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ORIGINAL ARTICLE

Lipocalin-2 regulates adult neurogenesis and contextual discriminative behaviours

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In the adult mammalian brain, newborn granule cells are continuously integrated into hippocampal circuits, and the fine-tuning of this process is important for hippocampal function. Thus, the identification of factors that control adult neural stem cells (NSCs) maintenance, differentiation and integration is essential. Here we show that the deletion of the iron trafficking protein lipocalin-2 (LCN2) induces deficits in NSCs proliferation and commitment, with impact on the hippocampal-dependent contextual fear discriminative task. Mice deficient in LCN2 present an increase in the NSCs population, as a consequence of a G0/G1 cell cycle arrest induced by increased endogenous oxidative stress. Of notice, supplementation with the iron-chelating agent deferoxamine rescues NSCs oxidative stress, promotes cell cycle progression and improves contextual fear conditioning. LCN2 is, therefore, a novel key modulator of neurogenesis that, through iron, controls NSCs cell cycle progression and death, self-renewal, proliferation and differentiation and, ultimately, hippocampal function.

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INTRODUCTION

In the adult mammalian brain, at the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus,[1] resident neural progenitor cells give rise to new functional neurons that integrate into the pre-existing neuronal circuitry, and modulate local neural plasticity and network.[1,2] This integration is important for hippocampal integrity and function, such as the regulation of learning, memory and pattern separation,[3–5] emotion[6] and neurodegeneration.[7] As so, understanding the factors that control adult neural stem cells (NSCs) maintenance, differentiation and integration is critical.

Of interest, iron is necessary for cell growth and differentiation[8,9] and its acquisition by cells is essential for cell survival and maturation. Accordingly, several studies showed that iron controls cell cycle progression[10] or arrest,[11] and cell death-related genes.[12] On the other hand, iron is also potentially toxic due to its participation in redox reactions that lead to the formation of reactive oxygen species (ROS), thus creating an oxidative stress environment.[13] Noticeably, increasing evidence implicates ROS in the regulation of long-term potentiation (LTP) in hippocampal neurons,[14] and in neuropsychiatric pathologies and cognitive performances.[15–17]

Most mammalian cells acquire iron *via* receptor-mediated endocytosis of iron-loaded transferrin.[18] However, in recent years, lipocalin-2 (LCN2) has emerged as an alternative mechanism of physiological iron delivery and uptake.[19–21] As a member of the lipocalin family, the best described function for LCN2 concerns its secretion in the innate immune response to infection, to limit iron availability for bacterial growth.[22] Additionally, LCN2 has the ability to interact with iron through a mammalian catechol complex[19,23] and modulate iron cell content.[21] The existence of a fine-tuned mediation of intracellular iron by LCN2 is still poorly recognized, but in the context of the brain, the iron-binding capability of LCN2 was shown to be important in the regulation of hippocampal neuronal dendritic spine density and morphology,[24] with impact on structural plasticity and function. In fact, *Lcn2* deletion is associated with increased anxiety, depressive-like behaviour, spatial reference memory impairments and decreased LTP.[25] Nevertheless, the contribution of LCN2 in the regulation of cell proliferation, putatively through iron trafficking and iron-mediated oxidative stress has never been explored in the brain.

In this study, we addressed the role of LCN2 in the adult mammalian brain cell genesis, and its relevance for NSCs homeostasis maintenance, as well as for the integrity of hippocampal plasticity and function.

MATERIAL AND METHODS

Animal experiments

Experiments were performed in 2-months old male mice lacking *Lcn2* expression (LCN2-null) and the respective wild-type (Wt) littermate controls, in a C57BL/6J background. All animal procedures were conducted in accordance with EU Directive 2010/63/EU and were approved by the Portuguese national authority for animal experimentation (ID: DGV9457). Mice were intraperitoneally injected with 50 mg kg^{-1} of BrdU (Sigma Aldrich, St Louis, MO, USA) 24 h before they were killed (for fast proliferation protocol), or twice a day for five consecutive days, followed by a chase period of 28 days (for slow dividing cells analysis). To test the effect of iron chelation in cell cycle regulation, mice were intraperitoneally injected with 200 μM of deferoxamine (DFO; Sigma), along with BrdU, and killed 24 h later. To assess the capacity of iron chelation in contextual discrimination, mice were chronically treated with DFO (200 μM) by intraperitoneally injection every other day for 28 days, and in the end tested for contextual fear conditioning. Animals were also pharmacologically treated with the antioxidant agent N-acetylcysteine (Sigma) by intraperitoneally injection with 300 mg kg^{-1}, and killed 24 h later. The respective control groups were injected with saline.

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Immunohistochemistry and imaging

Brains were fixed by transcardial perfusion with 4% paraformaldehyde, sectioned in a cryostat and incubated overnight with primary antibodies, followed by the respective fluorescent secondary antibodies. Fluorescence images were acquired with the Olympus Fluoview FV1000 confocal microscope (Olympus, Hamburg, Germany) and the number of double-positive cells calculated using Olympus Fluoview FV1000 software.

Contextual fear conditioning

Contextual fear conditioning was conducted for 2 days and each test session was scored. On the conditioning day, mice were placed in a chamber with a stainless grid floor and pairings between light and shock (1 s, 0.5 mA) were delivered, spared from each other with an interval of 20 s (Figure 3a). On day 2, mice were re-exposed to the conditioning chamber for 3 min for contextual fear memory and 2 h later presented to a novel context, where the grid was removed, black plastic inserts covered the floor and the walls of the chamber, which was also scented with a paper towel dabbed with vanilla extract. The session consisted of a 3-min trial and animal freezing was scored for the entire session and defined as the complete absence of motion for a minimum of 1 s.

NSCs culture

Stem cells were cultured from 1–3 days-old Wt and LCN2-null mice SGZ, as an adaptation of the protocol described elsewhere.[26] Parameters analysed included cell proliferation, self-renewal, cell cycle, cellular viability and death, and ROS levels. Cell treatments included the exposure of LCN2-null-derived neurospheres to recombinant mouse LCN2 protein (R&D Systems, Minneapolis, MN, USA), at a concentration of 100 ng ml^{-1} for 24 or 72 h, and to 2 μM of DFO for 24 h. Also, Wt cultures were treated with 500 μM of ferric ammonium citrate (Sigma) for 24 h.

For a more detailed description of the experimental procedures, please see Supplementary information.

Statistical analyses

All experiments were performed and analysed by the same experimenter, blind to the animals' genotype or group treatment under assessment. Animals were assigned to groups according to their genotypes, and no method of randomization was applied. Sample sizes were determined by power analyses based on previously published studies.[25] Variables followed a Gaussian distribution as revealed by the D'Agostino and Pearson normality test. Data are reported as mean ± standard error (s.e.m.). Statistical significant differences between groups were determined using two-tailed Student's *t*-test for two comparisons, and two-way analysis of variance, followed by Bonferroni's multiple comparison test for multiple comparisons. Values were considered to be statistically significant for $P \leqslant 0.05$ (* or $^{\#}$), $P \leqslant 0.01$ (** or $^{\#\#}$), $P \leqslant 0.001$ (*** or $^{\#\#\#}$) and $P \leqslant 0.0001$ (**** or $^{\#\#\#\#}$). For the *in vitro* analysis, the number of independent experiments is specified in the legend of each figure.

RESULTS

The absence of LCN2 leads to decreased rates of cell proliferation

In order to understand the role of LCN2 in the process of adult neurogenesis, we first analysed the rates of proliferating cells in the SGZ of the DG in LCN2-null mice. For that, the number of cells entering the S-phase was analysed by injecting animals once with BrdU (Figure 1a) and 24 h later, quantification of the total number of BrdU$^+$ cells showed a significant reduction ($P = 0.004$) in LCN2-null mice hippocampus when compared to Wt littermate controls (Figures 1b and c).

Interestingly, when we analysed the levels of LCN2 mRNA, by RT-PCR (not shown), and protein expression by western blot and immunohistochemistry in the DG of adult mice, we were not able to detect expression of LCN2 (Supplementary Figures S1a and b), which is in accordance with other reports.[27,28] Nevertheless, analysis of protein expression revealed that LCN2 is highly present in the serum (Supplementary Figure S1a), suggesting that a possible regulation of adult neurogenesis by LCN2 may occur extrinsically to the brain, through its delivery by the blood vessels that surrounds the neurogenic niches, which needs further clarification.

The subsequent analysis of the endogenous Ki67 marker of proliferation revealed that LCN2-null mice also presented a significant reduction ($P = 0.012$) in the number of Ki67$^+$ cells (Figures 1b and c). Also, the effect of LCN2's absence on cell proliferation at the adult subventricular zone (SVZ) was studied and, similarly, LCN2' absence impaired cell proliferation (Supplementary Figures S2a–c).

LCN2-null mice present deficits in adult progenitors differentiation

The impact of LCN2 in the generation of newborn mature cells in the neurogenic niches was next investigated. Twenty-four hours upon BrdU injection, quantification of the number of proliferating neuroblasts (DCX$^+$ BrdU$^+$ cells; Figure 1d) revealed a significant reduction ($P = 0.01$) in the SGZ of LCN2-null mice (Figures 1e and h). Concomitantly, analysis of newborn neurons (Calb$^+$ BrdU$^+$ cells; $P = 0.05$) and of new mature astrocytes (S100β$^+$ BrdU$^+$ cells; $P = 0.04$; Figures 1f–h), showed significant decrease in LCN2-null mice. Similarly, the analysis of the DCX$^+$ BrdU$^+$ neuronal precursor cells, in LCN2-null mice SVZ, revealed a significant decrease (Supplementary Figures S3a–c). Nevertheless, the proportion of 28-days old newborn neurons that migrated into the olfactory bulb was similar between animals (Supplementary Figures S3d–f).

The absence of LCN2 impairs NSCs proliferation and survival

Since LCN2's absence affected progenitors proliferation and differentiation, we next analysed if such impairments were dependent on the misregulation at the level of the stem cells pool. Firstly, we identified radial glia-like type-1 stem cells, as GFAP$^+$ BrdU$^+$ cells (Figure 1d), and observed a marked increase ($P = 0.009$) in LCN2-null mice SGZ (Figures 1i and l). Consistently, for the SVZ, an increase in type-B1 stem cells was observed (Supplementary Figures S4a and b). Moreover, the transcription factor Sox2 was used to identify nonradial type-1 stem cells (Sox2$^+$ Ki67$^-$; Figure 1d) and type-2 amplifying progenitor cells (Sox2$^+$ Ki67$^+$). Even though the total number of Sox2$^+$ cells in the SGZ of LCN2-null mice was similar to the number observed in Wt littermate controls (Supplementary Figure S5e), analysis of the nonradial type-1 stem cells confirmed that LCN2' absence leads to a significant ($P = 0.04$) increase in this population (Figures 1j and l). On the other hand, quantification of type-2 stem cells (Sox2$^+$ Ki67$^+$) revealed a prominent decrease ($P = 0.04$) in LCN2-null mice SGZ (Figures 1k and l).

Overall, and despite the total number of cells being similar, a consistent increase in quiescent type-1 stem cells (radial and nonradial types) and a prominent depletion of type-2 amplifying progenitor cells was evident in the SGZ of LCN2-null mice, which suggests the existence of a misregulation in cell transition from type-1 to type-2 progenitors when LCN2 is absent, with subsequent impact on neuronal and glial lineages.

LCN2 deletion affects progenitors cycling, promoting cell cycle exit and death

Data presented suggested the requirement of LCN2 for the specific regulation of the NSCs pool maintenance and differentiation, which next lead us to analyse the cell cycle regulation in LCN2-null mice SGZ. The number of cells exiting and re-entering the cell cycle at the SGZ within 24 h after the BrdU pulse were indexed (Figure 2a) and revealed that the proportion of cell cycle exit was significantly increased ($P = 0.003$) in LCN2-null mice; in contrast, a significant lower proportion of cells were observed to re-entry the cycle ($P = 0.03$; Figures 2b, c and e). Interestingly, a similar regulation of the cell cycle at the SVZ was observed (Supplementary Figures S4a–c).

This increased cell cycle exit in LCN2-null DG could, at least in part, explain the observed decrease in the number of dividing progenitors and, consequently, of newborn mature cells; however, it did not clarify which cells exit the cycle. Quantification of the percentage of $Sox2^+$ cells in cycle showed a clear decrease ($P = 0.002$; Figures 2d and e) when LCN2 was absent, further supporting the idea that LCN2 regulates cell cycle in type-1 stem cells and the transition to type-2 in the DG. Also, LCN2-null mice

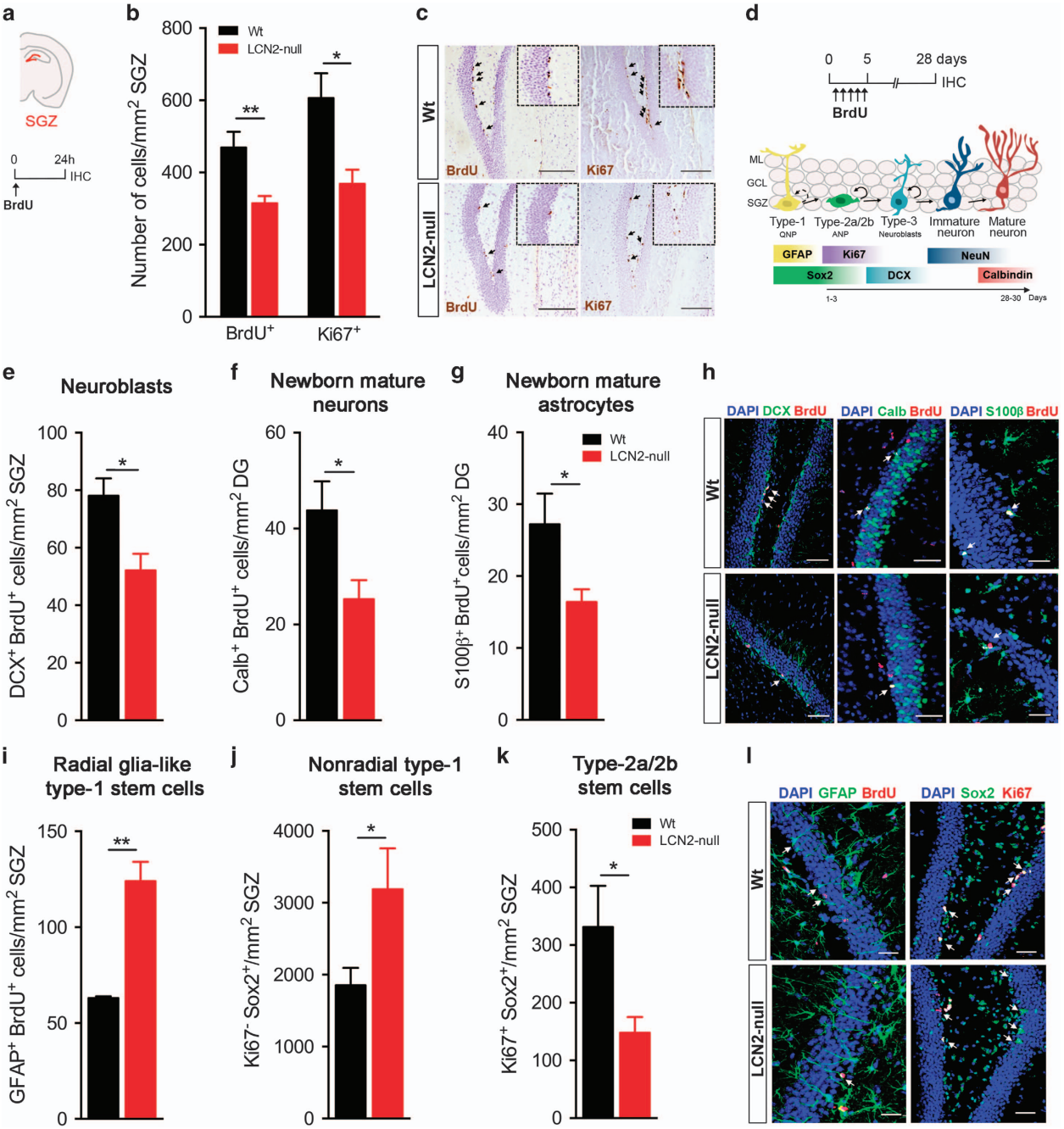

Figure 1. LCN2-null mice present reduced rates of cell proliferation and impaired NSCs proliferation, survival and maturation in the adult hippocampus. (**a**) Schematic diagram of the SGZ of the DG at the hippocampus and of the BrdU protocol used. (**b**) Quantification analysis of $BrdU^+$ and $Ki67^+$ cells in the SGZ ($n = 5$ per group). (**c**) Representative images of BrdU and Ki67 immunostaining at SGZ (indicated by black arrows; scale bar, 50 μm). (**d**) Schematic diagram of the experimental protocol of BrdU used and illustration of the hippocampal neurogenesis process, including cellular types and their specific markers. (**e**–**g**) Reduction of DCX^+ $BrdU^+$ neuronal precursors, of newborn mature neurons ($Calb^+$ $BrdU^+$) and astrocytes ($S100\beta^+$ $BrdU^+$) in LCN2-null SGZ, compared to Wt mice ($n = 6$ per group). (**h**) Confocal images of DCX/BrdU, Calb/BrdU and S100β/BrdU co-labelling. (**i**,**j**) Increased number of radial glia-like $GFAP^+$ $BrdU^+$ type-1 stem cells and of nonradial $Ki67^-$ $Sox2^+$ type-1 stem cells in LCN2-null mice ($n = 5$ per group). (**k**) Decreased number of $Ki67^+$ $Sox2^+$ type-2 progenitor cells in LCN2-null SGZ ($n = 5$ per group). (**l**) Representative co-immunostaining of GFAP/BrdU and Ki67/Sox2 at DG (identified by black arrows; scale bars, 50 μm). Data are presented as mean ± SEM analysed by two-tailed Student's *t*-test and are representative of two independent experiments. $^*P \leqslant 0.05$, $^{**}P \leqslant 0.01$. ANP, amplifying neural progenitor; Calb, calbindin; DCX, doublecortin; DG, dentate gyrus; GCL, granular cell layer; IHC, immunohistochemistry; LCN2, lipocalin-2; ML, molecular layer; NSC, neural stem cells; QNP, quiescent neural progenitor; SGZ, subgranular zone. See also Supplementary Figures S2 and S3.

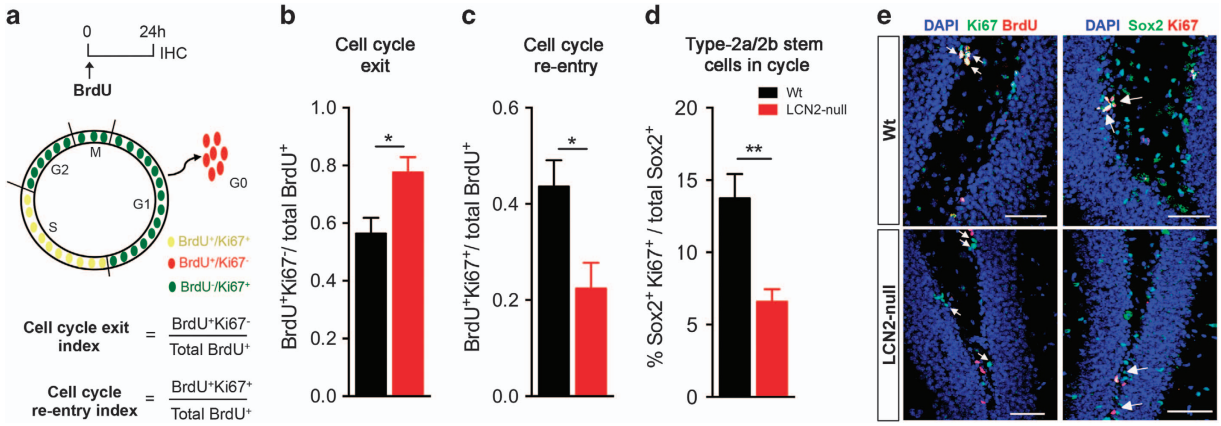

Figure 2. LCN2 deletion impairs the number of cycling DG progenitors by favouring cell cycle exit. (**a**) Schematic representations of the BrdU protocol, of the cell cycle phases that BrdU and Ki67 co-labelling correspond to and of the indexes used for cell cycle exit and re-entry. (**b**) Increased proportion of cells exiting the cell cycle at the SGZ in the absence of LCN2 ($n = 5$ per group). (**c**) Reduced number of cells re-entering the cell cycle in the SGZ of LCN2-null mice ($n = 5$ per group). (**d**) Decreased percentage of type-2 progenitor cells in cycle at the SGZ ($n = 5$ per group) in the absence of LCN2. (**e**) Representative confocal immunostaining images of BrdU/Ki67 and Sox2/Ki67 co-labelling, indicated by white arrows; scale bar, 100 μm. Data presented as mean ± SEM analysed by two-tailed Student's *t*-test and are representative of two independent experiments. $^{*}P \leqslant 0.05$, $^{**}P \leqslant 0.01$. See also Supplementary Figure S4. DFO, deferoxamine; DG, dentate gyrus; IHC, immunohistochemistry; LCN2, lipocalin-2; SGZ, subgranular zone.

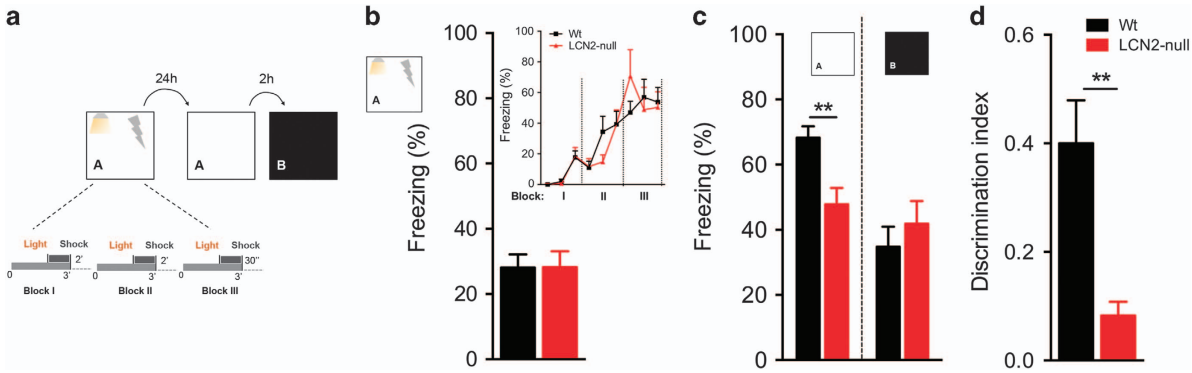

Figure 3. Impaired contextual fear conditioning in LCN2-null mice. (**a**) Experimental scheme of the used paradigm for contextual fear condition test. (**b**) Freezing behaviour during the training session, as total percentage of freezing and of freezing acquired along the trials of shock exposure (Block I-III). (**c**) Impaired contextual retrieval by LCN2-null mice at context A, but similar freezing between genotypes at context B ($n = 10$ per group). (**d**) Discrimination index is impaired in LCN2-null mice. Data are presented as mean ± SEM analysed by two-tailed Student's *t*-test and are representative of two independent experiments. $^{**}P \leqslant 0.01$. LCN2, lipocalin-2.

displayed increased hippocampal cell death, as quantified by the number of positive cells for activated caspase-3 ($Casp3^+$) ($P = 0.048$), and further co-immunostaining analysis revealed that the number of radial type-1 stem cells expressing $Casp3^+$ was significantly increased in LCN2-null mice ($P = 0.03$), when compared to Wt (Supplementary Figures S5a–d).

As so, the data presented suggest that the impaired cell genesis in the adult hippocampus in LCN2-null mice is the consequence of a deficient cell cycle regulation and of substantial apoptosis of type-1 NSCs. More importantly, this seems to be due to a lack of an antioxidant regulation at the level of stem cells resultant from the LCN2's absence, since a significant ($P = 0.009$) decrease of glutathione peroxidase-4 (Gpx4) expression by $Sox2^+$ stem cells was observed in LCN2-null mice (Supplementary Figures S5d and e).

LCN2-null mice impaired hippocampal neurogenesis affects contextual discrimination behaviour

Consistent with the current knowledge that adult hippocampal neurogenesis contributes to enhance the extent of information encoded by the DG and improves pattern separation and contextual discrimination, the ability of LCN2-null mice to discriminate between overlapping contextual representations in a contextual fear conditioning task was next investigated. Assessment of animals' behaviour during training revealed that animals presented similar percentage of freezing (Block I-III; Figure 3b). Upon re-exposure to the conditioning context, 24 h after the training and with no cue presentation, LCN2-null mice showed a significant ($P = 0.004$) decreased percentage of freezing (Figure 3c). Subsequent analysis of contextual discrimination, by exposing the animals to a novel context 2 h later, revealed that LCN2-null mice presented similar levels of freezing behaviour when compared to the training context, while Wt mice presented substantially less freezing behaviour upon exposure to the novel context (Figure 3c). This altered behaviour translated into a lack of context discrimination capacity by LCN2-null mice (discrimination index: $P = 0.002$; Figures 3c and d). Overall, this reveals the importance of the observed impaired adult hippocampal neurogenesis in LCN2-null mice, since it translated into deficits in hippocampal function.

The absence of LCN2 impairs *in vitro* neural stem cell proliferation and self-expansion

In order to mechanistically understand how the absence of LCN2 affects hippocampal precursors' proliferation and survival, with

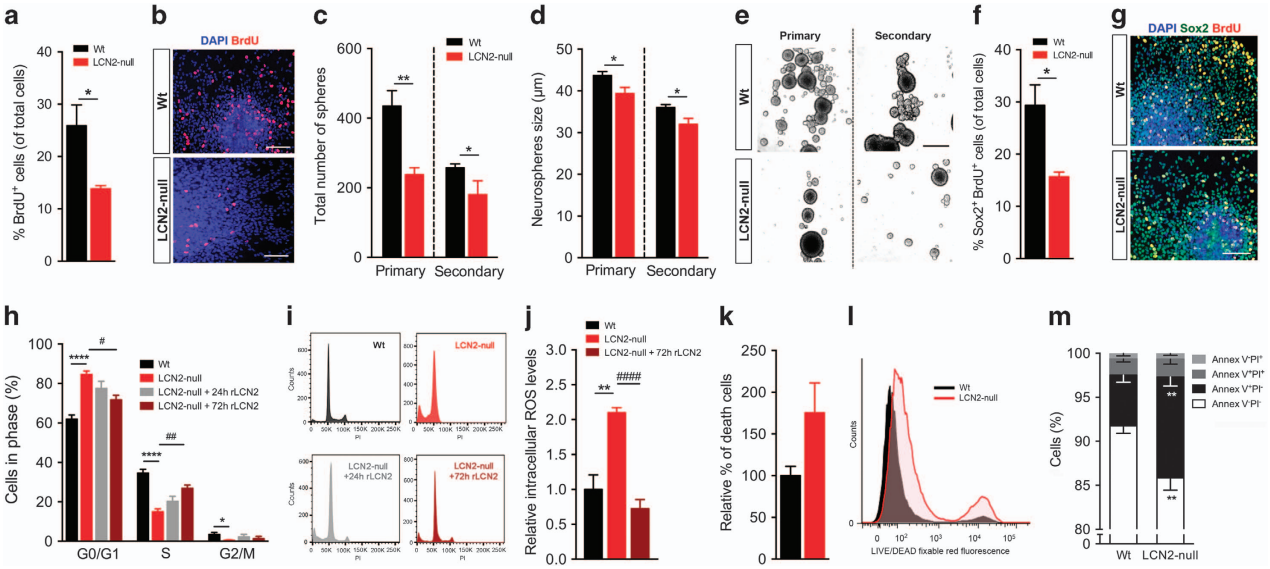

Figure 4. LCN2 is required for NSCs G0/G1 cell cycle transition, through the control of endogenous redox cellular status, and its absence impairs proliferation and self-renewal and promotes cell apoptosis. (**a**,**b**) Reduction of proliferating neurospheres represented by $BrdU^+$ cells/total cells in LCN2-null derived cells. (**c**) Reduction of the number of primary and secondary neurospheres in the absence of LCN2. (**d**) Average diameter of primary and secondary neurospheres. (**e**) Cultured primary and secondary spheres from Wt and LCN2-null SGZ. (**f**,**g**) NSCs sphere-renewal impaired in LCN2-null as quantified by $Sox2^+$ $BrdU^+$ cells. Scale bars, 100 µm. (**h**,**i**) Increased percentage of NSCs at G0/G1 phase of the cell cycle in LCN2-null mice, rescued upon delivery of exogenous rLCN2 protein. (**j**) Two-fold increased levels of intracellular ROS in LCN2-null derived neurospheres, restored upon rLCN2 treatment. (**k**,**l**) Live and dead assay by flow cytometry. (**m**) Increased NSCs apoptosis in LCN2-null mice. Data are presented as mean ± SEM, analysed by two-tailed Student's *t*-test and are representative of three independent experiments. $^*P \leqslant 0.05$, $^{**}P \leqslant 0.01$, $^{****}P \leqslant 0.0001$ for Wt vs LCN2-null; $^{\#}P \leqslant 0.05$, $^{\#\#}P \leqslant 0.01$, $^{\#\#\#\#}P \leqslant 0.0001$ for treatment vs LCN2-null alone. rLCN2, recombinant LCN2. LCN2, lipocalin-2; NSC, neural stem cells; ROS, reactive oxygen species; SGZ, subgranular zone.

important consequences at the behavioural level, a further detailed characterization was performed using *in vitro* culture assays.

Analysis of $BrdU^+$ cells [over total positive nuclei ($DAPI^+$)] revealed that LCN2-null-derived neurospheres presented a significant ($P = 0.03$) decrease in the percentage of proliferative cells (Figures 4a and b). Moreover, the clonal neurosphere assay showed that the total number of primary and secondary neurospheres generated from LCN2-null NSCs were significantly lower when compared to Wt (Figures 4c and e). Similarly, analysis of the mean size of the neurospheres revealed that both primary ($P = 0.03$) and secondary neurospheres ($P = 0.03$) obtained from LCN2-null SGZ were smaller (Figures 4d and e).

The effect of LCN2's absence on the self-renewal of SGZ cells was subsequently tested by adhering neurospheres for 48 h under proliferative conditions, and further labelled for Sox2 and BrdU. Quantification of the percentage of $Sox2^+$ $BrdU^+$ cells revealed a significant decrease in LCN2-null-derived NSCs ($P = 0.03$), thus confirming a significant effect on self-renewal capabilities in NSCs from LCN2-null SGZ (Figures 4f and g). We anticipated that LCN2 exerted such particular modulation through its specific cellular receptor 24p3R.[21] In fact, *in vivo* analysis of 24p3R expression by immunofluorescence at the DG, in Wt animals, revealed that the receptor was present throughout the gyrus and in the hilus (Supplementary Figures S6a and a'). Specifically, additional co-labelling assessments showed that NSCs positive for Sox2 and Nestin (Supplementary Figures S6b and c) co-expressed 24p3R, as well as labelled-retaining $BrdU^+$ cells at the SGZ (Supplementary Figure S6d). In addition, analysis of 24p3R expression in cultured neurospheres revealed that NSCs under proliferative conditions express the LCN2 receptor (Supplementary Figures S6e and f).

LCN2 regulates NSCs endogenous levels of ROS, which is required for a proper cell cycle progression

To assess the influence of LCN2 in neurospheres cell cycle progression, the proportion of NSCs at the different cell cycle phases were analysed by flow cytometry. After labelling 7 days-old cultured neurospheres with propidium iodide, the analysis of the DNA content in each genotype revealed that the percentage of cells at G0/G1 phase was significantly ($P < 0.0001$) higher in LCN2-null cells (Figures 4h and i). In accordance, a significant ($P < 0.0001$) decrease in the proportion of cells at S phase was observed, as well as for the G2/M phase ($P = 0.01$; Figures 4h and i).

Interestingly, when LCN2-null neurospheres were treated with exogenous recombinant LCN2 (rLCN2) for 24 and 72 h, we found that 72 h of treatment significantly ($P = 0.02$) decreased the percentage of cells at G0/G1, when compared to LCN2-null neurospheres alone, and that it allowed NSCs cell cycle progression, since it significantly ($P = 0.008$) increased the percentage of cells at S phase (Figures 4h and i). These observations did not, however, provide information on how LCN2 determines the control of NSCs cell cycle progression. In recent years, the modulation of cellular redox states has been suggested to be important for the balance between self-renewal and differentiation in progenitor cells. Of notice, and consistent with the idea of LCN2' role in iron trafficking,[19,20] we further considered the putative contribution of LCN2's absence to a ROS-dependent cell cycle arrest and death. In fact, analysis of intracellular ROS levels in NSCs revealed a significant ($P = 0.03$) increase in LCN2-null-derived cells, which was successfully reverted by the addition of rLCN2 for 72 h (Figure 4j). Moreover, we observed that the overproduction of ROS in the absence of LCN2 sensitized NSCs to cell death, which was demonstrated by the increase in the percentage of non-viable cells in LCN2-null ($P = 0.06$; Figures 4k and l) and of the percentage of apoptosis (Annexin V^+PI^-; $P = 0.002$) (Figure 4m). Moreover, *in vivo* treatment of

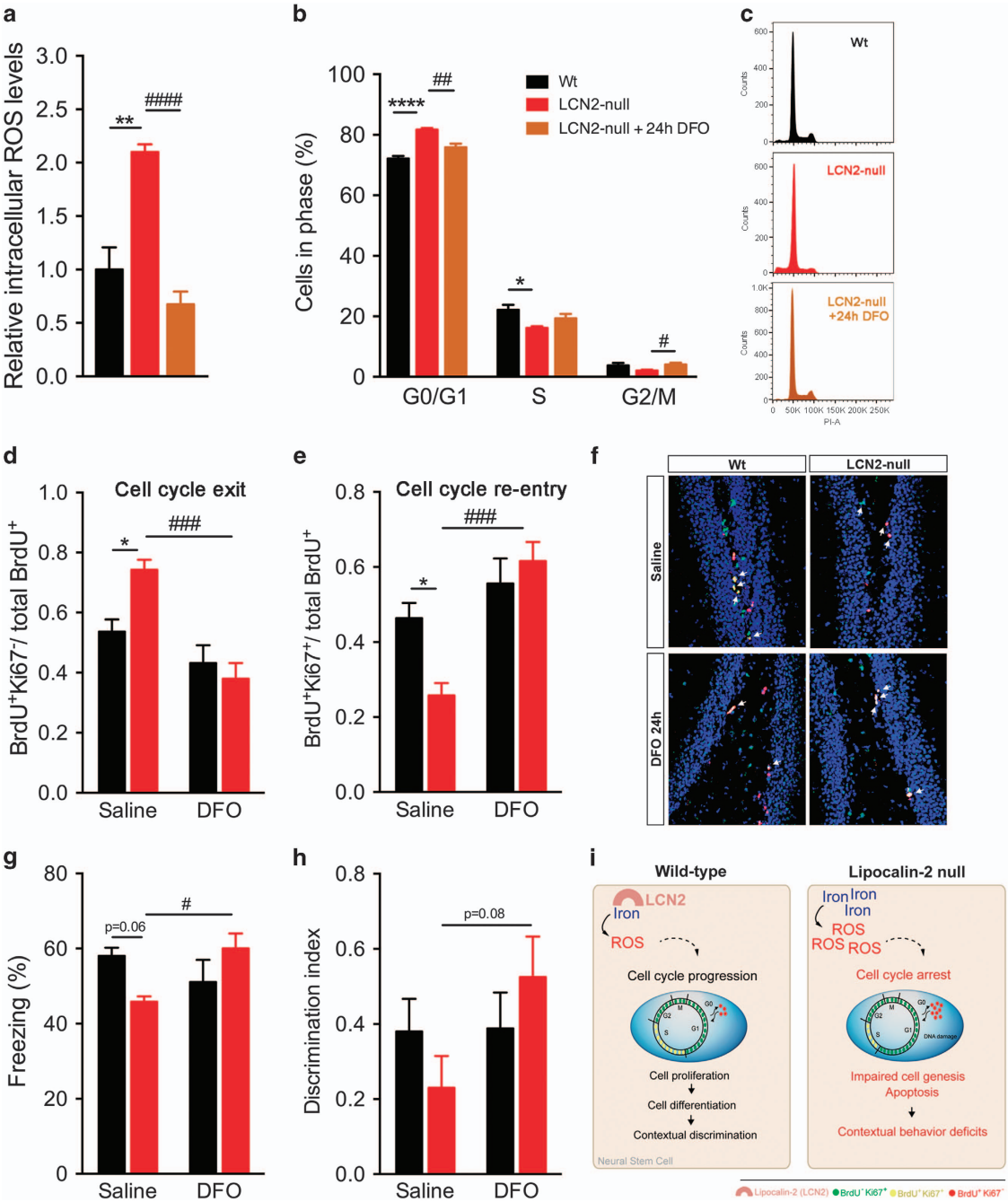

Figure 5. LCN2 absence imposed oxidative damage and cell cycle arrest is dependent on intracellular iron regulation. (**a**) Increased oxidative damage of LCN2-null is rescued by DFO. (**b,c**) Cell cycle arrest in LCN2-null mice derived neurospheres is restored after DFO treatment. (**d,e**) *In vivo* impaired cell cycle exit and re-entry in LCN2-null SGZ is normalized after the DFO treatment. (**f**) Representative confocal immunostaining images of BrdU/Ki67 co-labelling. Indicated by white arrows; scale bar, 50 μm. (**g**) Impaired contextual retrieval by LCN2-null mice at context A restored upon DFO chronic treatment ($n = 6$ per group). (**h**) Discrimination index is improved after DFO in LCN2-null mice. (**i**) Proposed mechanism for the role of LCN2 in the control of NSCs cell cycle progression through intracellular iron regulation and ROS signalling, for contextual discriminative behaviours. Data are presented as mean ± SEM and are representative of two independent experiments. (**a,b**) two-tailed Student's *t*-test; $^{*}P \leqslant 0.05$, $^{**}P \leqslant 0.01$, $^{****}P \leqslant 0.0001$, Wt vs LCN2-null; $^{\#}P \leqslant 0.05$, $^{\#\#}P \leqslant 0.01$, $^{\#\#\#\#}P \leqslant 0.0001$, treatment vs LCN2-null alone. (**d,e,g,h**) two-way analysis of variance analysis with Bonferroni's multiple comparison test; $^{*}P \leqslant 0.05$ vs saline Wt; $^{\#}P \leqslant 0.05$, $^{\#\#\#}P \leqslant 0.001$ vs saline LCN2-null mice. DFO, deferoxamine; LCN2, lipocalin-2; NSC, neural stem cells; ROS, reactive oxygen species; SGZ, subgranular zone.

LCN2-null mice for 24 h with the antioxidant agent N-acetylcysteine (300 mg kg^{-1})[29] significantly reduced ($P = 0.02$) the levels of $Casp3^{+}$ in the SGZ, when compared to the saline control group (Supplementary Figure S5h), which suggests that ROS accumulation in the absence of LCN2 is likely the inducer of the observed increased cell death.

Oxidative damage induced by LCN2 absence is iron-mediated

Recognizing that excessive intracellular iron promotes oxidative stress, the connection between iron regulation and ROS, by LCN2 and specifically in NSCs, was next studied. For that, NSCs intracellular iron levels were depleted by adding the membrane-permeable iron chelator DFO to LCN2-null neurospheres for 24 h

[2 μM;[30]]. Noticeably, relative to LCN2-null cultures alone, DFO treatment significantly ($P < 0.0001$) reduced the intracellular levels of ROS in neurospheres, to levels similar to those occurring in Wt animals (Figure 5a). Additionally, cell cycle analysis in LCN2-null neurospheres treated with DFO and labelled with propidium iodide, revealed that DFO significantly decreased the percentage of cells arrested at G0/G1, when compared to LCN2-null neurospheres alone ($P = 0.004$; Figures 5b and c), and allowed the progression of NSCs in the cycle, since it increased the percentage of cells at S ($P = 0.09$) and G2 ($P = 0.02$) phases.

These results suggest that intracellular iron accumulation in NSCs, due to the absence of LCN2, is the key underlying mechanism promoting oxidative stress and cell cycle arrest. In fact, and interestingly, treatment of cultured Wt NSCs with an iron source (ferric ammonium citrate) for 24 h [500 μM;[31]] similarly induced a significant ($P = 0.02$) increase in the production of ROS, when compared to control non-treated cultures (Supplementary Figure S7a). Importantly, this iron-mediated imposed oxidative stress also induced cell cycle arrest, as ferric ammonium citrate significantly increased the proportion of cells at G0/G1 phase ($P = 0.007$), not allowing for cells to progress in cycle (S phase: $P = 0.01$; Supplementary Figures S7b and c), which largely resembles the phenotype observed to occur in the absence of LCN2.

Moreover, *in vivo* analysis of cell cycle indexes of exit and re-entry, after DFO treatment for 24 h [200 μM;[32]], showed that iron chelation was sufficient to improve progenitors cell cycle regulation in the SGZ of LCN2-null mice (treatment effect: $F_{1,13} = 19.3$, $P < 0.0001$). Specifically, DFO significantly normalized cell cycle exit in LCN2-null SGZ ($P = 0.0005$), promoting progenitors cell cycle re-entry ($P = 0.0007$), to indexes similar to those occurring in Wt animals (Figures 5d–f). In fact, a significant increase in cell proliferation was observed in LCN2-null mice DG after treatment with DFO, of both $BrdU^+$ (treatment effect: $F_{1,14} = 20.5$, $P = 0.0005$) and $Ki67^+$ cells (treatment effect: $F_{1,15} = 10.9$, $P = 0.005$; Supplementary Figures S7a and b), with an additional prominent decrease ($P = 0.03$) in the number of $Casp3^+$ cells in the SGZ of LCN2-null mice (Supplementary Figure S8c). Importantly, the additional chronic administration of DFO for 28 days significantly improved the performance of LCN2-null mice in the contextual fear conditioning. The observed decreased percentage of freezing presented by LCN2-null mice was significantly improved after DFO treatment ($P = 0.02$), when compared to saline-treated LCN2-null animals (Figure 5g). Moreover, context discriminatory indexes were also recovered in LCN2-null mice after DFO treatment ($P = 0.08$; Figure 5h).

DISCUSSION

This study provides *in vivo* and *in vitro* evidence for a novel role for LCN2 as a modulator of neurogenesis in the adult mammalian brain. It shows that, *in vivo*, LCN2 deletion induces deficits in neural progenitors proliferation, differentiation and maturation, with impact on contextual discriminative behaviours. Specifically, the absence of LCN2 leads to an accumulation of type-1 (radial and nonradial) stem cells, due to a misregulation of the cell cycle, with a higher proportion of progenitor cells exiting the cycle towards apoptotic cell death. Moreover, NSCs cultured *in vitro* allowed to mechanistically disclose that the deletion of LCN2 significantly induces endogenous oxidative stress, cell cycle arrest and death, an effect that is iron-mediated since it is restored by the addition of the iron chelator DFO. Altogether, the data presented imply that LCN2 is required to control NSCs redox status, functioning as an antioxidant agent in the maintenance of a proper oxidative environment for cell cycle progression and stem cells' self-renewal. This mechanism of cell cycle regulation is crucial for hippocampal integrity and function, as the absence of LCN2 impacts on contextual fear discriminative behaviours.

Iron is an essential element required by almost all organisms, playing critical physiological roles that include oxygen transportation, energy metabolism and cellular proliferation. On the other hand, iron is a highly reactive element, having a catalytic role in a multitude of redox reactions.[13] Iron homeostasis dysfunction is the major source of oxidative stress via the generation of ROS by the Fenton's reaction and the aberrant cellular accumulation of iron, ROS, or both, is linked to DNA damage, cell cycle arrest and death.[13] In recent years, LCN2, primarily considered to be exclusively an innate immune protein, was shown to have multifaceted roles in cellular iron transport and homeostasis.[19,21,23] Importantly, the isolation and identification of 24p3R by Devireddy and colleagues (2005) paved the way to propose the existence of an iron delivery mechanism that is LCN2-mediated and transferrin-independent.[19,21] Specifically, it was proposed that upon binding to 24p3R, iron-containing LCN2 is internalized, traffics to the endosomes being iron released from LCN2, thereby increasing intracellular iron.[21] On the other hand, the binding and internalization of iron-free LCN2 chelates intracellular iron and may transfer it to the extracellular milieu, thus reducing intracellular iron.[21] In line with this, herein we propose LCN2 as an iron sequester in NSCs, with impact on cell cycle regulation and cell death and, ultimately, animal behaviour. Importantly, LCN2-mediated iron delivery to NSCs is most likely occurring by its internalization through the specific 24p3R, shown here to be present in neurospheres and stem cells *in vivo*. In fact, previous studies in kidney cells demonstrated the internalization of LCN2 and its localization in vesicles scattered throughout the cytoplasm, and in distinct intracellular compartments of that of internalized transferrin.[19] This is a mechanism that should be next further studied in the context of NSCs. Still, altogether, it is increasingly clearer that LCN2 seems to actively participate in cell physiology in several organs.

The regulation of NSCs physiology by LCN2 was primarily observed by the loss of general proliferation rates in the absence of LCN2, when using both BrdU and Ki67 proliferative markers. Moreover, the absence of LCN2 was sufficient to induce the accumulation, expansion and apoptosis of type-1 stem cells (radial and nonradial type of cells), with a subsequent reduction in the proliferation of type-2 and of cell genesis commitment, maturation and integration. Specifically, cell cycle transition from G1 to S phase was compromised in LCN2-null mice, with NSCs being arrested at G0/G1 phase and exiting more the cell cycle. Mechanistically, the current observations support the notion that the lack of LCN2 in NSCs leads to endogenous iron accumulation, which in turn increases intracellular ROS, thus creating an oxidative environment that is boosting cell cycle arrest and cell death. Actually, the observation that treatment of LCN2-null mice with the well-known antioxidant agent N-acetylcysteine[29,33] decreases apoptotic cell death clearly indicates that ROS, increased as the consequence of intracellular iron accumulation by the absence of LCN2, is the likely inducer of cell death in the DG of LCN2-null mice. This is a novel mechanism of NSC commitment that, ultimately, impairs animal behaviour, as shown here (Figure 5i). Consistent with the known ability of LCN2 to traffic iron within the cells, the *in vitro* reversion of the oxidative environment and of cell cycle progression with DFO treatment, along with the *in vivo* prevention of an exacerbated cell cycle exit and apoptotic cell death at the SGZ, as well as the improvement in the contextual behaviour, strengths such hypothesis. This finding is novel as it shows that LCN2 acts in the regulation of endogenous ROS levels in NSCs by controlling its cellular content. Also, and since the addition of rLCN2 restores ROS levels and promotes cell cycle progression, it is possible to assume that this treatment, likewise, restores endogenous levels of iron in LCN2-null neurospheres. Interestingly, and of notice, others have shown that LCN2-null mice have increased intracellular[34] and serum[32] iron levels, which renders them more susceptible to lipopolysaccharide-mediated oxidative stress.[32] In addition,

DFO treatment in LCN2-null mice was effective in rescuing lipopolysaccharide-induced toxicity, including the reduction of cleaved caspase-3 levels.[32] It is possible that, also in the brain and specifically in the neurogenic niches, LCN2-null mice accumulate iron in neural progenitors, which is impacting on ROS levels, and on the normal progress of cell cycle and adult neurogenesis sequence. Importantly, the fact that the addition of iron to Wt NSCs induced oxidative stress and cell cycle arrest, thus mimetizing the impairments observed in NSCs of LCN2-null mice, strengths this hypothesis.

Of interest, LCN2 has been extensively described in the brain to be mainly expressed in response to injury or inflammation, so it can engage critical roles in the progression and establishment of inflammation.[35] Particularly, others have shown LCN2 secretion by astrocytes to target and modulate microglia activation and polarization into M1 phenotype, and also to target astrocytes in an autocrine fashion manner for pro-inflammatory activation.[35] Interestingly, astroglial cells can sharply affect neurogenesis, through the release of inflammatory cytokines, by regulating progenitors proliferation, differentiation and the survival of new adult-born neurons.[36] Noticeably, LCN2 could, through this route, and in case of neuroinflammation, impact on neurogenesis by regulating progenitors proliferation and differentiation, and the survival of new adult-born neurons. Still, herein, we believe that, in physiological conditions, regulation of adult neurogenesis by LCN2 may arise from the external systemic environment. While in the physiological brain most reports are unanimous in not detecting LCN2 expression,[27,28,37] with the exception for a few conflicting reports,[24] LCN2 is described to be highly present in the serum at physiological conditions,[38] in accordance with our current observations. As so, it is possible that regulation of adult neurogenesis by LCN2 may occur through its delivery by the blood vessels located within the niche and that surround neural progenitors. Of notice, others have shown, particularly in aging, that the systemic milieu and its circulating factors can regulate neurogenesis and cognitive function.[39] Future studies should consider the potential role of the cells at the periphery that produce LCN2 (for example, neutrophils, hepatocytes) in the regulation of brain cell genesis, and even of the cells in the central nervous system (for example, neurons, astrocytes), despite the production of LCN2 in the brain, under basal conditions, being at levels below detection with the current available methods.

Stem cells usually reside in an environment of reduced redox status, which favours self-renewal and differentiation,[40] highlighting the importance of a proper redox balance. In line with this, and in case of oxidative stress, all cells have a complex antioxidant system to detoxify endogenous ROS.[41] The glutathione peroxidase group of antioxidant enzymes acts in concert to remove ROS and, among them, Gpx4 is considered the primary enzymatic defence system against oxidative damage.[41] In line with this, we assessed the number of neural stem precursors $Sox2^+$ that expressed Gpx4. The fact that significantly less $Sox2^+$ cells in LCN2-null mice DG co-expressed Gpx4, along with the *in vitro* data of increased intracellular levels of ROS in NSCs, confirms the inefficient antioxidant regulatory system of LCN2-null mice. This is, most likely, contributing to further impaired hippocampal function in contextual fear conditioning tasks, as the ultimate consequence of impaired cell differentiation. This imbalanced redox regulation in LCN2-null mice, resultant of iron trafficking misregulation, can also be the critical mediator of previous observed impairments in hippocampal plasticity and animal behaviour.[25] Specifically, we have previously shown that LCN2-null mice display anxious- and depressive-like behaviours and spatial learning impairments, along with an altered hippocampal neuronal cytoarchitecture and synaptic plasticity.[25]

Importantly, herein, we cannot exclude the limitation of a full knockout system in disclosing important regulatory mechanisms of adult neurogenesis. LCN2 is certainly not the only key mediator of the process we here describe. The present data support that a further understanding on whether LCN2 acts in a context- and cellular-dependent manner, through the use of conditional-null models, will provide clues on whether its modulation may impact neurogenesis in health and in disease. Nevertheless, and in summary, the present study provides the detailed sequence of events for the role of LCN2 as a novel modulator of iron-mediated oxidative stress in the orchestration of neurogenesis for hippocampal plasticity and behavioural function. As such, these findings add an important piece into the current knowledge on the mechanisms modulating adult mammalian neurogenesis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Molecular Psychiatry website (http://www.nature.com/mp)