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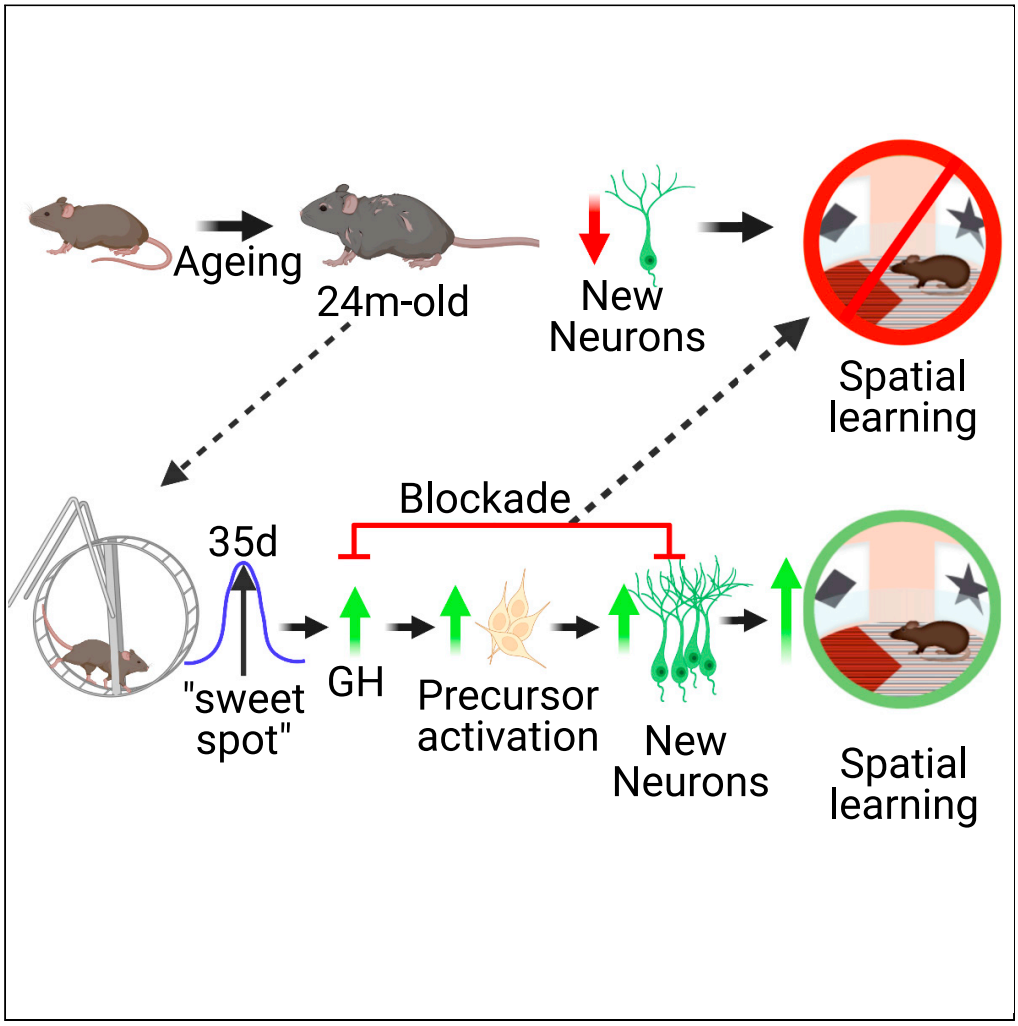

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Highlights

Only specific periods of exercise restores spatial learning in aged mice

Increased growth hormone (GH) mediates improved learning by activating neurogenesis

Neurogenesis is required for exercise-mediated improvement in learning

Identification of mechanisms regulating GH-mediated improvements in learning

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An exercise "sweet spot" reverses cognitive deficits of aging by growth-hormone-induced neurogenesis

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SUMMARY

Hippocampal function is critical for spatial and contextual learning, and its decline with age contributes to cognitive impairment. Exercise can improve hippocampal function, however, the amount of exercise and mechanisms mediating improvement remain largely unknown. Here, we show exercise reverses learning deficits in aged (24 months) female mice but only when it occurs for a specific duration, with longer or shorter periods proving ineffective. A spike in the levels of growth hormone (GH) and a corresponding increase in neurogenesis during this sweet spot mediate this effect because blocking GH receptor with a competitive antagonist or depleting newborn neurons abrogates the exercise-induced cognitive improvement. Moreover, raising GH levels with GH-releasing hormone agonist improved cognition in nonrunners. We show that GH stimulates neural precursors directly, indicating the link between raised GH and neurogenesis is the basis for the substantially improved learning in aged animals.

INTRODUCTION

Advanced age is typically associated with a decrease in cognitive function, including the formation and retention of new memories. The hippocampus is critical for a range of learning and memory processes, especially spatial learning (Deng et al., 2010). It is also one of the two main regions in the mammalian brain in which new neurons continue to be generated in adulthood (Altman and Das, 1965; Eriksson et al., 1998; Reynolds and Weiss, 1992; Richards et al., 1992). This adult hippocampal neurogenesis occurs within the dentate gyrus (DG) in a multistep process that culminates in the integration of adult-born neurons into the existing neurocircuitry where they directly contribute to hippocampal function (van Praag et al., 2002). Unfortunately, however, the hippocampus is particularly sensitive to aging, with each stage of neurogenesis being detrimentally affected over time, leading to a reduced number of activatable endogenous neural stem cells (Kuhn et al., 1996; Walker et al., 2008), a decrease in overall neurogenesis (Ben Abdallah et al., 2010; Wu et al., 2008), and spatial learning deficits (Benice et al., 2006; van Praag et al., 2005).

One of the strongest positive physiological modulators of hippocampal function is physical exercise, where it has been shown to increase hippocampal cell proliferation, neurogenesis, and spatial learning in mice (van Praag et al., 1999a,b). In aged animals, however, the effects of physical exercise have proven less consistent, with exercise reported to be either beneficial (van Praag et al., 2005; Wu et al., 2015) or refractory (Creer et al., 2010), possibly due to variations in the methodologies and approaches used.

Although several theories have been proposed to explain how exercise improves brain health, the precise mechanism(s) remains largely unknown. One unifying proposal is that alterations in systemic circulating factors mediate both age-dependent deterioration and exercise-induced improvements in hippocampal function. In support of this, parabiosis experiments, resulting in a shared blood circulation between young and aged mice, reduced hippocampal neurogenesis and learning ability in young adult mice, as did injections of plasma from aged into young animals (Villeda et al., 2011). However, plasma from exercised aged mice abolished these deficits in sedentary aged animals (Horowitz et al., 2020). Previously, we have shown that the circulating factor, growth hormone (GH), is responsible for the stimulation of neurogenic precursors in the subventricular zone (SVZ) in mice after exercise (Blackmore et al., 2012) and in humans, GH is negatively affected by age (Ho et al., 1987), whereas exercise increases circulating GH levels (Felsing et al., 1992; Pritzlaff et al., 1999). There is growing evidence that GH is involved in cognitive function (Falleti et al., 2006), including a positive correlation between GH secretion and cognitive health in aged human

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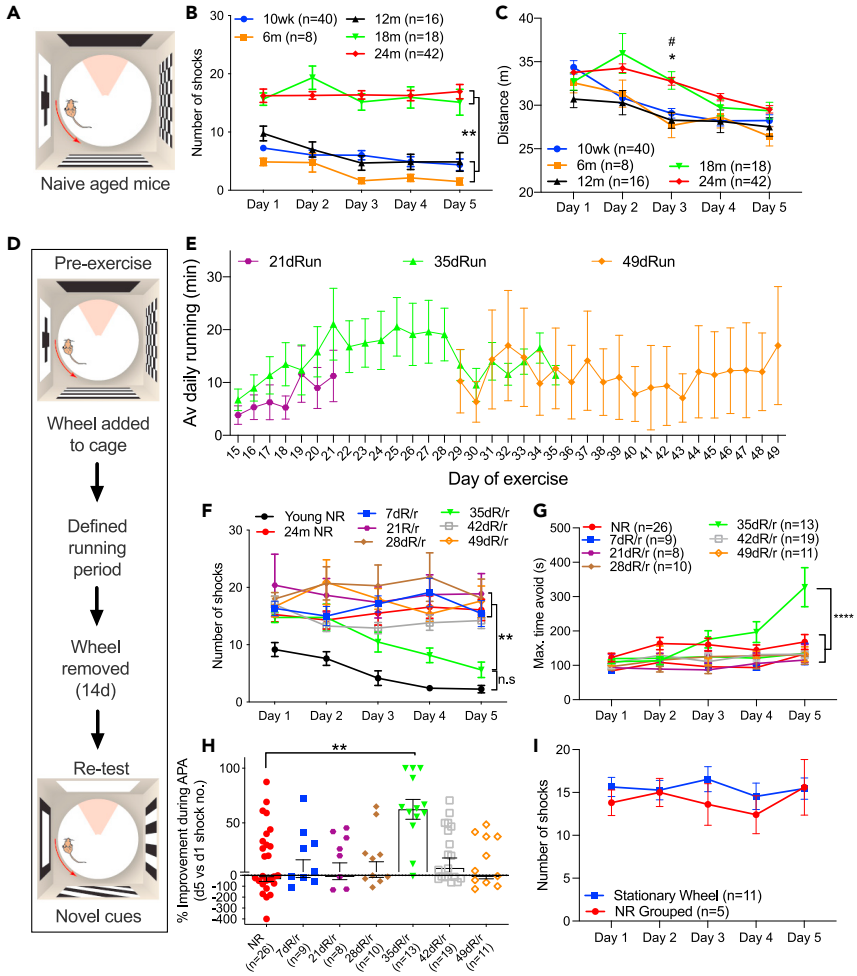

Figure 1. Aged animals display a spatial learning impairment that is ameliorated by an optimized period of physical exercise

(A) Representation of the APA apparatus. Each mouse is placed on a rotating grid and must use the spatial cues located around the room to avoid the stationary shock zone (shown in red).

(B) Naïve mice from 10 weeks to 24 months of age were tested on the APA task. The 18- and 24-month-old animals were unable to learn as evidenced by the lack of change in shock number over the test period (mean ± SE; two-way RM-ANOVA [age effect $F(4, 119) = 50.79$; $p < 0.0001$] with Bonferroni post hoc tests).

(C) During APA testing, there was a significant difference in the total distance traveled for the different age groups; however, on the final day, there were no significant differences between the groups (mean ± SE; two-way RM-ANOVA age effect [$F(4,119) = 1.750$; $p = 0.1435$], with Bonferroni post hoc tests. * represents significance between 10 weeks and 18 months old; # represents significance between 12 months old and 18 months old).

(D) Schematic representation of the experimental design. All 24-month-old animals were tested for spatial learning ability prior to exercise. At the completion of the exercise period, the running wheels were removed. Two weeks later, novel cues were used in the second test of spatial learning.

(E) The running time for group-housed animals for 21 d, 35 d, or 49 d revealed no difference in average daily running time during the exercise intervention (mean ± SE). Analysis of running activity was calculated between the hours of 7 pm and 3 am, when the majority of exercise took place.

(F) Only the 24-month-old animals that underwent the optimized exercise period of 35 days improved during APA testing, to a similar level to that of young animals (mean ± SE; two-way RM-ANOVA [exercise effect $F(7, 100) = 9.208$; $p < 0.0001$] with Bonferroni post hoc tests).

(G) There was a significant increase in maximal time spent avoiding the shock zone for the 35-d run group, and on the final day, this was significantly different from the time recorded for all other groups (mean ± SE; two-way RM-ANOVA [exercise effect [$F(6,89) = 4.723$, $p = 0.0003$], with Bonferroni post hoc tests).

(H) The learning ability following exercise was calculated by comparing the number of shocks on the first day of testing to the number of shocks on the last day of testing, which was represented as a percentage (mean ± SE; one-way ANOVA

Figure 1. *Continued*
[F(6,89) = 3.092; p = 0.0085], with Bonferroni post hoc tests). This revealed that the animals that ran for 35 days significantly improved whereas those that underwent other exercise periods did not.
(l) There was no difference and no improvement in the number of shocks received during APA testing for animals that had access to a stationary running wheel (mean ± SE; two-way RM-ANOVA, with Bonferroni *post hoc* tests). *p < 0.05, **p < 0.01, ****p < 0.0001. NR, no run, R/r, Run/rest where rest is 14 days. See also Figure S1.

individuals (Quik et al., 2012). Furthermore, peripheral injection of GH has been shown to increase cell proliferation in the hippocampus of rodents (Aberg et al., 2010). This led us to first investigate if fluctuating levels of circulating GH during exercise represent an efficient biomarker to measure successful exercise duration. Second, we aimed to determine if GH plays a causal role in the timing and activation of exercise-mediated hippocampal function of aged mice.

Here we report that an optimal period of exercise in 24-month-old mice significantly improves spatial learning in a neurogenesis-dependent manner and that a spike in the circulating GH level corresponds with this increase. We also show that pharmacological induction of GH release is able to mimic the beneficial effects of exercise on the spatial learning of old animals. Finally, as we observed a tight temporal window for both exercise-mediated improvements and GH levels, we determined that the circulating level of somatostatin, the main inhibitor of GH secretion, is critical in the exercise-dependent hippocampal responses in the aged mouse.

RESULTS

Spatial learning deficits occur in aged mice

To assess how aging affects hippocampus-dependent spatial learning, we tested mice aged from 10 weeks to 24 months on the allothetic active place avoidance (APA) task. We chose this task to examine hippocampus-dependent learning as hippocampal inactivation via tetrodotoxin (Cimadevilla et al., 2000; Wesierska et al., 2005), lesion studies (Codd et al., 2020), and specific ablation of immature neurons within the DG (Vukovic et al., 2013), all result in almost complete loss of spatial learning during APA testing. This task also has the advantage that the amygdala is unlikely to be involved as there is no evidence of increased stress and fear, which the amygdala is heavily involved in mediating, as cortisol levels are not increased during APA testing (Lesburgueres et al., 2016). During APA testing, the animals are placed on a rotating platform and are required to use surrounding distal cues to determine their position and navigate away from a stable shock zone (Stuchlik et al., 2013) (Figure 1A). It is important to note that as the platform is rotating, the mouse is unable to use an idiothetic approach for navigation, nor can it use exteroceptive cues such as scent marks because these cues rotate with the platform while the shock zone remains stable (Stuchlik et al., 2013). The 18- and 24-month-old animals performed significantly worse than their younger counterparts on each of the 5 days of behavioral testing, as indicated by the increased total number of shocks received (Figure 1B), with no significant improvement being observed from the first to the final day of behavioral testing. This inability of the older mice to learn appeared to be unrelated to their mobility, as aged animals covered similar distances to young animals during testing, and even traveled further on day 3 than young animals (Figure 1C). Nevertheless, although the older animals were equally or more active, their movements did not appear to be influenced by the use of visual spatial cues to avoid the shock zone. It is therefore unlikely that the deficits in spatial learning ability were due to a reduction in general mobility.

An optimized exercise duration improves cognitive function

Prior to the initiation of defined exercise periods, 24-month-old animals were tested using the APA paradigm to ensure there were no differences between groups in learning ability (Figures S1A and S1B). Animals were then housed three to a cage with *ad libitum* access to a single running wheel (Figures 1D and S1C). During the exercise period, we recorded a subset of cages and conducted a detailed video analysis for 8 h each day, covering the entirety of the running periods we investigated. This included analyzing the last 7 days of an acute 21-day running paradigm and the last 21 days of longer periods including 35 and 49 days of exercise. The analysis period was from 7 pm to 3 am as this was when the majority of running activity occurred. Although all animals accessed the running wheel daily during their exercise intervention, there was some variation in the time spent per day on the running wheel. On average, the daily time (Figure 1E) and distance traveled (Figure S1D) on the running wheel remained stable until the end of the exercise paradigm, confirming previous reports that mice show very little, if any, habituation to the running

wheel over time (Kronenberg et al., 2006). At the completion of the defined exercise period, the running wheel was removed for a period of 2 weeks prior to behavioral retesting as a similar delay has been shown to improve cognitive outcomes following exercise (Berchtold et al., 2010). This delay was to allow the exercise-activated precursor cells to differentiate into doublecortin-positive (DCX^{+ve}) immature neurons (Lucassen et al., 2010), which we and others have shown to be important for hippocampal spatial learning (Vukovic et al., 2013). Novel visual cues were then used during APA retesting (Figure 1D). Only the mice that had run for 35 days showed a significant improvement in learning following exercise, including a reduced number of shocks and an improved ability to avoid the shock zone during testing (Figures 1F–1H), whereas shorter or longer periods of exercise had no discernible effect. Furthermore, aged animals that ran for 35 days showed improved spatial learning to a level that approximated that of nonrunning young animals (Figure 1F). We also excluded the possibility that improvements in spatial learning were the result of an enriched environment owing to the presence of the running wheel as there was no improvement in spatial learning for animals that had *ad libitum* access to a locked running wheel for 35 days during APA testing (Figure 1I).

To confirm the importance of the "sweet spot" of exercise, we tested an additional cohort using a different hippocampus-dependent test, the Barnes maze (Figure 2) (Harrison et al., 2009; Kennard and Woodruff-Pak, 2011), which has previously been demonstrated to require neurogenesis to successfully complete (Raber et al., 2004). Barnes maze testing consisted of each mouse undergoing three trials a day for three consecutive days. The room was brightly lit to encourage escape behavior. The mouse was placed in a novel quadrant for each trial, and the mice had to use the spatial cues located around the room to determine their position and find the darkened escape tunnel (Figure 2A). There was no difference between groups in distance traveled during the test (Figure 2B), and only those animals that had run for 35 days showed a reduced escape latency and reduced error number during testing (Figures 2C and 2D, respectively). Heat maps reveal that the 35-day runner group (Figure 2G) had a more focused strategy to find the escape tunnel on the final day of testing than the other groups (Figures 2E–2H), with this being the only group to exhibit improved performance, as measured by comparing escape latency from the first day of testing to the last, expressed as a percentage during the Barnes maze testing period (Figure 2I).

The restricted period of the exercise "sweet spot" was particularly surprising, leading us to query whether it reflected activation of a tightly regulated physiological process or whether it was merely due to the length of time between beginning exercise and the assessment of APA performance. To address this, we exercised 24-month-old animals for 21 days, removed the running wheel, and waited for 28 days prior to retesting the animals, which is equivalent to the 35-day paradigm; however, in this instance, we found no evidence of improved performance (Figures S1E–S1G). Conversely, animals that underwent 35 days of exercise and were retested 6 weeks later significantly outperformed the control nonrunning aged mice (Figures S1H–S1J). Taken together, these results indicate that the exercise period of 35 days is critical for improving spatial learning in 24-month-old mice.

Exercise-mediated neurogenesis is responsible for improved spatial learning

One mechanism that regulates spatial learning is the production of new neurons in the DG (Clark et al., 2008; Kee et al., 2007; van Praag et al., 2005), with their depletion in young animals resulting in impaired APA performance similar to that observed in 24-month-old animals (Vukovic et al., 2013). The number of newborn DCX^{+ve} immature neurons was significantly decreased in the DG of mice aged 6 months or older when compared with 10-week-old animals (Figure 3A), and by 24 months of age, the production of DCX^{+ve} cells had almost ceased. As exercise has been shown to activate the production of new neurons (Kronenberg et al., 2006; van Praag et al., 1999a), we investigated whether a change in neurogenesis was associated with the exercise "sweet spot." Analysis revealed that, following cognitive testing, only those aged animals that exercised for 35 days displayed an increase in DCX^{+ve} cell numbers (Figures 3B–3E). Branching pattern analysis of the DCX^{+ve} cells following exercise also revealed a significant increase in branching complexity in those animals that ran for 35 days compared with no-run controls (Figure 3F). We also found a significant correlation between DCX^{+ve} cell number and spatial learning ability following exercise (Figures S2A–S2D). *In vivo* labeling with the thymidine analog BrdU for 3 days before sacrifice at 35 days was conducted to provide a snapshot of proliferation at the optimal period of exercise for 24-month-old mice. This showed a doubling in the number of DCX^{+ve} cells colabeled with BrdU, which represented the majority of the $BrdU^{+ve}$ cells observed (Figures S2E–S2G). The production of $NeuN^{+ve}$ mature neurons was also examined in a separate cohort of animals that underwent 35 days of exercise versus nonrunners. For this, BrdU was added

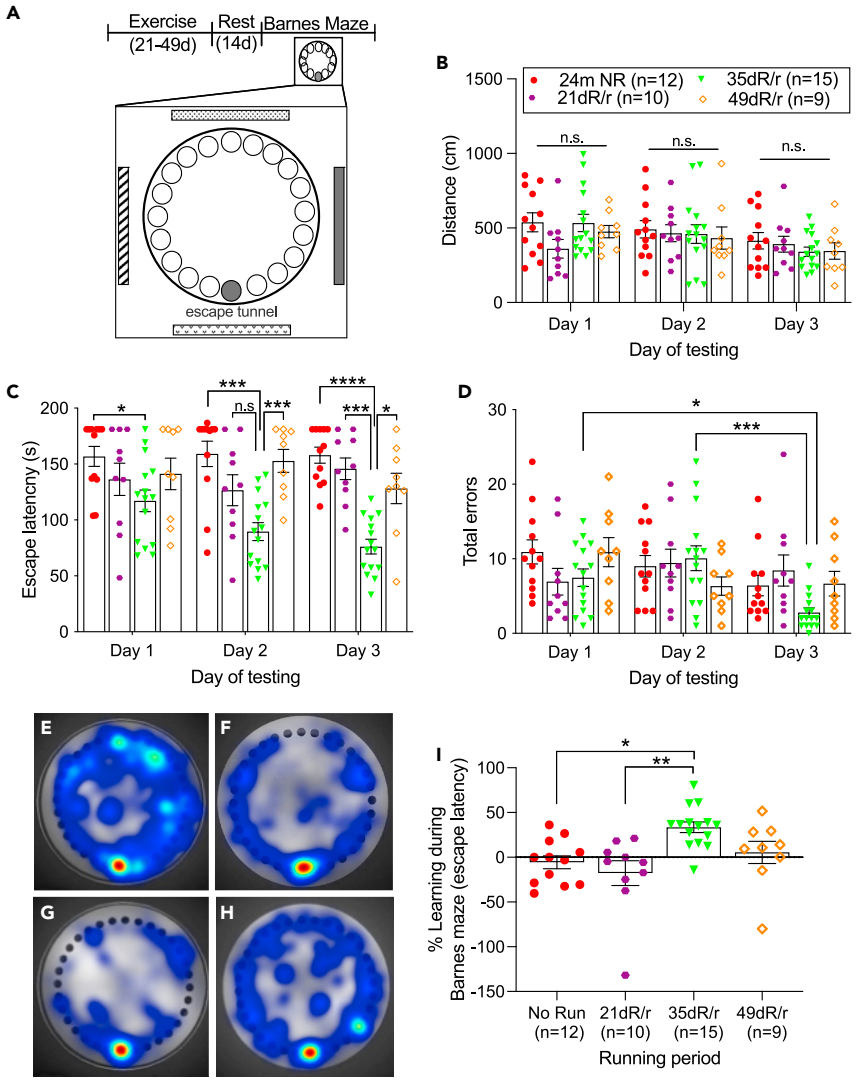

Figure 2. The optimal period of exercise improves Barnes maze performance in 24-month-old animals

(A) Schematic of the experimental approach to test animals following exercise with the Barnes maze. Each animal used spatial cues placed evenly around the room to find and enter the escape tunnel (marked in gray). Three trials per day for 3 days were conducted, with each animal placed in a different quadrant to start each trial.

(B) There was no difference between groups for the total distance traveled during testing.

(C) The 35-d runners were the only group to exhibit a significant reduction in the time to enter the escape tunnel during the course of testing (mean ± SE; two-way RM-ANOVA [exercise effect $F(3,42) = 13.32$ $p < 0.0001$], with Bonferroni post hoc tests).

(D) Only the 35-d runner group exhibited significantly fewer errors during the testing period (mean ± SE; two-way RM-ANOVA [$F(6,84) = 2.77$; $p = 0.0163$], with Bonferroni post hoc tests).

(E–H) Heat maps reveal differences in the areas of the Barnes maze explored prior to escape on the final day of testing for (E) no run, (F) 21-d run, (G) 35-d run, and (H) 49-d run animals, with hotter colors representing more time spent in that position.

(I) There was a significant increase in learning ability for animals that ran for 35 d (mean ± SE; one-way ANOVA [$F(3,42) = 6.109$; $p = 0.0015$], with Bonferroni post hoc tests). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. NR, no run, R/r, Run/rest where rest is 14 days.

to the drinking water for a period of two weeks that began on day 28 of exercise. Two weeks after running had been completed, we found a significant increase in the number of $BrdU^{+ve}/NeuN^{+ve}$ cells in the runners (Figures S2H–S2J).

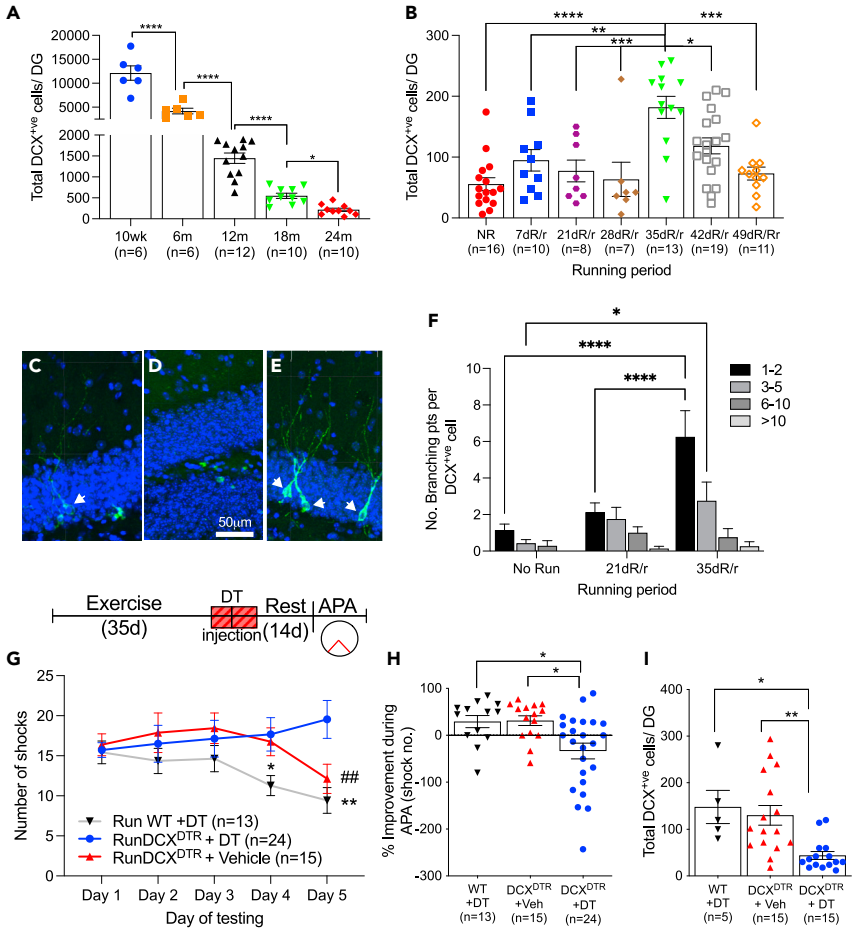

Figure 3. Exercise increases the frequency of immature neurons required for spatial learning in the aged brain

(A) The total number of DCX^{+ve} cells in the DG decreases with age (mean ± SE; one-way ANOVA [$F(4,39) = 80.55$, $p < 0.0001$] with Bonferroni post hoc tests).

(B) Following cognitive testing, only those 24-month-old animals that underwent exercise for 35 d showed a significant increase in DCX^{+ve} cells in the DG relative to controls and all other running periods (mean ± SE; one-way ANOVA [$F(6,77) = 8.255$; $p < 0.0001$] with Bonferroni post hoc tests).

(C–E) Representative photomicrographs of the DG of (C) 24-m no-run, (D) 24-m 21-d run, or (E) 24-m 35-d run mice, labeled for DCX (green) and DAPI (blue). Scale bar = 50 µm. Arrowheads point to DCX^{+ve} cells.

(F) 24m 35dR/14dr animals show an increase in branching morphology compared with age-matched no run controls (mean ± SE; two-way ANOVA [$F(6,64) = 4.939$; $p < 0.0003$] with Bonferroni post hoc tests).

(G) Schematic representation of the experimental design to specifically ablate DCX^{+ve} cells following exercise. At the end of the running period, animals were injected with either vehicle or DT and then tested on the APA task. The number of shocks received by DCX^{DTR} animals injected with DT did not decrease during APA testing, whereas the number received by mice injected with vehicle did (mean ± SE; two-way RM-ANOVA [effect of treatment; $F(2,49) = 3.767$; $p = 0.0301$], with Bonferroni post hoc tests). There was a significant difference in the shock number on the final day of testing between Run DCX^{DTR} injected with DT and DCX^{DTR} run animals injected with vehicle and WT run animals injected with DT.

(H and I) (H) 24-month-old DCX^{DTR} animals injected with DT failed to learn and had significantly fewer DCX^{+ve} cells in the DG compared with control animals (I) (mean ± SE; one-way ANOVA [$F(2,49) = 6.381$, $p = 0.0034$] and [$F(2,33) = 8.101$; $p = 0.0014$], respectively, with Bonferroni post hoc tests). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. NR, no run; R/r Run/rest where rest is 14 days. See also Figures S2 and S3.

To determine whether the recorded increase in neurogenesis underlies the cognitive effect of exercise, we used a knock-in DCX^{DTR} mouse line (Vukovic et al., 2013), in which the diphtheria toxin receptor (DTR) is under the control of the DCX promoter, to enable ablation of DCX^{+ve} cells following the administration of diphtheria toxin (DT). Using this approach, 24-month-old DCX^{DTR} animals that exercised for 35 days and were injected with DT immediately following this period showed no improvement in spatial learning

in the APA task, whereas aged-matched control animals (wild-type [WT] animals injected with DT or DCX^{DTR} mice injected with the vehicle) showed improved spatial learning (Figures 3G and 3H). As expected, the number of DCX^{+ve} cells was significantly reduced in the DG of DCX^{DTR} animals injected with DT compared with control animals (Figure 3I), demonstrating that DT in the absence of its receptor does not adversely affect neurogenesis. To confirm the central importance of new neuron production, we ablated newly formed neuronal precursors in 24-month-old WT animals following exercise by administering a single dose of 5-Gy gamma irradiation focused on the head (Figure S3). Similar to the DCX^{DTR} mice injected with DT, animals irradiated following exercise failed to show an improvement in spatial learning compared with sham-irradiated, exercised mice (Figures S3A and S3B) and also had decreased DCX^{+ve} cell numbers in the DG (Figure S3C).

The dependency of cognitive improvement on neuronal production suggested that the exercise "sweet spot" may be due to the activation of neurogenic precursors. As expected from the rate of neuronal production, the number of neural precursors detected in the hippocampus declines with age (Walker et al., 2008); however, we have shown that a quiescent pool remains and can be activated by different factors in vitro (Jhaveri et al., 2015; Walker et al., 2008) and by exercise in vivo (Vukovic et al., 2012). We therefore determined the number of activated precursor cells using a neurosphere assay following different periods of exercise in 24-month-old animals, performing the assay immediately after exercise as this response should precede the increase in DCX^{+ve} cells. This analysis revealed a peak in neurosphere numbers after 35 days of exercise (Figure 4A).

Increasing GH levels via exercise increases neural precursor cell activation

To explore why such a precise period of exercise leads to the activation of neurogenic precursors, neuronal production, and subsequent cognitive improvement, we first examined whether fluctuating levels of GH play a role, as we have previously demonstrated the involvement of GH in regulating precursor number in the subventricular zone following exercise (Blackmore et al., 2009, 2012). To investigate whether circulating levels of GH change with exercise, we obtained tail blood samples from 24-month-old animals at defined periods during the course of exercise. To take into account the diurnal variation in pulsatile GH release (Ho et al., 1987), we standardized our sampling by collecting whole blood at the same time of day. Using an ultrasensitive enzyme-linked immunosorbent assay (ELISA) (Steyn et al., 2011), we found that there was only a significant elevation in circulating GH levels after 35 days of running compared with "no-run" controls (Figure 4B). As exercise has been described as a physiological stressor, especially for longer exercise durations (Naylor et al., 2005), we examined if corticosterone levels were altered following defined periods of exercise and may account for the generation of the observed sweet spot. We found that there was a general decrease in corticosterone following exercise, reaching significance at 21- and 49-day running periods (Figure S4A).

Although the majority of the study was conducted on group-housed mice owing to concerns regarding social isolation of 24-month-old mice, we wanted to determine if individually housed mice had a similar response and timing to exercise. We did this by comparing a separate cohort of individually housed and group-housed mice with access to a running wheel following defined periods of exercise (Figures S4B–S4H). For these cohorts, we also examined longitudinal changes in GH during exercise using weekly GH blood samples and determined how these changes related to spatial learning (Figure S4B). Individually housed animals that ran for 35 days improved during APA testing, reaching a level comparable with that of age-matched, group-housed 35-day runners (Figure S4C). Once again, we found a peak of GH for the 35-day running period (Figure S4D). Animals that ran for 35 days (either individually housed or group-housed) did not show a correlation between average daily running time and GH levels (Figure S4E). Critically, however, comparison of individual animals' circulating GH level on the final day of exercise with its APA performance revealed positive correlations between higher blood GH concentration and better performance in the APA task (Figures S4F–S4I). We did not find a significant correlation between average daily running time and APA performance (Figure S4J). To determine whether the changes in GH levels also mirrored precursor activation in younger animals, we exercised 10-week-old mice and again found that the exercise-dependent increase in precursor cells reflected an increase in circulating GH levels (Figures S4K–S4M), but with a much earlier time course. This result is consistent with the previously reported early neurogenic responses seen in young animals (Kronenberg et al., 2006) and further suggests that GH may play a central role in regulating the neurogenic response in the hippocampus. It may also explain the increased time required for precursor cell activation to be initiated in aged animals, as circulating GH levels

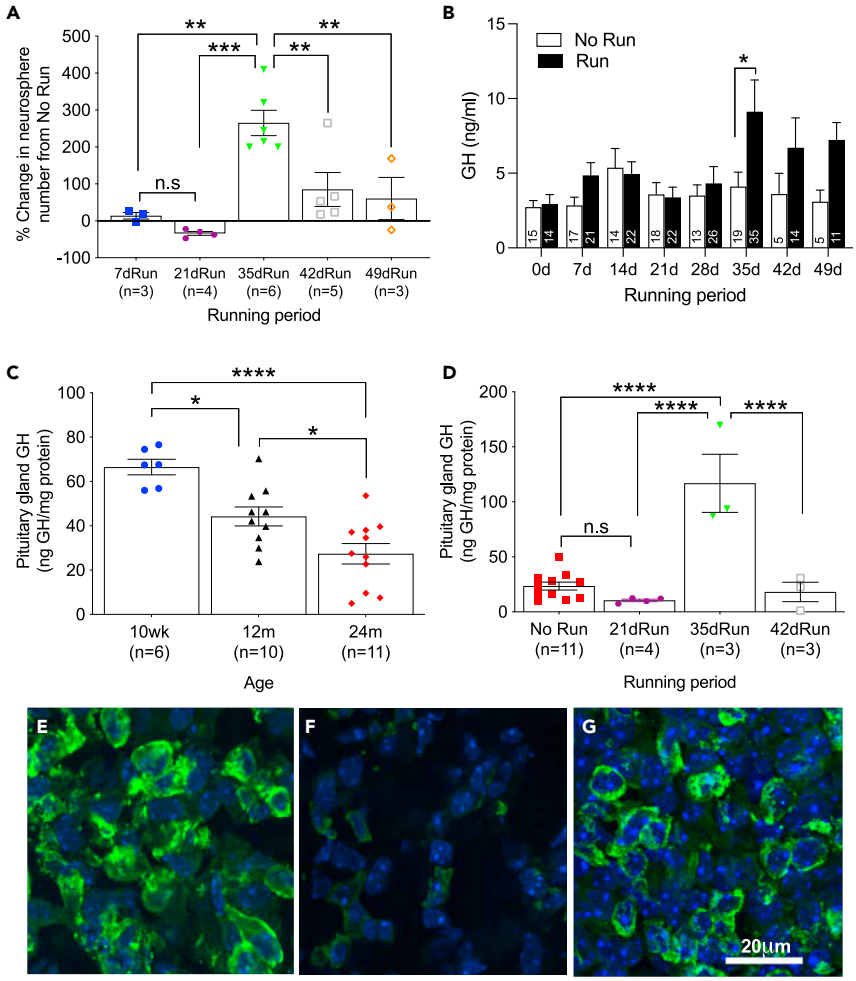

Figure 4. Changes in growth hormone levels during exercise mirror the timing for the activation of endogenous hippocampal precursor cells

(A) 35 d of exercise significantly increased hippocampal neurosphere numbers relative to age-matched no-run controls and for all other running periods (no-run controls standardized to zero, mean ± SE; one-way ANOVA [$F(5,21) = 13.32$; $p < 0.0001$] with Bonferroni post hoc test).

(B) There was a significant increase in GH in the blood of 24-month-old animals following 35 d of exercise (mean + SE; multiple t tests; n values are given in each column of the graph).

(C) Naïve pituitary gland GH levels decreased during aging (mean ± SE; one-way ANOVA [$F(2,24) = 16.57$; $p < 0.0001$] with Bonferroni post hoc tests).

(D) Pituitary gland GH levels showed a significant increase following 35 d of exercise in 24-month-old mice (mean ± SE; one-way ANOVA [$F(3,17) = 22.99$, $p < 0.0001$] with Bonferroni post hoc tests).

(E–G) Representative photomicrographs of pituitary gland cells labeled for GH (green) and DAPI (blue) in (E) 10-wk naive, (F) 24-m naive and (G) 24-m 35-d run mice (Scale bar = 20 µm). *$p < 0.05$, **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. See also Figures S4–S6.

decrease with age (Steyn et al., 2016). Finally, we showed a low-resistance exercise paradigm using a running disc (Figure S5A) does not induce GH release (Figure S5B), or result in improvement in spatial learning (Figure S5C), again demonstrating the link between raised GH levels and cognitive performance.

As the pituitary gland is the primary source of GH (Steyn et al., 2016), we also examined its GH content during aging and found a significant reduction in 24-month-old mice compared with both 10-week-old and 12-month-old animals (Figure 4C). In response to exercise, however, we observed a substantial and significant rise in GH content after 35 days of running but no change at 21 or 42 days when compared with "no-run" controls (Figure 4D). The change in GH content following exercise was most strikingly illustrated when

histological sections of the pituitary gland were immunostained with anti-GH antibodies. The 24-month-old nonrunners showed low levels of GH in somatotroph cells (Figures 4F and S6A), whereas the 35-day runners (Figures 4G and S6B) displayed levels similar to those observed in 10-week-old animals (Figure 4E). Indeed, both the number of cells (Figure S6C) and the number of cells containing GH in the pituitary gland (Figure S6D) increased following exercise relative to 24-month-old nonexercised animals and reached levels comparable with those of nonexercising young animals. The observed increase in cell number in the 35-day runners was not related to an increase in pituitary weight (Figure S6F), as there was a significant decrease when compared with the nonrun animals. Rather, as shown in Figure S6A, the large interstitial spaces observed in the nonrun animals were absent in the pituitary of the 35-day runners (Figure S6B). Thus, the most parsimonious interpretation of our results is that the increase in cell number is due to an increase in cell density.

As brain-derived neurotrophic factor (BDNF) and insulin-like growth factor 1 (IGF-1) have also been associated with exercise-mediated responses in the hippocampus, we wished to examine these in 24-month-old animals following exercise. We found that circulating BDNF levels were negligible in sedentary mice, as previously described (Radka et al., 1996), but peaked at the optimized period of exercise (Figures S7A and S7B) with no change in circulating IGF-1 levels (Figures S7C and S7D). Changes to inflammatory markers following exercise have also been reported (Lezi et al., 2014), so we examined IFN-γ, IL-1β, IL-10, and IL-12 in aged mice but found no significant change in any of these inflammatory markers (Figures S7E–S7H), suggesting that they are not involved in regulating the observed cognitive improvement.

Pharmacologically increasing GH release improves spatial learning

As the temporal pattern of GH release in response to exercise corresponded with the other positive effects of running, we next tested whether the rise in circulating GH levels mediated the enhancement of learning. First, we stimulated GH release in aged mice using a GH-releasing hormone (GHRH) analog, JI-38 (Izdebski et al., 1995), to mimic the GH-elevating effects of exercise. GHRH is predominantly synthesized within cells of the arcuate nucleus of the hypothalamus (Kuwahara et al., 2004), which in turn initiates the release of GH into the circulation from somatotrophs in the pituitary gland (Kuwahara et al., 2004). Induction of endogenous GH release in this way typically results in fewer side effects than exogenous GH treatment (Hersch and Merriam, 2008). JI-38, a long-lasting GHRH receptor agonist, normalizes the growth of GHRH-deficient animals (Alba et al., 2005). Daily subcutaneous injection of JI-38 into 24 month-old mice for 28 days resulted in a significant increase in the blood levels of GH in these animals 30 min post injection at all weekly time points. There was also a progressive increase in the level of GH released as treatment progressed (Figure S8A). As significant increases in GH levels were observed in the first week, we also tested spatial learning ability after 6 days of JI-38 injection but found no evidence of improved performance (Figures S8F and S8G). However, when we tested the mice after 28 days of JI-38 injection, they showed a significant improvement compared with controls (Figures 5A, S8B, and S8C), and there was a positive correlation between higher blood GH concentration and better performance in the APA task of individual mice (Figure 5B). This improvement in cognitive function was sustained even at 42 days following the final injection (Figures S8D and S8E).

Blockade of GH activation prevents exercise-mediated improvements

Next, we examined whether blocking GH signaling during exercise with a competitive GH receptor antagonist, G118R, affected learning. Intraventricular infusion of G118R over the course of the exercise period completely ablated the cognitive improvements seen after 35 days of exercise, whereas vehicle-infused controls showed improvement (Figures 5C and S6E). Having demonstrated that treatment with G118R also blocked the production of hippocampal DCX^{+ve} neurons after exercise (Figure 5D), we examined how GH regulates the activation of neural precursors, which appears to be the first step in the recovery of cognitive function in aged animals. To test whether GH directly activates precursor cells, we added recombinant GH to in vitro cultures of hippocampal cells from 10-week-old and 24-month-old mice and assessed the changes in the number of neurospheres formed (Jhaveri et al., 2010; Walker et al., 2008). This analysis revealed a dose response to GH, with double the number of neurospheres being produced in the presence of 100 ng/mL of GH for both young and old populations (Figure 5E), although there was no effect on neurosphere size (Figure 5F). The GH-dependent increase in hippocampal neurospheres was, however, completely abrogated by the addition of G118R (Figure 5G). To determine whether the GH receptor (GHR) was present on the precursor cells, we enriched these precursors using a method previously reported (Jhaveri et al., 2015). RNA-seq analysis of this near-pure population of Nestin- $GFP^{+ve}/EGFR^{+ve}$

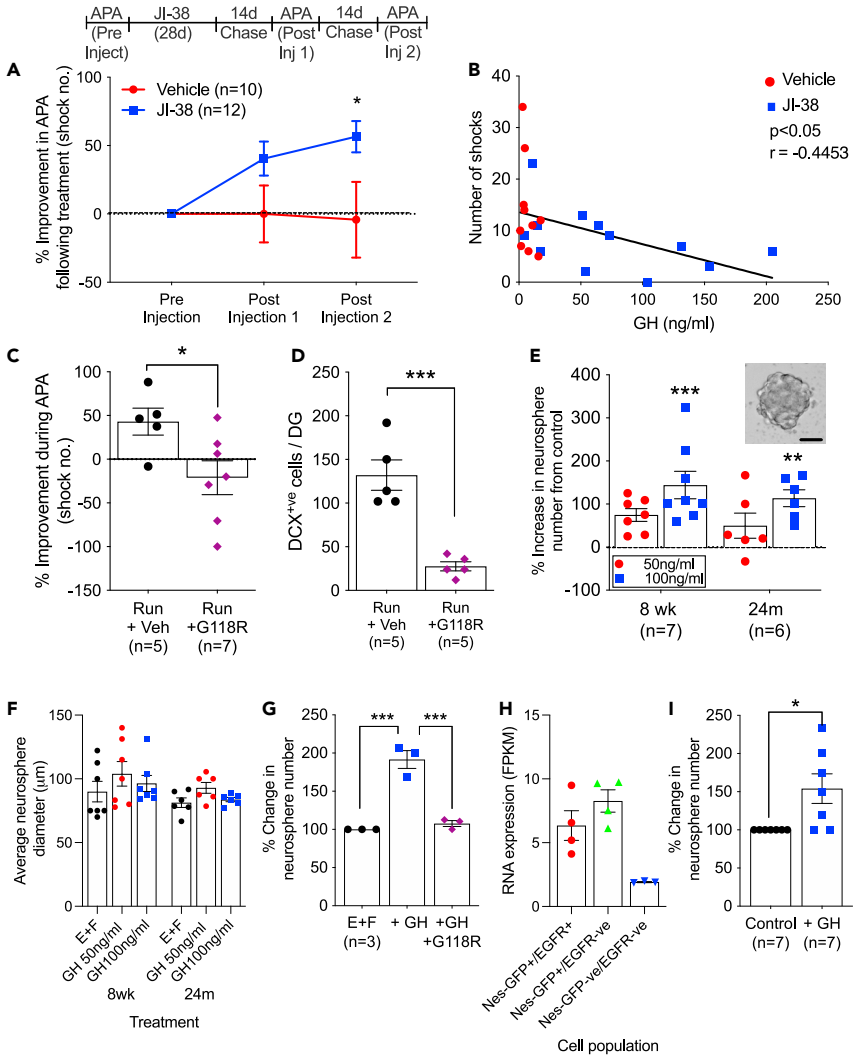

Figure 5. Pharmacological induction of GH release with JI-38 results in improved cognition in aged animals

(A) Comparison of the learning ability prior to treatment and following JI-38 administration revealed a continued improvement in spatial learning, reaching significance at the second postinjection test period (mean ± SE; two-way RM-ANOVA [F(2,40) = 3.243; p = 0.0495] with Bonferroni post hoc tests).

(B) Following JI-38 treatment, there was a significant correlation, with higher circulating GH levels correlating to a lower number of shocks received on the final day of APA testing.

(C) Animals infused with the GH antagonist G118R during exercise showed no improvement in APA testing (mean ± SE; Student's t test; t score = 2.421, p = 0.036).

(D) Infusion of G118R during exercise prevented an increase in DCX^{+ve} cell number in the DG (mean ± SE; Student's t test; t score = 5.749, p = 0.0004).

(E) The addition of 100 ng/mL of GH significantly increased primary hippocampal neurosphere numbers for both young and 24-month-old animals (mean ± SE; one-way ANOVA [F(2,19) = 11.17; p = 0.0006 and [F(2, 15) = 7.907; p = 0.0045], relative to control (E + F) conditions respectively, with Bonferroni post hoc tests). Insert shows a representative neurosphere. Scale bar = 50 μm.

(F) There was no change in neurosphere diameter with the addition of GH.

(G) The GH antagonist G118R prevented the GH-dependent increase in hippocampal neurospheres (mean ± SE; one-way ANOVA [F(2,6) = 50.87; p = 0.0002] with Bonferroni post hoc tests).

(H) GHR RNA was expressed on the purified, precursor cell population ($NesGFP^{+ve}/EGFR^{+ve}$).

(I) Addition of 100 ng/mL of GH to the purified, clonal hippocampal precursor cells significantly increased neurosphere number (mean ± SE; Student's t test; t score = 2.782, p = 0.0166). *p < 0.05, **p < 0.01, ***p < 0.001. See also Figure S8.

hippocampal neural precursor cells revealed preferential expression of GHR (6.34 ± 1.1 FPKM, fragments per kilobase of transcript per million mapped reads) (Figure 5H). To confirm that GH acts directly on the enriched precursor population, we added it to clonal cultures of the purified precursor population and found that it produced a significant increase in the number of neurospheres (Figure 5I). Although GH has previously been shown to influence precursor differentiation during development (Ajo et al., 2003), this is the first evidence that it can act directly on hippocampal precursors to initiate a neurogenic response.

Somatostatin is a critical regulator of GH release during exercise

The regulation of the process that begins with precursor activation and culminates in cognitive improvement appears to be dependent on both an increase in the level of GH and the duration of the elevation. The direct correlation between the serum levels reached following GHRH analog injections and the degree of cognitive improvement suggests that a critical level of GH is required to initiate this process and that this is reached following 35 days of running. However, a raised level alone appears to be insufficient, as the animals run beyond 35 days showed no cognitive improvement or changes in precursor activity or neurogenesis, and their GH levels returned to control levels. Another feature of the 35-day run period was that the increase in GH levels was maintained for an extended period of time compared with longer runs within the pituitary gland, cerebrospinal fluid (CSF), and blood (Figures S6G–S6I, respectively). Prolonged elevation of GH was also required to initiate the process when we injected the GHRH analog to mimic the effects of exercise (Figures 5A, 5B, and S8). The downregulation of GH observed in runners that went beyond the optimal period of exercise suggested a strong inhibitory feedback response. It is known that somatostatin (SRIF), particularly the age-associated form, SRIF-28 (Sonntag et al., 1986), is a potent inhibitor of GH release from the pituitary and is upregulated in response to raised GH levels (Giustina and Veldhuis, 1998). Previous studies have also shown that GH release in response to exercise is mediated by lowered somatostatin release (Thompson et al., 1993). Considering that the loss of GH secretory ability (somatopause) during physiological aging is mainly attributed to an increased sensitivity to SRIF, as it has been reported to inhibit both GH and GHRH *in vivo* (Zheng et al., 1997), we examined the levels of circulating somatostatin following different periods of exercise to determine whether this could explain the observed differences in GH levels. Our results revealed that mice that ran for 28 days or 49 days had similar levels of somatostatin to nonrun controls, whereas those animals that ran for 35 or 42 days had a significantly lower concentration (Figure 6A). However, when measured again at the time of behavioral testing, only the 35-day run animals had maintained the lower level of somatostatin (Figure 6B).

To test directly whether decreasing the levels of somatostatin in animals run for periods longer than 35 days simulated the cognitive performance seen after the 35-day run, we injected the acetylcholinesterase inhibitor (AChEI) donepezil. AChEIs such as pyridostigmine and donepezil have been proposed to suppress somatostatin secretion from the hypothalamus and thus increase GH secretion (de Vries et al., 2002; Obermayr et al., 2005). We therefore injected donepezil into animals daily for the last 14 days of a 49-day running period. We found that GH levels in the donepezil-treated animals were significantly elevated (Figure 6C) compared with those of 49-day runners. In addition, the animals treated with donepezil showed a significant improvement in the APA test when compared with untreated or nonrun controls (Figures 6D and S9A), and the number of DCX^{+ve} neurons in the DG of donepezil-treated animals (Figure 6E) was significantly increased relative to nonrun controls. There was also a significant correlation between the number of DCX^{+ve} cells and improved learning ability (Figure S9B). In a separate experiment, we confirmed that donepezil treatment increases GH and reduces somatostatin levels following a 28-day injection paradigm (Figures S9C and S9D). Comparing the change in GH levels following treatment to spatial learning ability revealed a positive correlation for these parameters (Figure S9E). Finally, we injected 24-month-old animals with a specific antagonist to the somatostatin receptor 5 subtype (sstr5) BIM23056 (Wilkinson et al., 1997) and found a correlation in terminal circulating somatostatin levels and spatial learning ability (Figure S9F). Taken together, these results indicate that the exercise-induced change in somatostatin levels is the critical regulator of the GH level, which in turn is responsible for improved spatial learning.

DISCUSSION

Our results reveal that an optimal period of voluntary exercise in 24-month-old mice improved key components of hippocampal function. This included hippocampus-dependent spatial learning, as evidenced by improvements in both active place avoidance and Barnes maze tasks. We went on to demonstrate that these improvements were dependent on an exercise-mediated increase in hippocampal neurogenesis. Finally, having observed that an increase in circulating GH during exercise mirrored the timing of

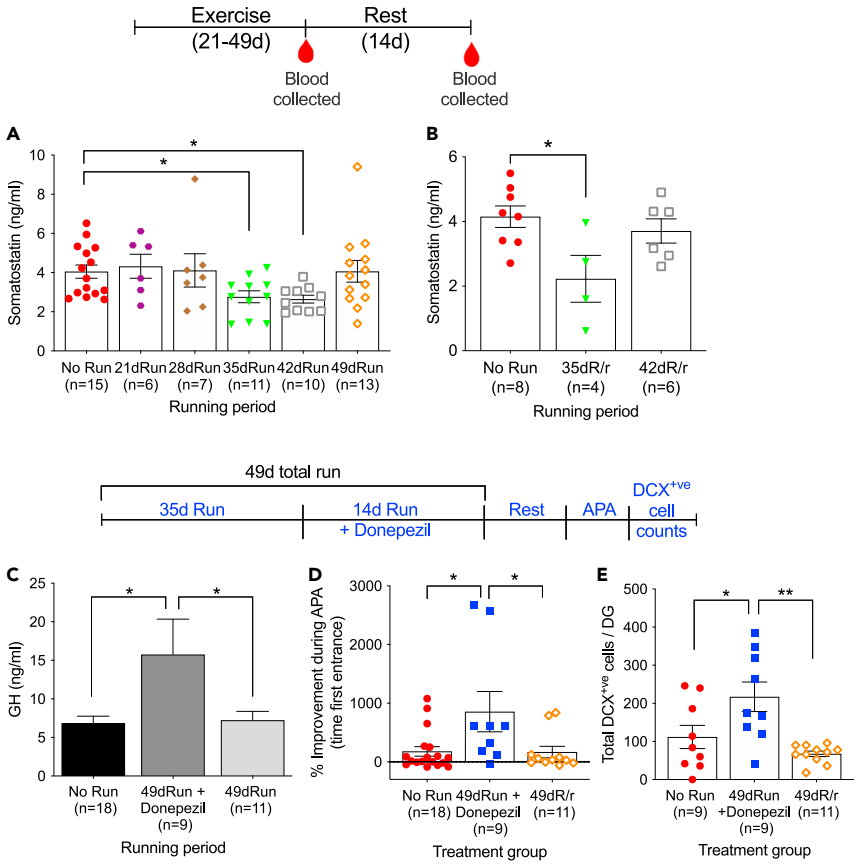

Figure 6. Manipulation of somatostatin alters growth hormone levels and behavior in 24-month-old mice

(A) Circulating somatostatin levels decreased after 35 and 42 d of exercise but returned to control levels after 49 d of running (mean ± SE; one-way ANOVA [F(5,56) = 2.436; p = 0.045], with Bonferroni post hoc tests).

(B) Somatostatin levels were reduced 2 weeks after the optimized exercise period relative to no run controls and animals that ran beyond 35 d (mean ± SE; one-way ANOVA [F(2,15) = 4.5; p = 0.0295], with Bonferroni post hoc tests).

(C) GH levels were elevated at 49 d with continued running when animals were injected daily for 14 d with donepezil (mean + SE; one-way ANOVA [F(2,35) = 4.858; p = 0.0137], with Bonferroni post hoc tests) compared with noninjected 49-d runners (49-d run GH data from Figure 4).

(D) Analysis of performance during APA testing revealed a significant improvement only for those animals injected with donepezil during exercise (mean ± SE; one-way ANOVA [F2,35) = 4.8; p = 0.014], with Bonferroni post hoc tests).

(E) There was a significant increase in DCX^{+ve} cell number in animals that received donepezil during the final 14 days of exercise (mean ± SE; one-way ANOVA [F2,26) = 8.239; p = 0.0017], with Bonferroni post hoc tests). *p < 0.05 Run/r: Run/rest where r refers to a rest period of 14 days. See also Figure S9.

hippocampal activation, we manipulated its release to demonstrate that GH is critically involved in these processes.

While others have reported that a 5-week period of daily voluntary exercise results in the improvement of hippocampal cognitive function in aged mice (van Praag et al., 2005; Wu et al., 2015), surprisingly, our results reveal that if we extended this period of exercise, there was abrogation of cognitive improvement. The discovery of a "sweet spot" of exercise duration critical for both neurogenic activation and improved spatial learning may help explain previous conflicting reports of the efficacy of exercise in improving cognitive improvement in aged mice. For example, the report that 43 days of exercise in aged mice failed to improve hippocampus-dependent pattern separation also revealed no increase in neurogenesis levels in aged mice (Creer et al., 2010). Improvement in learning ability following prolonged exercise periods has also been mixed. For example, the study by Marlatt et al. (2012) first shows that a "month" (the number of days not stipulated) of exercise does not improve spatial learning in 10-month-old animals. Continued exercise with these animals for an additional 6 months only showed a difference in the immediate Morris water

maze probe trial conducted 4 h following the acquisition; however, this was absent at the 24-h probe trial (Marlatt et al., 2012), indicating that any improvement observed in these animals was minimal. Of course, this does not mean that all previous experiments would have been successful if they had chosen this period of running but that determining the effects of exercise on hippocampus-based function requires a comprehensive and sequential examination over an extended period of time. So it is not surprising with strain-dependent differences in exercise response (Clark et al., 2011; Hamilton et al., 2015), and variations in methodology, including the range of ages examined (Creer et al., 2010; Kronenberg et al., 2006; van Praag et al., 2005), or duration of exercise (Marlatt et al., 2012), the literature is replete with conflicting reports.

Having established the optimal period of exercise required for improved spatial learning in 24-month-old mice, we then moved to identify the causal factor(s) that drives this phenomenon in aged mice. Previous studies in which exercise-dependent improvements in hippocampal cognitive function occurs have demonstrated that they correspond to a period of increased hippocampal neurogenesis in adult animals (van Praag et al., 1999a). The duration required for exercise-induced neurogenesis in aged mice, however, has been inconsistent across studies, with one group observing an increase in neurogenesis following an acute 10-day period (Kronenberg et al., 2006). However, as only a single exercise period was examined for 24-month-old mice, it is impossible to determine if the increased neurogenesis rate was a maximal response, although this is unlikely considering that a biphasic response, with a maximal increase in DCX^{+ve} cells observed after 32 days of exercise in young adult animals in the same study. We also observed a small but significant increase in DCX^{+ve} cells when directly comparing no-run animals with 7-day runners; however, our peak in exercise-dependent neurogenesis occurred after a more prolonged 35-day period. Importantly, as with prolonged exercise abrogating spatial learning ability, we found extending exercise beyond the sweet spot resulted in DCX^{+ve} cell number returning to control levels. Comparing DCX^{+ve} cells to learning ability following these exercise periods also revealed a strong correlation between the two components. To confirm the pivotal role of exercise-mediated neurogenesis in hippocampal spatial learning, we specifically ablated DCX^{+ve} cells in animals at the time of maximal exercise-mediated neurogenesis. This resulted in a threefold reduction in DCX^{+ve} cells and the complete abrogation of the improvement in spatial learning, thus confirming the central importance of neurogenesis to this paradigm of spatial learning shown in earlier studies in younger mice by ourselves and others (Clark et al., 2008; Codd et al., 2020; Naylor et al., 2008; Vukovic et al., 2013). Although the exercise-mediated increase in DCX^{+ve} cells relative to age-matched nonrunners we observe is large, the total number of DCX^{+ve} cells this represents is modest. This raises the question as to how these exercise-mediated newborn neurons contribute to improved cognitive function. It is likely that these cells are recruited into existing neuronal networks and circuitry to aid in hippocampal cognitive improvement as previously suggested (Vivar et al., 2016), especially as we observed a concomitant increase in branching complexity of these cells. Furthermore, we previously showed that the ablation of DCX^{+ve} cells resulted in a significant decrease in Arc^{+ve} cells, which are associated with memory consolidation. Repopulation of DCX^{+ve} cells restored spatial learning ability and returned Arc^{+ve} cells to control levels (Vukovic et al., 2013). Taken together, these changes are all likely to contribute to improved hippocampal function.

The next question was to identify how exercise influenced neurogenesis and ultimately hippocampal function through the regulation of blood factors. Changes in the level of one circulating factor that has been associated with aging, cognition, and exercise is GH. The age-associated decrease in circulating GH in humans has long been recognized (Ho et al., 1987; Rudman et al., 1981), with levels being almost negligible beyond 60 years of age (Corpas et al., 1993), and emulate some of the hallmarks of clinically defined adult-onset GH deficiency (Hersch and Merriam, 2008). GH-deficient patients exhibit deficits in a multitude of cognitive processes, including attention and memory (Maruff and Falleti, 2005), but show an improvement in cognition following treatment with recombinant human GH (Reimunde et al., 2011). The clinical relevance of GH levels in physiologically aged individuals is further highlighted by the fact that GH levels positively correlate with cognitive health (Quik et al., 2012) and that GH treatment in healthy aged men can reverse certain markers of epigenetic aging (Fahy et al., 2019). It has also been shown that exercise increases circulating GH levels in humans (Felsing et al., 1992), and we have previously shown in mice of various ages up to 18 months that exercise activation of neuronal precursors in the SVZ was dependent on GH because blocking GH activation with anti-GHR effectively blocked this response (Blackmore et al., 2009). Here we extend these findings by showing an age-dependent decrease in both pituitary gland and circulating GH levels in naive animals that are ameliorated during the course of exercise. Specifically, our results show that the exercise-induced increase in hippocampal precursor cell activation and

neurogenesis corresponded to a peak in circulating GH levels. We went on to show that the resulting GH levels following exercise positively correlated with spatial learning ability. Moreover, exercise using low-resistance running discs (Creer et al., 2010) failed to increase GH or improve spatial learning, while the blockade of GH signaling using a competitive GH receptor antagonist inhibited hippocampal precursor cell activation in vitro and prevented exercise-mediated spatial learning and neurogenesis levels in vivo. By demonstrating a direct effect of GH on a purified population of hippocampal stem cells, we add critical insight into the specific role(s) of GH in hippocampal function, with previous reports demonstrating that the peripheral injection of GH increases hippocampal cell proliferation (Aberg et al., 2010) and that overexpression of hippocampal GH improves both spatial learning and increased spine density in the CA1 region of the hippocampus (Haugland et al., 2020).

We next investigated if other circulating factors may be involved in the exercise-dependent changes in spatial learning. One such factor, corticosterone, is known to increase in aged mice (van Olst et al., 2018), with high corticosterone levels reported to negatively influence hippocampal proliferation (Cameron and McKay, 1999; Yau et al., 2011). As corticosterone is also known to modulate both GH synthesis and secretion, typically in an inhibitory fashion at higher doses (Mazziotti and Giustina, 2013), and the recent finding that glucocorticoid oscillations are involved in regulating hippocampal precursor cell proliferation, especially in the aged brain (Schouten et al., 2020), we examined corticosterone levels following defined exercise periods. Similar to the previous work (Kannangara et al., 2011), we found a general decrease in corticosterone levels following exercise. Although the observed decrease in corticosterone could provide a more permissive environment and allow GH secretion, this did not correlate with the peak of GH levels, nor with its time course. Therefore, while exercise influenced glucocorticoids levels, we do not believe it is a major regulator in this instance.

Having demonstrated the tight relationship between GH levels and hippocampal function during exercise, we next investigated the effect of inducing GH release pharmacologically. This is medically relevant as inducing GH release via GH secretagogues produces a more physiological response, reducing both occurrence and severity of side effects including fluid retention, peripheral edema, and arthralgias associated with bolus treatment of GH (Hersch and Merriam, 2008). We found that the injection of the GHRH agonist JI-38 pharmacologically increased circulating GH levels approximately fourfold during treatment and had similar effects on learning in nonrunners as was observed in their exercise counterparts. The importance of critical levels of GH to learning was demonstrated by the close correlation between it and cognitive performance following both the pharmacological induction of GH release and exercise.

The tight temporal window for optimal exercise we observed in 24-month-old animals was intriguing, especially in relation to the decreases in hippocampal neurogenesis, spatial learning, and circulating GH levels following prolonged exercise. We reasoned that as somatostatin, the main inhibitor of GH release, has been reported to decrease during exercise in humans (Thompson et al., 1993), it may be responsible for the biphasic response to exercise in aged mice. We found the optimal period of exercise, as identified by increased hippocampal activation and circulating GH, also revealed a decrease in circulating somatostatin levels. The rapid decrease in circulating GH levels and the subsequent hippocampal response we observed in 24-month-old animals following extended exercise is likely due to the age-dependent shift in somatostatin from SRIF-14 to SRIF-28. This shift in somatostatin isoform prevalence is proposed to be a key regulator in the decrease in GH release during aging, as SRIF-28 is more bioreactive (Sonntag et al., 1986). We then reasoned that if increased circulating somatostatin was responsible for the decline in hippocampal function beyond the "sweet spot" of exercise, then the inhibition of somatostatin levels during prolonged exercise would lead to the maintenance of elevated GH levels, thereby extending the positive period of exercise in aged animals. We therefore injected the AChEI donepezil in both nonrun animals and during prolonged exercise and found an increase in GH and a decrease in circulating somatostatin levels. Although it has been proposed that one mechanism of action for AChEIs is to increase GH secretion via inhibition of somatostatin secretion (de Vries et al., 2002; Obermayr et al., 2005), we believe that we are the first to confirm this mechanism in vivo. Importantly, increasing GH release in this manner once again increased neurogenesis and improved spatial learning ability in aged mice.

Recent studies have attempted to mirror the beneficial effects of exercise through the injection or overexpression of their molecule of choice, with one approach focusing on the exercise-mediated increase in glycosylphosphatidylinositol (GPI)-specific phospholipase D1 (Gpld1) to improve cognitive function

(Horowitz et al., 2020). Gpld1 is a GPI-degrading enzyme that is synthesized in the liver and is unable to cross the blood-brain barrier. Treatment instead initiates downstream regulatory cascades, resulting in multiple altered proteins, including a decrease in liver-derived IGF-1 which is involved in the inhibition of GH release (Steyn et al., 2016). Another recent study demonstrated that increased neurogenesis and BDNF are sufficient to mimic exercise-mediated improvements in cognitive ability in a mouse model of Alzheimer's disease (Choi et al., 2018). This accords with our findings, particularly in light of recent work demonstrating that GH directly induces BDNF expression (Martinez-Moreno et al., 2018) which would explain the increase in BDNF we observe after the optimized period of exercise.

We do not contend the optimized exercise period of 35 days reported here is a sine qua non for hippocampal activation under all conditions and for all ages. Rather, we demonstrate that a comprehensive examination of different exercise periods is crucial to understand the mechanisms underlying the cognitive improvements which follow exercise. This is highlighted when summarizing the multiple changes in circulating factors, neurogenesis, and spatial learning ability following defined running periods and interventions (Figure S10). The identification of the mechanism controlling the "sweet spot" in aged animals may indeed be common to other situations and provide a marker for future exercise studies in other species including man. This appears to be true, as we demonstrate in this article that in young animals GH levels rise much earlier after exercise in concordance with increases in neurogenesis and previously published reports. Although there has been recent intense discussion about the level of neurogenesis in the adult hippocampus in humans (Boldrini et al., 2018; Moreno-Jimenez et al., 2019; Sorrells et al., 2018), our studies reveal that even if the level decreases dramatically with age, as has been reported, its potential to reverse cognitive loss remains a tantalizing prospect. With the burden of age-associated cognitive decline and dementia increasing in our community, physical exercise remains the most promising public health measure to counteract this, especially if the precise parameters and mechanisms of action can be determined. Our studies indicate that GH may act as a functional biomarker to determine the optimal period of exercise and that the pharmacological regulation of GH release may be effective in mimicking exercise-dependent improvements in hippocampal function.

Limitations of the study

Only healthy, aged female C57BL/6 mice were used. These experiments would therefore need to be repeated in males to determine if our findings can be generalized to both sexes. The exact timing of physical exercise may be different between the sexes.

Although the present study is true for mice, the timing of exercise and response in humans needs to be confirmed. However, human studies have indicated the importance of GH in both exercise response and contribution to cognitive health in aging individuals.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.103275.

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AUTHOR CONTRIBUTIONS

D.B., F.S, A.C, I.O'K., K-Y.V., X.Z., O.L., D.J., and J.V. performed the experiments; M.W. provided reagents; D.B. and P.F.B. conceived the experiments, analyzed the data, and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Alexa Fluor-647-streptavidin	Life Technologies	A20186
4′,6-diamidino-2-phenylindole (DAPI)	Sigma-Aldrich	D9542
Guinea pig anti-DCX	Merck Millipore	Cat# AB2253: RRID:AB_1586992
Goat anti-guinea pig Alexa Fluor-488	Invitrogen	Cat# A-11073:RRID:AB_2534117
Rat anti-BrdU	Accurate Chem	Cat# OBT0030: RRID:AB_2313756
Rabbit anti-NeuN	Merck Millipore	Cat# MAB377: RRID:AB_2298772
Goat anti-rat Alexa Fluor 568	Life Technologies	Cat# A11077: RRID:AB_141874
Donkey anti-rabbit Alexa Fluor-647	Life Technologies	A11011 Cat# A32795: RRID:AB_2762835
Rabbit anti-GH	Purchased from the Parlow Lab, UCLA, USA	AFP5641801
Goat anti-rabbit Alexa Fluor-488	Life Technologies	Cat# A11008: RRID:AB_143165
Chemicals, peptides, and recombinant proteins		
Papain	Worthington	3120
Neurocult NSC Basal Medium-plus Proliferation Supplement	Stem Cell Technologies	05701
Epidermal growth factor	PeproTech	AF-315-09
Basic fibroblast growth factor	PeproTech	AF-100-18B
Heparin	Sigma-Aldrich	3149-100KU
Trypan blue	Sigma	T8154-100ML
recombinant mouse GH	Prospec Bio	cyt-540
DMEM/F-12 medium	GIBCO	113300-032
Ketamine	Provet	Keti1
Xylazine	Provet	Xylaz2
5-bromo-2′-deoxyuridine	Sigma	B5002
Fluorescence anti-fade mounting medium	DAKO	S302380
JI-38	Phoenix Pharmaceuticals	PH03103
Donepezil	Sigma-Aldrich	D6821
G118R	Laboratory of M.J. Waters	N/A
BIM 23056	Sigma-Aldrich	B4310
Diphtheria toxin	Sigma-Aldrich	D0564
DMSO	Sigma	D2650
NGS	Sigma	G9023
BSA	Sigma	A8412
Triton-X100	Sigma	T9284
Tween 20	Sigma	P1379
NE	Sigma	A9512
KCl	Sigma	P9541
Critical commercial assays		
Somatostatin ELISA	Phoenix Pharmaceuticals	EK-060-03
BDNF ELISA	R & D Systems	DY248 with DY008

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Corticosterone ELISA	Enzo Life Sciences	ADI-900-097
IGF-1 ELISA	R & D Systems	MG100
Magpix high sensitivity cytokine panel	Millipore/ Merck	MHSTCMAG-70K
Experimental models: Organisms/strains		
Nestin-GFP mice	Laboratory of S.Kernie (UT Southwestern)	Yu et al. (2005) Genesis 41:147–153.
C57BL/6J	The Jackson Laboratory	JAX-00664
DCX^{DTR} knock-in mouse	Ozgene	Vukovic et al. (2013) J.Neurosci 33(15):6603-13
Software and algorithms		
EthoVision	Noldus	XT13
Microsoft Excel	Microsoft	16.53
Prism	GraphPad	9.0
Other		
Running disc	Med Associates	ENV-047
PAP pen	Abcam	Ab2601
APA apparatus	Biosignal	N/A
Osmotic mini pumps	ALZET	1004
Infusion cannula	Plastics One	3280PM-SPC OP CONN

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Perry Bartlett (p.bartlett@uq.edu.au).

Materials availability

This study did not generate unique reagents.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report the original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals

Adult female C57BL/6J mice up to 24 months of age were used for the majority of the study unless otherwise stated. We have an established aged colony of female C57BL/6 animals on site where cohorts of mice are continuously added to the colony at 6 months of age. Animals are maintained, aged, and used when they reach the appropriate age. This allows all animals to be housed in the same conditions for the majority of their lives. The DCX^{DTR} mouse we described previously (Vukovic et al., 2013) was used for adult neuron ablation experiments. Nestin-GFP mice (Yu et al., 2005), as previously described (Jhaveri et al., 2013), were bred on a C57BL/6J background and used for the cell-sorting experiments. All animals were tested in accordance with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*. The University of Queensland Animal Ethics Committee approved all experiments. Animals were housed three or four per home cage unless otherwise stated. All animals had ad libitum access to food and water and were housed in a 12 h light/dark cycle.

METHOD DETAILS

Exercise paradigm

Unless otherwise stated, animals were housed three to a cage during exercise, in boxes that were 50 cm long × 20 cm wide × 12.5 cm deep, with test animals given ad libitum access to a single hanging running wheel (Able Scientific) for defined periods of time. These exercise ("run") periods were 7, 21, 28, 35, 42, or 49 days. The majority of control ("no run") animals did not have a locked running wheel placed in the cage as this may have induced climbing exercise and/or investigation, and hence may have qualified as environmental enrichment (Clark et al., 2008). However, one cohort of group-housed animals was provided with a single locked wheel for a period of 35 days. The running wheel was removed following defined exercise periods for two weeks prior to behavioral testing to allow precursor cells to differentiate into doublecortin-positive (DCX^{+ve}) immature neurons, which we and others have shown to be important for active place avoidance (APA) learning (Vukovic et al., 2013). A subset of animals housed either individually or three to a cage with access to a running wheel were videos recorded during the exercise period. Tails were uniquely marked to allow for easy identification. Detailed video analysis of time spent by each animal on the running wheel was conducted for the last 21 days of their respective exercise period of either 35 or 49 days. The daily 8 h analysis period was from 7 pm to 3 am as this was when the majority of running activity occurred. A subset of animals were given access to low resistance Running discs (Med Associates). These were placed in home cages for 35 days with three animals in a cage.

Primary neurosphere tissue culture

Primary neurospheres were cultured as previously described (Walker et al., 2008). Briefly, mice were sacrificed via cervical dislocation, after which the brains were removed and prepared for tissue culture. Curved forceps were used to peel away the cortex and remove the exposed hippocampus. This tissue was finely diced using a scalpel, transferred to a 15 ml tube in 2 ml of papain (Worthington), and placed in a 37°C water bath for 16 min with gentle trituration twice during this period. Following centrifugation at 100 × g for 5 min, the cells were re-suspended in 1 ml of Neurocult NSC Basal Medium-plus Proliferation Supplement (Stem Cell Technologies), supplemented with 20 ng/ml epidermal growth factor (EGF; BD Biosciences), 10 ng/ml basic fibroblast growth factor (bFGF; Roche) and 20 ng/ml heparin (Sigma-Aldrich), referred to as the control (E + F) condition. The cells were then again centrifuged prior to resuspension in 1 ml of the above medium. The number of viable cells was counted using a 1:1 Trypan blue mix, with the total number of cells being calculated. The cells were then equally distributed into the respective conditions and 200 μl of cell suspension was added to each well of a 96 well plate. Plates were incubated at 37°C with 5% CO_2 for 14 days. When examining the effect of growth hormone (GH) on primary hippocampal cells, recombinant mouse GH (rmGH; Prospec Bio) was added at a concentration of either 50 or 100 ng/ml. The total number of neurospheres obtained in each treatment group was determined on day 14, normalized to that of the control (E + F) group, and plotted as a percentage of control.

Fluorescence-activated cell sorting of clonal hippocampal neural precursor cells

Brains were removed from Nestin-GFP mice, their hippocampi dissected as described above, and sorting was conducted as previously described (Jhaveri et al., 2015). Briefly, dissociated cells were incubated with biotinylated EGF conjugated with Alexa Fluor-647-streptavidin (EGF-647; 2 μg/ml; Life Technologies) for 30 min at 4°C. Cells were washed in excess DMEM/F-12 medium prior to sorting. Dead cells were excluded by labeling with propidium iodide (1 μg/ml) and the cells were sorted and analyzed on a FACS Aria sorter (Becton Dickinson). The GFP-positive gate was set relative to basal fluorescence levels obtained from wild-type (WT) littermates. Single cells were plated into individual wells in 96 well plates in complete neurosphere medium (E + F) with or without 100 ng/ml rmGH. The following day, the wells were examined to confirm that a single cell per well was obtained. The total number of neurospheres obtained in each treatment group was determined on day 14, normalized to that of the control (E + F) group, and plotted as a percentage of control.

Tail vein blood collection

Tail vein blood collection was conducted as previously described (Steyn et al., 2011). Samples were collected at 7 day intervals unless otherwise stated and the start time for blood collection was 8 am. Briefly, the distal 1 mm portion of the tail tip of each mouse was cut using a razor and gentle finger pressure was applied in the direction moving from the base of the tail to the tip. A 4 μl whole blood sample was collected

using a fine pipette, added to 116 μl of phosphate-buffered saline (PBS) + 0.05% Tween 20, mixed thoroughly, and then snap frozen in dry ice. Samples were stored at −80°C until used.

For experiments involving exercise, blood samples were collected from control and exercising animals at a consistent time of 8am. However, as the exercise paradigm in this study was voluntary wheel running there was no way to efficiently determine when each animal last exercised prior to blood collection. Therefore, the results shown in Figures 4, S4, and S6 represent differences in baseline circulating levels of GH between no run controls and exercised animals for the time point measured.

For experiments investigating the induction of GH release, two samples were taken weekly from each animal. Blood was collected immediately prior to vehicle or treatment injection (T = 0 min) and following injection (T = 30 min). In these experiments, the timing of treatment and blood collection was controlled, such that the T = 30 min samples represented a pulse of GH release.

CSF collection

Animals were anesthetized via intraperitoneal injection of a ketamine/xylazine mixture (50 mg/kg and 8 mg/kg body weight, respectively). Once anesthetized, the mice were mounted onto a stereotaxic frame and a 1.5 cm incision was made to expose the skull. A single hole was drilled in the skull (1.25 mm diameter) directly above the lateral ventricle (anterior/posterior: +0.2 mm, mediolateral: ±0.9 mm relative to bregma). A fine glass pipette connected to a Hamilton syringe was gently inserted to a depth of 2.5 mm. CSF (4–8 μl) was then slowly extracted, snap frozen on dry ice and stored at −80°C until used.

Enzyme-linked immunoassay Whole tail blood samples and pituitary glands were used to conduct GH analysis. This was performed using an in-house mGH ELISA, as characterized previously (Steyn et al., 2011). The inter- and intra-assay coefficients of variation for this assay were 7.2% and 0.8%, respectively. Both plasma and serum blood samples were obtained from cardiac puncture. Plasma was obtained by collecting the blood in EDTA-containing tubes (Sarstedt) placed on ice, followed by centrifugation at 10,000 rpm for 10 min. Serum was collected by collecting whole blood, allowing it to clot and then centrifuging it at 10,000 rpm for 10 min. These samples were collected, aliquoted, and stored at −80°C prior to use. The somatostatin ELISAs were conducted as per the manufacturer's instructions (Phoenix Pharmaceuticals) using a 1:50 dilution for plasma samples. The BDNF ELISAs (R & D Systems) were conducted using a 1:25 dilution for plasma samples as per the manufacturer's instructions. Corticosterone ELISAs (Enzo Life Sciences) were conducted using a 1:40 dilution for serum samples. IGF-1 ELISAs (R & D Systems) were conducted using a 1:500 dilution for plasma samples. The Magpix high sensitivity cytokine panel (Millipore/Merck) for IFN-γ, IL-1β, IL-10, and IL-12 was conducted using plasma samples at a final dilution of 1:4.

Tissue processing

Animals were deeply anesthetized with sodium pentobarbitone then transcardially perfused with ice-cold PBS followed by 4% paraformaldehyde in PBS (pH 7.4). Brains and pituitary glands were removed, post-fixed overnight at 4°C in 4% paraformaldehyde, and then placed in 30% sucrose solution. Serial brain coronal sections were cut at a thickness of 50 μm and placed in a six-series configuration. All sections were placed in PBS, washed, and stored at 4°C in PBS + 0.01% sodium azide until used.

BrdU delivery

A stock solution of BrdU (5-bromo-2′-deoxyuridine, Sigma) at a concentration of 10 mg/ml was made up in 0.9% saline. This stock solution was placed in a 50°C water bath and vortexed every two minutes until the BrdU had dissolved. It was then aliquoted and stored at −20°C until needed. Mice were weighed and injected intraperitoneally daily for three days with a working solution of BrdU (50 mg/kg). Animals were sacrificed 2h after the final injection. For studies involving BrdU in drinking water; 100 mg/ml of BrdU stock aliquots in saline were made and frozen until needed. Fresh BrdU in drinking water was made to a final concentration of 0.8 mg/ml. Water consumption was measured daily.

Immunohistochemistry

Brain tissue sections were processed and labeled for the detection of DCX and 4′,6-diamidino-2-phenylindole (DAPI). Six to eight sections containing the hippocampus for each animal were used for all immunohistochemical analyses. Sections were washed three times with PBS prior to incubation with blocking

solution (PBS/0.2% Triton-X100/2% normal goat serum (NGS)) for 60 min at room temperature (RT). The sections were then washed three times in PBS and incubated overnight at 4°C on a shaking platform with guinea pig anti-DCX (1:1,000; Merck), after which they were washed three times prior to the addition of goat anti-guinea pig Alexa Fluor-488 (1:1,000; Invitrogen) for 2 h on a shaking platform at RT in the dark. The sections were washed and DAPI (1:10,000) was added after the second wash. The sections were then mounted on Superfrost Plus slides and, once excess liquid had dried, fluorescence anti-fade mounting medium (DAKO) was added and a coverslip applied. The sections were sealed with clear nail varnish and viewed using a ×20 objective on an upright Zeiss Axio Imager Z1 fluorescence microscope. Brain tissue sections processed and labeled for the detection of BrdU with either DCX or NeuN were washed three times with PBS prior to incubation in 1M pre-heated HCl for 30 min at 37°C. The sections were washed five times with PBS for 10 min. This was followed by a wash in dH_2O for 5 min prior to incubation with blocking solution (PBS/0.2% Triton-X100/2% NGS) at RT for 1 h. The sections were then washed three times in PBS and incubated overnight at 4°C on a shaking platform with guinea pig anti-DCX (1:1,000; Merck), and rat anti-BrdU (1:500; Accurate Chem) for DCX labeling, or rabbit anti-NeuN (1:1000; Abcam) and rat anti-BrdU (1:500; Accurate Chem) for NeuN labeling, after which they were washed three times prior to the addition of either goat anti-guinea pig Alexa Fluor-488 (1:1,000; Invitrogen) and goat anti-rat Alexa Flour 568 (Life Technologies) for DCX/BrdU detection or donkey anti-rabbit Alexa Fluor-647 (1:1,000; Invitrogen) and goat anti-rat Alexa Flour 568 (Life Technologies) for NeuN/BrdU detection overnight on a shaking platform at RT in the dark. The sections were then washed and processed as described above. For calculation of cell counts one complete series of these sections comprising the whole dentate gyrus (DG) was labeled and DCX^{+ve} cells were counted from the point at which both blades of the DG were visible (approximately bregma −1.34). Due to the very low numbers of DCX^{+ve} cells present, we employed the same technique used previously by others where stereological counting frames were not used (Kempermann et al., 2002), but rather every DCX^{+ve} cell observed was counted. The total number of cells in the DG was then derived by multiplying the value by 6 as every sixth section had been used.

A hydrophobic barrier was drawn around pituitary gland sections (4 μm thick) mounted on Superfrost Plus slides using a PAP pen (Abcam). Slides were washed three times in PBS prior to blocking in blocking solution (PBS/0.2% Triton-X100/2% NGS) for 60 min at RT. Following incubation, the slides were washed three times with PBS, after which 200 μl rabbit anti-GH (1:5,000 purchased from the Parlow Lab, UCLA, USA) was added to each slide. Sections were incubated overnight at 4°C on a shaking platform, then washed three times with PBS prior to the addition of 200 μl goat anti-rabbit Alexa Fluor-488 (1:2,000). The sections were then incubated in a dark humid chamber for 2 h at RT, washed three times with PBS and stained with DAPI (1:10,000) which was added after the second wash. Once excess liquid had dried, fluorescence anti-fade mounting medium (Dako) was added and a coverslip applied. Sections were sealed with clear nail varnish and then viewed using a ×60/1.4 NA oil-immersion objective (Nikon) on an inverted Diskovery spinning disk confocal fluorescence microscope (Spectral Applied Research) built around a Nikon TiE body equipped with two sCMOS cameras (Andor Zyla 4.2, 2048 × 2048 pixels) and controlled by Nikon NIS software. Four fields of view of the pituitary gland were captured randomly for each animal for pituitary cell counting. The average of these counts across these four images was then calculated to represent each animal.

Active place avoidance (APA) task

Mice were handled daily for 4 days prior to the initiation of behavioral tests. The APA apparatus (Biosignal) consisted of a gridded platform on which a clear plastic cylinder (1 meter diameter) was placed to contain the mouse. The gridded platform rotated at 1 rpm during the course of the task. A stationary 60° area was denoted by the tracking computer which we refer to as the shock zone. This zone did not rotate and remained in the same location for the entirety of the testing period. Four novel, high contrast spatial cues were placed evenly around the room at eye level to the mouse. The mice were tracked automatically and if an animal entered this shock zone it received a 0.5 mA shock with a 500 ms latency. A 5 min habituation session with no shocks was conducted on the first day, with 10 min testing sessions being conducted on each of the following 5 days. The inter-shock latency was 1,500 ms. The animal was placed into the arena at the same position on each day of testing. At the conclusion of testing, the mice were randomly assigned to control ("no run" or vehicle injection) or treatment (running wheel or injection) groups. At the end of the experimental paradigms, the animals were again tested on the APA platform. In the retest phase, visual cues were replaced with novel cues but the shock zone remained in the same location. For retesting, the habituation day was omitted and there were 5 testing days. Visual data collection for the animals was accomplished via a ceiling-mounted video camera connected to the tracking and shock computer.

Between testing of each animal, the grid and platform were thoroughly cleaned with 70% ethanol. Parameters including distance traveled, speed and number of shocks received, first time to receive a shock, and maximum time spent avoiding the shock zone were all recorded automatically. Learning ability during the test period was also examined by comparing the parameters on the first day to those on the final day of testing for each animal and representing this as a percentage. Prior to all experiments, any animal with cataracts was excluded. Improvement in APA was calculated as a percentage change for each animal based on the number of shocks received on the first and last days of the testing paradigm.

Barnes maze

Mice were handled daily for 4 days prior to the initiation of behavioral tests in a room which was 2 m^2 in size, with four novel high contrast visual cues spaced evenly around the room. A 32 hole Barnes maze 1m in diameter was placed in the middle of the room on an elevated pedestal at a height of 1m. Lighting intensity was set at 1000 Lux to encourage animals to seek the darkened escape tunnel. On the habituation day, mice were placed in the escape tunnel with a clean tissue for a period of 1 min. The animals were then removed from the escape tunnel and gently placed at the center of the Barnes maze arena and allowed to search the arena for 3 min or until they escaped into the darkened tunnel. If an animal did not escape during the allocated time it was gently moved to the escape tunnel and allowed to remain there for a period of 20 s. The animal was then removed and placed back in its home cage. Between animals both the escape tunnel and the arena were thoroughly cleaned with 80% ethanol, a fresh piece of tissue paper was placed in the escape tunnel. On the first day of acquisition, the escape tunnel was rotated 90° and it remained in that position for the entirety of the acquisition period. Animals were tested with 3 trials per day for 3 days. Mice were gently put in a darkened start chamber and placed in the center of one of the four quadrants of the maze. After 15 s the start chamber was removed and the trial commenced. The trial stopped after 3 min had elapsed or if the animal entered the escape tunnel. If the animal did not escape, it was moved to the escape tunnel for a period of 20 s. The animal was then removed and placed back in its home cage. Between animals, both the escape tunnel and the arena were thoroughly cleaned with 80% ethanol and a fresh piece of tissue paper was placed in the escape tunnel. Each subsequent animal underwent the same procedure; however the start location was rotated through the different quadrants to minimize any inherent location bias. Visual data collection for the animals was accomplished via a ceiling-mounted video camera connected to the tracking computer. Escape latency was calculated and heat maps were generated using EthoVision XT13.

GH-releasing hormone agonist injections

The growth hormone releasing hormone (GHRH) agonist JI-38 (Phoenix Pharmaceuticals) was diluted in vehicle solution (Milli-Q water with 0.2% bovine serum albumin (BSA) and 0.01% dimethyl sulfoxide) to a working solution of 10 μg/ml. Animals were weighed and then subcutaneously injected daily for 28 days with either vehicle or JI-38 at a concentration of 40 μg/kg body weight.

Somatostatin inhibitor injections

Donepezil (Sigma-Aldrich) stock was made using Milli-Q water to a concentration of 20 mg/ml, aliquoted, and frozen until required. A working solution was made daily using saline and injected intraperitoneally at a concentration of 1.5 mg/kg body weight of the animal. A stock solution of the somatostatin receptor 5 (sstr5) antagonist BIM 23056 (Sigma-Aldrich) was made using Milli-Q water to a concentration of 1 mg/ml, aliquoted and frozen until required. A working solution was made daily using saline and injected intraperitoneally at a concentration of 0.5 mg/kg body weight.

Intracerebroventricular infusion

The day before surgery, osmotic mini pumps with a flow rate of 0.11 μl/h (ALZET #1004) were loaded with either GH antagonist G118R (400 μg/ml; provided by Prof. M. Waters) or vehicle solution (0.9% sterile physiological saline/0.2% BSA), and attached to the infusion cannula. The entire apparatus was then stored overnight in PBS. Pumps were incubated in a 37°C water bath until surgery the next day. Adult C57BL/6J mice were anesthetized via intraperitoneal injection using a ketamine/xylazine mixture (50 mg/kg and 8 mg/kg body weight, respectively). Once anesthetized, the mice were mounted on a stereotaxic frame and a 1.5 cm incision was made to expose the skull. A single hole was drilled in the skull (1.25 mm diameter) directly above the lateral ventricle (anterior/posterior: +0.2 mm, mediolateral: ±0.9 mm, dorsoventral: −2.5 mm relative to bregma), and a 30 gauge cannula was lowered and fixed into place using cyanoacrylate adhesive, to enable infusion directly into the ventricle. Following surgery, the animals

were housed individually to prevent grooming and potential cannula removal or chewing on the tubing connecting the osmotic pumps. The animals were allowed to recover for a day prior to the addition of a running wheel to each cage.

Specific ablation of DCX^{+ve} cells

The previously described DCX^{DTR} knock-in mouse model (Vukovic et al., 2013) was used to investigate the effect of specific ablation of newborn neurons following voluntary exercise. Briefly, animals (DCX^{DTR} mice and WT littermate controls) underwent an initial round of APA testing to obtain their baseline spatial learning ability. The mice were then housed three to a cage and given ad libitum access to a running wheel for 35 days. After 28 days of exercise, diphtheria toxin (DT; Sigma-Aldrich) injections were initiated with 10 ng DT/g body weight being injected intraperitoneally every third day for a total of 6 injections. Body weight was measured daily. After 35 days of exercise, the running wheels were removed for 2 weeks prior to retesting of spatial learning ability using the APA task with novel cues.

Irradiation

Mice were restrained within a plastic chamber and placed in a lead-shielded container (reducing exposure by 95%), leaving only the head exposed for irradiation as described previously (Blackmore et al., 2009). A single dose of irradiation was given by exposure to a Co^{60} source in a Gamma Cell 200 irradiator until a 5 Gy dose had been applied. Non-irradiated animals were similarly restrained but were not exposed to the irradiation source. After irradiation, animals were allowed to recover for 1 day before the addition of a running wheel to their cage.

QUANTIFICATION AND STATISTICAL ANALYSIS

Factorial design one-way and repeated measures (RM) for two-way analysis of variance (ANOVA) with Bonferroni's multiple comparison post hoc test, a Student's t-test, or correlation analysis were used to analyze data, as appropriate. Parameters were analyzed in Microsoft Excel and Prism 9 (GraphPad Software Inc.). Data for individual experiments are shown as dot plots representing data from individual animals and all values were expressed as mean ± standard error of the mean unless otherwise indicated. Comprehensive statistical descriptions and results for every figure and supplementary figure are found in Table S1. Sample sizes for power calculations were conducted using the power/sample size calculator website generated by the University of British Columbia (Canada): https://www.stat.ubc.ca/~rollin/stasts/ssize/. Sample sizes were based on the mean of populations 1 and 2, common standard deviation (sigma value), an alpha value setting of 0.05, and a desired power value of 0.80.