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Immuno-characterization of the switch of peptide elongation factors eEF1A-1/EF-1α and eEF1A-2/S1 in the central nervous system during mouse development

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Abstract

During early postnatal development, a switch occurs between eEF1A-1/EF-1α and eEF1A-2/S1, homologous peptide elongation factors, in brain, heart, and skeletal muscle; eEF1A-2/S1 becomes the major form expressed in maturity. By immunofluorescent labeling, we detected both homologues in the developing brains of wild-type and *wasted* mutant mice, carrying a deletion in the *eEF1A-2/S1* gene; we found that brain expression of eEF1A-2/S1 protein is restricted to mature, terminally differentiated neurons, and coincides with the disappearance of eEF1A-1/EF-1α 20 days after birth. Furthermore, no elongation factor 1A is present in *wasted* mutant mice neurons following the developmental switch, indicating that the genetic regulation silencing eEF1A-1/EF-1α is still functional.

Keywords

Elongation factor 1A; Neuron; Central nervous system; Antibody; Neurofilament; Protein translation; Wasted mutant mouse; Developmental regulation; Immunohistochemistry

1. Introduction

The translation of mRNA into protein, essential for the survival of all organisms, is divided into three steps: initiation, elongation, and termination. Translation is initiated when an mRNA associates with ribosomal subunits and initiation factors to form mRNA/ribosomal complexes. Following this assembly, elongation factors participate in the successive addition of amino acids to nascent polypeptide chains. More specifically, eukaryotic peptide elongation factor 1A (eEF1A) translocates, in a GTP-dependent manner, the aminoacyl–tRNA complex from the aminoacyl synthetase to the aminoacyl site (A-site) of the ribosome, in a codon-dependent manner [13]. Once the chains are completed, translation is terminated and mRNA/ribosome/nascent polypeptide chain complexes are disassembled.

During postnatal development, a transition in elongation factor 1A from eEF1A-1/EF-1α to eEF1A-2/S1 occurs in brain, heart, and skeletal muscle [7,10,12]. In mice and rats, eEF1A-1/

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EF-1α is expressed during embryonic development, as well as the first two postnatal weeks. Thereafter, eEF1A-1/EF-1α is replaced in the brain, heart, and skeletal muscles by its homologue, eEF1A-2/S1; the reason for this transition, as well as the localization of eEF1A-2/S1 in different cell populations of the central nervous system (CNS), remain to be characterized. Absence of eEF1A-2/S1 caused by partial deletion of the gene, characterized in spontaneous *wasted* (*wst*/*wst*) mutant mice, is lethal in less than 30 days after birth [2], and is postulated to occur as a result of the absence of elongation factor 1A in brain, heart, and skeletal muscle following the developmental switch [7]. Heterozygous mice (+/*wst*) do not display any phenotype or abrogated longevity and have a normal pattern of protein expression, similar to wild-type (+/+) animals.

Two forms of elongation factor 1A, eEF1A-1/EF-1α and eEF1A-2/S1, share 92% homology in their amino acid compositions, and have been shown to function similarly during the elongation step of protein translation [8,9]. Since they are highly homologous in their composition and elongation activity, it is likely that the two factors do not share the same non-canonical functions, in order to explain the developmental switch. Evidence supporting the presence of distinct non-canonical functions between the two proteins has already started to accumulate. For instance, eEF1A-1/EF-1α exerts pro-apoptotic functions, accelerating the cell death rate, whereas eEF1A-2/S1 protects differentiated myotubes from apoptotic cell death [3,5,15]. Among other non-canonical functions, eEF1A-1/EF-1α cleaves actin cytoskeleton and microtubules [14,16], and interacts with two nuclear proteins, RNA polymerase and zinc finger protein ZPR1, thus enabling cells to successfully cycle through the G_2/M phase transition [4,6].

In order to better characterize the timing of the switch between eEF1A-1/EF-1α and eEF1A-2/S1, as well as to localize eEF1A-2/S1 in the brain, we investigated, using immunofluorescence, the in situ localization and expression patterns of the two homologous proteins in wild-type and *wasted* mutant mice brains during early postnatal development. We show that eEF1A-2/S1 is only expressed in the soma of terminally differentiated neurons of wild-type mice following the developmental disappearance of eEF1A-1/EF-1α. No elongation factor 1A is present in the neurons of *wasted* mutant mice following the timed developmental switch, indicating that the regulation of the *eEF1a-1*/*EF-1α* gene is still functional in these mutant mice, despite the absence of eEF1A-2/S1 protein.

For the purpose of this study, heterozygous mice for the *eEF1A-2*/*S1* gene (+/*wst*, B6CBFEa/a) of C57BL/6J background were purchased from the Jackson Laboratory (Bar Harbor, ME) and bred in our institutional animal facilities, to generate wild-type (+/+), heterozygous (+/*wst*) and *wasted* mutant (*wst*/*wst*) animals. Offspring were genotyped using multiplex polymerase chain reaction (multiplex PCR), as described below. *Wasted* mutant mice (*wst*/*wst*) exhibit muscle wasting, neurological impairment, immunological abnormalities, and tremors beginning at 21 days of age. The study was conducted in accordance with guidelines of the Canadian Council of Animal Care and the Lady Davis Institute Animal Care Committee.

For mouse genotyping, DNA extraction from mouse tails was carried out as previously described [7]. Briefly, approximately 0.5 cm of tail was digested overnight in DNA lysis buffer (100 mM Tris, pH 7.6, 5 mM EDTA, 0.2% SDS, 200 mM NaCl and 100 μg/ml proteinase K) at 55 °C. Digestion was followed by phenol/chloroform extraction and precipitation with isopropyl alcohol; DNA was dissolved in TE buffer (10 mM Tris pH 8.0 and 1 mM EDTA). Approximately 0.5 μg of mouse genomic DNA was amplified by PCR using the following reagents: PCR buffer (20 mM Tris, pH 8.4, 50 mM KCl, 2 mM $MgCl_2$, 0.1% Triton X-100 final concentration) to which was added 1 mM dNTP, 100 ng each of two pairs of primers (P1 and P2) and Taq polymerase (laboratory clone), in a total volume of 50 μl. Reactions were carried out in a Perkin-Elmer PCR apparatus as follows: hot start at 95 °C for 5 min, followed

by 28 cycles of amplification (denaturation at 94 °C for 28 s, annealing at 57 °C for 25 s, and elongation at 72 °C for 25 s), and finally extension for 10 min at 72 °C. Primer pair 1 (P1) (sense: 5′ TCTGCTGCCTCTGTCATT and antisense: 5′ CGCCATTCTTGTATTGTTTG) yields a fragment of 456 bp, while primer pair 2 (P2) (sense: 5′ TAGTGGCTCCTTGGAACAG and antisense: 5′ CTACTCTCCCTGAATGCCTT) yields a fragment of 304 bp (GenBank accession number: AJ223837). Ten microliters of each product were visualized on a 1.2% agarose gel stained with ethidium bromide, using a UV transluminator.

These two new pairs of specific oligonucleotide primers to mouse *eEF1A-2/S1* gene were designed to rapidly genotype target mice at early age in a single PCR reaction. This multiplex PCR [1] reaction allows the rapid identification of the partial *eEF1A-2/S1* gene deletion present in *wasted* mutant mice (Fig. 1A). P1 primers amplify a 456 base pair (bp) fragment in wild-type (+/+) and heterozygous (+/*wst*) animals, but not in *wasted* mutant mice (*wst*/*wst*), which lack the region amplified by these primers. Unlike P1 primers, P2 primers were designed to identify the presence of the *wasted* deletion of *eEF1A-2/S1* gene. In wild-type animals possessing the full-length *eEF1A-2*/S1 gene, P2 sense and antisense primer binding sites are separated by 15.8 kb, but in heterozygous (+/*wst*) and *wasted* mutants (*wst*/*wst*), as a result of the *wasted* deletion, only 304 bp separates them. Thus, in heterozygous and *wasted* mutant mice, a 304 bp fragment is generated during the multiplex PCR reaction. Consequently, when multiplex PCR using P1 and P2 primer pairs are used, three results may be obtained. First, two fragments are amplified in heterozygous mice: one of 456 bp, indicating the presence of one full-length *eEF1A-2/S1* gene, and one of 304 bp, indicating the presence of a truncated *eEF1A-2/S1* gene. Second, only one 456 bp band is generated in wild-type mice, as both *eEF1A-2/S1* alleles are complete. Third, only one 304 bp band is generated in homozygous *wasted* mice, since their genome shows partial deletion of the *eEF1A-2/S1* gene (Fig. 1B and C). The genotype of homozygous *wasted* mutant mice is phenotypically validated when the animals enter the fourth week of life, as they begin displaying pathological symptoms, such as tremors and uncoordinated body movements, characteristic of the absence of *eEF1A-2/S1*.

Brains from wild-type, heterozygous and *wasted* mutant mice of embryonic day 16 (E16), as well as postnatal (P) days 1, 7, 14, 20, and 26, as well as 1-year-old wild-type mice, were used for the immunohistochemical analysis. Since no differences were observed between wild-type and heterozygous (+/*wst*) mice, both were used without discrimination as controls in the preparation of the figures. Therefore, in the description of the results, wild-type refers to the phenotype instead of the genotype. For this study, one set of brains was fixed overnight at 4 °C in a phosphate-buffered saline (PBS) solution containing 4% paraformaldehyde (W/V). Fixed brains were washed twice for 12 h at 4 °C in PBS containing 15% and 30% sucrose, respectively; specimens were kept frozen at −80 °C until cryosectioned. The other set of brains was directly embedded in Tissue-Tek's Optimum cutting temperature (OCT) medium, and kept frozen at −80 °C until sectioning. Cryosections were prepared on a cryotome (Leica) by serial coronal sections of 4–6 μm. One set of sections was stained with haematoxylin and eosin for histological examination, while the remaining sections were used for immunofluorescence analysis.

Immunohistochemistry was carried out using rabbit polyclonal antibodies HT7 and CB5, specific to eEF1A-1/EF-1α and eEF1A-2/S1, respectively, generated and purified in our laboratory, as previously described [7,15]. Neuron-specific monoclonal antibody to neurofilament NF-68 and rabbit anti-glial fibrillary acidic protein (GFAP) were purchased from Sigma-Aldrich.

For immunofluorescence analysis, fixed and unfixed brain sections were washed as described above, and incubated with primary antibodies HT7 (eEF1A-1/EF-1α 1:500), CB5 (eEF1A-2/S1 1:100), anti-NF-68 (1: 100), or anti-GFAP (1:1000), overnight at 4 °C. After washing in

PBS, sections were incubated with fluorescein-conjugated (FITC) goat anti-rabbit IgG or rhodamine-conjugated (RITC) sheep anti-mouse IgG secondary antibody. Following incubation, sections were washed, mounted and then visualized by confocal microscopy. Controls were generated using pre-immune sera instead of primary antibodies. Mounted sections were visualized on a laser scanning confocal imaging system (BioRad MRC-600), equipped with a 15 mW krypton/argon lamp. The excitation filter wheel was used in the dual 488 and 568 nm wavelengths excitatory position. Emission wavelengths were detected using the K1 and K2 filters block set, to permit the detection of wavelengths between 522 and 545 nm on the green channel and 585 or greater on the red channel. Both channels were open at 70%, and images scanned on both channels were merged to produce a single profile of co-localization, identified by a yellow color. A 60 ×/1.4 oil immersion objective was used for all examinations (600 × total magnification).

We found that during embryonic development, the expression of eEF1A-1/EF-1α in the brain is restricted to the perikaryon of developing neurons (Fig. 2A). After birth, eEF1A-1/EF-1α is highly expressed in mice at 1 day of age, then its expression slowly declines (Fig. 2B and C). By 14 days of age, only residual expression of eEF1A-1/EF-1α remains visible inside neurons (Fig. 2D); complete disappearance of eEF1A-1/EF-1α protein is observed in the neurons of 20-day-old mice (Fig. 2E). Neurons remain negative for eEF1A-1/EF-1α afterward (Fig. 2F and data not shown). The neuronal marker NF68, the low molecular weight neurofilament, was used in co-localization experiments with eEF1A-1/EF-1α, to confirm neuronal identity (Fig. 2B, D–F). Our results parallel those of Khalyfa et al. [7], showing by Western blot a slow postnatal decrease in eEF1A-1/EF-1α protein abundance in whole brain extract. Identical patterns in the postnatal expression of eEF1A-1/EF-1α protein are observed between heterozygous (+/*wst*) and wild-type (+/+) mouse brains.

As seen in wild-type and heterozygous mice, the developing brain of *wasted* mutant mice (*wst*/*wst*) embryos (E16) expresses eEF1A-1/EF-1α. When co-localization experiments were performed using antibodies against eEF1A-1/EF-1α and NF68, it was found that eEF1A-1/EF-1α protein is located in the perikaryon of neurons (Fig. 3A). During early postnatal development, the expression of eEF1A-1/EF-1α protein decreases in a fashion similar to that observed in wild-type animals. Diminished expression of eEF1A-1/EF-1α protein in P14 *wasted* mutant mice is shown in Fig. 3B; the protein eventually disappears by 20 days of age (data not shown). These results indicate that the developmental disappearance of eEF1A-1/EF-1α protein observed in wild-type and heterozygous mice brain neurons also occurs in *wasted* mutant mice.

Unlike its homologue eEF1A-1/EF-1α, eEF1A-2/S1 protein is absent from mouse brain in early postnatal development (Fig. 4A). Concomitant with the decrease in eEF1A-1/EF-1α protein in neurons, expression of eEF1A-2/S1 becomes detectable in the brain of 14-day-old mice (Fig. 4B), as revealed by neuronal co-localization with NF68. Thereafter, eEF1A-2/S1 protein abundance slowly increases in mouse brain neurons, and becomes readily visible by 20 days after birth (Fig. 4C). At this age, eEF1A-2/S1 replaces eEF1A-1/EF-1α, and becomes the major elongation factor 1A present in neurons. By P26, eEF1A-2/S1 is abundant in brain neurons (Fig. 4D) and, as expected, remains highly expressed in adult mice (Fig. 4E). The expression of eEF1A-2/S1 appears to be limited to neuronal bodies, as co-localization of eEF1A-2/S1 with glial fibrillary acidic protein (GFAP), an astrocyte marker, is not observed in the white matter (Fig. 4F). Thus in brain, eEF1A-2/S1 protein is exclusively expressed in neurons. A similar pattern of expression was observed for eEF1A-1/EF-1α and eEF1A-2/S1 in the cerebellum and spinal cord (data not shown), confirming the presence of eEF1A-2/S1 protein in the central nervous system.

Unlike the pattern seen in wild-type mice, where eEF1A-2/S1 and NF68 proteins co-localize in brain neurons (Fig. 5A), co-immunolocalization of both proteins is never observed in *wasted* mutant mice (Fig. 5B–D). The time points (P14, P20, P26) shown correspond to a period when eEF1A-2/S1 protein is expressed in normal brain neurons. Thus, during development in *wasted* mutant mice, eEF1A-2/S1 protein fails to appear in brain neurons before death. Furthermore, since the CB5 antibody recognizes the carboxy terminal portion of the protein, a part encoded by a region of the gene not affected by the *wasted* deletion, complete absence of eEF1A-2/S1 protein confirms that, in fact, the *wasted* mutation is a null mutation for *eEF1A-2/S1*.

As previously mentioned, a developmental switch between eEF1A-1/EF-1α and eEF1A-2/S1 occurs in brain, heart, and skeletal muscle a few days after birth. Although previous studies have shown that *eEF1A-2/S1* mRNA is present in neuronal cultures and brain during development [11,12], no studies have yet documented the expression of eEF1A-2/S1 protein in the neurons of the central nervous system. In order to determine the localization of eEF1A-2/S1 protein in the CNS, and document the timing of this developmental switch in vivo, we performed immunofluorescent studies of eEF1A-1/EF-1α and eEF1A-2/S1 in brains of wild-type, heterozygous and *wasted* mutant mice, during late embryonic as well as early postnatal development. *Wasted* mutant mice die before 30 days of age, as they fail to express eEF1A-2/S1 protein following the disappearance of its homologue eEF1A-1/EF-1α. This failure results from the deletion of a large part of the promoter region and the first coding exon of the *eEF1A-2/S1* gene [2].

Previous studies have shown that eEF1A-2/S1 is the major elongation factor 1A present in brain, heart, and skeletal muscle. We show that in the brain, eEF1A-1/EF-1α and eEF1A-2/S1 localize predominantly in the soma of neurons, as expected, since the protein synthesis machinery is mostly present in the perikaryon. During embryonic development, only eEF1A-1/EF-1α is found in mouse brain. Using neurofilament (NF68), a specific marker for neurons, we show that eEF1A-1/EF-1α is present in embryonic and early postnatal brain neurons. During postnatal development, eEF1A-1/EF-1α progressively disappears from brain neurons, becoming absent by 20 days after birth. Simultaneously with the decline in eEF1A-1/EF-1α protein, eEF1A-2/S1 protein expression increases. Consequently, eEF1A-2/S1 protein first appears in the brain 7 days after birth, and completely replaces eEF1A-1/EF-1α by 20 days. We show that eEF1A-2/S1 expression is limited to neurons, as it strictly co-localizes with NF68, but not with other brain markers such as the astrocyte-specific protein GFAP. The results presented herein show definitely that eEF1A-2/S1 in vivo is only expressed in long-lived, terminally differentiated cells; similar results were obtained in skeletal muscle and heart (data not shown). The switching event is important during development, to maintain long-lived terminally differentiated neurons, cardiomyocytes, and myofibers in a non-replicating stage, protect them from apoptosis, and possibly also from debilitating effects of microtubule severing and myofiber bundling [3–6,14–16].

Analysis of elongation factor 1A expression in *wasted* mutant mouse brain reveals that the timely appearance of eEF1A-2/S1 does not occur in neurons. This is not surprising, since *wasted* mutant mice carry a lethal deletion in the *eEF1A-2/S1* gene. As the *eEF1A-1/EF-1α* gene is intact in *wasted* mutant mice, the disappearance of eEF1A-1/EF-1α, occurring in the wild-type mice, is also observed in *wasted* mutant mice, and follows the same schedule. This result indicates that the normal genetic events regulating the silencing of *eEF1A-1/EF-1α* gene in neurons are still in place, despite the absence of eEF1A-2/S1 protein. Furthermore, it indicates that eEF1A-2/S1 does not regulate the silencing of *eEF1A-1/EF-1α*, as absence of eEF1A-2/S1 protein does not prevent the down-regulation, eventually leading to the complete absence of eEF1A-1/EF-1α expression in neurons. Complete disappearance of eEF1A-1/EF-1α from *wasted* mutant mouse neurons also reveals that the genetic events leading to

eEF1A-2/S1 activation parallel those that inactivate *eEF1A-1/EF-1α* expression, and are not affected by the *wasted* mutation. Although we describe the expression of elongation factors 1A in mouse brains, phenotypic absence of eEF1A-2/S1 protein is similarly observed in skeletal muscles and heart of *wasted* mutant mice (data not shown).

Disappearance of eEF1A-1/EF-1α from *wasted* mouse neuron, heart, and skeletal muscle 20 days after birth results in complete absence of elongation factor 1A in these tissues. It is thus probable that protein synthesis is drastically impaired in these tissues. Interestingly, the onset of the *wasted* mutant mice phenotype, characterized by tremors and lethargy, occurs simultaneously with complete disappearance of eEF1A-1/EF-1α, and absence of eEF1A-2/S1 proteins, from neurons, heart, and skeletal muscles. Thus, it appears that the absence of protein synthesis from certain tissues is associated with pathological conditions incompatible with life. The genetic events leading to the activation and inactivation of both elongation factor 1A genes, as well as regulatory elements, are important to understand the regulation of both genes during development. Future experiments will test whether introduction of a functional *eEF1A-2/S1* gene (knock-in) can rescue *wasted* mutant mice.

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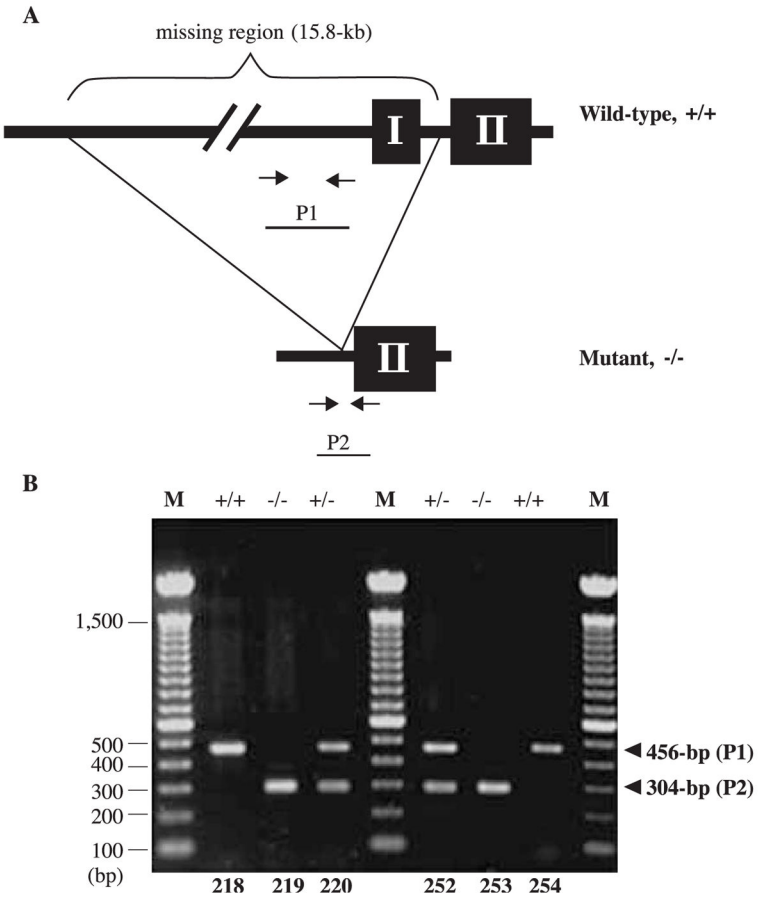

Fig. 1.

Multiplex PCR genotyping of *wasted* mutant mice. (A) Schematic diagram of the *eEF1A-2*/*S1* gene locus of wild-type and *wasted* mutant mice. *Wasted* mutant mice have a deletion of the *eEF1A-2*/*S1* gene in its promoter region as well as the first exon (black box I). The arrows indicate the binding sites of the two sets of primers. (B) Multiplex PCR genotyping of 1-week-old pups from heterozygous (+/*wst*) parent cross-breeds reveals the presence of a single 456 base pair (bp) fragment in wild-type animals (+/+) (mice #218 and 254), two bands at 456 and 304 bp, respectively, for heterozygous animals (+/*wst*) (mice #220 and 252), and a single 304 bp band for *wasted* mutant animals (*wst*/*wst*) (mice #219 and 253).

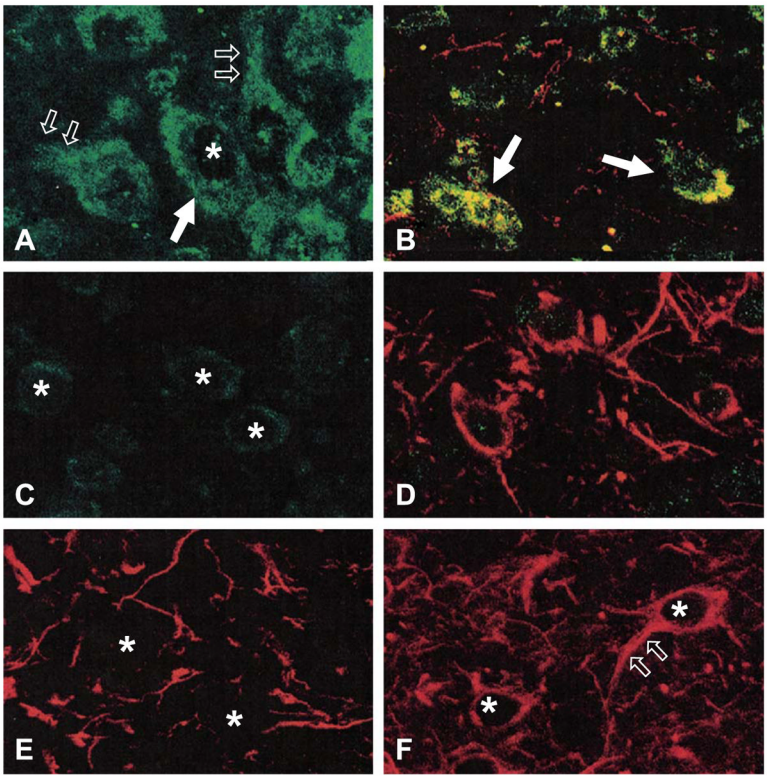

Fig. 2.

Laser confocal micrographs of eEF1A-1/EF-1α in the central nervous system of mice during development. (A) At embryonic day 16, eEF1A-1/EF-1α is widely expressed in the cytoplasm of the neuronal soma (solid arrows), but not in the nucleus (*). (B) In the brain of 1-day-old (P1) mice, co-immunolocalization of eEF1A-1/EF-1α (green) and neurofilament NF68, a neuronal marker (red), reveals that eEF1A-1/EF-1α is present in neurons. Co-localization of both proteins appears as yellow. (C) Seven days after birth (P7), a reduction in eEF1A-1/EF-1α expression is observed in brain neurons. (D) Confocal co-localization reveals that at P14, there is an almost complete disappearance of eEF1A-1/EF-1α (green) in brain neurons. Neuronal phenotype was confirmed by the use of NF68 antibodies (red). (E, F) Co-immunolocalization of eEF1A-1/EF-1α (green) and NF68 (red) at P20 and P26, respectively, fails to detect the presence of eEF1A-1/EF-1α in neurons. Solid arrows indicate neuronal soma, open arrows indicate axons, and asterisks indicate nuclei.

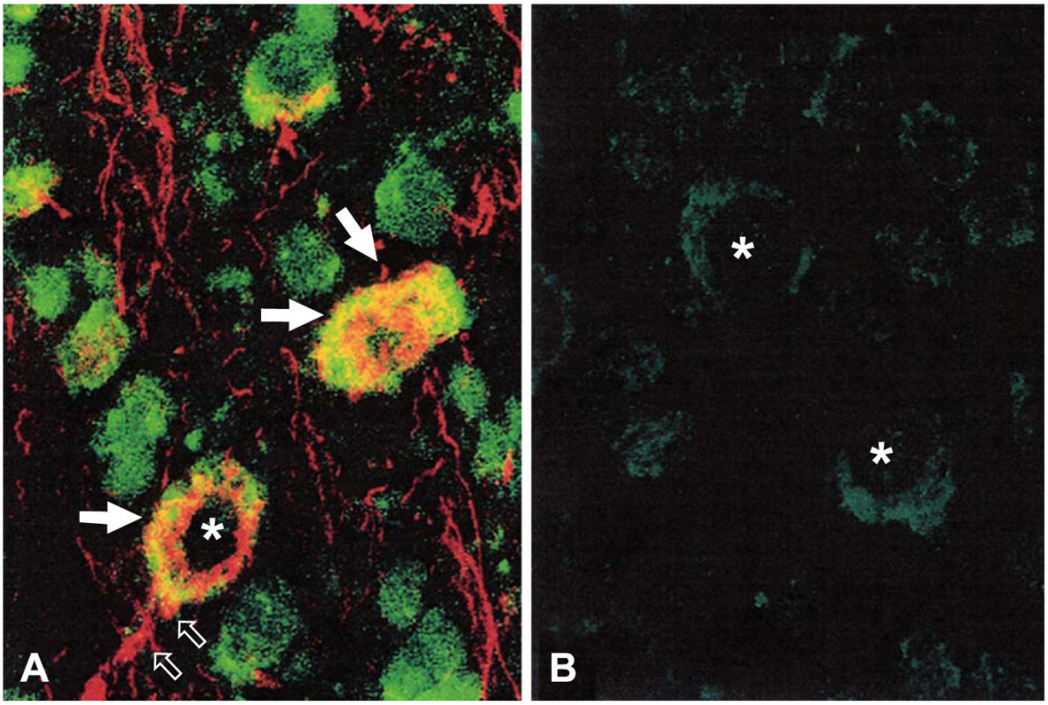

Fig. 3.

Confocal micrographs of eEF1A-1/EF-1α in *wasted* mutant mouse neurons. (A) Brain of 16-day-old *wasted* mutant mice embryos (E16) reveals the presence of eEF1A-1/EF-1α (green) in neurons. NF68 (red) is used as a neuronal marker. Co-localization of eEF1A-1/EF-1α and NF68 results in the yellow color. (B) Expression of eEF1-A1/EF-1α in neurons is greatly diminished in *wasted* mutant mice 14 days after birth. Solid arrows indicate neuronal soma, open arrows indicate axons, and asterisks indicate nuclei.

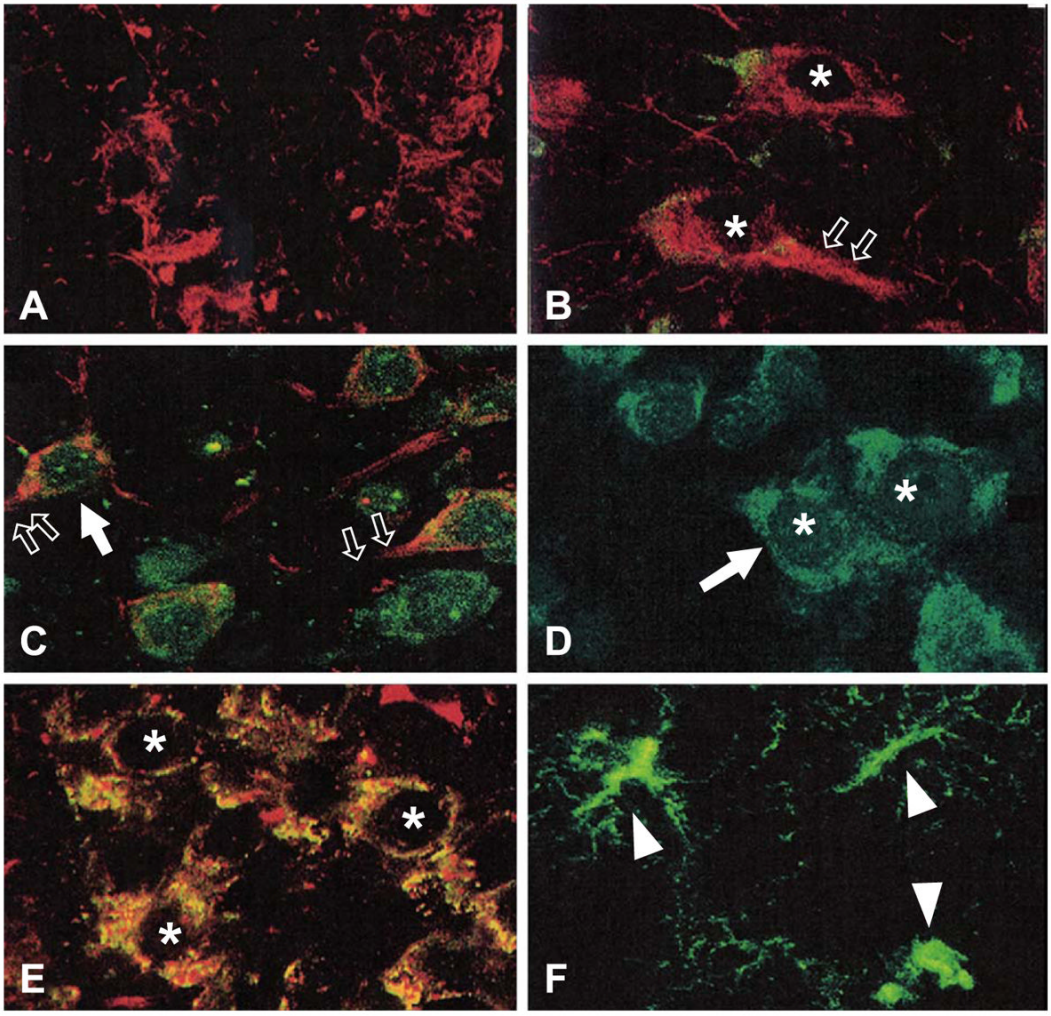

Fig. 4.

Confocal analysis of eEF1A-2/S1 during murine brain development. (A) One day after birth (P1), co-immunolocalization between neurofilament NF68 (red) and eEF1A-2/S1 (green) reveals absence of eEF1A-2/S1 in neurons. (B) Later in development at P14, co-immunolocalization reveals that both eEF1A-2/S1 (green) and NF68 (red) co-localize in neurons. However, eEF1A-2/S1 expression is weak. (C) The same co-localization (yellow) 20 days after birth (P20) reveals an increase in eEF1A-2/S1 abundance in the neuronal soma. (D) At P26, eEF1A-2/S1 (green) is clearly visible in the soma of neurons. (E) Twelve-month-old mouse brain shows intense co-localization (yellow) between EF1A-2/S1 and NF68 (red). (F) Co-immunolocalization of eEF1A-2/S1 (red) and GFAP (green) reveals that eEF1A-2/S1 is not expressed in astrocytes. Solid arrows indicate neuronal soma, open arrows indicate axons, and asterisks indicate nuclei.

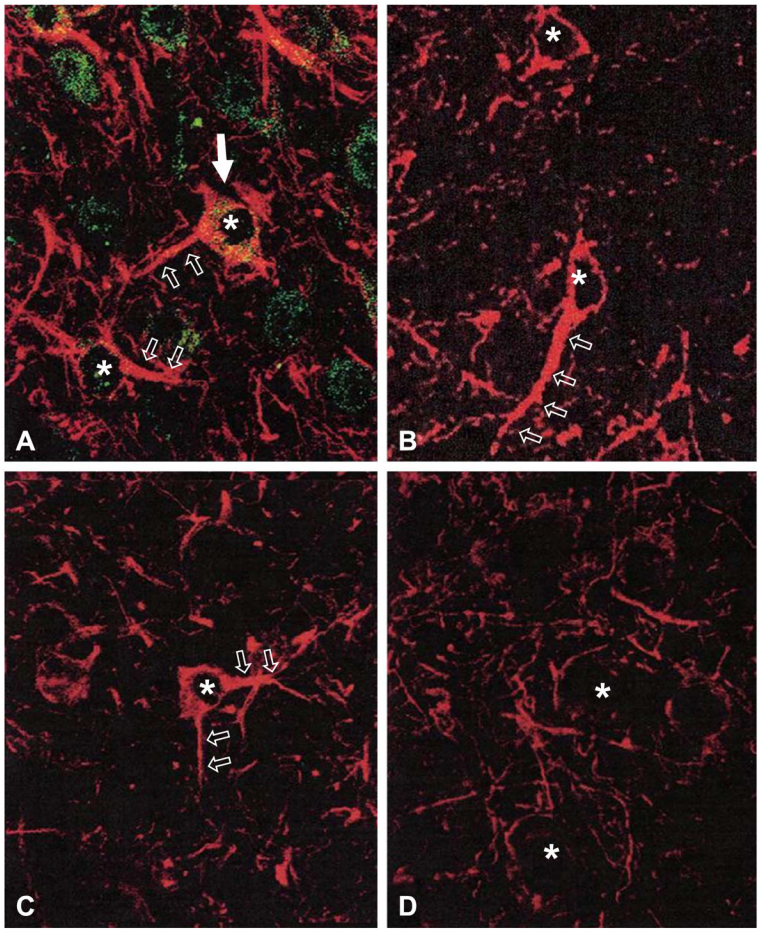

Fig. 5.

Confocal analysis of eEF1A-2/S1 expression in the brain of *wasted* mutant mice. (A) This wild-type phenotype control (+/+ or +/*wst*), at P26 reveals the co-localization (yellow) of eEF1A-2/S1 (green) and NF68 (red) in brain neurons (bold head arrow). (B–D) In these sections from P14, P20 and P26 *wasted* mutant mouse brains, respectively; complete absence of eEF1A-2/S1 protein is noted. Neurons are revealed by NF68 (red), a neuronal marker. Solid arrow indicates the neuronal soma, open arrows indicate axons, and asterisks indicate nuclei.