression, UPR induction and oligodendrocyte death is quite sharply defined.

5.3 Detection of oligodendrocytes in which the genetic switch has been triggered

In several of the applications of the GST, it is not of great importance to identify the cells that induce the UPR, only the regions where pathology is emerging. For example, repeated single doses of tamoxifen every 1–2 weeks for several months will induce successive waves of oligodendrocyte death in the CNS. Each wave will be accompanied by focal demyelinating lesions which will subsequently be remyelinated by the expansion, migration and differentiation of oligodendrocyte progenitors from local stem cell pools. The purpose of this experiment would be to examine the demyelination or remyelination processes after the oligodendrocytes had undergone apoptosis.

For applications of the genetic switch that are focused on characterizing the different aspects of the UPR signaling cascade, the in vivo identification of individual stressed cells will be critical. To accomplish this task, oligodendrocytes in which the UPR has been activated will also require a detectable tag, such as green fluorescent protein. For this purpose, a transgene driving expression of a loxP-activated membrane-tethered EGFP reporter is ideal (e.g. *Tomato* mice). Thus, a timecourse experiment would begin with a single dose of tamoxifen given to a cohort of mice at time zero, and the harvesting of CNS from several animals every 12–24 hours thereafter. The tissues would be processed by cryostat sectioning and laser capture to isolate fluorescent oligodendrocytes. Alternatively, the tissue would be homogenized for cell purification using fluorescence-activated cell sorting. The samples would then be processed for microarray analysis.

5.4 Alternative knockin constructs for simultaneous UPR induction and labeling of oligodendrocytes

A limitation of the experimental design in Section 5.3 is the number of alleles that need to come together in individual mice to carry out the experiment: the *Neo^{msd}* allele, the *MCreER^{T2}G* transgene and the *Tomato* allele. In addition, the mice must be male to ensure mutant *Plp1* gene (on the X-chromosome) expression in oligodendrocytes. From such a breeding colony, the frequency of generating these mice is 1-in-16. The colony can be simplified somewhat by breeding the *Tomato* allele to homozygosity, which will increase

the frequency to 1-in-8. Control mice for this experiment, which do not harbor the *Neo^{msd}* allele, will also be generated with a frequency of 1-in-8.

The frequency of the required genotypes for experiment can be further improved to 1-in-4 by combining the *Neo^{msd}* and an EGFP reporter in a single locus, as illustrated in Fig. 4. Thus, the spliced *Plp1* transcript that is generated by activating the *Neo^{msd:egfp}* allele will be bicistronic. Ribosomes will be recruited to the 5' cap to synthesize mutant PLP1 and DM20, and also to the internal ribosome entry site (IRES) located in the 3' untranslated region of the *Plp1* gene to synthesize the EGFP reporter. Some studies report that the efficiency of IRES-dependent translation in different cell types varies according to the translation initiation factors available. In addition, the efficiency of IRES- and 5' cap-dependent translation can shift under conditions of metabolic stress (reviewed by Fitzgerald and Semler, 2009). Accordingly, an alternative approach is to fuse the EGFP reporter to the carboxyl-terminus of the *Plp1* open reading frame in the targeting construct.

5.5 Expected phenotypes in GST mice

Analogous to humans, the phenotype of male mice lacking a functional *Plp1* gene is very mild. Indeed, male *Plp1*-null mice develop normally, synthesize myelin of appropriate thickness and to be phenotypically normal until 1 – 1.5 years of age (Klugmann et al., 1997; Rosenbluth et al., 1996; Stecca et al., 2000). Abnormal neuronal metabolism at the level of vesicular trafficking in axons is increasingly apparent from approximately 2 months of age (Griffiths et al., 1998).

In contrast to the *Plp1*-null mice, the phenotype of male *msd* mice is very severe. Clinical signs comprising intention tremors and whole body shudders are apparent from 11–12 days of age and these symptoms intensify over the following two weeks. By 20–25 days, these mice develop stimulus-induced seizures, which intensify in severity and frequency until the death of the animals at 25–30 days. Histology of the CNS at the time of death reveals the virtual absence of myelin membrane and oligodendrocyte lineage cells.

Depending on the size and frequency of the tamoxifen dose, the phenotype of the *Neo^{msd}* mice will range from near wild type to approximately *msd*. Some variability in the phenotype might be expected because the precise number and neuroanatomical locations of the demyelinating lesions will vary in different mice. Thus, if a significant demyelinating lesion develops in the brainstem after tamoxifen treatment, it will be more likely to cause significant motor disability or kill the mouse than would a similar size lesion in the optic nerve.

5.6 Applications of GST to the study of UPR diseases and neurodegeneration

Perhaps the simplest application of the GST in *Neo^{msd}* mice is to generate a timeline for UPR signaling in oligodendrocytes in vivo. Following a single dose of tamoxifen, oligodendrocytes will begin to express mutant PLP1 and DM20, which will accumulate in the ER and trigger the UPR. This pathology will evolve gradually over a few days or so, which will provide us with a number of timepoints to purify RNA from the cells and determine their expression profile (e.g. every 12 hours). Changes in the expression profiles of known UPR genes will be used to rank order the genes in the UPR pathway. In addition, we use these profiles to identify novel candidate genes that are coordinately regulated.

A second application for these mice is to examine the demyelination/ remyelination cycle in adults. Thus, a single dose of tamoxifen, or a tightly-clustered series of doses over several days, would trigger demyelination throughout the CNS. The evolution and dissolution of these lesions would be examined with respect to cell death, the infiltration of oligodendrocyte progenitors, the involvement of the immune system. A number of

experimental demyelinating models are available in mice, but most are relatively complex to initiate. For example, some models require stereotactic injections of toxins into the parenchyma or extensive treatment with toxins in the diet, while others require specific genetic background strains for susceptibility to autoimmune demyelination (Blakemore and Franklin, 2008; Gao et al., 2000; Reynolds et al., 1996).

An extension to the single dose strategy to induce demyelination would be repetitive tamoxifen doses (every 1–2 weeks) to drive repeated demyelination/ remyelination cycles in the mice. This model would be useful for studying the long term affects of oligodendrocyte cell death on the integrity and function of large white matter tracts such as corpus callosum or brainstem. A great deal of anecdotal evidence in the clinical literature suggests that patients with multiple sclerosis eventually develop behavioral disorders, presumably stemming from the cumulative affects of remodeling white matter tracts following multiple demyelinating lesions (reviewed in Fields, 2008; Stewart and Davis, 2004).

6. GENERALIZING GST TO STUDY OTHER UPR DISEASES

The current chapter draws upon the research program in my laboratory to characterize the UPR in oligodendrocytes expressing a mutant allele of the *Plp1* gene. However, GST is easily generalized to generate mouse models of other genetic forms of UPR disease. In this regard, it is important to consider that UPR diseases principally arise from gain-of-function mechanisms and that one or two alleles of the targeted gene will induce a UPR in activated cells (Gow and Sharma, 2003). Accordingly, the design of GST targeting constructs described here will be applicable to most genes and cell types and the uses for these animals will be similar to *Neo^{msd}* mice. For targeted mutations of autosomal genes, there may be significant advantage to comparing the phenotype caused by activation of one or two mutant alleles (i.e. heterozygotes or homozygotes for the mutant allele) in terms of the strength of UPR induction. In the case of the *Plp1* gene, only one allele is expressed in any cell as a result of X-inactivation in females or the presence of the Y-chromosome in males.

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Figure 1.

Essential features of the PERK signaling temporal delay circuit. UPR-induced dimerization and transautophosphorylation of PERK in the ER results in phosphorylation of eIF2 α , which inhibits global translation in the cell by suppressing ribosome assembly, induces the specific translation of the ATF4 transcription factor. ATF4 activates expression of other transcription factors including ATF3 and CHOP. CHOP induces expression of GADD34, which is a regulatory subunit of the protein phosphatase, PP1, and targets this complex to effect the dephosphorylation of phospho-eIF2 α . The time taken to express this set of proteins constitutes the time delay, during which the cell reduces protein accumulation in the ER by dislocation of the misfolded nascent chains and proteolysis through the ubiquitin-proteasome pathway.

Figure 2.

Summary of wild type or mutant PLP1 expression and intracellular trafficking in oligodendrocytes. (a) Wild type PLP1 and DM20 are synthesized on the rough ER and transported in vesicles along microtubules for incorporation into myelin sheaths. Oligodendrocytes (cell with four processes to myelin sheaths) simultaneously synthesize myelin sheaths around multiple axons and generate several hundred-fold more membrane within a few days than the surface area of the cell body. (b) Missense and nonsense mutations in the *PLP1* gene destabilize the primary structure of PLP1 and DM20, causing ER accumulation, metabolic stress and activation of the UPR. Global protein translation is temporarily arrested via PERK pathway signaling. ER-associated protein degradation clears the accumulated protein to shut down the UPR and protein translation resumes. Mutant PLP1 and DM20 again accumulate in the ER leading to additional cycles of UPR activation. In each cycle, the cell can resolve the stress but there is an increased risk of undergoing apoptosis when protein expression resumes (Gow and Wrabetz, 2009).

Figure 3.

Components of the GST used to generate an in vivo model of a UPR disease in the CNS. (A) A mouse *Plp1* gene targeting construct for homologous recombination in embryonic stem cells. A portion of the wild type *Plp1* gene comprising exons 2 – 7 is shown for comparison with a targeting construct of this region. A missense mutation, which specifies the *msd* allele, as well as a neutral polymorphism to generate an *Eco* RI site (for genotyping purposes) is inserted into exon 6. An antisense *PGKneo* selectable marker gene is cloned into the 5' end of intron 6 to allow neomycin resistance selection of homologous recombinant ES cells as well as to confer antisense-mediated suppression of *Plp1* gene expression in mice during development. The *PGKneo* cassette is flanked by loxP sites to allow Cre recombinase-mediated recombination. Excision of the *PGKneo* cassette activates expression of the mutant *msd* allele of *Plp1*, which induces the UPR. Locations are shown for the external 5' and 3' genomic probes used to verify homologous recombination in ES cells. (B) A transgene, designated *MCreERT²G*, containing 1.9kb of the mouse myelin basic protein promoter and the human β -globin splice and polyadenylation signals, is used to drive expression of a tamoxifen-activated Cre recombinase (CreER^{T2}) in mature myelinating oligodendrocytes. In the presence of tamoxifen, CreER^{T2} translocates to the nucleus and excises the *PGKneo* cassette to activate mutant *Plp1* gene expression.

Figure 4.

Variation on the targeting construct to enable fluorescence-based detection of cells expressing the *msd* mutant *Plp1* gene. In addition to insertion of the *msd* mutation in exon 6 and the *PGKneo* cassette in intron 6, an internal ribosome entry site-EGFP cassette has been inserted into exon 7 in the 3' untranslated region. Expression of this allele after Cre-mediated recombination yields a bicistronic mRNA that encodes both mutant PLP1 and DM20 as well as EGFP. Locations are shown for the external 5' and 3' genomic probes used to verify homologous recombination in ES cells.