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Research

Late gestation modulation of fetal glucocorticoid effects requires the receptor for leukemia inhibitory factor: an observational study

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Abstract

Background: Ablation of the low-affinity receptor subunit for leukemia inhibitory factor (LIFR) causes multi-systemic defects in the late gestation fetus. Because corticosterone is known to have a broad range of effects and LIF function has been associated with the hypothalamo-pituitary-adrenal axis, this study was designed to determine the role for LIFR in the fetus when exposed to the elevated maternal glucocorticoid levels of late gestation. Uncovering a requirement for LIFR in appropriate glucocorticoid response will further understanding of control of glucocorticoid function.

Methods: Maternal adrenalectomy or RU486 administration were used to determine the impact of the maternal glucocorticoid surge on fetal development in the absence of LIFR. The mice were analyzed by a variety of histological techniques including immunolabeling and staining techniques (hematoxylin and eosin, Alizarin red S and alcian blue). Plasma corticosterone was assayed using radioimmunoassay.

Results: Maternal adrenalectomy does not improve the prognosis for LIFR null pups and exacerbates the effects of LIFR loss. RU486 noticeably improves many of the tissues affected by LIFR loss: bone density, skeletal muscle integrity and glial cell formation. LIFR null pups exposed during late gestation to RU486 in utero survive natural delivery, unlike LIFR null pups from untreated litters. But RU486 treated LIFR null pups succumb within the first day after birth, presumably due to neural deficit resulting in an inability to suckle.

Conclusion: LIFR plays an integral role in modulating the fetal response to elevated maternal glucocorticoids during late gestation. This role is likely to be mediated through the glucocorticoid receptor and has implications for adult homeostasis as a direct tie between immune, neural and hormone function.

Background

Glucocorticoids (GC's) are the effectors of stress and necessarily impact multiple target tissues during normal function. Regulation of GC levels occurs through the hypothalamo-pituitary-adrenal axis (HPA). Recent evidence indicates that the cytokine, leukemia inhibitory factor (LIF) and its functional binding to the high affinity receptor, a heterodimer between low-affinity LIFR and glycoprotein 130 (LIFR:gp130), are players within the HPA cascade of events. The ligands ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1) and a novel neurotrophin set, known alternatively as NNT-1/BSF3 and CLF/

CLC, also function through the high affinity LIFR:gp130 heterodimeric receptor but require a third ligand-binding but non-signaling subunit [1–4]. Oncostatin M (OSM) utilizes LIFR:gp130 as an alternative receptor complex for OSM signaling in humans [5], but not in mice [6]. Thus, in mice LIFR:gp130 is obligatory for signaling induced by LIF, CNTF, CT-1 and CLF/CLC. Signaling proceeds through the Janus kinase/signal transducers and activators of transcription (JAK/STAT) or the ras-mitogen-activated protein (MAP) kinase pathways [for a review see [7]].

LIF and LIFR are functionally expressed in rodent tissues that are central to GC production: the hypothalamus [8] and pituitary [9–12]. LIF works in synergy with corticotropin-releasing hormone (CRH) in the hypothalamus to increase pituitary pro-opiomelanocortin (POMC) and, ultimately, pituitary adrenocorticotrophin hormone (ACTH) [9,11,13–16]. LIF also stimulates POMC gene expression directly in the pituitary [9,11,15,17]. LIF upregulates pituitary prohormone convertase (PC1). Subsequently, PC1 goes on to facilitate POMC processing to ACTH [18] within the pituitary. LIF promotes survival of magnocellular vasopressinergic neurons in the hypothalamus [19] which ultimately also positively influences ACTH level. Thus, it is well documented in rodents that signaling through the LIFR:gp130 heterodimer in the hypothalamus and pituitary leads to adrenal GC production via elevated ACTH. Other LIF effects that may influence the stress response through actions in the rodent nervous system are a LIF induced increase in acetylcholine [20] and LIF induced decreases in neuropeptide Y and tyrosine hydroxylase [21]. LIFR:gp130 signaling has strong effects in systems peripheral to but influenced by the HPA including reproductive, skeletal, nervous, neuromuscular, cardiovascular, hematopoietic, immune and metabolic systems, with influences on both development and adult homeostasis [for reviews see [7,22–27]].

Animal models of LIF over expression and ablation have confirmed importance in stress regulation. Adult mouse LIF over expression mediated by transfer of LIF over expressing hematopoietic cells results in a lethal, multisystemic phenotype that includes small adrenals with a loss of the innermost cortical layer [28]. Pituitary specific LIF over expression leads to a Cushing's Syndrome-like condition of GC hyperactivity [29,30]. LIF null adult mice have normal or slightly reduced basal ACTH and GC concentrations, and are unable to respond to acute stress by increased ACTH. Basal POMC is reduced, but inducible upon stress [16]. Thus, an obligatory function of LIF may be in the processing of POMC to ACTH during acute stress.

Null mutation of LIFR results in neonatal death and defects in many systems including absence of glial cells, osteoporosis, glycogen hyper-synthesis, placental defects [31] and loss of motor neuronal subsets that cripple suckling ability [32]. In all of the systems affected as a biological consequence of LIFR loss, an explanation of the mechanism of action can be designed around direct action of LIFR in each system. However, when considered as a whole, direct, non-redundant obligatory LIFR function in such diverse systems makes little evolutionary sense. LIFR function within the stress response system could be a unifying element in many of the diverse biological consequences of LIFR loss. Among the defects seen upon developmental LIFR loss, all but the loss of suckling ability and placental changes mirror reported effects of GC excess in the adult. This study explores the effect of alteration of the GC environment during late gestation, with the expectation that suppression of GC effects will improve the LIFR null newborn phenotype and identify LIFR as an obligatory player in concert with the maternal GC surge during late gestation development. To link LIFR function with GC function we have manipulated the late gestation maternal steroid profile by maternal adrenalectomy or RU486 administration to determine if the maternal GC surge impacts fetal well being in the absence of LIFR and functional LIFR:gp130 signaling. RU486 administration during late gestation results in improved development of bone, skeletal muscle, and glial cells in LIFR null fetuses.

Methods

Mice

The mice used in this study were first described in 1995 [31]. Disruption of the first coding exon with a promterless β-galactosidase/neomycin resistance fusion cassette [33] created a null allele for *Lifr*. AB1 ES cells, on a129S7/SvEvBrd-*Hprt*$^{b\text{-}m2}$ background, were used for targeting *Lifr*. The mice were sent to the Induced Mutant Resource of the Jackson Laboratory in 1994, from where one heterozygous (*Lifr* +/-) male was received in 1997 to re-establish the colony at the University of Washington. Since the initial germ line chimera, the line has been bred back to C57BL/6 for over 5 generations and there have been several generations of sibling matings. Thus, although exact records were not kept, the mice are on a predominantly C57BL/6 background (>96%). All work was carried out following the Institutional Animal Care and Use Committee guidelines within an Association for Assessment and Accreditation of Laboratory Animal Care approved specific pathogen-free facility.

Lifr +/- females were mated to *Lifr* +/- males and the day of plug (E0.5) recorded. Pups were either born naturally or derived by Cesarean section (C-section) on E18.5 as described [34]. The E18.5 pups were placed in a dish on ice to induce hypothermia and sleep followed by euthanasia through decapitation. Tail snips were used for

genotyping by polymerase chain reaction as previously described [31].

Hormone manipulation

E16.5 was chosen as the starting point for each treatment since no phenotypic differences among the *Lifr* genotypes was detected at this point. Noticeable differences were seen beginning on E17.5 [31]. Also, fetal feedback control of GC production is established on E16.5 coincident with the initiation of the maternal surge [35]. Adrenalectomy of E16.5 pregnant mice was performed as previously described [36]. Briefly, adult mice were anesthetized using ketamine and xylazine. The site was prepped and a small incision (~1 cm) made on the right side of the spine over the upper kidney. The adrenal gland was located and excised, with care taken to avoid damage of blood vessels. Following removal of the right adrenal, one suture was placed to close the muscle incision. The adrenalectomy procedure was repeated on the left side. The skin was closed using a 9 mm wound clip. No hormonal support was provided. Pups were born following C-section on E18.5. Two litters containing in total 3 *Lifr* +/+, 6 *Lifr* +/- and 5 *Lifr* -/- pups were studied. Within litter *Lifr* +/+ and +/- pups served as controls since there has been no detectable gene dose effect between *Lifr* +/+ and +/- at this stage of development. No mock surgeries were performed.

Lyophilized RU486 (National Hormone and Peptide Program) was dissolved to 20 mg/ml in ethanol and diluted to 25 μg/ml in peanut oil. RU486 was administered as a subcutaneous injection of 2.5 μg/mouse in 0.1 ml of peanut oil on E16.5 and E17.5. Pups were born by natural delivery on E18.5. Three litters containing in total 5 *Lifr* +/+, 10 *Lifr* +/- and 4 *Lifr* -/- pups were studied. These were compared with their littermates and to control groups that experienced no hormone intervention. C-section delivery of untreated control animals was performed on E18.5. Three untreated control litters containing in total 6 *Lifr* +/+, 3 *Lifr* +/- and 7 *Lifr* -/- pups were studied. Comparisons were made between *Lifr* -/- versus *Lifr* +/+ and +/- littermate controls.

Histology

E18.5 fetuses were fixed in 4% paraformaldehyde in PBS for paraffin sectioning. Paraffin blocks were cut in 5–6 μm sections, deparaffinized and hydrated to water prior to staining. Sections were stained with hematoxylin and eosin (H&E) or by periodic acid-Schiff (PAS). For PAS, slides were placed in 0.5% periodic acid (Sigma) for 10 minutes. They were rinsed in water and placed in Schiff reagent (Sigma) for 30 minutes, washed and counterstained with Gill 3 hematoxylin (Sigma). Slides treated with diastase to digest sugars were compared with untreated slides to control for specificity of PAS staining. Immunohistochemistry was performed using antibodies to ACTH (rabbit polyclonal anti-ACTH; Chemicon International) or glial fibrillary acid protein (rabbit polyclonal anti-GFAP; Chemicon International). Antibody binding was visualized using the Universal Quick Kit with NovaRed (Vector).

Skeletal preparations followed a combination of the protocols of Gendron-Maguire et al. [37] and Rijli et al. [38]. Briefly, the fetuses were eviscerated, the skin was removed and they were fixed in 100% ethanol for four days followed by three days in acetone. They were rinsed with water and transferred to staining solution which contained 1 volume of 0.1% Alizarin red S (Sigma), 1 volume of 3% alcian blue (Sigma), 1 volume acetic acid and 17 volumes ethanol. After staining for 10 days, the skeletons were rinsed with water, followed by immersion in 20% glycerol in 1% potassium hydroxide overnight at 37°C and held at room temperature until cleared. At this point they were passed through 50, 80 and 100% glycerol for storage.

Only within litter mice were compared. Following detection of differences, an array of slides and tissues were presented to a pathologist blinded to genotype for confirmation. The numbers of pups observed for each parameter are listed in Table 1.

Table 1: LIFR Genotypes of Tissues Assessed by Immunolabeling and Histochemistry

	No of animals								
	Control			Adrenalectomy			RU486		
Tissue	+/+	+/-	-/-	+/+	+/-	-/-	+/+	+/-	-/-
Bone	6	3	6	3	6	5	5	10	4
Spinal cord	1	1	2				1	1	2
Skeletal muscle	2	1	6				1	1	2
Pituitary	1	1	2				1	1	2
Adrenal	4	1	7	4		3	1	1	2

Radio-immune assay (RIA) for corticosterone

Samples were run at a dilution of 1:200 using a kit (ICN) in the recommended diluent using the kit protocol. The standards spanned 0.125–5.00 ng/ml. All samples were run in the same assay with high and low controls. Samples, standards and controls were run in duplicate and results averaged. Blanks were run in quadruplicate and results averaged. The standard curve was calculated using a four-parameter logistic curve fit. All samples were between 25–87% bound. The coefficient of variance between duplicate samples was 5% or less for all samples, standards and controls.

Results

Consistent Phenotype

The phenotype due to LIFR loss has remained consistent throughout the transition through three separate institutions and between the transition from, 129S7/SvEvBrd-*Hprt*$^{b\text{-}m2}$ to greater than 96% C57BL/6. In particular, LIFR null mutation remains a neonatal lethal with associated osteopenia and an absence of glial cells in late gestation, as described below.

Effect of Altered GC Action

Two approaches were used to attempt to reduce the effects of the maternal GC surge on the fetus. The first approach toward manipulating the maternal GC surge was maternal adrenalectomy on E16.5, without further GC support. Control *Lifr* -/- pups could not be identified by gross morphology or behavior. *Lifr* -/- pups from adrenalectomized mothers were noticeably smaller than the littermates and did not move vigorously. The gross morphology and behavior could accurately identify the 4 *Lifr* -/- pups following treatment that presumably heightened the fetal exposure to GC's. Thus, the impact of maternal adrenalectomy on the fetuses appeared to have a selective negative effect on the development of the *Lifr* -/- pups.

The second approach was to administer RU486 on E16.5 and E17.5 to block the effects of the GC surge at the GR in the mother and in the fetus, following placental transport [39]. We wanted to find a dose of RU486 that allowed litters to be born on E18.5 by natural delivery since E18.5 is the earliest day of natural birth seen in unmanipulated pregnancy. A dose of 5–10 μg per mouse generally, but not consistently, caused abortion on E17.5. Pups were born after E18.5 following a dose of 1.0 μg RU486 per mouse per day. A 2.5 μg per mouse dose induced birth on E18.5. However, response throughout was individual.

A pregnant mouse was treated with RU486 on E16.5 and 17.5. Following natural delivery on E18.5, the one *Lifr* -/- pup was indistinguishable from the littermates both by size and vigor, but succumbed to the soporific effect of hypothermia before its littermates. Subsequently, a further four litters similarly treated with RU486 were born by natural delivery and left with their mothers for four weeks. Of the 37 pups that survived to weaning, none were *Lifr* -/-. No pups were excluded from the litters on the day of birth; however, the remains of dead pups were found the following day. Only one pup found dead on the day after birth was of sufficient quality to genotype, and, as expected, it was *Lifr* -/-. Two more litters of RU486 treated pups were born by natural delivery on E18.5. These litters were taken on E18.5 for analysis. The mothers accepted all the pups into the litters. Four pups had not suckled, as evidenced by the lack of milk in their stomachs, while the other 8 had. All were otherwise clinically normal. Of the four with no milk, three succumbed to hypothermia more rapidly than the rest. These three proved to be *Lifr* -/-. The one additional pup with no milk in its stomach, but that resisted the effect of hypothermia the longest of the four was *Lifr* +/-. All the littermates that had suckled and had detectable milk in their stomachs were *Lifr* +/+ (2 pups) or *Lifr* +/- (6 pups). Consequently, RU486 allowed the *Lifr* -/- pups to survive natural delivery and maternal culling, although they apparently succumbed due to an inability to suckle.

Bone

There was no loss of density at the growth plate following any of the treatments in the *Lifr* +/+ and +/- animals (Figure 1A,1C,1E). The expected loss of density was seen at the growth plate in the untreated control and maternal adrenalectomy exposed *Lifr* -/- pups (Figure 1B,1D). Fragility may be increased in the adrenalectomy group as evidenced by broken mineralized bone at the growth plate, presumably due to osteopenia combined with handling post mortem. Of the 5 *Lifr* -/- pups from pregnancies where the mother was adrenalectomized, 4 had broken bones once the staining was complete, whereas, no stained bones from the *Lifr* +/+ and +/- littermates were broken. In addition, all bones from both control and RU486 exposed litters remained intact throughout the staining process. RU486 treatment of the mother noticeably protected *Lifr* -/- pups from bone loss (Figure 1F), although modest thinning could still be seen, suggesting that protection was incomplete.

Glial Cell Formation

Consistent with previous findings, no GFAP positive cells could be detected in the spinal cord of either of the *Lifr* -/- untreated control animals. Ordinarily very few GFAP positive cells are seen at this stage of development in wild type animals, making detection of GFAP positive cells dependent on particular spinal cord section viewed. Seven control *Lifr* +/+, 5 control *Lifr* -/-, 8 RU486 exposed *Lifr* +/+ and +/- and 11 RU486 exposed *Lifr* -/- spinal cord sections were immunolabeled. GFAP became detectable in the sections from the two E18.5 *Lifr* -/- animals analyzed

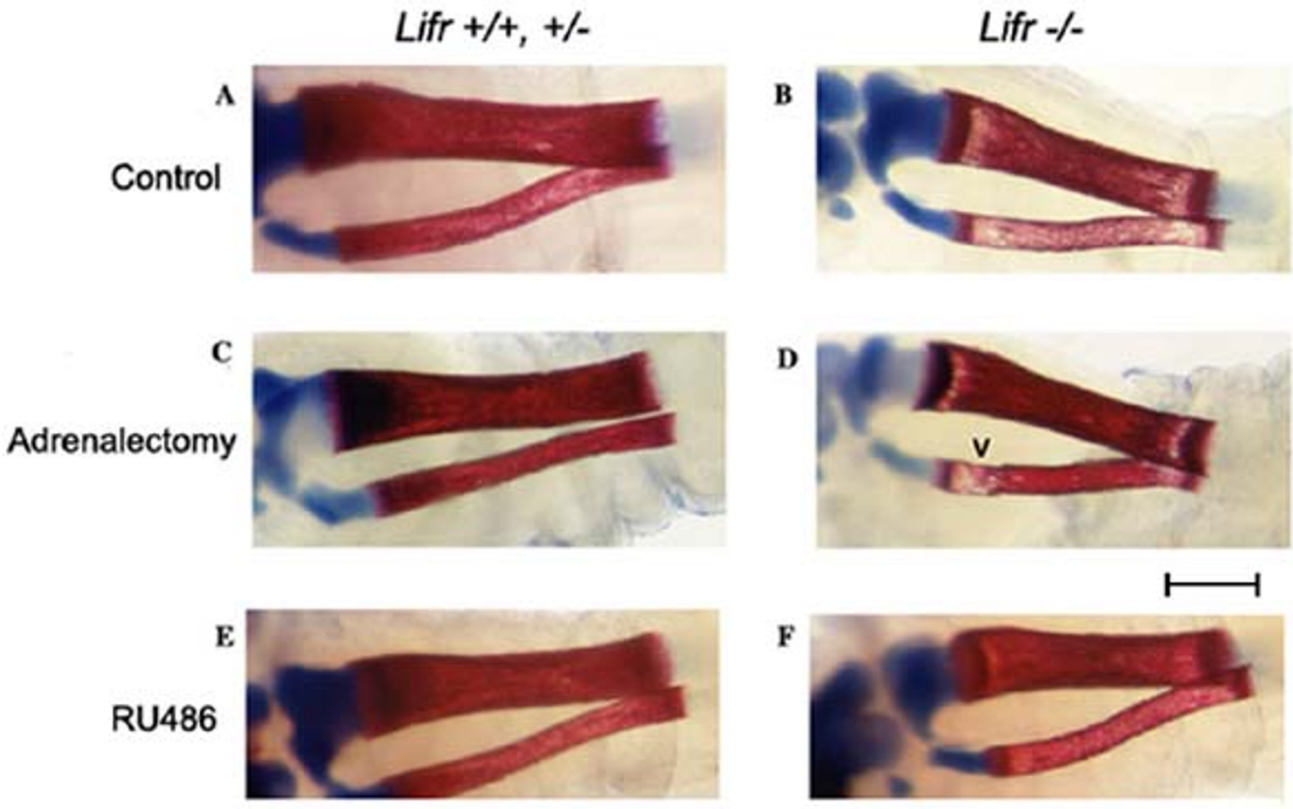

Figure 1

Fetal protection from osteopenia through RU486 administration during late gestation. Mineralized bone is visualized with Alizarin red S and cartilage with alcian blue. A. Tibia and fibula of an E18.5 *Lifr* +/+ untreated control mouse. Bone mineralization occurs throughout the length of the bone, including the epiphysis. The same can be seen for *Lifr* +/+ and *Lifr* +/- mice whether the mother was adrenalectomized on E16.5 of gestation (C) or received RU486 on E16.5 and E17.5 (E). B. An E18.5 *Lifr* -/- untreated control mouse littermate of the pup shown in A demonstrates mineralized bone loss at the growth plate. D. An E18.5 *Lifr* -/- littermate of the pup shown in C where the mother was adrenalectomized on E16.5 displays similar bone loss at the epiphysis as seen in B, but bones of the *Lifr* -/- mice in this group tend to be more fragile and show breaks within areas of osteopenia (arrow). F. A *Lifr* -/- E18.5 pup that was exposed to RU486 on E16.5 and E17.5. The epiphysis is modestly thinner than that of the wild type littermate (E), but mineralization at the growth plate is improved relative to the untreated control (B) or adrenalectomy treated (D) *Lifr* -/- pups. The size bar indicates 1 cm.

when the mother was given RU486 on E16.5 and 17.5 of gestation (Figure 2). Although, immunolabeling was reduced relative to that seen in wild type. GFAP immunopositive cells were often associated with blood vessels in the spinal cord. Although staining was relatively spare in all sections, complete absence of any labeling near vessels was apparent in the control *Lifr* -/- spinal cord (Figure 2B).

Skeletal Muscle

Skeletal muscle integrity appeared compromised in the control *Lifr* -/- animals relative to wild type (Figure 3A and 3B). This was evidenced by a frayed, granular appearance with loss of staining intensity of muscle fibers in *Lifr* -/- untreated controls. On closer examination, the individual muscle fibers contained fine vacuoles formed within the sarcoplasm (Figure 4B, arrows). *Lifr* -/- pups treated with RU486 completely lacked this phenotype and appeared normal. The characteristic appearance of skeletal muscle in *Lifr* -/- mice may be due to altered metabolism as evidenced by glycogen accumulation. Modest, but inappropriate glycogen accumulation was also seen in *Lifr* -/- periodic acid-Schiff stained skeletal muscle (Figure 3B). Glycogen was barely detectable in comparable tissues from *Lifr* +/+ or +/- littermates (Figure 3A).

Pituitary ACTH

Immuno-staining for ACTH in the pituitary showed no profound difference due to LIFR loss (Figure 4A and 4B).

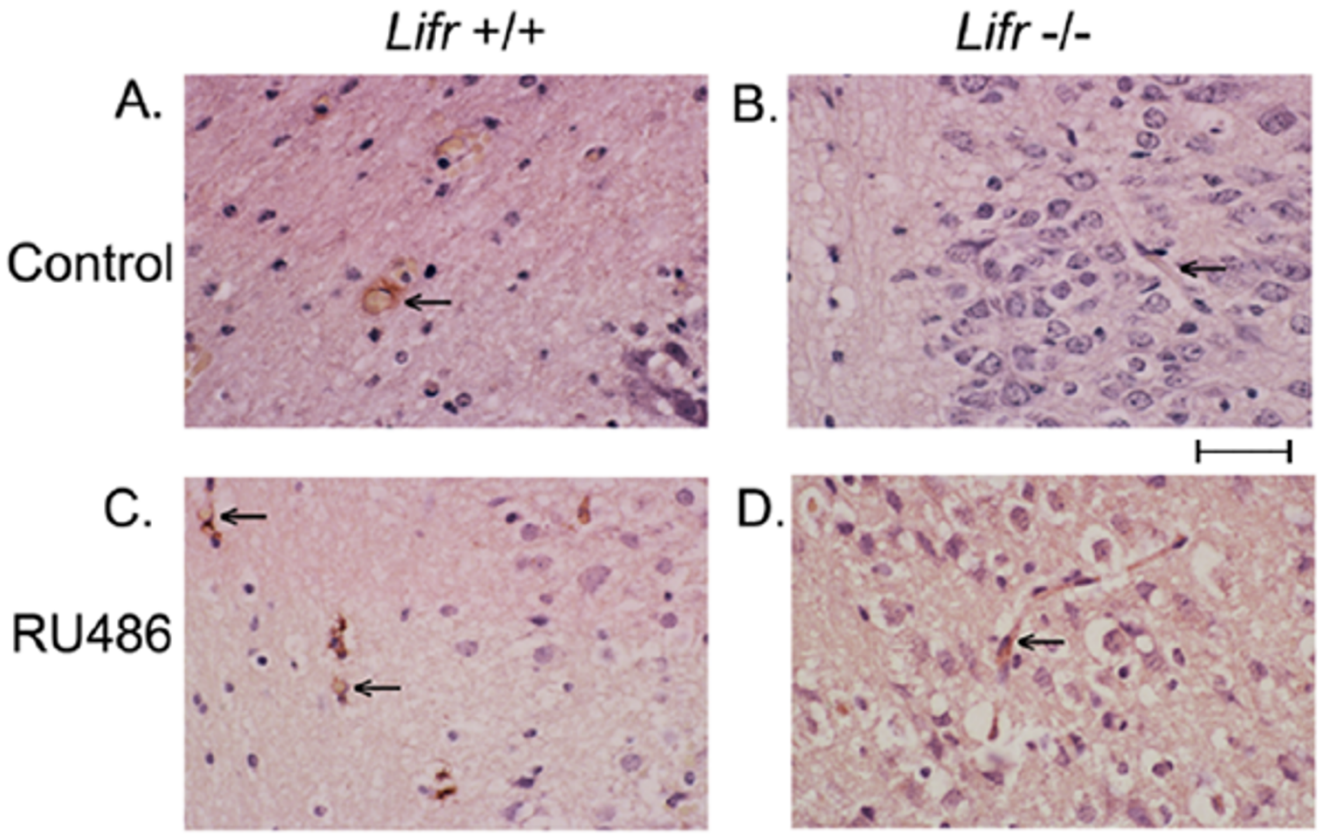

Figure 2
GFAP expression in the spinal cord. A polyclonal antibody was used to label GFAP antigen expressing cells red. A. The spinal cord of an E18.5 *Lifr* +/+ untreated control mouse immunolabeled GFAP expressing cells surrounding a blood vessel in cross-section (arrow). B. Immunolabeling of a *Lifr* -/- control littermate of the pup in A. There are no detectable positive cells. The arrow indicates a blood vessel. C. A *Lifr* +/+ pup exposed to RU486. The arrows indicate positive cells associated with blood vessels. D. A *Lifr* -/- pup exposed to RU486 (littermate to the pup in C. The arrow indicates positive staining surrounding a blood vessel. The size bar indicates 50 μm.

Treatment with RU486 resulted in apparent pituitary ACTH up-regulation as evidenced by increased immunolabeling regardless of genotype (Figure 4C and 4D). The strongest ACTH immunolabeling was seen in the pars intermedia of the anterior pituitary with scattered labeling in the pars distalis. This was an appropriate pattern for the corticotroph population. Thus, the corticotrophs did not seem altered by LIFR loss using this method of assessment. GC's induce negative feedback regulation on pituitary ACTH production, as a mechanism for protecting against GC overproduction. Because RU486 is a GR antagonist, it is known that administration can cause increased pituitary ACTH production [41]. RU486 exposure increased pituitary ACTH levels in both the *Lifr* +/+ and -/- animals, as judged by the apparent increase in labeling density through immunohistochemistry. Increased pituitary ACTH indicates that, in the absence of LIFR, pituitary ACTH could still be induced. However, this will require measurement of POMC by quantitative methods.

Lung

Lung development in all the pups looked normal as viewed on H&E stained thin sections. The lungs had inflated and were indistinguishable between treatments and genotypes (Figure 5).

Serum Corticosterone

Corticosterone levels in blood collected from untreated control pups or pups from pregnancies where the mother was adrenalectomized on E16.5 had no evident

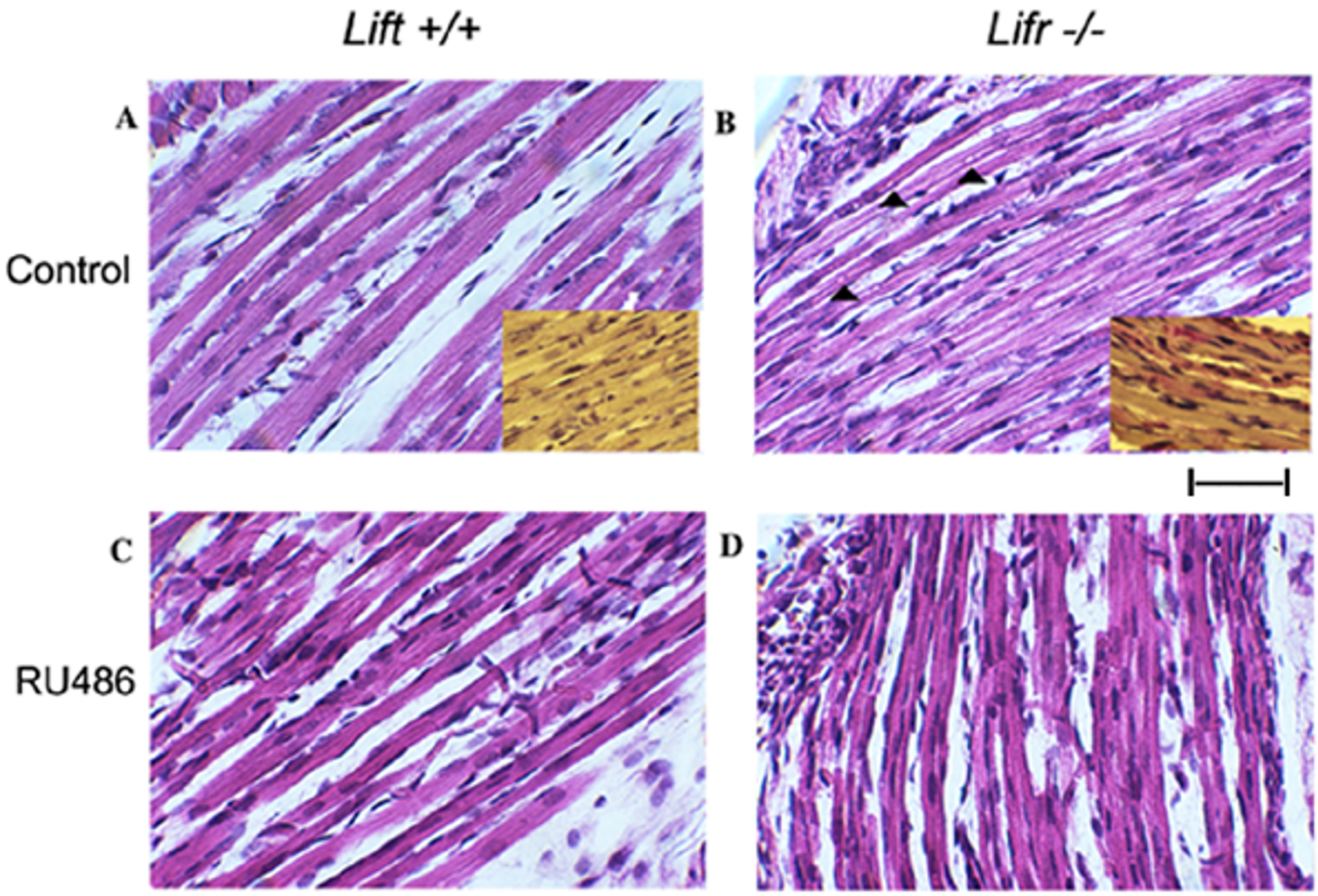

Figure 3
Skeletal muscle integrity. The primary views in all panels are stained by hematoxylin and eosin. A. Skeletal muscle is shown from an E18.5 untreated control *Lifr* +/- pup. The insert in the lower right is a PAS stain of a similar view in a *Lifr* +/+ E18.5 untreated control. Note the absence of fuchsia staining glycogen stores. B. *Lifr* -/- skeletal muscle from an untreated control littermate displays a separation of the muscle fibers that reveal small vacuoles present within the sarcoplasm (arrows). The insert in the lower right is a PAS stain of a similar view of a *Lifr* -/- E18.5 untreated control pup. Note fuchsia staining glycogen stores. C. Skeletal muscle from an E18.5 *Lifr* +/+ pup from a litter where the mother received RU486 on E16.5 and E17.5. D. Skeletal muscle from a RU486 treated *Lifr* -/- littermate to the pup viewed in panel C. The size bar indicates 100 μm.

correlation to *Lifr* genotype, as evidenced by the trial results from serum collected from only a few pups (Table 2.). Pups in the control and adrenalectomy groups were born by C-section on E18.5 and the blood collected within one hour of birth. The two pups in the RU486 group were born by natural delivery and were ex utero for greater than one hour prior to euthanasia and blood collection.

Discussion

The results implicate excess fetal GC response in late gestation as the reason for the multi-systemic phenotypes in the absence of LIFR. Maternal adrenalectomy worsens the osteopenia, while maternal hormone suppression through RU486 improves the presumed GC related phenotypes: osteopenia, skeletal muscle integrity and glial development. This was an improvement over the poor prognosis for *Lifr* -/- newborns described initially [31]. In that study, *Lifr*-/- pups rarely survived natural delivery, but when they did, they were excluded from the litter and died at variable times beginning one hour following birth. No pups survived through the night following birth. The survival of *Lifr* -/- pups following RU486 treatment was similar to the phenotype seen in CNTFR null mice where neuronal development was disrupted which disallowed suckling [40]. This indicates that at least some of the problems encountered with neuronal development that affect normal suckling were not ameliorated

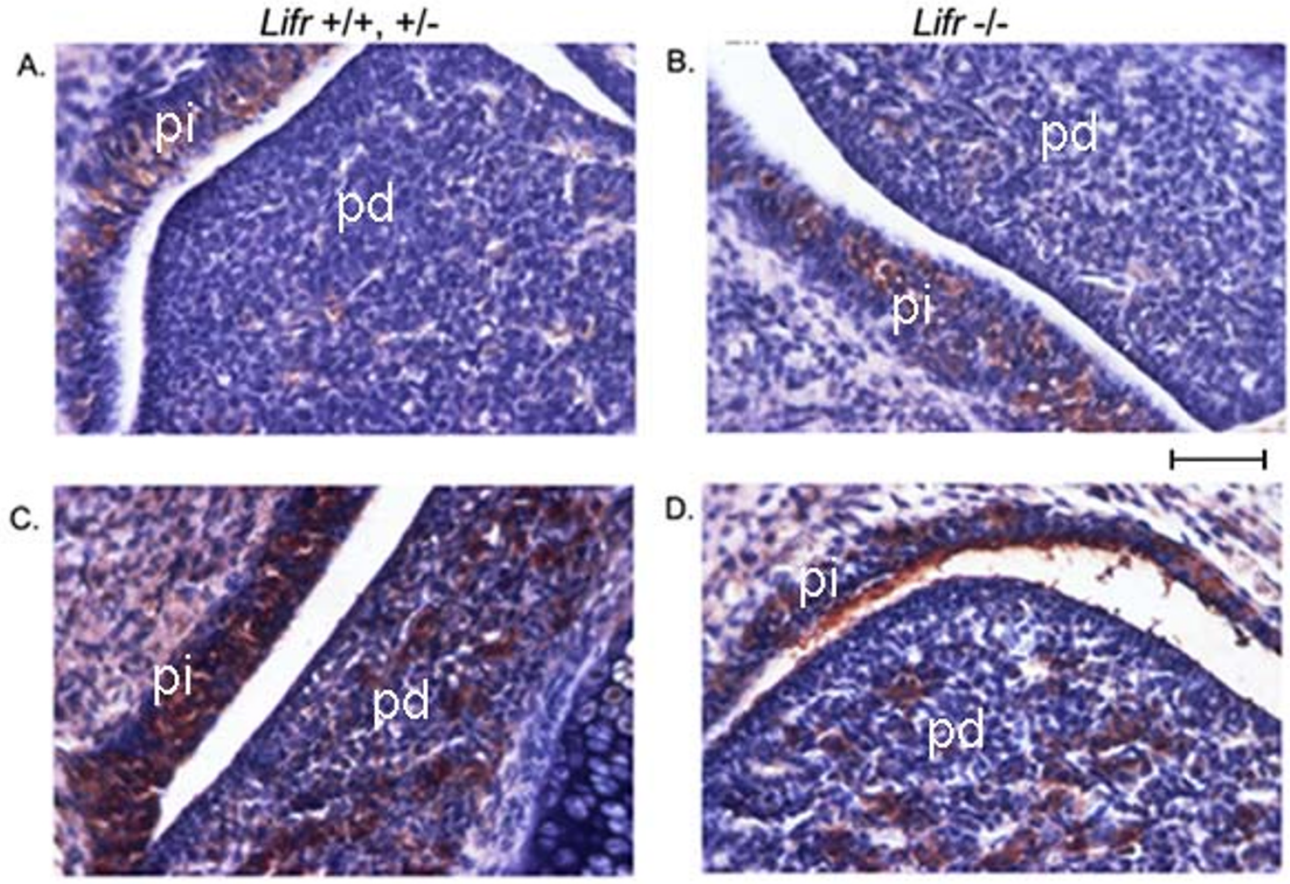

Figure 4
ACTH expression in the pituitary. Immunolabeled ACTH positive cells are red. A. Anterior pituitary section from a *Lifr* +/- untreated control mouse. The pars intermedia (pi) appears as a band to the left that contains many immunolabeled cells. The pars distalis (pd) contains scattered ACTH immuno-positive cells. B. Anterior pituitary from a *Lifr* -/- littermate to the pup in panel A. C. Anterior pituitary section from a *Lifr* +/+ pup where the mother received RU486 on E16.5 and E17.5. ACTH immunolabeling is increased relative to panel A. D. An anterior pituitary section from a RU486 treated *Lifr* -/- littermate of the pup in panel C. All sections were processed by immunohistochemistry at the same time. The size bar indicates 200 μm.

by RU486. Neither adrenalectomy nor RU486 treatment impact the apparent well-being of the *Lifr* +/+ or +/- littermates. Excess GC activity due to LIFR loss is an unexpected result since LIF gain-of-function mice develop a Cushing's-like syndrome [29,30]. Thus, if the relationship were simple, loss of LIFR function would predict development of GC insufficiency. The observed GC activity excess suggests that LIF and LIFR are integral to GC regulation, but that the relationship is complex and that alterations in GC response are likely to result from altered balance of the GC response cascade induced both by gain and loss of LIFR function.

There is a maternal GC surge beginning on E16.5, where E0.5 is the day of vaginal plug following mating. A primary function of this surge is to allow development to switch from growth to maturation of a number of systems that will allow the pup to survive on separation from the mother. Lung surfactant synthesis and connective tissue maturation allow the lungs to become distensible and capable of coping with high surface tension. In addition, the maternal GC surge effectively leads to fetal glycogen accumulation in the liver for energy demands at birth, increased fetal bioavailable tri-iodothyronine from thyroxine to allow for an increased metabolic rate and thermogenesis required at birth, maturation of the fetal small gut to prepare for digestion, heightened fetal adrenal

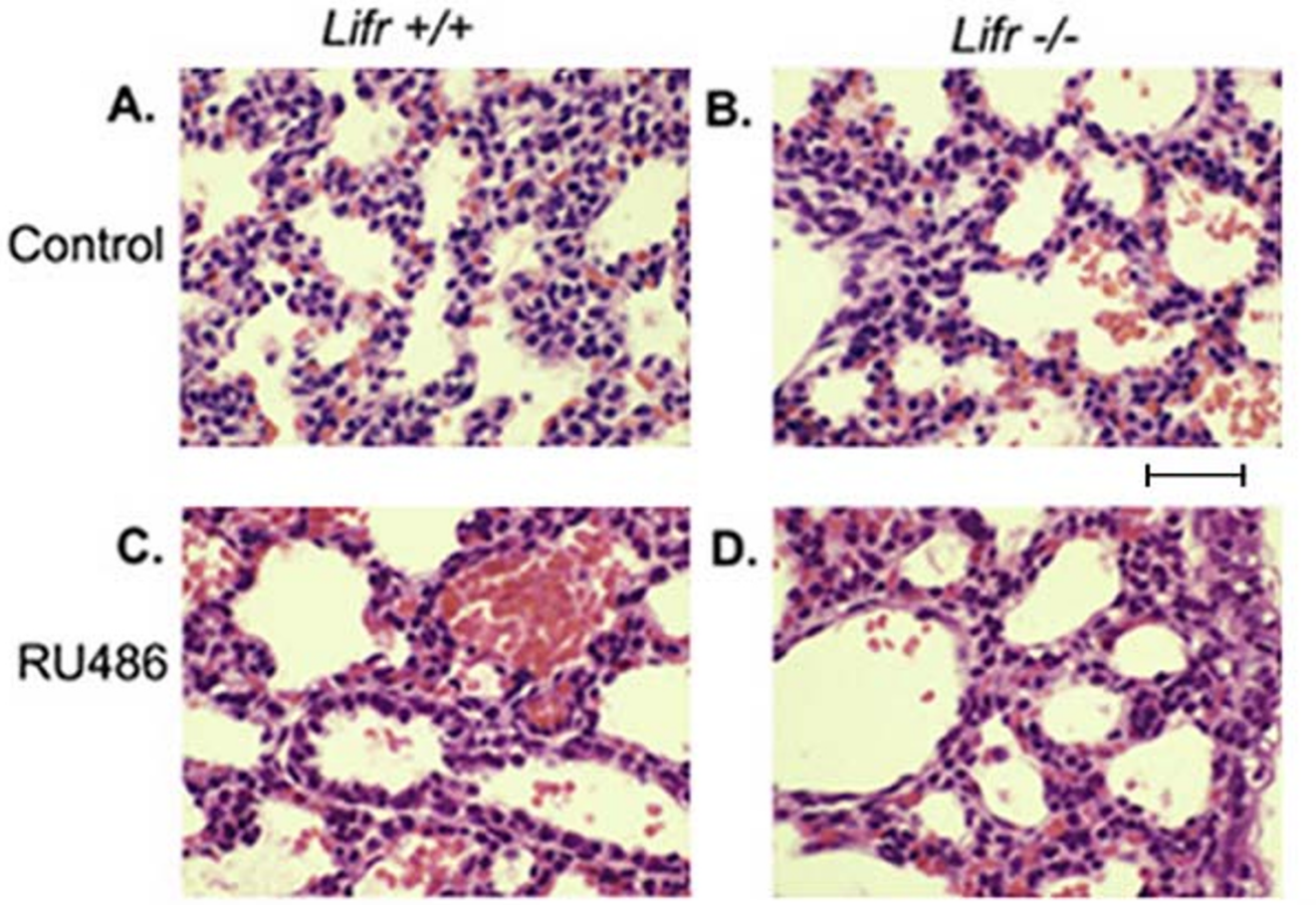

Figure 5
Lungs of E18.5 pups. All panels are stained by hematoxylin and eosin and indicate normal lung inflation in newborn pups. A. C-section derived wild type control pup. B. C-section derived *Lifr* -/- control pup. C. Lung of a wild type pup naturally delivered following exposure to RU486 in utero. D. Lung of a *Lifr* -/- pup naturally delivered following exposure to RU486 in utero. The size bar indicates 100 μm.

Table 2: Plasma Corticosterone Levels in E18.5 Pups (ng/ml)

Genotype	Control	Adrenalectomy	RU486
		(number of animals assayed)	
Lifr +/+	145 (1)	67 (2)	20 (1)
Lifr +/-	77 (1)	69 (2)	-
Lifr -/-	74 (2)	92 (4)	75 (1)

medullar catecholamine release for control of the above processes, maturation of fetal kidney function and a switch to bone marrow hematopoiesis from the fetal liver [reviewed by [41]]. The timing of this maternal surge suggests that LIFR function is required at this point in development as a moderator of the consequences of excess GC level.

Mock adrenal surgeries were not performed on the mice. Therefore, we do not know if stress of surgery alone is enough to impact well-being in LIFR null mice. Progesterone was administered in peanut oil following the same regimen as RU486, but resulted in bone loss reminiscent of the results following adrenalectomy (unpublished observation). Thus, peanut oil alone is not responsible for the improvements seen in the RU486 group and the effect of excess progesterone provides further evidence that the

LIFR phenotype can worsen through endocrine manipulation.

We previously reported that placental integrity is disrupted at the LIFR null maternal-fetal interface [31]. In that study, the maternal response to disorganized fetal placental tissue was sufficient to protect the fetus from malnutrition, since there was no detectable disruption of fetal erythropoiesis. However, what this meant for relative transport of RU486 or maternal mediators of fetal hormone balance upon adrenalectomy is unknown. Maternal adrenalectomy on E16.5 was a naïve approach toward blocking the maternal GC surge. It likely had the effect of increasing maternal ACTH levels [42] that would, in turn, act on the fetal adrenal to synthesize corticosterone. This may have been the source of the modestly elevated corticosterone level seen among the *Lifr* null pups in the adrenalectomy group. Although anecdotal, it would appear the RU486 exposed wildtype animal cleared residual late gestation corticosterone following birth more effectively than did the *Lifr* -/- littermate. Previous studies measuring circulating insulin and glucose levels in C-section derived E18.5 pups bore no apparent correlation to *Lifr* genotype (unpublished observation). At the time it appeared that plasma measurements in pups immediately following C-section were an unreliable reflection of individual condition and were likely to be a reflection of maternal levels. An accurate picture of corticosterone levels will probably require separation from the mother for several hours.

RU486 was first explored as a GR antagonist and subsequently found to be both a GR and progesterone receptor (PR) antagonist, with mild antagonistic effects on the androgen receptor. In certain cases it can function as a mild agonist. RU486 can bind to the hormone-binding domain of both GR and PR. Although still able to localize to the nucleus, it prevents subsequent transcription of GC and progesterone responsive genes. It does not directly antagonize the mineralocorticoid receptor (MR) [reviewed by [43]].

Because RU486, along with antagonizing the GR antagonizes the PR, some of the RU486 benefit may be due to antagonism of progesterone effects. Progesterone is integral to the hormonal milieu. It is processed to GC's through the actions of 21 hydroxylase and 11 β-hydroxylase [44] and it is an anti-GC [45–49] and an anti-mineralocorticoid (MC) [50–53]. Therefore, PR antagonism by RU486 could increase GC and MC effects, although this is the opposite of the phenotype seen. Progesterone interacts synergistically with estrogen in bone remodeling [54], while GC excess is implicated in osteoporosis. LIFR is known to be important in the balance between bone resorption and bone formation acting directly on osteoblasts [55–57]. Bone loss in the untreated control *Lifr* -/- pups suggests a heightened response to GC rather than a heightened response to the bone-remodeling effects of progesterone. Whereas, protection by RU486 implies antagonism of GR mediated bone loss, rather than antagonism of PR mediated bone remodeling. In addition, a progesterone receptor knockout mouse model affects only female mice with reported effects limited to organs of reproduction causing anovulation, uterine hyperplasia and inflammation, limited mammary development and impaired sexual behavior [58]. There was no noticeable difference in phenotype in the *Lifr* -/- pups due to gender following any of the treatments (data not shown). Since apparent defects due to LIFR loss arise during the late gestation GC surge, while maternal progesterone levels are declining in anticipation of birth and because phenotypes that have been attributed in the literature to GC excess are lessened through RU486 and the apparent heightened GC response in *Lifr* -/- mice affects both sexes equally, it is likely that RU486 attenuation of associated phenotypes is primarily mediated through GR antagonism. However, the interconnectedness of hormone action may also include RU486 mediated PR antagonism as a means of impacting the extent of GR-related effects.

The primary clinical utility of RU486 is PR antagonism to induce early pregnancy abortion. Since the data were collected from pups that were born early by natural delivery (E18.5 versus E19.5–21.5) following RU486 treatment of pregnant females, it can be assumed that the RU486 used was an active PR antagonist, and, by association, an active GR antagonist. The effective dose used in this study was somewhere between 0.1 and 0.2 μg/gram/day for two days. The dose was not administered by weight since litters vary in size and will have a large impact on maternal weight in late gestation. 0.3 μg/gram/day has been reported to induce abortion in 5% of mice during early gestation following 3 days of administration [59]. The dosage used in this study was able to induce pre-term birth by natural delivery at a time when the pups were able to survive ex utero (E18.5), but was well below the dose used to achieve clinical abortion.

The apparent GC hyper-responsiveness in LIFR null pups is probably not directly due to excess circulating corticosterone in the absence of LIFR. Instead, expression of GR and subsequent GR induced transcription may be more relevant than circulating GC level. Another consideration is that the benefit of RU486 administration may be either central within the HPA or can be due to peripheral GR antagonism. Cardiac dysfunction has not yet been explored in the LIFR null model. However, many of the defects caused by gp130 loss are shared by mice with a null mutation of LIFR, reflecting the heterodimeric relationship of LIFR and gp130 for functional LIFR:gp130 sig-

naling. A recent cardiac muscle specific gp130 knockout model develops normally [60]. Whereas, non-tissue specific gp130 knockout results in hypotrophic cardiac muscle apparent by E15.5 [61]. This suggests that the effect of gp130 knockout on cardiac development is mediated outside of cardiac tissue and the primary developmental defect is peripheral to the heart. Given the improvement in skeletal muscle noted in this study following RU486, it would be reasonable to explore a central HPA defect following gp130 loss as the mediator of hypotrophic cardiac development.

Muscle regeneration begins through the muscle precursor satellite cells. Activation is followed by proliferation and fusion with other satellite cells to form new myotubes. Muscle regeneration is a dynamic process necessary in the maintenance of muscle integrity. LIF and CNTF stimulate muscle regeneration in vivo [62,63], as do other growth factors; such as insulin-like growth factor [64,65]. The power of LIF to regenerate muscle is seen in dystrophin null mdx mice, where exogenous LIF regenerates atrophied diaphragm muscle [66]. LIF induced signaling appears to be essential in muscle development as seen by muscle atrophy in the absence of LIFR on E18.5. In the absence of LIFR, muscle atrophy may be caused by GC induced alteration of metabolism, which leads to glycogen accumulation, inhibition of protein synthesis and stimulation of protein degradation [67,68]. Because low levels of RU486 can fully protect *Lifr* -/- fetal muscle from the late gestation GC surge, the balance between hormone function and muscle integrity appears both delicate and direct.

GR expression is specific but widespread within neuronal and glial cell populations. LIF is also responsible for increased GFAP expression [69], while signaling through LIFR:gp130 is critical in fostering the differentiation of neuronal precursors into astrocytes [70] mediated through STAT3 [71–74]. Glial cells respond to GC, which, in turn, can affect transcriptional control of GFAP level either positively or negatively [74]. The present study shows that GC's can play a critical role in hindering astrocyte development in the absence of LIFR as seen through the partial abrogation of this defect following exposure to RU486 in *Lifr* -/- mice. RU486 crosses the blood-brain-barrier and is present at only 28% of levels seen in the serum [75]. Perhaps glial development would be further improved in the presence of higher levels of RU486 were these levels not abortive. The RU486 effect is not likely to be mediated through PR in the *Lifr* -/- astrocyte precursors, since astrocytes have low levels of PR's that are only detectable in females [76].

The vigor displayed at birth by RU486 treated *Lifr* -/- pups is striking and suggests sweeping improvement in well-being. Pups of all *Lifr* genotypes are able to breathe well at birth and *Lifr* +/+ and +/- pups thrive with no apparent lung handicap. This indicates that the improvement due to RU486 is implemented through modest alteration in GC regulation since complete loss of GR function leads to incomplete lung development at birth and impaired survival through atelectasis [77]. The neural compartment may have been under-protected by RU486 due to partial exclusion by the blood-brain-barrier, while bone and skeletal muscle, tissues exposed to higher levels of RU486, were clearly protected. Hypothalamus and anterior pituitary are also protected by the blood-brain-barrier. Elevation of ACTH in response to RU486 was apparent. Consequently, very low levels of RU486 appear to mediate partial neural normalization. Loss of neuronal subpopulations integral to suckling need to be explored in the RU486 late gestation pups, but the data suggest that the health of this population is not directly influenced by altered GC response.

Conclusions

In conclusion, many of the multi-systemic defects during late gestation brought about by LIFR loss were attenuated using maternal RU486 administration. Osteopenia is reduced, muscle integrity is normal and glial cells are forming. The mother can no longer detect abnormalities in the LIFR null pups severe enough to stimulate culling behavior. However, motor neuron deficit appears functionally unaffected and the newborn pups still succumb through inability to suckle. These results indicate that signaling through LIFR utilizing the LIFR:gp130 heterodimer is essential for an appropriate GC response during development.

Authors' contributions

CBW conceived of the study and participated in all aspects including coordination, mouse husbandry, tissue collection, genotyping, histology, immunohistochemistry and data analysis. AMN assisted with mouse husbandry and tissue collection. DL provided pathology expertise and valued discussion. All authors read and approved the final manuscript.

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