L-lactate induces neurogenesis in the mouse ventricular-subventricular zone via the lactate receptor HCA_1

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Short title: HCA_1 dependent neurogenesis in SVZ

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Abstract

Aim: Adult neurogenesis occurs in two major niches in the brain: the subgranular zone of the hippocampal formation and the ventricular-subventricular zone. Neurogenesis in both niches is reduced in aging and neurological disease involving dementia. Exercise can rescue memory by enhancing hippocampal neurogenesis, but whether exercise affects adult neurogenesis in the ventricular-subventricular zone remains unresolved. Previously, we reported that exercise induces angiogenesis through activation of the lactate receptor HCA1. The aim of the present study is to investigate HCA_1-dependent effects on neurogenesis in the two main neurogenic niches. **Methods:** Wildtype and HCA_1 knock-out mice received high intensity interval exercise, subcutaneous injections of L-lactate, or saline injections, five days per week for seven weeks. Well-established markers for proliferating cells (Ki-67) and immature neurons (doublecortin), were used to investigate neurogenesis in subgranular zone and ventricular-subventricular zone. **Results:** We demonstrated that neurogenesis in the ventricular-subventricular zone is enhanced by HCA_1 activation: Treatment with exercise or lactate resulted in increased neurogenesis in wild type, but not in HCA_1 knock-out mice. In the subgranular zone, neurogenesis was induced by exercise in both genotypes, but unaffected by lactate treatment. **Conclusion:** Our study demonstrates that neurogenesis in the two main neurogenic niches in the brain is regulated differently: Neurogenesis in both niches was induced by exercise, but only in the ventricular-subventricular zone was neurogenesis induced by lactate through HCA_1 activation. This opens for a role of HCA1 in the physiological control of neurogenesis, and potentially in counteracting age-related cognitive decline.

Keywords: adult neurogenesis, exercise, GPR81, HCA_1, HCAR1, lactate.

Introduction

The prevalence of age-related memory decline and age-related neurodegenerative diseases is likely to escalate in the years to come, in pace with the rapid ageing of the population. In humans, evidence from both long-term prospective studies [1,2] and cross-sectional studies [3-5] demonstrate that exercise may reduce cognitive decline and the risk to suffer from dementia [6]. Physical exercise has shown beneficial effects in young people as well as in elderly people with mild cognitive decline[7]. The mechanisms underlying these exercise-induced effects are not completely understood. Exercise may improve executive function and attention through direct actions on the brain: for instance exercise may counteract the decline in cerebral blood flow seen in ageing and age-related neurodegenerative diseases [8-10]. In addition to direct effect of the brain, exercise may affect brain function indirectly though peripheral mechanisms, including regulation of glucose metabolism, and reduction of obesity and type 2 diabetes; all of which have secondary effect on cognitive functions (for review, see [11]). Physical exercise, especially running, also enhances hippocampal adult neurogenesis in rodents [12-14]. Neurogenesis, the generation of new neurons from neuronal stem cells, and their integration into the neuronal circuits may enhance memory function. Most studies also find exercise to be as efficient as pharmacological antidepressant therapy at treating mild to moderate depression[15-24]. The antidepressant effect of exercise, as well as that of antidepressants, also appears to involve neurogenesis[22,25-30]. For this reason, adult neurogenesis has gained interest as a potential target for manipulation in the combat against depressive symptoms, as well as against neurodegeneration and memory loss associated with ageing and age-related diseases.

In the adult brain, neurogenesis occurs primarily in two niches: One niche is the subgranular zone (SGZ) of the dentate gyrus of the hippocampus. Cells from this niche migrate throughout the dentate granular layer of the hippocampal formation, serving a role in memory function [31]. The second neurogenic niche is the ventricular-subventricular zone (V-SVZ), which is located

alongside the ependymal cells that line the lateral ventricles [32]. In rodents, progenitor cells originating from this niche migrate predominantly anteriorly to the olfactory bulb where they differentiate into interneurons [33]. In this process, activated type B neuronal stem cells divide and give rise to transit amplifying type C cells, which again give rise to type A neuroblasts (type A cells) (figure 1). Eventually, the type A cells migrate along the rostral migratory stream to the olfactory bulb, where they are incorporated into the existing circuitry and mature into GABAergic or dopaminergic neurons [34-37].

[insert Figure 1.]

To what degree hippocampal neurogenesis occurs in the adult human hippocampus has been a hot topic for debate during the last years, due to opposing findings [38-40]. There is a consensus that neurogenesis in the SGZ is reduced over the lifespan: some researchers find this decrease to take place rapidly after birth, resulting in undetectable neurogenesis during adulthood and ageing [39]. Other researchers find that neurogenesis persists throughout life [41], but at a progressively slower rate as we age [42]. Neurogenesis in rodents and non-human primates is important for memory and learning as well as in the prevention of mood disorders like depression. Ageing of the neurogenic niches, resulting in declining neurogenesis is not fully understood. Exhaustion of the neural stem cell pool and age-related changes in the circulatory milieu are probably important [43,44] but a broader 'niche aging' mechanism may also depend on other factors, for instance vascular ageing [45].

Neurogenesis in the V-SVZ of adult humans seems to be less controversial than in the SGZ, but the role of this stem cell niche in memory and learning is less obvious. In the ageing V-SVZ, a reduction of the stem cell pool has been demonstrated to be paralleled by a loss of the characteristic cytoarchitectural hallmark: the pinwheel structure where the apical cilium of radial glia is surrounded by ependymal cells [46]. Neurogenesis in the V-SVZ and the migration,

maturation and integration of the newborn neurons into the olfactory bulb circuitry is of importance for odour-recognition/olfactory memory [47,48]. Olfactory memory is distinct from the hippocampus-dependent spatio-visual memory but may provide contextual clues to the hippocampal neurons. As discussed by Eichenbaum and Robitsek [49], odour-recognition may be essential for higher-order cognitive processing and reduced olfactory memory may therefore correlate with cognitive decline in ageing. In humans [50] and non-human primates [51-53], V-SVZ-derived neurons appear to migrate to the striatum, where they may serve to protect against Parkinson's disease [54,55]. Similarly, a reduction of adult-born neurons in the striatum has been implemented in Huntington's disease [50], where cognitive decline is an early symptom.

Physical exercise decelerates the age-related decline in cell proliferation and neuroplasticity. In fact, exercise affects hippocampal adult neurogenesis at multiple levels: Exercise increases the proliferation and differentiation of neurons [12-14] but also increases the integration of newly formed neurons into the functional hippocampal network [56-59]. Hippocampal neurogenesis is accompanied by plasticity-related changes in neuronal morphology, such as increased spine density and arborisation of the dendritic tree, as well as long-term potentiation [60,61]. In line with this, exercise efficiently delays some of the functional declines associated with brain ageing. At the functional level, exercise enhances learning, memory and problem solving, and may thus be particularly conducive to prolonging autonomy during ageing. Exercise counteracts the pathology of Alzheimer's disease (AD) in several ways: In mouse models where mutated human genes that increase the accumulations of β-amyloid and/or tau are inserted, running has been reported to reduce the hyper-phosphorylation of β-amyloid and tau [62,63], which triggers protein aggregation. Interestingly, high intensity exercise was shown to more efficiently reduce β-amyloid levels than exercise at lower intensities, presumably due to an upregulation of several proteases involved in β-amyloid clearance [64]. Whether lactate,

which is enhanced in blood and brain in response to high intensity exercise, is involved in the regulation of β-amyloid degradation, is not known. In humans, β-amyloid aggregation in the brain cannot be measured directly (except post mortem), but one study reported an inverse correlation between physical activity and the level of β-amyloid 42 in the cerebrospinal fluid [65]. Supporting this finding, β-amyloid burden was lower and hippocampal volume was greater in old people who were physically active compared with an age matched inactive group [66]. Mouse models for AD consistently show an association between physical activity or exercise and preserved cognitive functions[67-70]. In line with this, exercise increases dendritic arborization [71], maturation of dendritic spines [72,73], hippocampal neurogenesis, and reduces the AD-related loss of spatial memory [74].

Whether exercise also induces neurogenesis in the V-SVZ remains controversial. While some studies report exercise-induced neurogenesis in the V-SVZ, others fail to detect it. Recent data suggest that exercise induces neurogenesis in additional smaller niches, including the hypothalamus and ependymal lining of the third ventricle, and this has been linked to the recovery of homeostatic functions after brain injury [75]. Furthermore, the number of V-SVZ-derived neurospheres has been shown to increase in response to running exercise in aged mice [76]. Lee and colleagues [77] found that corticosterone-induced reduction in V-SVZ neurogenesis could be rescued by running. Along the same lines, Mastrorilli and colleagues [78] showed that 12 days of voluntary running wheel exercise restored the impaired neurogenesis in the V-SVZ in mice lacking the antiproliferative gene Btg1. In the Btg1 knock-out (KO) mice, an exercise-induced shortening of the cell cycle caused type B cells to accumulate, and hence led to a re-establishment of the physiological progression of neurogenesis the SVZ. This effect was not observed in the wildtype (WT) mice [78]. Nevertheless, the idea that exercise may regulate cell cycle kinetics is in line with previous studies from the hippocampus [56,79].

Although exercise is known to induce neurogenesis in the hippocampus and is likely to do so in the SVZ as well, the initial molecular signal involved in this process remains unresolved. L-lactate (hereafter referred to as lactate) increases in the blood in response to strenuous exercise and may activate the lactate receptor hydroxycarboxylic acid receptor 1 (HCA_1, also known as HCAR1 or GPR81) [80]. HCA_1 is a G_i-protein coupled receptor expressed at the plasma membrane, with the lactate binding site facing extracellulary. Hence activation of this receptor is distinct from the metabolic effects of lactate and does not require entry of lactate into cells via the monocarboxylate transporters (MCTs) [81]. A recent study showed that lactate induced neurogenesis in the hippocampus [82]. This effect was presumably through a metabolic action, as blocking the lactate transporter MCT2 prevented lactate-induced neurogenesis. In line with this, lactate administration prior to learning has repeatedly been shown to enhance memory [83,84]. Lactate signaling though HCA_1, however, also directly affects the excitability and firing properties of neurons independently of metabolic action [85,86]. Recently, a biphasic action of lactate in memory was suggested, where metabolic lactate enhanced memory when administrated before or during the learning process, while HCA_1 signaling mainly enhanced memory consolidation [87]. Hence, evidence for a role of HCA_1 activation on memory is emerging. Furthermore, lactate has been suggested to mediate some of the beneficial effects on neurobioenergetics, and hence to act as an exercise mimetic [88]. We have previously presented that seven weeks of exercise or lactate injections, up-regulates cerebral vascular endothelial growth factor A (VEGFA) levels and angiogenesis in the hippocampus and neocortex through activation of HCA_1 [89]. Recent progress in the field suggests that increased angiogenesis correlates with an increase in hippocampal neurogenesis in humans and rodents [38,90]. In these studies, neurogenesis occurs predominantly in neuro-angiogenic foci where neuronal, glial, and endothelial precursors divide into tight clusters. The formation of these clusters near newly formed capillaries also ensures a steady supply of nutrients, oxygen and

growth factors to maintain the proliferation of the stem and precursor cells. Taken together, these data suggest that angiogenesis and neurogenesis might be mechanistically linked.

Based on our previous demonstration of HCA_1-dependent angiogenesis and the known stimulation of neurogenesis by exercise, we here investigate whether lactate induces HCA_1-dependent neurogenesis in the SGZ of the hippocampus and/or in the V-SVZ.

Results

HCA_1 activation, by exercise or exogenous lactate, induces neurogenesis in the V-SVZ. To investigate whether activation of HCA_1 could be an initial event leading to neurogenesis, we exposed WT mice and HCA_1 KO mice to high intensity interval exercise (HIIT), five consecutive days per week for seven weeks[89]. In the V-SVZ of WT animals, the exercise regime resulted in an increased density of DCX positive neuroblasts compared to what was found in sedentary controls (Fig. 2b vs. 2c; quantified in 2h). A similar trend was seen in the HCA_1 KO animals exposed to exercise but did not reach statistical significance (Fig. 2e vs. 2f; quantified in 2h). The observed control density of DCX positive cells was higher in KO than in WT, perhaps reflecting compensatory changes that may blunt the effects of interventions in the KO. This did not reach statistical significance ($p=0.23$). Interestingly, injections of L-lactate (2 g kg^{-1} bodyweight; 200mg ml^{-1}; 18mmol kg^{-1}; pH 7.4) five days a week for seven weeks, increased neurogenesis in WT mice, to a larger degree than what was found in mice exposed to exercise (Fig. 2b vs. 2d; quantified in 2h). In HCA_1 KO mice, on the other hand, lactate injections did not increase the density of DCX-positive cells above control levels (Fig. 2e vs. 2g; quantified in 2h). The lactate treatment used in the present study increases serum lactate to about 10 mM [89]. The dependency of enhanced neurogenesis on HCA_1-activation in the V-SVZ, as determined by the quantification of DCX positive cells after lactate injections in WT and KO mice, was confirmed by quantification of Ki-67 positive cells (Fig. 1i), and of

cells co-expressing DCX and Ki-67 (Fig. 2j). DCX and Ki-67 represent cells at different stages of neurogenesis; DCX identifies neuroblasts (type A cells) while Ki-67 identifies type C cells.

[insert Figure 2.]

Hippocampal SGZ neurogenesis is induced by high-intensity interval exercise but is independent of HCA_1. Since neurogenesis in the V-SVZ was shown to be HCA_1-dependent, we also investigate whether activation of HCA_1 could initiate neurogenesis also in the SGZ of the hippocampus. In the SGZ (Fig. 3a), exercise resulted in an increased density of DCX positive neuroblasts in both genotypes compared to sedentary controls, i.e., independently of HCA_1 (Fig. 3b vs. 3c; and Fig. 3e vs. 3f; quantified in 3h). In this region, lactate injections did not induce neurogenesis above control levels (Fig. 3b vs. 3d; and Fig. 3e vs. 3g quantified in 3h).

[insert Figure 3.]

HCA_1 activation leads to activation of the Akt/PKB pathway. Data from the HCA_1-mRFP reporter mouse line show that HCA_1 is localized in leptomeningeal fibroblasts [89]. In a recent study [91], we show that HCA_1 is highly enriched also in fibroblast-like cells in the dorsal part of the wall of the third ventricle, in parts of the choroid plexus and in the tela choroidea. We do not find high levels of HCA_1-mRFP in cells of the SGZ or the SVZ, suggesting that HCA_1-containing leptomeningeal fibroblasts represent the primary site of action for lactate via HCA_1. Release of growth factors from these cells in response to HCA_1 activation may underlie the selective effect in the SVZ. In line with this, we demonstrate that primary cultures of leptomeningeal fibroblasts isolated from the HCA_1 (WT) mouse line responded to lactate by an increased phosphorylation of Akt/PKB at 5 minutes (Fig. 4a). This effect was not seen after 15 minutes (Fig. 4a). After longer exposure times to lactate, p-Akt levels were

reduced compared to control (Fig 4b). The latter finding likely represents internalization of the HCA_1 receptor [92], because the effect on p-Akt is nullified by co-incubation with the selective β-arrestin/β2-adaptin interaction (endocytosis) inhibitor, barbadin (Fig 4b). Furthermore, the exogenous HCA_1 agonist 3-chloro-5-hydroxy benzoic acid (3-Cl-5-OH) [93] produced a similar effect as did lactate, highlighting that the observed effect results from receptor activation, distinct from metabolic effects of lactate.

[insert Figure 4.]

Despite three independent attempts (3-6 mice in individual wells per attempt) to culture leptomeningeal fibroblasts from the HCA_1 KO mouse line in parallel with the WT fibroblast cultures, we did not succeed in obtaining KO fibroblast cultures. The KO fibroblasts stopped proliferating and/or died prior to reaching confluency (data not shown), suggesting that HCA_1 is important for viability in these cells.

Discussion

The present study demonstrates that in the V-SVZ, injections of lactate, five days a week for seven weeks, induced neurogenesis in WT mice, as evidenced by increased densities of cells expressing markers of newly formed neuroblasts (DCX) and/or earlier developmental stages (Ki-67) (Fig. 2). This effect was abolished in HCA_1 KO mice, indicating that the lactate receptor regulates V-SVZ neurogenesis (Fig 2). The lactate treatment regime increases plasma lactate to about 10 mM in 5-15 minutes, returning to basic levels within 45-60 minutes [89]. High intensity interval exercise, which increases lactate to a similar, but somewhat lesser extent (see [89]), tended to induce neurogenesis in both genotypes, reaching statistical significance only for DCX in WT, but not for Ki-67 or double labelled DCX+Ki-67 cells in either genotype. While exercise did not increase the density of DCX labelled cells in KO, it should be noted that KO mice had a control level of DCX labelling that was slightly higher

than in the WT. This difference did not reach statistical significance, but still, it opens for the possibility that the lack of HCA_1 could have caused compensatory changes, masking an exercise induced increase of DCX density in KO mice. Based on these results we cannot conclude whether the effect of lactate injections in HCA_1 WT animals is an additional effect on top of a smaller exercise-induced neurogenesis (occurring through HCA_1-independent mechanisms), or whether the effect is induced by HCA_1-activation alone. Nevertheless, our data demonstrate that HCA_1 is a pivotal regulator of neurogenesis in the V-SVZ.

The HCA_1 dependency of neurogenesis in this niche was not caused by differences in running speed or exercise intensity, as the knock-out and wild type mice performed equally well in maximal exercise-capacity tests, which were performed every second week throughout the exercise period [89]. As reported previously, the mice rapidly increased their maximal exercise capacity during the first weeks of exercise, reaching a plateau at about 140% after 5 weeks, as predicted for HIIT [89].

In the other main neurogenic niche of the brain, the SGZ of the hippocampus, exercise induce neurogenesis in both genotypes, but this effect could not be replicated by lactate injections (Fig 3). This is in line with previous studies demonstrating that even mild exercise is enough to induce hippocampal neurogenesis, through several mechanisms [94].

Neurogenesis has been suggested to occur in close proximity to newly formed capillaries[45]. We have previously shown that angiogenesis in the dentate gyrus is increased in response to exercise or lactate injections in an HCA_1-dependent manner [89]. Since these analyses were performed in the same animals as used in the present study, we can conclude that the regulation of hippocampal neurogenesis, being independent of HCA_1, is not secondary to an HCA_1-dependent angiogenesis. Hence, in the present study, neurogenesis occurred independently of angiogenesis.

Which HCA_1-expressing cells may be the primary sites for the action of circulating lactate resulting in HCA_1-induced increase in V-SVZ neurogenesis? Using a HCA_1-mRFP reporter mouse line, we have previously demonstrated that HCA_1 is expressed at high levels in the pia mater, where it localizes on fibroblast-like cells accumulated along pial blood vessels [89]. The pial localization of HCA_1 was confirmed by qPCR analysis, and is consistent with data presented in the Allen Brain Atlas (http://mouse.brain-map.org/gene/show/89056; http://mouse.brain-map.org/experiment/siv?id=77464856&imageId=77469798&initImage=expression&contrast=0.5,0.5,0,255,4). The HCA_1-mRFP labelling was stronger along arteries than veins, and did also line some of the larger blood vessels penetrating into the brain parenchyma [89]. This labelling could be consistent with a layer of pial fibroblasts reported to continue along the glia limitans along the branching arteries that penetrate into the brain parenchyma [95,96]. Growth factors like VEGF [97] and IGF-1, all of which can be released from fibroblasts, are known to regulate neurogenesis: The proper regulation of the neurogenesis process depends on VEGFA [97], or the coordinated action of fibroblast-growth factor 2 (FGF2) and insulin-like growth factor 1 (IGF1) [98]. Fibroblasts are known to release all of these growth factors, opening for the possibility that pial fibroblasts are the main site of action for lactate via HCA_1. In fact, we have previously shown that hippocampal VEGFA levels increased in response to HCA_1 activation in vivo. Interestingly, we recently showed that pial fibroblast-like cells, expressing HCA_1, are also found along the dorsal region of the wall of the third ventricle, and the adjacent parts of the choroid plexus and the tela choroidea[91]. In the present study, we demonstrate that primary leptomeningeal fibroblasts respond to HCA_1 activation by enhanced phosphorylation of Akt/PKB. A possible result of this increase in p-Akt is an increase in CREB, leading to enhanced expression of growth factors [99]. Various growth factors are also known to regulate the Akt-pathway though activations of their respective plasma membrane

receptors, but given the fact that our data were obtained in monocultures of fibroblast and that increased p-Akt is detected within five minutes, an indirect effect via growth factor release is less likely. We therefore believe that the activation of the Akt-pathway occurs downstream of HCA_1 activation, in line with previous evidence from different cell types [100,101]. The results on p-Akt in leptomeningeal fibroblasts expand on the previous observation [92] that HCA_1 activation over time (minutes) leads to downregulation of the response to below basal levels, due to internalization of the receptor. The observations indicate that intermittent stimulation, like by lactate generated in high intensity interval exercise, will act differently from continuous stimulation, such as by administration of an exogenous agonist with a longer half-life. This phenomenon has important implications for using HCA_1 as a target of therapeutic intervention. It may also serve to explain apparently contradictory results of HCA_1 activation. Our data suggest that the HCA_1-containing fibroblasts and ependymal cells have the ability to respond to alterations in brain, blood and CSF lactate to release their growth factors directly into the CSF where they may gain access to the cells of the subventricular zone. This remains to be investigated but could theoretically represent an explanation of why neurogenesis in the V-SVZ, but not in the SGZ, is sensitive to lactate/HCA_1-regulated neurogenesis. In this context, it should be noted that the walls of the lateral ventricles, where the V-SVZ is located, do not contain pial fibroblasts, so a local action within the ventricle wall is not likely. In our previous study [91], we also found HCA_1-mRFP in cells along smaller vessels in the neural tissue. Based on their perivascular localization and their expression of platelet-derived growth factor receptor beta (PDGFR-β), we suggested that these HCA_1-containing cells were immature pericytes, but PDGFR-β is also expressed by immature fibroblasts. Although not detected explicitly in the HCA_1-mRFP reporter mice, the localization of HCA_1 on other cell types in the brain has been suggested. For instance, Jean-Yves Chatton's lab have convincingly shown HCA_1-dependent electrophysiological effects in

cultured neurons, indicating that neurons express HCA_1 [85]. An astrocytic localization of HCA_1 has also been suggested [102]. Localization of HCA_1 on neural stem cells has not been reported but cannot be excluded. Consequently, we cannot conclude as to which cell type is responsible for the HCA_1-mediated influence on neurogenesis in the V-SVZ, but lactate-sensing fibroblast is a likely option.

Taken together, our results demonstrate that neurogenesis in the two main neurogenic niches of the brain are regulated differently: while physical exercise is important for SGZ neurogenesis, it appears to serve a smaller role in V-SVZ neurogenesis. In contrast, HCA_1-dependent lactate-induced neurogenesis was only detected in the V-SVZ, and not in the SGZ. HCA_1 may therefore represent a potential target for future drugs aiming to increase neurogenesis in the V-SVZ niche selectively. The functional consequences of V-SVZ neurogenesis remain unresolved. Nevertheless, recent data suggest that V-SVZ neurogenesis and the insertion of newborn neurons into the olfactory bulb circuitry plays a role in odour-recognition/olfactory memory, [47,48] which may be important for higher-order cognitive processing and olfactory memory [49]. Although distinct from the spatio-visual memory, which is dependent on the hippocampus, olfactory memory may affect hippocampal memory by providing contextual clues. While hippocampal neurogenesis is important in memory recollection and spatio-visual memory, some researchers claim that olfactory memory deficit may be the rodent equivalent of human age-related cognitive decline [49]. Furthermore, in humans [50] and non-human primates [51-53], V-SVZ-derived neurons appear to migrate to the striatum where they may serve to protect against Parkinson’s disease [54,55] and Huntington’s disease [50]. Therefore, it is not unlikely that an HCA_1-regulated increase in neurogenesis may decelerate age-related cognitive decline.

Materials and methods

Animals and animal treatment: The study was performed in strict accordance with the national and regional ethical guidelines, including the directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. All animal handling and experiments were performed by Federation of European Laboratory Animal Science Associations (FELASA) certified personnel and approved by the Animal Use and Care Committee of the Institute of Basic Medical Sciences, The Faculty of Medicine, University of Oslo, and by the Norwegian Animal Research Authority (FOTS6292, FOTS6505, FOTS6590, FOTS6720, FOTS6758, and FOTS8243). The experiments are reported in accordance with the Animal Research: Reporting in Vivo Experiments (ARRIVE) guidelines [103] and in agreement with the recommendations for good publishing practice in physiology [104].

The HCA_1 knock-out (KO) mouse line was generated as described [105]. At seven weeks of age, HCA_1 KO or wild-type (WT) mice were semi-randomized into three groups: high-intensity interval training (HIIT), sodium L-lactate injections (LAC), or saline injections (control), ensuring an equal distribution of males and females in each group. The HIIT regime used in the present study was designed for optimal gain in cardiovascular function and to reach about 90 % of VO_{2max} [106,107] and consisted of interval exercise as described [89], five consecutive days per week for seven weeks. Each session consisted of 10 min warm-up at 5 m min^{-1}, followed by 10 x 4 minutes of high-intensity intervals on a treadmill (Columbus Instruments, USA) at a 25 degrees incline. Between the intervals there were two minutes of active rest at 8.6 m min^{-1} 25 degrees incline. Every second week throughout the exercise period, a maximal exercise capacity test (MECT) was performed for each individual mouse, to adjust the running speed of the intervals. The MECT was performed as follows: After a 15 minute warm up-period at 9.6 m min^{-1}, the band speed was increased by 1.8 m min^{-1} every 2 min until exhaustion, i.e.,

the mice refused to run further, despite being manually pushed on to the band or receiving electrical stimuli (<1.5 mA, maximally 1-2 per day by the intrinsic device of the treadmill).

The mice that were treated with lactate, received a subcutaneous injection of sodium L-lactate (2 g kg^{-1} body weight; 200 mg ml^{-1} dissolved in 0.9 % saline; pH adjusted to 7.4; i.e., 18 mmol kg^{-1}). The control mice received the same volume (per kg body weight) of 0.9 % saline. The lactate or saline injections were administered subcutaneously five days a week for seven weeks. The mice were weighed every week for dosage-adjustment.

At 6 h after the end of the exercise or 6 hours after the last dose of lactate or saline, the mice were anaesthetized with zolazepam 3.3 mg ml^{-1}, tiletamine 3.3 mg ml^{-1}, xylazine 0.5 mg ml^{-1}, fentanyl 2.6 µg ml^{-1}; 0.01 ml g^{-1} body weight, intraperitoneally (i.p.) and perfused transcardially with 4 % formaldehyde in 0.1 M sodium phosphate buffer pH 7.4 (NaPi) for 8 min. After perfusion with the fixative, the brains were gently removed from the skull, and stored in 4 % formaldehyde at 4 °C overnight, before the brains were transferred to a 1:10 dilution of the fixative. During the experiment, one KO mouse exposed to exercise was excluded after 5 weeks, as it suddenly performed worse than expected (based on the previous performance of the same mouse) during two consecutive exercise section. It was therefore considered injured or sick. Two mice were excluded due to stereotypical behavior (one KO saline, and one KO exercise). Two mice were excluded due to sub-optimal perfusion fixation (1 WT saline; 1 KO exercise). The remaining animals were WT: 7, 7, 6 (saline, exercise, lactate) and KO: 5, 4, 6 (saline, exercise, lactate).

Immunofluorescence:

Before sectioning, the brains were transferred to 30 % sucrose in 0.1 M NaPi and allowed to sink in this solution overnight. Sagittal sections (20 µm thickness) were cut on a sliding microtome. Free-floating sections obtained 0.75-0.80 mm laterally from the midline were

used. Immunofluorescence was performed largely as described [89]. Free-floating brain sections were rinsed three times in PBS (10 mM NaPi, 0.9 % NaCl) and antigen retrieval was performed by incubation of the section in citrate buffer (0.01 M, pH 8.7) for 30 minutes at 80 °C. The sections were then rinsed in PBS and unspecific antibody binding sites were blocked by incubating with 10 % newborn calf serum and 0.5 % Triton X-100 in PBS for 2 h. The sections were then incubated with primary antibodies (rabbit anti-Ki-67, Abcam AB15580; final dilution 1:500) and guinea pig anti-doublecortin (DCX, Merck AB2253, final dilution 1:2000), overnight at room temperature under careful shaking. Then, the sections were rinsed in PBS and incubated with secondary antibodies: Alexa Fluor 594 Anti Guinea Pig (Invitrogen A11076) and Alexa Fluor 647 Anti Rabbit (Invitrogen A21241), both at a dilution of 1:500, for 2 h at room temperature. From this step on, the sections were covered from light. After incubation with the secondary antibodies, the sections were rinsed, stained with DAPI (Thermo Fisher, UK) for 15 minutes and rinsed again. Finally, the sections were mounted with Prolong Gold Antifade Mounting Reagent (Thermo Fisher, UK) and coverslipped (Assistant, Germany).

Confocal microscopy was performed with Zeiss LSM880 with Airyscan detector on 20x magnification with each Z-stack slice 0.5 μm, stacked together with maximum intensity projection option. The cells were counted manually by an observer who was blinded to the genotypes and treatments, and the length of the SVZ or SGZ was measured in the Fiji distribution of ImageJ [108] (version 2.0.0-rc-69/1.52p; Java 1.8.0_172, ImageJ, RRID:SCR_003070) or in ZenBlue 2.3. The density of newborn neurons were calculated as the number of DCX or Ki-67 positive cells divided by the measured length of the corresponding SVZ or SGZ for each animal. For the SVZ, six sections were excluded from the analysis, either because they had artefacts in the region of interest, or because they were

not in the narrow anatomical region included in this study. The excluded sections were two WT saline, two WT lactate and one KO saline.

Isolation of primary leptomeningeal fibroblast:

Leptomeningeal fibroblasts were isolated [109] from WT (n=5) and HCA_1 KO (n=6) mice. The mice were deeply anesthetized with isoflurane and euthanized by decapitation. The brain was gently removed from the scull and put on ice cold PBS. A thin layer of the neocortex was dissected loose, transferred sterile culture medium, and cut into smaller pieces. The pieces of cortical tissue from each animal were distributed into 3 of the wells of a 6-well plate (VWR, United Kingdom). Growth media (DMEM/F12 GlutaMAX (Thermo Fisher Scientific), with 10 % Fetal Bovine Serum (FBS) (Thermo Fisher Scientific, USA) and 1 % Penicillin-Streptomycin (Thermo Fisher Scientific, USA)) was added at a volume that prevented the tissue from floating yet kept them moist (about 850 µL in each well). The tissue pieces were grown at 37 °C with 5 % CO_2. The media was gently replaced twice a week. When a confluent layer of leptomeningeal fibroblasts covered the entire well after approximately four weeks), the remaining tissue pieces and the medium was removed. During this four-weeks period, all the KO cells stopped growing and/or died. The WT cells were washed with trypsin-EDTA (0.25 %; Sigma-Aldrich, USA) in PBS and the detached cells were pipetted into 15 ml falcon type. To inactivate the trypsin, growth media containing 10 % FBS was added, and the cells were centrifuged (Heraeus Megafuge 1.0 R, Thermo-Fisher Scientific, USA) at 1000 g for about 5 minutes. The resulting cell pellets from all animals were pooled and grown in the T75 flask until confluency. For experiments, the cells were split to several 6-well Tissue Culture plates, with 2 mL growth medium in each well. The cells used in the present study were generation 7-11 *ex vivo.*

Exposure of cells to HCA_1 agonists:

At 24 hours before the experiment, the medium was replaced by either growth medium

containing 10 % FBS or medium without FBS. The following day, the cells were exposed either sodium L-lactate (Sigma-Aldrich, USA) 20 mM in PBS or vehicle, for five, 15, 30 or 60 minutes. To study whether HCA_1 was internalized in response to receptor activation, other cells were exposed to sodium L-lactate (20 mM), the HCA_1 agonist 3-chloro-5-hydroxybenzoic acid (3-Cl-5-OH: Sigma-Aldrich, USA; 40 μM), or PBS (control), in the absence or presence of the β-arrestin/AP2-dependent G-protein-coupled (GPCR) endocytosis inhibitor, barbadin [110]. In the cultures treated with barbadin, barbadin was added at 30 minutes prior to the HCA_1 agonists. Since 3-Cl-5-OH was dissolved in DMSO, all wells were supplemented with DMSO 1 μL per 2mL PBS. The exposure times for the latter experiment were 60 and 120 minutes. The cells were harvested 100 μL ice-cold RIPA buffer with protease- and phosphatase inhibitor (Sigma-Aldrich, USA), transferred to Eppendorf tubes and snap frozen in liquid nitrogen before they were stored in -80 °C.

Western blotting:

The protein content of the harvested fibroblast samples was measured with the Bicinchoninic acid (BCA) assay (Thermo-Fisher Sientific, USA). The samples were heated at 95°C for 2 minutes, and diluted in 25 % 4X Laemmli buffer (94 mM Tris HCl pH 6.8, 10 % (w/v) SDS, 25 % (v/v) glycerol, 0005 % (w/v) bromophenol blue, in milli-Q water) with 5 % 2-Mercaptoethanol (Sigma-Aldrich, USA) to a final concentration of 1 μg protein μL^{-1}. The samples 10 μg/lane) was applied to a Bio-Rad Laboratories, USA, precast gradient (4-15 % or 4-20 %) gel. The electrophoresis was run on 150 volts for approximately one hour. The proteins were then blotted onto a nitrocellulose membrane (Bio-Rad Laboratories, USA) at 2.5 A and 25 V in the Trans-Blot® Turbo Transfer System (Bio-Rad Laboratories, USA) for 10 minutes. The quality of the transfer of the proteins, was confirmed by Ponceau S staining.

The membrane was incubated with 5 % dry nonfatty milk for one hour to block unspecific protein-interactions sites. Then the membrane was incubated with primary antibody (Phospho-Akt (Ser473) (D9E) XP® Rabbit IgG, catalogue number: 4060 (Cell Signaling Technology, USA; final dilution 1:2000) overnight. The next day, the membrane was washed 3 x 10 minutes in TBST and exposed to species-specific secondary antibodies (Anti-Rabbit IgG, Horseradish Peroxidase linked whole antibody (from donkey), catalogue number: NA934 (GE Healthcare Life Sciences, United Kingdom); final dilution 1:5000) for one hour. The membrane was washed 3 x 10 minutes and the exposure to an HRP-substrate (Luminata™ Classico Western HRP Substrate; Merck Millipore, USA) for 5 minutes. The protein bands were visualized and imaged (Syngene Chemi Genius 2 Bio Imaging System, USA). Finally, the membrane was rinsed (3 x 10 minutes), blocked for 1 hour with 5 % dry milk and incubated with an HRP-coupled α-tubulin antibody for 3 hours. Then the blots were exposed to the HRP-substrate again and imaged as described.

The p-Akt antibody produced one predominant band at the correct molecular mass (60 kDa) with no other visual bands. Quantitative analysis of the chemiluminescence signals of the protein bands, representing p-Akt (60 kDa), was performed using the Image Studio Lite Version 5.2.5 (LI-COR Biosciences, USA) program. Measured chemiluminescence signal for protein binding of the above proteins was normalized against measured chemiluminescence signal for protein-binding of the loading control, α-tubulin (50 kDa) from the same membrane. For the shorter exposure times (figure 4a), seven separate cell experiments and Western blots, each with two replicates, were performed. For the longer exposure times, four independent cell experiments and Western blots, each with two replicates, were performed.

Statistical Analysis:

Data were analyzed with pair ways student´s *t*-test for the Western blot data presented in

figure 4a, or by one-way ANOVA; LSD post-hoc test in SPSS (SPSS, RRID:SCR_002865) for all other data sets.

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Author contributions

L.H.B., J.S.-M. and C.M. designed the study. C.M., K.A.A., Ø.P.H. and A.H. performed the in vivo studies. C.M. and K.A.A. planned these studies and harvested organs. L.T.Ø., M.L., and C.M. performed immunohistochemistry. L.T.Ø. performed the analysis of neurogenesis in the SGZ (supervised by C.M). M.L. performed confocal imaging of neurogenesis in V-SVZ and performed the analysis (supervised by S.G. and C.M.). M.S.H. performed the cell culture and Western blot experiments. C.M. wrote the draft manuscript. L.T.Ø., M.L. and C.M. prepared the figures. M.L., L.T.Ø. and J.S.-M. revised the draft manuscript. All authors discussed the results, critically revised the manuscript and approved the final version of the manuscript. M.L. and L.T.Ø. contributed equally to the paper.

Conflict of interests:

The authors declare no competing financial interests.

Data availability:

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure legends

Figure 1: (a) The location of the two major neurogenic niches in a sagittal section of a mouse brain; the V-SVZ and the SGZ. (b) Schematic drawing of the neurogenic process of the V-SVZ niche. Quiescent type B neural stem cells (NSCs; blue) send an apical process through a layer of ependymal cells (light grey) that line the ventricle (dark grey). A basal process of the type B cell makes contact with the blood vessels of the vascular plexus. Upon activation, the type B cells divide to give rise to type C transit amplifying cells (green). Type C cells rapidly divide and differentiate to type A neuroblasts (red), which migrate to the olfactory bulb. Resident mature astrocytes (blue) and microglia (orange) also inhabit the V-SVZ neurogenic niche. (c) Schematic drawing of the neurogenic process of the SGZ niche and the migration into the granular cell layer (light grey cells). Type 1 neuronal stem cells (radial glia; blue) may give rise to neurons (right-pointing arrow) or astrocytes (left-pointing arrow). In neurogenesis, type 1 cells give rise to non-radial type 2 cells (green), which further give rise to intermediate progenitor cells (not shown) that proliferate to give rise to type 3 neuroblasts (pink). As the neuroblasts differentiate to form immature neurons (red) and later fully mature granule neurons (orange), they migrate into the granule cell layer. The stages where Ki-67 and doublecortin (DCX) are expressed, are indicated.

Figure 2. The effect of HCA_1 on SVZ neurogenesis on the dorsal part of the lateral ventricle. (a) Schematic view of the SVZ. (b-g) Confocal micrographs of WT (b-d) and HCA_1 KO (e-g) showing labelling for DCX (red), Ki-67 (yellow), and DAPI (blue). (h) The density of DCX-labeled cells increased with high intensity exercise (blue bars) in both genotypes (compared to their respective untreated control groups; white bars), but this only reached significance in the WT ($p=0.039$). In the WT mice, lactate treatment (grey bar) lead to an even larger increase in

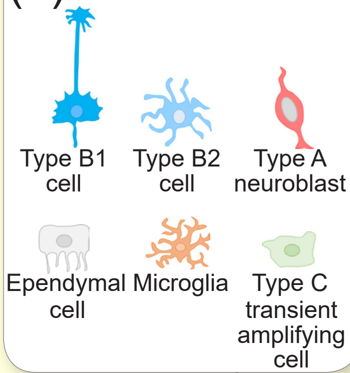
DCX-positive cells (p=0.0004). The effect of lactate on the density of DCX positive cells was not present in the KO mice (grey bar vs. white bar). (i) The HCA_1-dependent neurogenic effect of lactate was reproduced by quantification of Ki-67-positive cells. (j) Cells which are positive for both Ki-67 and DCX. As for DCX, the number of Ki-67-positive cells increased in response to lactate treatment (grey bar vs white bar) in the WT mice (p=0.003), but not in the HCA_1 KO mice. * $p<0.05$, † $p<0.01$; one way ANOVA; LSD post-hoc test, n=5,7,4; KO n=4,4,6). Scale bar=100µm

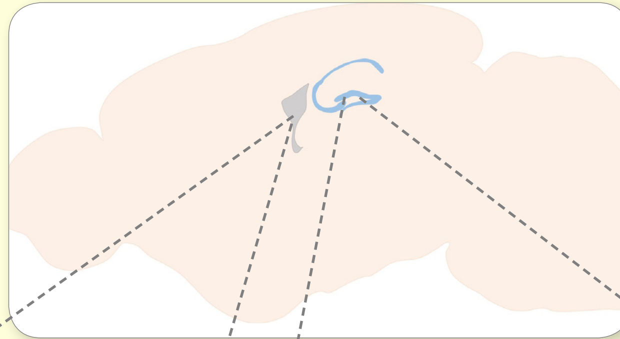
Figure 3: The effect of HCA_1 on SGZ neurogenesis on the granule cell layer of the hippocampal formation. (a) Schematic view of the SGZ neurogenic niche. (b-g) Confocal micrographs of WT (b-d) and HCA_1 KO (e-g) showing labelling for DCX (red), and DAPI (blue). (h) The density of DCX-labeled cells (cells per mm subgranular zone length) increased in response to high intensity exercise (blue bars) in both genotypes (compared to their respective untreated control groups, white bars); WT (p=0.003); KO (p=0.038). Contrary to what was seen in the SVZ, lactate treatment (grey bars) did not lead to increased density of DCX-positive cells neither in the WT mice nor in the KO mice. Data are average ± SD; * $p<0.05$, ‡ $p<0.005$; one way ANOVA; LSD post-hoc test, n=7,7,6; KO n=5,4,6). Scale bar=100µm

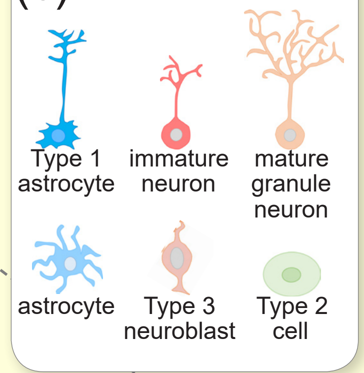
Figure 4. The effect of HCA_1 activation on the level of phosphorylated Akt (p-Akt) in leptomeningeal fibroblasts. (a) Representative Western blots for p-Akt (band at 60 kDa) and α-tubulin (band at 50 kDa) after exposure of leptomeningeal fibroblasts to PBS (control) or PBS with sodium L- lactate (20 mM) for 5, 15, 30 or 60 minutes. Below: Quantitation assessment of the immunoluminescence intensity of the p-Akt band, relative to the loading

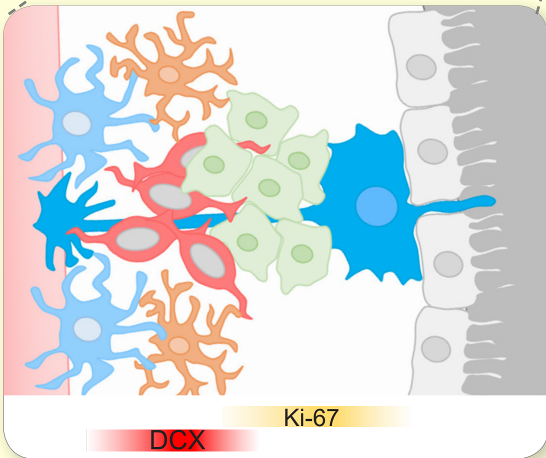
control. To be able to combine results from different experiments (n=7), the results were normalized to the average of all the control samples on the same blot. The results are presented as mean±SD. Exposure to lactate (blue bars) resulted in an increased level of p-Akt compared to control (white bars) after five minutes ($p=0.00002$; paired Student´s *t*-test). Ths effect was not seen after 15 minutes. After longer exposure times, 30 and 60 minutes, p-Akt levels were reduced in response to lactate treatment (blue bars) compared to control (white bars; $p< 0.005$ for both comparisons; paired Student´s *t*-test for each time point). (b) Representative Western blots for p-Akt after exposure of leptomeningeal fibroblasts to PBS (control), PBS with sodium L-lactate (20 mM) or 3-Cl-5-OH (3C5H; 40 µM) in PBS in the absence or presence of the endocytosis blocker barbadin for 60 or 120 minutes. Quantitative results shown below are immunoluminescence intensity of the p-Akt band, relative to the loading control. To be able to combine results from different experiments (n=4), the results were normalized to the average of all the control samples on the same blot. As expected, lactate (blue bars) produced a reduction in the p-Akt levels after 60 or 120 minutes compared to the respective controls (white bars; $p< 0.005$; one-way ANOVA for each time point; LSD posthoc test). These findings were reproduced by exposure to the exogenous HCA_1 agonist, 3-Cl-5-OH (grey bars; $p< 0.05$; one-way ANOVA; LSD posthoc test) compared to the respective controls. Barbadin (B) completely inhibited the effects of the HCA_1 agonists, suggesting that the reduction on p-Akt produced by lactate and 3-Cl-5-OH after 60 or 120 minutes were due to internalization of the HCA_1 receptor. * $p<0.05$; † $p<0.01$; ‡ $p<0.005$.

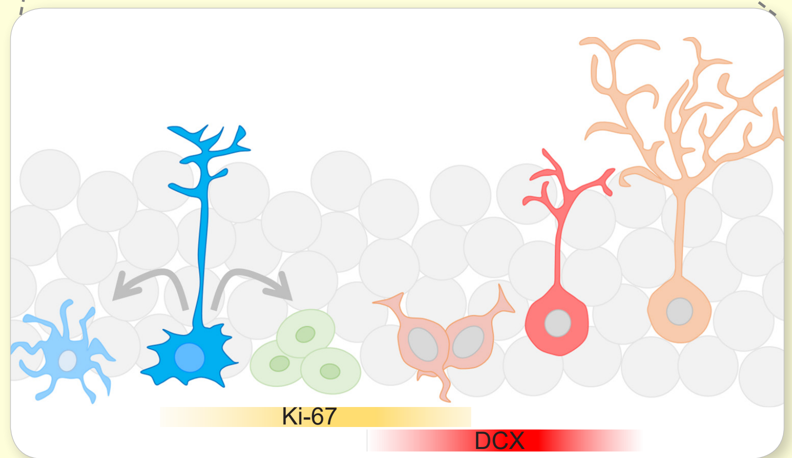
(a) Neurogenic niches in the rodent brain

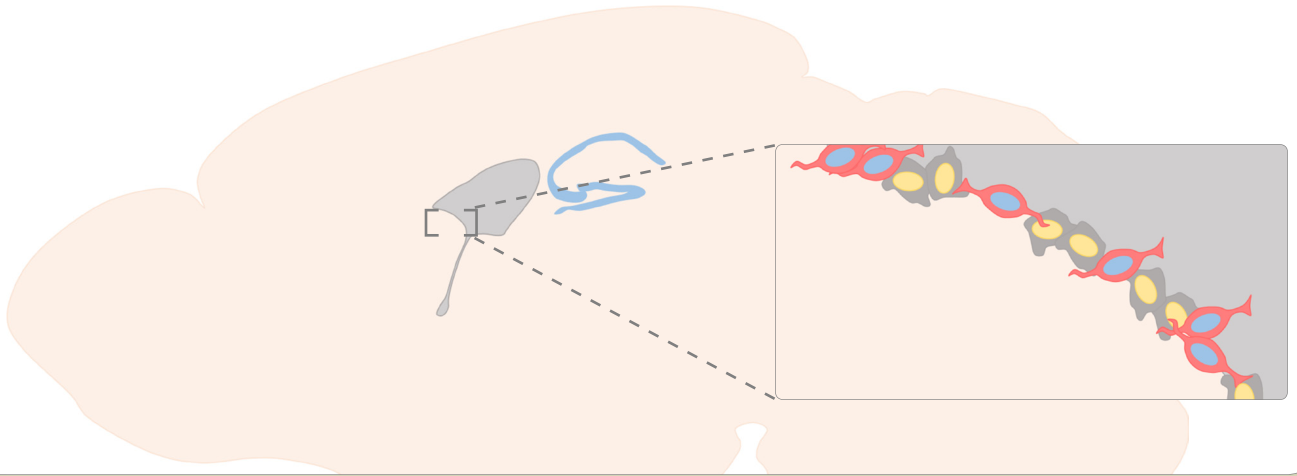


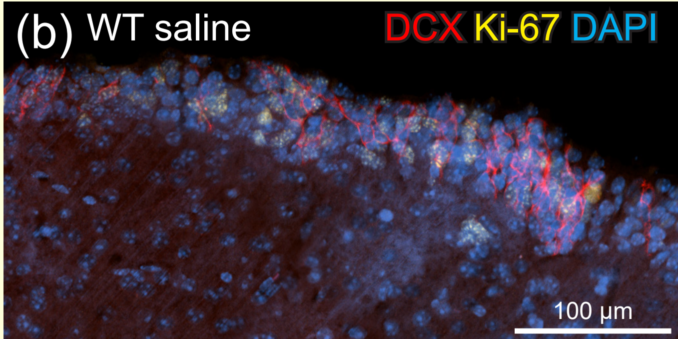


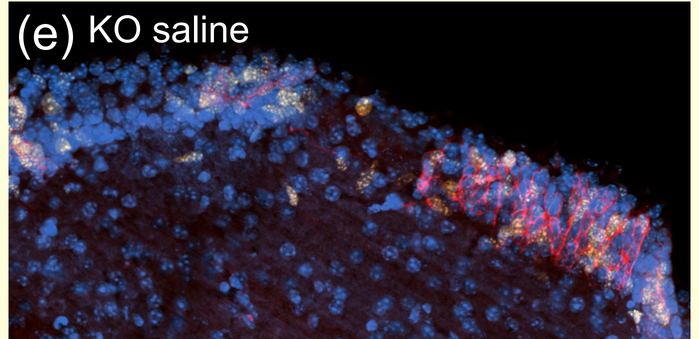


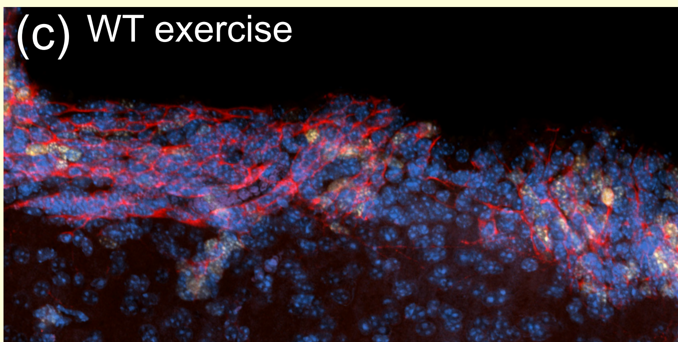





(a)


(b) WT saline
DCX Ki-67 DAPI
100 µm


(e) KO saline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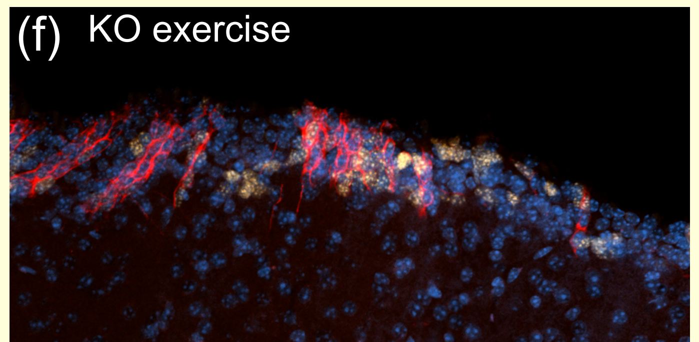
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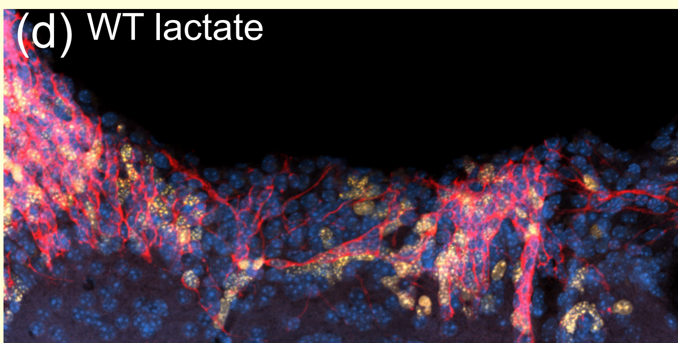
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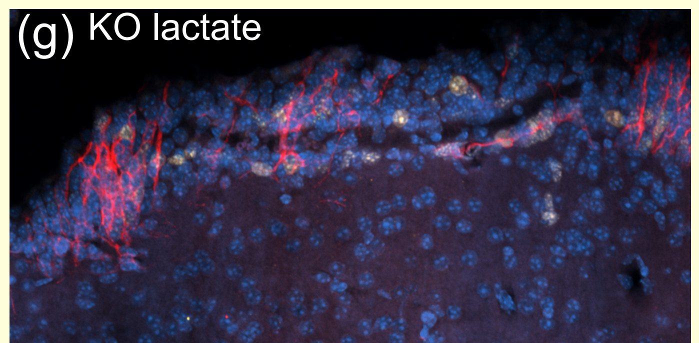
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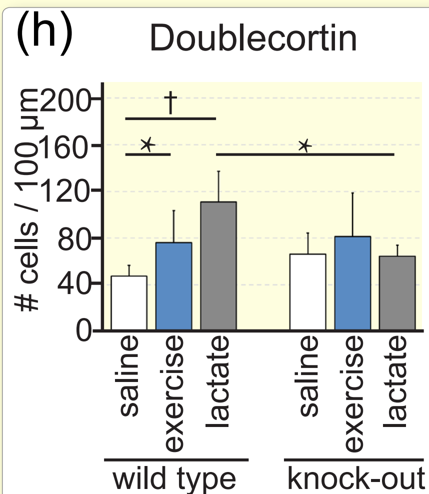
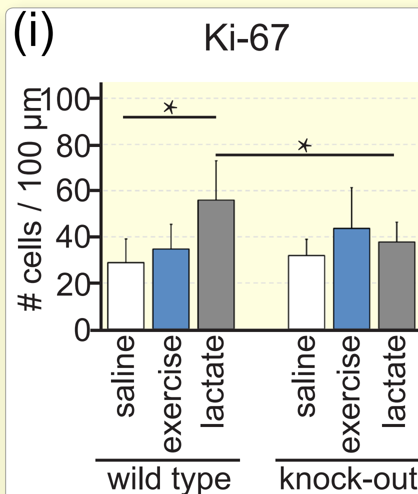
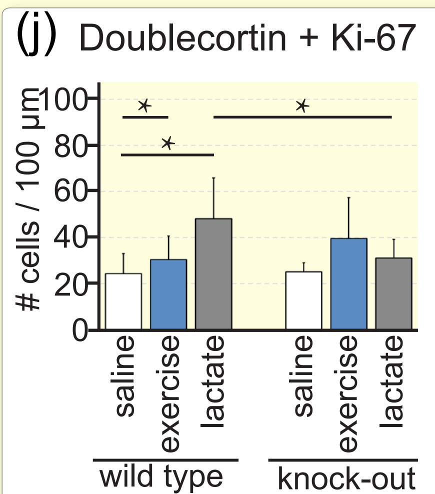
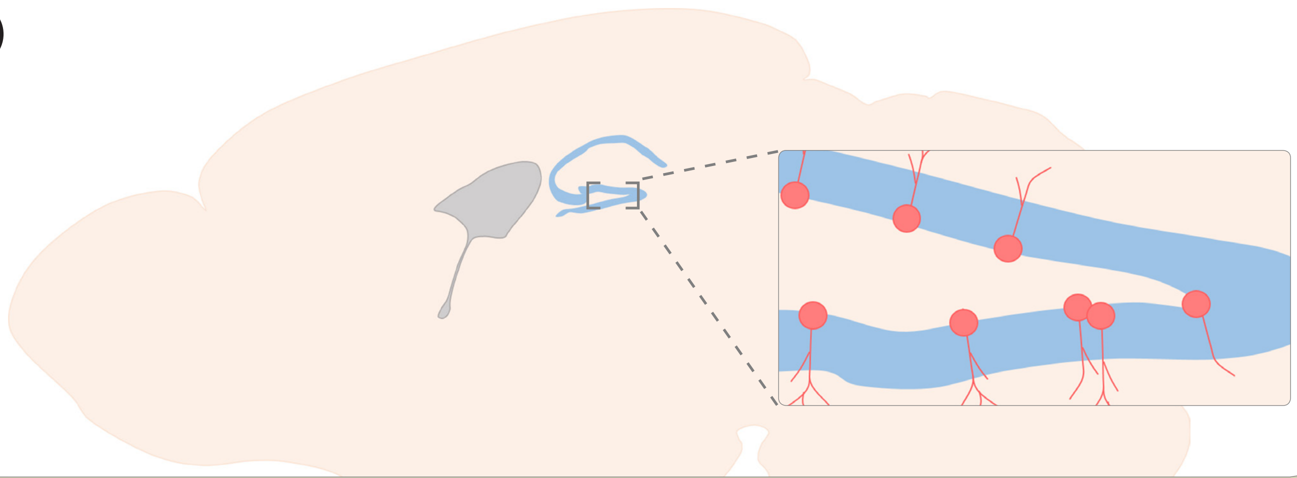

(h) Doublecortin
cells / 100 µm
200
160
120
80
40
0
†
*
*
saline
exercise
lactate
saline
exercise
lactate
wild type
knock-out


(i) Ki-67
cells / 100 µm
100
80
60
40
20
0
*
*
saline
exercise
lactate
saline
exercise
lactate
wild type
knock-out


(j) Doublecortin + Ki-67
cells / 100 µm
100
80
60
40
20
0
*
*
*
saline
exercise
lactate
saline
exercise
lactate
wild type
knock-out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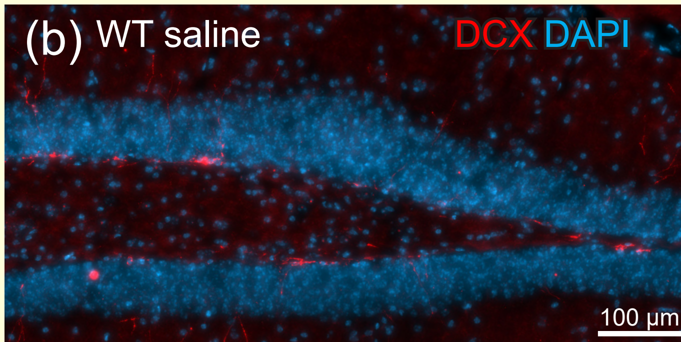
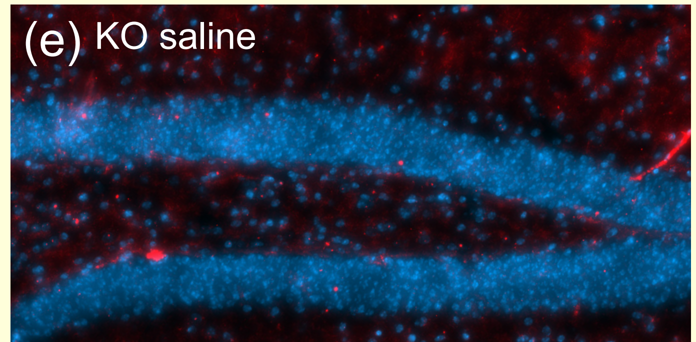
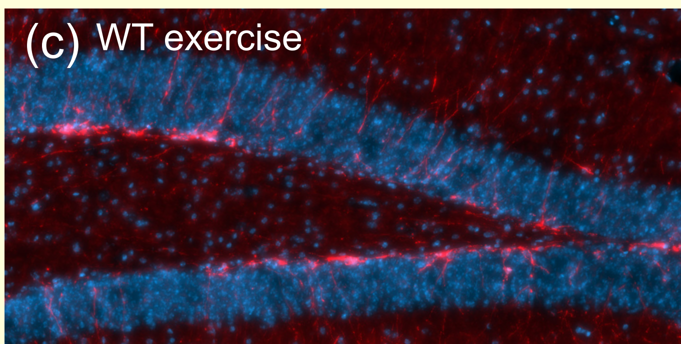
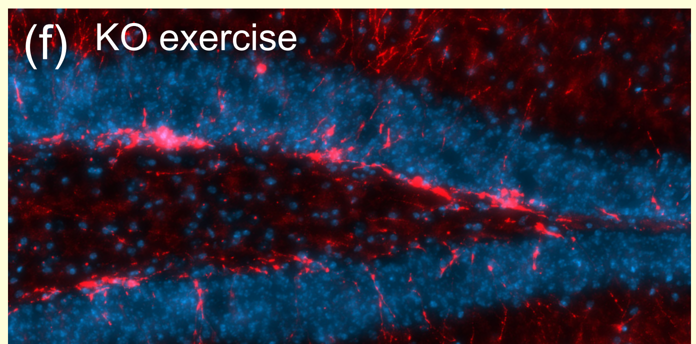
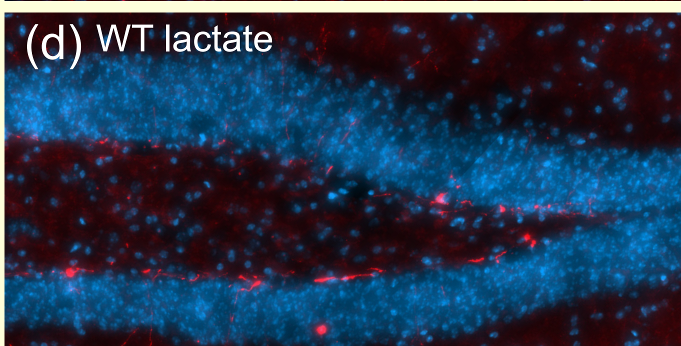
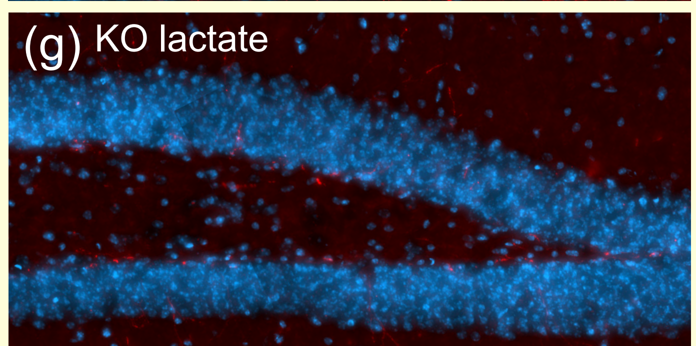

(b) WT saline
DCX DAPI
100 µm

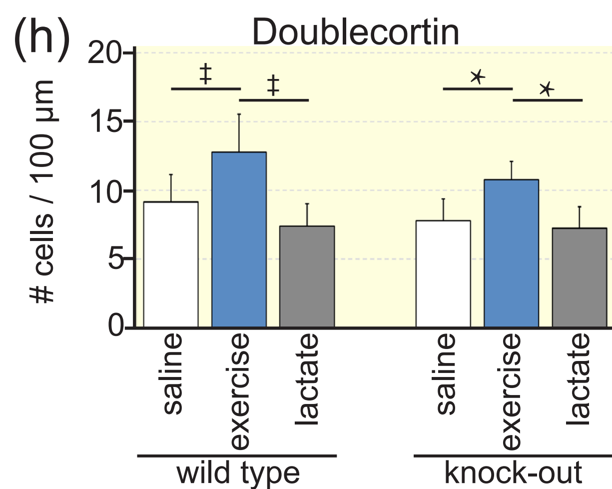

(e) KO saline


(c) WT exercise

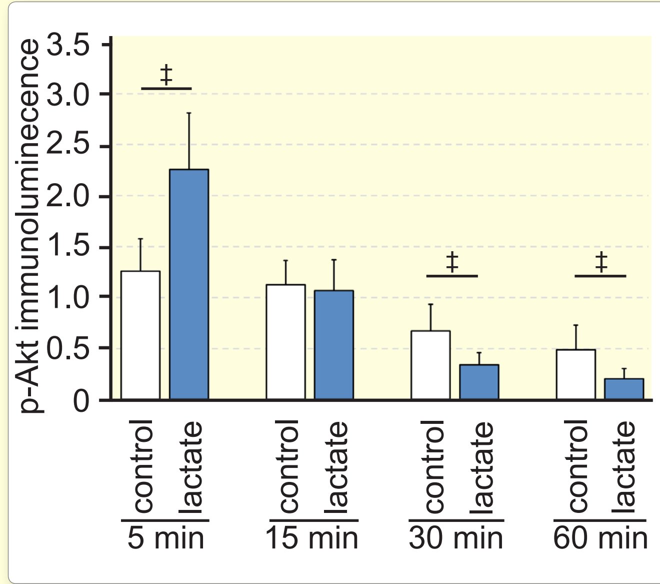

(f) KO exercise

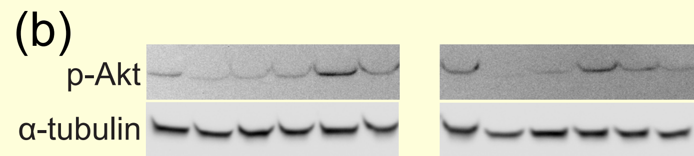

(d) WT lactate

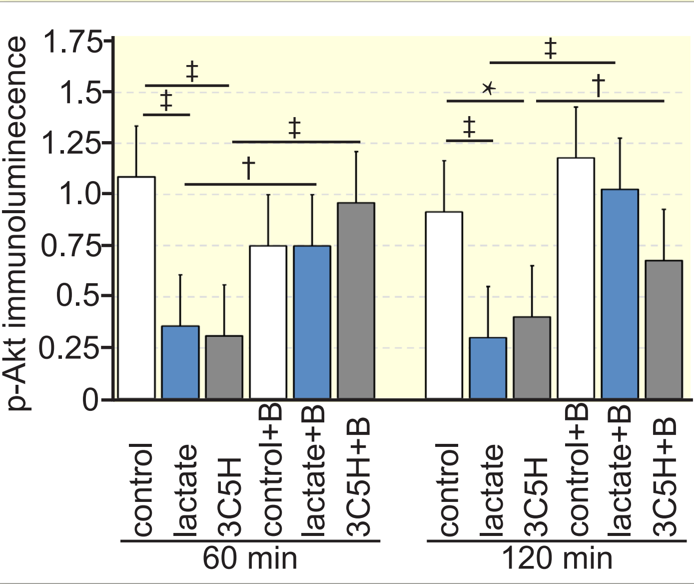

(g) KO lactate


(h)
Doublecortin
cells / 100 µm
20
15
10
5
0
‡
‡
*
*
saline
exercise
lactate
saline
exercise
lactate
wild type
knock-out

(a)
p-Akt
α-tubulin


p-Akt immunoluminecence
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
‡
‡
‡
control
lactate
5 min
15 min
30 min
60 min


(b)
p-Akt
α-tubulin


p-Akt immunoluminecence
1.75
1.5
1.25
1.0
0.75
0.5
0.25
0
‡
†
*
control
lactate
3C5H
control+B
lactate+B
3C5H+B
60 min
120 min