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The Transcriptional Response to Hypoxic Insult Controlled by FRA-2

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FRA-2 is involved in cellular differentiation and is also upregulated in response to ischemic injury to the brain. To shed light on the function of this transcription factor, a novel microarray analysis was utilized to identify FRA-2-dependent gene expression increased in the hypoxic response. Genes were identified that were upregulated by exposure of neuronally differentiated PC12 cells to hypoxia. Using a dominant negative construct to block FRA-2, a second subset of genes that were FRA-2 dependent was found. Cross comparison then allowed isolation of a list of genes that were induced in response to hypoxia in a FRA-2-dependent manner. These data suggest that FRA-2 is involved in the transcriptional control of neuroprotective genes and in the switch from aerobic to anaerobic metabolism.

Key words: Rat pheochromocytoma; PC12; AP-1; Neuroprotection

FOS-RELATED antigen 2 (FRA-2) is a member of the family of AP-1 proteins but demonstrates differences from other family members both in its regulation and kinetics of induction in response to stimulation (14). While most AP-1 proteins have been implicated in both survival and death pathways (18), the few studies conducted to date demonstrate FRA-2 in association with protective signaling. We have reported expression of FRA-2 in surviving neurons following stroke in rats (8). It is also induced, along with a general increase in AP-1 DNA binding, as a protective response by the pheochromocytoma line (PC12) following serum starvation and rescue with nerve growth factor (NGF) (32).

The PC12 cell line models the hypoxic response (2) and, in a neuronally differentiated state, this is relevant to our understanding of the response of neurons to ischemic injury in vivo. NGF drives the differentiation of PC12 cells to exhibit a neuronal phenotype with similarities to both sensory and sympathetic neurons (15). In the PC12 cell line, pathways activated by hypoxia in this cell line have been identified in both an undifferentiated (29) and differentiated (9,16) state. However, the details of these pathways have yet to be elucidated.

There has been a recent move to investigate neuroprotective- or neurodegenerative-associated signaling using microarray analysis (20,31). Importantly, these analyses have demonstrated generalities in the signaling induced in response to different insults, highlighting their usefulness to the study of brain injury (31). We chose to examine the broad response of differentiated PC12 cells to hypoxic insult as well as gene expression specifically driven by the transcription factor FRA-2 using microarray analysis.

MATERIALS AND METHODS

Cell Culture, Transfections, and Hypoxic Treatment

PC12 cells were maintained in RPMI supplemented with 10% horse serum and 5% calf serum. The cells were adhered on collagen-coated (Aldrich) plates and differentiated with 100 ng/ml NGF (Alomone Labs) for 24 h. Hypoxic exposure was performed for appropriate times in a humidified chamber (CBS Scientific) flushed with 95% N_2, 5% CO_2, 1% O_2 and then allowed to recover in standard culture conditions (referred to as normoxia). Plasmids used were pGFP-C3 (GFP; Clontech) and a dominant negative FRA-2 [DNF2; a gift from Dr. Ruben Baler

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(30)]. They were introduced into PC12 cells using Lipofectamine 2000 (Invitrogen). Following transfection, cells were plated for differentiation as described above.

FACS Sorting and Harvesting

PC12 cells were cotransfected with DNF2 and pGFP-C3 in a 3:1 ratio, maximizing the number of transfected cells that contained both plasmids (13). Cells were sorted using a FACS Vantage SE Diva (BD Biosciences) and both GFP-positive and -negative cells were collected. Thus, two populations were derived from a single mixed sample. GFP^- cells also lacked the DNF2 plasmid and were FRA-2 expressing, while GFP^+ cells, which contained the DNF2 plasmid, did not possess transcriptionally active FRA-2. Data acquisition and analysis were conducted using Cell Quest software. Forward angle and side-scatter light gating was used to exclude dead cells and debris, and purity of the two populations was confirmed before subsequent manipulation. Decreased FRA-2 expression in the GFP/$DNF2^+$ relative to the GFP/$DNF2^-$ population was confirmed by Western analysis in preliminary experiments.

RNA Extraction and Affymetrix Chip Analysis

Gene chip-based analysis of expression was performed on four groups representing two experiments. The first compared FACS sorted GFP/$DNF2^+$ and GFP/$DNF2^-$ groups. In the second, differentiated PC12 cells exposed to 16 h of hypoxia and allowed to recover for 3 h were compared to a normoxic control group. Total RNA was prepared using Trizol (Invitrogen). Subsequent RNA processing and analysis was conducted by the H. Lee Moffitt Microarray Core Facility using standard protocols established by Affymetrix and four rat U34A chips. MAS 5.0 software was used to determine presence of a transcript, calculate its abundance, and determine the probability of change. Excel was then employed for the comparative analysis across all four groups. A detailed description of the U34A chips and use of the Affymetrix Gene chip software has been given by others (31).

Western Blot Analysis

Cell extracts were prepared and protein concentration determined with Bio-Rad protein assay dye. Aliquots of 35 μg were electrophoresed on 10% SDS-PAGE gels. Following transfer to nitrocellulose membranes (Bio-Rad), equal protein loading was confirmed by staining with Ponceau S (Sigma-Aldrich). Membranes were destained and protein detection was performed using antibodies against FRA-2 (Santa Cruz Biotech.), pErk1/2 (Cell Signalling), rat transferrin receptor (Zymed Labs.), neuron-specific enolase (Polysciences), cytokeratin 18, and exportin-1 (BD Biosciences). Horseradish peroxidase-conjugated secondary antibodies were purchased from Vector laboratories. The blots were developed using Amersham's ECL kit following the recommended protocol. Western data were derived from at least three independent experiments.

RESULTS

FRA-2 expression and activity is enhanced during NGF-induced differentiation of PC12 cells. It has been suggested that this is mediated by Erk phosphorylation (5). We confirmed concurrent upregulation of FRA-2 and activation of the Erk pathway, measured by increased levels of its phosphorylated form (pErk1/2) in our model (Fig. 1A). Here, the upregulation extended over a longer time course than was previously described.

Susceptibility of PC12 cells to hypoxic-induced death is profoundly affected by differentiation (9). In the predifferentiated state, these cells are resistant to hypoxia. However, when differentiated with NGF, they exhibit sensitivity (Fig. 1B) more analogous to that seen in neurons (data not shown). For this reason, all analyses were performed on cells that had first been differentiated with NGF. Initial experiments tested the effect of hypoxic exposure times on the expression of FRA-2 protein and demonstrated upregulation of FRA-2 expression that correlated with the severity of insult (Fig. 1C). Interestingly, although the FRA-2 protein ran at a size suggesting a normal phosphorylation state, consistent expression of pErk1/2 was not seen (Fig. 1D). The temporal changes in pErk1/2 levels may reflect cycling initiated after return to normoxia or a multiphasic response to hypoxic treatment.

The maximal effect on FRA-2 expression was seen following 16 h of hypoxic exposure (Fig. 1C) yet many cells survived the injury (Fig. 1B), providing an ideal model for identification of protective signaling. Therefore, this treatment time was used for subsequent experiments. The temporal course of expression of FRA-2 was assessed at times extending from immediately after 16 h of hypoxic insult through to 48 h later. FRA-2 protein was already highly expressed in cells that were not allowed any recovery time and slowly subsided to basal levels by 48 h post-treatment (Fig. 1D).

To aid insight into FRA-2's function in differentiation and the response to injury, we sought to identify

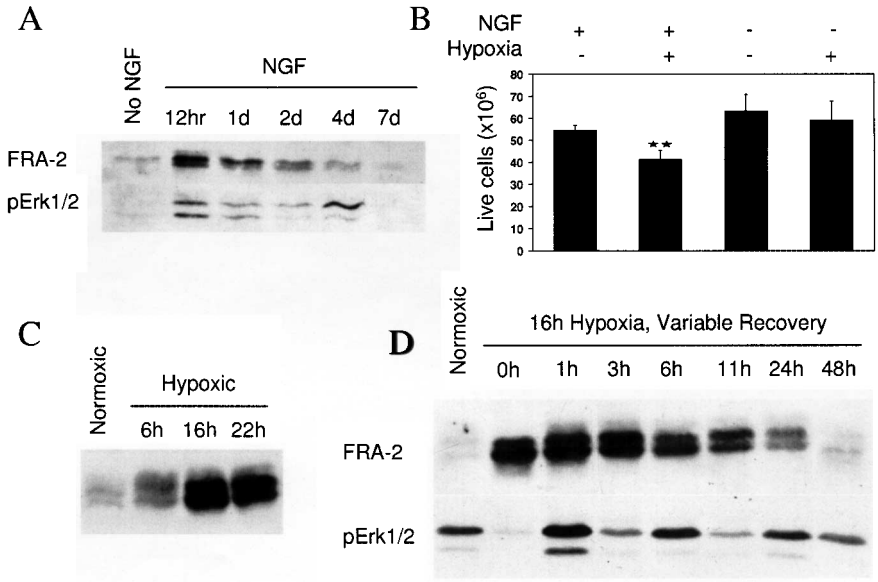

Figure 1. (A) PC12 cells were treated with NGF for various times and FRA-2 expression analyzed by Western blotting. NGF-induced upregulation of FRA-2 was prolonged, lasting up to 4 days. Levels of phosphorylated Erk1/2 largely correlated with that of FRA-2. (B) The effect of NGF differentiation on survival of PC12 cells following hypoxia was assessed. Hypoxic treatment lasted 16 h followed by a 24-h recovery period and live cells were counted by trypan blue staining. NGF-differentiated cells displayed a significant (Student's *t*-test: $p < 0.01$) reduction in the number of live cells while nondifferentiated cells were unaffected. (C) Western analysis of FRA-2 protein levels in response to increasing lengths of hypoxic exposure demonstrated a maximal response when 16 h of hypoxia was used. (D) FRA-2 expression during normoxic recovery following 16 h of hypoxic treatment is markedly upregulated at the 0-h time point and gradually declines to baseline levels over the next 24 h. Reprobing shows that levels of phosphorylated Erk1/2 do not correlate with those of FRA-2 expression.

genes regulated by FRA-2 during differentiation with NGF and in response to hypoxia by gene array analysis. This was achieved by comparison amongst four treatment groups: 1) PC12 cells exposed to hypoxia and allowed to recover for 3 h (in the range of the maximal expression of FRA-2), 2) normoxic control cells, 3) GFP/DNF2$^+$ PC12 cells, and 3) GFP/DNF2$^-$ PC12 cells. Thus, two experimental groups are represented: transcription related to hypoxic injury and transcription affected by inhibition of FRA-2.

The data obtained from the array analysis were first analyzed for each of the two major experiments separately to obtain two lists of affected genes. A total of 1442 genes were affected by hypoxic treatment and 977 genes were affected by inhibition of FRA-2 activity. These numbers included genes for which transcription was increased as well as those that were decreased.

The ongoing study focused on genes that were transcriptionally upregulated. The analysis was limited to known genes that were shown to increase at least twofold relative to the baseline value and had a reported baseline signal of at least 1000. Thus, it was found that 26 genes were more highly expressed in PC12 cells submitted to hypoxia relative to their normoxic controls (Table 1) and 5 genes were more highly expressed in FRA-2-expressing cells relative to those in which FRA-2 activity had been inhibited (Table 2).

Next, genes were identified that were potentially FRA-2 dependent in the hypoxic response. This was achieved by comparison of the 1442 genes affected by hypoxia and the 977 genes affected by inhibition of FRA-2 and identified 68 genes that were increased in both paradigms and 23 genes that were decreased. Through direct comparison of signal values for each gene, focus was placed on genes that displayed an expression pattern across the four groups that could be clearly interpreted as being both FRA-2 regulated and affected by hypoxic exposure. Only upregulated genes were considered, as this has been suggested to be more relevant than downregulated genes in analysis of the general injury response (11). Refining the list in this way revealed 11 candidate genes that were potentially transcribed in a FRA-2-dependent fashion in response to hypoxia. The signal data for these genes are detailed in Figure 2A.

Of the 11 upregulated genes, 6 have published promoter sequences and most of these are at least partly controlled by AP-1 regulatory elements (Table 3). Further, one of the genes, tissue plasminogen activator (tPA), is known to be regulated specifically by FRA-2 (1). A subset of the candidates was tested to measure protein levels in response to hypoxic insult

TABLE 1
GENES UPREGULATED IN RESPONSE TO HYPOXIC INJURY

Accession No.	Gene Expression Increased by Hypoxia (Alternate Names)	Fold Change	Maximum Signal
NM 019371	factor responsive smooth muscle protein (SM-20; Egln3)	8.6	3180
NM 138863	dithiolethione-inducible gene 1 (DIG-1)	5.5	3713
NM 172055	melanocyte-specific gene 1 protein (Msg1)	5.2	2985
NM 019144	acid phosphatase 5 (Acp5; TTRRAP; TRAP)	4.5	5330
NM 017070	steroid 5 α-reductase 1 (Srd5a1)	4.4	2751
NM 139324	pincher (Ehd4; Past2)	4.1	1561
NM 022278	glutaredoxin 1 (thioltransferase) (Glrx1)	3.9	1078
NM 017200	tissue factor pathway inhibitor (Tfpi; LAC1)	3.3	1187
NM 139192	stearoyl-CoA desaturase 1 (Scd1)	3.3	2905
NM 133514	matrix metalloproteinase 10 (Mmp10; stromelysin 2)	3.0	2750
NM 017014	glutathione-*S*-transferase, μ1 (Gstm1; GSTA3; GST1)	2.7	7041
NM 053827	procollagen-lysine, 2-oxoglutarate 5-dioxygenase (Plod)	2.6	1931
NM 053698	Cbp/p300-interacting transactivator, 2 (Cited2; MRG1)	2.6	2600
NM 080888	BCL2/adenovirus E1B 19 kDa-interacting protein 3-like (Bnip3l; Nlx)	2.6	2197
NM 133390	gonadotropin-inducible ovarian transcription factor 2 (GIOT2)	2.5	1124
BM383531	metallothonein-2 and metallothionein-1 (MT-I, MT-II)	2.4	1544
NM 030834	monocarboxylate transporter (Mct3; now MCT4)	2.4	3045
NM 031753	activated leukocyte cell adhesion molecule (Alcam; MEMD; CD166)	2.3	2860
NM 017076	tumor-associated antigen 1 (Taa1; Tage4)	2.3	1388
NM_178334	EGL nine homolog 1 (Egln1; PHD2)	2.3	3450
NM 022585	ornithine decarboxylase antizyme inhibitor (Oazi)	2.2	3362
NM 017258	B-cell translocation gene 1 (Btg1; Hugo)	2.2	1541
NM 017126	ferredoxin 1 (Fdx1)	2.1	1600
M58040	transferrin receptor (Tfrc; TfR; p90; CD71)	2.1	1407
NM 031606	phosphatase and tensin homolog (Pten; Mmac)	2.1	1184
NM 139325	neuron-specific enolase (enolase 2, γ; Eno2; RNEN3; NSE)	2.0	2640

and confirm the gene chip results in a functional manner. They were the transferrin receptor (Tfrc), cytokeratin 18 (Krt1-18), exportin-1 (Xpo1), and neuron-specific enolase (NSE). As for the FRA-2 analysis, protein expression was assessed over a time course of normoxic recovery following 16 h of hypoxia and compared with normoxic controls.

All proteins tested underwent upregulation in cells exposed to hypoxia, although the response was less marked than that of FRA-2 (Fig. 2B). With the exception of NSE, protein levels returned to baseline by the 24–48-h time points, when FRA-2 was also no longer highly expressed. This offers support for a role for FRA-2 in their transcriptional control.

DISCUSSION

Recent data from this laboratory demonstrated upregulation of FRA-2 protein levels in the brains of rats that had been subjected to stroke (8). Here, we demonstrate that FRA-2 expression is also induced in response to hypoxic injury in vitro. This protein has not been widely studied, but the emerging evidence supports a neuroprotective or survival-associated role in a variety of stress and injury settings (19,26,28, 32). Given the fact that the different AP-1 family members demonstrate diverse roles in the response to injury, an association with survival signaling for FRA-2 is highly significant.

TABLE 2
GENES DEPENDENT ON FRA-2 EXPRESSION DURING NGF DIFFERENTIATION

Accession No.	Gene Expression Increased by FRA-2 (Alternate Names)	Fold Change	Maximum Signal
NM 012903	acid nuclear phosphoprotein 32 family, member A (Anp32a; LANP; PP32)	3.8	1016
BG379338	neural precursor cell expressed, developmentally downregulated gene 4A (Nedd4a)	2.1	3003
NM 024128	brain-specific mRNA B (clone p1a75) (Bsmrb; PEP1)	2.1	5362
NM 017029	neurofilament 3, medium (Nef3; Nfm, Nefm)	2.1	3759
NM 019296	cell division cycle 2 homolog A (Cdc2a)	2.0	1162

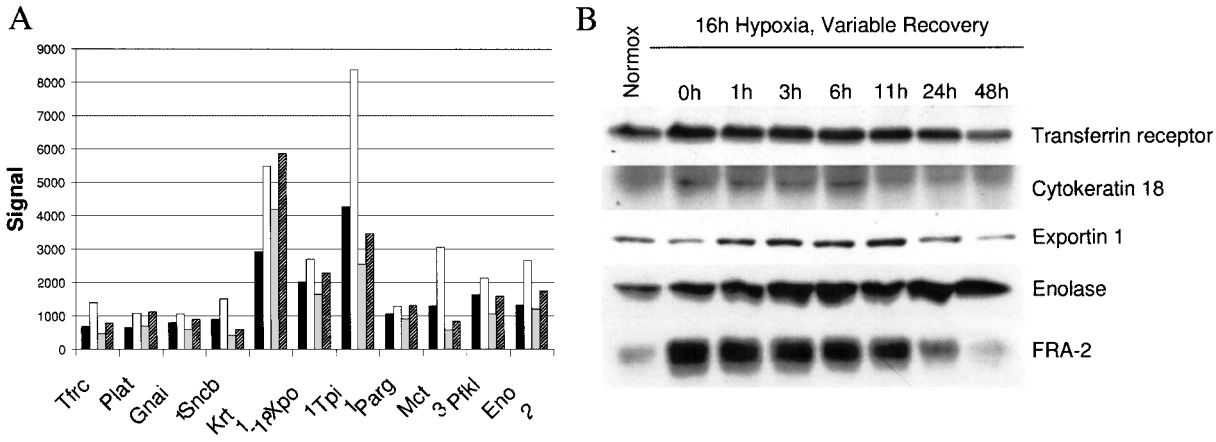

Figure 2. (A) Signal data for genes selected from the four-way analysis (see Table 3) representing candidate genes for regulation by FRA-2 and hypoxia. Normoxic (black bars), hypoxic (white bars), DNF2$^+$ (gray bars), DNF2$^-$ (striped bars). (B) Western analysis of the shortlisted gene products demonstrates upregulation of protein expression that in most cases correlates with FRA-2 levels. Shown are representative Westerns of three independent experiments.

The successful inhibition of FRA-2 in GFP/DNF2 cotransfected cells was confirmed in two ways. First, FRA-2 expression was highly reduced in GFP/DNF2$^+$ relative to GFP/DNF2$^-$ cells (data not shown). Second, two genes that have previously been demonstrated to be NGF responsive and AP-1 regulated in PC12 cells [see (5)] were more highly expressed in the DNF2/GFP$^-$ group. These were the neurofilament light chain and tyrosine hydroxylase (data not shown). Other genes identified that were FRA-2 dependent (Table 2) support its differentiation role. Anp32a, also known as leucine-rich acidic nuclear protein (LANP), expression correlates with differentiation of cerebellar neurons (25). Neurofilament proteins, including the medium subunit, are classic neuronal markers that are upregulated during differentiation of PC12 cells into a neuronal phenotype (11).

The current study, to the authors' knowledge, represents the first microarray analysis of differentiated PC12s exposed to hypoxia. However, hypoxic treatment and microarray analysis of whole brain has been conducted (31). Consistent with the referenced study, induction of genes associated with iron homeostasis (transferrin receptor) and glycolytic enzymes (NSE)

TABLE 3
GENES EXHIBITING FRA-2-DEPENDENT REGULATION IN RESPONSE TO HYPOXIC TREATMENT

Accession No.	Increased Hypoxia and FRA-2	Function (Reference)	Regulation at Promoter
M58040	rat transferrin receptor (Tfrc; TfR)	iron homeostasis (15); neuronal dendritic protein (16)	Sp1, AP-1/CRE (17), Ets (18)
NM 013151	tissue plasminogen activator (Plat; tPA)	fibrin dissolution; neuritogenesis in PC12 cells (19)	Sp1, AP-1 (FRA-2 specific) (20)
NM 013145	guanine nucleotide binding protein, α inhibiting 1 (Gnai)	receptor-mediated signaling; largely neuronal (21)	
AI145381	synuclein, β (Sncb)	synaptic protein; neuronal plasticity (22)	
BI286012	cytokeratin 18 (Krt1-18)	epithelial structural protein; expressed in PC12 cells (23)	Sp1, TATA (24), AP-1 (25)
AW533924	exportin 1 (Xpo1; Crm1)	nuclear export (26)	
NM 022922	triose-phosphate isomerase 1 (Tpi1)	glycolysis; deficiency causes neurological disorder (27) and neuronal death in vitro (28)	Sp1, TATA (min. promoter) (29)
AB019366	poly(ADP-ribose) glycohydrolase (PARG)	catabolism of poly(ADP-ribose), implicated in DNA repair and apoptosis (30)	
NM 030834	monocarboxylate transporter (MCT4)	lactate transport; inhibition enhances neurodegeneration after stroke in rats (31)	
NM 013190	phosphofructokinase, B-type (Pfkl)	glycolysis (32)	Sp1, AP-1-like (32)
AF019973	neuron-specific enolase (NSE; Eno2)	glycolysis; carbohydrate transport and metabolism, neuronal marker (33)	Sp1, AP-1, AP-2, CRE (34)

were found in the current investigation. Other survival associated genes identified in this model include the metallothonein proteins, which act as antioxidants and are essential to CNS repair (27), and MCT4, involved in lactic acid transport and metabolism (17). Not surprisingly, genes that function in cell death were also included in the list. The Egln proteins are prolylhydroxylases that target HIF α proteins for degradation (6) and Cited2 acts as a competitive inhibitor of HIF α (4). Along with the Bcl-2 family member Bnip3l (10), these proteins are proapoptotic.

To aid insight into the function of FRA-2, genes regulated by this transcription factor during differentiation with NGF and those produced in response to hypoxia were identified. The four-way comparison used, however, is limited by the experimental design. That is, comparison of the restricted gene expression profile in a differentiation setting versus that of a larger hypoxic response only allows identification of genes that have function in both of these distinct processes. Nevertheless, NGF does control protective signaling (7,34) and, for this reason, overlap in the two processes is expected. This is highlighted by the fact that genes were found in the hypoxic analysis that produce proteins involved in PC12 cell differentiation, such as Pten (24) and the Btg proteins (3).

The four-way gene expression analysis largely demonstrated genes with neuroprotective characteristics (Table 3). Anaerobic glycolysis is pivotal to cellular survival during loss of oxidative phosphorylation, and proteins involved in the maintenance of energy metabolism are likely to be involved generally in stress responses (23). Glycolytic proteins highlighted by this study include triosephosphate isomerase (TPI), monocarboxylate transporter, phosphofructokinase, and NSE. TPI catalyzes the interconversion of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate in the initial phase of glycolysis. By enhancing function of the Na, K-ATPase pump it may help to maintain intracellular Na^+ levels (21), and in ischemia and reperfusion of the heart, this protein appears to be involved in the protective response to oxidative stress (12).

Analogous protein upregulation was demonstrated for shortlisted genes that were analyzed by Western blotting. Some temporal differences in their expression were seen that may result from changes in FRA-2 dimer composition and, therefore, specificity of gene target (22). The dependence upon FRA-2 for expression of these proteins in the hypoxic response must be confirmed in future experiments. Exportin 1, through its function in nucleocytoplasmic shuttling, may be involved in various cell stress responses (33) and forms part of the PC12 hypoxic response in this analysis. However, regulatory regions in the exportin 1 promoter have not been analyzed and this study represents the first evidence for FRA-2-dependent transcription. The neuronal marker NSE displayed a temporal expression that did not correlate with the other proteins or the late downregulation of FRA-2. This highlights the need to confirm the results of array analyses in a functional manner.

In conclusion, while the disparate roles of AP-1 proteins in cellular signaling are well known, there is a paucity of information focusing on individual family members and their downstream gene targets. A clearer understanding of this aspect of signaling specificity will greatly enhance our understanding of the injury response. This study aims to initiate development of methods to address this question and provides evidence for FRA-2-mediated signaling in neuronal survival and adaptation following hypoxic injury.

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