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Heme Deficiency is Associated with Senescence and Causes Suppression of NMDA Receptor Subunits Expression in Primary Cortical Neurons

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Running title**: Heme depletion and NMDA receptor expression**

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ABBREVIATIONS: ALAS1, 5-aminolevulinate synthase 1; HMOX, heme oxygenase; NMDA, N-methyl-D-aspartate; NMP, N-methyl protoporphyrin IX; SA, succinyl acetone; NF-L, neurofilament light polypeptide.

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ABSTRACT

Heme is a crucial component of many pharmacological and toxicological processes and studies have suggested that heme deficiency may play a role in cellular ageing. A model of ageing neurons was established using prolonged cultures of BALB/c mouse primary cortical neurons. Aged neurons displayed a senescent phenotype and a marked up-regulation of cathepsin-L expression. Down-regulation of the candidate neuron-specific genes for N-methyl-D-aspartate (NMDA) receptor subunits (NMDAζ1 and ε2) and neurofilament light peptide (NF-L) were found to be characteristic of the aging process as reported *in vivo* (Magnusson et al., 2002; Ossowska et al., 2001). In contrast, the genes for the controlling enzymes of heme synthesis and degradation (5-aminolevulinate synthase 1 and heme oxygenase 1 respectively) were up-regulated implicating depletion of a regulatory heme pool. Inhibition of heme synthesis (by 70-80%) at different enzymic steps by succinyl acetone (SA) and N-methylprotoporphyrin IX (NMP) resulted in the earlier lowered expression of NMDAζ1 and ε2 and NF-L. Exogenous hemin added to heme-depleted cells rescued the expression of these neuron-specific genes. Culture of cortical neurons from BALB/c *Fech*^{m1Pas} mutant mice demonstrating depressed heme synthesis showed premature senescence and reduced expression of NMDAζ1 and ε2 receptor subunits and NF-L compared with wild type cells. Our findings suggest that reduced availability of heme in neurons associated with senescence may have significant effects on synaptic function.

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Heme serves as the prosthetic moiety of numerous hemoproteins. As well as acting as the key component of hemoglobin and cytochromes (including those of drug metabolism), heme regulates many processes of pharmacological importance by controlling pathways through interaction with key regulatory proteins (Ogawa et al., 2001; Taoka et al., 2002). Brain is a high oxygen consumer and mitochondrial cytochromes are particularly vital for maintaining normal neural metabolic function. In addition, hemoproteins are important constituents of signalling processes in the brain. Circadian clock and heme biosynthesis are reciprocally regulated with heme acting via core clock mechanism member NPAS2 (Kaasik and Lee, 2004). Both the production of nitric oxide and some of its actions are mediated through hemoproteins, such as guanylyl cyclase (Boehning and Snyder, 2003). Similarly, the signalling function of carbon monoxide as a neurotransmitter/neuromodulator (Boehning and Snyder, 2003; Ingi et al., 1996) is utterly dependent on heme. The only known biosynthetic source of CO in the brain is as a cleavage product of heme catalysed by heme oxygenases (HMOX) (Ingi et al., 1996). Type 1 HMOX can be markedly induced under conditions of stress thereby increasing the requirement for substrate (Sassa, 2004; Sassa and Nagai, 1996). In non-erythroid tissue, including brain, increased requirement for intracellular heme can be detected as up-regulation of the gene *Alas1* for aminolevulinate synthase 1 (ALAS1), the first step of heme synthesis, and commonly accepted as a response to a lowered regulatory heme pool (De Matteis and Ray, 1982; De Matteis et al., 1981; Sassa and Nagai, 1996). The availability of heme may also be a limiting factor in the ability of neuronal cytochrome P450 enzymes to metabolise drugs and chemicals (Meyer et al., 2002). In contrast, larger amounts of exogenous heme, produced in hemorrhage of stroke, may be toxic to neurons (Goldstein et al., 2003).

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Some genetic defects in heme synthesis in humans are associated with diseases with neurological symptoms and the inappropriate accumulation of heme precursors perhaps contributes to development of neuropathy (Lindberg et al., 1999; Rank et al., 1993). Alternatively, neuropathy may be the consequence of heme deficiency causing dysfunction of crucial signalling pathways and functioning of hemoproteins such as cytochrome P450 (Lindberg et al., 1999). In the liver, many drugs and chemicals affect heme synthesis and this may also occur in the brain (De Matteis and Ray, 1982). Furthermore, ALAS1 activity in brain, as with liver, declines with age (Bitar and Shapiro, 1987; Paterniti et al., 1978) and the binding of heme to amyloid β in Alzheimer's disease may be an additional limitation on its availability (Atamna and Frey, 2004). Aging has an implication for every part of the brain but the frontal cortex and parts of the hippocampal system appear especially affected which have been linked to expression of synaptic N-methyl-D-aspartate (NMDA) receptor subunits ζ1 and ε2 that are important for learning and memory (Eckles-Smith et al., 2000; Magnusson et al., 2002; Ossowska et al., 2001; Uylings and de Brabander, 2002). Experimentally, proliferation in cultured astrocytoma and differentiation of neuroblastoma cell lines can be restricted by heme deficiency that may lead to compromised mitochondrial function (Atamna et al., 2002). Differentiation of pheochromocytoma-derived (PC12) cells induced by nerve growth factor is also affected by heme deficiency (Sengupta et al., 2005; Zhu et al., 2002) whereas conversely, neurite outgrowth in neuroblastoma cells is stimulated by exogenous heme (Ishii and Maniatis, 1978).

In this study we established that cultures of primary cortical neurons exhibited increasing senescence and up-regulation of the genes for ALAS1 and HMOX1 suggesting a state of relative heme deficiency and down-regulation of expression of the neuron-specific genes for NMDA receptor subunits ζ1 and ε2. A relationship between expression of NMDA receptor

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subunits and heme synthesis was confirmed with inhibitor and mutant models of heme depletion and restored by exogenous heme.

Materials and Methods

Primary Cell Culture. Primary cortical neurons were prepared from male and female 14 - day-old fetuses of the BALB/c mouse strain bred in house. The BALB/c *Fech*^{m1Pas} mouse strain was obtained from the Jackson Laboratories, Bar Harbor, Maine. The *Fech*^{m1Pas} mutant (abbreviated here to *Fech* mouse) contains a point mutation in the ferrochelatase gene (Davies et al., 2005; Tutois et al., 1991). Mice were bred by homozygous mating and maintained in a negative pressure isolator at 21°C under reduced light to protect from skin lesions. Isolated brain cortex of embryos was gently dissociated to release the neurons which were washed twice in Neurobasal Medium (GIBCO) supplemented with 10% fetal calf serum. Cell suspensions were plated on poly-L-lysine coated 35mm plates at a density of 2 million cells per dish as described previously (Sangerman et al., 2001). After attachment of the cells, the plating medium was changed to culture medium containing 96% (v/v) Neurobasal Medium (GIBCO), 2mM glutamax, 2% B-27 supplement (GIBCO/InVitrogen, Paisely, UK), 100 µg/ml streptomycin and100 U/ml penicillin. Viability of the cells was estimated by the trypan blue exclusion assay and was >80%. After 5 days 10 µM cytosine arabinoside was added to the culture medium for 3 days to stop proliferation of glial cells or fibroblasts. The cells were grown in a humidified incubator at 37 C (95% room air, 5% CO2) for up to 25 days.

Inhibition and Measurement of Heme Synthesis. To inhibit heme synthesis, cells were cultured in serum free medium with 0.5mM succinyl acetone (Sigma-Aldrich, Dorset, UK) or with 1µM N-methylprotoporphyrin IX (Frontier Scientific Porphyrin Products, Logan, UT) continuously for the duration of the experiments. For measurement of heme synthesis, cells

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were incubated with 0.4μCi [3,5-^{3}H] ALA (2.6 Ci/mmol) (Perkin Elmer, Boston, MA) for 24 hours. Heme was extracted from the cells by acetone-HCl and diethyl ether. The amount of radioactivity in extracted heme was measured by scintillation counting as described (Shedlofsky et al., 1987). Total recovery of radioactivity from all fractions was the same for treated and untreated cells. Ferrochelatase activity of cortical tissue was measured as reported for lymphocytes (Rossi et al., 1988). For heme recovery experiments heme as hemin (0.1μM) was added to culture medium in the presence of bovine serum albumin in a 1:1 molar ratio (Taketani et al., 1998).

Necrosis, Apoptosis and Senescence. Cell viability in heme depletion experiments was estimated by using SYTOX/Hoechst double staining method. To determine the amount of necrosis or apoptosis in the neurons, the cultures were stained with a mixture of the membrane-permeable dye Hoechst-33342 (500 ng/ml) and the membrane-impermeable dye SYTOX (500nM) for 5min at 37 °C. The amount of normal, necrotic (damaged/SYTOX-permeable membrane, normal nuclei) and apoptotic (impermeable membrane, condensed/fragmented nuclei) cells were scored with a fluorescence microscope and no significant difference was observed at any time between controls and any of the treatments performed in this study. Identification of senescent cells was performed with Senescent Cells Staining Kit (Sigma-Aldrich, Dorset, UK) in accordance with manufacturer's instructions by detection of ß-galactosidase histochemically at pH 6 (Dimri et al., 1995).

RNA Extraction And Quantitative Real-Time PCR Analysis. Treated and untreated cells at different time point were collected and total RNA was isolated by using TRI-reagent (Sigma-Aldrich). cDNA synthesis was carried out using random primers and Superscript II (Invitrogen). PCR primers were selected using the Primer Express v2.0 Software program (Applied Biosystems, Foster City, CA).

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Primers sequences were as follows:

β2-microglobulin forward primer 5'-CATACGCCTGCAGAGTTAAGCA-3', reverse primer 5'-GATCACATGTCTGCATCCCAGTAG-3'; β-actin forward primer 5'-GATTACTGCTCTGGCTCCTAGCA-3', reverse primer 5'-GTGGACAGTGAGGCCAGGAT-3'; ALAS1 forward primer 5'-TCTTCCGCAAGGCCAGTCT-3', reverse primer 5'-TGGGCTTGAGCAGCCTCTT-3'; NF-L forward primer 5'-CACCAGCGTGGGTAGCATAA-3', reverse primer 5'-GTAAGCAGAACGGCCGAAGA-3'; NMDA ζ1 forward primer 5'-GGGCTGATGACCCGAATGTC-3', reverse 5'-GTGGTACGGTGCGAAGGAA-3'; NMDAε2 subunit forward primer 5'-CTTAATCTGTCCGCCTAGAGCTTT-3', reverse 5'-TGCGCTGGGCTTCATCTT-3'; HMOX1 forward primer 5'-CACTTCGTCAGAGGCCTGCTA-3', reverse 5'-GTCTGGGATGAGCTAGTGCTGAT-3'; HMOX2 forward primer 5'-GGCCTCCTCAAGTCTTTTATTCAG-3', reverse 5'-GGTCCCAGGTGCACTGTGA-3'; cathepsin-L forward primer 5'-GGGTTGTGTGACTCCTGTGAAG- 3', reverse 5'-CGCTAAACGCCCAACAAGAC- 3', γ- aminobutyric acid (GABA) A α 1 5'- GCCTAATAAGCTCCTGCGTATCA-3', reverse 5'- TTCAGCTCTCACGGTCAACCT-3'. Primers were designed to cross exon-exon boundaries and the concentration optimised (300-900nM) to ensure that the efficiency of the target amplification and the efficiency of the endogenous reference amplification are approximately equal. PCR was performed using SYBR Green PCR Master Mix, primers and 100ng of reverse transcribed cDNA in the ABI PRISM 7700 Sequence Detection System, the thermal-cycler protocol was: stage one, 50 °C for 2 min; stage two, 95 °C for 10 min; stage three, 40 cycles at 95 °C for 15 sec and 60 °C for 1 min. Each sample was run in triplicate. The CT (threshold cycle when fluorescence intensity exceeds 10 times the standard deviation

of the baseline fluorescence) value for the target amplicon and endogenous control (β2-microglobulin or β-actin) were determined for each sample. Quantification was performed using the comparative CT method (ΔΔCT). Data are presented as the mean ± S.D. (n = 3–7 for each group). Statistical significance was assessed as $P < 0.05$ using one-way analysis of variance.

Immunoblotting. Proteins were extracted from primary neurons after 14 and 21 days of culture using lysis buffer (7M urea, 50mMTris-HCl, pH7.5; 5mM DTT) followed by brief sonication. SDS electrophoresis and immunoblotting were performed (Davies et al., 2005) using chemiluminescence detection (ECL, Amersham Pharmacia) and primary antibodies from the following sources: HMOX1 AND HMOX2 from Stressgen (Victoria, Canada), NMDAζ1, NF-L and α-tubulin from Santa Cruz (CA, USA). Results were quantified using densitometry and Image Quant 5.2 software. Statistical significance of data was estimated using two-tailed student's t-test.

Results

Senescence of Primary Cortical Neurons. To establish the aging of primary neurons in culture over time we examined the changes associated with senescence. After 20 days in vitro morphological changes of the aged cells were observed, namely an adoption of enlarged shape which is characteristic for the senescent phenotype (Campisi, 2005). Electron microscopy revealed signs of swollen appearance of the cells and reduced density of neurites on day 25 (data not shown). β-Galactosidase activity detected by histochemical staining is a measure of increased residual lysosomal activity at a sub-optimal pH and has been used to identify senescent human cells in culture and *in vivo* (Dimri et al., 1995; Kurz et al., 2000).

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The proportion of the senescent cells steadily increased in neuron cultures (Fig. 1A-1C, Fig. 2A). We also detected a significant increase in cathepsin L expression over time (Fig. 2B). Up-regulation of proteases such as cathepsin L is associated with the development of senescence phenotypes causing a disruption of tissue integrity and function (Varela et al., 2005). The proportion of cells detected showing apoptosis and necrosis was not significantly changed during this time.

Comparison of Heme-Related and Neuron-Specific Gene Expression. The temporal pattern of gene expression during the ageing of cultures of neurons for up to 25 days was established by real time RT PCR (Fig. 3) of selected genes known to be critically associated either with heme metabolism or neuronal function. Greater expression of the gene for ALAS1 (*Alas1*) reflects an increased requirement for heme whereas induction of the *Hmox1* gene associated with the heme catabolism enzyme HMOX1 may reflect potential increased degradation possibly as a stress response (Sassa, 2004). There was initial down-regulation of all the genes in comparison with fresh embryonic cells after 1 or 2 days prior to differentiation of the cultures. However, between days 2 and 6 the expressions began to recover and by day 10 were comparable with freshly isolated cells. However, at later stages of culture (days 21 and day 25) ALAS1 expression was markedly increased. The increase in ALAS 1 expression probably reflected a negative feedback from a lowered regulatory heme pool as cells aged and was compatible with a marked increase in the amount of HMOX1 mRNA (80-fold by day 25). In contrast, expression of HMOX2 showed much lower induction.

Increased expression of ALAS1 and HMOX1 was preceded by decreased expression of the neuron-specific genes for NMDA receptor subunits ζ1 and ε2, and neurofilament light polypeptide (NF-L) that had recovered from plating and were induced to the greatest level on day 14 (Fig. 3D-F) reflecting maximum neurite networking. For instance, an increase in

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NMDAζ1 mRNA was observed from day two and by day 14 reached a significant maximal 22-fold but had declined to only 5-fold higher on day 25. Complementary changes in HMOX1, HMOX2 and NMDA ζ1 protein expression were demonstrated by immunoblotting (Fig. 3G). Thus lower expression of the genes associated with neurons was associated with aging of the cells and inversely related to up-regulation of the genes for ALAS1 and HMOX1. NMDAε2 temporal expression pattern was similar to that of NMDAζ 1, but with less marked changes.

Heme Deficiency Results in Down-Regulation of Neuron-Specific Genes. To test whether heme deficiency *per se* could effect neuronal gene expression, cells were treated continuously with succinyl acetone (SA), a specific inhibitor of ALA dehydratase (Tschudy et al., 1981), or NMP, a specific inhibitor of ferrochelatase(De Matteis and Marks, 1996). Heme synthesis in cultures was significantly depressed by both treatments (Fig. 4A). On day 12 of culture in the presence of SA, ALAS1 expression was significantly higher than in control cells as well as after 18 days of culture (Fig. 4B, C). The expression of HMOX1 was similarly induced while no difference between heme depleted and control cells was observed in the expression of constitutive HMOX2. Increased HMOX1 protein, but not HMOX2, was confirmed by western blotting (Fig 4E). Inhibition of heme synthesis by NMP resulted in similar changes in ALAS1 and HMOX1 expressions (Fig. 4D). In contrast, in both heme-depleted cultures, expression of NMDA receptor subunits ζ1 and ε2 and NF-L were significantly depressed compared with neurons not exposed to SA or NMP (Fig. 5). By day 18 when the control culture was also displaying signs of aging, expression of NF-L was detected at similar levels in treated and untreated neurons. A similar pattern but with more profound changes was detected in NMP-treated cells (Fig. 5D). Thus disruption of heme

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synthesis with SA and NMP resulted in premature changes in expression of genes associated with aging of untreated cells.

Protection by Heme. Neurons treated with SA to depress heme synthesis were co-cultured with heme at a level (0.1μM as hemin) added in albumin thought to balance heme synthesis without inducing heme degradation (Sassa, 2004; Taketani et al., 1998). As previously, SA induced up-regulation of the genes for ALAS1 and HMOX1 and down-regulation of NMDAζ 1 and NMDAε2. In the presence of additional heme this was mostly reversed so that only small changes in expression of NMDAζ 1 and NMDAε2 were observed and ALAS1 and HMOX1 were little affected (Fig. 6 A-D). In order to compare this finding with the effect of heme deficiency on an inhibitory neurotransmitter type receptor we investigated the expression of the GABA A receptor. The most common receptor configuration includes the α1 subunit (Wassef et al., 2003). We did not detect marked changes in the expression of GABA A α1 in SA-treated neurons and additional heme did not alter expression of the gene (Fig 6E).

Premature Senescence of *Fech* Neurons. The *Fech* mutant BALB/c mouse contains a point mutation in the ferrochelatase gene; the resulting enzyme exhibits less than 5% of normal ferrochelatase activity in the liver and spleen. This is associated with insufficiency of erythropoietic heme synthesis, haemolytic anemia, splenic enlargement and marked dysfunction of liver metabolism (Davies et al., 2005; Tutois et al., 1991). Similarly, ferrochelatase activity of brain cortex from *Fech* mice was measured at <3% of that in wild-type BALB/c brain (Fig. 7A). In addition, when heme synthesis in cultured neurons was estimated it was decreased by 47% compared with that of BALB/c primary cells (Fig. 7B). *Fech* neurons were more sensitive to the stress of plating and had less ability to survive long

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term in primary culture than wild type BALB/c neurons. Staining for β-galactosidase activity showed earlier signs of senescence compared to control BALB/c neurons so that by day 12, 89.1 % ± 3.2 (n=5) of the *Fech* cells were identified as senescent (Fig 1D) whereas this degree of senescence was not observed until much later with control BALB/c cells.

Down-regulation of Neuron-Specific Genes in *Fech* Neurons. Heme related gene expression patterns in cultured *Fech* neurons (Fig. 7C, D) showed changes similar to heme-depleted cultures caused by treatment with SA and NMP. Increased expression of ALAS1 was already detected on day 14 not later as observed with BALB/c neurons. Elevated expression of HMOX 1 at the time of preparation of primary culture reflected that the *Fech* embryo cells were already under stress at the time of isolation. Neuron-specific genes were expressed at lower levels than control BALB/c neurons during differentiation of the culture. NMDAζ1 expression in *Fech* neurons was less than 20% of that in a control culture on day 6 and 30% on day 14. NMDA ε2 detected in *Fech* neurons was 57% and 53 % on days 6 and 14 of culture respectively compared to the wild-type cells. Treatment with exogenous heme (0.1μM hemin) largely rescued expression of MNDAζ1 in *Fech* neurons (Fig. 6 F).

DISCUSSION

In these studies we demonstrated that, subsequent to their differentiation, primary cortical neurons in prolonged culture develop characteristics of senescence, using a method which is unique for senescent cells (Dimri et al., 1995). In addition, the expression of genes for synaptic NMDA receptor subunits, which are important for learning and development, and that for NF-L associated with healthy neurite growth and networking, reached a maximum after 14 days but began to decline rapidly by day 21. Expressions of NMDA receptor subunits detected at the later stages of neuron culture were less than a half of that identified on day 14. The decrease in NMDAζ1 and ε2 subunit expressions appeared to be directly

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associated with aging of the cortical neurons after initial differentiation. The decline of expressions of NMDA receptor subunits NMDAζ1 and ε2 (NR1 and NR2A in the rat; NMDA1 and NMDA2 in humans) in an age-dependent manner *in vivo* is well established and may also have effects on agonist and antagonist binding to the intact receptor (Eckles-Smith et al., 2000; Magnusson et al., 2002; Ossowska et al., 2001). Thus the use of primary mouse cortical neurons rather than cell-lines not only provided a more physiologically relevant model, but the possibility to compare gene expression patterns in the aging process. The NMDA receptors are important in neuronal cell signalling by their role in intracellular Ca^{2+} homeostasis and their dysfunction can be linked to loss of neuronal integrity and death (Nicotera, 2003). The maximum expression of the gene for NF-L at day 14 reflected a demand for cytoskeleton protein during intense development of the neurites. Again, subsequent lowered expression of NF-L by day 21 may be linked with degenerative processes in older cortical neurons. In additional studies (Chernova and Smith, unpublished data), gene array analysis demonstrated that other synapse-linked genes may also be down-regulated in older cell cultures whereas at the peak of differentiation many genes were expressed to a similar degree to adult frontal brain cortex. On the other hand, we have found little evidence for marked changes in the expression of inhibitory receptor GABA A subunit α1 over time.

In sharp contrast to genes associated with neuronal function, expression of genes associated with control of heme synthesis and catabolism (those for ALAS1 and HMOX1) were markedly induced in older cultures of neurons on day 21 and longer, by which time the proportion of senescent neurons in cultures was estimated as approximately 86%. *Alas1*, the gene for the rate-controlling enzyme of nonerythroid heme synthesis, is usually considered to be up-regulated in liver by repression of a negative feedback mechanism as a consequence of a depleted regulatory heme pool (Sassa and Nagai, 1996). Our findings agree with *in vivo*

studies that this mechanism probably also operates in the brain (De Matteis and Ray, 1982, De Matteis et al., 1981). Up-regulation of *Alas1* in neurons may be evidence of limitation of heme supply with aging of the cells. This was probably compounded by the simultaneous greater expression of the heme degrading enzyme HMOX1 that is often associated with conditions of cellular stress and may be an attempt to generate the antioxidant bilirubin as well as the signalling molecule CO (Sassa, 2004). However, this mechanism must depend on the synthesis of considerable amounts of extra heme as a substrate in addition to that required for normal cellular functions since there is probably little free heme available for this purpose in cells (Sassa, 2004). Elevated activity of heme oxygenases may exacerbate a problem, already present, of inadequate heme supply in aging cells.

Although the changes in older culture of primary neurons were consistent with heme depletion partly associated with induction of HMOX1, and also a decline in expression of NMDA receptor subunit genes, it was not clear whether there was any link between these systems. To address this point differentiating cultures of neurons displaying maximum expression of NMDA subunits were treated with SA or NMP to inhibit different steps in heme synthesis thereby potentially producing heme-depleted cells (De Matteis et al., 1980; Sassa and Nagai, 1996; Tschudy et al., 1981). Treated cells did indeed show significantly depressed heme synthesis with patterns of *Alas1* and *Hmox1* up-regulation observed in much older cultures of untreated cells. In contrast, there was decreased expression of NMDA receptor subunits and NF-L especially with NMP. This could be considered as premature aging of the primary neurons and seemed to indicate that there could be a direct relationship between heme supply and the expression of these neuron-specific genes. We were unable to exclude from this the possibility that SA or NMP might themselves affect gene expression or some other process that might influence that of the subunits of NMDA receptor. However, when

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heme was added back at physiologically relevant levels to SA treated cells expression of the *Alas1* and *Hmox1* genes were decreased and expression of those for NMDA receptor subunits was mostly restored.

The use of primary cortical neurons from *Fech* mice obviated the need for chemical intervention to cause a depleted heme supply (Tutois et al., 1991). This allowed investigation of whether up-regulation of HMOX1 in a stressed environment was the primary event with subsequent up-regulation of *Alas1*, or that heme depletion *per se* occurred first and was compounded by induction of HMOX1 activity as cells aged. Not only was *Alas1* up-regulated to a greater degree in *Fech* primary culture than wild-type BALB/c cells at any time point as a result of defective heme synthesis but neurons displayed signs of premature senescence. Modest induction of HMOX1 expression in *Fech* embryonic cells was possibly associated with providing defence against oxidative stress but at the same time enhancing the potential for heme depletion. Importantly, after an initial increased expression over that in BALB/c cells, NMDA receptor genes, especially for NMDAζ1, were expressed significantly less and suggest that heme itself could affect expression of these neuron-specific genes. An interesting finding of different effect of heme deficiency on GABA neurotransmitter receptor subunit and glutamate receptor NMDA subunit may lead to better understanding why heme distorted metabolism results in certain clinical manifestations in patients. One of the possible explanation of this distinctive difference in effects for NMDA and GABA receptors could be related to regulation by different signalling pathways (Kumar et al., 2005; Zhu Y, 2002).

What could be the mechanism of the senescence of cortical neurons being affected by heme supply? Studies of human brain cell lines have shown that inhibitors of heme synthesis can activate NO synthesis, alter zinc and iron metabolism and cells fail to differentiate or

undergo a successful cell cycle (Atamna et al., 2002). This might partly be due to a decrease in mitochondrial function especially complex IV, as consequence of specific disruption in the synthesis of heme *a*. However, hepatic mitochondrial respiratory chain activities, including complex IV (cytochrome oxidase), remained unchanged or were increased in *Fech* mice (Navarro et al., 2005). Observations of neurite outgrowth has indicated that heme may act by regulation of kinases concerned with structural proteins and receptors (Ishii and Maniatis, 1978). It is interesting that inhibition of heme synthesis interferes neuron growth factor-induced outgrowth of PC12 cells by diminishing a subset of neuron specific genes expressed via the Ras-mitogen activated protein kinase signalling pathway including NF-L (Sengupta et al., 2005; Zhu et al., 2002).

In summary, long-term primary cultures of mouse cortical neurons displayed senescence and decreased expression of NMDA receptor subunits and characteristics of heme deficiency as has been observed *in vivo* for aging. Down-regulation of NMDA receptor subunits expression was potentiated by both inhibitor and mutant models of heme depletion, strongly suggesting a mechanistic link between these metabolic processes.

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Figure legends

Figure 1. Detection of senescence-associated β-galactosidase activity in primary culture of BALB/c cortical neurons (A) on day 7 (7%), (B) on day 16 (57%), (C) on day 22 (86%) and (D) in cortical neurons isolated from *Fech* mouse on day 12 (>89%). These estimates and those illustrated were deemed representative of repeated cultures from different preparations of neurons.

Figure 2. Changes in senescence-associated β-galactosidase activity during whole culture period (A). Rise in cathepsin L expression with the age of culture relative to β-actin expression detected by real time PCR (B).

Figure 3. Changes in gene expression in prolonged culture of primary cortical neurons. Heme-related (A-C) and neuron-specific (D-F) gene expression relative to β-actin expression estimated by real time RT PCR. (G) Detection of HMOX1, HMOX2, NMDAζ1 receptor subunit and NF-L proteins by immunoblotting in representative cultures of cortical neurons on day 21 compared with day 14. Equal amounts of protein were loaded on to the gels. All were significantly different.

Figure 4. Effects of inhibitors on heme synthesis and gene expression in primary cortical neurons. Neurons were cultured in the presence of 0.5mM succinyl acetone (SA) or 1μM N-methylprotoporphyrin IX (NMP) for 12 or 18 days. (A) Treated and control cells were incubated with ^{3}H-ALA for 24 hours on day 12 and then labelled heme was extracted and measured (n = 5). (B and C) Expression of ALAS1, HMOX1 and HMOX2 in succinyl

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acetone-treated and control cultures on day 12 and day 18, estimated by real time RT PCR. (D) ALAS1, HMOX1 and HMOX2 expressions in NMP-treated primary cortical neurons and control cells on day 12. * Statistically different from control group $p < 0.05$. (E) Detection of HMOX1 and HMOX2 proteins in representative cultures of succinyl acetone-treated and control neurons on day 12.

Figure 5. Effects of inhibition of heme synthesis on NMDAζ1 and NMDAε2 receptor subunits and neurofilament light peptide (NF-L) expression in cortical neurons. (A-C) Effects of succinyl acetone (SA) at day 12 and at day 18 estimated by real time RT PCR. (D) Gene expression in N-methylprotoporphyrin IX (NMP) treated neurons and control cells at day 12 (D). 1, NMDAζ1; 2, NMDAε2; 3, NF-L. * Statistically different from untreated control group $p < 0.05$.

Figure 6. Rescue of gene expression in succinyl acetone (SA) -treated neurons by heme. Heme added back for 24 hr to neurons after treatment with SA for 12 days eliminated up-regulation of ALAS1 (A) and HMOX1 (B). Recovery effects of added heme on NMDA receptor subunit expression in BALB/c primary neurons (C, D) and in *Fech* primary neurons (F) estimated by real time RT PCR. Expression of GABA $_A\alpha 1$ subunit in primary neurons treated with SA and heme (E). * Statistically different from untreated control group $p < 0.05$.

Figure 7. Heme synthesis and gene expression in primary cortical neurons from *Fech* mice compared with time-matched BALB/c cells. (A) Reduced ferrochelatase activity in brain cortex from adult mice. (B) Heme synthesis in cortical neurons from *Fech* mice compared

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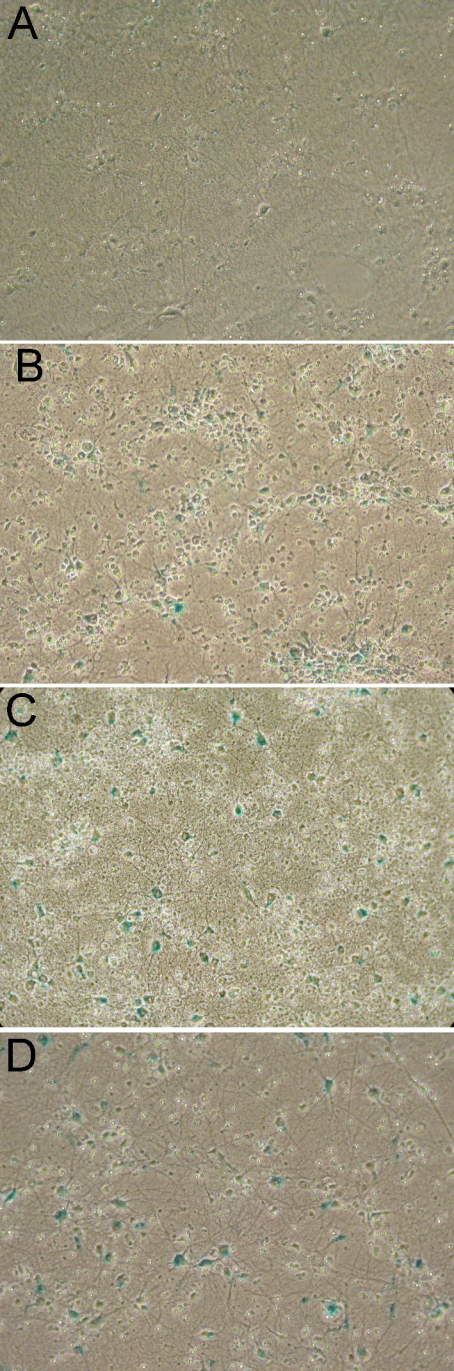
with BALB/c mice after incubation with ^{3}H-ALA for 24 hr on day 12 of culture as in Figure 3 (n = 3). Heme related (C-E) and neuron-specific (F-H) gene expression in BALB/c and *Fech* neurons estimated by real time RT PCR on days 6 and 14 of culture. * Statistically different from control group $p < 0.05$.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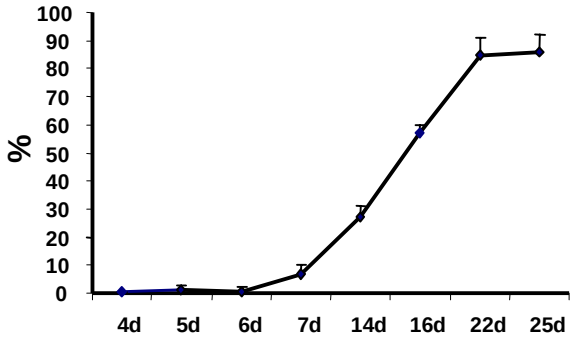

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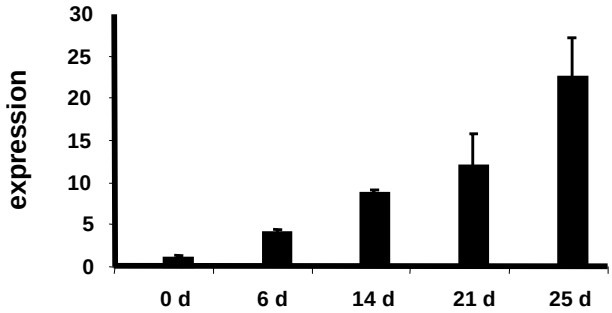

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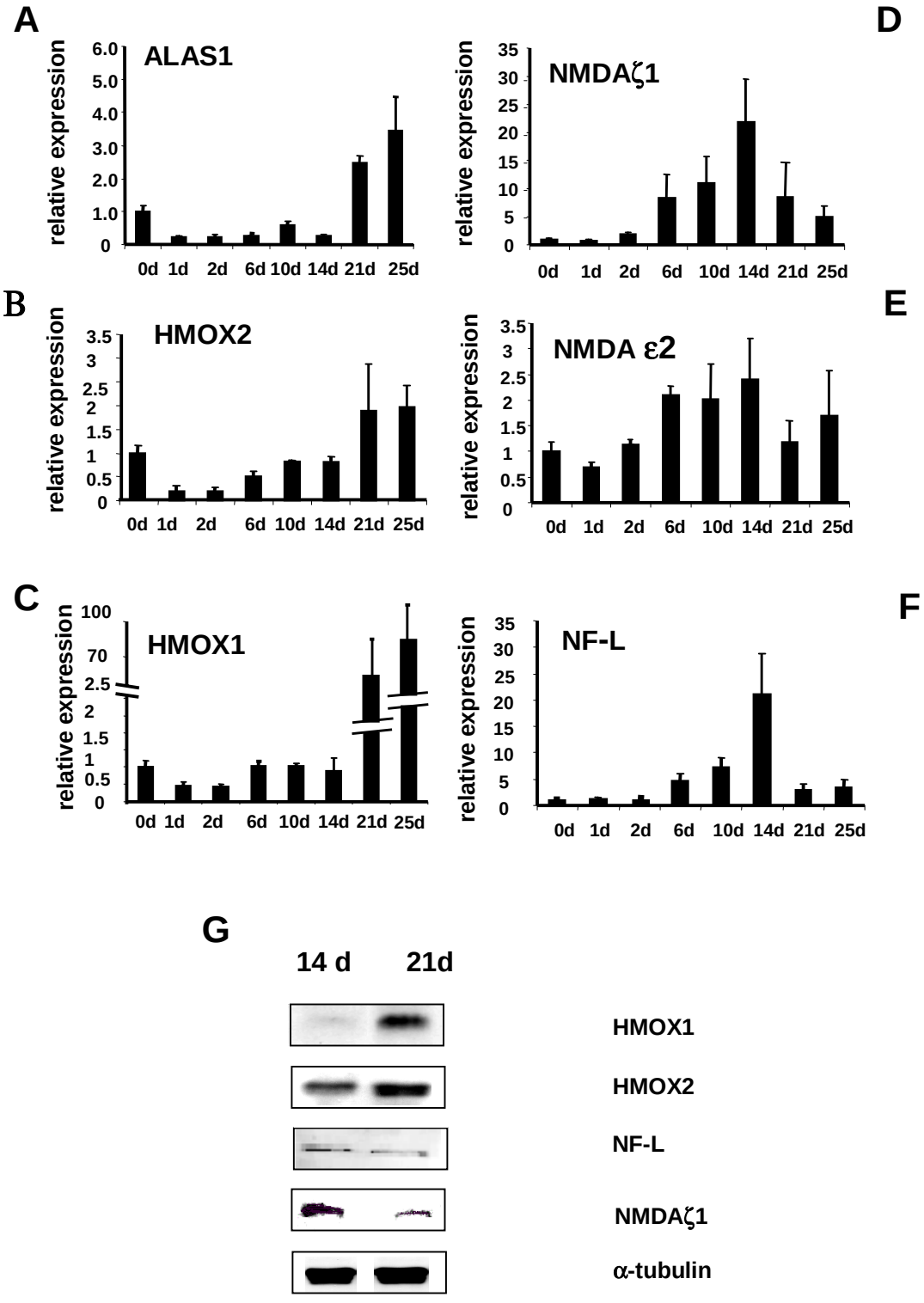

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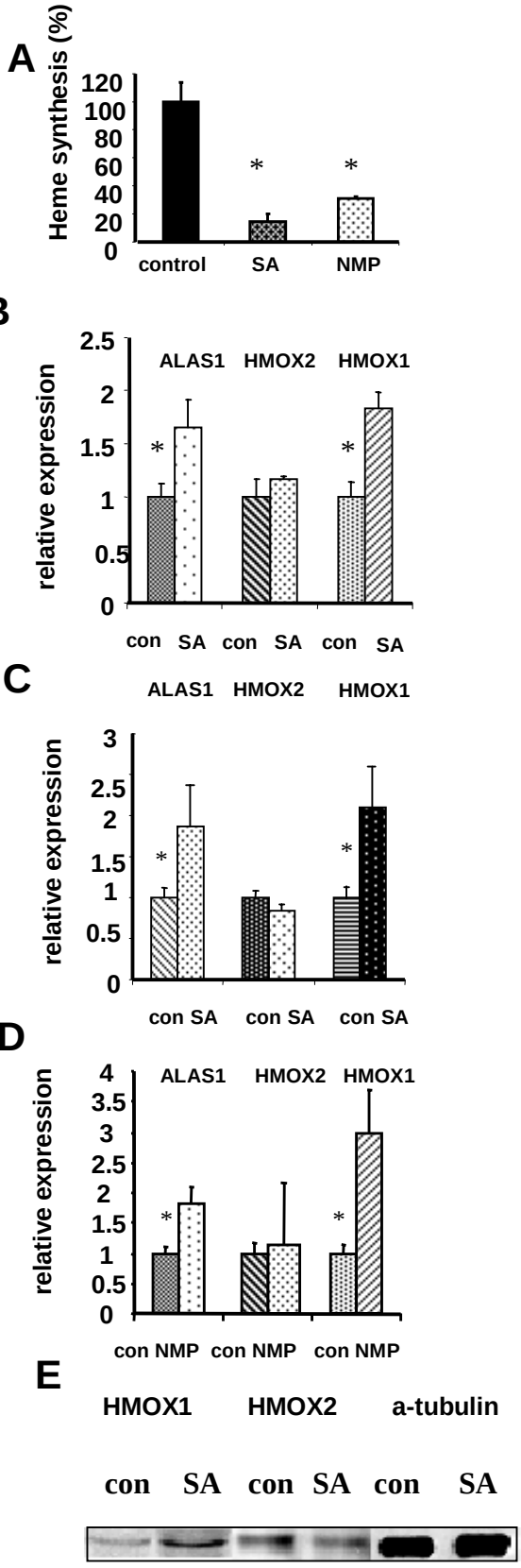

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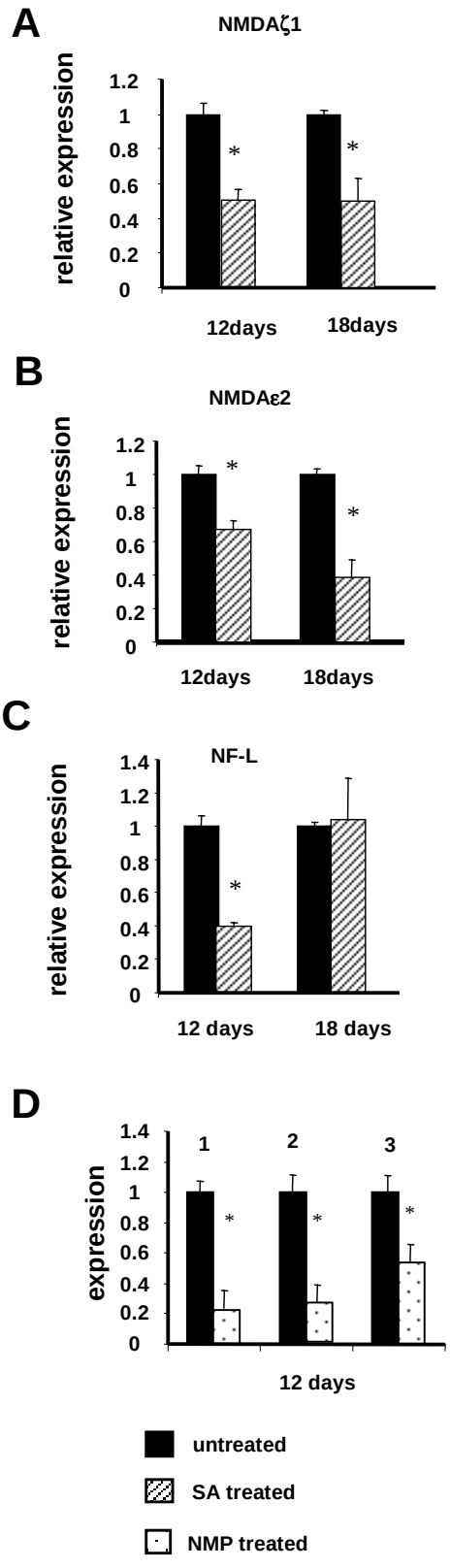

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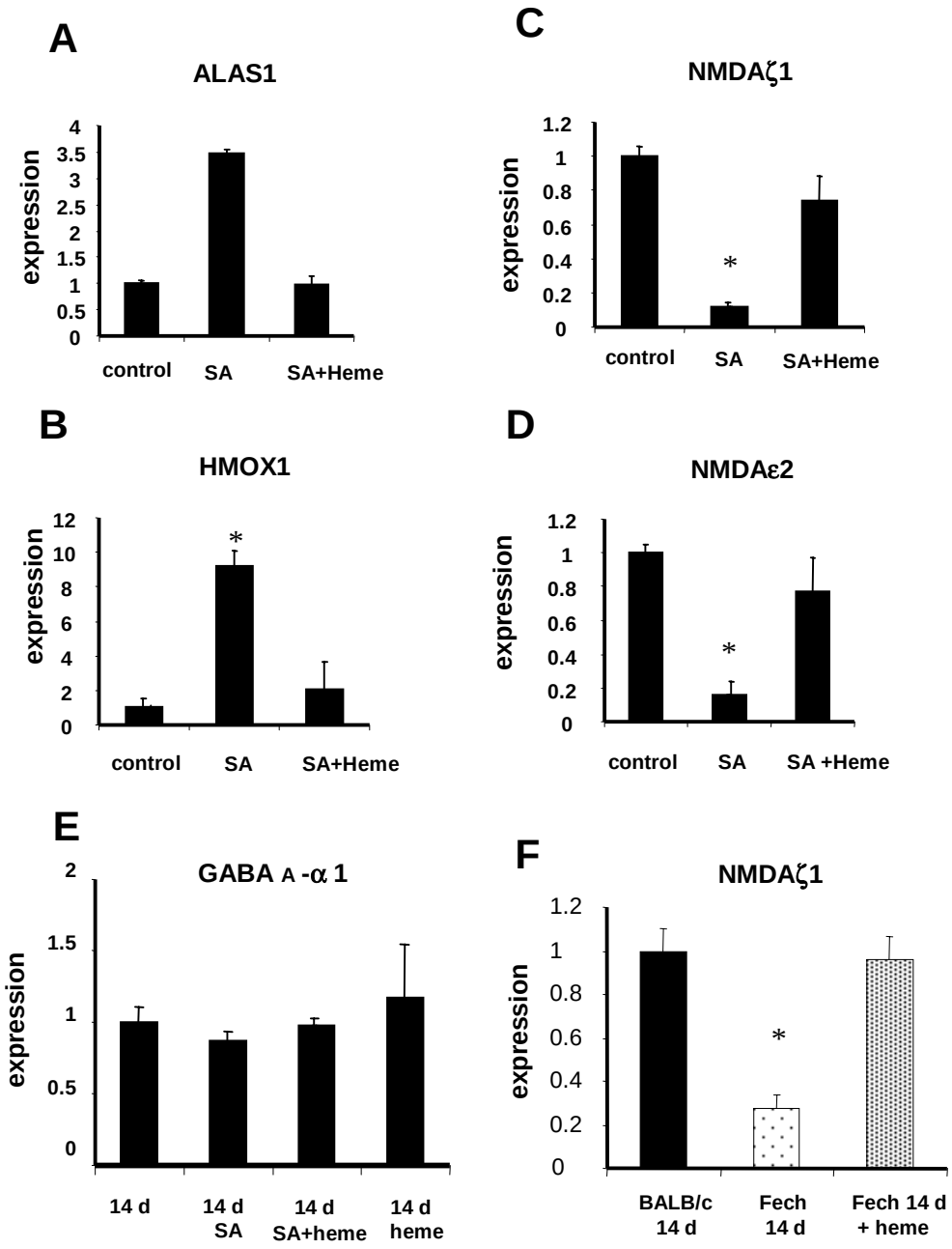

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