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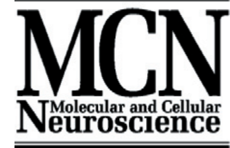

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The effects of exercise on spatial learning and anxiety-like behavior are mediated by an IGF-I-dependent mechanism related to hippocampal neurogenesis

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Knowledge about the effects of physical exercise on brain is accumulating although the mechanisms through which exercise exerts these actions remain largely unknown. A possible involvement of adult hippocampal neurogenesis (AHN) in the effects of exercise is debated while the physiological and pathological significance of AHN is under intense scrutiny. Recently, both neurogenesis-dependent and independent mechanisms have been shown to mediate the effects of physical exercise on spatial learning and anxiety-like behaviors. Taking advantage that the stimulating effects of exercise on AHN depend among others, on serum insulin-like growth factor I (IGF-I), we now examined whether the behavioral effects of running exercise are related to variations in hippocampal neurogenesis, by either increasing or decreasing it according to serum IGF-I levels. Mutant mice with low levels of serum IGF-I (LID mice) had reduced AHN together with impaired spatial learning. These deficits were not improved by running. However, administration of exogenous IGF-I ameliorated the cognitive deficit and restored AHN in LID mice. We also examined the effect of exercise in LID mice in the novelty-suppressed feeding test, a measure of anxiety-like behavior in laboratory animals. Normal mice, but not LID mice, showed reduced anxiety after exercise in this test. However, after exercise, LID mice did show improvement in the forced swim test, a measure of behavioral despair. Thus, many, but not all of the beneficial effects of exercise on brain function depend on circulating levels of IGF-I and are associated to increased hippocampal neurogenesis, including improved cognition and reduced anxiety.
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Keywords: Adult neurogenesis; Spatial learning; Exercise; Anxiety; Insulin-like growth factor I; Treadmill; Morris water maze

Introduction

Exercise has antidepressant (Farmer et al., 1988; Greenwood et al., 2003; Lawlor and Hopker, 2001) and anxiolytic effects (Dunn et al., 2001) as well as increases neurogenesis in rodents (van Praag et al., 1999a,b), possibly through the activity of some growth factors, such as local factors (Fabel et al., 2003; Cao et al., 2004) and the exercise-increased entrance of circulating IGF-I into the brain (Trejo et al., 2001, 2002). Indeed, anxiolytic treatments such as those involving either antidepressant drugs or electroconvulsive treatment, are accompanied by an increase in neurogenesis (Madsen et al., 2000; Scott et al., 2000). In the same way, exercise modulates cognition (Tomporowski and Ellis, 1985; Kramer et al., 2006) and improves performance in spatial learning tests concomitantly with increased neurogenesis (van Praag et al., 1999a,b). Indeed, spatial learning modulates neurogenesis by a mechanism not fully understood (Dobrossy et al., 2003; Ehninger and Kempermann, 2006).

Adult hippocampal neurogenesis (AHN) is an intriguing feature of the brain that has been related with many aspects of both normal and pathological hippocampal function (Ming and Song, 2005). By furthering our understanding of how mature neurons are normally generated in an adult brain, we will know how this process can be manipulated and whether it may serve as a putative target for therapeutic interventions after insults which produce neuronal loss (Emsley et al., 2005). Nevertheless, despite the clear link that exists between AHN and some hippocampal-dependent behaviors (Shors et al., 2001), many of the purported roles of AHN still remain to be demonstrated. There is evidence that many different events, both internal and external to the organism, affect the rate of AHN, including stress (Gould and Tanapat, 1999); enriched environments (van Praag et al., 2000); estrous cycle (Tanapat et al., 1999); seasonal activity (Barker et al., 2005); hierarchy encounters (Kozorovitskiy and Gould, 2004); hormone levels (Ming and Song, 2005); circadian rhythms (Holmes et al., 2004) and many others. These events influence either the proliferation, determination, differentiation, or maturation of the newborn neurons in the dentate gyrus.

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Several recent studies have addressed how exercise affects AHN and consequently how it influences hippocampal-dependent behaviors (for a review see for example Ernst et al., 2006). It appears that AHN is always related to both the stimuli and the associated behavior. We tested the hypothesis that serum insulin-like growth factor 1 (IGF-I) is mediating the effects of running exercise on spatial memory/learning and anxiety-like behaviors, and analyzed the involvement of adult neurogenesis and its regulation by physical exercise in these effects. We report herein a paradigm in which forced exercise induces changes in spatial learning and anxiety-like behavior parameters only when AHN is increased.

Results

We aimed to test the relationship between serum insulin-like growth factor 1 (IGF-I) and adult hippocampal neurogenesis (AHN) in hippocampal physiology after exercise. We tested the hypothesis that serum IGF-I is mediating the effects of running exercise on spatial memory/learning and anxiety-like behaviors, and examined the involvement of AHN and its regulation by exercise in these effects. Thus, the influence of both an increase and a decrease in serum IGF-I (and presumably a higher or lower entrance into the brain) and AHN was analyzed on the performance of mice in a hippocampal-dependent task such as spatial memory and learning, or in anxiety-like behaviors, both modulated by exercise.

To examine the effect of exercise in this paradigm, we used liver-IGF-I deficient (LID) mice, a model of adult low blood IGF-I levels. These animals performed normally in a set of neurological tests for behavioral phenotyping (according to protocols by Crawley, 2000), such as motor coordination in the rotarod test (control littermates 240 s±22 vs. LID 220±23; $p=0.421$; $N=6$); exploratory deambulation in the activity cage (considered as percentage of control littermates (100%); horizontal movements: controls 100±14 vs. LID 117±23, $p=0.55$; vertical movements: controls 100±20 vs. LID 124±40, $p=0.61$; $N=8$); and brightness choice in a visual discrimination task (control littermates 3.8±0.3 vs. LID 4.3±0.6, $p=0.66$; $N=8$). We first began by characterizing the hippocampal neurogenesis and the granule cell layer of these animals during development, and found no differences between LID and control littermates neither in the $BrdU^+$ cell proliferation nor in the total number of mature granule neurons, after weaning at postnatal day 20, ruling out developmental defects (BrdU cell densities: wild-type 2,076 cells/mm^3±195 vs. LID 1,991±120; $p=0.32$; Granule neuron numbers: wild-type 450,933 cells/hemisphere±2,010 vs. LID 396,677±3,150; $p=0.66$. $N=8$ for both groups; in every group, individuals came randomly from different litters). However, in the adults, basal AHN was significantly reduced (Fig. 1). Other brain regions that project to the hippocampus did not show any relevant alteration neither in the cytoarchitecture of the region (septum) nor in the total number of neurons (entorhinal cortex layer II: control littermates 14,354.5 cells±1,030.4 vs. LID 13,448.6±885; $p=0.686$. $N=5$). Specifically, we observed that LID mice show a reduced number of new dentate cells that survive for either 7 days or 1 month after BrdU administration (Fig. 1B). In contrast, no differences in proliferating cells were found (2-h protocol, Fig. 1B). The time points of 7 days and 1 month survival were analyzed to see the percentage of $BrdU^+$ cells that appeared double labeled with βIII-tubulin and no differences were found in cell lineage between genotypes or treatments (7-day survival: wild-type 76.8%±0.6; LID 80.1±2.4; LID+IGF-I 79.8±1.6; ANOVA F, 1.867, $p=0.166$; $N=9$ in every group except for the LID+IGF-I pump (8)). Similar results were found when considering the total number of differentiating neurons in these mice (Fig. 1C; total number of $BrdU^+$/βIII-tubulin$^+$ cells in wild-type mice at 1-month survival 195.6 cells±15.1—considered as 100% in the chart). The reduction in AHN is dependent on IGF-I, since systemic administration of IGF-I to LID mice for either 7 days or 1-month restored AHN to the levels of control littermates (Figs. 1B, C). Contrarywise, and in agreement with previous results (Trejo et al., 2001), exercise did not increase levels of AHN in the absence of a normal IGF-I input (Fig. 1C, LID mice+

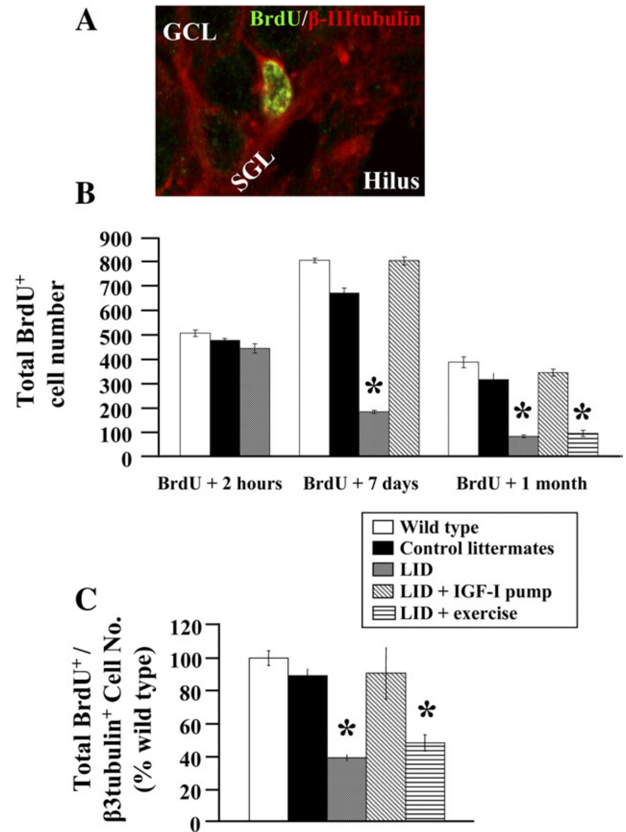

Fig. 1. Adult neurogenesis in LID mice with low serum levels of IGF-I (LID/wild-type comparison experiments 1 to 3). A group of C57BL6 mice was included only as a reference. (A) Representative image of a cell double-labeled for BrdU (green) and βIII-tubulin (red) in the dentate granule layer as those used for cell counts. (B) Proliferation and survival of new neurons in the LID mice. Data are total cell number of $BrdU^+$ cells counted in the DG of 3 to 4 months old mice. No significant differences were found in proliferation, but after 1-week survival LID mice showed a dramatic decrease in total cell numbers, still detected 1 month after BrdU. In LID with IGF-I osmotic mini-pumps, the total number of the $BrdU^+$ cells recovered both after 7 days and 1 month survival, while exercise did not recover the cell number (*$p<0.0001$). (C) The total number of $BrdU^+$/βIII-tubulin$^+$ neurons (1-month survival) in the DG as percentage of C57BL6 controls. A LID mice group trained in the treadmill 1 month is included. We found a 60% decrease in $BrdU^+$ cell numbers in LID when compared with wild-type control mice. Note that while IGF-I mini-pumps were able to restore the cell numbers in LID animals after administration over 1 month, forced exercise during the same time did not recover the cell number (*$p<0.05$). $N=9$ in every group except for the LID+IGF-I pump (8) for 7 days survival experiment. $N=6$ for c57, $N=5$ for the rest of groups for 1 month survival experiment.

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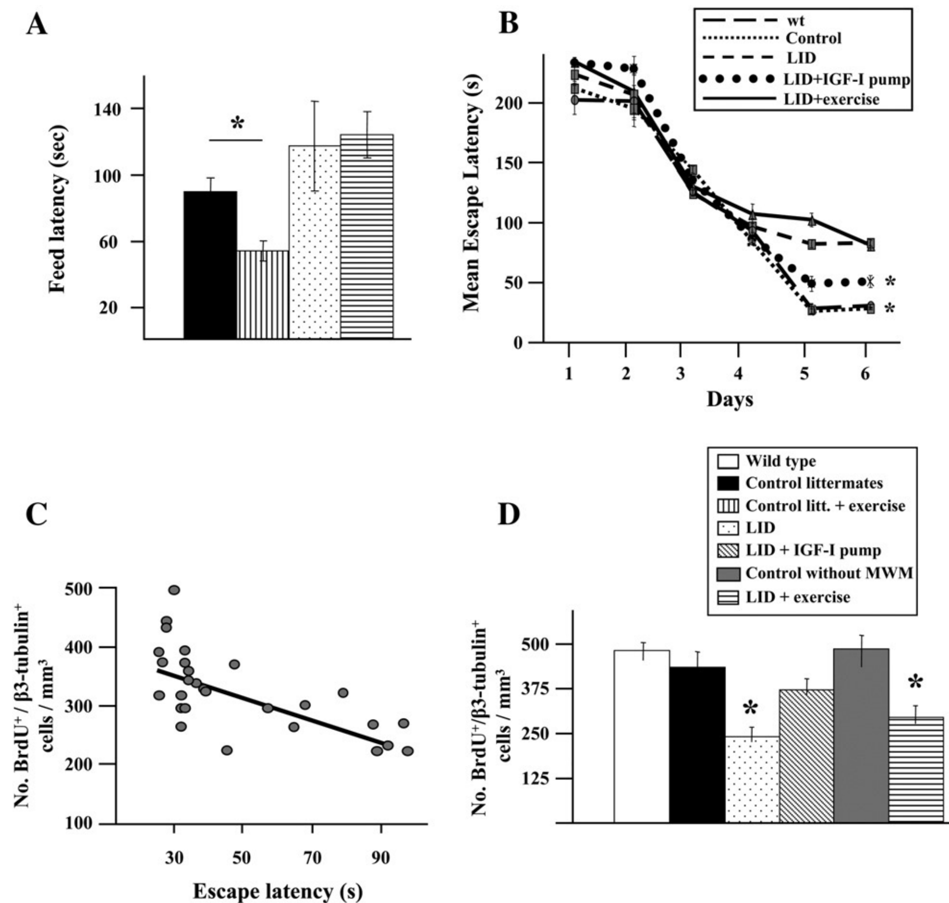

Fig. 2. Correlation between hippocampal neurogenesis, feed latency in the NSF test, and escape latency in the water maze test (LID/wild-type comparison experiments 1 to 3). (A) Effect of exercise on the novelty-suppressed feeding in LID mice. Exercised control mice showed significantly lower feed latencies. On the contrary, exercise training did not modify the performance in any LID group. (* $p<0.05$). (B) Acquisition phase of place learning in a water maze test. The data are the sum of 4 trials and the escape latency in the LID mouse is significantly higher than in the wild-type controls. Administration of IGF-I through osmotic minipumps for 1 month before the test partially recovered performance. However, 1 month training on the treadmill did not decrease the score of the animals, in contrast to the effects in the Porsolt test (asterisks represent significant differences between experimental groups as analyzed both by mean escape latency and learning curve mean slope; however, only escape latencies are represented; * $p<0.05$). (C) When neurogenesis of the animals of water maze test was evaluated 7 days after BrdU injection, a significant correlation was found between the "density of BrdU$^+$/βIII-tubulin$^+$ cells" and "escape latency" on the last day of the test (day 6). Thus, the higher the density of BrdU$^+$ cells, the lower the escape time to the hidden platform. Control littermate mice, LID, and LID + IGF-I animals are shown. Significant correlation was found both across groups for all animals and between groups. Regression line for all animals is charted for clarity of the representation. (D) The total number of BrdU$^+$/βIII-tubulin$^+$ labeled cells in the same animals performing the water maze test was calculated to consider the effect of the water maze test itself. The data show that LID mice have fewer BrdU$^+$ labeled cells than wild-type controls. Cell numbers recovered after 1 month infusion of IGF-I through mini-pumps in LID mice, but not after exercise. A control group that was not subjected to the water maze test was included to determine the effect of the water maze test itself on the survival of BrdU$^+$ labeled cells after 7 days. No significant differences were found with the groups that were subjected to the water maze. (* $p<0.05$). The sample size was $N=9$ for every group except for control littermates and control without MWM (8).

exercise). As no significant differences in the volume of the structures were found between control littermate and the rest of groups, neither in the hippocampal formation nor in the dentate gyrus granule cell layer, similar significant differences in cell counts were found both in the cell density and in the total number of cells per hemisphere (volume of the granule cell layer of dentate gyrus: C57BL/6 461.5 ± 21.5, control littermates 432.2 μm^3 ± 24.7, LID 394.2 ± 5.2, LID + IGF-I minipump 454.8 ± 14, LID + exercise 387.8 ± 6.8; F, 4.037, $p=0.014$, the only difference being between c57 and LID + exercise, $p=0.035$; $N=6$ for c57, $N=5$ for the rest of groups). The figures for 1-month BrdU$^+$ cell density data were C57BL/6, 838.3 cells/mm^3 ± 45.8; control littermates, 732 μm^3 ± 41.5; LID, 223.6 ± 24.8; LID + IGF-I minipump, 762.8 ± 32.4; LID + exercise, 258.4 ± 16.3; F, 73.028 $p<0.0001$, LID and LID + exercise groups being different from the rest of groups $p<0.0001$ ($N=6$ for c57, $N=5$ for the rest of groups).

We next tested how LID mice, with abnormally low AHN levels, score in mouse models of anxiety-like behavior and spatial learning. In the novelty-suppressed feeding test, we found a non-significant tendency to higher scores of sedentary LID mice compared to sedentary controls (Fig. 2A). The relevant finding is that while exercised controls showed a significant decrease in the feed latency compared with sedentary (anxiolytic effects of exercise have been previously reported in rats; Chaouloff, 1994; Fulk et al., 2004), exercise had no effect on LID mice. This test is a conflict test based on two parameters: the fear to a brightly lit, open field, and the hunger after a 24-h fast. The genotype of the animals or sedentary/exercise condition had a non-significant tendency to different values for the parameter "food intake/animal/day" (for the last week of training, sedentary control, 4.16 g ± 0.37; exercised control, 4.44 ± 0.13; LIDs, 3.56 ± 0.02; LID + exercise, 3.71 ± 0.8;

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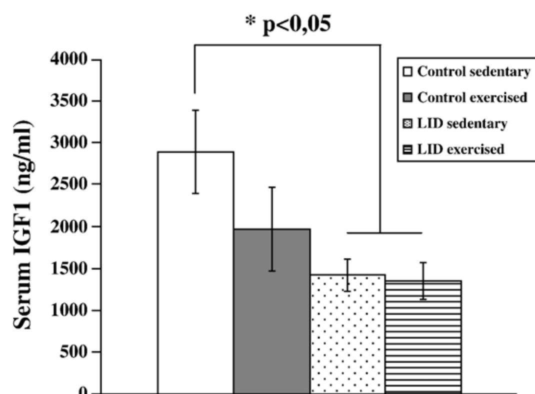

Fig. 3. IGF-I protein levels in serum after exercise (ELISA). Serum IGF-I levels of LID mice are significantly 50% lower than those of controls. Exercise was not able to restore IGF-I levels (both groups of controls, $N=7$; both groups of LIDs, $N=8$).

ANOVA F, 0.925, $p=0.347$; sample size was $N=9$ for every group), and non-significant effects in the parameter “weight before fasting”. In the same way, the weight loss after fasting was similar between groups and genotypes (sedentary control, 3.26 g±0.69; exercised control, 3.77±0.72; LIDs, 2.5±0.34; LID+exercise, 2.68±0.26; ANOVA F, 0.043, $p=0.839$). All this points to similar levels of hunger after the period of training. The effective level of hunger at the time of the test was shown by the parameter “food intake after fasting”. No significant effects of genotype and experimental group were found (sedentary control, 71 mg±10; exercised control, 112±15; LIDs, 100.5±20; LID+exercise, 120±16; ANOVA F, 0.041, $p=0.842$). Similarly, the data from the activity cage test revealed no significant differences between groups (sedentary control, 29.9 arbitrary units±5.6; exercised control, 34.02 ±4.6; LIDs, 38.2±9.7; LID+exercise, 35.9±8.2; ANOVA F, 0.177, $p=0.678$). In these animals, we determined the levels of serum IGF-I in the different groups. We found that LID animals showed significantly lower concentrations compared to controls and exercise did not restore these levels (Fig. 3).

Next, we tested whether the disturbed performance of LID mice in the water maze test (Trejo et al., 2007) could be ameliorated by exercise. We found that exercise did not improve water maze performance in LID mice (Fig. 2B). To rule out possible irreversible learning disturbances in LID mice, we treated them with systemic IGF-I and determine their performance in the water maze. In this case, IGF-I treatment resulted in recovery of performance levels (Fig. 2B). Both escape latencies and swim distance showed similar statistical differences between groups. Swim speed showed no significant differences between groups (wild-type, 21.32 cm/s± 5.26; LIDs, 25.21±6.47; LIDs+IGF-I, 21.47±6.6; ANOVA F, 2.307, $p=0.111$; sample size was $N=9$ for every group), therefore being the differences in the escape latencies only attributable to significantly higher path lengths in the LID and LID+exercise groups. Learning curve slopes also showed the same differences than the mean escape latency chart shown in Fig. 2B. No significant differences were found neither in sensorimotor and motivational

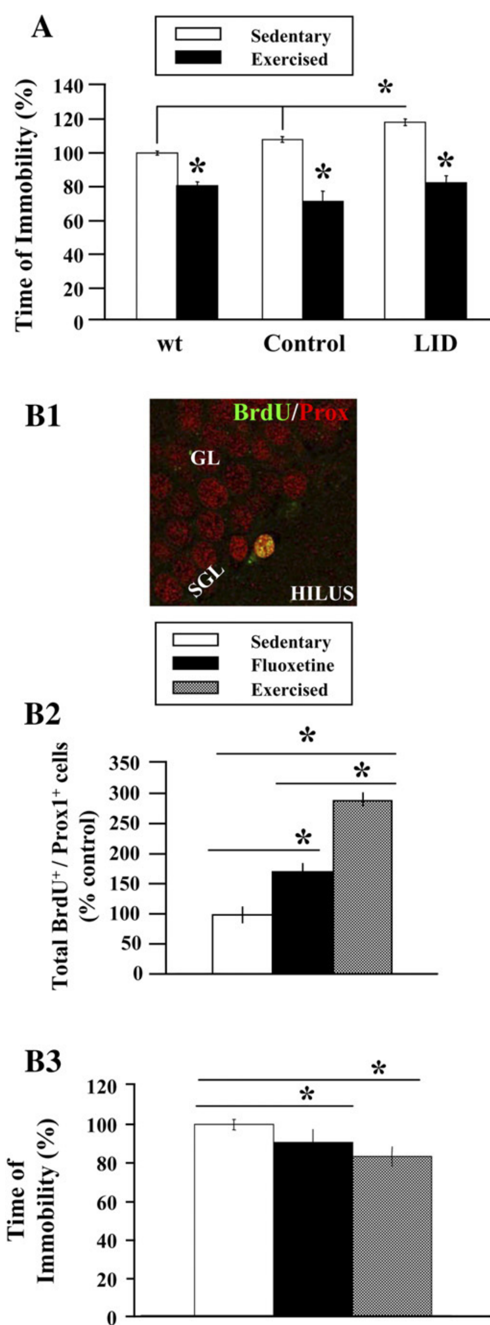

Fig. 4. Effect of exercise on the Porsolt test and neurogenesis. (A) Comparison of the effect of exercise between C57BL6 and LID mice. The data are the percentage with respect to C57BL6 controls and show that LID mice performed significantly higher than wild-type controls in the Porsolt test. In the three groups of animals, 3 weeks of exercise decreased the Porsolt scores and after exercise, no differences were observed between LID and wild-type controls in this test. The sample size was $N=9$. (B) Correlation between hippocampal neurogenesis and the immobility time in the Porsolt test in C57BL6 mice. (B1) Representative image of a cell double labeled for BrdU (green) and Prox1 (red) in the dentate granule layer as those used for cell counts. (B2) Numbers of $BrdU^+/Prox1^+$ new neurons in the DG showed that the effects of exercise were considerably higher than those of fluoxetine (150% vs. 50%, respectively). Since 99% of the cells inside the subgranular zone (SGZ) of the dentate gyrus that incorporated BrdU were $Prox1^+$, we considered the number of $BrdU^+/Prox1^+$ cells to represent the most reliable indicator of adult neurogenesis of dentate granule cells. (B3) Immobility times of C57BL6 mice showed that 3 weeks of exercise had a similar antidepressant effect as a 3-week treatment of fluoxetine when compared with sedentarism. The data are the percentage with respect to C57BL6. Sedentary, $N=7$; fluoxetine, $N=7$; exercised, $N=9$.

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status of the animals in the cued version nor in the swim distance and speed in the probe trial. Although all groups spent significantly longer times in the platform quadrant (PQ) compared with the other three quadrants (OPQ, RQ, LQ) during the probe trial (for LID mice: PQ, 21.2 s±1.3; OPQ, 9.1±0.7; RQ, 12.6±2.1; LQ, 17.1±0.8; ANOVA F, 4.989 $p=0.011$; PQ, significantly different from the other quadrants, $p<0.05$), the LID and LID+exercise groups spent significantly shorter times in the platform quadrant compared with the rest of groups (wild-type, 48.3%±1.1; control, 49.5%± 1.8; LIDs, 35.3%±2.3; LID+IGF-I, 44.1%±0.9; LID+exercise, 36.9%±0.8; ANOVA F, 5.095 ($p=0.01$); LID and LID+exercise groups significantly different from the rest of groups, $p<0.001$).

To see how the variations in behavior were related with variations in hippocampal neurogenesis in the same animals, we used the same protocol of water maze test. For this goal, we analyzed the correlation of water maze scores with varying levels of AHN (control littermate mice, LID, and LID±IGF-I treatment) using additional groups of animals, and found a significant correlation ($p<0.0001$; $r=0.681$) between both parameters (Fig. 2C), both across groups for all animals, and between groups. Since the number of $BrdU^+$ cells was analyzed immediately after the water maze test, an additional control group that did not perform the water maze test was included to examine the possible effects of the test itself on AHN. No differences were found between these animals when compared with wild-type or control littermates tested in the water maze (Fig. 2D).

As neither novelty-suppressed feeding nor water maze tests showed any variation after exercise in LID mice, we next evaluated whether LID animals were able to show any sensitivity to exercise using a different behavioral test. For this, we analyzed the performance of animals in the Porsolt forced swim test. Sedentary LID mice showed a significantly lower score in this test compared to controls (Fig. 4A, "sedentary" groups), indicating mood disturbances in low serum IGF-I mice. However, by using a similar exercise training protocol than that used previously for the novelty-suppressed feeding test, LID mice increased Porsolt scores to the same extent than in control exercised mice (Fig. 4A, "exercised" groups), indicating that LID mice retain the ability to improve mood (as measured by the Porsolt test) to the same extent as control mice.

Finally, as the Porsolt forced swim test scoring was restored to "control" levels in LID mice after exercise, while these animals did not recover "basal" levels of hippocampal neurogenesis after exercise, we evaluated if in control mice our exercise protocol was raising both neurogenesis and Porsolt scores in the same animals. For this purpose, we trained control mice in a treadmill over 3 weeks. Exercise training is known to increase adult hippocampal neurogenesis (AHN) and to decrease the mean score of animals in the Porsolt test (Greenwood et al., 2003). As an additional control way to enhance AHN, we administered fluoxetine (Borsini, 1995). The extent of AHN was measured by counting the number of BrdU/Prox1 cells (Fig. 4B1) in the hippocampal dentate gyrus. We found that the increases in AHN after exercise were significantly higher than after fluoxetine (Fig. 4B2), while performance in the Porsolt test was improved after exercise or fluoxetine to a similar extent (Fig. 4B3).

Discussion

Physical exercise exerts behavioral effects that may be manifested through multiple mechanisms. Among these, those involving hippocampal-dependent learning and memory or anxiety have received particular attention. These behaviors are also currently analyzed on the basis of the participation of adult hippocampal neurogenesis (AHN) as a possible mechanism mediating the effects of environmental enrichment and other interventions. Accumulating evidence supports the idea that both neurogenesis-dependent and -independent mechanisms are involved in these effects (Scharfman and Hen, 2007). We need to gain further insight into the mechanisms (whether neurogenesis-dependent or not) involved in exercise effects because of the importance of physical activity to maintain proper brain function (reviewed in Cotman and Engesser-Cesar, 2002; Trejo et al., 2002). We herein report that in mice the effects of moderate treadmill running on spatial learning and on anxiety-like behavior depend on IGF-I and are associated to AHN while those on depressive-like behaviors appear independent of these factors.

We have found that serum IGF-I deficient mutant (LID) mice showed impaired spatial learning together with reduced AHN, with no other apparent differences relevant to the task. In old, but not in young mice with low serum IGF-I obtained through a different genetic manipulation, water maze performance was also impaired (Svensson et al., 2005). In addition, we observed that C57BL/6 adult male mice showed lower latencies in the NSF test after moderate exercise. Similar results were previously obtained in rats (Chaouloff, 1994; Fulk et al., 2004). However, in LID mice, spatial learning, anxiety-like behavior, and AHN remained impaired after running. This is probably due to the inability of exercise to raise the entrance of serum IGF-I into the brain of LID mice. The latter is in all probability related to the fact that LID mice have very low levels of blood IGF-I. Thus, stimulation of the entrance of serum IGF-I into the brain in response to exercise probably requires a higher availability of this circulating growth factor to elicit beneficial effects. These results confirm our previous findings that low levels of serum IGF-I input induce low levels of AHN (Trejo et al., 2001) and extend them illustrating the impact of reduced serum IGF-I on behavioral traits related to anxiety. Similarly, the inability of physical exercise to enhance AHN levels in serum IGF-I deficient mice suggests that the production of IGF-I from tissues other than the liver is not enough to compensate the lack of liver IGF-I in LID mice. This observation also reinforces the need to enhance entrance of serum IGF-I into the brain to increase neurogenesis after exercise (Torres-Alemán, 2005; Trejo et al., 2002). Furthermore, modulation by exercise of spatial memory and anxiety-like behavior depends on serum IGF-I because in LID mice this modulation is absent. This interpretation is reinforced by the fact that LID mice can respond to exercise because performance in the forced swim test was normalized in LID mice after running.

LID mice constitute a good model to test the response of AHN and diverse behavioral traits to exercise. The lack of effect of exercise in LID mice is not the result of permanent changes in hippocampal structure or other irreversible general physiological defects. When exogenous IGF-I was infused to the animals, LID mice partially recovered water maze performance and hippocampal neurogenesis. The behavioral phenotype cannot be attributable to developmental defects (infant LID mice show no developmental defects, see data from P20 animals) or to metabolic/hormonal alterations because no differences were found in physical strength as assessed by equal swimming speed or total distance covered during the probe trial, or performance in the rotarod test or activity test. Similarly, data from the cued trials of the water maze and from the visual discrimination task let us discard any visual defect to

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explain the differences in water maze performance. In addition, in a completely different paradigm (forced swim test), LID mice were able to improve after exercise. It is noteworthy that physical exercise may induce effects on spatial learning and anxiety-like behaviors through processes different from those induced with a related intervention such as environmental enrichment (Meshi et al., 2006). As long as most enrichment protocols include wheel running, the results in the latter works are probably due to mixed effects. In studies separating the influence of exercise and enrichment, different effects have been reported for each paradigm on hippocampal neurogenesis (Olson et al., 2006). It is not known yet if the specific effects of an enriched environment might influence the effects of exercise when both are considered together in standard enrichment cages.

Recently, it has been shown that a water maze protocol was unaffected after ablation of AHN (Saxe et al., 2006). Previous works have also shown similar results, but either in rats (Shors et al., 2002) or with different spatial learning paradigms (Winocur et al., 2006). These reports add support to the notion that not all hippocampus-dependent behaviors depend on hippocampal neurogenesis. However, in the former study as well as in Meshi et al. (2006), AHN-depleted animals were tested in a version of the water maze test (first cued, later hidden) different to that used in the present work (hidden version). Searching strategies vary considerably in each version of the water maze (Cain et al., 2006; Redhead et al., 1997), even as the test progresses. These differences may be sufficient to explain the discrepancy in the results. The variations in IGF-I-dependent behaviors as shown here, presented significant correlations with AHN levels. On the contrary, improvements in the forced swim test similar to those seen after exercise in LID mice have been shown to take place after fluoxetine administration independently of AHN (Vollmayr et al., 2003), or IGF-I (Cryan et al., 2002; Engesser-Cesar et al., 2007).

The effects reported here clearly demonstrate a role of peripheral IGF-I in brain plasticity and cognition. In addition, the fact that forebrain-specific IGF-I deleted mice showed only minor detectable changes in the brain (Dávila et al., 2007), reinforces the notion that circulating IGF-I is necessary for the beneficial effects of moderate exercise. This suggests a direct action of blood IGF-I inside the brain, rather than a simply permissive action, such as facilitation of the entrance of other beneficial factors. Nevertheless, regulation of the traffic through the blood–brain barriers by IGF-I is an additional intriguing possibility as glial end-feet covering brain endothelial cells and choroid plexus cells contain IGF-I receptors (García-Segura et al., 1997). For a putative direct action, hippocampal newborn cells and granule precursors are possible targets of IGF-I, as $BrdU^{+}$ cells located in the adult granule cell layer express IGF-I receptors (Trejo et al., 2001).

The biology of IGF-I in the brain is still far to be fully characterized (see for example Russo et al., 2005). An intriguing possibility is that some actions of IGF-I inside the brain take place differently at distinct time frames. In this regard, while blocking IGF-I input to the brain (either by blocking brain IGF-I receptors – Ding et al., 2006 – or blocking circulating IGF-I – LLorens-Martín, Torres-Alemán and Trejo, unpublished results) does not block exercise-driven enhancement of learning at short/medium term, blocking peripheral IGF-I at long term impairs the acquisition of a spatial memory (present results). IGF-I plays in the brain of both sedentary and exercised animals pleiotropic actions at short and long terms and its modulation through time merits further investigation.

We have found that the effects of moderate physical exercise on spatial learning and anxiety-like behavior depend on serum IGF-I levels and the entrance of blood IGF-I into the brain, and that hippocampal newborn neurons are regulated as expected if they are involved in the effects of exercise and IGF-I on these behaviors. The evidence presented allows us to indicate that a neurogenesis-dependent mechanism participates in several, but not all the beneficial effects of exercise in the brain. However, more experiments are needed to firmly establish whether the underlying mechanisms are neurogenesis-dependent.

The effects of exercise on neuronal activity and neuroprotection against several insults have been reported to be mediated at least in part by IGF-I (Carro et al., 2000, 2001). Now we report evidences for an IGF-I-mediated effect of moderate exercise on various hippocampus-dependent behaviors in healthy animals. The relevance of these findings lies in the therapeutic potential of unveiling the ways exercise helps to maintain brain health, elaborating on a welcomed insight that holds moderate exercise as the normal condition, while sedentarism is the harmful abnormal situation.

Experimental methods

Animals

Adult C57/BL6 mice, serum IGF-I deficient mutant mice (LID mice, congenic with C57/BL6), and their control littermates were used. LID mice were generated as described elsewhere; they present low levels of serum IGF-I due to the disruption of the liver IGF-I gene by the postnatal activation of the albumin-Cre/Lox system (Yakar et al., 1999). Serum IGF-I deficient mice have normal body and brain weights and they do not show any developmental defects (Sjogren et al., 1999; Yakar et al., 1999). The mutant mice have normal serum IGF-I levels during the first weeks of life but soon after, liver IGF-I production starts to decline and by 2 months of age LID mice show a ~60% decrease in serum IGF-I when compared to control littermates (Carro et al., 2002). In contrast, the levels of IGF-I in tissue are normal, even in the brain (Yakar et al., 1999). All the animals used were 3-month-old males, housed at 22 ± 1 °C with a light/dark cycle of 12/12 h and with *ad libitum* access to food and water. The animals were kept under standard laboratory conditions in accordance with the guidelines established by the European Community Council (directive 86/609/EEC). Prior to the beginning of the experiments, an independent group of LID mice was compared with control littermates in the rotarod and the activity cage as described (Carro et al., 2001), and in a visual discrimination test (Myhrer and Johannesen, 1995); see protocols below. Similarly, a group of 20-day-old LIDs and control littermates was injected with BrdU and sacrificed 24 h after injection to determine the total number of new $BrdU^{+}$ cells in the dentate gyrus by immunohistochemistry (see below), and the total number of mature granule neurons by a stereological procedure described in detail elsewhere (Llorens-Martin et al., 2006); data shown as mean ± S.E.M. and analyzed with Mann–Whitney *U* test.

Experimental design

We used 4 experimental approaches to investigate the relationship between exercise, performance in the novelty-suppressed feeding test, the forced swim test (Porsolt test), and a spatial learning test (Morris maze test), and its relation with neurogenesis. In the first experiment, we analyzed the effect of exercise on adult hippocampal neurogenesis in LID mice with low levels of circulating IGF-I and low basal levels of neurogenesis. We also examined the effect of IGF-I administration in this model. We analyzed both cell proliferation (24 h after BrdU injection) and survival at two time points after BrdU injection (7 days and 1 month). In this way, we studied a neurogenesis model amenable to experimental modulation (Fig. 5A; results of

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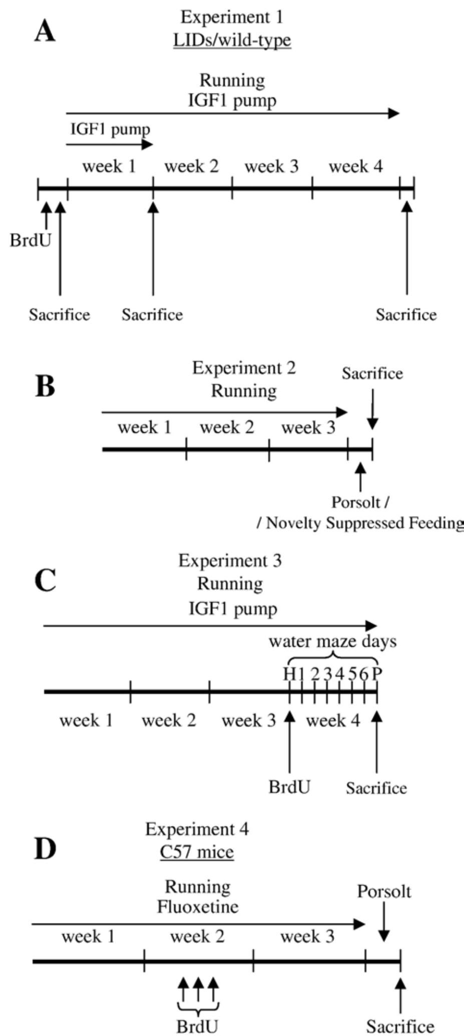

Fig. 5. Timeline and design of experiments. (A–C) The three LIDs/wild-type comparison experiments. Experiment 1 in panel A corresponds to data of Fig. 1; experiment 2 in panel B to data of Figs. 2A and 4A; experiment 3 in panel C to data of Figs. 2B–D. BrdU/βIII-tubulin immunohistochemistry was employed. (D) The exercise/Porsolt experiment (experiment 4) in c57BL/6 mice, corresponding to data of Figs. 4B2, B3. BrdU/Prox1 immunohistochemistry was employed. In experiment 3, H means habituation, P means probe trial.

Fig. 1). In the second experiment, we analyzed the effect of running on the performance of LID mice in the novelty suppressed feeding test and in the forced swim test (Fig. 5B, results of Figs. 2A and 4A, respectively). With this approach, we aimed to detect behavioral differences in the response to exercise between animals with different levels of hippocampal neurogenesis. In the third experiment, we analyzed both the effect of running on the performance of LID mice in the water maze test, and the correlation between exercise-induced or IGF-induced changes in neurogenesis with the changes in the water maze test (by using the same group of animals; Fig. 5C; results of Figs. 2B–D). We used a 7-day survival BrdU protocol to have an adequate number of positive new neurons to correlate with behavioral scoring. As in the Porsolt test, exercise changed the behavior of LID mice without modifying neurogenesis, in the fourth experiment (the exercise/Porsolt experiment) we analyzed in normal animals the correlation between exercise-induced increase in neurogenesis and the exercise-induced improvement in the Porsolt test (Fig. 5D, results of Figs. 4B2, B3). The target of this experiment was to know if our exercise protocol was able to modulate the behavior in the Porsolt test and at the same time produce variations in neurogenesis in normal animals. A group of mice administered with fluoxetine were used as a positive control group in the Porsolt test. A BrdU injection protocol was used that maximized the effect of running at the time of sacrifice. Animals ran for 3 weeks, were evaluated in the Porsolt test, and sacrificed the day after. We scored the number of $BrdU^+/Prox1^+$ cells to detect all new cells committed to the granule neuron lineage. We used C57BL6 mice for this purpose. For the rest of experiments (LID/control comparisons), we compare LID mice and control littermates, and using C57BL6 mice as a reference. For these ones, we use a standard protocol of evaluation of the number of $BrdU^+$/βIII-$tubulin^+$ cells.

Exercise protocol

Animals were habituated to the treadmill apparatus for 15 min (Cibertec, Spain). The training of exercised animals included gradual adaptation to the running schedule (Carro et al., 2000). The mice ran at 0.2 m/s, 5 days a week, for 40 min/day over 3 weeks in the exercise/Porsolt experiments (experiment 4). A 40 min daily running schedule is thought to be particularly effective in providing beneficial effects to male mice, because it minimizes the impact of the increment in stress hormones (Coleman et al., 1998). In the rest of experiments, LID and control mice were subjected to a schedule of 1 h/day over 4 weeks to maximize the effects of exercise in LID/control comparisons. Sedentary or fluoxetine animals remained on the treadmill without running for the same periods of time, respectively.

Treatments

A group of animals received a daily intraperitoneal injection of fluoxetine (5 mg kg^{-1} body weight) over 3 weeks, while control and exercised animals received 0.9% saline. Recombinant human IGF-I (GroPep, Australia) was administered through a subcutaneous osmotic mini-pump to normal and LID mice for 1 month (Alzet 2001; 50 μg kg^{-1} day^{-1}, as described (Fernandez et al., 1998), while control animals received saline. Administration of IGF-I through minipumps produced a significant >2-fold increase in serum IGF-I levels (Fernandez et al., 1998). BrdU (50 mg kg^{-1}) was administered by intraperitoneal injection. In the exercise/Porsolt protocol (experiment 4), BrdU was injected at the middle of the experiment (Greenwood et al., 2003), and the animals were sacrificed 13 days after the last BrdU injection. As for the LID/control comparisons, a standard injection protocol was employed where three distinct groups of animals were used and sacrificed either 2 h, 1 week, or 1 month after a single BrdU injection (see Fig. 5 for details). This protocol allowed us to measure differences in proliferation, as well as the short-term and long-term survival of newborn cells between genotypes.

Behavioral tests

Different groups of animals were employed for each type of test.

Novelty-suppressed feeding (NSF) test

Animals housed in each cage were from the same genotype and experimental group, and similar in age (4–6 months old, 3–4 animals per cage, $N=30$). Total amount of food was weighed daily for each cage, and daily intake averaged by number of animals in each cage. At the end of every week, the parameter "food intake/animal/day" was obtained as an average of the week. One day after the last day of training, the animals were weighed to measure the parameter "weight before fasting" and then they were fasted during 24 h before the NSF test. Food pellets were removed from the cages at intervals of about 10–15 min in the same order than the cages entered the next day into the behavior room to guarantee that all animals had a 24-h fast. Therefore, NSF was conducted 2 days after the last day of training. Immediately after the NSF test, each animal was weighed to measure the parameter "weight after fasting". The animal was then placed into an empty cage (no mates) containing a weighed food pellet and allowed to stay during 5 min. After this, the rests of the pellets were weighed to obtain the parameter

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"food intake after fasting". All animals ate during the 5 min period. The animal was then tested in a standard activity cage to determine "horizontal and vertical activity" and "time spent in center/margin". Finally, the animal was put back to its cage. In the NSF test, we used a brightly lit round arena (50 cm diameter × 10 cm, ~6800 lx) with the floor covered with 1–2 cm bedding. Two food pellets were located upon a white round paper (5 cm diameter) over the bedding. The animal entered the arena facing the wall and placed in a random point of the perimeter. The experimenter was blind to experimental group and measured the latency to feed. It was considered feeding when the mice bit the pellet holding it with its forepaws and not only if the animal merely dragged or pushed the pellet. All animals tested showed a thigmotaxic behavior in the first seconds of the test, and all bit the pellet before 6 min.

Water maze test

For this test, we followed standard procedures (Morris, 1984) with minor modifications previously described in detail (Carro et al., 2001, 2006). Briefly, after a 1-d habituation trial (day 0) in which preferences between quadrants in the different experimental groups were ruled out, the animals learned to find a hidden platform over the following 1–6 days, through 4 trials/day, 60 s each trial, plus 20 s in the platform. If an animal fails to reach the platform, it is placed on it by the experimenter. Subsequently, the animals were subjected to two trials on the 7th day, the first without the platform to assess possible differences in swimming speed and preference for the platform quadrant. And in the second, a cued version protocol using a visible platform was conducted to determine sensorimotor and motivational status of the animals. In the water maze charts, asterisks represent significant differences between experimental groups, as analyzed both by mean escape latency (two-way repeated measures ANOVA) and learning curve mean slope (one-way ANOVA). However, only escape latencies are represented in the chart.

Porsolt test

Two days after the last exercise session or fluoxetine injection, the animals were placed in a tank filled with water at 23 °C in which they remained for 6 min each time. The behavior of the animals was video recorded (JVC Mod QZ MG 20), and evaluated on a computer screen as previously detailed (Llorens-Martin et al., 2007). Briefly, the data are presented as a factor of the time of immobility considering the last 5 min of the test. The time spent in the different behaviors was measured double-blind using PowerDirector™ NE Express software (Cyberlink): While each clip was played on the computer in timeline mode, the experimenter divided the clip every time the behavior of the animal changed, by using the timeline of the clip forwards and backwards with the sliding control of the software. When the 5 min finished, the graphic script mode yields the time spent for each behavior. Each behavior was assigned to one of four categories (Detke et al., 1995): climbing, swimming, staying afloat, and immobility. As absolute immobility is hardly achieved, "staying afloat" was considered the smooth movement necessary to equilibrate the animal's postural control. To avoid that these displacements through inertia were considered "swimming", the difference was easily determined considering swimming as the time during which the displacement was maintained through at least one lap of the cylinder. When the movement was interrupted, it was considered as staying afloat. For these purposes, we took into consideration the movement of the legs as suggested previously (Gersner et al., 2005), rather than the more classical estimate of simple body displacement through virtual quadrants in the water surface. The data are presented as the time in the immobile state plus staying afloat.

As for the preliminary behavioral tests, motor coordination was evaluated with animals in a rotarod apparatus (Ugo Basile, Italy) with increasing acceleration. The apparatus consisted of a horizontal motor-driven rotating rod in which the animals were placed perpendicular to the long axis of the rod, with the head directed against the direction of rotation so that the mouse has to progress forward to avoid falling. The trial was stopped when the animal fell down or after a maximum of 5 min. The time spent in the rotating rod was recorded for each animal and trial. Animals received a pretraining session to familiarize them with the procedure before evaluation. Thereafter, a total of six consecutive trials were done for every animal. Only the average results from the fifth and sixth trials for each animal were used for statistical comparison. Data are presented as the mean time spent before falling from the apparatus ± S.E.M. For activity behavior, we used a Hamilton–Kinder Photobeam System computerized recording of animal activity over a designated period of time. Exploratory locomotor activity is recorded in an open field measuring 40 × 40 cm over a 10 min period. Infrared beams automatically record horizontal movements and rearing in the open field. The task provides measures of locomotor activity by including number of beams broken every 5 min. The mean number of broken beams of control animals is used as 100%. Data are shown as percentage of control animals ± S.E.M. For visual discrimination test, mice were deprived of water for 24 h and trained to distinguish a target cylinder (i.e., black vs. white non-target cylinders) containing water in a small well, in the absence of olfactory cues. The position of the cylinders is changed randomly during a 10 trial-session. The test was performed during two consecutive days and results were expressed as number of wrong choices before reaching the target in the second day. Data are shown as number of errors before correct performance ± S.E.M. In these three tests, Mann–Whitney's *U* analysis was employed to compare data.

Immunohistochemistry

After the different experimental protocols, animals were deeply anaesthetized with pentobarbital and transcardially perfused with saline followed by 4% paraformaldehyde in PB. The brain was removed and postfixed overnight at 4 °C. Coronal sections (50 μm width) were collected individually in a 96-multiwell culture plate. One-in-8 random series were collected for immunohistochemistry and the slices were preincubated in PB with Triton X-100 0.5% and BSA 0.1% (PBTBSA). For BrdU immunohistochemistry, DNA was denatured with a 30 min incubation in HCl 2N at room temperature. Double immunohistochemistry was performed as described previously (Trejo and Pons, 2001). For one experiment, sections were incubated with a mouse anti-BrdU antibody (Developmental Hybridoma Bank 1:15,000) and a rabbit anti-Prox1 (a transcription factor marker for granule neuron lineage in dentate gyrus, Pleasure et al., 2000; Chemicon, 1:1,000) for 3 days at 4 °C, both diluted in PBTBSA, followed by a 594 alexa-conjugated donkey anti-rabbit and a 488 alexa-conjugated donkey anti-mouse antibody. For the other experiments, the sections were labeled with a rat anti-BrdU (Accurate Scientific, Westbury, NY 1:2,000) and a mouse anti-βIII-tubulin (Promega, Madison, WI 1:8,000), that were detected with a 488 alexa-conjugated goat anti-rat and a 594 alexa-conjugated donkey anti-mouse antibody (Alexa-conjugated antibodies from Molecular Probes, 1:1,000). Sections were analyzed and photographed with a confocal microscope Leitz DMIRB/TCS4D. Neuronal granule cell populations were counted in sections of the subgranular and granule cell layer following BrdU/Prox1 immunohistochemistry for the exercise/Porsolt experiment (experiment 4, data are presented as the total number of cells), or single BrdU labeling and BrdU/βIII-tubulin immunohistochemistry for the LID/control comparison (data are presented as either single $BrdU^+$ total cell number in Fig. 1B because it includes proliferation (24 h after injection) or the number of $BrdU^+$/βIII-$tubulin^+$ neurons when survival is considered (Figs. 1C and 2)). In experiment 1 (see Fig. 5), both $BrdU^+$ total cell number for the three times of sacrifice, and the double $BrdU^+$/βIII-$tubulin^+$ cell density for 7 days and 1 month survival were estimated. For total number of cells, volume of the dentate granule cell layer was calculated by means of the Cavalieri method as described (Llorens-Martin et al., 2006). The data of the total number of cells are obtained by multiplying the mean cell density by the mean GCL volume for each individual separately and then obtaining the group mean. The mean cell density of each individual is calculated by using a standard optical dissector protocol as published previously (Trejo et al., 2001).

Statistical analysis

Data from the cell counting and behavioral tests were analyzed either by one-way ANOVA for comparisons between control animals, or by two-way ANOVA for comparisons between control and LID animals. Water maze data were compared either by repeated measures ANOVA for escape latencies or

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one-way ANOVA for learning curve slopes. Tukey test was employed for post hoc analysis. When simple comparisons between control littermates and LID were made, Mann–Whitney *U* test was employed. The statistical software packages used were either SPSS 13 (SPSS Inc. 1989; Apache Software Foundation) or Statgraphics Plus 5.0 (Statistical Graphics Corp., 1994).

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