LIFRβ plays a major role in neuronal identity determination and glial differentiation in the mouse facial nucleus

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Received for publication 16 April 2007; revised 16 October 2007; accepted 17 October 2007
Available online 24 October 2007

Abstract

In the hindbrain, generation of the facial nucleus involves complex developmental processes that will lead to the formation of a structure composed of motor neurons, astrocytes and oligodendrocytes. The implication of LIF-related cytokines in the development of this nucleus came to light with the analysis of mice mutant for the receptor of these cytokines, LIFRβ, which exhibit a massive loss of facial branchiomotor (fbm) neurons at birth and a severe decrease in GFAP expression, a marker of astrocytes. To uncover the cellular mechanisms regulated by LIFRβ during facial nucleus development, we first analyzed its expression pattern in the hindbrain. lifrβ is first expressed at E11.5 in the hindbrain neuroepithelium. The receptor is absent during the migration of fbm post-mitotic neurons but is strongly expressed when fbm neurons have reached rhombomere 6 at E12.5, and its expression is maintained until E18.5. From the analysis of lifrβ mutant embryos, we established that LIFRβ is necessary for fbm neurons’ identity determination. We also show that LIFRβ is implicated in astrocyte and oligodendrocyte differentiation, specifically within the facial nucleus.

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Keywords: Cytokine; Motor neuron; Astrocyte; Oligodendrocyte; lifrβ mutant mice; Facial nucleus; Hindbrain development

Introduction

Development of the central nervous system (CNS) is governed by multi-step processes in which neurons, astrocytes and oligodendrocytes are generated from common neural stem cells. Specification and differentiation of these populations result from an interplay between intrinsic genetic mechanisms and extrinsic cues such as growth factors. Among these factors, members of the IL-6 cytokine family including LIF, CNTF, CLC and CT-1, also called LIF-related cytokines, have been shown to play a central role in the developing nervous system (Bauer et al., 2007). These cytokines signal through a receptor complex consisting of the heterodimerization of LIFRβ and gp130 (for review, see Ip and Yancopoulos, 1996; Murphy et al., 1997). This dimerization triggers the activation of several intracellular signal transduction cascades including the JAK/STAT and MAP kinase pathways (for review, see Turnley and Bartlett, 2000).

LIFRβ functional implication in neuronal and glial development has been studied both in vivo and in vitro. Mutant mice for LIFRβ show a massive loss of motor neurons at birth both in the facial nucleus and the spinal cord (Li et al., 1995), highlighting a major role for these cytokines and their receptors in CNS neuronal development. Nevertheless, little is known about the exact processes affected during motor neuron development in these mutant mice. Further analyses have clearly established that LIFRβ is also implicated in the development of astrocytes (Barnabe-Heider et al., 2005; Ware et al., 1995). These studies demonstrate that LIFRβ mutant embryos completely lack GFAP expression, a marker of mature astrocyte, in spinal cord and hindbrain (Ware et al., 1995). This phenotype is due to the implication of LIFRβ signalling pathway in the initiation of GFAP expression, but not in the determination of astrocyte cell fate (Bonaguidi et al., 2005). A further analysis has also established that CT-1, one of LIFRβ ligands, is essential for the initiation of gliogenesis in vitro (Barnabe-Heider et al., 2005).
Although the implication of LIFRβ signalling during astrocyte development has been investigated, little is known about the exact role of this pathway during development of the other major subtype of glial cells, oligodendrocytes. Most of the studies have been performed in the adult, revealing the role of the IL-6 cytokine family in oligodendrocyte survival and remyelination in case of injury (Azari et al., 2006; Bauer et al., 2007; Butzkueven et al., 2002, 2006; Ishibashi et al., 2006; Kerr and Patterson, 2005). The implication of these cytokines during oligodendrocyte development is thus still to be discovered.

To understand the role of LIFRβ in multi-step developmental processes leading to the generation of neuronal and glial lineages, we examined lifβ expression pattern and studied its role during facial nucleus development in mouse. Here we show that, at late developmental stages, lifβ is expressed in mature motor neurons, but not in glial cells. However, via the analysis of lifβ mutant embryos, we establish that LIFRβ is necessary for motor neuron, astrocyte and oligodendrocyte development in the facial nucleus at late embryonic stages.

Materials and methods

Animals and genotyping analysis

All procedures involving the use of animals were performed in accordance with the European Community Council Directive of 24 November 1986 on the protection of animals used for experimental purposes (86/609/EEC). Heterozygous and wild type embryos was performed as described in Arce et al. (1999). The vaginal plug was recorded as E0.5. Genotype analysis of zygous and wild type embryos was performed as described in Arce et al. (1998).

For immunolabelling on tissue sections, the slides were briefly washed in PBS, followed by 1-h incubation at RT in blocking buffer (10% FCS, 0.2% BSA, 0.1% Triton X-100 in PBS). Tissue sections were then incubated overnight at 4 °C in primary antibody diluted in the blocking solution. The sections were subsequently washed and incubated with the appropriate fluorescein labelled secondary antibodies (1:1000; Jackson Immunoresearch Laboratories, West Grove, PA, USA) for 1–2 h at room temperature. Cell nuclei were stained with Hoechst 33342 (1:1000; Sigma).

Following in situ hybridization, staining with antibodies were performed on the same sections. The slides were washed three times 10 min in PBS, followed by 1-h incubation at RT in blocking buffer (10% FCS, 0.2% BSA, 0.1% Triton X-100 in PBS). The slides are incubated overnight at 4 °C with the appropriate primary antibody. Sections are washed three times 10 min in PBS (RT) and then incubated with the appropriate fluorescently labelled secondary antibodies (1:1000; Jackson Immunoresearch Laboratories) for 1–2 h at room temperature. Cell nuclei were stained with Hoechst 33342 (1:1000; Sigma).

For immunolabelling on whole brainstems, brainstems were dissected in cold PBS and fixed 2 h at 4 °C in 4% PFA. The samples were washed in PBS, followed by 1-h incubation at RT in blocking buffer (10% FCS, 0.2% BSA, 0.1% Triton X-100 in PBS). The samples were then incubated 1 h at 4 °C in a primary antibody solution (primary antibody diluted in the blocking solution). Brainstems were subsequently washed for 24 h at 4 °C in PBS/0.1% Triton X-100 and incubated with the appropriate fluorescently labelled secondary antibodies (1:1000; Jackson Immunoresearch Laboratories) for 48 h at 4 °C. Samples were then washed for 24 h at 4 °C in PBS, 0.1% Triton X-100. Cell nuclei were stained with Hoechst 33342 (1:1000; Sigma). Brainstems are then incubated overnight at 4 °C in PBS/80% glycerol and flat mounted in this medium.

Fluorescent microscopy, quantification and statistical analysis

Whole brainstems were analysed using confocal imaging with the LSM 510 imaging system (Zeiss). Brainstems cryostat sections were examined using confocal imaging with the Apotome imaging system (Zeiss). All motor neurons counting were performed on digital sections taken every 6 μm from whole mount preparations. All glial cells counting were performed on confocal images from cryostat sections. The number of positive cells for a given marker was counted on 1 out of 3 sections per embryo. All quantifications were performed on at least 3 embryos per genotype. Each positive cell was examined by two independent observers, and scored as positive for the marker under examination. Counts are presented as mean±standard error of the mean (S.E.M.). Statistical analyses were performed with t-tests using the SigmaStat software (SPSS, USA).

Results

lifβ is expressed during facial nucleus development

During facial nucleus formation, fbm neural progenitors are generated between E9.5 and E12.5 (Altman and Bayer, 1980; Auclair et al., 1996; Pierce, 1973). Then, fbm neurons undergo a complex migration in the mouse embryonic hindbrain, that we followed using isl1, a marker of post-mitotic fbm neurons (Pfaff and Kimter, 1998; Figs. 1C, E, G, I, and K). From E10, fbm neuron cell bodies start migrating from r4 tangentially along the lateral margin of the floor plate, reaching first r5 and subsequently r6 (not shown and Figs. 1C and E). In a second wave of migration, fbm neurons start a lateral migration, moving away from the rhombencephalon ventricular zone (VZ),...
facial motor nucleus migration is completed around E14.5 (Fig. 1G; Garel et al., 2000; Studer, 2001; Cordes, 2001; Chandrasekhar, 2004). Subsequently, fbm neurons start to segregate into motor neuron pools called sub-nuclei (Figs. 1G, I and K; Ashwell and Watson, 1983).

In order to determine lifrβ expression pattern during facial nucleus formation, we performed in situ hybridization (ISH) on whole brainstems using a lifrβ probe (Figs. 1A, B, D, F, H, J and L) and compared it to isl1 staining (Figs. 1C, E, G, I and K). lifrβ is clearly not expressed in the hindbrain at E9.5 (Fig. 1A) and E10.5 (Fig. 1B). Its expression was first detected at E11.5, labelling a narrow column in the rhombencephalon VZ (Fig. 1D), all along the anterior–posterior (A–P) axis. In contrast, isl1 was expressed in post-mitotic fbm neurons but not in the floor plate (Figs. 1C and E; Pfaff and Kintner, 1998). At E12.5, lifrβ was still detected in the VZ, but also in r6, where fbm neurons start to form the facial nucleus (Fig. 1F). From E14.5 to E18.5, lifrβ was expressed in the facial nucleus, but stronger in its intermediate part (Figs. 1H, J and L).

These data show that lifrβ is dynamically expressed in the hindbrain during facial nucleus development. It is first detected as a narrow column in the VZ at E11.5, then absent during the r4–r5 migration of post-mitotic neurons, and finally strongly expressed at E12.5 in r6, where fbm neurons will form the facial nucleus. lifrβ expression is then maintained while fbm neurons maturate, generating the facial sub-nuclei.

Lifrβ is expressed in mature motor neurons but is not detected in mature glial cells in the E18.5 facial nucleus

To uncover the role of LIFRβ during facial nucleus formation, we have focused our work on late stages, when lifrβ is strongly expressed in the developing facial nucleus.

From E14.5, fbm neurons start to segregate, generating sub-nuclei that will innervate specific muscular targets (Ashwell and Watson, 1983). At E18.5, we can thus distinguish the lateral, intermediate and medial sub-nuclei (Fig. 2A). We wished to identify the cells in which lifrβ was expressed at late developmental stages on transverse section of the E18.5 facial nucleus (Fig. 2). We showed co-expression of lifrβ with Isl1 in all mature fbm neurons, mainly in the intermediate sub-nucleus, and weaker in medial and lateral sub-nuclei at this stage (Figs. 2B–C). Combined labelling for lifrβ and either S100β (marker of astrocytes; Schmidt-Kastner and Smyzynska, 1990), or pdgfrα (marker of oligodendrocytes; Liu et al., 2002), revealed no lifrβ staining in glial cells at E18.5 (Figs. 2D–G). Thus, these data show that, at late developmental stages of the facial nucleus, lifrβ is expressed in mature motor neurons, but is not detected in glial cells.

LIFRβ signalling is not necessary for the generation of fbm neurons

A previous study described a loss of 40% of fbm neurons in newborns lacking LIFRβ (Li et al., 1995), suggesting a role for this receptor in fbm neurons’ development. lifrβ was first detected at E11.5 in the ventricular zone (Fig. 1D). It could
therefore play a role in a late phase of fbm neurons’ production. We thus analysed the expression pattern of various markers of neuronal progenitors and post-mitotic neurons at E11.5 and E12.5 in r4 (Fig. 3). We performed double staining on r4 hindbrain sections at E11.5 (Figs. 3A and B) and E12.5 (data not shown) using nKX2.2 and Phox2b. We observed that, at both stages, the expression pattern of these two markers was not modified in \textit{lifr}^{-/-} compared to \textit{lifr}^{+/+} embryos. Whole mount hindbrain ISH with nKX2.2 (Figs. 3C and D), phox2b (Figs. 3E and F), phox2a (Figs. 3G and H) and isl1 (Figs. 3I and J) at E12.5 revealed no difference at this stage in mutant compared to control embryos. Consistent with these data, no significant changes in the total number of Isl1+ fbm neurons were observed in the absence of LIFR\textbeta{} signalling at E12.5 (Fig. 4A). Interestingly, using phox2b (Figs. 3E and F) and isl1 (Figs. 3I and J) staining at this specific stage, no modification in the stream of migrating neurons from r4 to r6 was observed in \textit{lifr}^{-/-} compared to \textit{lifr}^{+/+} embryos. Altogether, these data suggest that generation of fbm neurons in absence of LIFR\textbeta{} signalling is not affected.

\textit{LIFR}\textbeta{} signalling is necessary for fbm neurons’ identity determination

To elucidate the implication of LIFR\textbeta{} signalling in fbm neurons’ identity determination, we first studied the expression patterns of two markers of fbm neurons, Isl1 and Phox2b, in \textit{lifr}^{-/-} and \textit{lifr}^{+/+} embryos between E12.5 and E18.5. Immunofluorescent staining with Isl1 on whole brainstems revealed no obvious difference in the expression of this marker from E12.5 to E18.5 (Supplementary Figs. 1A–H). Consistent with these data, counting on optical sectioning of these preparations showed no significant change in the total number of Isl1+ fbm neurons in the absence of LIFR\textbeta{} signalling at these stages (Fig. 4A).

Using Phox2b as a second marker for fbm neurons, no obvious defects were observed at E12.5 in \textit{lifr}^{-/-} compared to \textit{lifr}^{+/+} embryos (Supplementary Figs. 1I and J). Surprisingly, although Phox2b is known to be a marker of all fbm neurons (Pattyn et al., 1997, 2000), a remarkable decrease in Phox2b expression was observed in E14.5 and E16.5 \textit{lifr}^{-/-} embryos, compared to \textit{lifr}^{+/+} at the same stages (Supplementary Figs. 1K–N). In line with these results, the expression of Phox2a, a marker of fbm neurons that is downstream of Phox2b in its signalling pathway (Pattyn et al., 2000), was decreased in \textit{lifr}^{-/-} embryos compared to their wild type littermates at the same stages (Figs. 4B–E).

To examine the expression pattern of Phox2a and Phox2b specifically in \textit{lifr}^{+/+} cells, we took advantage of the fact that the \textit{lifr}^{β−} deleted gene was replaced by a lacZ/neO cassette in the \textit{lifr}^{β−} mouse line (Ware et al., 1995). We thus performed combined labelling for β-galactosidase and Phox2a on E14.5 facial nucleus transverse sections. These experiments revealed a severe reduction of Phox2a expression in \textit{lifr}^{β−} compared to \textit{lifr}^{β+/+} cells (Figs. 4F–I). Similarly, co-labelling for β-galactosidase and Phox2b on E14.5 transverse sections showed a reduction of Phox2b expression in \textit{lifr}^{β−} compared to \textit{lifr}^{β+/+} cells (Figs. 4J–M). Consistent with these results, using double staining for Isl1 and either Phox2a (Figs. 4N–Q), or Phox2b (Figs. 4R–U), on E16.5 transverse sections, we observed a
reduction in Phox2a and Phox2b expression in Isl1+ cells from lifrβ−/− compared to lifrβ+/+ embryos.

Overall, these results show that, in the absence of LIFRβ signalling, whereas the correct number of Isl1+ neurons is present in the facial nucleus, Phox2a and Phox2b expressions are severely altered once all the fbm neurons have reached r6 and start to segregate into sub-nuclei. These data suggest a modification in the combination of genes expressed by fbm neurons during their maturation in absence of LIFRβ signalling.

To go further with these results and to better understand how fbm neurons develop in a LIFRβ mutant context, we examined the expression pattern of specific markers of motor neuron sub-populations in the facial nucleus.

To this end, we analyzed the expression patterns of ER81, a transcription factor of the ETS gene family (Arber et al., 2000), and Lhx4, a LIM homeobox-containing transcription factor (Sharma et al., 1998), two markers known to be expressed in fbm neurons’ sub-populations (Gavalas et al., 2003). In contrast to Lhx4 that is not altered in the mutant (Figs. 5E–H, J and K–L), ER81 expression domain is expanded towards a medial position (Figs. 5A–D and K–L) as seen by immunohistochemistry at E16.5 and E18.5.

To examine the ER81+ sub-population during development, we counted ER81+ cells on confocal optical sections from whole mount preparations at different embryonic stages (Fig. 5I). At E14.5, there were no differences between lifrβ−/− and lifrβ+/+ facial nuclei whereas later on at E16.5, the number of ER81+ cells in the facial nucleus of lifrβ−/− embryos (602 ± 90, n = 4) was increased by 85% relative to lifrβ+/+ embryos (323 ± 69, n = 4, p < 0.05). At E18.5, the number of ER81+ cells decreased in both genotypes but there was still a 70% increase in lifrβ−/− compared to lifrβ+/+ facial nuclei (333 ± 19 and 193 ± 53, respectively; n = 4; p < 0.05). Consistent with these results, immunofluorescent staining performed on E16.5 transverse sections revealed that Isl1-positive cells that do not express ER81 in lifrβ+/+ embryos (Fig. 5K), express this gene in lifrβ−/− ones (Fig. 5L). Moreover, double staining for ER81 and Lhx4 on these sections (Figs. 5K and L) did not show any intermingling between ER81+ and Lhx4+ cells in a lifrβ mutant context, suggesting that, concerning the analysed motor pools, fbm neurons’ migration is not affected during sub-nuclei segregation in lifrβ−/− embryos compared to lifrβ+/+.

In conclusion, although the total number of fbm neurons does not change during development in absence of lifrβ, the expression of specific motor neuron markers can be drastically altered (Phox2a, Phox2b and ER81) or unchanged (Isl1, Lhx4). Therefore, there is a modification in the combination of genes
expressed in fbm neurons of \( lifr \beta^{−/−} \) embryos. Together, these data show that LIFR\( \beta \) signalling plays a crucial role in the determination of fbm neurons’ identity during their maturation.

The number of glial cells is reduced in the E18.5 facial nucleus of \( lifr \beta \)-deficient embryos

In order to analyse the development of glial cell populations in absence of LIFR\( \beta \) signalling in the facial nucleus, we examined both astrocyte and oligodendrocyte lineages in late \( lifr \beta^{−/−} \) embryos compared to \( lifr \beta^{+/+} \) (Fig. 6 and Table 1). We quantified at E16.5 and E18.5 the number of Olig2\( ^/+ \), pdgfr\( \alpha \)\( ^{−/−} \) and sox10\( ^{−/−} \) cells, three oligodendrocyte-specific markers and the number of S100\( \beta \)\( ^{−/−} \) cells, identified as astrocytes. No changes in the expression and number of S100\( \beta \)\( ^{−/−} \) cells and Olig2\( ^{−/−} \) cells were observed comparing E16.5 \( lifr \beta^{−/−} \) and \( lifr \beta^{+/+} \) embryos (Table 1 and Figs. 6A and B).

Remarkably, we observed 40% less oligodendrocytes in \( lifr \beta^{−/−} \) E18.5 facial nuclei compared to \( lifr \beta^{+/+} \) littermates (Olig2\( ^{−/−} \), sox10\( ^{−/−} \) and pdgfr\( \alpha^{−/−} \) cells, Table 1 and Figs. 6C and D). The number of S100\( \beta \)\( ^{−/−} \) astrocytes was also reduced by 27% in \( lifr \beta^{−/−} \) E18.5 facial nuclei compared to \( lifr \beta^{+/+} \) (Table 1; Figs. 6E and F) in accordance with a significant decrease in GFAP expression (Figs. 6G and H; Ware et al., 1995).

These results show that both astrocyte and oligodendrocyte development are impaired in \( lifr \beta^{−/−} \) embryos compared to \( lifr \beta^{+/+} \), and suggest a defect in glial differentiation in the absence of LIFR\( \beta \) signalling.

Strikingly, these data reveal a very paradoxical situation: Although \( lifr \beta \) is not expressed at E18.5 in astrocytes and oligodendrocytes, the defects due to the absence of \( lifr \beta \) are only observed at this stage. This phenotype could be explained either by an early quiescent modification in glial precursor cells, or to a late indirect effect of \( lifr \beta \) expressing fbm neurons on glial cells. To test these hypotheses, we counted the number of sox10\( ^{−/−} \), pdgfr\( \alpha^{−/−} \) and S100\( \beta^{−/−} \) cells at the same A–P level as the facial nucleus, but outside this structure. Indeed, since glial cells migrate radially from the VZ (Thomas et al., 2000), if the precursors are altered, the defects should be observed in all glial cells coming from the r6 VZ. Remarkably, in these regions of the E18.5 hindbrain outside the facial nucleus, no differences in the number of sox10\( ^{−/−} \), pdgfr\( \alpha^{−/−} \) and S100\( \beta^{−/−} \) cells were observed (Table 1).

Overall, we show that, at this given A–P level of the hindbrain originating from r6, the number of both astrocytes and oligodendrocytes is specifically reduced in the facial nucleus of E18.5 \( lifr \beta^{−/−} \) embryos compared to \( lifr \beta^{+/+} \). In sharp contrast, gliogenesis is correctly occurring at the same level of the hindbrain but outside the facial nucleus, the number of glial cells being unaffected in E18.5 \( lifr \beta^{−/−} \) embryos compared to \( lifr \beta^{+/+} \). This suggests that the effect of LIFR\( \beta \) signalling upon glial differentiation is indirect.

Discussion

In this study, we have established that LIFR\( \beta \) is a key regulator of specific processes involved both in neuronal and glial development leading to facial nucleus formation in the mouse hindbrain. We showed that LIFR\( \beta \) signalling is not required for the specification of motor neuron, astrocyte and oligodendrocyte, in a first phase of facial nucleus development. However, in a second step, fbm neurons’ identity is modified and the number of generated astrocytes and oligodendrocytes is reduced in absence of LIFR\( \beta \) signalling. Our study thus shows that signalling via LIFR\( \beta \) exerts important influences upon motor neuron identity determination and glial differentiation during mouse facial nucleus development.

LIFR\( \beta \) signalling is not required for neuronal and glial early specification in the facial nucleus

Previous studies showed that \( lifr \beta \) is expressed in brain neuroepithelium (Gregg and Weiss, 2005), but is not involved in neural stem cell proliferation and survival (Shimazaki et al., 2001; Pitman et al., 2004; Ohtani et al., 2000). Here, we confirmed that \( lifr \beta \) is not functionally involved in the specification of neuronal and glial cells. Indeed, in the absence of LIFR\( \beta \) signalling, neither the number of fbm neurons nor the number of immature glial cells generated was altered during facial nucleus formation. These in vivo data demonstrate that LIFR\( \beta \) is not involved in the early steps of neurogenesis and gliogenesis during facial nucleus development.

LIFR\( \beta \) signalling is necessary for the determination of motor neuron identity within the facial nucleus

Immediately after exit from the neuroepithelium in r4, fbm neurons migrate tangentially to their final position in r6. Here we show that fbm neurons do not express LIFR\( \beta \) during this migration step and this process is not affected in mutant embryos. These results indicate that LIFR\( \beta \) signalling pathway is not necessary for fbm neurons’ early specification and migration towards their final destination in r6. During the next phase of facial nucleus formation, at a time when sub-nuclei

Fig. 4. Phox2a and Phox2b expressions are affected in the facial nucleus of \( lifr \beta^{−/−} \) embryos. (A) Quantification of the number of Isl1 positive cells during facial nucleus development, from E12.5 to E18.5. Cell counting was performed on confocal (LSM 510, Zeiss) optical sectioning from whole brains. No significant difference in the number of Isl1 positive cells was observed during facial nucleus development between \( lifr \beta^{−/−} \) and \( lifr \beta^{+/+} \) embryos. (B–E) Whole mount confocal views showing a severe reduction in Phox2a expression in \( lifr \beta^{−/−} \) facial nuclei compared to wild type both at E14.5 (B, C) and E16.5 (D, E). (F–M) Fluorescent ISH for \( \beta \)-gal combined with immunohistochemistry either for Phox2a (F–I) or for Phox2b (J–M) on E14.5 transverse sections of the facial nucleus. In \( lifr \beta^{−/−} \) embryos, \( lifr \beta^{−/−} \) positive cells (\( \beta \)-gal\(^{-} \) cells) express Phox2a (F, white arrow in panel G) and Phox2b (J, white arrow in panel K) whereas \( lifr \beta^{−/−} \) cells show a total loss of Phox2a (H, white arrow in panel I) and a partial loss of Phox2b (L, white arrow in panel M). (N–U) Immunofluorescent staining for Isl1 with either Phox2a (N–Q) or Phox2b (R–U) on E16.5 facial nucleus transverse sections. In \( lifr \beta^{−/−} \) embryos, Isl1\(^{−/−} \) cells express Phox2a (N, O, white arrow) and Phox2b (R, S, white arrow), whereas in \( lifr \beta^{−/−} \) embryos, Isl1\(^{−/−} \) cells show a loss of Phox2a (P, Q, white arrow) and a reduction in Phox2b expression (T, U, white arrow). (B–E, F, H, J, L, N, P, R, T) Lateral is left and medial is right. Scale bars: 50 μm.
Fig. 5. fbm neurons’ identity is altered in the absence of LIFRβ signalling. (A–H) Whole mount confocal views showing immunofluorescent staining for ER81 (A–D) and Lhx4 (E–H) in the facial nucleus of lifrβ+/+ (A, C, E, G) and lifrβ−/− (B, D, F, H) embryos. (A–D) ER81 expression domain is extended in the facial nucleus of lifrβ−/− embryos (B, D) compared to their lifrβ+/+ littermates (A, C) at E16.5 (A, B) and E18.5 (C, D). (E–H) No obvious difference in the expression of Lhx4 is noticed in lifrβ−/− embryos (F, H) compared to wild type (E, G) during development. (I, J) ER81+ and Lhx4+ cells were quantified during the development of the facial nucleus on confocal image stacks (LSM 510, Zeiss) after immunohistochemistry on whole brainstems. The number of ER81+ cells is considerably increased in lifrβ−/− facial nuclei compared to lifrβ+/+ (I, 86.3% and 72.6% more ER81+ cells in lifrβ−/− facial nuclei at E16 and E18, respectively; t test, p<0.05). No significant difference in the number of Lhx4+ cells is observed comparing lifrβ−/− to wild type (J). (K, L) Immunofluorescent staining for ER81, Isl1 and Lhx4 on E16.5 facial nucleus transverse sections shows that Isl1+ cells that do not express ER81 in a lifrβ+/+ context (K) express this gene in lifrβ−/− embryos (L, white arrows). (A–H, K, L) Lateral is left and medial is right. Scale bars: 50 μm.
segregation occurs, \( \text{lifr}\beta \) is expressed by fbm neurons. During this process, the combinatorial expression of transcription factors will define motor neuron identity. We observed that expression of motor neuron markers was either altered (Phox2a, Phox2b and ER81) or unaffected ( Isl1, Lhx4 ) in the mutant context during this second phase of facial nucleus development. For instance, in the absence of \( \text{lifr}\beta \) signalling, we observed that the number of ER81+ cells is significantly increased, leading to the expression of this gene in Isl1-positive fbm neurons that do not express ER81 in a wild type context.

Thus, our data show that LIFR\( \beta \) controls the determination of motor neuron identity characterized by the combinatorial expression of transcription factors.

Many studies have described the implication of LIFR\( \beta \) in motor neuron survival (Li et al., 1995; Oppenheim et al., 2001; Forger et al., 2003), based on the massive cell death observed in \( \text{lifr}\beta \) mutants at birth. Here, we suggest that the alteration of motor neuron identity in mutant embryos could lead to the cell death observed at birth, probably due to the elimination of mis-specified fbm neurons, at a time when facial nucleus is com-

Fig. 6. Glial differentiation is affected in \( \text{lifr}\beta^- \) embryos. (A–D) Analysis of oligodendrocyte differentiation in the facial nucleus of \( \text{lifr}\beta^- \) embryos (B, D) compared to wild type (A, C) at E16.5 (A, B) and E18.5 (C, D). Whereas Olig2 expression is not affected in \( \text{lifr}\beta^- \) embryos at E16.5 (A, B), a reduction in Olig2 expression is obvious in \( \text{lifr}\beta^- \) embryos (D) compared to wild type (C) at E18.5. (E–H) Analysis of astrocyte differentiation in the facial nucleus of \( \text{lifr}\beta^- \) embryos at E18.5. At E18.5 in the mutant facial nucleus as compared to wild type, S100\( \beta \) expression is slightly reduced (E, F) whereas GFAP expression is severely reduced (G, H). (A–H) Lateral is left and medial is right. Scale bars: 50 \( \mu \)m.
The values shown are expressed in %±S.E.M., using the number of cells in Table 1

<table>
<thead>
<tr>
<th>S100β+ cells</th>
<th>Olig2+ cells</th>
<th>pdgfra+ cells</th>
<th>sox10+ cells</th>
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<tr>
<td>In the facial nucleus</td>
<td>E16.5</td>
<td>16.1%±6.2</td>
<td>-25.7%±4.3*</td>
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<tr>
<td>In the facial nucleus</td>
<td>E18.5</td>
<td>11.7%±20</td>
<td>-44.6%±4**</td>
</tr>
<tr>
<td>Outside the facial nucleus</td>
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The number of oligodendrocytes (Olig2+, pdgfra+ and sox10+ cells) and astrocytes (S100+ cells) is reduced in the E18.5 facial nucleus of lifr−/− embryos compared to lifr+/+ but is unchanged in the E16.5 facial nucleus or at E18.5 in a region of the hindbrain at the same A-P level as the facial nucleus, but outside this structure.

The values shown are expressed in %±S.E.M., using the number of cells in lifr−/− embryos as 100%. Counting was performed on three embryos in each group.

* p < 0.01.
** p < 0.003.
*** p < 0.001.

LIFRβ signalling is necessary for glial lineages differentiation within the facial nucleus

Several groups have reported that LIF-related cytokines influence gliogenesis (Barnabe-Heider et al., 2005; Bonni et al., 1997; Koblar et al., 1998; Nakashima et al., 1999). In this study, besides the known function of LIFRβ in the initiation of GFAP expression (Bonaguidi et al., 2005), we demonstrate that S100β expression is normal at E16.5, but is altered at E18.5 in the mutant context, favouring a role for LIFRβ in astrocyte differentiation and/or maturation. Consistent with our in vivo data, the cortex of ct-lifr−/− newborns exhibits reduced levels of both CD44, a marker of early astrocytes, and GFAP (Barnabe-Heider et al., 2005).

Remarkably, our data give new insights into the role of LIFRβ signalling on the oligodendroglial lineage in vivo. We demonstrate that although cells have acquired their oligodendrocyte fate in the mutant, the number of oligodendrocytes is reduced at E18.5, suggesting that oligodendrocyte differentiation is altered in mutant facial nucleus. In line with this result, it has been reported that LIF-related cytokines are involved in the differentiation of immature oligodendrocytes (Mayer et al., 1994) and that cultured precursor cells from lifr−/− embryos generate fewer O4+ oligodendrocytes (Pitman et al., 2004). This highlights a major role for LIFRβ signalling in oligodendrocyte differentiation and/or maturation during facial nucleus development in vivo.

We further show that the defects in glial development within the facial nucleus are not observed in adjacent structures in the hindbrain, where oligodendrocytes and astrocytes that derive from the same pool of progenitors mature correctly. In line with our observation, Koblar et al. (1998) have shown that lifr−/− cells isolated from late embryos and cultured with BMP-2 can be induced to express GFAP. This strongly suggests that other mechanisms can support the differentiation process and that the absence of LIFRβ signalling does not irreversibly affect the maturation of astrocytes. Overall, these data support the notion that the impairment in glial maturation in the absence of LIFRβ signalling is contextual.

Herein, we show that no defects were observed in both astrocyte and oligodendrocyte lineages at E16.5 in lifr−/− compared to lifr+/+. However, we observe a significant loss of both glial cell lineages specifically in the facial nucleus at E18.5. This could suggest a direct role of LIFRβ in glial cell development. Nevertheless, these defects are observed at a stage when lifrβ is not expressed in these cells. Thus, one possibility could be that LIFRβ signalling acts on astrocyte and oligodendrocyte differentiation via a non cell-autonomous mechanism during late facial nucleus development. In this study, we demonstrate that in a lifrβ+/− context, motor neuron identity is altered in the facial nucleus from E14.5. This event could change the factors secreted and/or expressed at the membrane by these neurons and thus modify the environment of differentiating glia. Indeed, several groups have reported a direct cross talk between neurons and glia (see Simons and Trajkovic, 2006; for review). As an example, secretion of TGFβ1 by cortical neurons has been shown to enhance the number of generated astrocytes and induce GFAP expression in these cells (de Sampaio e Spohr et al., 2002). In the case of a direct interaction between neurons and oligodendrocytes, numerous growth factors (PDGF, FGF2, IGF1 and NT3) have been implicated in oligodendrocyte proliferation and differentiation (for review see Simons and Trajkovic, 2006). A change in neuron identity could also, for instance, alter the expression along the axons of molecules known to be involved in oligodendrocyte maturation such as NCAM (Charles et al., 2000).

Our observation could also be explained by complex indirect interactions between neurons, astrocytes and oligodendrocytes. Since many growth factors are secreted both by neurons and astrocytes, the effect on oligodendrocyte development could occur via an astrocyte/oligodendrocyte interaction and thus be a secondary effect of motor neuron mis-specification. Other populations present in the facial nucleus could also be implicated in these complex interactions. As an example, Kerr and Patterson (2005) have described that LIF can stimulate secretion of trophic factors such as IGF-1 by ancillary cells and indirectly promote oligodendrocyte survival.

Conclusion

Overall, our study based on a detailed analysis of lifrβ expression pattern and the phenotype of lifrβ-deficient mice
uncover new prospective functions for LIFRβ during development. We specifically highlight a potential role for LIFRβ in the determination of motor neuron identity, and a new role for LIF-related cytokines and their receptors, which besides their survival activity, also play an instructive role for the maturation of neuronal subtypes in the CNS.

Acknowledgments

We thank members of the Durbec’s lab and the INSERM U623 for helpful comments on this work. The expert help of S. Corby and V. Girod-David with animal care and genotyping is acknowledged. We are grateful to B. Pettmann, K. Dudley and E. Dessaud for stimulating discussions and critical reading of the manuscript. We thank Drs. C. Goridis, T. Jessell, B. Zalc, M. Wegner and R. Lu for the kind gift of probes and antibodies. This work was funded by INSERM, CNRS and the Association Française contre les Myopathies (AFM) grant 9212. F.A was supported by the French Ministry of Research and further by AFM.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.10.020.

References


