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HALO, a Novel bHLH-PAS Protein Induced by Neuronal Preconditioning and Ischemia, Mediates Cytotoxicity Through BAX Gene Upregulation

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By

Ian Wayland Hester

B.Sc. University of Ottawa, 2004

THESIS

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ABSTRACT

Cortical spreading depression (CSD) induces waves of neuronal depolarization that confer neuroprotection to subsequent ischemic events in the rat brain. To gain insights into the molecular mechanisms elicited by CSD, we used representational difference analysis (RDA) to identify mRNA induced by potassium depolarization *in vivo*. We have isolated a cDNA encoding a novel bHLH-PAS protein distantly related to SIM2, termed HALO. Our results confirm that HALO mRNA and protein are rapidly and transiently expressed in cortical neurons following CSD but not following short duration ischemia, another form of pre-ischemic conditioning. In the untreated adult brain, HALO is expressed at low levels but is highly expressed during embryonic development in neuronal lineages. Surprisingly, delayed HALO expression is also observed following middle cerebral artery occlusion (MCAO) in rats. Reporter assays show that HALO is a transcriptional activator that associates with the bHLH-PAS sub-class co-factor ARNT2. Adenovirus-mediated expression of epitope-tagged HALO results in the direct induction of the Bax gene and sensitization of cultured cells to cytotoxic stress. Together, our data indicate that HALO is a novel bHLH-PAS transactivator transiently induced by preconditioning and that its sustained expression is detrimental. The identification of HALO may represent an important step in our understanding of the molecular mechanisms of brain preconditioning and injury.

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List of Abbreviations

ΔTAD – deleted TransActivational Domain

AMPA – alpha-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid

ARNT1/2 – Aryl-Hydrocarbon Receptor Nuclear Translocator 1/2

bHLH-PAS – basic Helix-Loop-Helix/ Per Arnt Sim

CCN – Cortical Cultured Neurons

CGN – Cerebellar Granule Neurons

ChIP – Chromatin Immunoprecipitation

CME – CNS Midline Element

CME-v – CME-variant

CNS – Central Nervous System

CSD – Cortical Spreading Depression

Dpc – Days post coitum

DRG – Dorsal Root Ganglia

EMSA – Electro-Mobility Shift Assay

EPO – Erythropoietin

GFAP – Glial Fibrillary Acidic Protein

GFP – Green Fluorescent Protein

HALO – bHLH-PAS Activated Left-side ORF

HIF-1_ – Hypoxia Indicible Factor-1_

HRE – Hypoxic Response Element

IgY/IgG – Immunoglobulin Y/G

OGD – Oxygen/Glucose Deprivation

MCAO – Middle Cerebral Artery Occlusion

MOI – Multiplicity of Infection

NMDA – N-methyl-D-aspartate

RDA – Representational Difference Analysis

SIM1/2 – Single Minded Protein 1/2

TF – Transcription Factor

Tuj-1 – anti-beta III Tubulin

VEGF – Vascular Endothelial Growth Factor

VHL – von Hippel-Lindau protein

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Firstly, I would like to thank Dr. Charlie Thompson, for sharing with me his intelligence on research, politics and football. Thank you to all the members of my lab and the NRI, we worked long and hard but we had some good time along the way. I would like to thank both my sets of parents for believing in me and encouraging me throughout this endeavour. I could not have completed this achievement had it not have been for the love and support of my wife, who is as beautiful as she is kind hearted.

And lastly I would like to thank my supervisor Dr. Luc Sabourin who not only shared his wisdom, knowledge and guidance over the years but also shared his friendship. Thank you truly.

I) INTRODUCTION

Stroke is characterized by the interruption of the blood flow (ischemic stroke) or the rupture of blood vessels (hemorrhagic stroke) in an organ to cause a sudden loss of function. The brain is the most susceptible organ to stroke as neuronal death may occur after approximately 5 minutes of ischemia compared to other organs which can take 4 times as long or more to undergo cell death (Lee J.M. *et al.,* 2000). The most sensitive regions of the brain affected in short ischemic events are the hippocampus, the cerebral cortex and the striatum. The brain's vulnerability paired with the characteristic mitotically arrested state of neurons brings to light the importance of stroke research.

An important and exciting finding is the delayed neuronal death characteristic of a stroke. When a stroke occurs there are two regions that are affected in two different manners i) the ischemic core which typically is associated with irreversible necrotic cell death and ii) the penumbra area which typically takes 2-3 days to mature to infarction (Kirino, 2000) (Figure 1). The discovery of the penumbra revealed an opportunity where potential therapeutics may be generated and applied in order to reverse the delayed neuronal death process within the 2-3 day time frame.

Excitotoxicity and Apoptosis

Investigation into the molecular mechanisms associated with delayed cell death has unveiled the hypothesis of excitotoxicity. The normal function of the brain requires the use of neurotransmitters to communicate to adjacent neurons

across the synaptic cleft. Glutamate is the major excitatory neurotransmitter of the mammalian central nervous system capable of activating ligand gated ionotropic receptors such as N-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid (AMPA) and kainate receptors. In brief, NMDA receptors, when stimulated by neurotransmitters function to depolarize the cells by causing a conformational change of the receptor, which opens the channel releasing Mg^{2+} and therefore allowing the influx of Ca^{2+} and Na+ in exchange for an efflux of K^{+}. The uncontrolled release of glutamate and activation of the NMDA receptor and non-NMDA receptors (AMPA and Kainate receptors) is also essential in the disease state of ischemia. Antagonists to the NMDA receptors were found to protect neurons *in vitro* (Rothman, 1984) and *in vivo* (Simon et al., 1984) against hypoxic damage and ischemic damage respectively. Soon after the implication of NMDA receptors, calcium was also found to play an important role as a mediator in glutamate neurotoxicity. A group lead by Randall and colleagues (1992) determined that there are three phases of glutamate-induced cell death with varying concentrations of calcium. After a short 5 minute exposure to glutamate the phases proceed as follows: 1) there is an increase in intracellular calcium which persists for a short duration of 5-10 minutes. 2) The dormant phase begins where calcium levels are attenuated to average levels for approximately 2 hours. 3) A steady increase in the concentration increases to an overload concentration plateau which is associated with cell death. The increased levels of calcium may also produce, as a consequence, the reported ATP depletion that is associated with ischemia

causing cell death. Mitochondria have the ability to sequester calcium from the cytosol, and in the case where mitochondria take up calcium in excess would cause a decrease in the mitochondria membrane potential. Mitochondria dysfunction would result as a consequence and the cell would cease to produce ATP (reviewed in Nicotera et al., 1990).

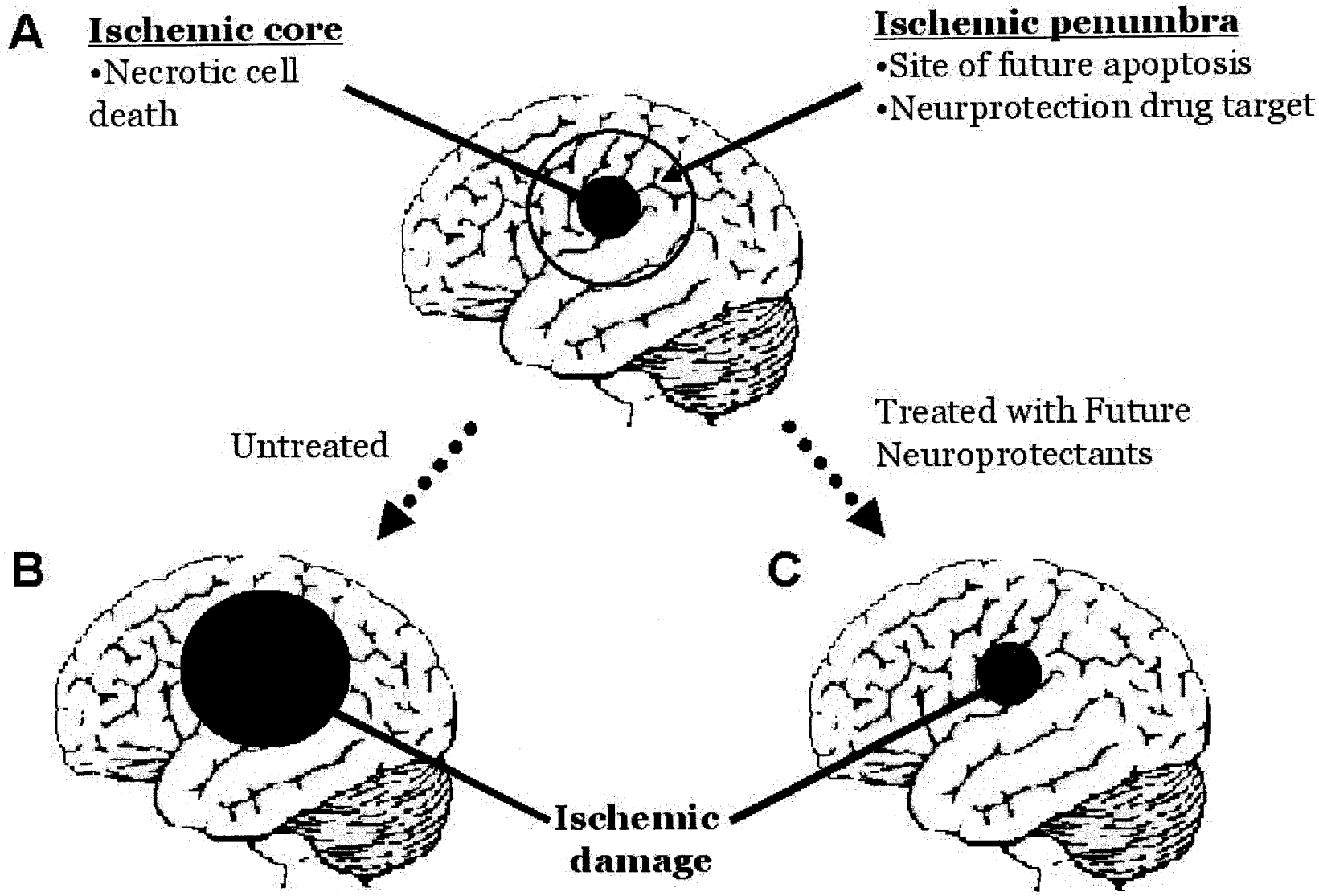
To date the exact mechanism of glutamate induced neurotoxicity in ischemia is not yet defined. However, increased calcium mediates the activation of enzymes such as protein kinases, phospholipases, nitric oxide synthases, and proteases and causes the inhibition of protein synthesis as well as free radical formation. It has been proposed that the mechanism of excitotoxicity may be attributed to the uncontrolled and therefore potentially lethal activation of one or more of the processes initiated by the influx of calcium (reviewed in Beal, 1992).

Delayed neuronal death and the discovery of the penumbra have emerged as a great opportunity in research and the production of novel therapeutic approaches to prevent extensive neuronal loss. Neuronal death following focal ischemia manifest itself within 24 hours of the traumatic event and the infarction was demonstrated by Du and colleagues (1996) to be predominantly due to excitotoxic necrosis. Alternatively, following the elicitation of a mild ischemic episode whereby neuronal death was not immediately observed, the group subsequently described a delayed neuronal death. Du and colleagues showed that 1 day following transient ischemia no death was observed. However 3 days later an infarct had developed and at 2 weeks following the mild ischemia the volume of neuronal death was similar to the severe ischemia model (Du et al.,

1996). These findings suggest that even if excitotoxic necrosis is improved, apoptosis may compensate and/or possibly be promoted. Caspase activation has been subsequently found to be augmented within 9 hours following brief ischemia (30 minutes). Furthermore, following ischemia and reperfusion, treatment with zDEVD-fmk (a caspase inhibitor) reduced ischemic damage which lasted for the 3 week observation period (Fink et al. 1998) and treatment against stroke with apoptotic inhibitors has provided evidence that targeting apoptosis holds promise (reviewed in Kreuter et al., 2004). However the area of stroke recovery and stroke therapy has some major hurdles to overcome in research and in clinical trials (Gladstone et al. 2002). In present day therapeutic setting, there are no treatments that target the delayed neuronal death phenotype induced by ischemia.

Currently, the best treatment minimizing the effects of stroke uses recombinant Tissue Plasminogen Activator (r-TPA) which is used in some patients to restore the cerebral circulation. The National Institute of Neurological Disorders and Stroke (NINDS) produced a study that showed minimal to no disability in 30% of patients using the r-TPA properly screened and dispensed in a 3 hour time frame from the initiation of the stroke (Del Zoppo *et al.*, 1992). The r-TPA treatment demonstrates that cells in the penumbra can be rescued but the treatment itself is not ideal as there is the restriction of the 3 hour window of time. Research directed at understanding the pathophysiology of a stroke and the molecular mechanisms underlying neuroprotection will serve in the generation of therapy to prevent and reduce ischemic damage.

Figure 1. The areas of infarct following ischemia. (A) Focal ischemia or a stroke causes necrotic death and an infarction occurs (red circle). At this state the penumbra (A) contains cells that are still viable. (B) Without treatment the infarction increases in volume as quickly as 3 days. (C) Future drugs targeting the penumbra, will provide neuroprotection to the area in an attempt to limit the ischemic damage and stop excitotoxicity.


A
Ischemic core
•Necrotic cell death
Ischemic penumbra
•Site of future apoptosis
•Neurprotection drug target
Untreated
Treated with Future Neuroprotectants
B
C
Ischemic damage

Preconditioning

Single celled organisms exposed to environmental sublethal stresses acquire transient tolerance to subsequent environmental stress that would otherwise be lethal. Based on this model of single cell induced tolerance, research in coronary occlusion was first conducted by Murray and his colleagues (1986), which uncovered an induced transient tolerance of the whole organ, the heart, to a subsequent ischemic event. The revolutionary discovery led the team to describe the transient tolerance as "pre-conditioning". Following to the research accomplished by Murray's group, sublethal doses of brief ischemia administered to the brain, showed a similar induced tolerance of the vulnerable hippocampal CA1 neurons causing a protection that has never been previously reported in the brain (Kitagawa *et al.*, 1990). In addition, when the induced tolerance of the brain is compared to that of the heart, the brain will maintain a tolerance over a period of approximately 3 days while the heart will loose the tolerance within a few hours. The prolonged tolerance characteristic to the brain effect is known as a delayed tolerance while the weaning of tolerance observed in the heart is known as immediate tolerance.

Preconditioning induced ischemic tolerance is unlikely to ever become a useful means of increasing the brain's resistance to subsequent damage in a clinical setting. However, preconditioning does provide an experimental model to comprehend the responses involved in neuroprotection from cerebral ischemia.

Since the first report of induced tolerance by brief episodes of sublethal ischemia in 1990, other methods of preconditioning have emerged with similar

protection to the hippocampus results (reviewed in Kirino, 2002) (Table 1) but with less damaging effects. Induced tolerances from noxious stress other than ischemic insults are known as cross-tolerance as the origin of stress is different from the disease type. Although many preconditioning treatments have been found, the important molecular mechanism by which any of these treatments generates a neuroprotective effect is poorly understood. However, increasing evidence indicates that preconditioning induced ischemic tolerance is dependent on *de novo* protein synthesis and gene expression (Barone *et al.*, 1998).

A potential approach to the treatment of stroke is to harness the molecular events elicited by neuroprotective treatments. To date there have been many advances regarding the recognition of genes that have demonstrated their potential as therapeutic neuroprotective factors by correlating their expression during preconditioning with the neuroprotective time window. These genes include but are not limited to stress proteins such as hsp70 and hsp72 (Kitagawa *et al.*, 1991), stress response transcription factors such as Nuclear Factor-κB (Blondeau *et al.*, 2001), proinflammatory genes such as COX-2 (Yrjänheikki *et al.*, 2000) and immediate early genes such as c-Jun and c-Fos (Hermann et al., 1998).

Cortical Spreading Depression

Our studies have focused on a preconditioning treatment called Cortical Spreading Depression (CSD) characterized by the depolarization of neurons and glia across the cerebral cortex with a massive redistribution of ions (Lauritzen,

Table 1. Various preconditioning methods previously described to induce tolerance to a subsequent ischemic event. Cross-tolerance refers to the nature of the stress differing from ischemia, but has been reported to provide protection against ischemia. Cortical Spreading Depression (CSD) is represented as spreading depression within the Table.

Table 1 - Inducers of Ischemic Tolerance

Inducers of tolerance
Brief transient focal ischemia
Sublethal global (forebrain) ischemia
Inducers of Cross-tolerance
Hyperthermia (heat stress)
Anoxia/Hypoxia
Spreading depression
Epilepsy
Inhibition of oxidative Phosphorylation
Oxidative Stress
Lipopolysaccharide
Traumatic brain injury
Hypothermia

Adapted from Kirino, T (2002) J Cereb Blood Flow Metab, Vol. 22, No. 11. (Review)

2001). CSD was first described more than 60 years ago (Leao *et al.* 1944) and has subsequently been shown to be induced experimentally by mechanical trauma, electrical simulation, the application of high potassium or a variety of other chemical agents (Somjen, 2001). CSDs may also occur as a natural phenomenon most commonly in patients suffering from: i) head injury, ii) epileptic activity, iii) migraine headaches and iv) infarct tissue (Gorji, 2001).

Interestingly, the relationship between CSD and ischemia may be of a protective nature as well as a destructive nature. These reported opposite effects are determined by the timing of the CSD wave in relation to the ischemic event and therefore the state of the neurons. CSD waves may naturally occur at the periphery of an ischemic core, up to 2 hours following an ischemic event. Although neurons in the penumbra at this time point are still viable, they are in a vulnerable state due to oxygen/glucose deprivation. The onset of CSD increases the release of glutamate in these metabolically compromised cells, initiating an excitotoxicity demonstrated by an increase in ischemic volume and the deterioration in the penumbra (Koroleva *et al.*, 1996). Opposite to this effect, preconditioning the neocortex of a rat with CSD prior to an ischemic event has revealed its neuroprotective properties by showing a decreased infarct volume following subsequent middle cerebral artery occlusion (MCAO) (Pérez-Pinzón M., *et al*, 1997). As few as two waves of depolarization may be sufficient to produce neuroprotection (Chow *et al.*, 2002) and maximal protection offered by CSD preconditioning can occur up to 3 days after treatment (Osuga *et al.*, 1996). The maximum length of time offering protection after a prolonged treatment of CSD

has been reported to last up to 15 days in one study (Yanamoto *et al.*, 1997). In addition to neuroprotection, CSD causes a prolonged increase in local blood flow and tissue oxygenation (Mies and Paschen, 1984; and Wolf *et al.*, 1997) and does not provoke morphological or metabolic damage in normal brain tissue (Gorelova *et al.*, 1987; Somjen *et al.*, 1990).

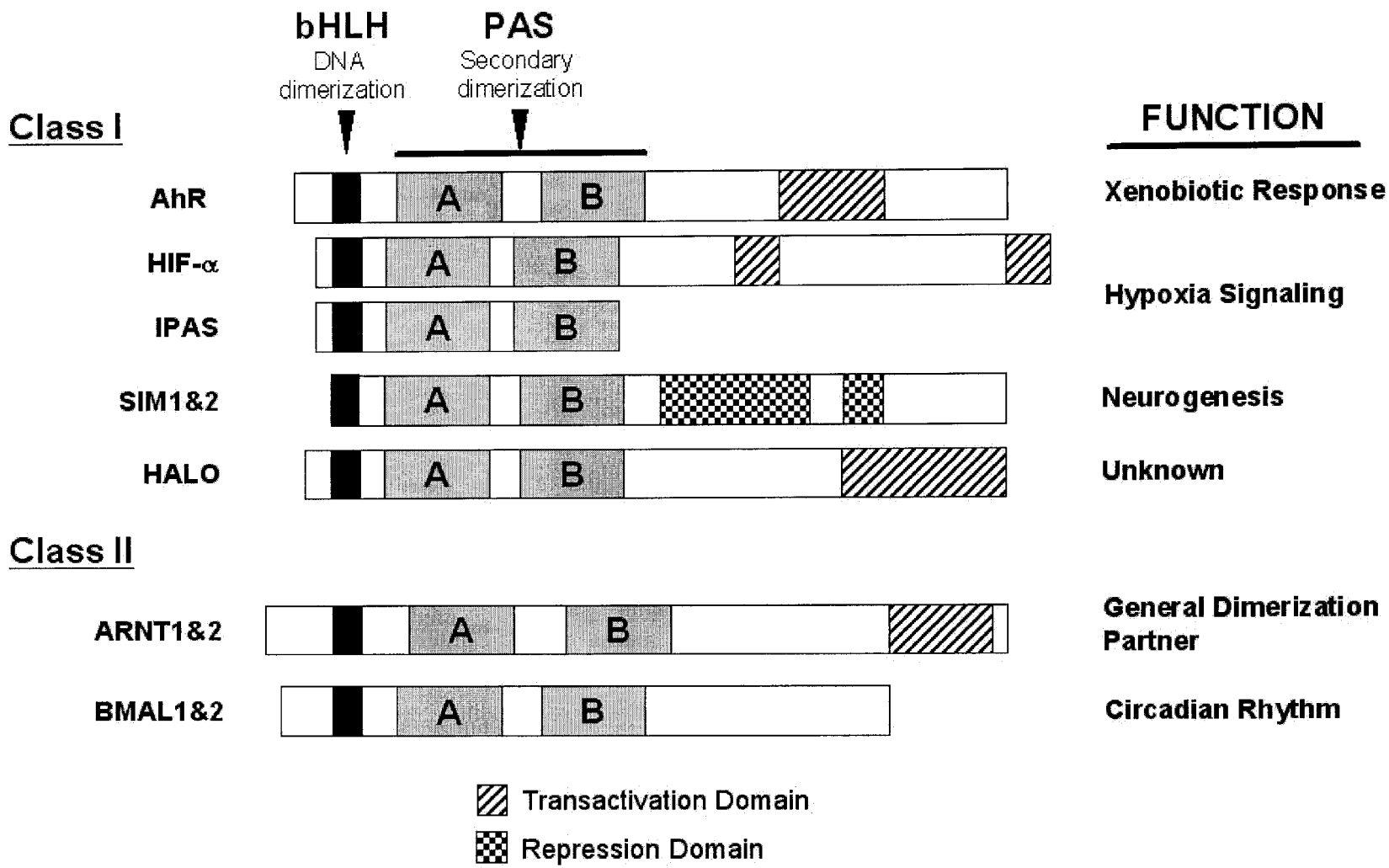
CSD represents a more attractive model for studying induced tolerance than the brief ischemic episode model as it is not as harmful to the brain and CSD will elicit a tolerance over the whole cortex compared to the induced tolerance observed in only the area of the brief ischemic episode. If this phenomenon is to be clinically useful, the molecular mechanisms mediating the neuroprotective response elicited by CSD must be identified and exploited.

CSD induced gene expression has long been established with examples of gene products including genes that have also been associated with neuroprotection and ischemic tolerance (Candelario-jalil, 2002, Chow *et al.*, 2002, Rangel *et al.*, 2001, Whitfield *et al.*, 1999). Examples of genes that have neuroprotective properties and who's expression are induced by CSD include HSP-72 (Plumier, 1997), COX-2 (Yrjänheikki, 2000) c-fos (Kitahara, 2001) and c-jun (Koistinaho, 1999). These findings suggests that CSD triggers an extensive genetic response and that understanding the molecular basis of this response is critical to the therapeutic manipulation of neuroprotective signalling system.

bHLH-PAS Transcription Factors

Transcription factors may have the most potential as neuroprotectants response as they have the capability to regulate a multitude of gene elements that could cause an overall protective environment for the cell to survive. Basic helix-loop-helix (bHLH)-PAS proteins are a subfamily of the bHLH transcription factors (TF) typically containing two structurally conserved PAS (Per, Arnt, Sim) domains. Transcription regulators of this family carry out multiple roles within various eukaryotic regulatory systems such as the circadian clock system (Clock gene), physiological stress response such as hypoxia (HIF-α genes), central nervous system formation in Drosophila (Sim genes) and metabolism of xenobiotics (AhR and ARNT) (Reviewed in Kewley, 2004). There are two distinct classes of bHLH-PAS TF distinguished by their ability to dimerize with other bHLH-PAS transcription factors. Most of the bHLH-PAS transcription regulators fall into the first class represented by their ability to form a functionally active heterodimer complex with class II factors. Class II TFs, (which includes BMAL1 & 2 and ARNT1 & 2) have the ability to form functionally active homodimers as well as herterodimers (Figure 2). Upon dimerization of the bHLH-PAS TFs, the complex is then capable of binding to known DNA elements (See Table 2). In addition to DNA, each monomer subunit binds a core sequence within the element. For example class II ARNT proteins have the ability to bind 5'- GTG -3' sequence within the HRE element when dimerized with HIF-α (Table 2 underlined). HIF-α in turn binds the adjacent 5'-TAC-3' sequence. Therefore ARNT in a homerdimerization complex has the ability to bind 5'CACGTG-3' which is a form of the E-Box motif.

Figure 2. Schematic representation of a sample of the bHLH-PAS transcription regulators. Familly membes include transactivators as well as transcriptional repressors. Class I bHLH-PAS members form active complexes by heterodimerizing only with class II bHLH-PAS TFs. Class II members form active complexes by heterodimerization or homodimerization with class I or II factors.



Adapted from Kewley et al. (2004) Int. J. Bioch. Cell Biol. Vol 36, No. 2 (Review)

Table 2. A list of some of the known bHLH-PAS transcription factor family members and their reported binding elements. CME – Central Midline Enhancer; HRE – Hypoxia Responsive Element; XRE – Xenobiotic Response Element; CME-v – CME-variant.

Table 2 - Binding Elements Associated with bHLH-PAS Transcription Regulators

bHLH-PAS Transcription Regulator	Binding Element	Binding Element Sequence	Reference
SIM1/2	CME	G/ATACGTGA	Pelletier J. and Moffett P. (2000) FEBS Lett, Vol. 466, p. 80
HIF-1/2/3 _	HRE	TACGTG	Michel G et al. (2002) Biochem Biophys Acta. Vol. 1578 p. 73
AhR	XRE	NGCGTG	Fujisawa-sehara et al. (1988) PNAS USA Vol 85, p. 5859
NXF/HALO	CME-v	NTCGTG	Ooe N. et al. (2004) Mol. Cell Biol. Vol. 24 p. 608
ARNT1/2[1]	E-Box	CACGTG	Kewley R.J. and Whitelaw, M.L. (2005) Biochem Biophys Res Commun Vol. 338, p. 660

[1] – ARNT 1/2 have the ability to bind the 5'- GTG -3' (underlined) core sequence when it forms functionally active dimmer complexes with any of the bHLH-PAS TFs listed above.

bHLH-PAS transcription factors' important role in development can be illustrated by the various knockout mice generated to specific bHLH-PAS genes such as HIF-1&2 α, SIM 1&2 and ARNT. HIF-1α knock out mice die *in utero* by embryonic day 10.5 due to poor vascularization throughout the embryo. Three different HIF-2α (also known as Endothelial PAS or EPAS) targeted knock out mice have been developed and have associated phenotypes. The first knockout demonstrated a decrease in levels of catecholamines, a tyrosine based neurotransmitters of the sympathetic nervous system consisting of epinephrine, norepinephrine and dopamine. The mid-gestation lethality of these knockout mice could be rescued by treatment with a norepinephrine precursor known as L-threo-3,4-dihydroxyphenylserine (DOPS). The other two HIF-2α knockout mice have varying degrees of vascularization deformities and die as neonates of respiratory distress syndrome due to immature development of the lungs.

SIM1 and 2 transcription regulators have been reported to be repressors within the mammalian system even though their Drosophila counterpart has been reported to have transactivational properties. mSIM1-/- mice die during the period around birth while the mSIM2 knockout mice die within 3 days of birth. Similar to the HIF-2α knockout mice, the SIM2 knockout mice die due to breathing failure caused by immature development of the lung wall.

Not surprisingly, similar to the HIF-1α knockout mice, the ARNT knockouts die *in utero* by embryonic day 10.5 with defects in vascularization of the placenta and solid tissue. Notably, the ARNT knockout mice are similar to the phenotype

found in mice deficient in vascular endothelial growth factor (VEGF), a target of HIF-1α /ARNT.

The wide spectrum of genetic regulation such as erythropoietin (EPO), VEGF, circadian rhythm controlled genes and a variety of drug metabolising enzymes further establishes the bHLH-PAS family of TFs as having important functions relating to the cells response to environmental stress. Although the bHLH-PAS family covers a wide field of regulatory systems, its role is both deliberate and select regarding the downstream expression events it controls.

The most notable of all bHLH-PAS proteins in relation to ischemia is the Hypoxia-inducible Factor 1 (HIF-1α). HIF-1α is translated under a normal state of oxygen availability (normoxia) by the cell but is degraded by the proteosome. This is due to the recognition of the hydroxylated proline residues found within the oxygen degradation domain by the von Hippel-Lindau (VHL) protein, an E3 ubiquitin ligase. Under hypoxic conditions, the proline residues are not hydroxylated, due to the inactivity of the oxygen-dependent hydroxylases. In this state, HIF-1α is allowed to accumulate, and become functionally active by heterodimerizing with ARNT to activate its target genes. With over 40 target genes identified to date, HIF-1α has a variety of roles in the body including vasomotor regulation, angiogenic signalling, energy metabolism, hormonal regulation, growth and apoptosis and other (reviewed in Schofield and Ratcliffe, 2004). It is not surprising that HIF-1α is induced in a number of manners in relation to focal ischemic stroke: 1) HIF-1α is induced following a permanent focal ischemic stroke due to the continued hypoxic state of the penumbra. 2) is

induced following transient global cerebral ischemia, characterized by neuronal loss within brain regions such as the hippocampus and the cortex, and 3) is induced 1 hour following transient global ischemia by cardiac arrest but the accumulation, most interestingly lies within the rat cerebral cortex. Furthermore, these high levels of HIF-1α remain in the cortex for up to 7 days following treatment. Considering that blood flow returns within 20 minutes and that surrounding tissue return to normoxia within 2 days, other mechanisms may be causing the stabilization of the HIF-1α protein (reviewed in Sharp and Bernaudin, 2004).

HIF1 has been demonstrated to play a role in induced tolerance to ischemia by preconditioning rat cortex with sublethal doses of hypoxia which requires the synthesis of RNA and protein products (reviewed in Sharp et al. 2004). HIF-1α target genes that have been reported to provide protection in this model include, but are not limited to: EPO, VEGF, adrenomedullin, MT-1 and others (Bernaudin et al., 2002). Not surprisingly, induced tolerance to ischemia by preconditioning a rat brain with sublethal dose of hypoxia induces a sustained expression of HIF-1α. In addition to inducing HIF-1α by preconditioning with hypoxia, preconditioning by pharmacological induction of HIF-1α, with iron chelators (such as CoCl), provides induced tolerance to brain ischemia. Therefore, the induction of HIF-1α and its target genes are thought to be responsible in part for providing the reported protection against subsequent ischemic events. (Bergeron et al. 1999).

Recently, a novel bHLH-PAS transcription regulator, termed NXF, was reported with distant sequence similarity within this family of proteins (Ooe, *et al.*, 2004). A functional role for NXF has presently not been established. However, northern hybridization analysis demonstrates the low level expression of NXF to be unique within the adult human brain suggesting a potential neuro-physiological function. Using a subtractive hybridization technique known as Representational Difference Analysis (RDA) (Hubank and Shatz, 1994) we have isolated NXF in rat cortex treated with CSD.

As a bHLH-PAS transcription factor, NXF appears to be a good candidate in becoming a neuroprotective gene as this family of transcription factors have already demonstrated their implication in various physiological stresses such as hypoxia (HIF), hypoglycaemia (ARNT) and toxic compound stresses (AhR). The nature by which NXF was recovered demonstrates further its candidature as a gene with neuroprotective properties.

Hypothesis and Rationale

De novo protein synthesis and gene expression have been shown to contribute an important part of the underlying molecular mechanisms, which may confer neuroprotection. The observation of preconditioning induced ischemic tolerance can be exploited to become clinically relevant by examining the genetic profile of the preconditioning treatment, which forms the basis of the delayed type protection.

Apoptosis, or programmed cell death, is thought to contribute to the underlying mechanism of the characteristic delayed neuronal death within the penumbra. Good candidate genes targeting and inhibiting this process would be the Inhibitor of Apoptosis Proteins (AIPs) such as XIAP and NIAP. Preconditioning with CSD reveals however, that this family of protein are not induced by CSD, suggesting that protection is mediated by other molecular mechanisms. Therefore, the role of other families of genes that may or may not be involved with apoptosis need to be explored.

CSD promotes the expression of immediate-early genes of the Activator Protein-1 (AP-1) family such as junB, c-jun and most notably c-fos (Hermann, 1998). This family of transcription factors are involved in neuronal function, cellular defence mechanisms and apoptotic cell death. Their activation by CSD has proven to be transient as the expression levels of mRNA are attenuated 45 to 75 minutes after a CSD elicitation. Similarly, the Extracellular signal-Regulated Kinase 1 and 2 (ERK1/2) pathway known to activate immediate early gene products, has also been reported to be phosphorylated and therefore activated by CSD (Chow, 2002). The phosphorylation of Erk1/2 was reported to be transient as well, attenuating after 45 minutes of activity.

Transcription factors are promising candidates that have the potential in regulating many genes products resulting in protection. Researching novel transcription factors that are induced by CSD may prove to be a useful strategy to help uncover mechanisms underlying CSD-induced neuroprotection.

We hypothesized that CSD preconditioning acts to modulate gene expression of transcription regulators, resulting in downstream targeting and gene expression of putative neuroprotectants.

Our Objectives were to:

1) Isolate novel gene induced at early time points of CSD elicitation.
2) Determine the functional characteristics of the novel proteins.
3) *In vitro* analysis of cells over expressing the novel gene with respect to protection against apoptotic treatments.

While there are most likely a myriad of factors that contribute to ischemic tolerance, the investigation of novel genes has the potential to reveal novel neuroprotective mechanisms either by unveiling unique pathways or linking known pathways to neuroprotection.

II) MATERIALS AND METHODS

Cortical Spreading Depression Surgery

All surgical procedures followed the guidelines of the Canadian Council for Animal Care and were approved by the Animal Care Committee of the University of Ottawa. Male Sprague Dawley rats were obtained from Charles River (Montreal, Canada) weighing between 250-275 grams. They were allowed to habituate to their surroundings for five days before manipulation. At the time of the surgery, rats weighed between 290-330 grams. All surgical procedures and decapitations were performed under halothane anesthesia (4% induction, 0.75% maintenance). Body temperature of the animals was monitored at all times and maintained between 36.5°C and 37.5°C, using a homeothermic feedback blanket system (Harvard Apparatus, South Natick, MA, USA)

After anaesthetization, the tail artery of every animal was catheterized using polyethylene tubing to monitor mean arterial blood pressure (MAP), blood gases, and blood glucose during the experiment. The animals were then mounted in a stereotaxic apparatus (Stoelteing Co., Wood Dale IL, USA). A 2 mm burr hole was drilled in the skull over the left occipital cortex (Bregma AP -6.8mm, ML 5.6mm) without damaging the dura mater for pledget placement. A 0.5 mm diameter burr hole was placed in the left frontal cortex (Bregma AP -2.0 mm, ML 5.0 mm) without damaging the dura for platinum electrode placement. The platinum electrode was inserted 2 mm into the frontal cortex and then secured to the skull with dental cement. This electrode was used t record the CSD waves. A copper reference electrode was threaded though the skin on the back of the

animal's neck. Our follow up studies used silver/silver chloride electrodes as both the recording and reference electrodes. CSDs in the left hemisphere were induced for 2 hours by application of a cotton pledget soaked in 0.5 M KCl. 0.5 M NaCl was placed on the intact dura of the pledget was replaced every 15 minutes for the 2 hour time frame. In addition, blood pressure and renal temperature was monitored and recorded every 15 minutes and blood gases and blood glucose measurements obtained every 60 minutes. Two hours after the first pledget placement, the last pledget is removed, the dura rinsed with normal saline, and the skin over the skull sutured. The tail cannula was also removed and the tail sutured. Rectal temperature was monitored and maintained between 36.5°C and 37.5°C until animals recovered from anaesthesia, at which point they were returned to their cages. Three days post-CSD, experimental animals were deeply anaesthetized with 4% halothane, decapitated and the brain rapidly removed for plasma membrane extraction.

RNA extraction and Northern Blot Hybridization

Total RNA was extracted from various rat tissues using 4M guanidinium thiocyanate RNA isolation described as follows. Tissues were homogenized in 5 mL of GIT solution (4M Guanidine thiocyanate, 10mM EDTA, 50mM Tris.HCl pH 7.5, 8% (v/v) 2-mercaptoethanol.) 500 μl of 2M NaOAc, 5 mL of phenol and 1 ml of chloroform, shaken vigorously to extract lipids and centrifuged at 4000g for 10 minutes. The top aqueous phase was recovered and one volume of isopropanol was added and then centrifuged. The RNA pellet was resuspended in 400 μl

RES-1 buffer (0.5M LiCl, 1M Urea, 20 mM citrate, 5 mM EDTA and 1% SDS, pH 6.8)) and sonicated. 4 μL proteinase K (10 mg/mL) was added and the solution was incubated for 30 min at 50°C. Following the incubation period 26 μL of 2 M NaOAc and 1 mL of EtOH was added and the solution was centrifuged. 400 μL of RES-1 was added to the pellet and the proteinase K digestion step was repeated. After the second digestion, 100 μL of phenol/chloroform mixture (1:1) was added and centrifuged. Chloroform was added to the extracted top phase and was centrifuged. The top phase was recovered, 400 μL of 5M LiCl and 4 μL of 2 N acetic acid were added and the solution was incubated overnight on ice (0°C). The following day the solution was centrifuged and after discarding the supernatant the RNA was redissolved in 400 μL of CCS (1 mM citrate, 1 mM EDTA and 0.1% SDS, pH 6.8), 4 μL 2M NaOAc and 1 mL of EtOH. After repeating this step the RNA pellet was redissolved in CCS buffer and the final concentration was calculated based on 1 OD_{260} = 40 μg/mL RNA. Following RNA extraction, samples were subjected to Northern blot analysis described as follows. 20 μg of total-RNA were subjected to electrophoresis in a 1% formaldehyde gel (1 X MOPS buffer (0.2M morpholinopropanesulphonic acid, 50mM sodium acetate, 5mM EDTA, pH 7.0), 2.2M formaldehyde and 1% (v/v) agarose). Immediately prior to loading, 20 μg RNA was diluted to 10 μL with CCS solution, 10 μL denaturation buffer was added (6.6M formamide, 10% formaldehyde in 1 x MOPS buffer) in addition to 2 μL loading dye (50% Glycerol, 1 mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol). Samples were heated to 65°C for approximately 10 minutes to denature any secondary

structure and cooled on ice for 2 minutes. Following electrophoresis, the gel was washed in 1 μg/ml ethidium bromide for 30 minutes to aid visualization of RNA. RNA was transferred onto a nitrocellulose membrane (Eppindorf) by capillary transfer overnight. The following day the membrane was washed in twice 2 x SSC for 15 minutes and allowed to incubate for one hour at 65°C with prehybridization solution (5 x Denhardt's, 1 M Na-phosphate, 250 μL ssDNA, 6 x SSC, 1% SDS and 5% dextran). The BamHI-HindIII digested HALO probe was radiolabeled with the Random Prime labelling Kit (Amersham) as per the manufacturer's instructions. ^{32}P-radiolabeled probe was added to the prehybridization solution and membrane and incubated overnight at 65°C. The following day, the membrane was washed at room temperature with 2 x SSC, and then washed at 65°C with 2 x SSC, 0.1% SDS for 1 hour. Depending on the signal, the blot was washed with 0.1 x SSC and 0.1% SDS at 65°C as needed. The membrane was then exposed to Kodak Biomax XAR film overnight to two weeks (depending on signal) at -80°C.

Cloning

All PCR and RT-PCR amplifications employed the Eppendorf MasterCycler gradient thermal cycler. Full length HALO coding region was recovered by RT-PCR (up:5'-ATGTACCGATCCACCAAGGGC-3'; down:5'-TCAAAACGTTGGTTCCCCTCC-3', based on accession no. AB050103) using 1 μg of total RNA from CSD-treated brain. Products were subcloned into pGEM-T Easy and sequenced. No differences were found between rat HALO and the

reported rat NXF sequence. GAL4 fusions were generated by subcloning HALO restriction fragments in-frame with the yeast GAL4 DNA binding domain (pcDNA3-GAL4-DBD; provided by Michael Rudnicki, Ottawa). For reporter assays, a luciferase reporter construct bearing 4 GAL4 binding sites (GAL4-Luc), 3 hypoxic response elements (HRE-Luc; kindly provided by Carine Michiels, Namur, Belgium) or 2 central midline enhancers (CME-Luc; provided by Jerry Pelletier, Montreal, Canada) upstream of the minimal SV40 promoter was used in co-transfection experiments.

Cloning of the Human BAX promoter regions employed genomic DNA extracted from HeLa cells with the Qiagen DNeasy extraction kit (cat # 69504). Each piece of the BAX promoter (region 1 upstream 5'-aagcttagagccccgctgaac-3', downstream 5'- cagcccgggaattccagactg-3'; region 2 upstream 5'-cagtctggaattcccgggctg-3' and downstream 5'-gtccaagagatcttcctgacac-3'; region 3 upstream 5'- gtgtcaggaagatctcttggac-3' and downstream 5'-gggtctcaccatggtacagcc-3') was amplified with the Vent polymerase system (NEB) containing a proofreading capability and subcloned into the pGEM-T for sequence analysis. No difference between the targeted HALO binding sites were from the reported promoter sequence were identified.

Cell Cultures, Transfections and Viral Infections

HeLa, N1E-115, 293 cells were maintained in DMEM with 10% foetal calf serum at 37°C in a humidified atmosphere containing 5% CO_2. Cultures were transfected with HA-tagged HALO, or HA-HIF1α (a gift from Stephen Lee,

Ottawa), and Myc-tagged ARNT2 (provided by Oliver Hankinson, UCLA) expression vectors using lipofectamine 2000 according to the manufacturer's instructions.

Cortical cultured neurons (CCN) were prepared from 14-15 day old Sprague Dawley rat embryos. The embryos were dissected in 1 x Hanks Basic Salt Solution (HBSS; Gibco) and freshly dissected cortices were dissociated by mechanical disruption and by incubation at 37°C for 20 min in neurobasal (Gibco) solution containing 0.3 mg/mL trypsin. Equal volumes of neurobasal solution containing 0.1mg/mL trypsin inhibitor and 0.125 mg/mL DNAse1 were added and mixed by inversion for 2 minutes and then centrifuged. Supernatant was aspirated and 5 mL neurobasal solution with 0.5 mg/mL trypsin inhibitor and 0.7 DNase1 was added and allowed to settle for 5 minutes. The supernatant was recovered and the step was repeated. The supernatants were combined and centrifuged for 5 minutes at 1000 rpm and resuspended in culturing media. CCNs were counted on a hemocytometer and plated at a density of 7.5×10^5 cells/mL on plates coated with 20 µg/ml poly-D-lysine (PDL) and incubated at 37°C with 5% CO_2. Cells were cultured in Neurobasal (GIBCO) containing, 1.25 mL L-glutamine (sigma), 2.5 mL penicillin and streptomycin (GIBCO), and completed with N2 and B27 (GIBCO). Cultures were allowed to mature to 7 days before all experiments were preformed.

Cerebella granule neurons (CGN) were extracted from 7-9 day old mice. Freshly dissected cerebella were dissociated by mechanical disruption and by incubation at 37°C for 20 min in solution B (solution 1; DMEM (sigma), 14.5 mM

D-(+)-glucose, 25 mM Hepes Buffer and 3mg/mL BSA V) containing 0.3 mg/ml trypsin. Trypsinization was ended by adding equal volumes of Solution C (Solution 1 with 0.17 mg/mL trypsin inhibitor and 0.25 mg/mL DNAse 1). The mixture was centrifuged at 1000 rpm for 5 minutes and the supernatant was recovered. 2 mL of solution D (solution 1 containing 1mg/mL trypsin inhibitor and 0.75 mg/mL DNAse1) was added to the recovered supernatant, titrated 10 times and allowed to settle for an additional 5 minutes. The supernatant was placed in a separate vial and the titration step involving solution D was repeated three more times. The supernatants were combined in a fresh tube and 0.3 mL of solution E (solution 1 with 0.01 mM CaCl) was added for every mL of supernatant recovered. This blend was mixed and allowed to settle for 10 minutes. The supernatant was recovered, centrifuged for 5 minutes at 1000 rpm. Fresh media was added to the pellet and the cells were counted on a hemocytometer (Fisher). They were plated at a density of 1.5×10^6 cells/ml on plastic culture wells coated with 20 µg/ml poly-D-lysine (PDL) and incubated at 37°C with 5% CO_2. Cells were cultured in Neurobasal (GIBCO) containing, 25 mM KCl, penicillin, and streptomycin (GIBCO), and completed with N2 and B27 (GIBCO). Cells were immediately infected with adenovirus (MOI=50) upon plating on a poly-D-lysine coated plate in growth medium without antibiotics. Antibiotics were added the following day. Five days after plating, propidium iodide (PI) in a final concentration of 0.02 mg/ml was added to the medium, washed twice with 1 x PBS, fixed in 4% PFA washed twice with 1 x PBS and then stained with DAPI. Images were captured by immunofluorescence on an Zeiss Axiovert S100

inverted microscope with a Sony power HAD 3ccd color video camera (Zeiss) to evaluate the CGN infections. For each experiment 4 wells were infected and the number of cells were based on the average of 3 images taken of each well. Each experiment was repeated in triplicate.

In Situ Hybridization

For whole mount in situ hybridization, a 397bp ApaI-BamHI mouse genomic fragment corresponding to exon 8 was subcloned into pBluescript (Stratagene) and used as sense and anti-sense template. Mouse embryos were dissected out and placed in cold PBS solution. A hole was punctured into the back of the brain cavity prior to adding 0.3% hydrogen peroxide and incubated at room temperature for 30 minutes. The embryos were washed with PBS with 0.1% Tween-20 (PBST) and dehydrated in methanol/PBS solution series as follows: 25%, 50%, 75% then 100% methanol. Embryos were stored at -20°C until used. Embryos were rehydrated in reverse sequence series as follows: 75%, 50%, 25% methanol and then placed in PBST. The embryos were washed three more times for 5 minutes in PBST and then in placed in a solution containing 4.5 μg/ml proteinase K/PBST for 15 minutes. The proteinase K digesting was stopped by replacement with PBST containing 2mg/ml glycine and then rinsed three more times in PBST for 5 minutes. Embryos were fixed in 4% paraformaldehyde and 0.2% glutaraldehyde in PBST for 15 minutes and then rinsed three times with PBST for 5 minutes. Embryos were then washed in hybridization solution (50 ml solution : 0.5 g Boehringer Block, 25 ml formamide,

12.5 ml 20X SSC, 100 µl 50mg/ml heparin, 250 µl 20% Tween-20, 500 µl 0.5 M EDTA, 6 ml H_2O) for 5 minutes and then prehybridized for 1 hour in prehybridizing solution (hybridization solution plus blocking agent) at 65°C. Probe was added to the hybridization solution and mixed with the embryos and hybridized overnight at 70°C. Probe/hybridization solution was removed and prehybridization solution was added and incubated at 70°C for 30 minutes twice. A 1:1 mixture of TBST and hybridization solution was added and incubated at 70°C for 20 minutes. Embryos were then rinsed three times at 70°C with TBST for 30 minutes each and then blocked with PBST containing 10% serum for 3 hours at room temperature. Embryo was then incubated with pre-absorbed anti-digoxygenin antibody diluted to a final concentration of 1:2000 overnight in the cold room (4°C). The following day, the embryo was quickly rinsed three times with TBST and then washed three times in TBST for 1 hour each wash. Embryos were then washed in alkaline phosphatase buffer (100mM Tris, 50mM MgCl2, 100mM NaCl, 0.1% TWEEN 20, 5mM Levamisole) for 1 hour. 4.5 µL of NBT and 3.5 µL of BCIP were added for every mL of alkaline phosphatase buffer present and incubated in the dark for various amount of time. Reaction was stopped by quickly rinsing in alkaline phosphatase buffer for 5 minutes twice and then washed three times in PBST for 1 hour. Embryos were sequentially diluted in PBST containing glycerol in series as follows: 25% 50% 75% then 100% glycerol.

Immunostaining, Embryo Staining

Following CSD, rats were perfused with 0.9% sodium chloride in 10 mM sodium phosphate buffer (pH 7.2, PBS) followed by Lana's fixative consisting of 4% paraformaldehyde and 0.2% saturated picric acid in 0.16 M sodium phosphate buffer at a pH of 6.9 (27) and the brains were surgically removed. Following sucrose perfusion (10% sucrose in 100 mM phosphate buffer) brain tissues were frozen in isopentane and 12μm cryosections were subjected to immunostaining. Embryos were collected following caesarean sections of timed matings of Balb/c mice and fixed for 1h in Lana's fixative, perfused in sucrose and cryosectioned as above. Primary neurons were fixed for 10 min in 4% PFA and washed in PBS prior to immunostaining. Immunofluorescence analysis was performed using a chicken IgY polyclonal antibody (Washington Biotech) raised against the last 19 C-terminal amino acids of HALO. Pre-adsorption to HALO peptide antigen prior to immunostaining resulted in signal loss. Double immunostaining was performed in combination with anti-βIII-tubulin antibodies (Tuj.1; 1:5) or anti-NeuN (clone A60; 1:5). Primary antibodies were detected using anti-mouse TRITC and anti-chicken FITC secondary antibodies (Sigma).

For focal ischemia, male Sprague-Dawley rats (Charles River, Montreal, PQ, Canada) weighing between 200 and 280 grams were anaesthetized with 4.0% halothane for induction and maintained with 1.0% in a mixture of 70% oxygen and 30% nitrous oxide. Focal ischemia was induced for 2 hours using the method of middle cerebral artery occlusion (MCAO) described previously by Longa et al (1989). Rectal temperature was monitored and maintained at 37.0 $\pm$ 0.5 ^{O} C for the duration of surgery using a homeothermic blanket and heating

lamp. Following the ischemic insult, animals were reperfused for the indicated times and processed for immunofluorescence as described above. Primary cortical neuron cultures were established from 14.5dpc embryos as described above and were maintained until mature 7 day old cultures were established. These cultures were rinsed in 1 x PBS and fixed in 4% paraformaldehyde (PFA) for 10 minutes and then washed three times with PBS. Fixed cells were then incubated at room temperature with 0.3% triton-X solution in PBS for 5 minutes washed twice with PBS for 5 minutes each and then incubated with 7% goat serum for 30 minutes. Cells were then quickly rinsed with 1 x PBS and then the primary antibody in appropriate dilution (anti-HALO and anti-GFAP 1:200) in PBS was added to the cells and incubated at room temperature for 1 hour. Cells were then rinsed twice with 1 x PBS and the secondary antibody (anti-Chicken FITC 1:1000 and anti-mouse TRITC 1:1000) dilution was incubated with the cells for an additional hour. Following the secondary antibody, 1 x DAPI (invitrogen) DNA stain was added to the cells for 5 minutes and then rinsed three times in 1 x PBS and the primary cortical cultured neurons were mounted in antifade (Invitrogen).

Western and Immunoprecipitation

Western blotting was carried out as follows: equal amounts of protein, as determined by the Bradford A_{595} protein assay, were applied to a 4% polyacrylamide stacking gel, followed by an 8 -12% polyacrylamide separating gel. Proteins were then electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (PerkinElmer), which were prewet in methanol for 1 minute,

using 30 mAmps for 75 minutes. Before the PVDF membrane containing the bound protein was blocked with Tris-Buffered Saline Tween-20 (TBST; 500mM NaCl, 20mM Tris, 1% Tween-20) containing 5% w/v powdered skim milk for 1 hour, the PVDF membranes were rinsed in TBST for 5 minutes. The blocking solution was rinsed off with TBST and then incubated overnight at 4°C with the appropriate primary antibody diluted in TBST with 1% w/v powdered skim milk. The following day the membranes were rinsed in TBST twice for 10 minutes before incubation at room temperature with 1:5000 dilution of horseradish peroxidase conjugated secondary antibody for a minimum of one hour. The membranes were rinsed three times in TBST for 5 minutes and twice with 1 x phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4) for 5 minutes before washing for one minute with enhanced chemiluminescence (ECL) substrate. The membranes are then exposed to Kodak biomax XAR film for varying lengths of time.

For co-immunoprecipitation studies, 293 cells were transfected with the indicated expression vectors and whole cell lysates were collected 24 hours later in modified RIPA buffer (50 mM Tris-HCl (pH 7.4), 150mM NaCl, 1mM EDTA, 1% Triton X-100, 0.5 % sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS), 1% NP40 and protease inhibitors; Sigma-Aldrich inhibitor cocktail) as described (25). Lysates (400μg) were subjected to immunoprecipitation using an anti-HA (12CA5) monoclonal and protein A agarose (20μl), washed with NETN buffer (150mM NaCl, 50mM Tris [pH 7.5], 1mM EDTA and 0,1% NP-40) and analysed by western blot using anti-myc (9E10) antibodies. Total lysates were

also evaluated for HALO, HIF and ARNT2 expression prior to immunoprecipitation by western blotting.

Electrophoretic mobility shift assays (EMSA)

Electrophoretic mobility shift assays (EMSA) were performed as previously described (26) using nuclear extracts from transfected 293 and HeLa cells prepared as previously described (Hoppe-Seyler et al., 1991). Cells were lysed directly in the culture dish using a gentle lysis buffer (0.6% NP40 0.15 M NaCl, 10 mM Tris pH 7.9 and 1 mM EDTA) and incubated on ice for 5 minutes. Cells were transferred to a 2 ml Eppendorf tube and centrifuged (1250 g for 5 minutes). 100 μL of nuclear extraction buffer (10mM HEPES pH 7.9, 0.1 mM EGTA, 0.1 mM EDTA, 1.5 $MgCl_2$, 420 mM NaCl, 0.5 mM DTT, 0.5 mM PMSF and 25% glycerol) was added to the pelleted nuclei and incubated on ice for 20 minutes. Following incubation and centrifugation (1250 g for 5 minutes) supernatant containing the nuclear extracts was removed and the protein was quantified using the Bradford protein assay (A_{595}).

The oligonucleotide was prepared as follows. Oligonucleotide (1 μg) was incubated for 30 minutes at 37°C in 1 x PNK buffer containing α-^{32}P dATP (3000Ci/mmol), 1 unit of T4 Polynucleotide Kinase. Equal volumes of Chlolorform:Isoamyl alcohol (24:1) was added to eliminate the protein by centrifugation. The top phase containing the labelled oligonucleotide was added to a g-50 to purify the labelled oligonucleotide away from unincorporated

nucleotides. The radioactivity (CPM/ μL) was counted using a scintillation counter and the labelled oligonucleotide was diluted to around 50 000 CPM/ μL.

The binding assay was carried out as follows: 1 μg dI:dC (Amersham), 10 μg BSA (NEB), 5 μg nuclear extract was mixed in a 1x Bandshift buffer (250mM HEPES pH 7.6, 50 mM $MgCl_2$, 340 mM KCl). 300 x mutant or wild type competitor was added to mix and allowed to bind on ice for 15 minutes. ^{32}P labelled oligonucleotide was added to the mix and incubated on ice for an additional 15 minutes. 2 μL of Bandshift dye (20% Ficoll 400, 0.1% Bromphenol blue) was added and each sample was electrophoresed for 4 hours at 150 volts in a 6% acrylamide gel containing 0.25% TBE. The gel was made using the Joey gel casting system (Owl) and was pre-electrophorized for 30-60 minutes at 100 volts. Following electrophoresis the gel was fixed in solution containing 10% acetic acid and 20% ethanol. The gel was dried on a gel dryer (Labconco Corporation, Kansas City, Missouri USA) and exposed to Kodak biomax XAR film for 16 hours to one week, depending on signal, incubated at -80°C.

The shifts were performed on the CNS midline enhancer (CME: 5'-acaggcaaaccacgaccattctga-3') variant. Mutant competitor was mutCME (5'-acaggcaaaccTTgaccattctga). The shifts performed on the BAX promoter from region 3 were on the CME-v (5' gagctgagatcgtgaccttgc 3') and the Mutant competitor was (5' gagctgagatTTtgaccttgc 3'). The shifts performed on the BAX promoter from region 2 were performed on the CME-v (5' gccgagatcgtgccattgcac 3') and mutant competitor used was (5' gccgagatAAtgccattgcac 3').

Chromatin Immunoprecipitation Assay

Chip assays were performed using HeLa cells transiently transfected with an HA tag fused HALO (HA-HALO) and Myc tag fused with ARNT2 plasmid over night. Verification of HALO and ARNT2 expression were performed using western blot analysis (see above). Standard protocol was performed with the ChIP kit (Upstate biotechnology) with PCR primer pairs for BAX upper 5' ttgtggaaattttgggtggatgaa 3' and BAX lower 5' gaggcaggggaatcgtttgaatct 3'. PCR primer pairs for Nkx2.2 upper 5' tgactcccaagacccgcccacacg 3' and Nkx2.2 lower 5' gccattgcccgagcgatcagtccat 3' which amplifies a portion of the promoter of the randomly selected human gene Nkx2.2 (chromosome 20p11) as the negative control. The buffers and procedures used were as described by the manufacturer.

Cytotoxic Assays

For Hydrogen Peroxide (H_2O_2) and Oxygen Glucose Deprivation (OGD) sensitivity assays, the cells were plated on 60mm plates at 7.5 x 10^5 cells/plate. The following day cells were infected with either a MOI=10 (HeLa) or MOI=50 (N1E-115) and then seeded into 96 well plates at 10^4 cells/well 16-24h prior to treatment with either H_2O_2 or OGD. Cells were either treated with H_2O_2 as indicated for 30 minutes in complete growth medium or oxygen-glucose deprived at indicated time intervals in Basic Salt Solution (BSS; pH 7.4) containing 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$ 10 mM HEPES and 0.03 mM Glycine. Cells were first washed three times with PBS and then incubated in the OGD chamber

(Coy laboratories) in BSS. Both PBS and BSS solution were atmospherically equilibrated to 0% O_2 for 30 minutes prior to use with an aerator stone. After either treatment, cells were then incubated for an additional 16-24 hour period and then analyzed using 10% tetrazolium salt WST-1 for 2-4 hours. The plates were scanned in a SpectraMax 340 plate reader (Molecular Devices) at 450 and 650 nm to determine the metabolic activity of the cells. Percent survival was calculated relative to the vehicle treated (GFP) only wells, which were assigned to one hundred percent survival. All experiments were done in triplicate in three independent experiments with similar results. Adenoviral vectors consisted in HA-tagged HALO or HA-tagged HALO with a deletion of the bHLH-PAS domain (ΔTAD). All vectors were generated in AdTrack vectors expressing GFP from a second CMV promoter. Infections were confirmed by both immunoblot using anti-HA (12CA5) and by GFP immunofluorescence. Infection efficiency ranged from 70-90%. HeLa cells were plated at a density of 2×10^5 in a 12 well plate. Once HeLa cells were settled (4 hours later) they were then infected with adenovirus. Infection was >98% efficient. At each time point, cells were lifted off the plate with 1 x Trypsin-EDTA solution (GIBCO), and then resuspended in DMEM containing 10% FBS. Trypan Blue was added in a 1:1 ratio and cells were counted on a hemocytometer factoring in the dilution.

III) RESULTS

Gene upregulation has been clearly established following various preconditioning treatments, but the underlying molecular mechanisms that afford protection are still poorly understood. To isolate novel genes induced by CSD, ***R***epresentational ***D***ifference ***A***nalysis (RDA: Hubank, and Schatz, 1994), a PCR based process was used as a method of subtracting the difference products between CSD treated left cortex and the contralateral untreated right side. This approach successfully isolated cDNA fragments upregulated following CSD treatment in rat brain, prior to my arrival. Briefly, the left cortex of adult male Sprague Dawley rats were depolarized for 2 hours as previously described (Douen et al., 2000) and double stranded cDNA pools were generated from poly-A+ RNA isolated from CSD-treated left cortices and control untreated right cortices. Two rounds of subtraction were performed using right side cortex cDNAs as the driver population and confidence was achieved with the isolation of previously reported genes such as, c-fos, c-jun, egr-1 and cycloxygenase-2 (COX-2).

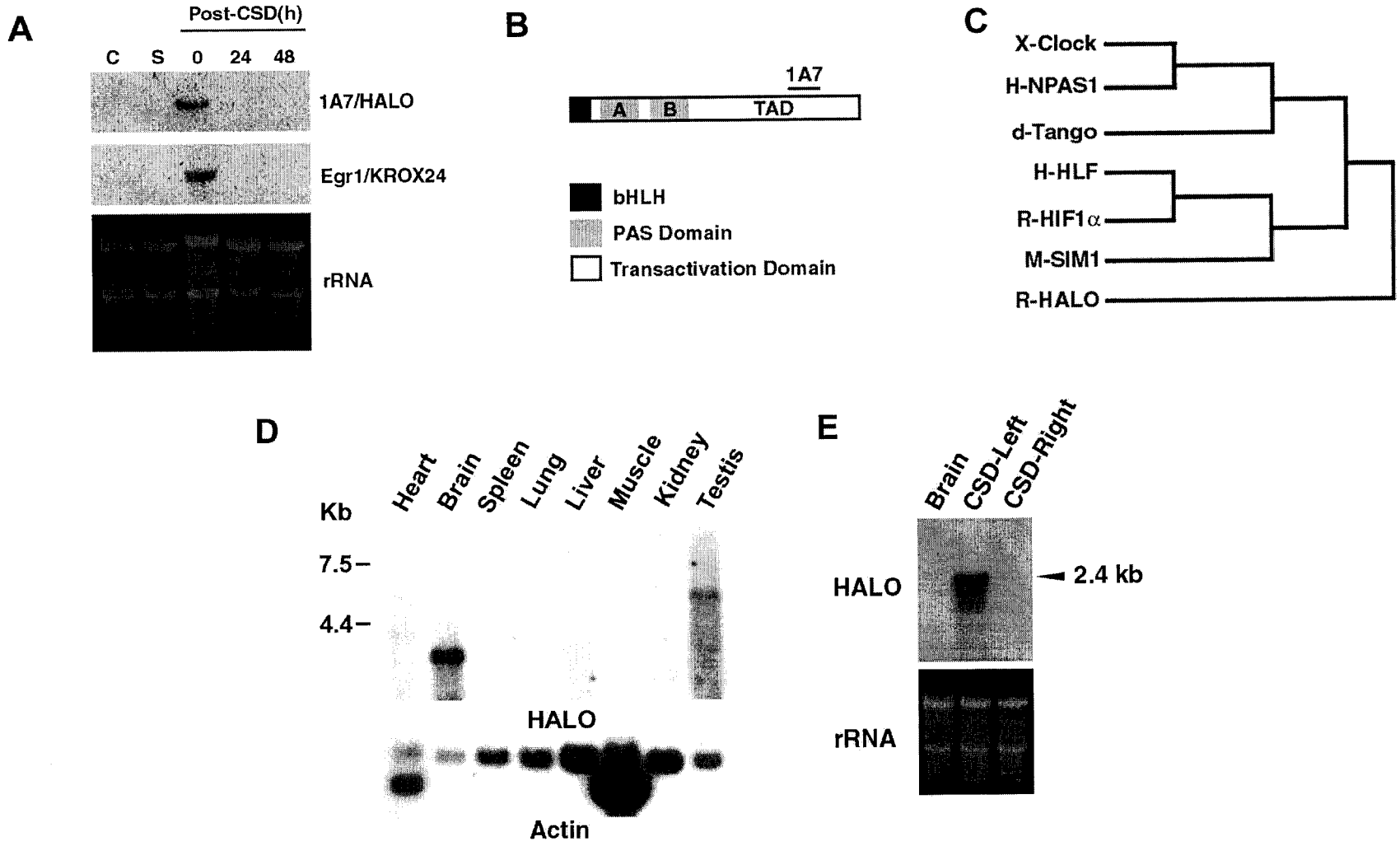
Isolation and Structure Analysis of HALO/NXF

Following RDA, one clone, RDA tag 1A7, was found to represent a fragment of a recently described cDNA encoding NXF (accession no. AB050103), a member of the bHLH-PAS family of proteins characterized by their role in neurogenesis, angiogenesis, response to xenobiotics and the control of circadian rhythm. To validate the isolated RDA tag 1A7 as a CSD induced gene,

the 200 bp fragment was used as a probe in Northern hybridization blot containing total RNA isolated from a CSD time course over a 48 hour period (Figure 3A). Robust expression of the 1A7 tag was observed in the left cortex immediately after the CSD elicitation and the signal was subsequently abolished 24 and 48 hours later. The contralateral hemisphere and sham controls did not demonstrate expression of the 1A7 tag. The previously reported CSD regulated Egr1/KROX24 gene was also isolated from the RDA subtraction (Figure 3A), and used as a control for comparison.

The full-length 1A7 coding region was subsequently obtained by RT-PCR using total RNA from CSD-treated rat brains. Because of the pre-existence of the large number of ***N***uclear e***X***port ***F***actors already labelled as NXF (1 through 7), the 802 amino acid protein encoded by the 2409 bp full length cDNA has been termed and will be referred to hence forth as HALO for b***H***LH-PAS ***A***ctivated ***L***eft-side ***O***RF. Phylogenetic tree analysis (DNAStar; MegAlign) representing a subset of bHLH-PAS factors shows that HALO is distantly related to HIF-1α and is a close relative of the ***Si***ngle ***M***inded (SIM) proteins (Figure 3C) and the recently characterized *dysfusion* gene (Jiang and Crews, 2003). Sequence alignment analysis showed that HALO shared significant homology with SIM1 (29%) and SIM2 (34%) as well as NPAS (23%) in the bHLH and PAS domains. No similarity was observed in the carboxy-terminal transactivation domain.

Figure 3. Expression of the bHLH-PAS HALO in adult tissues and following CSD. (A) Northern blot analysis of 10mg total RNA isolated from control right cortex, C, NaCl sham treated, S, or CSD treated left cortex. Blots were probed with the 1A7 RDA clone and compared to Egr-1/KROX24, previously shown to be induced by CSD. A marked upregulation of HALO was observed shortly and transiently after CSD. (B) Schematic representation of the HALO coding sequence, showing the bHLH region, the PAS and transactivation domains (TAD). The region corresponding to the 1A7 RDA tag is depicted in the TAD. (C) Phylogenetic tree analysis of selected bHLH-PAS family member showing that HALO is one of the most distant family member but related to the SIM proteins. X= Xenopus, M= mouse, H= human, d= Drosophila, R= rat. (D) Tissue distribution of HALO mRNA. A rat Multiple tissue Northern (2mg poly-A+; Clontech) was probed with a c-terminal HALO fragment. HALO is preferentially expressed in adult brain as a 2.4 kb mRNA and in testes a 6 kb transcript. For comparison, total RNA from a CSD-treated brain is shown in E.


A
Post-CSD(h)
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S
0
24
48
1A7/HALO
Egr1/KROX24
rRNA
B
1A7
A
B
TAD
bHLH
PAS Domain
Transactivation Domain
C
X-Clock
H-NPAS1
d-Tango
H-HLF
R-HIF1α
M-SIM1
R-HALO
D
Kb
Heart
Brain
Spleen
Lung
Liver
Muscle
Kidney
Testis
7.5
4.4
HALO
Actin
E
Brain
CSD-Left
CSD-Right
HALO
2.4 kb
rRNA

HALO Expression Pattern in the Adult Rat

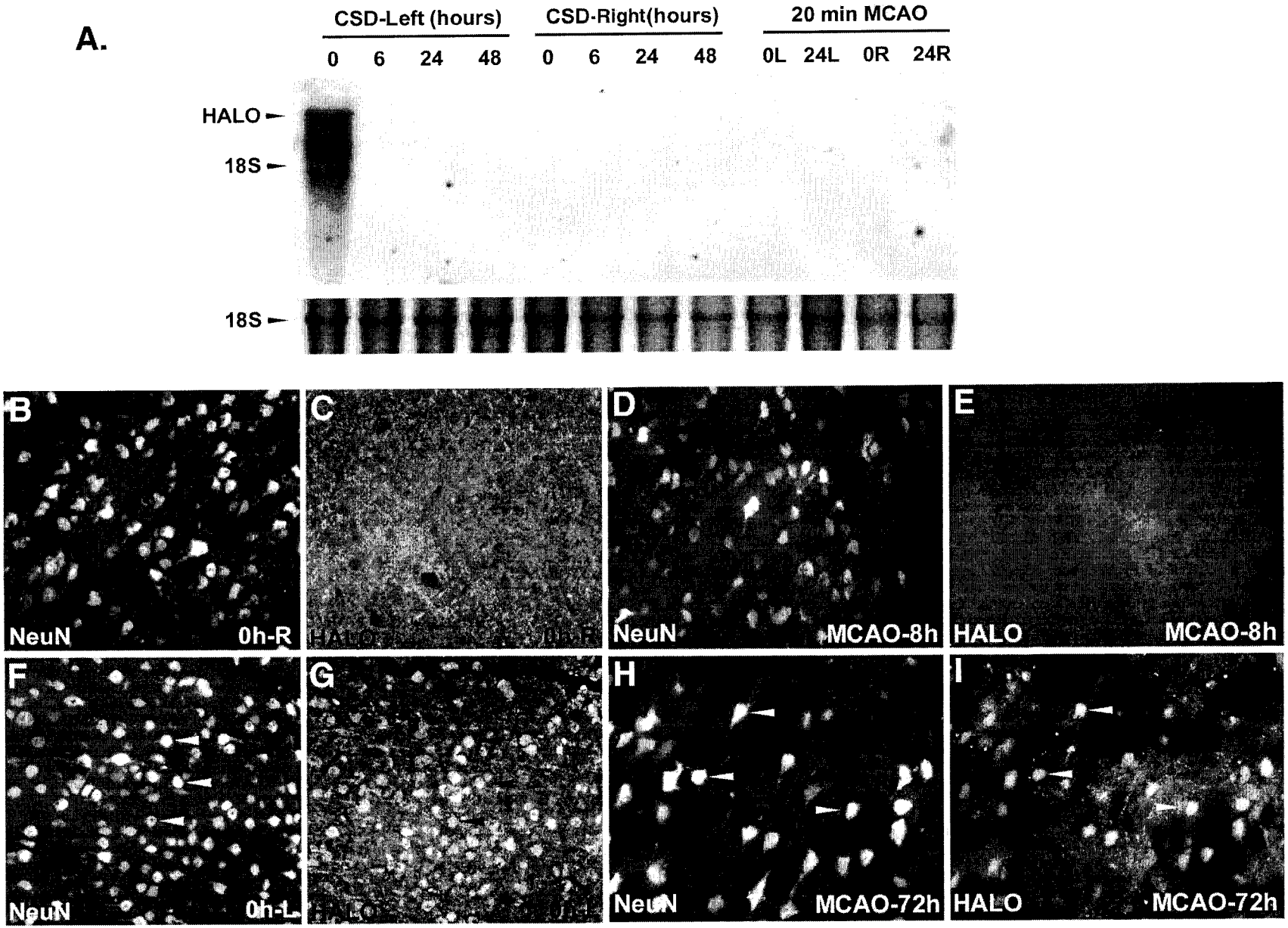
To gain insights into the role of HALO, various rat tissues were screened by Northern blot hybridization to determine its expression profile. A probe designed from the distinctive carboxy-terminus was isolated from the full-length HALO cDNA and used against various adult rat tissues on a poly-A+ RNA membranes (Clonetech, MTN). HALO was preferentially expressed in the brain and to a lesser extent the testes as previously reported (Figure 3D; Ooe *et al.*, 2004; Moser *et al.*, 2004). The lack of expression in the muscle and kidneys as observed by Ooe and colleagues may be attributed to the specificity of the unique probe used in the screening in comparison to Ooe's group, who probed with the entire HALO cDNA, possibly cross hybridizing to other bHLH-PAS RNA.

The relative induction of CSD induced HALO expression was determined by Northern blot hybridization. Comparison analysis between total RNA isolated from rat CSD treated left cortices, CSD untreated contralateral right cortices and Sham NaCl-treated cortices showed that HALO mRNA expression levels were highly induced in CSD-treated cortices but was undetectable in control or sham NaCl-treated cortices (Figure 3E). The contrast between expression levels of each sample demonstrates that HALO expression is dramatically upregulated by spreading depression (>100 fold). In addition, the failure of NaCl-treated cortices to induce HALO expression, suggests a link between potassium specific depolarization and HALO induction.

HALO Protein Expression is Specific to CSD Preconditioning and in the Ischemic Recovery Phase in the Adult Rat

To further define HALO as a neuroprotective gene product other methods of preconditioning were analysed for HALO induction. The previously reported preconditioning by sublethal ischemic event (20 minutes) was applied to rat cortex and the expression pattern of HALO was compared to preconditioning by CSD. HALO induction was apparent in the CSD treated left cortex only immediately after CSD elicitation and the signal was subsequently abolished at later time points (Figure 4A). Both the untreated contralateral and preconditioning by sublethal ischemia did not induce HALO mRNA expression. Double immunolabelling with a neuronal marker NeuN, and the chicken anti-HALO IgY was used to analyse the protein expression of HALO in rat cortices treated with CSD. Similar to the mRNA expression pattern, HALO protein expression was observed in the CSD treated rat left cortex but not the untreated right cortex immediately following CSD elicitation. HALO protein expression was further characterized to be expressed within neurons (Figure 4F-G) of the CSD treated cortex. Interestingly, although HALO is not induced by short term ischemic preconditioning methods, HALO appears to be induced 72 hours following a complete ischemic event (2 hours MCAO) in neurons (Figure 4H-I). Worth noting, this time point coincides with the beginning of the recovery phase of the brain after ischemia.

Figure 4. HALO is induced by CSD and ischemia. (A) Northern blot analysis of total RNA isolated from CSD-treated and control cortices. RNA samples were extracted at different times following CSD and analysed for HALO expression. HALO was found to be induced immediately following CSD and undetectable by 6 hours post-CSD. HALO mRNA was not induced by short duration ischemia (MCAO) another form of pre-conditioning. The 18S rRNA is shown as loading control. Following CSD, HALO protein is undetectable on the right side control (C) but is induced in NeuN-positive cells on the CSD-treated left side (F and G). HALO protein was detectable up to 6 hours post-CSD (not shown). Similarly, HALO protein was found to be induced in NeuN-positive cells following transient ischemia (D, E, H and I) but only at 72h of reperfusion.

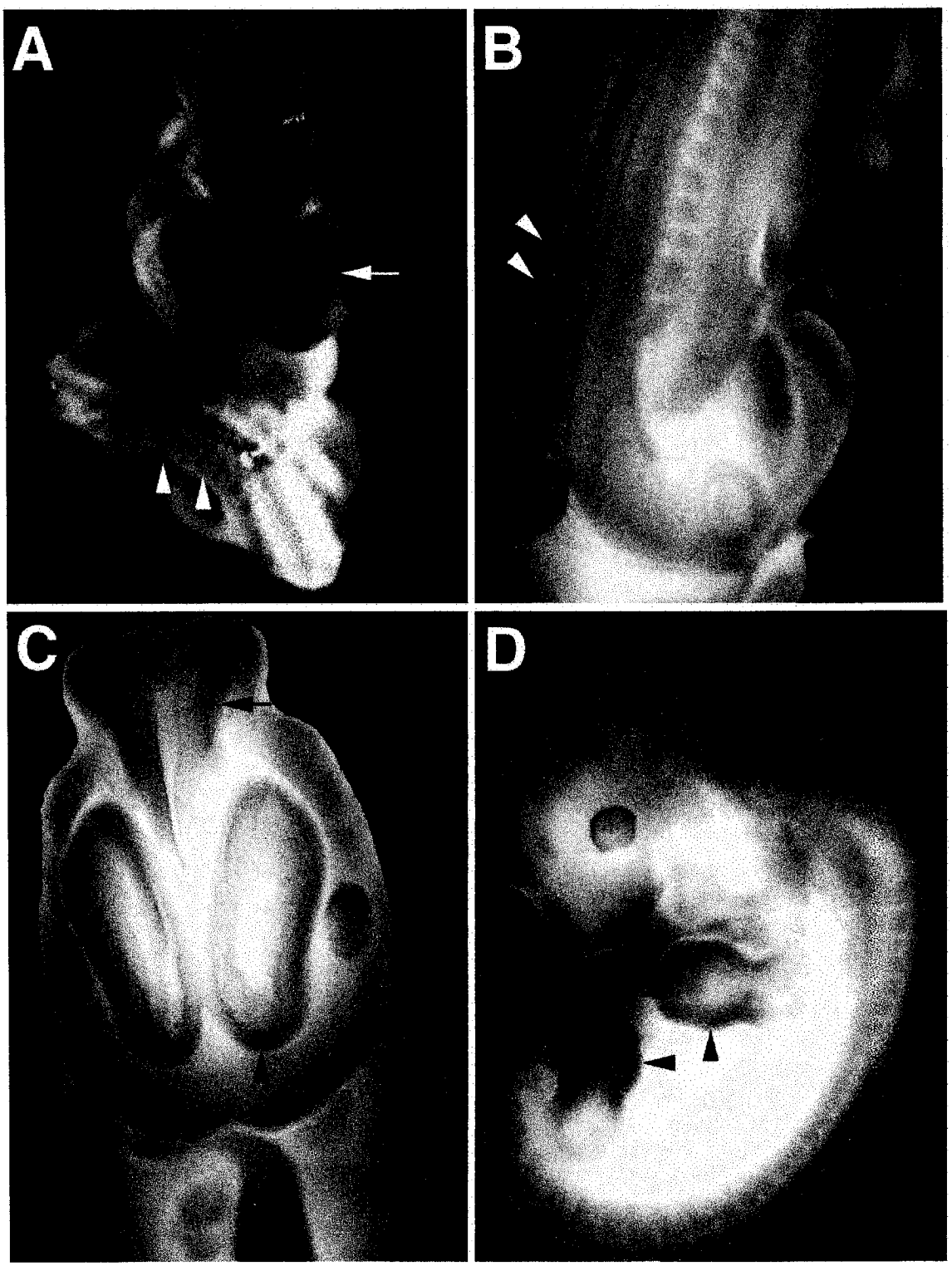

A.
CSD-Left (hours)
CSD-Right(hours)
20 min MCAO
0 6 24 48 0 6 24 48 0L 24L 0R 24R
HALO
18S
18S
B
NeuN
0h-R
C
D
NeuN
MCAO-8h
E
HALO
MCAO-8h
F
NeuN
0h-L
G
H
NeuN
MCAO-72h
I
HALO
MCAO-72h

Spatiotemporal mRNA Expression of HALO in the embryo

Previous work done by Ooe and colleagues originally identified HALO using a predicted exon database isolated from a human foetal brain cDNA library. Subsequently, the human HALO cDNA was isolation from a screen of the human foetal brain library (Ooe et al., 2004) suggesting expression of HALO during foetal development. Its role in development is consistent with the involvement of other bHLH-PAS in developmental processes such as neurogenesis and vascularization. The original isolation from foetal tissue coupled with the observation of low HALO expression levels in the adult rat suggests a potential developmental role for HALO. To investigate this further, whole mount *in situ* hybridization was performed on 10.5 dpc and 11.5 dpc mouse embryos using an RNA anti-sense probe designed from the HALO exon 8. *In situ* hybridization shows that HALO is most prominently expressed in the developing mouse mesencephalon and telencephalon (Figure 5C). Expression of HALO is also observed in the forebrain, olfactory bulbs (Figure 5A) as well as the dorsal root ganglia (DRG; Figure 5B), suggesting a role for HALO in the developing CNS. Interestingly, outside the CNS HALO mRNA expression is also detected in the forelimb and hind limb buds (Figure 5D), suggesting an additional role in mesenchymal development.

Spatiotemporal Protein Expression of HALO in Embryogenisis

To gain further insight into the expression profile of HALO and to validate the mRNA expression pattern in the mouse embryo, the protein expression


A
B
C
D

pattern was analysed. A chicken polyclonal IgY antibody was custom generated (Washington Biotech) against the last 19 amino acids of the C-terminus of HALO, which is conserved between human, mouse and rat. Sagital sections of 11.5 dpc and 13.5dpc mice were double immunolabelled with the newly generated anti-HALO in combination with the neuronal marker anti-βIII-tubulin (Tuj1). In accord with the whole mount *in situ* hybridization results, HALO protein expression was most prominent in the CNS with localization in the ventral telencephalon or forebrain (Figure 6A-C), the DRGs (Figure 6D-F), the olfactory system (Figure 6G-I), the anterior portion of the telencephalon (Figure 6J-L) and finally the mesencephalon or midbrain (Figure 6M-O). In contrast to Northern blot analysis, HALO protein expression was not detected in the mouse testes. HALO immuno-reactivity is detected in post-mitotic Tuj1-positive cells and detection of HALO protein expression was abolished by preadsorption of anti-HALO IgY with the HALO peptide (Figure 6D and J; inset). Taken together, the whole mount *in situ* hybridization and the immunofluorescence results show that both the transcriptional and translational HALO products are expressed in the developing embryo. Moreover, with the exception of the forelimb and hind limb, the expression appears to be specific within the CNS which is consistent with the original isolation of HALO as a foetal brain library clone.

In addition to the HALO expression found in the CNS of the developing embryo, we examined the localization of HALO in rat primary cultured neurons. Double immunolabelling of primary cortical cultured neurons (CCN) with mouse anti-Glial Fibrillary Acidic Protein (GFAP) and chicken anti-HALO demonstrates a

Figure 6. Detection of HALO reactivity in the developing embryo. 13.5 dpc murine embryos were cryosectioned and stained for HALO (A, D, G, J and M) and bIII tubulin (Tuj.1: B, E, H, K and N). HALO reactivity was observed in the forebrain neuroepithelium and Tuj.1-positive neurons (A-C) as well as in the DRGs (D-F). Similarly, double labeling was observed in the olfactory system (G-I), the roof of the forebrain (J-L) and midbrain (M-O). Preadsorbtion of anti-HALO IgY resulted in a loss of signal in the forebrain (J; inset) and HALO protein expression could not be detected within the testes (D; inset. Marked by asterisk).

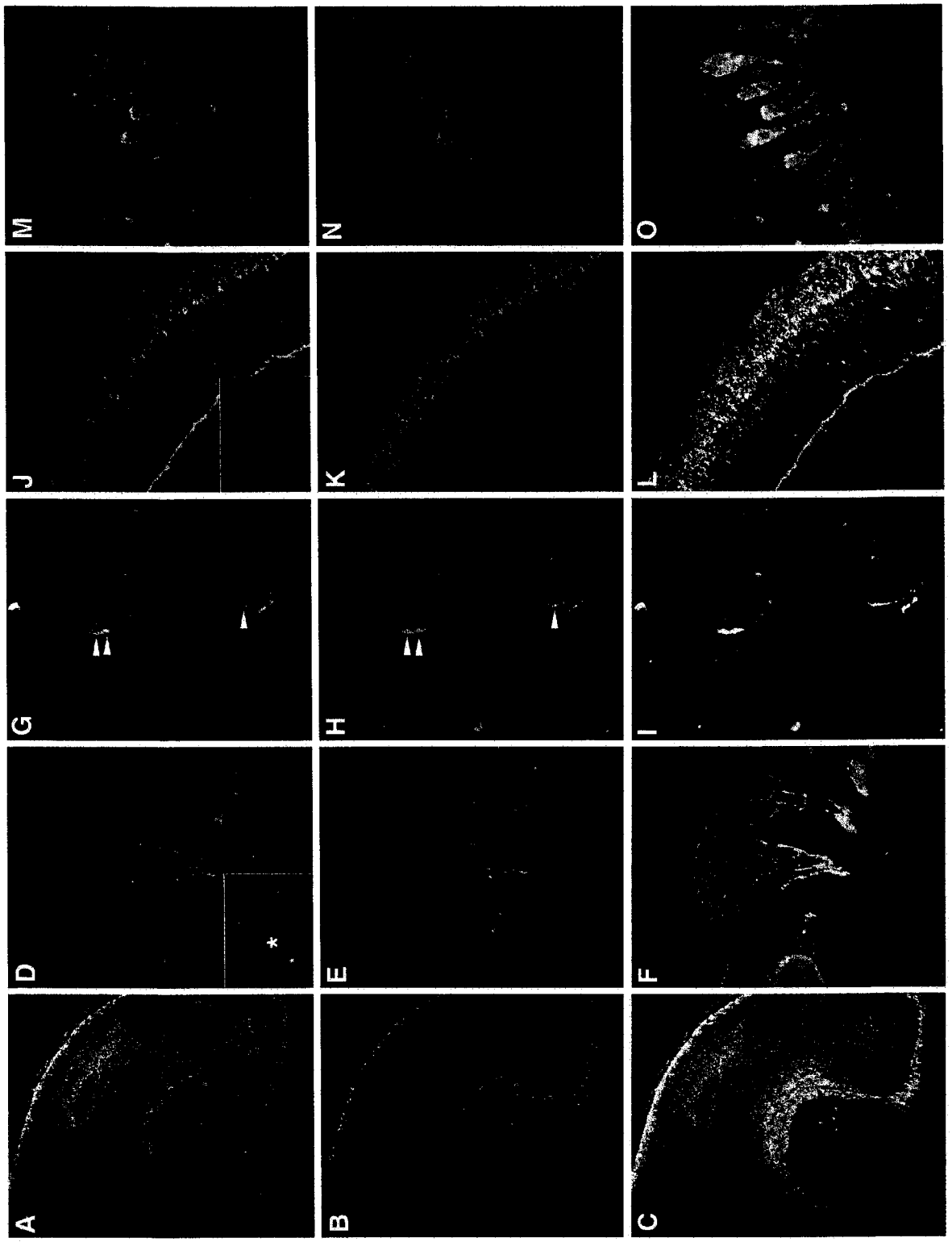
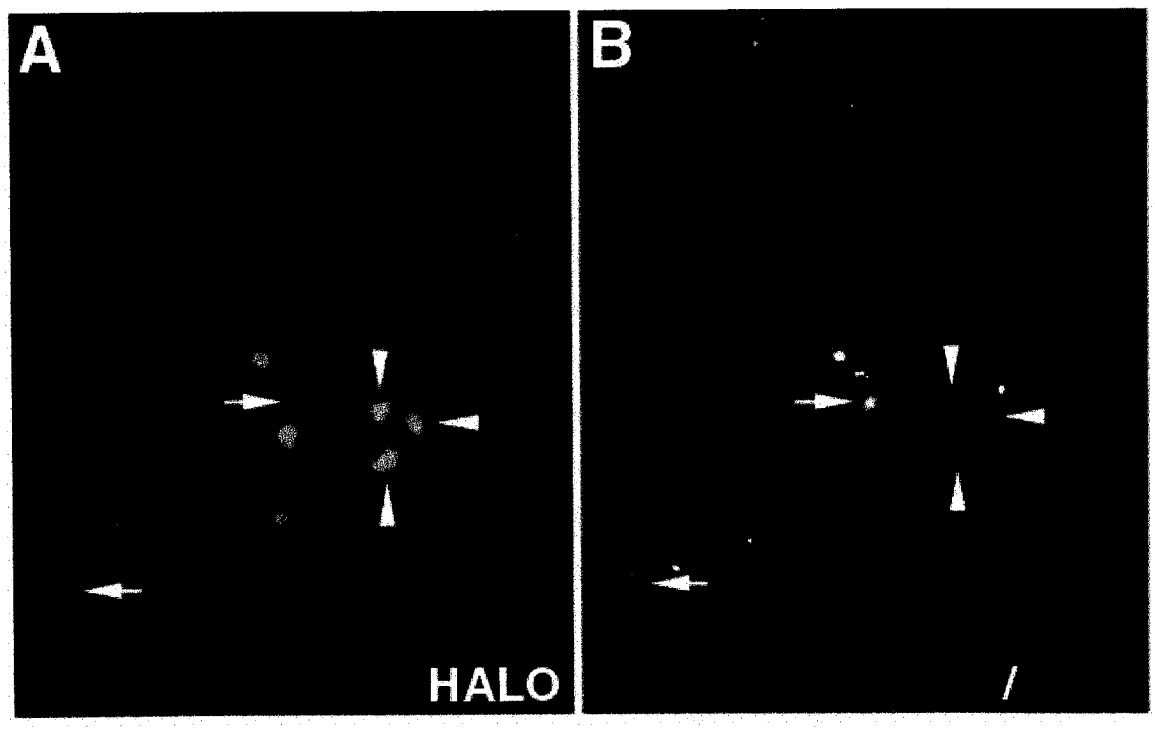
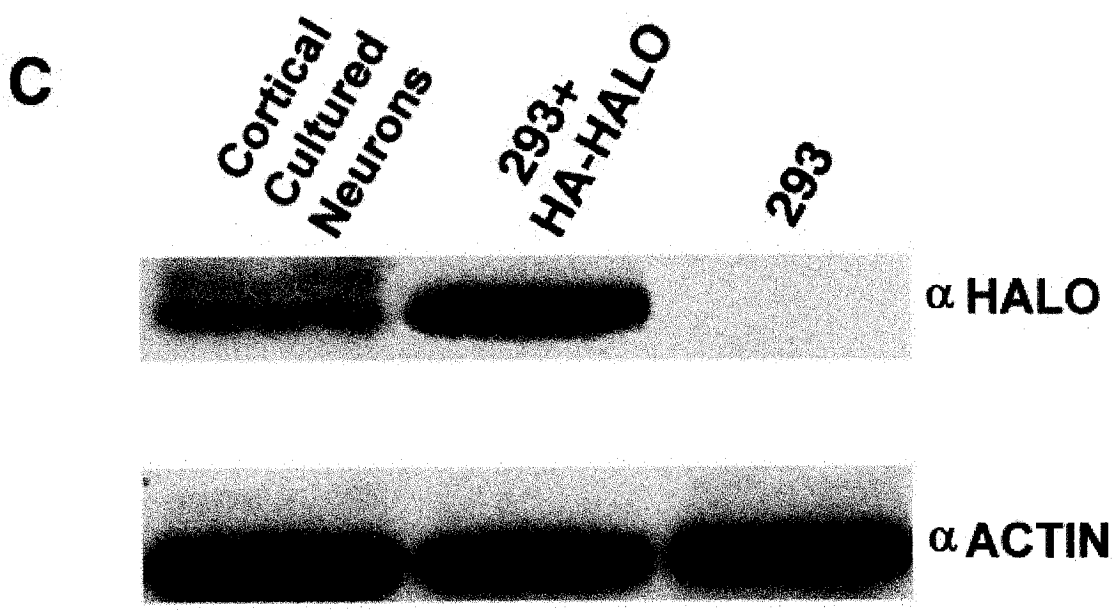

HALO
Tuj.1
Merged
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
*

Figure 7. Expression of HALO in cultured primary neurons. (A) Primary neurons (10 div) express detectable levels of HALO protein (arrowheads) that localize to both the nucleus and cytosol. No expression was found in GFAP-positive cells (arrows; B). (C) The polyclonal chicken IgY raised against the C-terminal 19 amino acids of rat HALO reacted with a 100kDa polypeptide that co-migrates with HA-HALO expressed in 293 cells on western blots.


A
HALO
B
/

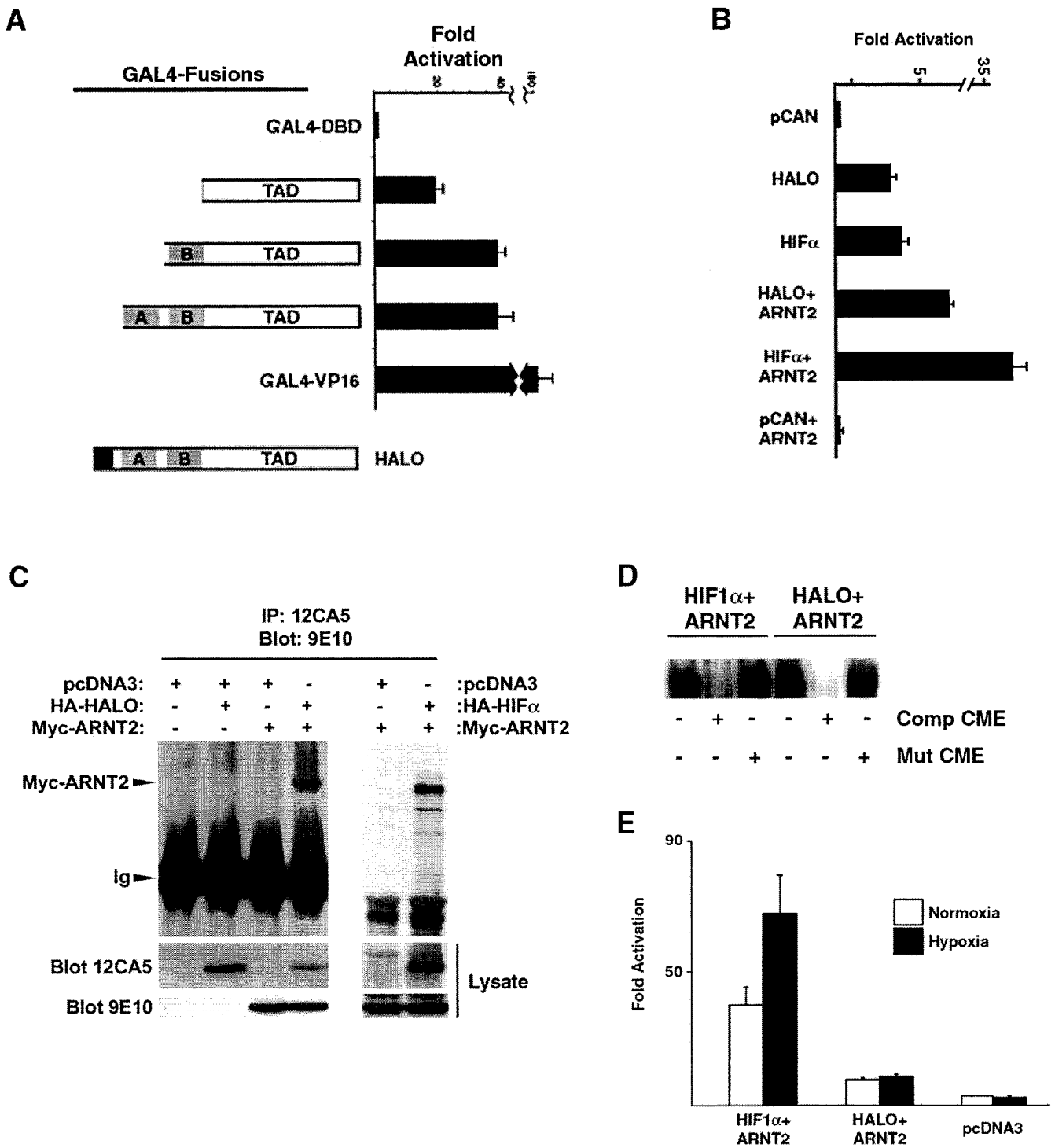

C
Cortical Cultured Neurons
293+ HA-HALO
293
α HALO
α ACTIN

nuclear localization of HALO to be specific within neurons and not Glia (Figure 7A-B). Endogenous HALO was also shown in pure cultures of primary CCNs by western blot analysis against nuclear extracts of 7 day cultures (Figure 7C). In combination with the expression profile in the developing embryo, these results strongly suggest a possible role in the developing neuron.

HALO/ARTN2 Heterodimerization is Required for Transactivation and DNA Binding

In contrast to other bHLH-PAS factors, the SIM2 protein has been shown to be a transcriptional repressor. To investigate whether HALO mediates the activation or repression of transcription, various deletions encompassing the bHLH-PAS and c-terminal regions were fused to the yeast GAL4 DNA-binding domain. Dual Luciferase assays (Promega; Stop and Glow) performed on a GAL4 binding site reporter showed that the c-terminal domain of HALO can activate transcription from the GAL4 reporter plasmid (Figure 8A). Maximal transactivation was observed when the PAS-B domain was present in the GAL4 fusion, suggesting that it is required for optimal activity of HALO. As most bHLH-PAS factors bind DNA elements containing the core sequence CGTG, we performed Luciferase assays using a reporter plasmid bearing 2 CNS midline enhancers (CME). Transient transfections showed that HALO or HIF-1α alone could induce transcription from the CME (4-5-fold) to a similar extent (Figure 8B). Although some bHLH-PAS factors can form homodimers, the most functional unit is composed of heterodimers with the ARNT subclass of bHLH-PAS. Because

Figure 8. Transcriptional activation by HALO. (A) HALO GAL4 fusions were co-transfected along with a GAL4-Luc reporter. Transcriptional activity was mapped to the C-terminal domain of HALO. Increased activity was observed when the PAS B region was present. (B) Activation of a CME-Luc by HALO. Co-transfection of HALO and CME-Luc induced transcriptional activiation of the reporter. This was further increased by co-expressing the class II bHLH-PAS ARNT2. For comparison, the activity of HIF1_ on CME is shown. In all assays luciferase activity was normalized internally to Renillia luciferase activity using Stop and Glow (Promega). (C) HALO can form heterodimers with the class II bHLH-PAS ARNT2. Co-transfected 293 cells were analysed by IP/Western for the interaction of HALO and ARNT2. These factors could be co-precipitated when co-expressed, suggesting that they interact. For comparison, the HIF-1_:ARNT2 interaction was used. Expression of all factors was detected in whole lysates prior to IP and blotting. (D) HALO binds directly to the CME. EMSA on the CME shows that the HALO:ARNT2 dimer binds with high affinity to a CME oligonucleotide (tCGTG), also bound by the HIF-1_:ARNT2 dimer. (E) HALO transcriptional activity is not induced by hypoxia. 293 cells co-transfected with HALO, ARNT2 and HRE-Luc were subjected to 1% oxygen 5% CO_2 for 24 hours (Coy OGD Chamber) and analysed by luciferase assays. The HIF-1_:ARNT2 dimer was used as a positive control. No change in HALO activity was observed under hypoxic conditions. Luciferase assays (A, B and E) were done in triplicate (n=3).


A
GAL4-Fusions
Fold Activation
GAL4-DBD
TAD
B TAD
A B TAD
GAL4-VP16
A B TAD
HALO
B
Fold Activation
pCAN
HALO
HIFα
HALO+ ARNT2
HIFα+ ARNT2
pCAN+ ARNT2
C
IP: 12CA5
Blot: 9E10
pcDNA3:
HA-HALO:
Myc-ARNT2:
:pcDNA3
:HA-HIFα
:Myc-ARNT2
Myc-ARNT2
Ig
Blot 12CA5
Blot 9E10
Lysate
D
HIF1α+ ARNT2
HALO+ ARNT2
Comp CME
Mut CME
E
Fold Activation
Normoxia
Hypoxia
HIF1α+ ARNT2
HALO+ ARNT2
pcDNA3

ARNT2 is preferentially expressed in the brain, we tested the possibility that HALO activity could be modulated by this factor in a reporter assay. As shown in figure 8B, co-transfection of ARNT2 with HALO further increased the transactivation ability of HALO to about 8 to 10-fold above background, suggesting that ARNT2 associates and potentiates HALO activity. Supporting this, ARNT2 could be co-immunoprecipitated with HALO from co-transfected 293 cells (Figure 8C). This interaction has also been confirmed by mammalian two-hybrid assays (Ooe et al, 2004).

In marked contrast to HIF-1α only an 8 to10-fold upregulation of the CME-Luc reporter was observed following HALO:ARNT2 co-transfection. However, both complexes appear to bind the CME with similar affinity in DNA gel shifts assays (Figure 8D), suggesting that the HALO:ARNT2 dimer may require neuronal specific co-factors.

The activity of several bHLH-PAS proteins has been shown to be upregulated under hypoxic conditions (Crews ST, 1998; Crews ST and Fan CM, 1999; Harris AL, 2002). To determine whether HALO is also responsive to oxygen deprivation, transcriptional assays were performed on an Epo hypoxic response element (HRE-Luc) reporter plasmid. In contrast to HIF-1α, no increase in activity was observed for HALO when the transfected cells were incubated under hypoxic conditions for 24 hours (Figure 8E), suggesting that its activity is not modulated by hypoxia.

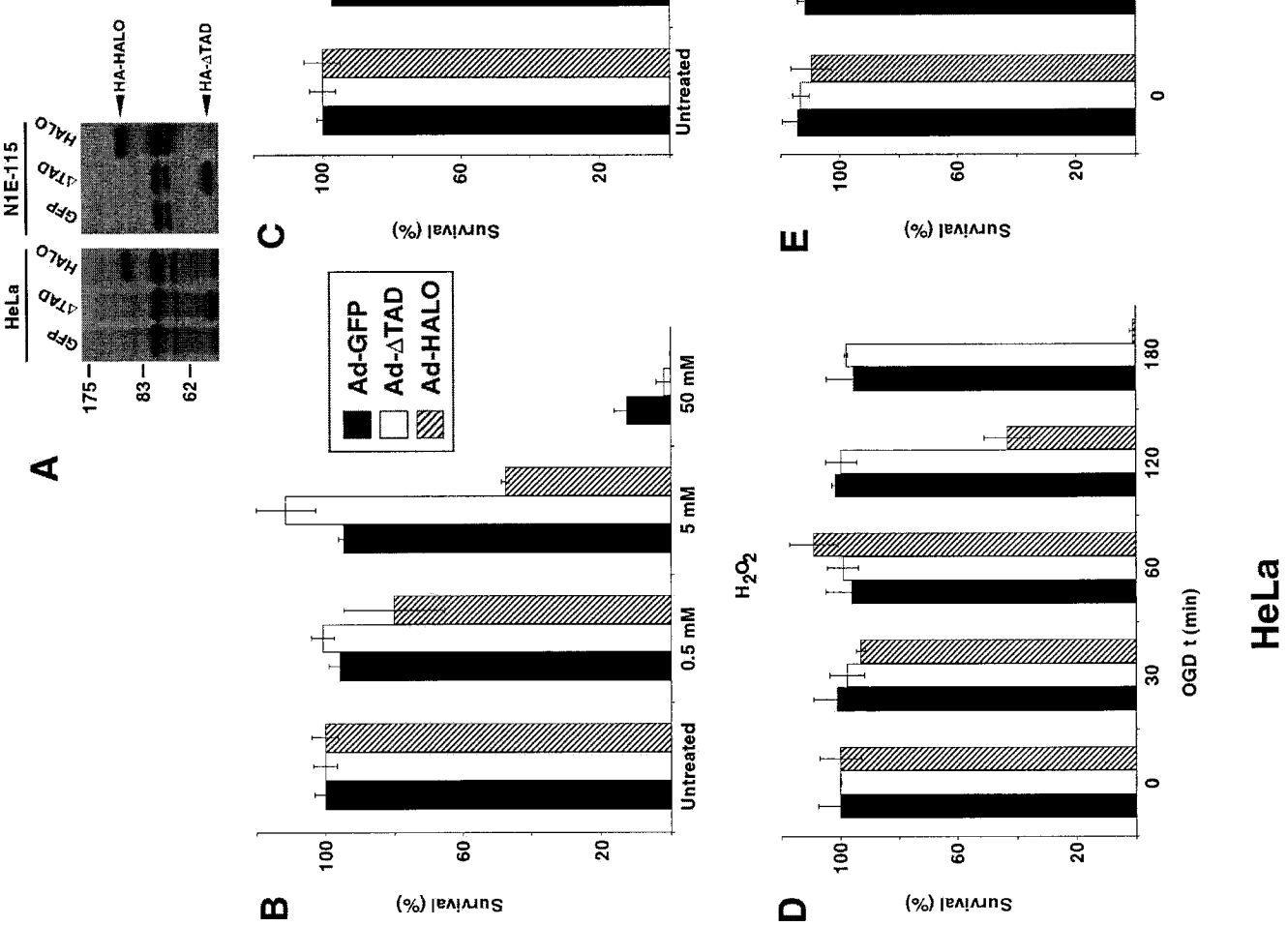
HALO Overexpression Sensitizes Cultured Cells to Apoptotic Triggers.

To investigate the potential role of HALO as a neuroprotectant, HALO was over expressed in neuronal and non-neuronal cell cultures and then subjected to the ischemic-like apoptosis inducing agents. To obtain robust expression of each assay, adenoviruses expressing HALO, ΔTAD (HALO lacking the transactivational domain) and GFP (termed Ad-HALO, Ad-ΔTAD, and Ad-GFP respectively) were constructed. All viruses also co-express GFP under a different CMV promoter. Therefore, each infection could be monitored directly by immunofluorescence of the CMV-GFP as well as by western blot analysis (Figure 9A). Infections were incubated for 90 minutes and the cells were subjected the following day to either Oxygen Glucose Deprivation (OGD) or Hydrogen Peroxide (H_2O_2). Survival was evaluated with the WST-1 proliferation assay system, 24 hours following treatment with the apoptosis inducing agents. Surprisingly, HALO failed to provide protection against OGD or H_2O_2 in either the non-neuronal HeLa cells or the N1E-115 neuroblastoma (Figure 9B-E). However, counter intuitively to the CSD protective model, the HeLa cells over expressing HALO developed increased sensitivity to both apoptotic targets (Figure 9 B and D).

Prolonged Expression of HALO Causes Death in Primary Neuron and Non-Neuronal Cultures

Illustrating HALO's endogenous association to the BAX gene promoter *in vivo* generates an important concern with the viability of a cell that stably expresses HALO. To address this issue, HeLa cells were infected with Ad-HALO

Figure 9. HALO overexpression sensitizes HeLa cells to oxidative stress. HeLa and N1E-115 cells overexpressing HALO or a transactivation domain deletion were subjected to hydrogen peroxyde (H_2O_2) toxicity or oxygen glucose deprivation (OGD) using a WST-1 based survival assay. (A) Western blot analysis showing expression of both HA-HALO constructs in HeLA and N1E-115 cells during WST-1 experiments. (B and C) H_2O_2 toxicity assay showing high sensitivity of HeLa cells at lower concentrations of H_2O_2. (D and E) Similarly, HeLa cells displayed high sensitivity to OGD at early time points. Interestingly, HALO expression had no effect on the neuroblastoma cells. These data indicate that HALO sensitizes HeLa cells but not N1E-115 to oxydative stress. All WST-1 survival assays were preformed in triplicate (n=3).


A
HeLa
N1E-115
GFP
ΔTAD
HALO
HA-HALO
HA-ΔTAD
175
83
62
B
C
D
E
Ad-GFP
Ad-ΔTAD
Ad-HALO
Survival (%)
Untreated
0.5 mM
5 mM
50 mM
1 mM
10 mM
H2O2
OGD t (min)
HeLa
N1E-115

and the overall cell viability was determined by employing the Trypan Blue Exclusion Assay at various time points post infection. HeLa cells infected with Ad-HALO show a decrease in the number of cells present over a 3 day period when compared to an expanding Ad-GFP infected population (Figure 10A).

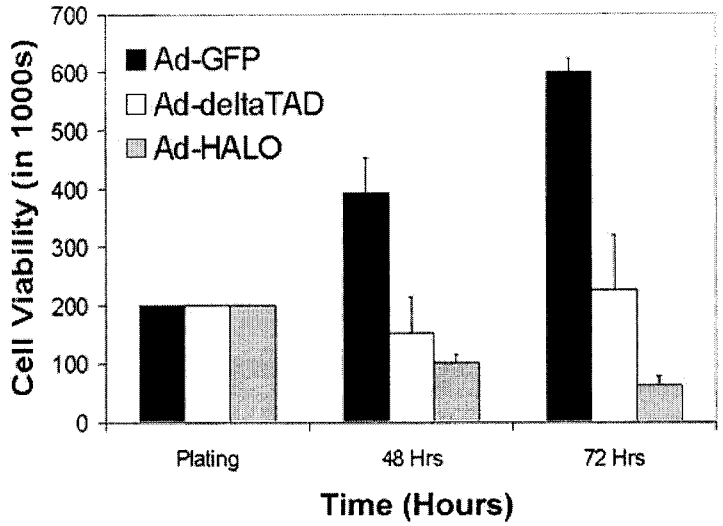
To identify the stable expression effect of HALO in a more *in vivo* model, we used Cerebella Granule Neurons (CGN), a primary neuronal cell culture, previously demonstrated to infect at a great capacity. Applying a time course over the period of 5 days, we found that the CGN population infected with Ad-HALO amounted to 20% of the CGN population infected with Ad-GFP on day 5 (Figure 10B-C). Furthermore, propidium iodide (PI) stain to examine the viability of the CGN on the final time point (day 5) demonstrates only a 15% viability of the remaining Ad-HALO infected CGN (Figure 10D and Figure 11). CGN infected with Ad-GFP were 75% viable, establishing a marked decrease in viability of stably expressing HALO CGN.

HALO/ARNT2 Heterodimer Complex Induces BAX Activity

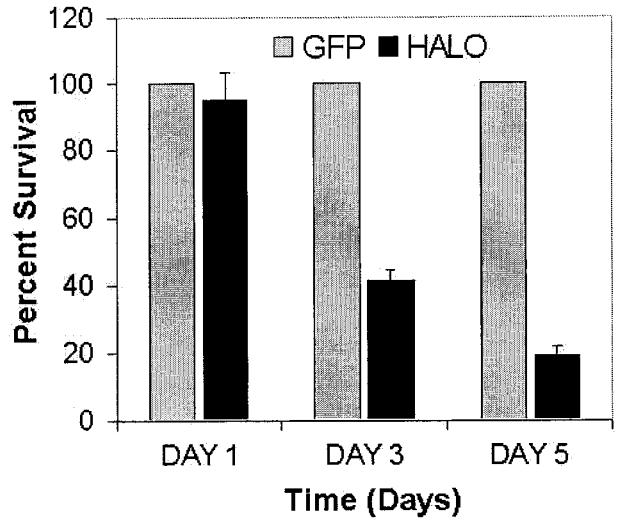
To investigate the molecular mechanisms mediating HALO-induced sensitization, we performed microarray analysis with Ad-HALO- and Ad-GFP-infected HeLa cells. One putative HALO target was found to be the proapoptotic gene BAX. To test whether the expression of HALO was sufficient to induce the expression of BAX, HeLa cells were infected with Ad-HALO or Ad-GFP and subjected to Western blot analysis. As shown in figure 5A, a marked increase in Bax protein levels was observed following Ad-HALO infection, supporting the Affimetryx data. Previous work by Ooe *et al.* (2004) has demonstrated high

Figure 10. Prolonged expression of HALO in non-neuronal and neuronal cells causes cell death. HALO expression in HeLa cells over a 3 day period results a decline of cell viability as compared to both Ad-GFP and Ad-DTAD infected populations (A). HALO expression mouse CGN population shows similar decline in cell population when compared with Ad-GFP control (B-C). Propidium Iodide (PI) staining of the Day 5 time point reveals the decrease in viability of cells by fluorescence microscopy (D). All cell counts (A, B and D) were preformed in triplicate (n=3) generating the indicated error.

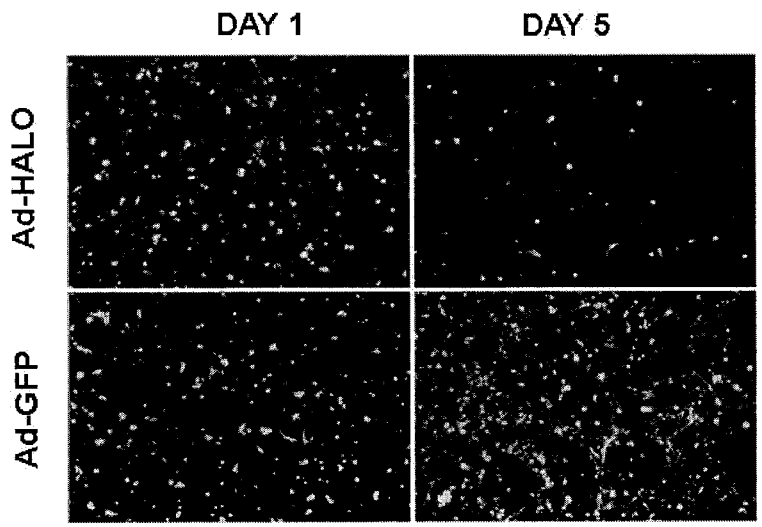
A)


HALO Expression Reduces Cell Viability in HeLa Cells over Time
Cell Viability (in 1000s)
700
600
500
400
300
200
100
0
Ad-GFP
Ad-deltaTAD
Ad-HALO
Plating
48 Hrs
72 Hrs
Time (Hours)

B)


HALO Expression Reduces Cell number in CGN Over Time
Percent Survival
120
100
80
60
40
20
0
GFP
HALO
DAY 1
DAY 3
DAY 5
Time (Days)

C)


DAY 1
DAY 5
Ad-HALO
Ad-GFP

D)

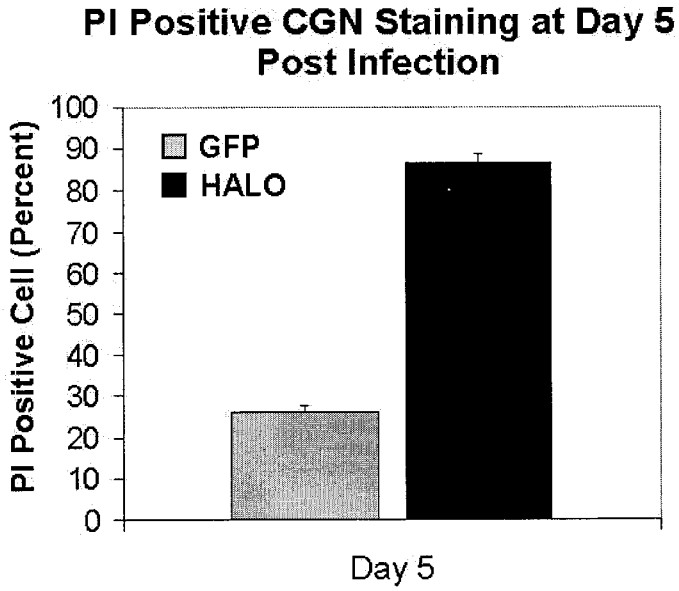
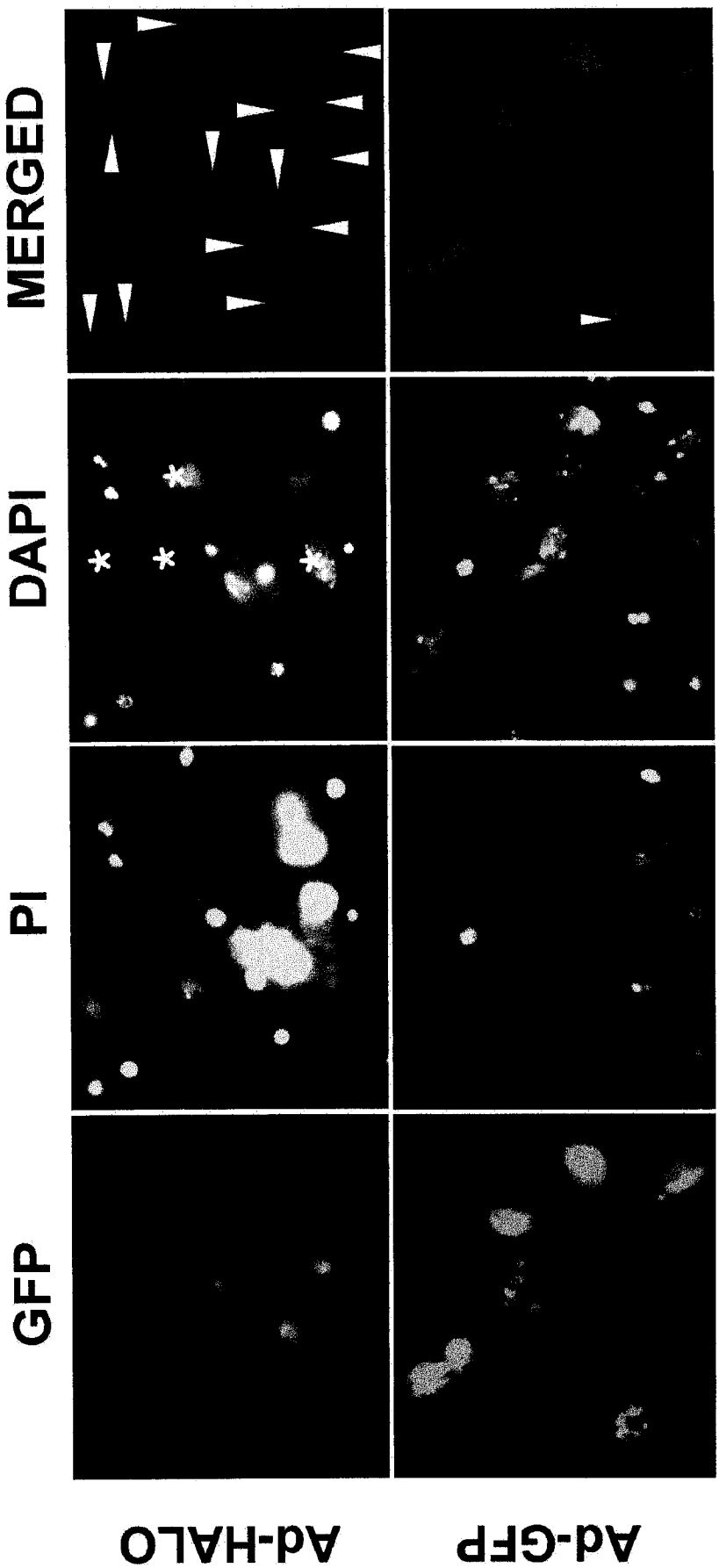

PI Positive CGN Staining at Day 5 Post Infection
PI Positive Cell (Percent)
100
90
80
70
60
50
40
30
20
10
0
GFP
HALO
Day 5

Figure 11. PI staining of CGNs shows a decrease in cell viability with cells infected with Ad-HALO. Fluorescence microscopy was used to examine CGN cells stained with PI five days following infection with Ad-GFP and Ad-HALO. Five days after plating, PI in a final concentration of 0.02 mg/ml was added to the medium, and following fixation the CGNs were stained with DAPI. Arrow heads indicate Ad-HALO-GFP or Ad-GFP positive cells stained with PI. CGNs marked by (*) are uninfected by Ad-HALO and unstained with PI.

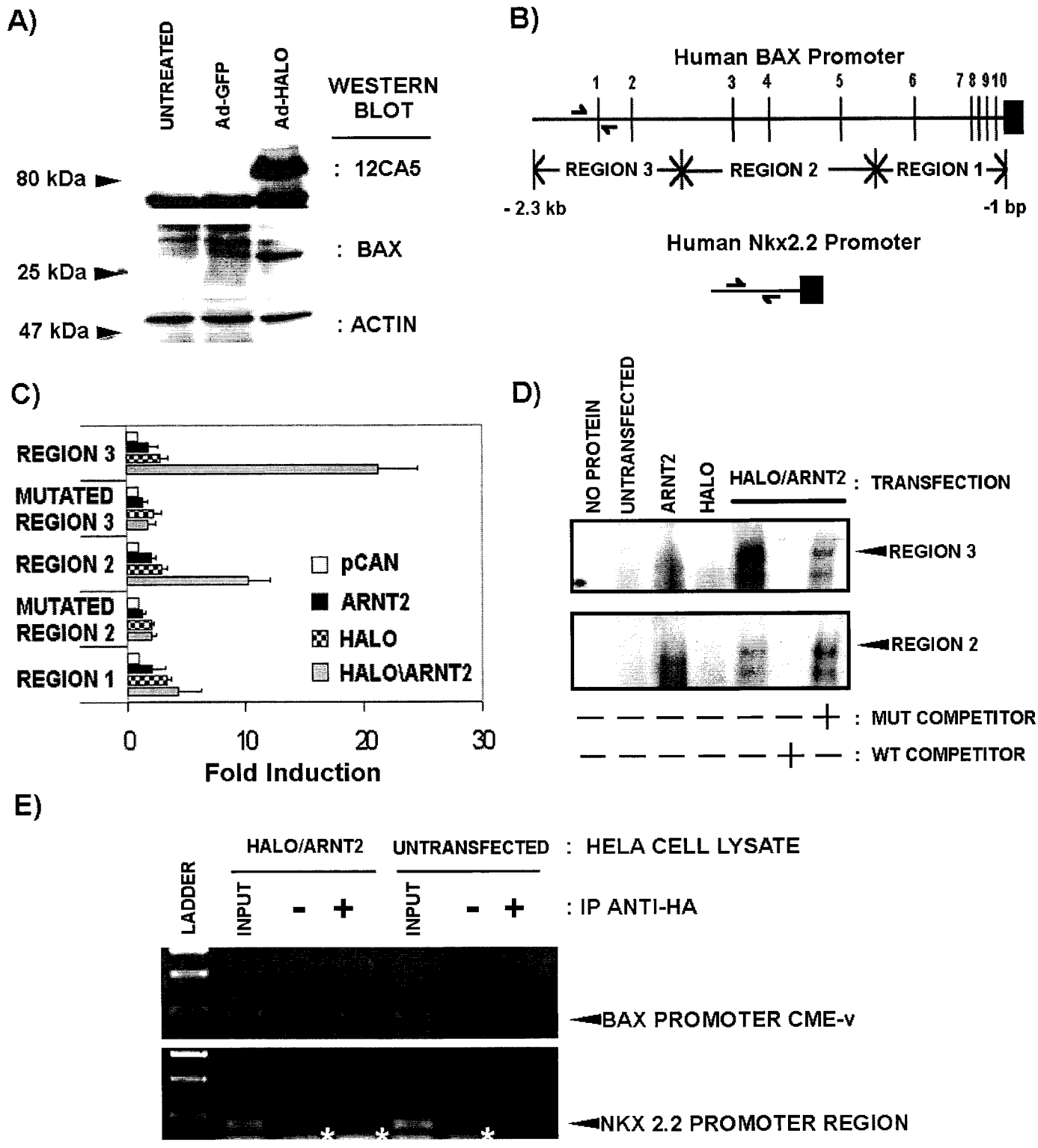

GFP
PI
DAPI
MERGED
Ad-HALO
Ad-GFP

affinity of HALO for a CGTG core sequence with highest affinity for a CNS midline enhancer variant (CME-v) element. Initial inspection of the 2.3kb human BAX promoter region, revealed 10 putative HALO binding sites (Fig 13B). The BAX promoter was analysed as 3 independent fragments, termed regions 1-3 (Fig. 13B), using luciferase reporter assays in the presence of HALO and ARNT2. Notably, regions 2 and 3 containing a CME-v element displayed high luciferase reporter activity compared to region 1 (Fig 13C). These observations suggest that both region 2 and 3 CME-v are binding sites for HALO in the BAX promoter. Supporting this, DNA gel shift assays with HALO/ARNT2 demonstrated that the wild-type CME-v, but not a mutant version, could bind the HALO/ARNT2 complex (Fig. 13D). Furthermore, mutation of these sites reduced luciferase activity to near background levels (Fig. 13C) in the presence of exogenous HALO and ARNT2, suggesting that they are the major HALO binding sites within the surveyed BAX fragment.

To investigate whether HALO can directly bind these sites in vivo, we performed Chromatin Immunoprecipitation (ChIP) assays. Because of its higher enhancer activity, primers were designed to amplify a 200bp fragment that included the CME-v sequence of region 3 (Fig 13B). HeLa cells were transiently transfected with HA-tagged HALO plasmid as well as ARNT2 and subjected to ChIP assays. As shown in figure 13E, a specific BAX promoter fragment could be amplified in the anti-HA immunoprecipitation but not in the control IP or anti-HA in the absence of HALO transfection. No product was observed in the Nkx2.2 negative control in the presence or absence of HALO (Fig 13E). These results

indicate that HALO specifically associates the endogenous BAX gene promoter region *in vivo.*

Figure 12. HALO induces BAX expression by binding to CME-variant of the BAX promoter. (A) Western blot shows an increase in BAX protein expression of the Ad-HALO infected HeLa cells compared to Ad-GFP infection and untreated cells. (B) Schematic representation of the 2.3 Kb human BAX promoter, outlining the potential HALO binding sites labeled 1-10, and separated into 3 regions for luciferase analysis (regions 1-3). (C) Luciferase analysis of the 3 regions of the BAX promoter and the respective mutation of the CME-v sites number 1 and 5 (as marked in B). (D) Bandshift analysis of the CME-v element of region 2 and 3 competing with 300x mutant competitor (Mut) and 300x wild type competitor (W.T.) as indicated by +. (E) ChIP analysis of HALO/ARNT2 transfected and untransfected HeLa cells immunoprecipitated with (+) or without (-) anti-HA. Primers of the BAX promoter and negative control Nkx2.2 promoter are as indicated in B and stars (*) indicate the formation of primer dimmers. Luciferase assays (C) were preformed in triplicate (n=3) generating the error bars as indicated.


A)
UNTREATED
Ad-GFP
Ad-HALO
WESTERN BLOT
80 kDa
: 12CA5
25 kDa
: BAX
47 kDa
: ACTIN
B)
Human BAX Promoter
1 2 3 4 5 6 7 8 9 10
REGION 3
REGION 2
REGION 1
- 2.3 kb
-1 bp
Human Nkx2.2 Promoter
C)
REGION 3
MUTATED REGION 3
REGION 2
MUTATED REGION 2
REGION 1
pCAN
ARNT2
HALO
HALO\ARNT2
0
10
20
30
Fold Induction
D)
NO PROTEIN
UNTRANSFECTED
ARNT2
HALO
HALO/ARNT2
: TRANSFECTION
REGION 3
REGION 2
: MUT COMPETITOR
: WT COMPETITOR
E)
HALO/ARNT2
UNTRANSFECTED
: HELA CELL LYSATE
LADDER
INPUT
- +
INPUT
- +
: IP ANTI-HA
BAX PROMOTER CME-v
NKX 2.2 PROMOTER REGION

IV) DISCUSSION

Current research investigating protective mechanisms to help in stroke is focused in part by the use of induced tolerance preconditioning techniques such as cortical spreading depression. CSD has been shown to induce a great level of gene expression, which may provide the infrastructure of induced tolerance system. We have isolated a bHLH-PAS transcription factor termed HALO, demonstrating a markedly increased expression immediately following CSD treatment. HALO is identical in sequence to the newly identified bHLH-PAS transcription factor NXF (Ooe et al., 2004) and has been characterized in Drosophila to be required in tracheal fusion (Jiang and Crews, 2003). Here we look at the functional characteristics of HALO in relation to CSD, ischemia and cell survival to help elucidate potential molecular mechanisms associated with CSD mediated neuroprotection.

Initial investigation of the tissue expression pattern of HALO demonstrates its expression to be found in the brain at low levels but is drastically induced following CSD. Subsequently, HALO protein expression was also confirmed to be highly expressed immediately following CSD treatment. In relation to HALOs drastic upregulation to the wave of depolarization over the cortex characterized by CSD, Flood and colleagues (2004) have demonstrated HALOs induced expression by seizure activity. Together, this data suggest that the induction of HALO expression may be attributed to electrical activity in the excitable cell. HALO was not found to be induced by sublethal ischemic preconditioning, another method of preconditioning inducing ischemic tolerance, but was induced

in the recovery phase following a complete ischemic event. Whether the induction of HALO following ischemia is due to brain recovery or to cell depolarization remains to be investigated. However, the lack of gene expression in the alternative preconditioning model provides further evidence for HALO as a cellular depolarization mediated gene product.

Preliminary sequence analysis outlines HALOs distant relation with SIM2, a bHLH-PAS repression factor implicated in murine and drosophila neuronal development (Crews et al., 1992). In accord with the sequence similarity, HALO mRNA and protein expression was found during murine embryogenesis in the DRGs, the telencephalon (forebrain), the mesencephalon (midbrain) and the olfactory bulbs. Additionally, HALO protein expression was most abundant in the outer layer within the more differentiated Tuj1 positive neurons, indicating a possible role for HALO in terminal differentiation of the neuroepithelium. Furthermore, *in vitro* analysis of protein expression in cortical cultured neurons did not detect HALO protein expression in cells positively labelled by GFAP antibody, but did detect HALO expression in neurons, suggesting a developmental role specific to neurons within the CNS. In contrast to Northern blot analysis, and *in situ* hybridization (Ooe et al., 2004; Moser et al., 2004; Flood et al., 2004) HALO protein expression was undetected by immunolabelling of adult rat brain slices suggesting a low level of translation or absence of translation.

In contrast to the SIM2 family members, the HALO C-terminus was shown to have transcriptional activation properties, which was further increased by the

PAS domains. Analysis of the CME element, a known SIM2 regulatory binding site, unveiled HALOs ability to bind the element by forming a heterodimer complex with the SIM2 binding partner ARNT2. Ooe et al. (2004) demonstrated the ability of SIM2 to repress HALO transcriptional activity in a competitive and repressive manner, against E-box motifs. Interestingly, SIM2 is found within the human critical Down syndrome region of chromosome 21 causing an increase in SIM2 expression in the disease state (reviewed in Engidawork and Lubec, 2001). Ooe and colleagues hypothesized that in the disease state, an increase in SIM2 would result in a sequestering of ARNT2 by the overabundant SIM2 protein and the repression of HALO CME-dependent targets. Ooe et al. reported Drebrin, a protein involved in proper dendritic spine formation as one such HALO target gene affected by the imbalance between HALO and SIM2 translational products. Collectively, these data suggest the existence of a possible competition for binding partners and for overlapping genetic targets, between HALO and SIM2 bHLH-PAS factors. This will require further investigation. Although HALO did demonstrate its ability to bind ARNT2 with equal affinity as HIF-1α, a bHLH-PAS protein that is induced with hypoxia, HALO did not respond to hypoxic conditions suggesting that it does not play a role with hypoxia.

Determination of a HALO mediated induced tolerant cell state by overexpressing HALO in HeLa cells followed by exposure to apoptotic triggers, lead to the unexpected opposite effect of inducing sensitization. Since a sensitized state was not observed in a neuroblastoma cell line two possibilities exist: i) HALO confers a cell type specific sensitization or ii) the neuroblastoma

cell line used in this study lacks a co-factor required for optimal HALO activity. Additionally, HALO single-handedly has no protective effect on neuronal-like cells and requires the activity of supplementary factors to confer neuroprotection. Furthermore, we report that the prolonged expression of HALO in HeLa cells and the primary CGN cultures resulted in the decrease in cell number and viability of both cell types. Investigation into the HeLa cell induced sensitization phenomenon led to the observation that BAX gene expression is induced by HALO. Western blot analysis confirmed the upregulation of BAX protein expression in HeLa cells following the overexpression of Ad-HALO. Sequence analysis of the BAX promoter revealed possible HALO DNA binding elements, which were confirmed to be direct targets regulated by HALO when examined by luciferase, EMSA and Chromatin Immunoprecipitation (ChIP). Previously, the synaptic protein drebrin has been reported as a HALO target gene in a neuroblastoma cell line (Ooe et al., 2004). Probing CSD treated cortices for Drebrin by western blot and immunolabeling analysis failed to show an increase in drebrin levels (results not shown) suggesting that specific gene targets maybe affected by pre-conditioning.

The regulation of BAX by HALO presents interesting possibilities in relation to the roles of HALO in development and as a neuroprotective element. In terms of development, comparison between the expression pattern of HALO and BAX in the developing mouse CNS reveals overlap in areas such as the olfactory system the cortical layers (or telencephalon) and particularly within the DRGs (Krajewska et al., 2002). BAX knockout mice (BAX $^{-/-}$) show a reduced

level of apoptosis within the DRGs alone and possess no other major deficit concerning decreased apoptotic events (Knudson et al., 1995). Therefore, the importance of HALO regulating BAX with respect to development, may lie in the DRG as this region requires BAX as the major proapoptotic factor (Lindsten et al., 2001). However, a study looking at double labelling still has to be performed.

It is rather surprising that HALO, a gene induced by preconditioning, appears to be an inducer of cell death. Interestingly, HALO is only transiently induced in cortical neurons following CSD treatment. Therefore, one possibility is that high sustained expression of HALO, as achieved in HeLa cells and CGNs, results in the binding of sites (and gene induction) that have moderate to low affinity for HALO, being cytotoxic (see Figure 13). Alternatively, pro-apoptotic genes maybe induced at low levels by CSD, "conditioning" the brain to subsequent ischemic event. However, microarray analysis provides no evidence for the induction of those genes following CSD (our unpublished results). Therefore, HALO may be necessary but not sufficient on its own for the full neuroprotective effect elicited by CSD. The identification of additional HALO target genes and further characterization of the genetic response to CSD will provide further insights into the mechanisms of HALO-mediated neuroprotection.

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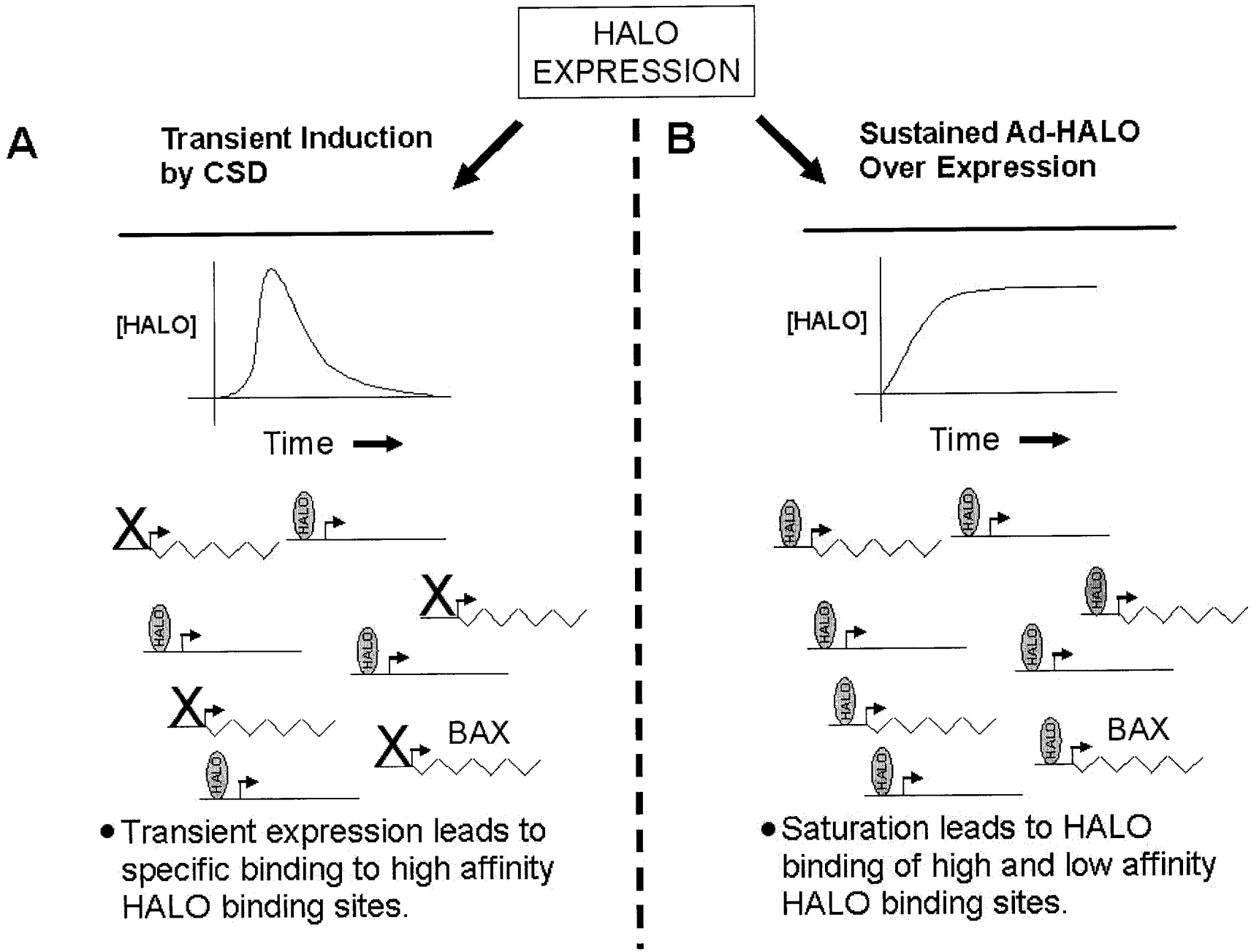
Overall, our data shows that HALO is a novel bHLH-PAS transcriptional activator that mediates BAX expression, functions within the CNS during

Figure 13. The opposite nature of transient and sustained HALO expression leads to different consequences. (A) The transient nature of HALO following CSD may implicate HALO as a neuroprotective gene by regulating appropriate target genes with high affinity binding sites. (B) Sustained expression may cause detrimental effects due to saturation of high and low affinity HALO binding sites leading to the upregulation of aberrant genes such as BAX.



- Transient expression leads to specific binding to high affinity HALO binding sites.

- Saturation leads to HALO binding of high and low affinity HALO binding sites.

embyogenesis and is transiently upregulated by neuronal depolarization, a form of ischemic preconditioning. The identification and preliminary characterization of HALO constitutes an important step into the identification of the molecular mechanisms underlying CSD-mediated neuroprotection. Further studies on HALO gene regulation and its genetic targets will provide insights into potential novel therapeutic approaches to stroke.

Future Experiments

Though these studies have revealed a counterintuitive and detrimental effect of HALOs stable expression in non-neuronal and primary neuronal cell lines, the potential of HALO-mediated neuroprotection still exist on a transient basis. In order to study neuroprotection in a more clinically useful setting, further HALO studies should be performed *in vivo*. Two models could be applied: 1) creating Adeno-Associated Viruses (AAV) containing HALO which would be injected into rat hippocampus prior to subjecting the rats to MCAO. 2) Creating mice with HALO controlled expression by a Tetracycline inducible promoter specifically within the hippocampus and the cortex. Neuroprotection would be analyzed in both models by inducing ischemia following the induced expression of HALO and comparing the volumes of infarction. The Tetracycline control of HALO could allow HALO to be expressed for varying amounts of time potentially validating our results with respect to the transient nature of HALO mediated neuroprotection.

Though the importance of HALO expression in Drosophila development has been attributed to tracheal formation (Jiang and Crews 2003), the function of HALO in vertebrate development has not been fully uncovered. Inhibition of HALO expression by creating a HALO knock out mouse ($HALO^{-/-}$), would substantiate the role for HALO development by examining the observable defects or maladies associated with the deletion. If our observations hold true, barring any compensatory mechanisms, the elimination of HALO expression would cause defects in relation to the CNS and possibly the forelimbs and hind limbs. Additionally, the previously reported SIM2 competition with HALO for the dimerization partner ARNT2 and the similar CME binding element may be an important factor in the HALO knockout mouse phenotype. A depletion of HALO protein in a knockout may exemplify an imbalance between HALO and SIM2 homeostasis towards SIM2 dominant function. Interestingly, chromosomal trisomy associated with overexpression of the critical Down syndrome region includes SIM2, creating an environment that may cause this shift. Therefore, the clinical potential of HALO-/- mouse may result in a slight Down syndrome phenotype, based on previous work by Ooe and colleagues (2004).

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