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Reduced wheel running and blunted effects of voluntary exercise in LPA_1-null mice: The importance of assessing the amount of running in transgenic mice studies

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ABSTRACT

This work was aimed to assess whether voluntary exercise rescued behavioral and hippocampal alterations in mice lacking the lysophosphatidic acid LPA_1 receptor (LPA_1-null mice), studying the potential relationship between the amount of exercise performed and its effects. Normal and LPA_1-null mice underwent 23 days of free wheel running and were tested for open-field behavior and adult hippocampal neurogenesis (cell proliferation, immature neurons, cell survival). Running decreased anxiety-like behavior in both genotypes but increased exploration only in the normal mice. While running affected all neurogenesis-related measures in normal mice (especially in the suprapyramidal blade of the dentate gyrus), only a moderate increase in cell survival was found in the mutants. Importantly, the LPA_1-nulls showed notably reduced running. Analysis suggested that defective running in the LPA_1-null mice could contribute to explain the scarce benefit of the voluntary exercise treatment. On the other hand, a literature review revealed that voluntary exercise is frequently used to modulate behavior and the hippocampus in transgenic mice, but half of the studies did not assess the quantity of running, overlooking any potential running impairments. This study adds evidence to the relevance of the quantity of exercise performed, emphasizing the importance of its assessment in transgenic mice research.

1. Introduction

Lysophosphatidic acid (LPA) is an endogenous lysophospolipid that acts through six G-protein coupled receptors (LPA_{1-6}) to regulate brain plasticity and behavior (Callaerts-Vegh et al., 2012; Choi and Chun, 2013; Choi et al., 2010; Dash et al., 2004; Estivill-Torrus et al., 2013; Shin et al., 2012). Among the LPA receptors, the LPA_1 is the most studied so far. It is required in the brain during neurodevelopment (Estivill-Torrus et al., 2008; Hecht et al., 1996) and also in the adulthood, when it shows expression both in white matter and hippocampal neurons (Choi and Chun, 2013; Handford et al., 2001; Noguchi et al., 2009; Pilpel and Segal, 2006; Weiner et al., 1998). Recently, the phenotype of the LPA_1-null mice has revealed an important role of the LPA_1 receptor for hippocampal functioning. The LPA_1-null mice show synaptic and neurochemical abnormalities in the hippocampus (Blanco et al., 2012; Musazzi et al., 2011; Roberts et al., 2005) in addition to a notable impairment in adult hippocampal neurogenesis, consisting of reduced cell proliferation, survival and number of immature neurons in the subgranular zone of the dentate gyrus (Matas-Rico et al., 2008). Considering the involvement of the adult-born hippocampal neurons in behavior (Castilla-Ortega et al., 2011b), the defective adult hippocampal neurogenesis in the LPA_1-nulls is likely to contribute to their behavioral abnormalities, such as impaired exploration, increased anxiety-like behavior and memory

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deficits (Blanco et al., 2012; Castilla-Ortega et al., 2010; Estivill-Torrus et al., 2013; Pedraza et al., 2013; Santin et al., 2009). Importantly, environmental enrichment (a protocol that included cognitive and social stimulation plus voluntary exercise in running wheels) has a blunted effect to enhance hippocampal neurogenesis in the LPA_1-null mice (Matas-Rico et al., 2008), and the impact of chronic stress on spatial memory and the hippocampus is aggravated in absence of the LPA_1 receptor (Castilla-Ortega et al., 2011a; Garcia-Fernandez et al., 2012). These results, suggest that the LPA_1 receptor modulates experience-dependent hippocampal plasticity in the adulthood.

Here we aimed to research the impact of 23 days of voluntary wheel running in the LPA_1-null mice, assessing exploratory and anxiety-like behavior in the open-field and adult hippocampal neurogenesis. Voluntary exercise exerts many beneficial effects in the brain (Cotman and Berchtold, 2002; Pietropaolo et al., 2008) and is frequently chosen as a therapeutic approach to rescue behavioral deficits and adult hippocampal neurogenesis in transgenic mice (Kida et al., 2013; Renoir et al., 2012; Rodriguez et al., 2011). However, it should be noted that voluntary running behavior is easily modulated by factors such as the genetic background, stress, sex or aging (Clark et al., 2011; Nakajima et al., 2010; Novak et al., 2012; Pietropaolo et al., 2008; Turner et al., 2005). The presence of wheel running impairments is likely aggravated in the case of transgenic mice, because their mutations, especially when they are life-long, may carry numerous physiological and behavioral alterations that would affect running (Brooks et al., 2012; Lalonde et al., 2012; Novak et al., 2012; Taylor et al., 2010; van den Buuse, 2010). An altered pattern of voluntary exercise is a relevant issue, since the quantity of exercise performed may account for its effects on behavior and the hippocampus (Pietropaolo et al., 2008). Therefore, for the first time we assessed the quantity of wheel running performed by the LPA_1-null mice, and studied its relationship with the effects of exercise. Because results pointed out a relevant role of the amount of running, a second part of this work reviewed the published literature that used voluntary running as a treatment for hippocampal and behavioral pathology in transgenic mice. Attention was given to determine whether studies assessed the amount of voluntary wheel running and the potential consequences of this decision on the results they reported.

2. Materials and methods

2.1. Animals

Experiments were performed in male C57BL/6J ×129X1/SvJ mice (wild-type, WT) and in male LPA_1-null mice (Null) from the same hybrid background. The LPA_1-nulls were obtained from the *Malaga* variant of the LPA_1-null mouse, that was spontaneously derived during the expansion of the original colony (Contos et al., 2000) and is extensively described in our previous works (Estivill-Torrus et al., 2008; Matas-Rico et al., 2008; Santin et al., 2009). Six animals per each genotype and experimental condition were used. All mice had approximately 12 weeks of age and the onset of the experiment and were housed on a 12-h light/dark cycle (lights on at 07:00 a.m.) with water and food provided ad libitum. All procedures were performed in accordance with European animal research laws (European Communities Council Directives 86/609/EEC, 98/81/CEE and 2003/65/CE; Commission Recommendation 2007/526/EC) and the Spanish National Guidelines for Animal Experimentation and the Use of Genetically Modified Organisms (Real Decreto 1205/2005 and 178/2004; Ley 32/2007 and 9/2003).

2.2. Bromodeoxyuridine administration and voluntary wheel running

On the first day of experiment, mice received four doses of 75 mg/kg of bromodeoxyuridine (BrdU, Sigma, St. Louis, USA) dissolved in saline and administered intraperitoneally at 2-h intervals, to mark a population of newly-born cells that will be later studied for cell survival. From the following day, mice of both genotypes were assigned to the control (*Ctrl*) or voluntary wheel running (*VRun*) treatments for 23 days (Fig. 1A). *Ctrl* mice were individually housed in standar laboratory cages (11 cm × 30 cm and 13 cm high) provided with shredded paper as nesting material, while *VRun* mice where individually housed in exercise cages (20 cm × 26 cm and 27 cm high) provided with two floors connected with a ladder, shredded paper as nesting material and a running wheel equipped with a magnetic counter (Dayang Pet Products, Foshan City, China). As a measure of the quantity of voluntary exercise, the distance ran on the wheel (number of rotations multiplied by the wheel perimeter) was monitored daily. The distance ran was averaged every four days and analyzed with a repeated measures ANOVAs ('genotype × day', with 'day' as a repeated measure) followed by post hoc Fisher's least significant difference (LSD) analysis. In addition, total distance ran was calculated as a mean per day and compared between genotypes by a Student's *t* test. The threshold for statistical significance was set at $p \leq 0.05$ for all analysis in this experiment, that were carried out with the statistical package SPSS 15.0 (SPSS Inc., Chicago, IL, USA).

2.3. Exploratory and anxiety-like behavior in the open-field and principal components factorial analysis

On day 24, WT and LPA_1-null mice were placed in the center of an open-field apparatus (40 cm × 40 cm × 40 cm, made of gray plexiglas) for 5 min, to assess the effects of voluntary running on exploratory and anxiety-like behavior on a novel environment. The session was recorded and the following behaviors were assessed:

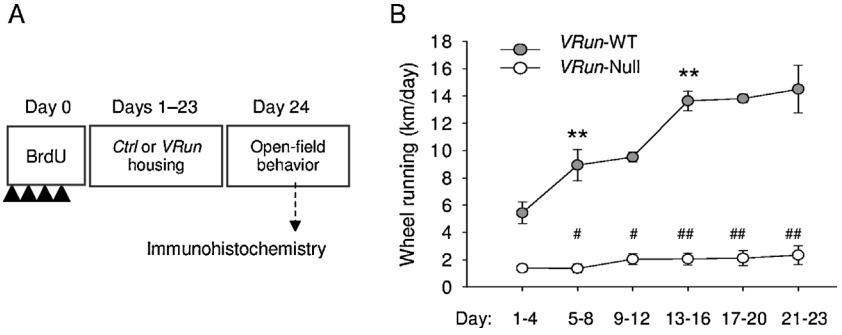


Fig. 1. Reduced voluntary wheel running in the LPA_1-null mice. (A) Protocol for BrdU administration, voluntary exercise and behavioral analysis. (B) Voluntary wheel running in WT and LPA_1-null mice. Means ± SEM. LSD: difference between genotypes: $^{\#}p < 0.05$; $^{\#\#}p < 0.001$; within-group difference from the previous day: $^{**}p < 0.001$.

locomotion (m travelled), thigmotaxis (percent of time spent by the animal in the periphery of the maze, defined as the 8 cm of arena in from the walls), supported rearing (SupRearing, the mouse stood on its hindpaws, with forepaws touching the walls), unsupported rearing (UnsRearing, the mouse stood on its hindpaws, with forepaws up in the air), jumping (the mouse jumped), risk assessment (the mouse stretched the front part of its body forward, and returned to its initial posture) and defecation (number of fecal boli laid in the arena). Locomotion and thigmotasis were analyzed by a video tracking software (Ethovision XT, Noldus, Wageningen, the Netherlands) while the other behaviors were registered observationally. Groups were compared by factorial ANOVAs ('genotype × running') followed by LSD.

Next, a principal components factorial analysis (PCA) was performed on the variables assessed in the open-field to reduce them to a smaller set of dimensions that would underlie animals' behavior (Budaev, 2010; Castilla-Ortega et al., 2010). The correlation matrix of the whole sample of subjects ($n = 24$) was used for the analysis and tested for sampling adequacy by the Bartlett sphericity and the Kaiser–Meyer–Olking (KMO) tests. PCA was followed by a varimax orthogonal rotation, which ensures that the extracted factors are independent one of the others. The resulting factors with the eigenvalue > 1 were selected, and the contribution of each behavioral variable to a factor (i.e. 'factor loading') was considered significant when it was more than 0.50 in absolute value. Finally, factor scores were calculated for each individual. To avoid negative values, factor scores were transformed from a typified scale of standard deviation = 1 and mean = 0 to a scale of standard deviation = 10 and mean = 50.

2.4. Immunohistochemistry and cell quantification

To study neurogenesis-related markers, 4 h after the behavioral task mice were deeply anesthetized with an intraperitonal dose of ketamine (100 mg/kg, Sigma) and then were intracardially perfused with 0.1 M phosphate-buffered saline pH 7.4 (PBS) and a periodate-lysine-paraformaldehyde solution (PLP) (McLean and Nakane, 1974) in PBS. Brains were post-fixed for 48 h in PLP. The left hippocampus was cut into coronal vibratome sections (50 μm) and serialized so one of every fourth hippocampal sections was used for each histological marker. Free-floating immunohistochemistry was performed by the peroxidase-conjugated extravidin method with diaminobenzidine (DAB) as the cromogen using the following primary antibodies: mouse anti-Proliferating Cell Nuclear Antigen (PCNA; Sigma; at 1:1000 dilution) for labelling of cells undergoing proliferation, goat anti-doublecortin (DCX; Santa Cruz Biotechnology, Santa Cruz, USA; 1:200) as immature neuron marker and mouse anti-BrdU (Developmental Studies Hybridoma Bank, Iowa, USA; 1:1000) for labelling the 24 days-old surviving cells. Secondary antibodies were biotin-conjugated rabbit anti-mouse or rabbit anti-goat (Dako, Glostrup, Danmark; 1:500), as appropriate. Immuhohistochemical procedures are detailed elsewhere (Castilla-Ortega et al., 2011a; Matas-Rico et al., 2008). Cell counting was carried out in the dorsal dentate gyrus (DG) from −1.22 to −2.54 mm from bregma (Paxinos and Franklin, 2001). The suprapyramidal (SupraDG) and the infrapyramidal (InfraDG) blades were counted separately, because they show distinct patterns of neurogenesis (Jinno, 2011) and could respond differently to exercise. Cell counting used an Olympus BX51 microscope equipped with an Olympus DP70 digital camera (Olympus, Glostrup, Denmark). High resolution photographs (4080 × 3072 pixels at 10× lens) of the whole dentate gyrus were taken and the total number of positively stained cells was manually quantified with software ImageJ (US National Institutes of Health, MD, USA). Countings were averaged on a mean value per section for each animal and analyzed by a repeated measures ANOVA ('genotype × running × blade', with 'blade' as a repeated measure) followed by LSD.

2.5. Relationship of the quantity of running with the exercise-induced changes on behavior and neurogenesis in WT and LPA_1-null mice

The importance of the quantity of running for the exercise-induced effects on behavior (the factors extracted from the open-field measures) and neurogenesis (the cells positive for PCNA, DCX and BrdU labelling in the SupraDG, that was the most responsive blade to exercise) was researched. For each variable and genotype, we quantified the percent of change of the *VRun* group from the *Ctrl* group (coefficients of change). The score of each *VRun* animal was multiplied by 100 and divided by the mean score of the corresponding Ctrl group. The resulting value was subtracted 100 so the absence of change from Ctrl would correspond to zero. These coefficients of change were then used for correlation (Pearson's) with the total distance ran and for backward regression analysis. Backward regression analysis tested the relative importance of the genotype and the distance ran to predict the exercise-induced change in each variable. A model included both the genotype and the distance ran as predictors of each coefficient of change, and the non-relevant predictors were then excluded on the basis of a statistical criteria (predictors were tested for exclusion starting from the one with lowest partial correlation to the dependent variable; the criterium for exclusion was the probability of the *F*-value > 0.10).

2.6. Quantitative review of literature

Finally, we aimed to assess whether the quantity of exercise performed by transgenic mice has been given importance in the existing literature. To this end, we reviewed publications that used voluntary wheel running, environmental enrichment or treadmill running as a treatment to modulate behavior and the hippocampus in transgenic mice. Research articles were searched through the PUBMED database (http://www.ncbi.nlm.nih.gov/pubmed) among publications from the last 6 years that contained relevant keywords within the title or abstract (e.g. 'running', 'exercise', 'transgenic', 'knockout', 'hippocampal neurogenesis', 'memory', etc.). Articles resulting for the search were checked out and those that did not use the environmental manipulation as a long-term treatment for mutants' behavior or hippocampus (e.g. used exercise as a mean to assess circadian activity or to induce an acute physiological response) were excluded. Those manuscripts to which we could not gain full access were excluded too.

For each of the resulting articles, we classified the environmental manipulation used into: (1) 'Voluntary running' (VRun), when only free access to running wheels was provided; (2) 'Environmental enrichment' (EE), when it consisted of novel stimuli that were changed from time to time; (3) 'Environmental enrichment plus voluntary running' (EE + VRun), when the stimular novelty was combined with free access to running wheels; or (4) 'Treadmill running' (TRun), when mice were forced to exercise in running wheels or other devices such as a rotarod. If an article contained several experiments including more than one environmental manipulation, those were treated as separate studies. For both the VRun and EE + VRun paradigms, we annotated whether the quantity of wheel running performed by the transgenic mice was clearly assessed and reported. Finally, to summarize the main results in the VRun studies, we examined the abstract (figures were also checked out when more information on a result was required) and annotated whether the key conclusions reported at least one relevant 'absent' or 'blunted' effect of the VRun treatment in the transgenic mice (i.e.

running failed to recover transgenic mice from behavioral and/or hippocampal pathology; and/or its effect was notably reduced in mutants compared to normal mice).

3. Results

3.1. LPA_1-null mice barely perform voluntary exercise

When the total amount of wheel running was analyzed across the experiment, averaged every four days, a repeated measures ANOVA revealed that LPA_1-null mice did not escalate the running behavior and showed a strikingly reduced amount of voluntary exercise compared to the WT genotype ['genotype': $F_{1,10} = 78.963$, $p = 0.000$; 'day': $F_{5,50} = 15.698$, $p = 0.000$; 'genotype × day': $F_{5,50} = 10.553$, $p = 0.000$; LSD in Fig. 1B]. After the exercise paradigm, the WT mice had ran a mean of 10.97 ± 0.79 km per day while the nulls only ran a daily mean of 1.88 ± 0.49 km. This measure reflects the total distance ran in the whole experiment averaged as a single value. A between-group comparison of this measure by a Student's t test, confirmed the result of the previous analysis revealing a notable difference according to the genotype [Student's t test: $t_{10} = 8.886$; $p = 0.000$].

3.2. Running reduced anxiety-like behavior in both genotypes, but increased exploration only in WT mice

After VRun or Ctrl treatments, mice were tested for exploratory and anxiety-like behavior in the open-field for 5 min. When locomotion and thigmotaxis were analyzed in the whole session, no effect of exercise was found, although the total locomotion was reduced in nulls (Supplementary Material). However, a detailed inspection of these measures revealed an effect of the exercise treatment on the first minute of the test, that represents the initial exploration of the novel environment before the rodent becomes familiar with it and habituates within the trial (Leussis and Bolivar, 2006). Therefore, on the first minute, running increased locomotion only in the WT mice ['genotype': $F_{1,20} = 13.145$, $p = 0.002$; 'running': $F_{1,20} = 4.894$, $p = 0.039$; 'genotype × running': $F_{1,20} = 6.243$, $P = 0.022$; LSD in Fig. 2A] and decreased thigmotaxis in both genotypes ['running': $F_{1,20} = 18.250$, $p = 0.000$; LSD in Fig. 2B]. Data of locomotion and thigmotaxis analyzed as a total per session or every 5 min are included in Supplementary material.

The rest of behavioral measures were analyzed through the whole session. In *Ctrl* conditions, LPA_1-null mice showed reduced SupRearing ['genotype': $F_{1,20} = 49.041$, $p = 0.000$; LSD in Fig. 2C], lacked jumping ['genotype': $F_{1,20} = 15.411$, $p = 0.001$; LSD in Fig. 2D] and increased risk assessment behavior ['genotype': $F_{1,20} = 5.237$, $p = 0.033$; LSD in Fig. 2E] compared to the *Ctrl*-WT mice. After running, SupRearing showed a tendency to reduction ['running': $F_{1,20} = 13.949$, $p = 0.001$], but UnsRearing and jumping were increased only in the *VRun*-WT mice [UnsRearing: 'genotype': $F_{1,20} = 7.440$, $p = 0.013$; 'running': $F_{1,20} = 9.152$, $p = 0.007$; 'genotype × running': $F_{1,20} = 4.134$, $p = 0.050$; jumping: 'running': $F_{1,20} = 5.548$, $p = 0.029$; 'genotype × running': $F_{1,20} = 5.548$, $p = 0.029$; LSD in Fig. 2C and D]. Running did not affect risk assessment or defecation (Fig. 2E and F).

The relationship among the open-field behaviors was studied by a PCA. Importantly, tests confirmed that the correlation matrix was appropriate to perform this analysis (Bartlett's sphericity test: $X^2 = 53.498$, $df = 21$, $p = 0.000$; KMO test for sample adequacy = 0.620, when a minimum score of 0.50 is considered necessary). PCA followed by a varimax rotated solution yielded 2 independent factors with eigenvalues > 1 that explained a 64.74% of the total variance (Fig. 2G). The variables best contributing to each factor were those that loaded high (>0.50) in that factor but low in the other. The first factor was composed of locomotion, UnsRearing, jumping and (negatively) risk assessment. It was named 'Exploration' because it suggested a dimension of general activity and investigation of the environment. The second factor was comprised by thigmotaxis and defecation, and likely represented 'Anxiety' (Castilla-Ortega et al., 2010). Interestingly, the SupRearing, a variable that involved peripheral exploration, partially contributed to both factors. Finally, factor scores were calculated and compared among groups. Factorial ANOVAs concluded that running reduced Anxiety in both genotypes ['running': $F_{1,20} = 22.866$, $p = 0.000$], but only the *VRun*-WT showed increased Exploration ['genotype': $F_{1,20} = 34.191$, $p = 0.000$; 'genotype × running': $F_{1,20} = 8.111$, $p = 0.010$; LSD in Fig. 2H].

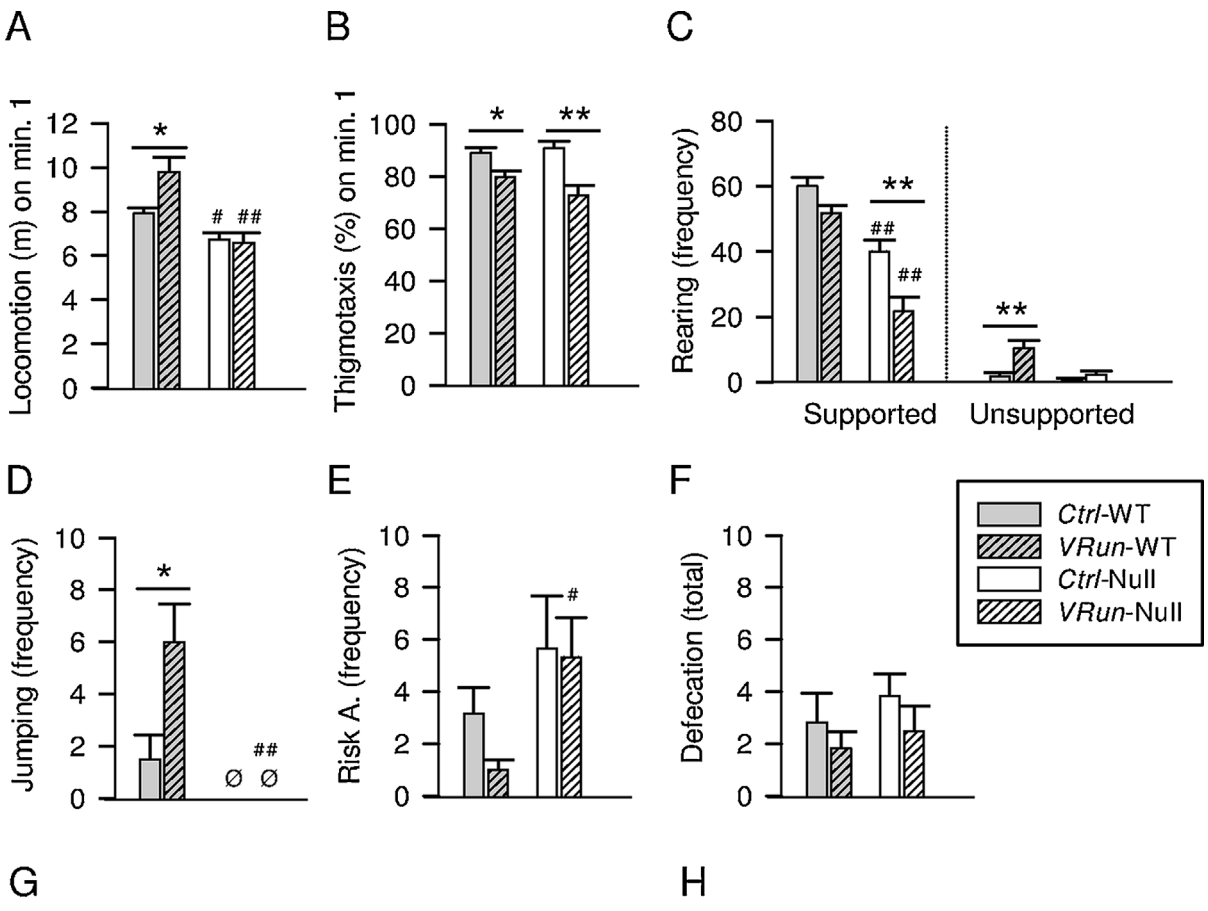
3.3. The effect of exercise on adult hippocampal neurogenesis is blunted in the LPA_1-nulls

Analysis of neurogenesis-related markers confirmed the previously reported deficit of adult hippocampal neurogenesis in the LPA_1-null mice (Castilla-Ortega et al., 2011a; Matas-Rico et al., 2008). In this way, the null genotype displayed a reduced number of proliferating cells marked with PCNA ['genotype': $F_{1,20} = 25.763$, $p = 0.000$; LSD in Fig. 3A], less adult-born immature neurons expressing DCX ['genotype': $F_{1,20} = 45.575$, $p = 0.000$; LSD in Fig. 3B] and a decreased number of 24 days-old surviving cells marked with BrdU ['genotype': $F_{1,20} = 15.582$, $p = 0.001$; LSD in Fig. 3C]. Interestingly, the effects of voluntary running were different for each genotype. In the *VRun*-WT mice, exercise induced a reduction of PCNA+ cells together with an increase in young DCX+ neurons and BrdU+ cells. However, the *VRun*-Nulls lacked those changes [PCNA: 'genotype × running × blade': $F_{1,20} = 15.404$, $p = 0.001$; DCX: 'genotype × running': $F_{1,20} = 10.079$, $p = 0.005$] or its magnitude was reduced in comparison to the *VRun*-WT mice [BrdU: 'genotype × running × blade': $F_{1,20} = 4.202$, $p = 0.050$].

Noteworthy, differences between the two blades of the DG were found. All the neurogenesis-related markers were more expressed in the SupraDG than in the InfraDG, in mice from both genotypes and treatments ['blade': PCNA: $F_{1,20} = 102.010$, $p = 0.000$; DCX: $F_{1,20} = 82.807$, $p = 0.000$; BrdU: $F_{1,20} = 57.890$, $p = 0.000$; LSD was < 0.05 when comparing both blades in a same experimental group, except for the *Ctrl*-WT in BrdU where it was > 0.05]. Moreover, the effect of running was more noticeable in the SupraDG than in the InfraDG for PCNA and BrdU ['running × blade': PCNA: $F_{1,20} = 4.799$, $p = 0.041$; BrdU: $F_{1,20} = 16.757$, $p = 0.001$].

3.4. The amount of voluntary running performed by each genotype predicts the running-induced change in neurogenesis and behavior

The total distance ran was significantly correlated with the exercise-induced change in Exploration and in all neurogenesis-related measures, so the change in Anxiety was the only one that not correlated with exercise (Fig. 4). Backward regression analysis excluded the genotype and retained the distance ran in the models that accounted for the change in Exploration [standardized $\beta = 0.840$, $t = 4.895$, $p = 0.001$; $R^2 = 0.676$] and BrdU [standardized $\beta = 0.628$, $t = 2.550$, $p = 0.029$; $R^2 = 0.394$]. On the contrary, only the genotype was retained to predict the change in PCNA [standardized $\beta = 0.832$, $t = 4.749$, $p = 0.001$; $R^2 = 0.693$] and DCX [standardized $\beta = -0.706$, $t = -3.252$, $p = 0.010$; $R^2 = 0.498$]. The change in Anxiety could not be predicted by the genotype nor by the distance ran, suggesting that it was independent of these variables.



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Variable:	Factor 1: Exploration	Factor 2: Anxiety
Locomotion (min 1)	**0.882**	0.024
Thigmotaxis (min 1)	0.003	**0.859**
Supp. Rearing	**0.657**	**0.649**
Unsupp. Rearing	**0.682**	-0.265
Jumping	**0.799**	-0.115
Risk Assesment	**-0.761**	-0.013
Defecation	-0.256	**0.574**
Variance explained:	42.265 %	22.471 %
Eigenvalue:	2.960	1.572

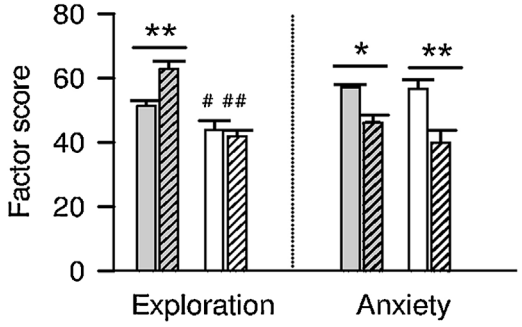


Fig. 2. Effect of voluntary running on open-field behavior. (A) Locomotion, (B) thigmotaxis, (C) supported and unsupported rearing, (D) jumping, (E) risk assessment and (F) defecation. Locomotion and thigmotaxis are assessed within the first minute, the rest of behaviors are assessed as a total frequency within the session. Behaviors are assessed as total frequency within the session. Means ± SEM. LSD: difference between genotypes: $^{\#}p < 0.05$; $^{\#\#}p < 0.001$; effect of running: $^{*}p < 0.05$; $^{**}p < 0.001$. (G) Behavioral factors resulting from the PCA. The PCA was followed by a varimax rotation to ensure that the extracted factors were independent of one another. The importance of a factor to explain the total variance was explored by means of the eigenvalues, so only factors with eigenvalue > 1 were selected. A variable was considered as included in a factor when its loading was > 0.5 in absolute value (highlighted in bold). (H) Factorial scores. Exercise reduced anxiety in both genotypes but increased exploration only in VRun-WT mice. The legend depicted in (F) is valid for the rest of graphs.

3.5. Voluntary exercise is widely applied in transgenic mice, but the quantity of running is frequently not assessed

In a second part of this work, we performed a literature review. After checking out the articles resulting from the search, a total of 104 experiments were reviewed. The oldest article dated from April 2006 and the most recent dated from March 2013. The studies evaluated and their outcomes are detailed in Supplementary material. The free access to a running wheel was revealed as a very popular approach to modulate behavior and the hippocampus in transgenic mice, as the VRun paradigms summed a 38% of the total followed by the EE + VRun treatments (35%). The use of EE (16%) or TRun (11%) was notably inferior (Fig. 5A). However, it was surprising that a half of the VRun experiments (48%) did not quantify the amount of running performed by the transgenic mice (Fig. 5B). Among those that assessed the quantity of wheel running, a 71% reported no differences between genotypes, a 19% reported defective running in the transgenic mice, and a 10% reported that transgenic mice ran more. Regarding the studies using EE + VRun, the assessment of the amount of running was nearly inexistent (Fig. 5B) and, moreover,

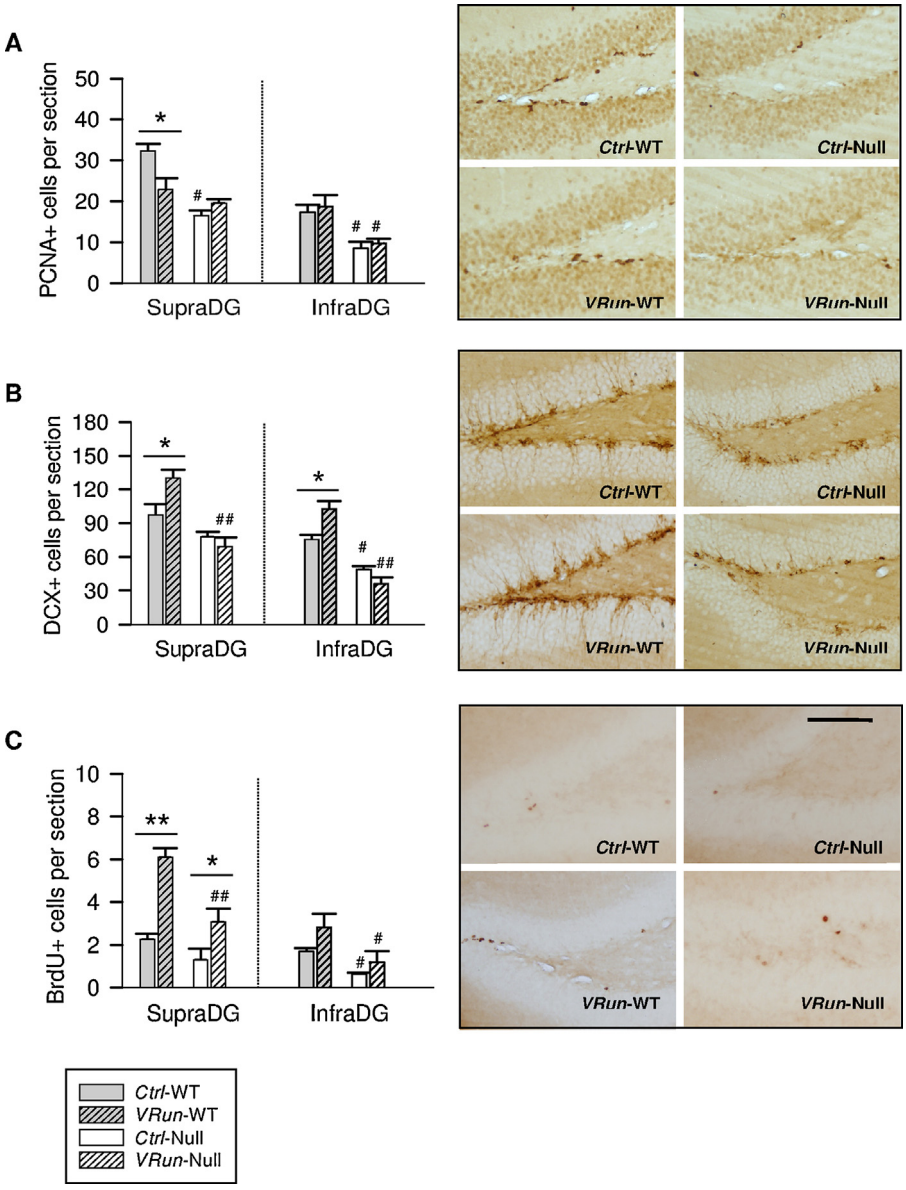


Fig. 3. Effect of voluntary running on neurogenesis-related markers in both blades of the DG. (A) Hippocampal cell proliferation, (B) adult-born immature neurons and (C) cell survival. Note that the effect of exercise was absent or notably reduced in the *VRun*-Nulls. Means ± SEM. LSD: difference between genotypes: $^{\#}p < 0.05$; $^{\#\#}p < 0.001$; effect of running: $^{*}p < 0.05$; $^{**}p < 0.001$. Scale: 90 μm.

few pointed out in the abstract that their enrichment protocol contained running wheels, so a detailed reading of the methods was necessary to find out this information.

On the other hand, an inspection of the main results of the VRun studies revealed that half of them reported absent or blunted effects of the treatment in the transgenic mice, on main behavioral or hippocampal variables researched (Fig. 5C; see Supplementary material for the detailed analysis). Remarkably, a notable proportion of these studies did not assess the quantity of running, so a potential wheel running alteration in the transgenic mice was not taken into account when interpreting results (Fig. 5C; the VRun studies that unassessed running and reported absent/blunted effects represented a 25% of the total sample of VRun studies). Finally, it should be noted that our results are likely underestimating the frequency of both wheel running alterations and absent/blunted effects of exercise in the transgenic mice. There is likely a publication bias, so VRun articles reporting running impairments or a lack of effect of exercise in the mutants could be more hardly published or less frequently submitted to publication. Moreover, some studies did not include a group of normal mice, avoiding comparison.

4. Discussion

In this work we have tested whether a paradigm of 23 days of free wheel running could recover LPA_1-null mice from behavioral and hippocampal pathology. Interestingly, the effects of running were different for the WT and the null genotype. Regarding the *VRun*-WT mice, they showed common changes reported after long-term voluntary exercise, such as increased exploration and anxiolytic behavior in the open-field (Dishman et al., 1996) and increased immature neurons and cell survival in the DG (Clark et al., 2010; Snyder et al., 2009), that in this case were accompanied by a small reduction in cell proliferation. Although exercise typically increases cell proliferation, absence of change or reduction are described after prolonged exercise and high running distances

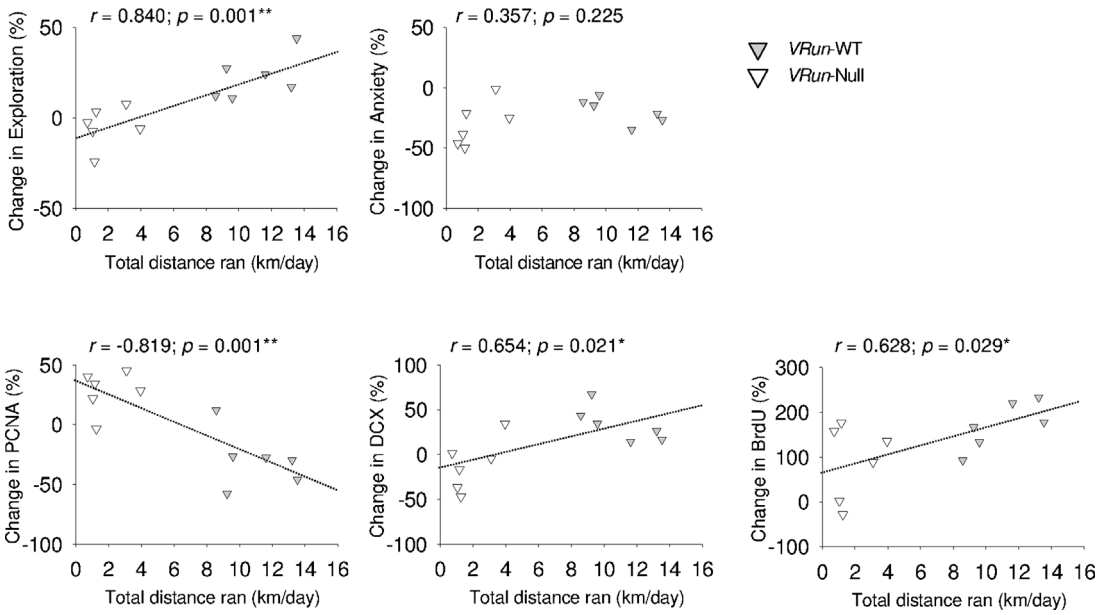
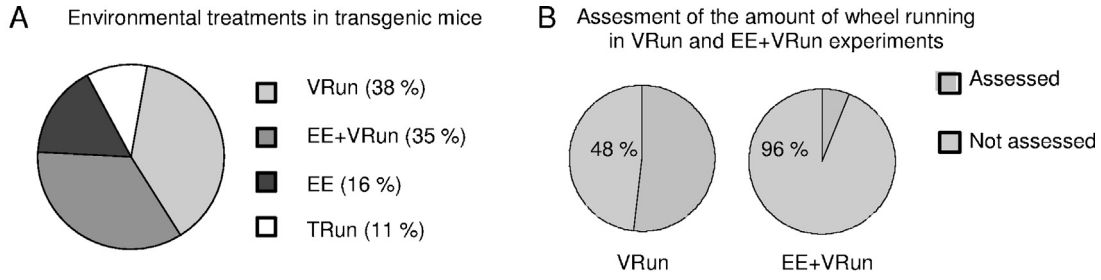


Fig. 4. Correlations of the coefficients of change (% of change in the running groups from controls) with the total amount of exercise performed by the animals (mean distance ran per day). The correlation was significant for the change in Exploration and in all neurogenesis-related measures, but not for Anxiety.

(Clark et al., 2010; Naylor et al., 2005), as is the case of the *VRun*-WT group. This reduction in cell proliferation may have functional consequences. Intriguingly, decreased proliferation or death of the younger hippocampal cells is reported after learning (Dupret et al., 2007; Pham et al., 2005), and it may favor the functional integration of other populations of newly-generated cells that are at a later stage of maturation. On the other hand, it is also interesting to note that exercise affected more the SupraDG than the InfraDG, supporting a dissociation of these areas. In the dorsal dentate gyrus of mice, but not in rats (Snyder et al., 2012), the suprapyramidal blade is described to have more neurogenesis than the infrapyramidal blade (Jinno, 2011), consistently with the increased expression of neurogenesis-related markers we found in the SupraDG for both genotypes. Moreover, the SupraDG generally shows greater experience-related activity than the InfraDG, both in basal conditions [reviewed in (Snyder et al., 2012)] and after exercise (Collins et al., 2009). Taken this together, the SupraDG seems the blade most involved in both the modulation of neurogenesis and the functional changes after exercise, although future studies should reveal whether there is a link between the increased number of newly-generated neurons and the increased functional activation.

Regarding mice lacking the LPA_1 receptor, they showed defective exploration in the open-field test as described previously



C

Assessment of running and effect of the running treatment in the VRun studies			
Assesment of running	Absent/blunted effects	No abs/blunt effects	Total
Running is unassessed	10 (25 %)	9 (22.5 %)	19 (48 %)
Transgenic mice ran normally	8 (20 %)	7 (17.5 %)	15 (37 %)
Transgenic mice ran less	2 (5 %)	2 (5 %)	4 (10 %)
Transgenic mice ran more	0 (0 %)	2 (5 %)	2 (5 %)
Total	20 (50 %)	20 (50 %)	40 (100 %)

Fig. 5. Review of the use of voluntary exercise in transgenic mice. (A) Environmental treatments used to modulate the hippocampus and/or behavior in transgenic mice, in a sample of 104 studies (years 2006–2013). (B) Frequency of VRun and EE + VRun experiments that did not assess the amount of voluntary wheel running in transgenic mice. (C) Number of VRun studies that reported absent and/or blunted effects of the running treatment on relevant behavioral and/or hippocampal measures in the transgenic mice. The outcome of the assessment of running is also reported. Note that a 25% of the VRun studies reported significant absent/blunted effects of the running treatment without controlling for running alterations in the transgenic mice. Studies and their main conclusions are detailed in Supplementary material. A total of 40 VRun studies were analyzed.

(Santin et al., 2009), and a reduction in all the neurogenesis-related markers (PCNA, DCX and BrdU), especially in the InfraDG. The defective adult hippocampal neurogenesis is already well reported in this genotype (Matas-Rico et al., 2008) and could be relevant to explain the hippocampal-dependent memory deficits that characterize these mutants (Blanco et al., 2012; Castilla-Ortega et al., 2010, 2011b, 2012; Pedraza et al., 2013; Santin et al., 2009). Therefore, the modulation of the hippocampal neurons by voluntary exercise seems of great interest to recover the pathological phenotype of the LPA_1-nulls. However, while exercise was effective to reduce anxiety and moderately increase cell survival in the *VRun*-Null mice, its overall effect on both behavior and neurogenesis was scarce compared to normal mice. Moreover, an almost six-fold reduction in the total running wheel distance was revealed in the LPA_1-null mice, indicating deficits in their time spent running and/or in its intensity. The potential reasons of reduced running are numerous and may include motor, emotional, hormonal, neurochemical or circadian alterations, that are frequently found in transgenic mice (Brooks et al., 2012; Lalonde et al., 2012; Novak et al., 2012; Taylor et al., 2010; van den Buuse, 2010). In the case of the LPA_1-null mice, their dramatic running impairment is likely contributed by motor deficits, because impaired neuromuscular strength, equilibrium and grasping reflexes are described in these mice (Santin et al., 2009), together with a disorganized neuronal architecture in the primary motor cortex (Estivill-Torrus et al., 2008). Nevertheless, considering that the absence of the LPA_1 receptor yields to profound neurodevelopmental deficits (Estivill-Torrus et al., 2008; Pedraza et al., 2013), additional alterations such as in the brain systems responsible of the rewarding effects of exercise cannot be ruled out.

Taking this into account, a main question is whether the blunted effects of the exercise treatment in nulls could be explained by the absence of the LPA_1 receptor or, instead, by the scarce wheel running performed by this genotype. Supporting the latter possibility, the total distance ran correlated with the exercise-induced changes in Exploration and in all neurogenesis-related measures. This agrees with previous evidence that has reported a positive correlation of the amount of exercise with both open-field activity (Pietropaolo et al., 2008) and adult hippocampal neurogenesis (Bednarczyk et al., 2009; Clark et al., 2011; Dubreucq et al., 2010; Holmes et al., 2004; Mustroph et al., 2012; Rhodes et al., 2003). Correlations found in our study, however, may be misleading, because they could have been generated just because we merged data from two groups (i.e. genotypes) that differed in the means of the two variables correlated (i.e. distance ran and the coefficients of change) (Lazic, 2012). Backward regression analyses were therefore performed, to test the relative importance of the genotype and the distance ran as predictors of the effects of the exercise treatment. We want to point out that other strategies, such as a causal modelling approach (Lazic, 2012) or correlational analysis carried out separately in each group, would be suitable to test the relationship of the amount of exercise with hippocampal and behavioral variables, excluding the potential influence of the genotype. However, these approaches are not used here due to the short number of animals in each group, which is somewhat a limitation in our study.

Although causality cannot be inferred by the backward regression analysis, they strongly suggested a relevant contribution of the amount of running to Exploration and BrdU measures, because the exercise-induced changes were more closely related to the total distance ran by an animal than to its genotype. A different result was found for PCNA and DCX, for which regression analysis selected the genotype, and not the distance ran, as a better predictor of the effects of exercise. Nevertheless, an important contribution of the total distance ran is still possible for these variables, as they could have a threshold-like relationship with exercise (Engesser-Cesar et al., 2007; Oliff et al., 1998; Pietropaolo et al., 2008; Soya et al., 2007). This means that surpassing a certain quantity of exercise could be necessary to affect these measures, but such relationship is not based on linear correlations and thus would be undetected by the analysis performed here. Therefore, Anxiety was the only variable clearly not influenced by null's reduced running, because it changed equally in *VRun* mice from both genotypes. It is possible that a low threshold of exercise was enough to induce the anxiolytic-like effects in the LPA_1-null mice, or Anxiety could have been modulated not by running itself but by other aspects of the running environment (Bednarczyk et al., 2011). These results support that the relationship established by exercise and a certain hippocampal or behavioral variable may be highly specific, explaining why the effect of running varies among the different measures assessed within a same study.

Therefore, while voluntary exercise could still exert some benefits in mice with wheel running alterations, main effects of exercise could be attenuated by the defective running. Because of this, VRun may not be the treatment of choice in transgenic mice with a running impairment such as the LPA_1-nulls. However, our review surprisingly found that half of the VRun studies in transgenic mice did not adequately assess the amount of running, including recent ones (Maesako et al., 2012; Malthankar-Phatak et al., 2012; Moreira et al., 2013; Renoir et al., 2012; Wu et al., 2012). While not every experiment that does not assess the quantity of exercise may yield inconsistent results, a substantial amount of studies reported absent or blunted effects of the VRun treatment without assessing running. Those studies may overlook a wheel running alteration in the transgenic mice that could explain the outcome. For example, studies that unassesed running have reported a fail of VRun to promote hippocampal plasticity in the R6/1 transgenic mouse model of Huntington's disease (Pang et al., 2006; Renoir et al., 2012; Zajac et al., 2010) but, intriguingly, it has recently been revealed that these mice show defective wheel running (Ransome and Hannan, 2013). In the experiment reported here, if wheel running had not been assessed we could have misinterpreted that exercise 'fails to' recover deficits in the LPA_1-nulls or, in other words, that the LPA_1 receptor is 'required for' some of the effects of exercise. Although the role of the LPA_1 receptor in the exercise-induced changes on hippocampal neurogenesis and behavior cannot be ruled out, it cannot be concluded from this experiment either, due to the drastical wheel running alteration in the nulls. Alternative approaches such as treadmill running in LPA_1-nulls, or a pharmacological modulation of the LPA_1 receptor in exercising WT mice, would be more suitable to answer this question. Finally, it is also important to note that the omission of the assessment of running was even more aggravated in studies that combined voluntary exercise with stimular novelty, which also seems inappropriate since wheel running could be a critical variable to affect hippocampal neurogenesis and memory in the EE + VRun paradigms (Mustroph et al., 2012). In conclusion, the assessment of the amount of wheel running should be strongly encouraged when transgenic mice are submitted to free running paradigms to modulate the hippocampus and behavior.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neures.2013.09.004.

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