Title: **Expression of Sox1, Sox2 and Sox9 is maintained in adult human cerebellar cortex.**

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ABSTRACT:

Neural stem cells (NSCs) have been found to reside in defined areas of the vertebrate brain, where they can be identified by the expression of specific markers such as Sox1, Sox2 and Sox9. In the mouse, expression of Sox1, Sox2 and Sox9 genes has recently been reported outside of these recognised NSC niches, in the Purkinje cell layer of the adult cerebellum. The present study establishes that expression of these marker genes is also found in the human cerebellum beyond the maturation phase. Expression of Sox1, Sox2 and Sox9 was detected at the mRNA level in both foetal and adult cerebellum samples, suggesting that the maintenance of these markers in adult tissue is also observed in the human cerebellum. Expression of these markers was further confirmed at the protein level on human tissue sections, as Sox1, Sox2 and Sox9 expression was detected in the Purkinje cell layer of the adult cerebellum. The present study demonstrates that Sox1 and Sox2 are expressed in the human adult cerebellum, outside of the characterised NSC niches.

Keywords: human brain, cerebellum, Sox genes, central nervous system, stem cell marker.

INTRODUCTION:

The vertebrate brain is known to contain discrete populations of neural stem cells (NSCs), which can generate both neuronal and glial derivatives. These somatic cell progenitors found in the developing tissue and in areas of the mature brain exhibit characteristic marker expression which distinguishes them from surrounding differentiated lineages. Members of the Sox gene family of transcription factors, in particular Sox1 and Sox2, are established markers for NSCs. Sox1 & Sox2 are reported to be amongst the earliest markers identifying neuroectodermal tissue during embryonic development [3, 29], and expression of Sox1, Sox2 as well as Sox9 is associated with NSC populations in the mature rodent brain [4, 6, 9, 12, 24].

The cerebellum is a multilayered region of the brain known to complete its development postnatally. Cerebellar maturation is completed by the first postnatal weeks in mouse and years in human, after which the tissue is considered to have low cell turnover [8, 27].

In adult mice, we have recently shown that in addition to areas harbouring neural stem cells such as the walls of the lateral ventricles and the dentate gyrus [2], the cerebellum also contains cells expressing Sox1, Sox2 and Sox9 [24]. This cell population located in the Purkinje cell layer, the Bergmann glia, exhibits radial glia phenotype and is closely associated with Purkinje cells. In the present study, we show that these marker genes are also expressed in the human cerebellum, and that their expression is also located to the Purkinje cell layer.

MATERIAL AND METHODS:

RT-PCR:

RT-PCR analysis was performed on RNA samples (AMS Biotech, Abingdon, UK)) following protocols described elsewhere [25] (primers available upon request). The PCR reactions were heated at 95ºC for 5min, before undergoing 35 cycles of [95ºC for 30s, 60ºC for 45s and 72ºC for 1min]. Clathrin primers were used as loading controls as previously described [25].

Immunohistochemistry:

Antibodies used were purchased from R&D Systems (anti-Sox1 antibody), Millipore (anti-Sox2 antibody and anti-Sox9 antibody), Sigma (anti-Calbindin), Dako (anti-GFAP) and Vector Laboratories (secondary antibodies). Paraffin-embedded tissue blocks prepared by the Pathology department (the University of Nottingham) were sectioned (10μm), dewaxed and rehydrated, before being washed in PBS containing 0.1% Tween-20 (Sigma, Gillingham, UK) and blocked for 1 hour in 1% blocking reagent (Roche, Burgess Hill, UK). After antigen retrieval treatment in citrate buffer, slides were incubated with a primary antibody diluted (1/200) in blocking reagent overnight at 4ºC. After extensive washing, samples were incubated in a peroxidise-conjugated secondary antibody (1/100) for 1 hour, washed in PBS containing 0.1% Tween-20, treated with a DAB peroxydase kit (Vector Laboratories) and mounted with dapi-containg Vectashield (Vector Laboratories).

RESULTS:

We analysed the expression of Sox1, Sox2 and Sox9 in cDNA prepared from RNA samples of human foetal and adult cerebellum to determine whether these genes were expressed at the RNA level. Samples prepared from whole foetal brain were analysed in parallel to provide a positive control for the detection of neural stem-cell marker expression. cDNA from the multipotent NTera cell line, which displays neuroprogenitor characteristics in vitro [7, 18], was also used as control (Fig.1). When analysed by RT-PCR, Sox1, Sox2 and Sox9 were all found to be strongly expressed in cDNA prepared from foetal brain, foetal cerebellum, and NTera cells as expected for samples containing immature neural progenitors [10, 22, 23, 26]. In the adult cerebellum, expression was found to be maintained for the three genes considered, indicating that their expression at the mRNA level is maintained beyond the phase of cerebellar maturation.

We compared this sustained level of Sox1, Sox2 and Sox9 expression in adult cerebellum with the signal obtained for Math1, a gene expressed in external granule cell precursors of the developing cerebellum and known to be downregulated in the mature cerebellar tissue [5, 21]. Although strong

expression of Math1 was detected in foetal brain and foetal cerebellum samples, the signal was clearly lost in the adult cerebellum as expected in mature tissue. For all of the markers analysed, genomic contamination in the human samples can be discounted as no signal was observed in the matching control samples where the reverse transcriptase enzyme was omitted (Fig.1).

To confirm the gene expression results obtained by RT-PCR and further elucidate the pattern of expression for Sox1, Sox2 and Sox9 in the human adult cerebellum, we analysed the presence of these gene products by immunostaining on tissue sections. The use of antibodies specific for Sox1, Sox2 and Sox9 revealed a pattern of expression similar for the 3 markers (Fig.2A-C). Additional staining with the Purkinje cell marker Calbindin and the glial marker GFAP confirmed the location of these Sox-positive cells between Purkinje cell bodies where the Bergmann glia resides (Fig.2D), establishing that these three markers are specifically found in the Purkinje cell layer of the human mature cerebellum.

DISCUSSION:

Our study shows that expression of Sox1, Sox2 and Sox9 is maintained in the Purkinje cell layer of the adult human cerebellum, matching our recent observation in mouse cerebellum [24]. These results show that, in both species, the adult cerebellum harbours a population of cells retaining expression of these neuroprogenitor marker genes. This finding is particularly topical as Sox1 and Sox2 represent a molecular signature increasingly relied upon to identify neural stem cells in the adult CNS [4, 6, 9]. The exposure of the adult cerebellum as an additional area of the brain expressing Sox1 and 2 thus is a significant contribution to the current debate on the existence of NSCs in the human cerebellum. There is evidence from rodent models that the postnatal cerebellum contains NSC-like cells [11, 14] although it is still unclear where these cells reside within the tissue [1], and whether such progenitor cells reported in mice also exist in the human adult cerebellum. The results produced here suggest that the Bergmann glia represent an interesting candidate based on expression of NSC markers Sox1, Sox2 and Sox9. Interestingly, the restricted distribution of the Sox-positive cells in the Bergmann glia population is also supported by the recent suggestion that the anti-glial nuclear antibody (AGNA), found in patients with paraneoplastic syndrome to react with Bergmann glia, may recognise Sox1 [19].

This distinctive gene expression pattern poses questions on the status of the Bergmann glia population in the mature cerebellum [1]. It is interesting to find expression of Sox1, Sox2 and Sox9 outside of typical stem cell niches, in the adult cerebellum, as the Bergmann glia is not considered as a stem cell population. Because of its supportive role for the correct architecture and biological function of Purkinje cells [15, 28], it will be important to clarify the molecular regulation of Bergmann glia and elucidate the role of Sox1, 2 and Sox9 expression in this particular cell population.

These findings highlight the need to fully map the cell populations sharing expression of Sox1 and Sox2 in the adult brain in order to re-evaluate the link between these markers and neural stem cell populations. Careful analysis of the nature and potential of the various Sox-positive cells in the adult CNS will be required to determine whether this molecular signature is a consistent marker of stem cell-ness, or whether this association is limited to specific brain areas. These results underline the wider need for a better molecular characterisation of stem cells within adult brain tissue, for both developmental biology and clinical research. There is growing evidence from cancer studies that members of the Sox gene family (including Sox9 and Sox2) are found to be expressed in CNS tumours [13, 17, 20]. Since the link between endogenous neural stem cells, cancer stem cells and cancer formation is increasingly suggested as a possible mechanism for tumour formation [16], the relationship between endogenous Sox-expressing populations, such as the Bergmann glia, and cancers found to arise in these brain areas is thus an important question to be addressed in the future.

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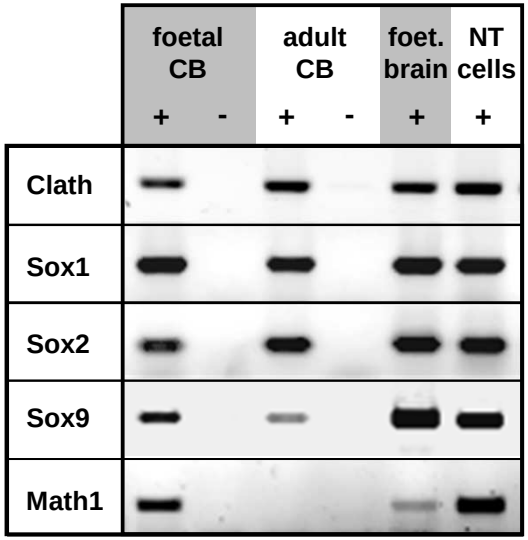
FIGURE LEGENDS:

Figure 1: RT-PCR analysis of gene expression in human cerebellum. PCR analysis of expression was carried out with cDNA samples from foetal cerebellum ('foetal CB'), adult cerebellum ('adult CB'), foetal brain ('foet. Brain') and NTera cells ('NT cells') prepared in the presence (+) or absence (-) of reverse transcriptase to establish the absence of genomic DNA contamination. Primers for clathrin (clath) were used as loading control.

Figure2: Analysis of Sox1 (A), Sox2 (B) and Sox9 (C) expression on sagittal sections of adult cerebellar tissue highlights small labelled Bergmann glia cells (arrows) around the body of Purkinje cells (asterisks). Top panel: brightfield view, bottom panel: dapi counterstain. (D) Overlaid pictures of double-staining for Sox2 (brown) and Calbindin (green, top panel), and for Sox2 (brown) and GFAP (green, bottom panel) confirming the Bergmann glia identity of the Sox-positive cells detected in the Purkinje cell layer. *ML: molecular layer, IGL: internal granule layer, PCL: Purkinje cell layer. Bar=50μm.*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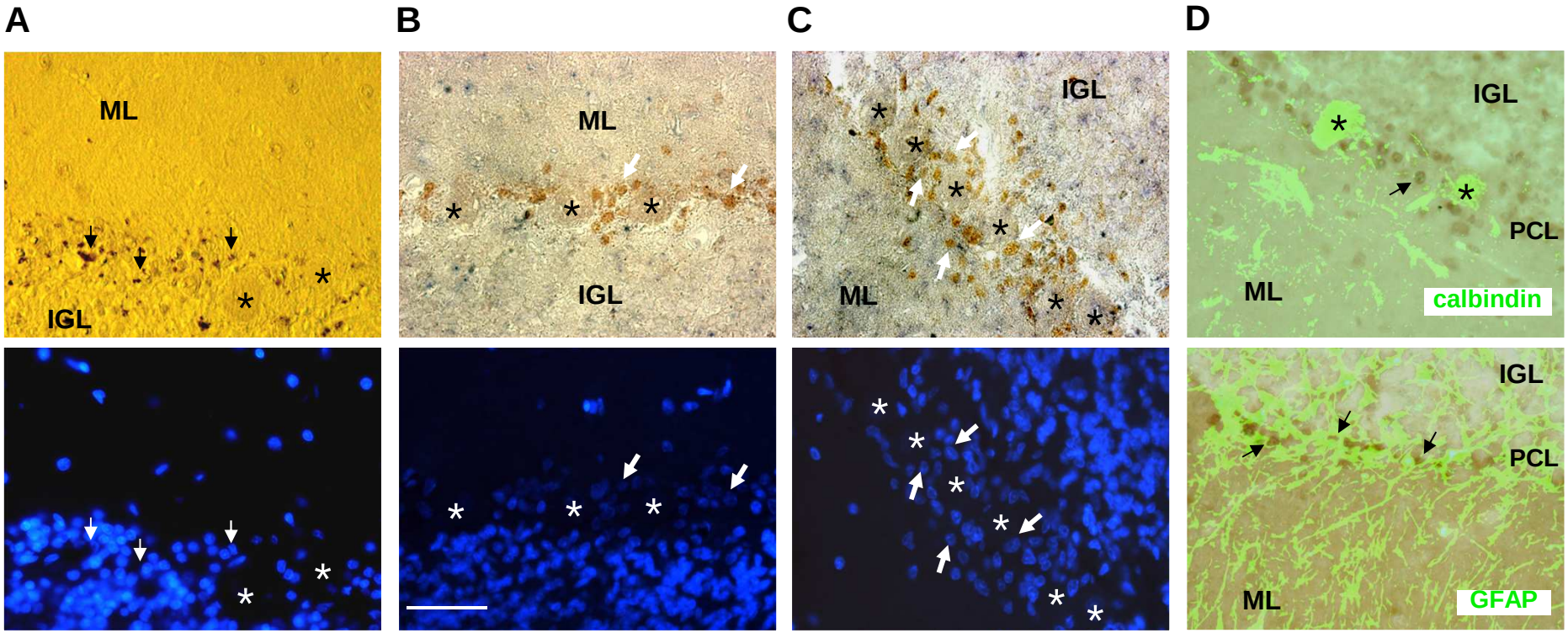
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