

# Sirtuins and neuropeptide y downregulation in Flinders Sensitive Line rat model of depression

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## Original Article

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### Abstract

Since the NAD<sup>+</sup>-dependent histone deacetylases sirtuin-1 (SIRT1) and sirtuin-2 (SIRT2) are critically involved in epigenetics, endocrinology and immunology and affect the longevity in model organisms, we investigated their expression in brains of 3-month-old and 14–15 months old rat model of depression Flinders Sensitive Line (FSL) and control Flinders Resistant Line (FRL) rats. In view of the dysregulated NPY system in depression, we also studied NPY in young and old FSL to explore the temporal trajectory of depressive-like-ageing interaction. *Sirt1*, *Sirt2* and *Npy* mRNA were determined using qRT-PCR in prefrontal cortex (PFC) from young and old FSL and FRL, and in hippocampi from young FSL and FRL. *PFC*: *Sirt1* expression was decreased in FSL ( $p = 0.001$ ). An interaction between age and genotype was found ( $p = 0.032$ ); young FSL had lower *Sirt1* with respect to both age ( $p = 0.026$ ) and genotype ( $p = 0.001$ ). *Sirt2* was lower in FSL ( $p = 0.003$ ). *Npy* mRNA was downregulated in FSL ( $p = 0.001$ ) but did not differ between the young and old rat groups. *Hippocampus*: *Sirt1* was reduced in young FSL compared to young FRL ( $p = 0.005$ ). There was no difference in *Sirt2* between FSL and FRL. *Npy* levels were decreased in hippocampus of young FSL compared to young FRL ( $p = 0.003$ ). Effects of ageing could not be investigated due to loss of samples. To conclude, i this is the first demonstration that SIRT1 and SIRT2 are changed in brain of FSL, a rat model of depression; ii the changes are age-dependent; iii sirtuins are potential targets for treatment of age-related neurodegenerative diseases.

### Significant outcomes

Major depressive disorder is the predominant cause of ‘Years of life lived with disability’ and ‘Years of life lost because of premature death’. Severity and prevalence of depression increase with age, but the molecular underpinnings of the disorder have only partially been elucidated. Since the NAD<sup>+</sup>-dependent histone deacetylases sirtuin-1 and sirtuin-2 affect the longevity in model organisms, we assessed *Sirt1* and *Sirt2* mRNA in the prefrontal cortex and hippocampus of young and old FSL rats, a well-characterised model of depression, and control FRL rats with the aim to explore the depressive-like ageing trajectory. *Sirt1* levels were decreased in the young FSL rats compared to the aged FSL as well as to the age-matched young FRL rats (cf Table 1); the former finding is of interest for depression-like-ageing interaction and the latter for elucidating depression pathology. Concluding, this is the first demonstration that SIRT1 and SIRT2 are changed in brain of the FSL rats and that the changes are age-dependent. Thus, sirtuins are potential targets for treatment of age-related neurodegenerative diseases.

### Limitations

(i) The small number of animals, (ii) due to lack of samples we could not determine the protein, and (iii) due to the loss of samples we could not compare hippocampal tissue from ‘young’ and ‘aged’ rats (cf Table 1).

### Introduction

Major depressive disorder (MDD) is a devastating, life-threatening disorder with a major and increasing burden on the public health (Greenberg et al., 2015). The understanding of MDD aetiology and pathophysiology is limited (McEwen et al., 2015) and optimal treatments are lacking. Using the currently available drugs, 30–40% of the patients fail to respond adequately

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**Table 1.** Significant outcomes and limitations

1. <i>Sirt1</i> expression was significantly decreased in the depressed FSL rats compared to the control FRL strain.
2. <i>Sirt1</i> expression was significantly increased in the 'old' FSL compared to the 'young' FSL.
3. <i>Sirt2</i> expression was significantly decreased in the depressed FSL rats compared to the control FRL strain.
4. <i>Npy</i> expression was significantly decreased in the depressed FSL rats compared to the control FRL strain.
5. The main limitations of the study are the small number of animals and that due to the loss of samples we could not compare hippocampal tissue from 'young' and 'old' rats

(Tundo et al., 2015). However, ketamine and esketamine (the latter approved by FDA and EMA in 2020 for treatment-resistant depression) have shown acute antidepressant effect (Henter et al., 2021). Moreover, recent papers translationally based on rat studies (Cohen et al., 2012) have shown that insufflated NPY alleviated MDD symptoms (Mathé et al., 2020) and PTSD symptoms (Sayed et al., 2018).

Individuals with depression have an enhanced risk of developing age-related diseases. Moreover, persons diagnosed with repeated episodes of MDD have decreased life expectancy, often due to cardiovascular disease and diabetes (Mezuk et al., 2008; Walker et al., 2015). In a longitudinal study of ageing (ELSA), an association between the duration of depressive symptoms and mortality risk has been demonstrated (White et al., 2016). However, the underlying biological mechanisms have only in part been elucidated. Of particular interest for our work that focuses on depression and ageing are sirtuins, a family of 7 NAD<sup>+</sup>-dependent histone deacetylases (HDACs) that alter the chromatin structure through deacetylation of histones resulting in altered gene expression (Sun et al., 2013; Lu et al., 2018). Sirtuins have several targets including the histone modifications H4K16ac and H3K18ac as well as H3K9m3 (Vaquero et al., 2004, 2006; Vaquero et al., 2007; Eskandarian et al., 2013). Thus, as HDACs, sirtuins regulate several essential cellular processes, such as mitochondrial activity, synaptic plasticity, apoptosis, cellular stress resistance, inflammation and memory processes through epigenetic marking (Brunet et al., 2004; Gao et al., 2010; Michan et al., 2010; Yoshizaki et al., 2010; Wątroba and Szukiewicz, 2016). With regard to ageing, sirtuins also alter the longevity in model organisms, where an extra copy of *Sirt2*, an orthologue to the mammalian sirtuin genes, extended the replicative lifespan in yeast by 50%, while a deletion of the gene shortened the lifespan (Haigis and Guarente, 2006). Thus, sirtuins may play an important role in ageing. Indeed, several studies suggest their role in the pathogenesis of many age-related diseases, including neurodegenerative diseases (reviewed in Wątroba and Szukiewicz, 2016).

Furthermore, several studies have shown that alterations of sirtuin levels have been associated with depression-like behaviour in animal models and depression in humans. For example, a sparse whole genome sequencing of a large population of depressed patients identified alterations of two single nucleotide polymorphisms (SNPs) that contributed to risk of MDD, with one of them located near the *SIRT1* gene (CONVERGE consortium, 2015). Moreover, a decrease in *SIRT1* and *SIRT2* expression in leukocytes was suggested to be a state marker in MDD and bipolar disorder patients (Abe et al., 2011). In accordance with this, another study

reported decreased levels of *SIRT1* in peripheral blood from MDD patients (McGrory et al., 2018). This was further supported by finding of reduced Sirt1 activity in the dentate gyrus in a mouse model of chronic stress. Results from rodent models regarding the role of Sirt1 in depression have been conflicting. A pharmacologic inhibition of hippocampal Sirt1 activity increased depression-like behaviour, while Sirt1 upregulation led to stress resilience, with corresponding effects on dendrite length and spine plasticity for dentate gyrus granule neurons (Abe-Higuchi et al., 2016). Ferland et al. (2013), in contrast to Abe-Higuchi, demonstrated that mice subjected to chronic stress had increased Sirt1 activity in dentate gyrus. In addition, mice with a *Sirt1* knockout in brain showed decreased anxiety and mice globally overexpressing *Sirt1* showed increased anxiety (Libert et al., 2011), and the Sirt1 activator Resveratrol had an antidepressant-like effect in rat models of depression. However, resveratrol is hypothesised to also act through other pathways leading to antidepressant-like effect (Howitz et al., 2003; Hurley et al., 2014; Liu et al., 2014). With regard to Sirt2, mice exposed to chronic social defeat stress had increased Sirt2 mRNA and protein levels in the prefrontal cortex (PFC), whereas repeated injections of imipramine reduced the Sirt2 levels (Erburu et al., 2015). Administration of a Sirt2 inhibitor into the mouse PFC reversed the chronic mild stress-induced elevated PFC Sirt2 levels and depression-like behaviour (Erburu et al., 2017).

Neuropeptide Y (NPY) is the most abundant peptide in mammalian brain and decreased NPY levels have been demonstrated in the cerebrospinal fluid of MDD and posttraumatic stress disorder (PTSD) patients as well as bipolar patients at risk for suicide. These findings have been replicated preclinically in models such as the Flinders Sensitive Line (FSL) an extensively characterised model of depression, displaying depressive-like behaviour, and Flinders Resistant Line (FRL) rats (Heilig et al., 2004; Overstreet et al., 2005; Wu et al., 2011; Cohen et al., 2012; Overstreet and Wegener, 2013; Sandberg et al., 2014; Thorsell and Mathé, 2017). FSL rats display several features that resemble the human depression, including elevated REM sleep, psychomotor retardation and increased immobility in the forced swim test (Overstreet et al., 2005; Overstreet and Wegener, 2013). Additionally, several molecular features are shared with human depression, such as elevated proinflammatory cytokine IL-6, lower central expression of the glial-specific protein S100B and complement factor C3 in several brain regions; shortened telomere length, dysregulated NPY system as well as abnormalities in the glutamatergic system (Jimenez-Vasquez et al., 2000, 2007; Hascup et al., 2011; Melas et al., 2012; Wei et al., 2014, 2015; Strenn et al., 2015; Du Jardin et al., 2017). Sirt2 has been shown to modulate the glutamate system (Erburu et al., 2017). Thus, in the PFC of the FSL rat, no differences in glutamate dynamics were observed in brains of young (3–6 months old) FRL and FSL rats, but a significant increase in resting glutamate levels was found in the aged (12–15 months old) FSL compared with the young FSL and age-matched FRL rats. In FSL, percent change in glutamate release during stress was also higher in the aged rat compared to younger rat (Hascup et al., 2011). These findings confirmed and extended interactions between depression and age in animal models. In view of these findings, we investigated the mRNA expression levels of *Sirt1*, *Sirt2* as well as *Npy* in the FSL and the control FRL rat strains. The behavioural and associated brain differences between FSL and

FRL, inter alia in NPY expression, are well documented and we measured NPY as a positive control. In addition, we have previously found NPY changes with age (Husum et al., 2006). The analyses were performed in the PFC and hippocampus, brain regions implicated in MDD and rodent depression models (Overstreet et al., 2005; Neumann et al., 2011; Overstreet and Wegener, 2013).

## Materials and methods

### Animals and tissue samples

FSL and FRL rats were maintained at the Animal Facility, Karolinska Institutet, Huddinge, under controlled conditions of temperature ( $22 \pm 1^\circ\text{C}$ ), relative humidity (45–55%) and daylight cycle (12:12 h, lights on at 6:00 am). Standard rat chow and tap water were available *ad libitum*. The PFC and hippocampi were dissected according to Glowinski and Iversen (1966) and stored at  $-80^\circ\text{C}$  until subsequent analyses. Expression levels of *Sirt1*, *Sirt2* and *Npy* were determined in the PFC of male ('young' = 3-month-old and 'aged' = 14–15 months old) FSL young  $n = 4$ , FSL aged  $n = 7$ , FRL young  $n = 5$  and FRL aged  $n = 9$ . Since hippocampal tissue from the aged rats was lost, we could only analyse tissue from the 3-month-old rats (FSL  $n = 8$ ; FRL  $n = 7$ ). All experimental work was approved by the Ethical Committee for protection of animals at the Karolinska Institutet.

### RNA extraction and reverse transcription

Tissue-Tearor (Biospec Products Inc., Bartlesville, OK, USA) was used to homogenise the brain samples. Total RNA was extracted using AllPrep DNA/RNA mini kit according to the manufacturer's protocol (Qiagen, Hilden, Germany) and was treated with DNase I to eliminate the contamination of DNA (Qiagen). The total RNA concentrations were measured spectrophotometrically using NanoDrop 2000 (Thermo Fisher Scientific, Rockford, IL, USA). The RNA quality of 10 samples was examined by Agilent 2100 BioAnalyzer (Agilent Technologies, Germany). Complementary DNA (cDNA) was synthesised by reverse transcription of total RNA using SuperScript III First-Strand Synthesis System for qPCR with random hexamers according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). cDNA was stored at  $-20^\circ\text{C}$  and RNA at  $-80^\circ\text{C}$ .

### Gene expression by quantitative RT-PCR

Quantitative RT-PCR was applied to measure mRNA levels of the genes *Sirt1*, *Sirt2* and *Npy* in PFC and hippocampal tissue. Amplifications of these genes and the housekeeping gene *Actin* were performed in triplicates of the cDNA samples using Power-SYBR Green (Applied Biosystems Inc., Foster City, CA, USA) and the QuantStudio™ flex 6/7 System (Thermo Fisher Scientific). Sample to sample variation in *Sirt1*, *Sirt2* and *Npy* expression levels were corrected for by normalising to that of *Actin*, and interplate variation was corrected for by including a positive control/calibrator sample in each plate. The relative gene expression, fold change, was calculated according to the comparative  $C_t$  method using the formula:  $2^{-\Delta\Delta C_t}$ . The primers sequences, restricting the amplification to mRNA, were (written 5' to 3') *Sirt1* Fw:TACCCCATGAAGTGCCTCAA, *Sirt1* Rv:AAGTTTGCCATACTCGCCAC, *Sirt2* Fw:GCAGGAATCCCTGACTTCCG, *Sirt2* Rv: TGCCAGGATAGAGCTCCTTA, *Npy* Fw:CGCTCTGCG

ACACTACATCA, *Npy* Rv:TGGGGGCATTTTCTGTGCTT, *beta-actin* Fw: TAAGGCCAACCGTGA AAAAGAT and *beta-actin* Rv: GTGGTACGACCAGAGGCATAC.

## Statistical analysis

The Levene's test and Shapiro–Wilk test were used for assessing the homogeneity of variance and the normality of the data, respectively. The statistical significance of differences in PFC gene expression between the four groups (young and aged FRL and FSL rats) were tested applying two way-ANOVA followed by a *post hoc* analysis with a Bonferroni-corrected independent sample *t*-test. As the beforehand mentioned assumptions were met for hippocampus, a two-tailed independent test was used. Statistical significance was assigned for  $p < 0.05$ . Outliers marked in the figure legends were identified based on the values below  $Q1 - 1.5 * IQR$  or above  $Q3 + 1.5 * IQR$ . The analyses were made in R (Rstudio version 1.2.1335).

## Results

### Prefrontal cortex

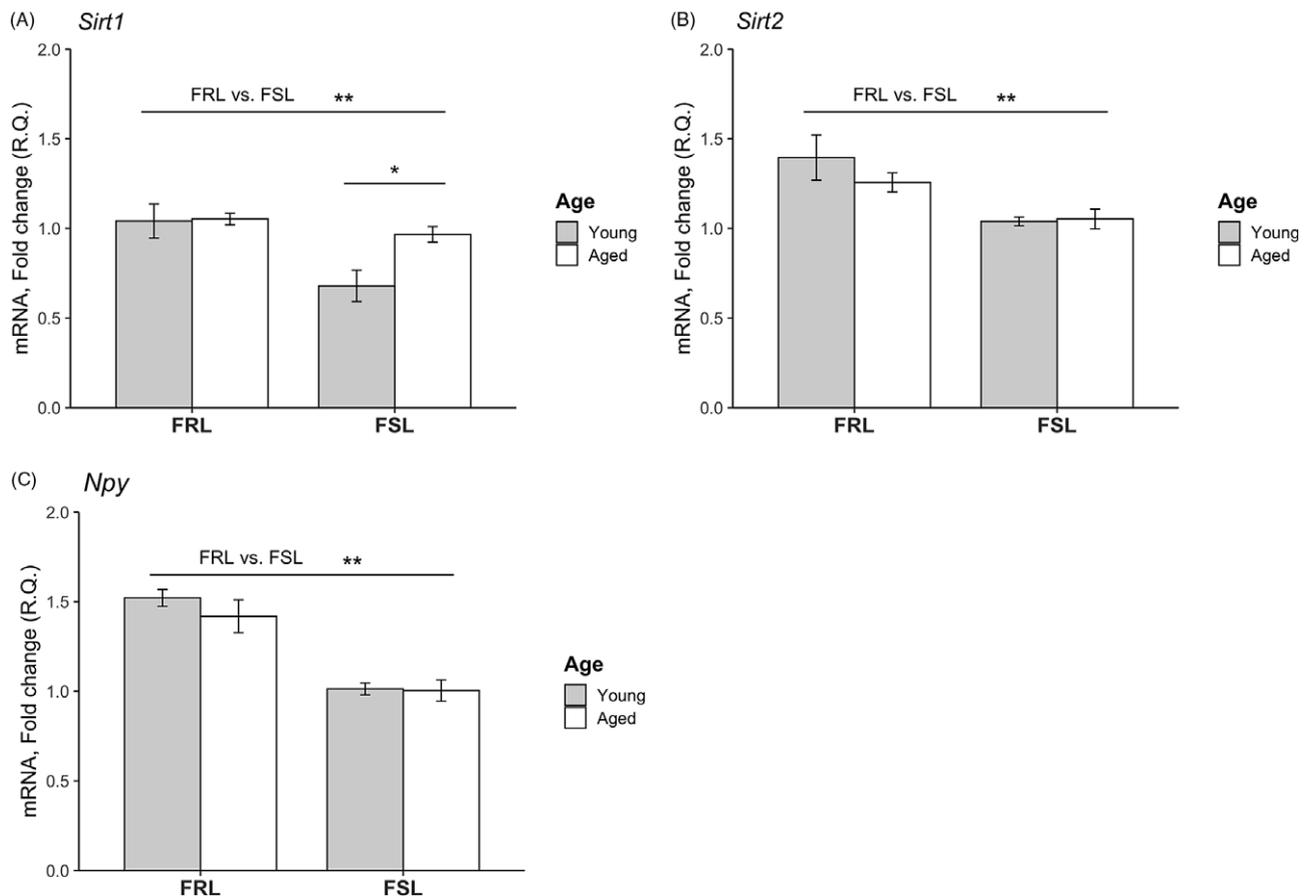
Expression levels of *Sirt1*, *Sirt2* and *Npy* in PFC of young and old FSL and FRL rats are depicted in Fig. 1. *Sirt1* levels were significantly decreased in the FSL compared to the FRL rats ( $F[1,21] = 13.8$ ;  $p = 0.001$ , two-way ANOVA). *Sirt1* levels were also significantly different between the age groups ( $F[1,21] = 6.22$ ;  $p = 0.022$ ), and a significant interaction between age and genotype was found ( $F[1,21] = 5.26$ ;  $p = 0.032$ ); the young FSL rats had a significantly lower *Sirt1* expression with respect to both age ( $p = 0.026$ , *post hoc* test) and genotype ( $p = 0.001$ , *post hoc* test). *Sirt2* levels were significantly lower in the FSL ( $F[1,21] = 14.1$ ;  $p = 0.001$ ) but did not differ between the age groups. With regard to *Npy*, in similarity to *Sirt2*, *Npy* mRNA levels in PFC were downregulated in the FSL compared to the FRL rats ( $F[1,21] = 31.3$ ;  $p < 0.001$ ) but did not differ between the age groups.

### Hippocampus

Expression levels of *Sirt1*, *Sirt2* and *Npy* in hippocampus of young FSL and FRL rats are depicted in Fig. 2. Since samples from the old FSL and FRL rats were lost in processing, effects of ageing could not be investigated. In similarity to findings in the PFC, hippocampal *Sirt1* levels were reduced in young FSL compared to young FRL rats ( $p = 0.005$ ). There was no detectable difference in *Sirt2* levels between the FSL and FRL. Lastly, *Npy* levels were decreased in the hippocampus of the young FSL compared to young FRL rats ( $p = 0.003$ ).

## Discussion

Our main findings are lower expressions of *Sirt1* and *Sirt2* in the PFC of the FSL compared to the FRL strain. Moreover, *Sirt1* was also decreased in young FSL rats. Relationships of these changes to pathology and the disease trajectory with age are not clear but point to new targets to elucidate depression pathophysiology and develop novel treatments. Vicissitudes of *Sirt1* and *Sirt2* expression have been reviewed in the Introduction section. Differences in species used (mouse and rat) as well as a variety of experimental procedures have led to divergent, sometimes conflicting results.

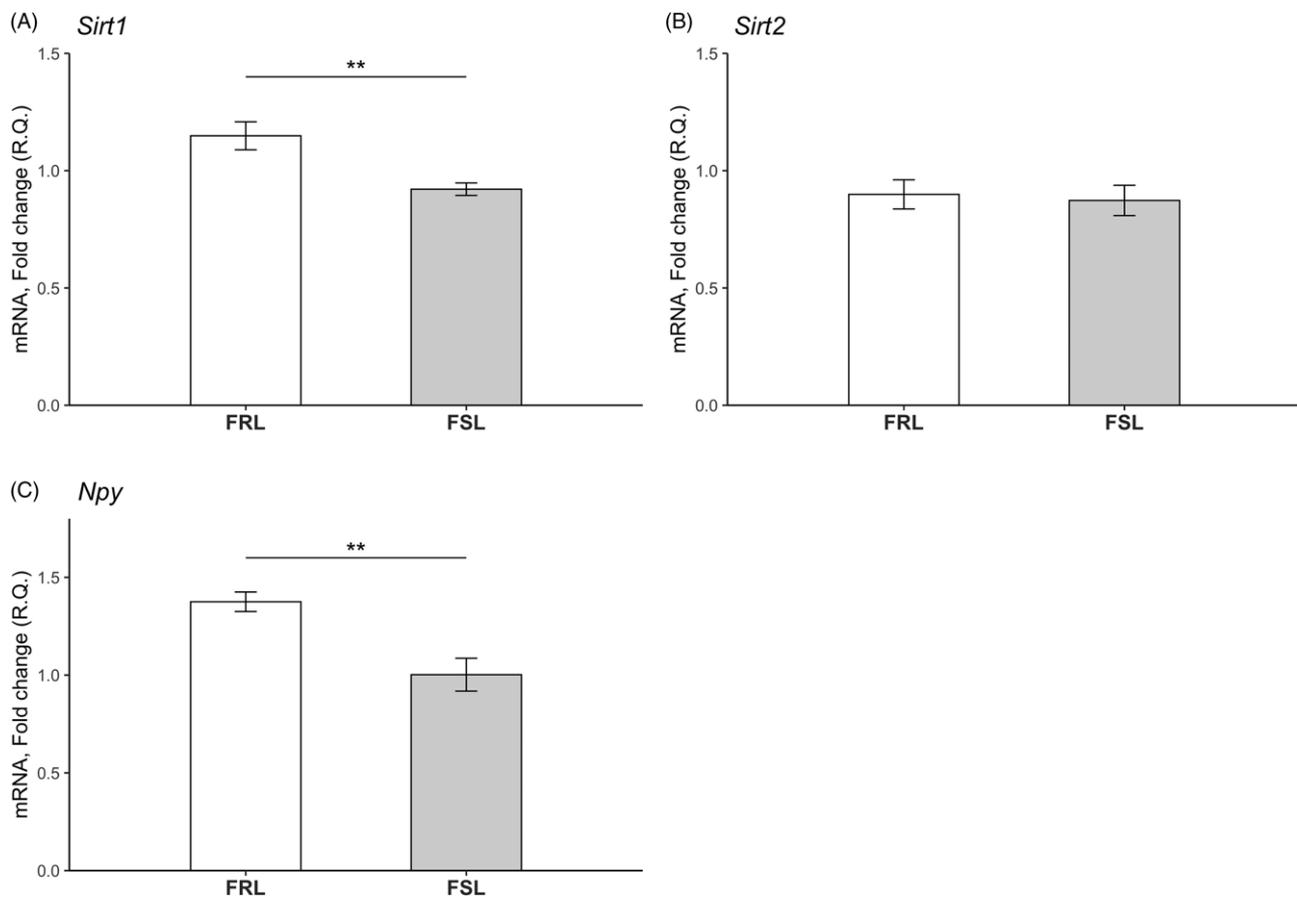


**Fig. 1.** Relative mRNA levels of the *Sirt1*, *Sirt2* and *Npy* genes in the prefrontal cortex (PFC) of young and aged FSL and FRL rats. (A) *Sirt1* levels were significantly decreased in FSL compared to FRL. Moreover, the levels were lower in the young FSL rats compared to the aged FSL. (B) *Sirt2* levels were lower in FSL compared to FRL. No difference with regard to age was found. (C) *Npy* levels were reduced in FSL compared to FRL. No difference with regard to age was found. The relative quantification (R.Q.) bars represent mean values and the error bars represent the standard error of the mean (SEM).  $n = 4$  young FSL;  $n = 7$  old FSL;  $n = 5$  young FRL;  $n = 9$  old FRL; \*  $p < 0.05$ , \*\*  $p < 0.01$ .

Nevertheless, a tentative conclusion is that reduced *Sirt1* and *Sirt2* expressions are associated with depression and anxiety-like behaviours. The downregulated *Sirt1* levels in PFC and hippocampus of young FSL rats were partly in agreement with reports showing a reduction of *Sirt1* mRNA levels in hippocampus but not in medial PFC using stress-induced behavioural models of depression in 2-month-old mice (Abe-Higuchi et al., 2016). In line with this, 2-month-old mice susceptible to chronic social defeat stress had reduced *Sirt1* mRNA levels in hippocampus compared to the controls and stress-resilient mice (Kim et al., 2016). With regard to the downregulated *Sirt2* levels, we found in the PFC of the FSL rats, studies in mice focusing on chronic stress and the disrupted vesicular glutamate transporter 1 (*VGLUT1*<sup>+/-</sup>) models reported a link between reduction in *Sirt2* mRNA and *Sirt2* activity in PFC (Erburu et al., 2015, 2017; Munoz-Cobo et al., 2017). In those experiments, both antidepressants and a selective *Sirt2* inhibitor reduced *Sirt2* levels in PFC as well as depression-like behaviour. Probable explanations are that the discrepancies are due to species differences and that FSL rats represent a genetic model of depression clearly different from the stress models and the model of disrupted VGLUT transporter. Furthermore, the differences between our *Sirt2* findings and the aforementioned studies in PFC could reflect the heterogeneity in underlying molecular pathways of depression-like behaviour. Another variable that has to be considered in interpreting the results is that of circadian rhythm. For instance, *SIRT1* expression also shows circadian rhythm (Chang

and Guarente, 2013) and, in a different context, we have shown that circadian rhythm fluctuations in NPYergic system and HPA axis underlie differences in vulnerability to stress responses (Cohen et al., 2015). However, since we controlled for the time of sacrifice, this factor can be ruled out with regard to results within our study but could have a bearing on comparison with other studies. The *Sirt1* mRNA expression in the PFC region was decreased in young FSL rats only, while the levels in the older group did not significantly differ between the FSL and FRL. These findings suggest that during the ageing trajectory, there are compensatory mechanisms that counteract the reduction of *Sirt1* levels found in young depressed FSL rats; elucidation of such mechanisms should contribute to understanding the depression-ageing interaction. Finally, our findings are consistent with the reports of reduced *SIRT1* and *SIRT2* levels in leukocytes from patients with MDD compared to healthy controls (Abe et al., 2011; McGrory et al., 2018).

Findings of reduced *Npy* levels in both PFC and hippocampus of the FSL rats are in agreement with previous results (Jiménez-Vasquez et al., 2000 and 2007; Wu et al., 2011; Thorsell and Mathé, 2017) and are thus a quality indicator of procedures used in this study. With regard to the effect of age, in one study (Husum et al., 2006), we found that ageing caused an exacerbated loss of NPY immunoreactive cells in the dentate gyrus in the FSL strain compared with FRL. The aged FSL rats also had shortened 5-HT-IR fibres in the dorsal hippocampus, indicative of an



**Fig. 2.** Relative mRNA levels of the *Sirt1*, *Sirt2* and *Npy* genes in the hippocampi of young FSL and FRL rats. (A) *Sirt1* levels were significantly decreased in young FSL compared to young FRL. (B) No differences in *Sirt2* levels between young FRL and young FSL were detected.  $n = 8$  FSL;  $n = 7$  FRL (one outlier in the FSL group in the *Sirt1* gene has been excluded) \* $p < 0.05$ , \*\* $p < 0.01$ . (C) *Npy* levels were significantly decreased in young FSL compared to young FRL. The relative quantification (R.Q.) bars represent mean values and the error bars represent the standard error of the mean (SEM)  $n = 8$  FSL;  $n = 7$  FRL (one outlier in the FSL group in the *Sirt1* gene has been excluded) \* $p < 0.05$ , \*\* $p < 0.01$ .

impaired 5-HT innervation of this area, compared with FRL. These results are not contradictory due to the basic difference in methodology and call for in depth study of ageing effect on the NPYergic system.

The strengths of the study – the first of its kind – are the findings of significant decrease in *Sirt1* and *Sirt2* expression in the FSL compared to the FRL strain as well as an increase in *Sirt1* with ageing in brain regions of relevance for depression. In addition, we confirmed the decreased *Npy* expression in FSL versus FRL strains. These findings contribute to mapping the molecular underpinnings of depression and the ageing–depression temporal trajectory and have a potential to contribute to development of novel therapeutic targets.

**Authors contributions.** AAM: concept and initiative, breeding of animals, preparation of brains, and tissue sample contribution; MS and PE: experimental work; all authors: data analysis and interpretation; MS and AAM: draughting the manuscript. MS, PE, CL, AL and AAM: final manuscript and approval.

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**Conflict of interest.** None.

**Statement of interest.** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Animal welfare ethical statement.** All experimental work was approved by the Ethical Committee for protection of animals at the Karolinska Institutet.

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