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J. Cell. Mol. Med. Vol 9, No 2, 2005 pp. 398-406

Age-dependent changes in Fibroblast Growth Factor 2 (FGF-2) expression in mouse cerebellar neurons

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Received: February 17, 2005; Accepted: March 16, 2005

Abstract

Fibroblast growth factor 2 (FGF-2) is a neurotrophic factor that regulates many neuronal functions and survival. We have characterised FGF-2 expression immunohistochemically in the cerebellum of young (4 months) and old (22 months) mice. About half of the population of the granule cells (GC), and all Purkinje cells (PC) expressed FGF-2 in all folia of the cerebellum at both ages. FGF-2 showed differential intracellular localization: predominantly localised to the nuclei of GC and present mainly in the cytosol of PC. There was a statistically significant ($P = 0.0028$) reduction in the number of FGF-2-positive GC in the cerebella of old (41.3±0.91%) compared to young (48.5±1.67%) mice, whereas no statistically significant age-dependent difference occurred in the number of FGF-2 positive PC. These results indicate a possible role of FGF-2 in cerebellar ageing.

Keywords: FGF-2 • growth factors • cerebellar granule neurons • Purkinje neurons • Bergmann glia • ageing

Introduction

With advancing age, there is a progressive reduction in cognitive function, learning and fine motor control. Instead of decreased neuronal numbers, normal brain ageing is associated with a number of subtler functional, metabolic and morphological changes [1, 2]. In the cerebellum, the cellular loss with age is minimal, in both humans [3] and in rat models [4, 5]. Instead, ageing is associated with a reduction in Purkinje cell (PC) volume [3, 5], a decrease in the number of synapses in the molecular layer (ML) (predominantly between the parallel fibers and PC dendrites) [6] and a significant shortening of the length of parallel fibers [7], resulting in a retracted connectivity. Neurogenesis is also reduced with age [8]. But can be restored in the ageing brain through various methods [9], including the administration of growth factors, such as FGF-2 [10]. These data indicate that there is no intrinsic age-dependent limitation of neurogenesis and point to the possibility that during ageing the availability of growth factors is decreased.

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Reprinted from:
Journal of Cellular and Molecular Medicine

Published by:
the CMM Foundation

Fibroblast growth factor (FGF-2) belongs to a large family of heparin-binding growth factors comprising at least 19 structurally related members [11]. Alternative splicing generates a range of FGF-2 isoforms. The 18 kD isoform is predominantly cytoplasmic and is the only FGF isoform exported from cells and stored in the extracellular matrix [12, 13]. The higher molecular weight isoforms (22, 22.5, and 24 kD in humans; [14]) localize to nuclear and ribosomal fractions [12, 15]. The low affinity heparan-sulphate (HS) proteoglycan receptor dimerizes on binding FGF-2, and interacts with the high-affinity transmembrane signalling receptor (FGFR). There are different splice variants of FGFR (FGFR1-FGFR4; [14, 16, 17]) that have intrinsic tyrosine kinase activity.

Initially identified as a mitogen with prominent angiogenic properties, FGF-2 is now recognised as a multifunctional growth factor with actions on neurons and glia. For example, in the brain FGF-2 is localized to both astrocytes and discrete neuronal populations [18], where it promotes neuronal survival, neuritogenesis, neurite branching, and synaptic transmission [19]. FGF-2 is not an essential survival factor for neurons, since FGF-2 knock-out mice are viable and fertile, and phenotypically identical to their FGF-2-expressing littermates [20], nonetheless, the trophic effects of FGF-2 on neurons justifies closer inspection for a possible involvement in neuronal ageing and, by use immunohistochemical techniques, we have tested the hypothesis that age-related changes in the cerebellum are correlated with reduced levels of FGF-2.

Experimental procedures

Tissue preparation

We used 3 of 4 months-old, and 3 of 22 months-old male CB57Bl/6 mice. Animals were killed with a halothane overdose, under local ethical and UK Home Office regulations. After intracardiac perfusion with 4% paraformaldehyde (PFA) (BDH, Poole, Dorset) for 5min, the cerebellum was dissected, divided by a mid-sagittal vermal incision, and placed in 4% PFA overnight at 4°C. Following washing with 0.01M phosphate buffered saline (PBS) (Sigma, Poole, Dorset) at pH 7.0, the tissue was dehydrated in increasing concentrations of ethanol (50% -100%, infiltrated with polyester wax at 40°C (99:1 of polyethyleneglycol distearate (Aldrich, Gillingham, Dorset): cetostearyl alcohol (BDH), to reduce antigen denaturation) and stored at 4°C. Para-sagittal sections (7μm thick) of the right and left cerebellar vermis were cut at 5°C using a cryostat (Bright Instruments Co., Huntingdon, Cambridgeshire), floated on a 1:100 dilution of Stay-on (Surgipath, St. Neots, Cambridgeshire) in milli-Q water, mounted in sequence on pre-cleaned glass microscope slides (Surgipath), air dried overnight at room temperature or at 30°C for 1h, and stored at 4°C until required.

Antibodies

Ab937 (from A. Baird, University of Birmingham) is a polyclonal rabbit antibody raised against a synthetic peptide of bovine FGF-2 [18], and was used at a concentration of 1:500. Neuronal nuclei were identified using a mouse monoclonal anti-neuron-specific nuclear protein (Neu-N) (MAB377, Chemicon, Harrow, Essex), and neuronal cytoplasm with either mouse monoclonal anti-βIII-tubulin (G7121, Promega, Southampton, Hampshire), or a mouse monoclonal anti-200kD neurofilament protein (RT-97) (MCA 1321, Serotec, Oxford, Oxfordshire). A polyclonal anti-GFAP (Z0334, DAKO Ltd. Ely, Cambridgeshire) was used to identify astrocytes and polyclonal anti-rabbit carbonic anhydrase II (CA II) to identify oligodendrocytes. All phenotypic cellular marking antibodies (Neu-N, βIII-tubulin, RT97, CA II and GFAP) were diluted to 1:500 in the appropriate medium.

Immunostaining for single antibody labelling

Mounted sections were dewaxed in 100% ethanol, rehydrated in graded solutions of ethanol and washed in 0.01M PBS. To improve antibody binding to FGF-2, sections were immersed in a 0.5 mg/ml solution of hyaluronidase (Sigma) in 0.1M sodium acetate solution and then in 0.3% hydrogen peroxide solution to inhibit endogenous peroxidases. They were then washed in PBS containing 0.3% Triton-X detergent (PBST) (Sigma) and blocked in 15μl/ml normal goat serum (Vector Laboratories, Peterborough, Lincolnshire). After overnight incubation with the primary antibody, sections were washed in PBST before adding secondary antibody, incubated for 1h at 4°C with goat polyclonal anti-rabbit biotinylated secondary antibody (Vector Laboratories) at a dilution of 1:200 (in blocking solution), and stained with the

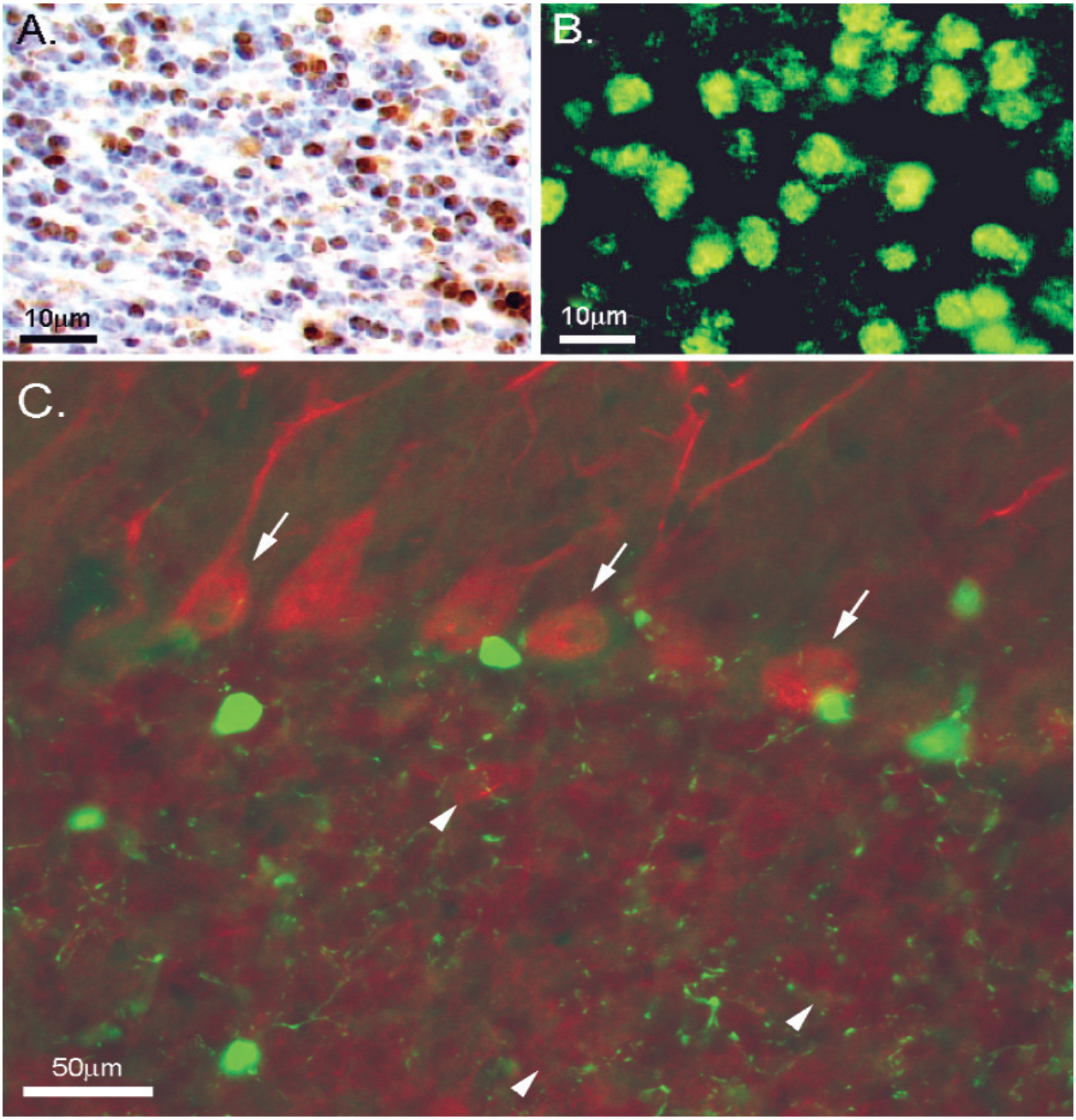

Fig. 1 FGF-2 expression in the GCL of the 4m-old mouse cerebellum. A. DAB/FGF-2-positive in the Mayer's Haemalum counter-stained GCL of the 4m-old mouse; note that FGF-2 is expressed in over 50% of GC, expression is of variable intensity and mostly nuclear. B. FITC image of FGF-2-positive GC showing nuclear and cytoplasmic expression similar to that seen in Fig. 1A. C. CAII-positive oligodendocytes (green) and FGF-2-positive PC (red) that project dendrites into the molecular layer; note that PC (arrows) express higher levels of FGF-2 than GC (arrowheads).

ABC complex solution (Vector Laboratories anti-rabbit ABC peroxidase kit) and counter-stained in Mayer's Haemalum (BDH, Poole, Dorset). All control sections failed to stain after incubation with secondary antibody in PBST with the primary antibody omitted. Although not illustrated, preabsorbed controls have been performed with this antibody to verify specificity of the antibody binding, as have Western blots with extracted mouse brain tissue to show detection by this antibody of all three isoforms of FGF-2.

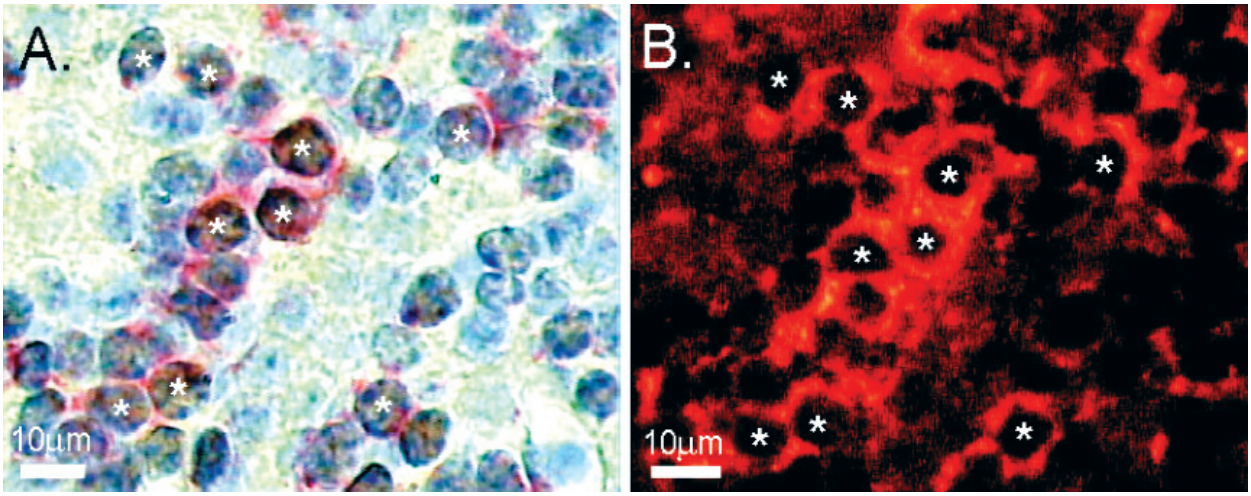

Fig. 2 FGF-2 and βIII-tubulin expression in the GCL of the 4m-old mouse cerebellum. A. GC double stained with FGF-2 (DAB brown precipitate) and βIII-tubulin (alkaline phosphatase Vector red). B. Fluorescence image of the same section shown in A, in which enhanced Vector red fluorescence of βIII-tubulin is seen exclusively. There is a strong cytosolic distribution of βIII tubulin (red) in GC and probably also in mossy fibre terminals and GC dendrites within GCL glomeruli. The GC nuclear FGF-2 DAB signal does not fluoresce. Asterisks mark identical GC in A and B.

Double labelling

All marker antibodies were mouse monoclonal with the exception of polyclonal anti-rabbit carbonic anhydrase II (CA II). Secondary anti-mouse antibody binds non-specifically to endogenous immunoglobulin (Ig) in the mouse tissue giving poor specificity. Accordingly, the M.O.M™ (Mouse on Mouse) kit (Vector Laboratories) was used to block endogenous mouse Ig. After FGF-2 staining, sections were washed in PBS and blocked with the Avidin-Biotin Blocking System (DAKO) and then incubated with either anti-Neu N, anti-βIII-tubulin, or anti-RT97 primary antibodies (1:500 in the diluent). Unbound antibody was removed by washing in PBS and sections were incubated in biotinylated anti-mouse Ig (1:250 in M.O.M™ diluent) and DAB stained as described above. For Vector red staining in DAB stained sections, an ABC-alkaline-phosphatase (AP) Vector Red kit (Vector Laboratories) was used and the sections were counter-stained with Mayer's Haemalum (BDH). For immunofluorescence, a secondary biotinylated anti-rabbit antibody was added to sections at a concentration of 1:100 in normal blocking solution for 1h. Sections were then incubated in Streptavidin-AlexaFluor 488 (Strept-AF) (Molecular Probes, Eugene, Oregon) for 1h at room temperature (diluted 1:100 in PBS). For double immunofluorescence, FGF-2 was visualised with fluorescein-isothiocyanate (FITC)-labelled secondary antibody, and GFAP/CA II/ βIII-tubulin/RT97 were developed using Texas red (Molecular Probes, Eugene, Oregon)-labelled secondary antibodies. All sections were visualised using a Zeiss Axioscope II Microscope (Zeiss, Welwyn Garden City, Hertfordshire).

Semi-quantitative methods

In cerebellar folia of 3 young and 3 old mice, the number of FGF-2-immunopositive PC/GC was determined in 7µm thick para-sagittal vermal sections taken through the cerebellum. Absolute numbers of PC were counted, and an estimate of GC was obtained using a 28µm x 21µm sampling grid and the percentage of FGF-2-positive cells calculated. Counts were analysed by univariate scattergram and Student's t-test.

Results

Distribution of FGF-2 in the cerebellum

Ab 937 was chosen to detect FGF-2 as it was optimal for detecting neuronal FGF-2, the focus of this study.

GC Layer (CGL): FGF-2 was localised to 40-50% GC nuclei (Fig. 1; Fig. 2). The density of FGF-2 nuclear immuno-staining varied from intense to relatively weak (Fig. 1A), suggesting the presence of variable amounts of FGF-2 in individual GC. There was cytoplasmic expression of FGF-2 in GC, and in glomeruli (Fig. 1 C; Fig. 2A, B), where mossy fibre terminals and GC dendrites were FGF-2-positive. FGF-2 was not detectable in GLC astrocytes/Bergmann glia using Ab 937 (Fig. 3A; Fig. 4B). The pattern of GCL FGF-2 staining was uniform throughout all folia.

PC Layer (PCL): Strong FGF-2 expression was confined to perinuclear and dendritic cytoplasm of all PC (Fig. 1C; Fig. 3A, B; Fig. 4A, B; Fig. 5B). FGF-2 was not detected in PC/basket cell axon arborisations (Fig. 3B), oligodendrocytes, and astrocytes/Bergmann glia and their processes in the PCL.

Molecular layer (ML): FGF-2-positive PC dendrite trees filled the ML (Fig. 1C; Fig. 3A, B; Fig. 4A, B). Climbing fibres (Fig. 4A) and Bergmann glia radial processes (Fig. 4B), parallel fibres, and both stellate and basket cells were unstained (Fig. 1C; Fig. 3A, B; Fig. 4A, B).

White matter (WM): The cerebellar WM was largely FGF-2 negative. In particular, FGF-2 was not detectable in axons (Fig. 5A) nor in oligodendrocytes (Fig. 5B) or astrocytes (not shown).

Age-related changes in expression of FGF-2

Semi-quantitative analysis of FGF-2 expression in the GCL and PCL: Analysis by univariate scattergram of the 18 specimens used in this study detected 2 distinct sample clusters. When samples were segregated by age (young versus old) (Fig. 6), 48.5±1.7% and 41.3±0.9% of GC were of FGF-2 positive in young and old mice, respectively. The 7.2% difference in the mean proportion of FGF-2 positive cells between the young and old animals was statistically significant ($P = 0.0028$). There was a smaller proportion of FGF-2 positive PC (44.4±15.8%) in the young animals compared with the old animals (52.0±6.2%), but the difference was not statistically significant ($P=0.473$).

Discussion

FGF-2 distribution in the mouse cerebellum

This is one of the first quantitative studies of FGF-2 in neurons of the mouse cerebellum. The variable expression of FGF-2 observed in neurons of the mouse cerebellum is consistent with that reported for rat cerebellum in earlier studies [18, 21]. Whereas, in the rat, 60% of GC and PC were

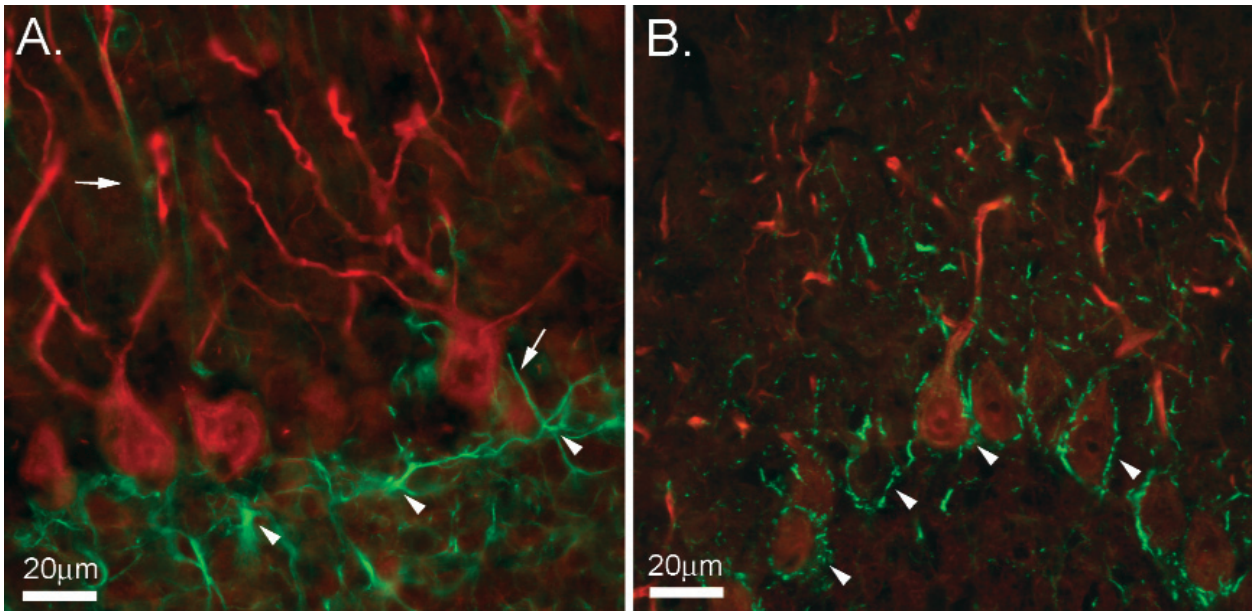

Fig. 3 FGF-2 localization in the PCL of the 4m-old mouse cerebellum. A & B. All PC strongly express FGF-2 (red), but GFAP-positive Bergmann glia somata (arrowsheads in A) (green) and processes (arrows in A) (green), and RT-97-positive basket cell axons (arrowheads in B) (green) do not express FGF-2 (red) in the PCL.

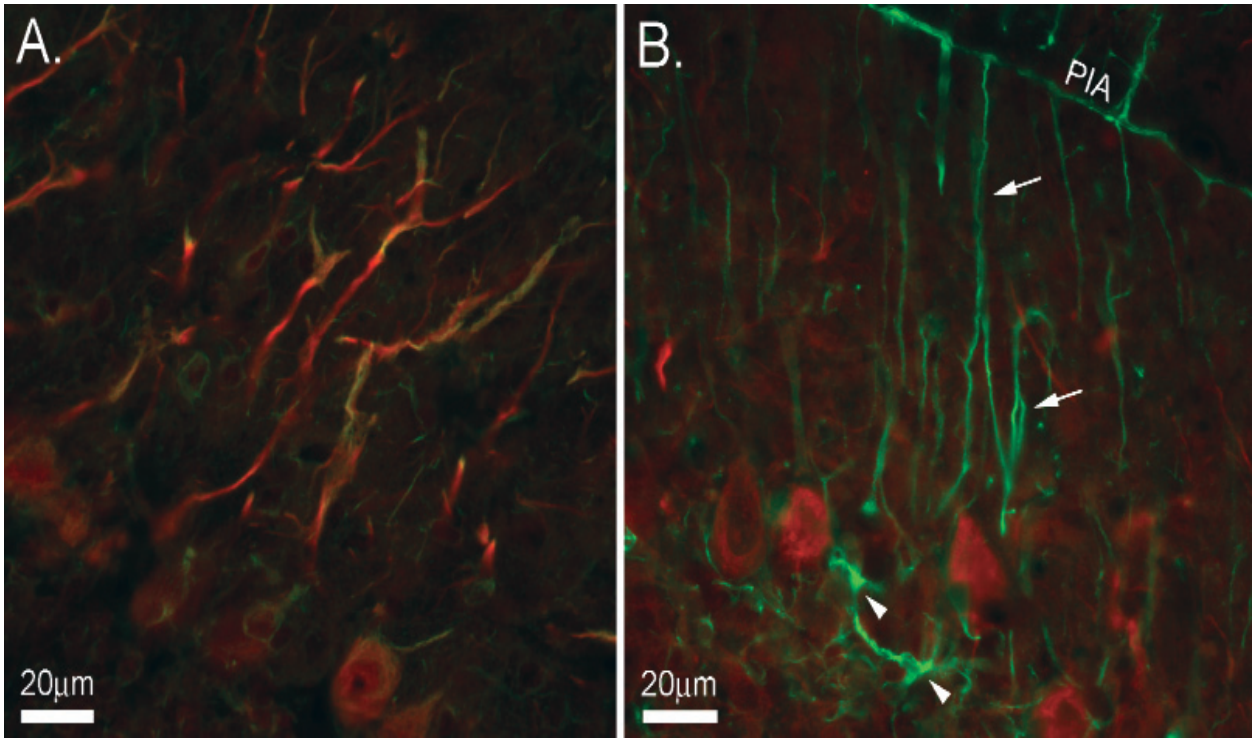

Fig. 4 FGF-2 localization in the ML of the 4m-old mouse cerebellum. A & B. PC and their dendritic trees are FGF-2-positive, but RT-97-positive climbing fibres (green in A) and GFAP-positive Bergmann glia somata (arrowheads in B, green) and processes (arrows in B, green) express FGF-2 (red). Note that stellate and basket cells are not FGF-2 positive in the ML. The pia is shown for the reference in panel B.

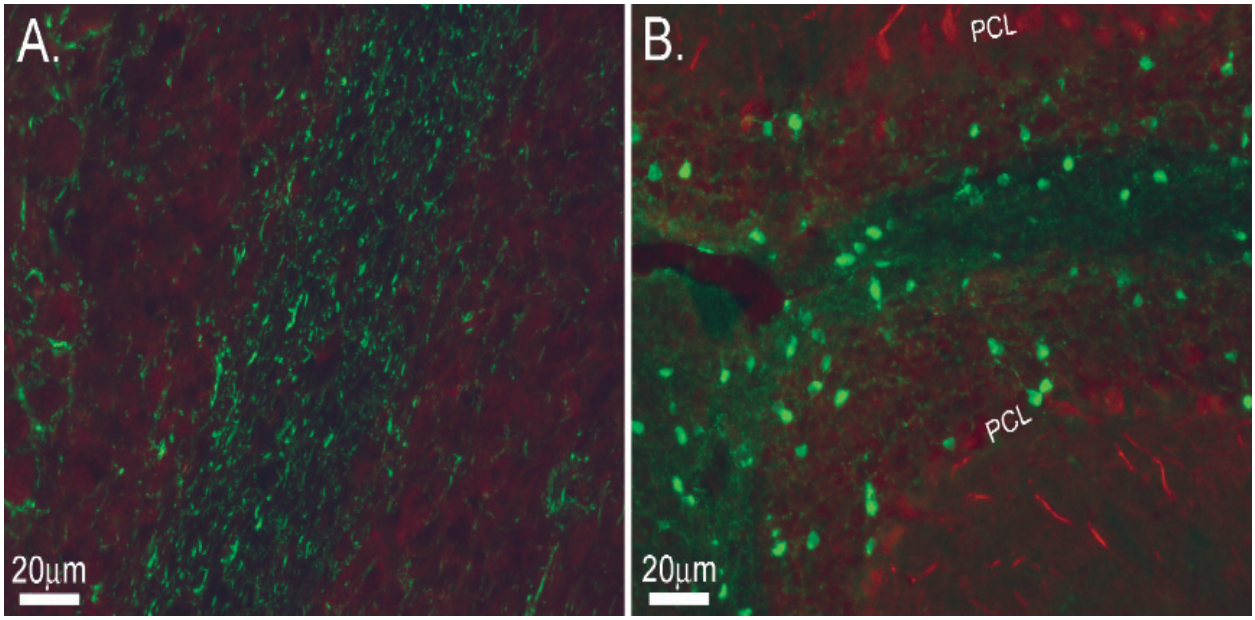

Fig. 5 FGF-2 localization in the WM of the 4m-old mouse cerebellum. Both RT-97-positive efferent and afferent axons (green in A) in the WM and CAII-positive oligodendrocytes, their processes and myelin sheaths (green in B) in the WM and GCL do not express FGF-2 (red). The PC layer is shown for reference (in panel B).

immunopositive for FGF-2 [18], the present study shows that about 50% of GC and PC express FGF-2 in the mouse.

Within the GCL, the terminals of mossy fibres in glomeruli were also FGF-2-positive, in agreement with previous results [18], possibly indicating a role for FGF-2 in the maintenance and modulation of glomerular synaptic function. This observation would support previous studies that have shown that FGF-2 enhances both glutamatergic [19] and GABAergic [22] synaptic function in hippocampal neurons. Interestingly, unlike mossy fibre terminals in glomeruli, BC terminals about PC somata, and those of climbing fibres about PC dendrites, do not express FGF-2.

Intracellular distribution of FGF-2

Significant differences were observed in the intracellular distribution of FGF-2 immunoreactivity in CG and PC. FGF-2 immunoreactivity was largely restricted to the nuclei of GC neurones, whilst in PC the staining was mostly cytoplasmic. Rat PC exhibit developmentally-induced changes in the intracellular localisation of FGF-2. PC nuclear staining peaks at P21 and largely disappears after P42, before the adult form of cytosolic staining appears [23]. FGF-2 exists in several isoforms derived from the same mRNA - a lower molecular weight (18kD) and several higher molecular weight isoforms [14]. Whereas all isoforms bind within the nucleus [24], the 18 kD isoform is also localized in the cytoplasm [12, 13]. Thus, the FGF-2-positive PC probably contain more of the 18kD FGF-2 isoform, and the FGF-2-positive GC mostly express the higher molecular weight (HMW) nuclear isoforms. The absence of significant levels of FGF-2 mRNA in rat GC and PC, revealed by *in situ* hybridisation studies, raises the question of the origin of the protein, particularly the HMW nuclear isoform. The externalisation of only the 18kD cytoplasmic form of FGF-2 implies that the protein observed in GC nuclei is endogenously synthesised, and the failure to detect rat GC FGF-2 mRNA reported in the previous studies [18] probably indicates a low FGF-2 mRNA turnover in rodents. Whilst rat PC are known to express FGFR1 mRNA, they appear not to synthesise significant levels of the FGF-2 protein [18], indicating that the FGF-2 localised in the PC cytosol is synthesised within cerebellar glia, and subsequently internalised by the PC *via* the high affinity FGF-2 receptor complex [18]. However, the actual glial source remains undefined without further *in situ* hybridisation studies in mice. Neighbouring Bergmann glia can be excluded since significant levels of staining was not seen in these cells.

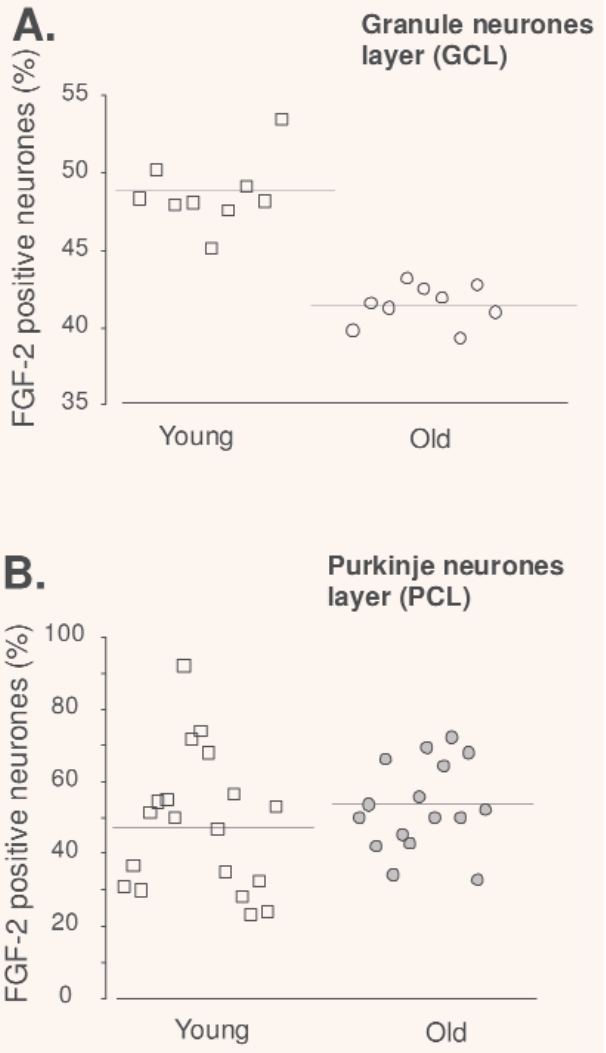

Fig. 6 Semi-quantitative analysis of the frequency of FGF-2 staining in GC and PC in the mouse cerebellum as a function of age of the animal. A. GC numbers in 4 m-old and 22 m-old) mice; the difference between the mean frequencies of GC at each age group was statistically significant (P=0.0028). B. PC numbers in young and old mice; there was no statistically significant difference between the means at each age (P= 0.473).

Although the distinct intracellular localization and differential regulation of the various FGF-2 isoforms indicate specific and unique biological roles, little is known about their differential biological effects. Specific overexpression of the HMW isoforms enhances the ability of various cell lines to grow in low/absent serum [25, 26]. Expression of either LMW or HMW in PC12 or immortalised Scwhann cells affect differentially cell growth and morphology, probably through nuclear binding sites [27]. The binding targets and the actual biological action of the cytosolic isoforms of FGF-2 are not known.

Age-related changes in cerebellar FGF-2

The important, although not indispensable, role of FGFs as regulators of neuronal development is well established. As recently reviewed by Dono [9], FGFs are active during the induction and early patterning of the neural plate and later on, particularly FGF-2, regulates the later phases of neuronal layering of the cortex [20]. Several studies have shown that FGFs and FGF-Rs are also expressed in the adult brain ([18, 28] and reviews [9, 29]), but there are only few reports of FGF expression during normal ageing. In the rat, in both the hippocampus and substantia nigra there was a 30-50% decrease in FGF-2, particularly in glia [30, 31, 32]. The current observation of a smaller decrease with age in the number of FGF-2-positive GC and PC in mice might reflect a species difference and/or indicate a differential effect of ageing on FGF-2 expression in various parts of the brain. Accumulating evidence indicate that FGF-2 signalling is mostly a reactive response. Thus, FGF-2 levels increase in the adult brain after ischemia [33], seizures [34, 35], CNS trauma [35]. Expanding on the earlier observations that FGF-2 is an extremely potent mitogen and survival signal for cultured neural stem cells [36, 37], FGF-2 restores neurogenesis in aged hippocampal dentate subgranular and subventricular zones [10]. Similarly, a kainate-evoked lesion of the hippocampus in aged rats was successfully treated by FGF-2 enriched fetal CA3 cell grafts [38]. Thus, in the aged brain, despite regionally specific reductions in FGF-2 expression, the capacity to respond to growth factor signalling with activation of neurogenesis is maintained. Taking into account the differences in intracellular localization and expression of FGF-2 labelling between the CG and PC reported here, it would be important to establish a dissociation of the FGF-2-reactive response between these two neuronal types.

Acknowledgements:

Part of the experimental work presented in this paper was supported by a grant from BBSRC to ECT.

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