Protective neuronal induction of ATF5 in endoplasmic reticulum stress induced by status epilepticus

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Activating transcription factor 5 (ATF5) is a basic-leucine-zipper transcription factor of the ATF/CREB family. The Atf5 gene generates two transcripts, Atf5α and Atf5β, of which Atf5α is known to be selectively translated upon endoplasmic reticulum stress response in non-neuronal cells. ATF5 is highly expressed in the developing brain where it modulates proliferation of neural progenitor cells. These cells show a high level of ATF5 that has to decrease to allow them to differentiate into mature neurons or glial cells. This has led to the extended notion that differentiated neural cells do not express ATF5 unless they undergo tumorigenic transformation. However, no systematic analysis of the distribution of ATF5 in adult brain or of its potential role in neuronal endoplasmic reticulum stress response has been reported. By immunostaining here we confirm highest ATF5 levels in neuroprogenitor cells of the embryonic and adult subventricular zone but also found ATF5 in a large variety of neurons in adult mouse brain. By combining Atf5 in situ hybridization and immunohistochemistry for the neuronal marker NeuN we further confirmed Atf5 messenger RNA in adult mouse neurons. Quantitative reverse transcriptase polymerase chain reaction demonstrated that Atf5α is the most abundant transcript in adult mouse encephalon and injection of the endoplasmic reticulum stress inducer tunicamycin into adult mouse brain increased neuronal ATF5 levels. Accordingly, ATF5 levels increased in hippocampal neurons of a mouse model of status epilepticus triggered by intra-amygdala injection of kainic acid, which leads to abnormal hippocampal neuronal activity and endoplasmic reticulum stress. Interestingly, ATF5 upregulation occurred mainly in hippocampal neuronal fields that do not undergo apoptosis in this status epilepticus model such as CA1 and dentate gyrus, thus suggesting a neuroprotective role. This was confirmed in a primary neuronal culture model in which ATF5 overexpression resulted in decreased endoplasmic reticulum stress-induced apoptosis and the opposite result was achieved by Atf5 RNA interference. Furthermore, in vivo administration of the eIF2α phosphatase inhibitor salubrinal resulted in increased ATF5 hippocampal levels and attenuated status epilepticus-induced neuronal death in the vulnerable CA3 subfield. In good agreement with the neuroprotective effect of increased ATF5, we found that apoptosis-resistant epileptogenic foci from patients with temporal lobe epilepsy also showed increased levels of ATF5. Thus, our results demonstrate that adult neurons express ATF5 and that they increase its levels upon endoplasmic reticulum stress as a pro-survival mechanism, thus opening a new field for neuroprotective strategies focused on ATF5 modulation.
Keywords: ATF5; endoplasmic reticulum stress; epilepsy; salubrinal; neuroprotection

Abbreviations: ATF = activating transcription factor; CREB = cAMP response-element binding protein; eIF = eukaryotic translation initiation factor; PERK = PKR-like ER kinase.; shRNAi = short hairpin RNA interference

Introduction

Activating transcription factor 5 (ATF5) (Nishizawa and Nagata, 1992) is a basic valine/leucine zipper transcription factor that belongs to the ATF/cAMP response-element binding protein (CREB) family (Vinson, 2002). Also known as ATF4 or ATF7 (Hai and Hartman, 2001), ATF5 promotes survival of different normal cell types such as lymphocytes (Persengiev et al., 2002) and cardiomyocytes (Wang et al., 2007). Its expression is also necessary for survival of some tumour cell types (Monaco et al., 2007; Dluzen et al., 2011) including neural ones (Angelastro et al., 2006; Li et al., 2009; Sheng et al., 2010; Dluzen et al., 2011; Arias et al., 2012). Accordingly, MCF-7 breast cancer and C6 glioblastoma cells are dependent on ATF5 expression (Dluzen et al., 2011).

By contrast, non-neoplastic breast cells and non-tumour reactive glial cells do not require ATF5 for their survival, suggesting that ATF5 exerts an anti-apoptotic effect in a cell-type dependent manner (Angelastro et al., 2006; Dluzen et al., 2011).

Expression of the Atf5 gene generates two transcripts, Atf5α and Atf5β (Hansen et al., 2002) that are identical in their coding regions but differ in the 5’ untranslated region (UTR) (Hansen et al., 2002). The leader UTR of Atf5α is very similar to that of Atf4/CREB2, another ATF/CREB family member. The leader UTR of Atf4 messenger RNA allows preferential translation upon endoplasmic reticulum stress response, which takes place when the endoplasmic reticulum suffers an overload of misfolded proteins (Harding et al., 2000a, b). When this occurs, the endoplasmic reticulum resident stress sensor PKR-like ER kinase (PERK), a eukaryotic translation initiation factor (eIF)-2α kinase, is activated thus increasing levels of phospho-eIF2α that in turn shuts off global translation. Paradoxically, phosphorylated eIF2α favours the selective translation of messenger RNAs able to bypass the phospho-eIF2α-dependent blockade, like Atf4 and Atf5α transcripts (Pascual et al., 2008; Watatani et al., 2008; Zhou et al., 2008).

In accordance, the endoplasmic reticulum stress inducer tunicamycin is known to increase protein levels of both ATF4 and ATF5 in cultured non-neuronal cells (Pascual et al., 2008; Watatani et al., 2008; Zhou et al., 2008). While an ATF4 increase has been implicated in neuronal endoplasmic reticulum stress response (Galehdar et al., 2010), ATF5 has been largely excluded from consideration as a potential mediator of neuronal endoplasmic reticulum stress response on the basis that ATF5 is not expressed in normal adult brain tissue (Greene et al., 2009).

Expression of ATF5 in neuroectodermal tissue was first described in the developing olfactory epithelium (Hansen et al., 2002) and its expression in brain has only been systematically studied during embryonic stages, with highest ATF5 levels being found in neuroprogenitor cells of the telencephalic subventricular zone (Angelastro et al., 2003). Evidence from experiments on cultured cells suggests that ATF5 promotes proliferation of these neuroprogenitor cells (Angelastro et al., 2003, 2005; Mason et al., 2005). Furthermore, ATF5 appears to inhibit differentiation of cultured neuroprogenitor cells (Angelastro et al., 2003, 2005; Mason et al., 2005), as dominant negative ATF5 and ATF5-short hairpin RNA interference (shRNAi) accelerate their differentiation into neurons or glial cells and ATF5 levels decay when neurite outgrowth takes place in PC12 pheochromocytoma cells upon treatment with nerve growth factor (Angelastro et al., 2003). However, there has been no in vivo systematic study to address ATF5 expression, distribution and function in the adult brain.

In the present study we aim to explore levels and distribution of ATF5 in adult brain and to test whether adult neurons increase ATF5 during physiopathological conditions that involve execution of the endoplasmic reticulum stress response such as status epilepticus. Finally, we study whether such an ATF5 induction would modulate neuronal survival after endoplasmic reticulum stress.

Materials and methods

All procedures are fully described in the online Supplementary material.

Animals

C57BL/6 male mice were housed at the Centro de Biología Molecular ‘Severo Ochoa’ animal facility in a colony room kept at 19–22 °C and 40–50% relative humidity on a 12 h light/dark cycle with food and water available ad libitum. All experiments were performed in accordance with institutional guidelines approved by the ethical committee of CSIC or Research Ethics Committee of the Royal College of Surgeons in Ireland and the principals of the European Communities Council Directive (86/609/EEC).

Status epilepticus model and intracerebroventricular injection of salubrinal

Status epilepticus was induced in adult isoflurane-anaesthetized mice by intra-amygdala injection of kainic acid (Sigma-Aldrich) as described (Engel et al., 2010). Salubrinal (Sigma-Aldrich) was intracerebroventricularly injected as described (Sokka et al., 2007).

Human brain tissue samples

This study was approved by the Ethics (Medical Research) Committee of Beaumont Hospital, Dublin (ERC/IRB 05/18) and written informed consent was obtained from all patients. Control (autopsy) temporal cortex (n = 5) was obtained from the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, MD. Patients (n = 3) were referred for surgical resection of the temporal lobe for the treatment of intractable temporal lobe epilepsy. Following temporal lobe resection the neocortex was obtained and frozen in liquid nitrogen and stored at −70 °C until use. Samples were then processed for protein analysis by western blot.
**Tissue preparation**

For free-floating immunohistochemistry-immunofluorescence and western blot brains were processed as previously described (Torres-Peraza et al., 2008).

**Fluoro-Jade B staining and cell counting**

Neurodegeneration was assessed using Fluoro-Jade B (Chemicon Europe Ltd) staining in frozen 12 μm sections, as described (Engel et al., 2010). Fluoro-Jade B-positive cells were counted in CA3 subfield.

**Quantitative real time reverse transcriptase polymerase chain reaction**

Levels were normalized with respect to total RNA content and then with respect to both Gadph and Anp32a transcript levels. To determine the ratio of Atf5a/Atf5β expression levels by absolute quantitative reverse transcriptase PCR, we used a pMA-RQ-ATF5a-β standard plasmid that contains one copy of each Atf5a, Atf5β and total Atf5 amplicons, which was provided by GeneART® (Life technologies).

**Cloning of Atf5**

The Atf5 protein coding sequence was amplified from a mouse complementary DNA library (Marathon®-Ready cDNA, Clonetech) by PCR and the Atf5 complementary DNA was then inserted into pGEMT plasmid (Addgene). Next, the MYC sequence was added to the 5’ end of Atf5 by PCR and the MYC-ATF5 complementary DNA was inserted into pCDNA3 (Addgene) to obtain the pCDNA3-MYC-ATF5 construct. The final construct was sequenced for verification.

**In situ hybridization**

Non-isotopic in situ hybridization was performed as described (Martin-Ibanez et al., 2012) and some sections were also processed for NeuN or GFAP immunohistochemistry.

**Culture, treatments and nucleofection of primary neurons**

Embryonic Day 18 primary cultures were prepared as described (Ortega et al., 2010). Tunicamycin (Sigma-Aldrich) and salubrinal were added to the culture medium at a final concentration of 4 μg/ml and 5 μM, respectively. Primary cultures were nucleofected before plating with 4 μg of each plasmid using the Primary Cell Nucleofector™ Kit (LONZA).

**Lentiviral vector preparation and Atf5-shRNA experiment**

The ATf5-shRNA self-inactivating lentiviral particles were produced by transient transfection of 293T cells with one of the pCMV puro transfer plasmids containing different ATf5-shRNA sequences (Mission shRNA Plasmid DNA, Sigma-Aldrich) together with the packaging and envelope pCMVdR8.2 and pMD2.G plasmids (Addgene) using Lipofectamine® Plus (Invitrogen, Life Technologies). High titre viral stock was produced and stored as described (Lopez-Hernandez et al., 2012).

**Results**

**ATF5 expression is maximal in neuroprogenitor areas of the embryonic brain but is also found in neuronally committed cells**

To explore the expression and distribution of ATF5 in brain tissue we first performed confocal immunofluorescence microscopy on mouse brain sections at embryonic Day 18. In agreement with others (Angelastro et al., 2003, 2005), highest levels of ATF5 immunoreactivity were found in the ventricular and subventricular zone of dorsal telencephalon (Fig. 1A and B). Quantification of fluorescence intensity showed that ATF5 immunoreactivity drops in the intermediate layers of the developing cerebral cortex to then increase in the outer layers, where newborn cortical neurons are located. As shown in Fig. 18, the mean fluorescence intensity in the area where newborn neurons are located (Bi) reaches 80% of the maximal levels observed in the subventricular zone (Bii and quantification according to schematic bar Biii). To confirm ATF5 expression in embryonic/early post-natal neocortical neurons, we performed double immunofluorescence of ATF5 and MAP2 on embryonic Day 18 telencephalic primary cultures and found that MAP2-positive neurons showed a clear ATF5 immunostaining (Fig. 1C). To confirm the specificity of the ATF5 antiserum we infected Neuro2A mouse neuroblastoma cells with lentiviral particles that confer expression of various ATf5-shRNAs and found by western blot that small hairpin RNAs 2 and 3 strongly knocked-down ATF5 (Fig. 1D, Clone 2: 12 ± 5% and Clone 3: 11 ± 3% with respect to control; P < 0.01). In good agreement, these were the clones that caused the highest decreases on ATf5 transcript level as determined by real time quantitative reverse transcriptase PCR (Fig. 1E: Clone 2: 0.55 ± 0.05 and Clone 3: 0.62 ± 0.08 fold respect to control; P < 0.05). We then nucleo-fected primary embryonic Day 18 cultures with a mixture of the validated clone 2 and clone 3 ATf5-shRNA plasmids and, as expected, we found that ATf5 protein and messenger RNA levels were decreased by western blot analysis (Fig. 1F, 39 ± 5% respect to control; P < 0.01) and quantitative reverse transcriptase PCR (Fig. 1G, 0.66 ± 0.07-fold with respect to control; P < 0.05). Accordingly, confocal analysis of immunofluorescence revealed that ATf5 was knocked-down in cells transfected with the mixture of clone 2 and clone 3 small hairpin RNAs that were visualized by GFP co-transfection and that also were MAP2-positive (Fig. 1H and I: 28 ± 0.6 versus 14 ± 0.5 in non-transfected and transfected cells, respectively; P < 0.01), thus demonstrating specificity of the antibody and ATf5 expression in cultured primary neurons. In good agreement, ATf5 and doublecortin (DCX) double immunofluorescence on embryonic Day 18 brain revealed co-localization in the outer layers, thus demonstrating ATf5 expression in the newly born neurons in vivo (Fig. 1J). To confirm that mature neurons also express ATf5, we then performed double immunofluorescence on post-natal Day 14 brain tissue for ATf5 and NeuN, a well-known marker of adult neurons. We found ATf5 immunoreactivity in virtually all NeuN-positive cells in...
Figure 1. ATF5 immunoreactivity in progenitor areas and in neuronally committed cells of embryonic Day 18 mouse brain and in mature differentiated neurons of post-natal brain. (A) Low magnification of a sagittal section of embryonic Day 18 mouse brain subjected to ATF5 immunofluorescence (green) and DAPI nuclear counterstaining (cyan) with amplified view of the indicated inset. (B) Higher magnification of the area corresponding to the inset shown in A; amplified view of insets (i) and (ii) and quantification of fluorescence intensity across the cerebral cortex (iii) are also provided. (C) Double immunofluorescence of ATF5 (green) and MAP2 (red) and DAPI nuclear counterstaining (cyan) on cultured embryonic Day 18 primary neurons at 4 days in vitro. (D and E) ATF5 protein levels (D) and ATF5 transcript (E) in control (Ctr) and ATF5-shRNA LV-transduced primary neurons. (F and G) ATF5 and Actin immunofluorescence in control (Ctr) and ATF5-shRNA LV-transduced primary neurons. (H and I) GFP reporter expression in control (Ctr) and ATF5-shRNA LV-transduced primary neurons. (J and K) DCX and NeuN immunofluorescence in control (Ctr) and ATF5-shRNA LV-transduced primary neurons.
ATF5 protects neurons against endoplasmic reticulum stress

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ATF5 is widely expressed in most neuronal fields of the adult encephalon

We then analysed the distribution of ATF5 protein in the adult mouse brain by immunohistochemistry. In agreement with previous reports (Arias et al., 2012), we detected strong ATF5 immunoreactivity in the subventricular zone (see arrowheads in Fig. 2A and C). However, we also detected a widespread neuronal pattern of ATF5 immunoreactivity across the different layers of brain cortex, the striatum (Fig. 2A–C), as well as in all other encephalic grey matter structures including the hippocampus (Fig. 2D) and the cerebellar cortex (Fig. 2E). Mesencephalic cells within substantia nigra pars compacta were also immunoreactive for ATF5 (data not shown). Specificity of ATF5 immunoreactivity was confirmed by using different antibodies raised against different portions of the ATF5 protein. Figure 2A, D and E show staining with an antibody raised against the N-terminal region of ATF5, while Fig. 2B and C were generated by using an antibody raised against the C-terminal region of Atf5. No staining was observed when primary antibody was omitted (data not shown). Specificity of ATF5 immunoreactivity was confirmed by using different antibodies raised against different portions of the ATF5 protein. Figure 2A, D and E show staining with an antibody raised against the N-terminal region of ATF5, while Fig. 2B and C were generated by using an antibody raised against the C-terminal region of Atf5. No staining was observed when primary antibody was omitted. For a more detailed analysis of ATF5 distribution and subcellular localization in adult brain we performed double immunofluorescence for ATF5 and NeuN. Confocal microscopy revealed that ATF5 was present in most NeuN-positive pyramidal neurons in layers II-III of cerebral cortex (Fig. 2F) and in the hippocampus (Fig. 2G) in 2-month-old mice. ATF5 and NeuN co-localization was also detected in cortical neurons of layer V, striatal projecting neurons and striatal cholinergic interneurons (data not shown). Subcellular localization of ATF5 was similar to that of other transcription factors of the ATF/CREB family including ATF4 (White et al., 2000; Vernon et al., 2001; Ritter et al., 2004). ATF5 was mainly found in the somata of neurons and, in some cases, it also extends to the apical dendrites of cortical and hippocampal pyramidal neurons. Further, a clear nuclear staining is also found in a significant proportion of neurons and this was more evident in cerebral cortex (Fig. 1K) while no signal was found when anti-ATF5 primary antibody was omitted (data not shown). Together, these results confirm maximal expression of ATF5 in the subventricular zone of embryonic Day 18 mouse brain but they also demonstrate expression in newly born and mature neurons of the embryonic and neonatal mouse brain.

In situ hybridization confirms neuronal expression of ATF5 in adult brain

To confirm the specificity of the neuronal pattern obtained with anti-ATF5 antibodies in adult brain tissue, we analysed the spatial distribution of ATF5 messenger RNA by in situ hybridization on sagittal sections of 2-month-old mouse brain by using a digoxigenin-labelled Atf5 antisense probe developed by immunohistochemistry with alkaline phosphatase-conjugated antidigoxigenin antibody. In accordance with the pattern of ATF5 immunoreactivity, the signal from the ATF5 antisense probe was detected particularly in the cerebral cortex, the cornus amonus (CA) fields of hippocampus (Fig. 3A), and the Purkinje cell layer of the cerebellum (Fig. 3B). The layered distribution of Atf5 in situ signal with maximal intensities in layer V of cerebral cortex, pyramidal cell layer of cornus amonus and also the granular cell layer of dentate gyrus suggests that Atf5 is expressed by neurons in those structures. Staining using an Atf5 sense probe, the negative control for the in situ hybridization experiment, did not result in any positive hybridization signal (Fig. 3A and B). In further support of the specificity of the ATF5 probe, we also detected ATF5 hybridization in the olfactory epithelium and the vomeronasal organ in embryonic Day 18 foetuses reported by others (Hansen et al., 2002) (data not shown).

**Figure 1 Continued**
Figure 2  ATF5 is expressed in a large variety of neurons along the adult encephalon, but not in glial cells. (A–E) ATF5 immunohistochemistry in 2-month-old adult mouse brain revealed widespread neuron-like ATF5 labelling. Two different anti-ATF5 antibodies, recognizing the N-terminus portion (A, D and E) or the C-terminus portion (B and C) of ATF5, yielded a neuron-like pattern in cerebral cortex (CTX), striatum (STR), cornus amonius (CA) and dentate gyrus (DG) of the hippocampus and in the Purkinje cell layer (PCL) of the cerebellum. CC = corpus callosum. Arrowheads indicate the subventricular zone. (F and G) Double immunofluorescence for ATF5 (green) and NeuN (red) and confocal imaging in layer II-III of the cerebral cortex (F) and in CA2 of the hippocampus (G). Orthogonal reconstructions of z-stacks (F and G) taken by confocal microscopy demonstrated co-localization in Z axis. (H) Double immunofluorescence for ATF5 (green) and NeuN (red) counterstained with DAPI (cyan) in layer II-III of the cerebral cortex. White arrowheads indicate nuclei of NeuN- and ATF5-negative cells. Occasionally, these cells can be found in perineuronal location (empty arrowhead). (I) Double immunofluorescence for ATF5 (green) and GFAP (red) counterstained with DAPI (cyan) and confocal imaging in CA2 of the hippocampus. White arrowheads indicate GFAP-positive cells and empty arrowheads indicate GFAP- and ATF5-negative cells.
To further validate these results, we performed \textit{Atf5} in situ hybridization combined with NeuN immunohistochemistry to confirm the presence of \textit{Atf5} messenger RNA in adult neurons. Using this approach we confirmed the presence of the \textit{Atf5} messenger RNA in NeuN-positive somata of the pyramidal cell layers of the hippocampus (Fig. 3C), in pyramidal neurons of layer V of cerebral cortex, and in the striatum (Fig. 3C). In contrast, when \textit{Atf5} in situ hybridization was combined with immunostaining for GFAP, no co-localization of signals was detected (see representative images of hippocampus and cerebral cortex, Fig. 3D). Taken together, these immunohistochemistry and \textit{in situ} hybridization studies demonstrate that \textit{ATF5} is expressed in neurons of the adult mouse brain.

\textbf{Predominant expression in brain of the \textit{Atf5} messenger RNA isoform that is preferentially translated upon endoplasmic reticulum stress}

\textit{Atf5} gene expression generates two types of transcripts, \textit{Atf5}α and \textit{Atf5}β, which are identical in their protein coding regions but differ in their 5'-UTR (Hansen et al., 2002) (Fig. 4A). Unlike \textit{Atf5}β, the 5'-UTR of \textit{Atf5}α contains two upstream open reading frames. These sequences favour the translation of \textit{Atf5}α messenger RNA during situations of cell stress (Watatani et al., 2008; Zhou et al., 2008) including endoplasmic reticulum stress (Pascual et al., 2008; Zhou et al., 2008). Because the probe used for the \textit{in situ} hybridization staining does not discriminate between \textit{Atf5}α and \textit{Atf5}β transcripts as it binds the protein coding sequence of \textit{Atf5}, we decided to perform quantitative real-time reverse transcription PCR experiments with a pair of primers that amplify total \textit{Atf5} and also with pairs of primers that specifically amplify exon-α or exon-β sequences of \textit{Atf5}α and \textit{Atf5}β, respectively. \textit{Atf5} messenger RNA was detected in all analysed regions: cortex, striatum, hippocampus and cerebellum, thus confirming our findings with the \textit{in situ} hybridization probe. Interestingly, relative expression of \textit{Atf5} total messenger RNA was higher in hippocampus (cortex: 100% ± 6; striatum: 92% ± 3; hippocampus: 114% ± 8; cerebellum: 86% ± 8, with respect to cortex; ANOVA \(P < 0.05\)). Next, we studied the expression pattern of \textit{Atf5}α and \textit{Atf5}β messenger RNA isoforms by using the specific primers and found that both isoforms were expressed in all analysed structures but with different expression patterns. Whereas \textit{Atf5}β messenger RNA showed similar levels across structures, \textit{Atf5}α isoform showed a very similar distribution to that of total \textit{Atf5} messenger RNA with the highest transcript expression being found in hippocampus (cortex: 100% ± 8; striatum: 89% ± 2; cerebellum: 86% ± 8, with respect to cortex; ANOVA \(P < 0.05\)).

\textbf{Figure 3} Atf5 messenger RNA is localized in neurons from several adult brain regions. (A and B) Low magnification pictures of \textit{Atf5} in \textit{situ} hybridization (ISH) show that \textit{Atf5} messenger RNA positive signal (violet) was detected in cortex (CTX), pyramidal cell layer of the cornus amonos (CA) and dentate girus (DG) of hippocampus (A). \textit{Atf5} messenger RNA was also detected by \textit{in situ} hybridization in the Purkinje cell layer (PCL) and in granular cell layer (GCL) of the cerebellum (B). Right panels in A–B correspond to the negative control using an \textit{Atf5} sense probe. (C) High magnification pictures of double \textit{Atf5} in \textit{situ} hybridization (violet) combined with NeuN immunohistochemistry (brown) confirms that ATF5-expressing cells in CA1 of hippocampus (HPC), cerebral cortex (CTX) and striatum (STR) are neurons. Cells with maximum ATF5 levels are indicated by filled arrows. (D) Double \textit{Atf5} in \textit{situ} hybridization (violet) combined with GFAP immunohistochemistry (brown) showed that astrocytes (white arrows-heads) did not express \textit{Atf5}, while \textit{Atf5} labelling was restricted to large GFAP-negative cells (black arrows). IHC = immunohistochemistry.
The endoplasmic reticulum-stress inducible isoform of Atf5 messenger RNA, Atf5α, is differentially distributed in brain structures and cerebellum. (A) Schematic representation of the structure of the 5’ end of mouse Atf5 gene and its different transcripts. Exons are represented as coloured boxes (red, grey and black), introns as white boxes and upstream open reading frames (uORF) as white triangles. (B) Relative expression of total Atf5 messenger RNA and of Atf5α and Atf5β isoforms was determined by quantitative real time reverse transcriptase PCR. Expression levels of each transcript were normalized with respect to the amount of RNA and then normalized with respect to both Gadph and Anp32a transcripts. The relative abundance of total Atf5 messenger RNA and of Atf5α and Atf5β isoforms was determined for each brain region by running in parallel a plasmid that contains a copy of each of the amplified fragments. Data are represented as mean ± standard error. Statistical analysis consisted of ANOVA followed by a least significant difference test. *P < 0.05 with respect to striatum and cerebellum. #P < 0.05 with respect all other structures; n = 7. (C and D) Representative images of ATF5 immunohistochemistry 1.5 h after intrastriatal injection of tunicamycin or vehicle into adult mouse brain. (D) Double immunofluorescence for ATF5 (green) and NeuN (red) counterstained with DAPI (cyan) in cerebral cortex of tunicamycin-injected brain. White arrowheads indicate nuclei of ATF5- and NeuN-negative cells. CTX = cortex; STR = striatum; HPC = hippocampus; CBL = cerebellum.
tunicamycin treatment (see arrowheads in Fig. 4D). Thus, only neurons respond to endoplasmic reticulum stress by increasing the levels of ATF5 within the analysed time frame.

**ATF5 levels increase in hippocampal neurons after status epilepticus**

Having demonstrated that ATF5 levels increase *in vivo* in neurons after tunicamycin-induced endoplasmic reticulum stress, we decided to explore ATF5 in a model of hippocampal endoplasmic reticulum stress induced by prolonged seizures. Status epilepticus was triggered in awake, freely moving adult mice by intramygdala injection of kainic acid (Fig. 5A) (Engel et al., 2010). Seizures were confirmed by surface EEG and were characterized by repeated behavioural manifestations, including Straub tail, rearing and falling and forelimb clonus (data not shown), as previously reported (Engel et al., 2010). We first measured, by western blot, the levels of endoplasmic reticulum stress markers, particularly those that are induced upon PERK activation. Thus, we found a rapid and transient increase in the levels of phospho-eIF2α and ATF4 that started 1 h after status epilepticus (Fig. 5B), thus confirming activation of PERK/eIF2α pathway. Interestingly, ATF5 levels also increased markedly and rapidly from 1 h after status epilepticus (Fig. 5C). Next, we analysed whether this ATF5 increase occurs differentially among hippocampal structures with different susceptibility to status epilepticus-induced neurodegeneration. For this, we performed ATF5 and ATF4 immunofluorescence in hippocampal sections of mice sacrificed 8 h after status epilepticus cessation, when the neurodegenerative process begins. In agreement with the western blot data, immunostaining showed that both ATF5 and ATF4 increase in hippocampus, but with a different subfield pattern. ATF5 increase took place mainly in CA1 and dentate gyrus neurons, which are the most resistant to status epilepticus-induced degeneration (Fig. 5D and E), whereas in the vulnerable CA3 region, only a reduced subset of neurons showed prominent ATF5 immunoreactivity (see arrowhead in Fig. 5D). On the contrary, the ATF4-increase mainly took place in the most vulnerable CA3 region (Fig. 5D), thus suggesting that ATF4 and ATF5 might play different roles on vulnerability to status epilepticus-induced degeneration with ATF5 upregulation correlating with resistance to status epilepticus-induced neuronal loss.

**Neuronal increase of ATF5 protects against endoplasmic reticulum stress-induced apoptosis in culture**

To explore whether increased ATF5 plays an anti-apoptotic role in the context of neuronal endoplasmic reticulum stress, we treated neuronal primary cultures with tunicamycin for 0.5 to 6 h. We first verified that the PERK/eIF2α-dependent signalling cascade of endoplasmic reticulum stress (Fig. 6A) is induced by this pharmacological approach. PERK activation can be demonstrated by increased levels of the low electrophoretic mobility band corresponding to phospho-PERK relative to its non-phosphorylated form by western blot (Bollo et al., 2010), and this was the case in our culture model (Fig. 6B). This rapid and sustained activation of PERK was accompanied by an increase in phosphorylation of its substrate eIF2α and increased levels of ATF4 (Fig. 6B), thus demonstrating efficient PERK/eIF2α-dependent signalling cascade in this neuronal culture model. Interestingly, primary neurons responded to tunicamycin by gradually increasing the levels of ATF5 within the same time frame (Fig. 6B). More detailed analysis showed that ATF5 levels reached 165 ± 16% with respect to control neurons 6 h after tunicamycin treatment (Fig. 6C). The tunicamycin-induced increase in phospho-eIF2α and ATF5 levels was also confirmed by immunofluorescence (Fig. 6D). Notably, cells with maximal levels of ATF5 in the tunicamycin-treated culture were NeuN-positive, indicating they were mature neurons (Fig. 6E). To further demonstrate that the phosphorylation of eIF2α controls ATF5 levels, we treated cells with the selective eIF2α phosphatase inhibitor salubrinal (Boyce et al., 2005). As expected, salubrinal treatment largely increased the levels of phospho-eIF2α (control: 100 ± 20%, salubrinal: 215 ± 24% with respect to control; Fig 6F and G). Interestingly, salubrinal treatment also strongly increased ATF5 levels (control: 100 ± 10%, salubrinal: 202 ± 38% with respect to control; Fig. 6F and G). Together, all these data indicate that the increase of ATF5 in response to tunicamycin-induced endoplasmic reticulum stress is due to activation of the PERK/eIF2α-dependent signalling cascade.

Next, to explore the potential anti-apoptotic role of ATF5 induction, we treated the primary neuronal cultures with tunicamycin or with tunicamycin + salubrinal over 24 h and quantified the number of apoptotic cells as evidenced by cleaved (activated) caspase 3 immunofluorescence and by analysis of nuclear morphology. Interestingly, the number of apoptotic cells was significantly lower in tunicamycin + salubrinal-treated cultures than in cultures treated with tunicamycin only (Fig. 6H and I; control: 19 ± 1%, tunicamycin: 34 ± 3%, tunicamycin + salubrinal: 25 ± 2%, P < 0.05 according to cleaved caspase 3 immunostaining and control: 26 ± 2%, tunicamycin: 36 ± 2%, tunicamycin + salubrinal: 28 ± 2%, P < 0.01 according to nuclear morphology). This suggests that the salubrinal-induced increase in ATF5 levels has a neuroprotective effect against tunicamycin-induced apoptosis. To fully demonstrate that the protective effect of salubrinal on tunicamycin-induced cell death is dependent on the ability of salubrinal to increase ATF5 levels, we performed similar experiments on neuronal cultures nucleofected with the plasmids expressing ATF5 shRNAi or the empty vector. As shown in Fig. 6J and K, ATF5 shRNAi-expressing cells are more sensitive to tunicamycin-induced apoptosis and the protective effect of salubrinal against tunicamycin-induced toxicity was absent in the ATF5 shRNAi-expressing cultures.

Finally, to fully prove that ATF5 upregulation plays a neuroprotective role, we tested the effect of ATF5 overexpression on tunicamycin-induced apoptosis. For this, we nucleofected primary neuronal cultures with an eukaryotic expression vector encoding MYC-ATF5 or a similar plasmid expressing GFP and quantified the endoplasmic reticulum stress-induced apoptosis at 24 h after tunicamycin treatment (Fig. 6L–N). We found that MYC-ATF5-nucleofected cultures showed significantly lower proportion of apoptotic cells as evidenced by cleaved caspase 3 immunostaining (Fig. 6M; control: 21 ± 3%; GFP/tunicamycin: 51 ± 4%; MYC-ATF5/tunicamycin: 32 ± 3%, P < 0.01) or by
Figure 5. ATF5 levels increase in hippocampal neurons upon status epilepticus-induced endoplasmic reticulum stress selectively in apoptosis-resistant structures. (A) Schematic representation of the status epilepticus model: kainic acid (KA) was injected into the amygdala thus leading first to focal/generalized seizures and then to neurodegeneration in CA3 24 h later. (B) Representative western blot of the endoplasmic reticulum stress markers P-eIF2α and ATF4 from hippocampus at indicated times after status epilepticus cessation. (C) ATF5 immunoblot from hippocampus at indicated times after status epilepticus cessation. (D) Representative images of the immunofluorescence for ATF4 (green) and double immunofluorescence for ATF5 (green) and NeuN (red) from control mice and kainic acid treated mice 8 h after status epilepticus cessation in CA1, dentate gyrus (DG) and CA3. (E) Histogram showing the quantification of the green (ATF4 or ATF5) fluorescence in D. Data represent mean ± SEM. *P < 0.05, **P < 0.01.
Figure 6 ATF5 overexpression protects primary neurons against endoplasmic reticulum stress-induced apoptosis. (A) Schematic representation of the PERK-mediated branch of the endoplasmic reticulum stress response. (B) Primary neurons were treated with tunicamycin (Tuni) during indicated times and then markers of endoplasmic reticulum stress [phospho (P)-PERK, P-eIF2α, ATF4 and ATF5] were determined by western blot. (C) Representative blot of ATF5 (top) and histogram of its quantification (bottom) 6 h after tunicamycin treatment. (D) Immunofluorescence of phospho-eIF2α and ATF5 in tunicamycin-treated primary cultures. Arrows show neurons with high levels of phospho-eIF2α or ATF5. (E) Double immunofluorescence of ATF5 (green) and NeuN (red) in primary neuronal culture treated with ATF5.
Modulating ATF5 levels might have therapeutic implications for endoplasmic reticulum stress associated neurodegeneration in epilepsy

Having shown that increasing ATF5 is neuroprotective in a cell model of endoplasmic reticulum stress and that ATF5 is increased in neurodegeneration-resistant neuronal fields in the mouse model of status epilepticus, we wondered whether pharmacological manipulations to increase ATF5 in vivo would also be protective in the mouse model of status epilepticus and whether human samples of epileptic foci resistant to neuronal apoptosis would also show increased levels of ATF5.

Thus, mice were intracerebroventricularly injected with salubrinal just before induction of status epilepticus. As expected, the salubrinal injection resulted in a strong increase in phospho-eIF2α levels in hippocampus as evidenced by western blot (Fig. 7A and B). The increase was significant at 8 h after injection but we also observed a tendency to increased levels by 4 h (vehicle: 100 ± 14, salubrinal 4 h: 120 ± 10, salubrinal 8 h: 161 ± 18; P < 0.001). Accordingly, ATF5 levels were also found significantly increased by western blot as early as 4 h after injection and more markedly after 8 h (vehicle: 100 ± 8; salubrinal 4 h: 126 ± 15; P < 0.01; salubrinal 8 h: 180 ± 33; P < 0.05). We then measured the density of Fluoro-Jade B-positive degenerating cells 24 h after seizure cessation. As expected, and in good agreement with others (Sokka et al., 2007), salubrinal treatment significantly reduced status epilepticus-induced degeneration of CA3 neurons (Fig. 7C and D, kainic acid + vehicle: 42 ± 6, kainic acid + salubrinal: 27 ± 1; P < 0.05). It is important to note that seizure time was equivalent in both animal groups (data not shown).

Finally, we determined ATF5 levels in cortical epileptic foci excised from patients with temporal lobe epilepsy. We found that these foci, which are known to be resistant to neuronal apoptosis, have significantly higher levels of ATF5 than control subjects (Fig. 7E and F, control subjects: 100 ± 19%, temporal lobe epilepsy patients: 288 ± 36% with respect to controls, P < 0.05).

Together, these results suggest that ATF5 modulating drugs such as salubrinal might be of therapeutic value for epilepsy-induced neurodegeneration.

Discussion

In the present study we demonstrate, for the first time, that ATF5 is widely expressed in neurons of the adult brain. We find ATF5 protein and messenger RNA, including the endoplasmic reticulum stress-preferentially translated transcript Atf5α, in a wide variety of neurons in adult mouse encephalon. In accordance, we demonstrate that mature neurons are able to increase ATF5 protein in response to endoplasmic reticulum stress-inducing stimuli, such as tunicamycin treatment or induction of status epilepticus, thus indicating that ATF5 is part of the neuronal endoplasmic reticulum stress response. Importantly, ATF5 overexpression protected primary neurons from suffering endoplasmic reticulum stress-induced apoptosis and Atf5 RNA interference rendered neurons more vulnerable. Accordingly, the high ATF5 levels detected in both epileptogenic foci of patients with temporal lobe epilepsy and in CA1 and dentate gyrus neurons after status epilepticus correlate with their resistance against seizure-induced degeneration. Furthermore, increased ATF5 levels as a consequence of the administration of the selective eIF2α phosphatase inhibitor salubrinal resulted in neuroprotection both in a cell model of endoplasmic reticulum stress and in the status epilepticus mouse model and, most importantly, Atf5-shRNAi administration in the cell model demonstrated that the protective effect of salubrinal is dependent on its ability to increase ATF5 levels. We thus can conclude that ATF5 is constitutively expressed in neurons of the adult CNS and that its translational activation contributes to neuroprotection in a variety of endoplasmic reticulum stress inducing conditions. Therefore, ATF5 may represent a novel target for the treatment
and prevention of neuronal injury in neurological disorders featuring endoplasmic reticulum stress.

ATF5 was initially reported to be undetectable or absent in post-natal brain neurons (Greene et al., 2009; Sheng et al., 2010) on the basis of studies showing ATF5 immunostaining to be restricted to choroidal cells and neuroprogenitor cells of the subventricular zone (Angelastro et al., 2003, 2006; Arias et al., 2012). Using various validated commercial antibodies raised against different portions of ATF5 we also detected maximal ATF5 immunostaining in the subventricular zone. However, we also detected widespread ATF5 immunoreactivity in neurons of the adult brain. It is possible that in the initial description of ATF5 brain distribution the relatively lower ATF5 immunostaining in a neuron-like pattern was overlooked. A possible explanation for this could be a threshold to consider a cell as positive too close to the maximal ATF5 levels found in the subventricular zone, choroidal cells and/or malignant glial cells, which express the highest levels of ATF5 (Angelastro et al., 2003, 2006; Arias et al., 2012). Here we irrefutably detected Atf5 messenger RNA in adult neurons by performing ATF5 in situ hybridization combined with NeuN immunohistochemistry, thus leaving no doubt about neuronal expression of ATF5 in adult mouse brain.

The levels of ATF4 and ATF5 are known to increase in non-neuronal cells treated with endoplasmic reticulum stress inducers (Pascual et al., 2008; Watatani et al., 2008; Zhou et al., 2008). Regarding neurons, until now only ATF4 had been shown to be induced by endoplasmic reticulum stress (Sokka et al., 2007; Galehdar et al., 2010). Here, we demonstrate for the first time that ATF5 is also induced in neurons subjected to endoplasmic reticulum stress. This happened upon tunicamycin-induced endoplasmic reticulum stress both in primary cultures and in vivo. Furthermore, we found that ATF5 is also selectively increased in hippocampal neurons after status epilepticus, a model of pathological brain activity which features induction of endoplasmic reticulum stress (Sokka et al., 2007). It is reasonable to think that this increase in ATF5 is part of the endoplasmic reticulum stress cascade as it occurs in parallel with the induction of other established endoplasmic reticulum stress mediators such as phospho-eIF2α. In support of this view, inhibition of the specific eIF2α-phosphatase with salubrinal strongly increased ATF5 levels in vivo and in cultured primary neurons. It is important to note that we are not the first to report that salubrinal protects neurons showing the quantification of blots in A. (C) Representative images of Fluoro-Jade B staining in the hippocampal CA3 subfield 24 h after seizure cessation in status epilepticus mouse model that were previously injected intracerebroventricularly with salubrinal or vehicle. (D) Histogram shows the quantification of the density of Fluoro-Jade B-positive cells. (Student t-test *P < 0.05, n = 13–14 per group). (E) Representative western blot showing levels of ATF5 in cortical epileptic focus from patients with temporal lobe epilepsy. (F) Histogram shows the quantification of ATF5 in cortical epileptic focus samples from patients with temporal lobe epilepsy as determined by immunoblot using tubulin as loading control (Student t-test *P < 0.05 with respect to controls).
from endoplasmic reticulum stress-induced injury (Sokka et al., 2007; Zhu et al., 2008). However, in the previous studies the mechanism by which salubrinal exerts its neuroprotective effect was not fully elucidated and they focused on very late downstream indicators of endoplasmic reticulum stress like activation of caspases 3 and caspase 12 (Sokka et al., 2007) or upregulation of CHOP/GADD153 (Zhu et al., 2008). Here we show that salubrinal treatment strongly increases ATF5 levels. Furthermore, we also show that ATF5 over-expression significantly reduces endoplasmic reticulum stress-induced neuronal apoptosis, that ATF5 RNA interference renders neurons more vulnerable and that the protective effect of salubrinal is dependent on its ability to increase ATF5 levels. Together, these data demonstrate that increasing neuronal ATF5 has a neuroprotective effect.

Regarding the transcriptional targets of ATF5 that might contribute to its anti-apoptotic effect, these comprise a spectrum of neuroprotective proteins such as Hspb1 (Hsp27) (O’Reilly et al., 2010), Bcl2 (Wang et al., 2007; Dluzen et al., 2011) and Mcl1 (Sheng et al., 2010). Interestingly, Mcl1 is rapidly upregulated after pilocarpine-induced status epilepticus (Mori et al., 2004). Since phospho-eIF2α levels increase after pilocarpine-induced status epilepticus (Carnevali et al., 2004) it is possible that Mcl1 would be a potential target of the phospho-eIF2α/ATF5 signalling cascade in hippocampal neurons after status epilepticus.

Endoplasmic reticulum stress occurs in several neurological and neurodegenerative disorders such as ischaemia/reperfusion injury (Kumar et al., 2001), Parkinson’s disease (Uehara et al., 2006), Alzheimer’s disease (Nakagawa et al., 2000; Hitomi et al., 2004) and others (Xu et al., 2005; Reijonen et al., 2008). In addition, endoplasmic reticulum stress is a pathological feature of chronic recurrent seizures in human temporal lobe epilepsy (Yamamoto et al., 2006). Accordingly, we found that epileptogenic foci excised from patients with temporal lobe epilepsy show increased ATF5 levels. Notably, neurons in these cortical foci do not display signs of apoptotic degeneration (McKieran et al., 2012; Engel et al., 2013). Similarly, CA1 and dentate gyrus neurons that do not degenerate after status epilepticus in our model, also show high ATF5 levels compared with the more vulnerable CA3 neurons. Thus, we postulate that neurons induce ATF5 as an anti-apoptotic mechanism in response to endoplasmic reticulum stress and that neuronal survival after an endoplasmic reticulum stress-inducing insult would in part depend on reaching a protective ATF5 threshold. More detailed analysis must be conducted to study the potential implication of ATF5 in other endoplasmic reticulum stress-related conditions.

Beyond the relevance of ATF5 upregulation upon endoplasmic reticulum stress, our results uncover constitutive neuronal expression of ATF5 under normal circumstances, suggesting that ATF5 is likely to play a role in normal neurophysiology. In this sense, both ATF4 and ATF5 bind GABA-B receptor subunit in vitro as demonstrated in yeast two hybrid assays (White et al., 2000), and ATF4:GABA-B receptor complexes favour the subcellular localization of ATF4 in neuronal somata rather than nucleus in vivo (White et al., 2000). Interestingly, we found that ATF5 resides in both neuronal soma and nucleus in adult brain. It is possible that ATF5 interacts with GABA-B receptor in vivo and that the ATF5:GABA-B receptor complexes would be relevant for neurophysiology.

We also report that the eIF2α-regulated Atf5 transcript (Atf5a) is enriched in hippocampal neurons compared with other encephalic structures, thus suggesting that the translational regulation of ATF5 would be especially relevant for hippocampal physiology. In support of this view, two genetically modified mouse lines with reduced levels of phospho-eIF2α, eIF2α+/−S51A and GCN2−/− mice, show facilitation of long-term potentiation and long-term memory (Costa-Mattioli et al., 2005, 2007). As we have shown that phospho-eIF2α induces ATF5 in neurons, it is possible that reduced ATF5 translation contributes to the long-term potentiation and long-term memory phenotypes observed in GCN2−/− and eIF2α+/−S51A mice. Thus, it would be possible that ATF5 might act as a modulator of hippocampal synaptic plasticity downstream to eIF2α phosphorylation.

Finally, it is important to note that anti-ATF5 approaches have been proposed as safe treatments for some glial tumours as they would not interfere with neuronal physiology (Greene et al., 2009; Sheng et al., 2010; Arias et al., 2012) since, until now, it was believed that ATF5 was not expressed by neurons. Given the presence of constitutive expression of ATF5 in neurons, reappraisal of the safety of antineoplastic anti-ATF5 strategies is warranted.

In summary, our results have established the neuronal expression of ATF5 in adult brain and show that neurons have the ability to induce ATF5 in pathological conditions that feature endoplasmic reticulum stress. Furthermore, we reduced endoplasmic reticulum stress-induced death of primary neurons by increasing ATF5 either by transfection of primary neurons or by treating mice and primary neurons with salubrinal. In addition, given that levels of ATF5 directly correlate with apoptosis-resistance in the status epilepticus-mouse model and in temporal lobe epilepsy brain samples, we postulate that the increase in ATF5 levels represents a protective mechanism activated by neurons to avoid neuronal death in circumstances of endoplasmic reticulum stress. These results open a new field for neuroprotective strategies focused on ATF5 modulation.

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Supplementary material

Supplementary material is available at Brain online.

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