Intergenerational transmission of the positive effects of physical exercise on brain and cognition


Physical exercise has positive effects on cognition, but very little is known about the inheritance of these effects to sedentary offspring and the mechanisms involved. Here, we use a patrilineal design in mice to test the transmission of effects from the same father (before or after training) and from different fathers to compare sedentary- and runner-father progenies. Behavioral, stereological, and whole-genome sequence analyses reveal that paternal cognition improvement is inherited by the offspring, along with increased adult neurogenesis, greater mitochondrial citrate synthase activity, and modulation of the adult hippocampal gene expression profile. These results demonstrate the inheritance of exercise-induced cognition enhancement through the germline, pointing to paternal physical activity as a direct factor driving offspring’s brain physiology and cognitive behavior.

Significance

Physical exercise is well known for its positive effects on general health (specifically, on brain function and health), and some mediating mechanisms are also known. A few reports have addressed intergenerational inheritance of some of these positive effects from exercised mothers or fathers to the progeny, but with scarce results in cognition. We report here the inheritance of moderate exercise-induced paternal traits in offspring’s cognition, neurogenesis, and enhanced mitochondrial activity. These changes were accompanied by specific gene expression changes, including gene sets regulated by microRNAs, as potential mediating mechanisms. We have also demonstrated a direct transmission of the exercise-induced effects through the fathers’ sperm, thus showing that paternal physical activity is a direct factor driving offspring’s brain physiology and cognitive behavior.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Published under the PNAS license.

Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, https://ncbi.nlm.nih.gov/geo (accession no. GSE123582).

1Á.F.-L. and J.L.T. contributed equally to this work.

2To whom correspondence may be addressed. Email: afontan@us.es or jltrejo@cajal.csic.es.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1816781116/-/DCSupplemental.

Published online April 22, 2019.

www.pnas.org/cgi/doi/10.1073/pnas.1816781116

PNAS | May 14, 2019 | vol. 116 | no. 20 | 10103–10112

Intergenerational inheritance of cognition traits | Moderate physical exercise | Adult hippocampal neurogenesis | Mitochondria
exercise are inherited by the progeny; (iii) the changes in the pattern of gene expression in the hippocampus of exercised fathers might also be inherited by the offspring; and (iv) the main characteristics of mitochondrial functioning (either number or organelle activation or both) are transmitted intergenerationally.

To achieve these goals, we performed a triple approach to patrilineal intergenerational inheritance to test the strength of the biological process at hand. First, we compared litters from sedentary males with litters from the same males after training (running). This approach was used to minimize interfather variability (the progenitor effect). Second, we compared litters from sedentary males with litters from different, exercised males to study intergenerational effects of exercise in nonrelated offspring. This approach was used to compare animals from experimental groups processed at the same time and without the potentially confounding factor of the order of the litter. Third, to study whether these intergenerational exercise-driven effects were germline dependent, we designed an experiment in which interactions between male and female progenitors were eliminated by generating the progeny through in vitro fertilization (IVF) and embryo transfer. Our experimental design followed main gold-standard guidelines (19). Because we were interested in the cognitive effects of physical activity, we tested the pure effects of physical exercise, separating these effects from the cognitive influence of an environmental enrichment. To minimize inter-subject variability, we employed moderately forced activity on a treadmill. Specific behavioral tests were used to analyze a possible enhancement of object recognition memory and spatial pattern separation. The novel object recognition (NOR) test is a common method used to assess the rodents’ ability to recognize a novel object in an environment without external cues and reinforcements. It is based on the rodent’s natural preference for novelty. When the animals are exposed to a familiar and a novel object, they spend more time exploring the novel object (20). There are several underlying neural circuits and brain structures involved in the NOR test (in which the hippocampal formation plays a key role) that support learning and memory processes, such as encoding, consolidation, and memory retrieval (21). On the other hand, pattern separation is a cognitive process that allows the formation of distinct representations out of similar inputs. A pattern separation task based on a novel object location by the mouse showed that mice can separate the novel object from the familiar object, which is greatly supported by the dentate gyrus and adult hippocampal neurogenesis (22). Both the NOR test and the pattern separation task can be evaluated by a discrimination index (DI), which expresses the difference in the exploration times of the novel and familiar objects (the moving and fixed objects in the pattern separation task), divided by the total exploration time.

To relate the exercise effects with the heritability of the changes in specific hippocampal neuronal populations (including neurogenic populations), we used ad hoc-designed stereological protocols. Exercise-induced changes in gene expression in the brains of fathers and offspring were also analyzed, as well as the changes in induced methylation in the parents’ sperm. Finally, exercise-induced changes in mitochondrial physiology and cellular energetics in the liver, cerebellum, and hippocampus of fathers and offspring were also analyzed.

Results

Experimental Design and Inheritance of Behavioral Effects of Exercise. Before exercise, we analyzed whether fathers had any cognitive differences (tested in a difficult NOR test), finding no significant differences between groups (Fig. 1 E and H). Next, one group underwent a moderate forced training protocol on a treadmill for 6 wk and the other group remained sedentary. After the application of the exercise protocol, all fathers were tested in a behavioral test battery based on Crawley (23), including the elevated plus maze (anxiety test) and a standard protocol of water maze in experiment A. These two tests revealed no significant differences between groups (SI Appendix, Fig. S14). Differences found in the rest of the tests are described below. In experiment A (SI Appendix, Fig. S14), litters of sedentary fathers were compared with litters born from the same fathers after exercising. In experiment B (SI Appendix, Fig. S1B), litters from sedentary males were compared with litters from different, exercised males. Finally, in experiment C (SI Appendix, Fig. S1C), to eliminate interactions between males and their mates, IVF and embryo transfer were used to produce litters from different exercised and sedentary males. In adulthood, they underwent the same behavioral protocols as litters in experiments A and B. In every experiment, all litters were sedentary—only groups of fathers underwent physical exercise during the experiments.

Litters in experiments A and B underwent a neurodevelopmental battery of tests (24), from postnatal day (P)2 to P15 (SI Appendix, Table S2). No relevant significant differences were found between groups (SI Appendix, Figs. S15 and S16). All animals in experiments A and B were tested at adult stages (3.5-mo-old litters, and 5.5-mo-old fathers).

First, the locomotor activity was analyzed using an activity cage in a novel and a known environment (day 1 and day 2, respectively). In experiment A (Fig. 1 A and B), a Wilcoxon signed-rank test indicated that exercised fathers (Z = −2.02, P = 0.043, r2 = 0.40) and both groups of litters (Z = −2.52, P = 0.012, r2 = 0.40) significantly reduced their activity on day 2. In experiment B (Fig. 1 C and D), exercised fathers showed significantly more activity than sedentary fathers on day 1 [t test, t (13) = −3.43, P = 0.004, g = 1.9, r2 = 0.50]. Both sedentary fathers [t (9) = 4.25, P = 0.002, dz = 1.34, r2 = 0.67] and exercised fathers [t (4) = 14.23, P < 0.001, dz = 6.3, r2 = 0.98] significantly reduced their activity on day 2. On the other hand, litters from exercised fathers tended to show more activity than litters from sedentary fathers (Mann–Whitney U test, U = 6, P = 0.089, r2 = 0.24) on day 1. A Wilcoxon signed-rank test revealed that both groups of litters significantly reduced their activity on day 2, but only litters from sedentary fathers showed a significant reduction (Z = −2.52, P = 0.012, r2 = 0.40 and Z = −1.83, P = 0.068, r2 = 0.41, respectively). In experiment C (Fig. 1O), a paired-sample t test indicated that litters from sedentary fathers [t (9) = 15.68, P < 0.001, dz = 4.96, r2 = 0.90] and litters from exercised fathers [t (9) = 12.02, dz = 5.3, r2 = 0.81] also reduced their activity on day 2. Overall, in experiment A, exercised fathers and both groups of litters reduced their activity in a known environment. In experiment B, exercised fathers showed more activity than sedentary fathers in a novel environment, and both groups of fathers reduced their activity in a known environment. Finally, litters from sedentary fathers reduced their activity in a known environment. For additional activity-cage parameters, see SI Appendix, Figs. S2 and S6A.

Second, NOR memory was assessed. To assess memory enhancement, difficult and easy protocols were designed by modifying the time spent during the training phase (SI Appendix, Fig. S11 A and B). The easy protocol had a longer training phase and, although animals showed low DI scores, no significant differences were found between groups in experiments A and B (SI Appendix, Fig. S3 A–D). In contrast, the difficult protocol allowed the animals limited time to explore the objects in the training phase. Before exercising, all groups of fathers were unable to discriminate the novel object in the difficult protocol (Fig. 1 E and H), but after 6 wk of physical exercise, exercised fathers and their offspring showed memory enhancement (Fig. 1 F–J). In experiment A (Fig. 1F), a Mann–Whitney U test indicated that exercised fathers showed significantly higher DI scores than sedentary males in the short-term memory (STM) phase (U = 0, P = 0.021, r2 = 0.67) and in the long-term memory (LTM) phase (U = 0, P = 0.02, r2 = 0.67). A Friedman test revealed significant differences in the performance of exercised
Fig. 1. Intergenerational inheritance of memory enhancement and pattern separation improvement through physical exercise. (A–D) Horizontal locomotor activity in a novel and a known environment. The locomotor activity was assessed using an activity cage in a novel and a known environment (day 1 and day 2, respectively). Charts represent the horizontal locomotor activity of fathers (A) and litters (B) in experiment A and of fathers (C) and litters (D) in experiment B. (E–J) NOR memory enhancement in exercised fathers and their offspring. Results represent a DI. A DI score of $>0.20$ was set ad hoc to determine proper novel-object discrimination; statistically significant within-group differences were also considered between the training phase and the test phases. Before exercising, all groups of fathers were unable to discriminate the novel object in a difficult protocol (E and H). After 6 wk of physical exercise, only exercised fathers and their sedentary offspring showed memory enhancement in experiment A (I and G) and experiment B (I and J). (K–N) Improved pattern separation performance of exercised fathers and their offspring in a low separation test. Results represent a DI, which allows the discrimination between the exploration of the objects in different positions. A DI score of $>0.20$ was set ad hoc to determine proper discrimination; statistically significant within-group differences were also considered between the training phase and the test phases. After 6 wk of physical exercise, only runner fathers and their sedentary offspring showed an improved pattern separation performance in experiment A (K and L) and B (M and N). (O–Q) Results were replicated in experiment C. Locomotor activity (O) of the offspring in the IVF experiment in a novel and a known environment (day 1 and day 2, respectively). The offspring of exercised fathers also showed enhanced object recognition memory (P) and an improved pattern separation performance in a low pattern separation test (Q). All data are shown as mean ± SEM. For comparisons between independent groups, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; tendencies $0.05 > P > 0.09$. Extreme values were removed from the analysis. SED A, $n = 4$; RUN A, $n = 5$; L. SED A, $n = 8$; L. RUN A, $n = 8$; SED B, $n = 10$; RUN B, $n = 5$; L. SED B, $n = 8$; L. RUN B, $n = 4$; L. SED C, $n = 10$; L. RUN C, $n = 13$. 

McGreevy et al. PNAS | May 14, 2019 | vol. 116 | no. 20 | 10105
fathers throughout the test ($X^2 = 6.5, P = 0.039$); post hoc analysis with Wilcoxon signed-rank tests showed significantly higher DI scores in both test phases ($Z = -2.023, P = 0.043, r^2 = 0.41$). To analyze the offspring’s performance (Fig. 1G), a mixed ANOVA was run, with the phase of the test (training, STM, or LTM) as a within-subjects factor and the fathers’ exercise protocol (sedentary or runner) as a between-subjects factor. There was a significant main effect of the phase of the test [$F(2,26) = 24.877, P < 0.001, \eta^2_p = 0.657$], as well as a significant interaction between the phase of the test and the exercise protocol [$F(2,26) = 10.779, P < 0.001, \eta^2_p = 0.453$]. Pairwise comparisons corrected by Bonferroni showed that only litters from exercised fathers obtained significantly higher DI scores in both test phases (STM and LTM) compared with the training phase ($P < 0.001$). There was also a significant main effect of the fathers’ exercise protocol [$F(1,13) = 45.021, P < 0.001, \eta^2_p = 0.776$]. Pairwise comparisons corrected by Bonferroni revealed that litters from exercised fathers obtained higher DI scores than litters from the same fathers before exercising in the STM phase ($P = 0.001$) and in the LTM phase ($P < 0.001$). In experiment B (Fig. 1J), a Mann–Whitney $U$ test indicated that exercised fathers showed significantly higher DI scores than sedentary fathers in the LTM phase of the test ($U = 2.5, P = 0.006, r^2 = 0.51$). A Friedman test revealed significant differences in the performance of exercised fathers throughout the test ($X^2 = 10, P = 0.007$). Post hoc analysis with Wilcoxon signed-rank tests indicated that exercised fathers showed significantly higher DI scores in both test phases ($Z = -2.023, P = 0.043, r^2 = 0.41$) compared with the training phase. Furthermore, litters from exercised fathers (Fig. 1J) showed significantly higher DI scores than litters from different sedentary fathers in the STM phase ($U = 2, P = 0.017, r^2 = 0.47$) and the LTM phase ($U = 0, P = 0.007, r^2 = 0.61$). Although the average DI scores were low, a Friedman test revealed significant differences in the performance of litters from sedentary fathers throughout the test ($X^2 = 13, P = 0.002$). Post hoc analysis revealed significantly higher DI scores in both test phases (Wilcoxon signed-rank test, $Z = -2.52, P = 0.012, r^2 = 0.40$). On the other hand, litters from different exercised fathers tended to vary their performance throughout the test (Friedman test, $X^2 = 6, P = 0.05$), and post hoc analysis with Wilcoxon signed-rank test indicated a tendency to show higher DI scores in both test phases ($Z = -1.93, P = 0.068, r^2 = 0.40$) but not significantly. Nevertheless, only litters from exercised males reached DI scores $>0.20$. Finally, in experiment C (Fig. 1P), a Mann–Whitney $U$ test indicated that litters from exercised fathers showed significantly higher DI scores compared with litters from sedentary fathers in the STM phase ($U = 7, P = 0.001, r^2 = 0.53$) and in the LTM phase ($U = 2, P < 0.001, r^2 = 0.65$). A Friedman test revealed that only litters from exercised fathers significantly varied their performance throughout the test ($X^2 = 12, P < 0.001$), and post hoc analysis with a Wilcoxon signed-rank test showed significantly higher DI scores in both the STM phase ($Z = -3.06, P = 0.002, r^2 = 0.39$) and the LTM phase ($Z = -2.94, P = 0.003, r^2 = 0.36$) compared with the training phase. The total exploration times of all NOR tests are shown in SI Appendix, Figs. S3 E–N and S6B.

Finally, to determine whether these differences were test dependent, a pattern separation test with customizable difficulty thresholds was designed (SI Appendix, Fig. S11 C and D). While the easy protocol of the test (high pattern separation) was achieved by all litters in experiments A and B (SI Appendix, Fig. S4 A and C), only the exercised fathers and their offspring performed outstandingly in the difficult protocol (low pattern separation). In experiment A (Fig. 1K and L), a Mann–Whitney $U$ test revealed that exercised fathers showed significantly higher DI scores than sedentary males in the training phase ($U = 2, P = 0.048, r^2 = 0.43$) and in the test phase ($U = 2, P = 0.049, r^2 = 0.43$). A Wilcoxon signed-rank test indicated that both groups showed higher DI scores in the test phase compared with the training phase, although this difference was only statistically significant for exercised fathers ($Z = -1.83, P = 0.068, r^2 = 0.42$ and $Z = -2.23, P = 0.043, r^2 = 0.41$, respectively). Furthermore, litters from exercised fathers showed significantly higher DI scores than litters from the same fathers before exercising in the training phase [$t = -3, P = 0.010, g = 1.5, r^2 = 0.41$] and in the test phase [$t = -9.17, P < 0.001, g = 4.46, r^2 = 0.87$]. A paired-sample $t$ test revealed that only litters from exercised fathers showed higher DI scores in the test phase compared with the training phase [$t = -6.07, P = 0.001, g = 2.15, r^2 = 0.84$]. In experiment B (Fig. 1M and N), exercised fathers tended to show higher DI scores than sedentary fathers in the test phase [$t = 1.47, P = 0.067, g = 0.08, r^2 = 0.24$] and showed significantly higher DI scores in the test phase compared with the training phase [$t = -6.93, P = 0.002, dz = 3.10, r^2 = 0.92$]. Moreover, litters from exercised fathers showed significantly higher DI scores than litters from different sedentary fathers in the test phase (Mann–Whitney $U$ test, $U = 2, P = 0.017, r^2 = 0.47$) and tended to show higher DI scores in the test phase compared with the training phase (Wilcoxon signed-rank test, $Z = -1.83, P = 0.068, r^2 = 0.42$). Finally, in experiment C (Fig. 1Q), only litters from exercised fathers showed significant differences between the training phase and the test phase [$t = -5.37, P = 0.001, dz = 1.79, r^2 = 0.78$] and significantly higher DI scores than sedentary litters in the test phase [$t = 2.56, P = 0.020, g = 1.18, r^2 = 0.28$]. Because the pattern separation test is based on an object location test (OLT), a standard OLT was used as a control to ensure correct object location performance. No significant differences were found between groups in the test phase of OLT (SI Appendix, Fig. S4 D–G). The total exploration times of all pattern separation tests and OLT in experiments A and B are shown in SI Appendix, Fig. S4 H–R and in SI Appendix, Fig. S6C for experiment C.

Because the NOR and pattern separation tests were part of a behavioral phenotyping battery, we previously studied whether continuous exposure to complex testing had an effect on the animals’ DI scores in the easy protocols. To verify this, a control experiment was designed with naïve animals performing only the easy protocols of these tests. All animals—sedentary and runner—learned to discriminate normally in the control experiment without intergroup differences, thereby validating the easy and difficult experimental designs and explaining the low DI scores found in the data of the behavioral test battery (SI Appendix, Fig. S17).

All of the mentioned effects in fathers and litters were observed, both when analyzing data considering all siblings from a given litter as one sample to avoid litter effects and when analyzing data considering all animals from all litters inside each experimental group as independent samples (SI Appendix, Fig. S5). In experiment C, the litter effect was minimized by applying a mixed weaning strategy on P21 (SI Appendix, Fig. S1C).

Exercise-Induced Increase of Hippocampal Cell Proliferation and Immature Neuron Number Is Mimicked by Sedentary Litters Raised from Exercised Parents. Nonspatial NOR and spatial pattern separation have been closely related to the hippocampal formation and impact on adult hippocampal neurogenesis (17, 25–27). For this reason, we analyzed adult hippocampal neural stem cell (NSC) number, cell proliferation, and differentiation. We measured cell proliferation and neurogenesis by immunohistochemistry of double staining of SOX2/GFAP, phosphohistone 3 (pH3), BrdU, and double staining of doublecortin/calretinin (DCX/CLR) (Fig. 2) in neural progenitors and their adult progeny within the hippocampal dentate gyrus. Fathers and litters of both experiments were killed after 24 h of survival time after BrdU injection. No significant variation was found in SOX2/GFAP cells, suggesting that the NSC number was not affected (Fig. 2A–C). A
Increased neurogenesis in the offspring of exercised males. (A–O) Assessment of neural stem cells (NSCs) in the hippocampus. Representative image (A) of SOX2/GFAP+ cells in the dentate gyrus (DG) (Left) and a magnification image showing the morphology of these cells (Right). Only cells that had their cell bodies in the subgranular zone (SGZ) and displayed a radial glial-like morphology with a long process across the granular cell layer (GCL) were taken into account. No significant differences were found in the total SOX2/GFAP+ cells (B and C) in any of the experimental groups (D–G) Cell proliferation. Representative image (D) of pH3+ cells in the GCL and SGZ of the hippocampus (Left) and a magnification image showing the morphology of these cells (Right). Only exercised males showed a significantly increased total number of pH3+ cells (G), while no significant differences were found in the offspring (F); as the variability in the litters was higher than the group mean variability (no litter effect), we also show the independent sample analysis where litters from runner fathers showed an increased number of pH3+ compared to controls (G). (H–J) Cell proliferation and 24-h cell survival. Representative image (H) of BrdU+ cells in the GCL and SGZ of the hippocampus (Left) and a magnification image showing the morphology of these cells (Right). Exercised fathers showed a significant increase in the total number of BrdU+ cells (I) compared to controls; no significant differences were found in the offspring (J). (K–T) Assessment of subpopulations of immature cells. Representative image of the total number of DCX+/CLR+ cells in the hippocampus of fathers (K) and litters (L) (Top) and magnification images showing the morphology of these cells (Bottom). Exercised fathers tended to show an increased total number of DCX+/CLR+ cells (M), while litters showed a significantly increased number of these cells compared to controls (N); no significant differences were found between groups in the total number of DCX+/CLR+ cells (O and P), in the total number of DCX+ cells (Q and R), and in the total number of CLR+ cells (S and T). Orange scale bars, 200 μm; white scale bars, 100 μm; yellow scale bars, 20 μm; H, Hilus; ML, molecular layer. For intergroup differences between two independent groups, the t test was applied (if the variable was not normally distributed, the Mann–Whitney U test was used), *P < 0.05, * *P < 0.01, * * *P < 0.001; tendencies 0.05 > P > 0.09 in Student’s t test or Mann–Whitney U test. For each test, extreme values were removed from the analysis. Data are shown as mean ± SEM. Group SED B, n = 10; RUN B, n = 5; group L. SED B, n = 8; L. Group RUN B, n = 4 (G, L. SED B, n = 36 and L. RUN B, n = 14).

Descriptive Analysis of Gene Expression Shows the Biological Processes Affected Differentially in Each Generation and Suggests a Possible Mechanism of Epigenetic Inheritance. We performed RNA sequencing (RNA-seq) analysis to compare (i) the hippocampal gene expression levels of exercised fathers with respect to sedentary ones and (ii) the hippocampal gene expression levels of litters from exercised fathers with respect to the litters of the sedentary ones. All samples used in this analysis were from animals from experiment B. The Database for Annotation, Visualization, and Integrated Discovery (DAVID) software (v6.8) was used as a first approach to functionally describe the gene expression changes.
in our groups. We took into account only significantly differentially expressed genes (sDEGs) for this analysis [adjusted P value (adj-P) < 0.05]. sDEGs of each comparison are listed in Fig. 3A. We generated the annotation chart for each list of sDEGs (the databases used were GOTERM_CC_DIRECT, GOTERM_BP_DIRECT, GOTERM_MF_DIRECT, and KEGG_PATHWAY). Nineteen annotation terms relevant for neural tissue were enriched on the fathers' list, and five were enriched on the litters' list (Fig. 3A). On the fathers' list, the terms were mostly related to synaptic transmission, whereas they were mostly related to transcription processes on the litters' list of sDEGs. We also generated an annotation table for each list to know all annotation terms related to our sDEGs (SI Appendix, Table S1).

To further expand the qualitative description of gene expression, we performed a preranked gene set enrichment analysis (GSEA) of the RNA-seq data (Fig. 3B). Because GSEA is a threshold-free method of analysis, we generated the ranked list using all genes and their log2FoldChange-associated value. Several gene sets from three Molecular Signatures Database collections (H: hallmark gene sets, MIR: microRNA targets, and CC: gene ontology cellular component) showed enrichment in both comparisons (exercised fathers B vs. sedentary fathers B, and litters from exercised fathers B vs. litters from sedentary fathers B). We found 10 gene sets related to cell cycle and cell proliferation positively enriched in the fathers' comparisons (SI Appendix, Table S1). A positive enrichment in this analysis indicates that overrepresented genes related to a specific function are found overexpressed in exercised fathers compared with the levels of expression in sedentary ones. Remarkably, all gene sets related

---

**Fig. 3.** Descriptive analysis of gene expression data. (A) Functional analysis for fathers' comparison (a) and litters' comparison (b). Boxes (Far Left) show the lists of sDEGs of each group (adj-P < 0.05). Graphs show the annotation terms enriched from each database consulted and the percentage of sDEGs associated with each term. P values indicate the significance of enrichment (EASE Score). (B) GSEA for fathers' comparison (a) and litters' comparison (b). Several gene sets related to metabolic processes, cell proliferation, cellular components, and microRNAs show enrichment in both groups. Graphs for Mitotic Spindle, P53 Pathway, MIR212_132, and Mitochondrial Matrix sets and their respective enrichment score (ES) and normalized enrichment score (NES) are shown to illustrate the most interesting findings. A positive enrichment score here indicates that genes related to a specific function show a trend to be overexpressed in exercised individuals; a negative enrichment score indicates that genes related to a specific function show a trend to be underexpressed in exercised individuals. Only expression data from experiment B was used in this analysis (exercised fathers, n = 4; sedentary fathers, n = 5; animals from exercised fathers, n = 7; animals from sedentary fathers, n = 8). ER, endoplasmic reticulum.
to cell cycle and cell proliferation were negatively enriched in the litters’ comparison (SI Appendix, Table S1). A negative enrichment in this analysis indicates that overrepresented genes related to a specific function are found underexpressed in litters from exercised fathers compared with sedentary fathers.

Moreover, we found sets of genes associated with microRNA activity enriched in both comparisons (197 microRNA sets enriched in fathers’ comparison and 200 in litters’ comparison). Interestingly, one of the negatively enriched sets in both comparisons was MIR212_132 (Fig. 3B and SI Appendix, Table S1). The negative enrichment in this case indicates that overrepresented genes that share putative target sites (seed matches) of this specific microRNA in their 3′ UTRs are underexpressed in exercised fathers compared with sedentary fathers, and in litters from exercised fathers compared with litters of the sedentary ones. This is interesting, as Benito et al. (16) implicated these RNAs in the intergenerational inheritance of environmental enrichment effects.

Parallel to other findings in this work, mitochondrial-related gene sets were found positively enriched in both comparisons (SI Appendix, Table S1). The positive enrichment in this case indicates that overrepresented genes that are related to these mitochondrial components are overexpressed in exercised fathers compared with sedentary fathers and in litters from exercised fathers compared with litters of the sedentary ones. For additional GSEA plots of enriched gene sets, see SI Appendix, Fig. S8. Heatmaps of sDEGs are shown in SI Appendix, Figs. S9 and S10.

The complete descriptive analysis shows that the replication of the cognitive advantage in the second generation is not due to a replication of the gene expression profile of the first one. Annotation terms enriched in sDEGs from fathers point to exercise affecting mostly synaptic transmission, whereas annotation terms enriched in sDEGs from litters point to changes in the way hippocampal cells regulate transcription. GSEA analysis shows that multitudes of biological processes are commonly affected in both generations, including processes related to other results in this paper (i.e., cell cycle, cell proliferation, and mitochondria), but these affects are not a perfect replication from the first generation to the second one. The change of expression in genes related to microRNAs, as GSEA shows, suggests them as a potential mechanism for epigenetic inheritance. These data gain interest, since we found no exercise-induced changes in methyltransferase activity of male sperm DNA in a methylated DNA immunoprecipitation sequencing analysis (SI Appendix, Fig. S13 and Table S4), suggesting that intergenerational effects were not mediated by altered DNA methylation in spermatozoa. Further investigation is needed to clarify this hypothesis.

The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, https://ncbi.nlm.nih.gov/geo (accession no. GSE123582) (28).

The Progeny of Exercised Mice Exhibit Augmented Markers of Mitochondrial Function in the Hippocampus. We next examined exercise-induced effects in mitochondrial physiology and cellular energetics in the liver, cerebellum, and hippocampus of the fathers and their respective offspring. The liver was selected because it is directly related to the production of insulin-like growth factor 1 after exercise. The cerebellum was selected because the increased motion associated with physical exercise may well change the activity of the cerebellum, and the hippocampus was selected because variations in physical activity are directly associated with changes in hippocampal neurogenesis rate. The ratio of mitochondrial DNA (mtDNA) to nuclear DNA (nDNA) copy number was not affected in the different experimental groups (Fig. 4 A–F), suggesting that mitochondrial number per cell is not affected. To determine possible modulations in mitochondrial performance, we analyzed the activity of citrate synthase, a marker of mitochondrial functionality (29) encoded in the nDNA, which participates in the tricarboxylic acid cycle in the matrix of the mitochondria. Citrate synthase activity was significantly greater in the cerebellum of exercised parents [Fig. 4 I; t test, t (8) = –2.88, P = 0.022, g = 1.79, r² = 0.50], while no modulations were detected in the liver or hippocampus.

![Fig. 4.](image-url) Paternal exercise enhances markers of mitochondrial function in the hippocampus of the offspring. (A–F) mtDNA/nDNA ratio was determined by real-time PCR in the liver of fathers (A) and offspring (B), in the cerebellum of fathers (C) and litters (D), and in the hippocampus of fathers (E) and litters (F). (G–L) Citrate synthase activity measured in the liver of fathers (G) and litters (H), in the cerebellum of fathers (I) and litters (J), and in the hippocampus of fathers (K) and litters (L). All data are shown as mean ± SEM. For comparisons, *P < 0.05 for SED vs. RUN and L. SED vs. L. RUN in unpaired Student’s t test. SED B and RUN B, n = 5 per group; L. SED B, n = 7 to 8; L. RUN B, n = 6 to 8.
hippocampus and the liver of these mice (Fig. 4 G and K). Interestingly, citrate synthase activity was significantly increased in the hippocampus of the offspring from exercised parents [Fig. 4L; t (11) = −2.86, P = 0.016, g = 1.6, r² = 0.41], while no effects were found in liver and cerebellum lysates from the same individuals (Fig. 4 H and J).

**Discussion**

Previous studies have addressed inter- or transgenerational inheritance of activity-induced effects on behavior (13–16). In these studies, limited results were found. No changes were observed in anxiety or depression-like behaviors of the filial (F) 1 generation after using environmental enrichment (14). Exercise alone only suppressed reinstatement of juvenile fear memory in the study by Short et al. (13), and isolated animals were tested only at juvenile stages after weaning (15). Benito et al. (16) reported an enhancement of synaptic plasticity after environmental enrichment, with limited results in cognition. The present study shows a significant, large effect of fathers’ pure physical activity on both the NOR memory and the spatial pattern separation of their adult offspring as compared with the offspring of sedentary fathers or the offspring of the same fathers before exercising.

We have also found that offspring significantly replicated the exercise effects on the immature neuron subpopulation found in the fathers’ hippocampus. However, differences in cell proliferation were not replicated. This is not surprising, as pH3+ and 24-h survival BrdU+ cells are different subpopulations usually under compensating regulation. Our results indicate that specific subpopulations of cycling progenitors and immature, differentiating neurons in the dentate gyrus GCL are changed in sedentary brains because of the exercise program performed by their fathers.

Despite this replication of cognitive advantage and effects in the immature neuron subpopulation, we did not find a similar gene expression profile in both generations. There were no matches between sDEGs from the fathers’ comparison and from the litters’ comparison. DAVID analysis of RNA-seq data showed that different annotation terms were enriched for each sDEG list, whereas GSEA analysis showed that some relevant biological processes were affected in the same direction in both generations (i.e., mitochondrial processes), but others in opposite directions (i.e., cell cycle, cell proliferation). These results indicate that different gene expression profiles are mediating the same cognitive, cellular, and molecular outcomes in fathers and their offspring. Moreover, as for the neurogenesis-related sets, an exercise-induced increase in the proliferation of neural progenitors can be the final outcome of the intervention both in fathers and offspring, even though the gene expression patterns are different, due to different compensatory mechanisms in parents and offspring. Our work provides an extensive and detailed functional analysis of the gene expression changes induced by physical exercise done in two generations: the exercised one and their offspring. Extensive descriptive analyses were previously made by other groups (30), but they were restricted to the exercised animals and not their offspring.

The findings on mitochondrial proteins suggest that paternal exercise produces a specific reprogramming of hippocampal mitochondria in the offspring. In particular, we found that citrate synthase activity was enhanced, while mtDNA copy number per cell was unaffected. These data are suggestive of increased mitochondrial function and/or content in this specific area of the brain, which may have beneficial effects for the offspring. This finding is reinforced by our GSEA analysis, in which we found several gene sets related to mitochondria, enriched both in the fathers’ comparison and the litters’ comparison, including mitochondrial matrix set (where citrate synthase is located). The enhanced mitochondrial activity in the offspring might contribute to the cellular and behavioral changes observed in the present study. This is supported by recent works reporting that mitochondrial integrity is crucial for cell differentiation and dendritogenesis of newborn neurons (31) and for efficient lineage progression of adult NSCs in adult and aged hippocampus (32), as well as the finding that acute activation or deactivation of certain mitochondrial receptors is sufficient to modify memory abilities in adult mice (33). We believe that the fact that the mtDNA/nDNA ratio is not altered in the hippocampus of the offspring but that differences are found in the same samples in citrate synthase activity is remarkable and might reflect differences in mitochondrial functionality rather than differences in the number of mitochondria.

Therefore, our data demonstrate that the specific brain effects of a physical activity program can be intergenerationally inherited. These transmitted effects include (i) enhancing the performance of nonspatial and spatial cognitive tasks; (ii) increasing the number of specific cell populations of adult hippocampal neurogenesis, inducing changes in hippocampal gene expression; and lastly (iii) increasing hippocampal mitochondrial citrate synthase activity. We found no exercise-induced changes in methylating of male sperm DNA, suggesting that intergenerational effects were not mediated by altered DNA methylation in spermatozoa. Our GSEA suggests a possible mechanism of epigenetic inheritance, since we found a huge number of enriched sets related to microRNA activity. This indicates that many of the genes that are microRNA targets show a tendency to be found overexpressed or underexpressed in the hippocampus of exercised fathers and their offspring compared with sedentary groups. It has been reported that paternal sperm microRNAs drive the changes in the progeny of stressed fathers (34, 35). Some of the gene sets that were enriched in our study are the target of microRNAs that have been proved as key in the epigenetic inheritance of an LTP improvement [e.g., the key role of microRNA 212/132 reported by Benito et al. (16)]. Therefore, the paternal sperm microRNAs of exercised fathers could well be originating the changes we observed here in mitochondria, neurogenesis, and behavior. We cannot discard other epigenetic marks such as histone methylation (36) or H3 retention sites (37) that may have mediated phenotype transmission.

Our data suggest that the intergenerational transmission of these exercise effects is pleiotropic. Multiple mechanisms involved at different levels of the hippocampus mediate these effects. First, we found that specific gene sets were modified in exercised fathers and their sedentary offspring; second, on an organelle level, an increased mitochondrial function in the hippocampus of sedentary offspring of runner fathers was found; finally, at a tissue level, we found increased proliferation of hippocampal cells in both generations. Our gene expression analysis suggests mitochondrial and cell cycle-related genes as potential mechanisms mediating these effects in the hippocampus, whereas some of the microRNAs that were differentially regulated in the hippocampus of fathers and offspring are involved in the germline transmission of these changes (16). Further experiments would be worth carrying out to demonstrate whether transgenerational effects are also inherited (by examining the F2 generation).

Most importantly, we have shown that the cognitive effects are germline dependent, because the main behavioral results were robust after IVF and embryo transfer in a patrilineal design. These findings demonstrate a patrilineal intergenerational inheritance of improved cognitive abilities in adult progeny, pointing to the physical activity levels of fathers as an unexpected, relevant factor in the brain physiology and cognitive performance of their descendants.

**Materials and Methods**

**Subjects.** C57/BL6J mice (Harlan Laboratories) were housed under standard laboratory conditions, with ad libitum access to food and water, in accordance with European Union Directive 2010/63/EU. All experiments were...
performed according to the European Community Guidelines (Directive 2010/63/EU) and Spanish Guidelines (Real Decreto 53/2013), and have been approved by the Committee of Ethics and Animal Experimentation of the Cajal Institute (2005/2016), Ethics Committee (Subcommittee of Ethics) of the Spanish Research Council (07/27/2016) and the Animal Protection Area of the Ministry of Environment of the Community of Madrid (10/26/2016).

**Male progenitors (F0).** In both experiments (A and B), animals were randomly assigned to the experimental conditions. Ten subjects were used in experiment A [referred to as group sedentary (SED) A and runner (RUN) A] and 15 subjects in experiment B [referred to as group SED B and RUN B]. A total of 50 offspring per group shared a home cage with two dams during mating periods and were housed individually immediately afterward. Animals were 3.5 mo old at the start of the preexercise behavioral assessment and 5.5 mo old at the beginning of the behavioral battery, and they were killed at 7 mo of age. All comparisons were made on subjects of the same age for each comparison.

**Male offspring (F1).** Only male offspring were used. To prevent litter effects, each dam was considered the experimental unit, and all siblings from a given litter were considered as one sample. In experiment A, litters from sedentary fathers (L. SED A) and litters from the same fathers after exercising (L. RUN A) resulted in a sample size of eight subjects in each group. In experiment B, litters from sedentary fathers (L. SED B) resulted in a sample size of eight, while litters from different exercised males (L. RUN B) resulted in a sample size of four. After weaning, subjects were housed with their respective siblings.

Animals were 3 mo old at the start of the behavioral assessment and were killed at 5.5 mo of age. All comparisons were made on subjects of the same age for each comparison.

**Experiment Design (Experiments A and B).** A polyamorous trio was selected as a breeding strategy, always housing one male with two females per cage during a whole week (different females were selected for the following trios). Dams were separated when visibly pregnant to prevent overcrowding and to keep records of which pups belong to which female. A cross-fostering strategy was implemented to minimize the impact of the mother on the offspring. Siblings from a given litter remained together and were culled to generate as balanced numbers as possible within and between experimental groups.

**Experiment C Design.** Experiment C was implemented to test the transmission of the positive effects of exercise in cognition through the germline, eliminating de novo effects between male and female progenitors by IVF and embryo transfer. In adulthood, litters underwent the same experimental protocols as litters in experiments A and B.

IVF and embryo transfer were conducted at the Mouse Embryo Cryopreservation Facility of the National Centre for Biotechnology, Spanish National Research Council and using described methods (38–40). C57BL/6J/OlaHsd females were superovulated (41). The IVF protocols used are available through the Center for Animal Resources and Development website (card.medic.kumamoto-u.ac.jp/card/english/sigen/index.html). The process produced 10 males from sedentary fathers (referred to as L. SED C) and 13 males from runner fathers (referred to as L. RUN C). Animals were 3 mo old at the start of the behavioral assessment and were killed at 4.5 mo of age.

**Control Experiment Design.** A control experiment was carried out to test whether continuous exposure to complex testing had an effect on the animals’ performance in easy behavioral protocols. Two groups of six adult males (sedentary and runner) exclusively underwent easy protocols of NOR and pattern separation.

**Exercise Protocol (All Experiments).** The exercise protocol that was used was modified from Trejo et al. (42). Mice ran at 1,200 cm/min for 40 min, 5 d a week. Sedentary mice remained in the same room without running throughout the duration of the protocol.

**Behavioral Assessment.**

**Activity assessment.** To study the spontaneous locomotor activity in an open field arena, a VersaMax Legacy Open Field activity box (Omnitech Electronics) was used. Animals underwent a two-day protocol (5 min in the activity cage per day).

**NOR protocols.** To assess memory enhancement, difficult and easy protocols were designed by modifying [from the original description (20) and recent modifications of the test (21, 43)] the time spent during the training phase (SI Appendix, Figs. S11 A and B and S12).

**Pattern separation.** A modified version of the pattern separation test was used to study pattern separation performance enhancement. To do this, animals underwent two different protocols (SI Appendix, Fig. S11 C and D), referred to as low separation and high separation.

**Brdu Injections.** Male experimental animals in experiments A and B received one i.p. injection of BrdU (50 mg/kg body weight; Sigma-Aldrich) 24 h before being killed.

**Tissue Collection.** All male experimental animals were deeply anesthetized with pentobarbital (Euta-Lender). Each animal was transcardially perfused with 0.9% saline. The right hemisphere was fixed by immersion in 4% paraformaldehyde for histology.

**Histology.**Coronal sections (50-μm width) were obtained on a Leica VT1000S vibratome. One random series was chosen for each immunohistochemistry as described previously (44). Slices were incubated for single or double staining (SI Appendix, Table S3). The Cavaleri method was used as described previously (45).

**Stereology.** Brdu- and pH3-labeled cells were counted by the optical fractionator method. The physical-dissector method, adapted to confocal microscopy as previously described (46), was used to estimate the total number of SOX2+/GFAP+ cells, DCX+, and CLR- cells. GFAP expression was analyzed in the dentate gyrus.

**RNA-seq.** Total RNA extraction from hippocampal tissue. The QuickGene RNA Tissue Kit (RT-S2) and the QG-Mini80 (Kurabo) was used to extract total RNA from hippocampal tissue of a random selection of fathers (n = 10) and of eight animals representing each litter per group (n = 16) in experiment B. The final number of useful samples for analysis was n = 9 fathers (sedentary fathers, n = 5; exercised fathers, n = 4) and n = 15 offspring (animals from sedentary fathers, n = 8; animals from exercised fathers, n = 7). Stranded mRNA library preparation and sequencing. RNA-seq libraries were made with the TruSeq Stranded mRNA LT Sample Prep Kit (cat. no. 5031047 Rev. E, October 2013; Illumina). The libraries were sequenced on HiSeq2000 (Illumina) using TruSeq SBS Kit v4. Image analysis, base calling, and quality scoring of the run were processed using the manufacturer’s software Real Time Analysis (RTA 1.18.66.3) and followed by generation of FASTQ sequence files by CASSAVA.

**RNA-seq data processing and analysis.** RNA-seq reads were mapped with STAR version 2.5.2a (ENCODENP parameters for long RNA), and genes were quantified with RSEM version 1.2.28 (with default parameters). Normalization and differential expression were performed with DESeq2 version 1.10. We considered significant genes with a false discovery rate (FDR) of <5%.

**Bioinformatic analysis of RNA-seq results of hippocampal tissue.** Only animals of experiment B were used in this analysis. DAVID v6.8 was used for the functional description of the sDEGs of each comparison (exercised fathers vs. sedentary fathers and litters from exercised fathers vs. litters from sedentary ones). sDEGs have adjusted P values associated with their log2Fold-Change of <0.05. Four databases were chosen for the extraction of terms: GOTERM_BP_DIRECT, GOTERM_CC_DIRECT, KEGG_PATHWAY, and GOTERM_MF_DIRECT, and an EASE Score of 0.05 was set as a threshold. GSEA of RNA-seq data was performed with GSEA (Broad Institute, v3.0). A pre-ranked analysis was performed using log2FoldChange as a ranking metric. Only gene sets with an FDR of <25% were considered for descriptive analysis following the guidelines set by the Broad Institute (https://software.broadinstitute.org/gsea/doc/GSEAUserGuideFrame.html).

**Mitochondrial Assessment in Liver, Cerebellum, and Hippocampus.**

**mtDNA and mtDNA ratio analysis.** Total DNA was extracted with the DNeasy Blood and Tissue Kit (QIAGEN). mtDNA was amplified using primers specific for the mitochondrial NADH dehydrogenase (ND1) gene. Primer sequences can be found in SI Appendix, Table S5. The RT-PCR was performed on individual DNAs by using iTAQ universal SYBR Green (Bio-Rad Laboratories). The relative DNA content was calculated by the 2−ΔΔCt method.

**Citrate synthase activity.** Citrate synthase activity was determined in 50 μg of protein lysates following the method described by Spinaazi et al. (29). Citrate synthase was determined by spectrophotometric methods.

**Statistical Analysis.** Depending on the type of comparison and the parameter analyzed, we used either the t test, the Mann–Whitney U test, the paired-sample t test, the Wilcoxon signed-ranked test, a repeated-measures ANOVA, a mixed ANOVA, a Friedman test followed by a post hoc Wilcoxon test.


