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Endogenous epitope tagging of eEF1A2 in mice reveals early embryonic expression of eEF1A2 and subcellular compartmentalisation of neuronal eEF1A1 and eEF1A2

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ABSTRACT

All vertebrate species express two independently-encoded forms of translation elongation factor eEF1A. In humans and mice eEF1A1 and eEF1A2 are 92% identical at the amino acid level, but the well conserved developmental switch between the two variants in specific tissues suggests the existence of important functional differences. Heterozygous mutations in eEF1A2 result in neurodevelopmental disorders in humans; the mechanism of pathogenicity is unclear, but one hypothesis is that there is a dominant negative effect on eEF1A1 during development. The high degree of similarity between the eEF1A proteins has complicated expression analysis in the past; here we describe a gene edited mouse line in which we have introduced a V5 tag in the gene encoding eEF1A2. Expression analysis using anti-V5 and anti-eEF1A1 antibodies demonstrates that, in contrast to the prevailing view that eEF1A2 is only expressed postnatally, it is expressed from as early as E11.5 in the developing neural tube. Two colour immunofluorescence also reveals coordinated switching between eEF1A1 and eEF1A2 in different regions of postnatal brain. Completely reciprocal expression of the two variants is seen in post-weaning mouse brain with eEF1A1 expressed in oligodendrocytes and astrocytes and eEF1A2 in neuronal soma. Although eEF1A1 is absent from neuronal cell bodies after development, it is widely expressed in axons. This expression does not appear to coincide with myelin sheaths originating from oligodendrocytes but rather results from localised translation within the axon, suggesting that both variants are transcribed in neurons but show completely distinct subcellular localisation at the protein level. These findings will form an underlying framework for understanding how missense mutations in eEF1A2 result in neurodevelopmental disorders.

1. Introduction

Neurons depend on protein synthesis not only for survival, but to enable a rapid response to environmental signals. Since the majority of mRNAs have a relatively long half-life, rapid changes in gene expression are more efficiently accomplished by changes in rates of protein synthesis and degradation. Translational control not only enables cells to respond rapidly to signalling, but for subsets of mRNAs to be translated in specific subcellular compartments. This system is particularly crucial for neuronal function; here the translational machinery is compartmentalised in the soma, dendrites and axons, and at synapses, permitting these often very physically distant compartments to respond to distinct signals (Holt et al., 2019). Localised translation in response to synaptic activity is essential for synaptic plasticity and memory consolidation, and axonal translation is now widely accepted as an important mechanism for establishment and maintenance of neuronal networks (Lin et al., 2021).

Translation elongation factor eEF1A plays a pivotal role in protein synthesis where it is responsible for the delivery of aminoacylated tRNAs to the ribosome (Merrick, 1992). This is a GTP dependent step, facilitated by the GTP exchange complex eEF1B (Le Sourd et al., 2006). In all known vertebrate species eEF1A exists as two independently encoded...
variants with distinct expression patterns; these variants are called eEF1A1 and eEF1A2. There is often confusion between the two in the literature due to the high degree of homology they exhibit at the amino acid level (92 % identity, 98 % similarity in human (Soares et al., 2009)). Most commercially available antibodies and many nucleic acid probes recognise both variant forms, complicating our understanding of their expression. However, multiple studies have shown that the eEF1A variants are differentially expressed in a developmental- and tissue-specific manner. Whilst eEF1A1 is expressed ubiquitously during development, and thereafter in almost all cell types, it becomes downregulated postnatally in brain and muscle in all vertebrates so far studied. In mice and rats eEF1A1 is downregulated to undetectable levels in muscle soon after weaning, at about 3 weeks of age. During this postnatal period, eEF1A2 becomes upregulated until it peaks at about 3 weeks and the switch is complete. In both mouse and rat this process seems to be regulated at the RNA level, as both mRNAs and protein expression levels change in parallel (Lee et al., 1992; Chambers et al., 1998; Khalyfa et al., 2001).

In neurons, in contrast, the postnatal switch from eEF1A1 to eEF1A2 has been less well characterised, largely because expression analysis in brain is complicated by the fact that glia express eEF1A1 at high levels, rendering whole tissue analysis difficult. However, Westerns of whole mouse brain in situ hybridisation and immunohistochemistry have been used to study expression of both variants, reaching the conclusion that in brain and spinal cord of post-weaning animals eEF1A1 is no longer expressed in neurons but is replaced with the closely related variant eEF1A2 (Khalyfa et al., 2001; Pan et al., 2004). Studies of early development have been limited to showing that eEF1A2 is not detectable by Western blotting in brain lysates at embryonic day 18 (Khalyfa et al., 2003), but can be detected in brain by immunofluorescence at postnatal day 1 (P1; Pan et al., 2004). In contrast, eEF1A1 mRNA and protein have been shown to be the major form in prenatal and early postnatal neurons (Chambers et al., 1998; Khalyfa et al., 2001; Pan et al., 2004), and throughout life in glia and white matter (Lee et al., 1995) (Newbery et al., 2007). Khalyfa et al. reported a lack of expression of eEF1A1 in dendrites of postnatal animals (Khalyfa et al., 2003).

In spite of this earlier work, it is still unknown whether all neurons in adult animals, in every region of the brain, express only eEF1A2. Furthermore, numerous proteomic and transcriptomic analyses in both humans and rodents have demonstrated the presence of both eEF1A1 and eEF1A2 in synaptosomes, repeatedly identifying eEF1A1 as a synaptic mRNA or protein in both developing and fully mature organisms (Bayes et al., 2011; Bayes et al., 2012; Cajigas et al., 2012). The obvious caveat here is that eEF1A1 is strongly expressed in glia, which can contain postsynaptic density or neuripil props, but this has largely been corrected for in the analyses performed (Cajigas et al., 2012). In addition, many more recent studies have reported expression of eEF1A1 in dendrites of adult animals, including Perez et al. who showed dendritic enrichment of eEF1A1 mRNA in GABAergic interneurons (Perez et al., 2021).

The functional consequences of eEF1A variant switching during development are still unknown, though Mendoza et al. (2021) showed that phosphorylation of specific sites on eEF1A2 (that are not shared with eEF1A1) modulates structural plasticity in dendritic spines, coordinating protein synthesis with actin dynamics. One of these sites, Ser358, had previously been shown by Gandin et al. to regulate stress-induced degradation of newly synthesised polypeptides, implying an additional role for eEF1A2 specifically in quality control of translation (Gandin et al., 2013). EEF1A2 is assumed to be essentially absent from early in development (since it has been shown to be essential in lower organisms like yeast (Cottrelle et al., 1985)), but mice with complete absence of eEF1A2 survive until ~4 weeks postnatal, dying of motor neuron degeneration (Chambers et al., 1998; Newbery et al., 2005).

No humans with null mutations in eEF1A2 have been reported, but a wide range of de novo heterozygous missense mutations in the EEF1A2 gene have been found to result in often severe neurodevelopmental disorders including early onset epilepsy and intellectual disability (Nakajima et al., 2015; Lam et al., 2016; Carvill et al., 2020). Affected children frequently also exhibit autistic behaviours and sleep disorders, and many have movement disorders or undergo a degenerative course (Carvill et al., 2020). The only known cases with homozygous mutations in EEF1A2 were seen in one family where three children died before the age of 5 with dilated cardiomyopathy and severe epileptic encephalopathy (Cao et al., 2017). Recapitulation of pathogenic mutations in the mouse suggests that at least some of these missense mutations represent a gain of function (Davies et al., 2017; Davies et al., 2020).

A detailed description of the patterns of expression of the two eEF1A variants during brain development is key for understanding and treating disorders resulting from mutations in eEF1A2, but very little work has been published using two colour immunofluorescence for eEF1A1 and eEF1A2 simultaneously (Pan et al., 2004; Newbery et al., 2007). Although there are many antibodies that are able to distinguish eEF1A1 and eEF1A2 on Westerns, and that are also isofrom-specific when used individually for immunofluorescence on tissue sections, their use in combination for two colour immunofluorescence has been problematical largely due to issues with crossreactivity and the need to demonstrate antibody specificity. In order to address this gap in knowledge we introduced an epitope tag at the C terminus of the endogenous eEF1A2 protein in mice by gene editing, to allow us to detect eEF1A2 specifically when in combination with eEF1A1. We show that eEF1A2 is expressed from as early as E11.5 in some cell types, demonstrate the switch from eEF1A1 to eEF1A2 in different areas of the brain leading to non-overlapping expression patterns. Further, we show that in neurons eEF1A1 expression is not switched off throughout the cell, but rather switches from the soma to axons once development is complete.

2. Materials and methods

2.1. Transgenic mouse generation

Mice were housed in the Biomedical Research Facility (BRF) at the University of Edinburgh. All mice were maintained in accordance with Home Office regulations and all protocols had been approved by the local ethics committee of the University of Edinburgh. All methods were performed in accordance with the relevant guidelines and regulations. Embryo transfer was carried out with short term recovery anaesthesia and analgesia where needed post-operatively. Mutant mice were closely observed for overall clinical condition and were euthanized where necessary to avoid suffering.

Transgenic mice were made using the EASI-CRISPR method first described by Quadros et al. (2017). gRNA (sequence TTCTTTAGTTA-GACCAACTAGG) was designed to cut the genome in intron 7 of Eef1a2 to minimise chances of unwanted indels around the cut site affecting gene function. To insert the V5 tag into the 3’ end of Eef1a2 a 720 bp repair template was designed. As well as adding the V5 tag, the template made a number of other silent mutations; this was necessary as much of the locus proved too GC-rich to synthesise. Eef1a2 intron 7 was replaced with human EEF1A1 intron 7 in the template, and the Eef1a2 3′ UTR was replaced with the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) and SV40 polyA. Homology arms were 67 bp long (see Supplementary data Fig. 1A).

The microinjection mix was prepared with components (all from IDT) at the following concentrations: crRNA/tracrRNA complex at 27 ng/μl, ssDNA megamer at 10 ng/μl, Alt-R s.p. HiF1 Cas9 nuclease V3 (50ng/μl). Freshly prepared microinjection mix was introduced by pronuclear injection into oocytes derived from C57BL/6JCr mice.

Mice were housed in single sex, mixed genotype groups of between 2 and 5 animals. Both male and female mice were used for expression analysis. All observational phenotyping was carried out blind to genotype.
2.2. Genotyping

Ear notches were taken at 14 days after birth, DNA extracted and used for PCR genotyping, initially by direct sequencing when identifying founders, then after establishment of colonies by analysis of band sizes of PCR products on a 1 % agarose gel where a wild-type allele gave a band size of 176 bp and a V5 knock-in 762 bp. PCR primers used were 5′-3′ TCTCTTTTGAGAAGAAGCGGCT and GCTTTAAGCGTCTGTCCGGT and PCR was carried out with annealing temperature of 62 °C using Platinum SuperFi polymerase (Thermofisher) for 34 cycles.

2.3. Protein analysis

Whole brains and hind limb muscles were dissected, frozen on dry ice and stored at −70 °C until use. They were homogenized in 10 µl/mg tissue 0.32 M sucrose containing protease inhibitor (cOmplete Mini, Roche) then centrifuged at 17,000g at 4 °C for 30 min. The supernatant was removed, mixed with an equal volume of 2× Laemmli buffer, and then heated to 100 °C for 5 min. 10 % (v/v) of 1 M diethioctetol was then added to each sample. Lysates were loaded onto 10 % acrylamide gels and run at 100 V in 1× TBS buffer before being transferred onto PVDF membrane (Amershams Hybond) in NuPAGE transfer buffer. Total protein was visualized using REVERT (LI-COR). Blots were then destained (according to manufacturer instructions) then blocked for 1 h at room temperature using Intercept protein-free blocking buffer (LI-COR). Primary antibodies (mouse anti-V5 1:5000 (Invitrogen R960-25) and rabbit anti-eEF1A2 1:2000) (custom made by Proteintech, equivalent to eEF1A1-1 in Newbery et al. (2007)), were applied to blots in blocking buffer overnight at 4 °C. After 3 PBS washes slides were treated with 1× TrueBlue or 2× TrueBlue Plus (Biotium) as per manufacturer’s instructions, then incubated with secondary antibody diluted 1:500–1:1000 in 1 % serum in PBS for 1 h. After another 3 PBS washes slides were coverslipped using Vectashield Vibrance + DAPI and left to cure overnight at room temperature.

Immunofluorescence images were acquired using either A: a Zeiss Axio Scan Z1 slide scanning microscope (Carl Zeiss UK, Cambridge, UK) with Fluor or Plan Apochromat objective lenses (2.5×, 5×, 10×, 20×, 40×), a Zeiss Colibri 7 LED light source and a Zeiss Axioscam 506 m monochrome CCD camera and Zeiss 90 HE, 92 HE, 96 HE, 38 HE and 43 HE fluorescent filter sets, with image capture performed using Zeiss Zen 3.5 Slidescan acquisition software. Or B: a Photometrics Prime BSI CMOS camera (Photometrics, Tuscon, AZ) fitted to a Zeiss Axiolmager M2 fluorescence microscope with Plan-Apochromat objectives, using either 1. A Zeiss Colibri 7 LED light source, together with Zeiss filter sets 90 HE, 92 HE, 96 HE, 38 HE and 43 HE (Carl Zeiss UK, Cambridge, UK), with image capture performed in Zeiss Zen 3.5 software, or 2. A Mercury Halide fluorescent light source (Exfo Excite 120, Excelitas Technologies) and Chroma #89014ET three colour filter set (Chroma Technology Corp., Rockingham, VT), with single excitation and emission filters installed in motorised filter wheels (Prior Scientific Instruments, Cambridge, UK) and image capture performed using Micromanager (Version 1.4).

Confocal images were acquired using a Multimodal Imaging Platform Dragonfly (Andor technologies, Belfast UK) equipped with 405, 445, 488, 514, 561, 640 and 680 nm lasers built on a Nikon Eclipse Ti-E inverted microscope body with Perfect focus system (Nikon Instruments, Japan). Data were collected in Spinning Disk 40 µm pinhole mode with a 100× objective on the iXon 888 EMCCD/Zyla 4.2 eCMOS camera using a Bin of 1×1 and frame averaging of 1 using Andor Fusion acquisition software.

2.6. Immunofluorescence

Mice were humanely killed and tissues taken and rinsed in ice-cold PBS and fixed overnight at 4 °C in at least 15 volumes of 4 % paraformaldehyde. Tissues were stored in 50 % ethanol until processing by dehydration and paraffin embedding, and were then cut into 10 µm sections and mounted on glass slides. Slides were deparaffinised with xylene, rehydrated and microwaved in citric acid pH 6 for 20 min. They were then blocked for 1 h in a 1:5 dilution in PBS of sheep or donkey serum, using the species in which the secondary antibody was raised. Primary antibodies were applied in 1 % serum in PBS with 0.1 % triton-X and incubated overnight at 4 °C. After 3 PBS washes slides were treated with 1× TrueBlue or 2× TrueBlue Plus (Biotium) as per manufacturer’s instructions, then incubated with secondary antibody diluted 1:500–1:1000 in 1 % animal serum in PBS for 1 h. After another 3 PBS washes slides were coverslipped using Vectashield Vibrance + DAPI and left to cure overnight at room temperature.

2.4. RNA analysis

Brain tissue was homogenized in Qiazol using a bead mill homogeniser (Precellys). RNA was isolated using the QIAGEN RNeasy mini kit and treated with QIAGEN RNase-Free DNase Set. RNA quality was assessed using a Bioanalyzer, and only RNA with a RIN above 7.8 was used for cDNA synthesis. cDNA was then synthesised using the Agilent (PrimerDesign). Brilliant II SYBR Green QPCR master mix (Agilent) was used for PCR products on a 1 % agarose gel where a wild-type allele gave a band size of 176 bp and a V5 knock-in 762 bp. PCR primers used were 5′-3′ TCTCTTTTGAGAAGAAGCGGCT and GCTTTAAGCGTCTGTCCGGT and PCR was carried out with annealing temperature of 62 °C using Platinum SuperFi polymerase (Thermofisher) for 34 cycles.

2.5. Timed matings

Proven males and 8–12 week old females were co-housed in the late afternoon. Females were checked for the presence of vaginal plug every subsequent morning, and weight of dam recorded once a plug was found. The morning the plug was found was taken to be day 0.5 post-fertilisation. Weight gain of >1.75 g on day of collection indicated probable pregnancy.
3. Results

3.1. Engineering an epitope tag into the mouse Eef1a2 gene

Differential expression analysis of eEF1A1 and eEF1A2 is non-trivial using standard antibodies because of the high chance of cross-reactivity resulting from the 92% identity between the two proteins. We therefore opted to introduce a V5 epitope tag at the 3’ end of the endogenous Eef1a2 mouse gene using CRISPR/Cas9 gene editing, with the aim of producing a line of mice expressing eEF1A2 protein with a C-terminal V5 tag that could be specifically detected in immunofluorescence. We designed a single-stranded oligonucleotide repair template of 720 nucleotides that included the whole coding portion of exon 8 of the Eef1a2 gene with a V5 tag in-frame. This was followed by the WPRE element (which is widely used in gene therapy and here used as a replacement for the 3’UTR of eEF1A2, which could not be synthesised), all flanked by homology arms; see Fig. 1A and Supplementary information. It also proved impossible to synthesise intron 7 so this was replaced by the less GC-rich intron 7 from eEF1A1. The repair template was introduced into fertilised oocytes together with pre-assembled crRNA + tracrRNA + Cas9 ribonucleoprotein (ctRNP) complexes using Easi-CRISPR (Quadros et al., 2017) and the resulting live-born mice genotyped by sequencing. Of 27 founders born, 16 had incorporated the repair template on one allele and 8 were homozygous for the V5 tag with only 8 mice showing no indication of a knock-in.

A line was generated from one of the mice heterozygous for the knock-in and expression analysis performed. Whilst the levels of tagged eEF1A2 mRNA were equivalent to those in wild-type (WT) littermates (Fig. 1D) in both heterozygous and homozygous V5 mice, Western blotting showed clearly that V5 carrying mice were producing a single band of the correct size, whether detected with an anti-eEF1A2 or anti-V5 antibody, although levels are reduced by two thirds in V5/V5 homozygous mice.

Fig. 1. A) Schematic of the CRISPR design used to create the eEF1A2-V5 mouse line, with the cut site indicated by scissors and the V5 and WPRE parts of the repair template in orange and blue respectively. Exons are shown as boxes, with solid boxes corresponding to coding regions. B) Western blot showing a single band (red) of the correct size detected in brain with an anti-V5 antibody in heterozygous and homozygous mice (and the absence of a signal in the negative control, from a wild-type mouse) and a slightly smaller band (blue) representing total eEF1A2. D) qPCR results showing that mice both heterozygous and homozygous for the V5 insertion express similar levels of eEF1A2 mRNA to those seen in WT mice. E) Quantification of protein detected by anti-eEF1A2 on the Western blot showing that the V5 tagged eEF1A2 protein shows reduced expression in V5/V5 homozygous mice.
homozygotes (Fig. 1B, E). Homozygous knock-in mice died by ~4 weeks, at a similar time to eEF1A2-null mice (Chambers et al., 1998) consistent with the low levels of expression seen. It is unclear whether the tag or the replacement of the 3’UTR with the WPRE element caused the reduction in expression, but the normal mRNA levels suggest that the tag might interfere with protein stability. Nevertheless, the heterozygotes were phenotypically normal in terms of weight and survival and expression of the tagged protein was sufficiently high for the resulting line to be used for assessing expression of eEF1A2 in tissues throughout development. These mice are referred to as V5/+ (heterozygotes) and V5/V5 (homozygotes) throughout, for ease of reading.

3.2. Expression of the tagged eEF1A2 protein reflects that of endogenous eEF1A2

Previous work using immunofluorescence imaging studies have showed eEF1A2 and eEF1A1 to be expressed reciprocally in different cell types of the brain. Broadly speaking, eEF1A2 is found in neuronal cell bodies, and eEF1A1 in glial cells and white matter tracts (Pan et al., 2004; Newbery et al., 2007). In contrast, RNA-seq studies have shown both eEF1A1 and eEF1A2 transcripts in neuronal cells, indicating that the expression patterns of eEF1A isoforms in brain may be more nuanced and complex than first thought (Bayes et al., 2011; Bayes et al., 2012; Cajigas et al., 2012).

We initially carried out immunofluorescence on sections of the cerebellum as Purkinje cells (PC) are known to express high levels of eEF1A2 (Newbery et al., 2007). Throughout, we refer to expression detected by the anti-V5 antibody as eEF1A2-V5 and expression detected by anti-eEF1A2 as eEF1A2 (which could be endogenous, V5-tagged on both depending on the genotype of the mice used). Antibodies to eEF1A1 and eEF1A2 have previously been shown to be isoform-specific on Western blots (Newbery et al., 2007). We examined expression of V5-tagged eEF1A2 in the cerebellum of post-weaning mice. Staining with an anti-V5 antibody revealed strong, specific expression in PC in heterozygous (V5/+), and equally specific expression, albeit at lower levels, in V5/V5 animals (Fig. 2A). Co-immunofluorescence with anti-eEF1A2 revealed strong, specific expression in V5/V5 mice and no detectable expression in the WT mouse.
Fig. 3. A) Immunofluorescence of a transverse section of an E11.5 embryo stained with anti-V5 antibody and B) showing expression of V5-tagged eEF1A2 in the mantle layer of the basal plate of the developing spinal cord (white dashed lines). On the right is a control using secondary antibody only, showing some non-specific staining/autofluorescence in other regions. C) More widespread expression of V5-tagged eEF1A2 by E13.5. D) Sections of cerebellum at different stages, all stained with anti-V5 antibody, showing developing expression of eEF1A2 in Purkinje cells. Nuclei stained blue with DAPI in all images.
Fig. 4. A) Composite image of whole brain of a V5/+ mouse at P25 stained with anti-V5 and B) a pan-eEF1A antibody that detects both eEF1A1 and eEF1A2, to demonstrate reduced expression of eEF1A in the striatum. C) Co-immunofluorescence of eEF1A1 and V5-tagged eEF1A2 (stained with anti-V5 antibody) shows predominant expression of eEF1A1 in the pallium at E17.5, to the right are images separated by channel. D) Co-immunofluorescence of eEF1A1 and V5-tagged eEF1A2 (stained with anti-V5 antibody) shows a gradient of switching from eEF1A1 to eEF1A2 in the hippocampal pyramidal layer at P5. C) E) Co-immunofluorescence of eEF1A2-V5 and eEF1A1 in the hippocampus of a P25 mouse expressing the V5 tagged form of eEF1A2, showing non-overlapping expression patterns of the two variant forms of eEF1A. F) Co-immunofluorescence of eEF1A1 and V5-tagged eEF1A2 in thalamus (zoomed in region of thalamus from Fig. 4E) at P25 showing clear staining of eEF1A1 in axons.
eEF1A2 and anti-V5 antibodies showed completely overlapping expression in PC of a V5/V5 mouse, suggesting that neither the tag nor the 3′UTR had affected location of the V5 tagged eEF1A2. There were some minor areas of non-specific background staining with the anti-V5 antibody in non-neuronal areas but these were also seen in the WT mouse not expressing V5; Fig. 2B. Staining of neuronal cell bodies also revealed overlapping expression of the tagged and total eEF1A2 protein at the subcellular level (Fig. 2C).

### 3.3. eEF1A2 is expressed as early as E11.5

We then examined expression of eEF1A2-V5 in V5/+ mice between the ages of E11.5 and P25. Timed matings in the V5 tagged line were used to collect embryos for sectioning at different stages. Although previous reports in the literature have suggested that eEF1A2 is not expressed until after birth, Fig. 3A shows that as early as E11.5, V5-tagged eEF1A2 is detectable at low levels in the basal plate of the embryonic spinal cord. By E13.5 (Fig. 3B) there is more widespread expression throughout the spinal cord and brain, with strong expression seen in heart, dorsal root ganglia, trigeminal ganglia and olfactory bulb. Analysis of V5 staining in the cerebellum at embryonic and postnatal stages shows eEF1A2 expression developing in Purkinje cells (PC). We show that eEF1A2 is found in cerebellar cell types as early as E13.5, around the time when PC are first differentiated in mouse (E11-E13) (Sotelo, 2004) and track the migration of PC through the developing cerebellum (Fig. 3C).

### 3.4. Non-overlapping expression of eEF1A1 and eEF1A2 throughout the brain by P25

We next examined expression of eEF1A2 throughout the brain at P25. Fig. 4A shows mouse brain at P25 with eEF1A2 detected by anti-V5. Strong expression of eEF1A2-V5 can clearly be seen in neurons of multiple brain regions by this age. eEF1A2 is clearly expressed throughout the brain, with staining throughout the cortex, and is particularly strongly expressed in hippocampus and cerebellum. However, whilst expression of eEF1A2 is generally widespread, expression is low in the striatum. In order to establish whether this low staining level could be an artefact resulting from the tagging of eEF1A2 we carried out immunofluorescence using a commercial antibody which detects both eEF1A1 and eEF1A2 (Fig. 4B). This shows clearly that in fact expression of either form of eEF1A is markedly lower in the striatum than in the surrounding areas of the brain, consistent with the findings of Ayola et al. who found reduced rates of protein synthesis in the striatum (Ayola et al., 1988).

Co-immunofluorescence of V5-tagged eEF1A2 and endogenous eEF1A1 reveals changing patterns of the two variant forms of eEF1A in neurons during development. Fig. 4C shows a section of hippocampus at E17.5 with predominant staining of eEF1A1 throughout (particularly in presumed neuronal progenitor cells) but with a focused band of strong staining of eEF1A2-V5 in the differentiating CA neurons. In Fig. 4D a section of hippocampus from mice aged P5 can be seen: here eEF1A1 is evenly expressed throughout pyramidal cells of CA3, whilst eEF1A2 shows a gradient of expression across deep and superficial layers, with stronger expression in the deep (more differentiated) pyramidal cells. By 25 days postnatal (Fig. 4E), analysis of expression of eEF1A1 and eEF1A2-V5 in hippocampus shows a dense band of expression of eEF1A2 in dorsal granule and pyramidal cells, and in dorsal CA1, CA2 and CA3 layers. By this stage of development there is no apparent overlapping expression of the two eEF1A variant proteins and eEF1A1 expression appears to be confined to axons under the cortex. A similarly mutually exclusive pattern of expression is seen in other brain areas (e.g. thalamus, Fig. 4F), with clear evidence of eEF1A1, but not eEF1A2, in axons.

### 3.5. eEF1A1 protein expression is confined to axons in mature neurons

It is clear from the literature and the results above (Fig. 4E and F)
that whilst eEF1A2 is highly expressed in neuronal soma, eEF1A1 is not expressed in mature neuronal cell bodies but is highly expressed in glial cells and white matter, including axon tracts. Since eEF1A1 is highly expressed in oligodendrocytes, it would be reasonable to assume that the staining seen in axons represents staining of the myelin sheath originating from oligodendrocytes. We set out to test this directly by performing co-immunofluorescence of eEF1A1 and myelin basic protein (MBP), a key component of the myelin sheath. Multiple sections through axon bundles in the cortex and striatum show clearly that anti-eEF1A1 and anti-MBP antibodies both stain axons (Fig. 5A–D).

Due to poor structural preservation of axons in formalin-fixed paraffin embedded sections, we were unable to fully resolve whether eEF1A1 was within axons. However, it was clear that there were areas of both overlapping and non-overlapping staining of eEF1A1 and MBP in transverse sections through subcortical axon bundles (Fig. 5D), suggesting that some eEF1A1 signal originated from within axons and not from non-neuronal cells.

Although we saw no overlapping expression between eEF1A1 and V5-tagged eEF1A2 in mature neurons, we wanted to exclude the possibility that the presence of the epitope tag had prevented eEF1A2 from being trafficked into axons. We carried out further immunofluorescence on sections from V5/+ mice (which therefore express both tagged and untagged eEF1A2) using three antibodies: anti-V5, anti-eEF1A2, and anti-MBP to stain the myelin sheath. Fig. 5E and F show the absence of both tagged and untagged eEF1A2 within axons.

4. Discussion

Epitope tagging of endogenous eEF1A2 has enabled us for the first time reliably to track the expression patterns of both eEF1A1 and eEF1A2 through development. This approach allowed us to circumvent issues of cross-reactivity of antibodies raised against the two closely related proteins, and the results form a valuable framework to underpin further studies investigating the functional consequences of the developmental switching from eEF1A1 to eEF1A2 in specific cell types and/or subcellular compartments. The EASI-CRISPR technique used to introduce the V5 tag worked extremely efficiently and could be applied to other proteins where antibody specificity cannot be achieved. We confirmed that although expression of V5 tagged eEF1A2 protein was lower than that of endogenous eEF1A2 (presumably due to the change of 3′UTR), staining with an anti-V5 antibody coincided perfectly with that seen using an antibody raised against eEF1A2, and that, crucially, the anti-V5 antibody was able to be used in co-immunofluorescence experiments with anti-eEF1A1.

We detected expression of eEF1A2 at E11.5, the earliest stage at which we looked. Expression was seen in heart and the central nervous system, increasing throughout development, and at later stages waves of switching between eEF1A1 and eEF1A2 could be observed in specific areas of brain, most notably in the cerebellum and hippocampus. In more mature brain, from postnatal day 25, a clear picture of non-overlapping expression of eEF1A1 and eEF1A2 emerges.

When stitched images of the whole brain stained with an antibody that detects both eEF1A1 and eEF1A2 are examined, it is clear that striatum shows very low levels of expression of both variants in relation to other areas of the brain (Fig. 4A). The striatum is largely made up of medium spiny neurons which are GABAergic and have unmyelinated axons. A study of protein synthesis rates in 21 different areas of mouse brain found that the lowest levels were seen in corpus callosum, closely followed by striatum (Qin et al., 2011). This correlates with the low levels of eEF1A1 expression, though the cause and result of this variation in expression across different regions of the brain has yet to be established.

Some regions of strong expression of eEF1A2 may explain the phenotypes seen in individuals with heterozygous missense mutations in EEF1A2. Clearly the high levels of expression in cortex and hippocampus correlate well with the known epilepsy and intellectual disability phenotypes, but we also see very strong expression in Purkinje cells in the cerebellum and dorsal root ganglia. A number of children with mutations in EEF1A2 who are able to walk have been found to have gait abnormalities such as ataxia; loss of Purkinje cells is well established to result in ataxia in both animal models and humans and has been often shown to be associated with defects in tRNA metabolism (Lee et al., 2006; Kapur et al., 2017). The expression of eEF1A2 in heart from early in development may additionally explain the fatal dilated cardiomyopathy seen in children homozygous for the P333L missense mutation in EEF1A2 (Cao et al., 2017), particularly in view of the recent report implicating eEF1A2 in cardiomyocyte differentiation (Lyu et al., 2022).

In addition to evidence for the mutually exclusive expression of eEF1A1 and eEF1A2 in the cell bodies of different cell types in the brain, we now show that eEF1A1 expression is not, as previously thought, shut down completely in mature neurons. Instead, it relocates from the neuronal soma, where it is highly expressed during embryonic and early postnatal development, to axons. This switching mechanism is in contrast to that seen in muscle where eEF1A1 is shut down transcriptionally around weaning in mice (Chambers et al., 1998; Khalyfa et al., 2009). Rather, transcription, is likely sustained at similar levels throughout development, but either the eEF1A1 mRNA or protein changes localisation. Although proteins can be directly targeted to specific subcellular locations by virtue of specific motifs, protein localisation in neurons is frequently controlled by targeting of the cognate mRNA, which is then translated locally, and this seems the most likely mechanism here. Indeed, a number of studies of both the mRNA content and translantome of axons have identified eEF1A1 but not eEF1A2 in axons of mice and rats (Taylor et al., 2009; Gummy et al., 2011; Shigeoka et al., 2016; Nijssen et al., 2018) and in dendrites, albeit in cultured cells (Wefers et al., 2022); other studies using TRAP and Ribosese methodological have shown enrichment of eEF1A1 in axons relative to cell bodies (Ostroff et al., 2019; Glock et al., 2021; Jung et al., 2023). It seems likely that there are one or more RNA binding proteins controlling the developmental switch resulting in the transport and subsequent translation of eEF1A1 in axons; the 3′UTRs of eEF1A1 and eEF1A2, unlike the coding regions, are largely dissimilar (41% identity in 3′UTRs compared with 80% in the coding regions).

A key question is why this switch between near-identical proteins exists, and what are the functional consequences of the change in subcellular localisation of eEF1A1 during neuronal maturation. Biochemically, the two variants have indistinguishable activity in an in vitro translation system, although with different affinities for GTP (eEF1A2 shows a greater affinity for GDP and eEF1A1 a greater affinity for GTP (Kahns et al., 1998)). The differences between eEF1A1 and eEF1A2 are highly conserved throughout vertebrate evolution, suggesting strong constraints on the amino acids that differ between variants, and a functional role for these differences. Nevertheless, the extent to which there is functional equivalence of the two isoforms, and the degree to which their activities differ, is still largely a mystery. However, some progress has been made by focusing on specific sites of variation between eEF1A1 and eEF1A2. At a number of locations, particularly at the C terminal end of eEF1A1, the amino acid differences between the two variant forms are alanine (eEF1A1) to serine (eEF1A2) substitutions, raising the possibility of eEF1A2-specific phosphorylation (Soares et al., 2009). Indeed, Gandin et al. (2013) demonstrated a role for serines 205 and 358 in regulating the degradation of newly synthesised proteins under conditions of stress, during which these serines are phosphorylated by the kinase JNK. Romaus-Sanjurjo et al. (2022), found that over-expression of eEF1A2, but not eEF1A1, in corticospinal tract neurons increased protein synthesis and actin rearrangements, leading to enhanced sprouting after experimentally induced neuronal injury (Romaus-Sanjurjo et al., 2022). Further, Mendoza et al. (2021) used phosphomimetic and nonphosphorylatable mutant forms of eEF1A2 transfected into hippocampal neurons to show differential effects of the mutant forms on actin binding and translation activity, suggesting a key role for eEF1A2 in coordination of translation and actin dynamics in
dendritic spines in response to metabotropic glutamate receptor signalling. Since this activity depends on the serines that are present in eEF1A2 and not eEF1A1, it is reasonable to assume that the two variant forms of eEF1A will have distinct roles in the different subcellular compartments, and manipulation of the two, targeting them to different regions, will be necessary to understand their distinct, specific functions in axons, dendrites, and at synapses (where there is evidence for both variant forms, though not necessarily within the same synapse).

Our results have implications for increased understanding of the ways in which missense mutations in eEF1A2 can result in often severe disease in axons, dendrites, and at synapses (where there is evidence for both eEF1A1 and eEF1A2 can heterodimerise (Singh et al., 2012; Crepin et al., 2014)). A mutation that affects the balance between monomers and homo- or heterodimers could in turn affect the balance in the cell between translation elongation (carried out by monomers) and cytoskeletal remodelling (performed by eEF1A dimers) (Bunai et al., 2006). Such a disturbance could ultimately affect spine maturation, remodelling, and/or pruning, leading to changes in circuit activity and ultimately to epilepsy and intellectual disability. Indeed, the clear compartmentalisation of eEF1A1 and eEF1A2 within the mature neuron might suggest the need to avoid heterodimerisation once development is complete. It will be interesting to establish how widespread this compartmentalisation is in species that have different variant forms of eEF1A. Ultimately, functional studies will be needed to examine the role of higher order complexes of eEF1A on neuronal function and dysfunction.

Finally, the strategy of endogenous tagging that allowed us to circumvent problems with antibody specificity could be applied to other proteins where similar issues exist and where detailed mapping of protein level expression is necessary to understand disease processes. Supplementary data to this article can be found online at https://doi.org/10.1016/j.mcn.2023.103879.

Abbreviations

eEF1A1/eEF1A2 eukaryotic translation elongation factor 1A1/2
WPRE woodchuck hepatitis virus post-transcriptional regulatory element
PC Purkinje cells
CA1-3 cortical area 1-3

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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