

## Estrogen-related receptor $\beta$ deficiency alters body composition and response to restraint stress

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# Estrogen-related receptor $\beta$ deficiency alters body composition and response to restraint stress

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## Abstract

**Background:** Estrogen-related receptors (ERRs) are orphan nuclear hormone receptors expressed in metabolically active tissues and modulate numerous homeostatic processes. ERRs do not bind the ligand estrogen, but they are able to bind the estrogen response element (ERE) embedded within the ERR response elements (ERREs) to regulate transcription of genes. Previous work has demonstrated that adult mice lacking *Err $\beta$*  have altered metabolism and meal patterns. To further understand the biological role of *Err $\beta$* , we characterized the stress response of mice deficient for one or both alleles of *Err $\beta$* .

**Results:** *Sox2-Cre:Err $\beta$*  mice lack *Err $\beta$*  expression in all tissues of the developing embryo. *Sox2-Cre:Err $\beta$ <sup>+/-lox</sup>* heterozygotes were obese, had increased *Npy* and *Agrp* gene expression in the arcuate nucleus of the hypothalamus, and secreted more corticosterone in response to stress. In contrast, *Sox2-Cre:Err $\beta$ <sup>lox/lox</sup>* homozygotes were lean and, despite increased *Npy* and *Agrp* gene expression, did not secrete more corticosterone in response to stress. *Sox2-Cre:Err $\beta$ <sup>+/-lox</sup>* and *Sox2-Cre:Err $\beta$ <sup>lox/lox</sup>* mice treated with the *Err $\beta$*  and *Err $\gamma$*  agonist DY131 demonstrated increased corticotropin-releasing hormone (*Crh*) expression in the paraventricular nucleus of the hypothalamus, although corticosterone levels were not affected. *Nes-Cre:Err $\beta$ <sup>lox/lox</sup>* mice, which selectively lack *Err $\beta$*  expression in the nervous system, also demonstrated elevated stress response during an acoustic startle response test and decreased expression of both *Crh* and corticotropin-releasing hormone receptor 2 (*Crhr2*).

**Conclusions:** Loss of *Err $\beta$*  affects body composition, neuropeptide levels, stress hormones, and centrally-modulated startle responses of mice. These results indicate that *Err $\beta$*  alters the function of the hypothalamic-pituitary-adrenocortical axis and indicates a role for *Err $\beta$*  in regulating stress response.

## Background

ERRs are nuclear hormone receptors that regulate multiple homeostatic processes throughout life [1]. ERRs were initially identified on the basis of sequence homology to estrogen receptors (ERs) [2]. The homology between Errs and Ers is 36% in the ligand binding domain and 68% in the DNA binding domain. ERRs bind both ERR response elements (ERREs) and the closely related estrogen response elements (EREs) embedded within an ERRE sequence on DNA to modulate transcription of target genes [3-8]. Errs activate gene transcription by binding to DNA, either as a monomer, homodimer, or a heterodimer complex, which includes two different Err isoforms [1,6,7,9,10]. While their

binding sites are similar to those of Ers, Errs do not bind estradiol and instead activate transcription in a ligand-independent manner, leading to their classification as orphan nuclear receptors. The three different *Err* genes,  $\alpha$ ,  $\beta$  and  $\gamma$ , have highly conserved ligand and DNA binding domains and thus may regulate homeostatic processes in a compensatory manner [11].

In mice, *Err $\beta$*  and *Err $\gamma$*  are selectively expressed in the brain and multiple peripheral tissues [2,12-14] and share the highest degree of sequence homology [11], suggesting that they may share overlapping functions. Since Errs recognize the same response elements, they are likely to regulate overlapping subsets of target genes [11].

We have previously reported that whole-body or central nervous system-specific deletion of *Err $\beta$*  increases expression of *Err $\gamma$*  and ultimately alters body composition, metabolism, meal patterns, and energy expenditure of mice [11]. Further, inhibition of *Err $\beta$*  or *Err $\gamma$*  alter

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metabolic parameters, whole-body energy balance (e.g. body composition, food intake and neuropeptide expression), while deletion of *Errβ* reciprocally modulates expression of *Errγ* (and vice versa) suggesting that balanced expression of *Errβ* and *Errγ* is important for control of energy balance and food intake [14-18].

Alterations in glucocorticoid signaling and whole-body energy balance positively correlate with one another, with increased glucocorticoid levels resulting in increased body weight [19-21]. *Errβ* suppresses glucocorticoid receptor activity in neuroblastoma and kidney cells in a dose-dependent manner, suggesting that it may also regulate metabolism at least in part through modulation of the hypothalamic-pituitary-adrenal (HPA) axis [22]. The HPA axis is regulated by corticotrophin-releasing hormone (Crh) released from neurosecretory cells of the hypothalamic paraventricular nucleus. Crh stimulates release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary, and ACTH, in turn, triggers glucocorticoid secretion from the adrenal gland. Negative feedback from ACTH and glucocorticoid secretion ultimately modulates *Crh* expression in the paraventricular nucleus via glucocorticoid receptors [23]. Disrupting glucocorticoid feedback loops can alter whole-body energy balance (e.g. body weight). Glucocorticoid excess (Cushing's disease) increases central fat deposition, whereas decreased body weight is associated with glucocorticoid insufficiency (Addison's disease) [19-21]. In addition to these effects on metabolism, alterations in the HPA axis can also influence anxiety and stress, which increase Neuropeptide Y (*Npy*) secretion. *Npy* further augments obesity susceptibility by inducing food intake and contributing to leptin resistance [23-25].

Consequently, we propose that *Errβ* modulates stress responses. Since *Errβ* suppresses glucocorticoid receptor activity [22], we hypothesized that the HPA axis may be altered in mice that carry heterozygous or homozygous loss of function mutations of *Errβ* in all somatic tissues [14,26,27]. The effects of *Errβ* deficiency on body weight, body composition, neuropeptide levels, stress hormones, and stress responses were examined in *Sox2-Cre:Errβ<sup>+lox</sup>* and *Sox2-Cre:Errβ<sup>lox/lox</sup>* mice, in which *Errβ* expression is disrupted in all somatic tissues. These results indicate that *Errβ* modulates stress responses, at least in part through central mechanisms.

## Results

### *Errβ* gene dosage alters body weight and body composition

*Sox2-Cre:Errβ<sup>+lox</sup>* heterozygous mice express one allele of *Errβ*, resulting in higher levels of *Errβ* expression relative to *Sox2-Cre:Errβ<sup>lox/lox</sup>* homozygous mice. Alterations in energy balance are observed in mice deficient for *Errβ* in all embryonic tissues (*Sox2-Cre:Errβ<sup>lox/lox</sup>*) [14]. Because

*Errβ* is proposed to modulate energy balance in a dose-dependent manner, we characterized *Sox2-Cre:Errβ<sup>lox/lox</sup>* and *Sox2-Cre:Errβ<sup>+lox</sup>* mice to determine whether gene dosage altered development of body weight and body composition. We previously showed that *Sox2-Cre:Errβ<sup>lox/lox</sup>* mice have decreased body weight and fat mass by nine months of age [8]. Body weight and body composition (fat mass and lean mass) were measured in *Sox2-Cre:Errβ<sup>lox/lox</sup>*, *Sox2-Cre:Errβ<sup>+lox</sup>*, and WT mice at three weeks and at nine months of age (Table 1). By three weeks, body composition differences began to emerge between the genotypes: *Sox2-Cre:Errβ<sup>+lox</sup>* mice significantly increased fat mass (fat mass:  $F_{1,8} = 9.32$ ,  $P = 0.05$ ), while *Sox2-Cre:Errβ<sup>lox/lox</sup>* mice trended toward decreased fat mass (fat mass:  $F_{1,10} = 4.95$ ,  $P = 0.05$ ) compared to WT mice. There was no difference in body weight among the genotypes at three weeks, implying that alterations in body composition arise prior to weight changes in *Errβ*-deficient mice.

At nine months of age, *Sox2-Cre:Errβ<sup>+lox</sup>* mice had increased fat mass and no change in lean mass relative to WT mice (fat mass:  $F_{1,9} = 35.90$ ,  $P = 0.002$ ). However, *Sox2-Cre:Errβ<sup>lox/lox</sup>* mice demonstrated the opposite trend in body composition, with decreases in both fat and lean mass (fat free mass) relative to WT mice (fat mass:  $F_{1,10} = 46.53$ ,  $P < 0.0001$ ; lean mass:  $F_{1,10} = 6.21$ ,  $P = 0.03$ ). Accordingly, body weight increased in *Sox2-Cre:Errβ<sup>+lox</sup>* mice ( $F_{1,9} = 32.31$ ,  $P < 0.000001$ ) and decreased in *Sox2-Cre:Errβ<sup>lox/lox</sup>* mice ( $F_{1,10} = 32.57$ ,  $P = 0.0004$ ) relative to WT mice. Given these differences, the *Sox2-Cre:Errβ<sup>+lox</sup>* mice surprisingly had a similar macrostructure of food intake as the *Sox2-Cre:Errβ<sup>lox/lox</sup>* [14], relative to WT mice. Specifically, after consuming a meal, the duration of time that the mouse was satiated was decreased (satiety ratio), the total number of pellets consumed was increased, and the duration of time between meals (intermeal interval, IMI) was not changed for *Sox2-Cre:Errβ<sup>+lox</sup>* mice, but IMI was decreased for *Sox2-Cre:Errβ<sup>lox/lox</sup>* mice (Table 1). The difference in IMI between the genotypes may be a compensatory change due to peripheral signals modulated by the increases in both body weight and fat mass observed in the *Sox2-Cre:Errβ<sup>+lox</sup>* mice.

### Hypothalamic neuropeptide expression in *Errβ* mutant mice

In the brain, *Errβ* is primarily expressed in the hindbrain, whereas *Errγ* is expressed in both the hindbrain and hypothalamus [14,28,29]. Nuclei of the hindbrain send primary projections to the hypothalamus (e.g., nucleus tractus solitarius to the paraventricular nucleus) and the amygdala, and activity in these nuclei can modulate hypothalamic gene expression [30-32]. Furthermore, in the absence of *Errβ*, *Errγ* can modulate food intake [14]. Since *Sox2-Cre:Errβ<sup>+lox</sup>* and *Sox2-Cre:Errβ<sup>lox/lox</sup>* mice demonstrated alterations in body weight and body composition relative to WT mice, we sought to determine if

**Table 1 Body weight and body composition, physical activity and meal patterns of wild type (WT), *Sox2-Cre:Errβ<sup>+lox</sup>*, and *Sox2-Cre:Errβ<sup>lox/lox</sup>* mice**

Genotype	Age	Body composition			Activity (beam breaks)	Meal patterns		
		Body weight (grams)	Fat mass (grams)	Lean mass (grams)		Pellets (number)	Satiety ratio (IMI/meal size)	IMI (minutes)
WT	3 weeks	13.1 ± 0.5	2.09 ± 0.09	10.06 ± 0.41				
<i>Sox2-Cre:Errβ<sup>+lox</sup></i>	3 weeks	14.0 ± 0.7	2.58 ± 0.16*	10.56 ± 0.62*				
<i>Sox2-Cre:Errβ<sup>lox/lox</sup></i>	3 weeks	12.9 ± 0.8	1.64 ± 0.17	10.51 ± 0.61				
WT	9 months	36.5 ± 0.9	12.19 ± 0.65	23.09 ± 0.46	67207 ± 8601	128 ± 15	8.3 ± 0.9	107 ± 17
<i>Sox2-Cre:Errβ<sup>+lox</sup></i>	9 months	46.1 ± 3.0*	21.43 ± 1.84*	24.00 ± 0.63	93599 ± 9879	238 ± 35* 5.6 ± 0.7 <sup>#</sup>	102 ± 10	
<i>Sox2-Cre:Errβ<sup>lox/lox</sup></i>	9 months	28.4 ± 1.3*	5.69 ± 0.74*	21.64 ± 0.40*	133741 ± 20533*	260 ± 57*	4.1 ± 0.7*	67 ± 11*

\**P* < 0.05 relative to WT.

<sup>#</sup>*P* = 0.05 relative to WT.

Activity is a measurement for the number of beam break, which represents horizontal physical activity that is parallel to the ground.

Meal patterns for *Sox2-Cre:Errβ<sup>lox/lox</sup>* mice are adapted from [14].

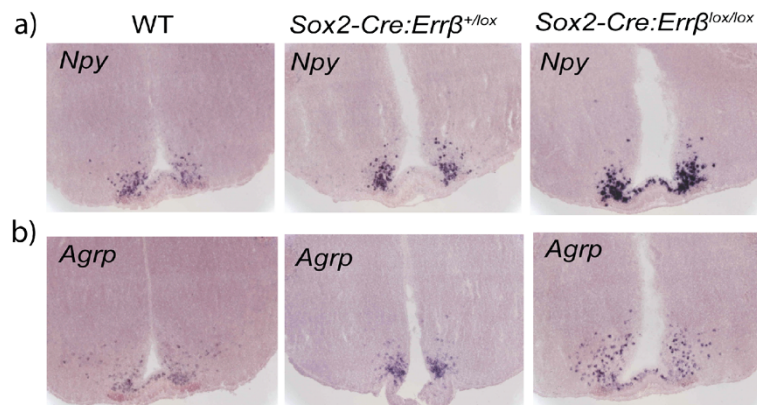
hypothalamic neuropeptides known to modulate energy balance, *Npy* and *Agrp*, were differentially expressed in the brains of these mutants. Brain tissue sections of three-week-old WT, *Sox2-Cre:Errβ<sup>+lox</sup>*, and *Sox2-Cre:Errβ<sup>lox/lox</sup>* mice were hybridized with cRNA probes specific to *Npy* and *Agrp* mRNA. *Npy* (Figure 1a) and *Agrp* (Figure 1b) staining were least intense in the hypothalamus of WT brain tissues, more intense in *Sox2-Cre:Errβ<sup>+lox</sup>* brain tissues, and most intense in *Sox2-Cre:Errβ<sup>lox/lox</sup>* brain tissues. Expression of *Npy* and *Agrp*, as determined by hypothalamic ISH staining, appears to correlate inversely with *Errβ* expression. Increased staining expression of *Npy* and *Agrp* may contribute to the increased fat mass of three-week-old *Sox2-Cre:Errβ<sup>+lox</sup>* mice; conversely, the high levels of *Npy* and *Agrp* in *Sox2-Cre:Errβ<sup>lox/lox</sup>* mice may be a downstream response to decreased fat mass.

*Sox2-Cre:Errβ<sup>lox/lox</sup>* mice show elevated activity levels due to defects in vestibular system development [14,26], which likely contribute to the body weight and body

composition differences observed at nine months of age. However, three-week-old *Sox2-Cre:Errβ<sup>+lox</sup>* mice are not hyperactive, suggesting that activity alone does not control hypothalamic neuropeptide levels (Table 1: *Sox2-Cre:Errβ<sup>lox/lox</sup>* vs. WT - *F*<sub>1,9</sub> = 16.43, *P* = 0.004).

#### ***Errβ* gene dosage alters expression of HPA axis components**

*Errβ* interacts with glucocorticoid receptors in neuroblastoma and kidney cells [22,33] and may also interact in the hindbrain where *Errβ* is expressed [14]. Since increased *Npy* expression is often associated with elevated levels of glucocorticoid release, which can influence adiposity [23-25], we hypothesized that *Errβ* deficiency may alter stress responsiveness via glucocorticoid secretion. Therefore, stress responses of WT, *Sox2-Cre:Errβ<sup>+lox</sup>*, and *Sox2-Cre:Errβ<sup>lox/lox</sup>* mice were measured by detecting alterations in HPA axis components, *Crh* expression and corticosterone.



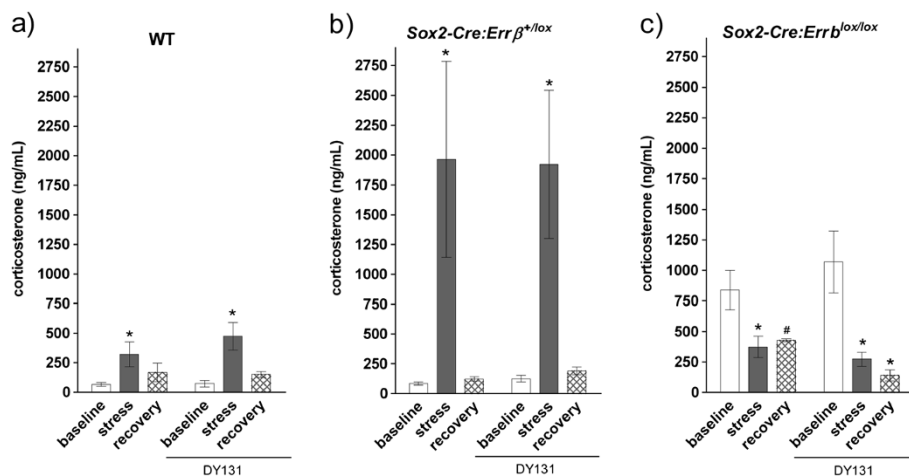
**Figure 1 Hypothalamic neuropeptide expression in wild-type (WT), *Sox2-Cre:Errβ<sup>+lox</sup>*, and *Sox2-Cre:Errβ<sup>lox/lox</sup>* mouse brains.** Brain tissues were harvested from three-week-old WT, *Sox2-Cre:Errβ<sup>+lox</sup>*, and *Sox2-Cre:Errβ<sup>lox/lox</sup>* mice; frozen tissue sections were hybridized *in situ* with cRNA probes to **a)** *Npy* and **b)** *Agrp* (n = 3/genotype).

To investigate the ability of Erry to compensate for Errβ deficiency, stress responses were investigated in the presence of synthetic agonists of Erry. Although agonists specific to individual Err isoforms are not commercially available, we were able to perform these studies using DY131, a selective agonist of both Errβ and Erry [34]. It has been previously determined that DY131 is able to readily penetrate the blood–brain barrier, as it is both hydrophobic and has a topological surface area (TPSA) less than 70 [14]. In *Sox2-Cre:Errβ<sup>lox/lox</sup>* null mice, DY131 would exclusively activate Erry and that this would result in alterations in HPA axis function (e.g. *Crh* expression or corticosterone levels). We utilized a restraint stress paradigm to measure corticosterone serum levels during baseline, stress, and recovery phases. WT mice demonstrated increased stress-induced corticosterone levels, which returned to baseline after one hour of recovery (Figure 2a) (baseline vs. stress:  $F_{1,8} = 7.82$ ,  $P = 0.03$ ). Similar results were measured in WT mice administered DY131 (DY131 WT, baseline vs stress:  $F_{1,8} = 6.46$ ,  $P = 0.03$ ; control WT; DY131 WT, stress vs recovery:  $F_{1,11} = 8.54$ ,  $P = 0.01$ ). *Sox2-Cre:Errβ<sup>+/lox</sup>* mice exhibited markedly elevated corticosterone levels during stress, which may arise from altered negative feedback mechanisms that modulate corticosterone secretion (e.g. enhanced *Crh* secretion from the brain). *Sox2-Cre:Errβ<sup>+/lox</sup>* mice exhibit normal recovery to baseline one hour after the stress test (Figure 2b – control *Sox2-Cre:Errβ<sup>+/lox</sup>*, baseline vs stress:  $F_{1,14} = 8.62$ ,  $P = 0.01$ ). Administration of DY131 yielded similar results (DY131 *Sox2-Cre:Errβ<sup>+/lox</sup>*, baseline vs stress:  $F_{1,14} = 7.02$ ,  $P = 0.02$ ; control *Sox2-Cre:Errβ<sup>+/lox</sup>*, stress vs recovery:  $F_{1,14} = 7.14$ ,  $P = 0.02$ ; DY131 *Sox2-Cre:Errβ<sup>+/lox</sup>*, stress vs recovery:  $F_{1,14} = 8.83$ ,  $P = 0.01$ ).

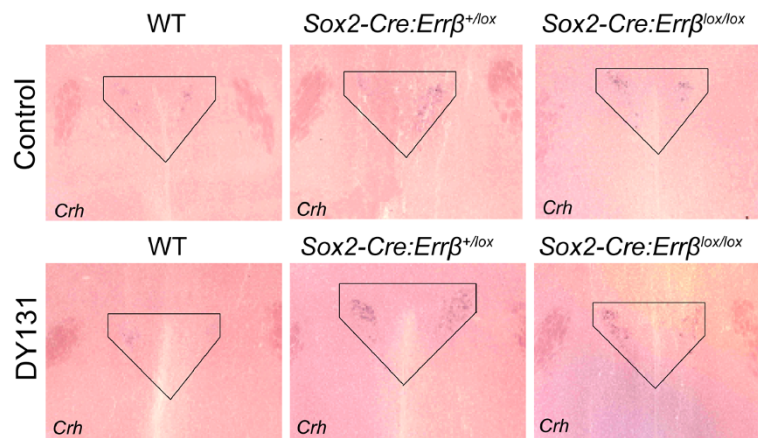
In contrast, *Sox2-Cre:Errβ<sup>lox/lox</sup>* mice had elevated baseline corticosterone levels but exhibited no increase with stress (Figure 2c – control *Sox2-Cre:Errβ<sup>lox/lox</sup>*, baseline vs stress:  $F_{1,8} = 10.86$ ,  $P = 0.02$ ; DY131 *Sox2-Cre:Errβ<sup>lox/lox</sup>*, baseline vs stress:  $F_{1,8} = 15.14$ ,  $P = 0.01$ ; control *Sox2-Cre:Errβ<sup>lox/lox</sup>*; DY131 *Sox2-Cre:Errβ<sup>lox/lox</sup>*, baseline vs recovery:  $F_{1,8} = 21.81$ ,  $P = 0.01$ ), suggesting that *Errβ:Sox2-Cre<sup>lox/lox</sup>* mice are unable to increase corticosterone levels in response to restraint stress. In fact, expression of *Crh*, as determined by ISH staining, was increased in the *Sox2-Cre:Errβ<sup>lox/lox</sup>* mice under baseline conditions, a modest increase in ISH staining was also seen in the *Sox2-Cre:Errβ<sup>+/lox</sup>* mice, with DY131 further increasing the ISH staining for *Crh*. This data suggests that Erry may modulate expression of *Crh* in a manner dependent on the level of *Errβ* expression (Figure 3).

### Neural progenitor-specific deletion of *Errβ* alters acoustic startle response

*Sox2-Cre:Errβ<sup>+/lox</sup>* and *Sox2-Cre:Errβ<sup>lox/lox</sup>* mice demonstrate differences in HPA activation, which may arise from central and/or peripheral mechanisms. In the central nervous system, *Errβ* expression is restricted to the hindbrain. *Nes-Cre:Errβ<sup>lox/lox</sup>* mice lack *Errβ* in neural progenitor cells, effectively resulting in selective loss of *Errβ* expression in the hindbrain [14]. Therefore, we investigated the central role of *Errβ* in modulating stress responses in *Nes-Cre:Errβ<sup>lox/lox</sup>* and WT mice using an acoustic startle test. The neuroanatomical and neurochemical basis of the acoustic startle response has been well mapped and involves neurons found in the amygdala, dorsomedial hypothalamus, and brainstem [35–39]. The amygdala elicits behavioral stress responses associated with the acoustic



**Figure 2** Glucocorticoid levels of wild-type (WT), *Sox2-Cre:Errβ<sup>+/lox</sup>*, and *Sox2-Cre:Errβ<sup>lox/lox</sup>* mice after restraint stress. **a)** Baseline, stress, and recovery glucocorticoid levels were measured in serum of WT mice and after treatment with Errβ/Erry agonist DY131 using a corticosterone radioimmunoassay. **b)** Baseline, stress, and recovery glucocorticoid levels were measured in serum of *Sox2-Cre:Errβ<sup>+/lox</sup>* mice and after treatment with Errβ/Erry agonist DY131 using a corticosterone radioimmunoassay. **c)** Baseline, stress, and recovery glucocorticoid levels were measured in serum of *Sox2-Cre:Errβ<sup>lox/lox</sup>* mice and after treatment with Errβ/Erry agonist DY131 using a corticosterone radioimmunoassay. \* $P < 0.05$ .



**Figure 3** *Crh* expression of wild-type (WT), *Sox2-Cre:Errβ<sup>+/lox</sup>*, and *Sox2-Cre:Errβ<sup>lox/lox</sup>* mice. Brain tissue of WT, *Sox2-Cre:Errβ<sup>+/lox</sup>*, and *Sox2-Cre:Errβ<sup>lox/lox</sup>* mice injected with saline (top) or *Errβ/Errγ* agonist DY131 (bottom) were stained for *Crh* by ISH (n = 2/genotype).

startle response and expresses the neuromodulators *Crh* and *Npy* [36,40]. *Nes-Cre:Errβ<sup>lox/lox</sup>* mice have decreased *Npy* expression in the hindbrain [14], which may modify neural circuitry activated by physical and psychological stress and, more specifically, the acoustic startle response.

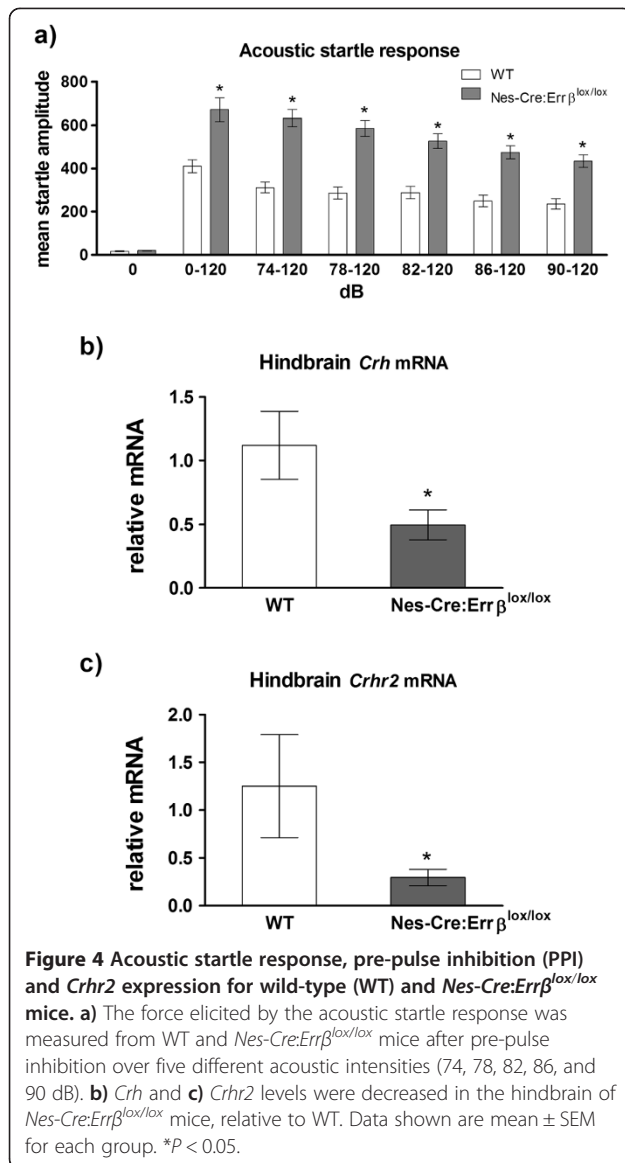
We measured PPI and the acoustic startle response to determine if *Nes-Cre:Errβ<sup>lox/lox</sup>* mice had alterations in stress responses that arise from dysfunction of the inhibitory hindbrain circuit associated with PPI or the excitatory circuit associated with the acoustic startle response [41]. The acoustic startle response was measured after delivery of a prepulse intensity signal (0, 74, 78, 82, 86, or 90 dB) followed by the lead interval to a strong auditory stimulus. We observed a greater startle response in *Nes-Cre:Errβ<sup>lox/lox</sup>* mice (n = 8, db120; 1081.5 ± 150) compared to WT mice (n = 12, db120; 475.8 ± 27) (Figure 4a, 0db -  $F_{1,20} = 0.05$ ,  $P = 0.81$ ; 0-120db -  $F_{1,20} = 9.25$ ,  $P = 0.006$ ; 74-120db -  $F_{1,20} = 15.13$ ,  $P = 0.001$ ; 78-120db -  $F_{1,20} = 15.63$ ,  $P = 0.0009$ ; 82-120db -  $F_{1,20} = 14.04$ ,  $P = 0.001$ ; 86-120db -  $F_{1,10} = 14.17$ ,  $P = 0.001$ ; 90-120db -  $F_{1,8} = 14.98$ ,  $P = 0.001$ ). However, the amplitude of the startle response decreased in *Nes-Cre:Errβ<sup>lox/lox</sup>* mice when the intensity of the prepulse tone increased. *Crh* expression was measured in the hindbrain of *Nes-Cre:Errβ<sup>lox/lox</sup>* mice and WT mice. Indeed, *Nes-Cre:Errβ<sup>lox/lox</sup>* mice have decreased expression of *Crh* and *Crhr2* relative to WT (Figure 4b,  $F_{1,10} = 6.54$ ,  $P = 0.03$  and Figure 4c,  $F_{1,10} = 6.23$ ,  $P = 0.03$ ). These results indicate alterations in the excitatory pathway that generates a startle response, but not the inhibitory pathway arising from the pedunculopontine tegmental nucleus associated with PPI [41-43]. The increased acoustic startle response in *Nes-Cre:Errβ<sup>lox/lox</sup>* mice may thus arise from altered activity of the excitatory pathway involving *Crh* and *Crhr2* expression and the pontine reticular nucleus, bed nucleus of the stria terminalis, amygdala, and hypothalamus [37-39,43-48]. The hindbrain excitatory pathways, which include

catecholaminergic projections to the paraventricular nucleus of the hypothalamus, increase *Crh* expression in the hypothalamus, suggesting that hindbrain signaling may alter the HPA-axis feedback loop [49].

## Discussion

ERRs are involved with energy balance and metabolism [14-18]. Using mice globally deficient for *Errβ*, we have shown that *Errβ* modulates body composition, stress signaling, and hypothalamic neuropeptide expression (Table 2). *Errβ* gene dosage affected body composition and stress response with increased fat mass and corticosterone levels in *Sox2-Cre:Errβ<sup>+/lox</sup>* mice and decreased fat mass and corticosterone levels in *Sox2-Cre:Errβ<sup>lox/lox</sup>* mice (Table 1 and Figure 2). Additionally, central nervous system-specific *Errβ* deletion alters stress associated with the acoustic startle response pathways (Figure 4).

Hypothalamic expression of *Npy* and *Agrp*, orexigenic factors that increase fat mass and food intake [50-52], increased in both *Sox2-Cre:Errβ<sup>+/lox</sup>* and *Sox2-Cre:Errβ<sup>lox/lox</sup>* mice (Figure 1). These results suggest that increased anabolic neuropeptide expression may be due to central or peripheral mechanisms that are activated following global deletion of *Errβ*. Increased *Npy* and *Agrp* expression may be due to differences in leptin levels from adipose mass. Increased fat mass and lean mass were measured in *Sox2-Cre:Errβ<sup>+/lox</sup>* mice, although decreased fat mass and lean mass were measured in highly-active *Sox2-Cre:Errβ<sup>lox/lox</sup>* mice at nine months of age (Table 1). Expression of *Npy* and *leptin* are coordinately regulated, as *Npy* blunts the effects of leptin and increased leptin levels decrease *Npy* expression [23,53-55]. Thus, *Sox2-Cre:Errβ<sup>+/lox</sup>* mice may consume more food and increase *Npy* expression and fat mass due to leptin resistance; *Sox2-Cre:Errβ<sup>lox/lox</sup>* mice may increase *Npy* expression to compensate for decreased fat mass arising from increased physical activity



(Figure 1 and Table 1). In support of this, *Nes-Cre:Errβ<sup>lox/lox</sup>* mice have increased lean mass, no change in physical activity and have decreased *Npy* expression in the hindbrain [14]. Changes in body composition emerged prior to changes in body weight, suggesting that both peripheral and central signals may be altered to regulate the development of increased fat mass (Table 1).

The opposite phenotypes that are seen in the *Sox2-Cre:Errβ<sup>+/lox</sup>* and *Sox2-Cre:Errβ<sup>lox/lox</sup>* mice may arise from the ability of *Errβ* or *Errγ* to regulate gene transcription as both homodimers and *Errβ/Errγ* heterodimers [1,6,7,9,10]. *Errβ/Errγ* heterodimers have been predicted to exist, but to our knowledge it has not been directly detected *in vivo* [1]. RIP140 is a nuclear receptor corepressor that regulates cellular metabolism [56–58]. RIP140 enhanced transcriptional activity for all three mouse *Err* genes [59]. Mice

**Table 2 Summary of phenotype difference between *Sox2-Cre:Errβ<sup>+/lox</sup>*, *Sox2-Cre:Errβ<sup>lox/lox</sup>* and *Nes2-Cre:Errβ<sup>lox/lox</sup>* mice, relative to wild type (WT)**

Phenotype	<i>Sox2-Cre:Errβ<sup>+/lox</sup></i>	<i>Sox2-Cre:Errβ<sup>lox/lox</sup></i>	<i>Nes2-Cre:Errβ<sup>lox/lox</sup></i>
Body Composition			
Body weight	↑,	↓,	↑
Fat mass	↑,	↓,	NC
Lean mass	↓,	↓,	↑
Hormone and Neuropeptides			
Corticosterone	↑,	↓,	NA
Corticosterone (DY131)	↑,	↓,	NA
<i>Crh</i> expression	↑,	↑,	↓
<i>Crh</i> expression (DY131)	↑↑,	↑↑,	NA
<i>Npy</i> expression	↑,	↑,	↓
<i>Agpr</i> expression	↑,	↑,	NA
Stress Behavior			
Acoustic startle response	NA,	NA,	↑
Meal patterns			
Total pellets consumed	↑,	↑,	NC
Inter Meal interval (IMI)	NC,	↓,	↓
Satiety Ratio	↓ <sup>#</sup> ,	↓,	↓

↑ – increase, ↓ – decrease, NC – no change, ↑↑ – increase relative to levels with no DY131 treatment, NA – not available, <sup>#</sup>*P* = 0.05 relative to WT; *Nes2-Cre:Errβ<sup>lox/lox</sup>* data adapted from [14].

lacking RIP140 are lean, with increased metabolic rate and insulin sensitivity [58]. Similarly, *Sox2-Cre:Errβ<sup>lox/lox</sup>* mice are lean with increased metabolic rate (Table 1 and [14]), and *Nes-Cre:Errβ<sup>lox/lox</sup>* have increased lean mass, increased metabolic rate and insulin sensitivity [14]. Since deletion of both *Errβ* and *RIP140* exhibit similar phenotypes, this suggests that increased lean mass relative to fat mass, metabolic rate and insulin sensitivity may arise from both the RIP140 corepressor and *Errβ* [59].

ChIP-seq analysis derived from embryonic stem cells revealed that *Errβ* binds the regulatory element of two genes associated with *Crh* activity — Corticotropin releasing hormone binding protein (*Crhbp*) and Corticotropin releasing hormone receptor 2 (*Crhr2*) — as well as one gene associated with whole-body energy balance and stress responses, Cholecystokinin B receptor (*Cckbr*) [60]. We hypothesize that *Errβ*, *Crhbp* and *Crhr2* may modulate stress signaling by altering the biological activity of *Crh* in extrahypothalamic sites and/or corticosterone feedback or secretion [32,39,48,61–64]. Disruption of *Errβ*-dependent regulation of expression of *Cckbr* and/or *Crhr2* may at least partially explain the abnormal meal patterns and stress behaviors (e.g. acoustic startle response, *Crh* expression or corticosterone levels) observed in *Sox2-Cre:Errβ<sup>lox/lox</sup>*, *Sox2-Cre:Errβ<sup>+/lox</sup>*, and *Nes-Cre:Errβ<sup>lox/lox</sup>* mice [14].

Crh is expressed in the paraventricular nucleus of the hypothalamus and initiates ACTH release from the pituitary [40,65]. Crh has since been found to be synthesized in extra-hypothalamic sites, where it also acts to modulate stress response and food intake [40,65-67]. ERR family members also modulate stress responses by regulating glucocorticoid receptor activity in muscle and neuroblastoma cell lines [22,33]. Further, *Errβ* and *Crh* are expressed in similar regions of the hindbrain [29]. Here we demonstrate that *Errβ* deletion modulates corticosterone levels after exposure to restraint stress, with increased levels in *Sox2-Cre:Errβ<sup>+lox</sup>* mice and decreased levels in *Sox2-Cre:Errβ<sup>lox/lox</sup>* mice relative to WT (Figure 2). Neural connections projecting to the hypothalamus from extra-hypothalamic sites, such as the hindbrain, may also regulate hypothalamic Crh release and *Crh* expression [30,49,68-70].

Biological activity of Crh is inhibited by Crhbp, and *Errβ* binds to the promoter region of the *Crhbp* gene [60,71], which contains three ERE half sites [72]. Mice that overexpress Crhbp have increased *Crh* expression, potentially resulting from a compensatory response aimed at ameliorating disruptions in stress response [73]. Similarly, increased *Crh* expression was observed when *Errβ* was reduced (*Sox2-Cre:Errβ<sup>+lox</sup>*) or eliminated (*Sox2-Cre:Errβ<sup>lox/lox</sup>*) in somatic tissue, and Erry was activated using DY131 (Figure 3). Therefore, we propose that partial or complete deletion of *Errβ* may alter *Crh* expression by modulating transcription of *Crhbp* or *Crhr2*, resulting in altered corticosterone secretion. Furthermore, *Sox2:Errβ<sup>lox/lox</sup>* mice lack corticosterone secretion after restraint stress (Figure 2), which may result from altered *Crhr2* expression (Figure 4c) and changes in negative feedback. Therefore, brain regions that express *Crhr2* may show reduced Crh signaling (Figure 4b and 4c), as in the hindbrain [64].

*Errβ* binds to *cis*-regulatory regions of the *Cckbr* gene [60], which is expressed in the hindbrain [29,74] and the corresponding gene maps to a genomic locus of the genome associated with obesity [75]. *Cckbr* deficient mice (*Cckbr<sup>-/-</sup>*) display a similar phenotype to *Sox2-Cre:Errβ<sup>+lox</sup>* mice, and have increased body weight and food intake, which may arise from changes in Cholecystokinin (Cck) signaling (e.g. satiety), and increased metabolism [74,76]. However, *Cckbr<sup>-/-</sup>* mice also have blunted stress responses associated with anxiety-like behavior [77] and increased *Npy* expression [78], which resembles the phenotype of *Sox2-Cre:Errβ<sup>lox/lox</sup>* mice (Figure 1 and Table 1). Therefore, heterodimers of *Errβ* alone, or *Errβ* in combination with *ERRγ*, may regulate *Cckbr* transcription, thereby partially accounting for the differences in the phenotypes seen in *Sox2-Cre:Errβ<sup>+lox</sup>* and *Sox2-Cre:Errβ<sup>lox/lox</sup>* mice (Table 2). Differences in developmental compensation arising from *Errβ* and/or *Erry* may

also contribute to the phenotype differences in *Sox2-Cre:Errβ<sup>+lox</sup>* and *Sox2-Cre:Errβ<sup>lox/lox</sup>* mice.

*Nes-Cre:Errβ<sup>lox/lox</sup>* mice show increased *Erry* expression relative to WT animals [14], while mice deficient for *Erry* show increased *Errβ* expression [17]. This suggests that homozygous mice have reciprocal patterns of *Errβ* and *Erry* expression, potentially arising from developmental compensation and heterozygous mice may partially lack this compensation, contributing to phenotype differences. The *Errβ/Erry* agonist (DY131) increased *Crh* expression more when *Errβ* expression was reduced (*Sox2:Errβ<sup>+lox</sup>* mice) than when *Errβ* expression was absent (*Sox2:Errβ<sup>lox/lox</sup>* mice) (Figure 3). These results suggest that the ratio of *Errβ* to *Erry* signaling may contribute to the observed difference in *Crh* expression, *Crhr2* expression and corticosterone secretion in the two genotypes examined.

*Sox2-Cre:Errβ<sup>lox/lox</sup>* and *Sox2-Cre:Errβ<sup>+lox</sup>* mice have alterations in the HPA axis (Figures 2 and 3). *Npy*, which modulates corticosterone levels [79], is altered in *Sox2-Cre:Errβ<sup>+lox</sup>* and *Sox2-Cre:Errβ<sup>lox/lox</sup>* (Figures 1 and 2). Both Crh and *Npy* have been implicated in modulating the acoustic startle response [32,36,40,80], which is altered in *Nes-Cre:Errβ<sup>lox/lox</sup>* mice (Figure 4 and [14]). Given the results reported here, the phenotype differences between *Sox2-Cre:Errβ<sup>+lox</sup>* and *Sox2-Cre:Errβ<sup>lox/lox</sup>* mice may specifically arise from altered *Crh* expression and corticosterone levels as a result of changes in *Errβ*-dependent regulation of *Crhbp* or *Crhr2* transcription, as well as through interactions of *Errβ* with *Erry*. However, since little is known about *Errβ/Erry* heterodimers or how different *Err* family homo and heterodimers may potentially regulate *Crhbp* or *Crhr2* transcription deserves further investigation.

Our data suggest that central *Errβ* modulates stress responses, food intake and body weight, although it remains to be determined whether peripheral *Errβ* also modulates components of the HPA axis and acoustic startle response. *Nes-Cre:Errβ<sup>lox/lox</sup>* mice lack *Errβ* in the hindbrain and have decreased expression of *Crh*, *Crhr2* and *Npy* [14], suggesting that neuromodulators involved with the acoustic startle response reside in the hindbrain to modulate stress and anxiety. However, other changes in neural circuitry (e.g. altered *Cckbr* expression) regulating the acoustic startle response in *Nes-Cre:Errβ<sup>lox/lox</sup>* mice are likely to exist and remain to be identified.

## Conclusions

Mice heterozygous for *Errβ* deletion have increased fat mass and stress hormone secretion after restraint stress, while those homozygous for *Errβ* deletion have decreased fat mass and secrete higher baseline levels of stress hormones. These effects may be modulated by components of the HPA axis, such as *Crh*, *Crhbp*, *Crhr2*, *Npy* or *Cckbr*. Central *Errβ* signaling influences stress associated



behavior (e.g. the acoustic startle response), possibly through regulation of *Npy*, *Crh* and *Crhr2* expression in the hindbrain or hypothalamic projections to the amygdala [32,62,63,80]. Since the neural circuitry controlling the acoustic startle response is well-conserved between rodents and humans [36,81], these data suggest that ERR $\beta$  or ERR $\gamma$  may be promising candidates for pharmacological treatment of excessive anxiety or stress levels in humans.

## Methods

### Animals, housing, food intake, and physical activity measurement

*Sox2-Cre:Err $\beta^{lox/lox}$* , *Sox2-Cre:Err $\beta^{+/lox}$* , and wild-type (WT) (*Err $\beta^{lox/lox}$* ) mice were generated as previously described [26]. Briefly, *Err $\beta$*  mice have a conditional allele, with loxP sites flanking exon 2 of the *Err $\beta$*  gene that encodes the DNA binding domain (exon 2) [26]. Expression of cre recombinase will excise the loxP-flanked exon 2 from the *Err $\beta$*  gene. *Sox2-Cre* deletes *Err $\beta$*  from all embryonic tissues and *Nestin-Cre* deletes *Err $\beta$*  from developing neural tissue. *Sox2-Cre:Err $\beta^{lox/lox}$*  mice completely lack functional *Err $\beta$*  because both alleles have been removed. *Sox2-Cre:Err $\beta^{+/lox}$*  have one wild-type allele of the *Err $\beta$*  gene, since the other allele has been excised by the loxP sites. These two mouse lines enable us to address possible phenotypic differences due to differences in gene dosage. Wild-type (WT) mice used for these studies were homozygous for the floxed *Err $\beta$*  allele. Mice were maintained on a 12:12 hour light–dark cycle in a temperature- and humidity-regulated vivarium and had *ad libitum* access to standard laboratory chow (2018, Harlan-Teklad, Harlan Laboratories, Frederick, MD, USA) and water at all times. Different cohorts of mice were analyzed at three weeks and nine months of age. Food intake data and physical activity levels were collected as previously described [14]. Physical activity levels were measured by detecting and counting horizontal beam breaks in a 40 cm  $\times$  40 cm  $\times$  30 cm plexiglass chamber (Digiscan, Accuscan Instruments, Columbus, OH). All experimental procedures were performed in accordance with the Johns Hopkins University School of Medicine Institutional Animal Care and Use Committee and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

### In situ hybridization assay (ISH) and quantitative real-time PCR

ISH was performed as previously described [14,82]. Briefly, digoxigenin cRNA probes to *Npy* and *agouti-related protein* (*Agrp*) were synthesized using the Brain Molecular Anatomy Project (BMAP) library containing sequence-verified expressed sequence tags. BMAP clones were purified using a PureLink plasmid miniprep kit per manufacturer's protocol (Invitrogen, Carlsbad, CA, USA) and synthesized using

a T3 or T7 RNA polymerase (Roche, Indianapolis, IN, USA). The riboprobe was purified using an RNA extraction kit per manufacturer's protocol (RNeasy, Qiagen, Valencia, CA, USA). Brains were collected from mice, fresh frozen in OCT compound (Tissue Tek, Fisher Scientific, Pittsburgh, PA, USA), and cut using a cryostat into 25- $\mu$ m sections onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA, USA). Hindbrain dissection, mRNA extraction and quantitative real-time PCR was conducted as previously described [14,83]. Briefly, RNA was extracted (RNeasy, Qiagen, Valencia, CA, USA) and cDNA was synthesized using 1  $\mu$ g of mRNA using Superscript II reverse transcriptase (Invitrogen) and random primers (Invitrogen). Quantitative PCR primer sequences were obtained from PrimerBank and conducted for *Crh*: fwd – 5' CCTCAGC CGTTCATGATCC 3' and rev – 5' GCGGAAAAAGTTA GCCGCAG 3'; *Crhr2*: fwd – 5' CATCCACCACGTCCGA GAC 3' and rev – 5' CTCGCCAGGATTGACAAAGAA 3' and *18S* fwd – 5' GCAATTATTCCCCATGAACG 3' and rev- 5' GGCTCACTAAACCATCCAA 3'. The Ct value generated was normalized to *18S* in order to obtain a  $\Delta$ Ct value, followed by generating the  $2^{-\Delta\Delta$ Ct value by normalizing the data to control animals as previously described [84].

### Restraint stress test, corticosterone radioimmunoassay, and DY131 injections

Baseline blood glucocorticoid levels were measured and mice were placed into a restraining tube (one mouse/tube) for one hour. Upon removal from the restraining tube, blood samples were collected again. Animals were then returned to their housing and blood samples were collected after a one-hour recovery period. Blood was collected in heparin-coated tubes and centrifuged at 3800 rpm for 20 min at 4°C. Corticosterone assays were performed with a radioimmunoassay kit for corticosterone per manufacturer's directions (MP Biomedicals, Solon, OH, USA). DY131 (Tocris, Bristol, BS11, United Kingdom) at a dose of 10  $\mu$ M/g body weight was injected, and data for meal patterns collected as previously described [14].

### Prepulse inhibition (PPI) of acoustic startle response

Startle reactivity and PPI were measured using two startle chambers located inside a sound-attenuating chamber (San Diego Instruments, San Diego, CA, USA). Mice were placed in a Plexiglass tube within the soundproof PPI box for a five-minute acclimation period, which provides exposure to a continuous background noise (70 dB) to elicit an increase in startle amplitude [43]. Mice were then exposed for five minutes without any startle stimulus. The PPI session then began and mice were randomly exposed to the following trials: pulse alone (120 dB), no stimulus, or five prepulse combinations (a 20 ms non-startling prepulse at 74, 78, 82, 86, or 90 dB, followed by an 80 ms

startle stimulus at 120 dB). The force from the startle reaction was recorded by an accelerometer with SR-LAB software (San Diego Instruments). Results were analyzed by PPI percentage, which was calculated as:

$$\frac{(\text{mean startle amplitude on pulse alone}) - (\text{mean startle amplitude on prepulse})}{(\text{mean startle amplitude on pulse alone})}$$

### Statistical analysis

All value comparisons were made using one-way ANOVA to identify individual differences between groups, and  $P < 0.05$  was considered significant (Statistica v.8.0, Tulsa, OK, USA).

### Abbreviations

ACTH: Adrenocorticotrophic hormone; Agrp: Agouti-related protein; Crh: Corticotropin-releasing hormone; Crhbp: Corticotropin releasing hormone binding protein; Crhr2: Corticotropin releasing hormone receptor 2; ERR: Estrogen-related receptor; ERRE: Estrogen-related receptor response element; HPA: Hypothalamic-pituitary-adrenal axis; IMI: Inter meal interval; ISH: *In situ* hybridization; Npy: Neuropeptide Y; PPI: Prepulse inhibition; WT: Wild-type.

### Competing interests

The authors declare that they have no competing interests.

### Authors' contributions

MSB, GWW, and SB; MSB conducted all research; RDS provided technical support for measuring corticosterone levels; MSB analyzed data; MSB drafted the manuscript and MSB, GWW, and SB edited the final version. All authors read and approved the final manuscript.

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