Alzheimer’s disease-related peptide PS2V plays ancient, conserved roles in suppression of the unfolded protein response under hypoxia and stimulation of $\gamma$-secretase activity

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Abstract

The PRESENLIN1 and PRESENLIN2 genes encode structurally related proteases essential for $\gamma$-secretase activity. Of nearly 200 PRESENLIN mutations causing early onset, familial Alzheimer’s disease (FAD) only the K115Efx10 mutation of PSEN2 causes truncation of the open reading frame. If translated, the truncated product would resemble a naturally occurring isoform of PSEN2 named PS2V that is induced by hypoxia and found at elevated levels in late onset Alzheimer’s disease (AD) brains. The function of PS2V is largely unexplored. We show that zebrafish possess a PS2V-like isoform, PS1IV, produced from the fish’s PSEN1 rather than PSEN2 orthologous gene. The molecular mechanism controlling formation of PS2V/PS1IV was probably present in the ancient common ancestor of the PSEN1 and PSEN2 genes. Human PS2V and zebrafish PS1IV have highly divergent structures but conserved abilities to stimulate $\gamma$-secretase activity and to suppress the unfolded protein response (UPR) under hypoxia. The putative protein truncation caused by K115Efx10 resembles PS2V in its ability to increase $\gamma$-secretase activity and suppress the UPR. This supports increased Aβ levels as a common link between K115Efx10 early onset AD and sporadic, late onset AD. The ability of mutant variants of PS2V to stimulate $\gamma$-secretase activity partially correlates with their ability to suppress the UPR. The cytosolic, transmembrane and luminal domains of PS2V are all critical to its $\gamma$-secretase and UPR-suppression activities. Our data support a model in which chronic hypoxia in aged brains promotes excessive Notch signalling and accumulation of Aβ that contribute to AD pathogenesis.

† The authors wish it to be known that, in their opinion, the first 3 authors should be regarded as joint First Authors.

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Introduction

PRESENILIN1 (PSEN1) is the major locus for autosomal dominant mutations causing familial Alzheimer’s disease (FAD). Most FAD cases show an early age of onset (younger than 65 years). The four known FAD loci are PSEN1, PSEN2, APP and SORL1 (1,2). The PSEN1 and PSEN2 genes encode two structurally related proteins that operate within γ-secretase complexes to cleave type 1 transmembrane proteins (Fig 1A, reviewed in 3). For this reason, they are essential to signalling by a wide variety of proteins such as Notch receptors, p75 and AMYLOID BETA A4 PRECURSOR PROTEIN (APP). The PRESENILINs also have other functions including autophagy (4), phosphorylation of β-catenin (5) and control of chromosome segregation (6,7).

At least 95% of Alzheimer’s disease (AD) is sporadic (SAD) with a late age of onset (65 years or older). SAD shares similar histopathology with FAD including deposition of plaques of Amyloid β-peptide (Aβ) that is produced from APP after cleavage by the β-secretase and γ-secretase enzyme activities (Fig. 1A). However, the causes and pathological mechanism behind SAD are hotly debated.

Evidence is accumulating that hypoxia may play important roles in AD pathogenesis via effects on the development and function of cerebrovasculature. Reduced cerebral blood flow and changes in cerebrovasculature (including microvasculature) have been implicated in AD (8–10). Indeed, Oresic et al. (11) saw that serum markers of hypoxia can be used to differentiate between people with mild cognitive impairment who progress to dementia and those who do not. Notch signalling is known to be important for vascular development (reviewed in 12) and is strongly influenced by hypoxia in this role (13). Interestingly, Luna et al. (14) have shown that loss of APP function in zebrafish causes deficient development of cerebrovasculature and that treatment of such embryos with Aβ can rescue these defects (14). Hypoxia induces HIF1α, a master regulator of cellular hypoxic responses (reviewed in 15). HIF1α binds directly to γ-secretase complexes to increase γ-secretase activity (16) that can then increase Notch signalling and production of the Aβ peptide from APP. Intriguingly, Gama Sosa et al. (10) observed that expression of FAD mutant forms of human PSEN1 exclusively in neurons in transgenic mouse brains caused a strong microvascular phenotype. This is consistent with APP acting in a form of communication between neurons and microvasculature (17).

Over 160 mutations in PSEN1 and ~20 mutations in PSEN2 are thought to cause FAD (1,18). All are mis-sense mutations, deletions or small insertions that preserve the C-terminal protein-coding part of the open reading frame. Recently, a unique exception to this rule was discovered. A frameshift mutation, K115Efx10, was identified in the PSEN2 gene of a woman with early onset AD but with no available family history to confirm FAD (18, Fig. 1B). If expressed this mutant allele would produce a truncated protein very similar to a truncated isoform of PSEN2 named PS2V that shows increased expression in SAD brains (19).

PS2V is a 124-amino acid residue PSEN2 peptide produced when alternative splicing of PSEN2 transcripts excludes exon 5 sequence and truncates the open reading frame (19,20) (Fig. 1B and C). PS2V has the remarkable property that it can increase γ-secretase activity and production of Aβ peptide from APP (20). Using human neuroblastoma SK-N-SH cells, Sato et al. (19) showed that PS2V formation is induced by hypoxia but not other forms of cellular stress. Under hypoxia, expression of a protein, HMGA1a, is induced in neural tissue and then binds to a sequence in exon 5 of PSEN2 transcripts. HMGA1a then interferes with spliceosome complex function at the exon 5 donor

Figure 1. PRESENILIN’s γ-secretase activity, structure and PS2V formation. (A) PRESENILIN act within γ-secretase complexes to cleave Type 1 transmembrane domain proteins such as Notch and APP within lipid bilayers. Before γ-secretase cleavage can occur the proteins must be truncated in luminal spaces or extracellularly by other proteases such as the α- or β-secretase activities that cleave APP. When APP is cleaved by β-secretase, subsequent γ-secretase cleavage produces the Aβ peptide involved in AD. Many γ-secretase substrates have cytosolic domains that migrate into the nucleus when released to modulate gene activity as part of transcription regulatory complexes. (B) Diagram illustrating the multiple transmembrane domain structure of Presenilin proteins. Protein sequence derived from successive exons is illustrated by alternating thick and thin lines. The coding exons are numbered according to the human PSEN1 and PSEN2 genes. N- and C-termini are indicated as well as the site of the endoproteolysis event (arrowhead) that is thought to activate γ-secretase activity. ‘D’ indicates essential aspartate residues at the catalytic site (star). The position of the K115Efx10 mutation in the PSEN2 coding sequence is indicated. This is the only AD mutation known that truncates a PRESENILIN coding sequence. (All other mutations are in-frame and preserve C-terminal sequences). If translated, K115Efx10 mutant transcripts would produce a truncated protein similar to the PS2V isoform of PSEN2 that is generated after an alternative splicing event that excludes exon 5 sequences (dashed) from transcripts. (C) Hypoxia causes mitochondria to generate reactive oxygen species (ROS) that induce expression of HMGA1a in neurons. HMGA1a binds to human PSEN2 transcripts at the 3’ end of exon 5 and excludes splicing factors. Consequently, exon 4 is ligated to exon 6 resulting in a frameshift, early termination of the coding sequence and translation of the truncated protein isoform PS2V.
site causing exon 4 to be ligated to exon 6 and excluding exon 5 sequence from the mRNA (21), (see Fig. 1C).

PS2V peptide had previously been described as ‘aberrant’, (22) probably because it had only been detected in human brain and not in mice or rats. The lack of a genetically malleable model organism has limited our ability to study PS2V’s normal physiological function and its role in AD. In this paper, we show that the alternative splicing mechanism producing PS2V in response to hypoxia is ancient and pre-dates the gene duplication event that formed PSEN1 and PSEN2 in vertebrates. In zebrafish (as in humans), hypoxia induces expression of the Hmga1a protein. However, this causes alternative splicing of transcripts of the zebrafish psen1 gene rather than psen2. The novel peptide product of the alternatively spliced mRNA, ‘PS1IV’, is far smaller than PS2V but, nevertheless, has retained PS2V’s abilities to stimulate γ-secretase activity and suppress the unfolded protein response (UPR). However, the molecular mechanisms by which these two peptides act may differ since human PS2V function requires protein domains that PS1IV does not possess. Thus, human PS2V and zebrafish PS1IV are a remarkable example of natural selection acting to conserve function, not structure. We use our zebrafish assays to examine activities of the putative peptide product of the K115Efx10 mutant allele of human PSEN2 that causes early onset AD. We show that the K115Efx10 peptide behaves like the PS2V peptide of SAD brains. Our data support a universal role of HC for PS2V function requires pro- (UPR). However, the molecular mechanisms by which these two peptides act may differ since human PS2V function requires protein domains that PS1IV does not possess. Thus, human PS2V and zebrafish PS1IV are a remarkable example of natural selection acting to conserve function, not structure. We use our zebrafish assays to examine activities of the putative peptide product of the K115Efx10 mutant allele of human PSEN2 that causes early onset AD. We show that the K115Efx10 peptide behaves like the PS2V peptide of SAD brains. Our data support a universal role of HC for PS2V function requires pro-

An ancient, conserved mechanism controls generation of human PS2V and zebrafish PS1IV

Manabe et al. (21,22) clearly defined the mechanism controlling loss of exon 5 sequence from human PSEN2 transcripts to form PS2V. In humans, transcription of HMGA1a (a splicing isoform of HMGA1) is induced by hypoxia. HMGA1a protein then binds to two sites at the 3′ end of exon 5 in PSEN2 transcripts (Fig. 2B and D). Binding of HMGA1a appears to block functional association of U1 snRNP with the exon 5 splice donor site of PSEN2 transcripts causing alternative splicing of exon 4 to exon 6 (21) (Fig. 2B). We have previously shown that the DNA binding domain of zebrafish Hmga1a protein is highly similar to that of human HMGA1a (24). However, the nucleotide sequence at the 3′ end of exon 5 in zebrafish psen2 is not highly conserved (24) (see Fig. 2D). This is consistent with the failure of zebrafish psen2 to produce a PS2V-like mRNA (24).

The PSEN1 and PSEN2 genes evolved from a single, ancestral PSEN gene. We reasoned that the HMGA1a-dependent mechanism controlling formation of human PS2V might also control formation of PS1IV from zebrafish psen1. Therefore, we examined the sequence of psen1 for potential HMGA1a-binding sites. Consistent with conservation of an HMGA1a-dependent mechanism, we discovered that the best match to the HMGA1a-binding site consensus was at the 3′ end of exon5 in psen1 (Fig. 2D). In fact, this site in psen1 is more similar to the HMGA1a-binding site in exon 5 of human PSEN2 than the related sequence in the zebrafish psen2 gene (Fig. 2D).

A binding site for Hmga1a appears to be conserved in exon 5 of zebrafish psen1 but hypoxia causes loss of exon 4, not exon 5, from psen1 transcripts. Current knowledge on the mechanisms controlling alternative splicing is limited and this makes it difficult to predict the outcomes of such events (e.g. see 27). Therefore, it was necessary to demonstrate that the putative HMGA1a-binding site in exon 5 of psen1 does, in fact, control the exclusion of exon 4 sequence from transcripts under hypoxia. To this end, we designed a morpholino antisense oligonucleotide, MoHmga1aBindBlock, to bind to psen1 transcripts and partially overlap the putative Hmga1a-binding site (see Fig. 2D). Since this morpholino binds far downstream of the Psen1 translation start codon, it should not block protein translation (28). MoHmga1aBindBlock binds at a distance of 19 nt upstream of the exon 5/intron 5 boundary. It should block binding of the Hmga1a protein to psen1 transcripts without interfering with normoxic splicing (Fig. 2D). Injection of this oligonucleotide into embryos followed by exposure to chemical mimicry of hypoxia (100 μM NaNO2) and RT-PCR analysis showed that MoHmga1aBindBlock greatly reduced exclusion of exon 4 from psen1 transcripts under hypoxia while the common, normoxic, splicing pattern still occurred (Fig. 3A).

Confirmation that Hmga1a protein is required for formation of zebrafish PS1IV

To confirm that Hmga1a is responsible for the exclusion of exon 4 from psen1 transcripts that gives PS1IV formation, we sought to
inhibit production of Hmga1a protein under hypoxia. A morpho-
lino antisense oligonucleotide (MoHmga1aTlnBlock, see Supple-
mentary Material, Supplemental Data File 2) was designed to
block translation of Hmga1a protein by binding over the start
codon of \( \text{hmga1a} \) mRNA. Injection of this morpholino followed
by chemical mimicry of hypoxia greatly reduced loss of exon 4
from \( \text{psen1} \) transcripts (Fig.3B). We could also force the formation
of PS1IV under normoxia by forcing expression of Hmga1a
protein. This was performed by injecting mRNA coding for
Hmga1a into zebra
fish embryos (Fig.3C1 and D). Furthermore,
the evolutionary conservation of this system controlling alterna-
tive splicing of \( \text{PRESENILIN} \) genes was demonstrated by forcing
expression of human HMGA1a protein in zebra
fish embryos by
mRNA injection under normoxia. This also led to formation of
\( \text{psen1} \) transcripts lacking exon 4 (Fig. 3C2). In both these cases
(forced expression of zebra
fish Hmga1a or human HMGA1a
under normoxia) the consequent loss of exon 4 could be inhibited
by co-injection of MoHmga1aBindBlock to prevent binding of
these proteins to nascent \( \text{psen1} \) transcripts (Fig. 3C1, C2 and D).
As a negative control, injection of a non-translatable mRNA
had no effect on \( \text{psen1} \) transcript splicing (Fig. 3C1, C2 and D).
These experiments clearly demonstrate evolutionary conserva-
tion of the mechanism controlling alternative splicing of zebra-
fish \( \text{psen1} \) and human PSEN2 under hypoxia, even though the
outcomes differ (i.e. different exons become excluded from the
mRNAs). Presumably, the ancient ancestral \( \text{PRESENILIN} \) gene
that was duplicated to form the modern \( \text{PSEN1} \) and \( \text{PSEN2} \) genes also displayed HMGA1a-dependent alternative splicing.
Prediction of hypoxia-inducible alternative splicing in other vertebrate lineages

The alternative splicing of PRESENILIN transcripts under hypoxia appears to depend on conservation of the HMGA1a-binding sites in their exon 5 sequence. The HMGA1a-binding sites in exon 5 of guinea pig Psen2 and zebrafish psen1 are highly similar to that in exon 5 of human PSEN2 (Fig. 2D, nucleotides shown as white on black differ from the human PSEN2 sequence) and transcripts of these genes show exon exclusion under hypoxia (23 and this study). In contrast, the equivalent sequences in exon 5 in zebrafish psen2 and in human PSEN1 show many nucleotide differences (Fig. 2D) and transcripts of these genes are not known to show exclusion of exons under hypoxia (24). Alignment of the 3’ region of exon 5 of PSEN1 and PSEN2 in various vertebrate species supports that most mammals possess hypoxia-inducible alternative splicing in PSEN2 transcripts but not in transcripts from PSEN1 (Fig. 2D). Interestingly, rats and mice (both species within the rodent subfamily Murinae) lack the ability to produce PS2V (23) and our tests have revealed no evidence for hypoxia-inducible splicing of Psen1 transcripts in these species (Supplementary Material, Supplemental Data File 3). The avian, Gallus gallus (chicken) and an amphibian, X. laevis (African clawed

Figure 3. Inhibition of PS1IV formation under hypoxia by blockage of Hmga1a function. (A) Mimicry of hypoxia by NaN3 exposure induces the alternative splicing event in psen1 transcripts that generates PS1IV as revealed by RT-PCR between exons 3 and 7. Injection of an inactive, negative control morpholino MoControl has no apparent effect on PS1IV formation (filled arrowhead) but injection of MoHmga1aBindBlock (MoHmga1aBB) that binds over part of the putative Hmga1a-binding site in exon 5 of psen1 transcripts inhibits PS1IV formation. (B) Injection into zebrafish embryos of a morpholino inhibiting translation of hmg1a mRNA, MoHmga1aTlnBlock (MoHmga1aTlnB), blocks formation of PS1IV under chemical mimicry of hypoxia (filled arrowhead). (C) Injection into zebrafish embryos of mRNA coding either for zebrafish Hmga1a protein (C1) or human HMGA1a protein (C2) can induce formation of PS1IV under normoxia (filled arrowheads). Co-injection of MoHmga1aBindBlock inhibits this induction. (D) qPCR detecting specifically PS1IV formation indicates at least a 3-fold induction of PS1IV by forced expression of zebrafish Hmga1a under normoxia. All PCRs were conducted on embryos at 48 hpf. Unfilled arrowheads indicate cDNA amplified from full-length transcripts.
frog) also show reduced conservation of the HMGA1a-binding site in exon 5 of their PSEN1 and PSEN2 genes (Fig. 2D). We have previously shown that PS2V expression can be induced in explanted guinea pig brain tissue by incubation in medium containing NaN3 (23). To test for PS2V-like alternative splicing events in chicken we performed similar brain explant experiments. We did not observe exclusion of exon 4 or exon 5 from transcripts of either chicken PSEN1 or PSEN2 (Supplementary Material, Supplemental Data File 3). Interestingly, the teleost Fugu rubripes (a puffer fish) does not have a well-conserved Hmg1a-binding site in exon 5 of either its psen1 or psen2 gene (Fig. 2D). The variability with which PS2V-like splicing events are conserved in various vertebrate lineages implies that, for some species, loss of the ability to form FS2V-like peptides under hypoxia is not severely detrimental to survival.

To test whether hypoxic control of alternative splicing exists in the single presenilin gene of an insect we exposed Drosophila melanogaster to severe hypoxia before extraction of mRNA and RT-PCR. However, no such alternative splicing was observed for transcripts of the Drosophila PRESENILIN orthologue Psn (Supplementary Material, Supplemental Data File 3).

Both human PS2V and zebrafish PS1IV can stimulate γ-secretase activity despite extreme structural divergence

The conservation of the mechanism controlling hypoxic induction of human PS2V and zebrafish PS1IV during 450 million years of evolutionary divergence (29) suggests that these peptides serve important, selectively advantageous functions despite their very different structures. The human PS2V peptide consists of 124 amino acid residues and is expected to possess one transmembrane domain (Fig. 2C). In contrast, the zebrafish PS1IV peptide is predicted to possess only 18 amino acid residues (12 amino acid residues encoded by exon 3 followed by 6 novel amino acid residues coded by exon 5) (Fig. 2C). Does this extreme structural divergence represent evolution towards different functions or are their functions conserved? To what extent can zebrafish PS1IV serve as a model of human PS2V? To assess this, we investigated (i) whether the known activities of human PS2V could be assayed in zebrafish embryos and (ii) whether PS1IV possesses these known activities. To understand the structure-function relationships of these activities, we also analyzed the activities of peptides derived from exon 3 (similar to PS1IV) and from exons 3 + 4 (similar to PS2V) of the zebrafish and human PRESENILIN genes.

Sato et al. (20) showed that forced expression of human PS2V increases Aβ levels in mouse neuro 2a cells, presumably by increasing γ-secretase cleavage of mouse APP protein. We have developed a unique, in vivo assay for γ-secretase activity based on cleavage of the protein product of the zebrafish appa gene (Appa), a co-ortholog of human APP (30). We exploited this assay to see whether forcing expression of human PS2V in zebrafish embryos could stimulate cleavage of zebrafish Appa. Messenger RNA coding for human PS2V (fused at its N-terminal to a FLAG antibody tag) was injected into fertilized zebrafish embryos together with the assay construct (see Materials and Methods and the legend to Fig. 4 for details). PS2V was observed to stimulate γ-secretase cleavage of the Appa assay construct (Fig. 4A). Thus, zebrafish embryos are a suitable system in which to analyse this activity of human PS2V. However, an equivalent truncation of the zebrafish Psen2 protein (zPsen2Δ-4; derived from exons 3 + 4) was unable to stimulate Appa-cleavage consistent with the fact that hypoxia does not cause alternative splicing of zebrafish psen2 transcripts (Fig. 4A). As previously published (31), PS2V-equivalent truncations of both zebrafish Psen1 (zPsen1Δ-4) and human PSEN1 (hPsen2Δ-4) are able to stimulate zebrafish Appa cleavage (Fig. 4A). Thus, protein structures exist within human PSEN1 and PSEN2 and within zebrafish Psen1 that can stimulate the γ-secretase cleavage of our Appa assay construct. Zebrafish Psen2 appears to have lost these structures after the divergence of the tetrapod and teleost (bony fish) evolutionary lineages while human PSEN1 has maintained them.

Zebrafish PS1IV is derived only from psen1’s exon3 sequence plus 6 amino acid residues from frameshifted sequence in exon 5. It resembles the zebrafish Psen1Δ-3 construct described in ref. (31) that includes only exon 3-derived sequence. In that paper, expression of Psen1Δ-3 proved too toxic to embryo development to allow us to assay its ability to stimulate γ-secretase activity. Subsequently, we found that we could reduce the toxicity of Psen1Δ-3 by reducing the concentration of the mRNA injected into embryos (see Materials and Methods). To test whether PS1IV shares PS2V’s ability to stimulate γ-secretase activity we tested the effect...

Figure 4. Appa-cleavage assay for stimulation of γ-secretase activity by PS2V-like peptides. Assays for γ-secretase cleavage of an Appa-derived substrate as described in ref. (30). Values below 1.0 (horizontal line) indicate increased γ-secretase cleavage of the substrate while values above 1.0 indicate decreased γ-secretase activity. (A) PS2V-like peptides including exon 3 and exon 4-derived sequence. All the peptides except that derived from zebrafish Psen2 can stimulate γ-secretase activity. (B) PS1IV-like peptides including exon 3-derived sequence. Only PS1IV (zPS1IV) and the almost identical peptide zPsen1Δ-3 (lacking only PS1IV’s exon 3-derived sequence) show an ability to stimulate γ-secretase cleavage. The exon 3-derived protein sequences of zebrafish Psen2 and human PSEN1 and PSEN2 lack this activity (except note ns* below). Values of P are from unpaired two-sample t-tests comparing peptide values with negative control mRNA values (see Supplementary Material, Supplemental Data File 7). ns, not significant ($\text{P} \geq 0.01$); ns*, hPSEN1Δ-3 did show a possibly significant increase in γ-secretase activity in comparison with negative control mRNA in a two-way ANOVA with the Dunnett Multiple Comparisons test (see Supplementary Material, Supplemental Data File 7). Error bars are standard errors of the means. DAPT indicates treatment with 100 μM of this γ-secretase inhibitor.
of co-injecting it with our Appa-cleavage assay construct. We also tested the effects on Appa cleavage of peptides derived from only the exon 3 sequences of zebrafish psen1 (zPsen1Δ>3), zebrafish psen2 (zPsen2Δ>3), human PSEN1 (hPSEN1Δ>3) and human PSEN2 (hPSEN2Δ>3). Interestingly, only PS1IV and zPsen1Δ>3 were able to stimulate the γ-secretase cleavage of our Appa-assay construct (although statistical analysis indicates the possibility of a lower level of stimulatory activity by hPSEN1Δ>3, see Fig. 4B). Thus, the ability of human PS2V to stimulate γ-secretase cleavage is conserved in zebrafish PS1IV meaning that PS1IV can be used to model this function of human PS2V. The zPsen1Δ>3 peptide is much smaller than PS1IV since it possesses only exon 3-derived amino acid residues. Nevertheless, the structure of zPsen1Δ>3 is sufficient to stimulate γ-secretase cleavage of Appa. However, if the exon 3-derived sequences of the other PRESENILIN proteins ever had this ability, they have lost it over evolutionary time. Alternatively, exon 3 of the zebrafish psen1 gene may have evolved this ability after the separation of the tetrapod and teleost lineages.

PS2V expression stimulates Notch signalling

While PS2V can stimulate γ-secretase cleavage of APP, it is not known whether this ability is specific to cleavage of APP or affects other protein substrates of γ-secretase more generally. After APP, NOTCH receptors are the next most intensively studied substrates of γ-secretase. Zebrafish possess orthologues of the human NOTCH1, 2 and 3 genes. There is not yet a direct assay of Notch cleavage available in zebrafish. The responsiveness of target genes to Notch signalling varies in different parts of the developing zebrafish embryo. This means that it is difficult to use quantitative reverse transcription PCR (qPCR) to assess Notch target gene expression. Instead, we have developed a Notch-signalling assay based on comparative assessment of staining in the trunk region of zebrafish embryos that have been subjected to whole mount in situ transcript hybridization to detect neurogenin1 transcripts (31,32). The assay is described in detail in ref. (32).

Briefly, staining for neurogenin1 expression in the individuals of a group of treated embryos is assessed as lighter than, similar to, or darker than that of an embryo from a control group. Staining in the other individuals of the control group is similarly assessed. The numbers of embryos in each class are used to populate a contingency table that is subjected to a $\chi^2$ test of independence for $k$ groups. The output of the assay is thus a value of $P$. Smaller values of $P$ indicate greater differences in apparent neurogenin1 staining between treated and control embryos and may possibly be correlated with more severe changes in Notch signal but this has not yet been confirmed experimentally.

To assess the ability of PS2V and PS1IV to stimulate Notch signalling (presumably via stimulation of γ-secretase cleavage of Notch receptors) we performed the above assay on embryos injected with mRNAs coding for these peptides (including N-terminal FLAG antibody tag fusions). We also tested peptides corresponding to the sequences of exon 3 and exons 3 + 4 of the zebrafish and human PRESENILIN genes as above (see Fig. 5). The results are largely consistent with our observations of cleavage of our Appa-assay construct. Thus PS2V, hPSEN2Δ>4, hPSEN1Δ>4 and zPSen1Δ>4 all stimulate Notch signalling. However, the equivalent truncation of zebrafish Psen2, zPsen2Δ>4, does not (Fig. 5A). Among the PS1IV-like peptides, only zPsen1Δ>3 was observed to be able to stimulate Notch signalling (Fig. 5B). The sequence of this peptide is included within PS1IV so it is unexpected that PS1IV was not observed to stimulate Notch signalling significantly. Possibly PS1IV is less stable than zPsen1Δ>3 or the exon 5-derived sequence that forms part of PS1IV suppresses the activity of the exon 3-derived region. Most likely our assay is too crude and insensitive to detect stimulation of Notch signalling by PS1IV.

Both human PS2V and zebrafish PS1IV suppress the UPR

Aside from stimulation of γ-secretase cleavage of APP, the other activity previously demonstrated for PS2V is its ability to suppress the UPR. Sato et al. (20) previously showed suppression of the UPR when PS2V expression is forced in human HEK293T cells (a kidney-derived cell line that does not normally express PS2V, 34). Induction of the UPR occurs via a change in the splicing of transcripts of the gene XBP1 to form the isoform XBP-1S (35). In zebrafish, we describe this gene and transcript isoform as xbp1 and xbp1s, respectively). Therefore, qPCR can be used to assay the relative UPR response in zebrafish embryos (35). To investigate further the conservation of function between PS2V and PS1IV despite their extreme structural divergence we assessed their ability to suppress the UPR in zebrafish embryos. The UPR can be stimulated in zebrafish embryos by treatment with the antibiotic tunicamycin. Embryos injected with mRNAs encoding PS2V or PS1IV and subsequently treated with tunicamycin show a much reduced UPR compared with embryos injected with a negative control (untranslatable) mRNA before tunicamycin treatment (Fig. 6). Thus, both PS2V and PS1IV suppress the UPR.

Suppression of the UPR is correlated with ability to stimulate γ-secretase activity

Katayama et al. (36) observed that the ability of PS2V to increase Aβ levels (presumably by stimulation of γ-secretase cleavage of APP)
An hypothesis for the function of PS2V-like truncations in the UPR

The ability of human PS2V and zebrafish PS1IV to suppress the UPR is conserved despite their radically different structures. What selective pressure could maintain this function during 450 million years of divergent evolution? A number of the chaperone proteins required for protein folding in the endoplasmic reticulum (ER) are ATP-dependent (reviewed in 37) and ATP-production by mitochondria is sensitive to oxygen levels. Also, the formation of disulphide bonds in the mitochondrial associated membranes of the ER (oxidative protein folding) requires oxygen (reviewed in 38). Therefore, hypoxia causes protein folding stress and stimulates the UPR (39,40). Excessive activation of the UPR causes cell death (reviewed in 41). Indeed, both signalling by the AD-associated protein APP (42) and Aβ production (43) (that would be stimulated by increased APP transcript levels (44) and increased γ-secretase activity (16) under hypoxia) can potentiate the cell death caused by ER stress. However, in cellular systems, negative feedbacks commonly exist to inhibit over-activation of particular activities. Therefore, we hypothesized that induction of PS2V-like peptide formation under hypoxia acts to dampen the UPR and inhibit cell death. To test this hypothesis, we first examined induction of the UPR under hypoxia in zebrafish and then the ability of PS2V-like peptides to modulate this response.

Hypoxia activates UPR signalling pathways in zebrafish

Three signalling pathways are known to mediate the UPR. The stress-sensing proteins that activate each of these signalling pathways are ATF6, EIF2AK3 (PERK) and ERN1 (IRE1α) (41). There are cross-regulatory relationships between these pathways (45). Increased expression of UPR genes including those encoding the proteins above can indicate induction of the UPR. To confirm that hypoxia induces the UPR in zebrafish, we used qPCR to examine the expression of the UPR genes ddit3 (chop), eif2ak3 (perk), ern1 (ire1), hspa5 (bip) and hsp90b1 (grp94) in the brains of adult zebrafish exposed to real hypoxia and in embryos subjected to mimicry of hypoxia using sodium azide. All these genes show very significantly increased expression under these conditions (Fig. 7A). This supports that hypoxia induces the UPR in zebrafish.

PS2V-like peptides suppress activation of the UPR and cell death under mimicry of hypoxia in zebrafish embryos

If PS2V-like peptides inhibit over-activation of the UPR under hypoxia then forced expression of such peptides under mimicry of hypoxia in zebrafish embryos should suppress the UPR. Injection of mRNA coding for PS2V or PS1IV led to dramatically decreased UPR activation and increased cell death. We assayed UPR activation under mimicry of hypoxia in embryos at 24 h post fertilization. Interestingly, embryos injected with mRNA coding for zPsen2Δ>4 showed the least ability to suppress UPR activation compared with embryos expressing other peptides (Fig. 7B).

According to our hypothesis, zebrafish embryos unable to express endogenous PS1IV under hypoxia should show over-activation of the UPR and increased cell death. We assayed UPR activation under mimicry of hypoxia in embryos that were either un.injected, or injected with the inactive negative control morpholino (MoControl), or injected with the morpholino blocking binding of Hmga1a protein to psen1 transcripts (MoHmga1aBind-Block) that inhibits PS1IV formation. As expected, embryos injected with MoHmga1aBindBlock showed significantly increased activation of the UPR relative to uninjected or MoControl-injected embryos under mimicry of hypoxia at 48 h post fertilization (see Fig. 7C). We also analyzed levels of cell death in embryos at 48 h post fertilization using our previously published method (46). Under normoxia, there was no significant difference in the levels of cell death between embryos injected with the negative control morpholino MoControl or the morpholino blocking PS1IV formation, MoHmga1aBindBlock. However, under mimicry of hypoxia, embryos lacking PS1IV formation showed significantly greater cell death than the control embryos (Fig. 7D). These results support that PS2V-like peptides function to modulate the UPR and inhibit excessive cell death under hypoxia.

Analysis of hPSEN2Δ>4/zPsen2Δ>4 chimeras indicates that stimulation of γ-secretase may be separable from UPR suppression

PS2V (and hPSEN2Δ>4 that lacks only PS2V’s novel exon 6-derived amino acid residues) can stimulate γ-secretase cleavage of APP and Notch signalling, while the equivalent truncation of the zebrafish Psen2 protein, zPsen2Δ>4, cannot. hPSEN2Δ>4 can also...
suppress the UPR when this is induced by either tunicamycin (Fig. 6) or mimicry of hypoxia (Fig. 7B), whereas zPsen2Δ>4 is very significantly less able to do this. To map the structural domains required for hPSEN2Δ>4’s ability to stimulate γ-secretase activity, we constructed cDNAs encoding chimeric proteins in which particular regions of hPSEN2Δ>4 were replaced with corresponding sequences from zPsen2Δ>4 (see Fig. 8A and B). Surprisingly, all of our chimeric proteins were inactive in our Appa-cleavage and Notch-signalling assays (see Supplementary Material, Supplemental Data File 4). Therefore, the overall structure of hPSEN2Δ>4—and thus human PS2V—appears to be important for its activity and structures affecting PS2V function exist in its cytosolic, transmembrane and luminal domains. This is remarkable when one considers that only two amino acid residues differ between the transmembrane domains of hPSEN2Δ>4 and zPsen2Δ>4 (see ‘Region 3’ in Fig. 8C). We interpret this to mean that interaction(s) must exist between the transmembrane domain and other domains of hPSEN2Δ>4 (PS2V) that are essential to its ability to stimulate γ-secretase activity.

The inability of zPsen2Δ>4 to stimulate γ-secretase activity corresponds with its relative inability to suppress the UPR. We showed earlier that the abilities to stimulate γ-secretase activity and suppress the UPR are correlated. As a further test of this idea, we asked whether the single amino acid changes in the transmembrane domain that abolish hPSEN2Δ>4’s ability to stimulate γ-secretase activity also abolish its ability to suppress the UPR. Analysis showed that one of the two mutations (m1) completely abolishes hPSEN2Δ>4’s ability to suppress the UPR while the
other mutation (m2) still allows partial UPR suppression (Fig. 8D). Thus, suppression of the UPR may not entirely explain the ability of PS2V-like molecules to stimulate γ-secretase activity. Alternatively, our assays may not be sufficiently sensitive to detect γ-secretase stimulation below certain thresholds.

The K115Efx10 mutation of human PSEN2 indicates that PS2V may be a molecular common link between sporadic AD and FAD

Around 200 dominant mutations in the human PRESENILIN genes are known to cause FAD. Most of these occur in PSEN1 but a number of mutations have been described in PSEN2 (1,18). Until recently all known FAD mutations in the PRESENILINs were mis-sense mutations or internal deletions/insertions that preserve C-terminal protein-coding sequences. Recently, a woman suffering early onset AD was reported to be heterozygous for a frameshift mutation in PSEN2, K115Efx10 (18). This mutation is a dinucleotide deletion in the PSEN2 coding sequence only six codons prior to the end of exon 4 resulting in a premature stop codon in exon 5 sequence (Fig. 1B). If transcripts of this gene are not degraded through nonsense-mediated decay, then they should generate a peptide similar in structure to PS2V and might act to increase Aβ production. They might also suppress the UPR which could promote accumulation of protein aggregates as is commonly seen in neurodegenerative diseases (47).
To test whether the putative truncated product of K115Efx10 increases γ-secretase activity we synthesised mRNA encoding it and injected this into zebrafish embryos. This was able to increase both cleavage of Appa (Fig. 4A) and Notch-signalling (Fig. 5A) consistent with K115Efx10 causing AD by increasing Aβ production. The putative-truncated product of K115Efx10 can also suppress the UPR with a potency similar to PS2V both in response to tunicamycin treatment (Fig. 6) and mimicry of hypoxia (Fig. 7B). This supports that the K115Efx10 mutation may cause early onset AD by forcing chronic expression of PS2V-like functions.

To show that the PS2V-like peptide putatively produced due to the K115Efx10 mutation in human PSEN2 could cause increased expression of Aβ in human cells, we cloned cDNA coding for this peptide into the pEGFP-N1 vector (CLONTECH Laboratories, Inc., Mountain View, CA, USA) and transfected this into human HEK-293 cells stably expressing the Swedish APP mutant (HEK-293) APPsw (48). However, consistent with our previous experience (31), we were unable to obtain sufficient expression of this construct in the cells to permit assessment of changes in Aβ production. Observation of the effect of a K115Efx10-derived peptide on Aβ expression in zebrafish must await characterization of zebrafish forms of Aβ and development of assays to measure these.

Discussion

The human PSEN1 and PSEN2 genes are major loci for dominant mutations causing FAD but genome-wide association studies (GWAS) have not linked variation at these loci to risk of sporadic, late onset AD. To date, the increased expression of truncated PSEN2 isoform PS2V in sporadic AD brains is the strongest evidence for involvement of the PRESENILIN genes in sporadic AD. However, research into the function of PS2V has been limited, possibly because PS2V was thought to be an ‘aberrant’ molecule resulting from AD pathological processes (20,22,49–51) and because laboratory mice and rats (Mus musculus and Rattus norvegicus) lack the ability to form PS2V (23). We recently showed that the inability to form PS2V is a peculiarity of at least some members of the rodent familyMuridaeand that other rodents and probably most mammals do, in fact, express PS2V (23).

Previously, Sato et al. (20) demonstrated that PS2V could suppress the UPR in cultured cells and increase the production of Aβ from APP. This apparent ability to increase γ-secretase activity may be linked to the suppression of the UPR, since Sato et al. were also able to increase Aβ production by suppression of the UPR in mouse Neuro 2a cells using a dominant-negative form of the ER stress monitoring protein ERN1 (IRE1) (20). In this paper, we have demonstrated conclusively that PS2V, does, in fact, up-regulate γ-secretase activity and that it does so not only for cleavage of APP but for other systems in which γ-secretase acts such as Notch signalling. We have also demonstrated that the mechanism controlling PS2V formation is truly ancient in vertebrates and probably functioned for the ancestral PRESENILIN gene that duplicated to form the PSEN1 and PSEN2 lineages over 450 million years ago (see later). Despite dramatic structural divergence between human PS2V and zebrafish PS11V, both molecules have retained the ability to suppress the UPR and stimulate γ-secretase activity (see Fig. 9). This indicates that PS2V formation is fundamentally a pro-survival cellular response to oxidative stress in sporadic AD brains. Such oxidative stress may be caused by hypoxia that would inhibit normal protein folding and create ER stress. Our results support that the induction of PS2V in humans and PS11V in zebrafish represents a negative feedback mechanism suppressing overactivation of the UPR under hypoxia to inhibit cell death. This is consistent with the pro-URP activities of both PRESENILIN (52) and APP (53) proteins.

In previous work (24,44), we analyzed the response to low oxygen levels of the genes encoding proteins (including the PRESENILINs) required for formation of the Aβ peptide that accumulates in the brains of people with AD (19). We showed that, similar to humans, expression of these genes appears to be up-regulated in zebrafish brains under hypoxia. Therefore, increased expression of Aβ under low oxygen is a selectively advantageous response that has been conserved during almost half a billion years of divergent vertebrate evolution. This supports that Aβ may be protective to neurons under stress (reviewed by 54) and is consistent with a number of analyses showing that Aβ can function as an antioxidant (55–59) (although Aβ complexed with metal ions appears capable of generating reactive oxygen species via the Fenton reaction 60,61).

Figure 9. The evolution of PS2V-like structure and function. The most parsimonious explanation for the divergent evolution of PS2V-like truncated PRESENILIN peptides is that these were produced by an ancestral PRESENILIN gene before the duplication event that formed the PSEN1 and PSEN2 genes. The ability to form PS2V-like truncated peptides under hypoxia was subsequently lost from PSEN1 in mammals and from psen2 in teleosts (bony fish). The fact that a PS2V-equivalent artificial truncation of human PSEN1 retains some PS2V-like activities suggests that both exon 3 (3′) and exon 4 (4′) sequences were required in the ancestral PRESENILIN for its PS2V-like activity. In teleosts, exon 3-derived sequences have evolved to perform these functions without the need for exon 4-derived sequences.
The work described in this paper presents additional evidence for an important role of PRESENLIN genes in cellular responses to low oxygen. Like human PS2V, zebrafish PS2V is produced in response to hypoxia as a result of induction of zebrafish Hmga1a protein that binds in transcribed psen1 sequence cognate with exon 5 of human PSEN2. Unexpectedly, however, this result in exclusion of exon 4 sequence rather than the exclusion of exon 5 sequence that occurs for human PSEN2 transcripts. There is no ready explanation for the difference in splicing outcomes controlled by the binding of human HMGA1a/zebrafish Hmga1a in exon 5 of the human PSEN2 and zebrafish psen1 transcripts, respectively. The different outcomes imply the existence of higher order structures formed during splicing allowing events at one intron–exon junction to affect events at another. The relative timing of the splicing events may also be important. Just as HMGA1a/Hmga1a produces very different outcomes by binding near the splice donor site of exon 5 in humans and zebrafish, so can attempts to manipulate the splicing of cognate exons of the DMD gene orthologues of humans and mice produce different splicing outcomes (27).

We recently showed that the ability of a PS2V-like molecule (2pSen1Δ4) to stimulate γ-secretase activity is reliant on the presence of full-length zebrafish Psen1 protein, apparently via a direct interaction (31). However, like Sato et al. (20), we cannot yet explain mechanistically how PS2V-like molecules increase Aβ/γ-secretase activity. It will be interesting to test whether, like 2pSen1Δ4, the shorter PS2V protein of zebrafish is dependent on full-length Psen1 to stimulate γ-secretase and, indeed, whether dominant-negative forms of ERN1 that suppress the UPR show similar dependency.

In possible contrast to our observations, Ohta et al. (62) saw that increased ER stress due to treatment with tunicamycin could stimulate γ-secretase activity in mouse kidney cells and human HEK293 cells (62). Tunicamycin treatment increased expression of the transcription factor ATF4 and this led to increased expression of PSEN1 mRNA and protein. Suppression of ER stress using the UPR inducer quercetin had the opposite effect (62). It will be important to test whether these effects can be replicated in zebrafish embryos and to differentiate between the effects on γ-secretase activity of ER stress (an excess of unfolded protein) and the cellular response to it (the UPR). In understanding Ohta et al.’s observations, it may also be relevant that mice do not express PS2V (this paper) and that HEK293 cells are known to be a cell type that does not express PS2V since they do not induce HMGA1a expression under hypoxia (22,34).

The work described in this paper includes the first structure/function analysis of PS2V-like molecules. The parallel activities of human PS2V and zebrafish PS1IV are a remarkable example of conservation of function in the face of extreme structural divergence. Only the exon 3–derived region of zebrafish Psen1 has the ability, by itself, to suppress the UPR and stimulate γ-secretase. The exon 3–derived sequences of human PSEN1 and PSEN2 do not. Also, no part of human hPSEN2Δ4 (equivalent to PS2V but lacking the normal C-terminal amino acid residues derived from exon 6), either cytosolic, transmembrane or luminal, could be substituted with the cognate region of zebrafish hPSEN2Δ4 while maintaining activity. Remarkably, even a single amino acid change in the transmembrane domain of hPSEN2Δ4 was sufficient to abolish its UPR suppression and γ-secretase stimulating activities. This implies that the transmembrane domain of hPSEN2Δ4 (and PS2V) has sequence-dependent interactions with its neighbouring domains during expression of its UPR suppression and γ-secretase stimulating activities. Interestingly, the positions of the two amino acids that differ between the transmembrane domains of human PSEN2 and zebrafish Psen2 are separated by 6 amino acid residues implying that they sit on the same face of these α-helical structures. Hardy and Crook (63) showed that FAD mutations in the α-helical transmembrane domains of the PRESENL1 protein, including transmembrane domain 1, tend to sit on the same face of these domains suggesting that they influence interactions between separate transmembrane domains within lipid bilayers.

We previously observed that the regulation of genes encoding proteins involved in Aβ production in response to hypoxia is conserved between humans and zebrafish (44). Production of PS2V through alternative splicing of PSEN2 transcripts in response to hypoxia appears to be an additional, simultaneous mechanism regulating Aβ synthesis (19,20). The conservation of PS2V function over nearly half a billion years is additional evidence that stimulation of Aβ production under low oxygen is selectively advantageous. The most parsimonious explanation for the conservation of this oxygen-sensitive alternative splicing event in the PSEN2 gene of mammals and the psen1 gene of teleosts is that the mechanism was present in the common ancestor of the PSEN1 and PSEN2 genes and was lost from one of the duplicate pair due to sub-functionalization after the divergence of the teleost and tetrapod lineages (Fig. 9). However, we found no evidence for formation of PS2V-related splicing isoforms in amphibians or birds and even some mammals (mice, rats) and teleosts (F. rubripes) appear to have lost this mechanism entirely. Deeper insight into the biological roles of PS2V and the existence of any redundant mechanisms will be required to understand why this mechanism is not consistently conserved across all vertebrate species.

Why is PS2V expressed at high levels in the brains of people with late onset, sporadic AD? Its presence is probably a marker of the high levels of oxidative stress known to occur in brains with overt AD (64,65) and suggests that this may be due to restricted oxygen levels. (Although the hypoxic response regulator HIF1α protein is reported to be down-regulated in overt AD post-mortem brains (66), HIF1α mRNA is observed to increase during normal brain aging (67). We suggest that PS2V and Aβ may be elements of a homeostatic mechanism for regulating levels of reactive oxygen species in the brain. Aβ production may be enhanced in cells to buffer increased ROS when oxygen levels fluctuate below normal (possibly due to temporarily increased neuronal activity). Aβ may also have a role in activating HIF1α expression to reduce oxidative stress by increasing the flow of glucose through glycolysis and the hexose monophosphate shunt (68). However, in an aging brain where vascular function is deteriorating (such that both oxygen delivery and Aβ clearance is inefficient) both PS2V and Aβ may become chronically up-regulated and Aβ may begin to oligomerize and aggregate. This would both reduce the levels of soluble Aβ antioxidant and possibly damage neurons and vasculature further by generating additional oxidative stress to further suppress oxidative phosphorylation. Such a model is consistent with numerous observations that suggest a role for hypoxia in development of AD (reviewed in 69) and would also explain the close correlation between the risk factors for AD and cardiovascular disease (70,71). Our observation that the putative-truncated protein produced by the K115Efx10 FAD mutation of PSEN2 is similar to PS2V in its ability to boost γ-secretase activity suggests that this mutation may be pathogenic via chronic up-regulation of Aβ production despite normal oxygen levels rather than due to decreased γ-secretase activity as previously suggested (18). We are currently engineering a homologous mutation into the psen1 gene of zebrafish so that its effects on the transcriptome can be compared with those of PS1IV.
Materials and Methods

Ethics statement and permits

All experimentation with animals and work with genetically modified organisms were conducted under the auspices of the University of Adelaide’s Animal Ethics Committee and Institutional Biosafety Committee.

Oligonucleotides

The sequences of all oligonucleotides (PCR primers, qPCR primers and morpholino oligonucleotides) used in this study are given in Supplementary Material, Supplemental Data File 2.

Construction and cloning of cDNAs for mRNA synthesis

cDNAs encoding PRESENLIN truncations (including truncations equivalent to the NTF) were synthesized by PCR from zebrafish pse1 and pse2 cDNAs or human PSEN1 and PSEN2 cDNAs (except for those marked * below which were synthesized by GenScript USA Inc., Piscataway, NJ, USA). All the zebrafish PRESENLIN truncations and the human PRESENLIN truncations hPSEN2Δ-4, hP52v and K115Ex10 are fused at their N-termini to the FLAG™ antibody tag, DYKDDDDK (72) that follows a start codon within a consensus Kozak sequence (73). The remaining human PSEN truncations are fused at their N-termini to an HA tag (YPYDVPDYA, 74) that follows a start codon within a consensus Kozak sequence (see also Supplementary Material, Supplemental Data File 2 for a table of gene constructs and tags). The cDNA encoding the putative protein product of the K115Ex10 mutation was synthesized by Mr. Gene GmbH, Regensburg, Germany. The construct for synthesis of the negative control mRNA was cDNA encoding for the N-terminal endoproteolytic fragment of zebrafish Psen1 including an N-terminal FLAG tag but with codon 4 of the Psen1-coding sequence now mutated to the stop codon TAA by QuikChange™ Site-Directed Mutagenesis (Stratagene Cloning Systems, La Jolla, CA, USA). All sequences were subcloned into the pcGlobin2 vector (75) between the BamHI and EcoRI restriction sites (for zebrafish cDNAs) and BamHI and XhoI restriction sites (for human cDNAs except see below) and their sequences confirmed by sequencing before their use for synthesis of mRNA as previously described (32). Chimeric hPSEN2Δ-4/zPsen2α-4 sequences and mutant cDNA derivatives of these were synthesized by GenScript USA Inc. and have an N-terminal FLAG™ antibody tag. These and constructs hPSEN2Δ-4, hP52v and K115Ex10 were subcloned between the BamHI and EcoRI sites of pcGlobin2. cDNAs encoding zebrafish Hmga1a and human HMGA1a were synthesized by GenScript USA Inc. and then subcloned into the pcGlobin2 vector between EcoRI and AgeI restriction sites.

Injection of morpholino oligonucleotides and mRNAs

Embryos were collected from natural mating, grown in embryo medium (E3) and staged. Morpholinos were synthesized by Gene Tools LLC (Corvallis, OR, USA) and are listed in Supplementary Material, Supplemental Data File 2. Fertilized zebrafish eggs were injected prior to cleavage. Embryos were always injected with morpholino solutions at total concentrations of 1 mm. Thus, injected solutions of MoHmga1aBindBlock or MoHmga1aTlnBlock included 0.5 mm of these morpholinos plus 0.5 mm of the inactive, negative control morpholino, MoControl, while injections of embryos with only MoControl were performed at a solution concentration of 1.0 mm.

In vitro-synthesised mRNAs were injected into one-cell stage embryos at concentrations of 100-200 ng/µl (each mRNA concentration is indicated in the table of neurogenin1-based Notch-signalling assay results, Supplementary Material, Supplemental Data File 5). Where possible, the ability of the injected mRNAs to produce proteins in zebrafish embryos was confirmed by detection of FLAG- or HA-tagged proteins of an appropriate size in embryo lysates by western immunoblotting (Supplementary Material, Supplemental Data Files 2 and 4). Note that protein fusions involving only exon-3 derived sequences were not detectable by this method due to their small size. As a control to show the ability of MoHmga1aTlnBlock to suppress hmgα1a mRNA translation, the first 135 bases of the hmgα1a coding sequence was fused in frame to enhanced green fluorescent protein (EGFP) in the N1-EGFP plasmid (BD Biosciences, NJ, USA) between the EcoRI/Agel sites to form plasmid, hmgα1a-135-EGFP. This plasmid was linearized with NotI prior to injection. 50 ng/µl of hmgα1a-135-EGFP plasmid was co-injected with either 0.5 mm of MoHmga1aTlnBlock mixed with MoControl or it was co-injected with MoControl alone. Embryos were then observed for GFP fluorescence at ~10 hpf using fluorescence microscopy (see Supplementary Material, Supplemental Data File 6).

Exposure of cells, embryos, explants and adult fish to hypoxia and NaN3

Danio rerio were bred and maintained at 28°C on a 14 h light/10 h dark cycle. Embryos were collected from natural mating, grown in embryo medium (E3, 76) and staged. For exposure of embryos to sodium azide (NaN3, Sigma-Aldrich CHEMIE GmbH, Steinheim, Germany), this was performed at 100 µM from 6 hpf until embryos reached developmental stages equivalent to those attained under normoxia at 48 hpf at 28.5°C (30 embryos were usually used for each concentration). In the experiments for exposure of adult brain tissue to sodium azide, this was performed at a concentration of 100 µM in DMEM medium after the tissue (from chicken and mouse brains) was cut into small fragments (e.g. ~1 mm diameter). This was not necessary for explanted zebrafish brains. In the experiments conducted on adult zebrafish under low oxygen conditions, oxygen was depleted by bubbling nitrogen gas through the medium. Oxygen concentrations were measured using a dissolved oxygen meter (DO 6+, EUTECH instruments, Singapore). The dissolved oxygen level in the hypoxia group was measured to be 1.20 ± 0.6 mg/l for adults; whereas the normal ambient oxygen levels were 6.6 ± 0.45 mg/l. For exposure of adult Drosophila melanogaster to hypoxia, healthy flies were exposed to low oxygen conditions (<1%, using AnaeroGen™, Oxoid, Basingstoke, UK) for 3 h, and immediately frozen for later RNA extraction as below. For analysis of rat cell responses to hypoxia, Rat phaeochromocytoma cells (Ordway PC12) displaying a semi-differentiated phenotype with neuronal projections were provided by Professor John Filetiz (Loyola University Medical Centre, IL, USA) and maintained in RPMI-1640 media with 5% fetal calf serum, 1% l-glutamine, 1% non-essential amino acids and 1% penicillin/streptomycin. Cells were seeded into 6-well plates at 2 × 10^5 cells per well in 1 ml fresh RPMI-1640 medium as above. PC12 cells were equilibrated for 24 h before treatment with NaN3 (1 mM final concentration) for varying periods of time as indicated (see Supplementary Material, Supplemental Data File 3).

RT-PCR assays for detection of splice events homologous to formation of human PS2V

Total RNA was extracted from samples mentioned above using the QIAGEN RNeasy mini kit (QIAGEN, GmbH, Hilden, Germany). Of note, 700 ng of total RNA were used to synthesize 25 µl of first-strand cDNA by reverse transcription (SuperScript III kit;
Invitrogen, Camarillo, USA). For PCR amplification, 5 μl of the cDNA was used with the following primer pairs as relevant: Chicken Psen2: Chic Psen2: Ex.3 F: (5′-ACGGGCACTGATCTACACC-3′) with Chic Psen2 Ex.7 R: (5′-GACCTTTCAGTGGATGCAA-3′); Chicken Psen1: Chic Psen1 Ex.3 F: (5′-CTACACCTCCCTCAAGA-3′); Chic Psen1 Ex.6 R: (5′-CAGGATAAGCCATCGCT-3′); Mouse Psen2: Mm Psen2 Ex.3 F: (5′-CAGGTTAGCTGACATGAGA-3′); Mouse Psen1 Ex.7 R: (5′-CTGCTGATCGAGGAAGGTGTG-3′); Mm Psen2 Ex.6 R: (5′-ACAGCCACTGCCACTGCCS′); Mouse Psen1: Mm Psen1 Ex.3 F: (5′-CTACACCCCATCACAAGA-3′); Mm Psen1 Ex.7 R: (5′-CTGCTGATCGAGGAAGGTGTG-3′); Rat Psen2: Rn Psen2 F: (5′-CTGTA TACGAGGAAAGGTTG-3′); Rn Psen2 Ex.7 R: (5′-CCTTCCTCCCAGGTAGATGTAGTGT-3′); Rat Psen1: Rn Psen1 Ex.3 F: (5′-CTGCTGATCGAGGAAGGTGTG-3′); Rn Psen1 Ex.7 R: (5′-CGCGGACATTGTTA GTCTTGTA-3′) and Drosophila melanogaster Psen: Dm Psen Ex.2 F: (5′-CTGCTGTGAATCTC-3′); Dm Psen Ex.5 R: (5′-TCCGACCCTCCAAAGTTCC-3′). PCR was performed using GoTaq polymerase (Promega, Madison, USA) for 35 cycles with an annealing temperature of 56°C for 30 s, an extension temperature of 72°C for 1 min and a denaturation temperature of 95°C for 40 s. The PCR products were cloned into the pGEM-T vector (Promega, Madison, USA) and sequenced.

Quantitative real-time PCR
Gene specific primers were designed for amplification of cDNA from the transcripts of genes of interest and from transcripts of the ubiquitously expressed reference gene ef1a1l1 that has previously been demonstrated to be suitable for this purpose (78). Each reaction consisted of 50 ng of cDNA, 18 μl of each of forward and reverse primers and Power SYBR green master mix PCR solution (Applied Biosystems, Thermo Fisher Scientific Inc., Waltham, MA, USA). The relative standard curve method for quantification was used to determine the relative levels of gene-of-interest transcripts and reference gene transcripts in experimental treatments compared with control treatments. To generate the standard curve, cDNA was serially diluted to provide 100, 50, 25 and 12.5 ng of cDNA per reaction. Amplification conditions were 2 min at 50°C followed by 10 min at 95°C and then 40–45 cycles of 15 s at 95°C and 1 min at 60°C. Amplification was performed on an ABI 7000 Sequence Detection System (Applied Biosystems) using 96-well plates. For each treatment, the PCRs for quantification of relative levels of the transcripts of a gene of interest and of the reference gene were conducted in triplicate. Each experimental treatment and control treatment was, itself, replicated three times independently. For the triplicate PCRs within each experimental treatment, gene of interest and reference gene transcript mean relative quantity was determined from the standard curve. The ratio of these means gave the relative level of the transcript of a gene of interest to the reference gene within an experimental treatment. The mean of the ratios was then determined for the three replicates of each experimental and control treatment. These means were then used to compare relative levels of transcripts of genes of interest in treatments and controls and for calculation of standard errors of the mean (SEM). For calculations of fold changes in transcript levels of genes-of-interest between replicated experimental treatments and replicated control treatments all mean and SEM values were scaled so that the control treatment means were normalized. Statistical comparison of the means from different experimental treatments was performed using Student’s t-test (two-tailed).

Appa-cleavage and Notch-signalling assays
Assays for γ-secretase cleavage of Appa (30) and Notch signalling (32) were performed as previously described (see Supplementary Material, Supplemental Data Files 7 and 5, respectively, for raw data and statistical analysis).

Assay of the UPR
Embryos were injected with mRNAs at the 1-cell stage. At 2 hpf fertilized injected embryos were split into two groups. For tunicamycin treatment, one group was left untreated and the other group was treated with 1.5 μg/ml tunicamycin. (Tunicamycin treatment causes ER stress and induces the expression of the spliced form of xbp1, here denoted xbp1s, 77). For NaNO₃ treatment of mRNA-injected embryos, the embryos were split into two groups at 6 h after fertilization and one group was left untreated while the other group was treated with 100 μM NaNO₃. For NaNO₃ treatment of morpholino-injected embryos, the embryos were split into two groups at 36 h after fertilization and one group was left untreated while the other group was treated with 100 μM NaNO₃. At 24 hpf (tunicamycin and NaNO₃-treated mRNA-injected embryos) or 48 hpf (NaNO₃-treated morpholino-injected embryos) total RNA was extracted from control and treated embryos and reversed transcribed into cDNA for comparison by qPCR of xbp1s levels. See Supplementary Material, Supplemental Data File 8 for qPCR data and analysis.

Assessment of relative cell death
Zebrafish embryos were injected at the one-cell stage with either the inactive, negative control morpholino MoControl at a concentration of 1 nM or with a mixture of 0.5 nM MoHmg1a1BindBlock plus 0.5 nM MoControl. At 36 hpf each batch of injected embryos was divided into two groups and NaNO₃ was added to one of the two groups to a concentration of 100 μM. Incubation of both groups then continued until development equivalent to 48 hpf. At 48 hpf, 10 embryos from each group were stained with acridine orange (Sigma) by placing them in a 2 μg/ml solution of the dye in E3 medium for 30 min. The embryos were then washed repeatedly with E3 medium before each group was photographed under UV illumination. All images were captured using identical exposure parameters. Images were manipulated and analyzed using ImageJ software as previously described (46). The experiment was performed in triplicate.

Supplementary Material
Supplementary Material is available at HMG online.

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