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Immunohistochemical study of Pax6 and Reissner fibre expression in the prenatal development of the mouse subcommissural organ

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SUMMARY

The subcommissural organ (SCO) releases glycoproteins into the ventricular cerebrospinal fluid (CSF), where they form Reissner´s fibre (RF) and also secretes a CSF-soluble material different from RF-material. Pax6 is a transcription factor important for the regulation of cell proliferation, migration and differentiation in the developing brain. In the present work, we studied wild-type, heterozygous and homozygous Sey mice to compare the expression of RF-antibody and Pax6 in the SCO and adjacent structures. In wild-type mice between E15 to E18, we observed Pax6 expression in cells surrounding the secretory cells of the SCO, and RF-immunoreactive material only in the SCO ependymal cell layer and its basal process. In the heterozygous mice, the neuroanatomical structure of the SCO was present, but RF-antibody staining and Pax6 expression was scarce or almost undetectable; in the homozygous mice neither SCO nor other epithalamic structures were found. We suggest that Pax6 expression at the periphery of the SCO is essential for the development and activity of the organ.

Key words: Subcommissural organ – Pax6 – AFRU – Ependymal layer

INTRODUCTION

The subcommissural organ (SCO) is a gland located in the epithalamus caudally to the pineal organ that undercover the posterior commissure (PC). The SCO is a highly differentiated ependyma that secretes glycoproteins into the cerebrospinal fluid (CSF), where its main part forms the Reissner´s fibre (RF) while the other one remains soluble in the CSF (Theiler, 1989; Vio et al., 2008). In vitro findings have shown that SCO secretions participate in neurite outgrowth, suggesting a close relationship between PC development and SCO glycoproteins (Gobron et al., 2000).

The homeobox gene Pax6, which plays a role in proliferation, migration and differentiation (Warren et al., 1999), is evolutionarily conserved (Callaerts et al., 1997) and almost exclusively expressed in the central nervous system and the eye (Walther and Gruss, 1991). A point mutation causes the small eye phenotype (Sey), in which there is no func-

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tional protein (Hill et al., 1991). Heterozygous mice have ocular abnormalities and nasal defects (Hogan et al., 1986) while homozygous mice die after birth with severe brain abnormalities in the CNS (Grindley et al., 1997; Stoykova et al., 1996). Human mutations of the Pax6 gene have been described in Peter's anomaly (Hanson et al., 1994) or the aniridia syndrome (Jordan et al., 1992), and recently the absence of the pineal gland (Mitchell et al., 2003).

Several authors have established changes in the expression of cell adhesion molecules in Sey mice (Stoykova et al., 1996) and these may

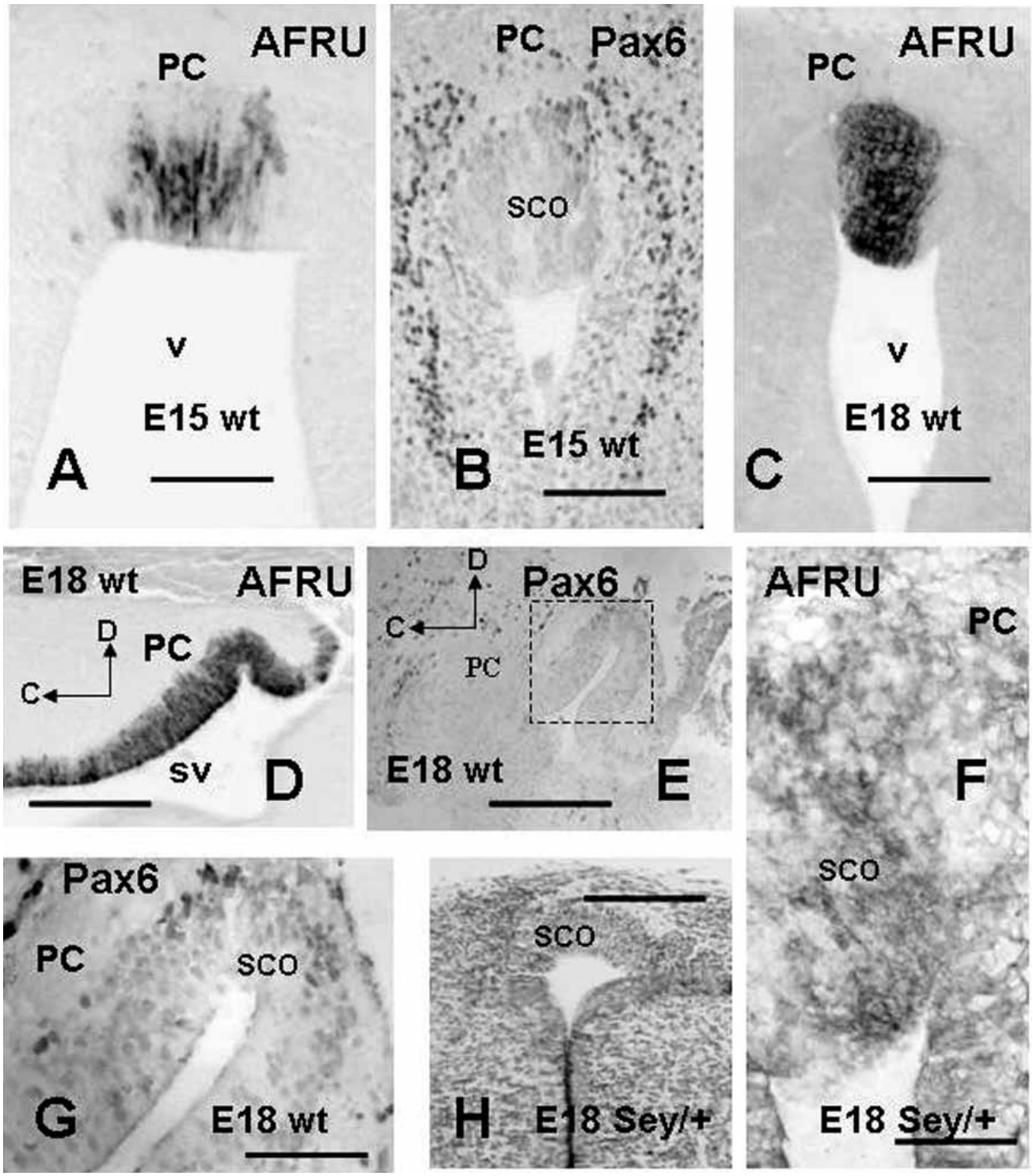

Figure 1. Subcommissural organ (SCO) immunostained with AFRU and Pax6.
Secretory cells of the (wt) mice SCO are AFRU immunoreactive positive in frontal sections of E15 wt (**A**) and E18 wt (**C**), whereas they are negative for Pax6 E15 wt (**B**). Sagittal section at E18 wt, showing the SCO immunostained with AFRU (**D**). Pax6 is expressed surrounding the posterior commissure (PC) and at the limit between the PC and the SCO on E15 wt (**B**) and E18 wt (**E**, **G**). Nissl staining showing the presence of the SCO in the heterozygous Sey/+ mice (**H**). AFRU-ir positive in the SCO of E18 Sey/+ mice frontal sections of E18 (**F**). Scale bar: A,B = 100 µm; C= 130 µm; D,E = 200 µm; F= 40 µm; G= 70 µm; H= 180 µm.
PC = posterior commissure, SCO = subcommissural organ, V = third ventricle, D = dorsal, C = caudal, SV = Sylvian aqueduct.

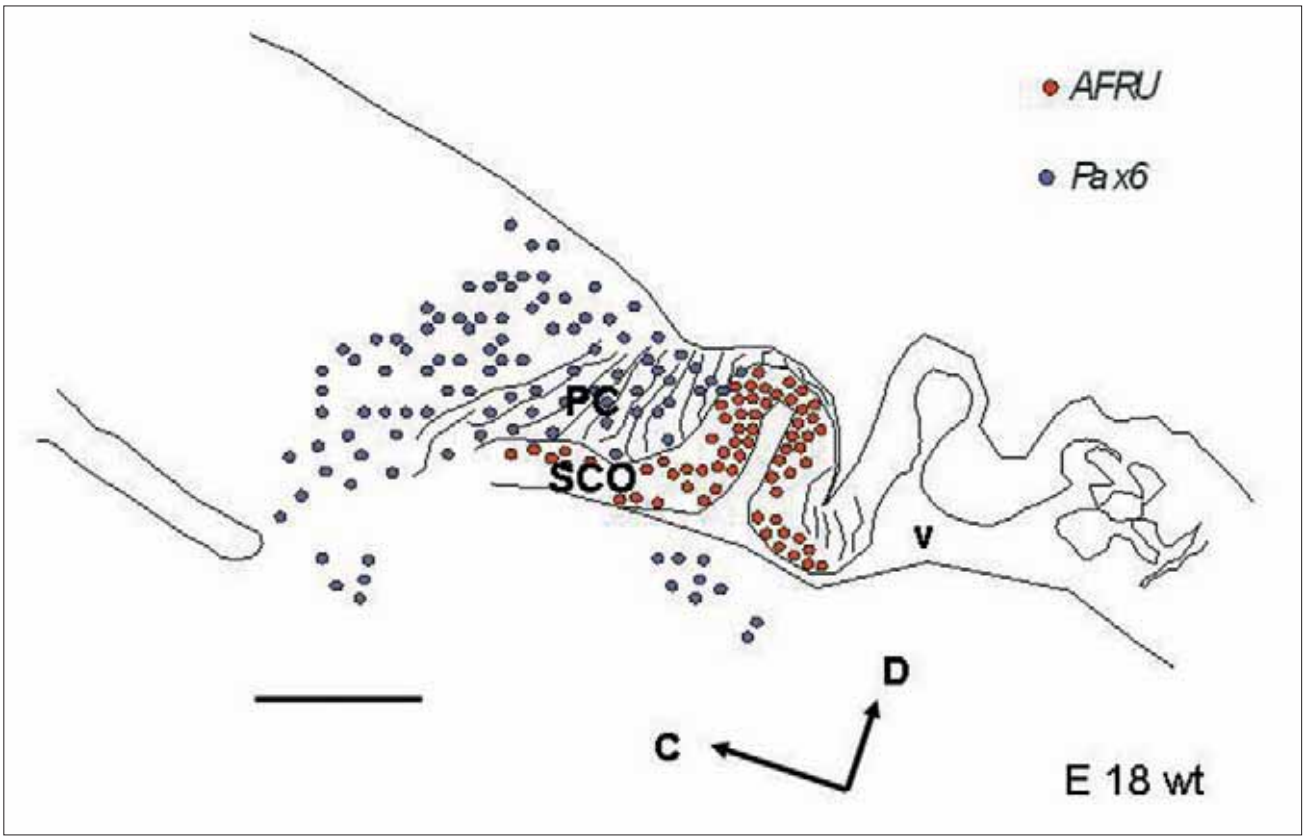

Figure 2. Diagrammatic representation summarizing Pax6 (blue dots) and Reissner fibre marker AFRU (red dots) expression in the subcommissural organ (SCO) and posterior commissure (PC). AFRU is exclusively confined to the SCO, whereas Pax6 is expressed in the PC and at the limit between the SCO and the PC. Scale bar: 200 µm.
PC = posterior commissure, SCO = subcommissural organ, V = third ventricle, D = dorsal, C = caudal.

underlie at least part of the Pax6 (Sey/Sey) phenotype. A recent study of mouse brain development has reported that Pax6 is expressed in specific epithalamic areas such as the pineal gland and the SCO (Estivill-Torrus et al., 2001).

In the present work, we examined Pax6 expression in the SCO of wild-type mice, comparing it with that of RF-antibody. A parallel study in heterozygous and homozygous Sey mice was performed to detect the SCO or a SCO-like structure.

Materials and Methods

Using immunohistochemistry, we have examined wild-type (wt), heterozygous (Sey/+) and homozygous (Sey/Sey) Pax6 mutant embryonic mice at stages 23 and 26 of Theiler (1989) (E15 and E18 prenatal days). The animals were perfused, fixed in Bouin (71.4% picric acid, 23.8% formaldehyde, 4.8% acetic acid) and Carnoy (60% ethanol, 30% chloroform, 10% acetic acid), embedded in paraffin, and cut in 10 µm-thick sections. The sections were boiled in citrate buffer followed by trypsin, and incubated with the primary antibodies overnight in a humid chamber at room temperature. After several washes, sections were incubated in the corresponding biotinylated secondary antibody (DAKO) and processed following routine protocols. The primary antibodies were a mouse monoclonal anti-Pax6 antibody (Developmental Studies Hybridoma Bank, Iowa, IA) and a rabbit polyclonal antibody against bovine Reissner fibre in buffer urea (AFRU) prepared by Rodriguez et al. (1984).

Embryos of Pax6 mutant mice of 15 (E15) and 18 (E18) prenatal days were used. The procedure for immunohistochemistry was the same as above.

Results

In the wild-type mice, we observed high levels of Pax6 immunoreactivity (ir) in cells of the pretectal and periaqueductal regions at E15 (Fig. 1B,G), whose expression decreased along development until E18 (Fig. 1E,G; Fig. 2), where the immunoreactivity was still present but at lower levels than E15. SCO secreto-

ry cells did not show Pax6 immunoreactivity at E15 (Fig. 1B) and only a few Pax6-ir cells were observed under the posterior commissure framing the SCO. At stage 26, Pax6 expression was lower than at stage 23, and in the SCO several ir-cells can be found located at the level of the hypendymal layer (Fig. 1E, G; fig. 2).

In the same areas of the heterozygous mice (Sey/+), Pax6 expression was lower than in wild-type mice at the same stages (not shown), and almost no immunoreactivity was observed at E18. The structure of the SCO was still present in the heterozygous mice (Fig. 1H), in contrast with the full absence of SCO in the knock-out mice.

The anti-Reissner fibre AFRU-ir was strongly present in the body, apical pole and peripheral prolongation of the wild-type SCO secretory cells of E15 and E18 mice (Fig. 1A, C, D).

Scarce AFRU staining was found in the heterozygous (Sey/+) mice (Fig. 1F), in contrast with a complete absence of staining in the knock-out (Sey/Sey) mice, probably due to an abnormal development of the brain structures, leading to an absence of the SCO and posterior commissure (PC).

Discussion

The cell activity of the mouse and rat subcommissural organ (SCO) begins in the ependymal layer of the SCO between E13 and E14 (Rodriguez et al., 1992), but its first morphological appearance is at E14 in mouse (Castañeyra-Perdomo et al., 1983). Nevertheless, the activity of the hypendymal layer begins postnatally (Köhl and Linderer, 1973; Rodriguez et al., 1992), but its morphological differentiation takes place during the second postnatal week in the mouse (Castañeyra-Perdomo et al., 1983). Glycoprotein secretory activity begins during the early stages of mouse development, when the SCO is not yet present.

In agreement with the findings of Estivill-Torrus et al. (2001), in our homozygous Pax6 mutant mice neither Pax6 nor AFRU immunoreactivity were expressed and the animals failed to develop the SCO, posterior commissure and pineal gland. Even in humans, with defined heterozygous Pax6 mutations, structural abnormalities, including the absence of the pineal gland, have also been described (Mitchell et al., 2003). In contrast, in wild-type mice between E15 to E18 we observed that the Pax6-ir was expressed in the cells framing the SCO, but not in the own secretory cells of the SCO. These Pax6 positive cells could be confused with hypendymal SCO secretory cells, which by this time, E15, are probably still not developed (Köhl and Linderer, 1973; Rodriguez et al., 1998).

We found AFRU-ir only in the ependymal cell layer and basal process of the SCO, which, in coronal sections, form a solid intraventricular protrusion below the PC, similar to a midsagittal crest and not extending laterally and dorsally, where the Pax6 positive cells were found.

However, in the heterozygous mice Nissl staining revealed the neuroanatomical presence of the SCO gland, but the AFRU staining was scarce and the Pax6 expression was almost undetectable at the periphery of the SCO. This suggests a down-regulated activity or a non-functional SCO gland. In contrast, a total absence of Pax6 expression gave rise a lack of SCO and PC. Thus, Pax6 expression seems to be necessary for AFRU expression as well as for the functionality of the SCO, although a role of Pax6 in the development of the SCO cannot be excluded either.

Taking in account the findings described above, we conclude that the presence of the Pax6 transcription factor is necessary for the normal development of the cells surrounding the SCO, whereas RF proteins seems to play a role in the correct development of epithalamic structures. Further studies should be performed for a better understanding of the relationship between Pax6 and the development of the SCO.

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